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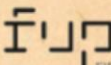
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Growth stimulation of *Gazania splendens* plants grown under lead and cadmium stress conditions using mycorrhiza fungus



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Key words: Chemical composition, flowering characters, *Gazania*, heavy metals, mycorrhiza, treasure flower, vegetative growth.

Abstract: A pot experiment was conducted at the Horticultural Research Institute Antonides in Alexandria (2022-2023 seasons) to evaluate the effect of arbuscular mycorrhizal fungi (AMF) on *Gazania splendens* under cadmium (2, 4, 8 ppm) and lead (150, 200, 400 ppm) stress conditions. A randomized block design was used, comprising thirteen treatments with three replications each. Results showed that the highest concentrations of Cd (8 ppm) and Pb (400 ppm) significantly inhibited vegetative growth, root development, and floral traits, while increasing metal accumulation in leaves and reducing carotene and proline content. In contrast, AMF inoculation (200 g/plant) significantly mitigated heavy metal stress, improving all studied growth and physiological parameters. AMF-treated plants showed lower heavy metal uptake and higher proline levels compared to non-inoculated stressed plants. The study demonstrates that AMF is an effective biotechnological tool for enhancing the tolerance of *Gazania splendens* in heavy metal-contaminated soils.

1. Introduction

Ornamental plants are among the natural elements that have a significant impact on human life, as they play an important role in psychological, aesthetic, cultural, health, and economic conditions (Pittenger *et al.*, 2001). The genus *Gazania* comprises 17 species and belongs to the family Asteraceae, specifically within the subfamily Cichorioideae and the tribe Arctotideae. Traditionally, *Gazania splendens* Moore has been used to relieve toothache, earache, urethral stricture, and to prevent miscarriage. *Gazania krebsiana* is valued as a soil stabilizer and is commonly used as a border plant. Additionally, an infusion prepared from the roots of *Gazania rigens* var. *uniflora* was traditionally

used by young Zulu men in a magical context during courtship rituals (Reddy *et al.*, 2014). *Gazania splendens* L., of the Asteraceae family, is a perennial herb that flowers in spring and summer and grows up to 15 cm in height. Recognized as a ground cover, its blooms open during the day and close at night. It is propagated by seeds or by dividing offshoots from the plant's base. Versatile and decorative, it works well as a potted plant or planted directly in soil (Sardoei and Shahdadneghad, 2014). *Gazania* plants are propagated by seed or division of old plants because they produce numerous offshoots within the same year and can be grown in pots or planted directly in the ground. Despite the effectiveness of chemical fertilizers in improving plant growth, it has been scientifically demonstrated that these substances are harmful to the environment and human health. Modern agricultural policies seek to provide nutrients that promote plant growth, limit environmental harm, and boost plant resilience to adverse conditions (Al-Taey *et al.*, 2019). In this context, growth stimulants - including amino acids, organic acids, plant hormones, plant extracts, and biofertilizers - have been widely adopted. These bio stimulants have proven effective in enhancing both growth and productivity in a wide range of horticultural crops (Al-Taey *et al.*, 2018).

In recent years, heavy metal (HM) contamination has become a widespread issue in both natural and anthropogenically impacted ecosystems, raising serious health and environmental concerns. These challenges have led to stricter regulations and increased interest in remediation methods. One such approach gaining traction is phytoremediation, which utilizes plants and their symbiotic organisms. Despite growing interest, the gap between fundamental mycorrhizal research and its practical applications remain significant (Crişan *et al.*, 2024).

Heavy metals (HMs) are among the most persistent environmental stressors and are known to reduce key soil ecosystem services globally (Rillig *et al.*, 2023). The most commonly encountered HM contaminants worldwide include arsenic (As), mercury (Hg), and lead (Pb) (FAO, 2021). In agricultural soils, arsenic (As), copper (Cu), and nickel (Ni) are prevalent (Tóth *et al.*, 2016), whereas urban soils are often contaminated with elevated levels of Cu, chromium (Cr), Ni, Pb, and zinc (Zn) (Binner *et al.*, 2023). Environmentally friendly or «green» solutions to HM pollution receive the highest levels of public support (Riaz *et al.*, 2022).

Broadly defined, HMs are metals and metalloids with high elemental densities and associated toxicities. However, some scholars have proposed narrower definitions or suggested using alternative terms such as “potentially toxic elements (PTEs)” (Ali and Khan, 2018; Pourret and Hursthouse, 2019).

The interaction between HMs and plants typically begins at the cell wall, which serves both as a barrier and a target. Functional groups in cell wall components can bind HMs, either detoxifying them or disrupting cell wall synthesis (Parrotta *et al.*, 2015). Among these components, pectin moieties play a significant role due to their chelating capacity, while lignin biosynthesis is often enhanced under HM stress (Kosakivska *et al.*, 2021). HM uptake by plants can occur passively through underground or aerial organs or actively via ion channels and transporters. Once absorbed, HMs can be transported systemically through the xylem and phloem, reaching various plant tissues (Ghuge *et al.*, 2023).

Cadmium (Cd) is considered one of the most phytotoxic HMs, as it significantly impairs plant growth and posing severe health risks through its accumulation in the food chain (Shah and Dubey, 1998). Excessive Cd in soil is known to cause a range of stress symptoms in plants, such as reduced root elongation, nutrient imbalances, and disrupted carbohydrate metabolism, ultimately leading to reduced biomass (Moya *et al.*, 1993). Lead (Pb) is another prevalent environmental contaminant and is one of the most abundant metals in the Earth's crust. Even at low concentrations, Pb can hinder critical plant processes, including seed germination, photosynthesis, mitosis, water uptake, mineral absorption, and enzymatic activity (Patra *et al.*, 2004). Pb exposure also disrupts nitrogen metabolism, carbohydrate utilization, and water relations in plants (Hamid *et al.*, 2010). Additionally, Pb contamination alters soil microbial communities, degrades soil fertility, and contributes to reduced plant productivity (Majer *et al.*, 2002).

The main objective of this study is to investigate the effect of mycorrhizal fungi in stimulating the growth of *Gazania splendens* plants cultivated under lead and cadmium stress conditions.

2. Materials and Methods

Plant material and experimental conditions

A pot experiment was carried out at the

Horticultural Research Institute - Antonides, Alexandria, during the 2022 and 2023 growing seasons to evaluate the effect of mycorrhizal application on the growth of *Gazania* plants under lead and cadmium stress conditions. To ensure the purity and accurate identification of the mycorrhizal species, the inoculum was sourced from the Namha Microbiology Laboratory, rather than using a commercial mixture.

Planting was conducted on April 15 in both growing seasons. Pots with a diameter of 16 cm were filled with 1.5 kg of soil whose chemical characterization, performed during both growing seasons, is reported in Table 1. According to the experimental treatments, cadmium at concentrations of 2, 4, or 8 ppm or lead at concentrations of 150 or 400 ppm, either alone or in combination with the addition of 200 g of mycorrhiza, were thoroughly mixed with the soil, as reported in Table 2.

A total of thirteen treatments were arranged in a Randomized Complete Block Design (RCBD) with three replicates.

Plant growth and flowering

Vegetative growth parameters were recorded throughout the experiment. Plant height was measured from the base of the stem at soil level to the shoot tip using a tape measure. The number of leaves per plant was measured by counting all fully expanded leaves on the selected plants, and the number of branches per plant was also recorded. Stem diameter was measured at the midpoint of the main stem. Fresh weight of the shoot system was measured by averaging the weights of five plants per experimental unit.

Root characteristics were evaluated by measuring root length, fresh weight, and dry weight. The roots were carefully harvested, and fresh weight was recorded immediately, while dry weight was determined after drying at 105°C for 72 hours until a constant weight.

Flower characteristics were recorded, including the number of flowers per plant, flower diameter,

flower fresh weight, flower duration (in days from opening to senescence), and days to flowering for each treatment.

Chlorophyll analysis

Chlorophyll content was measured at the end of the vegetative growth cycle, defined as the stage when plants reached full vegetative development prior to flowering. Total chlorophyll content in SPAD units was measured in fully expanded healthy leaves from each replicate using a Minolta SPAD meter following the method of Jemison and William (2006).

Chemical analysis

Leaf samples were oven-dried at 50°C to a constant dry weight prior to chemical analysis. For the determination of cadmium (Cd) and lead (Pb) concentrations, leaves were dried at 78°C, ground into a homogeneous powder, and digested using nitric acid (HNO₃) at 180°C under 20 atm pressure for 40 minutes using a microwave digestion system

Table 2 - Experimental treatments applied to *Gazania splendens* under cadmium and lead stress

Treatment code	Description
C	Control (plants grown in untreated soil without Cd, Pb, or mycorrhizal inoculation) ⁽²⁾
Cd2	Cd at 2 ppm
Cd4	Cd at 4 ppm
Cd8	Cd at 8 ppm
Pb150	Pb at 150 ppm
Pb200	Pb at 200 ppm
Pb400	Pb at 400 ppm
Cd2+M	Cd at 2 ppm + 200 g mycorrhiza
Cd4+M	Cd at 4 ppm + 200 g mycorrhiza
Cd8+M	Cd at 8 ppm + 200 g mycorrhiza
Pb150+M	Pb at 150 ppm + 200 g mycorrhiza
Pb200+M	Pb at 200 ppm + 200 g mycorrhiza
Pb400+M	Pb at 400 ppm + 200 g mycorrhiza

⁽²⁾ The control treatment consisted of plants grown under the same conditions but without heavy metal addition or mycorrhizal inoculation.

Table 1 - Chemical properties of the used sandy soil used during the two 2022 and 2023 growing seasons

Season	pH	EC dS/m	Water soluble cations (meq/l)				Water soluble anions (meq/l)		
			Ca ⁺⁺	Mg ⁺⁺	Na ⁺	K ⁺	HCO ₃ ⁻	Cl ⁻	SO ₄ ⁻⁻
2022	7.83	1.50	3.1	3.0	6.4	1.2	3.3	6.8	2.5
2023	7.91	1.53	3.3	3.4	6.6	1.3	3.5	6.6	2.3

(Mars-5, Candela, USA). The concentrations of Cd and Pb were subsequently measured spectrophotometrically using inductively coupled plasma (ICP) spectrometry (OPTIMA 2000 DV, Perkin-Elmer, USA).

Proline content (mg/g) in the leaves was determined according to the method described by Bates *et al.* (1973).

Carotene extraction was conducted based on the procedure outlined by Hagos *et al.* (2022) with minor modifications. Specifically, β -carotene was extracted by soaking 0.5 g of leaf tissue in 10 mL of acetone at room temperature in the dark to ensure complete extraction. The solution was magnetically stirred for 30 minutes.

Statistical analysis

All collected data were subjected to statistical analysis using the MSTAT software package to perform analysis of variance (ANOVA) as described by Gomez and Gomez (1984). Differences among treatment means were evaluated using the Least Significant Difference (LSD) test at a 0.05 level of significance.

3. Results

Vegetative growth

Results presented in Tables 3 and 4 indicate that the application of mycorrhizal fungi improved the growth of *G. splendens* plants under cadmium and

lead stress conditions. However, increasing concentrations of cadmium and lead decreased all vegetative growth parameters of *G. splendens* as compared to the control treatment, which recorded the highest values for all traits. The control treatment showed the highest values for all measured traits across the two examined seasons, including plant height (36.9 and 39.1 cm), number of leaves (111.6 and 112.7), number of branches (16.5 and 17.4), stem diameter (1.1 and 1.2 cm), total chlorophyll content (58.8 and 62.4 SPAD), and fresh weight (372.1 and 405.5 g). These were followed by plants treated with Cd (2 ppm) combined with 200 g of mycorrhiza, which exhibited slightly lower values of plant height (33.9 and 36.0 cm), number of leaves (100.4 and 101.4), number of branches (15.2 and 16.0), stem diameter (0.9 and 0.9 cm), total chlorophyll content (58.5 and 60.7 SPAD), and fresh weight (372.1 and 383.8 g). Similarly, plants treated with Pb (150 ppm) plus 200 g of mycorrhiza showed intermediate values: plant height (31.3 and 32.3 cm), number of leaves (92.4 and 93.3), number of branches (13.9 and 14.6), stem diameter (0.8 and 0.9 cm), total chlorophyll content (53.8 and 57.2 SPAD), and fresh weight (362.3 and 373.1 g). In contrast, plants exposed to the highest Pb concentration (400 ppm) recorded the lowest values across all parameters, including plant height (22.0 and 23.3 cm), number of leaves (57.1 and 59.3), number of branches (9.9 and 10.3), stem diameter (0.5 and 0.6 cm), total chlorophyll content (33.1 and 36.8 SPAD),

Table 3 - Effects of lead, cadmium, and mycorrhizal application on plant height, leaf number, and branch number of *G. splendens* during the 2022 (1st) and 2023 (2nd) growing seasons

Treatment	Plant height (cm)		Total leaves/plant (n)		Total branches/plant (n)	
	1 st	2 nd	1 st	2 nd	1 st	2 nd
Control	36.88	39.10	111.58	112.70	16.52	17.35
Cd2	29.51	28.15	82.13	82.95	13.87	14.61
Cd4	26.56	25.34	71.91	74.65	11.90	12.49
Cd8	23.90	21.28	65.52	67.19	10.94	11.40
Pb150	26.15	27.77	76.60	80.84	13.22	13.88
Pb200	24.43	25.90	62.24	61.46	12.16	12.77
Pb400	21.99	23.31	57.12	59.31	9.85	10.34
Cd2+M	33.93	35.97	100.43	101.43	15.20	15.96
Cd4+M	30.19	32.25	90.38	91.29	13.68	14.36
Cd8+M	25.88	26.67	83.24	84.08	11.31	11.93
Pb150+M	31.33	32.33	92.39	93.31	13.87	14.61
Pb200+M	27.54	28.37	83.15	83.98	12.38	12.50
Pb400+M	23.48	24.14	72.55	73.61	10.71	11.24
LSD _(0.05)	0.43	0.45	1.27	1.27	0.11	0.12

Table 4 - Effect of lead, cadmium, and mycorrhizal application on stem diameter, total chlorophyll, and total fresh weight of *G. splendens* during the 2022 (1st) and 2023 (2nd) growing seasons

Treatment	Stem diameter (cm)		Total chlorophyll (SPAD)		Fresh weight (g)	
	1 st	2 nd	1 st	2 nd	1 st	2 nd
Control	1.10	1.17	58.81	62.35	372.05	405.54
Cd2	0.71	0.75	42.12	46.33	297.64	324.43
Cd4	0.64	0.67	37.91	41.70	267.88	291.99
Cd8	0.57	0.61	32.25	35.47	241.09	262.79
Pb150	0.65	0.69	40.91	45.46	273.83	298.48
Pb200	0.58	0.62	36.46	40.51	246.45	268.63
Pb400	0.52	0.56	33.14	36.82	221.80	241.77
Cd2+M	0.88	0.93	58.50	60.69	372.08	383.77
Cd4+M	0.79	0.84	52.65	57.92	359.85	364.99
Cd8+M	0.72	0.76	47.39	52.12	301.36	312.49
Pb150+M	0.81	0.86	53.82	57.20	362.29	373.10
Pb200+M	0.73	0.77	48.44	53.28	328.06	335.79
Pb400+M	0.66	0.70	43.59	47.95	277.25	302.21
LSD _(0.05)	0.01	0.01	12.36	13.59	3.17	3.46

and fresh weight (221.8 and 241.8 g), consistently observed across both seasons examined. These results confirm that mycorrhizal inoculation helps mitigate the adverse effects of heavy metal stress, particularly at lower Cd and Pb concentrations.

