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Estimates of genetic variability and correlation coefficient for yield and its traits in black cumin (*Nigella sativa* L.) genotypes in South Wollo Ethiopia

OPEN ACCESS

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Abstract: Black cumin (*Nigella sativa* L.) is a valuable annual spice used in food, cosmetics, and industrial products. However, productivity is limited by the lack of improved varieties, mainly due to insufficient knowledge of the genetic diversity within Ethiopian black cumin populations. A study was conducted to assess genetic variability and interrelationships among traits related to seed yield in various black cumin genotypes. Twenty-five genotypes were tested using a 5x5 simple lattice design in Hara, Tehuledere district, Ethiopia during the 2022/2023 growing season. Analysis of variance revealed significant differences for most parameters, except stand count at harvest and thousand seed weight. Broad-sense heritability (H^2) and genetic advance as a percentage of the mean (GAM) ranged from 8.24% for days to 90% maturity to 89.56% for the number of tertiary branches per plant, and 4.51% for days to 90% maturity to 84.85% for the number of tertiary branches per plant, respectively. High to moderate estimates of genetic and phenotypic coefficients of variation (GCV and PCV), heritability, and GAM were observed for the number of tertiary branches per plant, number of flowers, number of capsules, biological yield, seed yield, and harvest index. The results also showed significant positive correlations between seed yield and various traits, including plant height, number of primary and tertiary branches, flowers, capsules, and seeds per capsule, biological yield, and harvest index, at both the genotypic and phenotypic levels. The study concluded that significant genetic variability exists, offering potential for targeted selection to enhance specific traits.

1. Introduction

Black cumin (*Nigella sativa* L.) belongs to the Ranunculaceae family within the Ranales order, which includes more than 70 genera and approximately 3,000 species, all having a diploid chromosome number of

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(2n = 2x = 12). It is one of the 14 annual herb species in the Nigella genus (Weiss, 2004). The name «Nigella» is derived from the Latin words «nigellus» or «niger,» meaning «black». Commonly known as black cumin, across the globe, this plant goes by many different names. In Ethiopia, it is called "tikurazmud" in Amharic and "awosetta" in Tigrinya (Jansen, 1981).

According to Nergiz and Otles (1993), black cumin features a slightly hairy stem, shiny green trifoliate leaves, and attractive flowers that bloom at the end of the stem. The flowers are typically milky white with a subtle blue or green tint at the tips. The seeds are housed in black, slightly curved seedpods with three edges. This annual crop grows to a height ranging from 15.04 to 30.08 cm (Nadaf *et al.,* 2015). black cumin is native to the Mediterranean region and has been cultivated for over 3,000 years (Khan, 2009). However, it is widely cultivated in Sub-Saharan Africa (Iqbal *et al.,* 2019).

Black cumin seeds are rich in nutrients, comprising 26.7% protein, 28.5% fat, 24.9% carbohydrates, 8.4% crude fiber, and 4.8% total ash. They also provide a significant quantity of various vitamins and minerals, including copper (Cu), phosphorus (P), zinc (Zn), and iron (Fe). The content and composition of the essential oil in black cumin can vary due to genetic factors, environmental conditions, and the plant's origin (Abera and Hirko, 2020). High acid and peroxide values are also characteristic of black cumin oils (Dabrowski *et al.,* 2024).

Black cumin is effective for curing a variety of health issues, including neurological and mental disorder cardiovascular diseases, cancer, diabetes, inflammatory conditions, infertility, and numerous infectious diseases caused by bacteria, fungi, parasites, and viruses (Yimer *et al.,* 2019). Additionally, some evidence suggests that components of black cumin seeds, such as thymoquinone, may offer significant therapeutic potential against COVID 19 (Badary *et al*., 2021). In Ethiopia, black cumin is utilized for flavoring bread and sauces and is a key ingredient in hot pepper spice mixtures, providing farmers with a better income and serving traditional medicinal purposes (Herms, 2015; Shimelis, 2021).

Studies indicated that black cumin contributes approximately 39.88% to farming income in certain regions of Ethiopia (Teshome and Anshiso, 2019). In terms of export capacity, it ranks third overall, and in value, it stands second to ginger (Yimer, 2010). In the 2013-2014 period, black cumin accounted for 9% of total spice exports, valued at approximately USD 2.4 million (Herms, 2015). The Ethiopian Ministry of Agriculture and Natural Resources reported that 21,550 hectares were dedicated to black cumin, yielding around 17,072 tons annually.

In the South Wollo region, smallholder farmers cultivate twelve spice species, with black cumin being the predominant one, followed by coriander, mustard, basil, and rue. To optimize crop performance and enhance systematic productivity, black cumin can be intercropped with pepper and other crops in Ethiopia (Gelaye, 2025).The production and productivity of black cumin in South Wollo are estimated at about 365 quintals per hectare and 4.93 quintals per hectare, respectively (Tiru *et al.,* 2017). Despite its potential, black cumin faces low productivity and production challenges, primarily due to a lack of improved varieties. This issue is intensified by limited information on the genetic variability of local populations of black cumin in Ethiopia, leading farmers to cultivate mainly landraces or traditional varieties. The restricted large-scale production and development of enhanced varieties result from minimal information concerning inter and intra-specific variability and genetic relationships among black cumin genotypes.

According to the Ministry of Agriculture and Natural Resources (MANR, 2016), only eight varieties of black cumin have been released from national and regional research centers in Ethiopia. This limited progress may result from insufficient knowledge about the genetic diversity of cultivated black cumin genotypes.

Moreover, few studies have explored the extent of genetic variation, phenotypic traits, trait relationships, and their effects on seed yield among Ethiopian black cumin genotypes. Therefore, this research aims to address the information gaps related to the genetic characteristics of black cumin and serve as a guide for selecting improved varieties. This is important for evaluating genetic variability and the association of traits among genotypes is essentiall for developing effective selection strategies. The objective of the study was to investigate the genetic variability of black cumin genotypes and estimate their genetic parameters concerning seed yield and its components.

2. Materials and Methods

Description of the study area

The experiment was conducted at the farmer field of Hara,Tehuledere Woreda, South Wollo, Amhara National Regional State, during the 2022 rainy season (Fig. 1).

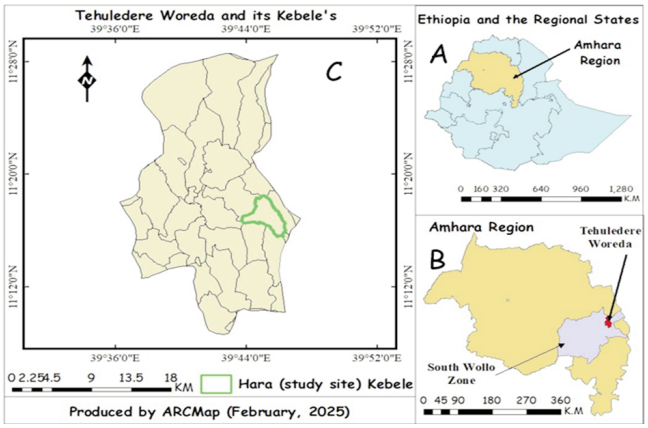

Fig. 1 - Map of the study area: where, A= Map of Ethiopia, B= Map of Amhara region, C= Map of the specific study area; ARCMap= A desktop geographic information system (GIS) application primarily used for mapping.

The site is situated in major cereal growing belts which are 448 km North East of Addis Ababa with a geographic co-ordinate of 11° 20'0» N and 39°44'0» E at an elevation of 2136 meter above sea level. The site has the minimum and maximum annual mean temperature of is 10°C and 25°C, respectively. The average annual rainfall for the site ranges from 900 to 1100 millimeter with a bimodal distribution. The soil of this area belongs to the group of well-drained vertisol soils.

Experimental materials

In total, 25 black cumin genotypes were used for this study, of which 24 were collected from various altitudes across the Amhara regional state.

The planting materials, along with supporting information like passport data were obtained from the Ethiopia Biodiversity Institute (EBI). Additionally, 'Dershaye' as a standard genotype that was supplied by Sirinka Agricultural Research Center (Table 1).

Experimental design and field management

The experiment was designed using a 5 x 5 simple

Table 1 - List of black cumin (*Nigella sativa* L.) genotypes collected from different locations of Amhara region and obtained from the database of the Ethiopian Biodiversity Institute

Genotype	Region	Zone	Latitude (North)	Longitude (East)	Altitude (m asl)
223072	Amhara	MirabGojam	11-07-02	37-11-32	2737
9069	Amhara	MirabGojam	10-38-48	37-05-09	2002
9071	Amhara	MirabGojam	10-38-21	37-05-13	1970
9068	Amhara	MirabGojam	11-45-40	37-05-4	1854
9067	Amhara	MirabGojam	11-41-08	37-01-12	1840
215319	Amhara	MisrakGojam	10-32-19	37-47-28	2754
223069	Amhara	MisrakGojam	11-00-08	37-00-11	2475
90505	Amhara	MisrakGojam	20-20-00	38-00-00	2024
90506	Amhara	MisrakGojam	20-20-00	38-00-00	2011
31114	Amhara	Semen Gonder	37-11-14	12-21-27	1834
31109	Amhara	Semen Gonder	37-22-51	12-25-31	1810
205167	Amhara	Semen Gonder	12-42-34	37-00-15	1289
31110	Amhara	Semen Gonder	37-23-55	12-23-41	1811
90507	Amhara	Semen Gonder	12-16-00	37-05-00	1760
207539	Amhara	Semen Gonder	12-20-00	37-14-00	1800
31112	Amhara	Semen Gonder	37-16-50	12-22-57	1868
19921	Amhara	Semen Gonder	12-33-20	37-06-40	1944
31111	Amhara	Semen Gonder	37-17-56	12-25-31	1821
207540	Amhara	Debub Gonder	12-10-59	39-32-20	1222
31108	Amhara	Debub Gonder	37-20-20	12-21-08	1800
212520	Amhara	Semen Shewa	10-27-00	39-15-00	2680
19590	Amhara	Semen Shewa	11-27-00	39-14-00	1993
242220	Amhara	Oromo spp zone	10-50-28	39-48-60	1480
242224	Amhara	Debub wollo	11-16-00	39-44-61	2170
Dershaye	Amhara	Semen Wollo	11-50-40	39-12-25	1800-2500

lattice layout. Each plot measured 1.5 m x 2 m (3 m²) and maintained distances of 0.5 m between plots and 1 m between blocks. Each plot consisted of eight rows, with a spacing of 25 cm between rows and 15 cm between plants. Ten holes were prepared in each lined row, and three hundred twenty seeds were dibbled in each plot, with four seeds placed in each hole. Treatments were randomly assigned within each block. Planting took place during the last week of June 2022 at a depth of 3 cm. After sowing, the seeds were immediately covered with loose soil and gently pressed down by hand. A fertilizer application rate of 60 kg ha^{-1} of nitrogen (N) and 40 kg ha^{-1} of phosphorus pentoxide (P_2O_5) was utilized, following the guidelines provided by Yimama *et al.* (2015). Additional agronomic practices were carried out in accordance with the recommendations for black cumin (Hammo, 2008). Thinning was done to regulate the number of seedlings in each row. This process was conductedby hand to achieve the desired density of 10 plants per row and a total of 80 plants per plot. All agronomic practices were consistently applied across all plots.

Data collection and analysis

Data collected included days to 50% flowering, days to 90% maturity, biomass yield (kg ha^{-1}), seed yield per hectare (kg ha^{-1}), and harvest index (%) on a plot basis. Additionally, plant-based measurements were taken for plant height (cm), number of flowers per plant, number of secondary branches per plant, number of tertiary branches per plant, number of capsules per plant, number of seeds per capsule, and 1000-seed weight (g).

Before the detailed analysis, the normality distribution of the data was cheeked using Shapiro test in R statistical software. Then, data for each quantitative trait was analyzed using one-way ANOVA with the general linear model. The analysis of variance for each characteristic was performed according to the standard statistical methods outlined by Gomez and Gomez (1984) and was conducted using R statistical software. Following the ANOVA, the least significant difference was calculated at a 5% probability level.

Estimates of genetic parameter

Variance components. Variance components, including phenotypic, genotypic, and environmental variations, were estimated using the method proposed by Burton and Vane (1953). The calculations for the genotypic, phenotypic, and environmental variance were conducted accordingly.

Genotypic variances: $\sigma^2 \ g = \frac{\mathrm{Msg}-\mathrm{Mse}}{\mathrm{r}}$ (1)

Where, Msg = mean square due to genotypes, Mse = error mean square, and r = number of replications.

Environmental variance: $\sigma^2 g = Mse$ (2)

Where, Mse = error mean square.

Phenotypic variance: $\sigma^2 p = \sigma^2 g + \sigma^2 e$ (3)

Where, $\sigma^2 p$= phenotypic variance, $\sigma^2 g$= genotypic variance and e= error mean square.

Estimates of coefficients of variations

The estimates of coefficient of variations was calculated using the following formulas.

Phenotypic CV: $\mathrm{PVC} = \frac{\sqrt{\sigma^2 \mathrm{p}}}{\mathrm{x}} \cdot 100$ (4)

Where, phenotypic variance; x= grand mean of the character

Genotypic CV: $\mathrm{GCV} = \frac{\sqrt{\sigma^2 \mathrm{g}}}{\mathrm{x}} \cdot 100$ (5)

Where, σ^2g = genotypic variance and x = grand mean of the character.

The values of Phenotypic Coefficient of Variation (PCV) and Genotypic Coefficient of Variation (GCV) were classified into low, moderate, and high categories according to the criteria established by Sivasubramaniah and Menon (1973).

Low = 0-10%, Moderate = 10-20% and High = >20%.

Heritability (in the broad sense)

Heritability in percent was computed based on the formula given by Allard (1960).

$$\mathrm{H}^2 = \frac{\sigma^2 \mathrm{g}}{\sigma^2 \mathrm{p}} \cdot 100 \quad (6)$$

In this context, H^2 represents heritability, σ^2g stands for the total genotypic variance, and σ^2p denotes the total phenotypic variance. According to Johnson *et al.* (1955), heritability percentages are classified as low (0-30%), moderate (30-60%), and high (greater than 60%). When the heritability of a trait is very high (60% or above), it is easier to select that trait because there is a strong correlation between genotype and phenotype, with the environmental influence on the phenotype being

relatively minimal. Conversely, selection may prove challenging for traits with low heritability (less than 30%) due to the masking effects of environmental factors.

Expected genetic advance under selection

The genetic advance in absolute units (GA) and as a percentage of the mean (GAM), based on selecting the top 5% of genotypes, was calculated using the methods described by Johnson *et al.* (1955).

The genetic advance was estimated as follows:

$$\mathrm{GA} = k\sqrt{\sigma^2 \mathrm{p}} \cdot \mathrm{H}^2 \quad (7)$$

where GA = Genetic advance, K= selection intensity at 5% (K= 2.063), $\sqrt{\sigma^2 p}$ = Phenotypic variance on a mean basis, H^2 =Heritability in the broad sense

Genetic advance as a percent of the mean: it was estimated as follows:

$$\mathrm{GAM} = \frac{\mathrm{GA}}{\mathrm{x}} \cdot 100 \quad (8)$$

Where, GAM= Genetic advance as percent of mean, GA=Genetic advance, and x= Population mean

Estimation of genotypic and phenotypic correlation coefficients

The genotypic and phenotypic correlation coefficients between yield and yield-attributing traits were computed as described by Singh *et al*. (2001) and using R statistical software.

I. Genotypic correlation:

$$\mathrm{Cov\ (g)\ (XY)} = \frac{\mathrm{cov(g)\ x\ y}}{\sqrt{\sigma^2\ \mathrm{(g)x} \cdot \sigma^2\ \mathrm{(g)y}}} \quad (9)$$

Where, Cov (g) (xy) = genotypic covariance between the variables x and y; σ^2 (g) x = genotypic variance of the variable x; σ^2 (g) y = genotypic variance of the variable y.

Genotypic correlation coefficients were tested for significance using the formula described by Robertson (1959)

$$t = \frac{\mathrm{rgxy}}{\mathrm{SErgxy}} \quad (10)$$

The calculated «t» value was compared with the tabulated «t» value at (n-2) degrees of freedom at a 5% level of significance.

Where, n = number of genotypes;

$$\mathrm{SEr}_{\mathrm{gxy}} = \sqrt{\frac{1-r^2 \mathrm{g}xy}{2\mathrm{Hx} \cdot \mathrm{Hy}}} \quad (11)$$

Where, H^2_x = Heritability of trait x; H^2_y = Heritability of trait y.

II. Phenotypic correlation:

$$\mathrm{Cov\ (ph)\ (xy)} = \frac{\mathrm{cov(ph)\ x\ y}}{\sqrt{\sigma^2\ \mathrm{(ph)x} \cdot \sigma^2\ \mathrm{(ph)y}}} \quad (12)$$

Where, Cov (p) (xy)=phenotypic covariance between the variables x and y. σ^2 (p) x=phenotypic variance of the variable x. σ^2 (p) y=phenotypic variance of the variable y.

The calculated phenotypic correlation value was tested for its significance using a t-test:

$$t = \frac{\mathrm{rp}}{\mathrm{SE\ (rp)}} \quad (13)$$

Where, r_p= phenotypic correlation; SE (r_p) = standard error of phenotypic correlation was obtained using the following procedure (Sharma, 1998).

The calculated absolute t-value was tested against the tabulated "t" value at n-2 degree of freedom at a 5% level of significance.

$$\mathrm{SE} = \sqrt{\frac{1-r^2 \mathrm{p}}{\mathrm{n}-2}} \quad (14)$$

Where, SE = standard error, n = the number of genotypes tested and r_p = the phenotypic correlation coefficient.

3. Results

Analysis of variance for seed yield-related traits

As shown in ANOVA Table 2, there were highly significant differences among genotypes (p<0.01) for several traits, including days to 50% flowering, days to 90% maturity, plant height, the number of primary, secondary, and tertiary branches, biological yield, seed yield, and harvest index. Additionally, significant variations were found among genotypes (p<0.05) for days to 50% emergence and the number of seeds per capsule. However, no significant variations were observed for stand count at harvest and thousand seed weight.

Genotypic and phenotypic coefficients of variation

Burton and Vane (1953) categorized PCV and GCV values greater than 20% as high, values below 10% as low, and those ranging from 10% to 20% as medium. Following this framework, high values were found for the number of tertiary branches (GCV at 45.34%, PCV

Table 2 - Mean squares for different traits evaluated in 25 black cumin genotypes evaluated at Hara, Tehuledere Woreda in 2022 GC

Traits	Treatment (Unadj) (Df= 24)	Rep (Df=1)	Block within rep (Df=8)	Intra block error (Df=16)	RCBD error	Efficiency of SL relative to RCBD	CV
DF	21.88 **	33.6	7.07	6.70	6.83	98.09	3
DM	31.50**	0.32	2.42	2.20	2.278	96.57	0.9
PH	36.78 **	26.06	5.08	4.21	4.51	93.34	3.6
NPB	2.80 **	2.51	0.86	0.49	0.61	80.32	8.9
NSB	8.51 **	0.0008	1.86	1.59	1.68	94.64	9.7
NTB	8.75 **	6.09	0.93	0.79	0.84	94.04	20.3
NF	120.44 **	0.01	4.03	8.08	6.74	119.88	5.4
NEC	126.17 **	5.9	3.20	10.29	7.93	129.76	6.3
NSC	84.84 *	1.62	21.60	28.84	26.43	109.11	6.5
TSW	0.61 NS	0.19	0.19	0.34	0.29	116.39	18.7
BY	472586**	6341	30770	23661	26030	90.89	6.1
SY	60649 **	4592	5832	3388	4203	80.60	8.9
HI	6.68 **	12.52	6.61	3.74	4.70	79.57	7.5

*, **, NS = significant at 5% probability level and highly significant at 1% probability level, non significant, respectively.
Df = Degree of freedom; Rep= Replication; RCBD = Randomized Complete Block Design; SL= Simple lattice design; CV= Coefficient of Variation.
DF= Days to 50% flowering, DM= Days to 90% maturity; PH= Plant height; NPB= Number of primary branches; NSB= Number of secondary branches; NTB= Number of tertiary branch; NF= Number of flowers; NEC= Number of effective capsules; NSC= Number of seed per capsule; TSW= Thousand seed weight; BY= Biological yield; SY= Seed yield; HI= Harvest index.

Fig. 2 - Pictures of black cumin genotypes during a field trial. A= Planting, B= Seedling stage, C= Vegetative stage, D= At Flowering, E= At maturity, F= Harvested Black cumin seeds.

at 49.92%) and seed yield (GCV at 25.67%, PCV at 27.25%). This high GCV shows that there is a substantial amount of genetic diversity present in the population.

Table 3 presents estimates of mean, range, and variance, genotypic and phenotypic coefficients of

variability, broad-sense heritability, and genetic advance as a percentage of the mean for the studied traits. Medium GCV and PCV values were noted for the primary branch (13.30%, 16.63%), secondary branch (14.26%, 17.43%), number of flowers (14.42%, 15.25%), number of effective capsules (15.12%, 16.1%), biological yield (18.70%, 19.76%), and harvest index (12.37%, 14.97%). Result suggests that these traits are less influenced by environmental factors, making phenotypic selection a viable breeding strategy for their improvement. Supporting this, Seid *et al.* (2013) found medium GCV and PCV values for the number of secondary branches (15.1% and 14.16%, respectively).

On the other hand, low GCV and PCV values were observed for days to 50% flowering (3.15%, 4.35%), days to 90% maturity (2.35%, 2.53%), plant height (7.02%, 7.94%), and number of seeds per capsule (6.53%, 9.02%) (Table 3). These low GCV and PCV values reflect limited potential for selection and improvement, indicating a low level of genetic variation among the genotypes for these traits. This indicates that these traits exhibit low variability and are relatively stable, with limited potential for improvement through selection. The small difference between GCV and PCV suggests minimal environmental influence, implying that the observed variability is largely genetic in origin. Similar findings were reported by Hika *et al.* (2015) and Tewodros *et al.* (2018), showing low GCV and PCV values for days to 50% flowering and days to 90% maturity, suggesting minimal environmental influence on the expression of these traits, which facilitates selection based on phenotypic characteristics.

Heritability in broad sense (H2)

According to Johnson *et al.* (1955), heritability is categorized as high when it is greater than or equal to 60%, medium when it falls between 30% and 60%, and low when it is below 30%. A heritability of 100% indicates that genotypic variance equals phenotypic variance, suggesting that phenotypic performance is a perfect indicator of genotypic value. Based on this classification, as shown in Table 3, high heritability estimates were found for several traits, including plant height (78.17%), number of primary branches (63.98%), number of secondary branches (66.78%), harvest index (68.35%), biological yield (89.56%), seed yield (87.04%), days to 90% maturity (86.51%), number of tertiary branches (82.5%), number of flowers (89.41%), and number of effective capsules (88.18%). In this study, moderate heritability estimates were observed for days to 50% flowering (52.44%), number of seeds per capsule (52.49%), and thousand seed weight (35.66%) (Table 3). The estimate of GA values ranged from 0.49 for thousand

Table 3 - Estimates of mean, range and variance, genotypic, phenotypic coefficients of variability, broad sense heritability and genetic advance as percent of the mean for the studied characters

Traits	Units	Mean	Range		σ^2 g	σ^2 p	GCV	PCV	H2	GA	GAM
			Min	Max			%	%	%		
DF	Days	86.94	76.93	92.93	7.53	14.35	3.15	4.35	52.44	4.09	4.70
DM	Days	162.24	151.87	166.3		16.89	2.35	2.53	86.51	7.32	4.51
PH	CM	57.21	49.60	62.87	7.02	20.64	7.02	7.94	78.17	7.31	12.78
NPB	Number	7.86	5.16	9.96	1.09	1.71	13.30	16.63	63.98	1.72	21.92
NSB	Number	12.95	9.42	17.37	3.41	5.09	14.26	17.43	66.98	3.11	24.05
NTB	Number	4.38	1.8	8	3.95	4.79	45.34	49.92	82.5	3.72	84.85
NF	Number	52.27	39.50	73.4	56.85	63.59	14.42	15.25	89.41	14.68	28.09
NC	Number	50.84	35.5	71.4	59.12	67.04	15.12	16.10	88.18	14.87	29.25
NSC	Number	82.69	67	104	29.20	55.63	6.53	9.02	52.49	8.06	9.75
TSW	Kg	3.11	2	4	0.16	0.45	12.89	21.59	35.66	0.49	15.86
BY	Kg	2526.3	1835.5	3520	223277.6	249308	18.70	19.76	89.56	921.17	36.46
SY	Kg	654.38	428.89	1026.6	28222.8	32425.8	25.67	27.5	87.04	322.86	49.33
HI	%	25.74	19.46	33.91	10.15	14.85	12.37	14.97	68.35	5.42	21.08

σ^2 g= Genotypic variation; σ^2p= Phenotypic variation; GCV= Genotypic coefficient of variation; PCV= Phenotypic coefficient of variation; H2= Broad sense heritability; GA= Genetic advance; GAM= Genetic advance as percent of mean;

DF= Days of 50% flowering; DM= Days to 90% maturity; PH= Plant height; NPB= Number of the primary branch; NSB= Number of the secondary branch; NTB= Number of the tertiary branch; NF= Number of flower; NC= Number of capsule; NSC= Number of seed per capsule; TSW= Thousand seed weight; BY= Biological yield; SY= Seed yield; HI= Harvest index.

seed weight to 921.17 for biological yield.

Genetic advance (GA)

Genetic advances show the degree of gain that can be made in a particular character (Ogunniyan and Olakojo, 2014). As indicated in Table 3, the highest value of genetic advance was shown on biological yield (921.17), followed by seed yield (322.86), but the lowest value was observed on thousand seed weight (0.49). The traits exhibited a moderate estimate of genetic advance, which was observed on a number of flowers (14.68%) and effective capsules (14.87%). Low genetic advance was obtained from days of 90% maturity, number of seed per capsule, plant height, harvest index, days of 50% flowering, number of secondary branches per plant, number of tertiary branches per plant, number of primary branches per plant and thousand seed weight (Table 3).

Genetic advance as percent of mean (GAM)

The expected genetic advance from selecting the best genotypes, expressed as a percentage of the mean, ranged from 4.51% for days to 90% maturity to 84.85% for tertiary branches (Table 3). According to Johnson *et al.* (1995), a genetic advance as a percentage of the mean (GAM) of less than 10% is considered low, between 10% and 20% is moderate, and above 20% is classified as high. Based on these categories, traits such as seed yield (49.3%), biological yield (36.4%), number of effective capsules (28.09%), number of flowers (28.09%), number of primary branches (21.92%), number of secondary branches (24.05%), and harvest index (21.08%) exhibited high GAM.

Intermediate genetic gain values were observed for thousand seed weight (15.86) and plant height (12.78); In contrast, low values were reported for the number of seeds per capsule, days to maturity, and days to flowering (Table 3). Generally, traits such as seed yield, biological yield, number of primary, secondary, and tertiary branches per plant, number of flowers, number of capsules, and harvest index demonstrated high heritability along with a high genetic advance as a percentage of the mean.

Additionally, medium heritability coupled with a high genetic advance as a percentage of the mean was observed for the harvest index (5.42 and 21.08), respectively, suggesting that selecting for these characteristics would enhance the performance of the genotypes. Conversely, traits such as days to 50% flowering, days to 90% maturity, and number of seeds per capsule exhibited medium to high heritability but low genetic advance and genetic advance as a percentage of the mean.

4. Discussion and Conclusions

Analysis of variance for seed yield-related traits

The analysis of variance revealed a highly significant difference among genotypes. This signifies that the differences observed in the studied trait(s) are statistically significant and not just a result of random variation or environmental interference. Such findings imply that specific genotypes exhibit markedly better or worse performance compared to others, thereby offering a robust foundation for selection in plant breeding initiatives. The existence of significant differences further emphasizes the opportunity for genetic enhancement by identifying and applying superior genotypes in forthcoming breeding activities. This finding is in line with the results of Zigyalew *et al.* (2020), who reported significant differences among black cumin genotypes for days to maturity, plant height, secondary branches per plant, and seed yield. Similarly, Seid *et al.* (2013) noted significant differences among Ethiopian caraway genotypes for days to 90% maturity, plant height, and secondary branches per plant, and seed yield. Another finding also revealed significant variation among accessions for various traits (Tilahun *et al.,* 2024).

Furthermore, Tewodros *et al.* (2018) also found significant differences among black cumin genotypes for phonological parameters such as days to 50% flowering and days to 90% maturity, growth traits like plant height and primary and secondary branches per plant, as well as yield components including the number of capsules per plant, seeds per capsule, and seed yield. Consistent with these findings, Iqbal *et al.* (2019) reported significant differences among genotypes for all evaluated traits. Adam and Getinet (2006), who assessed 28 black cumin genotypes in Adet and Woreta, noted highly significant variation among genotypes for days to flowering, days to maturity, plant height, and the number of primary and secondary branches per plant, making a similar observation. Breeders can take advantage of this variability for selection purposes and may apply it in hybridization and gene transfer to other genotypes.
The substantial variability observed could be

attributed to genetic diversity or variations in genetic makeup, as well as environmental factors influencing the phenotypic expressions (Sable *et al.,* 2020). In addition, Fikre (2023) reported that the differences in seed yield among genotypes were highly significant. This shows there is a potential genetic difference among the studied genotypes, which is very important to variety development of black cumin.

Genotypic and phenotypic coefficients of variation

To determine the variability present in the available materials, evaluating the genotypic and phenotypic coefficient of variation play a great role by showing the degree of variation among genotypes rather than calculating genetic variance alone (Kumar *et al.,* 2019). In this study, the individuals in the population differ significantly in their genetic makeup, leading to differences in how they express the trait (like seed yield). This may be due to crossbreeding, hybridisation, or natural genetic variation, which tends to exhibit higher genetic variability, leading to higher GCV. This occurs when diverse genetic backgrounds are included in the breeding population, increasing the potential for genetic differences (Begna and Teressa, 2024). High PCV indicates considerable variation in the observed phenotypes within the population. This data indicates that the trait is strongly influenced by environmental factors such as temperature, soil quality, water availability, and pest presence, which can strongly affect its expression. When environmental conditions vary significantly across populations or over time, they increase phenotypic variation (Dawa *et al.,* 2018).

Several studies have also reported high GCV and PCV for seed yield (Meena *et al.,* 2014; Hika *et al.,* 2015; Bitew, 2016; Preeti *et al.,* 2019). High GCV and PCV indicate sufficient variability within the gene pool, allowing for significant potential for genetic enhancement through selection in future breeding initiatives. This suggests that effective selection can be made on these traits, with their phenotypic expression serving as a reliable indicator of genetic potential (Hadru *et al.,* 2014). The high GCV values for these traits imply that they are relatively unaffected by environmental conditions. This means that the observed variation in that trait is largely due to genetic differences rather than environmental factors, suggesting greater potential for genetic improvement through selection. Conversely, while the thousand seed weight exhibited a high PCV (21.59%), its GCV was medium (12.89%), as shown in Table 3. This suggests that although there is substantial observable diversity in this characteristic, a considerable amount of it is affected by environmental influences. The significant difference between PCV and GCV demonstrates that environmental factors play a crucial role, while the genetic impact on the variability of the trait is moderate. Consequently, the selection for thousand seed weight might prove to be less effective unless the environmental factors are regulated.

Heritability in broad sense (H^2)

In this research high heritability estimates for characteristics such as plant height, the count of primary, secondary, and tertiary branches, harvest index, biological yield, seed yield, days to 90% maturity, number of flowers, and number of effective capsules suggest that the variation observed in these traits is predominantly attributable to genetic factors rather than environmental influences. This implies that these traits are consistently passed down from one generation to the next and can be effectively enhanced through selection in breeding programs. Consequently, breeders can anticipate considerable genetic improvements by concentrating on these traits, as their high heritability indicates robust genetic control and relatively minimal environmental variability. Previous research has reported high heritability in black cumin. Studies by Bairwa *et al.* (2015) and Iqbal *et al.* (2019) noted high heritability for seed yield and days to 90% maturity. Other researchers also identified high heritability for biological yield (87.92%), seed yield (99.81%), days to 90% maturity (95.32%), and the number of effective capsules (92.49%) (Mengesha and Getinet, 2011; Hika *et al.,* 2015). Similarly, Faravani *et al.* (2006) found maximum heritability for biological yield, harvest index, and stem branches in black cumin landraces, while high heritability was also reported for secondary branches of caraway (Seid *et al.,* 2013). Comparable findings were noted regarding the moderate heritability of the number of primary and secondary branches (Preeti *et al.,* 2019). High heritability trait indicate the expected response to selection in a segregating population. This suggests a reduced impact of environmental factors on the expression of these heritable traits, along with the predominance of additive gene action in their

inheritance (Kassa *et al.,* 2019). Therefore, these traits are suitable for straightforward selection processes. Gebremedin *et al.* (2024) also reported high broad sense heritability values, along with high to moderate genetic advance as a percentage of mean values, for the number of capsules per plant and plant height. This indicates possibilities for improving these traits through selection.

Genetic advance (GA)

High GA was recorded on biological yield and seed yield whereas low GA was showed on the rest of the studied trait. A high genetic advance (GA) for biological yield and seed yield indicates that these characteristics have significant potential for enhancement through selection, as they are likely governed by additive gene effects and show a favorable response to breeding initiatives. Conversely, the low genetic advance noted for the other examined traits implies that these traits might be more influenced by non-additive gene effects or environmental influences, making genetic enhancement through selection less efficient or slower for those traits. Consequently, prioritizing biological yield and seed yield in breeding programs is expected to yield quicker and more significant improvements. Gebremedin *et al.* (2024), reported similar results on the thousand seed weight and number of seeds per plant of black cumin genotypes. Another research in line with this finding reported high genetic advance for traits such as biological yield and seed yield of black caraway, which implies that these traits are primarily governed by additive gene action and can be effectively improved through selection. This suggests strong breeding potential for enhancing yield-related characteristics in black caraway (Faravani *et al.,* 2015).

Genetic advance as percent of mean (GAM)

The results from the studied genotypes indicated that the additive genetic effect had a more significant influence on most of the traits tested, while the non-additive effect played only a minor role. Given the higher estimated GAM for the genotype, the findings suggest that selecting for these traits can be an effective breeding strategy for improvement (Gebregergs and Mekbib, 2020). Intermediate genetic gain values were observed for thousand seed weight and plant height, while lower values were recorded for the number of seeds per capsule, days to maturity, and days to flowering (Table 3). This finding is consistent with the study by Zigyalew *et al.* (2020), which reported high genetic advance as a percentage of the mean for biological yield and seed yield in black cumin genotypes. Similarly, Meena *et al.* (2014) also found a high genetic advance as a percentage of the mean for plant height, number of primary branches per plant, number of secondary branches per plant, and seed yield in caraway genotypes.

In general, high heritability coupled with a high genetic advance as a percentage of the mean was observed for traits such as seed yield, biological yield, primary branches per plant, number of secondary branches per plant, number of tertiary branches per plant, number of flowers, number of capsules, and harvest index. Meena *et al.* (2014) reported similar findings for traits like primary branches per plant, number of secondary branches per plant, and seed yield. Additionally, medium heritability with high genetic advance as a percentage of the mean was noted for harvest index, suggesting that selection based on these traits could lead to improve genotype performance. In contrast, traits such as days to 50% flowering, days to 90% maturity, and number of seeds per capsule showed medium to high heritability but low genetic advance and genetic advance as a percentage of the mean. This indicates that phenotypic selection for these traits may not effectively predict genotype performance due to environmental influence on their phenotypic expression. Therefore, genetic improvement through selection is challenging due to the environmental masking effect on genotypic traits.

In conclusion, the analysis of variance revealed significant differences among the tested genotypes for all the traits evaluated, highlighting the variability present in these genotypes. The phenotypic coefficient of variation was slightly higher for all traits, suggesting a limited impact of environmental factors on the phenotypic characteristics of the crop. However, this also indicates that environmental factors do influence the phenotypic expression of these traits to some extent. Notably, high phenotypic coefficients of variation was observed for biomass yield per plot, grain yield per plot, and the number of unfilled grains per panicle. The heritability estimates were moderately high for days to 85% maturity and thousand-grain weight, while they were lower for the remaining quantitative traits in this study. Low heritability suggests that environmental factors play a significant role in the expression of these traits.

Relatively high genetic advance was noted for biomass yield, unfilled grains per panicle, grain yield per plot, and fertile tillers per plant. Additionally, the estimates of genetic advance (as a percentage of the mean) for thousand-grain weight, days to 50% heading, plant height, and panicle length were also significantly high. In this study, thousand-grain weight exhibited moderately high heritability and a high genetic advance as a percentage of the mean, indicating a favorable opportunity for improving this trait using the evaluated genotypes.

Phenotypic correlation among other traits

A positive and highly significant correlation was found between days to 50% flowering and days to 90% maturity at the phenotypic level. Tewodros *et al*. (2018) also observed a similar association, suggesting early-flowering genotypes mature earlier. Plant height showed significant positive correlations with primary, secondary, and tertiary branches, flowers, capsules, and biological yield, and a positive correlation with seeds per capsule (Table 4). This trend leads to morphological differences as taller plants produce more branches, flowers, capsules, and achieve higher biological yield due to pleiotropic gene action and the stem's role in producing nodes and internodes, which increase branches, flowers, seeds per capsule, and biological yield. Meena *et al.* (2014) reported similar findings in coriander genotypes, where plant height showed positive correlations with primary and secondary branches, flowering, capsules, and seeds per capsule, and maturity at the genotypic level.

Days to 50% flowering showed negative phenotypic correlations with most traits, except days to 90% maturity. Significant negative correlations were found with primary branches, secondary branches, seeds per capsule, and harvest index. This suggests that genotypes with more branches, seeds per capsule, and higher harvest index flower earlier. Negative, non-significant correlations were observed with plant height, flowers, effective capsules, tertiary branches, thousand seed weight, and biological yield. Days to 90% maturity showed negative significant correlations with primary branches, tertiary branches, flowers, and capsules. It showed negative, non-significant correlations with plant height, secondary branches, seeds per capsule, thousand-seed weight, biological yield, and harvest index. Additionally, it showed a positive, non-significant correlation with harvest index and a negative, non-significant correlation with thousand seed weight.

The number of primary branches showed a highly significant positive correlation with secondary and

Table 4 - Genotypic (below diagonal) and phenotypic (above diagonal) correlation coefficients of quantitative traits among 25 genotypes of *N. sativa*

	DE	DF	DM	PH	NPB	NSB	NTB	NF	NC	NSC	TSW	BY	SY	HI
DF	0.004		0.86 **	-0.23	-0.42 *	-0.42 *	-0.35	-0.24	-0.29	-0.49 *	-0.08	-0.19	-0.43 *	-0.62 **
DM	0.009	0.60 **		-0.38	-0.46 *	-0.30	-0.48 *	-0.39 *	-0.44 *	-0.34	-0.07	-0.34	-0.32	-0.14
PH	0.25	-0.25	-0.35 *		0.9 **	0.87 **	0.83 **	0.84 **	0.85 **	0.44 *	-0.25	0.75 **	0.60 **	0.14
NPB	0.30 *	-0.31 *	-0.319 *	0.82 **		0.97 **	0.60 **	0.75 **	0.77 **	0.69 **	0.10	0.61 **	0.45 *	0.05
NSB	0.29 *	-0.27	-0.25	0.80 **	0.83 **		0.57 **	0.72 **	0.75 **	0.57 **	0.06	0.50**	0.36	0.06
NTB	0.05	-0.19	-0.39 **	0.64 **	0.41 **	0.33 *		0.89 **	0.85 **	0.47 *	-0.40 *	0.90 **	0.91 **	0.51 **
NF	0.19	-0.18	-0.33 *	0.77 **	0.66 **	0.66 **	0.77 **		0.99 **	0.56 **	-0.29	0.97 **	0.87 **	0.35
NC	0.18	-0.24	-0.35 *	0.76 **	0.67 **	0.66 **	0.74 **	0.98 **		0.51 **	-0.24	0.96 **	0.85**	0.31
NSC	0.19	-0.25	-0.21	0.34 *	0.27	0.37 **	0.23	0.41 **	0.41 **		0.28	0.48 *	0.60 **	0.53 **
TSW	-0.10	-0.06	-0.08	-0.095	0.004	-0.005	-0.17	-0.18	-0.15	-0.03		-0.29	-0.29	-0.19
BY	0.13	-0.17	-0.27 *	0.64 **	0.48 **	0.49 **	0.77 **	0.88**	0.87 **	0.34 *	-0.14		0.88 **	0.72 **
SY	0.05	-0.25	-0.29 *	0.48 **	0.48 **	0.31 *	0.31 *	0.75 **	0.73 **	0.48 **	-0.11	0.84 **		0.72 **
HI	-0.07	-0.27	-0.15	0.07	0.31 *	0.31 *	0.71 **	0.23	0.21	0.44 **	-0.01	0.21	0.69 **	

*, **, significant at P<0.05 and P<0.01, respectively. DE= Days to 50% emergence; DF days of 50% flowering; DM=days to 90% maturity; PH=Plant height; NPB= primary branches; NSB= number of secondary branches; NTB= number of tertiary branches; NF= number of flower; NC= number of capsule; NSC= number of seed per capsule; TSW= 1000 seed weight; BY= biological yield; SY= seed yield; HI = harvest index.

tertiary branches, flowers, capsules, seeds per capsule, and biological yield (Table 2), indicating that these factors improve simultaneously through selection. It had a positive, non-significant correlation with thousand seed weight and harvest index. Preeti *et al.* (2019) and Zigyalew *et al.* (2020) reported similar positive associations with secondary branches, capsules, and biological yield.

Positive and highly significant associations were observed between the number of secondary branches per plant and the number of tertiary branches per plant, the number of flowers, the number of effective capsules, the number of seeds per capsule, and biological yield at the phenotypic level. This implies that improving secondary branches in black cumin genotypes will improve associated characters to some extent. This study was in agreement with Meena *et al.* (2014) and Preti *et al.* (2019), who reported that the number of secondary branches per plant had a positive and significant correlation with the number of effective capsules and biological yield at the phenotypic level. Tertiary branches showed highly significant positive correlations with flowers, capsules, biological yield, and harvest index. It showed a significant positive correlation with seeds per capsule and a significant negative correlation with thousand-seed weight.

