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Research Article

ENVI-met Simulations of the Effect of Different Landscape Design Scenarios on Pedestrian Thermal Comfort: Haydar Aliyev Street

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Haydar Aliyev Street,
Park,
Pedestrian road,
Thermal Comfort

Abstract: In the city of Erzurum, located in a cold climate region, it is important for pedestrian walkways and parks to be usable all year round. Haydar Aliyev Street, located on the city's new development axis, serves as both a pedestrian route and a park. Meteorological data was collected hourly throughout 2021 using a Vantage Pro 2 Plus device installed at a height of 1.5 m in the study area. The scenarios were analyzed using the ENVI-met BIO+ Science Software, with August (summer) and January (winter) identified as the hottest and coldest months, respectively. Sky View Factor (SVF) analysis was conducted using fisheye lens photos taken from different points in the area. Four different landscape design scenarios were created for the study area, consisting of plants, water surfaces, soil, and grass. It was found that the temperature decreased by an average of 0.2°C in the summer scenario when the number of plants was increased by 20%. Furthermore, it was determined that the deciduous tree scenario provided better thermal comfort compared to the treeless soil scenario for a pedestrian-friendly park during the winter months. The inactive water scenario for summer and winter was found to increase wind speed by a maximum of 1.3 m s^{-1}. The study concluded that different landscape design scenarios had an impact on outdoor thermal comfort and that further research was needed in this area. Such studies highlight the need for multidisciplinary teamwork to create healthy, sustainable, and livable urban environments in designing thermal-comfortable spaces.

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1. Introduction

In recent years, urban population growth has been rapidly occurring worldwide. According to the United Nations, the global urban population increased from 751 million in 1950 to 4.2 billion in 2018, a 4.6-fold increase. It is estimated that this number will further increase to 6.4 billion by 2050, with approximately 70% of the world's population living in cities. However, this rapid urbanization has led to the formation of Urban Heat Island (UHI), due to the increase of impermeable surfaces, decrease

of green areas, construction of more buildings and workplaces, and increased use of personal vehicles (Oke, 2002; Mirzaei and Haghighat, 2010; Oliveira et al., 2011; Oke et al., 2017).

As a result of this temperature increase, UHI has become a widespread urban problem that can cause health problems in living organisms and an increase in energy demand (Okumuş and Terzi, 2021; IPCC, 2022). Efforts are being made to develop planning and design strategies that provide more livable micro-climate conditions for living organisms. Academic studies have experimented with different materials naturally to reduce the effects of UHI These materials include plants (Tan et al., 2016; Irmak et al., 2018; Yang et al., 2019; Ma et al., 2020; Yucekaya and Uslu, 2020), water surface options (Grimm et al., 2008; Wu et al., 2019; Gupta et al., 2019), street orientation and angle studies (Ali-Toudert and Mayer, 2007; Qaid et al., 2016; Yilmaz et al., 2018; Mutlu et al., 2018), roof-vertical gardens (Taleghani et al., 2015), and different ground covering materials (Irmak et al., 2017; Bozdogan et al., 2021). Urban parks have become increasingly important in reducing the effects of UHI. Urban parks not only meet the recreational needs of people but also improve outdoor thermal comfort. In particular, it is seen that urban parks can have a positive impact on thermal comfort in hot climate regions (Georgi and Dimitriou, 2010; Jamali et al., 2021). The positive contribution of urban parks has been determined in all perception models (Yilmaz et al., 2023) as well as in reducing stress (Yilmaz, 2022).

Numerous climate software models are used in the determination of outdoor thermal comfort. According to a review conducted in this field, it has been found that ENVI-met software is the most commonly preferred in 77% of the studies conducted in the last five years (Tsoka et al., 2018). Scenario analyses were conducted using ENVI-met to improve thermal comfort for pedestrians on streets and in urban spaces (Middel et al., 2015; De and Mukherjee, 2016; Yilmaz et al., 2021; Weng et al., 2022). In Japan, measurements were made on a university campus in hot weather, and scenarios were developed to design comfortable spaces by reducing the urban heat island effect by covering the entire ground with grass and implementing green roofs on buildings. As a result of increasing green areas, a cooling effect of between 0.24°C and 2.29°C was observed (Srivanit and Hokao, 2013). De Munck et al. (2013) emphasized that environmental factors lead to a temperature increase of between 0.5°C and 2.0°C in the streets of Paris. This suggests that if planning is done correctly, with proper guidance and road widths, and if plants are used, it may be possible to reverse this effect and cool the space. Measurements were made in six different parks in urban areas by Lin and Lin (2016) to examine their effect on the thermal comfort of the city. ENVI-met analyses showed that parks with good greenery and larger areas contributed more positively to the climate. In areas with asphalt surfaces, the temperature was found to be 6.0°C higher than in areas with grass surfaces. In addition, it was found that the use of vehicles, incorrect street orientation, and insufficient and incorrect open-green areas for air circulation lead to an environment that is 1.0 - 4.0°C warmer (Girgis et al., 2016). It has been determined through an analysis using ENVI-met in an urban park that the scenario involving trees and greenery lowered the temperature by 0.5°C compared to the current state (Teshnehdel et al., 2022). Measurements were made using the ENVI-met model by taking a neighborhood unit in Austria/Melbourne for the summer. Comparisons were made using different methods by looking at the street direction, street width, and floor heights in proportion, and their effects on thermal comfort were emphasized (Jamei and Rajagopalan, 2017; Menteş et al., 2023).

The Sky View Factor (SVF) is an important factor that affects the thermal comfort of pedestrians and the street in an urban street canyon. In urban space planning, factors such as street width, street orientation, and the heights of surrounding buildings (Qaid et al., 2016) have been examined for hot cities. Similar analyses have also been conducted for cold cities (Xu et al., 2018; Chen et al., 2018). Generally, these studies have determined that appropriate street design can affect thermal comfort and reduce temperature stress by creating air corridors. Needle-leaved or broad-leaved trees, shrubs, and spaces designed according to different planting techniques have been analyzed with SVF, and their effects on thermal comfort have been examined (Tan et al., 2017).

In this context, it has been determined that the city of Erzurum has extreme features in terms of thermal comfort compared to other cities, due to its high altitude and harsh and long winter season (Yilmaz et al., 2022a). It is considered highly important for people to be able to use outdoor spaces comfortably throughout the year. With the aim of developing scenarios to increase pedestrian comfort year-round, Haydar Aliyev Street in Şükrü Paşa Neighborhood, which serves as both a park and pedestrian axis, located in the new development axis of Erzurum City, was Chosen. Different design scenarios were analyzed with ENVI-met to determine the most suitable design criteria. the results obtained are expected to be a valuable resource as they will be transferred to physical planning decisions prepared by local governments.

2. Material and Methods

2.1. Material

This research was conducted in the Şükrü Paşa neighborhood located in the north of the city of Erzurum, which is located in the Eastern Anatolia region. The Haydar Aliyev Park, located on Azerbaijan Boulevard, which is used as both a pedestrian axis and a park, was preferred as the study area. The area is approximately 2.8 km away from the city center and is surrounded by 5-story buildings. The park has a 2.80 m median strip in the middle of the two-lane road, approximately 20.4 m of road and green space on both sides of the traffic lane, and 4.80m pedestrian paths. The location map of the study area is given in Figure 1. The meteorological station established in this area is located at 39°55'29.26" N latitude, 41°16'2.65" E longitude, and an altitude of 1830 m.

Obtaining Meteorological Data on Site: In the study, a "Vantage Pro 2 plus" device was used to record microclimate data within the urban space. The meteorological data measurement device was mounted inside a 120x120 cm protected cage structure on Haydar Aliyev Avenue, next to the municipal and security unit. It was mounted on a metal rod 1.5 m high from the ground and placed on the ground (Morakinyo et al., 2019). Calibration was performed by the manufacturer and a meteorological engineer (within the scope of the TÜBİTAK 1001-TOVAG 119O479 project) (Figure 1). The recording device of the meteorological station was placed in a special security booth that provides security of the park and the electricity distribution company located within the study area. The meteorological station established in 2020 in the study area records the data on an hourly basis. 48-hour data representing hot and cold days recorded within the year 2021 were obtained from the collected data. 24 hours of these data were used in the ENVI-met analysis. The data obtained from the field included hourly air temperature (Ta-°C), humidity (RH -%), cloudiness (Octas), wind speed (m s^{-1}), and direction, which were processed in the software model.

Proposed Different Landscape Scenarios: Due to the fact that the workspace serves both as a park and a pedestrian path, there is a high demand for its use. Therefore, different landscape scenarios have been prepared in an area of 40250 m^2, including pergolas as landscape elements. The scenarios prepared for analysis are as follows: Firstly, an analysis of the current situation was conducted.

• A scenario with soil surface covering (10 000 m^2 throughout the space, covering 25% of it) was prepared.

• A scenario with grass surface covering (the entire surface covered with grass, with 10 000 m^2 of grass-covered area covering 25% of the space) was created.

• A scenario for increasing the tree-shrub ratio by 20% throughout the space; plant species commonly found in the region (*Pinus sylvestris* L. "Scots pine," *Betula verrucosa* L. (birch), *Ulmus glabra, Fraxinus excelsior, Cornus alba* "Siberian") were preferred. Scenario analyses were applied by increasing the number of existing trees by 20%.

• A scenario with a designed water surface within the ground (200 m^2 water surface) was planned.

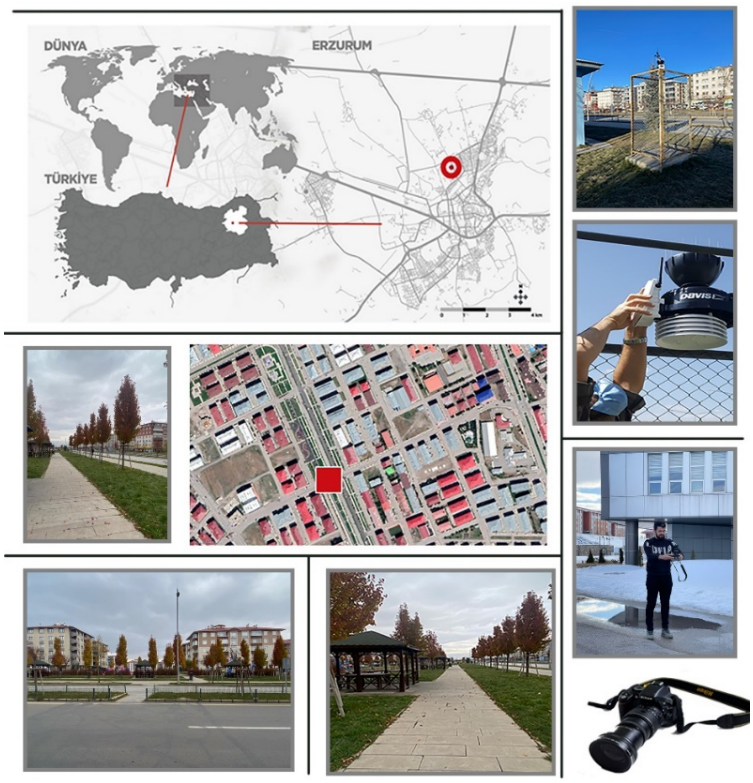

Figure 1. Haydar Aliyev Street study area location map (red square device location "Vantage Pro 2 Plus".

2.2. Methodology

Overview of the ENVI-met model: The ENVI-met BIO+ Science Software computer model is used to generate possible scenarios for the better planning of urban spaces in terms of climate-focused thermal comfort. Developed by Bruse and Fleer (1998), this model allows the use of climate data in planning, producing simulations for thermal comfort in various settings, from regional planning to urban planning and even alternative designs for a house garden. In urban planning, simulations can be conducted by changing the position of buildings, the type of plant species, street orientation, building height, roof gardens, or alterations in surface materials. The different scenarios produced can be analyzed using the ENVI-met + BIO Science model, which is purchased under the TÜBİTAK project for future simulations. This study had a few scenarios that were specifically designed for the summer and presented at a symposium. However, the accuracy analysis comparing the measured data and simulations was not conducted during the symposium presentation. After the symposium, these analyses were learned and simulations were re-performed by evaluating the winter version as well (Yilmaz et al., 2022b). The software, developed by Bruse (2022), can provide up to 250 grids for a single structure, with a resolution between 0.5 and 10 meters, and can perform simulations for surface air in small-scale atmospheres in urban areas, using 24-hour data. The hourly data used in the analyses can be either daily meteorological values or 24-hour data containing averages (Bruse, 2022; Guo et al., 2023). The study utilized a model with a horizontal resolution of 0.5-5 m and spatial microclimatic parameters of 2.00x2.00x2.00 m and an area of 175 m x 230 m x 36 m (Table 1). ENVI-met software is based on fluid mechanics and thermodynamic theories (Zhang et al., 2022), and is widely used by planning and landscape architecture researchers to improve thermal comfort and reduce the urban heat island effect (Wang et al., 2019). According to ENVI-met software, a portion of the study area covering 40250 m^2 was included within the measurement station boundaries. The measurement device should be located

within the study area for ENVI-met analyses, as this model simulates the area within a three-dimensional box that includes the ceiling, floor, and height (Bruse, 2022; Guo et al., 2023).

Table 1. ENVI-met model input data for winter and summer in 2021

Location	**Haydar Aliyev Street**	
Climate Type	Mountain Ecosystem	
Simulation Time	January and August	
Total Simulation Time	1st scenario (24 hours)	
Spatial Resolution	2m x 2m x 2m	
Field Size	175m x 230m x 36m	
	Model Angle	
	22.01.2021	29.08.2021
Basic Meteorological Input	Unshaded	Unshaded
Wind speed (m/s)	0.20	1.15
Wind direction	242,81 °C	176,25 °C
24-hour Air Temperature	+	+
24-hour Relative Humidity	+	+
Cloud cover (Octas)	0	0
Mina air temperature (Ta-°C) /h	-21,5 °C / 07:00	17,7 °C / 05:00
Max air temperature (Ta-°C) /h	-9,7 °C / 16:00	32,7 °C / 17:00
Min humidity (%)	% 65 / 16:00	%14 / 17:00
Max humidity (%)	% 86 / 05:00	%54 / 06:00
Sky View Factor (SVF)	Clear	Clear
Street and pedestrian road	Asphalt - Cement – Concrete – Granite – Grass - Soil	

Sky View Factor (SVF): The amount of solar radiation that affects any point depending on the settlement geometry is determined by a parameter called Sky View Factor (SVF). This value is calculated using the Rayman Pro 2.1 program and is expressed as a number between 0 and 1. In street canyons, as the value approaches 1, the space is wider, and as it approaches 0, the space becomes narrower. SVF is commonly used to prepare maps of thermal comfort streets, sidewalks, living spaces, and tree cover (Gülten, 2007; Algeciras et al., 2016; Li et al., 2020).

3. Results and Discussion

The thermal comfort of Haydar Aliyev Avenue, located in a densely populated modern residential area to the north of Erzurum city center, has been analyzed. This area stands out for its planned development on a slightly sloping area above agricultural land. The area consists of residential buildings along the road, with a ground plus five-floor structure on the front side and a ground plus seven-floor structure on the back side of the road. Each residential structure has green areas within its own parcel boundaries as per setback distances. The station is installed in an area suitable for the dominant detached and block construction styles prevalent in the Şükrü Paşa district. The residential areas are planned for balanced open and closed spaces with green areas and are planned for urban development. The street pattern is oriented in the southeast-northwest direction (TÜBİTAK 1st Development Report; Figure 2). SVF values above 0.9 were obtained for the main avenue and its immediate surroundings, indicating high openness and sky view factor in terms of SVF. In the backstreets, although not in many places, the value was found to drop below 0.5 (Figure 2) in terms of SVF.

ENVI-met Model Validation: The accuracy analyses of August 29, 2021 and January 22, 2021 were performed to represent hot and cold months from all data recorded within the study area. For the accuracy analysis of ENVI-met, 24 hours of 48 hours taken during summer and winter were used (Tables 2 and 3).

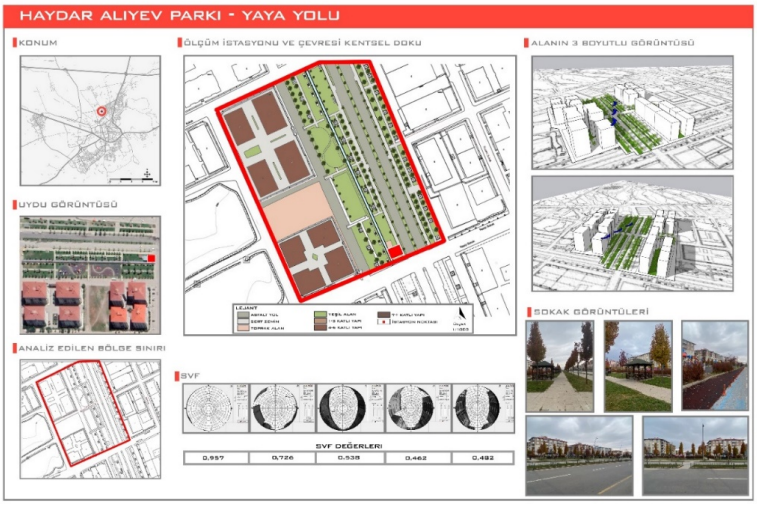

Figure 2. Haydar Aliyev Park and pedestrian road spatial texture analysis.

The temperature of the measurement days is one of the important variables used to verify the performance of the model. Seven statistical measures were used to calibrate the predicted (*P*) and observed (*O*) data of the street in the study area to evaluate the performance of the model. The statistical measures used are the determination coefficient (*R2*), efficiency coefficient (*E*), mean bias error (*MBE*), mean absolute error (MAE), root mean square error (*RMSE*), agreement index (*d*), and productivity coefficient (*E*). Agreement index (*d*) is a commonly used goodness-of-fit measure to evaluate model performance and is considered better than the determination coefficient (*R2*) (Qaid et al., 2016; Battista et al., 2016). Root-mean-square error (*RMSE*) and mean bias error (*MBE*) were evaluated regarding environmental prediction models. Simulation accuracy includes Willmott's (1982) agreement index (*d*). The accuracy of calculations depends largely on the grid size, model details, and input parameters. In the study, the calibration accuracy between the measured values and simulation analyses is given by the value of (*d*) and (*RMSE*). A value of (*d*) approaching 1 indicates that the simulation results are reliable (Qaid and Ossen, 2015; Yilmaz et al., 2021). *RMSE* is a measure that compares different prediction errors in the data set and indicates accuracy, showing that there are fewer errors in the prediction as the value decreases (Battista et al., 2016; Yavaş and Yilmaz, 2019).

Meanings of Abbreviations in the formula (Battista et al., 2016):
d : Index of agreement [–]
MAE : Mean absolute error [–]
MBE : Mean bias error [–]
ND : Number of analyzed data [–]
$\bar{O}$: Mean of the observed variable
Oj : Observed variables for each instant *j*
Pj : Model-predicted variables for each instant *j*

$$d = 1 - \left[\frac{\sum_{j=1}^{N_D}\left[\left(P_j - \bar{O}\right) - \left(O_j - \bar{O}\right)\right]^2}{\sum_{j=1}^{N_D}\left(\left|P_j - \bar{O}\right| + \left|O_j - \bar{O}\right|\right)^2}\right] \quad (1)$$

$$MBE = \frac{\sum_{j=1}^{N_D}\left(P_j - O_j\right)}{N_D} \quad (2)$$

$$MAE = \frac{\sum_{j=1}^{N_D}\left|P_j - O_j\right|}{N_D} \quad (3)$$

When the measured and simulated air temperature data for the current situation were evaluated for the summer months, the R^2 value was found to be 0.9897. The agreement index (*d*), which determines

the accuracy of the data, was 0.99 (Figure 3a), indicating that the data is reliable as it is close to 1. The root mean square error (*RMSE*) is an indicator used to measure the inconsistency between the values estimated by the model and the actual observed values (Yu et al., 2023). The result of 0.7323 obtained in the study indicates the accuracy of the analysis, as the value is small, indicating that the model simulation is accurate (Figure 3a). In the accuracy analysis conducted for the winter month data, the (*d*) value was calculated as 0.63 and the (*RMSE*) value was 3.2986. The reliability of the simulation analyses was found to be lower for the summer months compared to the winter months (Figure 3b). Based on the above analysis, software validation is statistically good and can be used for this study.

Analysis of ENVI-met Proposed Scenarios: Different landscape design scenarios were analyzed to provide outdoor thermal comfort in all seasons for urban residents to spend time in outdoor spaces. Studies in this field have shown that when landscape designs are created in harmony with the natural structure of the area, thermal comfort in the space can be improved (Jamei and Rajagopalan, 2017; Morakinyo et al., 2019; Yucekaya and Uslu, 2020; Lai et al., 2022; Okumuş and Terzi, 2022; Yu et al., 2023). It has been determined that the right decisions regarding land use can be taken with the right planning (Şatır and Berberoğlu, 2021). For this purpose, four different landscape scenarios were analyzed using the ENVI-met BIO+ Science model for summer and winter, along with the current situation, and the obtained data are presented in Table 4. Simulation visuals for summer and winter are presented in Figures 4 and 5, respectively.

Table 2. Meteorological data of 29 August 2021 (summer day)

	Time	**Observed air temperature (°C)**	**Simulated air temp. (°C)**	**Difference**	**Difference square**	**The root mean square error *(RMSE)***	**Index of agreement (*d*)**
29.08.2021	00.00	22.8	22.5	-0.3	0.09	0.732	0.99
	01.00	22.8	22.6	-0.16	0.025		
	02.00	20.2	20.8	0.6	0.36		
	03.00	20.3	20.6	0.3	0.12		
	04.00	21.4	21.3	-0.03	0.001		
	05.00	17.7	18.8	1.1	1..3		
	06.00	16.9	17.9	1.08	1.1		
	07.00	18.9	19.3	0.4	0.1		
	08.00	21.8	21.8	0.0	0.0		
	09.00	25.1	24.7	-0.3	0.10		
	10.00	25.8	25.9	0.1	0.01		
	11.00	27.9	27.4	-0.4	0.16		
	12.00	29.3	28.8	-0.4	0.21		
	13.00	31.6	30.4	-1.1	1.25		
	14.00	31.8	31.1	-0.6	0.42		
	15.00	32.6	31.7	-0.8	0.75		
	16.00	31.5	31.1	-0.3	0.09		
	17.00	32.7	31.3	-1.3	1.89		
	18.00	30.2	29.9	-0.2	0.07		
	19.00	28.2	28.3	0.1	0.03		
	20.00	27.4	27.4	0.0	0.0		
	21.00	24.4	25.3	0.9	0.83		
	22.00	22.1	23.4	1.3	1.75		
	23.00	20.8	22.2	1.4	1.96		

Table 3. Meteorological data of 22 January 2021 (winter day)

	Time	Observed air temperature (°C)	Simulated air temp. (°C)	Difference	Difference square	The root mean square error (*RMSE*)	Index of agreement (*d*)
22.01.2021	00.00	-17.1	-13.5	3.6	12.9	3.298	0.63
	01.00	-18.0	-13.6	4.3	19.1		
	02.00	-18.6	-14.1	4.4	19.4		
	03.00	-18.4	-14.4	3.9	15.2		
	04.00	-19.8	-14.8	4.9	24.6		
	05.00	-19.9	-15.1	4.8	23.1		
	06.00	-19.6	-15.2	4.3	19.1		
	07.00	-21.5	-15.5	5.9	35.8		
	08.00	-21.4	-15.7	5.6	32.1		
	09.00	-20.8	-15.8	4.9	24.8		
	10.00	-17.6	-15.4	2.1	4.6		
	11.00	-14.2	-14.5	-0.3	0.1		
	12.00	-14.3	-14.0	0.2	0.1		
	13.00	-12.8	-13.5	-0.7	0.5		
	14.00	-10.1	-12.6	-2.5	6.3		
	15.00	-10.5	-12.3	-1.8	3.3		
	16.00	-9.7	-12.7	-2.3	5.5		
	17.00	-12.0	-12.4	-0.4	0.2		
	18.00	-14.2	-13.1	1.1	1.1		
	19.00	-14.5	-13.4	1.1	1.2		
	20.00	-14.6	-13.5	1.0	1.1		
	21.00	-15.3	-13.7	1.5	2.4		
	22.00	-15.8	-13.9	1.8	3.5		
	23.00	-16.2	-14.1	2.1	4.3		

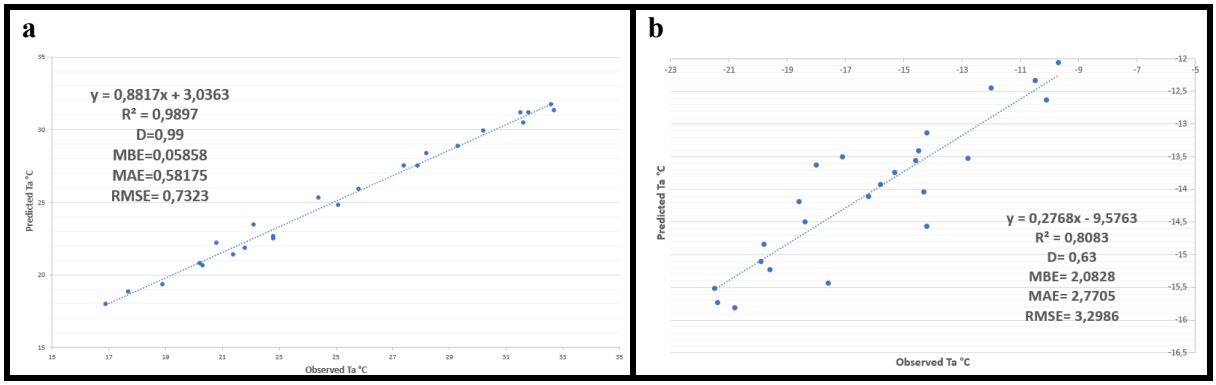

Figure 3. The observed (O) and the predicted (P) air temperature in summer (a) and winter (b).

In the analysis of the current situation, it was determined that there were no significant differences among the scenarios for the summer season. One of the important reasons for this is that the area has a dense and rich green area structure. The current situation was found to be 0.2°C warmer than the green area scenario for the summer season. In the summer season analysis, the scenario with the highest temperature of 30.9°C according to the mean values was found to be the scenario where green areas were increased. The analysis using ENVI-met showed that areas with dense vegetation had lower temperatures compared to other areas. In the summer analysis, the current condition was found to be only 0.2°C warmer than the green area scenario, with no significant differences among the scenarios. The scenario with increased greenery was found to be the coolest, with an average temperature of 30.9°C. Furthermore, it was found that areas with more plant material were cooler compared to other areas. In the summer analysis, the coolest area was found to be the one with the most vegetation cover.

In a study conducted on a city park using ENVI-met, it was found that the scenario with trees and green areas lowered the temperature by 0.5°C compared to the current condition. In the summer analysis, the soil scenario had the highest average temperature at 31.6°C and the lowest humidity at 20.2%. Similar results have been confirmed in various studies, which have identified the causes of differences in temperature, humidity, wind, and thermal comfort between areas with and without vegetation. These include the conversion of surface radiation into latent heat due to soil moisture, shade created by tree leaves, and the evapotranspiration effect of plants. For the winter months, the average temperature was found to be 1.3°C warmer on grass surfaces compared to the current condition. The relative humidity was found to be 107.5% in the grass scenario compared to 113.5% in the current condition.

Table 4. The temperature (°C) values of the scenarios for summer and winter with ENVI-met are as follows

Summer			
Scenarios / Temp. (°C)	**Min. temp. (°C)**	**Max. temp. (°C)**	**Mean temp. (°C)**
Current situation	29.2	33.0	31.1
Soil covered surface	29.9	33.3	31.6
Grass covered surface	29.8	33.2	31.5
Increasing the tree-shrub ratio by 20%	29.1	32.9	30.9
Water surface	29.2	33.0	31.1
Scenarios / Humidity (%)	**Min. humidity (%)**	**Max. humidity (%)**	**Mean humidity (%)**
Current situation	16.4	27.6	22.0
Soil covered surface	16.3	24.2	20.2
Grass covered surface	16.2	24.8	20.5
Increasing the tree-shrub ratio by 20%	16.4	28.0	22.2
Water surface	16.4	27.6	22.0
Scenarios / Wind	**Min. wind speed ($m\ s^{-1}$)**	**Max. wind change ratio (%)**	**Mean wind change ratio (%)**
Current situation	0.5	157.3	78.9
Soil covered surface	1.6	182.1	91.85
Grass covered surface	1.5	177.9	89.7
Increasing the tree-shrub ratio by 20%	0.5	156.9	78.7
Water surface	0.5	157.3	78.9
Winter			
Scenarios / Temp. (°C)	**Min. temp. (°C)**	**Max. temp. (°C)**	**Mean temp. (°C)**
Current situation	29.2	33.0	31.1
Soil covered surface	29.9	33.3	31.6
Grass covered surface	29.8	33.2	31.5
Increasing the tree-shrub ratio by 20%	29.1	32.9	30.9
Water surface	29.2	33.0	31.1
Scenarios / Humidity (%)	**Min. hum. (%)**	**Max. hum. (%)**	**Mean hum. (%)**
Current situation	16.4	27.6	22.0
Soil covered surface	16.3	24.2	20.2
Grass covered surface	16.2	24.8	20.5
Increasing the tree-shrub ratio by 20%	16.4	28.0	22.2
Water surface	16.4	27.6	22.0
Scenarios / Wind	**Min. wind speed ($m\ s^{-1}$)**	**Max. wind change ratio (%)**	**Mean wind change ratio (%)**
Current situation	0.5	157.3	78.9
Soil covered surface	1.6	182.1	91.85
Grass covered surface	1.5	177.9	89.7
Increasing the tree-shrub ratio by 20%	0.5	156.9	78.7
Water surface	0.5	157.3	78.9

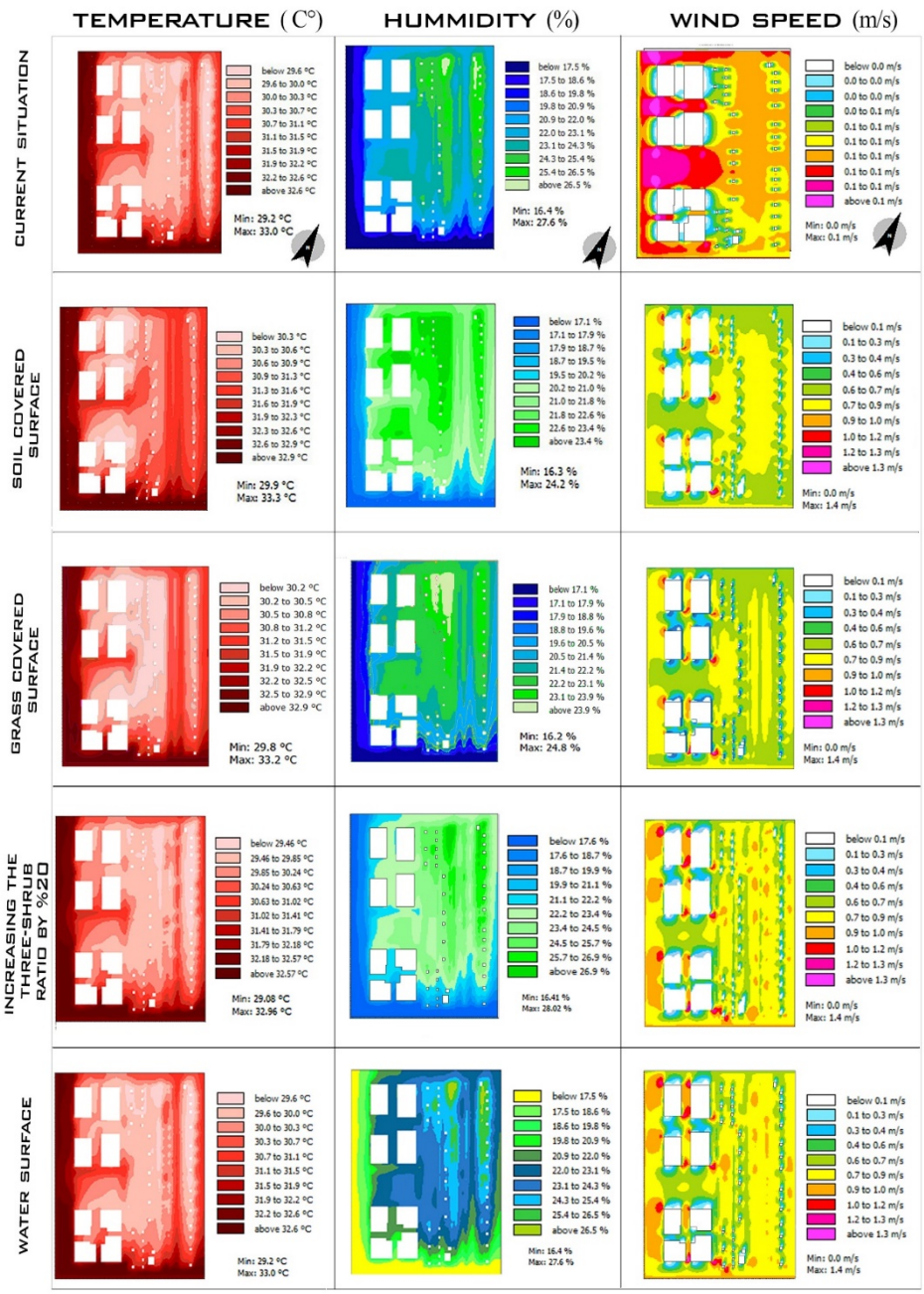

Figure 4. ENVI-met analysis for summer proposed scenarios.

According to the scenario-based analysis provided in Figure 6, it has been determined that the average wind speed change in the region with a high density of plant materials during the summer wind analysis has the lowest wind speed change with 78.7%. Similar analyses have shown that plants reduce wind speed by blocking wind circulation when they are not planted in a certain order (Vogel, 1989; Yilmaz et al., 2017; Chan and Chau, 2021; Orhan et al., 2022). In the winter analysis, the highest wind speed change rate in wind data was measured in the grass scenario with 227.9%, while the lowest change was determined in the soil scenario with 136.8%. This is due to the absence of any obstacles limiting wind movement in both scenarios. In the winter analysis, significant differences in the wind were also found between the current situation and the grass scenario, and it was determined that wind speed increased.

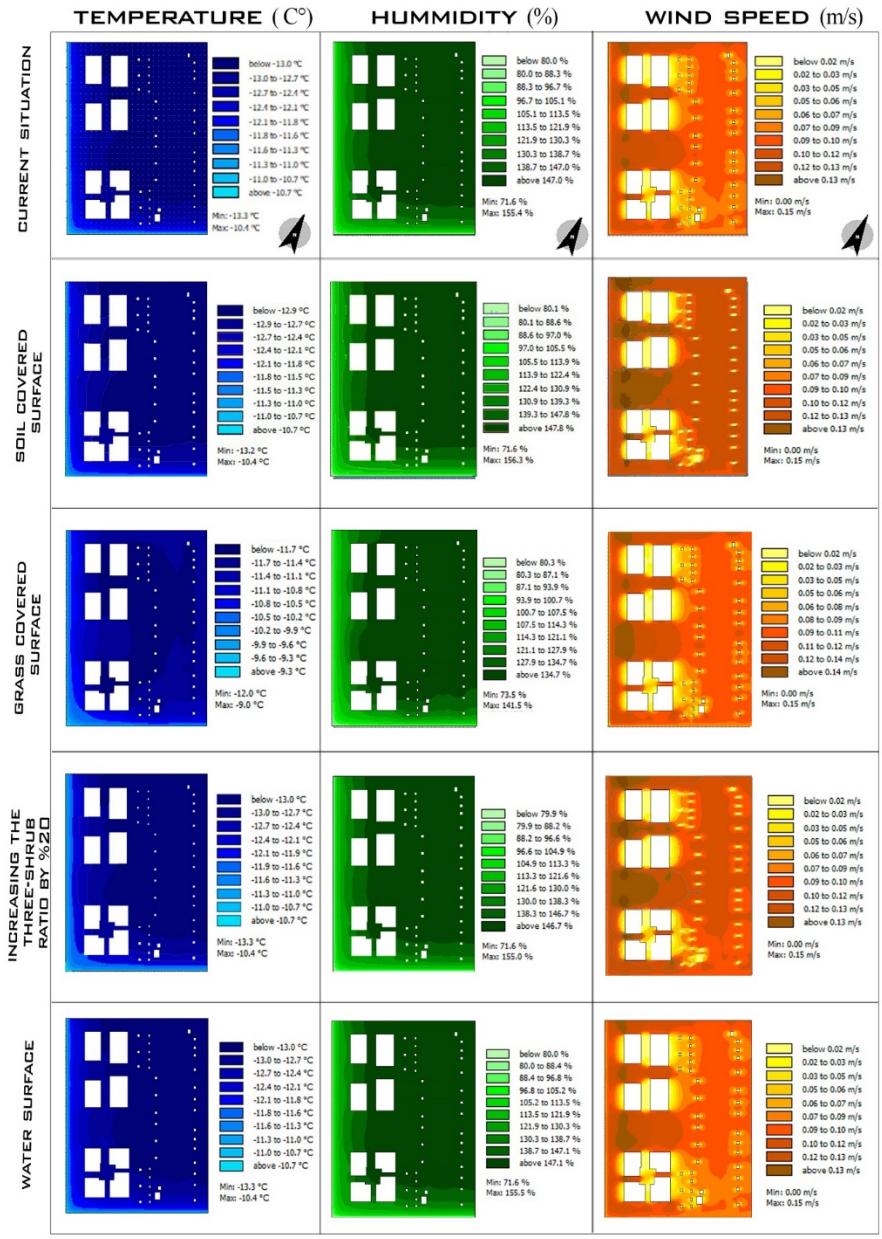

Figure 5. ENVI-met analysis for winter proposed scenarios.

The current wind speed change rate is 70.5%, while the change rate in the grass scenario has increased to 114.6%. In a study conducted in a park in the city of Qinhuangdao in China, it was found that scenarios prepared compared to the current situation improved the thermal comfort of the environment. According to the ENVI-met analysis conducted in this study, the temperature, relative humidity, and wind speed were 31.1°C, 49.70%, and 2.3 m/s, respectively, in the current situation, while in the scenario analysis, these values were improved to 29.7°C, 51.64%, and 1.0 m/s, respectively (Lai et al., 2022).

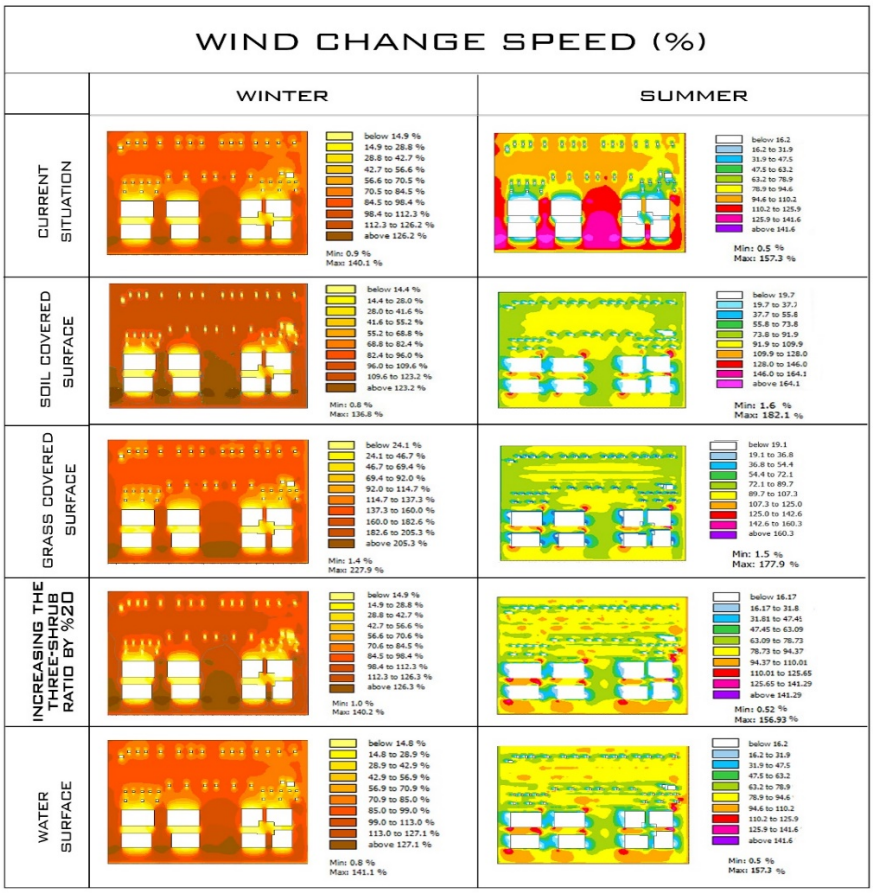

Figure 6. Wind change speed visualization with ENVI-met analysis in proposed scenarios.

In the winter scenario, it has been determined that the change in wind speed increases by an average of 0.4% in the water scenario. In the water scenario, it was observed that the temperature of the decorative pool was 0.7°C cooler than the soil scenario in summer analyzes. The cooling effect of the water has also been confirmed during the summer months (Yilmaz et al., 2021), and it has been found that the cooling effect of the water is also related to the size of the water surface (Wang et al., 2018). In a study, it was determined that the cooling effect of a large single water surface is greater than that of a fragmented water surface (Yücekaya et al., 2022). In fact, in an ENVI-met analysis conducted in a park in Valladolid, Spain, it was calculated that the scenario with water and plants was 4.3°C cooler than the current situation (Alves et al., 2022). Again, a cooling effect of between 0.8 and 1.3°C was determined in the water scenario during the summer months (Xu et al., 2019).

However, some ENVI-met studies have reported that the software does not provide the desired result in wind analysis when the wind speed is less than 2.0 m s^{-1} (Song et al., 2014; Acero and Arrizabalaga, 2018). It has been found that humidity and wind speed yield similar results in some scenarios and do not create significant differences. In fact, in a study, it was determined that water surfaces do not create a significant difference in wind speed up to a certain size (Yücekaya et al., 2022). In the winter scenario, there is no significant change in temperature and humidity, while there is a 0.4 m/s increase in average wind speed compared to the current situation. When scenarios were prepared by increasing the vegetation ratio by 20%, it was observed that the humidity in the region where the vegetation materials were dense decreased, and as a result, cool air was formed compared to the current situation analysis in summer analysis. When maximum humidity values in summer were examined, it was determined that the area with increased vegetation cover had the highest humidity with 28.0%, while the soil area had the lowest humidity with 24.2%. In summer analysis, it was observed that

humidity decreased compared to winter and varied in the region where vegetation material was dense. In the winter analysis of the scenario, it was observed that humidity increased compared to summer. At the same time, a homogeneous appearance was exhibited in the area where vegetation materials were dense. Similar results were found in all scenarios except for the grass scenario when winter humidity values were examined. In the winter scenario, the grass scenario had an average of 6% lower humidity compared to the current situation. According to the analysis results, it was observed that the vegetation cover or increased green area improved the thermal comfort of the environment more in summer compared to winter. Similarly, in similar academic studies, the effect of green areas on thermal comfort in winter was found to be less effective compared to summer (Gatto et al., 2020).

4. Conclusion

The results of this study have shown that improving outdoor thermal comfort can only be achieved by designing in accordance with the natural features of the area. Undoubtedly, plants are the most environmentally friendly landscape element for landscape architects. In the summer scenario where the number of plants was increased by 20%, it was determined that the temperature decreased by an average of 0.2ºC. The scenario with an increased number of trees was found to cool the environment in the summer and improve thermal comfort in the winter by reducing wind speed and allowing sunlight to enter the space. In the study area scenario, it was determined that the 200 m^2 water surface designed for summer and winter had no effect on temperature. However, the winter water surface scenario was found to increase wind speed by a maximum of 1.3 m s^{-1}. Low wind speed in the city center of Erzurum made it difficult to obtain accurate data in the analyses. In the future, it is aimed to produce locally specific scenarios for Erzurum to minimize the effects of climate change on the city. Ultimately, the data obtained will shed light on proposed new environmental planning schemes and will provide a foundation for creating climate-sensitive and thermally comfortable spaces.

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Research Article

Waterlogging Response of Lentil Cultivars Grown in Greenhouse Throughout The Early Vegetative and Recovery Period

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Recovery,
Waterlogging

Abstract: Under conditions of global climate change, the frequency of climate anomalies is predicted to increase. One of these issues is the problem of waterlogging in agricultural areas as a direct result of the unexpected and severe rainfall that has occurred over the last decades. In this study, the morphological responses to waterlogging stress and the recovery capacity of the lentil cultivars were investigated. A waterlogging stress study was conducted in small water pools with four different lentil varieties (Çağıl, Fırat 87, Kafkas and Kayı). Lentil cultivars were exposed to waterlogging stress for 7 and 14 days in the same greenhouse conditions. Measurements were taken at the end of 7 and 14 days of waterlogging (W-7 and W-14) and during the recovery period after flowering (R-7 and R-14). Lentil cultivars and plant traits were negatively affected by waterlogging stress applications (W-7 and W-14). According to the study, 14-day waterlogging had a greater impact on lentil cultivars than 7-day waterlogging. Total biomass measured after flowering at R-7 and R-14 waterlogging decreased by about 31.5% and 49.3%, respectively. Çağıl cultivar had a tolerance to waterlogging stress, but Kafkas cultivar was sensitive to waterlogging stress.

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1. Introduction

Legume crops are a cheap source of protein, minerals, and carbs. They also contain a wealth of secondary metabolites, or "bioactive substances," which have a positive impact on health by influencing cellular and physiological processes (Zeroual et al., 2022). Although the origin of the cultivated lentil (*Lens culinaris* Medik. subsp. *culinaris*) is in the southeastern region of Türkiye, it has a widespread area along the Mediterranean, from the north to Western Europe and from the south to Egypt and from the Nile to Ethiopia. This has led to the development of many lentil species that are adapted to different climates and soil conditions (Alo et al., 2011; Biçer et al., 2018). Canada is the world's leading producer of lentils, with 2.9 million tons, followed by India (1.2 million tons), Australia (0.5 million tons), and Türkiye (0.3 million tons) (FAO, 2022). The increase in various biotic and abiotic stresses in the expanding grain legumes cultivation areas in recent years creates fluctuations in yield and this brings along the problem of food security that threatens food safety. Lentil is a crop that suffers from abiotic stress factors such as drought, cold, frost, logging, salinity, and high temperature. These stress factors

affect the crop's physiological, biochemical, and molecular growth and development processes (Wiraguna et al., 2017). Food security is a major issue brought on by global climate change, but the nutritious and cheap lentil grain may serve an alternate role in addressing this issue.

Like animals, plants are what are known as obligatory aerobic beings, meaning they can't survive without oxygen. Some commercial cultivars cannot survive in waterlogged conditions due to the absence of aerenchyma tissue or adventitious roots in their roots. When water molecules replace air pores in soil cavities, waterlogging stress occurs in soils (Pan et al., 2021). Waterlogging is one of the most common causes of root zone oxygen deficiency. It causes more damage where there is poor drainage.

The waterlogging problem, which is commonly seen nowadays due to the changing global climate, deprives plant roots of oxygen, reducing or killing plant yields and threatening world food security. Increased porosity with the development of aerenchyma tissue in root systems facilitates oxygen movement from shoot to root by diffusion, increasing tolerance to waterlogging (Colmer, 2003). The plant's ability to survive and recover after waterlogging depends on the development stage, the duration of the waterlogging, number of days exposed to waterlogging, and the genotypic variance in waterlogging tolerance (Setter and Waters, 2003). Flooding problem is survived not only just along the coast where spring wheat crops are grown but also in other places where winter wheat is grown, because of sudden heavy rain and excessive irrigation in Türkiye (Ozseven and Genctan, 2018). Worldwide, approximately 10% of irrigated areas or approximately 22 million hectares, are exposed to waterlogging (Bowonder et al., 1987).

The most important reason why lentils cannot be grown on fertile lowlands in Türkiye is the stress of waterlogging or the accumulation of water in the soil. Because lentils are very susceptible to even short periods of waterlogging. Although excess water in lentil cultivation reduces the yield at any stage, the tolerance to waterlogging in the plant differs between and within species according to different growth stages. Poor lentil production is caused by the soils with poor drainage, such as fine textured with high clay and in subsoil compaction. The problem occurs seriously in long rainy weather conditions. It is impossible to achieve a high yield from lentils since they must be sown in stony, gravelly, and high-sloping lands that are lacking fertility (Malik et. al, 2015). Particularly waterlogging during germination can cause failed germination, late emergence, and prevention of root growth. In the flowering and pod-filling phase, a plant's ability to recover diminishes (Solaiman et al., 2007; Materne and Siddique, 2009; Wiraguna et al., 2017). In early flowering damage is most severe. In the damage, plants are stunted and turn yellow to red; wither, and eventually die. Root and collar portion rot are severe. In order to alleviate this problem, it is necessary to develop high-yielding, potential waterlogging resistant varieties and determine their genetic sources (Osman et al., 2013; Paudel et al., 2020).

The purpose of this research was to examine the impact that waterlogging caused on the development of different lentil cultivars when it was applied at varying times during the early stages of plant growth.

2. Material and Methods

This research was conducted under semi-controlled environments in the greenhouse of the Faculty of Agriculture, Dicle University.

The four lentil cultivars (Kayı, Kafkas,Fırat, and Çağıl) were used. Waterlogging treatments were given two times (7 days duration: W-7 and 14 days duration: W-14) in the begining of the 32nd day after seed emergence. The control plants were irrigated at the field capacity levels throughout the experiment.

The potting soil is clayey (79%), with low salt level (ds m^{-1} 0.85), slightly alkaline (pH 7.61), medium calcareous (6.43%), medium organic matter (2.88%), available phosphor (178.79 kg da^{-1}) and potassium (498.56 kg da^{-1}). Plastic pots were filled 1.76 kg with the soil. Eight seeds in each pot were sown to ensure good emergence on April 8, 2020, and after emergence, they were adjusted to four seeds per pot. Fertilization was applied 5.0 kg/da nitrogen and 9.0 kg/da phosphor by diluting with 30 ml/da water per pot before 5 days the waterlogging stress. The experiment arranged the randomized complete plots design in split plots in waterlogging treatments as the main factor and cultivars as subfactors with four replications.

Waterlogging treatments were initiated on the 32nd day of seed emergence. Plants were exposed to waterlogging during 7 (W-7) and 14 (W-14) days. The control plants were irrigated at the field capacity level throughout the experiment. Waterlogging stress was tested in a concrete brick-built pool that was filled to the pot's surface with water. It was covered with a thick plastic cover to prevent water leakage, and equipped with an electric air motor to assure the flow of oxygen.

The lentil plants to be subjected to waterlogging stress were cultivated under normal cultivation conditions for 32 days in pots. The pool was filled with water up to the surface of the pots. After 32 days, pots were transferred into the pool. At the end of the 7th and 14th days, all pots were removed from the poll and continued to be grown under normal conditions. After this stage, the plants were watered until the harvest. Plants were harvested 15 days after flowering dates.

Measurements were taken on W7 and W14 days of waterlogging and the fifteenth day (R7 and R14) of flowering. Plant height, root length, number of leaves per plant, plant diameter, above ground biomass, leaf weight, stem weight, fresh and dry root weight were measured. The dry weights of plant parts were determined after drying in an oven at 70 °C for 48 hours.

Cultivars and waterlogging treatments were tested using ANOVA. Data analyzed in the JMP statistical program (JMP Pro-13, SAS Institute).

3. Results and Discussion

The analysis of variance in two different waterlogging treatments for lentil cultivars is given in Table 1.

Table 1. Two-way ANOVA for all traits

Measurements after 7 days waterlogging													
Source of variation	**Df**	**PH (cm)**	**RL (cm)**	**FAGB (mg)**	**RFW (mg)**	**LFW (mg)**	**NLP $^{-1}$**	**SFW (mg)**	**TFB (mg)**	**LDW (mg)**	**SDW (mg)**	**RDW (mg)**	**TDB (mg)**
Cultivar (C)	3	71.25**	103.36*	151.00**	77.60	51.60**	14.70	17.80**	295.00**	2.21**	1.90**	1.39**	11.10**
W-7	1	52.57**	140.36**	2.60	422.00**	3.33	6.54	0.72	490.00**	0.80**	0.03	7.05**	13.70**
C*W-7	3	0.30	65.57*	4.60	56.00	7.20	5.82	1.21	60.20	0.12	0.16	2.16**	2.87*
Error	17	61.62	113.45	67.10	258.40	14.62	32.70	8.51	251.20	0.93	0.50	1.24	4.24
CV(%)		11.50	11.20	17.61	19.12	13.48	14.85	15.27	12.20	14.90	16.98	10.50	9.20
Measurements after 14 days waterlogging													
	Df	**PH (cm)**	**RL (cm)**	**FAGB (mg)**	**RFW (mg)**	**LFW (mg)**	**NLP $^{-1}$**	**SFW (mg)**	**TFB (mg)**	**LDW (mg)**	**SDW (mg)**	**RDW (mg)**	**TDB (mg)**
Cultivar (C)	3	163.90**	163.15*	47.00**	234.00**	44.40**	65.40**	18.30**	433.00**	2.90**	1.39**	10.02**	34.90**
W-14	1	38.93**	470.19**	56.00**	1782.00**	59.70**	59.04**	3.10	2473.00**	4.27**	0.00	14.70**	34.40**
C*W-14	3	6.57	55.89	49.00**	333.00**	21.80**	27.73	8.24	409.00**	0.91*	0.32	10.40**	17.20**
Error	17	46.36	203.32	51.40	140.00	15.80	60.85	16.78	183.00	1.18	0.75	3.31	9.36
CV(%)		10.50	16.60	19.90	15.40	19.77	21.20	23.60	11.90	20.70	20.80	22.95	17.42
Recovery measurements after flowering at 7 and 14 days waterlogging treatments													
	Df	**PH (cm)**	**LDW (mg)**	**SDW (mg)**	**RDW (mg)**	**TDB (mg)**	**PD (cm)**						
Cultivar (C)	3	1573.70**	3.10**	33.60**	7.20**	68.00**	0.22						
R	2	1075.40**	22.80**	39.90**	26.00**	236.00**	0.12						
C*R	6	2195.50**	12.00**	32.30**	9.50**	89.00**	0.41						
Error	33	491.20	6.15	11.06	1.94	19.40	0.95						
CV(%)		9.70	17.70	15.77	13.10	9.60	14.90						

W-7: Waterlogging 7 days, SV: Source of variation, Df: Degrees of freedom, R: Recover days: (R-0, R-7, R-14), PH: Plant Height, RL: Root Length, FAGB: Fresh Above Gound Biomass, RFW: Root Fresh Weight, LFW: Leaf Fresh Weight, NLP-1:Number of Leaves Plant-1, SFW: Stem Fresh Weight, TFB: Total Fresh Biomass, LDW: Leaf Dry Weight, SDW: Stem Dry Weight, RDW: Root Dry Weight, TDB: Total Dry Biomass, PD: Plant Diameter, Significant: $*P < 0.05$; $**P < 0.01$.

In the plants exposed to waterlogging for seven days, the differences among cultivars in all parameters except the number of leaves per plant were significant. The waterlogging application was significant for all traits except for the number of plant leaves and fresh above ground biomass, stem

fresh and dry weight, and number of leaves per plant. Cultivar x waterlogging application at the seventh day interaction was significant for root length, root dry weight, and total dry biomass.

In the plants exposed to waterlogging for 14 days, the differences among cultivars were significant for all parameters. The effect of waterlogging was significant on all properties except stem fresh and dry weight. Cultivar x waterlogging application at 14th day interaction was significant for all traits except for plant height, root length, number of leaves per plant, and stem fresh and dry weight.

The traits after the flowering time were evaluated as recovery in the control, 7th day and 14th day waterlogging treatments. The applications were called R0, R7, and R14, respectively. The analysis of variance for the recovery data reveals that cultivars, recovery, and cultivars x recovery interaction were significant for plant height, fresh and dry above ground biomass, stem, leaf and root dry weight (Table 1).

3.1. Measurements at 7 (W-7) and 14 days (W-14) waterlogging treatments

Plant height, root length, root fresh and dry weight, total fresh biomass, leaf dry weight, and total dry biomass decreased when lentil cultivars were subjected to seven days of waterlogging stress (W-7) in 32 days after sowing.

Compared to W-7, the effects of stress on the plant traits were increased when it was exposed to waterlogging for 14 days (W-14).

Plant height, root length, root fresh and dry weight, total fresh and dry biomass, and leaf dry weight were also adversely affected at W-14 compared to control and W-7. When the waterlogging stress duration was raised from 7 days to 14 days, the effect of stress often doubled. Similarly, Lake et al. (2021) stated that increasing the duration of waterlogging stress generates an increase in stress in specific features of the plant, which in turn causes a decline in plant growth.

Root weight characteristics affected the plant parts the most as a result of W-7 and W-14 applications, and significant losses occurred in the root area depending on the applications.The decrease in root fresh and dry weights was about 34-35% in W-7 and 55-60% in W-14, respectively (Table 2). Similar results were also reported by Yavas et al. (2012). Several metabolic processes, including respiration, are suppressed and ATP synthesis in anaerobic respiration is reduced when roots are deprived of oxygen as a result of waterlogging stress. Although this scenario primarily affects root development, it also has a negative impact on vegetative growth by limiting water and nutrient uptake (Mustroph ve ark., 2006; Mustroph ve ark., 2013).

Stem fresh and dry weights were not affected by W-7 and W-14 applications. The results showed that the plant through the stress gave priority to stem construction in the first developmental stages.

The significant losses for total fresh and dry biomass results from applications were determined, and the reductions were about 25% in W-7 and 40-50% in W-14, respectively.

Table 2. Investigated traits of seedling plants of lentil under control and 7 and 14 day waterlogging treatments

Traits	Control for 7 day waterlogging	7 day waterlogging	Loss at 7 day waterlogging (%)	Control for 14 day waterlogging	14 day waterlogging	Loss at 14 day waterlogging (%)
Plant height (cm)	18.29 a	15.33 b	16.20	17.31 a	14.76 b	14.70
Root length (cm)	25.75 a	20.91 b	18.80	26.01 a	17.16 b	34.00
Fresh above gound biomass (mg)	357.00 a	336.00 a	5.90	333.00 a	236.00 b	29.10
Root fresh weight (mg)	793.00 a	528.00 b	33.40	901.00 a	356.00 b	60.50
Leaf fresh weight (mg)	226.00 a	202.00 a	10.60	212.00 a	112.00 b	47.20
Number of leaves plant $^{-1}$	9.92 a	8.87 a	10.60	10.60 a	7.50 b	29.20
Stem fresh weight (mg)	135.00 a	146.00 a	-8.10	148.00 a	125.00 a	15.50
Total fresh biomass (mg)	1150.40 a	864.60 b	24.80	1235.00 a	593.00 b	52.00
Leaf dry weight (mg)	55.30 a	43.70 b	21.00	55.50 a	28.80 b	48.10
Stem dry weight (mg)	33.00 a	30.90 a	6.40	31.50 a	31.10 a	1.30
Root dry weight (mg)	101.30 a	67.00 b	33.90	89.30 a	39.70 b	55.50
Total dry biomass (mg)	189.80 a	141.80 b	25.30	175.90 a	100.10 b	43.10

In lentil plants exposed to waterlogging stress, the leaves fall from the lower leaves upwards, and the number of leaves and leaf weight were reduced. Moreover, the leaf losses were high in W-14. The rate of decrease in the total number of leaves raised from 10.6% in W-7 to 29.1% in W-14. The leaf

fresh and dry weights were more affected than the number of leaves by applications. This can be explained by the fact that the increase in the number of days the roots are without oxygen significantly affects the plant's growth potential. The effects of W-7 and W14 applications on cultivars were given in Fig. 1 and Fig 2. The Kafkas cultivar was negatively highly affected by W-7 for total fresh and dry weight. Kayı cultivar had the highest fresh and dry weight at W-7, W-14, and control conditions. However, Kayı cultivar lost unusually more dry and fresh weight in W-14 than the other cultivars, and it was unable to survive prolonged waterlogging.