Root characters

The results presented in Table 5 revealed that the root characteristics of *G. splendens* plants grown under cadmium (Cd) and lead (Pb) stress were notably improved when treated with mycorrhizal

fungi. However, increasing metal concentrations led to a progressive decline in all root parameters, with the control showing the highest values. The most notable improvements with 200 g of AMF were observed when combined with moderate levels of Cd (2 ppm) and Pb (150 ppm), whereas the highest Pb concentration (400 ppm) resulted in the poorest root development across both seasons.

Specifically, control p showed the highest values for root length (1.07 and 1.19 cm), root dry weight (44.54 and 49.43 g), and root fresh weight (11.14 and 12.36 g), and root fresh weight (11.14 and

Table 5 - Effect of lead, cadmium, and mycorrhizal application on root length, root fresh weight, and root dry weight of *G. splendens* during the 2022 (1st) and 2023 (2nd) growing seasons

Treatment	Root length (cm)		Root fresh weight (g)		Root dry weight (g)	
	1 st	2 nd	1 st	2 nd	1 st	2 nd
Control	1.07	1.19	44.54	49.43	11.14	12.36
Cd2	0.857	0.953	40.08	44.49	10.02	11.12
Cd4	0.690	0.760	35.27	39.15	8.82	9.79
Cd8	0.540	0.600	29.98	33.28	7.50	8.32
Pb150	0.933	1.03	36.88	40.94	9.21	10.24
Pb200	0.770	0.857	32.45	36.02	8.12	9.01
Pb400	0.520	0.583	27.58	30.62	6.90	7.66
Cd2+M	0.963	1.07	41.30	45.84	10.33	11.46
Cd4+M	0.747	0.827	36.35	40.35	9.09	10.09
Cd8+M	0.600	0.660	30.89	34.29	7.72	8.58
Pb150+M	0.840	0.933	42.65	47.34	10.66	11.84
Pb200+M	0.670	0.743	37.53	41.66	9.38	10.41
Pb400+M	0.540	0.597	31.90	35.41	7.98	8.85
LSD _(0.05)	0.01	0.01	0.17	0.19	0.04	0.05

12.36 g) in the two growing seasons. This was followed by plants treated with Cd (2 ppm) combined with 200 g of mycorrhiza, which showed root length values of 0.963 and 1.07 cm, root dry weight of 41.30 and 45.84 g, and root fresh weight of 10.33 and 11.46 g. Similarly, Pb (150 ppm) + 200 g mycorrhiza treatment resulted in intermediate values for root length (0.840 and 0.933 cm), root dry weight (42.65 and 47.34 g), and root fresh weight (10.66 and 11.84 g). In contrast, plants exposed to the highest Pb concentration (400 ppm) resulted in the lowest values for all root parameters, including root length (0.520 and 0.583 cm), root dry weight (27.58 and 30.62 g), and root fresh weight (6.90 and 7.66 g), consistently in both seasons.

Flower characters

The results presented in Table 6 demonstrate the impact of cadmium (Cd) and lead (Pb) stress on the flower characters of *Gazania splendens* when treated with mycorrhizal fungi. An increase in the concentration of Cd and Pb led to a noticeable decline in all studied floral traits compared to the control treatment, which consistently recorded the highest values.

In the control treatment, the highest values were observed for the number of flowers per plant (16.84 and 18.35), flower diameter (13.20 and 14.65 cm), flower fresh weight (4.64 and 4.88 g), and flower duration (10.77 and 11.63 days). This treatment also

recorded the shortest time to flowering (72.58 and 75.56 days) during both seasons. Following the control, the treatment with Pb (150 ppm) + 200 g mycorrhiza yielded comparably favorable results: number of flowers per plant (16.50 and 17.98), flower diameter (6.84 and 7.58 cm), flower fresh weight (3.13 and 3.29 g), flower longevity (9.05 and 9.77 days), and days to flowering (82.50 and 84.66), respectively, for the two seasons.

Chemical composition

Results in Table 7 show the chemical composition of *G. splendens* plants under cadmium and lead stress, with mycorrhizal fungi treatment. The data demonstrates that as cadmium and lead concentrations increased, carotene content decreased, while higher metal accumulation in the leaves led to higher levels of proline, cadmium, and lead compared to the control. For example, the control exhibited the lowest values of proline (0.517 and 0.563 mg/g), lead (3.02 and 2.90 mg/l), and cadmium (0.58 and 0.53 mg/l), as well as the highest carotene values (0.945 and 1.07 mg/100g fresh weight). The next best result was observed in the Pb (150 ppm) + 200 g mycorrhiza treatment (0.870 and 0.933 mg/100g fresh weight). These results underscore that the control consistently recorded the lowest stress-related values in both seasons, clearly linking increased metal stress to altered chemical composition in *G. splendens*.

Table 6 - Effect of lead, cadmium, and mycorrhizal application on some flower characters of *G. splendens* during the 2022 (1st) and 2023 (2nd) growing seasons

Treatments	No. of flower/plant		Flower diameter (cm)		Flower weight (g)		Flower duration (days)		No. of day to flowering	
	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
Control	16.84	18.35	13.20	14.65	4.64	4.88	10.77	11.63	72.58	75.56
Cd2	16.32	17.24	6.15	6.83	2.48	2.53	8.15	8.33	76.80	80.57
Cd4	15.23	16.60	5.51	5.56	2.27	2.30	6.74	6.36	83.04	84.18
Cd8	14.97	15.81	4.96	5.50	2.08	2.12	5.35	5.94	87.41	97.02
Pb150	15.52	16.92	6.30	6.66	2.50	2.67	7.69	8.09	81.40	84.80
Pb200	13.71	14.30	4.72	5.13	2.32	2.49	6.12	6.49	83.42	86.26
Pb400	12.47	12.78	3.19	3.99	2.16	2.31	4.76	5.30	87.65	92.30
Cd2+M	16.16	17.61	6.48	7.18	2.75	2.82	8.80	8.99	80.64	82.51
Cd4+M	14.54	15.94	4.47	5.81	2.52	2.55	6.91	6.54	85.27	86.42
Cd8+M	13.67	14.20	3.83	4.70	2.31	2.36	4.52	4.11	89.12	90.80
Pb150+M	16.50	17.98	6.84	7.58	3.13	3.29	9.05	9.77	82.50	84.66
Pb200+M	15.85	16.28	5.20	5.61	2.98	3.12	7.60	7.29	84.89	85.22
Pb400+M	13.62	14.64	4.30	4.66	2.82	2.97	5.17	5.82	86.35	89.18
LSD _(0.05)	0.25	0.27	0.27	0.30	0.01	0.01	0.07	0.07	1.64	1.83

Table 7 - Effect of lead, cadmium, and mycorrhizal application on chemical composition of *G. splendens* during the 2022 (1st) and 2023 (2nd) growing seasons

Treatment	Carotene (mg/ 100g f.w.)		Proline (mg/g)		Pb (mg/l)		Cd (mg/l)	
	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
Control	0.945	1.07	0.517	0.563	3.02	2.90	0.58	0.53
Cd2	0.756	0.780	0.620	0.670	8.74	7.78	1.16	1.20
Cd4	0.605	0.638	0.770	0.840	9.71	8.64	1.29	1.34
Cd8	0.484	0.510	0.967	1.05	10.79	9.60	1.61	1.79
Pb150	0.730	0.763	0.557	0.610	12.15	12.65	1.00	1.11
Pb200	0.544	0.580	0.693	0.760	13.17	13.50	1.16	1.23
Pb400	0.420	0.435	0.870	0.947	15.86	15.00	1.39	1.45
Cd2+M	0.783	0.840	0.540	0.587	5.32	5.44	0.99	1.09
Cd4+M	0.626	0.670	0.670	0.730	6.05	6.40	1.09	1.22
Cd8+M	0.501	0.540	0.810	0.843	7.55	6.72	1.37	1.52
Pb150+M	0.870	0.933	0.483	0.530	10.92	9.72	0.87	0.97
Pb200+M	0.696	0.747	0.603	0.660	12.14	10.80	0.97	1.07
Pb400+M	0.556	0.600	0.757	0.769	13.48	12.00	1.11	1.20
LSD _(0.05)	0.004	0.01	0.02	0.02	0.09	0.08	0.02	0.02

4. Discussion and Conclusions

As it has been clearly demonstrated that chlorophyll integrity - and thus photosynthetic efficiency - is affected by HM stress (Küpper and Spiller, 1996), chlorophyll measurements have been widely employed as reliable indicators to detect HM ion toxicity and plant damage (Chen *et al.*, 2019). Since the green tissues of land plants are the primary sites where organic compounds are synthesized to sustain plant growth and development (Lawson and Milliken, 2023), a well-balanced chlorophyll metabolism is essential throughout the plant's life cycle, highlighting the importance of related parameters. Overexcitation of the photosynthetic apparatus, chlorophyll degradation, or inhibition of its biosynthesis can impair other vital processes and have severe consequences for plant health (Hörtensteiner and Kräutler, 2011). Plants are biological solar panels that use photosynthetic pigments to absorb light in the 400-700 nm range to drive photosynthesis (McDonald, 2003). Chlorophyll molecules are bound to proteins embedded in the thylakoid membranes of the chloroplasts (Rantala *et al.*, 2020). They have a hydrophilic porphyrin head and a hydrophobic phytol tail. The pyrrole rings chelate an Mg^{2+} atom. In plants, chlorophyll a, chlorophyll b, and accessory pigments (such as carotenoids) complement each other to increase the range of light that can be absorbed. Chlorophyll a is 2-3 times more abundant than chlorophyll b

(McDonald, 2003) and is the pigment essential for photochemical reactions (Björn *et al.*, 2009), whereas chlorophyll b is important for the light acclimation process (Voitsekhovskaja and Tyutereva, 2015).

The effect of HMs on photosynthetic mechanisms depends on their reactivity and concentration. Replacement of Mg^{2+} in chlorophyll molecules by a heavy metal ion is one of the most important causes of metal-induced damage in plants (Küpper *et al.*, 2006). Other negative effects include inactivation of photosynthetic enzymes (such as RuBisCO) and oxidative damage caused by reactive oxygen species (ROS) in chloroplasts, all of which led to reduced chlorophyll concentration, destabilization of the chloroplast membranes, and decreased photosynthetic rates (Riyazuddin *et al.*, 2022) (Table 3 and 4). Heavy metal (HM) stress is known to impair root vitality and reduce plant biomass and chlorophyll content by inhibiting the uptake of essential macro- and micronutrients (Wu *et al.*, 2019; Zhao *et al.*, 2019). Consistent with our findings, *Iris wilsonii* plants inoculated with arbuscular mycorrhizal fungi (AMF) under chromium-contaminated water conditions showed improved chlorophyll content and biomass compared to non-inoculated controls (Hu *et al.*, 2020 b). AMF-inoculated plants are also more effective at removing HMs from their environment due to increased biomass and can mitigate chloroplast damage by restricting HM transport to aerial parts (Janeeshma and Puthur, 2020). One plausible explanation is that plants enhance their

physiological metabolism at low HM concentrations by increasing root vitality. The extensive mycelial-root network facilitates nutrient flow and helps prevent HMs from entering root cells (Alam *et al.*, 2019). However, high HM concentrations can damage root cell structures, reduce vitality, and cause metabolic dysfunction (Li and Yu, 2014). AMF play a crucial role by promoting HM accumulation in roots while limiting their translocation to shoots. For example, Huang *et al.* (2017) reported that AMF symbiosis in *Phragmites australis* improved cadmium tolerance by increasing BCF (bioconcentration factor) and lowering TF (translocation factor). Previous studies have shown that longer roots in mycorrhizal plants contribute to greater HM accumulation, with a preference for accumulation in fungal hyphae rather than root cells (Wu *et al.*, 2016). This may explain the increased HM uptake observed in AMF-treated plants. The variability in Pb, Zn, Cu, and Cd accumulation across studies (e.g., Fritioff and Greger, 2006) may be due to differences in plant species and metal characteristics.

AMF can secrete active compounds like glomalin-related soil proteins (GRSP), which bind HMs in the rhizosphere, reduce their mobility, and reshape microbial communities (Ma *et al.*, 2019; Wang and Feng, 2021). GRSP, a water-insoluble glycoprotein released from mycorrhizal hyphae and spore walls, has been shown to effectively chelate heavy metals (Wu *et al.*, 2014). Nafady and Elgharably (2018) confirmed that AMF inoculation significantly reduced concentrations of Cd, Pb, Zn, and Cu due to GRSP release.