Tertiary branches showed significant positive correlations with flowers, capsules, biological yield, and harvest index, as well as a negative correlation with thousand-seed weight. The number of seeds per capsule showed positive, significant correlations with biological yield and harvest index. Thousand-seed weight had a negative, non-significant correlation with both biological yield and harvest index, whereas biological yield was positively and significantly correlated with harvest index.

Genotypic correlation of seed yield with other yield component traits

The correlation of genotypes (Table 4) showed that seed yield per hectare had a highly significant ($p < 0.01$) and positive correlation with the number of flowers, number of effective capsules per plant, number of seeds per capsule, plant height, number of primary branches, harvest index, and biological yield. In addition, the significant correlations revealed that choices for such traits could be made to improve the development of high-yielding generic lines further. Therefore, by improving these traits, there is a possibility of enhancing the seed yield of black cumin, or selection of traits indirectly may be advisable. Haq *et al.* (2015) also reported a positive and significant genotypic correlation of seed yield of black cumin genotypes with thousand seed weight, number of capsules and number of seeds per capsule. Seid *et al.* (2013) in their study observed that seed yield was positively and significantly correlated with the number of primary branches per plant (0.48), the number of secondary branches per plant (0.5) and plant height (0.79).

Seed yield showed a negative, non-significant correlation with days to 90% maturity and seed weight, and a significant negative correlation with days to 50% flowering. As branches, plant height, flowers, capsules, seeds per capsule, biological yield, and harvest index increased, seed yield also increased, while days to maturity decreased. The delay in maturity is because flowers do not bloom and mature simultaneously. Early-flowering plants had more time for seed-bearing capsules to develop; however, longer germination times shortened the rainfall period for seed development, thereby reducing seed yield. This study is in agreement with the results of Sabaghnia (2025), who studied 27 different black cumin genotypes. The results showed that yield had a meaningfully positive association with most characters, except for leaf width, seed width, and thousand-seed weight. In addition, the analysis result coincides with Seid *et al.* (2013), who evaluated thirty-six local genotypes of Ethiopian caraway, and reported that seed yield was positive and highly significant with the number of primary branches per plant, number of secondary branches per plant, number of capsule per plant, number of seeds per capsule and plant height.

Haq *et al.* (2015) reported strong positive correlations between black cumin seed yield and various traits, including plant height, primary and secondary branches, seeds per capsule, capsules per plant, 1000-seed weight, and harvest index. Fufa (2016) found similar results in black cumin landraces, showing positive correlations between seed yield and plant height, capsules per plant, primary branches, and seeds per capsule, while seed yield was negatively correlated with days to flower and days to 90% maturity.

Genotypic correlation among other traits

Positive and significant correlations were

observed between the days to 50% flowering and the days to 90% maturity at the genotypic level. This indicates that days to bloom are shorter, allowing for earlier maturation. Genotypes that flower earlier can mature on time without facing environmental challenges such as moisture stress, so these traits can be selected simultaneously. It had a negative and significant correlation with the number of primary branches per plant. Days to 50% flowering also had a negative and non-significant association with plant height, number of secondary branches per plant, number of flowers, number of capsules, number of tertiary branches per plant, number of seeds per capsule, thousand seed weight, biological yield, and harvest index at the genotypic level. The negative correlation between the number of days to 50% flower and the number of capsules per plant may be due to a higher number of flowers aborting in earlier plants than in late-flowering plants, resulting in a greater number of capsules bearing seeds. Likewise, Tewodros *et al.* (2018) reported a positive and significant association between days of 50% flowering with days of 90% maturity of black cumin genotypes. Fufa (2016) also reported a positive correlation between days to flowering and days to 90% maturity.

Days to 90% maturity showed negative, significant correlations with plant height, primary and tertiary branches, flowers, effective capsules, and biological yield, indicating maturity variation among branches. Other traits, such as secondary branches, seeds per capsule, thousand-seed weight, and harvest index, had negative correlations at the genotypic level. Fufa (2016) also reported a negative correlation between primary branches and seeds per capsule with days to 90% maturity.

Plant height showed a positive and significant correlation with the number of primary, secondary and tertiary branches per plant, as well as the number of flowers, effective capsules, seeds per capsule, biological yield and seed yield at genotypic levels. This is because long height genotypes have a higher probability of producing more primary branches, which can bear capsules (flowers) and secondary and tertiary branches per plant, leading to an increment in biological yield and seed yield. So selecting this trait is important to improve the yield of black cumin genotypes. Positive and non-significant correlations were observed between plant height and harvest index. In contrast, negative and non-significant correlations were detected with seed weight.

In agreement with the present study, Zigyalew *et al.* (2020) noted that plant height was highly and significantly associated with the number of capsules per plant and seed yield ha^{-1}, but was negatively correlated with thousand seed weight. These findings are in agreement with (2014), who evaluated the character association of coriander genotypes and indicated that plant height showed positive and significant correlations with both the number of primary and secondary branches per plant.

Number of primary branches per plant had a positive and highly significant association with number of secondary branches per plant, number of tertiary branches per plant, number of flowers, number of capsules per plant and biological yield. This positive association of the number of primary branches per plant indicates that these traits improved simultaneously through selection. According to the results of studies by Preeti *et al.* (2019), there is a positive and significant association between the number of primary branches, the number of secondary branches, the number of capsules per plant, and the biological yield of black cumin genotypes. It had positive and non-significant associations with harvest index, thousand-seed weight, and number of seeds per capsule. Zigyalew *et al.* (2020) also reported that the number of primary branches per plant had a highly significant correlation with both the number of secondary branches per plant and the number of capsules per plant at the genotypic level. Fufa (2016) also reported that the number of primary branches per plant showed a highly significant and positive correlation with both the number of capsules per plant and the number of seeds per capsule, respectively. In addition, Yewubdinber (2020) reported that the number of primary branches per plant exhibited a positive and significant correlation with the number of capsule and plant height.

The number of secondary branches per plant showed a highly significant positive correlation with flowers, capsules, seeds per capsule, and biological yield. It was also positively correlated with tertiary branches and harvest index at the genotypic level. Thousand seed weight had a negative, non-significant correlation with secondary branches. This suggests that an increase in branches leads to a higher number of flowers, capsules, and a greater biological yield.

In addition, genotypes with more secondary

branches tend to have more tertiary branches per plant, resulting in variation in immaturity among branches due to differences in maturity days between number of secondary and tertiary branches per plant. In line with this result, Zigyalew *et al*. (2020) pointed out that the number of secondary branches per plant had a highly significant correlation with the number of capsules per plant. These findings are consistent with those of Meena *et al.* (2014), who reported that the number of secondary branches was positively associated with the number of capsules per plant and the number of seeds per capsule. It showed a significant correlation with biological yield, a positive correlation with harvesting index, and a negative correlation with thousand-seed weight.

The number of tertiary branches per plant showed a highly significant positive correlation with flowers, effective capsules, seeds per capsule, biological yield, and harvest index at the genotypic level, indicating that more tertiary branches lead to higher seed yield and biological yield. Indirect selection for this trait is advisable. Thousand seed weight had a negative, non-significant correlation. Zigyalew *et al.* (2020) reported similar findings. The number of flowers was positively correlated with effective capsules, seeds per capsule, and biological yield, while harvest index had a positive, non-significant association and a negative, significant correlation with thousand seed weight.

The number of capsules had a positive and significant association with the number of seeds per capsule and biological yield at the genotypic level. Similar results were reported by Bardideh *et al*. (2013). This suggests that selecting for a greater number of capsules could enhance the biological yield of the plant, which may result in increased overall productivity. The harvest index had a positive and weak association with the number of capsules. As the quantity of capsules rises, there is a corresponding tendency for the harvest index also to increase. Nevertheless, characterizing this relationship as a weak association suggests that, although plants with a greater number of capsules generally exhibit a higher harvest index, the correlation is not particularly robust. This is in line with Zigyalew *et al.* (2020), who noted the harvest index exhibited a positive yet weak correlation with the number of capsules. However, seed weight showed a negative and non-significant correlation. This implies that larger seeds do not automatically result in increased overall yield or greater seed production efficiency.

Positive and highly significant correlations were detected for number of seeds per capsule with harvest index. It had a positive and significant association with biological yield at the genotypic level. Plants with more seeds per capsule are more efficient in converting biomass into seeds, which is beneficial for improving overall seed production. Selecting seeds per capsule could result in a higher harvest index, making the plant more efficient in seed yield production. Zigyalew *et al.* (2020) also reported similar results. Thousand seed weight showed negative and non-significant associations with the number of seeds per capsule, biological yield, and harvest index at the genotypic level. Our results suggest that heavier seeds may not necessarily lead to an increase in biomass or an improved harvest index, indicating that selecting heavier seeds does not ensure enhanced overall plant improvement in these traits. Biological yield showed a positive and non-significantly correlated relationship with harvest index. This non-noticeable association is due to inversely proportionality of biological yield and harvest index. In general, a positive and significant association between a pair of traits at both the genotypic and phenotypic levels justifies the possibility of correlated response to selection. However, negative correlation prohibits simultaneous improvement of those traits.

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Morphometric analysis of *Fusarium* spp. isolates and relationship with pathogenic potential in banana Grande Naine (*Musa* sp. Cavendish) in Côte d'Ivoire

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Key words: Correlation fungus, *Fusarium*, pathogenicity, postharvest disease.

Abstract*: Fusarium* is one of the best-known plant pathogenic fungi, particularly in bananas. This study was conducted to determine the variability of *Fusarium* isolates and their potential impact on banana quality in Côte d'Ivoire. Apparently healthy bananas were collected from the seven production localities and used to isolate the associated fungi. Morphological characterization of the fungi included macroscopic (appearance, coloration and mycelial growth) and microscopic (presence or absence of septa, shape, size and conidial concentration) cultural characteristics. The pathogenicity of the isolates was assessed by the absence or presence of rotting symptoms on the bananas. The results showed that the coloration of the *Fusarium* isolates varied from white to yellow. Some isolates were cottony and thick, while others were flaky and carpeted. The mean macroconidia dimensions were 19.11-29.52 × 2.69-4.64 µm. The average number of septa in macroconidia ranged from 3-5 septa. Macroconidia were fusiform with pointed ends; microconidia were oval with one or two pointed ends. Among the morphometric characteristics of *Fusarium* isolates, the number and size of macroconidia was the most important discriminating traits for differentiating isolates. In terms of pathogenicity, all isolates caused a symptom except the one isolated in the Dabou area, which did not cause any symptoms. The incubation period varied from 10 to 16 days depending on the isolate.

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1. Introduction

Banana (*Musa accuminata* L.) is one of the most widely traded fruits in the world and plays a significant role in the economies and diets of many tropical and inter-tropical countries. It ranks 4th in the world after rice, wheat and maize in terms of global yield (Lassoudière, 2007). As well as playing a significant role in food security, bananas are a cash crop and a

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source of employment and income for local populations (Arias *et al*., 2003). In Côte d'Ivoire, the banana sector is one of the mainstays of the agricultural economy. With a cultivated area of 11,918 ha (FAO, 2022) and production of 531,382.44 tons in 2022 (FAO, 2022), Côte d'Ivoire ranks 2nd behind Cameroon in Africa and 13th in the world. Banana dessert industry in Côte d'Ivoire is based on a limited number of cultivars belonging to the Cavendish sub-group, and the varieties marketed in Côte d'Ivoire are 'Grande naine', 'Poyo' and 'Williams' (Kouassi, 2001; Kouame and Kanpigni, 2022). It accounts for 8% of agricultural GDP and provides 15,000 direct jobs and 30,000 indirect jobs (Chauvin *et al.*, 2025).

However, annual banana production and quality are severely reduced by pest and disease attacks. Losses caused by pests and diseases can reach 100%, affecting all organs of banana plant, from roots to flowers (Ploetz *et al*., 2003). In addition to field diseases, post-harvest fungal diseases are another major threat, especially for dessert bananas intended for export. These are crown rot and anthracnose, which cause significant damage and economic losses due to the deterioration of bananas during storage and transport (Nath *et al*., 2014; Kra *et al*., 2018).

Among the fungi associated with these post-harvest diseases, the genus *Fusarium* is among the most common and most damaging to fruit (Nelson *et al*., 1981). The *Fusarium* genus is a cosmopolitan fungus that can be transported by soil, air or plant debris. On bananas, this fungus is responsible for rotting (Ploetz, 2006). At least six species of *Fusarium* have been described as responsible for or associated with banana rots throughout the world. These are *Fusarium camptoceras*, *Fusarium concentricum*, *Fusarium proliferatum*, *Fusarium semitectum* and *Fusarium subglutinans* (Ploetz *et al*., 2003; Leslie and Summerell, 2006). This diversity of *Fusarium* species in bananas makes it difficult to identify the precise agent responsible for rotting (Ploetz, 2006). As a result, the pesticides used to control the disease are ineffective and targeted control strategies have failed. With a view to ensuring agricultural sustainability and boosting the competitiveness of banana exports, this study was conducted with the aim of understanding the variability that exists among species belonging to the *Fusarium* genus. It focuses on their involvement in the development of post-harvest diseases in bananas in Côte d'Ivoire in order to contribute to improving the quality of bananas for export.

2. Materials and Methods

Collection site

Banana dessert samples were collected from seven production areas in Côte d'Ivoire (Fig. 1): Abengourou (6°43' N,-3°29' W), Aboisso (5°55'N, -4°12'W), Agboville (5°55'N, -4°12'W), Azaguié (5°55'N, -4°12'W), Dabou (5°19' N, -4°22'W), Grand-Bassam (5°12'N, -3°44'W); and Tiassalé (5°53'N, -4°49'W). The sample collection was done every 6 months during one year

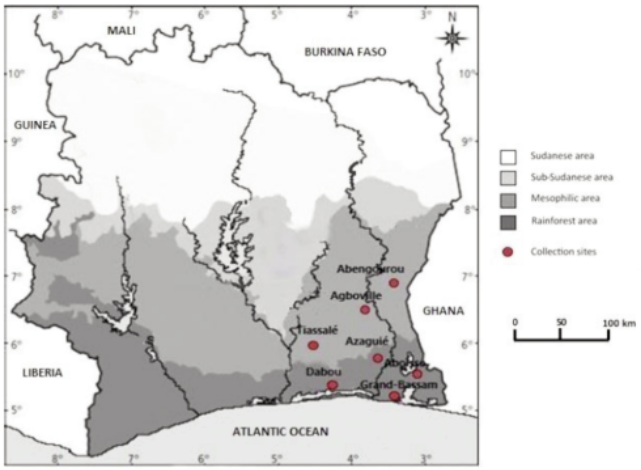


Fig. 1 - Map of banana sample collection sites in Côte d'Ivoire.

Plant material

The material consisted of ripe and apparently healthy bananas of the Grande Naine variety from the Cavendish subgroup. In each production stations, 65 banana fingers was collected each 6 months for isolation. A total of 2,080 banana fingers 18 weeks old were collected from all seven banana-producing localities in Côte d'Ivoire. For the pathogenicity test, 81 mature banana fingers were collected in the Dabou area. A total of 2161 banana fingers were used for the study.

Sampling of *banana*

Samples of 18-week-old bananas were collected at random from 16 production stations in seven production areas in Côte d'Ivoire. Three boxes (100 bananas/box) were taken from pallets of bananas ready for export at each production station. The bananas were incubated for 21 days at room temperature (27±1°C) until symptoms developed at the Plant Health Unit laboratory at Nangui Abrogoua University.

Isolation of fungal strains

Bananas showing symptoms of post-harvest diseases after 21 days of storage were used for the isolation of fungi. The surface of the bananas was disinfected with 1% diluted sodium hypochlorite for 5 minutes. Explant pieces measuring approximately 5 mm, taken from the edge of each symptom, were seeded on agar in Petri dishes (Meddah *et al*., 2010). The Petri dishes were incubated at laboratory temperature (27 ± 1°C) until fungal colonies developed. The colonies developed from each explant were transferred to Potato Dextrose Agar (PDA) medium and purified by single spore culture according to the method adapted from Choi *et al*. (1999).

Morphological characterization of fungal strains

Evaluation of mycelial growth. Colony diameter of isolated fungi was measured daily along two perpendicular axes drawn on the back of each Petri dish, and the mean of the two values was recorded. For each isolate, three plates were used, and the experiment was repeated three times. The mycelial diameter was calculated daily using the modified Dohou formula (Dohou *et al.*, 2004):

$$\mathrm{di} = \frac{\mathrm{d1}+\mathrm{d2}}{2}$$

where di is colony diameter of strain I, d1 is colony diameter of strain along axis 1 and d2 = colony diameter of strain along axis 2.

The mean growth diameter was then calculated for the three Petri dishes using the following formula:

$$D = \frac{1}{\mathrm{n}}\sum(\mathrm{di})$$

where *D* is mean diameter of the colony of strain I, di = diameter of the colony of strain I and n = 3

Macroscopic and microscopic description of fungal strains. Cultural characteristics, namely side and reverse coloration, mycelium aspect and isolate growth pattern, were observed on PDA medium. These parameters were evaluated on seven-day-old isolates.

Microscopic observation of isolates was performed after 15 days of incubation on PDA medium. Three plates were used for each isolate, and preparations were made and observed.

For each isolate, three boxes were used and mounts were prepared and observed between a slide and cover slip under an optical microscope fitted with a micrometer at 400× magnification (Optika, Italy). The conidia shape (macroconidia and microconidia) was described, and the dimensions and number of septa of the macroconidia were evaluated after observing 90 conidia per isolate.

Evaluation of fungal strain sporulation. The number of spores produced was assessed by measuring the spore concentration of 15-day-old *Fusarium* isolates. Three 6 mm diameter mycelium pellets were taken from the colonies and placed in 10 ml of distilled water. The solution was gently shaken for 30 seconds to separate the conidia from the conidiospores. The different conidial suspensions were obtained after being filtered through sterile Whatman paper.

The number of conidia in the solutions was determined after counts were made in 1 µl of spore suspension using a Malassez slide. Ten rectangles were considered. Three spore solutions were prepared per isolate and 10 counts were performed per spore solution. The average number of conidia per millilitre of solution was calculated using the formula developed by Duncan and Torrence (1992):

$$N = \frac{\sum(\mathrm{ni})}{\mathrm{a\ x\ V}}\ \mathrm{x\ Fd}$$

where N is mean number of conidia, ni is conidia number per rectangle i, a is number of rectangles considered, V is volume of a small rectangle (L×W×H = 0.01 mm) and Fd is dilution factor

Assessment of the pathogenicity of fungal strains

Production of fungi inoculum. Fusarium conidia suspension was obtained from 15-day-old cultures. Three mycelium pellets were taken and placed in 10 ml of sterile distilled water. The suspension obtained was filtered through sterile filter paper to separate the conidia and mycelial fragments. The spore solutions were adjusted to a final concentration of 10^6 conidia/ml with distilled water.

Soft inoculation of banana fruits. Bunch of apparently healthy bananas, ready to be packaged at the station, were collected at random on the day of the experiment. These bananas were cut to obtain several detached fingers. The surface of the crowns was refreshed with a sterile scalpel. The fruits were soaked in a 2% diluted sodium hypochlorite (NaClO) solution for 5 min. The fruits were then dried on sterile tissue paper under a laminar flow hood. The soft inoculation technique was used to evaluate the path-

ogenicity of the eight *Fusarium* isolates.

Sterile compresses were soaked in 50 µl of spore suspension from each isolate and placed on the crown, epicarp and distal end of each fruit in order to assess the sensitive part of the banana.

The inocula were kept on the fruit using sterile parafilm. The bananas were then incubated at room temperature (28±1°C). The pathogenicity of the *Fusarium* isolates was studied and demonstrated by the development of symptoms and the incubation time of the disease. Three bananas were used per isolate tested, and the experiment was repeated three times. Control bananas were inoculated with sterile distilled water and placed under the same conditions as the test bananas (Fig. 2).

Fig. 2 - Control and test bananas: Left= Control bananas inoculated with distilled water; Right= Test bananas inoculated with the spore suspension of the fungal

Statistical analyses

The data collected during this study were analyzed using Statistica software (version 7.1). A one-factor analysis of variance was performed to compare the average colony diameters on day 5 and the average number of partitions in the macroconidia of *Fusarium* isolates.

A two-factor analysis of variance was used to compare the average sizes of macroconidia and microconidia according to *Fusarium* isolates, as well as the average spore concentrations of macroconidia and microconidia from different *Fusarium* isolates.

Significant results at the 5% threshold were separated with least significant difference (LSD) test.

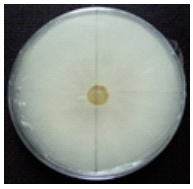
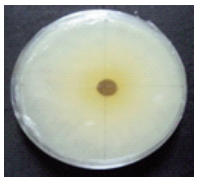
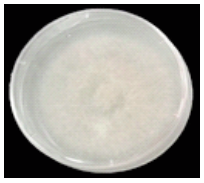
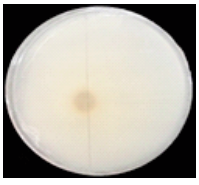
3. Results

Macroscopic characteristics of Fusarium isolates

Culture of isolates on PDA medium showed diversity in color, aspect and mode of mycelial growth. Three morphological groups were observed (Table 1).

Isolates F1 from the Abengourou area, F3 from

Table 1 - Cultural characteristics of the morphological groups of the eight *Fusarium* isolates

Morphological groups	Collection areas	Isolates	Coloration (front)	Coloration (reverse)	Colony aspect	Growth pattern
Creamy white colour (front and reverse)	Abengourou Abidjan Aboisso Dabou Grand-Tiassalé	F1 F3 F4 F6 F7 F8			carpet-like and cottony	diffuse
Ivory colour (front) Light yellow colour (reverse)	Abengourou	F2			dense and cottony	concentric circles
Light yellow colour (front and reverse)	Azaguié	F5			carpet-like and cottony	diffuse

(F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé.

Abidjan, F4 from Aboisso, F6 from Dabou, F7 from Grand-Bassam and F8 from Tiassale had white mycelium on the top and bottom of the Petri dish. The aspect of their mycelium was flaky and carpet-like, with a diffuse growth pattern. Isolate F2 from Abengourou had yellow-colored mycelium on the front. On the back, the mycelium was yellow in the centre and white at the margin. The mycelium was cottony and thick with concentric rings. Isolate F5 from Azaguié had yellow aerial mycelium and a pale yellow back. The mycelium was flaky and carpet-like. Isolate F5 from Azaguie had yellow aerial mycelium and a pale yellow underside. The mycelium was flaky and carpet-like in aspect. The growth pattern was diffuse.

Mycelial growth of Fusarium isolates

The average diameters of *Fusarium* isolates varied over time on PDA medium. Statistical analysis revealed a significant difference between the average colony diameters ($F = 10.120$ and $P < 0.001$). The mycelial growth curves of *Fusarium* isolates showed an initial adaptation period from day 1 to day 2, during which weak growth in colony diameter was observed for all isolates (from 0.85 to 1.84 cm). Then, a phase of rapid radial expansion from day 2 to day 4, during which the diameter of the colonies of isolates F1, F2, F3, F5, F6, F7 and F8 showed rapid growth (from 1.2 to 5 cm), except for isolate F4, which showed slow growth (from 0.86 to 1.64 cm). Finally a plateau phase from day 4 to day 9, during which the diameter of the colonies of isolates F1, F2, F3, F5, F6, F7 and F8 reached a plateau of around 5 to 5.5 cm, except for isolate F4, which grew slowly from 1.62 to 5 cm, reaching a plateau at the end of day 8 (Fig. 3).

Microscopic characteristics of Fusarium isolates

Two types of conidia were observed among the *Fusarium* isolates. Some conidia, called macroconidia, had partitions and were elongated, spindle-shaped, with both ends pointed. Other conidia, called microconidia, had no septa and were hyaline with different shapes (Table 2).

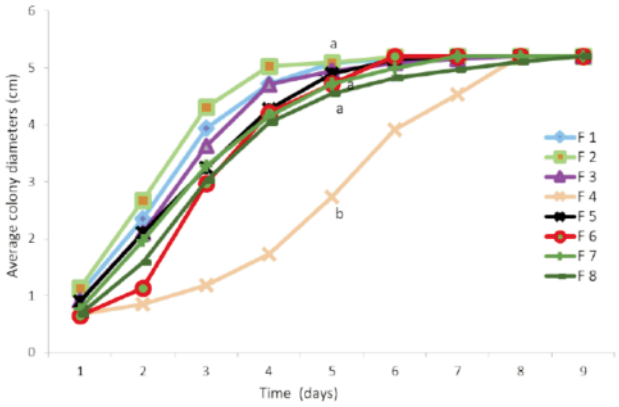
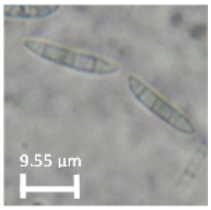
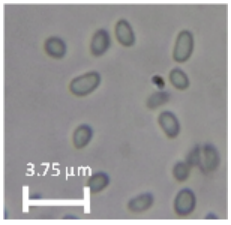
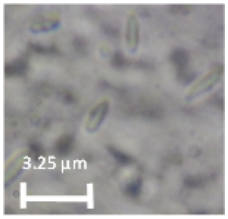


Fig. 3 - Evolution of the average diameters of *Fusarium* isolates over time: (F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé. Values assigned the same letter are identical according to Fisher's LSD test at the α= 5% threshold.

Table 2 - Different shapes of macroconidia and microconidia of *Fusarium* isolates

Fusarium isolates	Macroconidia		Microconidia	
F 1 , F 3, F 4, F 5, F 6, F 7, F 8	Fusiform (two pointed ends)		Oval (two rounded ends)	
F 2	Fusiform (two pointed ends)		Oval (one rounded end and one pointed end)	

(F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé.

All isolates had fusiform macroconidia with both ends pointed. Isolates F 1 from Abengourou, F3 from Abidjan, F4 from Aboisso, F5 from Azaguié, F6 from Dabou, F7 from Grand-Bassam and F8 from Tiassalé had oval-shaped microconidia with rounded ends.

Variability in the morphometric dimensions of macroconidia in Fusarium isolates

Diversity was observed in the average morphometric dimensions (length and width) of macroconidia depending on the *Fusarium* isolates (Fig. 4). Statistical analyses showed a significant difference (F = 39.75 and P < 0.001). The average length of macroconidia in the eight *Fusarium* isolates ranged from 19.11 µm to 29.52 µm. The average width of macroconidia in the eight *Fusarium* isolates ranged from 2.69 µm to 4.64 µm.

The F1 isolate from Abengourou and the F5 isolate from Azaguié had the longest macroconidia. The F3 isolate from Abidjan and the F6 isolate from Dabou had the smallest macroconidia. The F5 isolate from Azaguié and the F1 isolate from Abengourou had the widest macroconidia. The F4 isolate from Aboisso, the F6 isolate from Dabou and the F7 isolate from Grand-Bassam had the narrowest macroconidia.

Statistical analysis showed an interaction between the length and width of the macroconidia. Strains F1 and F5 had the longest and widest macroconidia. Isolates F3, F4 and F6 had shorter and narrower macroconidia. Morphometric dimensions of macroconidia (µm)

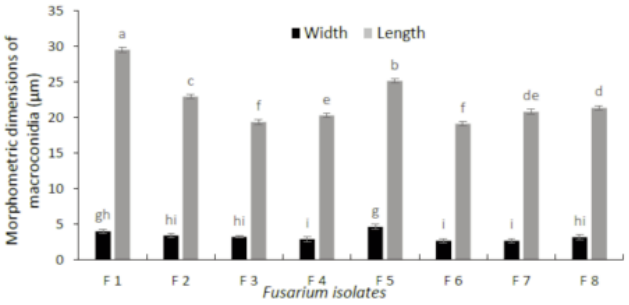


Fig. 4 - Variations in the average morphometric dimensions of *Fusarium* isolate macroconidia according to different collection areas G×400: (F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé. Values assigned the same letter are identical according to Fisher's LSD test at the α= 5% threshold.

Variability in the morphometric dimensions of microconidia in Fusarium isolates

Diversity was observed in the average morphometric dimensions (length and width) of microconidia depending on the *Fusarium* isolates (Fig. 5). Statistical analyses showed a highly significant difference (F = 39.86 and P < 0.001). The average length of microconidia in *Fusarium* isolates ranged from 6.11 µm to 10.53 µm. The average width of microconidia in the eight *Fusarium* isolates ranged from 2.5 µm to 3.00 µm. The F7 isolate from Grand-Bassam and the F3 isolate from Abidjan had the longest macroconidia. The F2 isolate from Abengourou and the F6 isolate from Dabou had the smallest microconidia. Isolate F5 from Azaguié had the widest microconidia. Isolates F2 from Abengourou, F3 from Abidjan, F4 from Aboisso and F6 from Dabou had the narrowest microconidia.

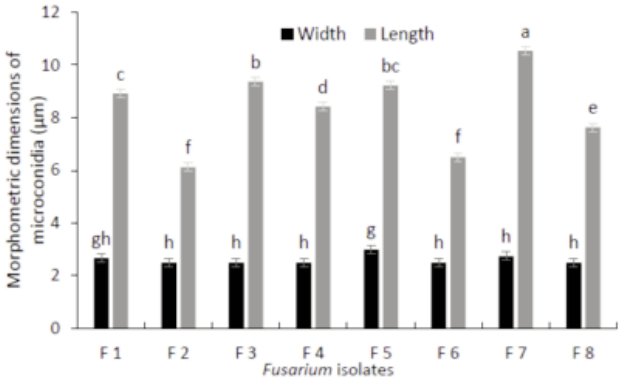


Fig. 5 - Variations in the average morphometric dimensions of *Fusarium* isolate macriconidia according to different collection areas G×400: (F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé.

Groups of macroconidia with a homogeneous number of septa

The number of septa in the macroconidia was ranged from 3 to 5. Statistical analysis showed a highly significant difference between the number of septa in *Fusarium* isolates depending on the collection area (F = 12.55 and P < 0.001). The two isolates from Abengourou and the one from Tiassalé had the highest number of partitions (4-5 septa), while the isolate from the Azaguié area had the lowest number of partitions (3-4 septa). The other isolates had an intermediate number of partitions between 3 and 4 septa (Fig. 6).

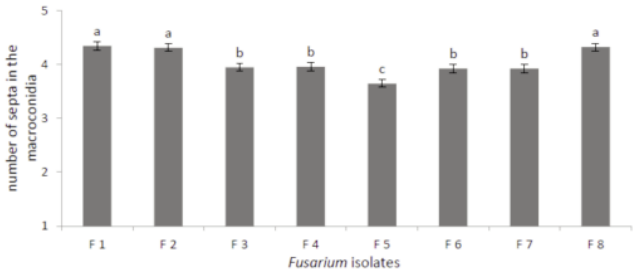


Fig. 6 - Average number of septa in macroconidia of *Fusarium* isolates in collection areas G×400: (F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé. Values assigned the same letter are identical according to Fisher's LSD test at the α= 5% threshold.

Groups of isolates with homogeneous conidia concentration

The average concentration of macroconidia was 3.5×10^5 conidia/ml, while that of microconidia was 3.1×10^5 conidia/ml (Fig. 7). The macroconidia concentration of *Fusarium* isolates ranged from 1.7×10^5 to 6.3×10^5 conidia/ml. The microconidia concentration ranged from 3.4×10^5 to 9.9×10^5 conidia/ml (Fig. 7).

The highest concentration of conidia (macroconidia and microconidia) was 7.10^5 conidia/ml. In contrast, the lowest concentration of conidia was 114.10^3 conidia/ml. Statistical analysis showed a highly significant difference (F = 260.213 and P < 0.001). Statistical analysis showed an interaction between the average concentrations of conidia and *Fusarium* isolates (F = 212.439 and P < 0.001).

The group composed of isolates from Abengourou, Abidjan, Azaguié, Aboisso and Dabou had more macroconidia than microconidia. The group composed of isolates from the Tiassalé and Grand-Bassam areas had more microconidia than macroconidia. Two patterns emerged in each of the two groups: in the first group, some isolates (Abengourou and Azaguié) produced more macroconidia than others (Abidjan, Aboisso and Dabou); in the second group, some isolates (Tiassalé) produced more microconidia than others (Grand-Bassam).

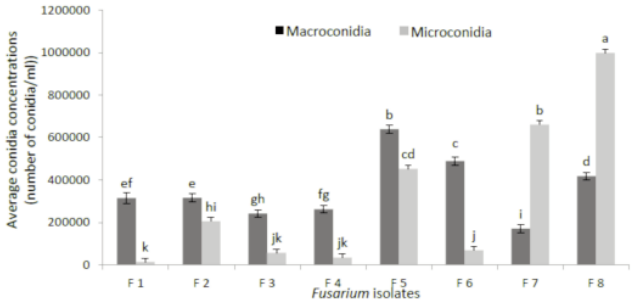


Fig. 7 - Variations in average spore concentrations in macroconidia and microconidia of *Fusarium* isolates in different collection areas: (F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé. Values assigned the same letter are identical according to Fisher's LSD test at the α= 5% threshold.

Correlation between the morphometric parameters of Fusarium isolates

Principal component analysis revealed the existence of two dimensions (dim1 and dim2) with respective inertia rates of 25.5% and 17.4% (Fig. 8). The variables macroconidia length (ML), macroconidia width (MW) and septa number (NS) are the variables that strongly influence (20 to 25%) the formation of the dim1 axis. The variable microconidia width (mW) contributes moderately (15%) to the formation of the dim1 axis. On the other hand, the variables macroconidia concentration (CM), microconidia concentration (Cm) and microconidia length (mL) strongly influence the formation of both axes. There is a

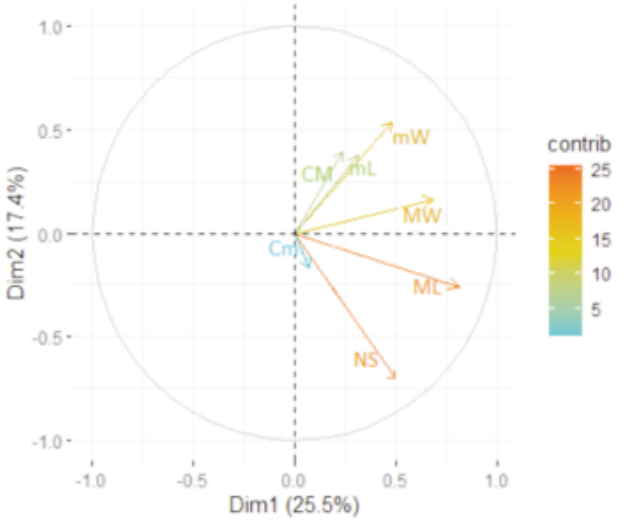


Fig. 8 - Correlation between the morphometric parameters of *Fusarium* isolates infecting bananas: (ML) Macroconidia length; (MW) Macroconidia width; (mW) Microconidia width; (Ml) Microconidia length; (NS) Septa number; (CM) Concentration of macroconidia, (Cm) Concentration of microconidia; (Dim 1) Dimension 1; (Dim 2) Dimension 2; (Contrib) Contribution.

very strong positive correlation between the ML, mW and NS variables. Similarly, the correlation between the mL and MW variables is strongly positive. However, there is no correlation between the variables CM and Cm. Thus, the morphometric dimensions and number of macroconidia are the most discriminating variables. The morphometric dimensions of microconidia are less discriminating.

Pathogenicity of Fusarium isolates

Symptoms observed. Soft inoculation of bananas with *Fusarium* isolates showed only one type of symptom depending on the part of the banana inoculated and the strain (Fig. 9). The symptom was characterised by soft brown rot of varying extent on the surface of the fruit. Considering the strain inoculated, all isolates caused soft rot, except for the Dabou strain, which caused no symptoms. The control bananas showed no symptoms. As for the inoculated part of the banana, the isolates from Abengourou, Abidjan, Aboisso, Azaguié and Grand-Bassam all caused symptoms at the crown of the infected bananas. The Tiassalé isolate caused symptoms at the distal end, and the Aboisso isolate caused symptoms on the crown and distal end of the inoculated bananas. However, no symptoms were observed on the epicarp, regardless of the strain inoculated.

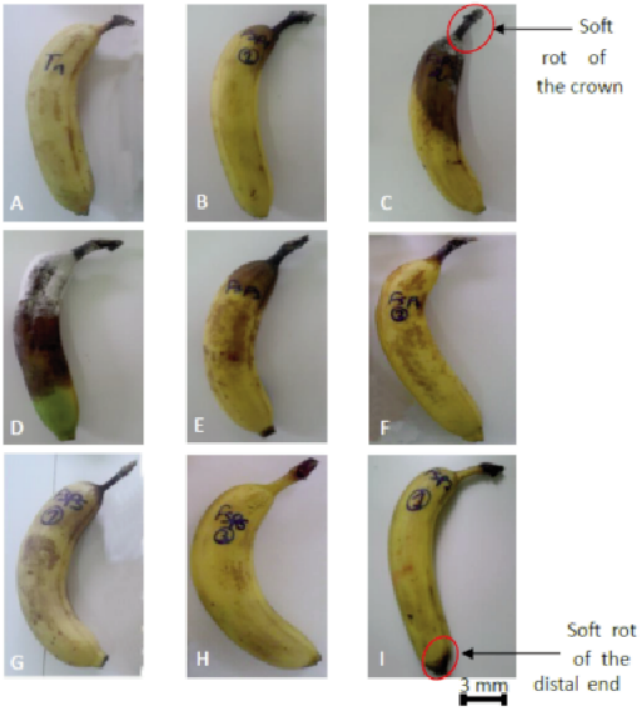


Fig. 9 - Variations in average spore concentrations in macroconidia and microconidia of *Fusarium* isolates in different collection areas: (F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé. Values assigned the same letter are identical according to Fisher's LSD test at the α= 5% threshold.

Incubation period. The time to symptom expression varied from 10 to 16 days depending on the isolates and inoculation sites (Table 3). The first symptom appeared 10 days after inoculation, caused by the Abidjan strain at the crown. The Aboisso strain induced the symptom 14 days after inoculation at the crown and 2 days later at the distal end. The F1 isolate induced the symptom 14 days after inoculation at the crown and 2 days later at the distal end. The F2 isolate induced the symptom 14 days after inoculation at the crown and 2 days later at the distal end. The F3 isolate induced the symptom 14 days after inoculation at the crown and 2 days later at the distal end.

The F4 isolate induced the symptom 14 The Aboisso strain induced the symptom 14 days after inoculation at the crown and at the distal end 2 days later. The Abengourou F1 isolate induced symptoms on the 12th day and the F2 isolate caused symptoms on the 13th day after inoculation. The Grand-Bassam and Tiassalé isolates induced symptoms 13 days after inoculation at the crown and distal end, respectively. The Azaguié strain caused symptoms 16 days after inoculation. The control fruits and those inoculated with the Dabou strain showed no symptoms during the incubation period.

Table 3 - Incubation time and duration of symptom appearance

Fusarium isolates	Incubation period (days)
F 3	10
F 1	12
F 2	13
F 7	13
F 8	13
F 4	14
F 5	16

(F 1) Isolate 1 from Abengourou; (F 2) Isolate 2 from Abengourou; (F 3) Isolate from Abidjan; (F 4) Isolate from Aboisso; (F 5) Isolate from Azaguié; (F 6) Isolate from Dabou; (F 7) Isolate from Grand-Bassam; (F 8) Isolate from Tiassalé.

4. Discussion and Conclusions

Macroscopic characterization of *Fusarium* isolates revealed variability in color and appearance depending on their origin. Three types of coloration were observed: white, ivory and pale yellow, and two types of aspect (cotton-like and downy).

This difference in cultural characteristics could be linked to the presence of different species within this population. However, this should be confirmed by further molecular analysis. Nevertheless, during their work on bananas in Morocco, Meddah *et al.* (2010) identified several species of *Fusarium* with different colors and appearances. With regard to the mycelial growth of *Fusarium* spp., the latency phase could correspond to an adaptation phase, during which the fungus establishes its mycelium growth elements by mitosis, leading to mycelium elongation and allowing the fungus to grow. Ruiz-Roldán *et al*. (2010), in their study on germination, sporulation and hyphal fusion in *Fusarium oxysporum*, showed that these different processes were carried out through mitosis. The exponential phase corresponds to the phase of active cell division and rapid vegetative growth of *Fusarium* isolates. This result would suggest that *Fusarium* isolates make optimal use of the energy resources available in the nutrient medium. The plateau phase indicates that the fungus has begun its reproductive or conservation phase due to a nutrient depletion in the environment or limited space in the Petri dish. To avoid death, the fungus undergoes conservation. In all *Fusarium* spp. isolates, it was observed approximatively three periods, except for isolate F4, which was characterized by slower mycelium growth. This could reflect lower virulence in this isolate, unlike the other isolates.

The present study highlighted two types of conidia: macroconidia (spores with septa) and microconidia (spores without septa), which are specific to fungi of the *Fusarium* genus (Heit, 2015). Morphometric variability in macroconidia and microconidia was observed between *Fusarium* isolates. Some isolates had large, broad conidia, while others, such as isolate F6 from Dabou, had the smallest conidia. This diversity in conidia size could reflect genetic diversity or ecological adaptation of these species.

In terms of spore concentrations, the variability observed between the numbers of macroconidia and microconidia may be related to the fact that some isolates produced more macroconidia than microconidia, unlike other isolates in which there were more microconidia than macroconidia.

The macroconidia were fusiform and the microconidia were oval with one or two rounded ends. Balali and Iranpoor in 2006 also observed variability in the shape of *Fusarium* species. According to these authors, this difference is due to genetic variability between *Fusarium* species. Furthermore, the strong positive correlation between macroconidia size and the number of septa suggests that *Fusarium* isolates with the longest macroconidia also have the widest and most septate macroconidia.