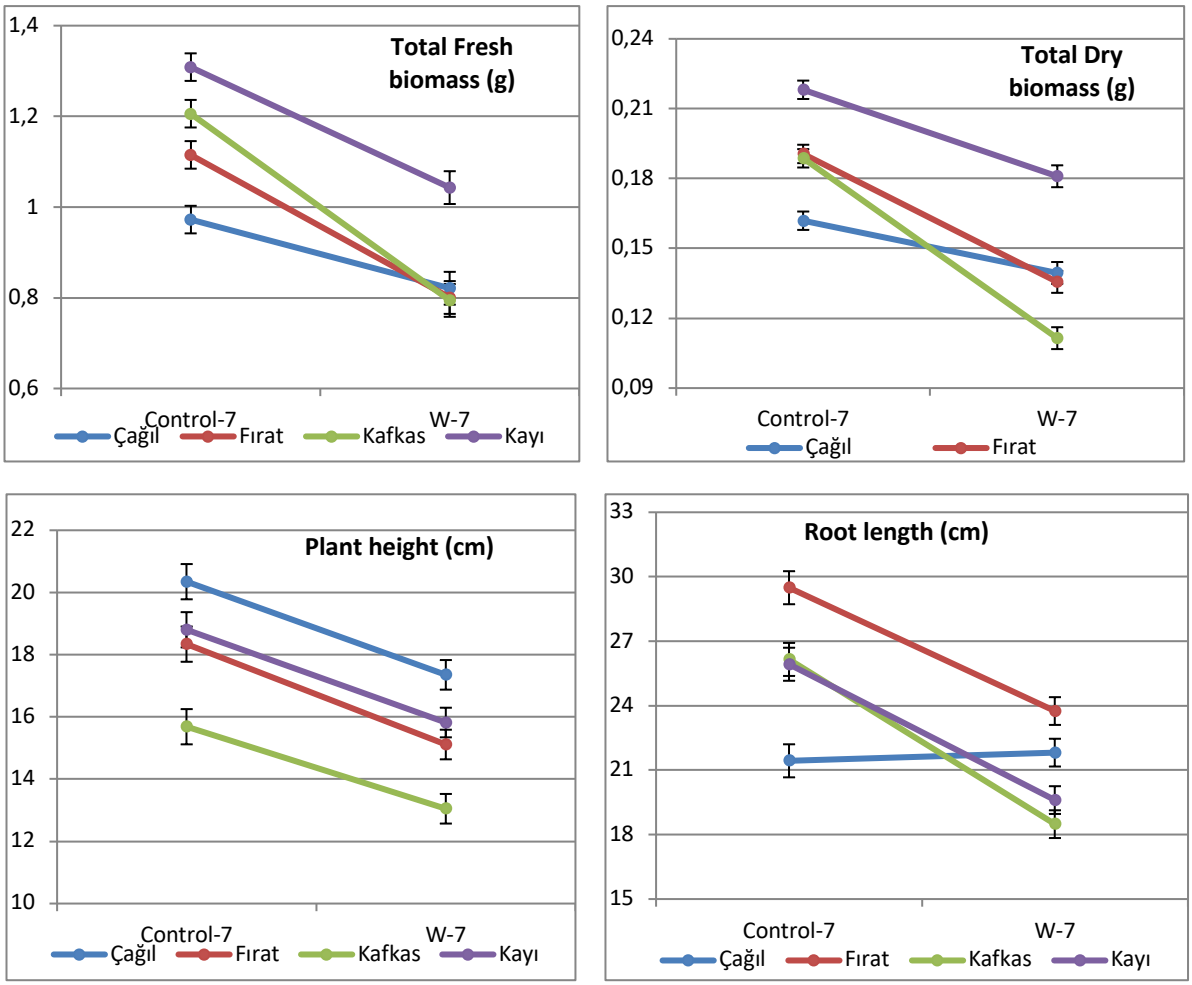

Figure 1. Total fresh biomass, total dry biomass, plant height, and root length in lentil genotypes at the end of 7 day waterlogging treatment. Vertical bars show ± SD of the mean.

Çağıl cultivar compared to other cultivars was low for total fresh and dry weight. However, it was proven to have a high tolerance, having the least amount of loss from waterlogging stress in W-7 and W-14. While plant height after W-14 stress was reduced by about the same amount in all lentil cultivars, plant height in the Çağıl cultivar was less affected (Figure 2). The root length loss of the Çağıl cultivar in W-7 and W-14 compared to other cultivars was low. It revealed that it may be a result of its overall tolerance.

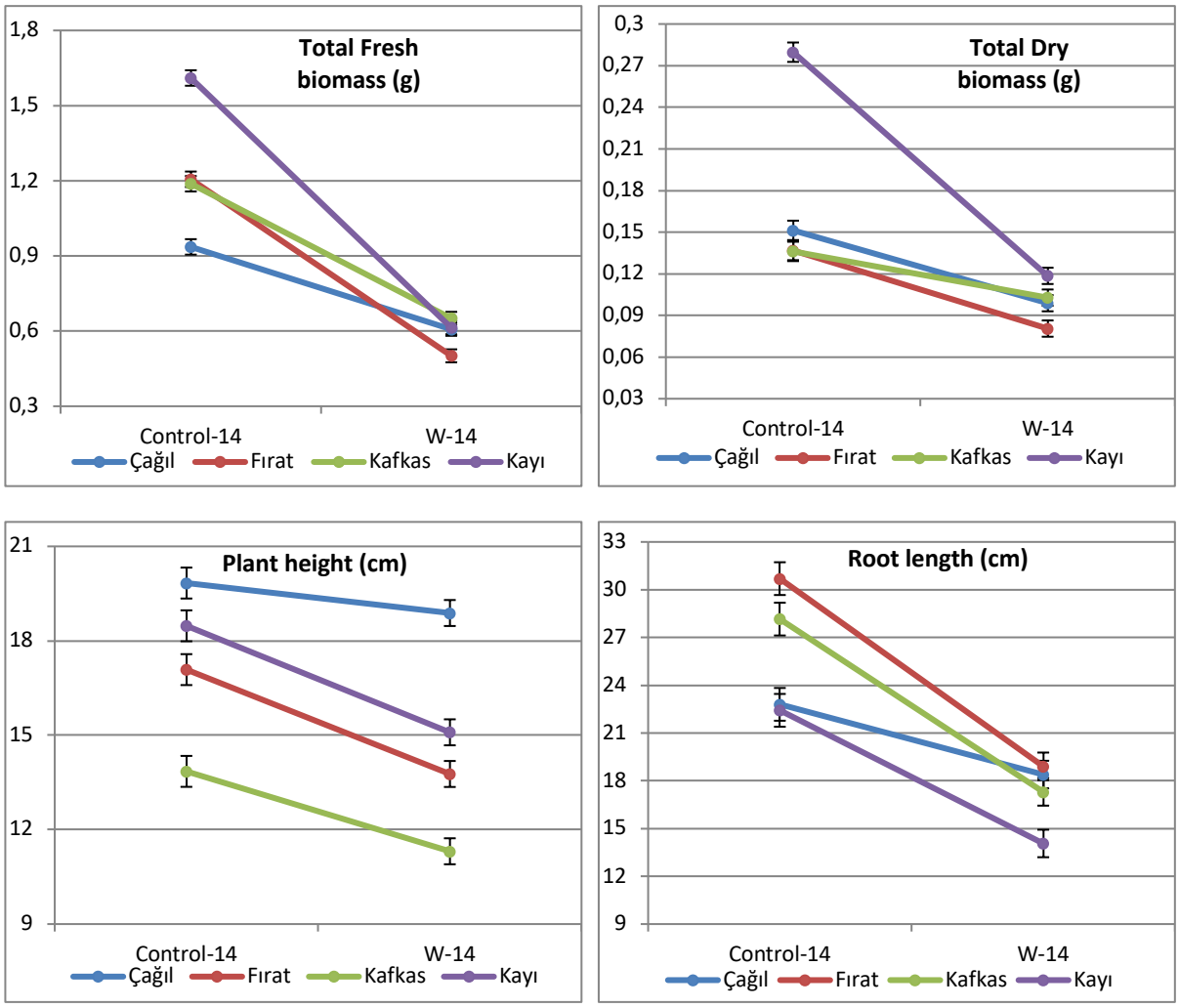

Figure 2. Total fresh biomass, total dry biomass, plant height, and root length in lentil genotypes at the end of 14 day waterlogging treatment. Vertical bars show ± standart deviation of mean

3. 2. Recovery measurements

The recovery capacity of lentil cultivars for all traits at the end of the treatment of waterlogging (W-7 and W-14) after the flowering period is given in Table 3. In the R-7 and R-14 recovery processes compared to the control were significantly reduced for all traits. In R-14, the recovery capacity of stem dry weight, root dry weight, and total dry biomass has deteriorated. Compared to the control, plant height was decreased by 18.9% in R-7 and 23.7% in R-14 (Table 3). Leaf dry weight decreased by 45.7% and 39.9%, respectively. Stem dry weight was not affected in the W-7 and W-14 applications compared to the control; however, it was reduced by 20.7% in R-7 and 46.9% in R-14. Root dry weight decreased in R-7 and R-14 applications, but it was as high as in W-7 and W-14 measurements. The root dry weight in R14 had the lowest recovery level of all parameters studied. The total dry biomass loss rate in the recovery period (R-7 and R-14) (31.5% and 49.3%, respectively) was higher than the loss rate in the W-7 and W-14 (25.3% and 43.1%, respectively). It showed that the healing process of the plant continued by getting worse (Prasanna and Rao, 2014). As a result, the plant metabolic activities and agronomic traits continue to deteriorate even after the waterlogging (W-7 and W-14) has ended.

Table 3. Investigated traits of lentil genotypes under control and 7 and 14 day duration waterlogging treatment followed by a recovery period until 15 days later of flowering

Traits	R-0	R-7	R-14	Loss at 7 day waterlogging (%)	Loss at 14 day waterlogging (%)
Plant height (cm)	46.20 a	37.50 b	35.80 b	18.90	23.70
Leaf Dry weight (mg)	107.50a	58.40 b	64.60 b	45.70	39.90
Stem Dry weight (mg)	150.20a	119.10 b	79.70 c	20.70	46.90
Root Dry weight (mg)	87.10a	58.75 b	30.56c	32.60	64.90
Total Dry biomass (mg)	344.90a	236.20 b	174.90 c	31.50	49.30
Plant Diameter (cm)	1.12	1.08	1.20	3.70	-7.20

R: Recover days: (R-0, R-7, R-14).

The recovery capacity of lentil cultivars at the end of the treatment of waterlogging (W-7 and W-14) after the flowering period were given in Figure 3.

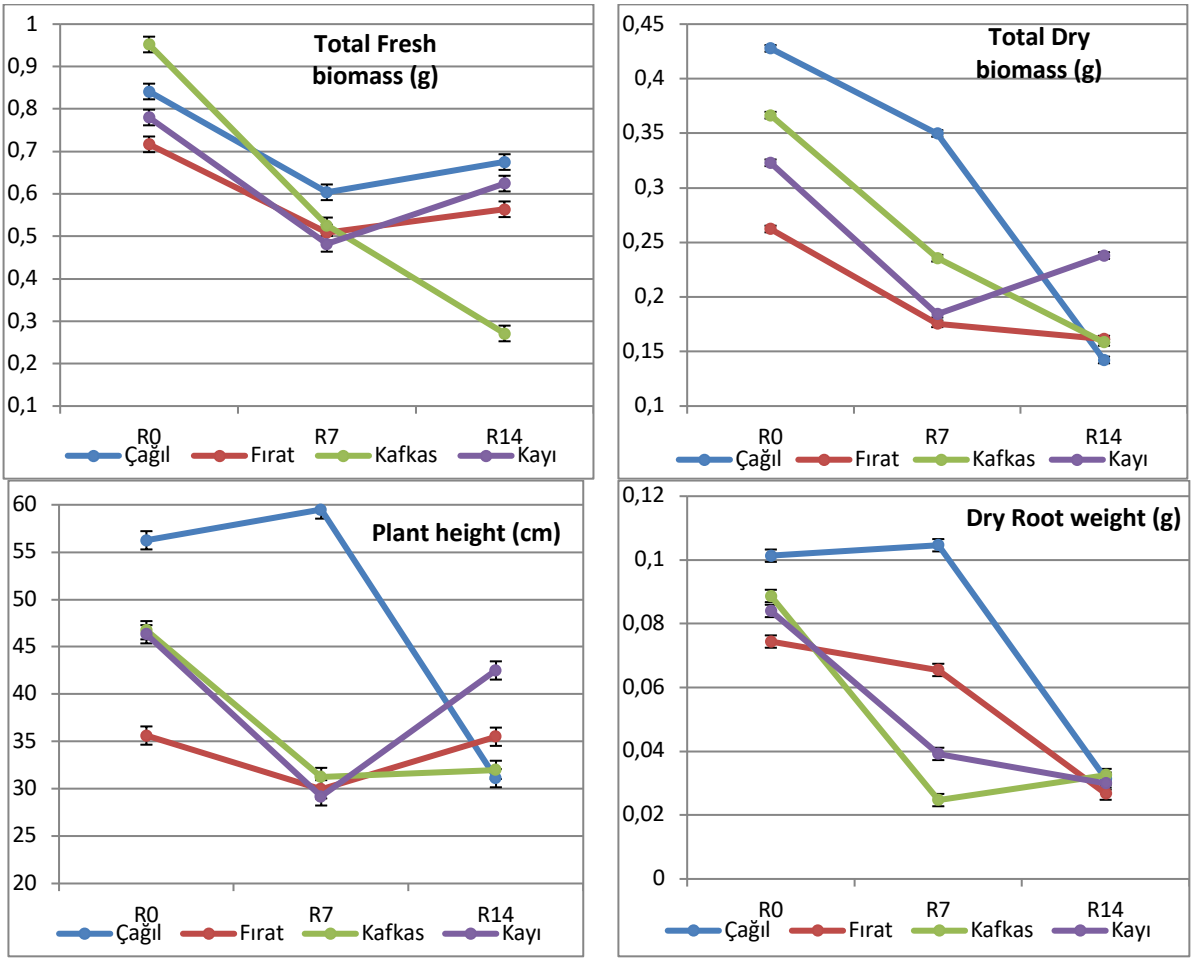

Figure 3. Total fresh biomass, total dry biomass, plant height, and dry root weight in lentil genotypes after waterlogging termination during recovery until 15 days after flowering, Vertical bars show ± standart deviation of mean.

Kafkas and Kayı cultivars for total fresh weight were more damaged under W-7 stress. In the W-14, the total fresh weight for all the cultivars except Kafkas was similar or slightly higher than in W-7. However, Kafkas cultivar was strongly negatively affected and lost more than 70% compared to the control (R-0). The total dry weight difference between cultivars was higher than the total fresh weight in R-0.

Kayı cultivar for all traits showed more loss in R-7 compared to other cultivars, but recovery in R-14 for the Kayı cultivar was high, unlike other cultivars.

Çağıl cultivar, which lost less in R-7 compared to other cultivars, lost excessive dry weight in R-14.

The waterlogging responses of lentil cultivars reveal that the Kafkas cultivar was sensitive to both waterlogging treatments. When compared to other cultivars, it might be possible to assert that Kayı had a higher tolerance to waterlogging conditions. In addition, the responses of cultivars to R-7 and R-14 treatments were different. For example, Çağıl was tolerating the R-7 application, but as the flooding continued, this situation changed in a decreasing direction. The tolerance or sensitivity to waterlogging of traits taken in the post-flowering stage (R-7 and R-14) were analyzed on the basis of cultivars. For this reason, it must be considered that the selection of resistant lentil cultivars in short-term flooding studies should be misleading, these results agree with Setter and Waters (2003).

Conclusion

To prevent major losses due to sudden flooding, which has grown increasingly common with global climate change, in lentil cultivation, it is vital to identify resistance mechanisms and resistant varieties. As the duration of waterlogging stress increases, the root zone remains oxygen-free for a longer time, preventing respiration and causing more damage to the plant. While the worst damage in lentils was particularly in roots and leaves, including all other vegetative parts. Recovery treatment in lentil increased the waterlogging damage as the stress duration (R-14) was extended. Lentil varieties respond differently to waterlogging stress and duration. The longer waterlogging duration caused more damage in lentil cultivars, and the cultivars that were able to resist short-term waterlogging weren't withstand long-term waterlogging. Also, it was not known that the responses of cultivars after waterlogging will not be the same as their responses in later development stages. This means that early detections might be misleading when trying to figure out which cultivars were tolerant or resistant. In this study, the Çağıl cultivar was generally tolerant to waterlogging, while the Kafkas cultivar was to be sensitive.

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Research Article

Effects of Data Augmentation Methods on YOLO v5s: Application of Deep Learning with Pytorch for Individual Cattle Identification

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Abstract: In this paper, we investigate the performance of the YOLO v5s (You Only Look Once) model for the identification of individual cattle in a cattle herd. The model is a popular method for real-time object detection, accuracy, and speed. However, since the videos obtained from the cattle herd consist of free space images, the number of frames in the data is unbalanced. This negatively affects the performance of the YOLOv5 model. First, we investigate the model performance on the unbalanced initial dataset obtained from raw images, then we stabilize the initial dataset using some data augmentation methods and obtain the model performance. Finally, we built the target detection model and achieved excellent model performance with an mAP (mean average precision) of 99.5% on the balanced dataset compared to the model on the unbalanced data (mAP of 95.8%). The experimental results show that YOLO v5s has a good potential for automatic cattle identification, but with the use of data augmentation methods, superior performance can be obtained from the model.

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1. Introduction

The increasing demand for animal products requires breeders to increase production without compromising animal welfare (Masebo et al., 2023). Therefore, it is important to solve on/off-farm management problems with less cost. Livestock farmers and producers are very concerned with the identification of their animals for management simplicity. It is known that most farms today use artificial farming methods and many of them utilize ear tags to count or identify the animals (Zhang et al., 2022). Traditional techniques for animal identification other than ear tags include microchips and Radio Frequency Identification (RFID) tags. All of these methods require direct contact and can cause varying degrees of injury to animals. Animal recognition and well-being evaluation utilizing non-invasive digital technology has received attention in agriculture in recent years, particularly for accuracy tracking (Dac et al., 2022; Zhang et al., 2022). Recently, a lot of work has been done in livestock management, including modern and intelligent methods using computer vision methods utilizing deep learning. Image processing and object detection have become very popular in these studies with the advancing graphics processing unit (GPU) technology. Object detection studies with computer vision are quite remarkable and have been extensively used in animal identification. Features such as body patterns, muzzle structure and posture are used to recognize individual farm animals. R-CNN object detection has been used to

detect the tail of a cow for body condition score (Huang et al., 2019) and to identify individual cattle (Andrew et al., 2017). Among the object detection methods, YOLO (You Only Look Once) is a remarkable and useful tool. The YOLO v5 algorithm has been used by different researchers for accurate identification of domestic cattle (Luo et al., 2022), cattle counting from UAV images (de Lima Weber et al., 2023), face identification in dairy cows (Dac et al., 2022), accurate and fast detection of goats (Zhang et al., 2022), and sheep behavior identification (Chen et al., 2022). Recent studies have shown that deep learning technologies that are non-invasive and do not affect animal welfare have the potential to provide ease of livestock identification and management (Bati and Ser, 2023; Subedi et al., 2023). In deep learning studies, unbalanced datasets are encountered, which affect network performance and are a chronic problem for almost every study. Especially in image classification or object detection studies in animal production, it is challenging to get the same amount of data such as images and videos for each class.

This study was designed to investigate the performance loss caused by unbalanced datasets in computer vision studies and to provide solutions. The rest of the paper, in which we use object detection from Holstein Friesian cattle images for individual cattle identification and improve the performance of the method with data augmentation methods, is arranged as follows; In the section two, the original dataset, the methodology used and experimental setup are presented. The third section presents the individual cattle identification results and the last two sections present the discussion and conclusion respectively.

2. Material and Methods

2.1. Original dataset

In this study, we used the FriesianCattle2017 (Andrew et al., 2016; Andrew et al., 2017) dataset, which consists of 940 RGB images of 89 different Holstein Friesian cattle. The data was captured in a real closed farm context, over a two-hour session, using a camera mounted on a walkway between pens and milking stations (Andrew et al., 2017). More detailed information on dataset acquisition can be found in Andrew et al. (2016) and Andrew et al. (2017). Sample frames of individuals in the dataset are presented in Figure 1.

Figure 1. Sample frames from the original dataset.

2.2. Deep neural architectures: YOLO v5s object detection algorithm

The YOLOv5 network model is one of the state-of-the-art object detection algorithms with high detection accuracy proposed by Glenn Jocher in 2020 (Jocher, 2020). The YOLO v5 network has the highest computational speed because the size of the weight file of the target detection network model is

small (Chen et al., 2022). This shows YOLO v5 is appropriate for applying real-time detection. We preferred the YOLO v5s network for cattle identification due to its advantages such as high detection accuracy, lightweight features, as well as detection speed (Yan et al., 2021). YOLO v5 network consists of four main components, namely the input, backbone (CSPDark net), neck(PANet), and head. Input terminal mainly includes data preprocessing, including mosaic data augmentation and adaptive image filling (Li et al., 2022). Once the image is input, it is aggregated in the backbone and generates image features at different image detail levels. It was based on the Cross-Stage-Partial-Network (CSPNet) (Jintasuttisak et al., 2022). The neck then combines the image features and passes them to the prediction layer. YOLO v5 uses the Path-Aggregation-Network (PANet) (Liu et al., 2018) in the model neck for extracting feature pyramids. The head estimates image features to create bounding boxes and predictive category (Chen et al., 2022; Jintasuttisak et al., 2022). The architecture of the YOLO v5 is presented in Figure 2.

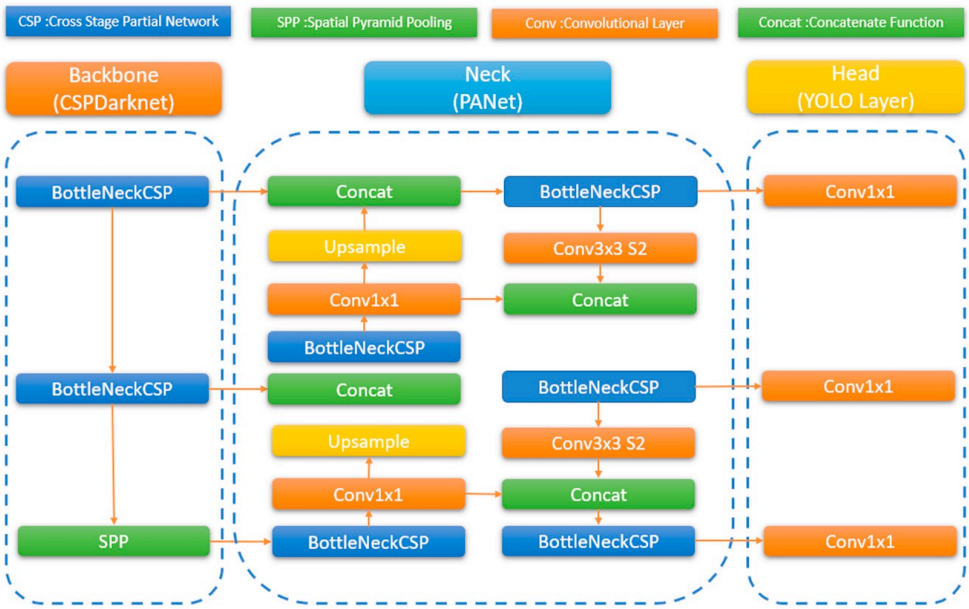

Figure 2. Architecture of the YOLO v5 (Egi et al., 2022).

2.2.1. Intersection over union (IoU)

This metric is used to find the match between each cattle's ground truth annotations and predicted bounding boxes to make a quantitative comparison of performance. As shown in Figure 3, we calculated the IoU as the area of the intersections between the predicted cattle bounding box and the ground truth divided by the area of the unions. The estimated bounding box with an IoU equal to or greater than 0.5 was considered the correct prediction of cattle. To evaluate the performance of the models comparatively, the number of accurate predictions was used in the calculation of performance metrics (Jintasuttisak et al., 2022).

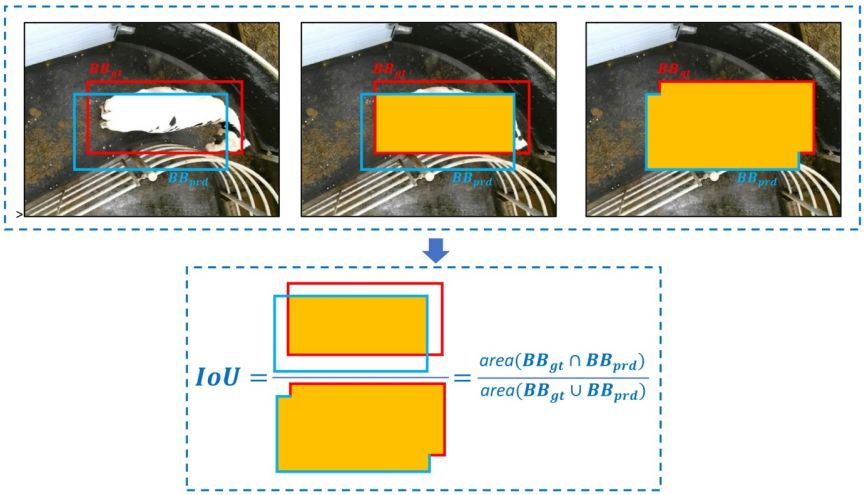

Figure 3. Calculation of IoU using predicted (BB_{prd}) and ground-truth bounding boxes (BB_{gt}). *Area* ($BB_{gt} \cap BB_{prd}$); Intersection of BB_{prd} and BB_{gt} *area* ($BB_{gt} \cup BB_{prd}$); Union of BB_{prd} and BB_{gt}.

2.3. Experimental setup

In this study, we followed the stages shown in Figure 4 for preprocessing, model training, and evaluation. In the first stage, we performed dataset creation (detailed description in section 2.3.1). In the second stage, we labeled and split the dataset (for training and validation). In the third stage, the mosaic data augmentation method was activated for YOLOv5, and training was performed. In the last stage, testing of the obtained models was performed.

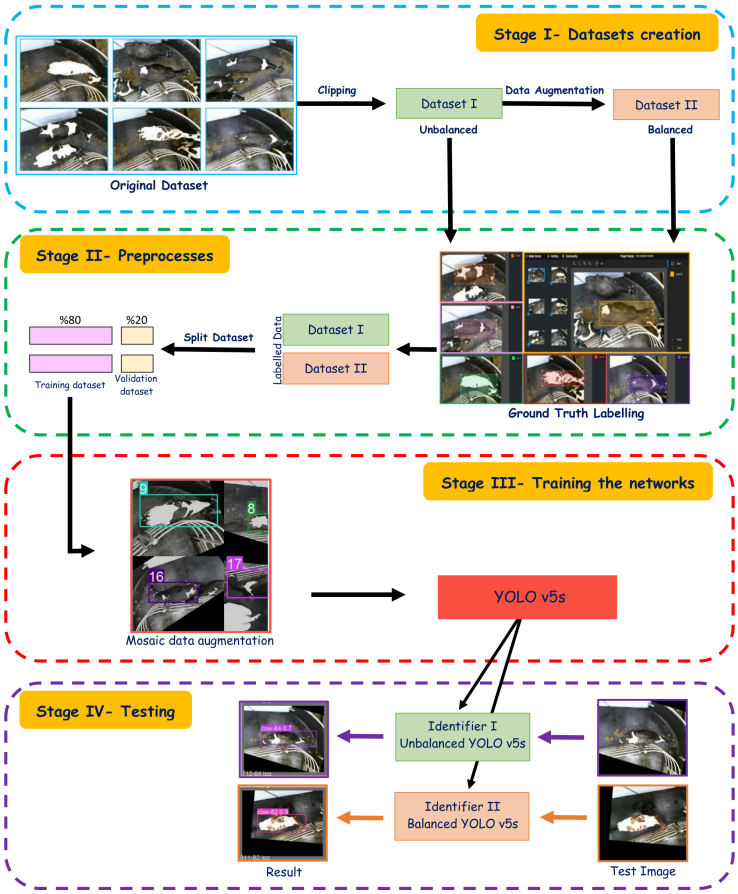

Figure 4. Data evaluation stages.

2.3.1. Experimental datasets

Considering the number of frames in Figure 5 for 89 different cattle in the original dataset, we used 20 Holstein Fresian cattle with 15 or more frames in our study and defined this dataset as Dataset I. Then, we created Dataset II by modifying Dataset I. A visualization showing the number of frames in both datasets is given in Figure 6.

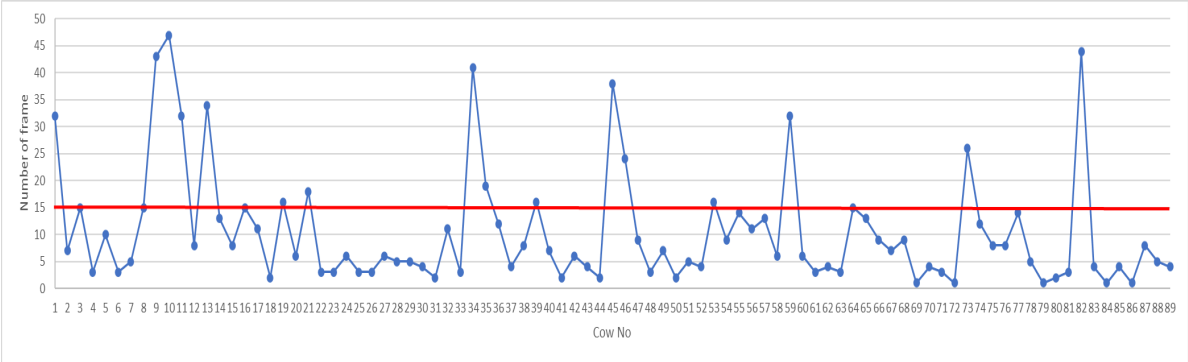

Figure 5. Number of frames in the original dataset.

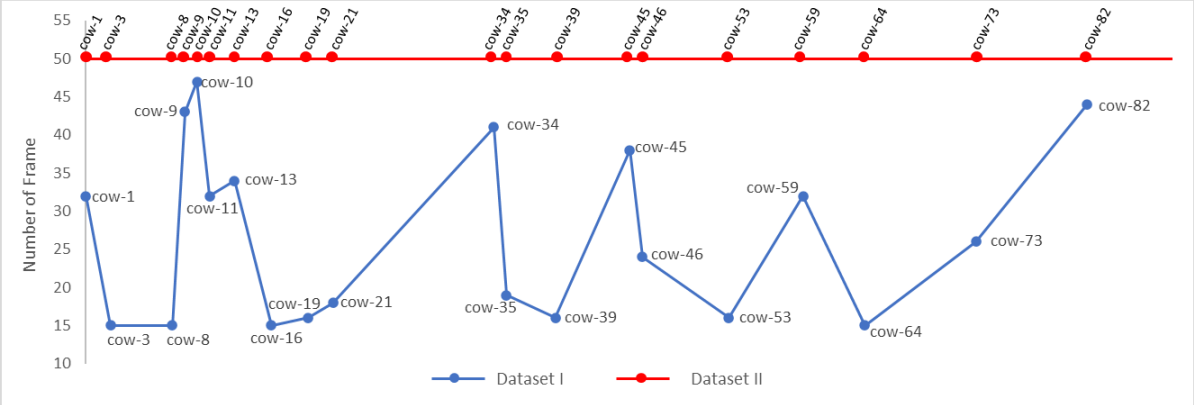

Figure 6. Number of frames in both datasets used in the study.

Detailed information on the construction of both datasets is provided.

Dataset I (Unbalanced): In this dataset, 539 images from videos of 20 cattle were used. While 432 of these images were used to train the models, 107 were used for testing. Figure 6 shows that the number of frames between classes is highly unbalanced due to the nature of the dataset. This is due to the fact that the animals were released on the walkway without any direction when the images were taken.

Dataset II (Balanced): In order to enrich the image data of the training set and to overcome the unbalance in Dataset I, we performed data augmentation using the "Augmentor" option in python. We used the data augmentation methods of rotate, zoom, skew, distortion, shear, and flip to arrange 50 frames in all classes of Dataset I. Thus, we balanced the dataset and used 800 frames for training and 200 frames for testing the models. Figure 7 shows examples of the application of data augmentation methods for Dataset I.

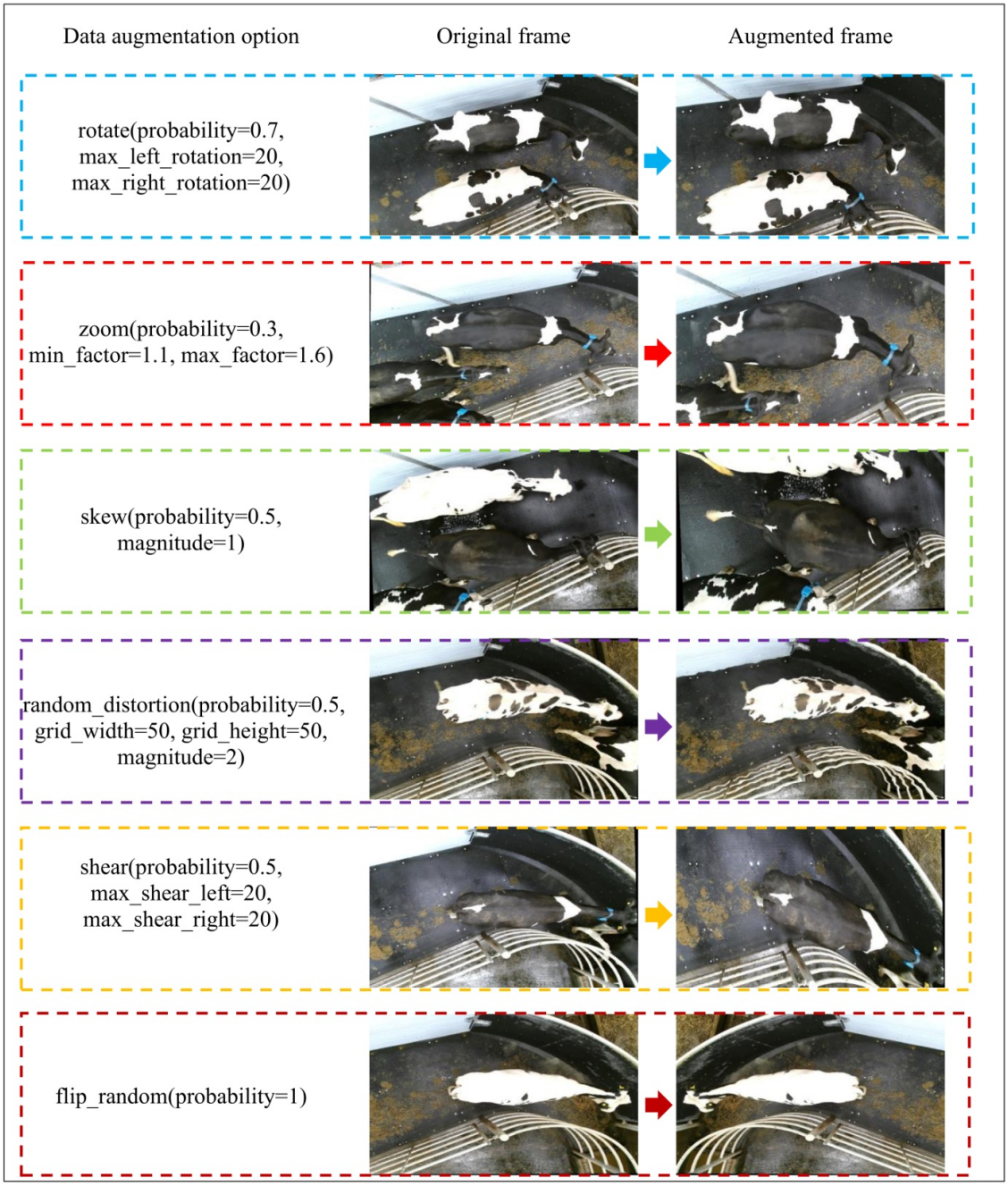

Figure 7. Implementation of data augmentation methods on the dataset I.

2.3.2. Ground truth labeling

In order to manually annotate cattle in the images, rectangular boxes were drawn to include the target cattle using the MakeSense (Skalski, 2019) image data annotation tool. The labeling process was completed by assigning cattle numbers to the drawn boxes. Afterward, the label package was created by saving the YOLO format files in zip format. The labeling process is given in Figure 8.

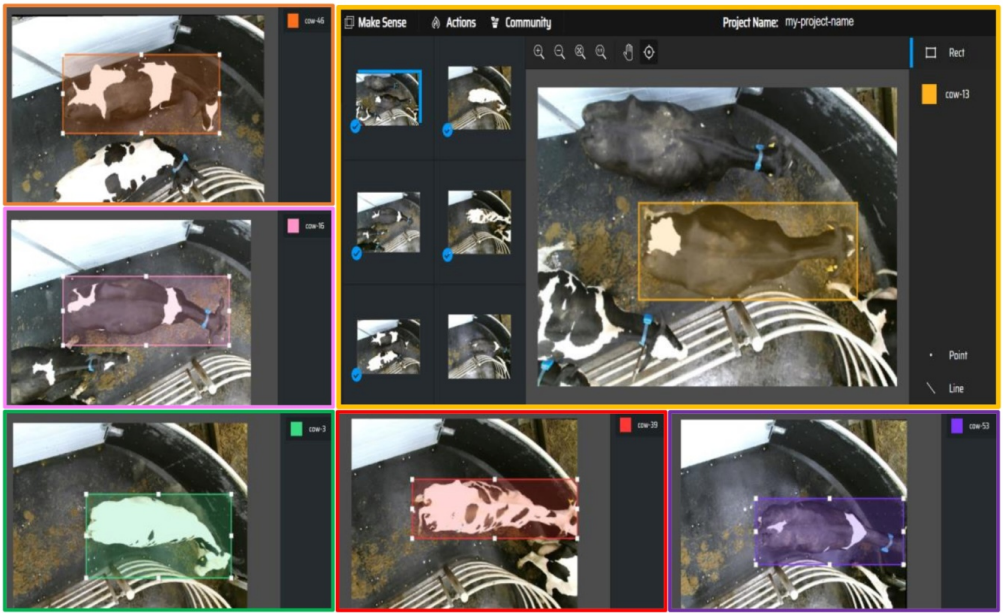

Figure 8. Ground truth labelling.

2.3.3. Network training parameters

In this paper, a ratio of 80%-20% is used for training and testing. The models have a batch size of 16, learning rate of 0.01, momentum of 0.937, epoch of 100, image size of 416, and optimization method of SGD. The open-source Python 3.8 (Van Rossum and Drake, 2009) package program and Pytorch 1.11.0 (Paszke et al., 2019), a high-performance deep learning library for YOLO v5s, were used in deep learning analyses. The information about some of the specific configurations we used is given in Table 1.

Table 1. Environment settings created for experimental processes

	Parameters	Configurations
Software	Operation system	Windows 10
	Framework for deep learning	Pytorch 1.11.0
	Langue of programming	Python 3.8
	IDE	Spyder 4.1.4
	GPU accelerated environment	CUDA 11.0
Hardware	GPU	GeForce RTX 2070, 8 GB GDDR6 Dedicated VRAM
	CPU	Intel Core i7-9750H @ 32 GB DDR4 2666 MHz

2.3.4. Performance evaluation

The criteria used to evaluate the performances of the models are Precision, Recall, and mAP (mean average precision). These metrics are calculated with tp (true positives), fp (false positives) and fn (false negatives) values obtained from the confusion matrix by Equation 1-3.

$$Precision = \frac{tp}{tp + fp} \quad (1)$$

$$Recall = \frac{tp}{tp + fn} \quad (2)$$

$$mAP = \frac{1}{N}\sum_{i=1}^{N} AP_i \quad (3)$$

Where;
AP: Average precision

3. Results

This section presents the performance results of the YOLO v5s algorithm for dataset I (unbalanced) and dataset II (balanced).

3.1. Dataset I results

Figure 9 shows the performance graphs of the YOLO v5s obtained from the training and validation dataset for the unbalanced dataset. The loss and mAP curves converge towards the 100th epoch. At the same time, the mAP value reached its highest value of 0.958 at epoch 76.

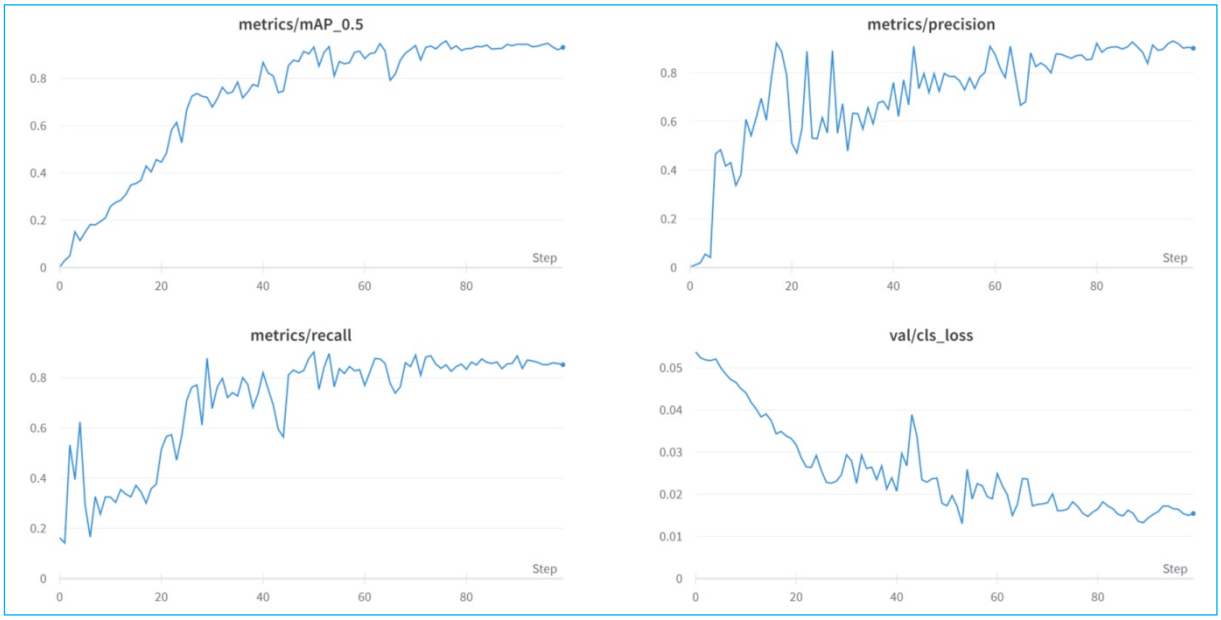

Figure 9. Model performance results trained on dataset I (Unbalanced).

The confusion matrix in Figure 10 demonstrates the functional ability of the YOLO v5s model to accurately predict the frames of cattle in images. The vertical and horizontal axes are the actual and predicted labels, respectively, and the diagonal represents the accurately identified frames. When the confusion matrix in Figure 10 is analyzed, all frames of 9 cattle are correctly identified. Only one of the 4 frames belonging to cow-35 was correctly identified, while one was identified as cow-39 and two were identified as background. It is understood that most of the frames that were actually cow-73 were identified as cow-53 and one frame that was actually cow-59 was identified as background. However, some frames that were actually cow-10, cow-19, cow-34 and background were predicted as cow-21. Images comparing some of the frames predicted by YOLO v5s with the actual labels are given in Figure 11.

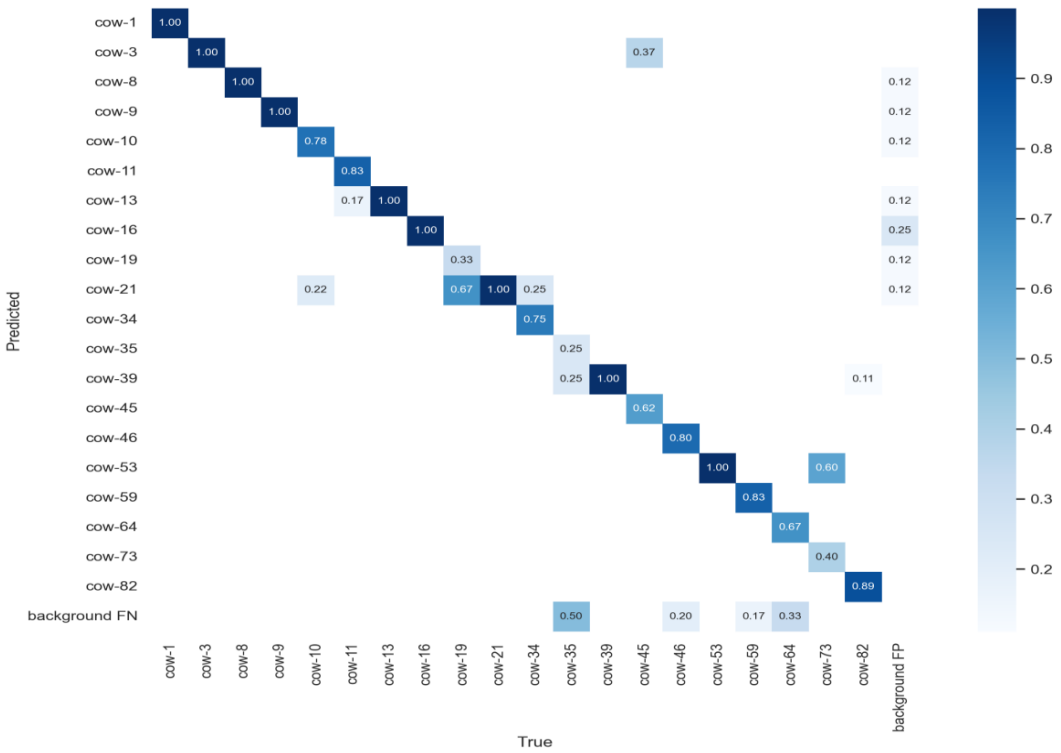

Figure 10. Confusion matrix of the model trained on dataset I (Unbalanced).

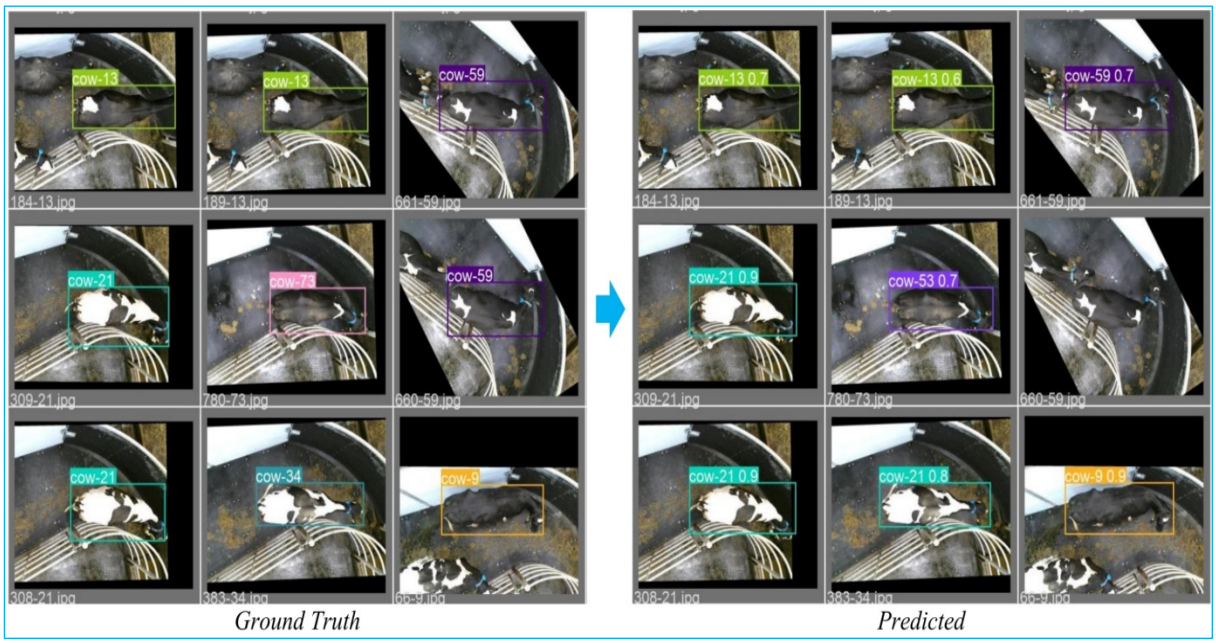

Figure 11. Comparison of ground truth and predicted frames for the model trained on the first dataset.

3.2. Dataset II results

The YOLO v5s model performance curves in the balanced dataset II are given in Figure 12. The model performance metric curves progressed smoothly. In contrast to the mAP curve in dataset I, here it reached high values after epoch 40 and reached its highest value of 0.995 at epoch 93.

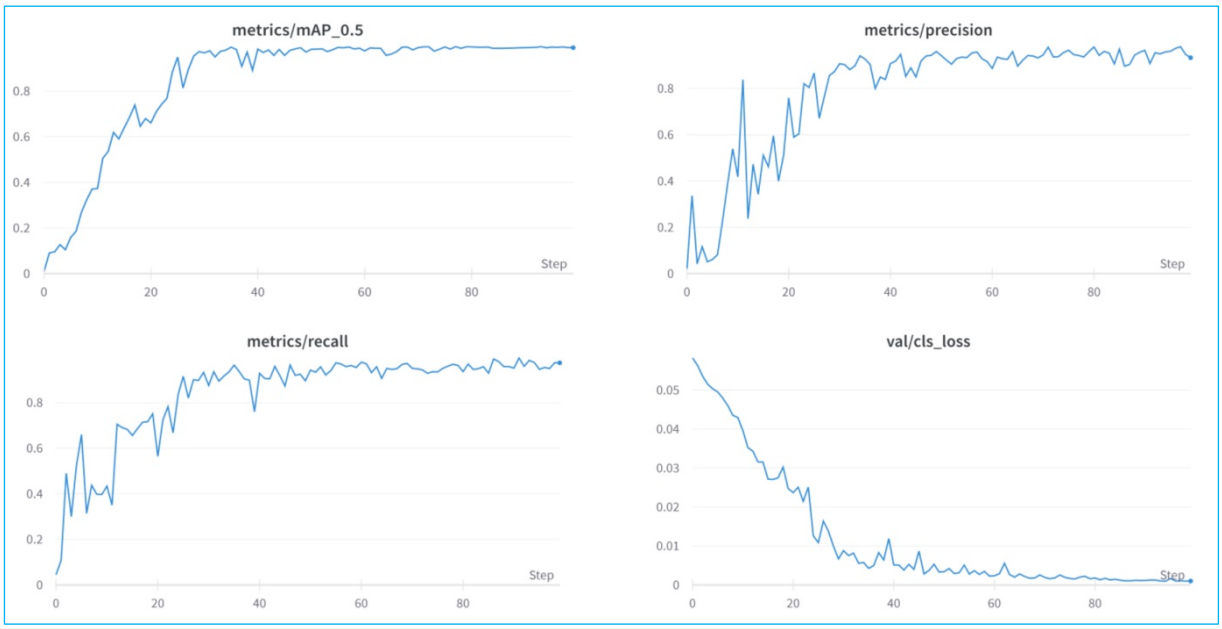

Figure 12. Model performance results trained on the second dataset (Balanced).

When the confusion matrix in Figure 13 is examined, it is understood that all images of all cattle except for only 3 cattle (cow-1, cow-46 and cow-59) are correctly identified. One image each of cow-1, cow-46 and cow-59 was identified as background. In addition, some of the images that were actually background (which could be an unlabeled cattle) were identified as cow-11 and cow-13. A comparison of some of the frames predicted by the model with the ground truth is shown in Figure 14.

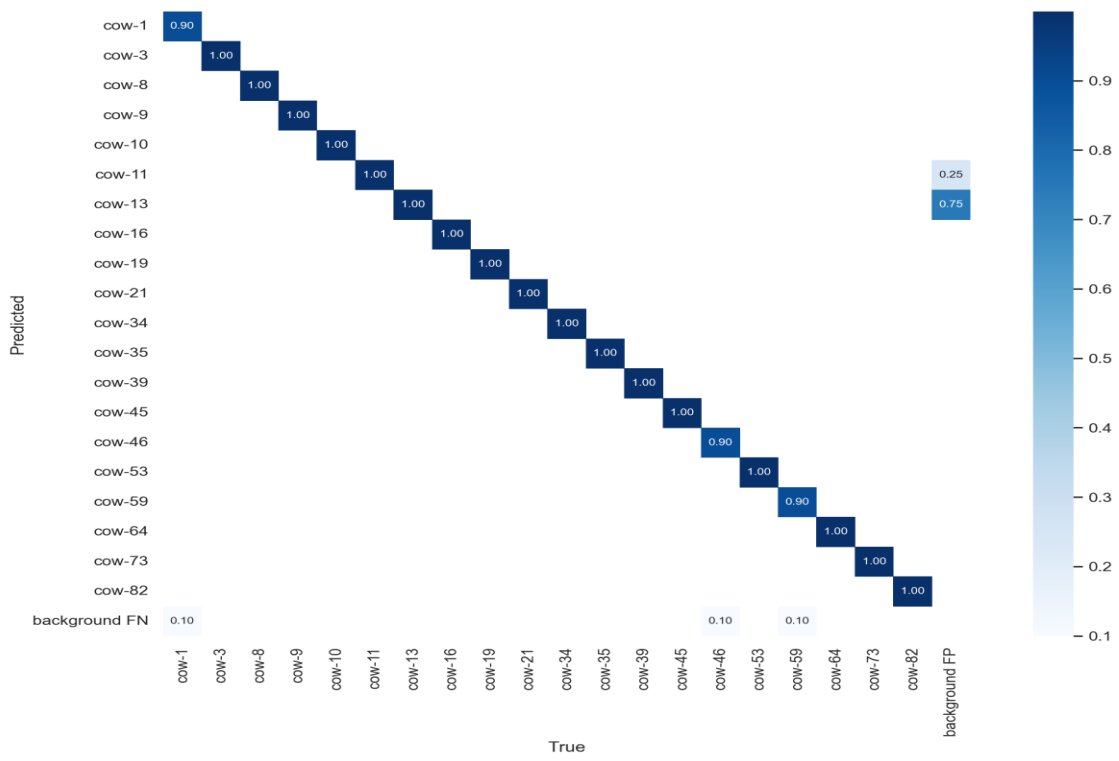

Figure 13. Confusion matrix of the model trained on dataset II (Balanced).

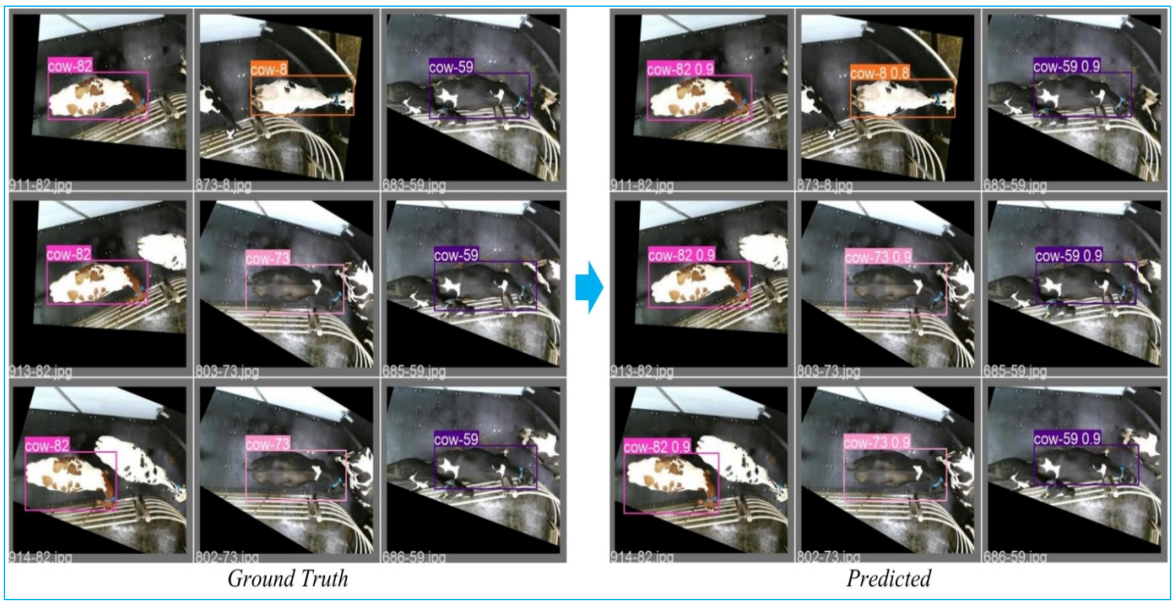

Figure 14. Comparison of ground truth and predicted frames for the model trained on the second dataset.

The performance metrics obtained from the YOLO V5s model to compare the classification accuracy for both dataset I and dataset II are given in Table 2. Balancing the dataset resulted in a significant increase in mAP (99.5%). In terms of recall and precision, balanced YOLO v5s gave the best performance. Comparing the training times, increasing the number of frames in the second dataset increased the training time by about 10 minutes.

Table 2. Individual cattle identification results

Networks	Training Time (mins)	Loss	Precision	Recall	mAP@0.5
Unbalanced YOLO v5s	16.467	0.015	0.871	0.851	0.958
Balanced YOLO v5s	26.600	0.001	0.950	0.985	0.995

4. Discussion

This study aims to determine the effect of data augmentation methods on YOLO v5s on an application dataset. For this purpose, the YOLO v5s object detection algorithm was applied to a dataset with unbalance in terms of the number of images between classes, and a second dataset was obtained by applying data augmentation methods and the results were discussed. Andrew et al. (2017), in their study on the original dataset of the study, achieved an mAP value of 0.993 in the cattle detection task and a mAP value of 0.861 in the individual cattle identification task with R-CNN networks. In the present study, we reduced the number of classes to 20 and obtained an mAP value of 0.995 with YOLO v5s in the dataset where we eliminated the unbalance.