Heavy metal accumulation in soil also negatively impacts microbial diversity and activity. AMF, as symbiotic microorganisms that interact directly with plant roots, play a key role in modulating the availability of mobile and immobile metal cations in the soil. Traditional remediation methods for heavy metal contamination - such as physical and chemical treatments - are often expensive and inefficient (Benavides *et al.*, 2005; Sharma and Dubey, 2005). AMF reduce HM concentration through various mechanisms, including binding to their chitin-rich cell walls (Hildebrandt *et al.*, 2007), secretion of glomalin (Gonzalez-Chavez *et al.*, 2004), and accumulation of non-toxic metal forms in root tissues (Joner and Leyval, 1997). Increased mycorrhizal colonization of the soil boosts plant biomass and shoot growth while reducing HM concentrations in both roots and shoots (Weissenhorn *et al.*, 1995). Furthermore, the root

density of AMF significantly affects HM tolerance, which varies based on the mycorrhizal inoculum used. AMF are highly effective at reducing metal transport from roots to shoots, especially in the early stages of HM uptake (Tonin *et al.*, 2001). Cadmium accumulation in agricultural soils poses serious risks, but symbiotic interactions between plant roots and soil microbes like AMF can help mitigate these effects. The use of mycorrhiza has shown potential in reducing heavy metal input, such as Cd, introduced through chemical fertilizers (Table 5).

These findings align with those reported by Asrar *et al.* (2012) and Vaingankar and Rodrigues (2012) who noted an increase in the number of flowers in *Pelargonium peltatum*, *Verbena sp.*, *Antirrhinum majus*, *Chrysanthemum morifolium*, and *Tagetes erecta*. Similarly, our observations, as can be seen in Table 5, are consistent with those of Püschel *et al.* (2014), who reported a tendency for increased flower production in *Gazania rigens*. Additionally, Asrar and Elhindi (2011) found that inoculation with arbuscular mycorrhizal fungi (AMF) enhanced flower size in *Chrysanthemum morifolium* and *Tagetes erecta*.

Higher Cd concentrations in the growing medium were found to accelerate flowering in non-mycorrhizal plants but had no significant effect on the flowering time of AMF-inoculated plants. However, increasing Cd levels slightly reduced the number of flowers overall. Growth inhibition due to Cd contamination is a well-documented phenomenon in other plant species (Rivera-Becerril *et al.*, 2002; Vassilev *et al.*, 2004). If not rapidly detoxified within plant tissues, cadmium can induce oxidative stress, which may inhibit growth and ultimately lead to cell death (Schutzendubel and Polle, 2002). Dunham *et al.* (2003) also reported that under greenhouse conditions, arbuscular mycorrhizal fungi (AMF) can reduce plant growth despite improved mineral nutrition and enhanced photosynthetic activity. The benefits of mycorrhization for Cd tolerance may be less evident in pot cultures compared to field conditions. According to Douds *et al.* (1988), in nutrient-rich media, mycorrhization can inhibit root and shoot growth due to sink competition for photosynthates, while in nutrient-poor substrates, AMF helps plants access more minerals through the external hyphae, positively influencing plant growth.

Lead is generally considered to have relatively low phytotoxicity and tends to accumulate in older

leaves, limiting its transport to younger tissues (Cseh *et al.*, 2000). Stunting, reduced biomass, and chlorosis are common symptoms in Pb-contaminated soils (Foy *et al.*, 1978). These effects are typically due to disrupted chlorophyll synthesis, lowered photosynthetic rates, metal toxicity, nutrient antagonism, or restricted root growth (Sharma and Dubey, 2005). In this study, Pb had minimal impact on the growth and flowering of scarlet sage, likely due to its low sensitivity to Pb. Different species show variable responses to Pb, suggesting species-specific biochemical mechanisms (Begonia, 1997). Increasing Cd doses resulted in higher Cd content in shoots of both mycorrhizal and non-mycorrhizal plants. Notably, at the highest Cd concentration, AMF-inoculated plants accumulated more Cd, corroborating findings by Guo *et al.* (1996) who showed that AM fungal hyphae can absorb and transport Cd. Similarly, Pb accumulation in scarlet sage increased with rising Pb concentration. This is consistent with reports that different plant species exhibit varying Pb uptake and transport efficiencies (Begonia, 1997). Lead can damage plant cellular structures, including cell walls, membranes, mitochondria, chloroplasts, and nuclei. Salazar and Pignata (2014) noted that this disruption impairs physiological processes like protein synthesis, respiration, cell division, and photosynthesis. The transfer factor of most treatments was below 1, indicating a relatively low physiological requirement for Pb, as seen in *Salvia splendens* (El-Shanhorey and Barakat, 2020). Plants manage Pb toxicity via internal tolerance or external exclusion mechanisms. External exclusion limits Pb entry and its accumulation in organelles, while internal tolerance involves the synthesis of organic Pb compounds like cysteine, glutathione, and phytochelatin that bind Pb into less toxic forms (Pourrut *et al.*, 2011; Sharma and Dubey, 2005). Metal transport from roots to shoots occurs via xylem loading and transpiration, as discussed by Blaylock and Huang (2000).

Findings are in agreement with Talebi *et al.*, (2014) for *Gazania* and Jaafari and Hadavi (2012) for *Ocimum basilicum* L., confirming the role of AMF in enhancing nutrient uptake, stress resistance, and rhizosphere microbial activity (Huang *et al.*, 2018; Riaz *et al.*, 2021). Despite low AMF colonization in wetlands due to limited oxygen (Calheiros *et al.*, 2019), AMF have shown promise in enhancing pollutant removal in constructed wetlands (Tondera *et al.*, 2021). Studies by Hu *et al.* (2020 a, b, 2021)

demonstrated that AMF improves plant performance in constructed wetlands, including heavy metal (HMs) remediation. However, the role of AMF in handling combined HMs pollution in aquatic environments remains underexplored. Aeration is another method used to increase oxygen and improve pollutant removal efficiency (Feng *et al.*, 2021). Gunathilakae *et al.* (2018) found that AMF had a stronger effect on removing high Cd concentrations (50 ppm) than lower concentrations, aligning with the current findings. According to Han *et al.* (2021) and Gao *et al.* (2021), AMF inoculation enhances Cd binding in cell walls by increasing polysaccharide and protein content, offering a primary barrier against Cd toxicity.

Additionally, AMF reduces the distribution of HMs in organelles, thus protecting plant structures (Huang *et al.*, 2018). Considine and Foyer (2021) highlighted oxygen as a growth signal that influences root-shoot development and HMs distribution. Chen *et al.* (2004, 2017) showed that AMF alters Cd dynamics in soil and supports photosynthesis under Cd stress by enhancing nutrient uptake and antioxidant capacity (Li *et al.*, 2016). Cd stress disrupts reactive oxygen species (ROS) balance, causing oxidative damage (Maiti *et al.*, 2012). AMF helps mitigate this via secretion of organic acids and chelators, restricting Cd transport to shoots (Wang *et al.*, 2010), and expanding root absorption area while promoting hormone production. Nonetheless, high Cd levels still impose toxicity even on AMF-inoculated plants. Zarei *et al.* (2008) found that AMF diversity decreased in zinc-contaminated soils. Tanaka and Yano (2010) reported improved nitrogen uptake in AMF-inoculated plants. Our findings show that *G. mosseae* increased nitrogen and phosphorus content in roots and leaves under Cd stress. This is likely due to AMF-mediated changes in soil microbial communities and root architecture, enhancing absorption of N, P, and minerals (Cuéllar *et al.*, 2015; Cruz *et al.*, 2017; Cui *et al.*, 2019) further observed that Cd-tolerant genotypes restricted Cd translocation to leaves by downregulating GmHMA18 expression, reducing leaf toxicity (Table 7).

The results of this study demonstrate that arbuscular mycorrhizal fungi (AMF) inoculation positively influenced the growth and physiological performance of *G. splendens* under Cd and Pb stress. AMF-inoculated plants exhibited increased plant height, leaf number, branch number, and chlorophyll content compared with non-inoculated controls.

Moreover, nutrient analysis showed higher nitrogen (N) and phosphorus (P) contents in both roots and leaves of inoculated plants, indicating that mycorrhizal symbiosis enhanced nutrient uptake and translocation to aboveground tissues. These improvements may be attributed to AMF-mediated modifications of root morphology, increased root surface area, and changes in the rhizosphere microbial community that promote mineral acquisition. In addition, AMF contributed to the sequestration of heavy metals in the roots, limiting their translocation to shoots and thereby reducing potential toxicity. Collectively, these findings highlight the dual role of AMF in enhancing nutrient uptake and mitigating heavy metal stress, underscoring their potential for *G. splendens* cultivation in contaminated soils.

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Growth and reproductive attributes of Mungbean (*Vigna radiata* L.) under organic and synthetic fertilization in Eastern Nepal

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Abstract: Mungbean (*Vigna radiata* L.) is a short-duration, protein-rich legume widely cultivated for its nutritional value and nitrogen-fixing ability, playing a vital role in sustainable agriculture. Despite its potential, mungbean productivity in Nepal remains limited due to suboptimal nutrient management. To evaluate the impact of various organic and inorganic nutrient sources on mungbean growth and yield, a field experiment was conducted at the Directorate of Agricultural Research, Tarahara, Sunsari, during the summer season (March-June 2023). The study employed a randomized complete block design (RCBD) with three replications and nine treatments: T1 (Control), T2 (Recommended dose of NPK: 20:40:20 kg/ha), T3 (Nano urea 2000 ml/acre per spray), T4 (Nano DAP 2000 ml/acre per spray), T5 (Farmyard manure 10 t/ha), T6 (½ Recommended NPK + ½ FYM), T7 (Seed treated with *Rhizobium* 25 ml/kg + 40:20 P₂O₅:K₂O kg/ha), T8 (Seed treated with PSB 25 ml/kg + 20 kg N/ha), and T9 (Seed treated with *Rhizobium* 25 ml/kg + PSB 25 ml/kg). Results showed significant effects on most growth and yield parameters. T4 recorded the highest grain yield (2030.02 kg/ha), pods per plant (28.83), straw yield (4026.04 kg/ha), branches per plant (5.60), and trifoliolate leaves (7.17), while T3 had the highest seeds per pod (11.47). T7 excelled in nodule formation. Regression analysis indicated strong yield correlations with pods per plant ($R^2 = 0.70$), test weight ($R^2 = 0.67$), and straw yield ($R^2 = 0.60$). Treatments T4 and T6 also had the highest benefit-cost ratios (2.24 and 2.23, respectively). These findings highlight the potential of nano and integrated nutrient management in enhancing productivity while promoting sustainable and resource-efficient mungbean cultivation for future food security.

1. Introduction

Mungbean (*Vigna radiata* L.), a short-duration grain legume of the

Fabaceae family, is an important crop for food and nutritional security across South and Southeast Asia (Tiwari *et al.*, 2022). It plays a crucial role in smallholder cropping systems due to its rapid growth cycle (60-75 days), capacity for biological nitrogen fixation, and adaptability to marginal soils and low-input conditions. Globally, mungbean is cultivated over an area of approximately 6 million hectares, producing about 3.5 million tonnes annually, with India alone accounting for nearly 50% of global production (Pratap *et al.*, 2020; Bam *et al.*, 2022). In Nepal, mungbean cultivation covers an estimated 12,000 hectares, predominantly in the Terai region, with an average productivity of 500 kg ha⁻¹ (Neupane *et al.*, 2023). Its integration into rice-wheat cropping systems as a catch crop during the spring fallow window offers a promising opportunity to intensify land use, improve soil fertility, and enhance income diversification in subtropical farming systems (Khanal *et al.*, 2004).

Beyond its agronomic versatility, mungbean is highly valued for its nutritional profile. The seeds are rich in digestible protein (~24%), essential amino acids such as lysine and tryptophan, vitamins including folate, vitamin A, and riboflavin, and minerals such as iron, calcium, and zinc (Muchira *et al.*, 2018; Tarafder *et al.*, 2020). Agronomically, mungbean improves soil health through nitrogen fixation contributing up to 42 kg N ha⁻¹ and leaves behind biologically enriched biomass that enhances organic matter and microbial activity (Verma *et al.*, 2017; Rijal *et al.*, 2023). Through its symbiotic association with *Rhizobium* species, mungbean forms root nodules that convert atmospheric nitrogen into plant-available forms, reducing the dependence on synthetic nitrogen fertilizers. This process not only sustains soil fertility but also benefits subsequent crops in rotation by improving nutrient availability and soil biological balance. Additionally, mungbean residues enhance soil organic carbon and promote beneficial microbial interactions, strengthening the agroecosystem's overall productivity and resilience.

It performs optimally in well-drained loam to sandy loam soils with a pH range of 6.2-7.2 and moderate temperatures, making it suitable for dryland agriculture and resource-poor regions. However, despite its agronomic potential, mungbean yields in many South Asian countries, including Nepal, remain well below the genetic potential due to suboptimal nutrient management and limited

input responsiveness. Conventional mungbean cultivation in South Asia has relied heavily on inorganic fertilizers such as urea and diammonium phosphate (DAP), which are often applied indiscriminately without consideration for nutrient losses, soil conditions, or long-term sustainability. In high-rainfall or poorly managed systems, nutrient use efficiency is alarmingly low-estimated losses reach 40-70% for nitrogen, 80-90% for phosphorus, and 50-90% for potassium- resulting in financial losses, eutrophication, and degradation of soil microbial communities (Mandal *et al.*, 2023; Tomar and Bhatnagar, 2023).