The *Fusarium* isolates tested developed soft rot symptoms on inoculated bananas, except for the Dabou isolate. These same symptoms were observed by Odame (2010) and Zakaria *et al.* (2012) after inoculating bananas with *Fusarium* isolates. These results also show that bananas are a host for this pathogen. The results obtained suggest that the *Fusarium* isolates that induced symptoms on bananas are pathogenic to bananas. The absence of symptoms in the presence of the Dabou isolate could be due to the fact that this isolate has smaller conidia than the other seven isolates, as demonstrated in this study. Several authors have demonstrated the potential role of fungal spore size in pathogenicity (Li *et al*., 2011). In fact, large spores don't need to expand further before the germination tube is produced, which happens quite quickly, allowing for rapid invasion of the host. Small spores, on the other hand, undergo a very long phase of volume increase, which delays the formation of the germ tube and thus slows down the infection or blocks it if conditions become unfavorable. Furthermore, of the three parts inoculated with the isolates, only the crown and the distal end of the bananas proved to be susceptible. Symptoms began to develop 10 days after inoculation of the fruit when they began to ripen. This late onset of symptoms could be related to the ripening of the fruit. Lassois *et al.* (2009) showed that the development of rot was linked to the physiological state of bananas after harvest. Coates and Jonhson (1997) stated that during the ripening of fruit and vegetables, respiration and water loss increase, chlorophyll decreases, aromas are secreted and the fruit loses rigidity. These conditions favor the development of fungi that were in a dormant state before the fruit was harvested.

Our findings demonstrate that Banana post-harvest rot can be caused by several isolates of

Fusarium. These isolates a very different in morphologic characters that affects their pathogenicity. These findings may contribute to the development of more effective strategies for the management of banana post-harvest diseases, thereby improving fruit quality.

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Efficacy of Aloe and Opuntia based composts on growth and phytochemical properties of *Salvia officinalis* and *Rosmarinus officinalis*

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Key words: Biostimulants, essential oil composition, organic cultivation, plant secondary metabolites, sustainable agriculture.

Abstract: The sustainable cultivation of medicinal and aromatic plants requires alternative substrates that reduce dependence on peat and synthetic fertilisers, while promoting acceptable plant growth and adequate phytochemical quality. This study evaluated the effects of five compost treatments on *Salvia officinalis* and *Rosmarinus officinalis* grown in greenhouses at CREA (Pescia, Italy). The treatments examined in this study include use of a peat-based control, commercial compost, monospecific composts derived from *Aloe arborescens*, *Aloe barbadensis* and *Opuntia ficus-indica*, plus a mixed Aloe-Opuntia compost. Growth parameters, photosynthetic performance, chlorophyll content, water and resource use efficiency, microbial biomass and secondary metabolites (phenols, flavonoids, essential oils) for both species were evaluated. For both species tested, the peat-based control supported consistently the best vegetative growth, highest phytochemical levels and was confirmed to be the best for physical support in greenhouse conditions. The mixed Aloe-Opuntia compost functioned similarly to the peat-based control, yet it showed a 5-8% reduction in vegetative growth, however, it still had a higher physiological/metabolic level than other compost based treatments. The results indicate that it is possible to use composts containing a combination of materials with complementary structural and biochemical properties to help overcome some of the limitations of single-species composts. On the other hand, composts made from *Aloe vera* alone exhibited the weakest agronomic and physiological performances likely due to having a higher electrical conductivity, high rapid decomposition rates, and having a poorly developed substrate structure even though it had a high total nutrient content. There was no difference in the safety of all composts; they caused no negative effects on plant survival, pest infestation, or disease occurrence. Such promising results were recorded particularly for mixed composts, but due to the fact that this was a greenhouse trial, there is a need for further replication to confirm findings. In conclusion, mixed plant composts could be a more sustainable solution, environmentally friendly for producing aromatic plants, and will reduce the reliance on the limited supply of peat.

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1. Introduction

There has been growing interest in the development of sustainable growing conditions for plants that have been requested for use as sources of medicinal and aromatic oils due to the worldwide increase in demand for these products from both the food and beverage industries as well as from pharmaceutical and cosmetics companies (Lange, 2002; Pirani *et al*., 2020; Chrysargyris *et al*., 2022). Due to their high bioactive monoterpenes (1,8-cineole, camphor and α-pinene) content, *Salvia officinalis* and *Rosmarinus officinalis* are of great importance due to their use as essential oils in many applications and as important parts of many herbal preparations (El Euch *et al*., 2019; Leporini *et al*., 2020). These monoterpenic substances have many health benefits, including antimicrobial, antioxidant and anti-inflammatory actions, and therefore are considered to be important components in the development of therapeutic products as well as in the preservation of natural food (Angioni *et al.,* 2004; Miguel, 2010; Shahina *et al.,* 2022). Cultivation practices that optimise both the quantity and quality of plants are crucial for sustaining the production and sales of MAPs such as *Salvia officinalis* and *Rosmarinus officinalis*, particularly when grown in controlled environments, such as greenhouses (Avasiloaiei *et al.,* 2025).

The advantages of using greenhouses as a method for growing crops are that they give producers a greater amount of control over their environment; however, the challenges of using alternative methods for managing substrates and providing nutrients to crops, as well as managing pests are many (Bu *et al.*, 2022; Chen *et al*., 2025). In addition, there has been considerable debate about the negative effects on both people's health and the environment related to the increasing number of inputs into agricultural production, including pesticides and synthetic fertilizers (Dhankhar and Kumar, 2023; Jamal *et al*., 2023). Due to this trend, many growers are beginning to make a shift away from the conventional style of agricultural production, which utilises an influx of synthetic materials, to a biodynamic approach to agricultural production (Brzozowski and Mazourek, 2018). Additionally, plant-based composts from renewable biomass are becoming increasingly common, as they provide both essential nutrients and bioactive compounds for promoting growth and increased resilience in plants (Salman *et al*., 2023; Ahmed *et al*., 2025). Composts formulated from *Aloe barbadensis* (aloe vera), *Aloe arborescens*, and *Opuntia ficus-indica* (prickly pear cactus) have recently attracted attention as bio enhancing soil amendments (Semerel *et al*., 2023; Bacchetta *et al*., 2024). These species are well-known for their medicinal properties, which are derived from their diverse chemical profiles that are rich in polysaccharides, phenolic compounds, organic acids, and glycoproteins (Di Palma *et al*., 2025). When incorporated into compost, these compounds may act as biostimulants, enhancing plant growth, improving tolerance to abiotic stress, and stimulating beneficial soil microbiota (Prisa and Gobbino, 2021).

Aloe species have been extensively studied for their antimicrobial, antifungal, and wound-healing effects in humans, and emerging evidence suggests that they may exert similar protective effects in soil-plant systems (Arsene *et al*., 2022). *Aloe barbadensis*, in particular, contains aloin, emodin, and acemannan compounds that can stimulate plant defense pathways and promote systemic resistance to pathogens (Ahmad *et al*., 2018). *Aloe arborescens*, though less commercially prominent, has shown superior antioxidant activity due to its higher phenolic content and greater diversity of bioactive metabolites (Maliehe *et al*., 2023). Likewise, *Opuntia ficus-indica*, a drought-tolerant cactus species, has shown potential as a compost base due to its high mucilage content, nutrient accumulation capacity, and ability to enhance soil water retention and microbial activity (Procacci *et al*., 2021).

Although plant-based composts are increasingly applied in open-field systems, their effects under greenhouse conditions remain underexplored, particularly for high-value aromatic crops (Oueld Lhaj *et al*., 2025). The confined root environment and controlled climate of greenhouses provide an ideal platform to test targeted compost formulations; however, their influence on growth performance, pest suppression, and secondary metabolite biosynthesis is not well understood (Zheng *et al*., 2020). Furthermore, the bioactive properties of *Salvia officinalis* and *Rosmarinus officinalis* essential oils are strongly influenced by environmental and nutritional factors, including substrate composition (Rapposelli *et al*., 2015). Previous studies on these two species have shown that organic amendments can modulate terpene biosynthesis pathways by altering both oil yield and chemical composition (Valiki and Ghanbari, 2015). Similarly, composts

enriched with phenolics or bioavailable micronutrients can enhance plant immunity and secondary metabolite accumulation in *S. officinalis* and *R. officinalis* (Naguib *et al*., 2012; Montoya *et al*., 2022).

However, most commercial composts used in organic horticulture are formulated from generic plant waste and lack the targeted bioactivity of the medicinal species. Composts made specifically from Aloe and Opuntia may provide added value by combining nutritional enrichment with biological protection (Procacci *et al*., 2021). Such dual actions could be especially advantageous in greenhouse cultivation, where disease pressure and nutrient competition are intensified (Di Palma *et al*., 2025). Integrating plant-based composts into MAP production also aligns with the principles of circular agriculture and zero-waste systems, particularly in the Mediterranean regions where Aloe and Opuntia are abundant and adapted to marginal soils (Zheng *et al*., 2020).

Despite their potential, scientific data comparing Aloe and Opuntia based composts with commercial products under greenhouse conditions are scarce. Few studies have simultaneously evaluated the effects of compost on plant growth, pest incidence, and essential oil yield, and even fewer have examined its impact on the chemical composition of essential oils, which is a critical determinant of both market value and biological efficacy (Di Palma *et al*., 2025).

Therefore, this study aimed to evaluate the agronomic performance, physiological responses, and secondary metabolite profiles of Salvia officinalis and Rosmarinus officinalis grown in greenhouse pot culture using composts derived from *Aloe barbadensis*, *Aloe arborescens*, and *Opuntia ficus-indica*, compared with commercial compost and an unfertilized control. It was hypothesized that plant-based composts formulated from bioactive species would enhance plant growth, physiological efficiency, and essential oil yield compared to the control and commercial composts, with mixed-species composts exhibiting superior effects over single-species formulations.

2. Materials and Methods

Experimental site and greenhouse conditions

The experiment was conducted in a controlled-environment greenhouse at the Council for Agricultural Research and Economics - Research Centre for Vegetable and Ornamental Crops (CREA-OF), Pescia, Tuscany, Italy (43.8912° N, 10.6856° E; 35 m asl) from January to December 2024. The greenhouse was equipped with an automated climate control system regulating temperature, humidity, and ventilation. Daytime temperatures were maintained between 24-28°C and nighttime temperatures between 16-18°C. Relative humidity ranged from 60-75%. Supplementary LED lighting provided approximately 250 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) for 12 h day^{-1}, which lies within the optimal range reported for *Salvia officinalis* and *Rosmarinus officinalis* (200-600 µmol m^{-2} s^{-1}) to sustain healthy growth and essential oil production (Hussain *et al*., 2010; Berkovich *et al*., 2017; Papafotiou *et al*., 2022).

Plant material and experimental design

Seedlings of *Salvia officinalis* L. and *Rosmarinus officinalis* L., propagated under identical nursery conditions, were transplanted at 60 days of age into 6 L plastic pots containing treatment-specific substrate mixtures. Each pot contained a single uniform seedling. The experimental design followed a completely randomized block (CRB) structure with six treatments with four replicates, with six plants per replicate and 24 plants per treatment. With a total of 144 plants per species.The six treatments were:

T0 (Control): Peat-based substrate without compost (baseline).
T1 (Commercial): Peat + 30% commercial green compost.
T2 (*Aloe barbadensis*): Peat + 30% compost derived from *Aloe barbadensis*.
T3 (*Aloe arborescens*): Peat + 30% compost derived from *Aloe arborescens*.
T4 (Opuntia): Peat + 30% compost derived from *Opuntia ficus-indica*.
T5 (Mixed): Peat + 10% compost from each of the three experimental composts (*Aloe barbadensis*, *Aloe arborescens*, *Opuntia ficus-indica*).

The experimental composts were produced through aerobic decomposition for 90 days using mature biomass from organically grown populations of *Aloe barbadensis*, *Aloe arborescens* and *Opuntia ficus-indica*. For the *Aloe* species, the compost

feedstock consisted mainly of senescent and pruned leaves, which are the main by-products generated during routine maintenance, while roots and stems were not included. For *Opuntia ficus-indica*, the composting material included pruned cladodes, collected after thinning and sanitary pruning; fruits, roots, and woody stems were excluded. All plant residues were shredded into 2-4 cm pieces prior to composting to ensure uniform decomposition. Prior to use, composts were characterized for pH, electrical conductivity, total nitrogen, organic matter content, and C:N ratio (Table 1). All plants were maintained under uniform irrigation, keeping the substrates at field capacity throughout the experimental period. No synthetic fertilizers or pesticides were applied during the trial.

Growth and morphological parameters

At 120 days after transplantation, various morphological traits were recorded for each plant. Growth and morphological parameters were monitored throughout the experiment. Plant height and leaf number were recorded biweekly, while final destructive measurements (leaf area, biomass, and root traits) were collected at 120 days after transplantation. Biweekly measurements allowed evaluation of growth trends over time, while final harvest data provided detailed information on biomass allocation and root development.

Plant height (cm) was measured from the substrate surface to the highest vegetative point using a flexible measuring tape, while the total number of leaves was counted manually for each plant. Leaf area (cm^2) was determined by collecting five fully expanded leaves per plant, scanning them, and analyzing the images using ImageJ software to calculate the mean surface area. For biomass assessment, plants were harvested, and roots were gently washed; the fresh biomass of both aboveground and belowground parts were recorded, followed by oven-drying at 65°C for 72 h to determine dry weight. Root length (cm) was measured as the longest root per plant using a digital caliper. Additionally, plant mortality (%) was recorded by counting the number of dead plants during the experimental period and calculating the mortality rate as a percentage of the total plants per treatment.

Photosynthetic and water-relations measurements

Photosynthetic parameters were measured biweekly, on clear days between 09:00 and 11:30 AM using a portable photosynthesis system (LI-6400XT, LI-COR Biosciences, Lincoln, NE, USA). Measurements were taken from the third fully expanded leaf from the shoot apex, avoiding shaded or damaged leaves. The recorded parameters included net photosynthetic rate (Pn, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (Gs, mol H_2O m^{-2} s^{-1}), and transpiration rate (E, mmol H_2O m^{-2} s^{-1}). Water use efficiency (WUE) was calculated as the ratio of photosynthesis to transpiration (Pn/E), indicating carbon gain per unit of water lost (Liu *et al*., 2020; Mingyang *et al*., 2022). All measurements were performed on three

Table 1 - Chemical and physical properties of composts used in the experiment

Parameter	Control (T0)	Commercial Compost (T1)	*Aloe barbadensis* (T2)	*Aloe arborescens* (T3)	*Opuntia . ficus-indica* (T4)	Mixed compost (T5)
pH (H_2O 1:5)	6.4	6.8	6.6	6.9	6.7	6.7
Electrical conductivity (dS m^{-1})	0.9	1.6	1.8	1.7	1.7	1.7
Organic matter (%)	28.7	48.6	50.1	45.7	48.1	48.1
Total nitrogen (N, %)	0.7	1.5	1.4	1.3	1.4	1.4
C/N ratio	20.5	16.4	15.9	17.1	16.5	16.5
Available phosphorus (P_2O_5, mg kg^{-1})	510	910	950	890	920	920
Exchangeable potassium (K_2O, mg kg^{-1})	620	1120	1170	1090	1130	1130
Moisture content (%)	29.6	35.2	34.6	36.5	35.4	35.4
Bulk density (g cm^{-3})	0.61	0.49	0.47	0.51	0.49	0.49
Microbial biomass (MBC, mg kg^{-1})	165	385	412	376	402	402

Values are means of three replicates. Composts were air-dried, sieved (≤10 mm), and analyzed according to standard soil and compost analysis protocols (EN 13039:2000 and ISO 14240-1 for MBC). Mixed compost (T5) was prepared by blending equal proportions (1:1:1) of T2, T3, and T4 by volume.

randomly selected plants per replicate, resulting in a total of 12 plants per treatment per species.

Chlorophyll content estimation

Relative chlorophyll content was measured biweekly using a SPAD 502 chlorophyll meter (Konica Minolta, Japan). Three SPAD readings were collected per plant from the same leaves used for gas-exchange measurements and averaged to obtain a representative chlorophyll index (Ling *et al*., 2011; Yuan *et al*., 2016).

Microbial biomass in substrate

At the end of the experiment, microbial biomass carbon (MBC) in the substrate was assessed using the chloroform fumigation-extraction method. Composite samples from each pot were extracted with 0.5 M K_2SO_4, and MBC was quantified via dichromate oxi-dation followed by spectro-photometric analysis. Data were expressed as mg MBC kg^{-1} dry substrate (Vance *et al*., 1987).

Leaf metabolite analysis

Leaf samples (~2 g fresh weight) were collected from each treatment, immediately flash-frozen in liquid nitrogen, and stored at -80°C until analysis. The samples were lyophilized and ground into a fine powder before extraction. Total phenolic content (TPC) was determined using the Folin-Ciocalteu method and expressed as mg gallic acid equivalents (GAE) g^{-1} DW, while total flavonoid content (TFC) was quantified via the aluminum chloride colorimetric assay and expressed as mg quercetin equivalents (QE) g^{-1} DW (Albayrak, 2013). Antioxidant capacity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay and expressed as IC_{50} (μg mL^{-1}). For essential oil analysis, dried leaves were subjected to hydro distillation using a Clevenger apparatus, and the resulting oil was analyzed by GC-MS, with compounds such as 1,8-cineole, camphor, and α-pinene identified based on retention indices and comparison with NIST library spectra (Farhadi *et al*., 2020).

Fertilization regime

To ensure a uniform nutrient baseline and prevent nutrient deficiency as a con-founding factor, all treatments received a standardized organic fertilization schedule. A liquid organic fertilizer (NPK 5-2-5), derived from plant extracts and permitted under European organic farming regulations (Reg. EC 834/2007), was applied biweekly. Each plant received 50 mL of a diluted solution (1:100 v/v) to the root zone, starting 14 days after transplanting and continuing until day 90.

Micronutrient supplementation (Fe, Zn, Mn) was performed once at day 40 using an organic-certified foliar product based on seaweed extract and chelated trace elements. Applications were carried out in the early morning to minimize evapotranspiration and photodegradation.

Biological pest and disease control

An integrated pest management (IPM) strategy was implemented throughout the cultivation period, combining preventive monitoring and biological control. Weekly visual scouting and yellow sticky traps were used to monitor aphid (*Myzus persicae*) and whitefly (*Trialeurodes vaporariorum*) populations. When pest thresholds exceeded two adults per plant, biological interventions were initiated. *Beauveria bassiana* (strain ATCC 74040) was sprayed on foliage at 10-day intervals (1×10^7 CFU mL^{-1}) during peak insect activity, while *Trichoderma harzianum* spores (10^8 CFU g^{-1}) were applied to the root zone at 30 and 60 days after transplanting to prevent fungal diseases such as *Botrytis cinerea* and *Rhizoctonia solani*. All biocontrol products used were certified for organic cultivation and applied according to manufacturer guidelines, with no synthetic pesticides or systemic chemicals used during the experiment.

Statistical analysis

All quantitative data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. When assumptions were met, differences among treatments were analyzed using one-way ANOVA, followed by Tukey's HSD post hoc test ($\alpha = 0.05$) for mean separation. In cases where data did not meet parametric assumptions, the Kruskal-Wallis test was applied as a non-parametric alterna-tive. Principal component analysis (PCA) and correlation plots were drawn to investigate the relationships among the studied variables using OriginPro Version 2024 (OriginLab Corporation, USA; www.originlab.com) software. Prior to PCA, the data were stand-ardized using z-score normalization (mean-centered and scaled to unit variance) by en-abling the "Standardize Variables" option in the PCA tool to ensure the equal contribution of all traits. PCA was performed using a

correlation matrix, and no rotation method was applied. Pearson's correlation coefficients were calculated to construct correlation heatmaps, which were visualized with color-coded ellipses indicating the strength and direction of the relationships among variables.

Quality assurance and replication

All measurements were performed in biological triplicates or quadruplicates as appropriate. Analytical assays were conducted in duplicate, and instruments were calibrated regularly. Substrate samples and plant tissues were handled with sterile equipment to avoid cross-contamination.

3. Results

Salvia officinalis

Agronomic traits

Compost treatments significantly influenced the agronomic performance of *Salvia officinalis* (Table 2 and Fig. 1). The control substrate (T0) supported the most vigorous growth, producing the tallest plants (53.4 ± 1.2 cm), the greatest number of leaves (140.0 ± 1.0), the largest leaf area (580 ± 10.3 cm²), and the highest shoot biomass (43.0 ± 1.1 g) (Fig. 1).

The commercial compost (T1) performed comparably to the control, with non-significant reductions in plant height (-2.8%), leaf number (-1.8%), leaf area (-2.6%), and shoot biomass (-3.5%), indicating its suitability as an alternative growth medium. Similarly, the mixed Aloe and Opuntia compost (T5) produced only slight decreases in plant height -5.6%), leaf number (-3.6%), and shoot biomass (-7.0%) relative to T0, suggesting its potential as an effective organic amendment (Fig. 1).

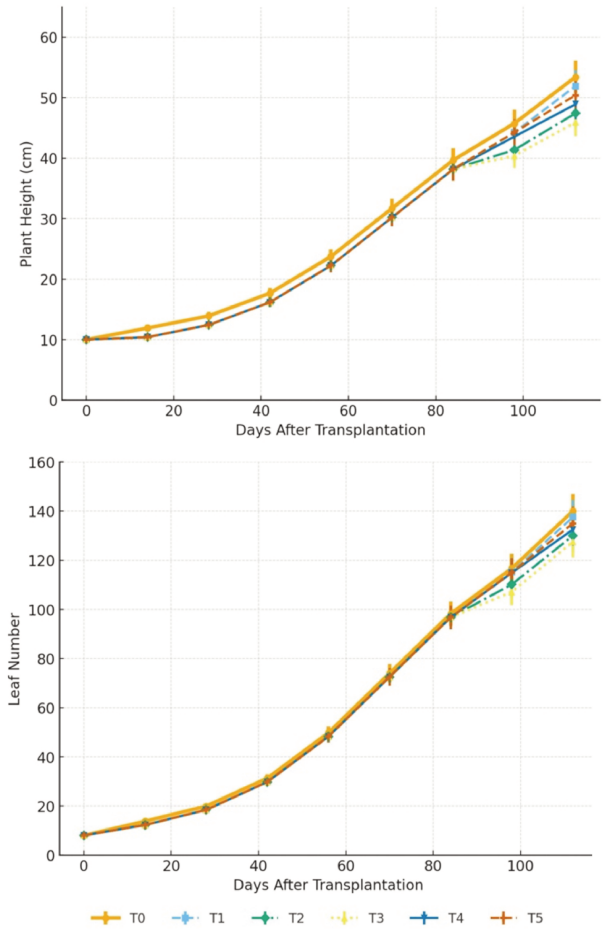

Fig. 1 - Biweekly growth (plant height and leaves number) dynamics of *Salvia officinalis* under different compost treatments.

In contrast, the Opuntia compost alone (T4) resulted in moderate reductions in plant height (-8.4%), leaf number (-5.4%), and shoot biomass (-10.7%) compared with T0. The Aloe-only treatments (T2 and T3) consistently underperformed. T2 plants exhibited an 11.2% reduction in height,

Table 2 - Agronomic parameters of *Salvia officinalis* under different compost treatments

Treatment	Plant height (cm)	Leaf number	Leaf area (cm²)	Shoot biomass (g)	Root biomass (g)	Root length cm
T0	53.4 ± 1.2 a	140.0 ± 1.0 a	580 ± 10.3 a	43.0 ± 1.1 a	14.2±1.8 a	21.3±1.8 a
T1	51.9 ± 1.3 ab	137.5 ± 1.3 ab	565 ± 10.6 ab	41.5 ± 0.9 ab	13.5±1.6 bc	20.2±1.8 b
T2	47.4 ± 0.8 c	130.0 ± 1.2 c	520 ± 13.8 c	37.0 ± 0.9 c	11.8±1.3 e	18.3±1.6 d
T3	45.9 ± 1.1 c	127.5 ± 1.5 c	505 ± 12.1 c	35.5 ± 1.0 c	12.5±1.7 d	18.9±1.7 cd
T4	48.9 ± 1.1 bc	132.5 ± 1.1 bc	535 ± 10.5 bc	38.5 ± 0.9 bc	13.0±1.6 cd	19.4±1.9 c
T5	50.4 ± 1.0 b	135.0 ± 1.2 b	550 ± 11.7 b	40.0 ± 1.1 b	14.1±1.4 ab	20.2±1.5 b

Values are mean ± SD with statistical groupings indicated by letters (Tukey's test, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

7.1% fewer leaves, and 14.0% lower shoot biomass than the control, while T3 showed even greater reductions (14.1% shorter height, 8.9% fewer leaves, and 17.4% lower shoot biomass, 12.0% reduced root biomass, and 11.3% shorter roots). Although the Aloe-based composts (T2 and T3) contained higher levels of organic matter, available phosphorus, exchangeable potassium, and microbial biomass compared with the control (Table 1), they consistently resulted in the lowest plant height, leaf area, shoot biomass, and root biomass (Table 2). Both Aloe treatments exhibited significantly reduced vegetative growth compared with the control and with the commercial (T1), Opuntia (T4), and mixed compost treatments (T5) (Fig. 2). Despite the elevated nutrient and organic matter content of T2 and T3, the plants grown in these substrates showed reductions of 11-18% in plant height and 14-20% in shoot biomass relative to the control. Physiological measurements also showed decreased chlorophyll content, lower photosynthetic rates, and reduced stomatal conductance under Aloe-only treatments. These results indicate that the beneficial chemical characteristics of Aloe composts did not translate into improved plant performance in *Salvia officinalis* or *Rosmarinus officinalis* under the conditions of this experiment.

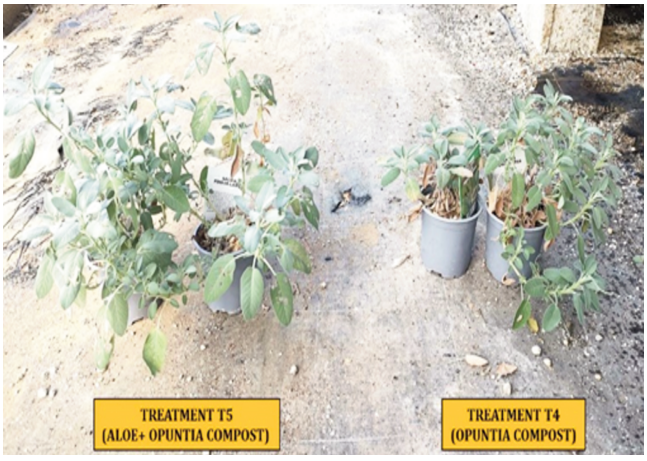

Fig. 2 - Effect of treatment T5 (Aloe + Opuntia compost) compared to T4 (Opuntia compost) on the height of Salvia plants.

These results demonstrate that while mixed or commercial composts can maintain growth comparable to the control substrate, single-source Aloe compost lacks the nutrient balance and structural benefits required for optimal plant development.

Physiological parameters

The physiological performance of *Salvia officinalis*, including chlorophyll content (SPAD), net photosynthetic rate (Pn), stomatal conductance (Gs), and water-use efficiency (WUE), varied significantly among treatments (Figs. 3, 4).

The highest SPAD index was observed in T0 (47), indicating maximum chlorophyll concentration. T1 (45) and T5 (44) exhibited only 4.3% and 6.4% lower SPAD values, re-spectively, compared with T0, whereas Aloe-only treatments (T2 and T3) recorded the lowest SPAD readings (38), representing a 19% reduction relative to the control (Fig. 3A).

A similar trend was found for photosynthetic activity. T0 achieved the highest Pn (32 µmol CO_2 m^{-2} s^{-1}), followed closely by T1 (30; -6.3%) and T5 (29; -9.4%). In contrast, T2 and T3 reduced photosynthetic rate to 24 µmol CO_2 m^{-2} s^{-1}, a 25% decline compared with the control (Fig. 3B), consistent with their reduced chlorophyll content and biomass production.

Stomatal conductance mirrored these patterns. T0

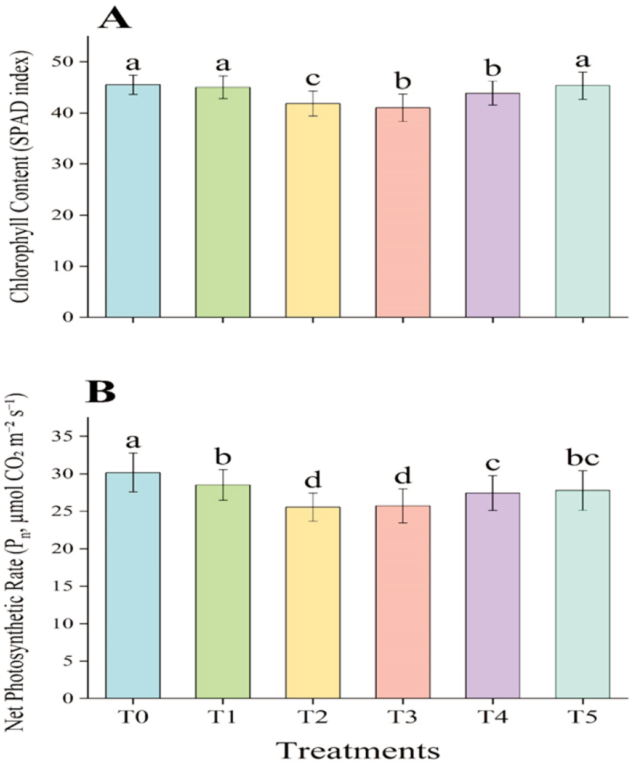

Fig. 3 - Chlorophyll content SPAD Index (A), and Photosynthetic Rate (Pn) (B) in *Salvia officinalis* under compost treatments. Note: Bars represent mean values (n = 4) with ±SD. Letters above the bars denote statistically different groups (Tukey's HSD, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

recorded the highest Gs (0.55 mol H_2O m^{-2} s^{-1}), while T1 (0.50; -9.1%) and T5 (0.49; −10.9%) showed moderate reductions. T2 and T3 further decreased Gs to 0.42 mol H_2O m^{-2} s^{-1}, a 23.6% reduction relative to T0 (Fig. 4A), indicating restricted stomatal aperture and reduced transpiration capacity.

Interestingly, water-use efficiency (WUE) exhibited a slightly different response. T5 recorded the highest WUE (2.9), representing a 3.6% improvement over the control (2.8), despite a modestly lower photosynthetic rate. T1 maintained a similar WUE (2.7; -3.6%), whereas T2 and T3 showed the lowest efficiency (2.3), reflecting a 17.9% decline compared with T0 (Fig. 4B).

Collectively, these findings suggest that while commercial and mixed composts (T1 and T5) sustain gas exchange and chlorophyll synthesis near optimal levels, Aloe-only composts (T2 and T3) limit photosynthetic performance through reduced nutrient availability and stomatal activity. Notably, T5 enhanced WUE, suggesting potential advantages for water-limited cultivation systems.

Secondary metabolites and essential oils

Compost treatments significantly influenced secondary metabolite accumulation and essential oil production in *Salvia officinalis* (Table 3). The mixed Aloe-Opuntia compost (T5) markedly enhanced phenolic (33.0 ± 0.7 mg GAE g^{-1}) and flavonoid content (18.0 ± 0.6 mg QE g^{-1}), showing an increase of 10.0% and 11.1%, respectively, compared with the control (T0). T4 (Opuntia compost) also improved these metabolites relative to Aloe-only treatments but remained slightly lower than T5. In contrast, T2 and T3 (Aloe-only composts) resulted in the lowest phenolic (-18.2% for T3) and flavonoid contents (-27.8% for T3) relative to T0.

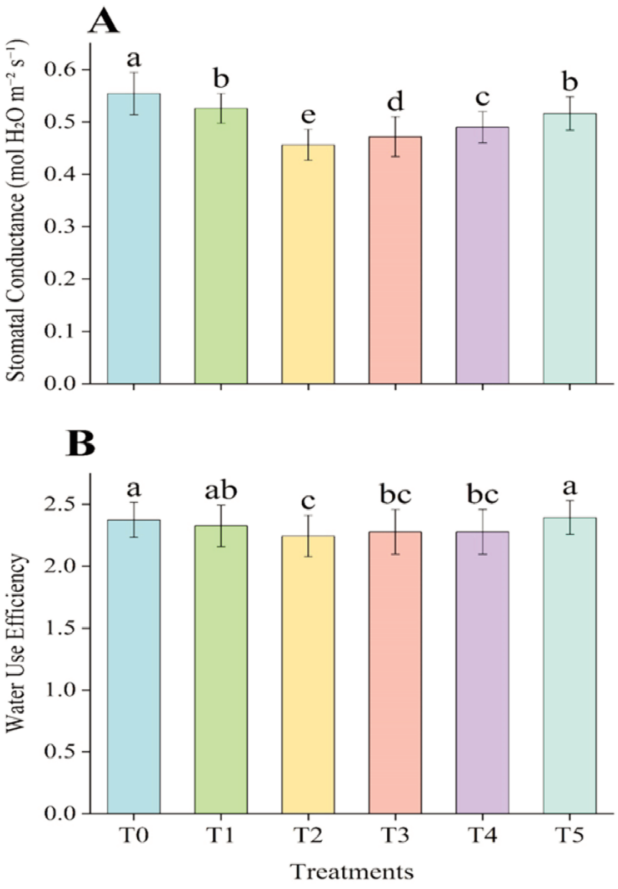

Fig. 4 - Stomatal conductance (A), and Water Use Efficiency (B) in *Salvia officinalis* under compost treatments. Note: Bars represent mean values (n = 4) with ±SD. Letters above the bars denote statistically different groups (Tukey's HSD, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

Antioxidant activity, assessed by IC_{50} values, showed that the control (T0) exhibited the strongest antioxidant capacity (IC_{50}= 5 0± 1.3 µg mL^{-1}), consistent with its higher phenolic and flavonoid

Table 3 - Metabolite content in *Salvia officinalis* under different compost treatments

Treatment	Phenolic content (mg GAE g^{-1})	Flavonoids (mg QE g^{-1})	Antioxidant activity (IC_{50} µg mL^{-1})	Essential oil Yield (%)	Microbial biomass (µg C/g soil)
T0	33.0 ± 0.7a	18 ± 0.6a	50 ± 1.3c	1.8± 0.1 a	260.2±20.5a
T1	31.5 ± 0.8a	17 ± 0.6a	52 ± 1.1c	1.6± 0.1 ab	249.3±23.4b
T2	27.0 ± 0.5d	14 ± 0.4bc	58 ± 1.0a	1.4± 0.1 bc	238.1±21.2c
T3	25.5 ± 0.8e	13 ± 0.6c	60 ± 1.3a	1.2± 0.1 c	237.4±19.6c
T4	28.5 ± 0.6c	15 ± 0.7ab	56 ± 1.0ab	1.4± 0.1 bc	244.5±19.5bc
T5	30.0 ± 0.7b	16 ± 0.7a	54 ± 1.1bc	1.6± 0.1 ab	250.3±19.3b

Values are mean ± SD with statistical groupings indicated by letters (Tukey's test, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

concentrations. Among compost treatments, T5 showed the best performance (54 ± 1.1 µg mL^{-1}), followed by T4 (56 ± 1.0 µg mL^{-1}). The Aloe-only treatments (T2 and T3) had the highest IC_{50} values (58-60 µg mL^{-1}), indicating the weakest antioxidant response. Thus, while mixed compost (T5) improved antioxidant capacity relative to other compost treatments, none surpassed the control substrate (T0).

Essential oil yield also followed this trend. T0 produced the highest oil content (1.8%), with T5 achieving a similar level (1.6%). T3 recorded the lowest yield (1.2%), representing a 33% reduction compared with T0.

Soil microbial biomass was highest in T0 and remained relatively high in T5, whereas Aloe-only composts (T2 and T3) showed an 8-9% reduction relative to the control.

Collectively, these results show that the mixed compost (T5) performed better than other compost treatments but did not exceed the control (T0) in antioxidant activity or essential oil yield.

Rosmarinus officinalis

Agronomic traits

Similar trends to *Salvia officinalis* were observed in *Rosmarinus officinalis* (Table 4, Fig. 5). The control treatment (T0) produced the most vigorous vegetative growth, yielding the tallest plants (52.0 ± 0.9 cm), the highest leaf number (138.0 ± 1.2), the largest leaf area (570 ± 11.4 cm^2), and the greatest shoot biomass (42.0 ± 1.0 g). Root traits also followed this pattern, with T0 showing the highest root biomass (14.0 ± 1.7 g) and longest roots (20.6 ± 1.8 cm).

Commercial compost (T1) performed comparably to the control, with only minor, non-significant

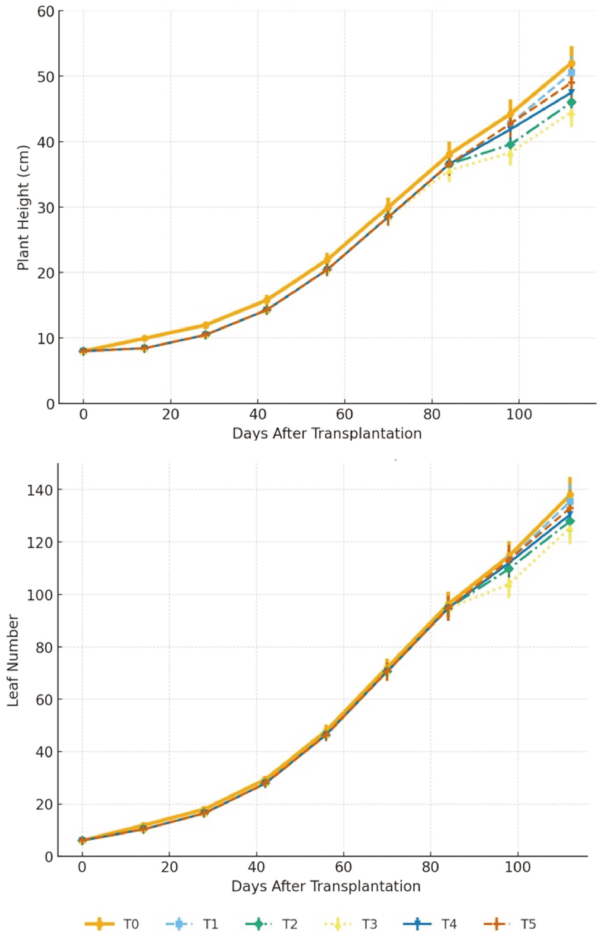

Fig. 5 - Biweekly growth dynamics of *Rosmarinus officinalis* under different compost treatments.

reductions in plant height (−2.9%), leaf number (−1.8%), leaf area (−2.6%), shoot biomass (−3.6%), root biomass (−4.3%), and root length (−3.9%). The mixed Aloe-Opuntia compost (T5) similarly supported substantial growth, showing 5.8% shorter plants, 3.6% fewer leaves, 5.3% smaller leaf area, and 7.1% lower shoot biomass, alongside moderate reductions

Table 4 - Agronomic parameters of *Rosmarinus officinalis*

Treatment	Plant height (cm)	Leaf number	Leaf area (cm^2)	Shoot biomass (g)	Root biomass (g)	Root length (cm)
T0	52.0 ± 0.9 a	138.0 ± 1.2 a	570 ± 11.4 a	42.0 ± 1.0 a	14.0±1.7 a	20.6±1.8 a
T1	50.5 ± 1.0 ab	135.5 ± 1.5 ab	555 ± 11.2 ab	40.5 ± 1.1 ab	13.4±1.5 ab	19.8±1.7 b
T2	46.0 ± 1.0 c	128.0 ± 1.4 c	510 ± 12.9 c	36.0 ± 1.3 c	11.1±1.4 d	17.6±1.4 d
T3	44.5 ± 1.2 c	125.5 ± 1.4 c	495 ± 12.8 c	34.5 ± 1.0 c	11.3±1.5 d	18.3±1.7 c
T4	47.5 ± 0.9 bc	130.5 ± 1.0 bc	525 ± 12.2 bc	37.5 ± 1.2 bc	12.6±1.3 c	19.4±1.6 b
T5	49.0 ± 1.2 b	133.0 ± 1.1 b	540 ± 11.2 b	39.0 ± 1.1 b	12.8±1.8 bc	19.7±1.6 b

Values are mean ± SD with statistical groupings indicated by letters (Tukey's test, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

in root biomass (−8.6%) and root length (−4.4%) compared with T0.

Moderate declines were recorded with Opuntia compost alone (T4), which resulted in 8.7% shorter plants (Fig. 6), 5.4% fewer leaves, 7.9% lower shoot biomass, 10.0% reduced root biomass, and 5.8% shorter roots relative to the control.

Fig. 6 - Effect of treatment T5 (Aloe + Opuntia compost) compared to T3 (Aloe compost) on the height of Rosmarinus plants.

In contrast, the Aloe-only treatments (T2 and T3) consistently produced the poorest growth. T2 reduced plant height by 11.5%, leaf number by 7.3%, leaf area by 10.5%, shoot biomass by 14.3%, root biomass by 20.7%, and root length by 14.6%. T3 showed even greater reductions, with 14.4% shorter plants, 9.0% fewer leaves, 13.2% smaller leaf area, 17.9% lower shoot biomass, 19.3% less root biomass, and 11.2% shorter roots compared with T0. These findings confirm that compost composition substantially affects both above- and below ground growth. Mixed or commercial composts provided a better nutrient balance and structural benefits, whereas Aloe-only composts were insufficient to support optimal root and shoot development.

Physiological parameters

Rosmarinus officinalis exhibited physiological responses similar to *Salvia officinalis* under different compost treatments (Figs. 7-8).

The SPAD index reached its maximum in control (T0, 47), indicating optimal chlo-rophyll content. T1 and T5 showed only 4-6% lower SPAD values, while Aloe-only treatments (T2 and T3) recorded the lowest readings (38), representing a 19% decline relative to T0 (Fig. 7A).

Net photosynthetic rate (Pn) peaked in T0 (31 µmol CO_2 m^{-2} s^{-1}). T1 and T5 main-tained near-optimal photosynthetic rates with only 3-6% reductions, whereas T2 and T3 exhibited the lowest rates (24 µmol CO_2 m^{-2} s^{-1}), corresponding to a 22.6% decline com-pared with the control (Fig. 7B).