In the present study, since the cattle identification performance obtained from the balanced dataset was approximately 100% (0.995 mAP), model training was performed with the default hyperparameters and no hyperparameter adjustments were made. However, previous studies on deep learning show that using various combinations of hyperparameters can result in architectures that are more suitable for the dataset (Ser and Bati, 2019; Altınbilek and Kızıl, 2022). Bocaj et al. (2020) used deep convolutional neural networks to identify the movements of horses and goats. In their study, they actively used hyperparameter tuning to optimize the overall accuracy. In another study, Zhang et al. (2022) developed a method based on YOLOv5 for goat head detection and automatic counting using combinations of data augmentation methods. In this study, they used combinations of mosaic, mixup, and RandAugment methods and it was reported that the most successful combination was obtained from YOLOv5 + Mixup + Mosaic + RandAugment. In addition, a convolutional neural network-based animal

identification method for cattle and sheep was proposed by Sun et al. (2021). As in the present study, the original image data were firstly augmented by random cropping, random angle inversion, and random horizontal undoing. Then, a binary classification model for cattle and sheep recognition based on VGG-16 convolutional neural network is constructed. In addition, the relevant hyperparameters were constantly adjusted to increase the number of iterations in the study, and finally, a higher recognition accuracy rate was achieved.

Data augmentation provides an efficient way to extend training data and overcome the unbalance between classes (Shorten and Khoshgoftaar, 2019). However, the construction of models based on deep learning is realized on the basis of training a large amount of image data (Yan et al., 2021; Shojaeipour et al., 2021). The larger the size of the dataset and the better the data quality, the greater the ability of the model to generalize. Yet, while collecting data for real-life situations, classes in the data may not be equally represented, often due to conditions such as different environmental conditions. The unbalance between classes is known to affect network performance (Kasfi et al., 2016). Lee et al. (2022) used a YOLO network to monitor the invasion of wildlife animals on farms. In this study, they proposed a subtraction-addition data augmentation method, noting that creating a training dataset for specific wild animals requires considerable time and effort. In a comparison between the object detector trained using the proposed data augmentation technique and the object detector trained using existing data augmentation techniques, they found that the mAP increased by ≥2.2%. The mosaic data augmentation method, introduced by Bochkovskiy et al. (2020) in YOLO v4, uses four images from the training data and adds the training data to a single image through flipping, color gamut modification, and scaling (Zhang et al., 2022; Chen et al., 2022). In our study, we used mosaic data augmentation to expand the image set before feeding the images to the YOLO v5s network model. However, although the mosaic data augmentation method has advantages such as enriching the object detection back-ground and tiny objects and reducing the dependency on the batch size, it is unable to overcome the unbalance between classes. Therefore, in this study, the images in the first dataset were also included in the data augmentation process and then fed into the YOLO v5s network (Figure 5). According to the experimental results, the balanced classes in the object detection task significantly improved the performance in the cattle identification task by increasing the mAP value from 0.958 to 0.995 (Table 2). The result of this research shows that the use of data augmentation techniques can improve the performance of object detection methods.

The fact that the cattle in the dataset used in this study pass through a walkway individually rather than as a group, that all images were taken at the same time period, that all images have the same backgrounds, and that the lighting and image quality are good is advantageous for the deep learning models used in this study. It should be kept in mind that testing these models on natural images of cattle in pens or pastures will slightly decrease the identification accuracy. In general, due to these results, we propose the following strategies for training new networks;

- In studies with computer vision technologies, the number of frames between classes in the dataset should be taken into account.
- In deep learning studies such as YOLO, it should be kept in mind that it may be useful to evaluate the hyper-parameters of the models used and to use different combinations of hyper-parameters if necessary without depending on default settings.
- The presence of images with different features (lighting differences, individual or group images, distant or close images, etc.) in the data set of the model to be trained will increase the identification capacity of the model.

Conclusion

In this paper, the performance of data augmentation methods on YOLO v5s has been tested against the problem of obtaining equal image or video data for each class in image classification or object detection studies in animal husbandry. According to the results of the study, the balanced data set resulted in a ~4% increase in the mAP value and superior performance (0.995 mAP) was obtained with the YOLOv5 model trained on this data set. For future work, studies on individual identification and identification of various specific behaviors in sheep, where individual identification with computer vision technologies is more difficult than in cattle, are planned.

Acknowledgements

The authors declare no conflicts of interest.

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Research Article

Correlates and Determinants of Involvement in Sweetpotato Production among Farming Households in Niger-Delta Area of Nigeria

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Abstract: Information on determinants of involvement in sweetpotato production (SPP) in Niger-Delta Area of Nigeria is scarce. Determinants of involvement in SPP among farming households in Niger-Delta Area of Nigeria were therefore assessed. Multi-stage sampling procedure was used to select 330 respondents. Data were collected through structured interview schedule and analysed using descriptive statistics, Chi-square, Pearson Product Moment Correlation-PPMC and logistic regression. Age and household size of respondents were 42.9±11.9 years and 8.7±5.5 persons, respectively. Most respondents were female (53.3%), while 94.5% had no extension contact. Farming experience and farm size were 21.1±12.9 years and 5.4±5.1 ha, respectively. Employed labour per sweetpotato (SP) production cycle, SP farm size and farming experience were 8±6 persons, 2.6±3.5 ha and 18±12 years, respectively. Income from SP, other crops enterprises and non-crop livelihood activities were ₦2 637 552.0±3 362 512.0 yearly, ₦5 283 845.0±6 147 413.0 yearly and ₦1 733 562.0±2 175 223.0 yearly, respectively. Most respondents (56.4%) produced above one cycle per year. Limited knowledge on processing of SP ($\bar{x}$=1.88) was the major constraint to SPP. Above half (51.8%) of the respondents had low involvement in SPP. Gender (x^2=6.79), household size (r=0.12), farm size (r=0.19), farming experience (r=0.12) and income from SP (r=0.19) were significantly related to level of involvement in SPP. Government and other stakeholders should organise intervention (training and workshop) on increasing income from sweet potato production.

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1.Introduction

Sweetpotato (*Ipomoea batatas* [L.] Lam) is an important storage root crop that is widely grown as a vital staple food in many part of the tropical and subtropical regions of the world, which includes many developing countries (Odebode et al., 2008). Empirical analyses have established diverse advantage of sweetpotato (SP) over other root and tuber crops (Onumah et al., 2012; Olapade and Ogunade, 2014). These, among others, include low demand on soil nutrient, tolerant of drought; ability of producing reasonable yields in agro-ecological zones where other crops would fail; low external input

requirement; flexibility in planting and harvesting period; ability to be cultivated thrice per annum; and potential for being used as substitute for yam, Irish-potato and cocoyam.

Apart from its advantage over other root and tuber crops, SP has nutritional and therapeutic benefits as well as economic benefits. Meludu (2010) reported that its leaves, shoots and storage roots are valuable sources of complex carbohydrates; proteins; oil; vitamins; beta-carotene; minerals which are essential for human health and body functioning; dietary insoluble fibre which helps to prevent constipation; soluble fibre which plays a role in reducing cholesterol; dietary fiber; and low crude fibre. Akoroda (2008) noted that it supplies raw materials to industries in diverse developed and developing countries. Its production by low-income farmers has served as source of income for approximately 600 million people in developing countries of the world (CIP, 2005). The foregoing, makes SPP to play a big diet, food security, economic, and poverty reducing role to farming households as well as poor and undernourished people in developing countries. Thus, SP has the potential of playing an important role improving the economy of developing countries.

Nigeria, realising the potential of SP to her economy, has vigorously promoted improved production of the crop through several efforts (in form of policies, projects and programmes). The efforts among others include establishment of Root and Tuber Expansion Programme (RTEP) *vis a vis* National Root Crops Research Institute (NRCRI). However, in spite of these efforts by the Nigeria government, SPP status is still low in the country. According to Nwanebo (2012), SP is still grossly under-explored in the country due to fact that its production status is low. Also, Adewumi and Adebayo (2008) noted that its level of production still remain low in the country. Nigeria produce approximately 3.46 – 3.92 million tonnes of SP per annum, which accounts for 3.7 percent of world production (FAOSTAT, 2017). China, in contrast to Nigeria, produces 71-76 million tonnes of SP per annum and accounts for 67-74 per cent of the world SPP (Sugri et al., 2017). These are indications of inadequate production status of SP in Nigeria.

In Nigeria, Niger-Delta Area of Nigeria is known for good agricultural soil as well as tropical climate, suitable for high production status in root and tuber crops. The forgoing, suggests that the area possesses advantageous environmental, ecological and climatic factors required for high level production of SP. Empirical analysis has shown there is a direct relationship between good environmental, ecological and climatic factors with high level production of crops (Oyibo, 2015). Hence, it is expected that Niger-Delta Area of Nigeria should rank as the leading producer of SP in the country. However, empirical analysis has shown that Niger-Delta Area of Nigeria ranks second, surpassed by North-Central region of Nigeria (NFRA, 2007; Egeonu, 2011). The region produces approximately 520 thousand tonnes of SP per annum and account for 15 per cent of the country SPP. In contrast to Niger-Delta Area of Nigeria, North-Central region of Nigeria produces approximately 140 thousand tonnes of SP per annum and account for 40.6 per cent of the country's SPP. Niger-Delta Area of Nigeria account for 0.49 percent of the world SPP. Sichuan province of China, in contrast to Niger-Delta Area of Nigeria, accounts for 18.5 per cent of world SPP and produces 19.4 million tonnes per annum (Ogundele et al., 2009). These are indications of inadequate and/or low production level of SP in Niger-Delta Area of Nigeria. This suggests that the SPP in Niger-Delta Area of Nigeria demands scientific investigation. Empirical analysis has shown a positive correlation between level of involvement (intensity)-LI in SPP and SPP status (Nwanebo, 2012). Hence, to combat low level of SPP in Nigeria *vis a vis* Niger-Delta Area of Nigeria, cognizance should be given to other variables such as LI in SPP, constraints to SPP and determinants of involvement in SPP.

While there is a number of studies and literature on SP, the LI of farming households (FHs) in SPP and the consequent factors associated with involvement in SPP, particularly, in Niger-Delta Area of Nigeria have not been well explored. For example, Aboajah et al. (2018) assessed SPP for poverty alleviation in Nasarawa State, Nigeria. Ahmed et al. (2014) assessed efficiency of SP farmers in Nigeria: potentials for food security and poverty alleviation. Nwanebo (2012) assessed the factors associated with SPP among rural farmers in Imo State. These studies run short of the determinants of involvement of FHs in SPP. Thus, there is a gap in fathoming the exact determinants of involvement in SPP in terms of empirical predictors of involvement status (IS) in SPP of FHs. This study has attempted to fill the gap by assessing the level of farming households' involvement in SPP in Niger-Delta Area of Nigeria, constraints to SPP and determinants of farming households' involvement in SPP.

The general objective of the study is to ascertain the determinants of involvement in SPP among FHs in Niger-Delta Area of Nigeria. The specific objectives were to: describe the demographic

characteristics of SP farmers; examine the enterprise characteristics of SP farmers; ascertain the constraints to SPP; and establish the LI in SPP. Based on the objectives of the study, the following hypothesis were tested: there is no significant relationship between selected demographic characteristics and LI in SPP; there is no significant relationship between selected enterprise characteristics and LI in SPP; and there is no significant relationship between constraints to SPP and LI in SPP; there is no significant contribution of selected independent variables to IS in SPP.

2. Material and Methods

2.1. Study area

The study was carried out in Niger-Delta Area of Nigeria. The study area comprises nine coastal southern Nigerian states, namely: Edo, Delta, Bayelsa, Rivers, Akwa Ibom, Cross Rivers, Ondo, Imo and Abia (UNDP, 2006).

2.2. Population and sampling procedure

The population of the study comprised all SP FHs. Multi-stage sampling procedure was used to select respondents. The first stage involved random selection of 3 states out of the nine states in the study area, the states sampled were Bayelsa, Delta and Edo. Each of the states has three agricultural development programmes-ADPs zones. The second stage involved the purposive sampling of five ADPs zones out of the nine ADP zones in the selected states based on predominance of SPP. Thus, Delta-Central and Delta-South from Delta state; Yenagoa and Sagbama from Bayelsa state; and Edo-North from Edo State were purposively selected. The third stage involved stratification of the blocks in each of the selected zones into SP and non-SP producing blocks. The SP producing blocks were five and four in Delta-Central and Delta-South zones, respectively; seven and three in Yenagoa and Sagbama zones, respectively; and six in Edo-North zone.

The fourth stage involved random sampling of 40% of the SP producing blocks in the selected zones. The blocks sampled were Ughelli-South and Ughelli-North from Delta-Central zone; Patani and Bomadi from Delta-South zone; Atissa, Epie and Gbarain from Yenagoa zone; Sagbama from Sagbama zone; and Agenebode and Ekperi from Edo-North zone. The cells that are known for SP production in each of the selected block were identified. Altogether, 52 cells were identified in the selected blocks. The fifth stage involved random sampling of 25% of the SP producing cells in the selected blocks. The final stage involved the random sampling of 20% SP FHs from each of the selected cells to give a total of 330 SP FHs (111 from Bayelsa State, 114 from Delta State, and 105 from Edo State). The farmers responsible for SPP were interviewed in each of the selected households.

2.3. Data collection

Primary data were obtained through the use of interview schedule. The interview schedule captured information on demographic and enterprise characteristics, constraints to SPP and level of involvement (intensity) in SPP.

2.4. Measurement of variables

Constraints to SPP was measured at interval level. A list of 23 possible constraints which inhibit SPP was presented to respondents. The severity of the 23 possible constraints to SPP was measured. Response was rated using a three-point rating scale of “Severe constraint (2)”, “Mild constraint (1)”, and “Not a constraint (0)”. A minimum score of 8 and maximum score of 36 were obtained from the 23 constraint items. The mean value of each item was computed and used to rank the constraints in order of severity. In addition, an index of constraints to SPP was generated by adding all the responses. The mean (26.00±5.00) of the index of constraints to SPP was used to categorise respondents into high (26.00–36.00) and low (8.00–25.99) constraints categories using the above and below the mean score criterion.

Involvement (intensity) in SPP was measured at interval level. Sweetpotato farm size, number of employees, years of involvement and number of production cycles were used to derive involvement by adapting the involvement index of Samuel (2020). Involvement (intensity) in SPP was

operationalised by standardising and adding together scores from; SP farm size, years of involvement, number of employees utilised in SPP, and number of SP cycles operated per annum, to give a composite involvement index score. The minimum values of 0.00 and maximum values of 39.20 were obtained from the involvement index. The mean ($\bar{x}$=15.15) was used to categorise respondents into: high involvement in SPP (15.15-39.17) and low involvement in SPP (0.00-15.14) using the above and below the mean score criterion, respectively.

2.5. Data analysis

The data collected were entered into Statistical Package for Social Science-SPSS (version 20), and were analysed using descriptive statistics (frequency counts, percentages, means and standard deviation) and inferential statistics (Chi-square, PPMC and logistic regression model). Chi-square and PPMC were used to test hypothesis one (H_{01}). The PPMC was also used to evaluate hypothesis two (H_{02}) and hypothesis three (H_{03}). Binary logistic regression was used to ascertain the significant determinants of involvement status. The logistic regression model used is expressed as follow:

$$P_i = P\,[Y_i = 1/x_i] = \frac{\exp\,(\beta_1+\beta_2 X_i)}{(1+\exp\,(\beta_1+\beta_2))^2} \quad (1)$$

Where, P lies between 0 and 1 ($0< P_i >1$).
The P_i is the dependent binary variable (1 for high involvement in SPP and 0 otherwise) and Xi is the independent variable. Where: i = 1, 2, 3…10.
X_1 = age of farmer (years), X_2 = sex (male = 1, female = 0), X_3 = household size (number of persons), X_4 = total farming experience (years), X_5 = total farm size (hectare), X_6 = income from SP, X_7 = income from other cultivated crops, X_8 = income from non-crop enterprise, X_9 = constraints to SPP, X_{10} = number of extension contact

3. Results

3.1. Demographic characteristics of respondents'

The result on age distribution of respondents reveals that the mean age was 43 ± 10 years (Table 1). A little above average (53.3%) of the respondents were female. The mean household size of respondents was 9 ± 6 persons. Also, only Few (4.4%) of SP producers had extension contact.

Table 1. Demographic characteristics of SP producers

Variables	Categories	%	Mean ± SD
Age (years)	≤ 20	0.6	
	21 – 30	16.1	
	31 – 40	33.6	42.90 ± 11.94 years
	41 – 50	26.1	
	> 50	23.7	
Gender of respondents	Male	46.7	
	Female	53.3	
Household size (persons)	1 – 5 persons	30.0	
	6 – 10 persons	50.0	8.7 ± 5.5 persons
	> 10 persons	20.0	
Extension contact	Yes	4.4	

3.2. Characteristics of enterprise

The average farm size was 5.43 ± 5.10 ha as shown in Table 2. The respondents cultivated an average SP farm size of 2.60 ± 3.49 ha. The mean years of farming experience was 21 ± 13 years. The mean years of SPP experience was 18 ± 12 years. A larger percent (56.4%) of the respondents were able to do above one SP cycle per annum. The mean number of employees per production cycle was 8 ± 6

persons. The mean annual income realized from SPP was ₦2 637 552.0 ± 3 362 512.0. This translates to ₦219 796.0 income made by the respondents per month from SPP. The mean annual farm income from other crop production was ₦5 283 845.0 ± 6 147 413.0. The respondents had mean yearly income of ₦1 733 562.0 ± 2 175 223.0 from non-crop livelihood activities.

Table 2. Distribution of respondents according to enterprise characteristics

Variables	Categories	%	Mean ± SD
Farm size possessed (hectares)	≤ 1	3.0	5.43 ± 5.10
	1.01 – 2.00	15.5	
	> 2	81.6	
Farm size cultivated with SP (hectares)	≤ 1	34.5	2.60 ± 3.49
	1.01 – 2.00	29.7	
	> 2	35.8	
Farming experience (years)	1 – 10	26.4	21.07 ± 12.85
	11 – 20	35.5	
	> 20	38.2	
Sweetpotato growing experience (years)	1 – 10	40.6	18 ± 12
	11 – 20	32.4	
	> 20	27.1	
Number of SPP cycles per year	One	43.6	
	Two	50.0	
	Three	6.4	
Number of hired hands/employees per SPP cycle (persons)	None	4.2	8 ± 6 person
	1 - 10	79.4	
	11 – 20	12.7	
	> 20	3.3	
Income from SP (₦)	≤ 800 000.0	23.6	2 637 552.0 ± 3 362 512.0
	800 000.1 – 1 600 000.0	25.2	
	> 1 600 000.0	51.3	
Income from other crop cultivated (₦)	None	2.1	5 283 845.0±6 147 413.0
	≤ 800 000.0	7.9	
	800 000.1 – 1 600 000.0	13.9	
	Above 1 600 000.0	76.0	
Income from non-crop livelihood activities (₦)	None	60.6	1 733 561.5±2 175 223.0
	≤ 800 000.0	17.6	
	800 000.1 – 1 600 000.0	8.5	
	Above 1 600 000.0	13.2	

3.3. Constraints to SPP

The results (Table 3) reveal that on the overall, limited knowledge of processing of SP ($\bar{x}$=1.88±0.38) ranked first as the most serious constraint. Inadequate capital ($\bar{x}$=1.86±0.40) and lack of credit facilities ($\bar{x}$=1.83±0.41) ranked second and third on the list of constraints, respectively.

Table 3. Constraints to SPP

Items	Mean	SD	Rank
Limited knowledge on processing of SP	1.88	0.38	1st
Inadequate capital	1.86	0.40	2nd
Lack of credit facilities	1.83	0.41	3rd
Flooding	1.77	0.58	4th
Sweetpotato pests	1.71	0.52	5th
Low cash value per unit of weight	1.68	0.59	6th
Poor extension services	1.65	0.73	7th
Difficulties associated with transportation in tropical condition	1.61	0.70	8th
Drought	1.57	0.74	9th
Few markets	1.56	0.74	10th
Poor storability	1.53	0.77	11th
High susceptibility to disease	1.40	0.76	12th
Lack of improved cultivars	1.19	0.87	13th
Inadequacy of seedling at planting time	1.15	0.86	14th
Low yield	1.13	0.81	15th
Sweetpotato is being overlooked by consumer	1.03	0.87	15th
Unavailability of land	0 .79	0.89	16th
Herders men attack	0.57	0.90	17th
Lack of chemical	0.04	0.29	18th
Inadequacy of labourers	0.03	0.25	19th
Lack of machine	0.01	0.11	20th
Lack of fertilizer	0 .01	0.11	20th
Lack of irrigation facilities	0.003	0.06	22nd

3.3.2. Categorisation of respondents based on constraints to SPP

Result of analysis of the constraints to SPP, as seen in Table 4, reveals that 57.0% of the respondents had high constraints to SPP.

Table 4. Categorisation of respondents based on constraints to SPP

Constraints to SPP	Freq.	%	Minimum	Maximum	Mean	SD
Low (8.00 – 25.97)	142	43.0	8.00	36.00	25.98	5.24
High (25.98 – 36.00)	188	57.0				

3.4. Involvement in SPP

Table 5 indicates the result of SP farming variables pooled together to measure involvement of FHs in SPP. Table 6 shows that a little above average (51.8%) of the FHs had low involvement in SPP.

Table 5. Distribution of farmers' involvement in SP farming enterprise

Variables	Categories	Percentages
Farm size involved (hectares)	≤ 1	34.5
	1.01 – 2.00	29.7
	> 2	35.8
Years of involvement	1 – 10	40.6
	11 – 20	32.4
	> 20	27.1
Number of SP cycles	One	43.6
	Two	50.0
	Three	6.4
Number of hired hands (persons)	None	4.2
	1 - 10	79.4
	11 – 20	12.7
	> 20	3.3

Table 6. Categorisation of farmers based on their involvement in SP farming enterprise

Involvement	%	Minimum score	Maximum score	Mean	SD
Low (0.00 – 15.14)	51.8	0.00	39.17	15.15	6.73
High (15.15 – 39.17)	48.2				

3.5. Hypothesis one (H_{01}): There is no significant relationship between selected demographic characteristics and LI in SPP of FHs

Results in Table 7 show that respondents gender (x^2=6.79) and household size (r=0.12) were significantly ($p < 0.05$) related to involvement in SPP.

The relationship between gender of respondents and IS in SPP depict that SP producers' gender influence their IS. Table 8 shows that most (54.1%) of the male farmers had high involvement, while the female farmers (60.2%) had low involvement.

Table 7. Chi-square and PPMC analyses of selected demographic characteristics and involvement in SPP

Variables	Df	x^2	r-value	p-value
Age	-	-	0.06	0.29
Sex	1	6.79*	-	0.01
Household size	-	-	0.12*	0.03
Membership of SP association	1	1.04	-	0.31
Extension contact	-	-	0.06	0.29

Note: df = Degree of Freedom, x^2= Chi-square Coefficient, r = Correlation coefficient. *Significant at $p \leq 0.05$.

Table 8. Crosstab analysis of seleted demographic characteristic and involvement in SPP

Variable	Categories	Involvement		Total
		Low	High	
Gender of respondent	Male	68 (39.8)	86 (54.1)	154 (46.7)
	Female	103 (60.2)	73 (45.9)	176 (53.3)

Note: Values in parentheses are percentage scores.

3.6. Hypothesis two (H_{02}): There is no significant relationship between selected enterprise characteristics and involvement in SPP of FHs

Pearson Product Moment Correlation results in Table 9 shows that farm size (r = 0.19), farming experience (r = 0.12) and income from SP (r = 0.19) were significantly ($p < 0.05$) associated with involvement in SPP.

Table 9. Pearson Product Moment Correlation analysis of selected enterprise characteristics and involvement in SPP

Variables	r-value	p-value
Farm size	0.188*	0.00
Farming experience	0.120*	0.03
Income from SP	0.190*	0.00
Income from other cultivated crops	0.103	0.06
Income from non-crop activities	-0.066	0.46

Note: r = Correlation coefficient, *Significant at $p \leq 0.05$.

3.7. Hypothesis three (H_{03}): There is no significant relationship between the constraints to SPP and LI in SPP

The result in Table 10 indicates that there was no significant correlation between constraints to SPP and involvement in SPP (r = -0.008, $p > 0.05$).

Table 10. Pearson Product Moment Correlation analysis between constraints to SPP and involvement in SPP

Variable	r-value	p-value
Constraints	-0.01	0.88

Note: r = Correlation coefficient, Significant at p≤0.05.

3.8. Hypothesis four (H_{04}): Selected independent variables have no significant contribution to IS in SPP of FHs

Table 11 reveals that none of the selected independent variables had positive and significant relationship with the likelihood of high involvement in SPP at 5% level of significance.

Table 11. Factors determining farmers' high involvement in SPP

Predictors	Coefficient	Std. Error	z	P>|z|
Age	-0.04	0.03	1.83	0.18
Gender (Male)	0.58	0.41	1.95	0.16
Household size	-0.01	0.04	0.12	0.73
Number of extension contact	0.37	0.22	2.83	0.09
Total crop farm size	-0.02	0.08	0.03	0.86
Total years of farming experience	0.03	0.03	1.16	0.28
Income from SP	0.00	0.00	1.66	0.20
Income from other cultivated crops	0.00	0.00	0.02	0.90
Income from non-crop enterprises	0.00	0.00	0.92	0.34
Index of constraints	0.04	0.04	1.01	0.32

Significant at 5%.

4. Discussion and Conclusion

The SP farmers were middle aged, which implies that they were predominantly in their economically productive ages and have the energy to meet the labour demands of SPP. Hence, respondents can actively and/or energetically engage in SPP. This corroborates Eforuoku (2018) who asserted that middle-aged people are the most actively involve farmers in agricultural production, which is rooted in their agile and energetic nature. Middle and active aged people holds more promise for high involvement in agricultural production as most middle aged farmers are not only mature and having streams of income from diverse income generating activities which aids their timely inputs procurement, but also have the vigour to work on their farms (Alabi, 2019). This result is in tandem with the findings of Ahmed et al. (2014) that majority (61.7%) of SP producers were between the age range of 30 and 49 years. It was observed that farmers' dominance in SPP is slightly skewed towards female. The slightly dominance of female over male is similar to the findings of Mmasa (2014); he found relatively more dominance of female in SPP. The dominance of female over male may be partly due to the fact that SP is considered as a minor crop as it does not command a place over cassava, yam or plantain in the market, hence, most males are not farming SP. This is in line with Nwanebo (2012) who reported that SP is regarded agriculturally as a minor crop. Furthermore, the sex distribution of respondents is likely to affect involvement level (IL), as female are likely to have less energy to meet up with the labour demand of SPP *vis a vis* access to productive resources as well as control over decision, income, asserts and choices, hence, may have low IS. This corroborate Nwanebo (2012) who asserted that the physical demand in SPP discourage female from highly involving in SPP.

It could be deduced that the respondents have a large household size, which is considerably high when compared to the average Nigeria household size of 6 persons in rural communities (NBS, 2016). The result agreed with Mbanaso (2011) and Chinedu et al. (2022) who found large household size among SP and rice FHs in South-Eastern Nigeria, respectively. The implication is that more family labour would likely be available to meet the labour requirement for high involvement in SPP by the respondents. This is in line with Eforuoku (2018) who posited that the larger the family size, the more the number of family members utilised as source of farm labour. The large household size could be as a result of the likely need for large family size, which serve as source labour in the farm. Oyibo (2020) posits that the need for family labour as source of farm labour has led to large family size by FHs.

Household size could influence respondents' IS, because household size serves as source of family labour, which influence size of land cultivated. The SP farmers generally had less extension contact. This is consistent with Abdulkarim and Yunana (2015) who reported that 91.7% of SP producers had no extension contact. The implication is that respondents are likely not to be exposed to relevant SPP and processing technologies disseminated by extension agents. Extension contact exposes farmers to agricultural innovations and technologies (Mbanaso, 2011). The non-extension contact could likely be due to non-membership in farmers association. Agwu (2000) posits that farmers' organisations offer an effective medium for extension contact.

It was observed that the mean of total farm size is not above 6 ha. This implied that irrespective of the crops cultivated, the SP producers are generally small holder or small scale farmers. The result agrees with the finding of Nwanebo (2012) that SP FHs practice crops production on a small-scale level. Mbanaso (2011) classified farm holders in Nigeria into three categories of large, medium and small farm size holders, representing greater than 10 ha, 6 - 9.99 ha and less than 6 ha, respectively. The total farm size distribution of respondents is likely to affect IS in SPP, as small scale farmers are more likely to practice land fragmentation. Thus, higher likelihood of respondents to allocate small farm size to SPP, thereby decreasing/reducing their SPP potential or capabilities, hence, may have inadequate IS. Earlier study by Nwanebo (2012) found that small scale farmers were moderately involved in SPP. She further found that majority (74.1%) of small farm size holders did not cultivate SP as a main crop.

The SP farmers were relatively experienced in agricultural production having been in farming business for over two decades. This support Alabi (2019) who found that crop farmers in Nigeria are seasoned with mean farming experience of 20±13 years. The high presence of respondents with crops production experience, suggests that they would have amassed a relatively degree of crops production knowledge over time that would make them capable of determining necessary action to take as regard their crop farming activities. This corroborates the position of Olajide (2014) that experience contributes to farmers' ability to improve on their farm activities. Furthermore, the farming experience of respondents is likely to affect IS in SPP, as farmers with high farming experience are more likely to be broaden in knowledge and/or experience in their diverse crop production enterprises (including SPP), hence, have adequate involvement in SPP. Study by Ezeh (2013) found that farming experience is directly proportional to knowledge acquired in tackling farm production constraints, thereby, enhancing high involvement in farming. There was dominance of SP farmers with above one SP cycle per annum; implying considerable maximization of SP cycles possible for SPP in a year. There is dominance of SP farmers with at least 8 employees, suggesting and confirming the dominance of small scale operation of SP farming amongst the farmers. The monthly income from SPP suggests that most of the SP farmers earned above the monthly minimum wage (₦30 000.0) of civil servants in Nigeria. The implication is that SP farming can be a good source of income for FHs. Furthermore, the income earned from SPP is likely to affect involvement in SPP, as farmers earning considerably high income from SP are more likely to commit more effort and resources to SPP, hence, have high involvement in SPP.

The yearly income realized from other crops production is fairly high. This suggests that non-SP crops enterprises are good source of income for the SP FHs. The implication is that apart from SP enterprise, the respondents realized considerably income from other crops production. Income derivable by SP FHs from other crops production will not only serve to better enhance their living standards but also go a long way in determining if they will have adequate involvement in SPP. The yearly income from non-crop livelihood activities suggests that most of the SP producers were not involved in non-crop livelihood activities. The result also indicates that majority of the respondents were not involved in any livelihood activities except crop enterprises. The implication is that SP farmers were committed to SP and non-SP crop productions and took crop farming as their major livelihood activities.

The respondents cited limited knowledge of processing of SP, inadequate capital and lack of credit facilities as major barrier limiting their SPP. It has been observed that majority of the rural farmers lack alternative SP processing knowledge apart from boiling as well as the simple slicing and frying. This majorly constitute a huge hindrance in earning high income from SP as farmers are unable to add value to the crop. This finding is in consonance with Nwanebo (2012) who also emphasised lack of knowledge on processing of SP as one of the prominent constraints faced by SP farmers. Constraints to SPP due to limited knowledge of processing of SP could be as a result of no contact with extension agents. Contact with extension agents leads to more efficient transmission of information on improved practices and new technology or innovation to farmers (Adewumi and Adebayo, 2008) as well as

enhancing their knowledge level on processing technology. It is noted that inadequate capital and lack of credit facilities are among the constraints to respondents' SPP. The issues of inadequate capital and lack of credit facilities are consistently recurring constraints to SP farmers. Study by Odebode et al. (2008) pointed out that inadequate financial resources and lack of credit facilities are major hindrance to SP producers. Inadequate capital is expected to hamper respondents because high SPP required capital or money as farmers needed money to acquire large farm land and pay farm labourers, which is usually not available and they tend to seek for assistance from government and its agencies in this regard for funds. A closer look at the constraints to SPP showed that SP farmers had high constraints to SPP. The implication is that SP farmers in Niger-Delta Area of Nigeria had high constraints to SPP, which invariably might negatively affect involvement in SPP.

A closer look at the IS in SPP showed that SP farmers had relatively low IS in SPP in terms of SP farm size and employees. The result corroborates Nwanebo (2012) who found that more (59.7%) of SP producers were lowly involved in SPP. This suggest that the farmers were not channelling enough energy (committing themselves) and resources into their SP farming enterprise as they faced obstacles that prevent them from highly involving in SP farming business. This corroborate Samuel (2019) who reported that constraints faced by farmers affected their LI. The low involvement in SPP might be to poor access to extension service and poor demand of sweet potato (Nwanebo, 2012).

The relationship between gender of respondents and IS in SPP depict that SP producers' gender influence IS. It was also observed that more of the male respondents had high LI, while the female respondents had low LI, which suggests that male farmers were more involved in SPP than female farmers. This implies that the males are more poised to having high involvement in SPP. The result disagreed with Nwanebo (2012) who reported that female farmers had high LI in SPP than the male farmers. It is noted that males have more control over decisions, incomes, choices, assets and productive resources (such as land and credit facilities) than female farmers (Oyesola and Ademola, 2012), hence they will be more likely to have high IS in SPP. The positive correlation between household size and IS implies that the larger the household size, the higher the IS in SPP. Household size influence the cultivated size of SP farm, number of times SP is produced per annum, SPP experience and paid employees, which can shape IS. This may be due to the fact that household members supply available family labour on the SP farms, which can positively influence the SP hectrages as well as numbers of SP cycles per annum. Also, large household size increased the number of members of the household that is drawn as family labour to assist in SPP which could likely translates into more SP income, and by extension, more resources or ability to increase the number of paid employees. In addition, increase in household size make it easy for farmers to deploy family labour from their household size for SPP, hence, farmers will be able to engage in yearly production of SP, which can shape production experience. The result disagreed with Bawa et al. (2010) who observed no significant correlation between household size and IS.

The positive correlation between total farm size and IL in SPP indicates that the larger the total farm, the higher the LI in SPP. This implies that the total farm size which translates to scale of agricultural enterprise or production can affect the LI in SPP. It is expected that with increased total farm size, there is likely to be higher income from produced crops, which can shape IL in SPP. Furthermore, the total farm size determines the availability of land for SPP as well as size of land allocated to SPP which influence number of utilised paid labour, personal involvement in production activities and continuous production, hence, shape IL. The positive correlation between total farming experience and IS in SPP depicts that LI of SPP farmers increases as their total farming experience increases. The result supports Bawa et al. (2010), and Tijani and Sanusi (2019); they found a positive and significant correlation between farming experience and LI. The implication of this finding is that LI in SPP can be increased and/or enhanced through improvement in farming experience. Increased farming experience suggests increase in marketing and production experience, which increases derivable income from SPP that allows and motivates the farmers to have high involvement (by investing more time, energy and resources) in SPP.

The positive correlation between income from SP and LI in SPP implies that the IS of SP producers' increases as their incomes from SP increases. This suggests that an increase in SP income would result in an increase in LI in SPP. Income derivable from SPP can influence farmers IS in SPP as farmers deriving high income would likely be more motivated to highly involve than those with low income. Farmers earning low income from SP may for instance perceive the commitment and dedication

of resources into SP enterprise as an unprofitable venture. It is noted that as SP income increases, farmers will be motivated to address enterprise expansion and increase their personal involvement in production activities, which shape IS. More so, when farmers are assured of higher incomes following production, they will be more likely to highly involve in production. Farmers with high income from a particular farm enterprise, would be favourably poised to invest more time, energy and resources (farm size, farming experience and paid labour) in the enterprise, which can further sustain and/or improve their income.

The involvement in SPP was marginally low relative to high involvement, which can be improve upon. Factors that include gender, household size, farm size, farming experience and income from SP were features that influenced high involvement of farmers in SPP.

Based on the conclusion, the following recommendations are proffered for high level involvement in SPP Niger-Delta Area of Nigeria: Agricultural programme and policies oriented towards increasing involvement in SPP should be promoted to emphasize SP income *vis a vis* increased income from SPP. Special intervention programmes aimed at female producers of sweetpotato should be designed and implemented by various stakeholders to tackle the dominance of male over the female in IS in SPP. Sweetpotato farm size, production experience, cycle and number of labour should be rigorously targeted during intervention programmes for female producers of sweetpotato. All of these will increase the LI of the SP producers and increase the production of SP in Nigeria.

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Research Article

Morphological Characteristics and Chlorophyll Content of Dominant Weed Leaves After Peatland Fires in Oil Palm Plantation Areas

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Abstract: The weeds are high adaptability plants after peatland fires. The response of the dominant weeds growing after the fire is an important factor in weed control efforts to increase the yield of oil palm plantations on post-fire peatlands. The present study was conducted at the Kurao oil palm plantation, Lubuk Basung, West Sumatra, Indonesia. The weeds were collected by using the Quadrat method with random placement of 20 plots in post-fire and unburnt locations. The leaf morphological characteristics were determined by the descriptive method. While the chlorophyll content of leaves was measured by using calorimetry in the spectrophotometer. A total of 25 species and 17 families of weeds were collected in the present study. The *Peperomia pellucida* is the most dominant species collected with an important value index of 36.41% and follows by *Ageratum conyzoides* with an important value index of 28.99%. In the present study, we confirmed the differences in all aspects of the morphological characteristics of *Peperomia pellucida* leaves between post-fire and unburnt locations. Meanwhile, in *Ageratum conyzoides* leaves only show differences in several aspects. Furthermore, there were differences in the chlorophyll content of *Peperomia pellucida*, while *Ageratum conyzoides* did not show any differences in chlorophyll content.

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1. Introduction

Indonesia has a 14.9 million hectares of peatland area which is located in Sumatra, Kalimantan, Papua, Sulawesi, and on several small islands (Maftu'ah and Indrayati, 2013; Masganti et al., 2014; Maftu'ah and Nursyamsi, 2019; Reflinur et al., 2019; Yuwati et al., 2021). The peatland degradation will be increasing in the coming decade due to human activities such as fires, mining, etc. (Masganti et al., 2014; Wahyunto and Dariah, 2014; Nelson et al., 2021; Yuwati et al., 2021). Along with the increasing population and decreasing availability of plantation land, this has encouraged the conversion of peatlands into plantation land, especially oil palm plantations (Irma et al., 2018).

The oil palm (*Elaeis guineensis*) is the biggest cultivated plant commodity in Indonesia and has provided a very important role in the economy and country's development, as increasing demand for oil

palm products (Prasetyo and Zaman, 2016). One of the post-fire peatlands was converted into an oil palm plantation located in Kurao, Lubuk Basung, West Sumatra, Indonesia. The post-fire area is generally overgrown with weeds. The weeds are unwanted, harmful, dangerous, or economically detrimental plants (Mokoginta et al., 2021). Anthropocentrically, the term weed is applied to species growing around cultivated plants and is considered detrimental to the growth and yield of cultivated plants (Moenandir, 2010). Weeds as active allelopathic plants are shown its activities through allelochemical exudation (Jabran et al., 2015). Allelochemicals in weeds can interfere growth of surrounding plants slowing growth and germination due to competitiveness with cultivated plants in obtaining resources (Siyar et al., 2019). The presence of weeds as competitors for cultivated plants can reduce oil palm production by up to 20% (Barus, 2003). Based on these factors, weed management is very important in increasing agricultural potential, because uncontrolled weeds in agricultural landscapes can reduce agricultural yields (Sonmez and Alp, 2019; MacLaren et al., 2020; Mujdeci et al., 2020; Suleymanov et al., 2020).

The weeds that grow on post-fire peatlands have a high adaptability to extreme environmental stress. Therefore, weed control is necessary. In controlling weeds, information is needed regarding biological factors, such as morphological characteristics and chlorophyll content which will show its adaptability to fire. In general, plants can recognize and regulate their own organ and tissue activities in response to environmental changes (Sugiura et al., 2016). The response of plants will be seen in the appearance of plants as a form of adaptation to the environment. After the fire, it will affect the anatomy, morphology, and physiology of a plant. The occurrence of fires will cause environmental changes such as increased light intensity, increased evaporation of leaves, and decreased water content in the soil. The leaves as plant organs have an important role in adaptation to environmental changes in the strategy of temperature regulation against abiotic stress (Lin et al., 2017). The plasticity of leaves is important as a place for the process of photosynthesis and the exchange of materials and energy with the external environment. The plant adaptation strategy depends on internal physiology and external morphology formed by leaf responses to environmental changes (Wang et al., 2016).

In connection with the negative effects caused by weeds on the peatlands the plantation crops, the study of weeds by using the biological approach is important to understand the peatlands after fires. Nowadays, the information on the biological aspects of weeds is still inadequate and limited, especially in morphological characteristics and physiological responses, especially in the form of chlorophyll content from weeds that predominantly grow in oil palm plantations of post-fire peatlands. Therefore, the aim of the present study is to understand the response of morphological characteristics and chlorophyll content of weed leaves that predominantly grow after peatland fires in oil palm plantation areas.

2. Material and Methods

This study was conducted in January-April 2021, in the oil palm plantation of Kurao, Lubuk Basung, West Sumatra, Indonesia. The Observation plots were made using the Quadrat method by randomly placing 20 plots with a size of 1×1 meter to record all types of weeds found on 2 hectares of oil palm plantations.

The weeds that grow dominantly are known based on the following important value index equation:

$$\text{Density} = \text{Number of individuals}/\text{Sample plot area} \quad (1)$$

$$\text{Relative Density (\%)} = \text{Density of a species}/\text{Density of all species} \times 100 \quad (2)$$

$$\text{Frequency} = \text{Number of plots found of a species}/\text{Number of all plots} \quad (3)$$

$$\text{Relative Frequency (\%)} = \text{Frequency of a species}/\text{Frequency of all species} \times 100 \quad (4)$$

$$\text{Important Value Index (\%)} = \text{Relative Density} + \text{Relative Frequency} \quad (5)$$

The sampling of weed leaves was selected from two dominant weed species growing in post-fire and unburnt locations. Leaf samples were taken from 20 individuals of each species. Leaves are taken using shears, then labeled according to the sampling location. The observation and measurement

of leaf morphological characters are as follows: leaf length, leaf width, petiole length, and number of leaf vein branches. Then the samples were put into plastic bags and then put into jet fresh (a container filled with ice) and immediately taken to the Plant Physiology Laboratory (Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang) to examine the chlorophyll content.

For the preparation of leaf extracts, leaf samples were cut into small pieces and weighed at 1 gram. The sample pieces were crushed in a mortar and then extracted with 96% ethanol. After all the leaf chlorophyll pigment has dissolved (marked with white pulp), the chlorophyll extract is filtered with filter paper and put into a 100 ml volumetric flask and 96% ethanol is added until the volume of the extract reaches the limit of 100 ml. Furthermore, testing the chlorophyll content of the leaves using Calorimetry with a Spectrophotometer. Measurement of chlorophyll content was carried out by measuring the absorbance of the chlorophyll extract of weed leaves using a cuvette on a spectrophotometer at a wavelength of 649 and 665 nm. After that, chlorophyll was measured using the formula of Wintermans and de Mots (1965) as follows:

$$\text{Chlorophyll a (mg L}^{-1}) = 13.7 \times (OD665) - 5.76 \times (OD649) \quad (6)$$

$$\text{Chlorophyll b (mg L}^{-1}) = 25.8 \times (OD649) - 7.7 \times (OD665) \quad (7)$$

$$\text{Total chlorophyll (mg L}^{-1}) = 20.2 \times (OD649) + 6.1 \times (OD665) \quad (8)$$

Data on morphological characteristics and chlorophyll content of dominant weed leaves were analyzed using the t-test with a 5% confidence level.

3. Results and Discussion

3.1. The species of weeds collected in the post-fire of oil palm plantation

A total of 25 species of weeds were collected, and it belonging to 17 families and 3 303 individuals (Table 1). *Peperomia pellucida* and *Ageratum conyzoides* were the two dominant weed species found in the present study with an important value index of 36.41% and 28.99% respectively.

Table 1. Weeds in the post-fire oil palm plantation area and their frequency and density

Family	Species	Total number of individuals of species	Number of quadrats in which the species occurred	Frequency	Relative Frequency (%)	Density	Relative Density (%)	Important Value Index (%)
Acanthaceae	*Asystasia gangetica*	12	4	0.20	2.84	0.60	0.36	3.20
Asteraceae	*Ageratum conyzoides*	606	15	0.75	10.64	30.30	18.35	28.99
	Cyanthilium cinereum	3	1	0.05	0.71	0.15	0.09	0.80
	Elephantosus tomentosus	45	6	0.30	4.26	2.25	1.36	5.62
	Praxelis clematidea	520	8	0.40	5.76	26.00	15.74	21.42
	Syndrella nodiflora	2	1	0.05	0.71	0.10	0.06	0.77
Cleomaceae	*Cleome rutidosperma*	2	2	0.10	1.42	0.10	0.06	1.48
Cyperaceae	*Cyperus aromaticus*	220	6	0.30	4.26	11.00	6.66	10.92
	Cyperus escullentus	22	3	0.15	2.13	1.10	0.67	2.79
Dryopteridaceae	*Dryopteris flix-mas*	53	12	0.60	8.51	2.65	1.60	10.12
Euphorbiaceae	*Chamaesyce hirta*	11	1	0.05	0.71	0.55	0.33	1.04
Lomariopsidaceae	*Neprolepis cordifolia*	26	4	0.20	2.84	1.30	0.79	3.62

Table 1. Weeds in the post-fire oil palm plantation area and their frequency and density (continued)

Family	Species	Total number of individuals of species	Number of quadrats in which the species occurred	Frequency	Relative Frequency (%)	Density	Relative Density (%)	Important Value Index (%)
Lygodiaceae	*Lygodium circinatum*	3	2	0.10	1.42	0.15	0.09	1.51
Lythraceae	*Cuphea carthagenensis*	42	5	0.25	3.55	2.10	1.27	4.82
Melastomaceae	*Melastoma malabathricum*	24	7	0.35	4.96	1.20	0.73	5.69
Phyllanthaceae	*Phyllanthus niruri*	24	9	0.45	6.38	1.20	0.73	7.11
Piperaceae	*Peperomia pellucida*	898	13	0.65	9.22	44.90	27.19	36.41
Plantaginaceae	*Veronica montana*	42	3	0.15	2.13	2.10	1.27	3.40
Poaceae	*Axonopus compressus*	151	9	0.45	6.38	7.55	4.57	10.95
	Digitaria sanguinalis	194	7	0.35	4.96	9.70	5.87	10.84
Pteridaceae	*Adiantum capillus-veneris*	2	1	0.05	0.71	0.10	0.06	0.77
Rubiaceae	*Mitracarpus hirtus*	218	8	0.40	5.67	10.90	6.60	12.27
	Oldenlandia corymbosa	1	1	0.05	0.71	0.05	0.03	0.74
	Spermacoce alata	181	12	0.60	8.51	9.05	5.48	13.99
Verbenaceae	*Stachytarpheta indica*	1	1	0.05	0.71	0.05	0.03	0.74
Total		3303	141	7.05	100	165.15	100	200

3.2. Morphological characteristics leaves of dominant leaves

The morphological characters of *Peperomia pellucida* leaves showed the differences in all aspects between post-fire and unburnt locations. On the other hand, the *Ageratum conyzoides* showed differences in the aspects of leaf length, leaf width, and petiole length (Table 2).

Table 2. Average leaf length, leaf width, petiole length, and number of branches of *Peperomia pellucida* and *Ageratum conyzoides* leaf veins at post-fire locations compared to unburned locations in Kurao, Lubuk Basung

Species	Location	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Number of leaf vein branches
Peperomia pellucida	Post-fire	2.66[a]	2.21[a]	1.02[a]	5.50[a]
	Unburnt	2.21[b]	2.16[b]	0.80[b]	4.80[b]
Ageratum conyzoides	Post-fire	5.80[a]	3.76[a]	1.96[a]	7.00
	Unburnt	5.83[b]	4.33[b]	1.40[b]	7.60

Notes: Numbers followed by the same letter are not significantly different at the 5% significance level.

The difference in leaf size in post-fire location is a response of plants to the environmental changes which is very influential on the growth and morphology of leaves. The leaves collected at the post-fire location are bigger than the leaves from the unburnt location. Huang et al., (2007) explained that fires generally increase post-fire nutrient availability and plants can respond by increasing leaf nutrient concentrations. The morphological characteristics of leaves is changes in response to the long-term adaptation of plants to different light capacities of the photosynthetic process (Pandey and Sinha, 2009). The plants respond to light stress conditions by increasing the leaf surface area to capture more sunlight (Francis and Gilman, 2019). This theory is in accordance with the results of the present study which is the leaf morphological characteristics, are higher in post-fire locations compared to unburnt locations.

Inside the leaf veins there is a transport network that functions to transport food so that with an increase in the number of branches of the leaf bones, the activity of the xylem and phloem also increases. The xylem in the vascular tissue functions to transport water and nutrients, while the phloem functions to transport the results of photosynthesis (Chatri, 2013). The differences in leaf growth are also caused by differences in environmental conditions in post-fire and unburnt locations, which will affect the physiological growth of plants. According to Schulze et al., (2005), plant growth is strongly influenced by chemical and physical environmental factors which include temperature, humidity, light intensity, rainfall, and nutrients in the soil.

3.3. The chlorophyll content of the dominant weed leaves grew

There was a difference in the chlorophyll content of *Peperomia pellucida* between post-fire and unburnt locations. In the unburned location, the chlorophyll content was higher, namely 17.022 mg g^{-1} and in the post-fire location, namely 14.572 mg g^{-1}. In *Ageratum conyzoides*, the chlorophyll content between post-fire and unburnt locations was not shown any differences, the results can be seen in Table 3.

The results of the previous study by Vauzia et al., (2019) showed that the chlorophyll content of Jabon leaves (*Anthocephalus cadamba* [Roxb] Miq.) at two different locations showed significant differences, with the parameters of the light intensity factor and soil factor in the Nyalo river area, South Coast and Lubuk Alung area, Padang Pariaman. However, in *Peperomia pellucida*, post-fire environmental changes cause changes in chlorophyll content. At post-burn locations, the chlorophyll content is lower because fires cause higher light intensity. The higher the light intensity, the higher the chlorophyll content of plants, but on the other hand, too high a light intensity can also reduce leaf chlorophyll levels (Salisbury and Ross, 1995). At high light intensity, plants have thicker leaves and mesophyll cells, longer palisade cells, and more spongy cells (Wang et al., 2016).

Table 3. T-test results for chlorophyll content of 2 dominant species in post-peatlands fire and not burning in oil palm plantation areas

Sample	Location	Chlorophyll a	Chlorophyll b	Average total chlorophyll content (mg L^{-1})
Peperomia pellucida	Post-fire	7.420	7.027	14.57[a]
	Unburnt	9.324	7.550	17.02[b]
Ageratum conyzoides	Post-fire	16.856	14.976	32.17[a]
	Unburnt	15.438	16.987	32.69[a]

Notes: Numbers followed by the same letter are not significantly different at the 5% significance level.

While *Ageratum conyzoides*, it is not show any difference in chlorophyll content between post-fire and unburnt locations. We assumed that this condition was correlated with the ability of *Ageratum conyzoides* to survive in environments with high light intensity. This result is similar to the previous study of Ariningsih (2009) which stated that the *Ageratum conyzoides* lives by requiring sufficient light intensity and they grow rapidly. The pH of the soil is one of the environmental factors that influence this condition. In post-fire locations, the soil pH is usually lower than normal and we recorded the value as five in the present study. The pH value in the present study is higher than the previous study of Hartatik et al. (2011). Generally, the peat soils in Indonesia have acidic pH smaller than four, due to their high organic matter content (Hartatik et al., 2011). The higher value in soil pH at post-fire location in the present study was caused by residual ash from burning which can increase cation exchange so that it tends to increase the soil pH (Putri et al., 2017). At the unburnt location, the pH of the soil is close to normal (6.2), due to the treatment of unburnt location with some fertilizers to increase the pH and the productivity of oil palm. The previous study of Ramadhan et al. (2018) have find a similar condition and they assumed that the addition of Dolomite lime fertilizer will affect increasing the soil pH. However, the adaptation of plants to post-fire conditions on peat soils is very diverse and it depends on the abilities of each species. The previous study of the characteristics of stomata and chlorophyll contents of *Anthocephalus cadamba*, *Terminalia catappa,* and *Mallotus leucodermis* regenerating with sprouts and seeds after burning at peat swamp forest in Batang Alin have different regeneration

mechanisms and showed different response on environmental changes after burning (Vauzia et al., 2016; Des et al., 2021).

The chlorophyll content in *Peperomia pellucida* and *Ageratum conyzoides* have different responses to post-fire environmental conditions. *Ageratum conyzoides* was able to adapt and compete in physiological aspects better than *Peperomia pellucida*. This means that different plants will respond to different chlorophyll content in post-fire environmental conditions.

Conclusion

There were 25 types of weeds consisting of 17 families that grew on post-fire peatlands. The dominant species growing were *Peperomia pellucida* with an important value index of 36.41% and *Ageratum conyzoides* with an important value index of 28.99%. The morphological characters and chlorophyll content of *Peperomia pellucida* leaves showed differences between post-fire and unburnt locations, while the *Ageratum conyzoides* species did not show any differences in chlorophyll content and morphological characters in the aspect of the number of leaf veins. However, it showed differences in the aspects of leaf length, leaf width, and leaf stalks in post-fire and unburnt locations.

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Research Article

Evaluation of Sesame (*Sesamum indicum* L.) Lines Under Salt Stress for 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. The experiments were applied in sandy conditions with drip irrigation 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	78.5

±: 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.

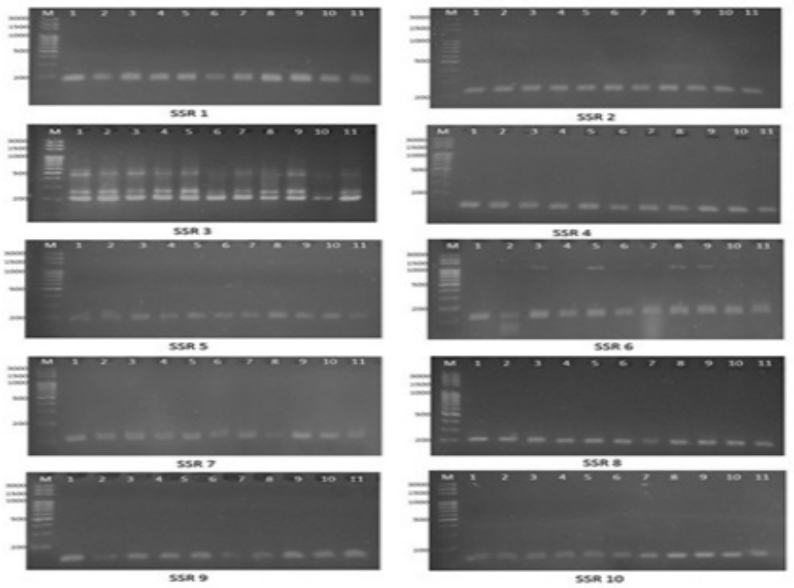

Figure 1. Amplification profile of SSR markers for eleven sesame genotypes.

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.

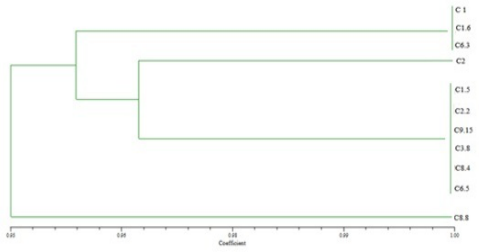

Figure 2. UPGMA dendrogram of the eleven genotypes based on the genetic similarity matrix.

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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Research Article

The Effect of Oregano (*Origanum onites*) Pulp to Quality Parameters of Meadow Silage

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Abstract: It was aimed to determine the effect of ensiling by adding Oregano pulp at different rates to the meadow grass on the physical quality properties of silages, silage fermentation and aerobic stability. In the study, dry oregano pulp at the rate of 3% (OP3) to 5% (OP5) by weight basis and without additives (control) was added to the first harvested of meadow grass in 2021 and it was ensiled as 3 groups. A total of 18 silage samples, 6 for each group, were left for fermentation in 1 liter glass jars for 60 days. Addition of oregano pulp in two different ratios decreased the DM content of silages compared to the control group ($p<0.05$). Control and silage groups with added oregano pulp were obtained in 2nd and 3rd roughage quality class, and their relative feed values were between 101.06 and 106.55. As a result of the physical properties of the silages, silages without additives were obtained as "satisfactory", while "good" quality silages were obtained from the groups with 3% and 5% oregano pulp. pH levels of silages were obtained between 4.37-4.89. The LA concentrates of the silage decreased significantly with the addition of oregano pulp, while the AA and BA levels of the silages were also decreased ($p<0.001$). As the ratio of oregano pulp increased from 3% to 5%, it was observed that the aerobic stability of the silages increased compared to the control group. As a result, it was concluded that oregano pulp can be evaluated as a silage additive.

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1. Introduction

Meadow and pasture forages are the most important roughage sources in our country. However, there are still significant quality problems in these areas due to problems such as harvesting and grazing (Özkan and Demirbağ, 2016). Making silage of meadow grass causes less nutrient loss compared to drying it. For this reason, it is reported that silages obtained from meadow grass provide significant savings in terms of both reducing labor and preventing nutrient loss (Akyıldız, 1986; Kaya et al., 2009).

The increase in animal production has led to the search for suitable feed additives as well as quality and cheap roughage sources from past to present. From additives, aromatic plants and/or essential oils obtained from these have important antimicrobial properties due to the active components

they contain, and are used as feed additives in both poultry and ruminant rations (Gladine et al., 2007; Cobellis et al., 2015). In recent studies, it is seen that natural aromatic plants or essential oils are used as silage additives (Kung et al. 2008; Hodjatpanah-Montazeri, 2016). As it is known, the purpose of using silage additives is to improve silage fermentation quality and allow ruminant animals to benefit at the maximum level (Filya 2000; Slottner and Bertilsson, 2006). It has been stated that the active ingredients of oregano herb have antimicrobial effects. It is due to the polyphenols in the antimicrobial in the plant, and the major active compounds are carvacrol and thymol (Mellencamp et al., 2011; Üstü and Uğurlu, 2018). Oil of oregano herb is also as important export source in Türkiye (Bozdemir, 2019). After oil is obtained from oregano herbs, the remaining pulp remains as a product processing waste (Abdollahzadeh et al., 2010). In addition, waste pulp, which can cause environmental pollution cannot be evaluated in terms of rich nutrient content. Oregano pulp, which is valuable in terms of its rich nutritional content and phenolic substances in its composition, is considered to be used as a silage additive in animal nutrition. The use of aromatic plant pulp as silage additives in animal nutrition is a new and current issue. There was limited research on the use of oregano itself and its essential oil as a silage additive, but no study was found, except for a single study on its pulp.