Prolonged use of chemical fertilizers has been associated with declining soil fertility, acidification, and loss of beneficial soil organisms. To address the inefficiencies of conventional urea, the introduction of Nano Urea, a novel liquid fertilizer containing nanoscale nitrogen particles, has emerged as a promising alternative. It enables higher nutrient absorption due to its increased surface area and targeted delivery, thereby reducing nitrogen losses and environmental contamination. According to recent studies, a 500 mL bottle of Nano Urea can potentially replace a 45 kg bag of conventional urea when applied properly, significantly improving nitrogen use efficiency and lowering input costs. In contrast, organic nutrient sources such as farmyard manure, compost, and crop residues contribute to improved soil aggregation, water-holding capacity, and microbial activity while supplying nutrients in synchrony with crop demand (Subedi and Yadav, 2013). Biofertilizers, especially *Rhizobium* and phosphate-solubilizing bacteria (PSB), enhance nutrient acquisition, root architecture, and stress tolerance in legumes. These microbial inputs improve nitrogen and phosphorus availability through biological fixation and mineral solubilization and help mitigate the adverse effects of chemical inputs on soil health and the environment (Kishor *et al.*, 2021; Chamuah *et al.*, 2023). Although various nutrient sources have been tested individually, limited research has focused on the comparative and interactive effects of organic, inorganic, and biological inputs on mungbean performance under field conditions. Given the urgent need to transition toward more sustainable crop nutrition strategies, understanding how mungbean responds to different nutrient regimes is essential for maximizing yield while conserving soil and ecological resources.

Therefore, the present study was conducted to

evaluate the effects of organic, inorganic, and biofertilizer nutrient sources on the growth, yield attributes, and productivity of mungbean (*Vigna radiata* L.).

2. Materials and Methods

Site selection

The field experiment was conducted at the Directorate of Agricultural Research (DoAR), Tarahara, Sunsari, under the Nepal Agricultural Research Council (NARC), located in the eastern Terai region of Nepal. The experimental site is situated at an altitude of 130 meters above sea level, with geographical coordinates of 26°70'45" North latitude and 87°28'11" East longitude. During the study period, the area received an average rainfall of 481.9 mm, and the maximum and minimum recorded temperatures were 38.7°C and 11°C, respectively.

Soil sample collection and analysis

Before sowing the crop, a composite soil sample was collected from the upper 0-25 cm depth of the experimental field to determine its initial physico-chemical properties. The sample was air-dried, ground, and passed through a 2-mm sieve before analysis. Standard laboratory protocols were followed to analyze various soil properties. Total nitrogen was determined using the Kjeldahl digestion method, while available phosphorus was assessed using the Olsen method. Available potassium was measured using the ammonium acetate extraction technique. Soil pH was measured using a 1:2.5 soil-to-water suspension with a potentiometer, and soil organic matter content was determined using the

Walkley and Black rapid titration method. The soil texture, including the percentage of sand, silt, and clay, was analyzed using the Bouyoucos hydrometer method. The general characteristics of the soil at the experimental site are presented in Table 1.

Table 1 - Soil chemical and physical properties with parameters and methods adopted for laboratory analysis at DoAR, Tarahara, Sunsari, 2024

Parameters	Units	Method	Analysis result
Total nitrogen	%	Kjeldahl Digestion	0.14
Available P ₂ O ₅	ppm	Olsen	13.6
Available K ₂ O	ppm	Ammonium acetate	38.5
pH	-	Potentiometric 1:2.5	6.37
Soil organic matter	%	Walkley and Black	2.73
Textural class	-	Bouyoucos Hydrometer	Silty clay loam

Source: DoAR, Tarahara, Sunsari, NARC.

Experimental design and layout details

The experiment was laid out using a Randomized Complete Block Design (RCBD) with three replications and nine treatments. A total of 27 experimental units were established, each measuring 3×2 m², within a total field area of 242 m². To minimize inter-plot interference, a spacing of 1 meter was maintained between replications and 50 centimetres between individual plots within each replication.

Treatment details

The experimental design consisted of nine treatments, each arranged in three replicates, as detailed in Table 2.

Table 2 - Treatments for field trial conducted at DoAR, Tarahara, Sunsari

SN	Treatments
T1	Control
T2	Recommended dose of PNs through urea, DAP and MOP (N: P ₂ O ₅ : K ₂ O @ 20: 40: 20 kg/ha)
T3	Foliar spray @ 2000 mL/acre per spray at 25 and 45 DAS with Nano urea
T4	Foliar spray @ 2000 mL/acre per spray at 25 and 45 DAS with Nano DAP
T5	Farmyard manure @ 10 t/ha
T6	1/2 of T2 +1/2 of T5
T7	<i>Rhizobium</i> @ 25 mL/kg seed + 100% phosphorus and potassium through SSP and MOP (N: P ₂ O ₅ : K ₂ O @ 0:40:20 kg/ha)
T8	PSB @ 25mL/kg seed + 100% nitrogen through urea (N@ 20 kg/ha)
T9	<i>Rhizobium</i> @ 25 mL/kg seed + Treatment with PSB @ 25 mL/kg seed

Cultural practices and treatment applications

After harvesting the previous crop, the experimental field was prepared by crushing soil clods and achieving a fine tilth using a rotavator. The land was levelled, and all weeds and stubbles were removed. The plots were laid out according to the experimental design, slightly raised to prevent waterlogging, and a surrounding furrow was dug to ensure proper drainage during rainfall.

Mungbean variety 'Pratigya' was used in this experiment, with breeder seeds sourced from the Grain Legume Research Program, NARC, Khajura, Banke.

Seeds were soaked in water for 12 hours, air-dried under shade for 3 hours, moistened with jaggery solution, and treated with *Rhizobium* and PSB @ 25 ml/kg seed, specifically for treatments 7, 8, and 9. Sowing was done by maintaining a spacing of 30 cm between rows and 10 cm between plants, with one plant per hill and 200 plants per plot. Replications were spaced at 1 meter and treatments at 50 cm. Irrigation was applied lightly from germination to pod development at 7-10-day intervals, totalling 3-4 irrigations depending on soil type and environmental conditions. Fertilizers were applied on a net plot basis, where different NPK doses were used in treatments 2, 6, 7, and 8 using granular Urea, MOP, and DAP. Foliar sprays of nano urea and nano DAP were applied at 25 and 45 DAS in treatments 3 and 4. FYM was applied as a basal dose in treatments 5 and 6. Details about the treatment applications are

presented in Table 3.

Data observation and collections

For data collection, five plants were randomly selected from each treatment plot, and observations were recorded on both vegetative and reproductive parameters. Vegetative parameters included plant height, number of branches, number of trifoliate leaves, total number of nodules, and number of effective nodules. Reproductive parameters included days to first flowering, days to 50% flowering, average number of pods per plant, average pod length, grain yield, straw yield, and biological yield. All data were recorded from field observations, and averages were calculated accordingly.

Statistical analysis

Data entry and preliminary processing were conducted using Microsoft Office Excel 2019 (Professional Plus). Statistical analyses were performed using RStudio (version 4.1.1), where Analysis of Variance (ANOVA) was employed to evaluate the significance of treatment effects, and mean separation was carried out using the Least Significant Difference (LSD) test at a 5% probability level. Additionally, correlation and regression analyses were conducted in RStudio to examine the strength and nature of relationships between dependent and independent variables, thereby providing deeper insights into treatment responses and underlying patterns in the data.

Table 3 - Description of treatments applied during field trial at DoAR, Tarahara, Sunsari

SN	Basal (Before sowing)	25 DAS	45 DAS
T1	-	-	-
T2	4.8 g Urea, 44.6 g DAP and 17.1 g MOP was applied in the soil before sowing.	-	-
T3	-	Nano urea was sprayed @ 2.53 ml/L water.	Nano urea was sprayed @ 2.53 ml/2L water.
T4	-	Nano DAP was sprayed @ 2.53 ml/L water.	Nano DAP was sprayed @ 2.53 ml/L × 2 water.
T5	17.1 kg FYM was applied in the soil before sowing.	-	-
T6	2.4 g Urea, 22.3 g DAP and 8.55 g MOP + 8.55 kg FYM was applied in the soil before sowing.	-	-
T7	Treatment with <i>Rhizobium</i> @ 0.35 ml/14 g seed + 128 g SSP and 17.1 g MOP was applied in the soil before sowing.	-	-
T8	Treatment with PSB @ 0.35 ml/14 g seed + 4.8 g Urea was applied in the soil before sowing.	-	-
T9	Treatment with PSB @ 0.35 ml per 14 g seed + Treatment with <i>Rhizobium</i> @ 0.35 ml per 14 g seed.	-	-

3. Results

Effect of different fertilizer combinations on vegetative and reproductive traits of mungbean

Plant height. The application of different nutrient management practices induced significant variation in plant height, reflecting the role of nutrient availability in driving vertical vegetative growth, as presented in Table 4. The tallest plants were recorded in the N:P:K treatment (64.87 cm), closely followed by $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (64.77 cm) and FYM (63.87 cm). These treatments outperformed all others, indicating that both chemical fertilizers and integrated nutrient management approaches significantly enhanced vertical growth. Other treatments such as Nano DAP (61.33 cm), *Rhizobium* + PSB (61.10 cm), Nano urea (60.63 cm), and *Rhizobium* + P:K (60.63 cm) showed intermediate results. PSB + N produced slightly shorter plants (58.90 cm), while the Control exhibited the lowest

These treatments encouraged vigorous branching, supporting the role of nano and integrated nutrient inputs in promoting lateral shoot development. Other effective treatments included FYM and N:P: K (both 5.27), *Rhizobium* + PSB (5.23), and PSB + N (5.13). The Control treatment had the lowest branch count (4.77), emphasizing the limited growth potential in the absence of supplemental nutrients.

Number of Trifoliolate leaves per plant. Although statistically non-significant, differences in trifoliolate leaf count across treatments reflect trends in canopy expansion and photosynthetic surface area in response to varying nutrient inputs. The highest values were recorded in Nano DAP (7.17), FYM (7.07), and PSB + N (6.87), suggesting a potential positive effect on leaf development. Other treatments with substantial leaf counts included *Rhizobium* + P:K (6.80), N:P:K (6.70), and *Rhizobium* + PSB (6.53). The Control had the lowest number of trifoliolate leaves (6.03), reflecting minimal canopy

Table 4 - Effect of different fertilizers on growth parameters of mungbean

Treatments	Plant height	No. of branches	No. of trifoliolate leaves	Total no. of nodules	No. of effective nodules
Control	55.37 d	4.77 c	6.03 b	21.33 e	16.73 c
N: P: K	64.87 a	5.27 ab	6.70 ab	23.20 de	22.70 bc
Nano urea	60.63 bc	5.53 ab	6.37 ab	29.53 cd	25.27 b
Nano DAP	61.33 b	5.60 a	7.17 a	32.93 bc	27.13 b
FYM	63.87 a	5.27 ab	7.07 a	26.00 cde	22.23 bc
$\frac{1}{2}$ N: P: K + $\frac{1}{2}$ FYM	64.77 a	5.33 ab	6.57 ab	31.93 bc	26.33 b
<i>Rhizobium</i> + P: K	60.63 bc	5.47 ab	6.80 ab	52.67 a	45.73 a
PSB + N	58.90 c	5.13 bc	6.87 a	32.00 bc	27.40 b
<i>Rhizobium</i> + PSB	61.10 bc	5.23 ab	6.53 ab	38.87 b	28.93 b
P value	<0.001	0.03	0.18	<0.001	<0.001
LSD (0.05)	2.37	0.45	0.81	7.48	7.81
SE _m ($\pm$)	0.26	0.05	0.09	0.83	0.87
CV (%)	2.24	4.86	7.03	13.49	16.75
Grand Mean	61.27	5.29	6.68	32.05	26.94

height (55.37 cm), suggesting that lack of supplementation substantially limits vegetative development.

Number of branches per plant. Nutrient treatments exhibited a moderate but significant effect on the development of lateral branches, indicating that specific nutrient regimes can influence plant architecture through enhanced axillary bud initiation. The highest branching was observed under Nano DAP (5.60), followed by Nano urea (5.53), *Rhizobium* + P:K (5.47), and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (5.33).

expansion without nutrient input. While these differences were not statistically significant, the trend suggests that enhanced nutrient availability, especially through biofertilizers and nano sources, may support greater foliar development.

Total number of nodules per plant. Nutrient management practices had a profound impact on total nodulation, with biological and integrated treatments demonstrating significantly enhanced symbiotic activity ($P < 0.001$). The highest nodule count was recorded under *Rhizobium* + P:K (52.67),

followed by *Rhizobium* + PSB (38.87). These results highlight the superior nodulation induced by microbial inoculants. Moderate nodulation was observed in treatments such as Nano DAP (32.93), PSB + N (32.00), and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (31.93), while Nano urea (29.53), FYM (26.00), and N:P:K (23.20) showed slightly lower counts. The Control treatment resulted in the fewest nodules (21.33), reinforcing the importance of both chemical and biological nutrient inputs in promoting effective rhizobial symbiosis.

Number of effective nodules per plant. The number of effective nodules, a critical parameter for evaluating biological nitrogen fixation, was markedly influenced by the applied nutrient regimes ($P < 0.001$). *Rhizobium* + P:K recorded the maximum number of effective nodules (45.73), clearly outperforming all other treatments. Other nutrient regimes with strong performance included *Rhizobium* + PSB (28.93), PSB + N (27.40), Nano DAP (27.13), and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (26.33). Slightly lower but still improved values were seen in Nano urea (25.27), N:P:K (22.70), and FYM (22.23). The Control again showed the lowest performance (16.73), indicating minimal nitrogen fixation potential without inoculation or nutrient application. These findings underscore the pivotal role of *Rhizobium* inoculation and integrated nutrient management in enhancing nodulation efficiency and biological nitrogen fixation.

Days to first flowering. Nutrient management practices significantly influenced the onset of reproductive development, as indicated by differences in the days to first flowering ($P = 0.02$). The earliest flowering was observed in the Control (31 days), *Rhizobium* + P:K (31 days), and Nano DAP (31 days), indicating that these treatments hastened the transition from vegetative to reproductive stages (Table 5). Slightly delayed flowering was recorded under Nano urea, PSB + N, and *Rhizobium* + PSB (all 32 days), suggesting a minimal extension of the vegetative phase. The most delayed flowering occurred with FYM (33 days) (Table 5), which differed significantly from the control, likely due to slower nutrient mineralization and availability from organic sources. These findings imply that nutrient form and bioavailability distinctly affect phenological shifts, with quicker nutrient uptake supporting earlier flowering.