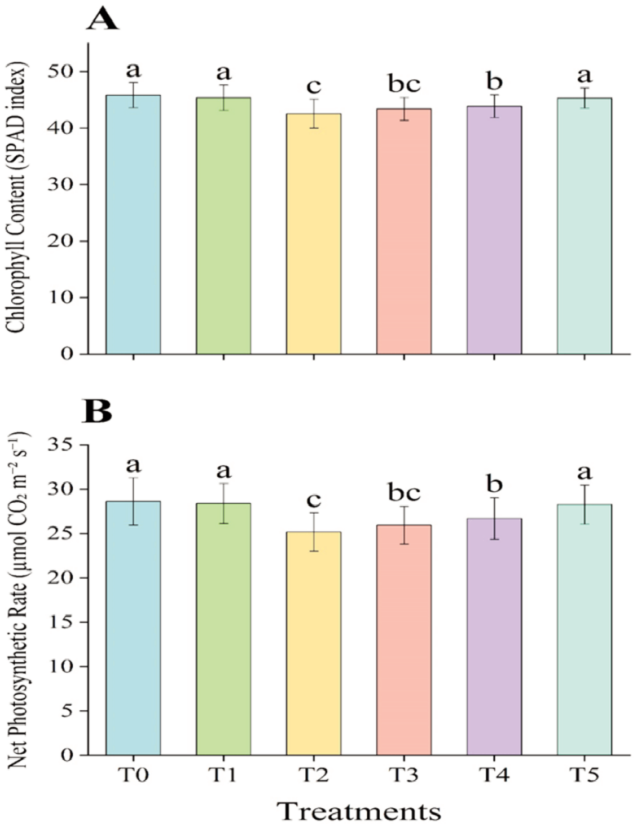

Fig. 7 - Chlorophyll content SPAD Index (A), and Photosynthetic Rate (B) in *Rosmarinus officinalis* under different compost treatments. Note: Bars represent mean values (n = 4) with ±SD. Letters above the bars denote statistically different groups (Tukey's HSD, $p<0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

Stomatal conductance (Gs) followed the same trend. T0 showed the highest con-ductance (0.55 mol H_2O m^{-2} s^{-1}), while T1 (0.50; −9%) and T5 (0.49; −11%) showed mod-erate reductions. T2 and T3 decreased Gs to 0.42 mol H_2O m^{-2} s^{-1}, a 23.6% reduction compared with T0 (Fig. 8A), suggesting limited stomatal aperture and reduced tran-spiration.

Interestingly, water-use efficiency (WUE) showed a slight advantage for the mixed compost. T5 achieved the highest WUE (2.9), a 3.6% improvement over T0 (2.8), while T1 maintained a similar efficiency

(2.7; −3.6%). T2 and T3 recorded the lowest WUE (2.3), a 17.9% decline relative to T0 (Fig. 8B).

These findings confirm that commercial (T1) and mixed composts (T5) sustain chlorophyll synthesis, gas exchange, and photosynthesis close to control levels, while Aloe-only composts significantly compromise physiological performance. Notably, the mixed compost (T5) enhanced WUE, indicating potential benefits under water-limited conditions.

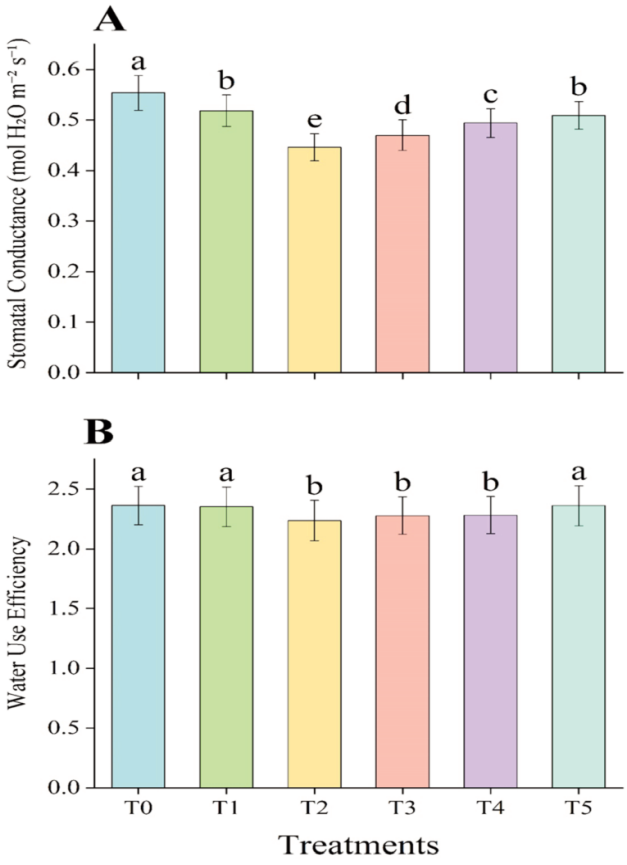

Fig. 8 - Stomatal conductance (A), and Water Use Efficiency (B) in *Rosmarinus officinalis* under different compost treatments. Note: Bars represent mean values (n = 4) with ±SD. Letters above the bars denote statistically different groups (Tukey's HSD, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

Metabolite content and essential oil yield

Compost treatments significantly affected secondary metabolite production and soil microbial biomass in *Rosmarinus officinalis* (Table 5). The control substrate (T0) yielded the highest phenolic content (32.0 ± 0.7 mg GAE g^{-1}) and flavonoid content (17.5 ± 0.6 mg QE g^{-1}).

The mixed Aloe-Opuntia compost (T5) performed slightly lower than the control, with −9.4% phenolics and −11.4% flavonoids, yet it was statistically superior to single-source composts. Opuntia compost alone (T4) followed a similar trend with −14.1% phenolic reduction compared with T0. In contrast, Aloe-only composts (T2 and T3) produced the lowest metabolite levels, reducing phenolic content by 18.8% (T2) and 23.4% (T3), and flavonoids by 22.9% and 28.6%, respectively, relative to the control.

Antioxidant activity, expressed as IC_{50}, showed the expected inverse trend, in which lower IC_{50} values indicate stronger antioxidant capacity. The control treatment (T0) exhibited the highest antioxidant activity (48 ± 1.3 µg mL^{-1}), followed by T1 (50 ± 1.4 µg mL^{-1}). The mixed compost (T5) showed moderate antioxidant performance (52 ± 1.4 µg mL^{-1}), whereas the Aloe-only treatments, particularly T3 (58 ± 1.0 µg mL^{-1}), recorded the weakest antioxidant responses. Thus, T3 showed a 20.8% reduction in antioxidant efficiency relative to T0, confirming that Aloe-only composts were less effective in promoting antioxidant metabolism in rosemary.

Essential oil yield followed a similar pattern. T0 produced the highest yield (1.7 ± 0.1%), while T5 (1.5 ± 0.1%) and T4 (1.4 ± 0.1%) showed modest declines (11.8% and 17.6% reductions, respectively). The Aloe-only treatments resulted in the lowest oil content, with T3 yielding only 1.2 ± 0.1%, a 29.4% reduction relative to the control.

Soil microbial biomass was highest under T0 (264.0 ± 20.7 µg C g^{-1}) but remained relatively high under T5 (249.6 ± 19.2 µg C g^{-1}; −5.5%). Aloe-only treatments significantly reduced microbial biomass, with T2 decreasing by 13.1% and T3 by 10.9% compared with the control (Table 5). No significant differences were observed in pest or disease incidence across treatments in either species. Preventive biological treatments (*Beauveria bassiana* and *Trichoderma harzianum*) maintained pest populations below damage thresholds throughout the trial. Thus, compost influence appeared limited to plant growth and metabolism rather than pest suppression. Collectively, these results indicate that mixed or *Opuntia*-based composts can partially sustain metabolite synthesis, antioxidant capacity, and essential oil production, whereas Aloe-only composts significantly compromise both plant biochemical quality and soil microbial activity.

Table 5 - Metabolite content in *Rosmarinus officinalis*

Treatment	Phenolic content (mg GAE g^{-1})	Flavonoids (mg QE g^{-1})	Antioxidant activity (IC_{50} µg mL^{-1})	Essential oil yield (%)	Microbial biomass (µg C/g soil)
T0	32.0 ± 0.7 a	17.5 ± 0.6 a	48 ± 1.3 c	1.7 ± 0.1 a	264.0±20.7 a
T1	30.5 ± 0.6 b	16.5 ± 0.7 a	50 ± 1.4 bc	1.6 ± 0.1 ab	252.1±22.5 b
T2	26.0 ± 0.7 e	13.5 ± 0.7 bc	56 ± 1.2 a	1.3 ± 0.1 bc	229.4±18.5 e
T3	24.5 ± 0.5 f	12.5 ± 0.7 bc	58 ± 1.0 a	1.2 ± 0.1 c	235.3±20.3 de
T4	27.5 ± 0.5 d	14.5 ± 0.8 ab	54 ± 1.2 ab	1.4 ± 0.1 bc	242.2±17.8 cd
T5	29.0 ± 0.7 c	15.5 ± 0.7 a	52 ± 1.4 ab	1.5 ± 0.1 ab	249.6±19.2 bc

Values are mean ± SD with statistical groupings indicated by letters (Tukey's test, $p < 0.05$). Treatments: T0= Control; T1= Commercial; T2= *Aloe barbadensis*; T3= *Aloe arborescens*; T4= Opuntia; T5= Mixed.

Principal component and correlation analysis

The principal component analysis (PCA) of morpho-physiological and biochemical traits in *Salvia officinalis* and *Rosmarinus officinalis* under different compost treatments revealed distinct clustering patterns among treatments and species (Fig. 9A). The first two principal components (PCs) explained 95.1% of the total variance, with PC1 accounting for 91.2% and PC2 contributing 3.9%. PC1 was positively associated with water-use efficiency (WUE), chlorophyll content (Chl), root length (RL), evapotranspiration (ET), root biomass (RB), stomatal conductance (gs), and net photosynthetic rate (Pn). In contrast, plant height (PH), leaf area (LA), leaf number (LN), shoot biomass (SB), essential oil yield (EOY), total phenolic content (TPC), and total flavonoid content (TFC) were negatively loaded on PC1. PC2 was predominantly defined by antioxidant activity (AOA), indicating its distinct response relative to other traits.

Control treatments (T0) clustered toward the positive axis of PC1, indicating superior physiological performance, whereas T2 and T3 treated plants shifted toward the negative side, showing higher accumulation of secondary metabolites such as TPC, TFC, and EOY. Moreover, *Rosmarinus officinalis* tended to group closer to the positive PC1 axis, reflecting its relatively higher photosynthetic efficiency compared with *Salvia officinalis* under the same compost regimes.

Correlation analysis (Fig. 9B) further supported the PCA findings, showing strong positive correlations ($r > 0.80$, $p < 0.001$) among growth-related parameters such as (PH), LN, LA, SB, and RB, demonstrating their coordinated response to compost amendments. Photosynthetic parameters (Pn, gs, ET, and WUE) were also significantly

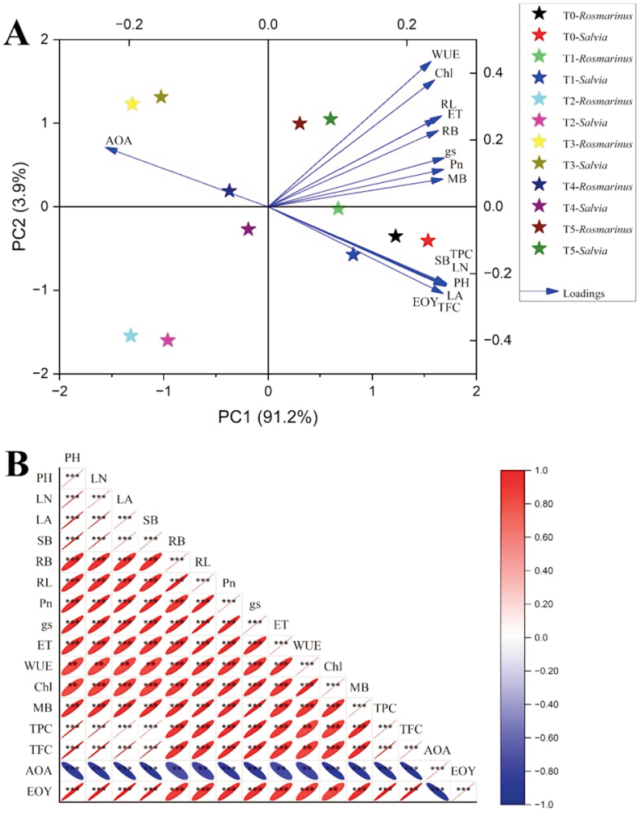

Fig. 9 - Principal component and correlation analysis of morpho-physiological and biochemical traits in *Salvia officinalis* and *Rosmarinus officinalis* under different compost treatments. (A) Principal component analysis (PCA) biplot showing the distribution of treatments and species along the first two principal components (PC1 = 91.2%, PC2 = 3.9%) with trait loadings indicated by blue arrows. (B) Pairwise correlation matrix of growth, physiological, and biochemical traits. The color scale indicates correlation coefficients (red = positive, blue = negative), with significance levels denoted as (*p ≤ 0.05, **p ≤ 0.01, **p ≤ 0.001).

correlated with Chl and biomass accumulation (MB), highlighting the role of compost in enhancing photosynthetic efficiency and growth. Conversely, secondary metabolite traits (TPC, TFC), AOA, and EOY

were positively interrelated but showed significant negative correlations ($r < -0.60$, $p < 0.01$) with growth and photosynthetic parameters, suggesting a trade-off between vegetative growth and secondary metabolite production.

Notably, AOA exhibited the strongest negative correlation with WUE, Chl, and MB, indicating that antioxidant defenses were enhanced under conditions limiting photosynthetic performance. Furthermore, TPC and TFC displayed a positive relationship with EOY, emphasizing their role in essential oil biosynthesis under compost-induced stress conditions.

4. Discussion and Conclusions

This study highlights the influence of compost composition on the growth, physiological behaviour and secondary metabolism of *Salvia officinalis* and *Rosmarinus officinalis* grown under controlled conditions in a greenhouse. While the peat-based control substrate (T0) consistently produced the highest levels of growth and metabolites, the Aloe-Opuntia mixed compost (T5) showed considerable potential as a renewable and environmentally sustainable alternative, despite its slightly lower agronomic performance. The results provide important insights into how specific compost raw materials, particularly plant species with contrasting biochemical profiles, influence substrate quality, plant physiology and secondary metabolic pathways (Muscolo *et al*., 2018; Veliu *et al*., 2025). The superior performance of the control substrate (T0) is not unexpected, given the well-documented physical advantages of peat, including optimal aeration, drainage, high porosity and a stable supply of nutrients (Zhang *et al*., 2025). These properties promote optimal root development, efficient water use and robust photosynthetic activity, which in turn lead to increased biomass production and secondary metabolite synthesis (Pan *et al*., 2025). As peat-based substrates continue to be the industry standard for container-grown aromatic and medicinal crops, they provide a natural benchmark for evaluating the effectiveness of alternative amendments (Atzori *et al*., 2021). However, environmental concerns related to peat extraction, including habitat destruction, carbon emissions and lack of renewability, necessitate the search for more sustainable substrate options (Räsänen *et al*., 2023; Patel *et al*., 2025).

In this context, the Aloe-Opuntia mixed compost (T5) emerged as the best performing organic soil amendment among those tested. Although it did not match T0 in terms of absolute growth, its performance remained relatively close, with reductions in vegetative traits generally ranging between 5 and 8%. Importantly, T5 consistently outperformed all other compost-based treatments in terms of agronomic, physiological, and biochemical parameters. This suggests that mixing aloe and opuntia biomass generates a substrate with more favourable physical and chemical characteristics than composts derived from a single raw material (Sortino *et al*., 2024). The improved performance of T5 is likely attributable to the complementary nature of its components. Opuntia residues are rich in mucilaginous polysaccharides, known to increase water retention and improve the moisture buffering capacity of the substrate (Semerel *et al*., 2023; Bacchetta *et al*., 2024). This effect helps maintain stable hydration around the root zone, minimising water-related stress fluctuations commonly experienced in organic substrates and facilitating more controlled stomatal behaviour. Meanwhile, aloe residues contribute bioactive compounds such as polysaccharides, phenols, and glycoproteins that can function as plant biostimulants, improving nutrient uptake efficiency, osmotic regulation, and tolerance to minor environmental stressors (Prisa and Gobbino, 2021). These combined effects likely contributed to the greater physiological stability observed in T5, including higher chlorophyll content, improved stomatal conductance, and greater water use efficiency compared to other compost-based substrates (Read and Gregory, 1997). In contrast, aloe-only composts (T2 and T3) resulted in the weakest plant performance, despite their relatively high nutrient content. This highlights the importance of distinguishing between total nutrient concentration and actual agronomic suitability. Several factors may explain the limited effectiveness of aloe-based composts (Jaramillo *et al*., 2025). First, both T2 and T3 showed the highest electrical conductivity values in the experiment (1.7-1.8 dS m^{-1}). It is well known that high salinity can limit water absorption by roots, reduce photosynthetic capacity and compromise stomatal function, reactions that are particularly evident in aromatic species native to Mediterranean regions (Tong *et al*., 2025). Therefore, the high EC may have caused physiological stress that overshadowed the potential benefits of the available

nutrients (Atiyeh *et al*., 2000).

Secondly, aloe biomass contains high levels of labile organic compounds, including mucilage, organic acids and simple sugars, which decompose rapidly and can temporarily create anaerobic microsites, organic acid accumulation and excessive microbial respiration (Maliehe *et al*., 2023; Di Palma *et al*., 2025). Such fluctuations can stress root systems, reduce oxygen availability, and create transient nutritional imbalances. This rapid decomposition dynamic is characteristic of monospecific composts derived from succulent plant material and has previously been associated with inconsistent nutrient release and reduced substrate stability (Gruber *et al*., 2013).

Thirdly, aloe species contain biologically active metabolites such as aloin, haemodin and anthraquinones. Although composting reduces their concentration, residual levels may still affect root physiology. Some studies report inhibitory effects of secondary compounds derived from aloe on seedling root elongation and early plant development (Radha and Laxmipriya, 2014). This may partly explain the reduction in root biomass and shorter root length observed in treatments with aloe-only compost (Tawaraya *et al*., 2007).

Finally, the physical structure of aloe tissue is characterised by low fibre content and high water content, which can result in compost with poor porosity, limited aeration and reduced structural stability. When used as the main component of the substrate, such composts can create compact or excessively moist environments, limiting root growth and compromising gas exchange (Dehouche *et al.*, 2020). The moderate performance of the Opuntia-only compost (T4) offers further insight into the influence of specific raw materials. Opuntia residues improved several metabolic characteristics and led to moderate but not optimal vegetative growth (Auteri *et al*., 2025). This pattern suggests that, although Opuntia contributes to favourable moisture retention, microbial stimulation and nutrient cycling, its physical and chemical properties alone are not sufficient to match the agronomic efficiency of mixed compost (Matheri *et al*., 2023). This reinforces the idea that substrate quality benefits from the biochemical and structural diversity of mixed raw materials, which improve nutrient release dynamics, microbial diversity, and physical stability (Naguib *et al*., 2012). Secondary metabolism was strongly influenced by substrate type. For both species, T0 produced the highest levels of phenols, flavonoids, and essential oils, likely due to the overall higher vigour of the plants and carbon assimilation. T5, although slightly lower than the control, consistently ranked above the other compost treatments, indicating that mixed compost maintained a physiological environment favourable to secondary metabolite production (Montoya *et al*., 2022). The performance of T5 suggests that mixed composts can support plant metabolic activity without inducing severe stress that would reduce secondary metabolite biosynthesis. In contrast, aloe-only composts showed the lowest accumulation of metabolites. Again, this may reflect the negative physiological effects of high EC and rapid decomposition dynamics, which impose sufficiently severe stress to limit carbon assimilation and reduce the metabolic resources available for secondary metabolite pathways (Biyada *et al*., 2022; Sarwari *et al*., 2024).

Importantly, none of the compost treatments adversely affected plant survival or increased susceptibility to pests or diseases. This suggests that all composts were sufficiently mature and free of phytotoxicity, supporting their potential use in organic greenhouse systems (Hadar and Papadopoulou, 2012). The absence of disease incidence is consistent with the well-known benefits of high-quality composts in suppressing soil-borne pathogens through competitive exclusion, microbial antagonism, and improved soil structure. Finally, the implications of these findings in terms of sustainability are significant (Pane *et al*., 2019). Aloe and opuntia are drought-resistant species widely cultivated in arid and Mediterranean regions, producing significant by-products during pruning and processing (Liontakis and Tzouramani, 2016). The reuse of this biomass in compost is in line with the principles of circular agriculture, reducing waste and providing growers with renewable, low-input substrate alternatives. Although peat remains agronomically superior, mixed aloe and opuntia composts represent a promising step towards more sustainable greenhouse production of aromatic crops (Campos *et al*., 2025).

Future research should aim to further characterise the microbial communities associated with each type of compost, as interactions between microbes and plants are increasingly recognised as determinants of nutrient availability, stress tolerance and secondary metabolite synthesis (Aguilar-Paredes *et al*., 2023;

Gil-Martínez and Madejón, 2025). Field validation across multiple seasons and environments would further strengthen the practical relevance of these findings. Furthermore, technical-economic analyses would help determine the feasibility of large-scale compost production using aloe and opuntia residues (Pergola *et al*., 2020; Gil-Martínez and Madejón, 2025).

The results of this research work confirmed that compost composition impacts the vegetative growth, physiological properties, and secondary metabolism of *Salvia officinalis* and *Rosmarinus officinalis* cultivated in a greenhouse environment. The control treatment containing peat as the growing substrate (T0) consistently provided greater growth and higher levels of phytochemical accumulation, indicating that it is a suitable growing medium for the conditions of this study. The mixed Aloe and Opuntia compost treatment (T5) produced less vegetative growth and phytochemical accumulation than the control treatment (T0) but had higher growth and phytochemical accumulation than the other compost treatments. This indicates that mixing plant residues can help lessen some of the disadvantages associated with using a compost consisting solely of one species of plant residue. While the differences seen between treatments should be approached cautiously, it is evident that more replicated trials in a variety of seasons/locations are needed to verify the clarity of the observed yield trends in this study, particularly for T5 moderate effectiveness and the lesser yielding ability of aloe-only composts. Although currently, peat (T0) is thought to be the best substrate, mixed plant-based composts such as T5 potentially present the opportunity for a more sustainable alternative. Their value lies not in the fact that they produce greater agronomic performance, but rather that they are renewable and consequently less harmful to the environment. When supported with further validation and additional levels of detailed evaluation, this research line may assist in the creation of environmentally sustainable composter for the farming of aromatherapy and medicinal crops.

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DNA barcoding-based assessment of genetic variation in selected Southwest Nigerian medicinal *Senna* species (Caesalpinoideae: Fabaceae)

OPEN ACCESS

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Abstract: *Senna* species in Southwest Nigeria possess significant medicinal and economic potential yet remain underutilized. This study aimed to employ DNA barcoding to characterize twenty *Senna* accessions and assess their genetic variation. Seeds from twenty accessions comprising six species (*S. hirsuta, S. obtusifolia, S. alata, S. siamea, S. acutifolia,* and *S. occidentalis*) were collected from Southwest Nigerian states. Fresh leaf samples underwent DNA extraction, PCR amplification of the psbA-trnH intergenic spacer region, and Sanger sequencing. Molecular analyses using Bioedit, MEGA 11, and NCBI BLAST yielded high-quality DNA sequences (312-414 bp). BLAST searches confirmed species identities with exceptional accuracy. Multiple alignments revealed inter-specific variations and genus-specific conserved regions. Genetic distance analysis showed moderate diversity (0.000-0.074) between species. Base composition analysis revealed characteristic A-T rich patterns: thymine showed highest variance, followed by adenine, while cytosine and guanine exhibited lower variation. Conserved sequences provided molecular evidence of monophyletic relationships within *Senna*, while base composition variations reflected evolutionary divergence and environmental adaptation. These findings establish a molecular framework for the identification and conservation of Nigerian Senna species, supporting their development for medicinal and agricultural applications.

1. Introduction

Senna Mill is a genus of plants belonging to the subfamily Caesalpinioideae of the family Fabaceae. The genus name was derived from the Arabic sanā, describing plants whose leaves and pods have cathartic and laxative properties (Monkheang, 2011). Originally, Hutchinson (1964) moved the subfamilies (Caesalpinioideae, Papilionioideae, and Mimosoideae) to the rank of families and placed them in order Leguminales. This division was primarily based on the

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characteristics of the corolla and the stamens (Dutta, 2001; Judd *et al*., 2002). However, more recent phylogenetic studies of many legume groups have unambiguously demonstrated the inadequacies of the tribal classifications proposed by Polhill and Raven (1981), Polhill (1994), and Lewis *et al*. (2005) because of the non-monophyly of most of the traditionally recognised tribes (LPWG, 2013). Currently, based on the phylogenetic structure of the family Leguminosae, the Legume Plant Working Group (LPWG, 2017) recognised six sub-families within Leguminosae (Fabaceae), and Caesalpinioideae was included.

The genus *Senna* (Fabaceae) contains around 300 species distributed pantropically across the Americas, Africa, Asia, and Oceania. The key morphological features include the lack of bracteoles on floral pedicels, clavoform to pyramidal extrafloral nectaries, and indehiscent fruits appearing as cylindrical pods or compressed legumes (Correia and Conceição, 2017). The genus is divided into six sections: Astroites, Chamefistula, Paradictyon, Peiranisia, Psilorhegma, and Senna (Irwin and Barneby, 1982).

In Nigeria, three *Senna* species hold particular ethnomedicinal and pharmacological significance. *Senna alata*, known as «Asunwon oyinbo» by the Yoruba, treats typhoid, diabetes, malaria, asthma, and skin infections (Oladeji *et al*., 2020), with decoctions used across regions for wounds, respiratory infections, and diarrhea. *Senna occidentalis* («Sanga-sanga» in Hausa, «Akidiagbara» in Igbo) serves as an antimalarial (Daskum *et al*., 2019) and remedy for hepatitis and liver diseases (Ibrahim *et al.,* 2022). *Senna siamea* leaves combine with *Carica papaya* and *Cymbopogon citratus* to combat malaria fever (Adewole *et al*., 2024). Pharmacological studies confirm antimicrobial (Oladeji *et al*., 2020; Tamasi, 2021), antimalarial (Daskum *et al*., 2019), hepatoprotective (Ibrahim *et al*., 2022), antioxidant properties (Atanu *et al.,* 2022), and multiple activities including antibacterial, antifungal, anticancer, and antidiabetic effects, attributed to secondary metabolites (Oladeji *et al.,* 2020).

DNA barcoding is one of the molecular techniques employed in recent times to resolve new evolutionary and taxonomical queries in plant diversity studies (Arif *et al.,* 2010). Molecular identification of plant species currently plays a key role in the assessment of conserving biodiversity especially in the phase of many plant species going into extinction (de Boer *et al*., 2022). DNA barcoding is a technique for characterizing species of organisms using a short DNA sequence from a standard and agreed-upon position in the genome (Kress and Erickson, 2008; Arif *et al.,* 2010; de Vere, 2015; Kenfack *et al.*, 2022). Accurate species identification is a prerequisite for conducting numerous basic and applied studies on monitoring and conserving natural resources, blocking the traffic of endangered and invasive species, as well as controlling the quality of pharmaceutical and food products. In this context, molecular markers are indispensable tools for measuring the diversity of plant species (Pang *et al*., 2012).

The *trnH-psbA* intergenic spacer region is a non-coding region located between the trnH and psbA genes in the chloroplast genome and described as a diagnostic barcode in distinguishing plant species. The trnH-psbA intergenic spacer is widely recognized as the most commonly used non-coding universal genetic marker in plant DNA barcoding and molecular phylogenetic studies, due to its high variability across plant species (CBOL, 2009; Hao *et al*., 2010; Hollingsworth *et al*., 2011; Monkheang *et al*. 2011; Pang *et al*., 2012; Feng *et al*., 2018; Loera-Sanchez *et al*., 2020; Hassan, 2023). Hassan (2023) evaluated a DNA-based assay that examined the resolution and sensitivity of the trnH-psbA intergenic spacer region as a DNA barcoding marker. Their findings suggest that these genetic markers could provide a novel method for understanding the evolutionary relationships and classification of closely related *Prunus* species.

The trnH-psbA region is widely used for plant DNA barcoding due to its variability and ease of amplification, offering significant discriminatory power for species identification and inferring genetic relationships (Damthongdee *et al*., 2024). It has been crucial in resolving inter-specific relationships and identifying cryptic diversity among closely related taxa (Hussain *et al*., 2019). In Fabaceae, trnH-psbA and other chloroplast markers (matK and rbcL) effectively resolve generic and species-level relationships, particularly in regions with inadequate molecular datasets (Bruneau *et al*., 2013). Although *Senna* is underrepresented in molecular studies from Nigeria, analogous research in legumes has established the importance of generating barcoding data for conservation planning and sustainable use.

Research on medicinal and economically valuable plants across Africa and Asia demonstrates that

integrating trnH-psbA data provides a robust framework for taxonomy, phylogeny, biogeographic tracing, and population structure analysis. Comparative chloroplast genome studies including the trnH-psbA region have identified mutational hotspots that serve as informative molecular markers for species discrimination and phylogenetic inference (Dong *et al*., 2021).

DNA barcoding in medicinal legumes like *Flemingia* and *Sophora* effectively resolves relationships in understudied regions, establishing baselines for population genetics and evolutionary studies (Duan *et al*., 2025; Wei *et al.,* 2025 b). Sub-Saharan investigations using DNA barcoding have revealed substantial intra-specific genetic diversity correlated with ecological and geographical factors (Akinro *et al*., 2019). A DNA barcode dataset for Nigerian *Senna* species could similarly uncover cryptic diversity and inform both in situ and ex situ conservation strategies.

Generating trnH-psbA molecular data from Nigerian *Senna* species would enable taxonomic clarification and accurate species identification (Zhao *et al*., 2021), baseline data for phylogeographic and population genetic analyses to identify unique genetic resources (Shi *et al.*, 2022), and reference barcodes supporting enforcement against adulteration and overharvesting for sustainable utilization (Wei *et al*., 2025 a).

Some molecular markers have been used for phylogenetic studies in *Senna* species (Mao *et al.,* 2017; Eldemerdash *et al*., 2022; Azeez *et al*., 2024). Previous studies of *Senna* in Nigeria examined seven species but with fewer accessions per species (Azeez *et al.,* 2024). However, studies have not reported the use of trnH-psbA intergenic spacer region in the barcoding of members of species of *Senna* in Nigeria. This study employs DNA barcoding using the trnH-psbA intergenic spacer to characterize twenty *Senna* accessions in Southwest Nigeria and assess their genetic diversity and phylogenetic relationships, addressing the region's rich biodiversity and the economic significance of these medicinal plants.

2. Materials and Methods

Plant data collection

Field collection of accessions of *Senna* species seeds was undertaken at various locations within the senatorial districts of Ogun, Oyo, and Ekiti States, South Western Nigeria. The coordinates of the sites are specified in Table 1. The collected *Senna* seeds included: six accessions of *Senna hirsute*, six accessions of *S. obtusifolia*, two accessions of *S. alata*, an accession of *S. siamea* and *S. acutifolia*, and four

Table 1 - Senatorial districts and coordinates of Senna species studied

S/N	Plant species	Senatorial district	State	Coordinate latitude N	Coordinate longitude E
1	*Senna hirsuta*	Ekiti south	Ekiti	7°37'30.94068"	3°54'39.70728"
2	*S. acutifolia*	Ekiti south	Ekiti	7°33'10.00332"	3°53'56.193"
3	*S. obtusifolia*	Ekiti south	Ekiti	7°32'56.45976"	3°53'41.00172"
4	*S. obtusifolia*	Ogun west	Ogun	6°52'46.18484"	3°11'27.08412"
5	*S. hirsuta*	Ogun west	Ogun	6°52'52.64400"	3°11'25.26170"
6	*S. occidentalis*	Ogun west	Ogun	6°53'15.486"	3°11'43.86688"
7	*S. hirsuta*	Oyo south	Oyo	7°23'45.7944"	3°51'29.93256"
8	*S. occidentalis*	Oyo south	Oyo	7°23'57.80292"	3°51'24.4152"
9	*S. hirsuta*	Ekiti north	Ekiti	7°33'5.02812"	3°53'47.51628"
10	*S. obtusifolia*	Ekiti north	Ekiti	7° 33'16.6554"	3°54'8 .35236"
11	*S. obtusifolia*	Ogun east	Ogun	7°37'30.89712"	3°54'40.14396"
12	*S. alata*	Ogun east	Ogun	7° 37'30.89712"	3°54'40.14396"
13	*S. hirsuta*	Ogun east	Ogun	7°37'24.73536"	3°54'50.53608"
14	*S. obtusifolia*	Oyo north	Oyo	7°35'30.2928"	3°53'37.221"
15	*S. hirsute*	Oyo north	Oyo	7°35'30.2766"	3°53'37.17384"
16	*S. occidentalis*	Oyo north	Oyo	7°33'12.3408"	3°54'0.36756"
17	*S. obtusifolia*	Ogun central	Ogun	7°13'54.57288"	3°26'1.9345"
18	*S. alata*	Ogun central	Ogun	6°52'46.18484"	3°11'2.08412"
19	*S. occidentalis*	Oyo town	Oyo	7°35'30.282"	3°53'37.35348"
20	*S. siamea*	Ekiti south	Ekiti	7°933'8.87796"	3°53'54.1878"

accessions of *S. occidentalis*. The collected seeds of each accession were planted in 10 L planting pots filled with humus soil and wet daily at the experimental plot of CAEDESE Federal University of Agriculture Abeokuta. Fresh leaves were collected from each plant accession and taken to Inqaba Biotec West Africa in Ibadan for DNA extraction and quantification, PCR amplification, and Sanger sequencing.

DNA extraction

Genomic DNA was extracted from each of the fresh leaf tissues of *Senna hirsute* (6), *S. obtusifolia* (6), *S. alata* (2), *S. siamea* and *S. acutifolia*, and *S. occidentalis* (4), following the protocols of Quick-DNA Plant/Seed Kits (Zymo Research, Catalogue No.D6020, California USA).

DNA quantification

The quality and quantity of the extracted DNA were measured using a nanodrop (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer). The system was blanked using 2 µL of DNA Elution Buffer. Afterwards 2 µL of the DNA was placed on the pedestal and measured. The concentration (ng/µL), A260/280 ratio and A260/230 ratio of the sample were noted.

PCR amplification

PCR total reaction volume was optimised to 25 µL using PCR master mix 12.5 µL (according to One Taq Quick-Load 2X Master Mix) (NEB, Catalogue No. M0486), 0.5 µL of 10 µM forward and reverse primers, 10.5 µL Nuclease-free water, and 1 µL genomic DNA as a template for the reaction. The reaction mixture was votexed and spun, then placed in the thermal cycler. PCR were performed by subjecting the samples to thermal cycling conditions following Eppendorf Mastercycler nexus gradient 230: initial denaturation at 95°C for 5 minutes followed by 35 cycles, denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute and 68°C for 90 secs with a final 10 mins extension step at 68°C and hold at 4°C. The primer sequences used for the amplification of trnH-psbA include:

psbA: 5'-GTTATGCATGAACGTAATGCTC-3' (Sang *et al*., 1997);

trnH: 5'-CGCGCATGGTGGATTCACAATCC-3' (Tate and Simpson, 2003).

Gel electrophoresis

After PCR amplification, 2 µL of PCR product was run on 1% agarose gel, stained with 1 µL of safe view Ethidium Bromide (EtBr) (10 mg/ml), and photographed using a gel documentation system (E-BOX, Vilber Lourmat, Italy). PCR products (amplicons) were cleaned using an enzymatic method (ExoSAP).

Sanger sequencing

The fragments were sequenced using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions (https://www.nimagen.com/products/Sequencing/Capillary-Electrophoresis/BrilliantDye-Terminator-Cycle-Sequencing-Kit/). The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up-Kit (Catalogue No. D4053). The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50 cm array, using POP7 (https://www.thermofisher.com/order/catalog/product/4406016, and sequence data collected).

Molecular data analysis

The sequences obtained were quality checked and edited using FinchTV version 1.4.0. The alignments were edited with BioEdit sequence alignment editor version 7.2.5 (Hall, 1999). The evolutionary history was inferred using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) according to Sneath and Sokal (1973). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed according to Felsenstein (1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The quantitative assessment of genetic relationships among the trnH-psbA genes of *Senna species* was done using genetic distance matrix. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are expressed in units of the number of base substitutions per site. This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 409 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

3. Results

Considerable variations were revealed among the DNA sequences of members of *Senna* species studied when they were aligned using the Multiple ClustalW algorithm in Bioedit software (Fig. 1). There is a significant variable region at the start of the sequence from positions 1 to 35. Specific variation in plant sample sequences 8_PSBA and 9_PSBA was observed at position 23; a small variable region occurs at positions 71 and 72, and a single variable position at 165. Meanwhile, a longer stretch of variable sequences was observed at the regions 151 to 165, and a large variable region at regions 200-

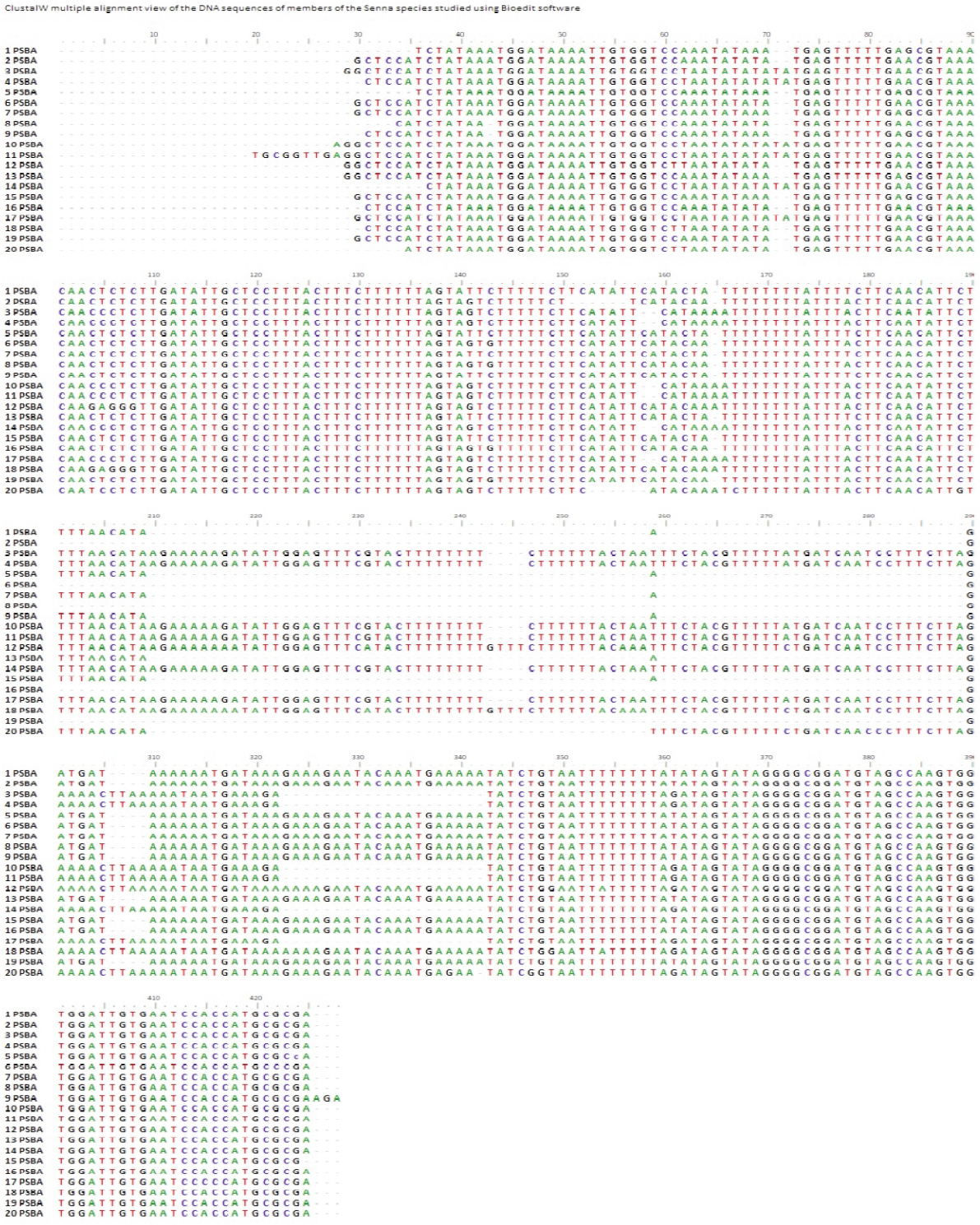

Fig. 1 - ClustalW multiple alignment of the DNA sequences of members of Senna species studied using Bioedit software.

289. Another substantial variable region is at positions 306 to 343, and lastly, variations are seen at the end of the sequence (positions 426 and 427). This implies the analyzed *Senna* species are unique individual species that exhibit significant variation or divergence.

Conserved regions identified in the DNA sequence alignment (Fig. 2) indicate shared genetic similarities among the *Senna* species, providing molecular evidence for their taxonomic grouping within the genus. They represent regions of DNA that have remained unchanged or very similar across different species within the genus *Senna*.

BLAST description of hits studied using MEGA software

The results from the Basic Local Alignment Search Tool (BLAST), a bioinformatics tool used to compare nucleotide or protein sequences against a sequence database and determine the statistical significance of matches, are presented in Table 2. The base pair lengths for the aligned DNA sequences of *Senna* species studied range from 312 to 414 bp. The results of the database sequences show very high-quality matches overall, with percent identity ranging from 99.02% to 100%, query coverage between 95-100%, and very low E-values (ranging from 0.0 to 4e-155).

Fig. 2 - Muscle multiple alignment of the DNA sequences of members of Senna species studied using MEGA software.