It has been reported that the pH levels of ensiled by adding thyme pulp to alfalfa and meadow grass changed between 4.34 and 4.62 and thyme pulp did not change the pH of the silages. In addition, It is reported that crude protein levels decreased, NDF and ADF levels increased, and thyme pulp changed silage microbiota population and their fermentation metabolites, and had many biologically active compounds such as polyphenols that could act as silage fermentation inhibitors. The researchers reported that the thyme pulp additive caused a certain increase in the acetic acid level in grass silage and the silage fermentation profile formed a heterofermentative fermentation as a result of the interaction between the phenolic compound and lactic acid bacteria (Aksu et al., 2017). In a another study using essential oil, oregano, cinnamon and their mixture were added to feed peas (400 mg kg^{-1}) and subjected to 60 days of fermentation. It was stated that the aerobic stability of silages was significantly improved on the 7th day of ensiling (Soycan-Önenç et al., 2015). The essential oils of mint, thymol, oregano and cinnamon were ensiled by adding 120 and 240 mg kg^{-1} DM to the corn forage. The chemical composition and aerobic stability of the silages after ensiling were determined. Accordingly, the contribution of oregano, cinnamon essential oils significantly decreased the NDF content of the silage compared to the control and increased the chemical composition and aerobic stability of the corn silage (Hodjatpanah-Montazeri, 2016).

This study was carried out to investigate silage fermentation, physical quality criteria of silages and especially aerobic endurance by adding oregano pulp, which is a waste material, to meadow grass in regions where oregano oil is grown in our country.

2. Material and Methods

2.1. Preparation of additive

Dry oregano pulp (*Origanum onites*) used as an additive was purchased from the producer company located in Antalya (İnan Tarım Ecodab). Then, the pulps were ground in a mill with a sieve diameter of 1 mm, and 3% and 5% on a weight basis was added meadow grass.

2.2. Preparation of silages

The feed material of the study was the first harvest of meadow forage grown in the meadow-pasture area in Edremit district of Van province. It is obtained from the natural meadow area where meadow grass, leguminous and grass grasses are grown mixed. After the meadow grass was harvested, it was cut into 2-4 cm sizes in Van Yüzüncü Yıl University Research and Application Farm and made ready for silage production. The meadow grass brought to the laboratory were made on nylon tarpaulin laid on a flat and clean surface, and was ensiled in 3 groups by adding 3% and 5% dry oregano pulp, no additives added to the control group. The silage samples were left to ferment for 60 days after being compressed in 1 liter glass jars with lids, 6 in each group, 18 in total, in accordance with the average weight standard of 950 g. Chemical composition of Oregano pulp and meadow grass are given in Table

Table 1. Nutrient composition of meadow grass and Oregano pulp before ensiling, DM%

Nutrient composition	Meadow grass	Oregano pulp
DM	25.70	93.91
Ash	11.13	7.99
CP	8.89	8.57
CF	1.82	1.89
NDF	50.27	41.23
ADF	36.19	31.89

DM: Dry matter; CP: Crude protein; CF: Crude fat; NDF: Neutral detergent fiber; ADF: Acid detergent fiber.

2.3. Physical analysis of silages

The physical properties such as color, odor and structure of the silages opened on the 60th day were determined by using the evaluation key recommended by the German Agriculture Organization (Alçiçek and Özkan, 1997). Flieg scores of silages were made according to the evaluation key reported by Kılıç (1986).

2.4. Chemical analysis of silages

At the end of fermentation, 25 g sample of each sample taken from the silages opened was mixed with 100 ml of distilled water and pH measurement was immediately made. Ammonia analysis of silages were determined by Kjeldahl distillation method immediately after pH measurement in silage samples (Markham, 1942). Then, the silage samples taken from each jar were dried in an oven at 65 °C for 48 hours to determine the dry matter content. Dried samples were ground in a mill with a sieve diameter of 1 mm and prepared for chemical analysis. Dry matter (DM), crude protein (CP) and ash contents of silages were determined according to the Weende analysis method (AOAC, 2000). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were analysed as described by Van Soest et al. (1991), and the crude fat (CF) content was measured according to ANKOM (2008) using an ANKOM XT15 device.

Phenolic compounds in dry oregano pulp were made according to the method specified by Singleton and Rossi (1965) and read in a gas chromatography device. Condensed tannin amount was determined spectrophotometrically according to the method reported by Makkar et al. (1995).

The relative feed value (RFV) index was used to determine the forage quality of the ensiled meadow grass by adding oregano pulp at two different rates. The RFV index, which is used as an important tool in the evaluation process of forage, was calculated according to Rivera and Parish (2010). In order to determine of RFV, first of all, the estimated digestible dry matter (DDM) amount was calculated over the ADF values of the silages. Depending on the body weight of the animal, dry matter intake percentage (DMI) was calculated based on the NDF value and the relative feed value was determined by replacing these values in the formula.

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

$$DMI\% = \frac{120}{NDF}\% \quad (2)$$

$$RFV = (DDM\%) * \frac{DMI\%}{1.29} \quad (3)$$

For volatile fatty acid (VFA) and lactic (LA) analyzes in silage liquids, the samples taken from the deep freezer were centrifuged at 3500 g for 15 minutes and then placed in the automatic sampler compartment of the high pressure liquid chromatography (HPLC) device to measure acetic, propionic, butyric and lactic acid measurements, and samples were made Van Yüzüncü Yıl University Science Research and Application Center (Leventini et al., 1990).

The aerobic stability test on silage samples was carried out according to Ashbell et al. (1991). The silages opened after the 60th day of ensiling were subjected to a 10-day aerobic stability test. For this, 250-300 g of fresh silage samples, 2 from each group, were taken and kept at room temperature for 10 days in a simple aerobic unit prepared. During this period, the aerobic endurance of the silages was

calculated with the help of the CO_2 amount measured after the titration with the precipitation of CO_2 gas.

2.5. Statistical Analyses

Statistical analysis of the data was carried out according to a completely random experiment design and was based on the following formula.

Analysis of variance was used to determine the relationships between the Oregano pulp additive and the physical, chemical and aerobic stability properties of silages, and Duncan's multiple comparison test was used SAS 9.4 to determine the differences (SAS, 2014).

$$Yij = \mu + ai + eij \quad (4)$$

$$\mu = overall\ average \quad (5)$$

$$Yij = i.level\ of\ Oregano\ pulp \quad (6)$$

$$ai = i.effect\ of\ level \quad (7)$$

$$eij = random\ error \quad (8)$$

3. Results and Discussions

The average phenolic compounds of oregano pulp used in the study were determined as 41.070 g kg^{-1}. Condensed tannin content in the samples was found to be 8.409 g kg^{-1} on average. The effect of ensiling the meadow grass with the addition of oregano pulp on the chemical composition is given in Table 2. Crude protein, CF, NDF and ADF contents of silages are not affected by oregano pulp additive, but differences between DM and ash contents were significant ($p<0.05$). The DM content of the silages decreased with the addition of oregano pulp at 2 different rates compared to the control group. It was observed that the DM content, which was 26.71% in the control, decreased to 23.94% with the contribution of OP3 and to 23.32% with the contribution of OP5. The ash content of the silages was observed to be higher in OP5 (15.80%) than the control (14.02%) and similar to OP3 (14.76%) on a DM% basis (Table 2; $p<0.05$).

Table 2. The effect of oregano pulp on the nutrient composition of meadow grass silage, DM%

	Groups of silages			
	Control	**OP3**	**OP5**	**p-value**
Item	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	
DM	26.71±0.53^{a}	23.94±0.66^{b}	23.32±0.45^{b}	0.0014
Ash	14.02±0.66^{b}	14.76±0.37^{ab}	15.80±0.10^{a}	0.039
CP	8.47±0.30	9.18±0.26	9.27±0.18	0.085
CF	1.58±0.32	1.87±0.15	1.65±0.17	0.650
NDF	52.16±0.88	53.05±0.75	50.66±0.83	0.148
ADF	40.19±0.50	40.24±0.53	39.76±0.31	0.718

There is a difference between the means in the same row and with different letters ($p<0.05$), OP3: meadow silage+dry oregano pulp of 3%; OP5: meadow silage+dry oregano pulp of 5%; DM: Dry matter, CP: Crude protein, CF: Crude fat, NDF: Neutral detergent fiber, ADF: Asit detergent fiber.

Soycan-Önenç and Turgud (2019) found that the DM contents of the silages did not change with the additive as a result of the addition of oregano essential oil to alfalfa silage, and the addition of lavender essential oil to alfalfa silage (Duru, 2019). In the study, although the silage material was ensiled and well preserved in the DM range recommended for silage feeds, DM loss occurred in the groups to which additives were added. This situation is thought to be related to the insufficient content of water-soluble carbohydrates in oregano pulp. It has been reported that feed materials are generally ensiled between 200-500 g kg^{-1} (Muck, 2010), and the higher the water-soluble carbohydrate (WSC) content of the silage material, the lower the dry matter loss that may occur during fermentation (Basmacıoğlu and Ergül, 2002). In terms of ash content, differences were found in the control and oregano pulp added groups ($p<0.039$). The use of tannin-rich gladia fruit in meadow silage was decreased the ash content

with the additive (Guven and Kalamak, 2021). It has been reported that the ash content of silages increased with the contribution of the gladia fruit to the sugar beet pulp (Özkan, 2012). In the current study, it was observed that 5% oregano pulp additive increased the ash content of silages compared to the control silage. It is thought that during the silage making of OP5 group, it is highly likely that sand, stone and especially dry branch pieces were mixed in the oregano pulp in large quantities. Örün and Erdoğan (2021) reported that the ash content may be an indicator of excessive amounts of mineral substances in the structure of the feed material or foreign substances (sand, stones, dry branches, etc.) mixed with the feed. In the study, CP, CY, NDF and ADF contents of the silages were not affected by the oregano pulp additive (Table 2).

In the study, the relative feed values of meadow silage did not change with the oregano pulp additive. In terms of forage quality class of silages, it is seen that 2nd quality silages from the OP5 group were obtained (Table 3). In the study, the relative feed values of meadow silage ensiled by adding oregano pulp varied between 101.06% and 106.55%. Ertekin et al. (2022) reported that the relative feed values in silages made with *Lolium multiflorum* L. varied between 78.1-88.2%. This result is quite lower than the study, and it was determined that the roughage quality class was at a good level in all silage groups in the our study. It is thought that is due to the fact that the plant composition of the meadow grass used in the study has a richer content in terms of legume grasses. When the results of the relative feed values are compared with the RFV of full bloom alfaalfa has a value of 100 (Rohweder et al., 1978), it is seen that the forage quality class is "2 and 3 quality". Indeed, Redfearn et al. (2006) reported that as the relative feed value falls below 100, the quality of the feed decreases, and if it is above 100, the feed value increases. All relative feed values obtained in the study were found above 100 (Table 3).

Table 3. Forage quality class and relative forage values of silages

Silage groups	N	DDM%	DMI%	RFV%	Forage class
		$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	
Control	6	57.59±0.39	2.30±0.04	102.84±1.65	3rd quality
OP3	6	57.55±0.42	2.26±0.03	101.06±2.05	3rd quality
OP5	6	57.93±0.24	2.37±0.04	106.55±2.07	2 nd quality
p-values		0.72	0.15	0.16	

There is a difference between the means in the same row and with different letters ($p<0.05$), OP3: meadow silage+dry oregano pulp of 3%; OP5: meadow silage+dry oregano pulp of 5%; DDM: digestible dry matter; DMI: dry matter intake; RFV: relative feed value.

The physical properties of the silages in terms of color, odor and stricture were examined. The total score of 14.67 in control group was 16.00 in the OP3 group and 16.83 in the OP5 group, and the highest total score was obtained from the OP5 silage. When the silages are examined in terms of quality classes, it is seen that satisfactory and good silages are obtained with the control and 2 different ratios of oregano pulp (Table 4). It was reported that the physical quality properties of silages prepared with the contribution to meadow grass of forage locust flakes increased (Atalay, 2015). It is stated that the silage quality criteria of lavender oil contribution to alfalfa forage were determined as satisfactory (Duru, 2019). Özturk et al. (2020) is reported that mixtures in hops with corn and forage soybean were obtained as "good" and "very good" silage quality classes. Our study is similar to these results.

According to the flieg scoring system were determined quality criteria of silages, and the highest flieg score was obtained from the control silage (83.75), while the lowest flieg score was obtained from the OP3 (57.42) silage. It is seen that the flieg scores of silages with 3% and 5% oregano pulp additive decreased compared to the control group (Table 5; $p<0.05$). As is known, the flieg score is calculated based on the pH and dry matter content of silages. In the study, it is thought that the decrease in the flieg score in the oregano pulp added meadow is related to the increase in pH compared to the control group and the decrease in the DM content in these groups. Gladicia fruit used as a silage additive did not cause a significant change in the pH of the wet sugar beet pulp, while the fleig score was slightly increased compared to the control (Özkan, 2012). Flieg scores of silages ensiled by adding thyme oil additive to sugar beet pulp did not change, and their flieg scores ranged between 90.81-98.33. Researchers have also reported that thyme oil can be added to silage as an additive if it balances the cost against losses due to mold and spoilage (Çayıroğlu et al., 2020).

Table 4. Scoring of the physical properties of oregano pulp added silages according to the method recommended by the German Agriculture Organization

	N	Smell	Stricture	Colour	Total point	Quality Class
Control	1	8	4	1	13	middle
	2	14	4	2	20	very well
	3	8	4	2	14	good
	4	8	4	1	13	middle
	5	8	4	2	14	good
	6	8	4	2	14	good
	Avg.	9	4	1.67	14.67	satisfactory
OP3	1	14	4	2	20	very well
	2	8	4	2	14	good
	3	8	4	2	14	good
	4	14	4	2	20	very well
	5	8	4	2	14	good
	6	8	4	2	14	good
	Avg.	10	4	2	16.00	very well
OP5	1	8	4	2	14	good
	2	14	4	2	20	good
	3	14	4	1	19	very well
	4	14	4	2	20	very well
	5	8	4	2	14	very well
	6	8	4	2	14	good
	Avg.	11	4	1.83	16.83	very well

OP3: meadow silage+dry oregano pulp of 3%; OP5: meadow silage+dry oregano pulp of 5%.

Table 5. Quality criteria of silage groups according to the flieg scoring system

	DM	pH	Flieg score
	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$
Control	26.71 ± 0.53^{a}	4.37 ± 0.18^{b}	83.75 ± 7.19^{a}
OP3	23.94 ± 0.66^{b}	4.89 ± 0.03^{a}	57.42 ± 2.20^{b}
OP5	23.32 ± 0.45^{b}	4.61 ± 0.13^{ab}	67.17 ± 5.70^{b}
p-value	0.0014	0.0393	0.0124

DM: Dry matter; OP3: Meadow silage+3% oregano pulp; OP5: Meadow silage+5% oregano pulp.

The successful completion of the fermentation process of the silage material is possible by following the silage making principles at the beginning. Muck (2010) reported that it is difficult to control the biological activity in the silo, therefore the ensiling process should be managed well and undesirable microorganisms may develop in silages where fermentation is not well managed. The results of the fermentation quality of the silage material ensiled with the addition of oregano pulp to meadow grass are given in Table 6. The pH levels of the silages were higher in the OP3 silage group than in the control group. The pH of meadow silage, which was 4.37 in the control, increased to 4.89 in the OP3 silage group (Table 6; $p<0.05$). The pH of the silages increased slightly in the OP5 silage group compared to the control group, but the difference was not statistically significant. In the study, it was seen that the additive of oregano pulp increased the pH of the silages. It has been reported that the pH levels of silages vary between 4.34 and 4.62 as a result of the contribution of thyme pulp to alfalfa and meadow grass, and thyme pulp does not change the pH of silages (Aksu et al., 2017). In a different study, it was reported that the addition of 400 mg kg^{-1} oregano oil (*Origanum onites*) to fresh peas did not change the silage pH compared to the control group, but the addition of cinnamon at the same level increased the silage pH (Soycan-Önenç et al., 2015). In our study, pH values of silages varied between 4.37-4.89. Kaya (2005) reported that the pH value varies between 3.8 and 4.8 in quality silages, while they reported that the Enterobacteria group microorganisms that cause deterioration are effective in the pH environment between 6-7 and lose their effectiveness at pH values below 5 (Filya, 2001). Therefore, it is possible to say that the pH range obtained in the study is in a pH range that may prevent the growth of unwanted microorganisms in the silo, and even within the recommended ranges. The increased pH

levels in the groups to which oregano pulp was added in the study can be explained by the fact that the oregano pulp did not have sufficient lactic acid content or had a lower amount of water-soluble carbohydrates compared to the control group. It has been reported that the rate of decrease in pH of silages with low water-soluble carbohydrate content is also slow (Merry et al. 1993). In addition, it is thought that phenolic compounds in oregano pulp may have realized a higher buffering capacity during silage fermentation.

The LA level, which was 14.51% in the control group, decreased to 1.35% in OP3 silage and 2.47% in OP5 silage (Table 6; $p<0.001$). As it is known, high LA level is desired in terms of silage fermentation quality. Alçiçek and Özkan (1997) reported that LA content should be over 2.0% in quality silages, Lorenzo and O'Kiely (2008) reported that LA levels should be 50-70 g kg^{-1} DM in quality silages. In the study, it is thought that the conversion of sugars to LA decreased in parallel with the insufficient content of water-soluble carbohydrates in the oregano pulp added groups. However, it can be thought that LA level was provided at a sufficient level to create a protective effect for the fermentation quality of the silages. It is seen that two different levels of oregano pulp additive significantly reduce the LA content of silages. With the realization of sufficient lactic acid production in silo feeds, the pH of the silage reaches the desired level. As a matter of fact, in the study, it was observed that the LA level decreased in the oregano pulp added groups, the pH levels of the silages increased. It has been reported that LA and enterobacteria levels of the silages did not change compared to the control, as a result of adding 0, 40 and 80 mg of essential oil to the feed per kg of wet weight on the quality of silage (Kung et al., 2008). In another study, it was reported that cumin (from *Cuminum cyminum* L.) stimulated the growth of Lactobacillus plantarum and lactic acid production (Kıvanç et al. 1991). Soycan-Önenç and Turan (2018) stated that the addition of 300 mg kg^{-1} cumin essential oil in the last harvest caused the cell membrane to break down by stimulating cell membrane-degrading enzymes. In the literature research, only one study was found on the use of thyme pulp additive as a silage additive. In this study by Aksu et al (2017), it was reported that dry thyme pulp used as a silage additive decreased the LA content in meadow silage group with 5% thyme pulp, and this result was found to be similar to the study conducted.

It was seen that the AA levels of the silages were decreased OP3 and OP5 compared to the control group (Table 6; $p<0.025$). It is observed that besides the production of lactic acid bacteria in the control group, some species belonging to the enterobacteria family also ferment water-soluble carbohydrates in the silo and cause acetic acid production, and so the addition of oregano pulp suppresses both LA and AA production. It is thought that this situation is caused by the antimicrobial effects of phenolic compounds in oregano pulp. Contrary to this situation, Aksu et al. (2017) reported that the addition of thyme pulp caused a certain increase in the acetic acid level in meadow silage and that the silage fermentation profile formed a heterofermentative fermentation as a result of the interaction between the phenolic compound and lactic acid bacteria.

In the study, the BA levels were decreased with the addition of oregano pulp compared to the control group. The BA level, which was 3.93% in the control group, decreased to 0.93% in the OP3 silage group and 2.22% in the OP5 silage group. In a similar study, it was stated that the fermentation quality of the silage varies according to the LA and BA content, and that good silage should contain high lactic acid content and low or no butyric acid content (Kiraz and Kutlu, 2016). Filya (2000) is stated that the use of water-soluble carbohydrates in silage by aerobic microorganisms initiates clostridial activity. It is known that high level of ammonia is formed by clostrida bacteria in the silo and thus is an important factor in the deterioration of silage quality. As a matter of fact, the NH_3-N level did not change in the study. In other words, it was observed that proteolysis did not occur in silages prepared by adding oregano pulp to meadow grass. It is thought that the decreased butyric acid level in the study is due to the phenolic substances in the oregano pulp. It has been reported that butyric acid bacteria are the most important competitors of acetic acid bacteria during silage fermentation, and butyric acid production causes significant loss of nutrients. It is stated that butyric acid bacteria either reduce or completely consume the nutrients they need by using the carbohydrates used by acetic acid bacteria, so butyric acid is not required in silages (Alçiçek and Özkan, 1997). When the fermentation properties of silages are evaluated in general, it is possible to say that the phenolic substances in the oregano pulp suppress the growth of desired microorganisms as well as unwanted microorganisms in the silage.

Table 6. Effect of oregano pulp additive on fermentation parameters of meadow silage

	Silage groups			
Fermentation parameter	**Control**	**OP3**	**OP5**	**p-value**
	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	
pH	4.37±0.18^{b}	4.89±0.03^{a}	4.61±0.13^{ab}	0.040
LA, DM%	14.51±1.14^{a}	1.35±0.09^{b}	2.47±0.30^{b}	0.001
AA, DM%	5.76±0.44^{a}	4.30±0.47^{b}	4.26±0.20^{b}	0.025
PA, DM%	5.04±0.42	4.52±0.34	4.36±0.31	0.391
BA, DM%	3.93±0.38^{a}	0.93±0.24^{c}	2.22”±0.38^{b}	0.001
NH_3-N, mg/100ml	0.20±0.02	0.29±0.03	0.30±0.06	0.208

There is a difference between the means with different letters in the same column ($p<0.001$); OP3: Meadow silage+3% oregano pulp; OP5: Meadow silage+5% oregano pulp; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid; NH_3-N: Ammonia.

Aerobic stability in silage feeds indicates the ability of oxygen-exposed silages to resist microbial growth (McDonald et al. 1991). Aerobic stability is one of the desired features in the silo, and it is reported that each silage has its own aerobic stability, and that this parameter is generally low in quality silages (Filya, 2004). In fact, after the silage is opened, it is inevitable that aerobic deterioration will occur in the silage (Çayıroğlu et al., 2016). However, the short duration of this period is very important for the animals to benefit from the silo feed to the maximum extent. In the study, the aerobic stability of meadow silage without oregano pulp was 1.75% in DM, and it was determined as 0.75% in the OP3 silage group and 0.35% in the OP5 silage group. As the ratio of oregano pulp increased from 3% to 5%, it was observed that the aerobic stability value decreased, that is, the durability of the silage increased (Figure 1). Kung et al. (2008) is stated that the effect of adding essential oil mixtures to corn silage on aerobic deterioration did not have an effect on the fermentation process of silages and aerobic deterioration. On the other hand, it has been reported that the contribution of cinnamon leaf, thyme and sweet orange essential oils in different amounts has a positive effect on 7 day aerobic stability in silage feeds (Chaves et al., 2012). Aksu et al. (2017) reported that in silages opened at the end of the 60 th day, the dry thyme pulp additive has a preventive effect on undesirable microorganisms such as enterobacter, yeast and mold without causing a decrease in the lactobacilli count of the silages compared to the control, and it can provide a very important advantage in terms of improving the aerobic stability of the silages. In a different study, it was stated that gladia fruit rich in phenolic compounds improves the aerobic stability of silos by reducing CO_2 production in silages (Canbolat et al., 2013). Filya et al. (2000) reported that the dry matter content of the ensiled material is an important factor affecting the aerobic stability and the aerobic stability of the silages made with the material with low dry matter content decreased. However, in this study, it was observed that aerobic deterioration was low in silages, despite a decrease in LA and AA levels of silages compared to the control group, and a decrease in dry matter content, especially in the OP5 silage group, in the groups to which oregano pulp was added. In the study, yeast and mold content was not detected. Therefore, it is very difficult to comment on this issue. However, in order to maximize the aerobic stability of the silage, it was thought that the risk of contamination with yeast and mold should be minimized during the harvest of the material to be ensiled. As a matter of fact, it can be predicted that the addition of oregano pulp at a level that will increase the durability of the oregano pulp in the silo may reduce the growth of yeast and mold.

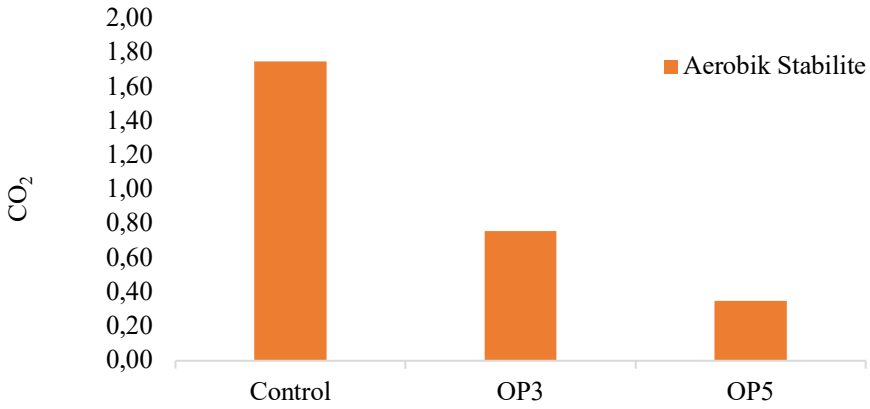

Figure 1. Effect of oregano pulp additive on aerobic stability of meadow silage (CO_2, g kg^{-1} DM).

Conclusion

As a result, DM content of silages ensiled by adding oregano pulp to meadow grass decreased compared to the control group, but in terms of forage quality class, 2nd quality feed value was obtained in the group in which 5% oregano pulp was added. It has been observed that good quality silages in terms of physical properties are obtained from silages that are ensiled with the addition of oregano pulp to the meadow grass. The NH_3-N level of the oregano pulp additive did not change and did not cause proteolysis in meadow silage. However, it decreased the LA levels of silages by suppressing the microbial fermentation in the silo in general. On the other hand, it was concluded that the incorporation of the pulp obtained after the distillation of oregano essential oils rich in phenolic compounds, which may accumulate and be waste into the meadow silage improves the sensory properties of the silages and increases aerobic endurance up to 5%. It is thought that waste pulp can be used as a silage additive in regions where oregano oil is obtained, especially in terms of increasing the aerobic quality of silages. In addition, it is thought that the use of oregano pulp as a silage additive together with water-soluble carbohydrate sources is important in terms of silage fermentation and aerobic stability and will contribute to future in vivo studies.

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Research Article

Fresh Semen Quality of *Bos taurus*, *Bos indicus* and *Bos sondaicus* Bulls in the Tropical Condition

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Motility,
Season

Abstract: This study aimed to assess the effect of genetic and seasonal interaction on semen quality in the Artificial Insemination Center. A total of 36,754 ejaculation records were evaluated. The dependent variable was semen quality which consisted of volume, pH, concentration, abnormalities, and motility of fresh semen. The independent variables consisted of age, season, and number of ejaculation. The mixed procedure with Tukey–Kramer multiple comparison test was used to analyze the effect of interaction between the variables. Seasonal factors significantly affected concentration, fresh semen motility, and abnormality, but did not affect pH and volume. Age factor had a significant effect on all semen quality variables. The ejaculation factor significantly affected pH, volume, concentration, and fresh semen motility, but did not affect abnormality. The species factor significantly affected pH, concentration, and fresh semen motility, but did not affect volume and abnormality.

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1. Introduction

Artificial insemination (AI) is still one of the technologies that play a very important role in optimizing the birth rate to fulfill the need for cattle commodities in Indonesia. Semen quality is one of reproductive potency which is the main factors in frozen semen production, where good quality semen also greatly influences the success rate of AI (reproductive performance) (Febriana et al., 2022).The quality of semen can be influenced by genetic and non-genetic factors including the environment and the technical aspects of semen storage. The age of the bull is one of the factors that has been frequently studied in various breeds of cattle (Snoj et al., 2013; Murphy et al., 2018; Konenda et al., 2020). Older bulls have semen with better quantity and quality than younger bulls (Ramajayan et al., 2023), although several studies have stated that there is an optimal point of semen quality at a certain age for bulls in each breed (Snoj et al., 2013). The effect of season on semen quality has been extensively studied in

several previous studies (Malama et al., 2017; Murphy et al., 2018; Ramajayan et al., 2023), but the results presented are contradictory.

Climatic conditions in each region are different, as well as the season, so season-based research needs to be carried out in certain areas with certain livestock. Livestock in the tropics is often characterized by variations in fertility for both males and females. This has been attributed to several external factors such as environmental factors, as well as internal factors such as genotype and their interactions (Landaeta-Hernández et al., 2020; Ramajayan et al., 2023; Tripathi et al., 2023). These factors are rarely considered to contribute to seasonal variations in male fertility (Landaeta-Hernández et al., 2020). Climatic conditions are not only different in each region but they are also known to change from time to time (Lee et al., 2016). Rainfall caused by climate change has increased, especially in the tropics. Increased rainfall intensity causes a longer rainy season period, resulting in a delay in the change of seasons. However, bulls in the tropics experience significant changes in semen quality as the seasons change. In the tropics, bulls with different genotypes also have different semen quality in each season (Landaeta-Hernández et al., 2020).

The mixed model in this study is a statistical model that has fixed effects (age and season) and random effects (name of the bulls). This model is useful in repeated measurements performed on the same statistical unit (longitudinal study), or in groups. Non-genetic and genetic factors have been studied both simultaneously and separately. Genetic factors can be seen in different breeds or species of cattle. In general, breeds represent certain characteristics at the lowest taxonomic level above individual genetic variation, while species include several breeds of cattle that have the same general characteristics and are located or originating in a particular region. Research that has been carried out generally focuses on one breed (Bhakat et al., 2014; Prastowo et al., 2019) and several breeds in the same species (Snoj et al., 2013; Konenda et al., 2020). Cattle commodities in Indonesia consist of three different species (*Bos sondaicus, Bos taurus, and Bos indicus*), and each species has different characteristics based on the environment of origin. The subject of this study was to assess the effect of genetic and seasonal interaction on semen quality in the Artificial Insemination Center.

2. Material and Methods

2.1. Ethical approval

Approval from the committee on the care and use of animals was not sought because no treatment and field experiments were conducted on animals.

2.2. Data collection

A total of 36,754 ejaculation records of 143 bulls were carried out by the Singosari National Artificial Insemination Center (SNAIC) of Indonesia. Five breeds included were Simmental, Limousine, Brahman, Ongole, grade, and Bali Cattle. The period of collecting data was between January 2018 and December 2021. The detail of the data used in this study is presented in Table 1.

The five parameters in the study were:

1). Volume (VOL) is the amount of semen in one ejaculation, calculated using a measuring cup with units of ml.

2). The pH is a degree of acidity for each ejaculation, tested by using Bromothymol Blue (BTB) indicator pH paper.

3). Concentration (CON) is the number of spermatozoa cells contained in semen, calculated by a spectrophotometer.

4). Abnormality (ABN) is the percentage of sperm cells that are considered not to have normal morphology.

5). Fresh Semen Motility (FSM) is the percentage of sperm cells that can move independently with a pattern a straight line or a large circle in fresh semen.

ABN and FSM were measured using Computer-Assisted Sperm Analysis (CASA).

2.3. Categories and Statistical Analysis

The bulls were grouped into three species: *Bos taurus* (Simmental and Limousine; n = 105), *Bos indicus* (Brahman and Ongole Grade; n = 17), and *Bos sondaicus* (Bali; n = 21). Age is the lifespan of

bulls to the time of semen collection, expressed in years. The age of bulls was categories to six groups: A1 (<3 years; n = 7848), A2 (≥3-<4 years; n = 10612), A3 (≥4-<5 years; n = 9208), A4 (≥5-< 6 years; n = 4621), A5 (≥6-<7 years; n = 1344), and A6 (≥7 years; n = 3121). The season was grouped based on the precipitation: low (rainfall 0 – 50 mm; n = 14259), medium (rainfall 51 – 150 mm; n = 13662), and high (rainfall 151 – 300 mm; n = 8833). The number of ejaculation was the frequency of taking semen from the same bull on the same day. The ejaculation used was the first ejaculation (n = 25137) and the second ejaculation (n = 10,151). The data was analyzed by using a mixed procedure of SAS On Demand for Academic (SAS, 2021). Species, age, season, and ejaculation were tested as fixed effects, then bulls were treated as a random effect. Tukey-Kramer multiple comparison was applied with a 5% significant level.

3. Results

The descriptive statistics for fresh semen quality from 36 715 ejaculates are presented in Table 1. The average of pH, VOL, CON, FSM, and ABN were 6.49; 5.77 mL; 1.16 10^9/mL; 77.12 and 5.39%, respectively. Bulls among species showed different quality of pH, CON, and FSM.

Table 1. Descriptive statistics of the data used in the study

Variable	Number	Mean	SE	Min.	Max.
pH	36715	6.49	0.01	6.00	7.60
VOL (mL)	36686	5.77	0.01	0.20	20.20
CON (10^9/mL)	35493	1.16	0.01	0.25	1.98
FSM (%)	35211	77.12	0.05	55.50	98.90
ABN (%)	24224	5.39	0.02	0.50	20.40

VOL, Volume; CON, Concentration; FSM, Fresh Semen Motility; ABN, Abnormality.

The significant values of bull traits can be seen in Table 2. The pH, CON, and FSM were affected by species. Age factor had a significant effect on all semen quality traits. Seasonal factors had a significant effect on CON, FSM, and ABN. The ejaculation factor had a significant effect on pH, VOL, CON, and FSM. The interaction between factors significantly affected semen quality traits, except pH, and ABN were not affected by the interactions species with the season, and species with ejaculation numbers, respectively.

Table 2. Type 3 tests of fixed effects for fresh semen quality

Factors	df	Traits									
		pH		VOL		CON		FSM		ABN	
		F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Species	2	10.42	<.0001	0.93	0.3947	10.84	<.0001	32.14	<.0001	1.43	0.2385
Age	5	7.48	<.0001	42.81	<.0001	43.86	<.0001	113.25	<.0001	7.93	<.0001
Season	2	0.45	0.6398	1.44	0.2376	27.43	<.0001	44.31	<.0001	6.38	0.0017
Ejaculation	1	37.71	<.0001	28.27	<.0001	175.34	<.0001	17.08	<.0001	1.34	0.2468
Species x Age	10	2.17	0.0168	3.40	0.0002	13.82	<.0001	15.62	<.0001	2.84	0.0024
Species x Season	4	2.09	0.0795	4.11	0.0025	2.60	0.0339	18.74	<.0001	2.74	0.0270
Species x Ejaculation	2	21.49	<.0001	11.33	<.0001	203.47	<.0001	159.03	<.0001	2.72	0.0659

VOL, Volume; CON, Concentration; FSM, Fresh Semen Motility; ABN, Abnormality.

Seasonal factors affected CON, FSM, and ABN ($p<0.01$), but did not affect pH and VOL. Age factor had a significant effect on all fresh semen quality. The ejaculation factor had a significant effect on pH, VOL, CON, and FSM.

As the age of bulls increased, the pH was declined but not statistically different across species. The pH of semen for *Bos taurus* was affected by season. pH was different among ejaculation in *Bos sondaicus* and *Bos taurus* bulls. pH of the first ejaculation was slightly lower than in the second one (Table 3).

Table 3. Interactions between species and age, between species and season, and between species and number of ejaculations for pH (mean ± SE)

Factors	Mean ± SE	Species		
		Bos indicus	***Bos sondaicus***	***Bos taurus***
Age				
A1	6.51 ± 0.01	6.48 ± 0.02^{bx}	6.63 ± 0.05^{cy}	6.51 ± 0.01^{bx}
A2	6.51 ± 0.01	6.47 ± 0.01^{bx}	6.53 ± 0.02^{by}	6.52 ± 0.01^{cy}
A3	6.50 ± 0.01	6.44 ± 0.01^{ax}	6.54 ± 0.02^{bcy}	6.51 ± 0.01^{ay}
A4	6.49 ± 0.01	6.42 ± 0.02^{ax}	6.53 ± 0.02^{by}	6.50 ± 0.01^{ay}
A5	6.48 ± 0.01	6.42 ± 0.02^{ax}	6.49 ± 0.01^{ay}	6.50 ± 0.01^{ay}
A6	6.48 ± 0.01	6.41 ± 0.05^{ax}	6.48 ± 0.01^{ay}	6.49 ± 0.01^{ay}
Season				
Low	6.50 ± 0.01	6.44 ± 0.01^{x}	6.52 ± 0.01^{y}	6.51 ± 0.01^{by}
Medium	6.49 ± 0.01	6.44 ± 0.01^{x}	6.53 ± 0.01^{z}	6.50 ± 0.01^{ay}
High	6.50 ± 0.01	6.44 ± 0.01^{x}	6.54 ± 0.01^{y}	6.51 ± 0.01^{by}
Ejaculation				
1	6.47 ± 0.01	6.44 ± 0.01^{x}	6.52 ± 0.01^{az}	6.47 ± 0.01^{ay}
2	6.52 ± 0.01	6.44 ± 0.02^{x}	6.54 ± 0.02^{by}	6.54 ± 0.01^{by}

^{a,b,c} Mean with different superscripts within a column are different ($P < 0.05$).
^{x,y,z} Mean with different superscripts within a row are different ($P < 0.05$).

The age of bulls had a significantly effect on VOL that increased with increasing Age. VOL in the season with low rainfall has the highest value compared to other seasons, except for *Bos sondaicus* which has a high VOL in the season with moderate rainfall. The first ejaculation tended to have a higher VOL than the second ejaculation, although in *Bos sondaicus* the difference was not significant. There was no significant interaction between species and the other factors for VOL (Table 4).

Table 4. Interactions between species and age, between species and season, and between species and number of ejaculations for VOL (mean ± SE)

Factors	Mean ± SE	Species		
		Bos indicus	***Bos sondaicus***	***Bos taurus***
Age				
A1	5.30 ± 0.14	5.34 ± 0.31^{a}	5.30 ± 0.50^{a}	5.28 ± 0.11^{a}
A2	5.59 ± 0.14	5.73 ± 0.30^{b}	5.40 ± 0.34^{a}	5.58 ± 0.11^{b}
A3	6.05 ± 0.14	6.49 ± 0.30^{c}	5.77 ± 0.34^{b}	6.01 ± 0.11^{c}
A4	6.24 ± 0.14	6.78 ± 0.31^{d}	6.21 ± 0.34^{c}	6.18 ± 0.12^{cd}
A5	6.22 ± 0.15	6.69 ± 0.32^{cd}	6.24 ± 0.33^{c}	6.11 ± 0.13^{d}
A6	6.27 ± 0.15	6.88 ± 0.36^{d}	6.28 ± 0.33^{c}	6.20 ± 0.14^{d}
Season				
Low	5.97 ± 0.14	6.44 ± 0.30^{b}	5.79 ± 0.33^{a}	5.92 ± 0.12^{b}
Medium	5.94 ± 0.14	6.29 ± 0.30^{a}	5.94 ± 0.33^{b}	5.88 ± 0.12^{a}
High	5.93 ± 0.14	6.22 ± 0.30^{a}	5.86 ± 0.33^{ab}	5.89 ± 0.12^{ab}
Ejaculation				
1	6.13 ± 0.14	6.44 ± 0.29^{b}	5.88 ± 0.33	6.09 ± 0.11^{b}
2	5.76 ± 0.14	6.19 ± 0.31^{a}	5.85 ± 0.33	5.70 ± 0.12^{a}

^{a,b,c,d}Mean with different superscripts within a column are different ($P < 0.05$).
^{x,y,z} Mean with different superscripts within a row are different ($P < 0.05$).

CON increased significantly with age in *Bos indicus* ($p<0.05$), whereas in *Bos sondaicus*, the highest CON was in the cattle group A5 and CON was highest for *Bos taurus* in the age group A6. The three species had significantly high CON in the season with low rainfall ($p<0.05$). CON in ejaculation 1 tended to be higher than in the second ejaculation, although in *Bos indicus* it was not significantly different. *Bos sondaicus* has a relatively lower CON when compared to *Bos indicus* and *Bos taurus* (Table 5).

Table 5. Interactions between species and age, between species and season, and between species and number of ejaculations for CON (mean ± SE)

Factors	Mean ± SE	Species		
		Bos indicus	***Bos sondaicus***	***Bos taurus***
Age				
A1	1.06 ± 0.02	1.15 ± 0.06^{a}	1.03 ± 0.08^{a}	1.09 ± 0.02^{b}
A2	1.01 ± 0.02	1.22 ± 0.05^{by}	1.09 ± 0.05^{abxy}	1.04 ± 0.02^{ax}
A3	1.09 ± 0.02	1.34 ± 0.05^{cy}	1.04 ± 0.05^{ax}	1.11 ± 0.02^{cx}
A4	1.16 ± 0.02	1.44 ± 0.06^{dz}	1.03 ± 0.05^{ax}	1.19 ± 0.02^{dy}
A5	1.21 ± 0.02	1.52 ± 0.06^{ey}	1.17 ± 0.04^{cx}	1.21 ± 0.02^{dx}
A6	1.25 ± 0.03	1.64 ± 0.07^{fz}	1.15 ± 0.04^{bcx}	1.31 ± 0.03^{ey}
Season				
Low	1.15 ± 0.02	1.42 ± 0.05^{by}	1.12 ± 0.04^{bx}	1.18 ± 0.02^{bx}
Medium	1.12 ± 0.02	1.36 ± 0.05^{ay}	1.07 ± 0.04^{ax}	1.15 ± 0.02^{ax}
High	1.12 ± 0.02	1.38 ± 0.05^{ay}	1.06 ± 0.04^{ax}	1.14 ± 0.02^{ax}
Ejaculation				
1	1.26 ± 0.02	1.40 ± 0.05^{y}	1.10 ± 0.04^{bx}	1.30 ± 0.02^{by}
2	1.00 ± 0.02	1.38 ± 0.06^{y}	1.07 ± 0.05^{ax}	1.02 ± 0.02^{ax}

^{a,b,c,d,e,f} Mean with different superscripts within a column are different ($P < 0.05$).
^{x,y,z} Mean with different superscripts within a row are different ($P < 0.05$).

FSM was significantly higher with increasing age ($p<0.05$) and then reached a maximum number in A6. FSM was significantly higher with increasing rainfall conditions ($p<0.05$) in *Bos sondaicus* and *Bos taurus*, whereas in *Bos indicus* FSM was not different among the season. FSM in the first ejaculation was higher except for *Bos taurus* where the second ejaculation was higher. Overall the highest FSM is *Bos taurus*, and the lowest is *Bos indicus* (Table 6).

Table 6. Interactions between species and age, between species and season, and between species and number of ejaculations for FSM (mean ± SE)

Factors	Mean ± SE	Species		
		Bos indicus	***Bos sondaicus***	***Bos taurus***
Age				
A1	70.32 ± 0.52	68.29 ± 1.23^{ay}	59.91 ± 2.66^{ax}	73.06 ± 0.40^{az}
A2	75.69 ± 0.52	73.00 ± 1.16^{by}	66.39 ± 1.54^{bx}	78.56 ± 0.40^{bz}
A3	77.04 ± 0.52	75.74 ± 1.17^{cy}	70.98 ± 1.48^{cx}	79.73 ± 0.40^{cz}
A4	77.73 ± 0.52	79.50 ± 1.23^{dy}	75.43 ± 1.52^{dx}	80.00 ± 0.42^{cy}
A5	80.88 ± 0.57	81.22 ± 1.35^{ey}	74.16 ± 1.44^{dx}	83.75 ± 0.53^{dy}
A6	85.38 ± 0.60	82.01 ± 1.63^{ex}	80.82 ± 1.40^{ex}	87.35 ± 0.64^{ey}
Season				
Low	77.10 ± 0.51	76.34 ± 1.19^{y}	68.99 ± 1.44^{ax}	79.88 ± 0.42^{az}
Medium	77.99 ± 0.51	76.94 ± 1.19^{y}	72.04 ± 1.44^{bx}	80.44 ± 0.41^{bz}
High	78.43 ± 0.51	76.59 ± 1.19^{y}	72.82 ± 1.44^{cx}	80.90 ± 0.42^{cz}
Ejaculation				
1	76.61 ± 0.50	77.77 ± 1.13^{by}	73.14 ± 1.42^{bx}	78.87 ± 0.41^{ay}
2	79.07 ± 0.51	75.48 ± 1.26^{ay}	69.42 ± 1.46^{ax}	81.95 ± 0.42^{bz}

^{a,b,c,d,e} Mean with different superscripts within a column are different ($P < 0.05$).
^{x,y,z} Mean with different superscripts within a row are different ($P < 0.05$).

ABN was significantly lower with age in *Bos indicus* and *Bos taurus* ($p<0.05$), whereas in *Bos sondaicus* they were not significantly different. ABN was lower in seasons with high rainfall in *Bos indicus* and *Bos taurus*, while there was no significant difference in *Bos sondaicus*. The ejaculation had no significant effect on ABN in *Bos indicus* and *Bos sondaicus*, while in *Bos taurus*, ABN in the second ejaculation was significantly lower ($p<0.05$). ABN between species did not differ significantly (Table 7).

Table 7. Interactions between species and age, between species and season, and between species and number of ejaculations for ABN (mean ± SE)

Factors	Mean ± SE	Species		
		Bos indicus	***Bos sondaicus***	***Bos taurus***
Age				
A1	6.22 ± 0.27	6.93 ± 0.62^{e}	-	5.88 ± 0.20^{d}
A2	6.12 ± 0.25	6.04 ± 0.53^{d}	6.60 ± 0.82^{b}	5.84 ± 0.19^{d}
A3	5.87 ± 0.25	5.54 ± 0.52^{c}	5.49 ± 0.72^{a}	5.62 ± 0.19^{c}
A4	5.52 ± 0.25	5.24 ± 0.54^{bc}	5.99 ± 0.69^{ab}	5.24 ± 0.20^{b}
A5	5.13 ± 0.27	4.66 ± 0.59^{bx}	6.33 ± 0.70^{by}	4.67 ± 0.26^{ax}
A6	4.71 ± 0.32	3.72 ± 0.72^{ax}	5.82 ± 0.65^{aby}	4.48 ± 0.43^{ax}
Season				
Low	5.76 ± 0.24	6.08 ± 0.52^{c}	5.81 ± 0.48	5.86 ± 0.20^{c}
Medium	5.68 ± 0.24	5.58 ± 0.53^{b}	5.92 ± 0.49	5.76 ± 0.20^{b}
High	5.34 ± 0.24	5.14 ± 0.53^{a}	5.90 ± 0.50	5.34 ± 0.20^{a}
Ejaculation				
1	5.76 ± 0.24	5.81 ± 0.49	5.71 ± 0.46	5.78 ± 0.19^{b}
2	5.43 ± 0.24	5.39 ± 0.58	6.04 ± 0.54	5.53 ± 0.20^{a}

^{a,b,c,d,e} Mean with different superscripts within a column are different ($P < 0.05$).
^{x,y,z} Mean with different superscripts within a row are different ($P < 0.05$).

4. Discussion

This study used a large number of ejaculation records regarding the evaluation of fresh semen from bulls of different species groups in one of the Artificial Insemination centers in Indonesia. Indonesia is a country that has a tropical climate. The average pH of fresh semen (6.49) in this study was in range of the previous studies reported pH of Bali Cattle ranged 6.47 – 6.57 (Prastowo et al., 2019) and 5.95 – 6.49 for Limousin cattle (Konenda et al., 2020), Affandhy et al. (2022) reported higher pH of fresh semen in Ongole cattle ranged 6.93 – 6.96.

The pH in this study was significantly influenced by age and tends to decrease in older bulls. This result is agreed with a previous study that reported the age of the bulls was affected the pH of fresh semen (Prastowo et al., 2019). Seasonal differences in this study did not affect the pH of fresh semen. This is in accordance with the explanation in the study of Konenda et al. (2020). Bhakat et al. (2015) reported that seasonal differences in pH may occur due to changes in feeding in certain seasons.

There was an increase in the VOL of semen produced along with the age of the bulls in each of the studied species. This is consistent with the results of studies on Limousine bulls (Konenda et al., 2020), Friesian Holstein (Murphy et al., 2018), and Czech Fleckvieh (Paldusová et al., 2016). The results of a study on Murrah buffalos showed that the best semen (including ejaculate volume) was produced by bulls over 12 years of age (Ramajayan et al., 2023). The increase in age associated with the increase in scrotal circumference and the volume of semen produced (Mahmood et al., 2014) can also be caused by an increase in testosterone (Konenda et al., 2020). The decrease in VOL that **is** influenced by the frequency of ejaculation is following the study by Sitanggang et al. (2020) that the frequency and interval of ejaculation greatly affect the characteristics of semen in Bali cattle.

Based on the results of this study, age had an effect on CON in *Bos indicus*. The increase in CON along with the age of the bulls contradicts the research conducted by Konenda et al. (2020) which showed that the highest concentration was found in bulls aged 2 years and the lowest in bulls aged 8 years. Increasing bulls' age has a negative correlation with spermatozoa concentrations (Mahmood et al., 2014), which may be possible due to the reduced function of bull reproductive organs in spermatogenesis (Vince et al., 2018). This study showed that there was a positive correlation between age and sperm concentration in *Bos indicus*. The CON in *Bos sondaicus* and *Bos taurus* in this study was in line with (Murphy et al., 2018) which shows CON after more than 1 year of age was constant, while the total number of spermatozoa increases with age. The increase in the total number of spermatozoa was driven by an increase in VOL of ejaculation until the age of 4 years.

This study showed a significantly higher CON in the season with low rainfall in each species. The results agreed with previous studies where sperm concentration and total sperm count were greater in the season with low rainfall (Snoj et al., 2013; Murphy et al., 2018). The ejaculation had a significant

effect on CON, where CON was higher in the first ejaculation than in the second ejaculation. These results were similar to a previous study, where the first ejaculation for bulls had a higher sperm concentration and total sperm count than the second ejaculation taken on the same day (Ramajayan et al., 2023).

FSM in this study tends to increase as increasing age of bulls in each species. Although the optimal point of sperm motility value has not been found in this study, several previous studies reported that the age factor had a significant effect on sperm motility (Boujenane & Boussaq, 2013; Sitanggang, 2018; Suyadi et al., 2020). The effect of seasons on FSM was consistent with previous studies on Simmental Bulls (Hapsari et al., 2022), Limousine Bulls (Konenda et al., 2020), Thapkar Bulls (Perumal et al., 2017), and Karan Fries Bulls (Bhakat et al, 2014). Other studies suggest a link between temperature and the continuity of spermatogenesis in terms of the effect of season on sperm motility (Konenda et al., 2020), where heat stress can reduce sperm motility (Selçuk et al., 2014). The increasing temperature resulted in a decrease in sperm motility when spermatozoa were in the epididymis (Konenda et al., 2020).

This study showed that the number of ejaculations has a significant effect on FSM where FSM in the first ejaculation was higher than in the second ejaculation. The process of spermatogenesis in the second ejaculation may not be perfect because of the difference in the short time lag when compared to the first ejaculation. Long intervals of semen collection resulted in better semen qualities (Boujenane & Boussaq, 2013; Murphy et al., 2018). A possible explanation for lower semen quality related to ejaculation frequency is the shorter meeting interval of the second ejaculation (Murphy et al., 2018).

ABN in this study was significantly influenced by season, where the highest ABN was found in seasons with low rainfall. This result agreed with the analysis of Quezada-Casasola et al. (2016) where the percentage of defective sperm was higher in European bulls during the summer and fall. Age significantly affects the ABN of *Bos indicus* and *Bos taurus*, where the abnormality tends to decrease with the increasing age of bulls. This result is in contrast to the results of the study where abnormalities increase with the increasing age of the bulls (Baharun et al., 2021). This is possible because this study used the age group from 3 to 7 years, whereas the results of the study by Baharun et al. (2021) stated that although abnormalities increased with age, semen abnormalities in the 4-5 and 6-7 age groups did not experience a significant increase. Based on this study, what might happen was as bulls mature or enter puberty, the scrotal circumference will also increase where Quezada-Casasola et al. (2016) agree, if bulls do not maintain their scrotal circumference, the result will be lower-quality sperm production.

Based on the results of this study, Bos sondaicus, which is a species native to Indonesia, showed a much higher pH than Bos indicus but was not significantly different from *Bos taurus. Bos indicus* has a much higher CON than other species, this may be because Bos indicus itself is a domestic species originating from the Indian continent which is a tropical area like Indonesia. *Bos indicus* has a higher concentration of semen, according to the results of a study by Landaeta-Hernández et al. (2020) when compared with Bos taurus. The highest FSM in this study was shown by the Bos taurus species with a significant difference when compared to other species. Despite originating from the subtropics, Bos taurus shows a high level of adaptation in the tropics (Landaeta-Hernández et al., 2020; Staiger et al., 2018). The result of this study showed that the concentration, abnormality, and motility of fresh semen were significantly affected by seasonal factors. The age factor significantly affects all semen quality variables observed in this study. The number of ejaculation significantly affects the pH, volume, concentration, and motility of fresh semen. The species factors present in this study showed a significant effect on pH, concentration, and motility in fresh semen.

Conclusion

This study shows that each bull with a different species gives a different response of semen quality to age, season, and ejaculation factors. Based on this study, species had a significant effect on the pH, concentration, and motility of fresh semen. These results indicate that genetic factors (species) also play a role in determining bull reproductive traits.

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Declaration of Competing Interest

The authors have no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Research Article

Determination of the Factors Affecting the Honey Production Per Coloney in Bingöl Beekeeping Enterprises

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Number of hive,
R^2 and adjusted R^2

Abstract: This study was carried out to determine the factors affecting the honey yield per colony in beekeeping enterprises in Bingöl province by regression analysis. The number of enterprises to be surveyed with the "proportional sampling method" was determined as 87 in the province of Bingöl. "T test", "Anova", "Chi-square", "Correlation and Regression" analyzes were used to evaluate the data. According to the research findings; the average age of the surveyed beekeepers was 46.14 years and the average beekeeping period was 15.5 years. The average number of hives owned by beekeepers was 219.5, while the average honey yield per beehive was calculated as 11.4 kg^{-1}. The coefficients of the variables in the model were found to be statistically significant. The R^2 value, which determines the explanatory power of the model, was found to be 0.323 and the adjusted R^2 value was found to be 0.203. The way beekeeping is done, the profession and the variables of getting information from PIKOM are determined as the variables that affect the honey yield. As a result; in conclusion; it has been concluded that there is an increase in honey yield per colony of the enterprises that are made by wandering beekeeping, beekeeping is done for the main income, the Caucasian bee race is used, the enterprises that receive training, support and information about beekeeping from picom and that are members of the union and that produce queen bees.. Considering Türkiye's ecological richness and existing rural economic conditions together, beekeeping; it should be done in an organizational, conscious and sustainable structure.

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1. Introduction

Beekeeping is a significant agricultural endeavor, particularly for the low-income settlements located in or near forests that have limited or no land. Also, it has socioeconomic significance in that it doesn't require additional labor or capital, is an agricultural activity that anybody can engage in, makes the best use of the family workforce, and produces money quickly (Küçük et al., 2022). Beekeeping is a significant agricultural activity, as evidenced by its low capital requirements, ability to generate the

farmer's primary and/or secondary source of income, role in the production of foods high in nutrients, contribution to alternative medical practices, assessment of unprocessed agricultural areas, and environmental sustainability due to its role in pollination (Günbey, 2007; Kızılaslan and Kızılaslan, 2007; Uzundumlu et al., 2011; Karakaya and Kızıloğlu, 2015; Aksoy et al., 2017; Terin et al., 2018; TEPGE, 2021). Türkiye is very fortunate to have the natural resources needed for beekeeping because of the following reasons.

- High honey yield,
- Existence of large flora areas,
- Experiencing the seasons suitable for flowering throughout the year,
- The existing topographic structure,
- Cultivation of common fruit species such as almonds and citrus,
- Cultivation of industrial plants such as cotton and sunflower,
- Owning high plateaus,
- Presence of meadows and pastures sufficiently,
- Developed pulse fields and forage crops cultivation,
- Many different types of trees and various scrubs (such as chestnut, acacia, linden, oleaster, eucalyptus, rhododendron) and pine forests.

The type and quantity of honey are also greatly influenced by the presence of such a diverse range of flora. Beekeeping has been one of Anatolia's oldest and most widely practiced production branches as a result of these regional characteristics (Burucu and Gülse Bal, 2017). The Turkish Statistical Institute (TUIK) reports that 96.344 tons of honey were produced in Türkiye in 2021. The results show that Türkiye now has 8984676 colonies, a rise of 2.87 percent over the previous year. The yield of honey increased by 19.40% from the previous year to 13.17 kg colony^{-1} (Table 1).

Table 1. Statistics on beekeeping in the world and Türkiye

Beekeeping statistics (2021)	Number of colonies (pieces)	Honey production (tonnes)	Honey yield (kg colony^{-1})
World	101624052	177194436	17.44
Türkiye	8733394	96344	11.03
Türkiye (2022)	8984676	118297	13.17
Index (2021=100)	102.87	122.78	119.40

In 2021, Türkiye will have a total of 8.7 million beehives. With 949 thousand hives, Muğla takes the top spot with a 10.8% part of all the beehives in Türkiye. Ordu is in second place with 604 thousand hives and a 6.9% stake, and Adana is in third place with 481 thousand hives. With a ratio of 5.5, it is ranked third. On the other hand, the province of Bingöl comes in at number thirteen with 157 thousand hives, or 1.8% of all the hives in Türkiye (Kadiroglu, 2022). When examining the province-based honey output, Bingöl has a 52.4% share of the total 1724 tons of honey produced in the TRB-1 region in 2021, placing it first overall. In 2021, the province of Bingöl produced 11 kg^{-1} of honey (TEPGE, 2022).

It has been concluded in many previous studies that honey yield should be increased in order for the beekeeping activity in Türkiye to reach the real value in the world (Çeliker, 2002; Fıratlı et al., 2005; Soysal et al., 2005; Kekeçoğlu et al., 2007; Parlakay et al., 2008; Sezgin and Kara, 2011; Uzundumlu et al., 2011; Aksoy and Öztürk, 2012; Söğüt et al., 2019a; Söğüt et al., 2019b). As a result, regression analysis was used in this study to identify the variables influencing honey yield per hive in beekeeping operations in the Bingöl province. The study's findings are believed to be crucial in illuminating the research that must be done in order to boost honey yield in Türkiye.

2. Material and Methods

In order to conduct this study, primary data were collected in March 2021 from 87 producers who were members of the Bingöl Provincial Beekeepers' Association. The provincial and district directorates of agriculture, online resources, general knowledge gathered from domestic and international sources, and relevant statistical data served as the study's secondary sources. By using the proportional sampling technique, sample volume was calculated (Newbold, 1995).

$$n = \frac{Np(1-p)}{(N-1)\sigma_{\hat{p}x}^{2} + p(1-p)} \quad (1)$$

Where;

n: Sample volume, N: Number of businesses in the population, p: the ratio of producers who have sufficient knowledge about beekeeping, (0.50 taken to reach the maximum sample volume), α_{px}^2: It gives the variance (0.0026).

Throughout the province, there are 857 beekeepers that are members of the union. 87 were found to be the sample size, with a 90% confidence range and a 10% error. Due to missing data in one questionnaire, 95 questionnaires were created, of which 94 were examined. This is a 10% increase in the total number of questionnaires. T.R. Bingöl University Scientific Research and Publication Ethics Committee has decided that it is suitable for research ethics for the study with the number 92342550/044/8375.