Days to 50% Flowering. A highly significant treatment effect was recorded for days to 50%

Table 5 - Response of different fertilizers on flowering days

Treatments	Days to first flowering	Days to 50% flowering
Control	31 c	35 d
N: P: K	32 bc	36 bcd
Nano urea	32 ab	36 cd
Nano DAP	31 bc	36 bcd
FYM	33 a	37 ab
$\frac{1}{2}$ N: P: K + $\frac{1}{2}$ FYM	32 bc	36 bcd
<i>Rhizobium</i> + P: K	31 bc	36 bcd
PSB + N	32 ab	37 abc
<i>Rhizobium</i> + PSB	32 ab	38 a
P value	0.02	<0.01
LSD (0.05)	1.07	1.42
SE _m ($\pm$)	0.12	0.16
CV (%)	1.95	2.25
Grand Mean	31.74	36.48

flowering ($P < 0.01$), suggesting that nutrient regimes considerably impact the synchrony and uniformity of flowering. The earliest attainment of 50% flowering was noted in the Control group (35 days), while the most delayed response was observed in *Rhizobium* + PSB (38 days) (Table 5). Treatments such as FYM (37 days) and PSB + N (37 days) also exhibited delayed flowering, likely reflecting the gradual nutrient release associated with organic or biofertilizer amendments. In contrast, quicker flowering responses were seen in N:P:K, Nano DAP, Nano urea, $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM, and *Rhizobium* + P: K, all of which achieved 50% flowering in 36 days (Table 5). These results highlight the nuanced influence of nutrient form and microbial activity on flowering dynamics. While quicker responses may be favored in short-duration crops, delayed and synchronized flowering from microbial-enhanced treatments could be advantageous for yield stability.

Pod length. Pod length was not significantly affected by the different nutrient management practices ($P = 0.62$), suggesting that this parameter may be genetically regulated and less responsive to nutrient variation under the given conditions. Despite the lack of statistical significance, numerical differences were evident. The longest pods were recorded under $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (8.52 cm), *Rhizobium* + PSB (8.44 cm), and Nano DAP (8.40 cm), which exceeded the grand mean of 8.11 cm. Treatments such as FYM (8.11 cm), *Rhizobium* + P:K (8.22 cm), and N:P:K (8.01 cm) also yielded relatively long pods. The shortest pod length was observed in

the Control (7.62 cm), underscoring a trend toward enhancement with nutrient supplementation, though not statistically confirmed (Table 6).

Number of pods per plant. Nutrient application significantly influenced the number of pods per plant ($P < 0.001$), indicating a strong response of reproductive capacity to improved nutrition. The highest pod count was observed under Nano DAP (28.83), followed by $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (27.87), both of which were statistically superior to the Control (20.40), which had the lowest pod number. Treatments such as N:P:K (26.93), *Rhizobium* + P:K (26.37), and *Rhizobium* + PSB (25.87) also performed well, with values above the grand mean (25.80) (Table 6). This suggests that integrated and nano-based nutrient sources play a pivotal role in enhancing reproductive output, likely due to better nutrient availability and physiological stimulation during flowering and pod development.

Number of seeds per pod. Seed number per pod exhibited significant variation among treatments ($P = 0.04$), reflecting a treatment effect on internal pod development and fertility. The maximum number of seeds per pod was observed in Nano urea (11.47), Nano DAP (11.40), and *Rhizobium* + P:K and PSB + N (both 10.53). These were followed by N:P:K and FYM (10.33), all of which surpassed the grand mean (10.47). Lower seed set was recorded under *Rhizobium* + PSB (9.80) and Control (9.53), the latter representing the statistically lowest value (Table 6). These results highlight the positive influence of nano-nitrogen and biofertilizer-based treatments in

enhancing seed formation within pods.

Test weight. Test weight was significantly influenced by nutrient management practices ($P < 0.001$), indicating a substantial impact on seed size and density. The highest test weight was recorded under $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (38.90 g), followed closely by Nano DAP (38.77 g), both of which were significantly superior to other treatments. N:P:K (37.83 g), Nano urea (37.57 g), and FYM and *Rhizobium* + P:K (both 37.50 g) showed comparable values around the grand mean (37.47 g) (Table 6). The Control treatment had the lowest seed weight (35.07 g), indicating that nutrient deprivation not only reduces reproductive output but also limits seed filling and maturity. These findings emphasize that combined or nano-based nutrient sources are more effective in improving seed quality through enhanced metabolic activity during grain filling.

Grain yield. Grain yield was significantly influenced by nutrient management treatments ($P < 0.001$), reflecting the strong impact of nutrient availability and uptake efficiency on reproductive productivity. The highest grain yield was recorded under Nano DAP (2030.02 kg/ha), followed by $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM (1912.00 kg/ha) and *Rhizobium* + P:K (1881.60 kg/ha). These yields were significantly higher than the Control (1094.85 kg/ha), which recorded the lowest performance. Other treatments such as N:P:K (1828.31 kg/ha), PSB + N (1775.39 kg/ha), *Rhizobium* + PSB (1771.63 kg/ha), and Nano urea (1755.67 kg/ha) also performed well, exceeding the grand mean of 1755.53 kg/ha (Table 7). These

Table 6 - Effect of different fertilizers on reproductive traits of mungbean

Treatment	Pod length (cm)	No. of pods/plant	No. of seeds/pod	Test weight (g)
Control	7.62 a	20.40 d	9.53 c	35.07 d
N: P: K	8.01 a	26.93 abc	10.33 abc	37.83 bc
Nano urea	7.94 a	25.00 c	11.47 a	37.57 c
Nano DAP	8.40 a	28.83 a	11.40 ab	38.77 ab
FYM	8.11 a	25.50 bc	10.33 abc	37.50 c
$\frac{1}{2}$ N: P: K + $\frac{1}{2}$ FYM	8.52 a	27.87 ab	10.27 bc	38.90 a
<i>Rhizobium</i> + P: K	8.22 a	26.37 abc	10.53 abc	37.50 c
PSB + N	7.72 a	25.40 bc	10.53 abc	37.07 c
<i>Rhizobium</i> + PSB	8.44 a	25.87 bc	9.80 c	37.07 c
P value	0.62	<0.001	0.04	<0.001
LSD (0.05)	1.07	2.64	1.17	1.05
SE _m ($\pm$)	0.12	0.29	0.13	0.12
CV (%)	7.68	5.92	6.44	1.62
Grand Mean	8.11	25.80	10.47	37.47

Table 7 - Effect of different fertilizers on yield traits of mungbean

Treatment	Grain yield (kg/ha)	Straw yield (kg/ha)	Harvest Index
Control	1094.85 e	1955.71 c	35.91 ab
N: P: K	1828.31 bcd	3992.74 a	31.41 c
Nano urea	1755.67 cd	3611.25 a	32.71 bc
Nano DAP	2030.02 a	4026.04 a	33.54 bc
FYM	1750.33 d	3610.46 a	32.65 bc
½ N: P: K + ½ FYM	1912.00 ab	3085.84 b	38.27 a
<i>Rhizobium</i> + P: K	1881.60 bc	3886.01 a	32.72 bc
PSB + N	1775.39 cd	2922.01 b	37.81 a
<i>Rhizobium</i> + PSB	1771.63 cd	3163.91 b	36.14 ab
P value	<0.001	<0.001	<0.01
LSD (0.05)	129.95	438.27	3.56
SEm (±)	14.45	48.73	0.40
CV (%)	4.28	7.53	5.95
Grand Mean	1755.53	3361.55	34.57

results underscore the effectiveness of combining nano-formulations, organic matter, and microbial inoculants in boosting grain yield.

Straw yield. Straw yield varied significantly across treatments ($P<0.001$), indicating differential impacts of nutrient regimes on vegetative biomass accumulation. The highest straw yields were observed under Nano DAP (4026.04 kg/ha), N:P:K (3992.74 kg/ha), and *Rhizobium* + P:K (3886.01 kg/ha). These treatments significantly outperformed the Control (1955.71 kg/ha), which had the lowest biomass. Nano urea (3611.25 kg/ha) and FYM (3610.46 kg/ha) also contributed to elevated straw

production, further suggesting that both mineral and microbial sources enhance total plant biomass. Lower straw yields in PSB + N (2922.01 kg/ha) and ½ N:P:K + ½ FYM (3085.84 kg/ha) were still statistically superior to the control, but below the highest performers (Table 7).

Harvest Index (HI). Harvest Index, representing the proportion of economic yield (grain) to total above-ground biomass, was also significantly influenced by nutrient regimes ($P<0.01$). The highest HI was observed in ½ N:P:K + ½ FYM (38.27) and PSB + N (37.81), suggesting more efficient partitioning of assimilates toward grain production. These were statistically superior to N:P:K (31.41), which showed the lowest HI despite its high biological yield, indicating a vegetative bias. Other treatments showed moderate HI values: *Rhizobium* + PSB (36.14), Control (35.91), Nano DAP (33.54), *Rhizobium* + P: K (32.72), Nano urea (32.71), and FYM (32.65) (Table 7). These values imply that integrated and biologically enriched nutrient practices can improve reproductive efficiency alongside yield enhancement.

Economic analysis

The gross return and B:C ratio varied significantly among treatments, reflecting the diverse economic responses to different nutrient management strategies, as presented in Table 8. The highest gross return was recorded under Nano DAP (Rs. 446,604.4), which also produced the highest B:C ratio of 2.24, establishing it as the most economically

Table 8 - Gross returns and B:C ratio under different fertilizer regimes

Treatments	Fertilizer cost (Rs)	Total Cost (Rs)	Gross returns (Rs)	Net returns (Rs)	B:C ratio
Control	0.00 i	180000.0 i	240866.3 e	60866.27 d	1.34 c
N: P: K	6687.50 f	186687.5 f	402228.9 bcd	215541.43 bc	2.15 ab
Nano urea	7907.09 e	187907.1 e	386248.9 cd	198341.78 c	2.06 b
Nano DAP	18977.02 a	198977.0 a	446604.4 a	247627.38 a	2.24 a
FYM	10000.00 c	190000.0 c	385072.6 d	195072.60 c	2.03 b
½ N: P: K + ½ FYM	8343.75 d	188343.8 d	420640.7 ab	232296.98 ab	2.23 a
<i>Rhizobium</i> + P:K	14274.36 b	194274.4 b	413952.0 bc	219677.64 abc	2.13 ab
PSB + N	346.86 g	180346.9 g	390585.8 cd	210238.94 bc	2.17 ab
<i>Rhizobium</i> + PSB	218.72 h	180218.7 h	389759.3 cd	209540.61 bc	2.16 ab
P value	<0.001	<0.001	<0.001	<0.001	<0.001
LSD (0.05)	0	0	28588.61	28588.61	0.1532
SEm (±)	0	0	3,18E+06	3,18E+06	0.017
CV (%)	6,11E-08	6.91	4.28	8.31	4.30
Grand Mean	7.417.256	187417.3	386217.7	198800.4	2.06

rewarding treatment. This was closely followed by $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM, which yielded a gross return of Rs. 420,640.7 and a B:C ratio of 2.23, statistically comparable to Nano DAP and economically efficient due to balanced input costs and strong yield performance. Other high-performing treatments included *Rhizobium* + P:K (Rs. 413,952.0, B:C 2.13), N:P:K (Rs. 402,228.9, B:C 2.15), PSB + N (Rs. 390,585.8, B:C 2.17), and *Rhizobium* + PSB (Rs. 389,759.3, B:C 2.16). These treatments demonstrated statistically similar B:C ratios ranging from 2.13 to 2.17, indicating high profitability per rupee invested, especially when biofertilizers were involved. Notably, PSB + N and *Rhizobium* + PSB achieved excellent economic returns at minimal fertilizer costs (Rs. 346.86 and Rs. 218.72, respectively), enhancing their appeal in low-input farming systems. Nano urea (Rs. 386,248.9, B:C 2.06) and FYM (Rs. 385,072.6, B:C 2.03) delivered moderate gross returns and profitability, slightly below the grand mean of gross return (Rs. 386,217.7) and B:C ratio (2.06), suggesting reasonable efficiency but not on par with integrated or nano-enhanced combinations. In contrast, the lowest economic performance was recorded in the Control treatment, with a gross return of only Rs. 240,866.3 and a B:C ratio of 1.34. This clearly indicates that the absence of fertilizer input severely limits productivity and economic gain, making it the least viable option for sustainable cultivation.