Table 2 - BLAST description of hits from the National Centre for Biotechnology Information (NCBI) and sequences producing significant alignments

S/N	Plant sample sequence	Base pair	Description of matched organism	Scientific name	Max score	Total score	Query cover %	E value	% Identity	Accession length	Alignment score	GenBank Accession
1	1_PSBA	323	*Senna hirsutaPsbA gene, partial cds*	*Senna hirsuta*	568	568	96	2,00E-157	99.68	349	>200	KC150886.1
2	2_PSBA	312	*Senna occidentalis chloroplast, complete genome*	*Senna occidentalis*	560	560	99	4,00E-155	99.35	159994	>200	OR478159.1
3	3_PBSA	390	*Senna tora voucher JKTM-1-000064 tRNA-His (trnH) gene*	*Senna tora*	710	710	98	0	100	426	>200	KP058332.1
4	4_PBSA	388	*Senna tora voucher JKTM-1-000064 tRNA-His (trnH) gene*	*Senna tora*	706	706	99	0	99.74	426	>200	KP058332.1
5	5_PSBA	323	*Senna hirsutaPsbA gene, partial cds*	*Senna hirsuta*	580	580	97	3,00E-161	100	349	>200	KC150886.1
6	6_PSBA	318	*Senna occidentalis isolate SCBGP509_1 photosystem II*	*Senna occidentalis*	568	568	99	2,00E-157	99.05	339	>200	KP095340.1
7	7_PBSA	329	*Senna hirsutaPsbA gene, partial cds*	*Senna hirsuta*	588	588	97	2,00E-163	100	349	>200	KC150886.1
8	8_PBSA	313	*Senna occidentalis isolate SCBGP509_1 photosystem II*	*Senna occidentalis*	547	547	97	3,00E-151	99.02	339	>200	KP095340.1
9	9_PSBA	330	*Senna hirsutaPsbA gene, partial cds*	*Senna hirsuta*	575	575	96	1,00E-159	99.37	349	>200	KC150886.1
10	10_PSBA	391	*Senna tora voucher JKTM-1-000064 tRNA-His (trnH) gene*	*Senna tora*	708	708	99	0	99.74	426	>200	KP058332.1
11	11_PBSA	399	*Senna tora voucher JKTM-1-000064 tRNA-His (trnH) gene*	*Senna tora*	706	706	96	0	99.74	426	>200	KP058332.1
12	12_PBSA	414	*Senna alata chloroplast, complete genome*	*Senna alata*	747	747	99	0	99.51	159176	>200	NC_065665.1
13	13_PSBA	330	*Senna hirsutaPsbA gene, partial cds*	*Senna hirsuta*	582	582	95	9,00E-162	100	349	>200	KC150886.1
14	14_PSBA	382	*Senna tora voucher JKTM-1-000064 tRNA-His (trnH) gene*	*Senna tora*	697	697	99	0	99.74	426	>200	KP058332.1
15	15_PBSA	328	*Senna hirsutaPsbA gene, partial cds*	*Senna hirsuta*	593	593	98	4,00E-165	100	349	>200	KC150886.1
16	16_PBSA	317	*Senna occidentalis isolate SCBGP509_1 photosystem II*	*Senna occidentalis*	568	568	99	2,00E-157	99.36	339	>200	KP095340.1
17	17_PSBA	389	*Senna tora voucher JKTM-1-000064 tRNA-His (trnH) gene,*	*Senna tora*	704	704	99	0	99.48	426	>200	KP058332.1
18	18_PSBA	412	*Senna alata chloroplast, complete genome*	*Senna alata*	747	747	100	0	99.51	159176	>200	NC_065665.1
19	19_PBSA	318	*Senna occidentalis isolate SCBGP509_1 photosystem II*	*Senna occidentalis*	573	573	99	5,00E-159	99.68	339	>200	KP095340.1
20	20_PBSA	352	*Senna siamea chloroplast, complete genome*	*Senna siamea*	638	638	98	2,00E-178	100	148437	>200	MN525772.1

This indicates highly significant matches between the query (aligned DNA sequences of *Senna species* studied) and that of the database with a consistent alignment score of >200. Notable matches occurred in KC150886.1 accession, which appears multiple times as a top match (plant codes PSBA 1, 5, 7, 9, 13, 15). KP058332.1 is another common match (plant codes PSBA 3, 4, 10, 11, 14, 17). Two plant codes (PSBA 12 and 18) matched to NC_065665.1, with the longest accession length of 159,176 bp. Best matches were observed in PBSA 3, 5, 7, 15, and 20, they achieved 100% identity with their matches. PSBA 12 and 18 had the highest max scores (747) while PSBA 18 achieved 100% query coverage. These results suggest that the sequences are very well conserved, and the sequencing quality was high. The consistent high scores and low E-values indicate that these matches are highly reliable or significant and not due to chance. The plant codes were identified as *Senna hirsute, S. occidentalis, S. tora, S. alata,* and *S. siamea* among the matches. A taxonomically significant observation emerged where field-identified *S. obtusifolia* and *S. acutifolia* accessions returned *S. tora* and *S. occidentalis* identifications, respectively.

Genetic distance matrix of the DNA sequences based on trnH-psbA intergenic spacer regions of members of Senna species studied

Estimates of the evolutionary divergence between DNA sequences of various members of the *Senna* species are presented in Table 3. The genetic distance values range from 0.000, indicating no

divergence, to a higher divergence value 0.074, which suggests significant evolutionary differences. This variation provides insights into how closely related or distantly related these species are. The lack of divergence was observed between the same species (PSBA 10 and 11; 1 and 13, 1 and 15, and others) irrespective of their locations. Higher divergence value observed between PSBA 1 and 17; 5 and 17 implies they are more distantly related as depicted also in the phylogenetic tree. Low divergence values observed range from 0.017 (between PSBA 2 and 13; 2 and 15) to 0.030 (between 10 and 17 PSBA). Divergence values were consistently high across all comparisons with 12 PSBA (*S. alata*). 5PSBA showed the greatest divergence (0.073), while 20 PSBA exhibited the lowest divergence (0.05). This range of 0.05- 0.073 suggests significant phylogenetic differentiation between the plant accessions. This implies that *S. alata* is distantly related to all other members of *Senna* species studied. Notwithstanding, it is more closely related or have identical DNA sequences with *S. alata* (18PSBA) from another location.

The phylogenetic tree analysis, using UPGMA based on the trnH-psbA intergenic spacer region, depicting four clades, is presented in figure 3. It shows the evolutionary relationships between and among different *Senna* species studied. *S. hirsuta*

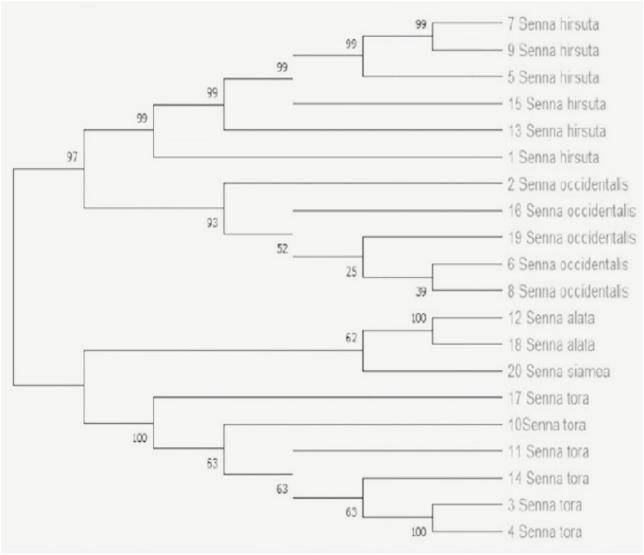

Fig. 3 - Phylogenetic relationships among members of Senna species studied using UPGMA based on trnH-pbsA gene sequences, depicting four clades.

Table 3 - Evolutionary divergence between DNA sequences based on trnH-psbA intergenic spacer regions of members of Senna species studied

	1	10	11	12	13	14	15	16	17	18	19	2	20	3	4	5	6	7	8	9
1																				
10	0.07																			
11	0.07	0																		
12	0.073	0.06	0.06																	
13	0	0.068	0.068	0.071																
14	0.07	0	0	0.062	0.07															
15	0	0.069	0.069	0.072	0	0.071														
16	0.021	0.057	0.057	0.06	0.02	0.058	0.021													
17	0.074	0.003	0.003	0.063	0.072	0.003	0.072	0.06												
18	0.073	0.06	0.06	0	0.072	0.062	0.072	0.06	0.064											
19	0.021	0.056	0.056	0.06	0.02	0.058	0.02	0	0.06	0.06										
2	0.018	0.054	0.054	0.057	0.017	0.055	0.017	0.003	0.058	0.057	0.003									
20	0.067	0.061	0.061	0.05	0.067	0.061	0.067	0.053	0.065	0.05	0.053	0.051								
3	0.07	0	0	0.06	0.068	0	0.069	0.057	0.003	0.06	0.056	0.054	0.061							
4	0.07	0	0	0.06	0.069	0	0.069	0.057	0.003	0.06	0.057	0.054	0.061	0						
5	0	0.07	0.07	0.073	0	0.071	0	0.021	0.074	0.073	0.021	0.018	0.067	0.07	0.07					
6	0.024	0.06	0.06	0.063	0.024	0.062	0.024	0.003	0.064	0.063	0.003	0.007	0.057	0.06	0.06	0.024				
7	0	0.069	0.069	0.072	0	0.07	0	0.02	0.072	0.072	0.02	0.017	0.067	0.069	0.069	0	0.024			
8	0.021	0.057	0.057	0.061	0.021	0.058	0.021	0	0.061	0.061	0	0.003	0.054	0.057	0.057	0.021	0.003	0.021		
9	0	0.069	0.069	0.072	0	0.071	0	0.021	0.073	0.072	0.021	0.017	0.067	0.069	0.069	0	0.024	0	0.021	0

1= *Senna hirsute*, 2= *S. occidental*, 3= *S. tora*, 4= *S. tora*, 5= *S. hirsute*, 6= *S. occidentalis*, 7= *S. hirsute*, 8= *S. occidentalis*, 9= *S. hirsute*, 10= *S. tora*, 11= *S. tora,* 12= *S. alata*, 13= *S. hirsute*, 14= *S. tora*, 15= *S. hirsute*, 16= *S. occidentalis*, 17= *S. tora*, 18= *S. alata*, 19= *S. occidentalis,* 20= *S. siamea.*

forms one distinct clade (PSBA 7, 9, 5, 15, 13, 1), *S. occidentalis* forms another clade (PSBA 2, 16, 19, 8, 6), *S. tora* clusters together (PSBA 10, 11, 14, 3, 4) while *S. alata* (PSBA 12, 18) and S. *siamea* (PSBA 20) form smaller groups. Bootstrap values reveal high confidence values (97-100%) at many major branching points. Some lower values (25-63%) indicate less certain relationships in those branches. The 99% values in the *S. hirsuta* clade suggest very strong confidence in the relationship within the taxa, and also support for the phylogenetic groupings while 39-52% bootstrap values in the *Senna occidentalis* clade indicate uncertainty and weak support for phylogenetic groupings. The tree shows clear separation between major species. Two main branches were observed: one is leading to *S. hirsuta/S. occidentalis* and another to *S. alata/S. siamea/S. tora*. Terminal branches with the same species accessions indicate recent common ancestry.

Nucleotide composition of some members of Senna species DNA sequences studied

The frequencies of each of the Nucleotide compositions and their total means in the DNA sequences of the members of the *Senna species* studied using MEGA 11 software are presented in Table 4. The Base composition patterns show Thymine has the highest variance ranging from 35.8% (2 PSBA) to 40.5% (14 PSBA), while Adenine has the second highest variance ranging from 31.0 % (11 PSBA) to 35.0% (5 and 8 PSBA). Cytosine ranges from 12.5% to 14.1%, and Guanine shows less variation ranging from 14.6% (4 PSBA) to 16.2% (20 PSBA). The mean total of Thymine, Cytosine, Adenine and Guanine is 38.1, 13.3, 33.3 and 15.4%, respectively, and the grand mean total for nucleotide composition for the twenty DNA sequences is 333.9%. Total A+T content is 71.4% (33.3% A + 38.1% T) consistently across all sequences while, total G+C content is 28.7% (15.4% G + 13.3% C). These imply that the nucleotide composition shows an AT-rich bias for *Senna species.* Sequence length varies from the shortest with 293 bases (2 PSBA) to the longest with 395 bases (12 PSBA). Notable length clusters were observed in ~300 bases (PSBA 1,2,5,6,8,16,19) and ~370 bases (PSBA 3,4,10,14,17).

Table 4 - Nucleotide frequencies of the DNA sequences of members of Senna species studied

Plant code	T (U)	C	A	G	Total
1 PSBA	37.5	12.5	34.9	15.1	304
2 PSBA	35.8	13.3	34.8	16	293
3 PSBA	40.2	13.7	31	15.1	371
4 PSBA	40.4	13.8	31.2	14.6	369
5 PSBA	37.6	12.5	35	14.9	303
6 PSBA	36.1	13.4	34.8	15.7	299
7 PSBA	37.1	13.2	34.5	15.2	310
8 PSBA	36.4	12.6	35	16	294
9 PSBA	37	13.2	34.7	15.1	311
10 PSBA	40.1	13.7	31.2	15.1	372
11 PSBA	40	13.7	30.5	15.8	380
12 PSBA	38.2	12.7	33.4	15.7	395
13 PSBA	37	13.2	34.4	15.4	311
14 PSBA	40.5	13.2	31.4	14.9	363
15 PSBA	37.2	13.3	34.3	15.2	309
16 PSBA	36.2	13.1	34.9	15.8	298
17 PSBA	40.3	14.1	30.8	14.9	370
18 PSBA	38.4	12.7	33.6	15.3	393
19 PSBA	36.1	13	34.8	16.1	299
20 PSBA	36.9	13.8	33	16.2	333
Mean	38.1	13.3	33.3	15.4	333.9

All frequencies are given in percent (%).
T (U)-Thyamine (Uracil), C- Cytosine, A- Adenine and G-Guanine.

4. Discussion and Conclusions

The gaps and variations observed in the ClustalW multiple alignments of the DNA sequences of members of the *Senna* species studied indicate genetic differences between the *Senna* species. The presence of multiple variable gene positions suggests these species have diverged enough to accumulate distinct genetic changes. The variations observed could represent different evolutionary paths, adaptations to different environments, and species-specific genetic characteristics. ClustalW identified these variations through multiple sequence alignment and the gaps indicate where insertions or deletions (indels) have occurred in some species relative to others. The presence of both short (single position) and long (spanning multiple positions) variable regions suggests a complex evolutionary history. Hassan (2023) suggested that trnH-psbA intergenic spacer regions as DNA barcoding markers could provide a novel method for understanding the evolutionary relationships and classification of closely related *Prunus* species. On the contrary, Olsson *et al*. (2022) observed the non-suitability of matK and psbA-trnH for species identification and phylogenetic analysis in closely related pines.

The BLAST result provides a strong molecular evidence for accurate species identification (99.02-100%) and high-quality genetic data, essential for taxonomic and evolutionary studies of *Senna* species using trnH-psbA intergenic spacer region. There is significant length polymorphism in the trnH-psbA chloroplast DNA region, with sequences ranging from 312 to 414 base pairs across the studied *Senna* species*;* Hassan (2023) also observed a varied length of base pairs in his study. The sequence length variation may be due to evolutionary studies in *Senna*. The psbA-trnH sequence lengths obtained in this study (312-414 bp) align closely with Indian populations (341-384 bp), where this marker demonstrated higher sequence length variation compared to conserved coding regions like rbcL (682/705 conserved sites) and matK (726/785 conserved sites) (Mishra *et al*., 2018).

The significant evolutionary differences observed between DNA sequences based on trnH-psbA intergenic spacer regions of various members of *Senna* species studied signify genetic divergence, which allows them to adapt to specific local environmental conditions. As populations evolve separately, they can develop unique traits that enhance their survival and reproductive success in distinct habitats (Chung *et al*., 2023). Higher genetic divergence typically correlates with increased genetic variation within a species. This variation is essential for adaptability, as it provides a broader pool of traits that can be selected for in response to changing environmental conditions. Genetic divergence can also influence phenotypic plasticity, the ability of a single genotype to produce different phenotypes in response to varying environmental conditions. Populations that have diverged genetically may exhibit different levels of plasticity, affecting how they respond to environmental changes. The lack of divergence between the same species collected from different locations may perhaps imply they are more closely related and that their sequences are not affected by changes in the environmental conditions. It confirms the existence of conserved regions in the sequence alignment.

The genetic distance among the studied *Senna* species (0.000-0.074) indicates moderate diversity, lower than that reported in other continental studies. Asian *Senna* species show substantially higher genetic variation, with Indian *S. italica* displaying intra-specific divergence of 0.77-16.03% and *S. auriculata* and *S. tora* sharing 0.14% maximum identity (Mishra *et al*., 2018). Analysis of 14 Thai *Senna* species using the psbA-trnH region demonstrated successful species discrimination comparable to our findings (Monkheang *et al*., 2011), validating the marker's utility across geographic populations.

The phylogenetic tree reveals evolutionary intra-specific and inter-specific diversity, and relationships in the species studied. This is demonstrated as *S. hirsuta* appears to be the most divergent group. *S. tora* shows internal variation but clear species cohesion, while *S. alata* and *S. siamea* appear to be more closely related to each other than to other species. The genetic distinctness observed in *S. hirsuta* accessions forms a well-supported clade with high bootstrap values (99%). This shows a significant genetic differentiation from other *Senna* species and occupies a distinct position at the top of the phylogenetic tree. The internal structure contains multiple subgroups (PSBA 7, 9, 5 vs. 15, 13, 1) which shows higher intra-genetic variation compared to other species and demonstrates a complex evolutionary history within the species. Evolutionarily, it may represent an earlier divergence from other *Senna species* or suggest longer independent evolutionary history. More so, it could indicate adaptation to different ecological niches and possibly might have undergone more extensive genetic changes over time.

Moreover, previous Nigerian studies from Ile-Ife using matK and rbcL markers (546-843 bp) reported similar phylogenetic relationships, particularly the close association between *S. occidentalis* and *S. hirsuta* (Azeez *et al*., 2024), corroborating our findings. Azeez *et al.* (2024) recorded chromosome counts of x=18 in *S. podocarpa* and *S. obtusifolia*, x=20 in *S. occidentalis*, x=22 in *S. alata* and *S. hirsuta*, and x=24 in *S. siamea* and *S. sophera*, demonstrating karyotypic diversity within Nigerian populations. However, the comparatively lower genetic diversity in Southwest Nigerian populations relative to Asian counterparts suggests recent colonization events, geographic isolation, or adaptation to uniform environmental conditions within the study region.

Notwithstanding, the intra-variation observed in the *S. tora* (PSBA 3, 4, 10, 11, and 14) show some genetic differences among them. This is seen in the slight branching patterns within the *S. tora* clade. Bootstrap values of 63% between some *S. tora* accessions indicate minor genetic differences. Despite these differences, all *S. tora* cluster together

in one distinct group. They form a monophyletic clade (sharing a common ancestor). There is a clear separation from other *Senna* species and strong bootstrap support (100%) for some branches. Genetic distances between *S. tora* accessions are relatively small. This pattern suggests that *S. tora* maintains its distinct species identity, shows normal population-level genetic diversity, all accessions are correctly identified as *S. tora* and natural variation exists within the species but not enough to question species boundaries. Thus, the phylogenetic tree has aided in resolving evolutionary relationships within the genus. It also supports the genetic distance data and provides a clear visual representation of the evolutionary relationships and divergence patterns within these *Senna* species. The closeness of *S. alata* and *S. siamea* might suggest species with potentially similar medicinal properties.

In contrast, the *S. occidentalis* clade displayed moderate to low bootstrap values (39-52%), these lower confidence values suggest greater intra-specific diversity within *S. occidentalis* studied, possibly reflecting multiple introduction events from different source populations, longer establishment history allowing accumulation of mutations, or potential historical gene flow with related species. The bootstrap values below the conventional 70% threshold indicate that additional molecular markers (ITS, matK, rbcL) are needed to clarify relationships within this clade and test for possible cryptic diversity or reticulate evolution.

Additionally, the nucleotide frequencies explain the phylogenetic relationships earlier discussed, with sequence length and base composition differences potentially reflecting evolutionary divergence patterns. The characteristic A-T richness (71.4%) observed in this study is consistent with typical chloroplast intergenic spacer regions globally, reflecting conserved evolutionary patterns within the genus. The A-T rich pattern provides pbsA-trnH intergenic spacer region as a valuable molecular markers for species identification and supports the taxonomic classification of these *Senna* species (Ravi *et al*., 2008). This is evidence in 99-100% BLAST identity, clear separation of all species and high confidence (99-100% bootstrap for *S. hirsute*). Functionally, A-T richness influences DNA stability and melting temperature, and may impact DNA replication efficiency.

Furthermore, the moderate genetic diversity observed in the *Senna* species (0.000-0.074) reflects adaptive evolutionary responses to environmental pressures (Alzahrani *et al*., 2020), enabling plants to adapt to climatic fluctuations (Li *et al*., 2022), though lower diversity compared to Asian species may constrain responses to rapid environmental changes (Mishra *et al*., 2018). Genetically diverse *Senna* species contain important metabolites with diverse pharmacological activities (Elbashir *et al*., 2021), and intra-specific genetic variation suggests potential variation in sennoside concentrations among Nigerian accessions similar to Indian studies (Kumar *et al*., 2024).

Meanwhile, the moderate genetic divergence observed may reflect evolutionary adaptation within similar ecological zones across Southwest Nigeria, contrasting with broader environmental gradients in Asian populations, and while psbA-trnH provides adequate species discrimination, future phylogenetic studies could benefit from multi-locus approaches combining this marker with nuclear markers to enhance resolution of population structure and evolutionary relationships (Mishra *et al*., 2018).

Nonetheless, market analysis in India revealed substantial adulteration in *Senna* herbal products (Seethapathy *et al*., 2015), highlighting the importance of DNA barcoding for authentication, while successful discrimination of Thai *Senna* species using trnH-psbA fragment polymorphism (Monkheang *et al.,* 2011) demonstrates the practical application of this marker for rapid species identification across geographic regions.

In addition, the presence of Shared conserved sequences provide molecular evidence for the monophyletic nature of *Senna* within Fabaceae subfamily Caesalpinioideae, validating genus-level separation from *Cassia* sensu stricto established by Irwin and Barneby (1982), while genetic distances (0.000-0.074) reveal moderate inter-specific divergence consistent with species-level differentiation and corroborate previous Nigerian studies documenting karyotypic diversity (x=18 to x=24) and the close relationship between *S. occidentalis* and *S. hirsuta* (Azeez *et al*., 2024), demonstrating concordance between cytogenetic, molecular, and morphological evidence characteristic of integrative taxonomy.

The amplified psbA-trnH intergenic spacer region reveals significant variations among the DNA sequences of *Senna* species studied. The presence of shared conserved sequences provides the molecular evidence of the monophyletic nature of these taxa

within *Senna*, while base composition variations reflect evolutionary divergence and adaptation of these species to different environmental pressures. These findings provide the molecular framework for Nigerian *Senna* species identification and conservation, supporting their potential development for medicinal and agricultural applications.

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The combined inoculation of *Trichoderma harzianum* and *Pseudomonas putida* as a microbial consortium enhances the growth and yield of pepper (*Capsicum annuum* L.)

OPEN ACCESS

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Abstract: A sustainable agricultural method to increase crop yield and reduce dependence on chemical inputs is the use of beneficial microbial species as bioinoculants, especially *Trichoderma* spp., *Pseudomonas* spp. and microbial consortia. This study evaluated the impact of single and combined inoculations of *Trichoderma harzianum* ITEM 3636 and *Pseudomonas putida* PCI2 on pepper plants in field assays. Fruits from consortium-inoculated plants were larger than those from single-strain treatments or uninoculated controls, showing pronounced increases in fruit width and fruit length. Additionally, yield gains were highest under consortium inoculation, whereas individual strain inoculations resulted in intermediate responses. These results suggest the potential use of the consortium as an effective bioinoculant for increasing pepper yield. An initial qualitative assessment of soil microbial taxonomic profiles was carried out. Results showed that root-associated soil from individual inoculations showed higher abundances of one dominant fungal taxon compared to the remaining taxa, in contrast to dual inoculation that displayed a more balanced fungal distribution. Also, bacterial taxa were more balanced in inoculated soils compared to the control. Overall, our work reports on a useful microbiological tool to improve sustainability in horticultural systems.

1. Introduction

The greatest economic importance of pepper (*Capsicum annuum* L.)

lies in the commercialization of its fruits, which are rich in provitamin A, vitamin B, vitamin C, and minerals such as calcium, phosphorus, potassium, and iron (Maboko *et al.,* 2012). This crop is among the seven most important vegetables in the world, with an estimated annual production of more than 30 million tons. It is consumed fresh, cooked, or as a condiment or «spice» in typical foods of various countries, and also in a range of industrial products (Barik *et al.*, 2022).

In order to meet the increased demand for food caused by the growing world population, crop production together with agricultural sustainability must rise. Plant growth-promoting microorganisms (PGPM) are a practical technology that can increase plant production, provide disease defense and have beneficial effects on agriculture (Vishwakarma *et al.*, 2020). Protection from pathogens, reduction of drought effects and stimulation of nutrient uptake are just a few of the many benefits that PGPM can provide. Additionally, microbial management can support the development of climate change resistant systems (Aguilar-Paredes *et al.*, 2020). The urge to look for natural methods that lessen the use of chemicals in agriculture is what has sparked interest in environmentally friendly solutions in contemporary agriculture. The employment of advantageous microorganisms together in consortia is extremely promising for enhancing crop productivity and quality, representing a dependable and eco-friendly approach that may address the issues for modern agriculture (Tabacchioni *et al.*, 2021).

Trichoderma plant symbionts live in a variety of environments, such as the rhizosphere and plant tissues (as endophytes). The use of several *Trichoderma* strains as biocontrol agents against phytopathogenic bacteria is very common (Abdullah *et al.*, 2021). *Trichoderma* spp. are also widely known for being important plant growth-promoting fungi (PGPF), as they boost the growth, development and yields of a variety of crops (Stewart and Hill, 2014; Abdullah *et al.*, 2021; Tyśkiewicz *et al.*, 2022). In prior investigations, we observed that strain ITEM 3636 of *T. harzianum* protected peanut plants against smut, caused by *Thecaphora frezii*. Additionally, *T. harzianum* ITEM 3636 increased crop yield without substantially altering the structure of soil microbial communities (Ganuza *et al.*, 2018; Ganuza *et al.*, 2019).

A group of bacteria known as plant growth promoting rhizobacteria (PGPR) interacts closely with the roots of plants to affect plant health and soil fertility. They provide a fantastic blend of qualities that are advantageous for promoting plant growth and controlling diseases. With their rapid growth, straightforward nutritional needs, capacity to utilize a variety of organic substrates, and mobility, fluorescent pseudomonads have emerged as the most prevalent and possibly the most promising group of PGPR. Numerous characteristics of *Pseudomonas* species make them suitable for use as PGPR. They are also a common type of bacteria in agricultural soils (Dorjey *et al.*, 2017). We isolated *Pseudomonas putida* PCI2 from the rhizosphere of a healthy tomato plant. *P. putida* PCI2 proved to be positive for phosphatase activity, solubilizes a high amount of P in Sperber broth medium and promotes tomato growth (Pastor *et al.*, 2014).

Microorganisms associated with plants have a significant positive impact on agricultural productivity. Several experiments have been conducted on inoculating seeds or plants with microorganisms, either individually or in consortia, to aid in the growth and development of plants. Even though multiple studies have shown that a single microorganism can benefit plants, it is becoming abundantly clear that when two or more microorganisms are participating in a microbial consortium, additive or synergistic effects can be anticipated (Vishwakarma *et al.*, 2020; Tabacchioni *et al.*, 2021). Agriculture can benefit from microbial consortia, which include bacteria and fungi combinations, in several ways. They can reduce the need for chemical pesticides by successfully controlling plant diseases and pathogens. Microbial consortia components can work together in a synergistic way to boost crop productivity and prevent disease in a more thorough and effective manner. By utilizing microbial consortia, farmers might support sustainable agricultural practices and reduce their reliance on chemical fertilizers. Since microbial consortia can affect the nutrient cycle and soil health, their effects on soil microbial populations should also be taken into account (Pastor *et al.*, 2023). Diverse microbial consortia (with different bacterial or fungal compositions) are regularly being released on the market as beneficial products. Thus, the impact of such formulations on agronomic and qualitative performance requires evaluation in open field experiments (Fusco *et al.*, 2023). In a previous study, we tested *T. harzianum* ITEM 3636 and *P.*

putida PCI2, alone and combined, for their effects on growth and yield of tomato under field conditions and observed that inoculation with the microbial consortium resulted in increases in yield, compared to single inoculation and the control treatment (Pastor *et al.*, 2024).

The effects of inoculating pepper plants during transplantation to the field with a *Trichoderma* strain and a *Pseudomonas* strain, alone or combined, have not been extensively studied. Duc *et al.* (2017), for instance, explored the impact of arbuscular mycorrhizal fungi alone or combined with *Trichoderma* and plant growth-promoting bacteria on defense enzymes and yield in the field. Additionally, Chemeltorit *et al.* (2017) tested a *Trichoderma* strain combined with a *Pseudomonas aeruginosa* strain in peppers, but with biocontrol purposes against *Phytophthora capsici*. The goal of this research was to assess the effectiveness of *T. harzianum* ITEM 3636 and *P. putida* PCI2, alone and combined, to promote pepper growth and fruit yield in a field setting.

2. Materials and Methods

Plant cultivar

Pepper seeds from the Fyuco INTA variety were used. The seeds were superficially disinfected using a 70% ethanol solution for 1 minute, followed by a 2% NaOCl solution for another minute. After disinfection, the seeds were washed with sterile distilled water (SDW) 8 times. The disinfected seeds were placed one per cell in trays containing a sterile soil:perlite mixture (2:1). To guarantee proper germination, trays were covered with a transparent nylon bag. Germination trays were placed in a growth chamber under controlled cycles of 14 h of light at 25°C and 10 h of dark at 20°C. The nylon bags were taken off once the seedlings had germinated, and they were maintained in the same environment until field tests.

Microorganisms

T. harzianum ITEM 3636, originally isolated from soil cropped with peanuts by researchers from the Plant Pathology Department, at UNRC, was deposited at the Institute of Toxins and Mycotoxins from Plant Parasites (Rojo *et al.*, 2007). The fungus was maintained in 15% glycerol at -80°C. To create the *T. harzianum* ITEM 3636 inocula, 7-day-old cultures on Petri plates with malt extract agar (MEA), at 28°C, were used. Each of the 10 plates was covered with 10 mL of SDW before the conidia suspensions were collected by scraping the surfaces of the cultures with a sterile glass spatula. The filtrates were obtained by filtering the suspensions through four layers of sterile gauze and the inoculum density was adjusted to 1 x 10^5 conidia mL^{-1} by adding SDW and counting with a hemocytometer. *P. putida* PCI2 was regularly cultivated on King's B medium at 28°C (King *et al.*, 1954) or Tryptic Soy Broth (TSB) and preserved at -20°C in TSB amended with 20% (v v^{-1}) glycerol. Bacterial cells were centrifuged after being incubated in liquid medium and then resuspended in sterile 0.9% saline solution to the adequate concentration (1 x 10^6 CFU mL^{-1}).

Field assays. Location of the assays

The field assays were conducted at the experimental field belonging to the National University of Río Cuarto, Córdoba, Argentina (33°06'26.0'' S, 64°17'52.5'' W) during the summer seasons of 2021-2022 and 2023-2024. Two days prior to transplanting, plots were abundantly watered to minimize tress and ensure good hydration of plants. The soils where seedlings were transplanted into during the experiments had, on average, a pH of 6.3, and 2.6% organic matter, 0.026 $mg \cdot g^{-1}$ of extractable phosphorus, 0.072 $mg \cdot g^{-1}$ of nitrates, 0.014 $mg \cdot g^{-1}$ of sulphates, 0.15% of total nitrogen, 7 meq 100 g^{-1} soil of Ca^{++}, 3 meq 100 g^{-1} soil of Mg^{++}, 0.12 meq 100 g^{-1} soil of Na^{+} and 1.8 meq 100 g^{-1} soil of K^{+}.

Treatments and plant handling

Raised ridges were prepared and then, 4-week-old plants were transplanted into double raised ridges (rows) with a spacing of 20-30 cm between plants and 70-100 cm between rows. Each treatment's surface area on the test plots was 8-10 m^2. The following treatments were tested: (1) immersion of roots in SDW (Control); (2) immersion of roots in a suspension of ITEM 3636 conidia; (3) immersion of roots in a suspension of PCI2 cells (4) immersion of roots in a mixed suspension containing ITEM 3636 conidia + PCI2 cells (Consortium). At the time of transplant, seedlings were inoculated by immersion of roots for 1 min in the corresponding single suspension or mixture. The concentration used for *Trichoderma* was 10^5 conidia mL^{-1} and that for *Pseudomonas* was 10^6 cfu mL^{-1}. SDW was applied to the roots of control seedlings. To supplement the water acquired by precipitation and meet the

summertime water needs of plants, a furrow irrigation system was implemented. In order to keep the plants adequately turgid and hydrated during the whole growth cycle, they were watered at regular intervals. After ransplanting, weeds were removed manually. No chemical herbicides were applied. Additionally, neither chemical fungicides nor insecticides were used. Plants from all treatments received a unique dose of 150 g (per furrow between rows) of a commercial fertilizer (TRIPLE 15), applied 1 month after transplanting. The fertilizer was incorporated through successive irrigations and with the water from precipitations. The composition of the applied fertilizer was as follows: nitrogen (15%), phosphorus (15%), Sulfur (3%), Calcium (6%), and potassium (15%). In experiment 1, fruits were collected when mature and plants (15 per treatment) were harvested at 120 days post transplanting (DPT) to the field. Assessments included: length and width of peppers, yield, dry weights of shoots, dry weights of roots and lengths of roots. In experiment 2, fruits were collected when mature and plants (20 per treatment) were harvested at 140 DPT. The parameters evaluated were: size of fruits, fruit yield, shoot area (using *ImageJ*), shoot dry weight and root dry weight.

Qualitative analysis of soil microbial profiles

Five plants were randomly selected and safely removed from their respective growing rows during harvest. Soil samples, including pits and soil detached from roots, were then collected from those zones. These samples were combined into 500 g samples using polyethylene bags, and promptly stored at 4 °C. For the following procedures, one sample per treatment was used. Following the guidelines and recommendations given, the commercial Highway® DNA PuriPrep-SOIL kit (K1210-50) was used to extract DNA from 250 mg of soil for each sample. After lyophilization, soil DNA samples were shipped to CD Genomics (Shirley, NY) for sequencing, PCR amplification, purification, and DNA library creation. The ITS1-1F-F and ITS1-1F-R primers were used to examine the spectrum of fungi, while a primer set that amplifies the hypervariable regions V3 and V4 of the 16S rRNA gene was used to investigate bacterial profiles. The Illumina Mi-Seq platform (Illumina Inc., San Diego, CA, USA) was used to sequence the amplicons. For the following bioinformatic analysis, the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software tool was utilized (Bolyen *et al.*, 2019; Estaki *et al.*, 2020).

Statistical analyses

All statistical analyses were conducted using R, *version 4.1.2*. Analyses of data from the field assays were performed using ANOVA, and differences were calculated using the Fisher's least significant difference (LSD) test ($P \leq 0.05$). The Kruskal-Wallis test was used when necessary.

3. Results

Field assays. Experiment 1

Plants were kept in the field for 120 DPT. At harvest, plants from all the treatments were collected, removing them individually being careful so as not to damage their root systems. Growth parameters were measured. There were statistically significant differences in shoot dry weight between treatments. The control presented the lowest average value, while inoculation with the consortium showed the highest. However, there was no statistically significant difference in root weight between the treatments. Plants inoculated with ITEM 3636 alone showed the highest values, generating an average value of 11.4 g. The rest of the treatments presented very similar values, slightly lower than those from ITEM 3636. Lastly, we found that the consortium inoculation resulted in the highest average root length value (19.3 cm). The control and PCI2 inoculation showed the lowest root length values, which did not differ significantly (Table 1).

Statistically significant differences were observed between treatments for average length of peppers. Peppers from plants treated with PCI2 showed the

Table 1 - Growth parameters of inoculated pepper plants from field assay 1

Treatment	Shoot dry weight (g)	Root dry weight (g)	Root length (cm)
Control	28.1 b	10.3 a	13.6 c
ITEM 3636	37.2 ab	11.4 a	17.3 ab
PCI2	40.7 a	10.7 a	14.5 bc
Consortium	44.5 a	10.4 a	19.3 a

The LSD test indicates that the mean values from each column with a distinct letter are significantly different ($P \leq 0.05$). Control= non-inoculated plants; PCI2= inoculated with *P. putida* PCI2 alone; ITEM 3636= inoculated with *T. harzianum* ITEM 3636 alone.

highest mean value (133.1 mm). The other treatments had values ranging between 115 and 121 mm and did not differ significantly. For fruit width, the treatment with the highest mean value was inoculation with the consortium. The other treatments did not present significant statistical differences between them. We also observed statistically significant differences between conditions regarding yield. Inoculation with the consortium generated the highest average value. The control and inoculation with ITEM 3636 alone presented very similar values, lower than inoculation with PCI2 alone and with the consortium, although without significantly differing from the first (Table 2).

Table 2 - Effect of *T. harzianum* ITEM 3636, P. putida PCI2 and their combination on pepper fruits and yield in field assay 1

Treatment	Length of peppers (mm)	Width of peppers (mm)	Yield (g/plant)
Control	120.6 b	77.2 b	352.3 b
ITEM 3636	115.3 b	77.8 b	299.7 b
PCI2	133.1 a	73.6 b	565.1 ab
Consortium	120.9 b	85.2 a	631.2 a

Significance in the differences between groups was assessed using the Fisher's LSD test. A different letter within each column indicates a significant difference between treatments at the P≤0.05 level.

Field assays. Experiment 2

We measured the shoot area at 60 DPT. It was observed that all variants of inoculation resulted in higher values compared with the control treatment. Additionally, the values obtained from plants inoculated with single microorganisms were higher at the time of measurement (Fig. 1A). Figure 1A also shows the average number of fruits per plant at that time. As shown, the highest average value was obtained from the inoculation with PCI2 treatment while the lowest was from the control. The other treatments showed intermediate average values. For shoot dry weight, no statistically significant differences were recorded between treatments at 140 DPT, the time of measurement after harvest (Fig. 1B). On the other hand, statistically significant differences were observed between treatments for average root dry weight. Inoculation with ITEM 3636 caused the highest value and the control the lowest (Fig. 1B). For the length of fruits, it was observed that peppers from plants inoculated with the

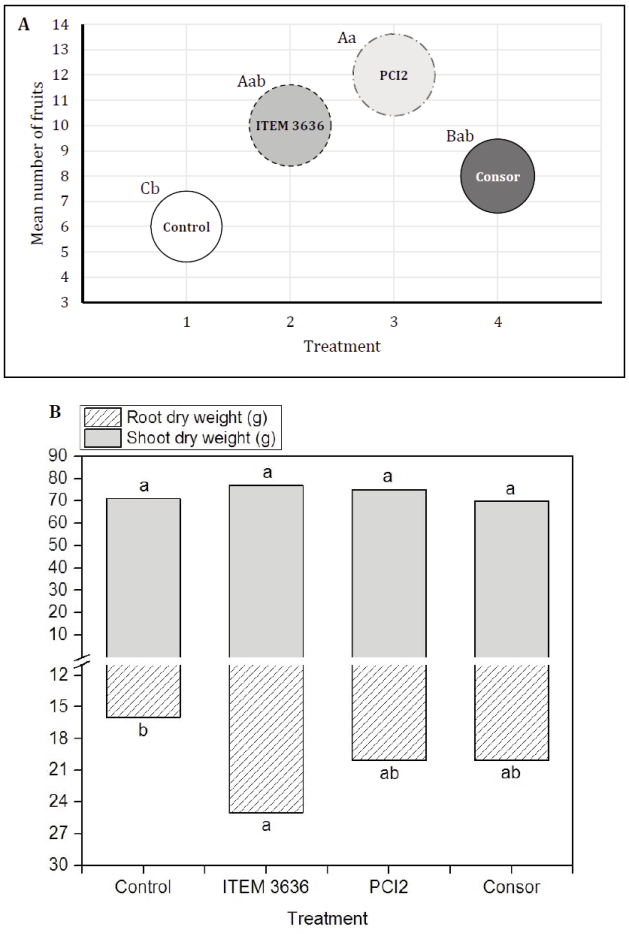

Fig. 1 - Growth parameters of inoculated pepper plants during experiment 2. (A) Bubbles with a different capital letter and size have a significantly different shoot area according to the LSD test (P≤0.05); Bubbles with a different lower case letter evidence a significantly different number of fruits per plant according to the LSD test (P≤0.05); (B) Mean values from each column with a different letter are significantly different according to the LSD test (P≤0.05). Control= non-inoculated plants; PCI2= inoculated with *P. putida* PCI2 alone; ITEM 3636= inoculated with *T. harzianum* ITEM 3636 alone. Shoot area and number of fruits values were assessed at 60 days. Dry weights were registered after harvest, at 140 days.

consortium showed the highest mean value (114 mm). The rest of the treatments presented values that oscillated around 100 mm and did not differ significantly. For fruit width, the treatments did not present significant statistical differences between them. Additionally, we observed that there were statistically significant differences between treatments regarding average yield. Specifically, the highest yield was caused by plants inoculated with the microbial consortium, compared to the control, while the treatments of inoculation with the

individual microbial strains caused intermediate values (Table 3).

Table 3 - Effect of *T. harzianum* ITEM 3636, *P. putida* PCI2 and their combination on pepper fruits and yield during experiment 2

Treatment	Length of peppers (mm)	Width of peppers (mm)	Yield (g/plant)
Control	103.32 b	76.16 a	480.31 b
ITEM 3636	103.84 b	76.28 a	639.68 ab
PCI2	99.54 b	78.04 a	594.36 ab
Consortium	114.47 a	79.85 a	703.42 a

The Kruskal-Wallis test was used to determine whether the differences between the groups were significant. A different letter within each column indicates a significant difference between treatments.

Global effects of treatments

After normalizing the values, the analysis of the overall impact of treatments on the various assessed parameters suggests that the development of the root system significantly improved in response to inoculation with strain ITEM 3636, both alone and in combination with PCI2 as a microbial consortium. The root development values were lowest in the control. On the other hand, inoculation in all its forms was successful in stimulating the aerial growth of pepper plants, especially in the case of strain PCI2. The consortium was superior in terms of enlarging peppers. When compared to fruits from plants treated with single strains or uninoculated controls, these fruits showed increased width and length, particularly width. Furthermore, yield measurements showed the highest gains under consortium inoculation, followed by individual strain inoculation (Fig. 2).