2.1. Linear regression analysis

Regression analysis uses data from any source to investigate the connection between the dependent variable and the independent variable or variables (Kutlar, 2009). Linear regression is used when there is a linear relationship between the variables. There is just one independent variable in the simplest linear model. According to this model, whether the value of the independent variable rises or falls, the real mean of the dependent variable changes at a constant pace (Oztürk, 2013). "Typically, x is used to represent the independent variable. It is the (explanatory) variable that is the cause of y or is considered to effect it, but is unaffected by any other variables. The standard symbol for the dependent variable is y. It is the variable that, depending on the variable X, can change or be influenced (explained). It's odd how many dependent variables there are. There may be more than one independent variable, though. In a simple linear regression analysis, there is only one independent variable (Anonymous, 2012; Oztürk, 2013). The independent variables listed below contribute to an explanation of honey yield per hive in the beekeeping industry.

$$V = f(ES, AGE, PE, BMP, P, RACE, NEH, QBRP, BW, ES, MS, SS, PIKOM, QBP) \quad (2)$$

In the equation: ES: Educational Status (years), AGE: Farmer age (years), PE: Professional Experience (years), BMP: Beekeeping Main Profession (Yes:1, No:0), P: Purpose (main livelihood:1, others:0), RACE: Race used (other races:1, caucasian:0), NEH: Number of Existing Hives (pieces), QBRP: Queen Bee Replacement Period (years), BW: Beekeeping Way (Migratory:1, Stationary:0), ES: Education Status (Yes:1 No:0), MS: Membership Status: (Yes:1 No:0), SS: Support Status: (Yes:1 No:0), PIKOM: The status of getting information from Pikom (Yes:1 No:0), QBR: Queen Bee Rearing (Yes:1 No:0)

The regression equation of the model is given below.

$$V=\beta_0+ESX_1+AGEX_2+PEX_3+BMPX_4+PX_5+RACEX_6+NEHX_7+QBRPX_8+BWX_9+ESX_{10}+MSX_{11}+SSX_{12}+PIKOMX_{13}+QBRX_{14}+\epsilon \quad (3)$$

2.2. Correlation analysis

It is a statistical technique used to establish the existence of a linear relationship between two numerical data and, if so, its strength and direction. If the data is regularly distributed, the "Pearson correlation coefficient" is used; otherwise, the "Spearman Rank correlation coefficient" is used. The p value needs to be lower than 0.05 in order to be considered as a correlation coefficient. If the correlation coefficient is negative, the two variables are said to have an inverse relationship, which means that when one variable rises, the other falls. It is said that "when one variable increases, the other increases" if the correlation coefficient is positive.

The following values are taken into account when interpreting the correlation coefficient (r). “If r<0.2, very weak correlation or no correlation between 0.2-0.4 weak correlation. Moderate correlation between 0.4-0.6 High correlation between, 0.6-0.8 0.8> is very high correlation” (Tatlıdil, 2008).

3. Results and Discussion

It can be seen that the breeders surveyed have a high average age (Table 2). The average number of available hives is 219.5, and the professional experience of the surveyed producers is 17.3 years. It was discovered that 63% of the breeders were migratory beekeepers, the average queen bee replacement period was three years, and 44% of the breeders did not work in beekeeping. The average honey yield per hive was 11.4 kg^{-1}, and the average individual education period was 9.11 years. It has been determined that half of those polled are beekeepers because it is their source of income. 82% of the individuals produced with Caucasian bees, 61% received beekeeping training, 89% were union members, 83% received beekeeping support, 29% received picom information, and 56% were in the main It was determined that they were raising bees. Uzundumlu et al. (2011) discovered in Bingöl that the average age of the beekeepers surveyed was 50.8 years old, the average number of hives was 115, the average honey yield was 16 kg $hive^{-1}$, and 51% of the beekeepers were wandering beekeepers. In a study conducted by Öztürk (2013) in Ordu province, the average age of beekeepers was 48.7 years, the average education level was 7.55 years, the average professional experience was 23.7 years, and the average queen bee replacement period was 1.75 years. It has been determined that 55% of beekeepers work outside of beekeeping, 79% are the primary source of income, 79% produce with Caucasian bees, and the average number of hives is 263.7. The average professional experience in the study conducted by Şeviş (2018) in Bingöl was 18 years, the yield was 11.12 kg^{-1}, the average age of the beekeepers was 47, the number of hives was 133, and the average queen bee replacement was 2 years. According to the same study, 84% of beekeepers were traveling beekeepers, 38% were solely beekeepers, 51% were their primary source of income, and 67% were producing with the Caucasian bee race. The study's findings are partially consistent with the findings of other literature reports.

Table 2. Description of variables and statistical summaries

Variables	Mean	Standart deviation
Honey Yield (kg $colony^{-1}$)	11.41	4.719
Age (years)	46.14	12.221
Professional experience (years)	17.35	8.528
Number of existing hives (pieces)	219.55	171.357
Queen bee change (year)	3.00	0.927
Beekeeping style (Traveler=1; Fixed=0)	0.63	0.486
Education level (years)	9.11	4.573
Is beekeeping the main occupation? (Yes=1; No=0)	0.56	0.499
Purpose of beekeeping (Main livelihood=1; Others=0)	0.50	0.503
Bee race (Caucasian=1; Others=0)	0.82	0.387
Education about beekeeping (Yes=1; No=0)	0.61	0.491
Status of being a member of a beekeeping association (Yes=1; No=0)	0.89	0.310
Status of receiving support for beekeeping (Yes=1; No=0)	0.83	0.378
Status of getting information from PIKOM (Yes=1; No=0)	0.29	0.455
Queen bee production status (Yes=1; No=0)	0.56	0.499

Table 3 shows the correlation values for the relationship between the dependent variable and the independent variables. If the correlation between the independent variables is 0.80 or higher, this is considered an indicator of the multicollinearity problem (Kalaycı, 2014; Çevrimli, 2017). When the correlation table was examined, it was determined that there was no high correlation between the independent variables and that the regression model created did not have a multicollinearity problem. Furthermore, significant relationships were discovered between the dependent variable (yield) and the independent variables (experience, beekeeping type, membership and race). There is no significant or very significant correlation between the dependent variable and the independent variables, or between the independent variables. The strongest correlation was found to be a moderately positive relationship between beekeeping support and union membership. Examining the tolerance and variance

magnification ratio VIF (Variance Inflation Factor) values is one method for determining whether there is a multicollinearity problem. In general, if the VIF criterion is greater than 10, it is assumed that the independent variables have a serious multicollinearity problem (Akdi, 2011; Gazibey et al., 2012). Furthermore, VIF and tolerance values greater than 10 and less than 0.2, respectively, indicate a multicollinearity problem (Gujarati, 2004; Tatldil and Ortunç, 2011; Gazibey et al., 2012). According to the results of both VIF and tolerance values for the variables in the model, it was determined that there was no multicollinearity problem in the study (Table 4). Figures 1, 2, and 3 show that the regression model's errors have a normal distribution and that there is no covariance problem in the model. Figure 4 depicts the visual of the multiple regression model.

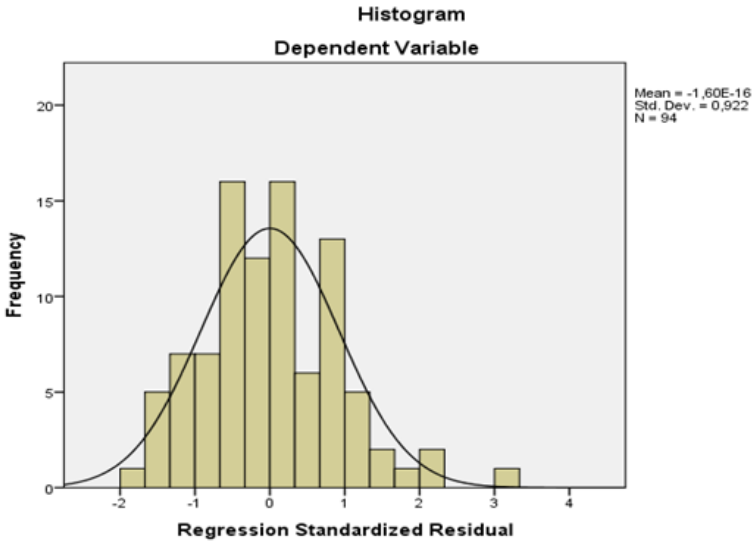

Figure 1. Graph of the normal distribution of errors.

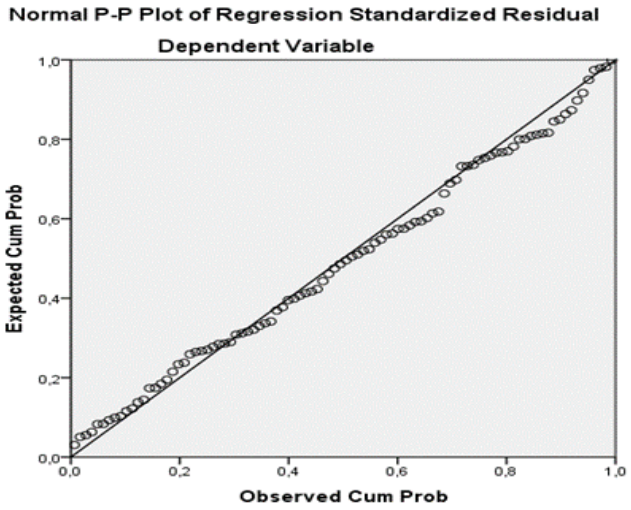

Figure 2. Normal distribution of errors.

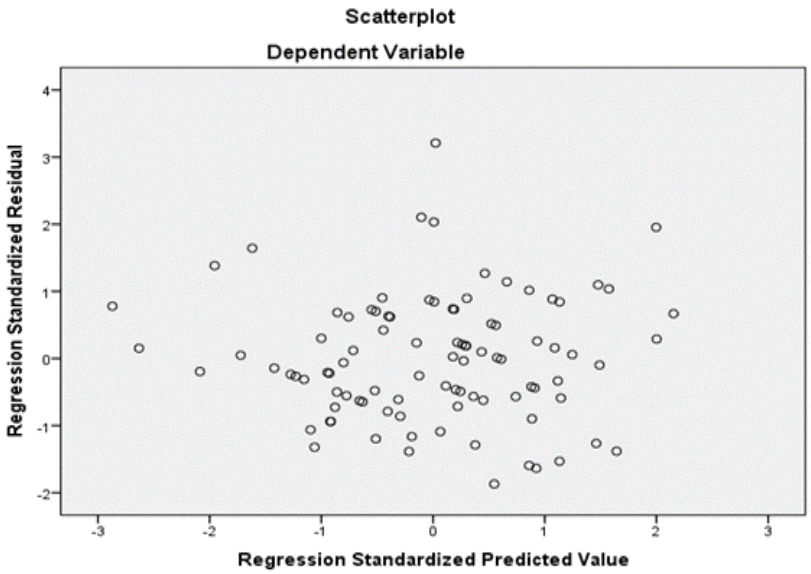

Figure 3. Graph of the covariance assumption.

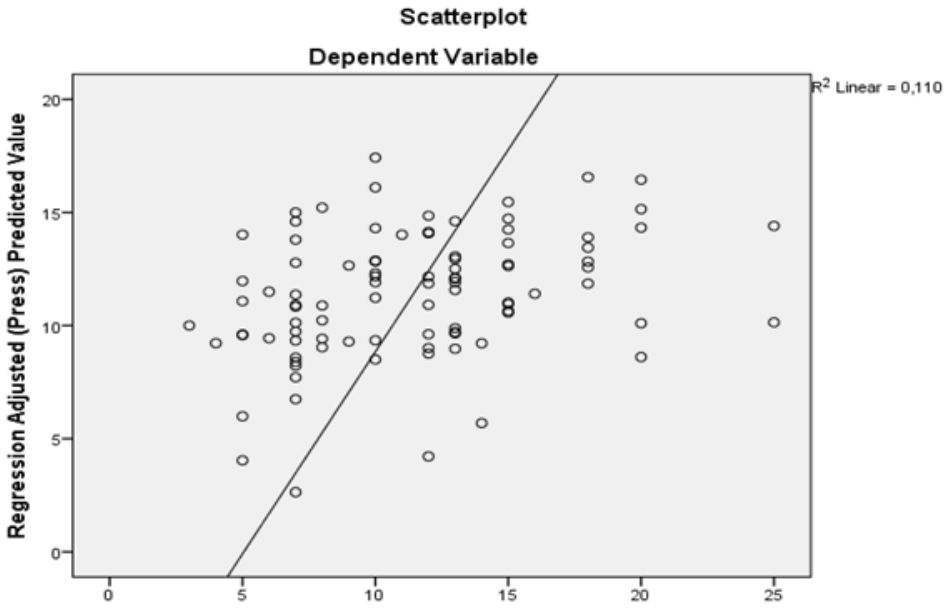

Figure 4. Multiple regression analysis visual.

Table 3. Relationship between dependent variable and independent variables

Variables	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Yield (1)	1	0.062	0.096	-0.064	0.213*	0.116	0.378*	0.104	0.090	0.273*	0.064	-0.146	0.212*	0.174*	0.029
Age (2)	0.062	1	-0.423*	0.324*	0.389*	0.179*	0.041	-0.017	-0.007	0.092	0.280*	-0.222*	-0.145	-0.161	-0.083
Education (3)	0.096	-0.423*	1	-0.446*	-0.232*	-0.070	0.018	-0.125	0.072	0.000	-0.276*	0.264*	0.084	-0.041	-0.139
Profession (4)	-0.064	0.324*	-0.446*	1	0.449*	0.193*	0.254*	0.171*	-0.050	0.184*	0.172	-0.153	-0.079	0.092	0.023
Experience (5)	0.213*	0.389*	-0.232*	0.449*	1	0.332*	0.463*	0.387*	-0.028	0.222*	0.106*	0.118*	-0.046	0.115	-0.022
Purpose (6)	0.116	0.179*	-0.070	0.193*	0.332*	1	0.330*	0.171*	0.022	0.138	0.000	-0.071	-0.083	-0.107	0.069
Beekeeping type (7)	0.378*	0.041	0.018	0.254*	0.463*	0.330*	1	0.505*	0.010	0.305*	0.120	0.051	0.038	0.299*	0.143
Number of hives (8)	0.104	-0.017	-0.125	0.171*	0.387*	0.171*	0.505*	1	-0.078	0.208*	0.107	0.198	-0.058	0.361*	0.238*
Getting an education (9)	0.090	-0.007	0.072	-0.050	-0.028	0.022	0.010	-0.078	1	0.146	0.157	0.223*	-0.039	0.082	0.094
Membership (10)	0.273*	0.092	0.000	0.184*	0.222*	0.138	0.305*	0.208*	0.146	1	0.578*	-0.010	0.107	0.392*	0.000
Support (11)	0.064	0.280*	-0.276*	0.172*	0.106	0.000	0.120	0.107	0.157	0.578*	1	-0.088	-0.066	0.230*	0.092
PIKOM (12)	-0.146	-0.222*	0.264*	-0.153	0.118	-0.071	0.051	0.198*	0.223*	-0.010	-0.088	1	-0.007	0.179*	-0.025
Race (13)	0.212*	-0.145	0.084	-0.079	-0.046	-0.083	0.038	-0.058	-0.039	0.107	-0.066	-0.007	1	0.144	-0.030
Queen bee production (14)	0.174*	-0.161	-0.041	0.092	0.115	-0.107	0.299*	0.361*	0.082	0.392*	0.230*	0.179*	0.144	1	0.116
Queen bee change (15)	0.029	-0.083	-0.139	0.023	-0.022	0.069	0.143	0.238*	0.094	0.000	0.092	-0.025	-0.030	0.116	1

*:$p \leq 0.05$.

Table 4. Linearity statistics

Variables	Linearity statistics	
Yield (fixed)	**Tolerance**	**VIF**
Age	0.599	1.670
Education	0.574	1.742
Is beekeeping the main occupation?	0.639	1.565
Experience	0.500	1.999
Purpose	0.770	1.299
Beekeeping type	0.553	1.810
Number of hives	0.577	1.734
Getting an education	0.862	1.160
Membership	0.490	2.042
Support	0.525	1.904
PIKOM	0.732	1.365
Race	0.912	1.096
Queen bee production	0.652	1.535
Queen bee change	0.859	1.164

The regression model, with honey yield per colony as the dependent variable, was attempted to explain with 14 independent variables. The coefficients of the variables in the model were found to be significant and significant. The R^2 value for the model's explanatory power was 0.323, and the corrected R^2 value was 0.203. (Table 5). The model can be interpreted as explaining 20.3% of the change in the dependent variable by the independent variables added to the model and the remaining 79.7% by the variables not included in the model via the error term. The presence of heteroskedasticity, which is common in cross-sectional data, has been investigated. The multicollinearity problem was investigated, and it was discovered that there was no problem because the VIF (variation inflation factor) values were less than 10. A specification test was performed on the model once more, and it was determined that quadratic terms were unnecessary. The Durbin-Watson coefficient was found to be 2.072, indicating that there was no auto-correlation in the model (Kalayc 2014; evrimli 2017). Table 24 shows the parameter values for the independent variables obtained from the regression analysis, as well as their t statistical values and explanatory coefficients. The variables influencing honey yield are beekeeping technique, profession, and information obtained from the picom, in that order.

The following is the equation derived from Table 24:

$$\begin{gathered}\text{Y (honey yield per hive)} = 3.928 + age * 0.030 + professional\ experience * 0.114 - \\ number\ of\ existing\ hives * 0.002 + queen\ bee\ replacement * 0.075 + type\ of\ beekeeping * \\ 3.190 + educational\ status * 0.071 - beekeeping\ purpose * 0.315 + bee\ race * 1.868 - \\ is\ beekeeping\ the\ main\ profession * 2.470 + receiving\ education * 1.121 + \\ membership\ status * 2.716 + receiving\ support * 1.263 + \\ receiving\ information\ from\ PIKOM * 2.726 + queen\ bee\ production * 0.751 + \varepsilon\end{gathered} \quad (4)$$

The interpretation of the regression equation created above will be as follows: "age" 0.030, "professional experience" 0.114, "type of beekeeping" 3,190, "educational status" 0.071, "bee breed" 1.868, "training" 1.121, "membership status" 2.716, "support" 1.263, "information from picom" "receiving" will increase by 2.726 and "queen production" will increase by 0.751. In terms of honey yield per hive, "existing number of hives" will result in a 0.002 decrease and a change of "queen bee to 0.075. According to Uzundumlu et al. (2011), in a study conducted in Bingöl, the variables affecting honey yield were the operator's age, the total number of hives, whether the beekeeper was a traveler or a fixed beekeeper, the number of bee hives extinguished the previous year, and non-agricultural income. It was discovered that the honey yield per hive increased with the farmer's age and the number of hives. It has been determined that mobile beekeeping has a statistically significant positive effect on honey yield. The findings of the study were partially similar and partially different from the findings of Uzundumlu et al (2011). The study's findings were partially similar in terms of farmer age, wandering beekeeping status, and honey yield, but different in terms of number of hives and honey yield. In a study conducted by Öztürk (2013), it was discovered that there was an inverse relationship between the yield per hive and the number of hives, with the yield decreasing as the number of hives increased. The reason for this situation is thought to be that dealing with these hives is difficult in enterprises where the number

of hives is large and the desired importance is not demonstrated. The reason why yield was lower in producers with fewer hives than others was interpreted as more stable beekeeping. The findings of the study were identical to the findings of the Öztürk (2013) study. While the farmer's age and education level were not found to be statistically significant on honey yield in the same study, professional experience was. It is expected that as professional experience grows, so will the yield per hive. However, the study found that those with more than 30 years of professional experience have lower productivity per hive. The reason for this is that elderly people are tired of their nomadic lifestyle and struggle with beekeeping (Öztürk 2013; Esen and Özmen Özbakır 2023). In this study, beekeepers' age, education level, and professional experience had no statistically significant effect on honey yield per hive, but all variables increased honey yield. In his study, Öztürk (2013) discovered that it is critical to replace the queen bee in beekeeping and that not changing the queen bee or changing it late has a negative impact on honey yield. The analysis revealed that as the queen replacement period increased, honey yield decreased, and there was an inverse and significant relationship between the queen bee replacement period and honey yield. The study's findings are partially similar to the findings of the Öztürk study (2013). According to Şeviş (2018)'s study in Bingöl, there is a positive significant relationship between professional experience and productivity per hive. There is a statistically significant, inverse relationship between the number of existing hives and the yield per hive, and the yield per hive decreases as the number of hives increases. The queen replacement period and honey yield have been found to have an inverse and significant relationship, with honey yield decreasing as the queen replacement period lengthens. Wandering beekeepers have a positive and statistically significant effect on honey yield. While the number of existing hives has a significant effect in explaining the model, it has been determined that the variables of beekeeping style, queen change, and the number of individuals in the family have a significant effect, and the variable of professional experience has a lesser effect. The study's findings were partly similar and partly different from the findings of Şeviş's study (2018). The following is the interpretation for dummy variables: It has been concluded that there is an increase in honey yield per hive of migratory beekeeping enterprises, beekeeping is seen as the main occupation and a source of livelihood, continuing the activity with the Caucasian bee race, training, support, and information about beekeeping from the picom and queen bee production that are union members.

Table 5. Regression analysis results

Variables	β	Std. Error	Standardized β	T calculation value	P value
Fixed	3.928	3.567		1.101	0.274
Age	0.030	0.046	0.077	0.646	0.520
Professional experience	0.114	0.072	0.205	1.569	0.121
Number of existing hives	-0.002	0.003	-0.069	0.565	0.573
Queen bee change	-0.075	0.508	0.015	0.148	0.883
Beekeeping type	3.190	1.209	0.329	2.638	0.010***
Educational status	0.071	0.126	0.069	0.563	0.575
Beekeeping purpose	0.315	0.991	-0.034	0.318	0.752
Bee race	1.868	1.182	0.153	1.580	0.118
Is beekeeping the main occupation?	2.470	1.096	-0.261	2.253	0.027**
Getting education	1.121	0.958	0.117	1.169	0.246
Membership status	2.716	2.014	0.178	1.348	0.181
Status of receiving support	1.263	1.596	0.101	0.791	0.431
Receiving information from PIKOM	2.726	1.122	0.263	2.429	0.017**
Queen bee production	0.751	1.086	0.079	0.692	0.491
R^2=0.323; Adjusted R^2= 0.203; F(14.79) = 2.688; P value = 0.003					
Breusch-Pagan Test = 10.116; P value = 0.066; Ramsey Reset Test = 1.254; P value = 0.132					
Durbin Watson test value = 2.072					

*: 0,10, **: 0,05, ***: 0,01; Std. Error: Standart Error.

4. Conclusion and Recommendations

The findings, evaluations, and recommendations developed in this study, which was conducted using a questionnaire, in order to determine the current situation of beekeeping activities of the producers engaged in beekeeping activities in Bingöl, to reveal problems and determine solutions, and to determine the factors affecting the honey yield per colony, are summarized below. The model's variable coefficients were found to be significant. The R^2 value indicating the model's explanatory power was found to be 0.323, and the corrected R^2 value was found to be 0.203. According to the model, the independent variables added to the model explain 20.3% of the change in the dependent variable, while the remaining 79.7% is explained by variables not included in the model via the error term. The variables influencing honey yield are beekeeping technique, profession, and information obtained from the PIKOM, in that order. Each independent variable will increase by one unit in terms of honey yield per colony; "age" 0.030, "professional experience" 0.114, "type of beekeeping" 3.190, "educational status" 0.071, "bee breed" 1.868, "training" 1.121, "membership status" 2.716, "support" 1.263, and "queen production" will increase by 0.751. In terms of honey yield per colony, "existing number of colony" will result in a 0.002 decrease and "replacement of queen bees" will result in a 0.075 decrease. One unit change in each independent variable in terms of honey yield per hive; "age" 0.030, "professional experience" 0.114, "type of beekeeping" 3.190, "educational status" 0.071, "bee breed" 1.868, "training" 1.121, "membership status" 2.716, "support" 1.263, "PIKOM" will provide an increase of 2,726 and "queen production" will provide an increase of 0.751. In terms of honey yield per hive, "existing number of hives" will result in a 0.002 decrease and "replacement of queen bees" will result in a 0.075 decrease. It has been concluded that there is an increase in honey yield per hive of migratory beekeeping enterprises, beekeeping is seen as the main occupation and a source of main income, continuing the activity with the Caucasian bee breed, training, support, and information on beekeeping from the queen bee, which are union members. One of the most important factors affecting honey yield is climate and flora characteristics. It is extremely important to analyze these factors by including them in the model. With the increased number of hives, it is possible that the COVID-19 pandemic, the lack of attention to the hives, the implementation of restrictions, and the current situation caused by the pandemic are all effective in reducing honey yield. Following are some recommendations based on these findings. It is critical to expand wandering beekeeping in Bingöl, to continue the activity with the Caucasian bee race, and to increase the number of enterprises that receive picom beekeeping training, support, and information, become union members, and produce queen bees. Efficiency in hive management and resource use should be ensured as a result of new production planning. Producers' queen bee needs can be met by establishing specific queen rearing centers or increasing the number of established centers in Bingöl province or neighboring provinces. Thus, even if not directly, an increase in honey yield can be achieved indirectly. Given Türkiye's ecological richness and existing rural economic conditions, beekeeping should be done in an organized, conscious, and sustainable manner.

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Research Article

Development and Detection of Antimicrobial Properties of Polyherbal Handwash

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Zone of inhibition

Abstract: Many of the medications marketed as traditional herbal medicines have unquestionably been used for a very long time. Hands have always been the dominant source of transmission of infection to patients. Thus, encouraging "personal hygiene" is the main objective of developing a herbal hand wash. The current study's goal was to develop a formulation of polyherbal handwash employing methanolic extracts of dried leaves of *Azadirachta indica*, *Ocimum gratissimum*, and *Coriandrum sativum*. Other ingredients entailed lemon juice, aloe vera, lavender oil, HPMC, triethanolamine, sodium lauryl sulphate (SLS), glycerine, and methylparaben. Four batches of hand wash formulations were prepared, and each batch was tested for stability, appearance, colour, grittiness, pH, viscosity, foam height, and other physical characteristics. Using the agar well diffusion method, the anti-microbial effectiveness of the prepared polyherbal hand wash was tested on a variety of bacteria, including *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Salmonella typhi, Aspergillus niger,* and *Candida albicans*. The results demonstrated that manufactured herbal handwash formulations, particularly F3, displayed a prominent zone of inhibition in comparison to standard commercial handwash, indicating that the extract of these phytoconstituents may be employed to manufacture handwash with antimicrobial properties. As a result, the research shows that the herbal handwash formulation is analogous to commercial handwash in reducing the amount of bacteria on hands and may be used as a replacement handwash made from natural sources without experiencing any undesirable effects.

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1. Introduction

Skin, the largest organ, is the most exposed organ of the human body. Due to the fact that hands are the major means by which infections spread, maintaining adequate health and hygiene, particularly with regard to hands, is quite essential (Acharya et al., 2018). Hand hygiene, the act of cleansing hands, is a very important, effective, simplest and affordable measure to prevent and decrease the transmission of harmful microbes and help fight the spread of the disease (Bagade et al., 2021). Hands', serving the maximum work for the human body, come in contact with dirt and harmful microbes acquired from the

soils, food, raw materials, equipments, etc. (Sinha et al., 2022). Thus, hand washing, the best way to maintain personal hygiene, is the most effective measure to help fight the spread of the disease and to protect the skin from harmful microbes (Singh et al., 2022).

Historically, plant species are the earliest and most good source of pharmacologically active molecules. The bioactive compounds, as well as the plant extracts, have been utilised for ages in the preparation of traditional and Ayurvedic medicines, food, natural dyes, and cosmetics, and in the treatment of different ailments (Powar et al., 2015). Herbal Plants, possessing a wide variety of bioactive compounds such as tannins, terpenoids, flavonoids, alkaloids, etc., are found to have potential *in-vitro* antimicrobial properties which are efficient against a wide spectrum of microbes (Takó et al. 2020; Vaou et al., 2021).

In the present research, herbal hand wash was formulated and prepared using different herbal plants like neem, tulsi, coriander, aloe vera, and lemon juice because of their ease of availability, less expensive, increased efficiency, and fewer side effects - benefits of using herbal handwash (Eshete and Molla, 2021). Neem (*Azadirachta indica*) belongs to the Meliaceae family and is also known as Margosa or Indian lilac. Different parts of the Neem such as leaves and barks hold therapeutic importance (Latif et al., 2020). The bioactive compounds, such as Azadirachtin, extracted possess antiviral, antifungal, antibacterial, insecticidal, and antiseptic properties (Islas et al., 2020). Also, these extracts are useful as folk medicine to control leprosy, respiratory diseases, and constipation (Ganguly et al., 2022). Nimma Tulsi, scientifically known as *Ocimum gratissimum* belonging to the Lamiaceae family, is widespread in India and South Africa. It is also grown across tropical regions on the globe (Bhavani et al., 2019). The leaves, flowers, stems, roots, seeds, fruits, and bark of this plant can all provide phytochemical constituents such as alkaloids, tannins, flavonoids, steroids, triterpenoids, and carbohydrates possessing pharmacological properties such as antimicrobial, antidiabetic, wound healing, etc. (Sharma and Upadhyaya, 2019). *Coriandrum sativum*, an annual herb belonging to the Apiaceae family, is commonly known as coriander, dhania, or cilantro. Though it is used as a flavouring agent in food preparation, it possesses both nutritional and medicinal properties (Wei et al., 2019). The most utilized part of the coriander plant is the dried ripe fruits, commonly known as coriander seeds, and leaves (Mahleyuddin et al., 2021). Major active constituents of *Coriander sativum* is fatty oil such as Linoleic acid, and oleic acid, and essential oils such as Linalool, Geraniol (Sobhani et al., 2022). Aloe vera, a cactus-like plant, also known as *Aloe barbadensis miller* belongs to the Liliaceae family (Hęś et al., 2019). It is utilized for its pharmacological properties such as anticancer, antimicrobial, antiviral, cleansing, and wound healing due to the presence of numerous bioactive compounds such as Vitamin B12, aloin, emodin, etc. (Sánchez et al., 2020). Thus, the herbal handwash prepared is in solution form which underwent several evaluation tests.

2. Material and Methods

2.1. Herbs, chemicals, and microbes collection

The herbal plants viz. *Azadirachta indica*, *Ocimum gratissimum*, *Coriandrum sativum,* and Aloe vera were collected from the herbal garden of the Brainware University Campus, Barasat, Kolkata, West Bengal, India. The lemon juice was collected from the lemon which was procured from the local market area of Barasat. All the chemicals, media, and reagents employed in the study were of analytical grade. The microbial strains (*Bacillus subtilis* ATCC 6633*, Escherichia coli* ATCC 8739*, Staphylococcus aureus* ATCC 6538*, Salmonella typhi* NCTC 786*, Pseudomonas aeruginosa* ATCC 9027*, Aspergillus niger* ATCC 16404*, Candida albicans* ATCC 10231) were procured from the Central Drug Laboratory, Kolkata, West Bengal, India.

2.2. Preparation of herbal extract

The dried leaves of *Azadirachta indica*, *Ocimum gratissimum,* and *Coriandrum sativum* were coarsely powdered. 5 grams of coarsely powdered leaves of each plant were soaked separately in 100 ml of methanol. The soaked leaves were kept for maceration for 4 days. The extract was filtered once the maceration was completed. The collected filtrate was used for manufacturing of herbal handwash (Bereksi et al., 2018; Kamalapurkar and Shendge, 2022; Rukari et al. 2022; Nurcholis et al., 2023).

2.3. Preparation of polyherbal handwash formulation

The formulation of the polyherbal handwash was prepared from the methanolic leaf extract of *Azadirachta indica*, *Ocimum gratissimum,* and *Coriandrum sativum,* with addition to lemon juice, aloe vera, Oil of lavender for perfume, using HPMC E-50 as gelling agent, Triethanolamine for adjusting the pH, Sodium Lauryl Sulphate (SLS) as foaming agent, glycerine for moisturizing and methyl paraben for preservative in various formulation batches (Table 1) (Bagade et al., 2021). The solution prepared, as per the standard procedure for the preparation of handwash, was made homogenous under room temperature and stored for further studies (Barman et al., 2020).

Table 1. Different formulations of polyherbal handwash

Composition	Formulation Batch (Quantities Taken)			
	F1	F2	F3	F4
Methanolic Extract of Neem	2 ml	2 ml	2 ml	2 ml
Methanolic Extract of Tulsi	2 ml	2 ml	2 ml	2 ml
Methanolic Extract of Coriander	2 ml	2 ml	2 ml	2 ml
Aloe vera	2 ml	2 ml	2 ml	2 ml
Lemon Juice	2 ml	2 ml	2 ml	2 ml
HPMC	0.6 gr	0.7 gr	0.8 gr	0.9 gr
Triethanolamine	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Glycerine	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Methyl Paraben	0.08 gr	0.08 gr	0.08 gr	0.08 gr
SLS	0.5 gr	0.6 gr	0.7 gr	0.8 gr
Oil of Lavender	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Purified Water q.s.*	q.s to 30 ml	q.s to 30 ml	q.s to 30 ml	q.s to 30 ml

*q.s. – as much as is sufficient.

2.4. Evaluation of polyherbal handwash

2.4.1. Physical evaluation

The polyherbal handwash prepared was visually inspected where the physical parameters such as colour, odour, texture, appearance, grittiness, and homogeneity were evaluated (Patel et al., 2017; Mulani et al., 2021).

2.4.2. pH

The pH of the formulations was examined using a standardised digital pH meter at room temperature (Mali et al., 2020).

2.4.3. Foam height

In 50 ml of distilled water, 1 gram of the prepared polyherbal handwash formulation was dispersed. It was then transferred into a stoppered measuring cylinder of 500 ml capacity, making up the volume to 100 ml with water. After applying 25 strokes, it was allowed to stand until the aqueous volume reached 100 ml. The foam height was then measured above the aqueous volume (Kuril et al., 2020).

2.4.4. Foam retention

In a 200 ml graduated cylinder, 50 ml of the prepared polyherbal handwash formulation was agitated 10 times. The quantity of the foam was measured for 4 minutes at 1-minute intervals ensuring the foam retained should be stable for at least 5 minutes (Chitkara et al., 2020).

2.4.5. Skin irritation test

The skin irritancy test was performed by applying the formulations on the skin and observed for 30 minutes (Wal et al., 2021).

2.4.6. Stability

The stability study was done by storing the formulations at different temperatures such as 17 ºC, 28 ºC, and 37 ºC for a period of one month (Rajalakshmi, 2019).

2.4.7. Antimicrobial activity of polyherbal handwash

The screening of antimicrobial activity of the prepared poly-herbal handwash formulation was performed against microorganisms such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa, Salmonella typhi, Aspergillus niger,* and *Candida albicans* using agar well diffusion method on Muller-Hinton agar and Potato Dextrose agar respectively (Sumaiya et al., 2017; Tunio et al., 2022). The microbes were stabbed over the Muller-Hinton agar plate and Potato Dextrose agar plate using sterile cotton swabs and 10 µl of the prepared polyherbal handwash formulation of each batch was poured into the wells on the plates. After 24 hours of incubation at 37 °C, the antimicrobial activity was evaluated by measuring the diameter of the zone of inhibition by taking triplicates (Donio et al., 2022).

3. Results

3.1. Physical evaluation

The polyherbal handwash prepared was inspected by visual observation where all formulations were found to be greenish in colour, mild lavender fragrant, sleek, pellucid, with no grittiness, and maintain homogeneous form respectively.

3.2. pH

The pH of all the formulations was determined by calibrated digital pH meter and it was found to be 6 to 7 (Table 2) which are near to the pH range of the human skin.

3.3. Foam height

After applying 25 strokes, it was allowed to stand until the aqueous volume reached 100 ml. The foam height of all the formulated herbal handwash was found to be as per the following Table 2. From the observation table, it is concluded that the F3 batch shows good foam height.

3.4. Foam retention

In a 50 ml graduated cylinder, 12.5 ml of the prepared polyherbal handwash formulation was agitated 10 times. The quantity of the foam was measured for 4 minutes at 1-minute intervals. The foam retention of all the formulated herbal handwash was found to be as per the following Table 2. From the observation table, it is concluded that the F3 batch shows good retention time than the others which was almost stable for 6 min thus it was best to be used as a herbal hand wash.

Table 2. pH, foam height, and foam retention of polyherbal handwash

Formulation batch	pH range	Foam height (cm)	Retention time (mins)
F1	6.8	8	2
F2	6.7	10	3
F3	6.9	12	6
F4	7.2	11	4

3.5. Skin irritation test

Ten final-year students voluntarily participated, and the formulation was applied to their skin for 30 minutes to test for skin irritation. Since the formulations' pH was within the acceptable range, no irritation or redness was found. This demonstrates that there was no chance for skin sensitivity.

3.6. Stability

A stability study was carried out at different temperatures such as 17 ºC, 28 ºC, and 37 ºC for a period of one month (Table 3), and found that there was not many changes in pH, physical evaluation, foam height & foam retention. From the observation table, it is concluded that F3 is a stable and more suitable batch than the others.

Table 3. Stability studies of polyherbal handwash

Formulation batch	Physical evaluation	pH	Foam height (cm)	Foam retention (mins)
F1	Slight change in color	6	5	2
F2	Slight change in color	6	7	2
F3	No change	6.7	11	6
F4	No change	6.2	9	4

3.7. Antimicrobial activity of polyherbal handwash

The antimicrobial activity of the prepared poly-herbal handwash formulation was performed against seven different microorganisms using the agar well diffusion method. The zone of inhibition indicates that the poly-herbal hand wash prepared from methanol extracts of the combined plant materials expressed significant antimicrobial activity. From the observation in Table 4, it is concluded that the F3 batch has shown good antibacterial activity against five different microorganisms.

Table 4. Antimicrobial activity of polyherbal handwash

Pathogens	Zone of Inhibition (cm)				
	Standard	F1	F2	F3	F4
Bacillus subtilis	2.3 ± 1.041	2 ± 0.194	2.3± 1.053	2± 1.064	1.8± 0.862
Escherichia coli	1.6 ± 0.764	1.5± 0.364	1.5± 0.684	1.6± 0.931	1.6± 0.485
Staphylococcus aureus	3.4 ± 0.414	2.4± 1.244	2± 1.912	2± 1.053	2± 1.154
Pseudomonas aeroginosa	2.7 ± 0.661	1.8± 0.743	1.9± 0.674	2± 1.041	1.8± 0.772
Salmonella typhi	3.2 ± 0.434	2.3± 1.037	2.1± 1.762	2.6± 2.00	2.1± 2.520
Aspergillus niger	1.8 ± 0.814	1.6 ± 0.672	1.6 ± 0.264	1.7 ± 0.392	1.7 ± 0.147
Candida albicans	1.6 ± 0.524	1.2 ± 0.533	1.4 ± 0.447	2 ± 1.113	1.8 ± 0.810

Values are expressed as Mean ± SD.

4. Discussion

The Covid-19 outbreak has raised consciousness about the significance that proper hand hygiene serves in preventing the spread of infection which many people were unaware (Natarajan et al., 2021). Bacteria, viruses, and other microbes are most commonly transmitted by hand. Thus, proper methods of hand washing can prevent the spread of infection (Ghurghure et al., 2019; Chen et al., 2022). In addition to restricting the spread of the infection, the way the world has embraced the appropriate utilisation of handwashes and hand sanitizers has also been crucial in minimizing the transmission of many other contagious diseases (Alzyood et al., 2020). In order to assess the medicinal potential of diverse herbs, a huge quantity of research has been done on the usage of traditional herbal products in south-east Asian nations (Kusarkar et al., 2022). A plethora of medicinal properties, as well as many medicinal plants, have been reported to be helpful for managing infectious diseases (Khan and Raghav, 2021). In the current scenario, natural plant-based products are widely in use for the management and prevention of different microbial infections and also for the betterment of life (Adhikari, 2021). In this era, the leading infection caused is skin infection which causes non-fatal diseases for a prolonged period of time resulting in expensive treatment (Sharma and Singh, 2020). As per different reports, herbal plants have effective pharmacological activities, especially in dermatological treatments (Rukari et al., 2022). To minimize and combat the bacterial pathogens which affect the skin, the most exposed part of the body, polyherbal handwash was formulated (Aware et al., 2022). The formulation was prepared having no or minimal side effect along with potential antimicrobial activity (Singh and Singh, 2022).

Ayurvedic, Unani, and homeopathic remedies frequently use neem, also known scientifically as *Azadirachta indica*. Neem has been reported to have anti-inflammatory, antifungal, antibacterial, and

antipyretic activities in its active components (Pallai et al., 2021; Reddy and Neelima, 2022). Native to the Indian subcontinent, Tulsi is known as the "Solution of Existence" in the Ayurvedic system. According to Sahoo et al. (2002), it has pharmacological properties including healing attributes, anticancer activity, antioxidant activity, anti-diabetic activity, anti-inflammatory activity, antibacterial activity, etc. The most popular culinary spice used globally is coriander, sometimes known as the herb of delight. The varied pharmacological effects of coriander include antibacterial, antioxidant, neuroprotective, migraine-relieving, and analgesic effects (Dhakshayani and Alias, 2022). Aloe vera, a succulent plant that resembles a cactus, is well known for its therapeutic properties and has been used for many years as a treatment for illnesses like sunburn, wounds, and skin issues. Aloe vera's pharmacological advantages are ascribed to its capacity to heal wounds as well as its immunomodulatory, anti-inflammatory, antioxidant, and antibacterial capabilities (Figueiredo et al., 2022). Lemon, a member of the Rutaceae family, has cancer-preventive, antibacterial, antifungal, and antidiabetic effects (Rukari et al., 2022). Neem, tulsi, coriander, aloe vera, and lemon juice are all common therapeutic herbs that are employed in the formulation of the polyherbal handwash in this study. The primary goal of incorporating all five herbal plants was to achieve a synergistic effect between the active components of various plants, which would aid in boosting the handwash formulation's antibacterial capability.

Four batches of polyherbal handwash were prepared for the present research. Based on their established pharmacological efficacy, inexpensive, compatibility with skin types, and ease of availability, the herbal plants used in the formulation were selected (Bhagwan et al., 2021). Every parameter, such as physical attributes, pH, foam retention, foam height, and stability, that was looked into for each batch of the polyherbal handwash was determined to be effective, but the third batch (F3) in particular showed that the formulation prepared had similar antibacterial properties to the formulation that was marketed. The handwash formulation was skin-friendly because it passed the test for skin irritation, and it can also be regarded as eco-friendly because it contained herbal plants. Microorganisms, mostly gram-positive ones, are abundant on our skin (Chindarkar, 2020). The antibacterial activity of the handwash preparations demonstrated a pronounced zone of inhibition against various pathogens. The F3 formulation produced the greatest results, demonstrating a synergistic response against the pathogens and demonstrating the presence of antibacterial properties. Hence, based on the findings and observations, it is possible to use polyherbal handwash and reduce the usage of handwash containing synthetic chemicals that might injure the skin.

The preparation and formulation of polyherbal handwash is thus a result of the growing demand for polyherbal formulations on the international market. The benefits of natural medicines are greater since they have fewer negative effects than synthetic ones (Giri et al, 2022).

Conclusion

When combined with lemon juice, aloe vera, and lavender oil, the methanolic extract of dried leaves of *Azadirachta indica*, *Ocimum gratissimum*, and *Coriandrum sativum* generate a significant zone of inhibition to fight against microorganisms similar to that of the commercial handwash employed in the study. In order to develop an environment-friendly and effective antibacterial handwash, these compounds could be isolated and incorporated to hand wash bases. The specifically formulated F3 hand wash demonstrates equivalent outcomes to the conventional hand wash in terms of physical and chemical parameters as well as close antibacterial activity against all tested microorganisms. The regular use of formulation can also assist in promoting good hygiene in both adults and children. As a result, a novel approach to combating pathogenic organisms' antibiotic resistance can be developed, allowing for the provision of safe and healthy living through the use of hands-free germs. Despite the fact that not all can be removed, a large portion may, and they can preserve their good health, which is a valuable resource for our everyday lives.

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Research Article

Tocopherol Content of *Euglena* sp. Isolated from Yogyakarta under Glucose and Ethanol Mixture Treatment

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Microalgae,
α-tocopherol

Abstract: *Euglena* sp. is a microalgae with significant potential for utilization as a high-value product because of the presence of protein, lipid, paramylon, and other compounds. Even though these microalgae may be found in freshwater, research on enhancing *Euglena* sp. cultivation is still limited in Indonesia. Tocopherols are antioxidants that can effectively protect against diseases caused by oxidative stress. The isomer of tocopherol with the highest biological activity is α-tocopherol. *Euglena* sp. cells had the highest levels of α-tocopherol compared to other microorganisms. Scientists are continuously trying to determine how to obtain a high α-tocopherol concentration and a significant *Euglena* cell biomass. Photosynthetic organisms culture has been found to boost α-tocopherol content in *Euglena* sp., although heterotrophic culture can potentially increase biomass. This study used photoheterotrophic culture with a mixture of glucose and ethanol to increase the α-tocopherol and biomass concentration inside the culture of the local strain of *Euglena* sp. The addition of treatments in a glucose and ethanol combination with levels of 3:2; 2.5: 2.5; 2: 2; and 0:0 (control) g L^{-1} was used in this study to assess the impact of *Euglena* sp. culture on growth, biomass, and α-tocopherol concentration. According to the findings of this study, the 3:2 treatment produced the most significant specific growth rate and biomass, including 0.992 (OD680/OD680/day) and 8.480 (g L^{-1}). In contrast, the 2.5:2.5 treatment produced the highest α-tocopherol content, specifically 7.09±0.096 mg L^{-1}.

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1. Introduction

During the Second World War, when Japan, America, and Germany struggled, microalgae cultivation as a global food source was heavily advocated (Potvin et al., 2010). Until now, microalgae are still used by the community as a source of protein, vitamins, and minerals, better known as a functional food. Microalgae outperform other microbes, such as yeasts and molds, regarding food safety.

In terms of efficiency and ease of production, microalgae are superior to mammalian single-cell proteins (Hardiyanto, 2012). Other benefits of microalgae over other microorganisms include a high biomass production per unit (Pradana et al., 2017; Yuarrina et al., 2019). Microalgae can be included in the classification of a functional food because it provides natural sources of protein, carbohydrates, and fats that act as energy sources in the body. More complex, microalgae can also function as a source of vitamins (Grimm et al., 2015). Microalgae as a food source have long been known. Some microalgae are also used as a source of drugs and are used in the pharmaceutical industry. For example, *Nannochloropsis* and *Chaetoceros* are ubiquitous microalgae used as natural feed for aquaculture animals. In addition, these microalgae can produce secondary metabolites utilized as antioxidants (Zulkarnain et al., 2020). Previous research by Tunio et al. (2022) also reported that cyanobacteria *Oscilatoria limosa* produced some bioactive compounds including phenolic acid, flavonoid, proteins, amino acid, and sugar.

Euglena is a freshwater protist that can thrive in various carbon sources, including glucose, glutamate, malate, pyruvate, lactate, and ethanol. These protists can also survive in high-stress environments, such as acidic waters, highly polluted rivers, and mining areas with high heavy metal content. Under extreme conditions, *Euglena* sp. was effectively isolated in the pH range of 2.5 – 3.5. Increasing the production of lipids and fatty acids in *Euglena* sp. through cultivation, metabolic, and genetic engineering are several methods to increase biofuel production (Erfianti et al., 2023). *Euglena* sp. contains paramylon (beta-1,3-glucan), which aids in producing several kinds of chemical components in industrial manufacturing processes. It plays a role in the medical field in synthesizing vitamin E (tocopherol) and 20 amino acids. In addition, *Euglena* is also commonly utilized as a raw material in the manufacture of biofuels, food and feed, and pharmaceutical. Furthermore, it can be used in environmental management, such as CO_2 reduction and water treatment. There is increasing interest in the commercialization of *Euglena* due to its durability and ability to synthesize a wide variety of unique bioproducts.

Euglena sp. is microalgae that have the potential to be utilized and can be found in various habitats such as fish ponds, rice fields, and polluted waters. As a result, *Euglena* sp. has the potential to be used since it can be isolated from a variety of environments. However, during the utilization process, researchers must recognize the potential negative consequences of by-products created during manufacture. Due to its ability to produce biofuel-synthesizable lipids, *Euglena* sp. has become increasingly popular in the industrial sector. Biodiesel could be made from microalgae, particularly in consortium cultures (Nur et al., 2023).

Tocopherol, often known as vitamin E, is an antioxidant that may help prevent various illnesses caused by oxidative stress (Rizvi et al., 2014). Tocopherol is a vitamin commonly used as a food preservative in the food business (Delgado et al., 2020). Tocopherols are obtained primarily by chemical synthesis and extraction from vegetable oils. However, extraction from vegetable oils comprises a combination of beta and gamma tocopherols. In reality, it must be refined to extract the active form of α-tocopherol for pharmaceutical uses (Ogbonna et al., 2019). Because of their considerable structural similarity, separating homologous mixtures of tocopherols is difficult. However, chromatographic techniques can be used. The tocopherol isomer with the highest biological activity is α-tocopherol. Although many other microbes may accumulate α-tocopherol compared to yeast, moulds, and macroalgae, the α-tocopherol concentration in *Euglena* sp. cells is considered the highest (Gissibl et al., 2019). *Euglena* sp. contains 97% α-tocopherol compared to other tocopherol isomers (Shigeoka et al., 1986). The demand for tocopherols in the market, especially α-tocopherol, is experiencing a rapid increase, so it is necessary to develop an efficient production system for chemically synthesized α-tocopherols by vegetable oils.

Due to a lack of genetic information on metabolic pathways that lead to diverse bioproducts, improved *Euglena* performance depends on improving culture conditions to enable the synthesis of intriguing chemicals, followed by increased culture volume. *Euglena* can be cultivated in heterotrophic, photoautotrophic, or mixotrophic. Depending on the culture method, the final biomass and cellular composition differ. Biomass might be optimized by changing growth determinants such as growth medium, temperature, light intensity, dissolved CO_2 concentration, cultivation procedure, and salinity (Sudibyo et al., 2018). Mix-culture cultivation could also enhance the optimization of algal growth and biomass (Suyono et al., 2016) and also improve the effectiveness of the harvesting process (Irawan et al., 2023). In this study, *Euglena* sp. is grown photoheterotrophically because cell development is not

entirely dependent on photosynthesis since light energy is not the only factor influencing growth; organic carbon substrate also plays a role (Sudibyo et al., 2018). To produce α-tocopherol commercially in *Euglena* sp. it is essential to obtain a high biomass concentration and a high concentration of α-tocopherol per unit cell. Photoheterotrophic culture, when organic carbon supplies and light energy are easily accessible for culture, can overcome problems in cultivation under photoautotrophic and photoheterotrophic circumstances. Glucose has been shown to enhance biomass content, whereas ethanol has been shown to increase the α-tocopherol range (Fujita et al., 2008). As a result, this study employs a combination of glucose and ethanol in various ratios. The amount of biomass is related to the amount of tocopherol. Therefore, if biomass production is high, the probability of tocopherol generation is likewise increased. This study aims to find the optimal glucose-ethanol ratio for enhancing tocopherol synthesis.

2. Material and Methods

2.1.Materials

In 2021, the current investigation was carried out in the Biotechnology Laboratory at Universitas Gadjah Mada in Yogyakarta, Indonesia. *Euglena* sp. strain Indonesia was obtained from the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada (isolated from a wild strain in Yogyakarta). Modified Cramers & Myers was used in the cultivation of *Euglena*, with the following materials: $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $Fe_2(SO_4)_3 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$ (Cramer and Myers, 1952). The medium was sterilized by autoclaving it for 15 minutes at 121ºC. After sterilization, filter-sterilized glucose and ethanol were added to the medium to avoid denaturation by high heat. Afterward, Vitamin B1 and Vitamin B12 were sterilized using a millipore filter.

2.2.Cultivation

Cultivation was carried out in 500 ml flasks using a combination of 300 ml modified CM media and 200 ml pre-cultured *Euglena* sp. cells. The cultivation was carried out in a photoheterotrophic condition, with aeration provided by a combination of 95% air and 5% CO_2 and lighting provided by LED lamps with a vlight intensity of around 1.000 lux). The effects of glucose and ethanol mixture were investigated with ratio: 0 g L^{-1} : 0 g L^{-1}; 3 g L^{-1}: 2 g L^{-1}; 2.5 g L^{-1}: 2.5 g L^{-1}; 2 g L^{-1}: 3 g L^{-1}. Pre-cultivation experiments showed that these concentrations of glucose and ethanol were not inhibitory to cell growth and α-tocopherol production. Three biological repetitions are used in this research (n=3).

2.3.Optical density and biomass

The optical density of the sample was determined by measuring absorbance at a wavelength of 680 nm (Suzuki et al., 2017). The culture biomass was determined by measuring the dry weight of the sample. The samples were centrifuged for 10 minutes at 4000 rpm. The supernatant is removed from the conical tube, leaving just the pellets. The leftover pellets were dried for 12 hours (overnight) in an oven at 36ºC until the weight was consistent (Ben-Amotz et al., 2004). Doubling time was calculated with the following formula:

$$Td = \frac{\ln 2t}{\ln(N_t/N_o)} \quad (1)$$

T = Time interval
Nt = The number of cells at the end of the exponential phase
N0 = The number of cells at the beginning of the exponential phase

The following equation was used to compute the specific growth rate:

$$\mu = \frac{\mathbf{0.693}}{\mathbf{td}} \quad (2)$$

Biomass productivity was calculated with the equation:

$$\text{Productivity (g L}^{-1}\text{ day}^{-1}\text{)} = \Delta x/t \quad (3)$$

Δx = difference in biomass on day t_1 and day t_0 (day 0)
T = time interval (day)

2.4.α-tocopherol measurement

The cells were extracted from the culture sample by centrifugation. According to Afiukwa et al (2007), the α-tocopherol content of the cell was removed. Quantitative analysis was performed using a spectrophotometric method with spectrophotometer UV-Vis and α-tocopherol standard. α-tocopherol quantification was carried out with a wavelength of 450 nm. Standard curves (r^2 value = 0.999) were made with standard α-tocopherol solutions with graded concentrations of 1 mg L^{-1}, 2 mg L^{-1}, 4 mg L^{-1}, 8 mg L^{-1}, and 16 mg L^{-1}. The absorbance of the *Euglena* sp. sample was calculated by the standard curve. Tocopherol productivity was calculated with the following equation:

$$\text{Productivity (mg/mL/day)} = \frac{\Delta x}{t} \quad (4)$$

Δx = difference in α-tocopherol on day t_1 and day t_0 (day 0)
T = time interval (day)

2.5.Statistical analysis

SPSS software was used to conduct all statistical analyses. Analysis of variance (ANOVA) and Duncan's multiple range tests at $p<0.05$ was used to compare the significant level between values. Statistical significance was defined as $p<0.05$ or above.

3. Results

3.1. Effect of glucose and ethanol on cell growth

Growth characteristics include specific growth rates and doubling time. The specific growth rate is the rate at which microalgae cells grow per unit of time and may be used to calculate the carrying capacity of nutrients for microalgae cell growth and division. Doubling time is the time necessary for microalgae cells to double in number. The fastest doubling time occurs in the logarithmic phase, which is the phase where the cells divide rapidly and constantly. Low doubling time values are correlated with high specific growth rate values and vice versa (Nurhanifah et al., 2019; Liu et al., 2011). Microalgae strains with a short doubling time and high specific growth rate are ideal for developing in-scale production. This is because the time from cultivation to harvest can be achieved with a shorter duration. Therefore the product can be more efficient.

According to Figure 1. It can be seen that the *Euglena* sp. culture, which had the highest absorbance, was the 3 g glucose: 2 g ethanol treatment. In contrast, the lowest absorbance was the control treatment—adding a glucose and ethanol combination enhanced cell development. Cultures with 3:2 and 2:3 treatment had the highest absorbance on the 4th day, respectively, namely 1.272 and 1.091, cultures with treatment 2.5:2.5 had the highest absorbance on the 5th day, which was 1.132, while the control with 0:0 treatment had the highest absorbance on the 6th day, which was 0.679. This information does not need to be deleted entirely; it is not interested in the results. These data and explanations describe the relationship between biomass productivity and cell growth, doubling time. These data lead to the conclusion that *Euglena* sp. in this study can be harvested quickly and effectively for further development (production scale).

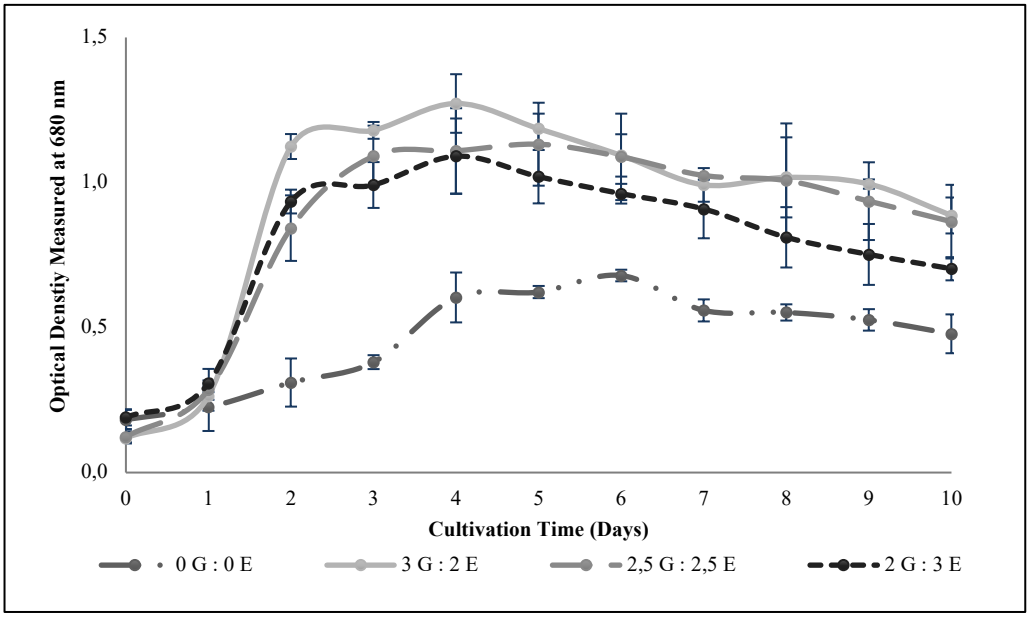

Figure 1. Effects of glucose and ethanol mixture with different ratios to cell growth of *Euglena* sp.

The treatment of a combination of glucose and ethanol with a ratio of 3:2 0.608 ± 0.049 resulted in the most incredible Specific growth rate (OD680 day-1), followed by 2.5:2.5 treatment with a value of 0.533 ± 0.008; treatment 2:3 worth 0.489 ± 0.005; and the last is control (0:0) worth 0.224 ± 0.014. The value obtained in this study is directly proportional to the absorbance results obtained, where the highest absorbance was achieved by the 3:2 treatment, and the lowest absorbance was performed by the control treatment (0:0).