Regression analysis

Figures 5 and 6 present twelve scatterplots (a-f in each), showing the fitted simple linear regression of grain yield (Y, in kg/ha) against various plant traits (X), each with its regression equation, R^2 value, and p-value to assess the strength and significance of the relationship. In Figure 5, plot (a) shows a moderately strong and significant positive relationship between plant height and yield ($R^2 = 0.4$, $p = 0.00038$), indicating that nutrient sources, particularly those enhancing vegetative growth, may improve yield by supporting taller, more vigorous plants. Plots (b) and (c) display weak, non-significant positive correlations between days to first flowering ($R^2 = 0.085$, $p = 0.14$) and days to 50% flowering ($R^2 = 0.12$, $p = 0.075$) with yield, suggesting that flowering time was minimally affected by nutrient treatments. This likely reflects that nutrient inputs influenced biomass and yield more than flowering behaviour, and that environmental conditions during flowering remained

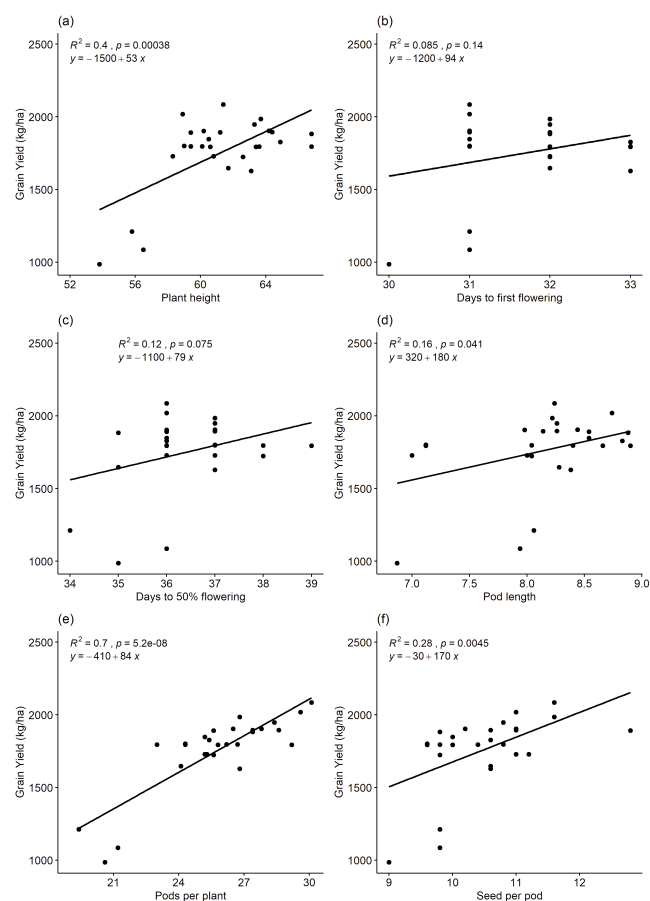


Fig. 5 - Coefficient of determination (R^2), Linear regression equation, and scatter diagram showing the fitted simple regression line of Y (Yield) on X (a) Plant height, (b) Days to 1st flowering, (c) Days to 50% flowering, (d) Pod length, (e) Pods per plant, (f) Seeds per pod.

relatively stable. Plot (d) shows a weak but significant positive relationship between pod length and yield ($R^2 = 0.16$, $p = 0.041$), implying a modest contribution of pod development to yield, possibly supported by improved nutrient uptake. Plot (e) reveals the strongest relationship, where pods per plant are highly correlated with yield ($R^2 = 0.7$, $p = 5.2e-08$), suggesting nutrient-driven enhancement of reproductive capacity. Plot (f) shows a moderate, significant positive relationship between seeds per pod and yield ($R^2 = 0.28$, $p = 0.0045$), indicating improved seed setting under nutrient-rich conditions. In figure 6, plot (a) indicates a moderately strong, significant positive correlation between branches per plant and yield ($R^2 = 0.46$, $p = 9.7e-05$), showing that nutrient sources promoting branching can boost photosynthetic area and yield potential. Plot (b) shows a strong, highly significant association between test weight and yield ($R^2 = 0.67$, $p = 1.8e-07$),

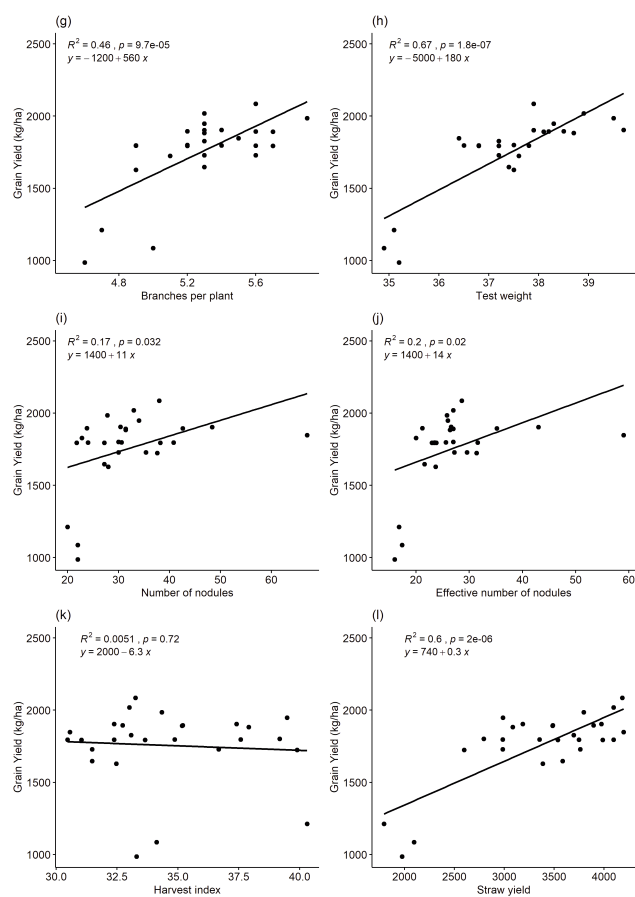


Fig. 6 - Coefficient of determination (R^2), Linear regression equation, and scatter diagram showing the fitted simple regression line of Y (Yield) on X (a) Branches per plant, (b) Test weight, (c) Number of nodules, (d) Effective number of nodules, (e) Harvest index, (f) Straw yield.

reflecting that better nutrient supply enhances seed filling and grain quality. Plots (c) and (d) show weak to moderate but significant positive correlations between number of nodules ($R^2 = 0.17$, $p = 0.032$) and effective nodules ($R^2 = 0.20$, $p = 0.02$) with yield, indicating that biofertilizers supporting root nodulation and nitrogen fixation contribute to productivity. Plot (e) shows a very weak, non-significant negative relationship between harvest index and yield ($R^2 = 0.0051$, $p = 0.72$), suggesting that nutrient sources affected both vegetative and reproductive growth similarly, resulting in little variation in partitioning. Finally, plot (f) presents a strong, significant positive correlation between straw yield and grain yield ($R^2 = 0.60$, $p = 2 \times 10^{-6}$), highlighting that higher total biomass, driven by improved nutrient availability, supports better grain output. Overall, the results indicate that nutrient sources, particularly those improving biomass,

branching, pod formation, and seed filling, play a critical role in enhancing mungbean yield, while traits such as flowering time and harvest index show limited responsiveness to nutrient-based interventions.

4. Discussion and Conclusions

The application of diverse nutrient management practices induced notable variation in the growth, phenology, and yield attributes of mungbean, reflecting the critical influence of nutrient form and availability on plant development. Treatments involving Nano DAP and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM produced substantial improvements in plant height, number of branches, and leaf count. The superior plant height under RDF and FYM can be attributed to the immediate nutrient availability in chemical fertilizers and the sustained release and microbial stimulation provided by organic amendments, enhancing photosynthetic activity and chlorophyll formation. Similar findings were reported by Kumar *et al.* (2015), and Rifhat *et al.* (2023), who observed that integrated nutrient management significantly enhanced vegetative growth in mungbean and other pulses. The integrated use of organic and inorganic fertilizers ensures both quick and sustained nutrient release, supporting continuous vegetative and root development. Moreover, the Nano DAP treatment recorded the highest number of lateral branches and trifoliolate leaves, likely due to its nanoscale particle size and large surface area that enhance nutrient penetration, enzymatic activity, and chloroplast function (Chamuah *et al.*, 2023). Although differences in leaf number were statistically non-significant, the numerical superiority of Nano DAP and FYM indicates improved canopy development and photosynthetic surface area. This observation agrees with Bam *et al.* (2022), who noted that balanced nutrient supply and nano-based interventions stimulate foliage expansion and carbohydrate accumulation in legumes. Root nodulation parameters, including total and effective nodules, were highest under biological treatments, particularly *Rhizobium* + P:K and *Rhizobium* + PSB. This outcome reflects successful symbiotic establishment and active nitrogen fixation under favorable microbial and nutrient conditions. Similar patterns were observed by Dipta *et al.* (2019), Bam *et al.* (2022), and Bhusal (2024), who demonstrated that *Rhizobium* inoculation with phosphorus and

potassium supplementation enhances nodulation efficiency, nodule biomass, and root elongation. The dominance of pink nodules in inoculated plants confirms active nitrogen fixation, while the white or brown nodules in control plots suggest inactive or senescent nodules, consistent with Razzaque *et al.* (2016). Phenological traits such as days to flowering and days to 50% flowering were significantly affected by nutrient regimes. Earlier flowering observed under Nano DAP and *Rhizobium* + P:K treatments may be due to enhanced early nutrient uptake, metabolic efficiency, and hormonal stimulation, whereas delayed flowering under FYM suggests slower nutrient mineralization. These findings are in accordance with Bhardwaj *et al.* (2021) and Tomar and Bhatnagar (2023), who linked phenological advancement with improved nutrient assimilation and faster physiological maturity. Reproductive and yield attributes were also influenced by integrated and nano-fertilizer treatments. The highest pod number per plant in Nano DAP and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM treatments may be attributed to improved nutrient partitioning, pollen viability, and pod retention. Similar yield improvements were documented by Dhakal *et al.* (2015), Verma *et al.* (2017), and Kishor *et al.* (2021), emphasizing the synergistic benefits of combined nutrient sources. Although pod length showed no significant variation, its numerical increase under Nano DAP and integrated treatments suggests partial enhancement due to nutrient-driven physiological vigor. Seed number per pod and test weight were significantly increased under Nano Urea, *Rhizobium* + P:K, and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM treatments. These effects likely result from balanced nutrient availability promoting fertilization, pod filling, and seed development. Enhanced test weight under integrated and nano-nutrient treatments indicates better seed maturity and density, consistent with the findings of Hou *et al.* (2019) and Muchira *et al.* (2018). Grain and straw yields followed similar trends, with Nano DAP, *Rhizobium* + P:K, and $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM outperforming the control due to improved nutrient uptake, biomass accumulation, and assimilate translocation. Comparable outcomes were reported by Neupane *et al.* (2023), and Rijal *et al.* (2023), who confirmed that combined nutrient inputs significantly enhance legume productivity and resource use efficiency. The highest harvest index (HI) observed under $\frac{1}{2}$ N:P: K + $\frac{1}{2}$ FYM and PSB + N treatments indicate superior partitioning of assimilates toward economic yield. This validates the

advantage of integrated nutrient management in optimizing both yield and nutrient use efficiency. From an economic standpoint, Nano DAP produced the highest benefit-cost ratio, followed by $\frac{1}{2}$ N:P:K + $\frac{1}{2}$ FYM, underscoring the profitability of integrating innovative and conventional nutrient strategies. Overall, the results highlight that while conventional fertilizers promote rapid initial growth, combining organic, biofertilizer, and nano-based sources provides a synergistic advantage by enhancing soil health, microbial activity, and nutrient use efficiency, leading to sustainable yield improvement. These findings reaffirm the conclusions of Dipta *et al.* (2019), Kishor *et al.* (2021), and Bam *et al.* (2022), emphasizing that integrated and nano-nutrient technologies can help achieve long-term soil fertility and productivity goals. Importantly, research on nano-based fertilizers in mungbean production within Nepal remains scarce. Future investigations should focus on optimizing nano-fertilizer formulations, dosages, and application timing under diverse agro-climatic conditions to validate their long-term effects on soil fertility, microbial dynamics, and crop productivity.

The field experiment demonstrated that nutrient management significantly influenced the growth, yield, and profitability of mungbean (*Vigna radiata* L.). Among the treatments, foliar application of nano Diammonium Phosphate (T4: 2000 ml/acre) produced the highest grain yield (2,030.02 kg/ha), pod number per plant (28.83), straw yield (4026.04 kg/ha), and benefit-cost ratio (2.24), highlighting its efficiency and high productivity potential. The integrated use of $\frac{1}{2}$ recommended N:P:K (10:20:10 kg/ha) with $\frac{1}{2}$ farmyard manure (5 t/ha) (T6) also performed well, offering a sustainable alternative that maintains high yields while improving soil fertility and microbial activity. Regression analysis confirmed that pod number per plant, test weight, and straw yield were strongly correlated with grain yield, emphasizing their importance as key determinants of productivity. These findings suggest that adopting integrated nutrient management, particularly combining nano-fertilizers or balanced chemical and organic sources, enhances crop performance and reduces environmental risks. It is recommended that farmers adopt T4 for rapid nutrient delivery and maximum yield, or T6 for sustainability and soil health benefits. Promotion through extension services, training, demonstration plots, and policy support can accelerate adoption.

Future research should validate these practices across multiple seasons and diverse agro-climatic zones to develop site-specific recommendations. Overall, integrated and eco-friendly fertilization approaches are essential for high-yielding, profitable, and sustainable mungbean production in Nepal.

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Performance of six genotypes of *Hibiscus sabdariffa* L. under cultural practices and jassids influence

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Key words: Agricultural practices, genotypes, jassids, organic fertilization, roselle.

Abstract: Roselle (*Hibiscus sabdariffa* L.), a plant in the Malvaceae family, is of vital importance because its leaves, calyxes and seeds are all consumed. However, this species is still absent from genetic improvement programs in Burkina Faso. The aim of this study was to gain a better understanding of its genetic potential and how this potential is expressed under agro-ecological intensification conditions in a real environment. To do this, six genotypes were tested with cropping practices such as organic fertilization and sowing densities. Qualitative and quantitative traits were measured, as well as the susceptibility of genotypes to attacks by jassids. The results revealed differences in the color, shape and physiological characteristics of the plants. Genotypes differed in growth cycle length, with HSE6 showing a shorter cycle and HSE1 a longer cycle. HSE4 and HSE5 were identified as the most productive. Cultivation practices had a significant influence: a sowing density of 0.4 m x 0.6 m and fertilization with poultry droppings (6000 kg/ha) gave the best performance. Conversely, the absence of fertilization adversely affected plant growth. Assessment of the damage caused by jassids shows uniformity of attack, with damage rates ranging from 26% to 50%. The impact of jassids should be studied in greater depth and more resistant genotypes should be explored.

1. Introduction

Traditional African vegetables, especially leafy vegetables, make a very

important contribution to the human diet because they are rich in minerals and vitamins. They therefore contribute to the resilience of populations to food and nutritional insecurity (Kahane *et al.*, 2005; Dansi *et al.*, 2008). However, these vegetables have been marginalized and under-exploited with the introduction of exotic vegetables from temperate regions such as lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*) and leek (*Allium porrum*) (Abukutsa-Onyango, 2010).