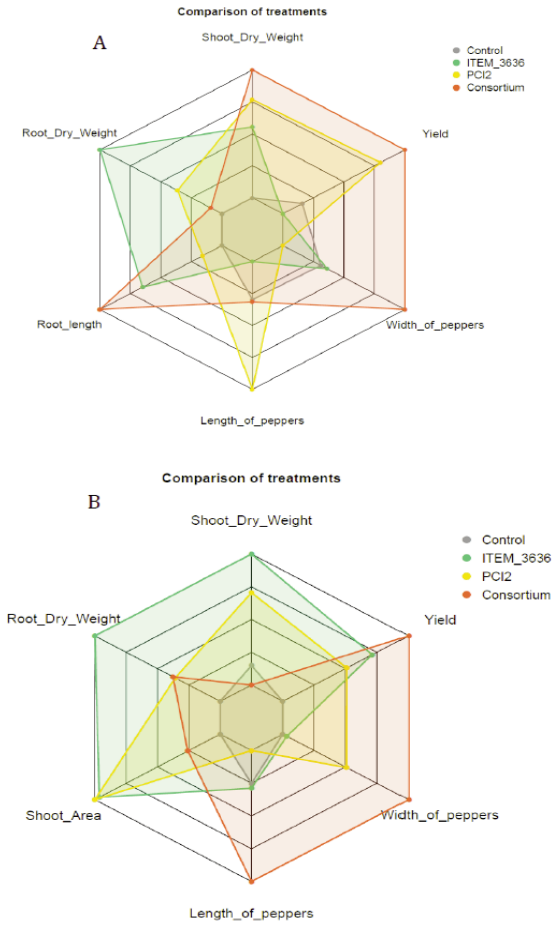

Fig. 2 - Global inoculation effects on pepper growth and yield in (A) Field assay 1 and (B) Field assay 2. Consortium = PCI2 + ITEM 3636; Control = non-inoculated plants; ITEM 3636 = inoculated with *T. harzianum* ITEM 3636 alone; PCI2 = inoculated with *P. putida* PCI2 alone.

Qualitative analysis of pepper soil microbial profiles

For this preliminary qualitative analysis, we used a single sample per treatment to obtain fungal and bacterial taxonomic profiles. In terms of fungal profiles, we focused on the 10 classified genera with the highest relative frequencies. Results are presented in figure 3A. Despite all profiles being composed of the same fungal genera, results suggest some differences, such as higher relative frequencies of one taxon in single inoculation treatments and a more balanced composition in the case of co-inoculation. The treatment of inoculation with ITEM 3636 showed the highest relative frequency for *Cladorrhinum* (49%), whereas the treatment of inoculation with PCI2 presented the highest for *Mortierella* (42.7%). Inoculation with ITEM 3636 also showed the lowest relative frequency for *Fusarium* (13.6%). The control profile suggests a dominant role of *Mortierella* (27.7%), *Fusarium* (23.4%) and *Thelonectria* (19.9%). Besides *Mortierella* (26.5%), the fungal profile for the treatment of inoculation with the consortium was also dominated by *Dichotomopilus* (20%) and *Trichoderma* (19.4%). For bacterial Class, it can be observed in figure 3B. that the detected taxa would exhibit a homogenous distribution in all of the inoculation treatments. Also, the control treatment appears to be most influenced by Actinobacteria (relative frequency of 36%). The taxonomic profiles of soil communities linked to

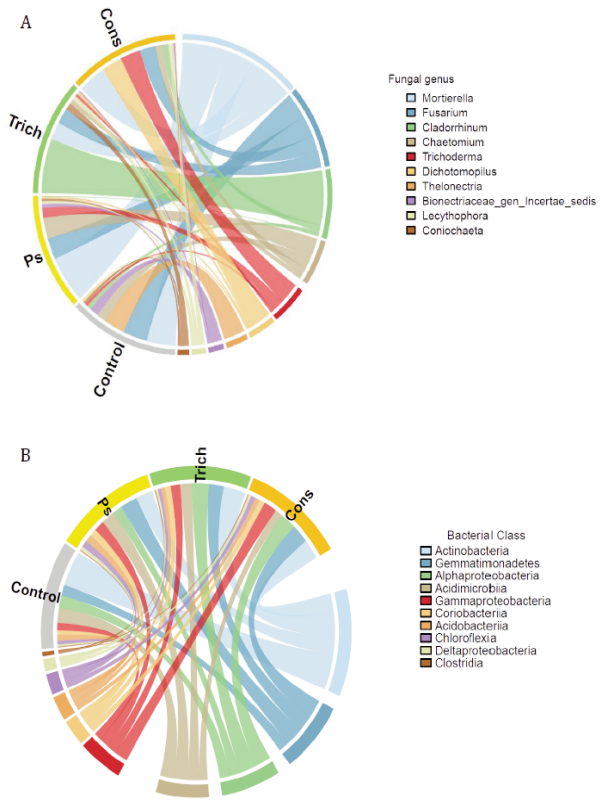

Fig. 3 - Circos/String plot of soil (A) fungal and (B) bacterial communities in the different treatments. Cons= consortium (PCI2 + ITEM 3636); Control= non-inoculated plants; Ps= inoculated with *P. putida* PCI2 alone; Trich= inoculated with *T. harzianum* ITEM 3636 alone.

inoculated pepper, as well as tomato, are currently the subject of more thorough and in-depth work based on these first explorations.

4. Discussion and Conclusions

In this paper, we propose that a consortium consisting of *T. harzianum* ITEM 3636 and *P. putida* PCI2 as a biostimulant could boost pepper yield. In a previous work, we observed that the strains under study are compatible and, in field experiments, contributed to increase tomato yield (Pastor *et al.*, 2024). When used in the field, potential biofertilizers that commonly perform well in the lab and greenhouse may not have the expected effects on plant development. Effective plant growth promotion under field settings cannot be ensured by merely screening axenic culture isolates for features that promote plant growth (Basu *et al.*, 2021). Bader *et al.* (2020) reported on *Trichoderma* strains that promoted the growth of tomato plants and hypothesized that this ability might be related to their capacity to produce phytohormones and to increase accessible phosphorus. Unpublished evidence suggests that *T. harzianum* ITEM 3636 solubilizes phosphates and raises the amount of phosphorus in the shoots of inoculated peanut plants. Conversely, we documented *P. putida* PCI2's capacity to produce indole-3-acetic acid (IAA) and to solubilize different sources of phosphate (Pastor *et al.*, 2012; Pastor *et al.*, 2014).

Inoculation with *P. putida* PCI2 alone during the first year of experimentation caused significant increases of 10% and 40% in the length of peppers and shoot dry weight, respectively, compared with control plants. Additionally, inoculation with *T. harzianum* ITEM 3636 alone resulted in a significant increase of 27% in root length when compared with the control during the first assay under field conditions. During experiment 2, we observed significant increases in shoot area values from single inoculation treatments, compared to the control. Results from this assay also demonstrated that inoculation with the consortium had a positive effect on fruit length, producing the largest fruits among all treatments. The dominance of inoculation with the consortium in this parameter underscores its potential as a promising treatment for enhancing fruit size and, hence, yield. Several studies have reported positive relationships between fruit size attributes and overall yield, suggesting that improvements in these parameters may translate into enhanced productive performance (Usman *et al.*, 2017; Sharma *et al.*, 2019; Deresa *et al.*, 2023).

Combinations of several *Trichoderma* species and bacteria have been shown to be more effective at promoting plant growth than single microorganisms (Pastor *et al.*, 2023). Our results showed that pepper yield increased significantly after inoculation under field conditions, particularly after inoculation with the microbial consortium. Results from this study showed that co-inoculation of pepper roots during transplant to the field produced significant increases in parameters such as root length and fruit size, compared with control plants, during the different experiments under field conditions. These findings concur with earlier studies on the advantages of inoculating horticultural crops with a beneficial

microbial consortium. A seed-coating formulation was created by Kumar *et al.* (2015) based on a microbial consortium that included *T. harzianum* OTPB3 and *B. subtilis* OTPB1 and exhibited significant improvements in growth parameters of many horticultural crops in greenhouse and field conditions. He *et al.* (2019) carried out a greenhouse study on tomato to compare the effects of co-inoculation with combinations of *B. pumilus*, *B. amyloliquefaciens*, *B. mojavensis* and *P. putida* on plant development and yield to single microbial treatments. According to the authors, the co-inoculation of beneficial microorganisms, at particular stages of plant development, improved plant performance more quickly than any other treatment. To promote tomato growth under drought stress, Krishna *et al.* (2022) created what they called a Hexa-PGPM consortium based on *B. megaterium* BHUPSB14, *P. fluorescens* BHUPSB06, *P. aeruginosa* BHUPSB01, *P. putida* BHUPSB04, *P. polymyxa* BHUPSB17 and a *T. harzianum* strain. This consortium was proven to enhance several growth and yield indices. The effectiveness of a consortium based on *B. pumilus* YSPMK11 and *B. subtilis* MK5 for promoting growth in bell pepper plants as well as for preventing the diseases damping off and anthracnose was assessed by Kaushal *et al.* (2019) in field conditions.

The consortium significantly improved growth, reduced the incidence of damping off and anthracnose disease, and increased fruit yield. Singh *et al.* (2019) also assessed *T. harzianum* and PGPRs as a microbial consortium for promoting mint growth and came to the result that using these microbes combined was more effective than using them individually for the improvement of plant growth. The yield measures from this study showed that consortium inoculation produced the greatest gains. According to these results, favorable benefits may be amplified by synergistic interactions among the microorganisms in the consortium, which could improve fruit set and overall productivity. More specifically, the improved performance observed under consortium inoculation may be associated with complementary effects of the microbial partners. In this sense, both *T. harzianum* ITEM 3636 and *P. putida* PCI2 have been reported to have phosphorus-solubilizing activity, and *P. putida* PCI2 has been demonstrated to produce IAA. Through improved plant-microbe interactions, these combined characteristics may affect fruit development and plant growth, potentially contributing to the observed increases in fruit size and yield-related traits under field conditions.

On the whole, the microbial consortium treatment displayed more root length. Increased root development enables plants to absorb more water and nutrients by growing their roots deeper. More nutrient and water absorption allows plants to develop biomass more rapidly and count with a fitter supply of assimilates for fruit production. Although further work is needed, our results suggest that pepper root-associated soil from the co-inoculation treatment contained higher abundances of fungal genera known to include beneficial members that lead to healthier plants and more resilient ecosystems, namely *Mortierella*, *Trichoderma* and *Dichotomopilus*. Worldwide research findings highlight the value of rhizospheric plant growth-promoting fungi by demonstrating their environmentally beneficial characteristics and potential for enhancing germination, the development of plant roots and shoots and crop yields (Adedayo and Babalola, 2023).

This study provides new information on the inoculation and co-inoculation of pepper roots with *T. harzianum* and *P. putida* strains during transplanting. Our results show that the strains enhance physiological processes that boost pepper yield, acting as effective biostimulants, especially when combined. This strategy reduces reliance on chemicals by providing an effective alternative to synthetic inputs. Future studies will examine how these studied microorganisms affect soil microbial populations and plant metabolic profiles in an effort to clarify the relationships and processes underlying enhanced pepper growth. Our goal is to further the creation of agricultural practices based on microorganisms that balance environmental care with productivity.

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Novel sweet potato hybrids with enhanced anthocyanin accumulation and resistance to Sweet Potato Feathery Mottle Virus (SPFMV)

OPEN ACCESS

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Abstract: Sweet potato, a top five global crop with over 95% of production in developing countries, surpasses wheat, rice, and cassava in yield, nutrition, adaptability, and stress tolerance, providing more edible energy per hectare and contributing significantly to food security. Purple-fleshed sweet potatoes (PFSP) have a rich, deep purple color due to the presence of anthocyanins, which are antioxidants that have numerous health benefits. The flavor is like other sweet potatoes but can have a slightly more earthy or nutty taste, depending on the variety. Purple sweet potatoes are nutritious, being high in fiber, vitamins, and antioxidants. They can be used in various dishes, from baked and mashed forms to soups, fries, and even desserts. Molecular characterization, using SSR markers, indicated significant genetic diversity among the genotypes. Specific markers for the types of anthocyanin and resistance to Sweet potato Feathery Mottle Virus (SPFMV) were utilized to screen for promising lines. Yield trials conducted over multiple seasons showed notable differences in root count and weight, with purple-fleshed genotypes generally outperforming others, particularly during the dry season. Biochemical analysis further confirmed the high anthocyanin content in these purple-fleshed varieties, which also exhibited better yields during the dry season. Fifteen high-yielding genotypes were screened for resistance to SPFMV. The results, confirmed by SPFMV2-specific markers, showed that these genotypes were tolerant to the virus. Despite the virus's presence, these genotypes continued to perform well, making them strong candidates for cultivation in regions affected by SPFMV. Based on their morphological traits, yield performance, biochemical properties, and virus resistance, 11 purple-fleshed genotypes were identified as top performers. These genotypes hold great potential for improving sweet potato production and food security in developing countries, where they can contribute to increased yields, enhanced nutritional value, and greater resilience to viral diseases.

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1. Introduction

Sweet potato plays an important role as a major food and feed source in developing countries, accounting for over 95% of its global production (CIP, 2023). It stands among the top five crops worldwide due to its high yield, nutritional value, adaptability to diverse geographical regions, short production cycle, and resistance to various production stresses like high temperatures, water deficit, pests, and diseases (CIP, 2023). This makes it not only a significant food source but also nutritionally superior to many other staple foods. Sweet potato is cultivated in more than 100 developing countries, surpassing other root or tuber crops. It provides more edible energy per hectare per day than staple crops like wheat, rice, or cassava. Purple-fleshed varieties are rich in anthocyanins, which have antioxidant properties and potential anti-cancer and antidiabetic effects (Ayeleso *et al.,* 2016).

Purple-fleshed sweet potatoes are rich in anthocyanins with antioxidant and anti-mutagenic properties (Ayeleso *et al.*, 2016; Khoo *et al.*, 2017). These pigments are structurally stable (Suda *et al.*, 2003), with up to 22 types identified-primarily acylated cyanidins and peonidins (Terahara *et al.*, 2004; Liao *et al.*, 2019). Advanced mass spectrometry has enabled efficient characterization, facilitating large-scale screening of varieties (Tian *et al.*, 2005). Compared to orange-fleshed types, purple sweet potatoes contain significantly higher anthocyanin and phenol levels, comparable to blueberries, blackberries, cranberries, and grapes, making them a low-cost, valuable source of natural pigments.

Consumer preference for sweet potato varieties has shifted over the years from white-fleshed, high-starch types to those with purple-fleshed varieties, which are higher in protein and anthocyanin (Chandrasekara and Kumar, 2016). Purple sweet potato is an important source of dietary fiber, minerals, and vitamins. The daily dietary structure of American residents revealed that the average daily anthocyanin intake per capita was approximately 12.5 mg/day (Wu *et al.*, 2006). Therefore, purple sweet potatoes can not only be used as a green food to meet people's daily intake of cereals, but also increase the daily intake of anthocyanins to achieve health effects (ASHS, 2007).

Sweet potato virus disease (SPVD), caused by co-infection of sweet potato feathery mottle virus (SPFMV) and sweet potato chlorotic stunt virus (SPCSV), is the most destructive viral disease of sweet potato, leading to severe stunting, curling, chlorosis, and yield losses of up to 80% (Gutiérrez *et al.*, 2003). SPFMV, the most widespread, is aphid-transmitted and often symptomless but becomes highly damaging in virus complexes (Kreuze, 2002; , Kreuze and Fuentes, 2008; Clark *et al*., 2012), while SPCSV, transmitted by whiteflies, was once mistaken for nutrient deficiency (Sim *et al.*, 2000). In the Philippines, SPVD ("kulot") is a major constraint, with incidence ranging from 10-60% (Prasanth and Hegde, 2008). Control relies mainly on resistant varieties, clean planting materials, and sanitation, as vector control is impractical and symptomless infections complicate selection. Maintaining sweet potato collections presents challenges such as genetic losses due to small plot sizes, virus infections, and significant losses during dry periods (Huaman, 1999). Cross-contamination and virus spread within clonal collections are common issues. To support the national government's food production program, efforts are directed towards assessing the genetic diversity in sweet potato germplasm collections using morphological and molecular markers and selecting varieties with high resistance to SPFMV.

The study aimed to select promising lines of sweet potato with high anthocyanin content, root yield, dry matter, and starch content, supporting commercial production and contributing to national food security. Specifically, this study aims to evaluate, select, and develop sweet potato varieties/ accessions with high anthocyanin and resistance to SPFMV suitable for commercial production.

2. Materials and Methods

Selection of purple-fleshed accession in the sweetpotato germplasm collection

A total of 27 purple sweet potato genotypes were studied. These accessions were characterized using the Revised Protocols for Sweetpotato Characterization in the Philippines, developed by NPGRL, CIP, and PhilRoots. Sixteen morphological traits related to shoots and leaves-such as leaf color and shape, as well as vine, petiole, and root morphology-were assessed in the sweet potato collection. From this collection, purple-fleshed sweet potato genotypes were selected for further evaluation in the field trial and as parents for the hybridization.

DNA isolation

Genomic DNA of the selected purple-fleshed sweet potato accessions were isolated using a modified Doyle and Doyle (1987) protocol. One gram of leaf tissue was ground with liquid nitrogen and PVP, then mixed with a CTAB extraction buffer and incubated at 65°C for an hour. The aqueous phase was treated with chloroform alcohol and centrifuged. The aqueous layer was mixed with NaCl and PEG solution, incubated at -4°C, and centrifuged again. DNA was precipitated with isopropanol, incubated overnight at -20°C, collected, washed with ethanol, and air-dried. The DNA pellet was resuspended in TE buffer with RNAse A and incubated at 37°C. DNA quality was assessed using a Biotek Epoch™ UV-VIS spectrophotometer and agarose gel electrophoresis and visualized under UV light with a Clinx GenoSens 1510 system.

Primer selection and polymerase chain reaction

Gene-specific molecular markers for anthocyanin biosynthesis (Table 1) were selected from published studies (Mano *et al.*, 2007; Park *et al.*, 2015; Khan *et al.*, 2016; Choudhury *et al.*, 2019) and synthesized for screening sweetpotato genotypes. Anthocyanin-related markers were amplified in purple-fleshed varieties, while β-carotene markers were amplified in orange-fleshed ones. DNA was normalized to 60 ng/µl, and PCR conditions were optimized based on reported protocols with annealing temperatures adjusted accordingly. Amplification products were resolved using 6% polyacrylamide gel electrophoresis (PAGE) stained with GelRed™ and visualized under UV light with the GenoSens 1510 system.

RNA Isolation for resistance to SPFMV screening

Symptomatic leaf tissues (100 g) were homogenized in a frozen mortar and pestle, and total RNA was extracted using Trizol® reagent following the manufacturer's protocol. After phase separation with chloroform and precipitation with isopropanol, RNA pellets were washed with ethanol, air-dried, and resuspended in RNase-free water. First-strand cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System with oligo(dT) primers, following standard conditions of denaturation, reverse transcription at 50°C, and termination at 85°C. The resulting cDNA was stored at -21°C and

Table 1 - List of gene-specific markers used for anthocyanin content screening across sweet potato (*Ipomoea batatas*) genotypes

Primer	Gene name	Gene function	Forward sequence	Reverse sequence	Ta (°C) literature	Ta (°C) Optimized	Product length (bp)	Literature cited
IbMYB1-FA/RA2	MYB proto-oncogene, transcription factor	codes for transcription regulator in anthocyanin synthesis	TATGGTCGGGATCGTCTTCG	TTCTGAAGATGGGTGTTCCAT	56	56	800	Mano *et al.*, 2007
CHS-F/R	chalcone synthase	flavonoid/isoflavonoid biosynthesis pathway	GGACTACCAGCTCACCAAGC	GTCCTCCACTTGGTCCAGAA	56	56	400-800	Mano *et al.*, 2007
CHI-F/R	chalcone isomerase	isomerization of naringenin chalcone into its corresponding (2*S*)-flavanones	GTTAAGTGGAACGGGAAAAG	GAGACGACCGTTTGTGGAAT	56	56	800	Mano *et al.*, 2007
F3H-F/R	flavanone-3-hydroxylase	flavonoid biosynthetic pathway	CGAGATTCCGGTGATATCGT	GGGGCATTTTGGGTAGAAAT-	56	56	900	Mano *et al.*, 2007
DFR-F/R	Dihydroflavonol 4-reductase	flavonoid biosynthetic pathway	TCCTGGGAACACAAAGAAGG	GAGCTTCGCAGAGATCATCC	56	56	1300	Mano *et al.*, 2007
ANS-F/R	anthocyanidin synthase	catalyzes the penultimate step in the biosynthesis of the anthocyanin	ATTTTCGCGGAGGAAAAGAT-	CTTCCTTCTCCAGCCTTCCT	56	56	800	Mano *et al.*, 2007
3GT-F/R	flavonoid 3-glucosyl-transferase	flavonoid biosynthesis	AAGTATCGATCGGCGAAATG	CACGATATGGCCTCCAGAGT	55	55	800	Mano *et al.*, 2007
VP24-F/R	encoding vacoular protein	Precursor in Anthocyanin-Producing Cells	CTTGACACTGCCCTCCAGTATG	ACGAGCAAGCTCCAACATAACA	56	56	800	Mano *et al.*, 2007
IT 4	sporamin	endopeptidase inhibitor activity	CCATACCAGCTCGGATTTGT3	TGGATGCCAACCTTAACTCC3	55	55	234	Choudhury *et al.*, 2019
IT 666	R2R3 type MYB gene	flavonoid 3'-hydroxylase of Ipomoea tricolor	GCGAATTTAGTCCCGATGAA	CGGTGTTTTCCGTGATTTCT3	52	52	479	Choudhury *et al.*, 2019

subsequently used for screening sweet potato genotypes for SPFMV resistance (Table 2).

Field evaluation of purple-fleshed sweet potato accessions

The twenty-seven purple-fleshed sweet potato genotypes were evaluated in preliminary yield trials using a randomized complete block design (RCBD) with three replicates. The first trial was conducted during the dry season (November 2020-March 2021) and harvested 105 days after planting. Storage roots were classified as marketable or nonmarketable, and root number and weight were recorded per replicate. Morphological traits (skin and flesh color) were assessed, while dry matter, starch, and anthocyanin contents were analyzed at the Analytical Service Laboratory, IPB, CAFS, UPLB. A second field evaluation was conducted during the wet season (May-September 2021) using the same design and procedures.

3. Results and Discussions

Morphological characterization of sweet potato genotypes

Morphological characterization for foliage and vine was conducted using sixteen descriptors based on the revised protocol for sweet potato characterization in the Philippines. Traits such as leaf color and shape, vine and petiole color pigmentation, and number and type of lobes were noted and scored. Some of the predominant phenotypic traits observed from leaves across genotypes were the triangular leaf outline and semi-elliptical central lobes. Leaf color variations were mostly green or purple, with veins that can also be green or strongly pigmented with anthocyanin. There are also genotypes with both green mature leaves and purple young leaves. Some genotypes even display a variation of leaf shape and lobe number on the same plant (Fig. 1).

Table 2 - List of gene-specific markers for screening of SPFMV-resistant sweet potato (*Ipomoea batatas*) genotypes

Primer	Forward sequence	Reverse sequence	Ta (°C)	Product length (bp)	Literature cited
SPFMV	CACTTCAGTGACGTTGCTGA	GCACACCCCTCATTCCTAAG	60	319	Sivparsad and Gubba, 2013
SPFMV	TGGGGTTATGATGAACTTCTTC	TTCTGGAATGRYTGCGGGTTG	60	400	Zhao *et al.*, 2020
NIB1536+	TAATGAAATGTAYGATGATAG	TTAAAGGCATACTAAAGATAA	60	1051	NCPN, 2016
SPFMV	TCTAATGAGAACACTGAATT	TTGCACACCCCTCATTCCTAAG	60	1051	Jiang *et al.*, 2018
CP1S	AGTGGGAAGGCACCATACATAGC	GCAGAGGATGTCCTATTGCACACC	-	960	Prasanth and Hegde, 2008
CP2S	TCTAGTGAACGTACTGAATTCAAAGA	ATTGCACACCCCTGATTCCTAAGA	-	960	Prasanth and Hegde, 2008

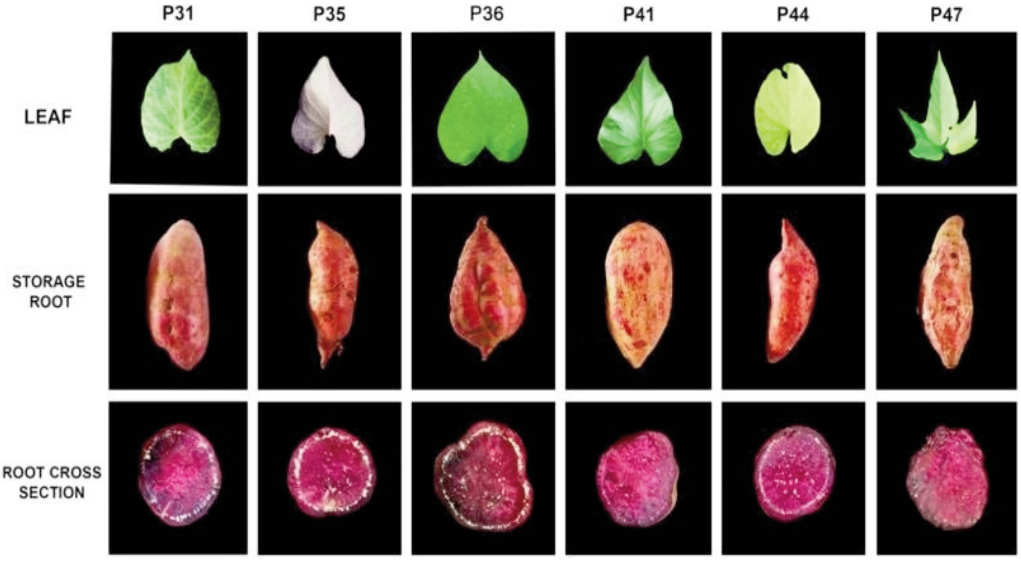

Fig. 1 - Morphology of top-performing purple-fleshed sweet potato genotypes subjected to general yield trial.

On the other hand, root morphological assessment was carried out after harvesting. The presence of purple pigmentation in the root flesh denotes high anthocyanin biosynthesis. Hence, flesh color was scored through their cross-sections. Most of the purple-fleshed genotypes were characterized as intermediate purple to strongly pigmented with anthocyanin, and a few individuals were recorded with pale purple color. Interestingly, similar to the check variety, genotypes that showed high pigmentation of purple color during the dry season were observed to have lighter flesh color or less pigmentation during the wet season. This might be associated with some environmental factors, such as excess amount of moisture during wet season considering that color intensity depends on environmental conditions (Huaman, 1999). For accurate quantification of dry matter, starch, and anthocyanin of the storage roots of each genotype per replicate were sent to Analytical Service Laboratory of Institute of Plant Breeding. Moreover, storage root shape, size, and skin color were also noted. Most genotypes have red purple to dark purple skin while the shape varies from elliptic, round elliptic, ovate, obovate, and oblong. Most genotypes showed normal root surface, but some showed some defects such as horizontal constriction (P25), longitudinal grooves (P36), and vein-like skin (P47). These defects occur normally as the result of environmental factors such as soil type or presence of excessive amount of water (Huaman, 1999).

Molecular characterization of sweet potato genotypes

Genomic DNA was isolated using the modified Doyle and Doyle (1987) protocol. Intact bands were generated using 1% AGE gel, indicating absence of degradation (Fig. 2). Moreover, quality and quantity assessment through spectrophotometry showed an A_{260}/A_{280} ratio reading of 1.8-2.0 indicating good quality DNA. Normalization of template concentration was done by dilution of DNA to a final concentration of 60ng for all samples. Moreover, primer selection and synthesis were done to carry out the molecular characterization of sweet potato genotypes. Fifty-four SSR markers were selected from the study of Meng *et al.* (2018), Amoanimaa-Dede *et al.* (2020), and Naidoo *et al.* (2022).

Fig. 2 - Genomic DNA of sweet potato representative genotypes resolved in 1% agarose gel.

DNA-based markers are crucial in expediting the timeline of any breeding program. Therefore, in the initial screening of sweet potato varieties for high anthocyanin, gene-specific markers were selected from the study of Mano *et al.* (2007), Park *et al.*, (2015), Khan *et al.* (2016) and Choudhury *et al.* (2019). Amplification of these genes was carried out across the advanced lines. Anthocyanin-related markers were amplified across purple-fleshed genotypes. Presence and absence of bands were recorded for each genotype. All genotypes generated positive amplification for each marker, with a few having one or two missing bands. This indicates the presence of anthocyanin biosynthesis across genotypes (Table 3). In addition, screening for SPFMV resistance was conducted after selecting the top-

Table 3 - Presence and absence of bands from purple-fleshed genotypes using anthocyanin biosynthesis gene specific primers

Accession Number	F3H	CHS	ANS	IBM	IT4
P25	+	+	+	+	+
P27	+	+	+	+	+
P28	+	+	+	+	+
P29	+	+	+	+	+
P31	+	+	+	+	+
P32	+	+	+	+	+
P33	+	+	+	+	+
P34	-	+	+	+	+
P35	+	+	+	+	+
P36	+	+	+	+	+
P37	+	+	+	+	+
P39	+	+	+	+	+
P40	+	+	+	+	+
P41	+	+	+	+	+
P42	+	+	+	+	+
P43	+	+	+	+	+
P44	+	+	+	+	+
P45	+	+	+	+	+
P46	+	+	+	+	+
P47	+	-	+	+	+
P48	+	+	+	+	+
P49	+	+	+	+	+
P50	+	+	+	+	+
P51	+	+	+	+	+

performing genotypes in the yield trial (Fig. 3). Gene-specific markers for SPFMV were selected from the study of Prasanth and Hegde (2008), Sivparsad and Gubba (2013), Jiang *et al.* (2018), and Zhao *et al.* (2020), and were subjected for synthesis.

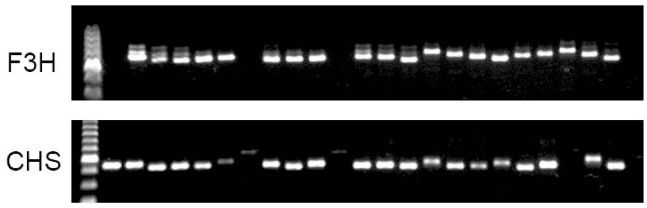

Fig. 3 - Representative gel of gene-specific markers for anthocyanin (F3H, CHS).

Yield trial of sweet potato genotypes

Yield for each individual genotype was counted and recorded for all replicates after harvesting. Analysis of variance was done for each recorded character (Table 4). Significant difference was observed from all parameters.

Significant increase in yield was observed during the dry season for both tuber weight and count (Fig. 4). Eight purple genotypes generated significantly higher values than the check variety for root storage count, with a range of 45.33 to 76.0 for dry season and 25.42 to 42.10 for tubers during the wet season. Eleven purple genotypes were accounted to have higher root weight values with 3.93 kg - 16.37 kg per 6 m plot. This is higher than the check variety with approximately 3.83 kg yield for dry season. Significant decrease was observed during the wet season, where 93% of the purple genotypes obtained lower values than the check variety. Highest yield performance was obtained from haponitaxhaponita[2] (P34) and HAPONITA X NSIC SP20 (P36) for dry and wet season, respectively.

Results showed that yield of purple genotypes were significantly higher during the dry season than

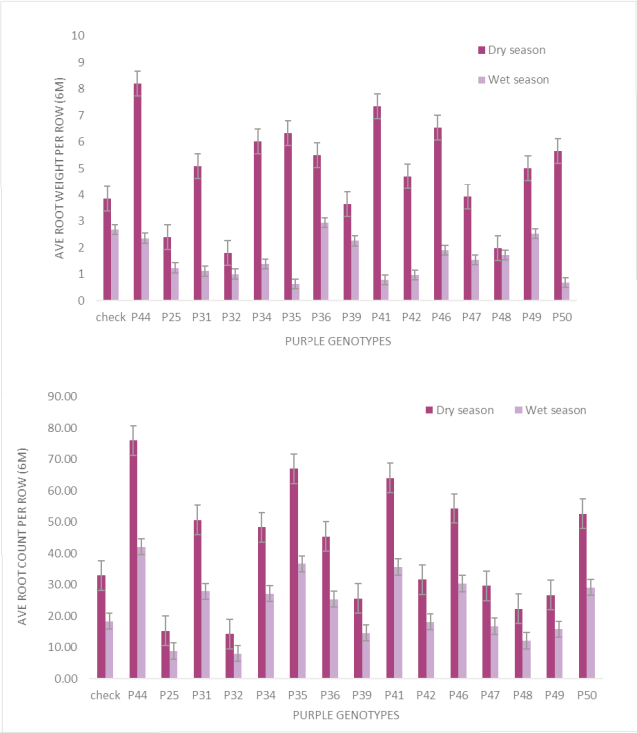

Fig. 4 - Relative root weight (top) and count (bottom) of purple genotypes harvested during dry and wet season

the wet season. However, it is important to note that sweet potato is of tropical origin and thrives better in dry season. This is why yield evaluation for wet season is a selection of at least reasonable quantity in contrast to dry season where excellent yield performance can be obtained and selected across genotypes (Wilson *et al.,* 1989). This proves that root formation of sweet potato is highly dependent on optimum environmental conditions. It was also evident in the morphology of several roots from wet season where defects in skin surfaces such as horizontal constriction, longitudinal groove, and vein-like skin were detected. This might be associated with factors like the relatively high moisture content

Table 4 - Analysis of variance for yield based on root count and weight per planting season

Purple genotype	Sum Sq	Mean Sq	F value	Pr(>F)
Root count dry season	16044.8	1069.65	5.1686	5.633e-05 ***
Root count wet season	2760.65	184.043	2.854	0.006617 **
Root weight dry season	164.220	10.9480	3.6479	0.001244**
Root weight wet season	25.5321	1.70214	3.094	0.003823 **

***, **, *, · means different significance for p≤0.001, 0.01, 0.05, 0.1, respectively.

present during wet season or even temperature differences.

Yield trial was conducted for the wet season across purple-fleshed sweet potato genotypes. Genotypes were significantly different for root weight (α=0.05), which ranges from 3.033 to 0.1 kg. Tukey's Honest Significant Differences (HSD) were carried out across accessions that grouped 16 purple individuals with SP34 and Inube check varieties. Among top performing genotypes, Nick732, P35, P44, P39, P47, and P41 obtained higher values than both purple check varieties (Fig. 5).

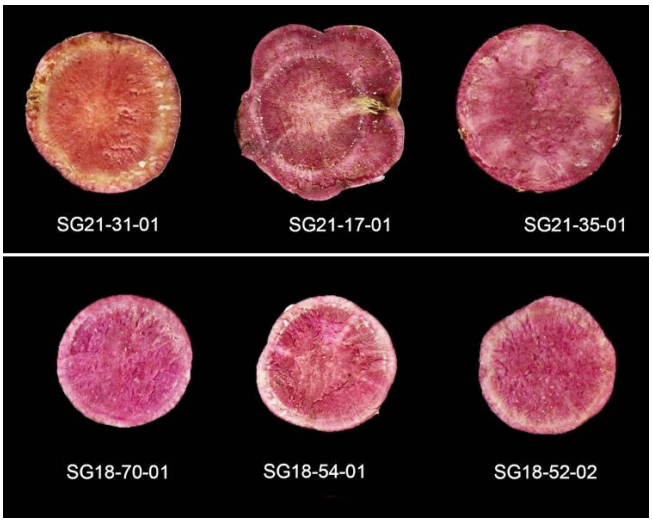

Fig. 5 - Root cross sections of selected, purple-fleshed sweet potato genotypes after field evaluation.

Evaluation of sweet potato genotypes for anthocyanin, dry matter and starch content

Results showed significant differences for all parameters except starch content for dry season (Table 5). Anthocyanin content of purple genotypes for dry season ranged from 12.35 mg L^{-1} to 138.044 mg L^{-1} with the highest value obtained (Table 6).

Table 6 - Biochemical profile of representative top performing purple-fleshed sweet potato lines for wet and dry season

CODE	Anthocyanin	%Dry matter	%Starch
Dry season			
P35	138.0441 a	25.4062	82.31325
P39	117.8663 ab	26.01484	79.35187
P41	91.45423 abc	29.23401	82.25589
P36	68.82724 bcd	29.60067	81.38915
P47	67.96446 bcde	31.05805	78.60501
P32	50.29147 cde	31.35445	80.13882
P44	48.8999 cde	33.96263	81.12375
P46	47.4805 cde	30.75377	80.54859
CHCK	42.24818 cde	33.42631	79.59258
P34	41.10709 cde	32.46437	82.35191
P48	33.89874 de	29.38918	81.73909
P31	33.45343 de	36.33059	81.9476
P42	28.9169 de	27.35473	81.76478
P50	22.79398 de	32.25506	82.12245
P25	16.36491 de	34.43242	80.99422
P49	12.35717 e	30.25301	81.86431
Wet season			
P35	65.29264 a	29.70628	63.52073
P34	39.40937 b	32.00372	68.95282
P44	36.8767 bc	34.96165	79.35083
P39	35.12332 bcde	30.64573	71.73507
P47	33.03596 bcdef	34.2485	78.36539
P41	31.36607 bcdef	31.46032	79.80127
P31	22.09819 bcdef	38.14614	77.95936
CHCK	17.45033 bcdef	34.02202	73.97153
P32	15.27948 bcdef	36.1835	73.27699
P36	15.22382 bcdef	26.48254	69.87282
P42	14.33321 cdef	32.28941	74.80796
P25	11.60572 def	37.52476	78.94269
P50	11.23 def	56.79378	49.16729
P49	96.01859 ef	31.08886	73.01925
P46	92.4005 ef	34.21978	78.75343
P48	74.86667 f	31.64359	78.61174

Means with the same letter are not significantly different.

Table 5 - Analysis of variance for yield based on root count and weight per planting season

Purple genotype	Sum Sq	Mean Sq	F value	Pr(>F)
Dry season				
Anthocyanin	55161	3677.4	11.3160	3.529e-08 ***
Dry matter	394.57	26.3047	8.9181	4.695e-07 ***
Starch	54.747	3.6498	0.7608	0.70612
Wet season				
Anthocyanin	10858.6	723.91	11.2722	2.494e-08 ***
Dry matter	409.15	27.277	2.3945	0.02128 *
Starch	952.60	63.507	2.0123	0.051803 ·

***, **, *, · means different significance for p≤0.001, 0.01, 0.05, 0.1, respectively.

Percent dry matter across hybrids ranges from 20.96 to 36.33 while percent starch content ranges from 76.23 to 82.35. Relatively lower anthocyanin content was obtained during the wet season. Anthocyanin content ranges from 9.60 to 65.29 mg L^{-1} for purple genotypes. Five accessions of purple genotypes (P35, P44, P39, P47, and P41) were consistently higher than the check varieties across two seasons. Results were consistent with the morphological scoring for flesh color, in which higher color pigmentation was observed during the dry season relative to results obtained during the wet season.

Selection of promising sweet potato lines

Accessions and hybrids with high yield, high dry matter, starch content and high anthocyanin levels were selected for an advanced yield trial which was carried out mainly on the basis of storage root weight and anthocyanin content across genotypes. Significant difference groupings (Tukey's test) based on root weight shown in Table 7 revealed the top yielding genotypes. Individuals with higher yield and/or in the same group as the check variety were considered. The advanced yield trial was then carried out in randomized complete block design (RCBD) in four replicates, each of which consists of two 6-meters plots. Each plot consists of 21 cuttings with 0.3-meter distance between hills and 1 meter distance between rows. Two check varieties namely SG08-09-11 and JK09-11-08 for purple genotypes were included in the trial. Figure 6 showed the morphology of the storage root and root cross section of representative genotypes that were selected for GYT.

Significant differences for root yield were observed across purple genotypes in dry and wet seasons trials (Table 8). Seven purple-fleshed genotypes obtained consistently higher root yield values as compared to check varieties across seasons.

Table 7 - Tukey's Honest significant difference groupings of top-performing purple genotypes based on root weight

Purple genotype code	Weight (kg)
Dry season	
P15	8.20 a
P41	7.33 ab
P34	6.91 abc
P46	6.53 abc
P35	6.33 abc
P50	5.67 abc
P36	5.50 abc
P31	5.10 abc
P49	5.00 abc
P42	4.70 abc
P47	3.93 abc
check	3.83 abc
P39	3.63 abc
P25	2.40 bc
P48	1.97 c
P32	1.80 c
Wet season	
P36	2.93 a
check	2.68 ab
P49	2.52 ab
P15	2.35 ab
P39	2.26 ab
P46	1.90 ab
P48	1.72 ab
P47	1.53 ab
P34	1.38 ab
P25	1.23 ab
P31	1.12 ab
P32	1.00 ab
P42	0.97 ab
P41	0.78 ab
P50	0.68 ab
P35	0.63 b

Means with the same letter are not significantly different.

Table 8 - Analysis of variance (ANOVA) on root weight across selected, purple-fleshed genotypes

	Sum Sq	Mean Sq	F value	Pr(>F)
Dry season				
Purple genotype	339.52	30.865	7.419	3.723e-05 ***
Rep	10.93	5.466	1.314	0.289
Wet season				
Purple genotype	49.385	44.895	6.309	0.000418 ***
Rep	8.698	43.492	61.116	0.010003 ***

***, **, *, · means different significance for p≤0.001, 0.01, 0.05, 0.1, respectively.

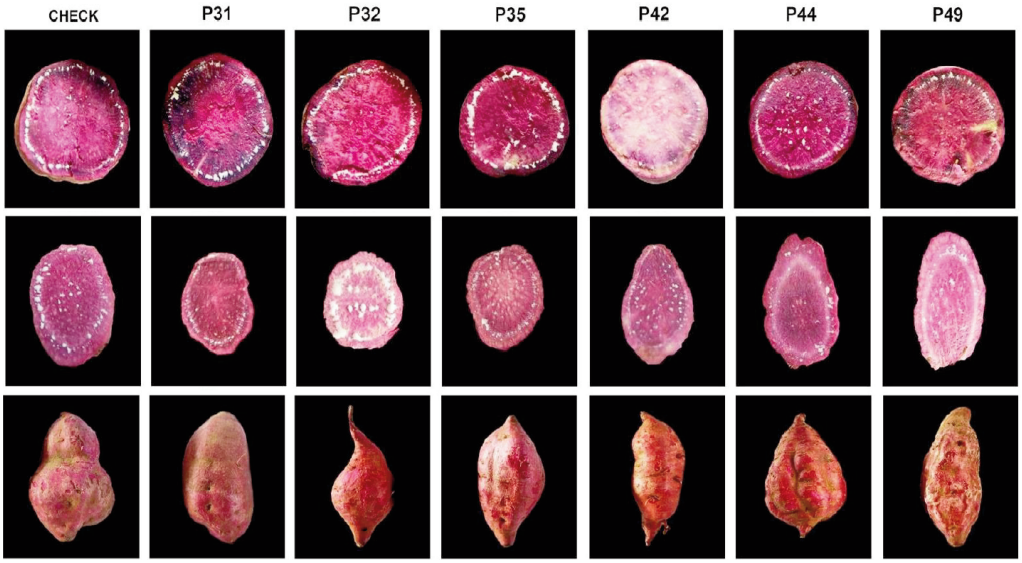

Fig. 6 - Root cross section of selected purple-fleshed sweet potato hybrids planted in the dry (upper) and wet (middle) season of 2022; bottom whole storage root.