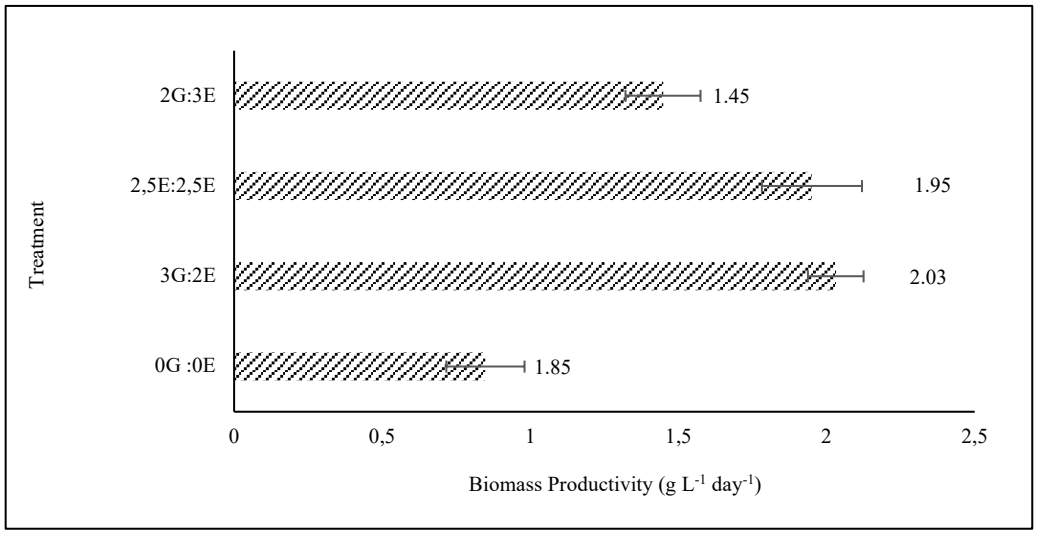

Figure 2. The specific growth rate of *Euglena* sp. in glucose and ethanol mixture with different ratios.

According to the data on Figure 2, the highest biomass productivity was achieved by 3:2 treatment of glucose: ethanol (2.03 g L^{-1} day^{-1}), followed by 2.5: 2.5 of glucose: ethanol (1.95 g $L^{-1}day^{-1}$), 2:3 of glucose: ethanol (1.45 g L^{-1} day^{-1}), and the lowest biomass productivity was 0:0 of glucose: ethanol (1.85 g L^{-1} day^{-1}). This result showed that microalgae successfully consumed the carbon source through glucose and ethanol. This finding was supported by a previous study by Afiukwa and James (2007) that the growth of *E. gracilis* was higher under the mixed carbon culture (ethanol and glucose), reaching 2.34 ± 0.109 x 10^7 mL for the cell density. Furthermore, the previous results indicated that the diverse carbon culture promoted cell growth, but antioxidant vitamin concentrations were insufficient.

Thus, the combined substrate system has a high potential for producing *Euglena* biomass on a large scale.

3.2. Effect of glucose and ethanol on dry weight (dry biomass)

Overall, from Figure 3, it can be seen that the highest trend in biomass was found in the 3 grams of glucose: 2 grams of ethanol treatment, while the lowest glucose trend was found in the control treatment. This trend is directly proportional to the increase in *Euglena* sp. cell density, where the mixture of glucose and ethanol enhanced growth. In all treatments, it was seen that the highest biomass was achieved on the 6th day, and on the 8th day, the biomass in all treatments decreased.

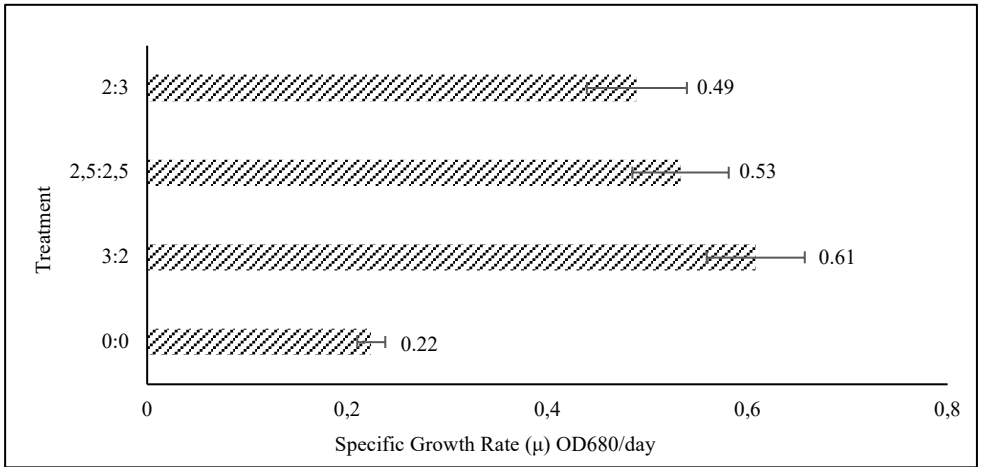

Figure 3. Effects of glucose and ethanol mixture in different ratios to the biomass of *Euglena* sp.

Figure 4 showed the biomass productivity of *Euglena* sp. in glucose and ethanol mixture with different ratios. The highest average of biomass in each treatment (3:2; 2.5:2.5; 2:3; and control) was 12.94 g L^{-1}, 12,32 g L^{-1}, 9.98 g L^{-1}, respectively. 6,36 g L^{-1}. The biomass produced on the last day of observation in each treatment (3:2, 2.5:2.5; 2:3, and control) was 9.40 g L^{-1}, and 10.32 g L^{-1}, respectively. 5,69 g L^{-1}, 5,69 g L^{-1}. The treatment of a combination of glucose and ethanol yielded the total biomass (g L^{-1} day^{-1}) with a ratio of 3:2 which was 8.48 ± 0.28, then 2.5:2.5 treatment which was 8.12 ± 0.55, treatment 2:3, which is 6.18 ± 0.7, and the lowest is the control treatment (0:0) which is 4.38 ± 0.23. Thus, this observation indicates that the 3:2 treatment is optimal for obtaining the highest biomass yield. This is in line with the productivity of the resulting biomass. These results indicate that the higher the glucose ratio increases the cell growth and biomass content.

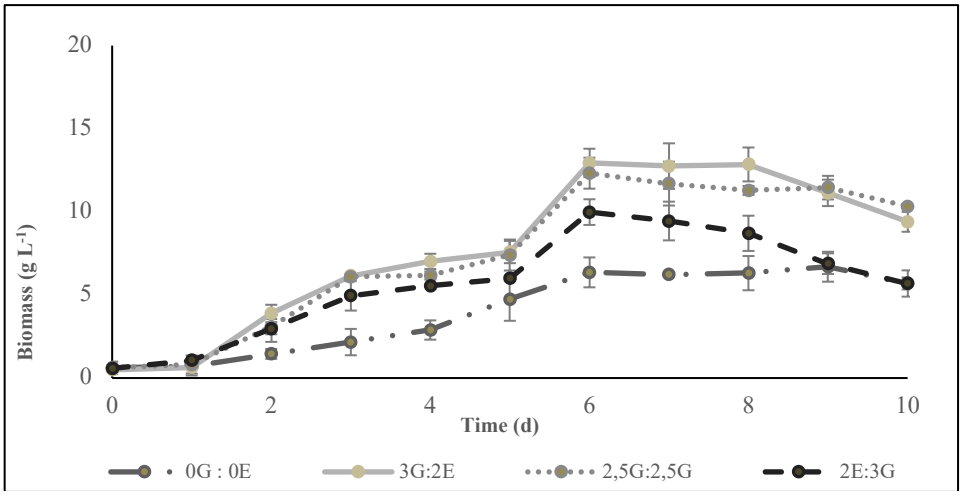

Figure 4. Biomass productivity of *Euglena* sp. in glucose and ethanol mixture with different ratios.

3.3. The effect of glucose and ethanol on the amount of α -tocopherol

Figure 5 showed that the α-tocopherol content increased from the beginning of cultivation until it reached the highest range on the 6th day when *Euglena* was at the end of the logarithmic phase. On the 8th day, α-tocopherol content decreased as the biomass and cell density of *Euglena* sp. The 2.5 glucose:2.5 ethanol culture treatment generally had the greatest α-tocopherol concentration. In the 3:2 and 2:3 treatments, the productivity of α-tocopherol was inversely proportional to the biomass produced by *Euglena* sp.

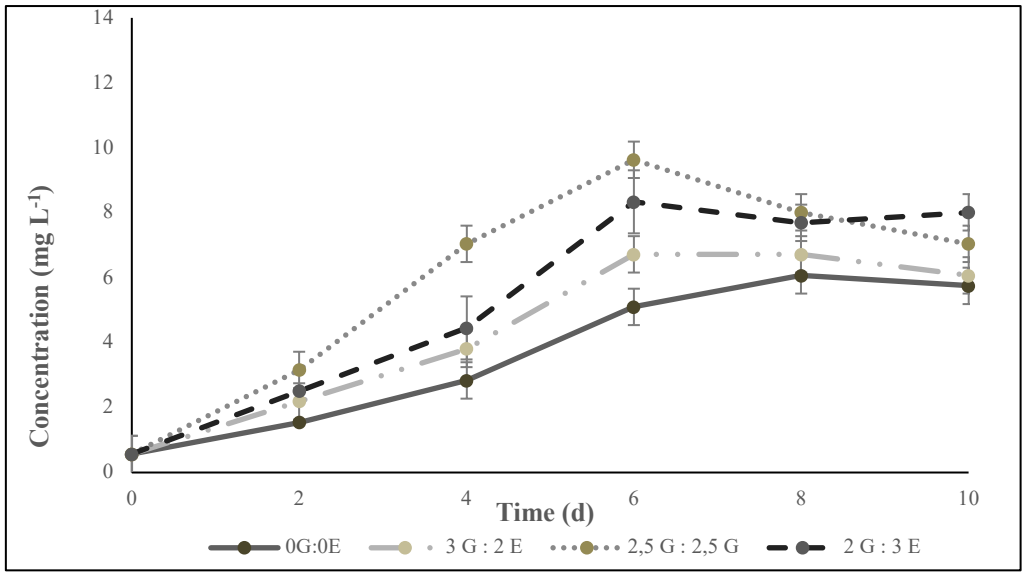

Figure 5. Effect of glucose and ethanol mixture with different ratios to α-tocopherol content of *Euglena* sp.

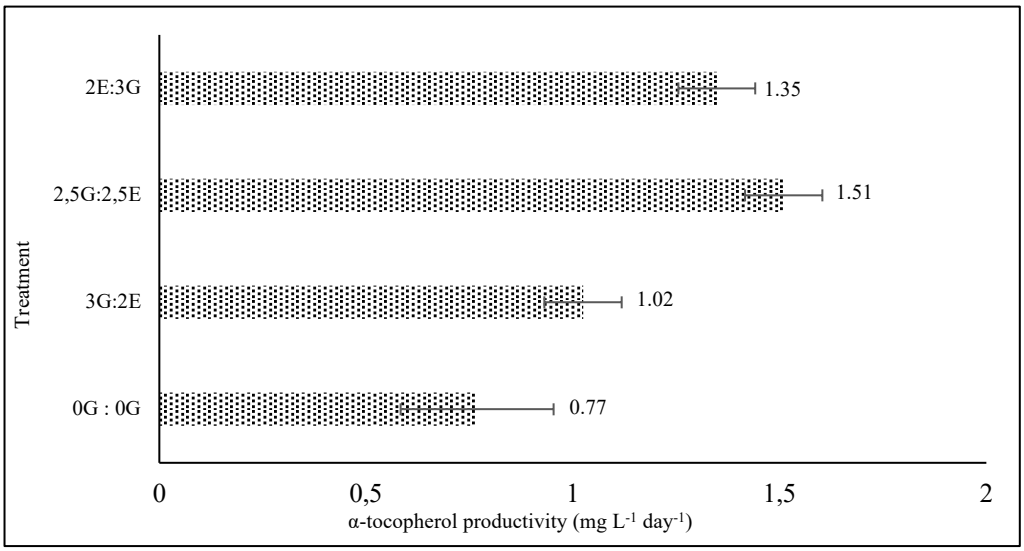

Figure 6. α-tocopherol productivity of *Euglena* sp. in glucose and ethanol mixture with different ratios.

Based on Figure 6, it can be seen the productivity of α-tocopherol produced by *Euglena* sp. in various treatment cultures. The highest α-tocopherol productivity was obtained in cultures treated with 2.5:2.5, which was 1.51 ± 0.09, followed by 2:3 treatment which was 1.35 ± 0.09, and 3:2 treatment was 1.02 ± 0.09. The lowest α-tocopherol productivity was obtained by the control treatment (0:0) of 0.77 ± 0.05. The value of α-tocopherol productivity was divided into three clusters, indicating significant differences between treatments, except for treatments 2.5:2.5 and 2:3, which were in the same notation.

Treatment of 2.5:2.5 resulted in the highest α-tocopherol productivity and average total tocopherol. The highest average total α-tocopherol per gram of cell biomass was produced in the

treatment 2.5:2.5, then followed by the control treatment (0:0); 3:2, and the last one is 2:3 with a successive content of 1.133 ± 0.215; 1.126 ± 0.078; 1.124 ± 0.134; and 0.812 ± 0.032 mg/g-cell. The mixed culture treatment of glucose and ethanol in a ratio of 2.5:2.5 produced the highest total α-tocopherol per gram of cell biomass because the total α-tocopherol produced had the highest content compared to other treatments, and the biomass produced was also high. The control (0:0) treatment delivered high total α-tocopherol per biomass; this was due to the low α-tocopherol content and low biomass obtained by this treatment, so the α-tocopherol content per gram cell was high, but the biomass produced was low. The treatment with the lowest total α-tocopherol per gram of biomass was the 2:3 treatment; in this treatment, the α-tocopherol content made was not too high compared to other treatments but produced the highest biomass in other treatments.

Table 1. The effect of a glucose-ethanol combination on biomass and α-tocopherol synthesis by *Euglena* sp.

Treatment	Average Biomass Production (g L^{-1})	Average α-tocopherol Production (mg L^{-1})	α-tocopherol per Cell Biomass (mg g^{-1}-cells)
0g glucose: 0g ethanol	$4.38^{a} \pm 0.02$	$4.37^{a} \pm 0.06$	$1.13^{b} \pm 0.08$
3g glucose: 2g ethanol	$8.48^{d} \pm 0.04$	$5.21^{b} \pm 0.09$	$0.81^{a} \pm 0.03$
2.5g glucose: 2.5g ethanol	$8.12^{c} \pm 0.05$	$7.09^{d} \pm 0.10$	$1.13^{a} \pm 0.04$
2g glucose: 3g ethanol	$6.18^{b} \pm 0.02$	$6.31^{c} \pm 0.09$	$1.12^{b} \pm 0.06$

[1]Significance at 95%.

4. Discussion

In this study, the growth of *Euglena* sp. was more significant when mixed carbon sources (glucose and ethanol) were added to the culture medium than when the carbon sources were not combined. In this study, adding carbon sources has enhanced cell development (Ogbonna et al., 1988; Afiukwa et al., 2007; Fujita et al., 2008). The highest cell density value was obtained in the 3:2 treatment, where the glucose ratio was higher than in ethanol. This follows the previous research, which states that glucose increases cell density up to 4 times compared to culture with ethanol. The previous study also proved that *Euglena* culture with a higher glucose ratio than ethanol produced higher cell density (Fujita et al., 2008). The occurrence of catabolite repression causes an increase in cell density by glucose. A previous study stated that glucose could affect the plastid *Euglena* sp. in the same way as bacteria and eukaryotes with the repression of these catabolites. Catabolite inhibition establishes the level of carbon resource utilization; before the energy required by the synthetase is consumed, the more efficient carbon source will be fully utilized to use the less efficient carbon source. Extensive research has been conducted on inhibiting catabolic metabolites in prokaryotes and eukaryotes (such as yeast), in which the synthesis of specific enzymes and all organelles, mitochondria, and microorganisms are synthesized inhibited by glucose (a fermentable carbon source). In photosynthetic autotrophic cultures, light acts through two photoreceptors to induce the formation of the enzymatic machinery necessary to use light and carbon dioxide as the sole source of carbon and energy for growth. This process is called chloroplast development (Görke et al., 2008). The regulation of catabolite inhibition involves the phosphotransferase system (PTS). Inactivation of PTS can reduce inhibition by increasing cAMP levels (Zhang et al., 2009). Glucose and Light allow CO_2 to be the only carbon source and energy source for the growth of *Euglena* sp.

Light induces enzymes for chloroplast development that occurs in photoautotrophic growth. Other carbon sources, such as ethanol, will form glyoxysomes when added. Glyoxisomes are organelles that contain enzymes needed for photoheterotrophic growth. Glucose content in the growth medium specifically represses photoinduction enzymes such as chloroplast valyl-t-RNA. The presence of glucose correlates with reduced adenosine 3':5'-monophosphate cAMP in cells. *Euglena* has cAMP and enzymes for its metabolism. The carbon dioxide produced by glucose metabolism is subsequently utilized in photosynthesis (Ogawa et al., 1981). The presence of a preferred carbon source inhibits the expression and, in many cases, the functioning of the catabolic system that facilitates the utilization of secondary substrates.

Adding a glucose and ethanol combination to the culture medium enhanced the α-tocopherol concentration more than the culture without the mixture. The activity of mitochondrial and chloroplasts

influences the formation of α-tocopherol. The mitochondria and cytoplasm contain most enzymes involved in the glycolytic pathway and ethanol metabolism in *Euglena* sp. In general, the light energy absorbed by chlorophyll is utilized for cell development and metabolite formation throughout the photosynthesis process (Schwelitz et al., 1978).

When ethanol is added to the culture medium as a carbon source, it activates the electron transport system in mitochondria and chloroplasts, producing Reactive Oxygen Species (ROS). Superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO) are reactive oxygen species consisting of radical and non-radical oxygen species generated by partial reduction of oxygen. Cellular ROS can be created endogenously, as in mitochondrial oxidative phosphorylation, or by contact with external sources. When ROS invades the cellular antioxidant defense system by raising ROS levels or decreasing cellular antioxidant capability, this is referred to as oxidative stress (Ray et al., 2012). In yeast, ROS is linked to damage to the mitochondrial and cell membranes (Jarboe et al., 2013). Under these conditions, α-tocopherol is synthesized as an antioxidant to prevent damage caused by these reactive oxygen species. Under photoheterotrophic conditions, the chlorophyll concentration was much lower than in photoautotrophic states. This is because adding a carbon source can inhibit the photoautotrophic mechanism so that light energy is not the primary energy source in the metabolism of *Euglena* sp. Thus the synthesis of α-tocopherol in the cultivation of *Euglena* sp. is more dependent on mitochondrial activity than chloroplast activity. Adding ethanol supports the production of α-tocopherol in *Euglena* sp. because it can increase mitochondrial activity, whereas when glucose becomes the primary carbon source, mitochondrial activity decreases sharply (Fujita et al., 2008; Rodriguez-Zavala et al., 2010). This study employs a combination of glucose, favorable for cell development, and ethanol to achieve practical- tocopherol synthesis.

Conclusion

Based on the findings of this study, it is possible that adding a glucose and ethanol combination to a photoheterotrophic culture might enhance the cell growth rate, biomass, and tocopherol content of *Euglena* sp. culture. The 3:2 treatment yielded the highest specific growth rate and biomass, with values of 0.99 (OD680/OD680/h) and 8.48 g L^{-1}, respectively. Meanwhile, the 2.5:2.5 treatment had the greatest tocopherol concentration, with a value of 7.09±0.096 mg L^{-1}.

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Research Article

Host-parasite Interactions between *Solanum aethiopicum, Meloidogyne incognita,* and *Fusarium oxysporum* f.sp. melongenae as Portrayed by Disease Traits and Crop Yield

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Abstract: *Solanum aethiopicum L.* cultivation is highly constrained by wilt disease induced by *Fusarium oxysporum* f.sp melongenae and *Meloidogyne incognita*. The effects of initial population densities of these pathogens on the crop were investigated to enhance knowledge of the host-parasite interactions. The 4 x 4 factorial set of treatments were laid out in the field using the randomized complete block design with three replications. Data were collected on plant vigour, vascular discoloration, fruit weight, shoot weight, root-gall index, final nematode population, disease incidence, and disease severity. The different initial population densities of *Fusarium* and/or *Meloidogyne* spp. had detrimental effects on the crop compared to the Control. The main effects of the pathogens on the crop/wilt showed the existence of cross-over interactions for all the disease parameters. The effects of the pathogens on yield (fruit weight and shoot weight) were partially directly proportional to population densities even though the effects were significantly different ($P \leq 0.05$) compared to the Control. All the disease parameters were positively correlated. Each of the pathogens was capable of causing severe damage to the crop in either single or concomitant infection.

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Footnote: Concomitant and single infection with *Fusarium oxysporum* and *Meloidogyne incognita* on wilt of African garden egg (*Solanum aethiopicum* L) and its management in Makurdi, Nigeria.

1. Introduction

In nature/agroecosystems, a crop is hardly ever exposed to the influence of a single pathogen. In some cases, the occurrence of disease is tied to the concomitant occurrence of two or more pathogens (vital tripartite inter-kingdom interactions). For instance, *Meloidogyne* Göldi (N.b: The order and family of this genus are presented in the discussion section due to the multiplicity of names in the literature related to the order and family), and *Fusarium* (Hypocreales; Nectriaceae) species occurred concurrently on coffee in Costa Rica leading to corchosis disease. The presence of *Meloidogyne arabicida* López & Salazar, or *Meloidogyne exigua* Goeldi alone caused a reduction in coffee shoot height and galling but no corchosis developed (Bertrand et al., 2000; Ndifon, 2019).

Nematodes often help to breach the endodermal barrier enabling the other pathogen(s) to drastically damage the host as was reported for potato cyst nematode and *V. dahliae* in different potato varieties. Back et al. (2002) in a review of disease complexes in plants emphasized the yield loss that results from the occurrence of these disease complexes, knowledge of which is inadequate for most host-parasite interactions. This inadequacy of knowledge on disease complex is still here with us.

Many mechanisms of disease complexes exist. One of which is the mechanism based on the utilization of wounds induced by nematodes for the entrance of soil-borne pathogens. This has as its basis the removal of physical impenetrable barriers that the fungi or bacteria pathogens could not by themselves breach. The type of wound depends on the feeding pattern of the nematode and its life cycle. The timing of inoculation of the second pathogen after the nematode is critical especially when the wounds are just micro-punctures or the pathogen depends more on the splitting of galls, or the presence of channels for the growth of hyphae, or cell proliferation (Back et al., 2002).

Finally, the pathogen may induce changes to the host plant, which may encourage the proliferation of the roots. An increase in number of galls and population of nematode juveniles recovered at the termination of experiments was reported by Back et al. (2002). The pathogen may increase the rate or the quantity of root exudates and the release of carbon dioxide that attracts the nematodes to the roots. The following factors affect disease complexes: i.e. the nematode species present, the initial population of the pathogens, the fungi genotypes, the environmental factors, and the abiotic factors (i.e.; soil pH, the soil structure, the soil moisture content, and the prevalent weather conditions).

African garden eggplant/African garden egg (*Solanum aethiopicum* L. (Solanales; Solanaceae)) is a very important constituent of the human diet and medicine. Cultivation of African garden eggplant is plagued by pests and diseases among which are *Fusarium* and *Meloidogyne* species. Sulaiman et al. (2019) showed that all the inoculum levels (500-8000) of *Meloidogyne incognita* reduced growth parameters leading to a corresponding decrease in the yield of eggplant (*Solanum* spp.). They indicated that the lowest nematode population used (500 eggs/juveniles of *M. incognita*) was capable of reducing the yield of the variety of eggplant used.

Research on diseases of this garden eggplant has not been carried out to the level of those of tomato and potato even though it is a good source of income and employment wherever it is cultivated. This study of the effect of the *Fusarium* /*Meloidogyne* wilt complex on African garden egg was necessitated by the paucity of information on this devastating disease. This study concentrated on the effects of the pathogen species and variation in their initial population densities on the wilt disease of African garden eggplant.

2. Material and Methods

2.1. The study site

The experiment was carried out at the Teaching and Research Farm of the College of Agronomy, Federal University of Agriculture, Makurdi (07°45″N by 08°37″E). Ibrahim and Idoga (2015) reported that the farm which is within the flood plain of River Benue is underlain by consolidated Makurdi sandstone, Turanian Eze-Aku shales, and Alluvium. They pointed out that superficial deposits such as weathered rocks, laterites, and alluvium extensively cover the study area. Furthermore, they observed that the soils were low in fertility and had a predominant sandy loam texture with a low total porosity, are moderate to slightly acidic (pH range of 5.56-6.17), are low in available P, total N, exchangeable K, Ca, while Mg and Na are moderate.

2.2. Experimental procedures

2.2.1. Sourcing, culturing, raising, and identification of the pathogens

F. oxysporum Schelecht ex Fries (Syn. & Hans) f.sp. melongenae Mauto and Ishigami (no races of this forma specialis have been reported so far (Edel-Hermann and Lecomte, 2019)) were isolated from *S. aethiopicum* plants (obtained from farmers' farms in Makurdi and the Research Farm), showing wilt symptoms. The fungus was isolated using Acetate Differential Agar (i.e. a Difco dehydrated medium) which was enriched with dextrose.

The African garden egg stems were thoroughly washed with tap water to reduce bacterial contamination. Pieces of the stem and roots (2 cm long) were surface sterilized using 1% sodium

hypochlorite for 20 minutes (Bertrand et al., 2000). Each medium was autoclaved at 15 psi for 15 minutes at 121 ^{o}C after which they were allowed to cool to about 50 ^{o}C and 50 mg L^{-1} of streptomycin sulphate in sterile distilled water was added to each of the media to minimize bacterial contamination. The fungal colonies were subcultured till pure cultures were obtained and identified using literature and microscopy. The fungal spores were counted using haemocytometry (Ndifon, 2019).

The *Meloidogyne incognita* (Kofold & White) Chitwood (race 1 is very common in the area), but the race was not determined as explained later. The nematode was obtained initially from *S. aethiopicum* roots (obtained from farmers' farms in Makurdi and the Research Farm). At the laboratory, the female nematode for each specific eggmass was identified by cutting the perennial pattern. Thereafter the egg masses were used to inoculate the sterilized soil in pots on which three-week-old *S. aethiopicum* seedlings were transplanted in the screenhouse. The *S. aethiopicum* plants were replaced when they stopped growing.

Estimating of the population of the nematodes in the soil before the experiments: The initial nematode population was estimated by collecting soil and plants from the inoculum that was being raised under an African garden egg (containing previously identified nematode specimens). These were taken to the laboratory for extraction using the modified Baermann tray method. All the galled roots were cut into tiny pieces (approximately 1 cm long). Any remaining egg masses in the soil that were dislodged from the female nematode were used as well to produce J2 larvae by allowing eggs to hatch for 6 days in Baerman trays.

This modified Baermann tray method for extraction of nematodes consisted of a coarse mesh sieve which was used to support the root/soil/eggmass sample when setting up the apparatus. On top of the sieve, a double layer 2-ply fine texture paper tissue was placed. The sieve was made to rest on top of a collecting tray and 200 cm^3 of finely crumbled soil (less than 8 mm particle size) or plant tissue was spread in the sieve setup. Tap water was gently added to the inside of the collecting tray until the soil layer was completely saturated.

The extracted nematodes were decanted after every 12 hours and the water in the collecting tray was topped up often to avoid desiccation until the 6th day after commencement of extraction. The nematodes being collected were stored at room temperature in shallow trays filled with water and stirred daily. The sieve was gently removed and the nematode suspension in the tray was poured into a tall 500 ml beaker. This was allowed to stand for four hours.

Then excess supernatant was gently decanted. The process ensured that the eggs were hatched and thus included in the count of the real population of nematodes applied during the study. The nematodes finally utilized for inoculation were referred to simply as nematodes. The final population (Pf) values consisted of the hatched J2 plus already present J2 plus any adult males present in the soil i.e. Pf = (J2 from egg masses + J2 juveniles already in plant/soil + adults in the soil if any) nematodes (Ndifon, 2019). The nematode larvae were counted by taking an aliquot and counting the larvae content of a counting dish under a stereomicroscope. Three counts were averaged to get the number of nematodes per litre of water.

2.2.2. Inoculation of the soil

Based on the field layout, the soil was inoculated at transplanting time using this *Meloidogyne* (Pf) extracted. After placing the seedling in the hole and the hole filled up with the soil scooped out of the hole, the required quantity of supernatant was poured using a pipette pump under the base of the plants in the plot at transplanting. Inoculation of the soil with *Fusarium* was carried out at transplanting time. The *Fusarium* inoculants were applied using sterile distilled water. The inoculum was obtained from 6 day-old-young culture by scooping the fungus mycelium off and smearing it to dislodge the spores then filtered through Whatmann No. 1 filter paper. Two shallow holes were made at the base of the transplanted seedling and the fungus inoculum was poured into the holes. The holes were then covered with the soil.

2.2.3. Experimental design

The research commenced with the sowing of the seeds on the 21st of June in both years. Transplanting of the seedlings was carried out on July 21st (28 days after sowing). The land was cleared, pegged and micro-plot beds were made manually. The seedlings were transplanted at a spacing of 25 cm intra-row in the centre of the micro-plot. The experiment was carried out using the microplot

technique (Ndifon, 2019). Each field microplot was 1.35 x 1.0 m^2. The inter-block furrow was 1.2 m wide and the spacing between treatments in a block was 0.45 m wide. The 4 x 4 factorial set of treatments used was arranged in a randomized complete block design and each treatment was replicated three times. The treatments consisted of four levels/population densities/population concentrations of *Fusarium* sp. (0, 1 x 10^4, 1 x 10^5, and 1 x 10^6 spores) and four levels/population densities/population concentrations of *Meloidogyne* sp. (0, 300, 600, and 900 nematodes) as shown in Table 1.

Table 1: The treatment set utilized for the experiment

Treatment	Description of the treatments
T1	Control (Meloidogyne 0* x Fusarium 0* spore)
T2	Meloidogyne 300 x Fusarium 0 spore
T3	Meloidogyne 600 x Fusarium 0 spore
T4	Meloidogyne 900 x Fusarium 0 spore
T5	Meloidogyne 0 x Fusarium 1x10^4 spores
T6	Meloidogyne 300 x Fusarium 1x10^4 spores
T7	Meloidogyne 600 x Fusarium 1x10^4 spores
T8	Meloidogyne 900 x Fusarium 1x10^4 spores
T9	Meloidogyne 0 x Fusarium 1x10^5 spores
T10	Meloidogyne 300 x Fusarium 1x10^5 spores
T11	Meloidogyne 600 x Fusarium 1x10^5 spores
T12	Meloidogyne 900 x Fusarium 1x10^5 spores
T13	Meloidogyne 0 x Fusarium 1x10^6 spores
T14	Meloidogyne 300 x Fusarium 1x10^6 spores
T15	Meloidogyne 600 x Fusarium 1x10^6 spores
T16	Meloidogyne 900 x Fusarium 1x10^6 spores

*The Figure behind *Meloidogyne* or *Fusarium* spp. refer to the population level/densities/population concentrations. In the text, references to *Fusarium* population densities are shortened by using only the exponent of the density without the 1x.

2.3. Data collection

Transplanting of the seedlings was carried out after four weeks (28 days) from germination. Five African garden egg seedlings were planted in each microplot along a row when the experiment was set up. The plants were cultivated for 75 days after inoculation/transplanting (DAT/DAS/DAI) before the termination of the experiment. The data that were collected included yield data (fruit weight and shoot weight) and disease data (root-gall index, number of nematodes per 500 cm^3 of soil, wilt discoloration index, disease incidence, and disease severity). Other plant growth data were presented elsewhere.

2.3.1.Disease incidence

This was calculated using Equation 1.

$$D.I = \{(\sum I_p)/\sum A_p\} \times 100\% \quad (1)$$

Where

D.I = Disease incidence
Ip = Number of infected plants (wilted, stunted, drooped leaves, epinastic leaves, chlorotic leaves, dead plants)
Ap = Number of assessed plants

Source: Ndifon (2019) as modified

2.3.2. Disease severity

Disease severity was obtained using Equation 2.

$$D.S = \{(\sum I_s)/3 \times \sum P_a\} \times 100\% \quad (2)$$

Where

D.S = Disease severity

3 = Highest severity score (Three (3) was the highest score on the individual disease severity scale below)
P_a = number of plants assessed
I_s = Individual disease severity scores (obtained using the modified individual disease severity scale below).
Source: Ndifon (2019) as modified

2.3.3. Modified individual disease severity scores

I_s as in equation 2
0 = No wilting at all, healthy plants
1 = Less than 1/3 of leaves wilted/dropped off
2 = More than 1/3 of leaves wilted/dropped off
3 = More than 2/3 of leaves wilted/dropped off or dead plants

2.3.4. Plant Vigour scale

Overall landscape performance scale
10 = Perfect foliage absolutely covered with blossoms/fruits, outstanding growth habit, flawless plants (very rare to attend).
9 = Very healthy foliage, an abundance of blossoms/fruits, and really nice growth habit.
8 = Healthy foliage with a significant number of blossoms/fruits, nice growth habit.
7 = Healthy foliage, only a few blossoms/fruits, nice growth habit.
6 = Healthy foliage, no blossoms/fruits, nice growth habit.
5 = 10% leaf drop, no blossoms/fruits
4 = 25% leaf drop, no blossoms/fruits
3 = 50% leaf drop, no blossoms/fruits
2 = 75% leaf drop, no blossoms/fruits
1 = 90% leaf drop, no blossoms/fruits
0 = Dead plants

Plus or minus a point

Use these adjustments with this National Earth-Kind Rose rating scale

- Good blossoms with fragrance = add 1 point
- If leaves intact but insect infected = minus 1 point
- If old sepals did not drop off = minus 0.5 point
- If poor growth habit overall = minus 1 point
- If significant chlorosis = minus 1 point
- If the disease in a replication = use zero in averaging e.g. (0+1+3)/3 = 4/3.

Scale as modified by Ndifon (2019) from National Earth-Kind Rose ratings (usually for use at blossom and fruiting stages of plant life)

2.3.5. Root knotting/root-gall index

Before putting the uprooted plants in polythene bags to take to the laboratory, nematode gall rating was carried out based on the following scale.

0 = Completely healthy root system, no infection.
1 = Very few small galls that can only be seen under close examination.
2 = Small galls as in "1" but more numerous and easy to detect.
3 = Numerous small galls, some galls coalesced, majority of roots still functioning.
4 = Numerous galls, a few big galls, root severely affected.
5 = 25% of root system severely galled.
6 = 50% of the root system severely galled.
7 = 75% of root system severely galled
8 = No healthy roots, plant still green.
9 = Completely galled, root system is rotting, dying.

10 = Plant and roots dead.
Sources: Ndifon (2019) as modified.

2.3.6. Fusarium vascular wilt discoloration rating

When plants were uprooted at the farms, the basal parts of the shoots were split longitudinally and discoloration of the vascular tissues was scored.

1 = No browning.
2 = Browning only around the base.
3 = Faint or patchy browning but limited below the first stem node.
4 = Strong browning but limited below the first stem node.
5 = Browning visible and extending above the first stem node.
6 = Browning extending above the first stem node and in up to half of the total number of nodes.
7 = Strong vascular browning in all but the uppermost internodes.
8 = Strong browning throughout the stem vascular tissue.
9 = Strong browning throughout the stem vascular tissue, branches wilted.
10 = Strong browning throughout the stem vascular tissue, dead plant.
Source: Ndifon (2019) as modified

2.3.7. Number of Meloidogyne sp. per 500 cm^3 of soil

Composite soil samples from the bases of the plants per plot were taken from the field to the laboratory and the nematodes were extracted at 75 days after inoculation (DAI) using the modified Baermann tray method.

2.3.8. Shoot and fruit weights

Shoot and fruit weights (g) per plant were taken at 75 DAI from three plants using an electronic balance and the mean weight was calculated.

2.4. Data analysis

Data were collected from the middle three plants per plot. The data collected were subjected to analysis of variance (ANOVA) using Genstat® statistical software (2nd edition, Discovery). Significant differences between means were separated using the new Duncan's multiple range test (DMRT) ($P \leq 0.05$).

3. Results

3.1. Determination of main effects of the population densities of these two pathogens using tests of between subjects effects in the 2014 cropping season

Tests of between subjects effects were used to determine the main effects of *Meloidogyne* sp. on vascular discoloration ($F(0.05) = 0.392$, $p = 0.759$) which was not significant though inoculation of soil with *Fusarium* sp. ($F(0.05) = 7.056$, $p = 0.00$) showed significant main effects of vascular discoloration. Analysis of incidence of wilt disease ($F(0.05) = 8.531$, $p = 0.00$), the severity of wilt disease ($F(0.05) = 3.969$, $p = 0.017$), root gall index ($F(0.05) = 36.081$, $p = 0.00$), and nematode count ($F(0.05) = 904.26$, $p = 0.000$) using tests of between subjects effects revealed the existence of significant main effects due to inoculation of soil with *Meloidogyne* sp. Analysis of the incidence of wilt disease ($F(0.05) = 5.990$, $p = 0.001$) and severity of wilt ($F(0.05) = 3.289$, $p = 0.023$) revealed that inoculation of soil with *Fusarium* sp. resulted in significant main effects.

Analysis of vigour ratings ($F(0.05) = 0.718$, $p = 0.549$) and fruit weights ($F(0.05) = 0.827$, $p = 0.489$) using tests of between-subjects effects revealed the existence of significant main effects due to inoculation of soil with *Meloidogyne* sp. Analysis of plant vigour ($F(0.05) = 1.168$, $p = 0.344$) and fruit weight ($F(0.05) = 0.680$, $p = 0.611$) revealed that inoculation of soil with *Fusarium* sp. resulted in significant main effects. Analysis of shoot weight revealed that inoculation of soil with *Meloidogyne* sp. ($F(0.05) = 0.177$, $p = 0.911$) and *Fusarium* sp. ($F(0.05) = 0.376$, $p = 0.824$) did not show significant main effects.

3.2. Determination of the main effects of these two pathogen species using tests of between subjects effects in the 2015 cropping season

Determination of the main effects using tests of between-subjects effects in 2015-cropping season revealed that incidence of wilt disease (F (0.05) = 2.313, p = 0.097), severity of wilt disease (F (0.05) = 4.369, p = 0.011), root gall index (F (0.05) = 10.109, p = 0.00), and nematode count (F (0.05) = 6.828, p = 0.001) showed the presence of significant main effects due to inoculation of the soil with *Meloidogyne* sp., while analysis of incidence of wilt disease (F (0.05) = 13.168, p = 0.000), severity of wilt disease (F (0.05) = 6.828, p = 0.001) using tests of between subjects effects revealed existence of significant main effects due to inoculation of the soil with *Fusarium* sp. Analysis of vascular discoloration scores showed that *Meloidogyne* sp. population densities had significant main effects (F (0.05) =2.665, p = 0.064), while *Fusarium* sp. population densities had significant main effects (F (0.05) = 37.896, p = 0.00).

Analysis of plant vigour ratings (F (0.05) = 3.274, p = 0.034), and fruit weight (F (0.05) = 0.936, p = 0.435) using tests of between subjects' effects revealed the existence of significant main effects due to inoculation of soil with *Meloidogyne* sp. While analysis of plant vigour (F (0.05) = 1.628, p = 0.202) revealed that *Fusarium* population densities had significant main effects but the fruit weight did not show significant main effects (F (0.05) = 0.137, p = 0.937) due to inoculation of the soil with *Fusarium* sp. Analysis of shoot weight revealed that *Meloidogyne* sp. population densities (F (0.05) = 0.097, p = 0.961) did not have significant main effects, but *Fusarium* sp. population densities (F (0.05) = 2.230, p = 0.104) had significant main effects.

3.3. Interaction of these two pathogen species at different population densities

3.3.1. Interaction of these two pathogen species at different population densities in 2014 cropping season

Interactions of different population densities of *Meloidogyne* sp. and *Fusarium* sp. on African garden egg in the 2014-cropping season are presented in Figure 1. There was a significant interaction between population densities of *Meloidogyne* sp. and those of *Fusarium* sp. as shown in the Figure for the incidence of wilting (F (0.05) = 1.178, p = 0.343), severity of wilt (F (0.05) = 2.966, p = 0.012), vascular discoloration (F (0.05) = 1.711, p = 0.129), root gall index (F (0.05) = 1.828, p = 0.104), and nematode count (F (0.05) = 2.166, p = 0.053). The type of interaction for all these parameters was cross-over interaction. For all the parameters assessed the interactions were such that when the pathogens were combined at lower population densities, lower parameter marginal mean scores were recorded while higher population densities led to higher parameter marginal mean scores compared to the lower population densities/combinations.

3.3.2. Interaction of these two pathogens at different population densities in 2015-cropping season

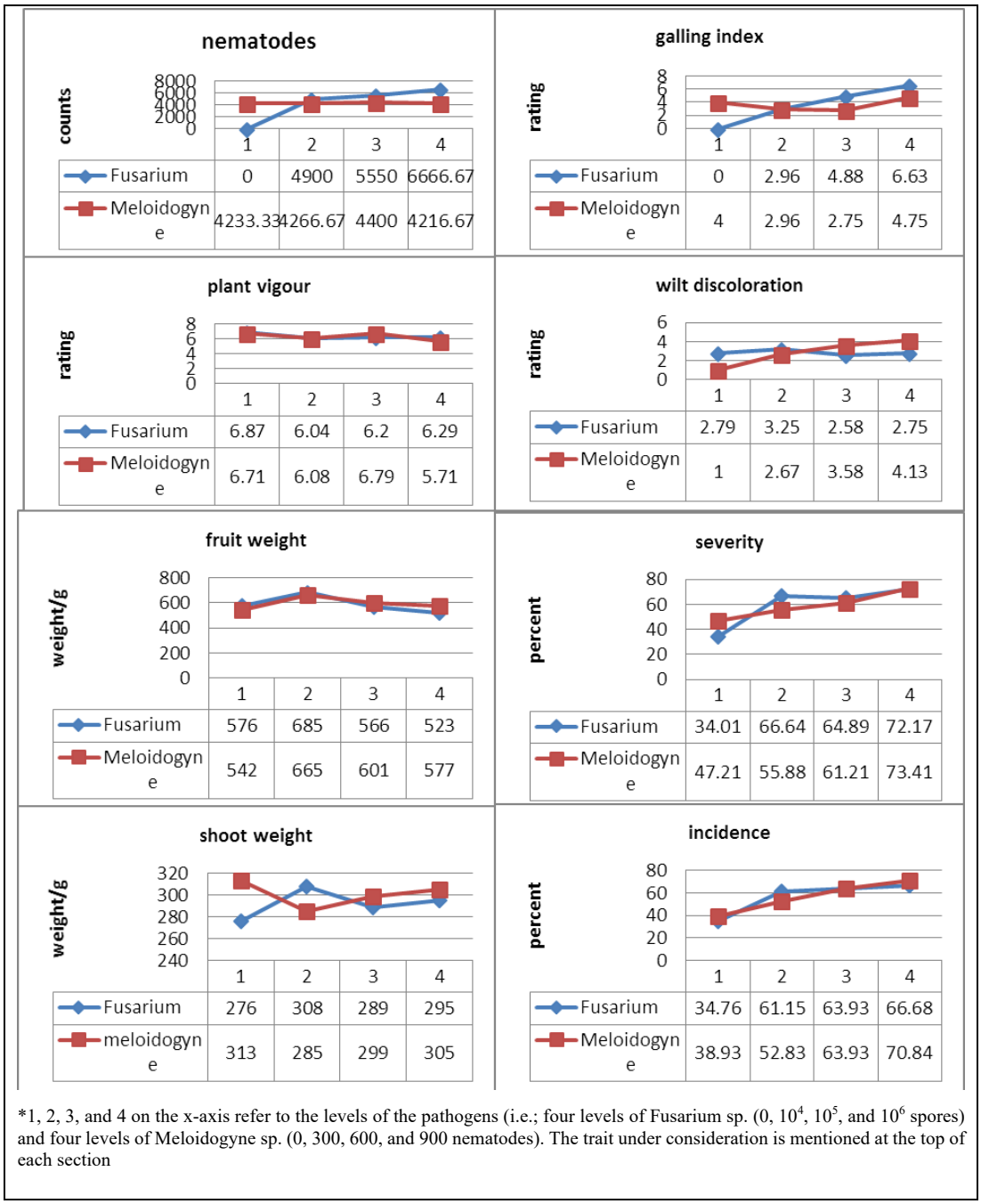
Interactions of different population densities of *Meloidogyne* and *Fusarium* spp. on African garden eggplant in the 2015-cropping season are presented in Figure 2. There was a significant interaction between population densities of *Meloidogyne* sp. and those of *Fusarium* sp. for incidence of wilting (F (0.05) =1.612, p = 0.158), vascular discoloration (F (0.05) = 0.772, p = 0.642), root gall index (F (0.05) = 1.652, p = 0.142), nematode count (F (0.05) = 2.853, p = 0.014), while for severity of wilt, the interaction was significant (F (0.05) = 1.342, p = 0.255).

There was a significant interaction between the population densities of *Meloidogyne* and those of *Fusarium* sp. for plant vigour (F (0.05) = 2.135, p = 0.056), fruit weight (F (0.05) = 0.705, p = 0.700), and shoot weight (F (0.05) = 1.091, p = 0.397). For growth parameters (plant vigour and shoot weight) the interactions were such that when the pathogens were combined at lower population densities, higher parameter marginal mean scores were recorded, while higher population densities led to lower growth parameter marginal mean scores.

The type of interaction for all these parameters was cross-over interaction. For disease parameters (incidence of wilting, vascular discoloration, root gall index, and nematode count) the interactions were such that when the pathogens were combined at lower population densities, lower parameter marginal mean scores were recorded, while higher population densities led to higher parameter marginal mean scores compared to the lower population densities.

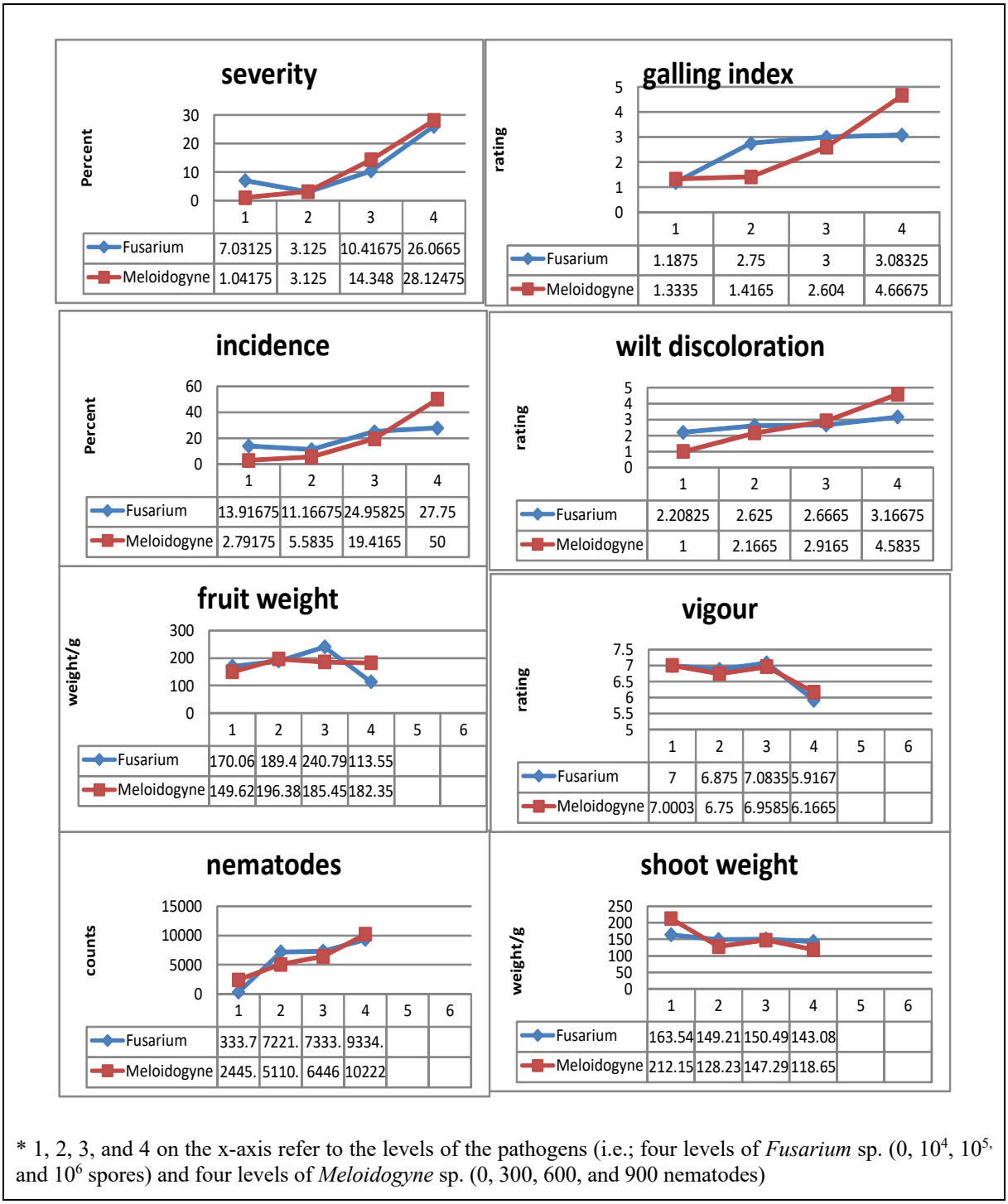
3.3.3. Correlation of disease parameters due to inoculation of soil with different population densities of these two pathogens during the two cropping seasons

The correlation of disease parameters due to inoculation of soil with different *Meloidogyne* sp. and *Fusarium* sp. concentrations in both cropping seasons are presented in Table 2 All the disease parameters (incidence of wilt, severity of wilt, vascular discoloration, root gall index, and nematode count) were positively correlated except root gall index in 2014 and 2015 (which was negatively correlated with vascular discoloration). All the parameters (vascular discoloration, root gall index, severity, and incidence of wilt disease) were positively correlated with 0.76, 0.03, 0.33, and 0.59 respectively in the first year and 0.02, 0.76, 0.33, and 0.57 respectively in the second year with nematode counts.



*1, 2, 3, and 4 on the x-axis refer to the levels of the pathogens (i.e.; four levels of Fusarium sp. (0, 10^4, 10^5, and 10^6 spores) and four levels of Meloidogyne sp. (0, 300, 600, and 900 nematodes). The trait under consideration is mentioned at the top of each section

Figure 1. Interaction of different population densities of *Meloidogyne* and *Fusarium* spp. on African garden egg at 75 DAS in 2014-cropping season.



* 1, 2, 3, and 4 on the x-axis refer to the levels of the pathogens (i.e.; four levels of *Fusarium* sp. (0, 10^4, 10^5, and 10^6 spores) and four levels of *Meloidogyne* sp. (0, 300, 600, and 900 nematodes)

Figure 2. Interaction of the different population densities of *Meloidogyne* sp. and *Fusarium* sp. on African garden egg at 75 DAS in the 2015-cropping season.

Table 2. Correlation of disease parameters due to inoculation of soil with different *Meloidogyne* and *Fusarium* spp. population densities in both cropping seasons

2014 cropping season at 75 DAS	Wilt incidence	Wilt severity	Root gall index	Wilt discoloration	Nematode count
Wilt incidence	1				
Wilt severity	0.32	1			
Root gall index	0.23	0.14	1		
Wilt discoloration	0.42	0.24	-0.01	1	
Nematode count	0.59	0.33	0.03	0.76	1
2015 cropping season at 75 DAS					
Wilt incidence	1				
Wilt severity	0.31*	1			
Root gall index	0.42**	0.24	1		
Wilt discoloration	0.25	0.13	-0.01	1	
Nematode count	0.57**	0.33*	0.76**	0.02	1

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

3.3.4. Interaction of the densities of these two pathogen species at 75 DAS in 2014

Interactions of varying population densities of *Fusarium* and *Meloidogyne* spp. inoculants on wilt disease, growth, and yield of African garden egg in the field during the 2014-cropping season are presented in Table 3. It was observed that infected treatments performed significantly worse than the uninfected control no matter the population density of *Meloidogyne* or *Fusarium* species based on the root gall index, incidence, and severity of wilt.

Vascular discoloration increased with population densities of *Meloidogyne* and *Fusarium* spp. thereby resulting in a higher level of discoloration which was directly proportional to the population density of the *Fusarium* sp. utilized. Vigour and fruit weight did not show clear trends in this study even though significant differences were observed between treatments. This observation was typical of wilt disease induced by *Fusarium* sp. which may result in sudden death of asymptomatic plants. Nematode count was higher in plots infected with *Meloidogyne* (900 nematodes) by *Fusarium* 10^4 spores, *Meloidogyne* 900 x *Fusarium* 10^5 spores, and *Meloidogyne* 900 *x Fusarium* 10^6 spores followed by treatments with *Meloidogyne* 600 nematodes at all levels of *Fusarium* sp. At *Fusarium* 0, 10^4, 10^5, and 10^6 spores, the severity of wilting was significantly higher in all infected plots compared to the control.

Incidence of African garden egg wilt was lowest in the control and was highest in *Meloidogyne* 900 nematodes x *Fusarium* 10^4 spores, *Meloidogyne* 300 x *Fusarium* 10^6 spores, *Meloidogyne* 600 x *Fusarium* 10^6 spores, and *Meloidogyne* 900 x *Fusarium* 10^6 spores. The severity of the wilt disease was significantly lowest in the control and nematode count was significantly higher in *Meloidogyne* 900 nematodes by *Fusarium* 0 spores.

The highest severity was recorded in *Meloidogyne* 900 nematodes x *Fusarium* 10^4 spores and *Meloidogyne* 300 x *Fusarium* 10^6 spores, although these were not significantly different from other infected treatments. Galling was significantly higher ($P \leq 0.05$) in plots infected with *Meloidogyne* 900 x *Fusarium* 10^6 spores compared with the other treatments except for *Meloidogyne* 600 x *Fusarium* 10^6 spores. The nematode count followed the same trend as the root gall index. The vascular discoloration was significantly ($P \leq 0.05$) lower in treatments without *Fusarium* sp. compared with the other treatments (except plots treated with *Meloidogyne* 900 x *Fusarium* 10^4 spores and in *Meloidogyne* 900 x *Fusarium* 10^6 spores).

Fruit weight was significantly ($P \leq 0.05$) lower in treatments infected with *Meloidogyne* 300 nematodes x *Fusarium* 10^4 spores compared with other treatments (except in *Meloidogyne* 900 x *Fusarium* 0 spore and *Meloidogyne* 300 x *Fusarium* 0 spore). Higher fruit weights were recorded in the control and treatments infected with *Meloidogyne* 600 x *Fusarium* 10^5 spores. The lowest plant vigour was recorded from *Meloidogyne* 300 nematodes x *Fusarium* 10^4 spores.

Table 3. Interaction of the varying population densities of *Fusarium* and *Meloidogyne* species inoculants on wilt disease and yield of African garden egg in the field during the 2014-cropping season

Treatment at 75 DAS	Disease severity (%)	Root galling index	Wilt discoloration	Nemato de count /500 cm^3	Plant vigour	Fruit weight (g)	Shoot weight (g)	Disease incidence (%)
Meloidogyne 0 nematode x *Fusarium 0* Spore (control)	0.0a	0.0a	1.0a	0.0a	8.2b	708ab	800	0.0d
Meloidogyne 300 x *Fusarium* 0 spore	65.9b	4.7bc	1.0a	4933bc	5.5ab	450a	338	44.5abc
Meloidogyne 600 x *Fusarium* 0 spore	70.1b	5.6bc	1.0a	5733def	6.8ab	692ab	327	44.5bc
Meloidogyne 900 x *Fusarium* 0 spore	62.5b	5.8bc	1.0a	6267fg	6.3ab	317a	296	66.7a
Meloidogyne 0 x *Fusarium* 10^4 spores	56.8b	0.0a	3.3bc	0.0a	6.8ab	583a	247	33.4c
Meloidogyne 300 x *Fusarium* 10^4 spores	78.4b	3.2b	4.3bc	4667b	6.0ab	1100b	354	55.6abc
Meloidogyne 600 *x Fusarium* 10^4 spores	74.3b	3.0b	2.0bc	5867ef	4.8a	343a	206	66.7ab
Meloidogyne 900 *x Fusarium* 10^4 spores	83.3b	5.7bc	1.0a	6533gh	6.7ab	683ab	333	55.6abc
Meloidogyne 0 x *Fusarium* 10^5 spores	65.9b	0.0a	4.7bc	5200bcd	7.2a	565a	275	66.7ab
Meloidogyne 300 *x Fusarium* 10^5 spores	79.2b	0.0a	2.3ab	0.0a	6.0ab	600ab	346	55.6abc
Meloidogyne 600 *x Fusarium* 10^5 spores	50.0b	4.7bc	4.0abc	5333cde	7.3ab	712ab	289	66.7ab
Meloidogyne 900 *x Fusarium* 10^5 spores	59.5b	6.3cd	3.3abc	7067h	6.7ab	527a	288	66.7ab
Meloidogyne 0 x *Fusarium* 10^6 spores	62.5b	0.0a	4.7bc	0.0a	6.2ab	592a	260	44.5bc
Meloidogyne 300 x *Fusarium* 10^6 spores	74.3b	4.0bc	3.0abc	4800bc	5.5ab	625ab	264	77.8a
Meloidogyne 600 x *Fusarium* 10^6 spores	65.2b	6.3cd	3.3abc	5267cd	5.8ab	517a	346	77.8a
Meloidogyne 900 x *Fusarium* 10^6 spores	83.3b	8.7d	5.7c	6800gh	5.5ab	567a	263	77.8a
SED	15.3	1.3	1.2	259	1.2	220	81	13.02

Means in the same column followed by the same letter(s) are statistically similar using DMRT (P≤0.05).

3.3.5. Interactions between population densities of these two pathogen species at 75 DAS in 2015

Interaction of varying population densities of *Fusarium* and *Meloidogyne* spp. inoculum on wilt disease, growth, and yield of African garden egg in Makurdi in the field during the 2015-cropping season are presented in Table 4. Incidence and severity of wilt disease increased with an increase in both *Meloidogyne* and *Fusarium* spp. population densities. Root gall index and nematode count increased with an increase in both *Meloidogyne* and *Fusarium* spp. population densities. Vascular wilt discoloration ratings were higher in the presence of *Fusarium* sp. and it increased with the population densities of both *Meloidogyne* and *Fusarium* spp.

When the plants were not infected with *Fusarium* (0 spores), root gall index, and nematode counts were significantly higher in infected treatments but when the plants were infected with *Fusarium* 10^4 spores, root gall index was significantly higher in *Meloidogyne* 600 nematodes by *Fusarium* 10^4 spores compared with the other treatments. Nematode count was significantly higher in *Meloidogyne* 300 by *Fusarium* 10^4 spores and *Meloidogyne* 900 nematodes by *Fusarium* 10^4 spores.

When the plants were infected with *Fusarium* 10^5 spores, the root gall index was significantly higher in all *Meloidogyne* infected treatments. When the plants were infected with *Fusarium* 10^5 spores, nematode count was significantly higher in *Meloidogyne* 600 by *Fusarium* 10^5 spores compared with other treatments. When the plants were infected with *Fusarium* 10^6 spores, root gall index, and nematode counts were significantly higher in *Meloidogyne* 300 x *Fusarium* 10^6 spores and *Meloidogyne* 900 nematodes x *Fusarium* 10^6 spores compared with the other treatments.