These vegetables include roselle (*Hibiscus sabdariffa* L.), a plant of the Malvaceae family widely grown in several tropical regions and distinguished by two botanical varieties: *Hibiscus sabdariffa* var. *Sabdariffa*, commonly known as Roselle, and *Hibiscus sabdariffa* var. *Altissima* known as Mesta. It is a very important plant in terms of diet, as almost all its parts are consumed (Hama-Ba *et al.*, 2017). The purple-red calyxes are used to produce 'bissap' in Wolof, a drink much appreciated by the local population. In the past, Roselle was grown by women on small areas and mainly in association with other cereal crops or pulses for self-consumption (Ouoba *et al.*, 2016).

Nowadays, roselle is attracting growing interest because of its significant economic potential for producers and processors and its nutritional and therapeutic benefits (Cissé *et al.*, 2009). Despite its many advantages, roselle cultivation in Burkina Faso faces a number of challenges, including a lack of varieties with high yield potential and cultivation practices adapted to the new environmental conditions imposed by climate change. Climate change has posed new challenges for agriculture in tropical regions, with rising temperatures having a negative impact on crop production due to limited water availability, resulting in sub-optimal fruiting (Gornall *et al.*, 2010). Also, the increase in humidity and temperature has made crops more vulnerable to infestations of insect pests that thrive in these conditions, resulting in the expansion of their geographical ranges and an increased risk of insect pest infestations on plants (Naeem-Ullah *et al.*, 2020). It was in such a hostile environment that, during the 2022/2023 wet season agricultural campaign, the Plant Protection Directorate made the chilling report of the appearance in Burkina Faso of a jassid species [*Amrasca biguttula* (Ishida) (Hemiptera: Cicadellidae)]. Over 617,000 hectares of cotton were infested, with huge losses. Other crops such as roselle and okra were also attacked, and the damage

was spectacular (Sawadogo *et al.*, 2023).

These many new constraints mean that production of this crop is unable to meet market demand, despite the growing popularity of the crop. Faced with the need to optimize yields while preserving ecosystems, agro-ecological intensification is emerging as a promising solution. The selection of genotypes with high yield potential adapted to the new environmental conditions, in particular parasitic and abiotic pressure, combined with agro-ecological intensification practices in agrosystems, is therefore necessary to relaunch bissap production in Burkina Faso. However, few studies have been carried out to assess the reaction of different *Hibiscus sabdariffa* genotypes to this approach in a Sudano-Sahelian environment. This is why knowledge of the genetic potential and the expression of this potential under agro-ecological intensification conditions in a real environment is becoming an important step in the development of innovative technological packages to increase the productivity of bissaps in Burkina Faso. Hence the interest of this study, which aims to assess the variability and agromorphological performance of the most widely grown genotypes in Burkina Faso in a real environment.

2. Materials and Methods

Site of study

This study was carried out at the Center for Environmental, Agricultural and Training Research (CREAF) in Kamboinsé (Ouagadougou) during 2024-2025 agricultural season. Geographical coordinates are 12°28 N latitude and 1°32 W longitude, with an altitude of around 296 m above sea level. The site is located in the northern Sudanese zone, with a long dry season from November to May and a short rainy season from June to October (Thiombiano and Kampmann, 2010). Average temperatures vary between 27°C and 31°C in the cold and hot periods respectively. During the 2024-2025 season of the trial, total rainfall was 970.7 mm. The Kamboinsé soils are classified as leached tropical ferruginous soils with a silty-clay or silty-sandy texture.

Plant material

The plant material used for this experiment consisted of six roselle genotypes. Five genotypes (HSE1 HSE2, HSE3, HSE4, and HSE5) came from

Burkina Faso and one genotype (HSE6) from Niger.

Experimental design

The experiment was arranged in a split-split-plot design with three replicates, spaced 1.5 m apart. Each replicate comprised two sowing densities [0.40×0.40 m (D1) and 0.60×0.40 m (D2)] which constituted the main plots. For D1, each main plot consisted of six sub-plots measuring 2×4 m, whereas for D2 each main plot consisted of six sub-plots measuring 3×4 m, giving a total of 12 sub-plots per replicate. Each sub-plot comprised six rows corresponding to the six genotypes, with 11 planting hills per row. The spacing between main plots and between sub-plots was 1.0 m and 0.8 m, respectively. Fertilization treatments (Table 1) were randomly assigned to the plots. The sowing was done with 6 holes per planting row, followed by thinning to one plant per hole at 21 days after sowing. The plots were regularly weeded as needed. Due to the early cessation of rainfall, supplementary irrigation was provided as needed until harvest.

Table 1 - Fertilizer regimes applied in the experiment

Fertilizer code	Description
F0	Unfertilized control
F3000	Poultry manure applied at 3000 kg ha ⁻¹
F4000	Poultry droppings graded at 4000 kg/ha
F5000	Poultry droppings graded at 5000 kg/ha
F6000	Poultry droppings graded at 6000 kg/ha
ASS	Intercropping with bambara groundnut (<i>Vigna subterranea</i>)

Data collection

A total of 25 variables were recorded. The choice of variables and data collection periods were based on some previous studies (Sharma *et al.*, 2016; Ouangraoua, 2023), and on the use of the roselle descriptor (Daudu *et al.*, 2019). These variables were classified into qualitative and quantitative variables. The various measurements were taken on five plants per row.

Qualitative parameters

Harvesting was carried out at physiological maturity. Qualitative traits were evaluated either in the field or after harvest, depending on the organ and trait considered. Vegetative traits, including leaf color, petiole color, leaf shape, leaf pubescence,

stem color, stem pubescence, calyx color, calyx shape and calyx pubescence, were visually assessed in the field at maturity. Flower color was visually observed at the flowering stage. Seed traits, namely seed shape, seed color, and seed pubescence, were assessed after harvest using an optical microscope. All color-related traits were evaluated by direct visual observation and were based roselle descriptor (Daudu *et al.*, 2019).

Quantitative parameters

Twelve quantitative traits were considered into three groups according to phenology, physiology and agronomic parameters. For phenology-related parameters, days to 50% flower bud formation (FBF50), days to first flowering (FFLO), days to 50% flowering (FLO50), and days to 50% calyx maturity (MAT50) were recorded. Physiological and agronomic parameters were measured on five plants per line and concerned plant height (PlanH), stem diameter (DiaSt), number of branches (NBR), calyx length (LogCal), calyx width (WiCal) as physiological parameters and the number of calyx per plant (CalP), dry calyx yield (YieCal) and seed yield (YieSd) as agronomic parameters. Yield was estimated using the formula:

$$\text{Rdt (kg/ha)} = [(10000 \text{ m}^2 / \text{density}) \times (\text{weight (calyx or seed) / NBR})] / 1000$$

Assessment of jassids damage on *Hibiscus sabdariffa* genotypes

Foliar damage caused by jassids on *Hibiscus sabdariffa* genotypes was assessed using a foliar damage scoring scale as proposed by Simon *et al.* (2018): 0= no foliar damage; 1= 25% of leaves damaged; 2= 26-50% of leaves damaged; 3= 70% of leaves damaged; 4= 100% of leaves damaged; 5= complete defoliation.

Statistical analysis

The data collected were entered and processed in Excel 2016. An analysis of variance (ANOVA) was performed using XLSTAT 2019 software to identify the traits that discriminate between genotypes. Means were compared using the Student Newman Keuls test with a threshold of 5%. Relationships between quantitative characteristics were estimated using the Pearson correlation matrix. Structuring of agromorphological diversity was estimated by principal component analysis (PCA) followed by

hierarchical ascending classification using Ward's aggregation method and Euclidean distance at the 5% threshold. These analyses of variance highlighted the influences of the various factors (genotypes, fertilization and sowing density) and their interactions on the agronomic performance of the genotypes studied.

3. Results

Variability of qualitative traits among roselle genotypes

The six genotypes showed high leaf variability (Table 2). In fact, these genotypes showed five

Table 2 - Frequency distribution of qualitative traits among the six roselle genotypes

Qualitative parameter	Category	Frequency (%)
Leaf color	Green-violet	33.33
	Green	50.00
	Light green	16.67
Leaf shape	Rounded	50.00
	High	50.00
Leaf pubescence	No	83.33
	Yes	16.67
Stem color	Red	33.33
	Light red	16.67
	Deep red	33.33
	Light green	16.67
Stem pubescence	No	83.33
	Yes	16.67
Petiole color	Red	33.33
	Light red	16.67
	Deep red	33.33
	Green	16.67
Calyx shape	Long	83.33
	Short	16.67
Calyx color	Red	33.33
	Light red	16.67
	Deep red	33.33
	White	16.67
Calyx pubescence	Yes	100
Seed color	Brown	66.67
	Black	33.33
Seed shape	Reniform	33.33
	Angular	66.67
Seed pubescence	Yes	100
Flower color	Pale violet with purple pigmentation at the base	16.66
	Violet with purple pigmentation at the base	50.00
	Pale yellow	16.66
	Deep violet with purple pigmentation at the base	16.66



Fig. 1 - Variability of floral organs and leaves in the six genotypes: a = rounded, non-pubescent violet-green leaves; b = acute, non-pubescent violet-green leaves; c = acute, non-pubescent green leaves; d = rounded, non-pubescent green leaves; e = violet flower with purple pigmentation at the base; f = pale violet flower with purple pigmentation at the base; g = pale yellow flower; h = dark violet flower with purple pigmentation at the base; i = red calyx; j = light red calyx; k = dark red calyx; l = white calyx.

different leaf characteristics: acute green leaves, not pubescent; rounded green leaves, not pubescent; rounded violet-green leaves, not pubescent; acute violet-green leaves, not pubescent; and rounded light green leaves, pubescent (Fig. 1). The petioles of these leaves were either red, deep red, light red or light green. They had four types of stems which were either red, deep red, light red or light green with or without pubescence. The calyxes of all the genotypes were pubescent and were either long or short. The difference between the calyxes of the different genotypes was basically in their color. Calyxes were either red, light red, deep red or white (Fig. 1). Flowers coloration exhibited considerably variability, ranging from violet with purple pigmentation at the base to dark violet with purple pigmentation at the base, including intermediate forms such as pale violet with basal purple pigmentation as well as pale yellow flowers (Fig. 1). The seeds were all pubescent, angular or kidney-shaped and black or brown in color (Table 2).

The colour of the leaves varied from green to purple-green, with green leaves predominating (50%), while the shape of the leaves was evenly distributed between rounded and tall shapes (50%

each). The majority of genotypes (83.33%) did not exhibit leaf pubescence, reflecting a general tendency towards glabrescence. With regard to the stem, marked variability in pigmentation was observed, with shades ranging from light red to dark red, while green stems remained in the minority. As with the foliage, non-pubescence dominates (83.33%). At the floral level, most genotypes (83.33%) had a long calyx, a dominant morphological characteristic within the studied group. The colour of the calyx showed wide variability, with a strong predominance of red hues (red, light red and dark red accounting for 83.33%), while white calyxes were rare (16.67%). On the other hand, calyx pubescence was a constant characteristic, observed in all genotypes (100%). Seed characteristics also revealed some diversity: brown was the dominant colour (66.67%), followed by black (33.33%), while angular shapes (66.67%) were more common than kidney-shaped ones (33.33%). Finally, flower color showed considerable variability, with purple pigmentation at the base (50%) being predominant, whereas lighter or darker shades (pale purple, deep purple or pale yellow) were less frequent.

Variability of quantitative traits among roselle genotypes

Figure 2 shows the results of the mean separation for the traits determining the growth cycle of the six genotypes, namely the timing of flower bud appearance, flowering and calyx maturity. Significant differences were observed for all variables

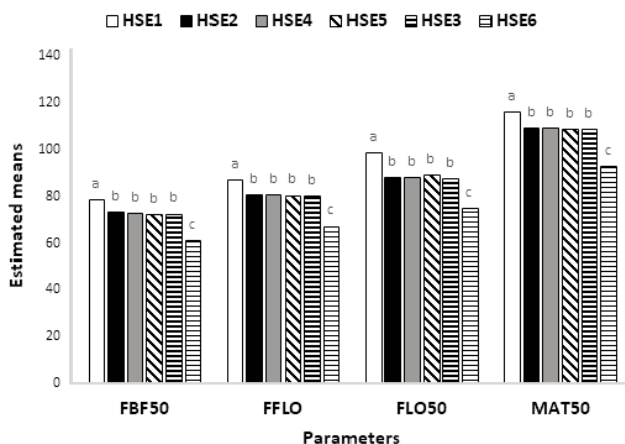


Fig. 2 - Variability in phenological traits of roselle genotypes. FBF50= days to 50% flower bud formation; FFLO= days to first flowering; FLO50= days to 50% flowering; MAT50= days to 50% calyx maturity.

($p < 0.0001$).

HSE6 was the earliest maturing genotype, with mean values of 61 days to 50% flower bud formation (FBF50), 75 days to first flowering (FFLO), and 92 days to 50% calyx maturity (MAT50). Genotype HSE1 had the latest flower bud formation (78 days), flowering (98 days) and calyx maturity (116 days). Genotypes HSE2, HSE3, HSE4 and HSE5 had intermediate periods of flower bud formation (72 days), flowering (88 days) and calyx maturity (108 days).

The results of mean separation for physiological traits are shown in figure 3. Highly significant differences were observed for all traits ($P < 0.0001$). The highest plant height (PlanH) and stem diameter (DiaSt) were reordered for genotype HSE4, with mean values of 86.9 cm and 11.2 mm, respectively, whereas the lowest values were observed in genotype HSE6 (71.2 cm and 9.4 mm, respectively). In terms of capsules size, genotype HSE1 had the longest capsules (46.4 mm) but the smallest diameter (22.8 mm). In contrast, genotype HSE6 showed the widest (25.9 mm) and shortest capsules (42.2 mm). Genotype HSE2 was the most branched with an average of 23.6 branches per plant, while genotype HSE6 was the least branched, with an average of 17.3 branches per plant.