With the consideration of their biochemical profile, a total of 11 purple-fleshed hybrids were considered high performing. Yield performance, morphological, biochemical analysis, and virus screening using PCR of the selected purple fleshed genotypes are summarized in Table 9.

Table 9 - Summary of general yield trial results and Tukey's Honest significant difference groupings of representative purple and orange genotypes based on root weight

Purple genotype	Ave. Yield (t/ha) dry season	Ave. Yield (t/ha) wet season
P31	1.9136 d	0.463 c
P34	4.6605 c	1.4815 b
P35	1.1728 d	0.2778 c
P36	12.2839 a	4.074 a
P41	3.2716 c	0.0972 c
P44	6.4814 bc	2.0988 ab
P46	5.5864 bc	1.2654 b
P47	3.5185 c	0.3241 c
P49	4.2592 c	0.2469 c
P50	6.2592 bc	0.2778 c
P15	5.9105 bc	0.3395 c
check1	8.1635 b	0.3395 c

Means with the same letter are not significantly different.

Screening for SPFMV resistance

Fifteen (15) high-performing, purple-fleshed sweet potato genotypes were selected and screened for resistance to Sweet Potato Feathery Mottle Virus (SPFMV) using specific primers. Initially, a field survey was conducted to identify vines exhibiting symptoms consistent with SPFMV infection, such as leaf curling and vein clearing. Samples from symptomatic plants were collected for further analysis. Detection of SPFMV was carried out using Reverse Transcription Polymerase Chain Reaction (RT-PCR). Plants testing positive for SPFMV were potted and maintained under greenhouse conditions to serve as positive controls. Concurrently, cuttings from the selected genotypes were propagated in plastic pots and placed in a screen cage for resistance screening.

SPFMV was isolated and inoculated into test plants via insect transmission using aphids (*Aphis gossypii*). The plants were incubated in sealed screen cages for two weeks to allow viral infection. After incubation, total RNA was extracted from infected plants using Trizol©, which yielded high-quality RNA isolates. Reverse transcription PCR was then performed to confirm the presence of the virus in each genotype. Complementary DNA (cDNA) synthesis was carried out following the protocol for the SuperScript™ III First-Strand Synthesis System (Invitrogen™, USA). Amplification of the cDNA was achieved using gene-specific primers. Among these, the SPFMV2 primers developed by Sivparsad and Gubba (2013), which target coat protein genes, produced positive amplification results.

All 15 genotypes evaluated in this study were identified as high-yielding and top-performing under

experimental conditions (Fig. 7). The detection of SPFMV in certain genotypes highlighted their tolerance to the virus, as these plants maintained strong performance despite infection (Fig. 7). Conversely, genotypes that tested negative for SPFMV were considered putatively resistant, as they showed no signs of infection during the testing process. Of the 15 genotypes, the presence of the viral gene was confirmed in several through the appearance of characteristic bands during PCR amplification.

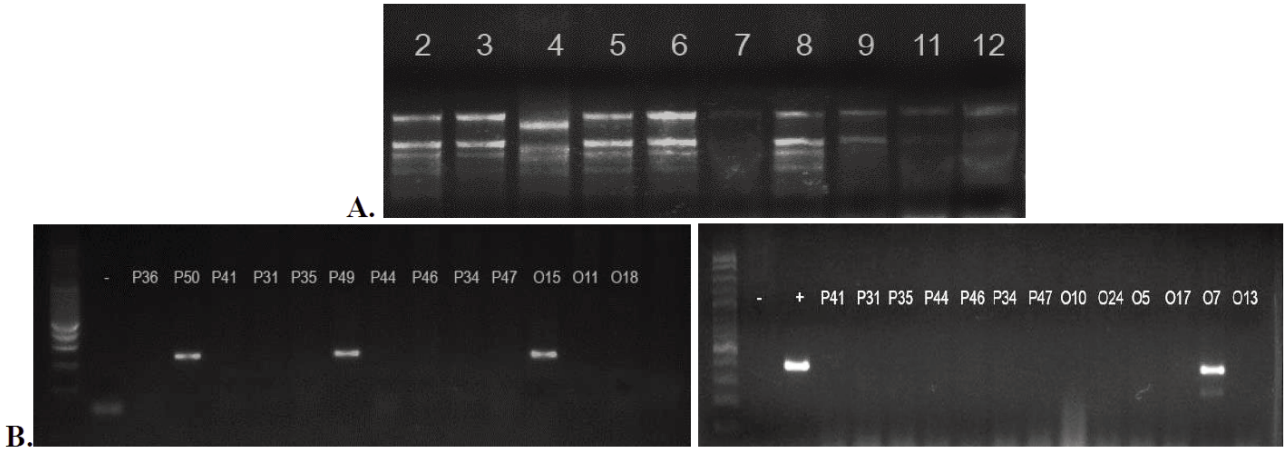

Fig. 7 - Representative total RNA (A) of sweet potato genotypes. Screening of purple-fleshed sweet potato genotypes for SPFMV (B) resistance using identified primers for the viral coat protein.

These findings underscore the importance of integrating both tolerance and resistance traits into sweet potato breeding programs. By doing so, the resilience and productivity of sweet potato crops can be significantly enhanced, ensuring better performance even under conditions of viral pressure or intensity and frequency of exposure of a plant to viruses and their vectors.

4. Conclusions

Sweet potato is recognized as one of the five leading crops globally in terms of production. The crop is increasingly popular, producing more edible energy per hectare per day than wheat, rice, or cassava. Despite its benefits, maintaining sweet potato collections in the field presents challenges. The maintenance of field genebanks is costly, and crops are exposed to diseases, pests, and environmental stresses. The Sweet Potato Virus Disease Complex (SPVD), caused by SPFMV and SPCS, is particularly devastating, reducing yields by up to 80%. This study aimed to select promising purple-fleshed sweet potato genotypes that are high yielding, with high dry matter, starch and anthocyanin contents and has resistance to SPFMV. The presence of purple pigmentation indicates high anthocyanin content. Gene-specific markers for anthocyanin, and SPFMV resistance were used to screen the sweet potato lines. Molecular characterization using these gene specific markers for anthocyanin showed high polymorphism, indicating significant genetic variation. Yield trials showed significant differences in root count and weight across genotypes. Purple and orange genotypes performed better in the dry season. Biochemical analysis revealed high anthocyanin and beta-carotene content, with dry season yields being higher. Fifteen high-yielding genotypes were screened for SPFMV resistance. Positive amplification of SPFMV2 markers indicated tolerance to the virus, allowing these genotypes to perform well even in the presence of the virus. Based on their morphological traits, yield, biochemical profiles, and SPFMV response, 11 top-performing, purple-fleshed genotypes were selected for future breeding programs (Table 10). These genotypes hold promise for enhancing sweetpotato production and supporting food security efforts.

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Table 10 - Summary of morphological characteristics, yield, biochemical profile, gene marker profile, and SPFMV response of the selected, purple-fleshed top performing genotypes

Accession purple genotypes	Morphological characteristics					Dry seaon				Gene markers for anthocyanins					
	Leaf lobing	Foliage color	Skin color	Flesh color	Maturity days	GYT (kg)	Anthocyanin	Dry matter	Starch	F3H	CHS	ANS	IBM	IT4	SPFMV reaction
P44	Semi-elliptic	Green leaf and vine	Purple red	Purple	105-120	6.481	48.900	33.963	81.124	+	+	+	+	+	-
P50	Semi-elliptic	Green leaf and vine	Purple red	Purple	105-120	6.259	22.794	32.255	82.122	+	+	+	+	+	+
P49	Semi-elliptic	Purple vine, green leaf	Purple red	Purple	105-120	4.259	12.357	30.253	81.864	+	+	+	+	+	+
P46	Semi-elliptic	Green leaf and vine	Purple red	Purple	105-120	5.586	47.480	30.754	80.549	+	+	+	+	+	-
P36	Semi-elliptic	Purple vine, green leaf	Purple red	Purple	105-120	12.284	68.827	29.601	81.389	+	+	+	+	+	-
P34	Semi-elliptic	Green leaf and vine	Purple red	Purple	105-120	4.660	41.107	32.464	82.352	+	+	+	+	+	-
check1	Triangular	Green	Purple red	Intermediate purple	105-120	8.164	42.248	33.426	79.593						
P5	Semi-elliptic	Green	Purple red	Intermediate purple	105-120	0.97	48.260	38.930	66.870	+	+	+	+	+	-
P16	Semi-elliptic	Yellow	Pink	Pale purple	105-120	2.00	23.787	25.563	59.063	+	+	+	+	+	-
P4	Toothed	Greyish green	Purple red	Intermediate purple	105-120	0.60	20.057	40.045	62.695	+	+	+	+	+	-
M4	Triangular	Green	Purple red	Intermediate purple	105-120	0.93	23.907	30.341	81.799	+	+	+	+	+	-
P9	Semi-elliptic	Green	Purple red	Intermediate purple	105-120	1.20	89.599	29.520	59.510	+	+	+	+	+	-
Check (INUBE)	Triangular	Green	Purple red	Dark purple	105-120	0.43	98.690	39.110	69.215						

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Non-destructive detection and quantification of embryo presence in *Acer monspessulanum* seeds using neural networks

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Key words: Embryo detection, Montpellier maple, seed classification, seed phenotyping, stepwise regression.

Abstract: This study aimed to develop a non-destructive method for predicting embryo presence and quantifying embryo biomass in *Acer monspessulanum* seeds using morphological and physical traits, combined with machine learning and statistical modeling approaches. Seeds were divided into two groups based on the presence or absence of embryos, and 26 morphological and physical traits were measured. Welch's t-tests were used to identify traits significantly different between seeds with and without embryos. A feedforward neural network classifier was trained on these traits to predict embryo presence. Additionally, forward stepwise regression models were constructed to identify key predictors of embryo fresh weight, dry weight, and water content in embryo-containing seeds. Statistically significant differences ($p<0.05$) were observed between embryo-containing and empty seeds in traits such as seed weight, length, roundness, Hue, and floating behavior. The neural network classifier achieved 91.03% accuracy, with strong precision (0.92) and recall (0.94) for identifying embryo-containing seeds. Regression models explained up to 56% of the variation in embryo dry weight and 50% in fresh weight, with seed weight, color parameters, and floatation traits emerging as primary predictors. Embryo water content was primarily predicted by the b* seed coat color parameter (31.6% variance), with additional contributions from buoyancy, perimeter, and post- scarification color traits, yielding a model explaining 45.5% of total variation. The integration of seed phenotyping with neural networks and multiple linear analysis provides a robust, non-destructive method for classifying embryo-containing seeds and predicting embryo development in *Acer monspessulanum*. This approach offers valuable applications in reforestation, seed banking, and ecological restoration initiatives.

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1. Introduction

The genus *Acer* includes numerous woody species distributed across temperate regions of the Northern Hemisphere, many of which are valued for their ecological roles, aesthetic features, and adaptability to diverse environments (van Gelderen *et al.*, 1994). Among them, *Acer monspessulanum* L. (Montpellier maple) is a small to medium-sized, stress-tolerant deciduous tree native to Southern Europe and Western Asia, including parts of Iran. Its compact crown, deeply lobed ornamental leaves, and distinct double samaras contribute to its visual appeal in natural and designed landscapes. In addition, *A. monspessulanum* exhibits high resilience to drought and poor soils (Teimouri *et al*., 2014), making it a suitable candidate for urban greening, reforestation, and restoration projects in arid and semiarid regions.

Despite its promising potential, propagation of *A. monspessulanum* from seed remains problematic due to a combination of physiological dormancy and seed quality heterogeneity. This species often exhibits double dormancy, which includes both an underdeveloped embryo and an inhibitory seed coat, requiring specific pre-treatments to overcome (Baskin and Baskin, 2014). Compounding this issue, a significant proportion of seeds collected from wild populations are often empty, lacking fully developed embryos. These empty seeds cannot be identified visually without destruction, which limits the efficiency of propagation programs.

Importantly, the proportion of empty seeds varies widely between years and populations and is strongly influenced by climatic and nutritional conditions during flowering, pollination, and seed development in the parent tree. Factors such as drought stress, high temperatures during flowering, and poor soil fertility can impair embryo formation, seed filling, and increase the number of non-viable or empty seeds (Fenner, 1992; Wulff, 1995; Gutterman, 2000; Adams, 2014; Mácová *et al.,* 2022; Dadlani and Yadava, 2023). These challenges underline the need for accurate, non-destructive methods to evaluate seed viability before germination trials or nursery sowing (Xu *et al*., 2024).

While classical methods such as X-ray radiography and tetrazolium (TTC) staining provide reliable embryo viability assessment (Ahmed *et al.,* 2018), they are either destructive, costly, or time-consuming. Building upon RGB-based software tools for external phenotyping (e.g., SmartGrain by Tanabata *et al.,* 2012; SeedExtractor by Zhu *et al.,* 2021), X-ray imaging methods (Gagliardi and Marcos-Filho, 2011; Gomes-Junior *et al.,* 2012), and infrared imaging (Agelet *et al.*, 2012) with chemometrics (Wakholi *et al.*, 2018) offer a complementary perspective by revealing internal seed structure and germination capacity. While advanced modalities like X-ray and NMR provide superior insight into internal anatomy, RGB imaging remains the more practical and accessible choice for high-throughput phenotyping due to its low cost, operational simplicity, and rapid data capture. This accessibility makes it ideally suited for large-scale studies focused on external morphological traits (Halcro *et al.*, 2020). Consequently, recent advances in digital phenotyping and artificial intelligence (AI) offer alternative approaches to evaluate internal seed quality based on external visual traits (Nguyen *et al.,* 2015; De Medeiros *et al.,* 2020 a, b). The application of machine learning techniques, such as Artificial Neural Networks (ANN), Random Forest (RF), Support Vector Machines (SVM), and k-means clustering, to agricultural problems is now widespread (Rehman *et al.,* 2019; Saha and Manickavasagan, 2021). By extracting features such as RGB color intensity, projected area, and seed shape from digital images, and combining them with machine learning tools like artificial neural networks (ANNs), researchers have successfully predicted seed viability in various species (Fraas *et al.,* 2014; Rousseau *et al.,* 2015; De Medeiros *et al.,* 2020 a, b).

In this study, we developed and tested a non-destructive method for detecting empty seeds in *Acer monspessulanum* using image-based features and a neural network classification model. Our aim was to determine whether morphological and colorimetric traits derived from 2D images could be used to predict the presence or absence of embryos with high reliability. This approach may enhance seed quality screening in undomesticated tree species with complex dormancy and contribute to the propagation and conservation of drought-tolerant ornamental species.

2. Materials and Methods

Seed collection and preparation

Mature seeds of *Acer monspessulanum* were obtained from the Natural Resources and Watershed Management Organization of North Khorasan

Province, Iran. The seeds were collected from naturally growing trees in their native habitat. A total of 200 seeds were randomly selected, manually dewinged, and labeled sequentially from 1 to 100 in duplicate.

Acid scarification and soaking

To facilitate seed coat removal and improve embryo extraction, seeds were immersed in 65% nitric acid (HNO_3) for 6 hours at room temperature. Following acid treatment, seeds were rinsed three times with distilled water (15 minutes per rinse) to remove residual acid. The seeds were then soaked in distilled water at 24±1°C on a rotary shaker. Floating and non-floating seeds were recorded after 24 and 48 hours of soaking. Embryos were extracted manually after 48 hours of soaking.

Embryo extraction and viability testing

Although nitric acid treatment improved seed coat removal, the viability of embryos were lost during extraction. Attempts to assess embryo viability using 2,3,5-triphenyltetrazolium chloride (TTC) staining failed during the main experiment. However, a separate viability test showed that 70% of the seeds contained embryos, while the remaining 30% were empty. The 200 seeds used in the main experiment followed approximately the same proportions of embryo-containing versus empty seeds, ensuring that the dataset accurately reflected embryo presence in the population.

Image-based morphological data collection

Before and after seed treatment and after embryo extraction, all 200 seeds and their respective embryos were placed on gridded paper and photographed under standardized lighting conditions (Fig. 1). Quantitative image-based traits of seeds and embryos - including projected area, perimeter, length, width, roundness, intensity, Hue, and RGB color intensity values (red, green, and blue channels) - were extracted using Digimizer software. RGB color intensity values were converted to CIELAB color space parameters (L*, a*, and b*) using Python (v 3.11) and the skimage library, after normalization of RGB values to the 0-1 range.

Embryo weight measurements

The fresh weight of the embryos was measured immediately after extraction. To determine dry

Fig. 1 - Seeds of *Acer monspessulanum* prior to treatment (left), following acid scarification and 48-hour water soaking (middle), and the corresponding extracted embryos (right).

weight, embryos were placed in a drying oven at 70°C for 48 hours, then weighed using a precision analytical balance.

Data analysis

Statistical analysis of seed traits based on embryo presence. To investigate relationships between the recorded traits, data were analyzed by grouping seeds according to embryo presence. Before selecting the t-test (Welch's t-test), we examined the homogeneity of variances. Statistical comparisons were performed in R using a two-sample Welch's t-test due to significant unequal variances in some traits and unequal sample sizes, with significance assessed at $p<0.05$. In addition, Fisher's Exact Test was used for the Seed Floating24 and Seed Floating48 traits because of their binary distribution.

Artificial neural network (ANN). Following the initial statistical analyses, the easily observable and well-defined traits that showed significant differences with in the t-test were selected for further modeling. To investigate feed-forward neural network potential of these traits in predicting embryo presence, a feedforward ANN model was developed using Python 3.11 with Keras (TensorFlow backend) libraries. The input layer consisted of seed length, seed roundness, seed weight, and seed floating after 24 and 48 hours, and the output layer was binary (1= embryo present, 0= empty seed). The dataset was randomly split into training (60%) and testing (40%) subsets. Prior to model training, all features were standardized to zero mean and unit variance using StandardScaler in order to improve convergence during neural network optimization. The architecture consisted of: An input layer with dimensionality equal to the number of features in X. A first hidden layer of 96 neurons with ReLU activation. A second hidden layer of 128 neurons with ReLU activation. A final output layer with a single neuron and sigmoid activation for binary classification. Alternative hyperparameter configurations were explored with Keras Tuner (Random Search), but the final reported model used fixed parameters based on preliminary results. The network was compiled using the Adam optimizer, with binary cross-entropy as the loss function and accuracy as the evaluation metric. Training was performed for 10 epochs with a batch size of 32, using 40% of the training data as an internal validation set. Model performance was assessed on both training and test sets. Metrics included: Accuracy, Confusion matrix AND Precision, recall, and F1-score (via classification_report). In addition, loss curves (training *vs* validation) were plotted across epochs using Matplotlib to monitor convergence.

Multiple linear regression. At the final stage, for seeds confirmed to contain an embryo, embryo fresh weight, embryo dry weight, and embryo water content were used as quantitative indices of embryo vigor. It should be noted that these indices provide information on embryo development but, as indirect measures, are most informative when interpreted together with physiological or performance-based vigor traits, such as germination rate, seedling growth, or TTC staining results. The processes related to dormancy breaking were not studied here, as the current work focuses on non-destructive embryo detection and phenotypic trait analysis. To evaluate how these vigor indices were explained by seed phenotypic features, multiple linear regression with stepwise (forward) selection was applied, using image-based traits and seed weight as explanatory variables (entry criterion: $p<0.05$; removal criterion: $p>0.10$). Statistical significance was accepted at $p\leq0.05$. All analyses were performed using SAS (version 9.4) software.

3. Results

Trait differences by embryo presence

To assess whether specific morphological and physical traits are related to embryo presence, seeds were divided into two groups: those containing an embryo and those without. A two-sample t-test assuming unequal variances (Welch's t-test) was performed to compare the mean values of the measured traits between the two groups (Table 1).

The analysis revealed statistically significant differences ($p<0.05$) in several traits, including perimeter (before scarification), color intensity (before scarification), length (before scarification), roundness (before scarification), Hue (after scarification), seed weight, and seed floating at 24 h and 48 h. Specifically, seeds containing embryos were significantly longer, rounder, heavier, and exhibited distinct Hue and intensity values, indicating morphological differentiation linked to embryo present. In addition, a significantly lower proportion of embryo-containing seeds remained afloat after 24 h and 48 h of soaking, indicating lower buoyancy and higher density, traits commonly of embryo-

Table 1 - Comparison of seed traits of *Acer monspessulanum* based on embryo presence using two-sample t-test (Unequal Variances)

Time	Seed Traits	With embryo		Without embryo		T-test
		Mean	SE	Mean	SE	(p-Value)
Before acid scarification	Seed weight	48.475	10.361	32.716	11.844	0
	Area	0.24	0.048	0.251	0.057	0.2
	Perimeter	1.782	0.172	1.845	0.208	0.047
	Intensity	0.24	0.094	0.273	0.107	0.046
	L*	27.432	11.276	31.239	12.471	0.051
	a*	14.55	6.362	15.883	6.356	0.189
	b*	2.728	2.759	3.159	3.135	0.371
	Hue	37.248	6.913	36.85	6.625	0.708
	Length	0.668	0.073	0.713	0.089	0.001
	Width	0.455	0.061	0.445	0.065	0.331
	Roundness	0.94	0.041	0.916	0.046	0.001
After acid scarification	Seed Floating24	0.362	0.482	1	0	0
	Seed Floating48	0.188	0.392	0.946	0.227	0
	Area	0.347	0.049	0.356	0.064	0.324
	Perimeter	2.113	0.153	2.144	0.2	0.298
	Intensity	0.275	0.055	0.271	0.055	0.584
	L*	33.596	6.746	32.916	6.914	0.533
	a*	34.418	5.466	33.9	6.126	0.584
	b*	24.992	3.038	26.03	4.245	0.1
	Length	0.754	0.074	0.773	0.089	0.162
	Width	0.584	0.051	0.584	0.064	0.955
	Hue	21.231	2.711	20.027	2.924	0.009
	Roundness	0.971	0.025	0.966	0.027	0.186

containing seeds. Conversely, no significant differences were detected in traits such as projected area, width, and several color parameters (e.g., b values), indicating that these characteristics may be less influenced by embryo presence. A marginally significant difference was observed in L* (before scarification; $p = 0.051$), suggesting a possible trend that may warrant further investigation. These findings demonstrate that a combination of morphological and physical parameters can serve as useful non-destructive indicators for identifying embryo-containing seeds of *Acer monspessulanum*.

Machine learning classification

In addition to statistical comparisons, a supervised machine learning approach was applied to classify *Acer monspessulanum* seeds based on embryo presence, using the same morphological and physical traits analyzed in the Welch's t-test and Fisher's Exact Test. A fully connected feedforward neural network was implemented using a sequential architecture composed of three dense layers: an input layer with 96 units, a hidden layer with 128 units, and an output layer with a single neuron for binary classification. The network architecture was selected based on iterative testing to maximize prediction accuracy while minimizing over fitting (Eryigit and Tugrul, 2021). The model included 13,121 trainable parameters.

The model achieved high predictive performance. On the test dataset, the model yielded an accuracy of 91.03% and a mean absolute error (MAE) of 0.91, with a minimum and maximum target value of 0.0 and 1.0, respectively. Training accuracy reached 96.55%, indicating strong learning capacity with minimal overfitting. Evaluation using a confusion matrix showed robust classification results, with 24 true negatives, 47 true positives, 4 false positives, and only 3 false negatives. The overall precision, recall, and F1-score were 0.91, 0.90, and 0.90, respectively, indicating balanced performance across both classes. Notably, seeds with embryos were predicted with high precision (0.92) and recall (0.94), reinforcing the model's reliability in detecting

embryo presence (Fig. 2).

These results confirm that the integration of morphological and physical seed traits - particularly those identified as statistically significant in the t-test analysis - can effectively support machine learning-based classification of embryo-containing seed. The deep learning model provides a promising non-destructive tool for embryo detection in *Acer monspessulanum* seeds.

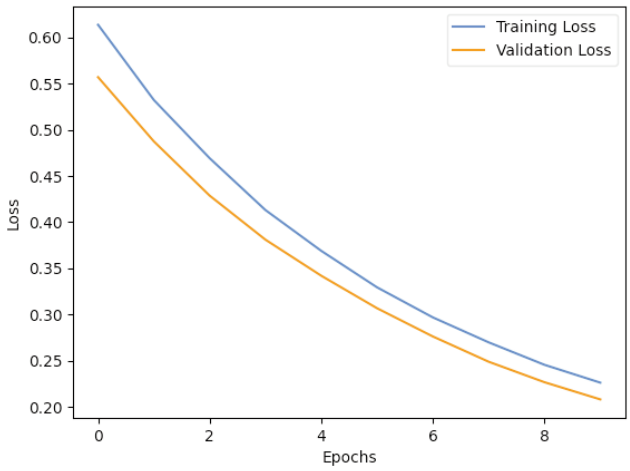

Fig. 2 - Training and validation loss curve of the Neural Network Model for embryo classification in *Acer monspessulanum* seeds.

Multiple linear regression models for predicting embryo biomass and water content

Stepwise forward multiple linear regression was conducted separately for embryo fresh weight, dry weight, and water content using only seeds confirmed to contain embryos, to identify the most predictive traits.

The predictive models showed moderate to strong agreement between observed and estimated embryo traits in *Acer monspessulanum* seeds (Fig. 3). In all three cases, data points (open blue circles) cluster around the 1:1 reference line (grey diagonal), indicating good agreement between predicted and measured values. For embryo fresh weight (left panel), the majority of values are concentrated between 10-25 mg, with only a few deviations above 30 mg. embryo dry weight (middle panel) exhibits a similar pattern, with predictions tightly grouped between 5-15 mg, though some scatter is evident at higher values. Embryo water content (right panel) shows a broader range (20-70%), with predictions capturing the general trend but with greater dispersion compared to embryo fresh weight and embryo dry weight. Overall, the models demonstrate strong predictive accuracy, particularly for embryo fresh weight and embryo dry weight, whereas embryo water content shows slightly higher

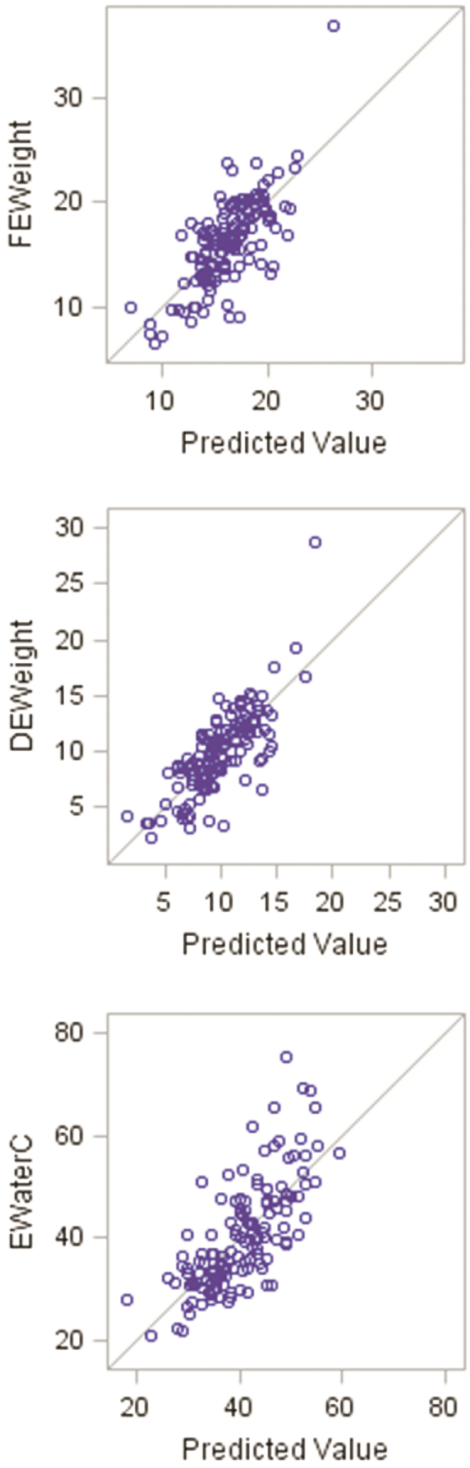

Fig. 3 - Relationship between observed and predicted values of embryo fresh weight (left, FEWeight), embryo dry weight (middle, DEWeight), and embryo water content (right, EWaterC) in *Acer monspessulanum* seeds. Each point represents and individual seed measurement, while the solid line denotes the 1:1 relationship.

variability. These results highlight the reliability of the predictive approach in estimating embryo traits critical for seed physiology studies.

Stepwise regression model for predicting embryo fresh weight

To identify the most influential predictors of embryo fresh weight, a forward stepwise selection procedure was applied using a multiple linear regression model. The results are summarized in Table 2. The model successively introduced variables that contributed the most to explaining variation in embryo fresh weight among seeds with embryos, based on partial R^2, F-value, and Mallows' C(p) criterion.

Seed weight was identified as the most significant predictor of embryo fresh weight, explaining 27.5% of the variation in the model (Partial R^2 = 0.2752). The inclusion of seed weight alone produced a strong and highly significant model (F = 51.65, p < 0.0001), indicating its primary role in determining embryo fresh weight in *Acer monspessulanum* seeds. The second variable included was the a* color parameter measured before acid scarification, which significantly improved the model by explaining an additional 13.5% of the variance (Partial R^2 = 0.1349). With this addition, the model R^2 increased to 0.4102 (F = 30.88, p < 0.0001). The intensity parameter, also measured before acid scarification, further enhanced the model fit, contributing an additional 6.9% to the explained variance (Partial R^2 = 0.0690), resulting in a cumulative R^2 of 0.4792 (F = 17.75, $p < 0.0001$). Seed floating after 48 hours of water immersion (Seed Floating48) was added at the fourth step and explained an additional 1.9% of the variance in embryo fresh weight (Partial R^2 = 0.0189), bringing the model R^2 close to 50% (0.4981). This variable showed a significant but smaller effect compared to the earlier entered predictors (F = 5.01, p = 0.0269). Subsequent variables, including intensity and a* color after acid scarification, width, roundness, Hue, and b* color parameter, contributed minimally to the model (each partial R^2 < 0.004), with none reaching statistical significance (all $p > 0.05$). These variables produced negligible improvements in the overall explained variance, indicating that the first four variables sufficiently capture the major variation in embryo fresh weight.

Stepwise regression model for predicting embryo dry weight

A forward stepwise regression analysis was conducted to identify the most influential predictors of embryo dry weight in *Acer monspessulanum*. Variables were sequentially added to the model based on their contribution to explained variance and statistical significance, as indicated by changes in partial R^2, model R^2, Mallows' C(p), and F-tests (Table 3).

Seed weight was the strongest single predictor of embryo dry weight, explaining 20.8% of the variance on its own (Partial R^2 = 0.2081). This variable produced a highly significant model (F = 35.74, $p < 0.0001$), underscoring its importance in determining embryo dry weight in *Acer monspessulanum*. The b* color parameter measured before acid scarification was the second variable selected and contributed an additional 22.3% to the explained variance (Partial R^2 = 0.2225). This substantially increased the model fit to an R^2 of 0.4306 (F = 52.76, $p < 0.0001$), highlighting the relevance of seed coat coloration in predicting dry weight. Adding the a* color parameter before acid scarification explained a further 4.1% of the

Table 2 - Summary of forward selection for predicting embryo fresh weight in *Acer monspessulanum*

Step	Variable entered	Number Vars In	Partial R^2	Model R^2	C(p)	F Value	Pr > F
1	Seed Weight	1	0.2752	0.2752	456.687	51.65	<.0001
1	a* (Before acid scarification)	2	0.1349	0.4102	142.181	30.88	<.0001
2	Intensity (Before acid scarification)	3	0.069	0.4792	-0.8864	17.75	<.0001
3	Seed Floating48	4	0.0189	0.4981	-35.691	5.01	0.0269
4	Intensity (After acid scarification)	5	0.0074	0.5055	-34.145	1.99	0.161
5	Width (Before acid scarification)	6	0.0037	0.5092	-2.342	1	0.3195
6	a* (After acid scarification)	7	0.0039	0.5131	-13.016	1.03	0.3112
7	Roundness (After acid scarifica-	8	0.0024	0.5156	0.0916	0.65	0.421
8	Hue (Before acid scarification)	9	0.0022	0.5178	15.458	0.58	0.446
9	b* (Before acid scarification)	10	0.0039	0.5216	2.587	1.03	0.3128

Table 3 - Summary of forward selection for predicting embryo dry weight in *Acer monspessulanum*

Step	Variable Entered	Number Vars In	Partial R^2	Model R^2	C(p)	F Value	Pr > F
1	Seed Weight	1	0.2081	0.2081	110.12	35.74	<.0001
2	b* (Before acid scarification)	2	0.2225	0.4306	43.52	52.76	<.0001
3	a* (Before acid scarification)	3	0.0406	0.4712	33.01	10.29	0.0017
4	Intensity (Before acid scarification)	4	0.0815	0.5527	9.9	24.22	<.0001
5	Seed Floating24	5	0.0197	0.5723	5.84	6.07	0.0151
6	Intensity (After acid scarification)	6	0.0102	0.5826	4.69	3.21	0.0755
7	a* (After acid scarification)	7	0.0066	0.5891	4.66	2.08	0.1512
8	Area (Before acid scarification)	8	0.0056	0.5947	4.94	1.77	0.186
9	Roundness (After acid scarifica-	9	0.0039	0.5986	5.75	1.24	0.268
10	Hue (Before acid scarification)	10	0.0023	0.6009	7.03	0.74	0.3911

variance (Partial R^2 = 0.0406), leading to a cumulative R^2 of 0.4712. This variable was also statistically significant (F = 10.29, p = 0.0017), indicating its complementary role alongside b* in characterizing seed coat traits linked to embryo dry weight. The intensity parameter measured before acid scarification added 8.1% to the model's explanatory power (Partial R^2 = 0.0815), bringing the total R^2 to 0.5527 (F = 24.22, p < 0.0001). This suggests that brightness or darkness of the seed coat further refines predictions of embryo dry weight. Seed buoyancy after 24 hours of immersion (Seed Floating24) contributed an additional 2.0% to the model (Partial R^2 = 0.0197), with the overall model R^2 reaching 0.5723 (F = 6.07, p = 0.0151). This variable showed a modest but statistically significant effect on embryo dry weight. Subsequent variables including intensity and a* after acid scarification, seed area, roundness after acid scarification, and Hue before acid scarification provided minor increments in explained variance (each partial R^2 < 0.011), none of which were statistically significant (p > 0.05), except for intensity after acid scarification, which approached marginal significance (p = 0.0755). This indicates that the major variation in embryo dry weight is explained predominantly by the first five variables included in the model.

Stepwise regression model for predicting embryo water content

To identify key variables influencing embryo water content in *Acer monspessulanum*, a forward stepwise regression analysis was performed. Variables were introduced into the model sequentially based on their individual contributions to explained variance, assessed through partial and cumulative R^2, Mallows' C(p), F-values, and associated p-values (Table 4).

The b* color parameter measured before acid scarification was the strongest predictor of embryo water content, explaining 31.6% of the variance (Partial R^2 = 0.3159). This variable alone resulted in a highly significant model (F= 62.79, p<0.0001), demonstrating the importance of seed coat color characteristics in determining embryo hydration levels. Seed buoyancy after 24 hours (Seed Floating24) contributed an additional 6.1% of explained variance (Partial R^2 = 0.0606), increasing the total model R^2 to 0.3765 (F= 13.12, p= 0.0004). This highlights seed floating capacity as a meaningful indicator of embryo water content. Perimeter measured before acid scarification added 3.7% to the explained variance (Partial R^2 = 0.0370), resulting in a model R^2 of 0.4134 (F= 8.45, p= 0.0043). This suggests seed size and shape features contribute to variations in embryo water content. Intensity after acid scarification increased the model fit by 1.4% (Partial R^2 = 0.0138), with a marginal significance (F= 3.20, p= 0.0758). Although this effect was weak, it indicates some influence of seed coat brightness after treatment on embryo hydration. The a* color parameter after acid scarification further explained 2.8% of variance (Partial R^2 = 0.0280), significantly improving the model to an R^2 of 0.4552 (F= 6.78, p= 0.0103). This highlights the role of seed color changes post-treatment in relation to embryo water content. Additional variables including seed weight, Hue before acid scarification, intensity before acid scarification, a* before acid scarification, L before acid scarification, area before acid scarification, and seed floating after 48 hours contributed small

Table 4 - Summary of forward selection for predicting embryo water content in *Acer monspessulanum*

Step	Variable entered	Number Vars In	Partial R^2	Model R^2	C(p)	F Value	Pr > F
1	b* (Before acid scarification)	1	0.3159	0.3159	379.098	62.79	<.0001
2	Seed Floating24	2	0.0606	0.3765	246.838	13.12	0.0004
3	Perimeter (Before acid scarifica-	3	0.037	0.4134	173.896	8.45	0.0043
4	Intensity (After acid scarification)	4	0.0138	0.4272	159.229	3.2	0.0758
5	a* (After acid scarification)	5	0.028	0.4552	108.876	6.78	0.0103
6	Seed Weight	6	0.0081	0.4634	108.429	1.99	0.1611
7	Hue (Before acid scarification)	7	0.0086	0.472	106.757	2.12	0.1475
8	Intensity (Before acid scarification)	8	0.01	0.482	101.634	2.49	0.117
9	a* (Before acid scarification)	9	0.0275	0.5095	52.557	7.17	0.0084
10	L (Before acid scarification)	10	0.0023	0.5118	66.874	0.59	0.4445
11	Area (Before acid scarification)	11	0.0029	0.5147	7.949	0.76	0.3841
12	Seed Floating48	12	0.0034	0.5181	90.934	0.88	0.3491

increments (each partial R^2 <0.028), with varying degrees of significance. Notably, a* before acid scarification was significant (*p= 0.0084*), whereas seed weight and several others were not statistically significant (*p>0.10*). These findings suggest that most variation in embryo water content is captured by the first five to nine variables entered into the model.

4. Discussion and Conclusions

This study demonstrates that a combination of morphological and physical traits can effectively distinguish embryo-containing seeds from empty ones in *Acer monspessulanum*. Using a two-sample t-test, several traits were found to be significant distinguishing traits for the presence of an embryo, including seed weight, length, roundness, Hue values, and soaking behavior (floating after 24 and 48 hours). This aligns with established seed physiology: filled seeds often exhibit greater tissue density, developmental maturity, and compositional differences compared to empty seeds (Bewley *et al.,* 2013), reflecting fundamental physiological distinctions.

Among the most discriminative features, seed weight emerged as a key indicator of embryo presence. This is consistent with studies demonstrating that seeds are significantly heavier than non-viable ones and are more likely to contain well-developed embryos (Daneshvar *et al.,* 2017; Domic *et al.,* 2020). Size-related traits are strongly positively correlated with seed weight, indicating that physical dimensions have a greater influence on weight than shape does (Duc *et al.,* 2023). Additionally, seed buoyancy, assessed through floating tests, proved to be a strong physiological marker. Techniques like incubation-drying-separation (IDS) and modified specific gravity (MSG) separation are widely used to remove empty seeds. These methods exploit the principle that viable seeds absorb and metabolically bind more water during soaking than dead seeds. This differential water uptake allows for highly effective sorting based on the viscosity of the flotation medium (Daneshvar *et al.,* 2017). Embryos are more prevalent in sunken seeds due to their higher density and structural integrity, both strong indicators of viability (Sautu *et al.,* 2006). The significant role of tissue density metrics as predictors in seed quality classification establishes a direct correlation between structural integrity and viability. This principle is operationalized in flotation tests, where the higher density of viable seeds causes them to consistently sediment (Daneshvar *et al.,* 2017). Consistent with research on hardwoods, flotation behavior serves as a standard proxy for seed viability (Schmidt, 2000; Bonner, 2008). This method's efficacy is confirmed in *Juniperus polycarpos*, where the sunken fraction contains a significantly higher proportion of viable seeds, solidifying its role as a reliable preliminary sorting technique. (Daneshvar *et al.,* 2017).

Significant associations were identified between

embryo presence and colorimetric variables, specifically Hue (post-scarification) and color intensity (pre-scarification). These optical traits may reflect underlying biochemical changes in the seed coat during embryogenesis, such as lignification and the accumulation of pigments (e.g., phenolic compounds), which alter light interaction. Consequently, spectral imaging emerges as a powerful tool for non-destructively quantifying these chemical compositions. The distinct spectral signatures of viable seeds highlight the potential of spectral and hyperspectral imaging and digital color analysis as rapid, non-destructive methods for assessing seed viability (Boelt *et al.,* 2018; ElMasry *et al.,* 2019; Wang *et al.,* 2022; Fan *et al.,* 2023).

Building upon our robust statistical findings, we implemented a neural network (NN) classifier to predict embryo presence, which achieved a high overall accuracy of 91.03%. The model demonstrated balanced performance, excelling particularly in the identification of embryo-containing seeds, as evidenced by strong precision (0.92) and recall (0.94) scores. This high predictive power indicates that the integration of key morphological and physical traits into a machine learning (ML) framework is a practical strategy for developing rapid, automated, and non-invasive seed sorting systems. Our results corroborate a growing body of research demonstrating the efficacy of artificial intelligence (AI) for seed quality assessment, with successful applications already established for plant species. The consistently high performance of various architectures - from CNN (92.06%; Xu *et al.,* 2024), 3DCNN (92.00%; Fan *et al.,* 2023), and LDA (95.8%; Jeong *et al.,* 2024) to exceptionally accurate 2DCNN models (99.96%; Fan *et al.,* 2023) across studies on maize and soybean - demonstrates the robustness of this approach. Notably, Artificial Neural Networks (ANNs) are repeatedly validated as a particularly effective algorithm for deciphering the complex spectral patterns indicative of seed physiological quality (De Medeiros *et al.,* 2020 a; Xu *et al.,* 2024; Jeong *et al.,* 2024). This convergence of evidence from independent research groups confirms that the integration of HSI with machine learning is a transformative methodology for rapid and precise seed classification.