Root gall index was significantly higher in plots infected with *Meloidogyne* 900 nematodes by *Fusarium*10^6 spores, and *Meloidogyne* 300 by *Fusarium* 10^6 spores. Root gall index was lower in plots infected with *Meloidogyne* 900 by *Fusarium* 10^4 spores, *Meloidogyne* 300 by *Fusarium* 10^4 spores,

Meloidogyne 900 x *Fusarium* 0 spores, and *Meloidogyne* 600 x *Fusarium* 0 spores. Nematode count was higher in plots infected with *Meloidogyne* 900 by *Fusarium* 10^6 spores, and *Meloidogyne* 300 by *Fusarium* 10^6 spores. Nematode count was lower in plots infected with *Meloidogyne* 300 x *Fusarium* 0 spores.

Generally, the incidence of wilt was lowest in the control and in the treatments infected with *Meloidogyne* 600 nematodes x *Fusarium* 0 spore, *Meloidogyne* 900 x *Fusarium* 0 spore, and *Meloidogyne* 300 x *Fusarium* 10^4 spores. *Meloidogyne* 600 x *Fusarium* 10^4 spores, *Meloidogyne* 0 x *Fusarium* 10^4 and *Meloidogyne* 300 x *Fusarium* 10^5 spores. The highest incidence was recorded in *Meloidogyne* 600 x *Fusarium* 10^6 spores and this was not different from those of *Meloidogyne* 900 x *Fusarium* 10^6 spores, *Meloidogyne* 300 x *Fusarium* 10^6 spores, *Meloidogyne* 0 x *Fusarium* 10^6 spores and *Meloidogyne* 900 x *Fusarium* 10^5 spores.

The severity of wilt was significantly ($P \leq 0.05$) higher in treatments infected with *Meloidogyne* 900 x *Fusarium* 10^6 spores compared with the other treatment (except *Meloidogyne* 900 x *Fusarium* 10^5 spores). No wilt was observed in the control and plots treated with *Meloidogyne* 300 x *Fusarium* 0 spore, *Meloidogyne* 600 x *Fusarium* 0 spore, *Meloidogyne* 900 x *Fusarium* 0 spore, *Meloidogyne* 300 *Fusarium* 10^4 spores, and *Meloidogyne* 600 x *Fusarium* 10^4 spores. The vascular discoloration was lowest in the control and treatments without *Fusarium* (0 spore).

Furthermore, nematode count was significantly ($P \leq 0.05$) higher in treatments infected with *Meloidogyne* 900 x *Fusarium* 10^6 spores compared with the other treatments, except *Meloidogyne* 300 x *Fusarium* 10^6 spores and *Meloidogyne* 600 x *Fusarium* 10^5 spores. Root gall index was significantly lowest in *Meloidogyne* 0 x *Fusarium* 10^4 spores, *Meloidogyne* 0 x *Fusarium* 10^5 spores, *Meloidogyne* 300 x *Fusarium* 10^5 spores, and *Meloidogyne* 0 x *Fusarium* 10^6 spores, but it was significantly higher in all other treatments.

The vascular discoloration was significantly ($P \leq 0.05$) higher in *Meloidogyne* 900 nematodes x *Fusarium* 10^6 spores compared with the other treatments. Fruit weight was highest in treatments infected with *Meloidogyne* 900 x *Fusarium* 10^4 spores, but this was not different from those of control, *Meloidogyne* 900 x *Fusarium* 10^6 spores and *Meloidogyne* 600 x *Fusarium* 10^6 spores, *Meloidogyne* 600 x *Fusarium* 10^5 spores *Meloidogyne* 300 x *Fusarium* 10^5 spores and *Meloidogyne* 300 x *Fusarium* 0 spore. The lowest fruit weight was recorded in plots infected with *Meloidogyne* 900 nematodes x *Fusarium* 0 spore and *Meloidogyne* 900 x *Fusarium* 10^6 spores.

Table 4. Interaction of the varying population densities of *Fusarium* and *Meloidogyne* inoculum on wilt disease and yield of African garden egg in the field during the 2015-cropping season

Treatment at 75 DAS	Disease severity (%)	Root gall index	Wilt discoloration	Nematode counts /500 cm^3	Plant vigour	Fruit weight (g)	Shoot weight (g)	Disease incidence (%)
Meloidogyne 0 x *Fusarium 0* spore (control)	0.0a	0.0a	1.0a	0.0a	7.6b	177	218ab	0.0a
Meloidogyne 300 x *Fusarium* 0 spore	4.2a	2.0bcd	1.0a	888a	7.7b	254	210ab	11.7ab
Meloidogyne 600 x *Fusarium* 0 spore	0.0a	1.7bc	1.0a	3557ab	6.0ab	45	159ab	0.0a
Meloidogyne 900 x *Fusarium* 0 spore	0.0a	1.7bc	1.0a	5334ab	6.7b	120	259b	0.0a
Meloidogyne 0 x *Fusarium* 10^4 spores	4.2a	0.0a	1.3ab	0.0a	7.0b	157	133ab	11.2ab
Meloidogyne 300 x *Fusarium* 10^4 spores	0.0a	1.3ab	2.3abc	8886bcd	6.0ab	4	42a	0.0a
Meloidogyne 600 *x Fusarium* 10^4 spores	8.3ab	3.0cde	2.3abc	2666ab	8.0b	385	206ab	0.0a
Meloidogyne 900 *x Fusarium* 10^4 spores	0.0a	1.3ab	2.7bcd	8890bcd	6.0ab	147	130ab	11.2ab
Meloidogyne 0 x *Fusarium* 10^5 spores	0.0a	3.0cde	3.0cd	4448ab	7.0b	237	174ab	0.0a
Meloidogyne 300 *x Fusarium* 10^5 spores	4.2a	0.0a	2.0abc	0.0a	6.7b	114	224ab	0.0a
Meloidogyne 600 *x Fusarium* 10^5 spores	16.7ab	3.3def	3.3cde	13331cd	7.7b	247	116ab	33.3abc
Meloidogyne 900 *x Fusarium* 10^5 spores	37.bc	3.3def	3.3cde	71114abc	6.7b	118	84ab	44.3bc
Meloidogyne 0 x *Fusarium* 10^6 spores	20.ab	4.0ef	4.0de	0.0a	6.3b	192	92ab	44.5bc
Meloidogyne 300 x *Fusarium* 10^6 spores	8.3ab	4.7fg	4.7ef	1510d	7.3b	183	165ab	22.3abc
Meloidogyne 600 x *Fusarium* 10^6 spores	25.0ab	4.0ef	4.0de	9778bcd	6.7b	284	118ab	22.3abc
Meloidogyne 900 x *Fusarium* 10^6 spores	58.3c	6.0g	5.7f	15999d	4.3a	68	97ab	55.5c
SED	13.2	0.7	0.7	2860	0.9	112	62	15.7

Means in the same column followed by the same letter(s) are statistically similar using DMRT ($P \leq 0.05$).

4. Discussion

Zhang et al. (2020) stated that vital tripartite inter-kingdom interactions between plants, bacteria, nematodes, and fungi are very common in nature which corroborated the findings of this study. Indeed, chemical signals (such as volatile organic compounds) released by organisms such as bacteria, nematodes, fungi, or plants have been detected to initiate interactions between fungi and nematodes. Plant root metabolites affect communication between plants and nematodes. This was the basic premise of this study and it was proven to be true. The pathogens co-existed in the plant and successfully completed their life cycles.

The results showed the presence of significant main effects of inoculation of the soil with either *Fusarium* or *Meloidogyne* spp. (single pathogen infection/alone) in both the 2014- and the 2015-cropping seasons. This implies that each pathogen (*Fusarium* or *Meloidogyne* spp.) could cause significant wilt disease in African garden egg everything being equal, as has been variously reported in the literature (Safikhani et al., 2013; Ndifon et al*.,* 2015; Feyisa et al., 2016; USDA, 2016; Göze Özdemir et al., 2022).

The effects of the pathogens on the crop/wilt disease showed the existence of cross over interaction for all the parameters (disease incidence, severity of wilt, wilt vascular discoloration, root gall index, and final nematode population). The effects of different concentrations of *Meloidogyne* species were corroborated by the findings of Abbasi and Hisamuddin (2014) who reported that by inoculating *Vigna radiata* (L.) R. Wilczek (Fabales; Fabaceae) with different population densities of *M. incognita* in a green-house, the leguminous plant showed a progressive decrease in growth and biochemical parameters**.**

It was also shown in this study that *Meloidogyne* sp. was not just an incitant or wound inducing agent for enhancing infection by *Fusarium* sp. (Agbenin, 2005). It was observed that African garden eggplant experienced less combined infection of these pathogens during the survey by Ndifon et al. (2015). The presence of other nematodes was however encountered during the survey along with *Fusarium* species. The association of other pathogens with nematodes or fungi may cause severe damage to vegetable crops in Benue State as was confirmed in another survey by Eche et al. (2018).

The findings of Sulaiman et al. (2019) showed that all the *M. incognita* inoculum concentrations (500-8000 juveniles) reduced the level of eggplant growth for all growth parameters with a corresponding decrease in the yield of eggplant (*Solanum* spp.) They revealed that the lowest nematode population used (500 eggs/juveniles of *M. İncognita* per plot) was capable of significantly reducing yield of the eggplant variety used. These findings corroborated the findings of this current study. Moreover one can observe that the levels of nematode population used in this current study were above and below 500 nematodes per plot. Thus this current study added much to our knowledge of the effect of inoculum potential especially in situations of disease complex.

The interaction of the two pathogens showed that they had a synergistic effect on the damage caused to the crop. This implies that different levels/population densities of *Meloidogyne* sp. had significant effects on different levels of *Fusarium* sp. which resulted in significant damage to African garden egg in the field. This was affirmed by the correlation results herein. The correlation of disease parameters (disease incidence, disease severity, vascular discoloration, root-gall index, and nematode counts) revealed a positive correlation among the disease parameters. Concomitant infection by *Fusarium* sp. and *Meloidogyne* sp. on tomato, African garden egg, pepper, and *Solanum melongena* L. (Solanales; Solanaceae) has been reported (Mehrotra and Aggarwal, 2010) which corroborates this current finding on concomitant infection by *Fusarium* sp. and *Meloidogyne* sp.

It was reported that tomato plants wilted faster and even died when inoculated simultaneously with root-knot nematode species and *Fusarium oxysporum* (Agbenin, 2004). Zhang et al. (2020) purported that in farms, the fungal species composition can vary, depending on whether the fields are infested by root-knot nematodes or not. İn fact, *F. oxysporum* (11% frequency) followed by *F. solani* (6% frequency) were found to be the most frequent fungal species associated with the presence of *Meloidogyne* spp., and fungal diversity played an important role in the interactions between host plants and soil microorganisms. This finding affirmed the results of this current study that revealed the capacity of these pathogens to co-exist successfully.

Another instance of an increase in disease severity due to the contemporaneous occurrence of pathogens has been reported involving potato cyst nematode and *Verticillium dahliae* Kleb.

(Hypocreales; Incertae-sedis) on potato (Ndifon, 2019). Both of these pathogens caused only mild disease when each occurred alone. Their combined effects were mostly synergistic although additive damage did occur, which corroborated the current findings on cross-interactions.

İt was reported that *Heterodera schachtii* Schmidt (Tylinchida; Heteroderidae), and *Rhizoctonia solani* Kühn Cantharellales; Ceratobasidiaceae) synergistically parasitized sugar beet (Ndifon, 2019). *M. incognita*, and *Thielaviopsis basicola* (Berk. & Broome) Ferraris (*Microascales; Ceratocystidaceae*) in cotton also show synergistic interaction, which converts the pathogens into more important pathogens than when each occurs alone on cotton (Back et al*.,* 2002; Ndifon, 2019).

Asari et al*.* (2022) established that plant parasitic nematodes are capable of inducing disease in plants whether singly or in combination with other pathogens. However, they noted that the combined effects of such relationships result in a greater level of damage produced compared to a single infection of any of the pathogens. The results of this present study agreed with this statement. Zhang et al. (2020) reported that fungi-nematode interactions in the soil can involve endophytic fungi triggering host plant defense against plant pathogenic nematodes or the plants may help the nematodes to escape fungal attacks through the production of metabolite complexes. These situations may have occurred in this current study based on the significant differences obtained between treatments.

Herczeg et al*.* (2021) insisted that these fungi and nematodes provide essential ecosystem services and play crucial roles in maintaining the stability of food-webs and facilitating nutrient cycling in the ecosystem. Besides that, tomato plants that were resistant to *F. oxysporum* lost some of their resistance in the presence of *Meloidogyne* species (Ndifon, 2019). However, Agbenin (2002) reported that simultaneous inoculation of resistant tomato varieties with *Fusarium* and *Meloidogyne* species did not result in the breakdown of the resistance to the *Fusarium* wilt pathogen. But pre-inoculation of the soil with *Meloidogyne* before *Fusarium* resulted in breaching of the resistance of the wilt resistant tomatoes (varieties Walter F and Petomech). İn this current study, it was observed that the inoculation of the pathogens simultaneously did not have an apparanent negative effect.

In this current experiment, the inoculation of the pathogens was carried out simultaneously at transplanting time, but the effect of time of inoculation was not studied herein. It was observed that fruit weight was highest in the middle of the nematode initial population density range. Zhang et al*.* (2020) reiterated the existence of a nematode–fungi disease complex in plants, whereby damage by *F. oxysporum* f. sp. *vasinfectum* was more severe in the presence of *Meloidogyne* spp. *Meloidogyne* species and cyst nematodes have been shown to interact with *Fusarium* wilt thereby negatively impacting a number of crops.

Entomopathogenic nematodes and pathogenic fungi are capable of generating additive effects which increase insect pest mortality. Besides in the rhizosphere, nematode attacks can lower the resistance of plants to pathogens and increase their susceptibility to infection by soil-borne fungal pathogens. In these situations, the physiological status of all three interacting partners plays a very important role in the outcome of such tripartite interactions.

Odeyemi et al*.* (2010) expounded that reproduction and root galling of *M. incognita* were significantly lower in cowpea varieties treated with *Glomus mosseae.* In fact, *G. mosseae* alleviated the damage induced by the root-knot nematode on all the cowpea varieties. Freire (1982) reported that *M. incognita* did not predispose black pepper (*Piper nigrum*) to infection by *Nectria haematococca*. However, *Radopholus similis* predisposed black pepper seedlings to attack by a less virulent isolate of *F. solani*. Thus interkingdom interactions do not always result in negative effects.

Zhang et al*.* (2020) reported that the time of inoculation of the pathogens seems to have varied effects on the disease complex. This affirmed the decision to inoculate the two pathogens utilized in this research simultaneously. However, Herczeg et al*.* (2021) revealed that the order of succession of different parasites and the time lag between exposure appear in many cases to fundamentally shape competition and disease pathogenesis/progression. They argued that intermediate mortality is obtained when exposure to the two agents is sequential, regardless of which pathogen was added first. This confirmed the fact that the pathogens studied herein both successfully caused significant disease severity. They said that the outcome of co-infection with the two chytrids studied appeared to depend on the relative timing of the exposures and the dose of the zoospores. These interactions they reported may result in both synergistic or antagonistic effects.

Herczeg et al*.* (2021), Göze Özdemir et al. (2022), and Rijal (2022) pointed out that direct or indirect relationships may result in either antagonism, parasitism, mutualism, or commensalism.

Different soil temperatures, moisture contents, textures, plant ages, and NPK nutrients did not alter the relationships between fungi and nematodes (Freire, 1982). Curtis (2008) stated that plant root exudates contain a range of compounds that mediate below ground interactions with pathogenic and beneficial soil organisms. The effect of population density (inoculum potential) was studied in this present research and it was shown to be effective in contributing positively to the amount of damage on the crop. Much work still needs to be carried out on other factors that enhance disease establishment and damage.

It has been stated that the interaction between nematodes and host plants is influenced by primary factors like crop species and the initial population density of nematodes at sowing time (Agbenin, 2002). Moreover, Herczeg et al. (2021) confirmed that simultaneous exposure to viral and fungal parasites tends to cause higher mortality than a sequential encounter with the same agents or single infections. However, the species composition of parasites and interestingly the degree of relatedness between them may significantly influence interactions and ultimately disease outcomes (Göze Özdemir et al., 2022). This also corroborated the findings of this current study.

Moreover, some microbes in the environments (internal and external) can exert antagonistic effects on both nematode and fungal pathogens, while others can form mutualistic interactions with plant pathogens (Toju and Tanaka, 2019; Göze Özdemir et al., 2022). For instance, continuous sole cropping of soybeans was observed to be effective in altering the composition of both bacterial and fungal communities in the rhizosphere and negatively impacted the rhizosphere microbiome in its ability to suppress the soybean cyst nematodes (*Heterodera* sp.) (Hamid et al., 2017). This shows how diverse the relationships between organisms may be. The host-multipathogens relationships reported herein are thus only the peak of the iceberg. We can sum up these views by stating that based on the perspective of the hosts, the presence of co-infecting parasites can cause disease synergisms via enhanced virulence (Rigaud et al*.,* 2010), even if the interactions among parasites are antagonistic (Malapi-Nelson et al., 2009).

Before rounding up this discourse, Unl.edu (Accessed 13/5/2023) expounded that nematodes have been classified into four different phyla with different Phyla names. There are two contending names for the phylum of nematodes (i.e. Nematoda and Nemata). In 1919, Cobb placed nematodes in their own phylum (i.e. the phylum Nemata). However, when nematodes were moved to the phylum Aschelminthes, they were classified as class Nematoda. In 1932, the class Nematoda was elevated to the rank of a phylum (leaving the name Nematoda the same). While both names have been used (and are still used today), many authors believe that Nemata is a more precise name. In addition, the name Nemata was used first and therefore should be given priority.

Al-Banna and Gardner (2022) recently did some work on this revived name - Nemata. At the family and order levels. GBIF (2023) has a classification that is of interest: Phylum Nematoda, Order Rhabditida, Family Meloidogynidae, with a Genus *Meloidogyne*. Yet Gebissa (2021) and Myers et al. (2023) presented another classification as follows: Phylum Nematoda, Order Tylenchida, and Family Heteroderidae, with the same Genus *Meloidogyne*. This work is not a taxonomy study but this could arouse our interest in the classification of this important genus. Finally, the issue of race and formae speciales is one that was left out of this work due to limited resources and after consulting the work of Edel-Hermann and Lecomte (2019). Once the effects of epigenomics and environment are fully determined and the naming of races sorted out then it can be easy to determine which race one is dealing with. Our laboratory normally identifies organisms to species epithet only, while forma specialis are determined based on host type and elimination of other host types that are not affected by the species.

5. Conclusion

A trial on the effects of the initial population densities of *Fusarium* and *Meloidogyne* species revealed that the different combinations of the pathogens had detrimental effects on the African garden eggplant compared to the uninfected Control. The growth, yield, and disease parameters assessed in tandem all showed these effects. The pathogens seemed to complement each other and co-existed in the rhizosphere amicably. The pathogens multiplied in the host effectively to the tone of several times above the initial populations used to inoculate the soil in the field. Thus the reproductive indices were very high. Gall rating was average to moderately high. The disease severity ranged from average to very high depending on the treatment and this was reflected in the reduction of the vigour of the treatments. Therefore it is recommended that the cultivation of African garden egg be carried out on land free from

Fusarium and *Meloidogyne* species to avoid economic damage to the crop. Future research on these eggplant pathogens should concentrate on concurrent integrated management of *Fusarium* and *Meloidogyne* species so as to reduce yield loss. The effect of climatic and environmental factors on the host multipathogens relationships needs to be studied as well.

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Research Article

Plant Growth Bio-stimulants of Seaweed Extract (*Sargasum boveanum*): Implications Towards Sustainable Production of Cucumber

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Abstract: The purpose of this experiment was to compare the growth and quality of cucumber fruits, in response to different concentrations (C_0, $C_{0.75}$, and $C_{1.5}$ g L^{-1}) and different application methods (M_1: foliar sprays, M_2: fertigation, M_3: combined foliar sprays and fertigation) of seaweed extract (SwE). The simultaneous use of the method and different concentration of SwE increased the fresh weight of the leaf, fruit weight, yield, number of leaves, evaporation, fruit length, fruit diameter and firmness, stomatal conductance, and nitrate concentration of fruit. On the other hand, the highest amount of fruit firmness (69.25 and 69.27 N) was observed in $M_2C_{0.75}$ and $M_2C_{1.5}$ compared to other treatments, respectively. The $M_1C_{0.75}$ treatment increased the fruit diameter by 26.52% more than the M_1C_0 treatment. Fruit weight, fruit length, and yield were in the following order in different treatments, $M_1C_{1.5}$> $M_1C_{0.75}$ > $M_3C_{1.5}$. So that only in the $M_1C_{1.5}$ treatment, fruit weight, yield, and fruit length were 25, 52.55, and 25.86% higher than the M_1C_0 treatment, respectively. Generally, the M_1 and M_3 in concentrations of 0.75 and 1.5 created better plant growth, fruit shape, and quality characteristics compared to the second method (M_2) and the C_0 treatment. Therefore, the concentration of 1.5 g L^{-1} and the use of foliar spraying methods, and the combination of foliar spraying and fertigation can be recommended to achieve the maximum yield and quality of cucumber fruits.

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1. Introduction

Recently, the increase in the use of chemical fertilizers has created concerns for the environment and human health. Therefore, considerable research efforts have been made to find new green cultivation technologies and increase the yield and quality of vegetables. In this regard, biostimulant applications are a valuable and environmentally friendly technology for improving the quality traits of vegetables (Consentino et al., 2020; Di Mola et al., 2021; La Bella et al., 2021; Sabatino et al., 2021).

The use of plant biostimulants (BS) or agricultural BS can increase plant growth due to the presence of different compounds and microorganisms, also BS has been considered an effective way to achieve sustainable agricultural production, and to maintain soil health (Rouphael and Colla, 2020; Shabani, 2023). The use of BS motivates natural processes to increase and improve nutrient absorption,

nutrient efficiency, product quality, and tolerance to abiotic/biotic stresses (Desoky et al., 2021). Seaweed extract (SwE) contains a complex mixture of polysaccharides, micronutrients, and plant growth hormones and has shown a stimulating effect on plant growth. The use of SwE extract improves the growth and flowering of the plant, as a result of which it increases the yield and quality of products in plants treated with SwE (Rodrigues et al., 2020).

SwE can be used in different ways, such as the application of irrigation fertilizer (near the plant roots), foliar spraying, and their combined application on a variety of flower, vegetable, and tree crops (Haider et al., 2012; Slatnar et al., 2019). The positive effects of SwE on plants under optimal, sub-optimal, or unfavorable conditions are attributed to several biochemical and physiological mechanisms, including stimulating enzymes involved in carbon and nitrogen metabolic pathways, stimulating the synthesis of plant hormones, improving the absorption and accumulation of minerals, and increasing the size of the root system (Consentino et al., 2021). Kocira et al. (2020) also reported that the biostimulants based on SwEs affect the development and resistance of particular parts of plants, such as rhizosphere (roots), phyllosphere (green parts and shoots), and spermosphere (flowers and fruit), and by this way determine the quantity and quality of crop yield produced.

The finding of Ozbay and Demirkiran (2019) on ornamental pepper plants showed that application of SwE improved stem diameter, plant height, number of leaves and leaf area, root and shoot fresh weight, and root and shoot dry weight compared to the control plants. On the other hand, Shafie et al. (2021) demonstrated that the application of different levels of SwE had positive effects on the growth indices, chlorophyll content, carotenoids, and the content of N, P, and K in the leaf of yarrow and the highest shoot dry weight was obtained by the application of SwE by 54% increase compared to the control. In addition, Meng et al. (2022) indicated that the use of SwE in the peanut plant increased the values of photosynthesis, main stem height, lateral branch length, and dry matter accumulation by 13.8%, 45.7%, 225.0%, and 8.7%, respectively. Recent findings showed that SwE in cucumber had a significant effect and increased most studied traits such as plant length, number of leaves, fruit weight, total soluble solids content (TSS), the number of fruits, and total yield (Hassan et al., 2020). Hassan et al. (2021) reported that by increasing the concentration of SwE (0, 1, and 2 g L^{-1}) in cucumber production, plant height, leaf area, leaf dry matter, plant yield, and fruit diameter were increased and leaf nitrogen (N) content was decreased. Also, the application of the SwE had no significant effect on the amount of phosphorus (P) in leaves and fruits. Ashour et al. (2020) findings in a similar study on Jew's Mallow (*Corchorus olitorius* L.) showed a decrease in N concentration and insignificant leaf P content.

Various studies have pointed out the role of SwE extracts on the fruit quality of some vegetables, for example, Colla et al. (2017) reported that foliar applications of SwE improved the total marketable yield of greenhouse tomatoes and Ca^{2+} concentration in the fruit tissue. Also, Mannino et al. (2020) showed that SwE improved tomato fruit development and quality, and by using SwE a two-week reduction of ripening times and a concomitant enhancement of the production percentage, in terms of both fruit yield (110%) and size (85%), were observed. In addition, various studies showed that SwE played an important role in increasing the fresh yield and P, potassium (K), N, and magnesium (Mg) levels of spinach leaves (Rouphael et al., 2018; La Bella et al., 2021). Haidar et al. (2012) showed that the foliar application of SwE concluded a positive response in potato plant growth and yield, moreover, a significant improvement in tuber quality of potato, TSS, N, and protein contents were observed. Similar results were reported in onion (Abbas et al., 2020).

Although there are many studies in the field of using SwE as a foliar application, there is little information about its use as an irrigation fertilizer. Therefore, investigating new methods and their impact on the growth process of plants can be the foundation of new information for researchers and producers. Despite the positive features that have been mentioned about the foliar application of SwE in different plants, disadvantages such as humidity increase in leaf surface and the possibility of fungal disease development in the foliar application method, creating a microscopic effect and the possibility of leaf burns and necrosis, and the difficulty of applying it by the user caused the possibility of using it near the root zone through irrigation fertilizer to be investigated. Therefore, the current research will investigate the growth, physiological, and qualitative characteristics of greenhouse cucumber under hydroponic conditions by studying two different methods of using SwE in different concentrations.

2. Material and Methods

This experiment was carried out in the fall and winter of 2021 in the research greenhouse of Shahid Chamran University of Ahvaz, Ahvaz, Iran, with the geographic location of latitude 31º20´N, and longitude 48º41´E. To conduct the experiment, greenhouse cucumber seeds (var. MRC07, Manier Company, Turkey) were germinated in a glass petri dish in laboratory conditions. In the next step, the germinated seeds were planted in greenhouse conditions under natural light conditions and a day temperature of 25±3 ºC, night temperature of 18±3 °C and humidity of 60±5%, and in disposable glasses. Since the time of seed planting until the emergence of cotyledon leaves, irrigation was done with potable water and after that with a concentration of one quarter of Resh (2013) nutrient solution. Seedlings were transferred to pots containing cocopeat-perlite (70:30 v/v) at the 4 true-leaf stage. Cucumber plants' nutrition during the growing season was carried out by Rash (2013) formula with the following concentrations of N (140), P (50), K (350), Mg (50), Ca (200), S (150), Fe (3), Mn (0.8), B (0.3), Zn (0.1), Cu (0.07), and Mo (0.03) mg L^{-1}. The pH of the nutrient solution was adjusted by 53% nitric acid in the range of 5.8-6. The watering of the plants started with 300 ml per plant and with the growth of the plant, it continued to 500, 700 ml, and finally one liter per plant.

The experiment was conducted based on the split plot randomized complete block design, with 3 replications and two observation plants in each replication. The experimental treatments included different methods of SwE treatment (foliar sprays, fertigation, combined foliar sprays, and fertigation) (as the main factor) and different concentrations (0, 0.75, and 1.5 g L^{-1}) (as the sub-factor) of seaweed extract (*Sargasum boveanum*). To prepare SwE extract, *Sargasum boveanum* seaweed was first collected from the shores of the Persian Gulf in Bushehr city and after the approval of the Persian Gulf Marine Biotechnology Research Center; it was transferred to the laboratory. In order to remove impurities, the washing operation was done several times with distilled water. Seaweed samples were air-dried in laboratory conditions and powdered by a grinder. 15 grams of seaweed powder was mixed with 300 ml of 70% ethanol and stirred on a shaker for 24 hours. After passing the extract through filter paper, the samples were placed in a rotary machine to remove ethanol. The supernatant containing seaweed extract was dried in an oven at 40 °C and the desired extracts were prepared using it (Mohkami and Habibi-Pirkoohi, 2019). Treatments were performed from the second week after transplanting to the tenth week (8 weeks in total). In the foliar application method, the plants were sprayed with 300 ml of the mentioned concentrations, and in the fertigation method, 300 ml of the prepared extract was poured on the pot and next to the cucumber plants, but in the combined method of foliar spraying and fertigation, 150 ml of the prepared concentration was poured on the pot and another 150 ml of the desired concentration was sprayed on the plant. Plant guidance, removal of lateral stems, and adjustment of EC and pH of nutrient solution were carried out regularly.

At the end of the experiment, for measurement of fresh weight (FW) of the root, stem, and leaves of the plants, they were cut from the bottom. The fresh weight of the samples was measured by digital weighing balance. The number of leaves in each experimental treatment was counted and a leaf area meter (LTD, Scientific Instrument, UK) was used to measure the leaf surface. The stem diameter was measured by a digital caliper. The dry weight (DW) of the samples was obtained after drying the roots, stems, leaves, and fruits at 70 °C oven after 48 hours. The dry matter percentage of root, stem, leaf, and fruit was obtained by dividing the dry weight by the fresh weight multiplied by 100. To measure photosynthetic indices such as net photosynthesis rate, stomatal conductance, and transpiration rate, a photosynthesis meter (LCi-SD, UK) was used on complete mature leaves. During the growing season, at each stage of fruit harvesting, the number of fruits was counted by the researcher, the weight of a single fruit was measured by a digital scale, and the length and diameter of the fruit were measured by a digital caliper. The total yield (sum of the weight of single fruits in the entire harvest period) was also recorded and reported at the end of the experiment. The firmness of the fruit was measured by a firmness meter (Santam, STM-1, Iran) and the total dissolved solid of the fruit (TSS) was measured by a refractometer (Atago, A-PAL-1, Japan). In order to evaluate the EC and pH value of the fruit extract, 10 grams of the crushed flesh of cucumber fruits are brought to a volume of 100 ml in a beaker, and after filtering the samples with filter paper, the values of the mentioned traits were recorded and reported (Tabatabai, 2013). Also, in this experiment, the amount of nitrate in the fruit was measured by the method of Cataldo et al. (1975), and nitrate absorbance was measured at 410 nm by a spectrophotometer (UV-1201, Shimadzu, Japan). Dry samples of cucumber leaves were used to determine mineral

elements. The plant material was ground with an electric mill and the concentration of P and K was measured by the vanadate-molybdate method using a spectrophotometer (UV-1201, Shimadzu, Japan) at a wavelength of 430 nm and a flame photometer, respectively. The data was evaluated by using SAS 9.1 software (SAS Institute, Cary, NC, USA). Data analysis was performed using the general linear model (GLM) procedure and means were compared using Tukey's test at $p \leq 0.05$ on each of the significant variables measured.

3. Results

3.1. Effect of the application method of seaweed extract

The results of this experiment showed that the application method of SwE caused a highly significant in fresh weight of leaf (FWL), dry weight of leaf (DWL), fruit weight, yield, number of leaves (Num. leaf) leaf area (LA), stomatal conductance, fruit length, fruit diameter, firmness and nitrate concentration of fruit ($p \leq 0.01$) (Tables 1, 2 and 3) (Figure 1). Also, it has been found out a significant increase in dry matter of leaf (DML), fresh weight of root (FWR), fruit dry matter (Fruit DM), and K concentration of leaf ($p \leq 0.05$) (Tables 1, 2, and 3). The highest and lowest FWL were measured in the M_1 (foliar spray) method (264.91 g/plant) and M_2 (fertigation) method (206.73 g plant^{-1}), respectively (Table 1). These results were exactly the same for the DWL with 37.70 g plant^{-1}in the M_1 method and 26.71 g plant^{-1} in the M_2 method, respectively (Table 1). Although the DML in M_2 and M_3 (combined foliar sprays and fertigation) showed no significant difference, but seaweed foliar spray caused an increase of about 9% compared to the other application methods (Table 1). Unlike other measured traits, the highest content of FWR (57.58 g plant^{-1}) and the highest level of cucumber fruit firmness were observed in M_2 (65.58 N) which was significantly higher than the M_1 and M_3 methods (Tables 1 and 3). Compared to the fertigation method, spraying cucumber plants with SwE increased the weight of single fruit by 8.13%, the dry matter of the fruit by 6.60% and the yield of the whole plant by 18.57% (Table 2) (Figure 1). Although the methods of using SwE showed no significant effect on stem diameter, photosynthesis, evaporation, and qualitative characteristics of fruit such as EC, pH, and TSS of the fruit extract, but it significantly affected the quantitative characteristics of leaves and fruits such as the number of leaves, leaf area, K content of leaves, stomatal conductance, length and diameter of fruits (Tables 2 and 3). One of the noticeable results of this experiment was the lower amounts of fruit nitrate in the foliar spray method compared to the M_2 and M_3 methods, so the highest amount of fruit nitrate was 149.54 mg kg^{-1} in the combined method (M_3) and 142.08 mg kg^{-1} in the fertigation method (M_2), respectively and the lowest amount of nitrate was recorded in the spray method with 116.39 mg kg^{-1} fruit fresh weight (Table 3).

3.2. Effects of different concentrations of seaweed

Findings of this experiment indicated that the use of different concentration of SwE had a significant effect on FWL, DWL, DML, fresh weight of stem (FWS), dry weight of stem (DWS), FWR, DWR, DMR, fruit weight, fruit DM, yield, stem diameter, LA, photosynthesis, stomatal conductance, evaporation, fruit length, fruit diameter, firmness, TSS, EC, nitrate and K concentration of cucumber ($p \leq 0.01$), also caused a significant increase in pH of the fruit extract ($p \leq 0.05$) (Tables 1, 2 and 3) (Figure 2). The findings of this study showed that there was a significant difference between different concentrations of SwE in all the quantitative and qualitative traits compared to the control treatment (Tables 1, 2, and 3). In such a way that in traits like FWR, stem and fruit diameter, the $C_{0.75}$ and in traits such as FWL and DWL, fruit weight, yield, number of leaves, stomatal conductance, evaporation, length and firmness of fruit, $C_{1.5}$ took higher values (Tables 1, 2 and 3) (Figure 2). While in other traits such as DML, FWS and DWS, DWR and DMR, fruit DM percentage, LA, photosynthesis rate, TSS, EC, and pH of fruit extract and leaf K percentage, no significant difference was observed between the concentrations of $C_{0.75}$ and $C_{1.5}$ (Tables 1, 2 and 3). No significant difference was observed between the control treatment and different concentrations of SwE in only two traits, the amount of leaf P and stem dry matter percentage (DMS) (Tables 1 and 2).

Table 1. The fresh and dry weight of leaf (FWL and DWL), dry matter of leaf (DML), fresh and dry weight of stem (FWS and DWS), dry matter of stem (DMS), fresh and dry weight of root (FWR and DWR), root dry matter (DMR) of cucumber in response to different concentration (C_0, $C_{0.75}$ and $C_{1.5}$ g L^{-1}) and different application method (M_1: foliar sprays, M_2: fertigation, M_3: combined foliar sprays and fertigation) of seaweed extract (SwE)

Treatments	FWL (g plant^{-1})	DWL (g plant^{-1})	DML (%)	FWS (g plant^{-1})	DWS (g plant^{-1})	DMS (%)	FWR (g plant^{-1})	DWR (g plant^{-1})	DMR (g plant^{-1})
Method (M)	**	**	*	NS	NS	NS	*	NS	NS
Concentration (C)	**	**	**	**	**	NS	**	**	**
M×C	**	NS	NS	NS	NS	NS	NS	NS	NS
Method									
Foliar sprays (M_1)	264.91 ^{a}	37.70 ^{a}	13.77 ^{a}	135.41	10.76	7.91	50.10 ^{ab}	7.81	15.22
Fertigation (M_2)	206.73 ^{b}	26.71 ^{b}	12.73 ^{b}	146.68	12.05	8.15	57.58 ^{a}	8.48	14.33
Combined (M_3)	210.92 ^{b}	27.09 ^{b}	12.63 ^{b}	135.12	10.70	7.88	48.94 ^{b}	7.26	14.41
Concentration(g L^{-1})									
0 (C_0)	152.76 ^{c}	17.94 ^{c}	11.72 ^{b}	106.64 ^{b}	8.28 ^{b}	7.76	39.22 ^{c}	4.63 ^{b}	11.81 ^{b}
0.75 ($C_{0.75}$)	242.42 ^{b}	32.99 ^{b}	13.41 ^{a}	163.99 ^{a}	13.37 ^{a}	8.12	62.12 ^{a}	10.01 ^{a}	16.07 ^{a}
1.5 ($C_{1.5}$)	287.39 ^{a}	40.57 ^{a}	14.00 ^{a}	146.57 ^{a}	11.87 ^{a}	8.06	55.27 ^{b}	8.91 ^{a}	16.09 ^{a}
M×C									
M_1C_0	154.40 ^{d}	18.41	11.91	105.06	8.25	7.85	38.90	4.59	11.79
$M_1C_{0.75}$	312.10 ^{a}	45.49	14.54	157.72	12.70	8.01	59.07	10.33	17.68
$M_1C_{1.5}$	328.24 ^{a}	49.19	14.88	143.45	11.34	7.87	52.33	8.52	16.20
M_2C_0	156.60 ^{d}	18.44	11.76	107.42	8.29	7.71	39.62	4.69	11.83
$M_2C_{0.75}$	198.82 ^{c}	25.79	12.89	168.66	14.04	8.32	68.57	10.80	15.74
$M_2C_{1.5}$	264.77 ^{b}	35.91	13.53	163.95	13.83	8.43	64.55	9.95	15.42
M_3C_0	147.28 ^{d}	16.96	11.51	107.45	8.29	7.72	39.14	4.62	11.80
$M_3C_{0.75}$	216.33 ^{c}	27.69	12.79	165.61	13.39	8.03	58.74	8.91	14.78
$M_3C_{1.5}$	269.16 ^{b}	36.63	13.60	132.32	10.43	7.88	48.93	8.26	16.64

NS= not significant, *= p≤0.05 and **= p≤0.01. Means followed by a different lowercase letters in a column were significantly different according to Duncan's multiple-range test (p≤0.05).

3.3. Combined effects of method and different concentrations of seaweed

The simultaneous use of the method and different concentration of SwE increased FWL, fruit weight, yield, number of leaves, evaporation, fruit length, fruit diameter, and firmness (p≤0.01) and also caused a significant increase in stomatal conductance and nitrate concentration of fruit (p≤0.05) (Tables 1, 2 and 3) (Figure 3). In other traits studied, no significant difference was observed in the methods and concentrations of SwE used on cucumber plants. The results of this experiment showed that the simultaneous use of $M_1C_{1.5}$ increased the amount of traits such as FWL, fruit weight, yield, number of leaves, stomatal conductance, and fruit length compared to other treatments. Also, the results showed that there is no significant difference between $M_1C_{0.75}$ and $M_1C_{1.5}$ treatments in traits such as FWL, number of leaves, and stomatal conductance (Tables 1, 2, and 3) (Figure 3). Compared to other treatments $M_2C_{1.5}$ treatment caused a significant increase in evaporation, and nitrate concentration of fruit (Tables 2 and 3). On the other hand, the highest amount of fruit firmness (69.25 and 69.27 N) was observed in $M_2C_{0.75}$ and $M_2C_{1.5}$ compared to other treatments, respectively (Table 3). The $M_1C_{0.75}$ treatment increased the fruit diameter by 26.52% more than the M_1C_0.treatment (Table 3). One of the important indicators of marketability and harvest is the fruit weight, fruit length, and the yield, which were in the following order in different treatments, $M_1C_{1.5}$> $M_1C_{0.75}$ > $M_3C_{1.5}$. So that only in the $M_1C_{1.5}$ treatment, fruit weight, yield, and fruit length were 25, 52.55, and 25.86% higher than the M_1C_0 treatment, respectively (Table 3) (Figure 3). In addition, M_1 and M_3 methods produced even better shaped fruits (Figure 4). Generally, in all three studied methods, the $C_{0.75}$ and $C_{1.5}$ produced better results in terms of plant growth and fruit quality characteristics compared to the C_0 treatment, so in most traits, the M_1 and M_3 in concentrations of 0.75 and 1.5 created better results compared to the second method (M_2) (Tables 1, 2 and 3) (Figures 3 and 4).

Table 2. Stem diameter, number of leaves (Num. leaf), leaf area (LA), photosynthesis, stomatal conductance, evaporation, phosphorus (P), and potassium level in leaves of cucumber in response to different concentrations (C_0, $C_{0.75,}$ and $C_{1.5}$ g L^{-1}) and different application method (M_1: foliar sprays, M_2: fertigation, M_3: combined foliar sprays and fertigation) of seaweed extract (SwE)

Treatments	Stem diameter (cm)	Num. leaf	LA (cm^2)	Photosynthesis ($\mu mol\ m^{-2}s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}s^{-1}$)	Evaporation ($mmol\ m^{-2}s^{-1}$)	P (% DW)	K (% DW)
Method (M)	NS	**	**	NS	**	NS	NS	*
Concentration (C)	**	**	**	**	**	**	NS	**
M×C	NS	**	NS	NS	*	**	NS	NS
Method								
Foliar sprays (M_1)	0.66	29.11 ^{a}	192.69 ^{a}	8.26	0.11 ^{a}	1.54	0.19	3.94 ^{ab}
Fertigation (M_2)	0.68	25.88 ^{b}	172.00 ^{b}	7.62	0.07 ^{b}	1.20	0.18	3.87 ^{b}
Combined (M_3)	0.65	25.77 ^{b}	174.47 ^{b}	7.55	0.06 ^{b}	1.08	0.22	4.25 ^{a}
Concentration (gL^{-1})								
0 (C_0)	0.57 ^{c}	22.11 ^{c}	142.92 ^{c}	5.22 ^{b}	0.05 ^{b}	0.86 ^{b}	0.19	3.57 ^{b}
0.75 ($C_{0.75}$)	0.72 ^{a}	28.33 ^{b}	193.79 ^{a}	8.78 ^{a}	0.07 ^{b}	1.21 ^{b}	0.20	4.31 ^{a}
1.5 ($C_{1.5}$)	0.69 ^{b}	30.33 ^{a}	202.45 ^{a}	9.43 ^{a}	0.11 ^{a}	1.75 ^{a}	0.21	4.17 ^{a}
M×C								
M_1C_0	0.57	22.33 ^{d}	144.00	5.25	0.07 ^{b}	1.01 ^{cd}	0.19	3.51
$M_1C_{0.75}$	0.71	32.00 ^{a}	212.21	9.20	0.13 ^{a}	1.77 ^{abc}	0.20	4.21
$M_1C_{1.5}$	0.70	33.00 ^{a}	221.87	10.33	0.14 ^{a}	1.83 ^{ab}	0.19	4.11
M_2C_0	0.58	22.66 ^{d}	142.41	5.20	0.05 ^{b}	0.80 ^{cd}	0.19	3.53
$M_2C_{0.75}$	0.74	26.00 ^{c}	182.02	8.73	0.02 ^{b}	0.53 ^{e}	0.17	4.26
$M_2C_{1.5}$	0.72	29.00 ^{b}	191.58	8.93	0.14 ^{a}	2.26 ^{a}	0.19	3.82
M_3C_0	0.56	21.33 ^{d}	142.35	5.20	0.04 ^{b}	0.76 ^{cd}	0.20	3.68
$M_3C_{0.75}$	0.72	27.00 ^{c}	187.14	8.42	0.07 ^{b}	1.32 ^{bcd}	0.23	4.48
$M_3C_{1.5}$	0.65	29.00 ^{b}	193.91	9.04	0.06 ^{b}	1.16 ^{cde}	0.23	4.59

NS= not significant, *= p≤0.05 and **= p≤0.01. Means followed by a different lowercase letters in a column were significantly different according to Duncan's multiple-range test (p≤0.05).

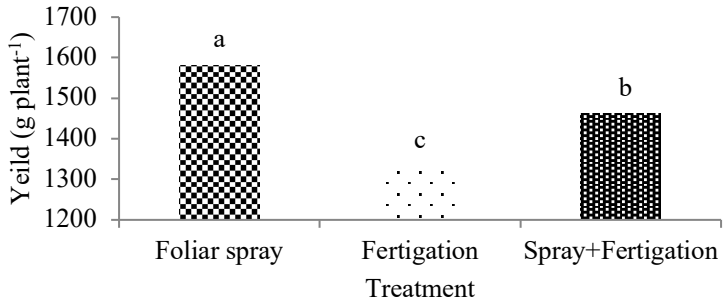

Figure 1. Effect of different application methods of seaweed (*Sargasum boveanum*) extract on cucumber yield.

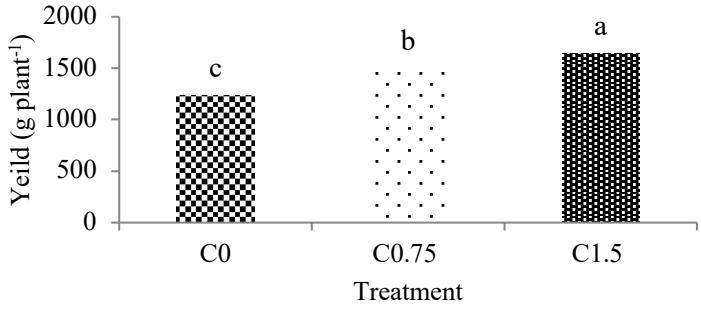

Figure 2. Effect of different concentrations of seaweed (*Sargasum boveanum*) extract on cucumber yield.

Table 3. Quantitative (fruit weight, fruit length, fruit diameter, firmness) and qualitative (fruit dry matter (Fruit DM), total soluble solid (TSS), EC, pH, and nitrate of fruit extract) changes of cucumber fruit in response to different concentration (C0, C0.75, and C1.5 g L-1) and different application method (M1: foliar sprays, M2: fertigation, M3: combined foliar sprays and fertigation) of seaweed extract (SwE)

Treatments	Fruit weight (g)	Fruit length (cm)	Fruit diameter (cm)	Firmness (N)	Fruit DM (%)	TSS (%)	EC (dS/m)	pH	Nitrate (mg kg^{-1})
Method (M)	**	**	**	**	*	NS	NS	NS	**
Concentration (C)	**	**	**	**	**	**	**	*	**
M×C	**	**	**	**	NS	NS	NS	NS	*
Method									
Foliar sprays (M_1)	90.11 ^{a}	14.76 ^{a}	2.69 ^{a}	63.80 ^{c}	3.71 ^{a}	3.04	1.61	5.16	116.39 ^{b}
Fertigation (M_2)	83.33 ^{b}	13.96 ^{c}	2.50 ^{c}	65.58 ^{a}	3.48 ^{b}	2.87	1.57	5.01	142.08 ^{a}
Combined (M_3)	85.88 ^{b}	14.39 ^{b}	2.58 ^{b}	65.28 ^{b}	3.56 ^{b}	2.94	1.58	5.23	149.54 ^{a}
Concentration (g L^{-1})									
0 (C_0)	80.33 ^{c}	12.57 ^{c}	2.30 ^{c}	58.01 ^{c}	3.42 ^{b}	2.73^{b}	1.50 ^{b}	4.95 ^{b}	86.59 ^{c}
0.75 ($C_{0.75}$)	87.33 ^{b}	15.18 ^{b}	2.76 ^{a}	67.79 ^{b}	3.59 ^{a}	3.01 ^{a}	1.62 ^{a}	5.27 ^{a}	150.14 ^{b}
1.5 ($C_{1.5}$)	91.66 ^{a}	15.36 ^{a}	2.72 ^{b}	68.87 ^{a}	3.74 ^{a}	3.12 ^{a}	1.64 ^{a}	5.17 ^{a}	171.27 ^{a}
M×C									
M_1C_0	80.00 ^{d}	12.68 ^{g}	2.30 ^{f}	57.66 ^{g}	3.42	2.73	1.49	4.94	81.26 ^{d}
$M_1C_{0.75}$	90.33 ^{b}	15.64 ^{b}	2.91 ^{a}	65.35 ^{e}	3.73	3.13	1.65	5.41	122.42 ^{c}
$M_1C_{1.5}$	100.00 ^{a}	15.96 ^{a}	2.85 ^{b}	68.45 ^{d}	3.99	3.26	1.68	5.12	145.48 ^{c}
M_2C_0	80.00 ^{d}	12.54 ^{h}	2.30 ^{f}	58.22 ^{f}	3.42	2.73	1.51	4.73	88.76 ^{d}
$M_2C_{0.75}$	85.00 ^{c}	14.71 ^{e}	2.61 ^{e}	69.25 ^{a}	3.52	2.93	1.61	5.13	153.30 ^{b}
$M_2C_{1.5}$	85.00 ^{c}	14.64 ^{f}	2.60 ^{e}	69.27 ^{a}	3.51	2.96	1.60	5.16	184.17 ^{a}
M_3C_0	81.00 ^{d}	12.49 ^{h}	2.31 ^{f}	58.22 ^{f}	3.43	2.73	1.52	5.18	89.74 ^{d}
$M_3C_{0.75}$	86.66 ^{bc}	15.20 ^{d}	2.75 ^{c}	68.76 ^{c}	3.54	2.96	1.60	5.27	174.71 ^{a}
$M_3C_{1.5}$	90.00 ^{b}	15.49 ^{c}	2.69 ^{d}	68.87 ^{b}	3.73	3.13	1.63	5.24	184.17 ^{a}

NS= not significant, *= p≤0.05 and **= p≤0.01. Means followed by a different lowercase letters in a column were significantly different according to Duncan's multiple-range test (p≤0.05).

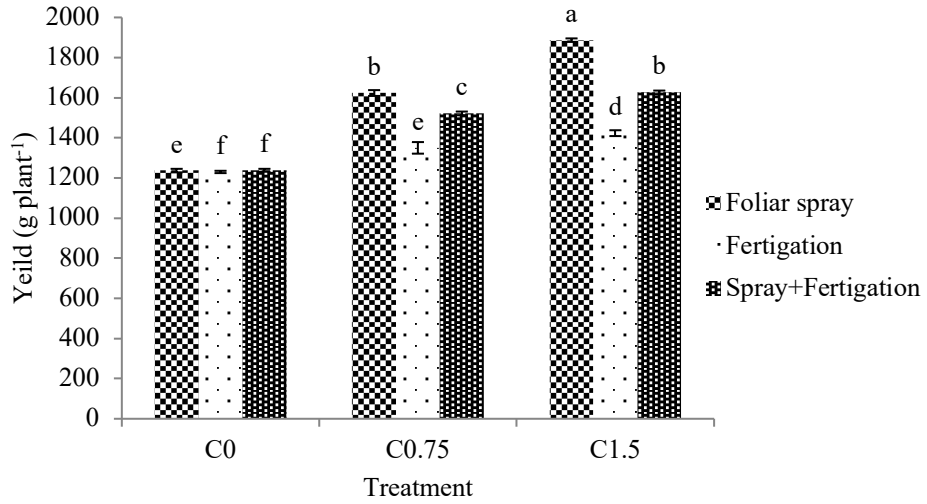

Figure 3. Effect of different application methods and concentration of seaweed (*Sargasum boveanum*) extract on cucumber yield.

Figure 4. Effect of different application methods of seaweed (*Sargasum boveanum*) extract on cucumber shape (Concentration= 1.5 g L^{-1}).

4. Discussion

Native seaweed species should be tested for their industrial and biotechnological potential as a source of bioactive compounds. The use of SwE has several advantages, such as biodegradability, harmlessness and compatibility with the environment, the absence of toxic residues, and resistance to disease due to the existence of a unique and exceptional biomolecular structure compared to chemical fertilizers (Ashour et al., 2020; Hassan et al., 2021). In the present study, the results indicated that the use of SwE obtained from *Sargassum bovianum* increased growth parameters, yield, and photosynthesis. Similar results have been reported by Hamed et al. (2018) and Hassan et al. (2021). These studies showed that SwE with adequate amounts of nutrients, rare-earth elements, vitamins, phytohormones, ascorbic acids, and many other bioactive compounds can increase the growth and yield of cucumber plants. Data are also consistent with La Bella et al. (2021). In this study, the marketable fruit increase prompted by the SwE application was due to a higher fruit mean mass rather than to the higher number of marketable fruits (Data not shown). Therefore, the use of SwE improves the plant's physiological responses, such as stimulating the absorption of nutrients, developing a strong root system, and increasing the leaf area, biomass, photosynthesis, and yield, and is a suitable option to minimize the use of conventional fertilizers (Bajpai, 2016; Abdel-Latif, 2018). Similar results were observed in the improvement of growth rate of *Kappaphycus striatus* plants using two liquid extracts from brown seaweeds, namely *Sargassum cristaefolium* and *Turbinaria conoides*, before out-planting (Tahiluddin et al., 2022). The findings of the researchers showed that the K content in SwE positively increases photosynthesis, meristem growth, and water content in treated plants. Moreover, P content activated root proliferation and increased the ratio of root/stem. Also, Calcium (Ca^{2+}) in SwE contributed to cell elongation, cell stability, and enzyme activation in treated plants (Ahmed et al., 2021).

Xu and Leskovar (2015), reported the application of SwE in spinach plants led to a large leaf area and high photosynthetic rate by improving leaf water relations, maintaining cell turgor pressure, and reducing stomatal limitation. Stimulation of the root system growth by SwE may result from the action of phytohormones like auxins and cytokinins. These compounds are important in the initiation of lateral and adventitious root development, as well as causing increases in total root biomass (Szczepanek et al., 2017). Change in root morphology is one of the major mechanisms by SwE that affect nutrient uptake. Previous findings showed that the increase in K absorption in cucumber plants treated by SwE may be related to several modes of action: (1) the presence of signaling molecules (free amino acids and soluble peptides) in SwE, (2) absorption, movement and accumulation of more nutrients that affect the stimulation of root system architecture, and (3) expression of genes encoding macronutrient transporters in the cell membrane (Lucini et al., 2018; Rouphael et al., 2018; Sestili et al., 2018). According to the results of the researchers, the use of SwE as a foliar spray is currently very useful and advantageous

because it can increase vegetative growth and yield in crops such as cereals, leafy vegetables, cucumber, tomato, and pepper (Ali et al., 2016; Rouphael et al., 2018; Trejo Valencia et al., 2018; Ashour et al., 2020). The trend of cucumber production in the future is organic cucumber production because it has high consumer demand. The pricing of these products in the foreign market can be higher than the pricing of normal products and as a result increase value of production and productivity (Trejo Valencia et al., 2018).

In this study, the use of SwE increased the concentration of K in cucumber, which is consistent with the results of Rouphael et al. (2018) and La Bella et al. (2021) in spinach. Results related to the increase of nitrogen, P, K, and Mg uptake in greenhouse cucumbers using SwE have already been reported (Valencia et al., 2018). Abdel-Mawgoud et al. (2010) reported that increasing vegetative growth by SwE was an effect on cucumber fruit growth, in which single fruit weight, fruit diameter, and fruit number were higher due to the absorption of more assimilates into fruits and conversion of fruits to high potential sink (Hassan et al., 2020). To explain these results, we can refer to the extract containing the potassium element, which is more absorbed by the plant than any other element, and is the dominant cation in the plant and its great importance in the process of cell division and regulating permeability. It regulates the membranes in the plant and the transport of sugar and protein, which had a positive effect on increasing the number of fruits, fruits weight, and the yield of cucumber plants (Hassan et al., 2020). Rouphael et al. (2010) study showed that increasing biomass production and crop yield of greenhouse cucumbers treated with SwE can be expected because the plants treated with SwE have a greater capacity to maintain a high rate of net photosynthesis and a better nutritional compounds (high concentration of P, K, magnesium, iron, zinc, and manganese) compared to untreated plants (Rouphael et al., 2010). It seems that in the interaction effects of this experiment, the positive changes in fruit weight, fruit length, and yield of cucumbers are more influenced by the absorbed elements such as K, free amino acids such as lysine, glycine, aspartic acid, etc., beneficial fatty acids and hormones and pseudo-hormones rather than the changes in root architecture and photosynthesis rate which these compounds have been mentioned in the review of the components of *Sargasum boveanum* (Khalifeh et al., 2021).

Mannino et al. (2020) reported that the improved productivity of tomato plants under SwE application can be related to signaling molecules including polysaccharides (alginates, fucoidan, and laminarins), soluble peptides, oligopeptides, and free amino acids, which are made up about 30–40% of the seaweed extract (on dry weight basis) and increase the promoting endogenous phytohormonal biosynthesis (auxin- and/or gibberellin-like activities) thus enhancing crop yield (Ertani et al., 2017; Rouphael et al., 2018).

In this experiment, apart from nitrate content, the biochemical characteristics of the cucumber fruit such as total soluble sugars (TSS), EC, and pH were more influenced by the main effects of the application method and the concentration of the SwE than by the interaction effects of the treatments. Kumari et al. (2011) reported a higher concentration of TSS in tomato crops with soil application and foliar application of *Sargasum johntonii* SwE. The increase in the amount of TSS in cucumber fruit can be directly related to the polysaccharide content of SwE which increases the synthesis of the main compounds of TSS such as organic acids, metabolites, and glucose (Sendur Kumaran, 2016; Mzibra et al., 2021). Trejo Valencia et al. (2018) reported that the use of SwE is very effective for improving fruit formation, fruit shelf life, yield, and increasing fruit quality in cucumbers. In recent studies, it has been shown that if biological stimulants such as SwE are used, made the larger xylem cells and phloem vascular bundles in the stems, and this phenomenon can help to transfer minerals and assimilates to the sink more effectively and increase the concentration of minerals in the fruit. The increased mineral concentration in the fruit of treated plants may also be due to a greater absorption of minerals through stimulation of root growth and the activity of nutrient transporters in cell membranes (Billard et al., 2014; Colla et al., 2017). In agreement with these findings, the results of this experiment clearly indicated the effects of different concentrations of seaweed on root growth, stem diameter, TSS, EC, and pH of fruit extract.

The results of this experiment showed that the SwE application enhanced fruit firmness in the fertigation method more than the foliar spray and combined method. This is consistent with previous research on the influence of plant-based biostimulants (Basile et al., 2021; Consentino et al., 2021). The effect of SwE on fruit firmness is likely related to more absorption and accumulation of Ca in plants treated with SwE compared to the control. The calcium-pectin crosslinks play an important role in the resistance of cell walls and thus the physical and structural characteristics of the fruit. Furthermore, since

it is assumed that auxins partake in the transport and uptake of fruit Ca^{2+}, the present study suggested that SwE may have an auxin-like action and improve Ca nutrition in cucumber fruits (Hocking et al., 2016; Basile et al., 2021; Consentino et al., 2021; Cozzolino et al., 2021). It seems that SwE has played a more effective role in fruit firmness by influencing the process of Ca transfer in the fertigation method compared to the foliar spraying and combined methods. In other words, the degree of firmness of the fruit is more influenced by the effective role of SwE in the transfer of Ca in the fertigation method than by the effect of the concentration of Ca in the SwE (foliar spraying method). To prove this claim, more studies are needed in the rhizosphere and at the cellular and molecular levels.