The regression analyses for embryo biomass components further validated the predictive value of specific traits. Seed weight consistently emerged as the strongest predictor across models for both embryo fresh and dry weights, underscoring its fundamental role in determining embryo biomass in *Acer monspessulanum*. This finding aligns with the well-established link between seed size and embryo development, where heavier seeds tend to harbor more substantial embryo reserves, contributing to seedling vigor and establishment potential.

For embryo fresh weight, seed weight alone explained 27.5% of the observed variation, with the model's explanatory power substantially improved by the inclusion of seed coat color traits, notably the a* parameter measured before acid scarification. The positive contribution of color parameters (a* and intensity) suggests that seed coat pigmentation and brightness are closely related to embryo development. These traits may reflect underlying biochemical or structural properties of the seed coat that influence nutrient allocation or protection of the embryo. Furthermore, seed buoyancy after 48 hours (Seed Floating48) contributed a smaller yet significant additional variance, potentially indicating seed coat permeability or structural integrity as factors modulating embryo fresh weight. Similarly, in predicting embryo dry weight, seed weight remained the dominant predictor, accounting for 20.8% of variance.

The b* color parameter, indicative of seed coat color along the yellow-blue spectrum, added substantial predictive power, raising the model R^2 to over 43%. This highlights the complementary role of seed coat color characteristics in predicting embryo dry mass, potentially reflecting biochemical composition linked to desiccation tolerance or storage compound accumulation. Other seed coat traits such as a* and intensity before acid scarification further improved the model, with seed buoyancy after 24 hours also contributing significantly, again pointing to physical seed coat properties affecting embryo biomass accumulation. In contrast to embryo biomass, embryo water content was most strongly predicted by the b* color parameter before acid scarification, explaining over 31% of variance, suggesting that seed coat coloration plays a crucial role in regulating embryo hydration. Seed buoyancy after 24 hours also contributed notably, supporting the hypothesis that seed coat permeability or water absorption capacity affects embryo water status. The influence of seed perimeter further implicates seed size and shape as factors in water retention. While seed weight was less predictive of water content, color parameters

both before and after acid scarification remained significant, indicating that dynamic changes in seed coat coloration and properties during acid treatment may reflect or influence water relations within the embryo.

Overall, the forward stepwise regression approach demonstrated that a relatively small set of seed traits - primarily seed weight, seed coat color parameters (a* and b*), seed coat brightness (intensity), and buoyancy measures - capture most of the variation in embryo fresh weight, dry weight, and water content. These findings emphasize the multifaceted role of seed morphological and physical traits in embryo development and hydration, which are critical for understanding seed quality and potential germination success in *Acer monspessulanum*. Future studies should investigate the mechanistic basis of these correlations, particularly the biochemical and structural seed coat changes linked to color variation and buoyancy, to better elucidate their impact on embryo growth and water regulation.

Collectively, these findings contribute to the growing body of literature advocating for non-destructive, image-based, and AI-driven methods in seed technology. While traditional x-ray or dissection methods remain reliable, they are labor-intensive and not scalable for large-scale screening in forestry or conservation programs (ISTA, 2020). By contrast, the approach demonstrated here offers a cost-effective and high-throughput alternative for selecting embryo-containing *Acer monspessulanum* seeds, with potential applications in other hard-seeded or recalcitrant species.

The non-destructive imaging-based approach combined with neural network analysis developed in this study is not limited to *Acer monspessulanum*. Given its reliance on general morphological and physical traits, this method is scalable and potentially transferable to seeds of other species, offering a cost-effective tool for seed quality assessment, reforestation programs, and conservation initiatives. Future studies could apply this framework to a wider range of species to evaluate its broader applicability and optimize species-specific prediction models.

This study demonstrates that a combination of morphological and physical seed traits can serve as reliable, non-destructive indicators of embryo presence and developmental status in *Acer monspessulanum*. Key traits such as seed weight, floating behavior, and colorimetric indices were significantly predictive of embryo viability and biomass. The successful application of a neural network classifier further highlights the potential of machine learning tools to accurately and efficiently differentiate embryo-containing from empty seeds based on external features. Additionally, multiple linear regression models provided insight into the predictive relationships between seed traits and embryo biomass components, enabling the estimation of internal seed quality without destructive testing. These findings support the development of scalable, cost-effective screening methods for assessing embryo presence in seeds in forestry, ecological restoration, and conservation programs, with potential applicability across a wide range of species.

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Exogenous application of humic acid or chitosan mitigates drought stress on *Paspalum vaginatum* turfgrass

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Abstract: The current study was carried out to examine the impact of humic acid or chitosan applications on morphology and physiology attributes of *Paspalum vaginatum* Swartz. cv. Salam grown under drought stress. Drought stress was enforced by various watering intervals (2, 5, 8 and 11). The plants subjected to various watering intervals were sprayed biweekly with either humic acid (HA) or chitosan (CHT) each at concentrations of 300 and 600 ppm, whereas the tap water was used as control. The findings indicated that drought stress decreased all growth traits (such as, sward height, lawn density and dry weights of clippings and underground parts), total chlorophylls, total carbohydrates, N, P and K%, while proline, phenols content and Enzyme activity (CAT, APX and SOD) were raised. The plants foliar sprayed with HA or CHT at higher doses led to boost in the tested growth traits, total chlorophylls, total carbohydrates, N, P and K%, proline, phenols and enzyme activity with superiority of HA. Based on the outcome of the present research it can be inferred that, foliar application of HA at 600 ppm can ameliorates the harmful impacts of drought stress on physiology and growth traits of *Paspalum vaginatum*.

1. Introduction

Seashore paspalum (*Paspalum vaginatum* Swartz.) is one of the most extensively utilized grasses for lawn establishment in Egypt. It widely utilized in new towns, as well as in golf courses, sports fields, coastal resorts, tourist villages, and home lawns. It is suited to warm subtropical and tropical regions. It produces a dark green turf that is thick and finely textured. It can be applied to golf course greens as well as sport turfs and utility lawns (Barsoom *et al*., 2024).

The primary environmental factor affecting the quality, growth, and production of turfgrass is water scarcity, which is a biotic stressor restricting agricultural productivity in the majority of countries, particularly in dry and semi-arid regions. Turfgrass undergo a number of

physiological and biochemical changes as a result of water stress, including a decrease in plant morphology (Cui *et al*., 2020; Katuwal *et al.*, 2021; Errickson *et al*., 2023; Taleb *et al*., 2023; Porcelli *et al*., 2024; Hejl *et al*., 2024), decreases in the absorption of nutrients (Shen *et al*., 2024), decreases in total chlorophyll content (Sheikh Mohammadi *et al*., 2017), rising levels of phenols and prolin (Fariaszewska *et al*., 2020), as well as rising levels of in CAT, SOD or APX (Bandurska and Jozwiak, 2010; Salehi *et al*., 2014; Katuwal *et al*., 2020).

Bio-stimulators have been used recently as a result of research into biological ways to prevent the use of chemical products and mitigate the negative effects of water scarcity in agriculture. Among the different types of bio-stimulators are Humic acid and chitosan. Humic acid (HA) is a natural polymer organic compound which can be utilized to improve plant growth, nutrient availability in the soil. Under normal conditions previous authors reported HA had a favorable impact on enhancing growth and nutrient uptake of turfgrass species (Shahin, *et al*., 2015; Taher *et al*., 2023). Under drought stresse conditions, HA has the ability to alleviate the deleterious impacts of drought by enhancing chlorophyll, and carotenoids, total sugars, indoles, phenols, nutrient uptake as well as antioxidant activities (El-Sayed *et al*., 2016; Aalipour *et al*., 2019).

Chitosan (CHT) is another bio- stimulators chitin derivative. It is a naturally occurring polymer that is environmentally benign and biodegraded by biological agents in agriculture (Shafiei-Masouleh, 2019). Under normal conditions, previous studies augmented CHT had a suitable impact like increase growth parameters, chlorophylls content, photosynthesis, and nutrient uptake (Byczyńska, 2018; Abd-El-Hady, 2020). Under stressed conditions, the detrimental impacts of drought can be effectively alleviated by CHT by increasing proline content, antioxidant activity, chlorophyll and carbohydrates (Pirbalouti *et al*., 2017; Zhao *et al*., 2019; Almeida *et al*., 2020; Abou dahab *et al*., 2023). The beneficial effects of CHT is increase stomatal closure through ABA synthesis, photosynthetic rate, and the synthesis of carbohydrates, amino acids, organic acids, and other metabolites that are necessary for energy metabolism under stress, osmotic adjustment, and stress signaling. They also stimulate antioxidant enzymes via the signaling pathways for hydrogen peroxide and nitric oxide (Hidangmayum, *et al*., 2019).

Although bio-stimulators have been shown to have positive effects on ornamental plants and to increase growth parameters, there is insufficient information on how they can mitigate the negative effects of drought on turfgrass. Therefore, the purpose of this study is to assess how foliar application of HA or CHT affects the quality of *Paspalum vaginatum* grown under drought stress.

2. Materials and Methods

The present experiment (8.5 months) was undertaken in the experimental nursery of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza, during the two growing season of 2023 and 2024. The aim of this work was to evaluate the response of seashore paspalum grown under drought stress to foliar application of HA or CHT.

Plant material

On 1st of March, 2023 and 2024 growing seasons, sods of seashore paspalum were obtained from a commercial turf nursery and planted in the experimental area which divided into sixty beds (1 m x 1 m), with distance 30-cm between them. Compost was incorporated into the soil to a depth of 12 to 15 cm at a rate of 2 m^3/100 m^2 to thoroughly prepare the beds. The physical and chemical characteristics of the experimental soil are presented in Table 1. The conditions of temperature, relative humidity and total rainfall from the beginning to the end of the experiment are reported in Table 2.

Experimental procedures

Starting from 15th of March in both seasons, respectively the beds were irrigated once every 2, 5, 8 and 11 days with 6 L of fresh water/bed for imposing drought stress. The beds were irrigated 127.5, 51, 32, and 23.2 times throughout the course of the study. This implies that when the experiment is finished (after 8.5 months), beds watered every 2, 5, 8 and 11 days interval were given 765, 306, 191.25and 139.09 water liters, in the order.

Stating from 31st March till to 15th October (in both seasons), the beds were sprayed every 2 weeks with either HA or CHT each at concentrations of 300 and 600 ppm, while only tap water was used to spray the control plants. Both HA and CHT were purchased from Tecknogreen company, Egypt. Using a plastic

Table 1 - Soil physical and chemical properties utilized for growing *Paspalum vaginatum* (mean of two seasons)

Soil properties	Data
Physical characteristics	
Soil texture	
Clay	55.2
Coarse sand	4.99
Fine sand	17.08
Silt	22.76
Chemical characteristics	
Soluble anions (meq/l)	
Cl-	3.13±0.03
SO4-	2.52±0.02
Ca++	7.39±0.04
Mg++	2.76±0.03
K+	0.39±0.01
Na+	5.15±0.05
N (ppm)	93.35±3.00
P (ppm)	21.13±0.13
O. m (%)	1.61±0.20
EC (dS/m)	1.54±0.12
pH	7.15±0.04

atomizer, the turfgrass was sprayed until the runoff threshold (100 ml/beds) was reached after adding 1 ml/L of bio-new film as a wetting agent. All the turfgrass beds were monthly fertilized with kristalon-quick fertilizer (NPK 20:20:20 + micronutrients) at a dose of 280 kg/ha. Additionally, manual picking of weeds, disease and pest control has also been carried out.

Layout of experimental

The layout of the experiment was a split-plot design with 20 treatments [4 watering frequency x 5 plant bio-stimulators (including the control)] with 3 blocks (replicates), each replicate consisting of 20 beds (1 bed/treatment). Irrigation frequency were assigned to the main plots in a randomized complete blocks design, while plant bio-stimulators treatments were randomly assigned to the sub-plots within irrigation frequency.

The data recorded

Vegetative growth traits. On the 15th of April till the end of the study (in two seasons, respectively), the turfgrass was mowed biweekly to a height of 3 cm using scissors. Sward height (cm) was recorded immediately before each mowing (every 2 weeks) using the method described by Dernoeden (1984), in which a ruler was set upright on the soil surface and a cardboard disk with a hole in its center was dropped freely over the ruler onto the top of the vegetative turf canopy. Sward height was calculated as the distance between soil surface and the disk, three sward height measurements were recorded in each plot, and the average sward height was calculated. Fresh weights of the clippings (g/m^2) after mowing (every 2 weeks) were collected manually and dried then the average of dry weights of the clippings (g/m^2) were calculated. At the end of each growing season, turf density (number of tillers/100 cm^2) was recorded using a 10 x 10 cm wooden frame which randomly tossed three times per plot, the number of tillers inside the frame was counted manually each time and the average lawn density was then calculated. Root length and dry weight of underground parts (g/m^2) were recorded according to the method of Hussein *et al*. (2012) in which the underground parts (roots + rhizomes) were taken from two sod and soil cores, each with an area of 400 cm^2 (20x20 cm) and a depth of 20 cm. The

Table 2 - Summary of forward selection for predicting embryo fresh weight in *Acer monspessulanum*

Period	Temperature Max. (°C)	Temperature Min. (°C)	Average of RH (%)	Total rain fall (mm)
March	23.5±1.50	11.6±0.06	53±2.50	1.9±0.20
April	28.3±1.30	14.6±2.30	47±1.00	0.9±0.20
May	32.0±1.00	17.7±0.07	46±1.00	0.5±0.10
June	33.9±1.30	20.1±2.10	49±2.00	0.1±0.00
July	34.7±0.07	22.0±1.50	58±±1.00	0±0.00
August	34.2±0.20	22.1±0.10	61±2.00	0±0.00
September	32.6±1.94	20.5±0.60	60±2.00	0±0.00
October	29.2±0.20	17.4±0.40	60±0.00	0.5±0.10

underground parts were washed and weighed, and then recorded weights were used to calculate the average dry weight of underground parts per square meter (g/m^2). Dry weight of the clippings and underground parts were assessed by allowing them to dry at 70°C until their weight was consistent.

Chemical Analysis. At the end of the growing seasons total chlorophylls (a + b) (mg/g fresh weight) in fresh clipping were determined according to the method of Lichtenthaler and Buschmann (2005). In accordance with Dubois *et al*. (1956), the total amount of carbohydrates in the clipping (as a percentage of dry matter) was determined. Nitrogen, phosphorus and potassium content in clipping were determined according to Estefan *et al*. (2013). Nitrogen (%) was determined by using the micro-Kjeldahl method. Phosphorus (%) was determined calorimetrically by using the chlorostannous molybdophosphoric blue colour method in sulphuric acid. Potassium (%) was determined by using the flame photometer apparatus (CORNING M 410, Germany). A method developed by Bates *et al*. (1973) was used to determine the proline content (µ moles/g fresh matter of clipping). According to Selim *et al*. (1978), the total phenol content of three grams of fresh clipping was measured after it was crushed and extracted with 80% ethanol at 0°C for seventy-two hours, with the ethanol being replaced every twenty-four hours. Antioxidant enzyme extraction were carried out using clipping at 40°C in a buffer solution (3: 1 buffer: fresh weight v/v) in a pastel. It was mortared with 100 mM potassium phosphate buffer (at pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinyl pyrolidone. The homogenates were centrifuged at 10000 *g* for 30 min and then the supernatants were stored in separate aliquots at 8°C. Antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were assayed as described by Haida and Hakiman, (2019). Activities of the enzymes were reported in units/min/mg protein.

Statistical analysis

Statistical analysis of variance (ANOVA) was performed on the mean of all collected data using a split plot design. Combined analysis was done on both growing seasons together. Snedecor and Cochran (1989) Duncan's multiple range tests at the 5% level was used to compare the data means.

3. Results and Discussion

Growth traits

Data presented on Table 3 showed that the tested growth traits (sward height, lawn density, dry weights of clippings and underground parts) were significantly affected by irrigation frequency, bio-stimulators treatment and their interaction. Data in Table 4 indicted that within each level of HA or CHT, in most instances, extending the intervals between irrigations daily from 2 to 5, 8 or 11 days resulted in gradual significant decrease in all evaluated growth traits compared to the brief interval (2 days). The detrimental effects of drought surrounding the underground parts, reduced soil moisture availability from water stress, and decreased root absorption of water and nutrients can all contribute to the

Table 3 - Mean square for the impact of irrigation frequency and bio-stimulators treatments and their interaction on vegetative growth traits of *Paspalum vaginatum*

Parameters	Source of variation						
	Treatment			Error		CV	
	Irrigation frequency (A)	Bio-stimulators (B)	(A × B)	(A)	(B)	(A)	(B)
Sward height (cm)	28.04 **	4.74 **	23.31 *	1.91	2.83	13.2	16.09
Lawn density (number of tillers/100 cm^2)	6.59 *	0.75 *	4.30 *	-	-	13.26	13.53
Root length (cm)	10.05 **	9.10 **	0.29 *	0.90	0.66	16.78	14.41
Dry weight of clippings (g/m^2/2 weeks)	159.62 **	41.27 *	101.93 *	4.75	22.56	7.29	15.89
Dry weight of underground parts (g/m^2/2 weeks)	1594.67 **	80.00 *	648.78 *	54.18	88.94	8.73	11.18

*, **, *** significant at P ≤ 0.05, P≤0.01, P≤0.001, respectively.

Table 4 - Sward height, Lawn density and dry weight of clippings and underground parts of *Paspalum vaginatum* as affected by the interactions between irrigation frequency and bio-stimulators treatments (mean of two seasons)

Irrigation frequency	Bio-stimulators	Sward height (cm)	Lawn density (number of tillers/100 cm^2)	Root length (cm)	Dry weight of clippings (g/m^2/2 weeks)	Dry weight of underground parts (g/m^2/2 weeks)
2 days	Control	8.28±0.46 e-g	204.23±8.75 a-d	5.04±1.01 e-g	25.85±0.98 f-h	81.33±3.78 d-f
	HA (1)	14.80±0.29 ab	207.57±10.82 a-d	7.03±0.1 a-c	36.08±4.29 a-d	96.90±8.64 bc
	HA (2)	16.05±0.66 a	220.90±14 a	8.11±0.53 a	40.82±1.1 a	114.30±2.69 a
	CHT (1)	10.52±1.72 de	212.72±19.37 a-c	7.03±0.49 a-c	36.90±2.64 a-c	92.70±5.3 b-e
	CHT (2)	13.55±1.12 a-c	208.92±12.2 a-d	6.50±0.25 b-d	34.62±0.66 a-e	100.5±4.71 ab
5 days	Control	8.10±0.74 e-g	161.03±11.74 e-g	4.44±0.7 fg	24.24±1.44 g-i	65.40±2.49 gh
	HA (1)	11.78±1.36 cd	204.92±24.87 a-d	5.47±0.95 d-f	29.99±3.03 c-h	91.57±1.36 b-e
	HA (2)	12.25±0.82 b-d	217.78±4.01 ab	7.16±0.47 ab	39.18±2.83 ab	100.07±7.89 ab
	CHT (1)	8.50±1.44 e-g	187.83±15.48 a-f	5.43±0.36 d-f	31.73±0.78 c-f	82.40±6.75 c-f
	CHT (2)	11.82±0.82 cd	202.23±11.48 a-e	5.44±0.33 d-f	32.01±3.36 b-f	89.60±2.13 b-e
8 days	Control	7.10±0.29 fg	136.70±2.2 gh	3.95±0.79 g	23.13±2.2 hi	59.70±4.24 hi
	HA (1)	11.73±1.4 cd	199.72±16.75 a-e	5.72±1.2 c-f	31.49±4.9 c-g	85.73±10.61 b-f
	HA (2)	11.78±1.26 cd	203.60±9.99 a-d	6.16±0.93 b-e	31.46±4.03 c-g	94.27±1.82 b-d
	CHT (1)	8.15±0.63 e-g	178.75±19.01 b-f	5.01±0.22 e-g	28.68±1.07 e-h	79.07±2.44 e-g
	CHT (2)	10.68±0.35 de	196.75±19.56 a-e	5.36±0.19 d-f	29.52±1.15 d-h	86.13±2.74 b-f
11 days	Control	6.85±0.47 g	112.00±3.45 h	3.68±0.87 g	18.49±1.17 i	47.83±3.13 i
	HA (1)	9.70±0.52 d-f	171.48±23.3 c-g	4.85±0.35 e-g	25.28±2.25 f-i	83.27±7.76 c-f
	HA (2)	11.38±0.97 cd	182.30±10.45 a-f	6.00±0.18 b-e	25.98±3.29 f-h	83.93±4.13 c-f
	CHT (1)	7.53±0.97 fg	153.10±8.88 f-h	4.93±0.22 e-g	24.70±1.66 f-i	78.47±2.28 e-g
	CHT (2)	8.48±0.64 e-g	169.95±19.24 d-g	5.41±0.27 d-f	27.72±0.88 e-h	74.13±6.07 f-h

HA (1) = Humic acid at 300 ppm, HA (2) = Humic acid at 600 ppm, CHT (1) = Chitosan at 300 ppm, CHT (2) = Chitosan at 600 ppm. The mean ± standard error of three replicates is represented by each value. Using the Duncan multiple range test, means in a column with various letters show a significant difference for each variable at the 5% level.

lowering of growth parameters in response to water scarcity, which ultimately results in a decrease in vegetative biomass (Rouphael *et al*., 2012). The results of reducing growth parameters of *paspalum vaginatum* owing to water deficient are analogy with those obtained by previous authors (Cui *et al*., 2020; Katuwal *et al.*, 2021; Errickson *et al*., 2023; Taleb *et al*., 2023; Porcelli *et al*., 2024; Hejl *et al*., 2024).

Data in Table 4 also showed that under each time between irrigations, in most cases spraying the plants with any dosage of HA or CHT caused a significant increase ($p<0.05$) in the tested growth traits in contrast to control plants (water-stressed pants without any HA or CHT treatments). The data also disclosed that in general with any one of the two tested bio-stimulators (HA or CHT), increasing the application rate resulted in steady increase in the recorded mean values compared to the control. Under the same HA or CHT level, HA was more effective for increasing studied growth parameters than CHT especially the highest concentration (600 ppm) since recorded the highest values in most cases. The results of boosting growth traits owing to CHT treatment are in sequence with the findings of prior reports (Dzung *et al*., 2011; Li *et al*., 2022; Cheng *et al*., 2024). While increasing the growth parameters due to HA treatments are in the same line of the findings of other researches (Shahin *et al*., 2015; El-Sayed *et al*., 2017; Abdou *et al*., 2020; Badran *et al*., 2023; Taher *et al*., 2023). In this regard El-Sayed *et al*. (2016) mentioned that application of HA has a beneficial impact on growth attributes of seashore paspalum turfgrass subjected to drought stress. The beneficial impact of HA on growth

parameters under drought stress is likely due to its capacity to increase soil water retention, boost nutrient availability, and support the plant's physiological response to stress. Under of water deficient stress, HA has the ability to improve soil structure and water-holding capacity that makes it an essential soil conditioner. It also improves uptake of nutrients, giving plants the tools they need to survive adversity. Moreover, protect plants from oxidative damage brought on by drought, HA can increase antioxidant activity (Chen *et al*., 2022). The useful effect of CHT on growth parameters may be attributed to an augmentation in the availability, water uptake and vital nutrients by modifying the cellular osmotic pressure and reducing the free radical accumulation by increasing the antioxidant enzymes activity (Pirbalouti *et al*., 2017).

Chemical constituents

Total Chlorophylls and carbohydrates contents. The data shown in Table 5 visualized that total chlorophylls and carbohydrates contents were significantly impacted by watering intervals, bio-stimulators treatment and interaction effects. Data in Table 6 emphasized that within each level of HA or CHT, the recorded mean values were decreased in parallel with prolonged watering intervals from 2 to 5, 8 or 11 days. The reductions in tested components as a result of water scarcity stress are in harmony with the finding of earlier reports (Chai et *al*., 2010; Shahidi *et al*., 2017; Sharaf El-Din *et al*., 2017; Sheikh Mohammadi *et al*., 2017; Wang *et al*., 2017; Gholamian *et al*., 2019; Fariaszewska *et al*., 2020; Katuwal *et al*., 2021; Shen *et al*., 2024). Reducing chlorophylls and photosynthetic activity may indirectly resulted a decrease in the amount of carbohydrates. The decrease in total chlorophylls caused by water deficiency may be linked to increased production of reactive oxygen species, which causes oxidative stress, damage to chloroplast structure, and chlorophyll losses. Additionally, low water helps abscisic acid for stomatal closure, which could lead to a decrease in net photosynthesis and the accumulation of carbohydrates (Baccari *et al*., 2020).

The data in the same table elucidated that, within each irrigation frequency, the plants sprayed with any concentration of HA or CHT had significantly higher values of total chlorophylls and carbohydrates content than those of the control. Generally, increasing the application rate of any of HA or CHT caused steady increase in the recorded mean values compared to control, with superiority of HA especially the highest on (600 ppm) which registered the highest mean values of tested components. These results are similar to those obtained by earlier studies which reported that application of CHT caused increase in total chlorophylls or carbohydrates content (Dzung *et al*., 2011; Li *et al*., 2022; Cheng *et al*., 2024). Whereas, the noticeable

Table 5 - Mean square for the impact of irrigation frequency and bio-stimulators treatments and their interaction on some chemical constituents of *Paspalum vaginatum*

Traits	Source of variation						
	Treatment			Error		CV	
	Irrigation frequency (A)	Bio-stimulators (B)	(A × B)	(A)	(B)	(A)	(B)
Total chlorophylls content (SPAD)	37.930 **	39.226 **	0.418 *	0.672	0.328	2.32	1.77
Total carbohydrates (% DW)	19.123 **	23.345 **	0.442 *	0.117	0.298	3.94	8.02
N (% DW)	0.911 **	0.632 **	0.005 *	0.002	0.005	1.91	2.61
P (% DW)	0.012 **	0.009 **	0.002 *	0.001	0.002	2.23	3.01
K (% DW)	0.003 *	0.005 **	0.002 *	0.001	0.002	7.30	10.13
Proline (µ moles/g fresh matter)	24.53 ***	9.88 **	0.92 **	0.11	0.14	7.60	9.33
Total Phenols (mg/100 g DW)	0.817 *	1.664 **	0.128 *	0.147	0.207	8.47	11.44
CAT (units mg protien) in clippings	6.275 *	1.122 **	2.752 **	0.093	0.044	8.00	5.22
SOD (units mg-1 protein) in clippings	4.275 *	1.003 **	1.912 **	0.068	0.033	7.00	3.52
Ascorbate peroxidase (APX)	2.994 **	0.437 **	1.032 *	0.036	0.018	3286	1833

*, **, *** significant at P ≤ 0.05, P≤0.01, P≤0.001, respectively.

increase in tested components due to HA treatments are in harmony with previous studies (Shahin *et al*., 2015; El-Sayed *et al*., 2017; Taher *et al*., 2023). Additionally El-Sayed *et al*. (2016) mentioned that application of HA has a valuable effect on the tested components of seashore paspalum turfgrass subjected to drought stress. The valuable effect HA on increasing total chlorophyll and carbohydrate content is likely due to its ability to improve nutrient uptake and transport, leading to enhanced photosynthetic efficiency. Furthermore, HA can impact enzymatic activity, decrease pH, and stimulate the biosynthesis of essential compounds such as chlorophyll and carbohydrates (Shaabani *et al*., 2022).

N, P and K (% of dry matter). It is evident from data in Table 6 that three nutrients (N, P and K %) were significantly impacted by interval between irrigations, bio-stimulators treatment and interaction effects. The data in Table 6 showed that within each level of the two tested HA or CHT, in most instances extending interval between irrigations from 2 to 5, 8 or 11 days resulted in reduction in N, P and K % compared to the brief intervals (2 days). The present reduction in the tested nutrients owing to stress caused by a drought stress has been reported by earlier studies (Shen *et al*., 2024).

The negative impact of drought stress on absorption and the three nutrients' buildup in plants could be caused by prolonging the irrigation intervals resulted in low soil moisture content, which impacts the elements' solubility and plant absorption capacity, hence reducing their accumulation in plant tissues. Furthermore, the roots absorb fewer nutrients and accumulate them in clippings as a result of reduced transpiration rates, compromised active transport, and poor membrane permeability (Filipović, 2021).

The data in the same table also elucidated that inside each irrigation frequency, spraying the plants with any concentrations of HA or CHT resulted in higher values than those of control plants. In most

Table 6 - Total chlorophylls, total carbohydrates, N, P, and K% as influenced by the interactions between irrigation frequency and bio-stimulators treatments (mean of two seasons)

Irrigation frequency	Bio-stimulators	Total chlorophylls content (mg/g FW)	Total carbohydrates (% DW)	N (% DW)	P (% DW)	K (% DW)
2 days	Control	1.85±0.14 e-h	17.93±3 d-f	1.71±0.05 a-d	0.17±0.01 e-h	1.69±0.09 a-d
	HA (1)	2.66±0.04 ab	22.88±1.4 a-c	2.02±0.21 ab	0.21±0.01 b-d	1.95±0.04 ab
	HA (2)	2.84±0.18 a	24.18±1.2 a	2.14±0.08 a	0.27±0.02 a	2.07±0.27 a
	CHT (1)	2.44±0.10 bc	23.60±1.3 ab	1.97±0.02 a-c	0.20±0.01 b-e	1.92±0.02 ab
	CHT (2)	2.35±0.12 b-d	23.45±0.2 ab	2.0±0.32 a-c	0.20±0.02 b-d	1.98±0.16 ab
5 days	Control	1.62±0.03 hi	16.70±1.7 e-g	1.29±0.16 d-f	0.16±0.01 f-h	1.42±0.15 c-e
	HA (1)	2.23±0.06 cd	20.76±0.3 a-d	1.73±0.17 a-d	0.19±0.02 b-f	1.71±0.07 a-d
	HA (2)	2.33±0.14 b-d	21.93±0.6 a-d	1.71±0.21 a-d	0.22±0.03 b	1.79±0.12 a-c
	CHT (1)	2.01±0.27 d-g	19.52±2 b-f	1.68±0.05 a-e	0.17±0.01 d-h	1.66±0.21 a-d
	CHT (2)	2.16±0.14 c-f	19.18±0.7 c-f	1.82±0.16 a-c	0.18±0.02 d-g	1.7±0.17 a-d
8 days	Control	1.45±0.04 ij	16.24±1.8 fg	1.19±0.27 ef	0.14±0.01 h	1.32±0.07 de
	HA (1)	2.11±0.13 c-g	20.69±0.2 a-e	1.69±0.1 a-e	0.16±0.01 f-h	1.66±0.1 a-d
	HA (2)	2.20±0.06 c-e	21.35±0.6 a-d	1.64±0.29 b-e	0.21±0.02 bc	1.73±0.01 a-d
	CHT (1)	1.84±0.15 f-h	18.81±2 c-f	1.59±0.23 b-e	0.16±0.01 e-h	1.60±0.04 b-d
	CHT (2)	2.07±0.06 d-g	18.55±1.9 d-f	1.72±0.31 a-d	0.16±0.02 e-h	1.66±0.25 a-d
11 days	Control	1.17±0.09 j	12.92±1.6 g	1.09±0.14 f	0.08±0.03 i	1.16±0.1 e
	HA (1)	2.01±0.04 d-g	18.61±2.8 d-f	1.50±0.18 c-f	0.16±0.01 e-h	1.65±0.17 a-d
	HA (2)	2.04±0.12 d-g	19.85±2.4 b-f	1.60±0.26 b-e	0.20±0.02 b-e	1.62±0.19 b-d
	CHT (1)	1.63±0.17 hi	18.92±0.4 c-f	1.58±0.24 b-f	0.15±0.01 gh	1.68±0.17 a-d
	CHT (2)	1.80±0.14 g-i	19.57±0.7 b-f	1.77±0.22 a-d	0.18±0.02 c-g	1.70±0.18 a-d

HA (1) = Humic acid at 300 ppm, HA (2) = Humic acid at 600 ppm, CHT (1) = Chitosan at 300 ppm, CHT (2) = Chitosan at 600 ppm. The mean ± standard error of three replicates is represented by each value. Using the Duncan multiple range test, means in a column with various letters show a significant difference for each variable at the 5% level.

cases, HA was preferable in its effect than CHT. The obtained increases in N, P and K % owing to CHT treatments are similar to those obtained by prior researche (Abou dahab *et al*., 2023) while, the increase as a result of HA application are in accordance with the results of earlier workers (Shahin *et al*., 2015; Taher *et al*., 2023). HA can mitigate unfavorable impacts of drought stress on plants, specifically by rising the availability and uptake of essential nutrients like N, P and K. This is accomplished by a number of methods, such as better soil structure, better water retention, and more accessible nutrients (El-Damarawy *et al*., 2025).

Proline and phenols content. Results in figure 1 revealed that within each level of the two tested HA or CHT, proline and phenols content were increased concurrently with rising irrigation intervals from 2 to 5, 8 or 11 days. In plants exposed to various stressors, the amino acid proline is quite helpful. Proline serves three primary functions during stress: as a metal chelator, an antioxidant defense molecule, and a signaling molecule, in addition to being a great osmolyte (Hayat *et al*., 2012). Additionally, In order to defend against oxidative damage brought on by reactive oxygen species (ROS), plants adapt to water-deficient stress like drought by raising their phenolic content (Albergaria *et al*., 2020).The results of increasing proline or phenols content due to drought stress are concordant with those obtained by previous studies (Bandurska and Jozwiak, 2010; Salehi *et al*., 2014; Marimuthu and Murali, 2018; Gholamian *et al*., 2019; Fariaszewska *et al*., 2020; Katuwal *et al*., 2020).

Results in figure 1 also showed that within each irrigation frequency, in most instances the plants sprayed with any concentrations of HA or CHT had notably greater values of proline or phenols than those of control plants. At the same level as the two bio stimulators that were tested, HA was more effective than CHT. The present increases in proline or phenols content owing to CHT treatments are in line with those findings of prior author (Cheng *et al*., 2024). Moreover earlier report (El-Sayed *et al*., 2016) stated the valuable effect of HA on enhancing phenols content of seashore paspalum subjected to drought stress. HA has the ability to enhance the plant's defense mechanisms against stress. Particularly, HA can enhance the expression of nitrogen metabolism and proline metabolism genes, and also improve the plant's ability to uptake vital

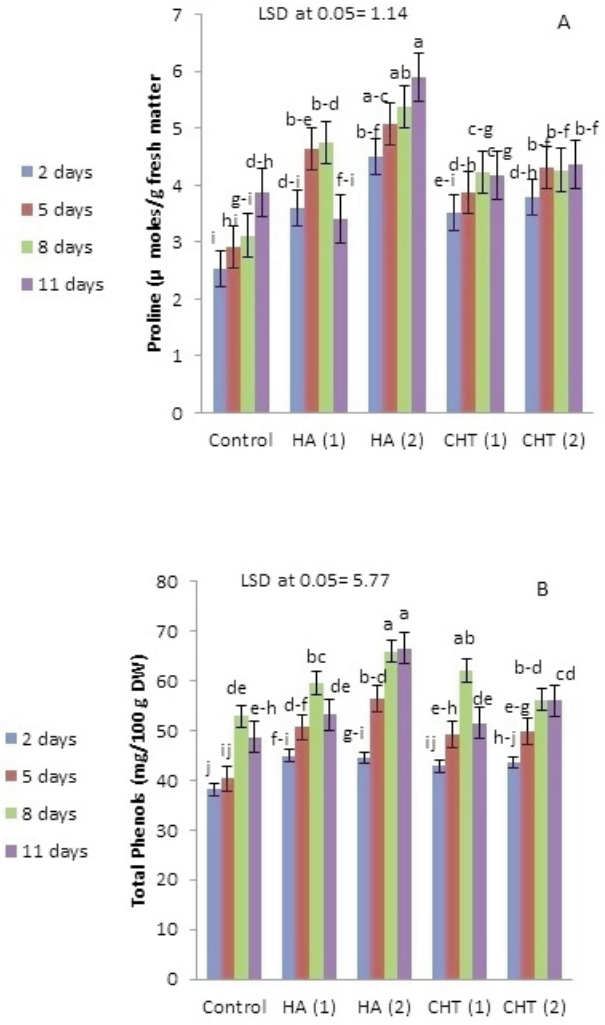

Fig. 1 - Proline content (A) and total phenols (B) as influenced by the interactions between irrigation frequency and bio-stimulators treatments (mean of two seasons). HA (1) = Humic acid at 300 ppm, HA (2) = Humic acid at 600 ppm, CHT (1) = chitosan at 300 ppm, CHT (2) = chitosan at 600 ppm. Column with different letters indicate a significant difference at 5% level. Three replicates' standard error (SE) is shown by vertical bars.

nutrients like iron and phosphorus, this improved nutritional status, thus lead to an increase in proline, which acts as an osmoprotectant and antioxidant under stress conditions (Bakry *et al*., 2014.)

Enzyme activity. It is clear from data in figure 2 that inside each of the two levels of HA or CHT, CAT, APX and SOD content were enhanced steadily with extending irrigation intervals from 2 to 5, 8 or 11 days. Although, previous report (Shahidi *et al*., 2017) indicated decrease in the activities of CAT and SOD

due to drought stress. The current results are consistent with those of (Bian and Jiang, 2009; Bandurska and Jozwiak, 2010; Salehi *et al*., 2014; Gholamian *et al*., 2019; Katuwal *et al*., 2020; Shen *et al*., 2024) who reported increase in CAT, APX or SOD due to water deficient stress. Under drought stress, raising the activity of antioxidant enzymes like CAT, SOD, and APX is a plant's defense mechanism to resist oxidative stress induced by the accumulation of reactive oxygen species (ROS). These enzymes function by scavenging ROS and stopping them from causing harm to the cellular constituents of the plant (Laxa *et al*., 2019).

Results in figure 2 also indicated that at each irrigation frequency, in most cases application of any concentrations of HA or CHT resulted in significant increase in CAT, APX and SOD compared to control plants (water-stressed pants without any biostimulator treatments). Under the same HA or CHT level, HA was Superior in its effect than CHT. Such results are in conformity with the findings of previous workers that mentioned increase in CAT, APX and SOD due to either CHT (Zhao *et al*., 2019; Liu *et al.*, 2020; Almeida *et al*., 2020) or HA treatments (Ozfidan-Konakci *et al*., 2018). Enhanced nutrient uptake, better soil qualities, and a direct effect on plant metabolism are some of the possible causes of the increase in enzyme activity in plants under drought stress, especially when humic acid (HA) therapy is applied. HA may also alleviate the harm caused by reactive oxygen species (ROS) by triggering the plant's enzymatic defense mechanism against stress (Ozfidan-Konakci *et al*., 2018).

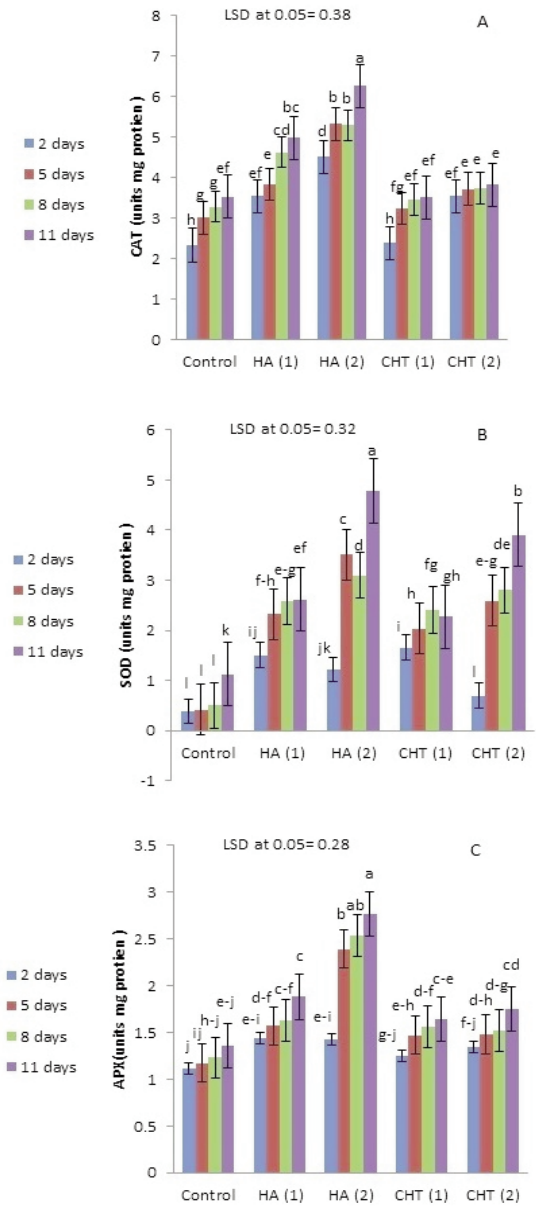

Fig. 2 - CAT (A), SOD (B) and APX (units mg protein) as influenced by the interactions between irrigation frequency and bio-stimulators treatments (mean of two seasons). HA (1) = Humic acid at 300 ppm, HA (2) = Humic acid at 600 ppm, CHT (1) = chitosan at 300 ppm, CHT (2) = chitosan at 600 ppm. Column with different letters indicate a significant difference at 5% level. Three replicates' standard error (SE) is shown by vertical bars.

4. Conclusions

Drought stress had an unfavorable impact on growth traits, total Chlorophylls, carbohydrates and nutrient uptake while, increased proline, phenols content and Enzyme activity (CAT, APX and SOD). Foliar application of HA or CHT at higher concentrations increased growth traits, total chlorophylls, total carbohydrates, nutrient uptake, proline, phenols and enzyme activity. HA was generally superior in its effect than CH. Based on the outcome of the present research it can be inferred that, application of HA at 600 ppm can ameliorates the harmful impacts of drought stress on *Paspalum vaginatum*.

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