As well as the use of SwE of *Sargasum boveanum* increased the nitrate concentration of cucumber fruit, but the combined use of $M_1C_{0.75}$ controlled the nitrate concentration of cucumber fruit according to the standard of World Health Organization (WHO) (150 mg kg^{-1} of fresh weight). Although, it has been more in the fertigation method than the foliar spraying method, like the firmness of the fruit. It seems that the role of SwE in the fertigation method is more related to the transfer of more ions from the substrate, which has been able to cause more firmness and more nitrates in the fruit. SwE contains amino acids and free peptides to prevent the accumulation of nitrates in the leaf tissue, if used correctly could be associated with an up-regulation of the key nitrogen assimilation genes like nitrate reductase, thus contributing to a higher assimilation of nitrates into amino acids (Tsouvaltzis et al., 2014; Rouphael et al., 2018). Therefore, it seems that the assimilation of nitrate in the foliar spraying method has been done in larger amounts compared to the fertigation and combination methods, which led to a lower concentration of nitrate in cucumber fruits. As a result, long fruits and small diameter in the $M_1C_{1.5}$ compared to the $M_1C_{0.75}$ can be affected by the high level of assimilation of chemical compounds, cell growth and development, and intercellular space in cucumber fruits under seaweed foliar application.

5. Conclusion

The findings of this research showed that although the use of common methods such as foliar spraying of seaweed extract can improve the growth and quality indicators of cucumber fruit in the greenhouse, the use of methods such as the combination of foliar spraying and fertigation can prevent the possible harms of foliar spraying and improve quantitative characteristics such as weight of single fruit, fruit length, fruit firmness and finally total yield. Also, the results of this experiment showed that in most of the traits studied, the concentration of 1.5 g L^{-1} of seaweed extract produced better results than other concentrations in improving indicators such as leaf fresh weight, number of leaves, fruit weight, fruit length, and total yield. It has been suggested for cucumber producers in greenhouses around the world. The fact that the simultaneous use of fertigation and foliar spraying could cause positive changes in the growth and quantitative and qualitative characteristics of cucumber requires more studies in the rhizosphere and the cellular and molecular level of the plant.

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Research Article

Exploring the Potential of Furofuran Lignans Isolated from *Beilschmiedia pulverulenta* for Drug Development: A Computational Approach

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Abstract: Natural products have played a significant role in drug discovery and continue to be an important source of lead for new drugs. In recent years, computer-based drug discovery methods have emerged as an effective approach for identifying small molecule leads with desirable pharmacokinetic and toxicity profiles. This study investigated the pharmacological and bioactivity of five furofuran lignans, namely, epiexcelsin, sesamin, sesartemin, syringaresinol, and yangambin, isolated from the plant *Beilschmiedia pulverulenta*. In silico studies were conducted to predict the pharmacological activities, toxicity, and drug likeliness properties of the lead compounds. The results showed that all compounds had promising pharmacokinetic activities, with epiexcelsin exhibiting strong binding affinity (-8.13 kcal mol^{-1}) and inhibitory activity (1.1 µM) against estrogen receptor-α, and predicted to be bioavailable and effective lead. The findings of this study provide important insights into the potential therapeutic uses of natural medicinal plants and emphasize the potential of combining traditional medicinal knowledge with modern scientific approaches in drug discovery. Overall, the furofuran lignans isolated from *Beilschmiedia pulverulenta* represent a promising source of natural compounds for the development of effective drugs.

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1. Introduction

Natural medicinal plants are rapidly being recognized as a rich source of bioactive chemicals with therapeutic potential. Among these, the genus *Beilschmiedia*, which contains about 250 species and is common in Africa and Asia, has attracted interest due to its various chemical compositions and therapeutic capabilities (Salleh et al., 2016a, 2019, 2020, and 2021). *Beilschmiedia pulverulenta*, also known as 'medang merah' in Malaysia and found in Peninsular Malaysia, Borneo, and Indonesia, is one such species that thrives in mixed dipterocarp forests on sandy loam soils (Salleh et al., 2016b). Previous phytochemical studies on *Beilschmiedia* species revealed the presence of a variety of natural products, including endiandric acid derivatives, alkaloids, flavonoids, terpenoids, lignans, neolignans, and

essential oils, some of which have antibacterial, antimalarial, and anti-tuberculosis activity (Salleh et al., 2016c, 2016d, and 2016e).

The process of discovering and developing novel drug leads from natural sources is a multistage activity. Unfortunately, many failures occur during the clinical phase, primarily due to pharmacokinetic and toxicity issues. With the capacity to predict potential inaccurate (off-target), identify negative effects, suggest new targets for medicines already in use, and assess affinity and selectivity among protein targets, in silico technologies have recently gained prominence as efficient methods for polypharmacological research (Shantier et al., 2023). Researchers can acquire significant insights into the mechanisms of action and toxicological profiles of natural compounds by utilizing computational approaches, aiding the selection and optimization of promising drug leads. These computational approaches have significantly contributed to the drug discovery field and can potentially drive the development of new drugs from natural sources in a more efficient and targeted manner (Sadybekov and Katritch, 2023).

Furofuran lignans are a class of natural compounds that have been isolated from various plant species. These compounds are characterized by the unique structural features of a furofuran ring system. They are typically found in plants belonging to the family Lauraceae, such as *Persea pyrifolia*, a plant native to South Brazil (Batista et al., 2010). Studies have shown that furofuran lignans possess various pharmacological properties, including anti-inflammatory, antitumor, and antiviral activities. Some furofuran lignans have been shown to inhibit the growth of cancer cells, making them potential candidates for the development of anticancer drugs. In addition, furofuran lignans have been found to have neuroprotective effects, potentially making them useful for the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Xu et al., 2018). Due to their interesting pharmacological properties, furofuran lignans have gained attention from the scientific community as potential lead compounds for drug discovery.

As part of drug discovery efforts from natural sources, five furofuran lignans, namely, epiexcelsin, sesamin, sesartemin, syringaresinol, and yangambin were isolated from *B. pulverulenta*. These furofuran lignans have notable anticholinesterase and anti-inflammatory effects (Salleh et al., 2016a). In this work, *in silico* studies were performed to estimate their pharmacological activity, toxicity, drug likeness, quality, pharmacokinetic (ADME) characteristics, and physicochemical properties in order to further evaluate their potential as drug candidates.

2. Material and Methods

2.1. Compound chemical structure format

To evaluate the pharmacokinetic and bioactivity properties of the isolated compounds from the stem bark of *B. pulverulenta*, various *in silico* tools were utilized through web-based platforms. The compounds were analyzed using both 3D SDF and isomeric simplified molecular input line entry system (SMILE) formats, which were obtained from the PubChem database.

2.2. Bioactivity properties

The Molinspiration online server (http://www.molinspiration.com/) (Molinspiration cheminformatics, 2006) was employed to assess the drug likeliness properties of the potential lead molecules. The tested molecules were analyzed for their ability to function as G-protein coupled receptor (GPCR) ligands, ion channel modulators (ICM), kinase inhibitors (KI), nuclear receptor ligands (NRL), protease inhibitors (PI), and enzyme inhibitors (EI) (Pushpa et al., 2022).

2.3. Pharmacological activities

To assess the potential pharmacological activities and toxicities of the lead compounds, they were submitted in SMILE format to the PASS Online tool (http://www.pharmaexpert.ru/passonline/). This tool utilizes a computational approach to predict the activity spectrum and possible toxic effects of chemical compounds based on their structural formula. By analyzing the compounds' molecular structure, the tool provides information on their possible pharmacological activities and potential toxicity (Lee et al., 2022).

2.4. ADME prediction and toxicity risk assessment

To predict the pharmacokinetic (ADME) and physicochemical properties of the lead compounds, we utilized the SwissADME web tool (http://www.swissadme.ch/) (Daina et al., 2017). To assess the potential toxicity risks of the compounds, both Molinspiration online server and OSIRIS Property Explorer open-source program (http://www.organicchemistry.org/prog/peo/) were employed in this study. While the Molinspiration server predicts the toxicity and drug-likeness properties of compounds, the OSIRIS Property Explorer program uses an extensive database of toxicity information to predict various toxicological endpoints, including mutagenicity, carcinogenicity, reproductive effects, and more (Mittal et al., 2021).

2.5. DFT computation and structural analysis

To calculate the structural and electronic properties of the five isolated compounds, we used the DFT/B3LYP method with a 6-31G* basis set in Spartan 14 (Spartan 14, 2013). Various parameters were evaluated, including the energies of the frontier molecular orbitals (HOMO, LUMO, and energy gap). These calculations provide valuable insights into the ligand-protein interactions in the active site of the target protein. Moreover, the electrostatic potential surfaces (EPs) of the molecules were obtained using population analysis computations and visualized with Spartan 14 (Umar and Uzairu, 2023).

2.6. Molecular docking

2.6.1. Preparation of ligands

The 3D structures of the compounds were obtained from the PubChem database in SDF format. To identify the bioactive conformer from the local minima, the energy minimization was performed using the MM2 force field in Spartan 14. Furthermore, optimization of the compounds was carried out using the DFT/B3LYP approach and 6-31G* basis set (Umar and Uzairu, 2023).

2.6.2. Identification of target

To determine the potential target for the selected lead compound, the ChemMapper and PharmMapper server (http://www.lilab-ecust.cn/chemmapper/ and https://www.lilab-ecust.cn/pharmmapper/), respectively utilized (Trosset, 2019; Srivastava et al., 2022). The lead compound was submitted in sdf format, and the target set was limited to human targets while all other parameters were kept as default.

2.6.3. Preparation of protein structure

The 3D structure of the target identified by the ChemMapper and PharmMapper servers was obtained from the Protein Data Bank (PDB ID: 3ERT; http://www.rcsb.org/). The protein files were prepared by removing all water molecules and hetero groups and adding polar hydrogen atoms using Discovery Studio Client software 2021 (BIOVIA Discovery Studio, 2019). It was then imported into PyRx Virtual screening tools which employ Mgltools to energy, minimize and as well apply appropriate and relevant charges (Salihu et al., 2023a).

2.6.4. In silico molecular docking and visualization

The AutoDock 4.0 software embedded in PyRx was utilized to perform molecular docking. The active residue of the protein was chosen for finding the most favorable binding site. All ligands were docked by selecting the specific residue in contact with the native ligand's, thereby generating a grid box (15.63Å each for X, Y, and Z dimensions at center 29.4152, -0.4138, 24.466 for X, Y, and Z respectively) which covered all binding pocket. The ligand with the highest binding energy (most negative) was considered to have the maximum binding affinity. Finally, the 2D interactions of the resulting docking file with poses exhibiting the lowest binding energies were visualized using DS Visualizer Client 2021 (Salihu et al., 2023b).

3. Results and Discussion

3.1. Bioactivity properties

Drug similarity analysis requires a delicate balance of considering different chemical attributes and structural factors to determine whether a molecule is similar to existing medications. To achieve this, Molinspiration employs a sophisticated Bayesian statistical method that compares the structures of active ligands for a specific target with those of inactive molecules. This approach helps to identify substructure features that are typical of active molecules and determine the physicochemical properties that contribute to drug activity (Alexey et al., 2014). This study evaluated epiexcelsin, sesartemin, sesamin, yangambin, and syringaresinol for their drug ability as GPCR ligands, ion channel modulators (ICM), kinase inhibitors (KI), nuclear receptor ligands (NRL), protease inhibitors (PI), and enzyme inhibitors (EI) and calculated their bioactivity scores. The higher the score value, the greater the likelihood of bioactivity (Paramashivam et al., 2015). Table 1 shows the drug-likeliness of the isolated lead compounds by Molinspiration. Based on the results, it appears that some of the compounds have higher bioactivity scores for certain drug properties compared to others. Syringaresinol has a high bioactivity score as a nuclear receptor ligand, indicating a higher probability that it could be effective as a drug that targets this receptor. Some compounds such as sesartemin and yangambin have negative scores for certain drug properties, indicating a lower probability of bioactivity for those properties. All compounds in the table, except sesartemin and epiexcelsin, have positive scores for enzyme inhibition, indicating that they have potential as inhibitors of enzyme activity.

Table 1. The drug likeliness of the isolated lead compounds by Molinspiration

Compound	Pubchem ID	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Epiexcelsin	489948	-0.03	-0.32	-0.32	-0.16	-0.21	-0.03
Sesartemin	342737	-0.04	-0.32	-0.31	-0.20	-0.22	-0.03
Yangambin	443028	-0.03	-0.25	-0.19	-0.10	-0.16	0.01
Syringaresinol	443023	-0.01	-0.23	-0.17	-0.01	-0.14	0.08
Sesamin	72307	0.02	-0.31	-0.27	-0.09	-0.15	0.03

3.2. Pharmacological activities

The PASS method is a computational tool that predicts the potential pharmacological activities and toxicity of molecules based on their chemical structure. It assumes that the activity of a compound is a function of its structure and uses original descriptors called multilevel neighborhoods of atoms (MNA) to define the chemical structure. This enables the prediction of whether a novel molecule will have a certain impact by comparing its structure to that of a well-known physiologically active drug. The server calculates two probability scores, Pa and Pi, for each studied compound, ranging from 0.000 to 1.000, indicating the compound's pharmacological activity. By predicting the different pharmacological activities of these lead compounds, PASS has proven to be a valuable tool in the estimation of the biological activity profiles of virtual molecules prior to their chemical synthesis and biological testing (Shantier et al., 2023). In this study, the PASS online tool was used to estimate the biological activity profiles of several lead compounds, namely, sesartemin, epiexcelsin, sesamin, syringaresinol, and yangambin. All compounds, including epiexcelsin, exhibited active antineoplastic activity, with epiexcelsin having the highest probability of pharmacological activity (Pa = 0.890) and a low probability of toxicity (Pi = 0.020). Furthermore, epiexcelsin, sesartemin, and sesamin have been shown to exhibit significant probabilities of caspase 3 stimulation (Pa = 0.887, Pi = 0.004 and Pa = 0.800, Pi = 0.005, respectively) and membrane integrity agonism (Pa = 0.802, Pi = 0.036 and Pa = 0.931, Pi = 0.005, respectively). Caspase 3 is an important enzyme involved in the initiation of programmed cell death (apoptosis), which plays a critical role in preventing the development and progression of cancer. Membrane integrity is essential for cellular homeostasis, and its impairment is associated with various pathological conditions.

Yangambin and syringaresinol were found to exhibit strong potential as inhibitors of Feruloyl esterase (Pa = 0.839, Pi = 0.008 and Pa = 0.865, Pi = 0.006, respectively) and Aspulvinone

dimethylallyltransferase (Pa = 0.823, Pi = 0.027 and Pa = 0.871, Pi = 0.015, respectively), based on their calculated probability scores from the PASS Online tool. The former enzyme is involved in the hydrolysis of ester linkages in plant cell wall materials, while the latter enzyme is important in the biosynthesis of secondary metabolites in fungi. These findings suggest that yangambin and syringaresinol may have potential applications in drug discovery as inhibitors of these enzymes.

Table 2. Predicted pharmacological activities of the isolated compound PASSonline

Compounds	Activities									
	Antineoplastic		Caspase 3 stimulant		Membrane integrity agonist		Feruloyl esterase inhibitor		Aspulvinone dimethylallyl-transferase inhibitor	
	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi
Epiexcelsin	0.890	0.020	0.849	0.004	0.866	0.020	-	-	-	-
Sesartemin	0.850	0.007	0.887	0.004	0.802	0.036	-	-	-	-
Sesamin	0.787	0.013	0.800	0.005	0.931	0.005	-	-	-	-
Yangambin	0.869	0.005	-	-	-	-	0.839	0.008	0.823	0.027
Syringaresinol	0.824	0.009	-	-	-	-	0.865	0.006	0.871	0.015

3.3. ADME prediction and toxicity risk assessment

In the process of drug discovery, it is crucial to identify promising hits or leads that specifically target the desired receptor, while also considering their physicochemical properties and toxicity risks. These factors are vital in determining the pharmacokinetic parameters and the eventual biological effects of lead compounds. Additionally, identifying promiscuous compounds that give false positives in high-throughput screening, known as pan assay interference compounds (PAINS), is important to avoid wasting time and resources on non-specific compounds. To assess the possibility of the tested compounds being PAINS, substructure filters were used in the SwissADME web tool. Fortunately, none of the compounds showed alerts for PAINS, indicating that they are promising leads. Furthermore, the SwissADME tool was also used to predict the pharmacokinetic (ADME) and physicochemical properties of the isolated compounds in the SMILE format, providing valuable information for drug design and optimization.

Table 3. Physicochemical properties and toxicity profile of the isolated compounds by SwissADME

Ligand	MW	nRB	nHA	nHD	TPSA	Log P	Water sol	RO5 Viol.
Epiexcelsin	414.4	4	8	0	73.84	2.75	Soluble	0
Sesartemin	430.4	6	8	0	73.84	2.89	Soluble	0
Yangambin	446.4	8	8	0	73.84	3.04	Moderately soluble	0
Syringaresinol	418.4	6	8	2	95.84	2.33	Soluble	0
Sesamin	354.3	2	6	0	55.38	2.79	Soluble	0

MW = molecular weight; nRB = number of rotatable bonds; nHD = number hydrogen bond donor; nHA = number hydrogen bond acceptors; TPSA = topological polar surface area; water sol= water solubility; RO5 viol. = lipinski's rule of five violations.

The physicochemical characteristics of drug-like molecules can be computed using Lipinski's rule of five, which takes into account factors such as molecular weight, hydrogen bond donors and acceptors, and logP. Compounds that break more than one of these criteria may have issues with bioavailability (Lipinski, 2000; Shams et al., 2022). In addition, Verber et al. (2002) noted that compounds with fewer than 10 rotatable bonds and a total polar surface area (TPSA) of 140 or less are more likely to have high bioavailability (Ramadan et al., 2022). Our study, summarized in Tables 3 and 4, revealed that all five isolated compounds adhered to these rules, including the number of hydrogen bonds and TPSA. They were also predicted to have molecular weights of less than 500 Da, making them easily transportable, diffusible, and absorbable compared to heavier molecules (Srimai et al., 2013). However, yangambin was predicted to have moderate solubility (Log P=3.04), while the remaining four compounds were found to be soluble and therefore have good bioavailability. It is worth noting that solubility can also influence the ADME profile, and the acceptable solubility profile of our compounds is a positive sign for their eventual success as drug candidates (Utomo et al., 2022).

Table 4. Predicted ADME properties of isolated compounds SwissADME webserver

Compound	A	B	C	D	E	F	G	H	I
Epiexcelsin	High	Yes	Yes	No	No	Yes	Yes	Yes	-6.97
Sesartemin	High	Yes	No	No	No	No	Yes	No	-6.97
Yangambin	High	Yes	No	No	No	No	Yes	No	-6.98
Syringaresinol	High	No	Yes	No	No	No	Yes	No	-7.27
Sesamin	High	Yes	No	No	Yes	No	Yes	Yes	-6.56

A= human gastrointestinal absorption; B = permeant; blood–brain barrier permeability; C = permeability glycoprotein substrate; D = CYP1A2 inhibitor; E = CYP2C19 inhibitor; F = CYP2C9 inhibitor; G = CYP2D6 inhibitor; H = CYP3A4 inhibitor; I = log Kp (cms-1): skin permeability coefficient.

3.4. DFT computation and structural analysis

Figure 1 shows that the geometry-optimized structures of epiexcelsin conform to a global minimum as obtained from DFT computations. The frontier molecular orbitals (HOMO and LUMO) play a crucial role in charge transfer interactions between the ligands and the active site of the target protein. The positive and negative regions of the orbitals are represented by blue and red colors, respectively, as shown in Figure 1. The frontier molecular orbitals, namely, HOMO and LUMO, are highly informative in predicting the most reactive position in π-electron systems and in explaining various types of reactions within the conjugated system. Additionally, the HOMO and LUMO energy levels can provide insights into the relative chemical stability and biological activity of the molecules. The difference in energy between these two orbitals, known as the HOMO-LUMO energy gap, can be utilized to determine the strength and stability of the molecule. Generally, a molecule with a smaller HOMO-LUMO energy gap is more polarizable and exhibits greater chemical reactivity (Shams et al., 2022; Umar & Uzairu, 2023).

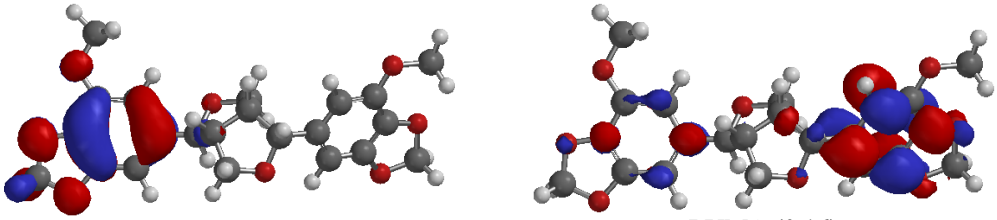
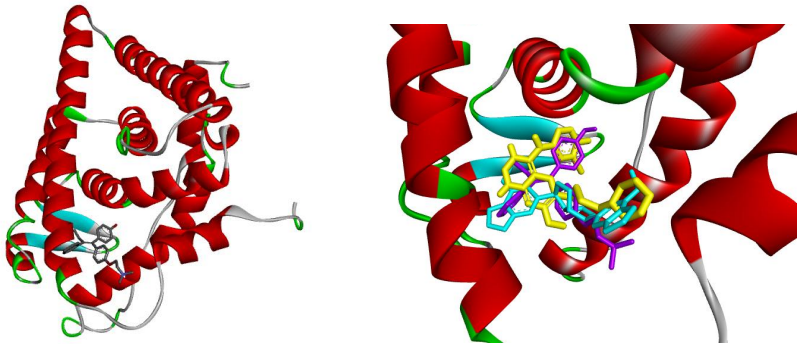


Figure 1. The 3D HOMO and LUMO frontier orbitals of the selected compound (Epiexcelsin).

3.5. Molecular docking

Upon completion of the analysis of the physicochemical and pharmacological properties of the isolated compounds, the potential target for these compounds was predicted using the ChemMapper and PharmMapper servers. The results revealed that the estrogen receptor-α could be a promising target for these compounds, with a fit score of 2.801 and a normalized fit score of 0.933. Moreover, the Estrogen Receptor-α (PDB ID: 3ERT) (Figure 2(A)) was downloaded from the protein data bank. The 3ERT target is particularly relevant to this study, as it has been shown to play a key role in the development and progression of breast cancer. Estrogen receptor-positive breast cancer is the most common type of breast cancer. It is often treated with drugs that target the estrogen receptor, such as tamoxifen and aromatase inhibitors (Yu et al., 2022). However, resistance to these drugs is a major clinical challenge, and there is a need for new therapies that can overcome this resistance (Tsoi et al., 2022). The potential of natural compounds to modulate the activity of the estrogen receptor and other proteins involved in hormone signaling pathways makes them promising candidates for developing new therapies for estrogen receptor-positive breast cancer and other hormone-dependent diseases (Khan et al., 2020; Talib et al., 2022).



(**A**) (**B**)

Figure 2. The 3D structure of (**A**) estrogen receptor-α together with native ligand, (**B**) redocked native ligand (magenta), Epiexcelsin (cyan), and gefitinib (yellow).

Table 5 presents the docking scoring results, inhibition constants (Ki), and the types of interactions formed by furofuran lignans and a known drug (Gefitinib) with the Estrogen Receptor-α (ER-α). The docking results show the binding affinity of each ligand, represented as the docking score, which is a measure of the predicted strength of ligand-receptor interaction. The lower the docking score, the higher the predicted binding affinity. The inhibition constants (Ki) are also reported, which provide an estimate of the potency of the ligands in inhibiting the activity of ER-α. Lower Ki values indicate higher potency. From the results, it can be observed that epiexcelsin has the lowest docking score (-8.13 kcal mol^{-1}) and the lowest Ki value (1.1 µM), suggesting it has the highest predicted binding affinity and potency among the tested furofuran lignans.

To validate the docking, we performed redocking of the native ligand and superimposed our test ligands in the identical binding conformation (Figure 2(B)). It can be seen that the compounds including gefitinib binds in the same pocket as the native ligand and in a similar conformation. The docking scores and interactions of furofuran lignan ligands with estrogen receptor-α were compared to gefitinib, an approved drug. Epiexcelsin showed the highest binding affinity with a docking score of -8.13 kcal mol^{-1}, surpassing gefitinib with a docking score of -7.04 kcal mol^{-1}, indicating a potentially stronger interaction with estrogen receptor-α. Epiexcelsin also had a lower inhibition constant (Ki) of 1.1 µM compared to gefitinib's Ki of 6.93 µM, suggesting higher potency in inhibiting estrogen receptor-α activity.

Both epiexcelsin and gefitinib formed hydrogen bonds with the receptor, but epiexcelsin formed three hydrogen bonds with His524, Leu346, and Ala350, while gefitinib formed two hydrogen bonds with Asp351 and Arg394. Additionally, epiexcelsin exhibited similarities with gefitinib in forming hydrophobic interactions with various residues, including Leu354, Leu387, Leu428, Leu525, Met421, Met388, and Ile424, as well as π-alkyl interactions with Leu525, His524, Ala350, and Trp383. However, epiexcelsin also showed unique interactions, such as a π-π stacking interaction with Trp383, which was not observed in gefitinib. This suggests that epiexcelsin may have additional binding modes or interactions that could contribute to its higher binding affinity and potency compared to gefitinib.

In our previous work, these five compounds were successfully isolated and displayed notable anticholinesterase and anti-inflammatory activities with IC_{50} ranging from 168.8 – 504.2 µM and 21.0 – 59.4 µM respectively (Salleh et al., 2016a). Recent study by Rabaan et al. (2023), epiexcelsin demonstrated a significantly high minimum binding energy of −7.4 kcal/mol in its best pose compared to all other compounds investigated against SARS-CoV-2 RdRp protein, and even its worst pose displayed a notable binding energy of −6.5 kcal/mol. This exceptional binding affinity of epiexcelsin highlights its potential importance as a lead compound for further exploration in drug discovery efforts. We are aware of the limits of our computational work and the critical need for additional experimental validation to support and expand the results reported in this study. Incorporating experimental data will improve the validity and precision of our findings while also shedding important light on the applicability and viability of our suggested approach. To improve the general validity and application of the findings in real-world circumstances, we recommend future studies to prioritize experimental verification.

Table 5. Docking scoring, inhibition constant, and the type of interactions formed by furofuran lignans with eastrogen receptor-α

Ligands	Binding affinity (kcal mol^{-1})	Inhibition constant (Ki)	Total number and residues involved in Hbonds	Hydrophobic bond and miscellaneous
Sesamin	-7.64	2.5μM	1 His524 (8vdw)	5 alkyl (Leu536, Leu539, Leu525, Met421, Ala350); 1 π- anion (Asp351); 1 π-sulphur (Met421) 1 π- δ (Met343); 4 π-alkyl (His524, Leu346, Leu525, Ala350)
Sesartemin	-6.88	9.02 μM	3 Thr347, Asp351, Glu419 (7vdw)	10 alkyl (Ala350, Leu525, Leu384, Leu354, Leu428, Met388, 2 x Met421, 2 x Ile424); 1 π- anion (Asp351) 2 π- π staking (Trp383); 4 π-alkyl (Leu346, Ala350 and 2 x Trp383)
Syringeresinol	-6.24	26.68 μM	3 Thr347, Leu346, Asp351 (6vdw)	6 alkyl (Ala350, Leu354, Leu391, Leu525, Met421, Ile424); 1 π- anion (Asp351); 1 π-sulphur (Met421); 1 π- δ (Trp383); 2 π-alkyl (Ala35 and Phe404)
Yangambin	-6.56	15.53 μM	2 Thr347, Asp351 (2vdw)	12 alkyl (Leu354, Leu536, Leu387, Leu428, Leu525, 2 x Leu391, Ala350, 2 x Met421, Met343); 1 π- anion (Asp351); 2 π-sulphur (Met421, Met343); 1 π- δ (leu346); 3 π-alkyl (Trp383, Phe404 and Ala350)
Epiexcelsin	-8.13	1.1 μM	3 His524, Leu346, Ala350 (7 Vdw)	7 alkyl (Leu354, Leu387, Leu428, Leu536, Met421, Met388, Ile424); 1 π- π staking (Trp383); 5 π-alkyl (Leu525,His524 , Ala350 and 2 x Trp383)
Gefitinib (approved drug)	-7.04	6.93 μM	2 Asp351, Arg394 (6vdw)	6 alkyl (Leu354, Leu346, Leu349, Leu525 and 2 x Ala350); 1 Sulphur-x (Met343); 4 π-alkyl (Leu387, Leu391, 2 x Trp383); 3 halogen (Arg394, Phe404 and Glu353)

vdw = vanderwaal interaction.

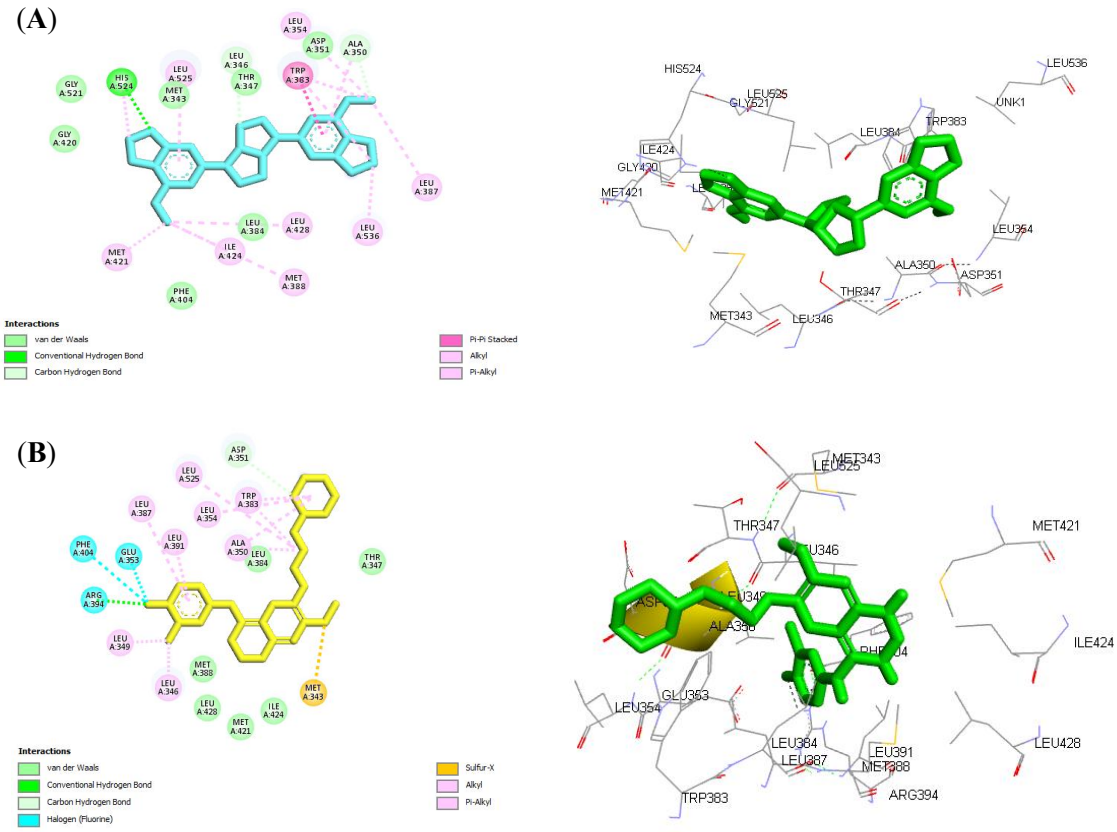


Figure 3. The 2D interactions and residue surrounding of (**A**) epiexcelsin and (**B**) gefitinib in the binding site of the eastrogen receptor-α.

4. Conclusion

This study utilized computer-based drug discovery to investigate the pharmacological and bioactivity of five isolated compounds, namely, epiexcelsin, sesamin, sesartemin, syringaresinol, and yangambin from *Beilschmiedia pulverulenta*. All compounds showed promising pharmacokinetic activities, with epiexcelsin exhibiting strong binding affinity and inhibitory activity against estrogen receptor-α. This furofuran lignan compound was predicted to be a bioavailable and effective lead, potentially outperforming gefitinib, an approved drug. The identification of these compounds highlights the potential of natural medicinal plants for drug development, and the use of computational methods in drug discovery to predict potential drug targets and estimate drug likeliness properties can help reduce the likelihood of failure in clinical phases. Therefore, this study contributes to the growing body of research on natural products as a source of drug lead, while also emphasizing the significance of integrating traditional medicinal knowledge with modern scientific approaches.

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Research Article

Callus Production in Geranium (*Pelargonium quercetorum* Agnew) Growing Naturally in Türkiye

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Abstract: *Pelargonium quercetorum* Agnew grows naturally in the Hakkari province of Türkiye. Although *P. quercetorum* Agnew has potential use as a medicine and ornamental plant, it is especially used as a medicinal plant for the cure of various diseases by local people. *In vitro* tissue culture methods are favorable for the propagation, conservation, and breeding of medicinal plants. We aimed in this study to achieve regeneration of *P. quercetorum* Agnew from different explant types. Seeds of *P. quercetorum* Agnew were germinated *in vitro* conditions and explants were taken from these germinated sterile plantlets. Totally four different experiments, containing three of them embryogenic and one of them organogenic culture, were established to achieve regeneration in *P. quercetorum* Agnew. Leaf, petiole, cotyledon, cotyledon stalk, and root collar disc were used as explant. Different concentrations of 1-Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic (2,4-D), 6-Benzylaminopurine (BA), 6-Furfurylaminopurine (Kinetin), 6-(γ,γ-Dimethylallylamino) purine (2iP), and Thidiazuron (TDZ) were used to induce embryogenic or organogenic regeneration. Explants were cultured in half-strength or full-strength Murashige and Skoog (MS) medium. In the embryogenic experiments, callus formation from different media ranged from 63.5% to 100%, and for explant types ranged from 39% to 100%. In the organogenic experiment, callus formation from different media ranged from 12.5% to 100%, and for explant types ranged from 71% to 93%. Also, embryo-like structures were obtained from embryogenic experiments. However, these structures could not grow more and transformed into plantlets.

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1. Introduction

Pelargonium quercetorum Agnew, most of which grows in and around South Africa, is included in the Geraniaceae family, which includes more than 220 species (Taherpour et al., 2008; Röschenbleck et al., 2014). One of the places where geranium grows naturally outside of South Africa is Anatolia. Another naturally grown geranium species was described by Agnew (1967) in the Mosul province of Iraq. This species, which was found around the Hakkari-Zap River in Türkiye, spreads in Southeastern Anatolia and is known as “Tolk” in the Gecitli village of Hakkari (Davis, 1967; Kaval et al., 2014).

The use of geranium plant, which has been used as a medicine among people for many years, is limited as an ornamental plant. Currently, naturally grown geranium plants do not have sufficient potential for use as ornamental plants. In order to increase the use of geranium as an ornamental plant and to gain a place in the ornamental plants' sector, it is necessary to develop the propagation methods of existing natural species. Ornamental plants can be propagated by seed or vegetatively by conventional methods, or by using tissue culture techniques.

In vitro, tissue culture techniques can be an effective alternative tool to classic techniques for propagation, conservation, and breeding of plants. One of the most frequently used techniques among tissue culture techniques is somatic embryogenesis. Somatic embryogenesis is the process of producing embryos from somatic tissues of the plant in a bipolar structure that retains the genotype of the parent plant. Somatic embryogenesis is one of the most effective techniques for mass vegetative propagation *in vitro* (Öktem and Yücel, 2012). These embryos consisting of somatic cells are called somatic embryos. Embryos can develop directly from somatic cells (direct somatic embryogenesis) or occur as non-embryogenic mitotic divisions (indirect somatic embryogenesis) before embryogenic structures appear (Rout et al., 2006). Significant differences are observed between species in terms of somatic embryo formation frequency, and embryo formation abilities of different genotypes and varieties within the same species are different. Somatic embryogenesis can be successfully applied in ornamental plants as well as in many plants. Somatic embryogenesis has been reported in cyclamen (Koçak et al., 2014), tulip (Bakhshaie et al., 2010), cloves (Pareek and Kothari, 2003), and begonia (Castillo and Smith, 1997).

Another propagation in tissue culture used widely is organogenesis. In many ornamental plants; *Lilium pumilum* (Zhang et al., 2016), *Camellia nitidissima* (Lü et al., 2013), *Echinacea purpurea* (Choffe et al., 2000), *Dendrobium kingianum* (Habiba et al., 2018), *Tulipa tarda* (Maślanka and Bach, 2014), *Polianthes tuberosa* (Daneshvar et al., 2022), *Chrysanthemum morifolium* (Kazeroonian et al., 2018) and *Begonia elatior* (Mendi et al., 2009) organogenesis has been reported.

Both somatic embryogenesis and organogenesis occur directly or with a callus phase. Callus is described as an undifferentiated cell mass. Callus cultures are used frequently for micropropagation, utilizing from variation and obtaining cell suspension cultures in *in vitro*. Callus is also a source for the production of secondary metabolites produced by plants for defense, protection, survival, and persistence (Altan and Duru, 2017). The utilization of callus culture has become widespread, serving as a crucial method in both experimental and commercial contexts for generating valuable therapeutic compounds. These compounds span a diverse range, including antibiotics designed to combat resilient infections, widely acknowledged anticancer agents, and medicinal nanoparticles (Benjamin et al., 2019).

There are papers reporting regeneration in the other some species of *Pelargonium*; *P. graveolens* (Sreedhar, 1999; Saxena et al., 2000; Benazir et al., 2013), *P. sidoides* (Kumar et al., 2015), *P. rapaceum* (Sukhumpinij et al., 2010), *P. radula* (Zuraida et al., 2014) and *P. odoratissimum* (Ebrahimzadeh et al., 2022). However, according to our best knowledge, there is no study in literature reporting *in vitro* regeneration or callus formation in *P. quercetorum* Agnew. Therefore, in this study, we aimed to produce callus in *P. quercetorum* Agnew by inducing embryogenic and organogenic regeneration with various plant growth regulators and different combinations-concentrations of them.

2. Material and Methods

In the study, seeds of *Pelargonium quercetorum* Agnew were collected from plants growing naturally around Hakkari. Seeds of *P. quercetorum* Agnew were sterilized and germinated in tissue culture conditions. Different parts of sterile plantlets (leaves, petioles, cotyledons, cotyledon stalks, and root collar discs) were used as initial explant for regeneration experiments.

2.1. Surface sterilization and germination of seeds

The seeds were kept in 70% ethanol for one minute afterward rinsing them in sterile distilled water. In the second step of sterilization, seeds were treated in 30% sodium hypocloride (Domestos® commercial product) for 30 minutes and rinsed with sterile distilled water until removal of sterilization solution. Before the transformation of sterile seeds into germination medium, they were dried on blotting paper. Sterile seeds were transferred to a culture tube containing ½ strength of MS and, cultured at 24 °C and under 16 hours light / 8 hours dark photoperiod.

2.2. Regeneration experiments

Four different experiments consisting of three of them inducing embryogenic regeneration and one of them inducing organogenic regeneration were established to provide regeneration in *P. quercetorum* Agnew.

Embryogenic culture 1 (E1): In this experiment leaf, petiole, cotyledon, and root collar disc were used as explant. To induce embryogenic regeneration, NAA, 2,4-D, BA, Kinetin, and 2iP were added to ½ MS medium (Table 1). Explants were cultured at 24 °C full dark condition. After four weeks from the initiation of culture and forming callus mass, subcultures were carried out to new media without hormones. In these nutrient media, explants were transferred to new culture media routinely every month.

Embryogenic culture 2 (E2): Calli obtained from embryogenic culture 1 were incubated in full-strength MS medium supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin (Table 1). All explants were cultured at 24 °C under 16 hours light / 8 hours dark photoperiod. After calli formation, explants were transferred to a hormone-free medium and subcultured per month with the same incubation conditions.

Embryogenic culture 3 (E3): This culture was established by culturing sterile leaf, petiole, cotyledon, and cotyledon stalk in a full-strength MS medium containing 0.1 g l^{-1} myo-inositol. Also, the effect of 0,02 g l^{-1} glutamine was tested. Various concentrations of NAA, 2,4-D, and TDZ were used as hormones to induce embryogenic callus formation (Table 1). After calli formation, explants were transferred to a hormone-free medium and subcultured per month. Explants were cultured at 24°C under 16 hours light / 8 hours dark photoperiod.

Organogenic culture (O): Leaf, petiole, and root collar disc were cultured in full-strength MS medium supplemented with various concentrations of NAA, 2,4-D, BA, and kinetin (Table 1). All explants were cultured at 24 °C under 16 hours light / 8 hours dark photoperiod.

Table 1. Concentrations and types of ingredients used in regeneration experiments

	Medium	NAA mg l^{-1}	2,4-D mg l^{-1}	BA mg l^{-1}	Kin. mg l^{-1}	2iP mg l^{-1}	TDZ mg l^{-1}	Glutamine
Embryogenesis	E1-1		2					
	E1-2		2	1				
	E1-3		2		1			
	E1-4		2			1		
	E1-5	2						
	E1-6	2		1				
	E1-7	2			1			
	E1-8	2				1		
	E2		2		0.5			
	E3-1	1					0.5	+
	E3-2	2					0.5	+
	E3-3		1				0.5	+
	E3-4		2				0.5	+
	E3-5	1					0.5	-
	E3-6	2					0.5	-
	E3-7		1				0.5	-
	E3-8		2				0.5	-
Organogenesis	O1	1		2				
	O2		1	2				
	O3				2			
	O4	1			2			
	O5		1		2			
	O6					2		
	O7	1				2		
	O8		1			2		

2.3. Experimental design and statistical analysis

The experiments were established as completed randomized plots (5 replicate/petri x 4 explants). All percentage data were transformed to arcsine value. Means were compared with an analysis of variance and significant differences were determined by performing an LSD test. All data analyses were carried out with the JMP® program (SAS Institute, Cary, NC) ver. 8.00.

3. Results

In the germination culture of the seed, no infection was observed. The germination rate was 83%, which suggested sterilization method did not decrease the germination capacity of seeds significantly. Seeds started to germinate in germination media in 3-4 days. Explants were taken from plantlets, grown, and covered inside growing tubes.

Embryogenic culture 1: Callus formation was observed ten days after the establishment of culture in all explant types (Figure 1). A high amount of callus formation was achieved from both all explant types and all media. Only E1-5 medium containing 2 mg l^{-1} NAA with 89 % of callus formation rate was significantly different from other media and it produced a lower level of callus. The other media presented no important differences statistically in terms of callus formation performance (Table 2).

Table 2. Callus formation percentage from explant types and different medium and assessment of medium-explant interaction in embryogenic culture 1

Medium	Leaf	Petiole	Root collar disc	Cotyledon	Mean of medium
E1-1	95 (84)a	100 (90)a	100 (90)a	93.75 (81.1)a	97.18 (86.2)A
E1-2	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
E1-3	100 (90)a	100 (90)a	100 (90)a	93.75 (81.1)a	98.43 (87.7)A
E1-4	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
E1-5	100 (90)a	90 (81)a	100 (90)a	66 (57.49)b	89 (79.62)B
E1-6	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
E1-7	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
E1-8	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
Mean of explant	100 (90)A	98.12 (88.12)A	100 (90)A	94.18 (83.71)B	

LSD explant = 3.75, LSD medium =5.3, LSD explant x medium = 10.61, ($P<0.05$).

Embryogenic culture 2: In this experiment, calli induced embryogenic regeneration previously, however not transformed embryos were induced embryogenic regeneration again with different conditions. Likewise, in the first embryogenic culture, embryo formation was not observed, however, callus mass-producing continued.

Embryogenic culture 3: In the experiment, callus production was obtained from both explant types and all different media. Callus formation rates from different explants were significantly different. Leaf and cotyledon explants were the best explants types with the rate of 91.25% and 92.5%. They were followed by petiole and cotyledon stalk with a rate of 54.25% and 39% respectively (Table 3). It is found that there was no effect of glutamine statistically for callus production.

Differences between callus production means rate of media was significant statistically. Although E3-1 (1 mg l^{-1} NAA) was the best medium with 82.5% callus production rate, the least callus production was obtained from E3-4 (2 mg l^{-1} 2,4-D) medium with 63.5% (Table 3).

Interaction between medium and explant for callus formation was found significantly different. Leaf x E3-6 (2 mg l NAA), cotyledon x E3-6 (2 mg l^{-1} NAA) and cotyledon x E3-8 (2 mg l^{-1} 2,4-D) interactions were the best medium-explant combinations with 100%. The least callus production rate was found in cotyledon stalk x E3-6 (2 mg l^{-1} NAA), cotyledon stalk x E3-5 (1 mg l^{-1} NAA), and cotyledon stalk x E3-7 (1 mg l^{-1} 2,4-D) interactions with the rate of 26% (Table 3).

Embryo-like structures were formed in all of the embryogenic cultures (Figure 2). However, the growth of these structures was blocked and they could not grow more and germinated.

Table 3. Callus formation percentage from explant types and different medium and assessment of medium-explant interaction in embryogenic culture 3

Medium	Leaf	Petiole	Cotyledon	Cotyledon stalk	Mean of medium
E3-1	96 (82.62)abc	82 (67.84)bcde	96 (84.68)ab	56 (48.46)fgh	82.5 (70.90)A
E3-2	96 (82.62)abc	62 (52.19)efg	80 (72.00)bcd	32 (33.81)hıj	67.5 (60.16)BC
E3-3	94 (83.35)ab	50 (45.00)fgh	84 (72.00)bcd	44 (41.31)fghı	68 (60.41)BC
E3-4	78 (65.95)cde	44 (40.89)fghıj	96 (84.68)ab	34 (35.39)ghıj	63.5 (56.73)C
E3-5	94 (81.00)abc	56 (47.91)fgh	90 (75.68)abc	26 (27.77)ıj	66.5 (58.09)BC
E3-6	100 (90.00)a	44 (41.31)fghı	100 (90)a	26 (24.04)j	67.5 (61.34)BC
E3-7	92 (79.67)abc	42 (40.33)fghıj	94 (83.35)ab	26 (27.59)ıj	63.5 (57.74)BC
E3-8	80 (69.46)bcd	54 (47.30)fgh	100 (90.00)a	68 (55.58)def	75.5 (65.50)AB
Mean of explant	91.25 (79.33)A	54.25 (47.85)B	92.5 (81.55)A	39 (36.74)C	

LSD $_{explant}$ = 6.05, LSD $_{medium}$ = 8.56, LSD $_{explant\ x\ medium}$ = 17.12, (P<0.05).

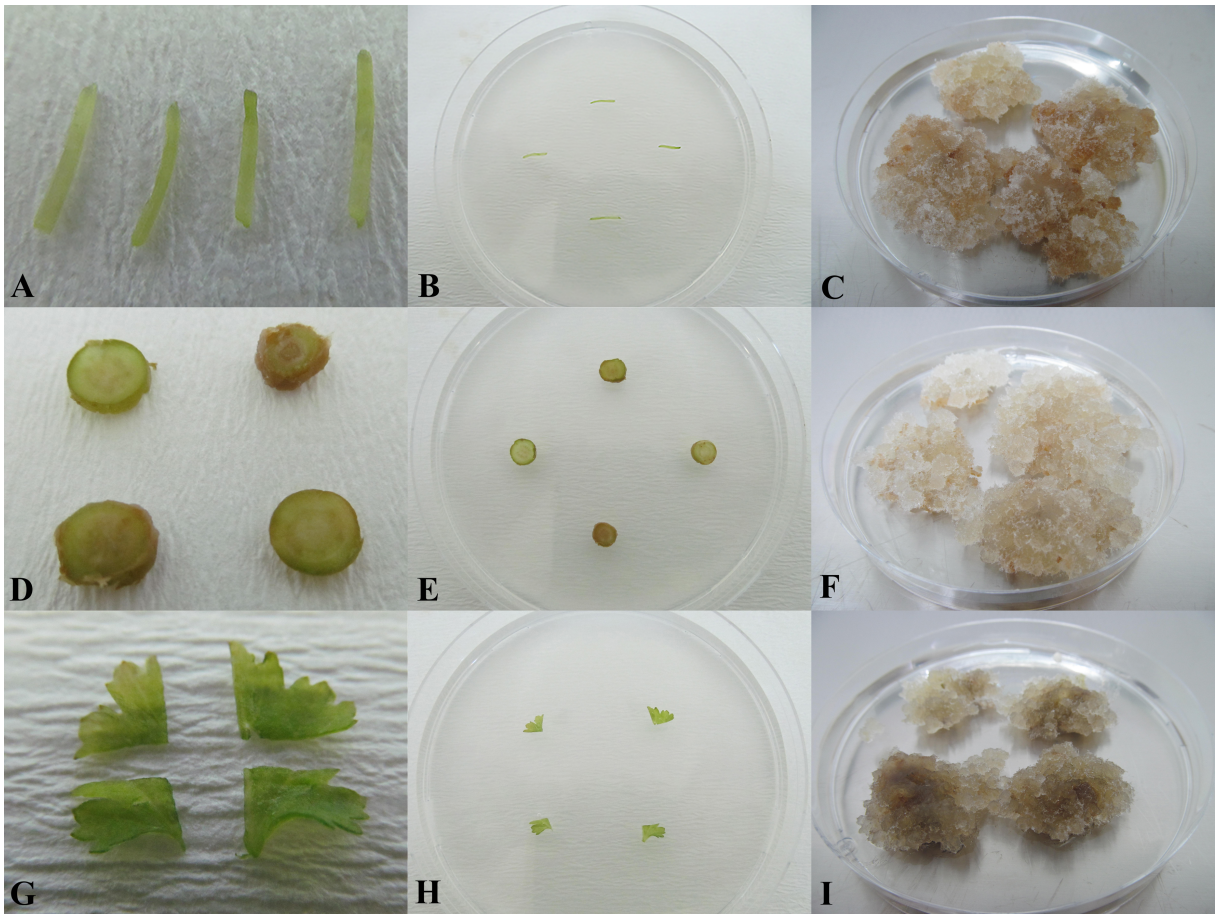

Figure 1. Explants and callus mass from explants A, B) Petiole, C) Callus from petiole, D, E) Root collar disc, F) Callus from root collar disc, G, H) Leaf, I) Callus from leaf.

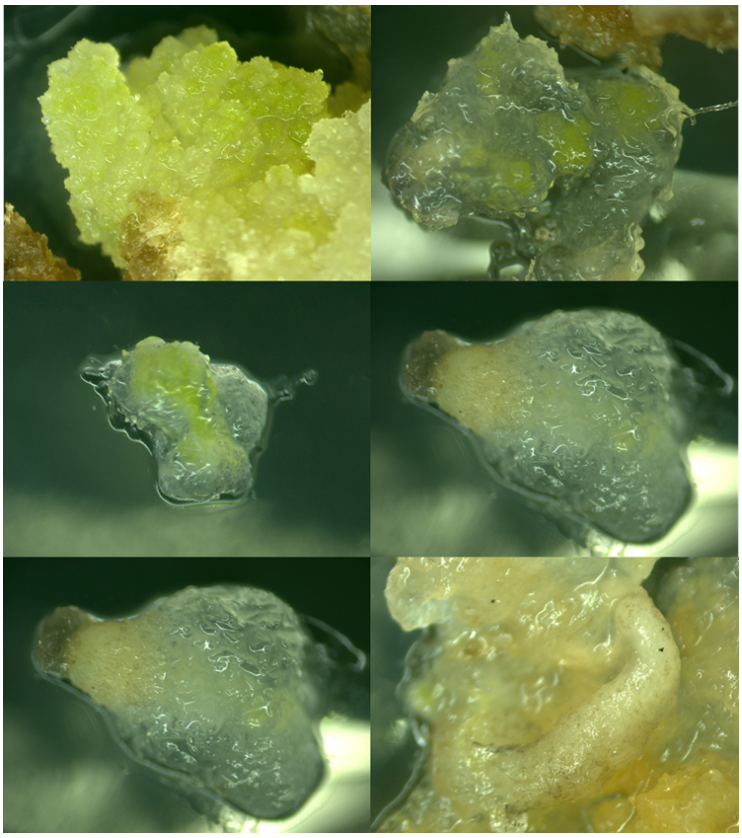

Figure 2. Callus formation and embryo-like structures in embryogenic cultures.

Organogenic culture: Callus formation was observed in a week. Some of the calluses with a color scale ranging from transparent-light color to brown in the first stages of culture showed green color formation with the effect of light in the later stages. According to both explant type and medium, callus formation rates were statistically important. The best callus formation rate observed in cultures from root collar discs with 93.75%. Cotyledon, leaf, and petiole followed it with the rate of 86.37%, 75%, and 71.25% respectively. The best callus formation obtained from O2 medium (1 mg l^{-1} 2,4-D + 2 mg l^{-1} BA) and O8 medium (1 mg l^{-1} 2,4-D + 2 mg l^{-1} 2iP) with the rate of 100%. The lowest callus yield was obtained from O3 medium (2 mg l^{-1} Kinetin) with 12.5%. Although most of the interactions between explant types and media gave a 100% callus formation rate, interactions of O3 (2 mg l^{-1} Kinetin) x leaf, O3 (mg l^{-1} Kinetin) x petiole, O3 (2 mg l^{-1} Kinetin) x cotyledon and O3 (mg l^{-1} 2iP) x leaf did not produce callus (Table 4).

Table 4. Callus formation percentage from explant types and different medium and assessment of medium-explant interaction in organogenic culture

Medium	Leaf	Petiole	Root collar disc	Cotyledon	Mean of medium
O1	100 (90)a	100 (90)a	100 (90)a	91 (76.6)bc	97.5 (86.65)AB
O2	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
O3	0 (0)	0 (0)	50 (45)d	0 (0)	12.5 (11.25)D
O4	100 (90)a	80 (69)c	100 (90)a	100 (90)a	95 (84.75)B
O5	100 (90)a	95 (84)ab	100 (90)a	100 (90)a	98.75 (88.5)AB
O6	0 (0)	5 (6)e	100 (90)a	100 (90)a	51.25 (46.5)C
O7	100 (90)a	90 (81)b	100 (90)a	100 (90)a	97.5 (87.75)AB
O8	100 (90)a	100 (90)a	100 (90)a	100 (90)a	100 (90)A
Mean of explant	75 (67.5)C	71.25 (63.75)D	93.75 (84.37)A	86.37 (77)B	

$LSD_{explant}$ = 2.98, LSD_{medium} = 4.63, $LSD_{explant\ x\ medium}$ = 8.43, ($P<0.05$).

4. Discussion

In this study, embryogenesis and organogenesis experiments of *Pelargonium quercetorum* Agnew were established for the first time and callus formation was achieved in all media and explant types used in all experiments.

Although *in vitro* studies have been reported in many *Pelargonium* species; *Pelargonium* × *Hortorum* L. H. Bailey (Vejsadová and Kuchtová-Jadrná 2008), *Pelargonium rapaceum* (L.) L'Hérit (Sukhumpinij et al., 2010), *Pelargonium graveolens* (Benazir et al., 2013), *Pelargonium radula* (Zuraida et al., 2014), *Pelargonium sidoides* DC (Kumar et al., 2015), there is no report *on Pelargonium quercetorum* Agnew. In the first experiment in this study, embryogenic cultures were established using combinations of 2,4-D and NAA as auxins and BA, Kinetin, and 2iP as cytokinins. Callus was obtained from all media. Brown and Charlwood (1986) also used 0.2 mg l^{-1} kinetin and 1 mg l^{-1} 2,4-D for callus production in their study on fragrant geranium species. Similarly, Benazir et al. (2013) obtained callus production in *P. graveolens* from an MS medium containing 20 µM NAA and 10 µM kinetin. However, in this study, callus production could not be obtained from media containing 10-60 µM 2,4-D.

The effect of glutamine on callus formation was found to be statistically insignificant. Similar results were reported by Amer et al. (2017). These researchers reported that glutamine had no effect on callus formation and shoot regeneration in two different varieties of rice plants. However, Pawar et al. (2015) reported that proline and glutamine had a positive effect on callus development in four different varieties of rice. These different results show that the effect of glutamine on callus formation *in vitro* may vary from species to species and even within species according to different varieties.

We observed that the best callus-forming medium was 1 mg l^{-1} NAA for *P. quercetorum* Agnew. In previous studies, it was determined that TDZ promoted callus formation for other *Pelargonium* species and varieties (Visser et al., 1992; Robichon et al., 1997). Qureshi and Saxena (1992) reported that regeneration type was determined by TDZ dose. Low concentrations (0.2 - 0.4 mg l^{-1}) stimulated adventitious shoot formation, while somatic embryos were observed at higher concentrations (2.2 mg l^{-1}). Sreedhar (1999) found that MS medium supplemented with various levels of 2,4-D was the primary medium for callus induction, however in contrast to reported studies in *P. graveolens*, the plant reacted poorly and showed no signs of growth. Sukhumpinij et al. (2010) found that the highest callus formation rate (100%) for *Pelargonium rapaceum* (L.) was observed in leaf explants cultured on media containing combinations of NAA and BAP, 2,4-D and BAP, and IAA and TDZ. In the presence of 2,4-D, none of the cultured explants formed shoots, and, in some cases, callus formed and then became necrotic and died. They reported that this was probably due to the toxic effects of auxin.

Agarwal and Ranu (2000) investigated *in vitro* plant regeneration potential of vegetatively propagated geraniums (*Pelargonium hortorum*). They found that combinations of zeatin and IAA or BA and IAA led to regeneration. They reported that zeatin and IAA combinations resulted in higher levels of regeneration and a number of regenerated shoots per explant compared to BA and IAA combinations.

Madden et al. (2005) examined modes of regeneration from hypocotyl explants with different cytokinin treatments [1 µM thidiazuron (TDZ), 4 µM TDZ or 8 µM N6-benzylaminopurine (BA), and 1 µM indole-3-acetic acid (IAA)] in *Pelargonium* x *hortorum* 'Scarlet Orbit' and three wild relatives *P. zonale*, *P. alchemilloides,* and *P. inquinans*. They found that *Pelargonium* x *hortorum* 'Scarlet Orbit' and *P. zonale* showed the same number of embryo-like structures in response to 1 µM TDZ. However, *P. alchemilloides* and *P. inquinans* showed weak embryogenic structures in response to all treatments. Similarly, in our study, embryo-like structures were formed in all embryogenic cultures, however, embryos did not grow.

Many studies have been conducted on different *Pelargonium* cultivars. Wojtania et al. (2004) compared the effects of meta-topoline and BA on *Pelargonium* × *hortorum* 'Bargpalais' proliferation. They found that regenerated shoots after meta-topolin treatment showed better quality and contained fewer abnormal shoots compared to shoots induced in the BA medium. Similar studies can be planned for *P. quercetorum* Agnew in the future.

Conclusion

We have established for the first time an effective callus production protocol for *Pelargonium quercetorum* Agnew. Preliminary studies to determine the appropriate conditions for somatic

embryogenesis have been successfully carried out. Based on this study, the development of new protocols can be aimed. The development and optimization of biotechnological approaches with the establishment of appropriate protocols for somatic embryogenesis will open some additional possibilities for further research in *Pelargonium quercetorum* Agnew. In the future, it is thought that important results will be obtained in areas such as the development of disease-resistant varieties, the application of techniques such as cell cultures, induced mutations and genetic transformation, and *in vitro* fertilization and embryo rescue studies.

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