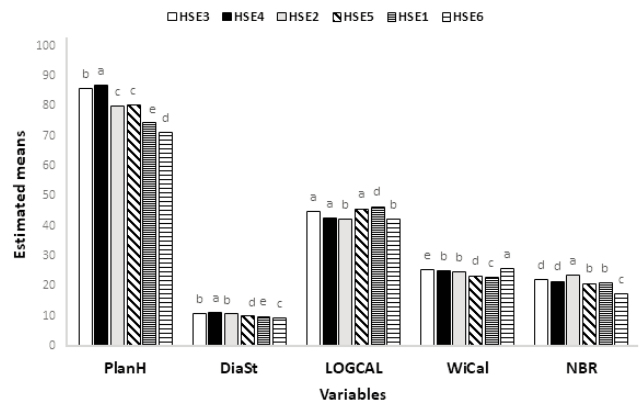


Fig. 3 - Variability in physiological traits of roselle genotypes. PlanH= plant height; DiaSt= stem diameter; LOGCAL= calyx length; WiCal= calyx width; NBR= number of branches.

Mean separation for agronomic traits are given in figure 4. Significant difference among the six genotypes were observed for the average number of capsules per plant (CalP) and grain yield ($p < 0.0001$). For the number of capsules per plant (CalP), genotypes HSE1 and HSE6 obtained the highest and

lowest values, with values of 14.72 and 8.85 respectively. For seeds yield (YieSd), the highest and lowest values were obtained by genotypes HSE5 (156.36 kg/ha) and HSE1 (98.29 kg/ha). Dry calyx yield (YieCal) showed no significant difference between genotypes.

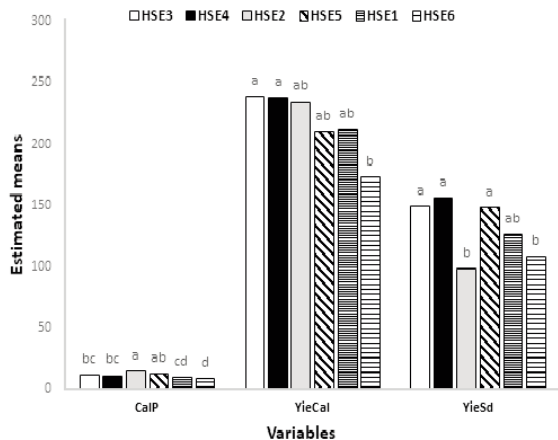


Fig. 4 - Variability in agronomic traits of roselle genotypes. CalP= number of capsules per plant; YieCal= dry calyx yield; YieSd= seed yield.

Structuring phenotypic variability

The results of the principal component analysis (PCA) indicated the contribution of each variable to the formation of the axes (Fig. 5, Table 3). Projection of the variables onto the F1/F2 plane (Fig. 5) showed that the first two axes explained 86.76% of the total variability (Fig. 5). Thus, the F1 axis, with 61.80% of the total

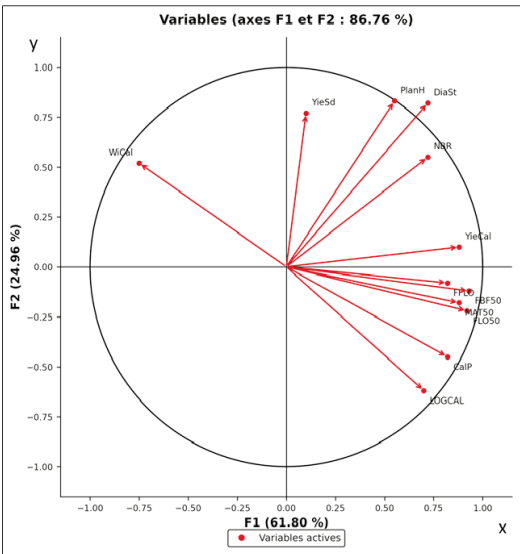


Fig. 5 - Projection of variables in the PCA F1/F2 plane. FBF50= days to 50% flower bud formation; FFLO= days to first flowering; FLO50= days to 50% flowering; MAT50= days to 50% calyx maturity; PlanH= plant height; DiaSt= stem diameter; LOGCAL= calyx length; WiCal= calyx width; NBR= number of branches per plant; CalP= number of calyx per plant; YieCal= dry calyx yield; YieSd = seed yield.

inertia, associates days to bud formation (FBF50) ($r=0.972$), days to appearance of the first flower (FFLO) ($r=0.980$), days to 50% flowering (FLO50) ($r=0.928$), days to 50% calyx maturity (MAT50) ($r=0.982$), the number of branches per plant (NBR) ($r=0.554$), calyx width (WiCal) ($r=0.609$), the number of capsules per plant (CalP) ($r=0.650$), and calyx yield (YieCal) ($r=0.824$). The F1 axis is therefore the productivity axis and the axis that defines the cycle.

Table 3 - Value of the cosines squared linking the twelve quantitative characteristics and the PCA factor

Variables	F1	F2	F3	F4	F5
FBF50	0.972	0.005	0.012	0.010	0.001
FFLO	0.980	0.006	0.007	0.007	0.001
FLO50	0.928	0.060	0.006	0.006	0.000
MAT50	0.982	0.004	0.005	0.009	0.001
PlanH	0.214	0.654	0.040	0.083	0.010
DiaSt	0.290	0.684	0.006	0.008	0.011
LOGCAL	0.349	0.339	0.227	0.061	0.024
WiCal	0.609	0.265	0.038	0.082	0.006
NBR	0.554	0.255	0.089	0.000	0.102
CalP	0.650	0.117	0.121	0.082	0.030
YieCal	0.824	0.007	0.092	0.011	0.066
YieSd	0.063	0.599	0.105	0.217	0.016

FBF50= days to 50% flower bud formation; FFLO= days to first flowering; FLO50= days to 50% flowering; MAT50= days to 50% calyx maturity; PlanH= plant height; DiaSt= stem diameter; LOGCAL= calyx length; WiCal= calyx width; NBR= number of branches per plant; CalP= number of calyx per plant; YieCal= dry calyx yield; YieSd= seed yield.

The F2 axis, which accounts for 24.96% of total inertia, has a positive association with height ($r=0.654$) and stem diameter (DiaSt) ($r=0.654$). This axis can be described as a growth axis.

Structure of the diversity of the six genotypes studied

Hierarchical and ascending classification (HEC) was used to structure the genotypes into 4 groups (Fig. 6). The first group consisted of HSE1, which is characterized by a to a longer growth cycle of 120 days after sowing and a large number of calyxes (14.720). The second group, made up of two genotypes, HSE2 and HSE3, was characterized by a cycle of 108 days, an average stem height of 79.943 cm and an average number of bolls of 12.310, with an average yield of 209.838 kg/ha of calyx. The third group was made up of two genotypes, HSE4 and HSE5, which were particularly characterized by their high calyx yield (237.500 kg/ha). The fourth and final group was characterized by its very early ripening days, low number of bolls (8.848) and low yield (173.206).

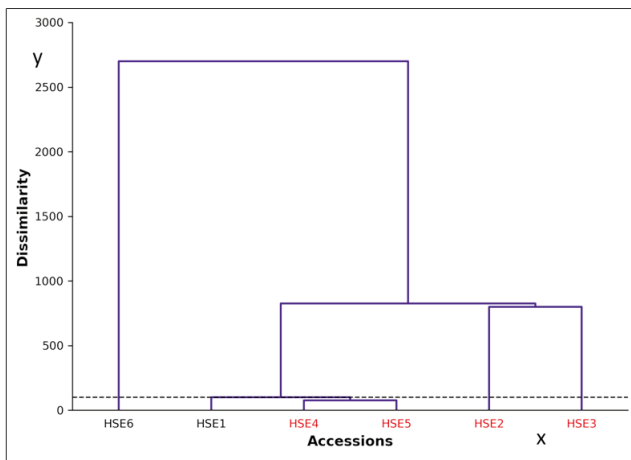


Fig. 6 - Genotype dendrogram.

Influence of cultural practices on genotypes performances

Analysis of variance indicated no significant effect of fertilization on phenological parameters at the 5% level. In contrast, fertilization had a significant effect on physiological traits, specifically plant height, stem diameter, and number of branches (Fig. 7) and agronomic traits (Fig. 8). The dose of 6000 kg/ha of poultry droppings (F6000) recorded the greatest height (87.703 cm) and diameter (11.517 mm), while the dose of 5000 kg/ha of poultry droppings (F5000) recorded the highest number of branches (23.263). On the other hand, the lowest values for height,

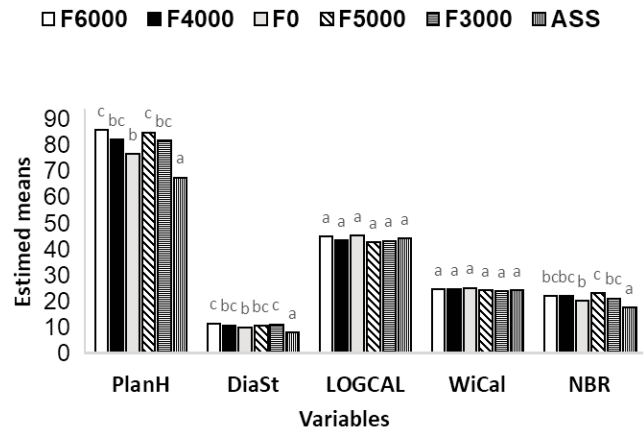


Fig. 7 - Performance of six genotypes for physiological traits under different organic fertilization regimes. PlanH= plant height ($p < 0.0001$); DiaSt= stem diameter ($p < 0.0001$); LOGCAL= calyx length ($p = 0.74$); WiCal= calyx width ($p = 0.885$); NBR= number of branches ($p < 0.0001$).

diameter and branching were observed in intercropping with Bambara groundnut (ASS), with values of 67.676 cm, 8.165 mm and 17.660 respectively. As for the length and width of the capsules, no significant differences were observed in any of the treatments.

The F6000 treatment resulted in the highest calyx and seed yields, reaching 256.973 kg ha⁻¹ and 151.833 kg ha⁻¹, respectively (Fig. 8). Conversely, intercropping with Bambara groundnut treatment produced the lowest yields, with calyx and seed yields of 143.248 kg ha⁻¹ and 89.178 kg ha⁻¹,

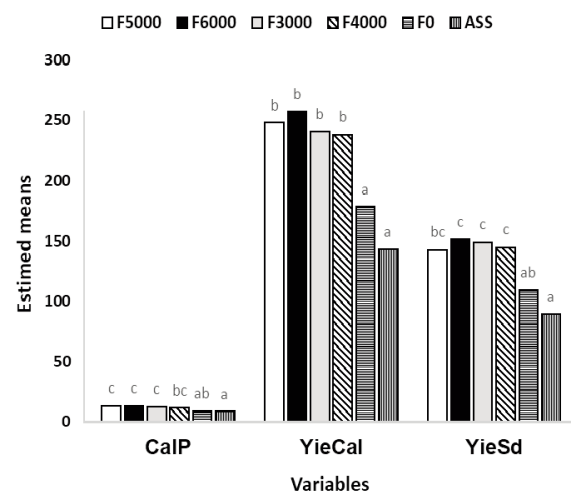


Fig. 8 - Performance of six genotypes for agronomic traits under different organic fertilization regimes. CalP= number of calyx per plant ($p < 0.0001$); YieCal= dry calyx yield ($p = 0.000$); YieSd= seed yield ($p = 0.000$).

respectively. Similarly, the average number of capsules per plant was maximized under F5000 and F6000 (13.5), whereas the lowest value was recorded under intercropping with Bambara groundnut (8.710).

Effect of sowing density on genotypes performances

Analysis of variance showed that sowing density had no significant effect on phenological parameters related to days to 50% flower bud formation (FBF50), days to first flowering (FFLO), days to 50% flowering (FLO50), days to 50% calyx maturity (MAT50) which showed respective p-values of 0.694, 0.524, 0.382 and 0.487. These results show that sowing density did not influence the duration of the growth cycle of the genotypes studied. In contrast, sowing density significantly affected several physiological traits (Fig. 9). Higher density (D2) resulted in greater calyx length and width, as well as a higher number of branches per plant, while plant height and stem diameter were not significantly influenced by density. Overall, plants grown at D2 exhibited more vigorous vegetative development.

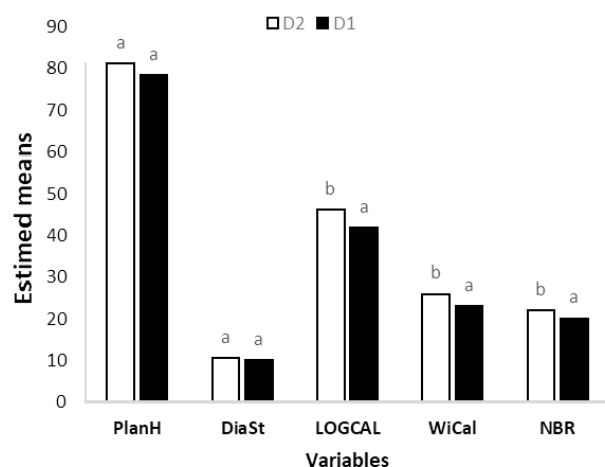


Fig. 9 - Effect of plant density on physiological parameters. PlanH= plant height ($p=0.139$); DiaSt= stem diameter ($p=0.117$); LOGCAL= calyx length ($p<0.0001$); WiCal= calyx width ($p<0.0001$); NBR= number of branches ($p=0.004$); G= genotype; D= sowing density.

Sowing density also had a significant impact on agronomic performance. The higher density (D2) consistently produced superior results, with an increased number of bolls per plant and higher calyx and seed yields compared with the lower density. These results indicate that increasing sowing density improved yield-related traits without affecting the phenological development of roselle genotypes.

For all agronomic parameters for the results of analyses of variance revealed significant differences between sowing densities at the 5 % threshold. Separation of the averages shows that the best yields were obtained with density D2, with values of 13.146 for the number of bolls, 243.789 kg/ha for calyx yield and 149.614 kg/ha for seed yield (Fig. 10).

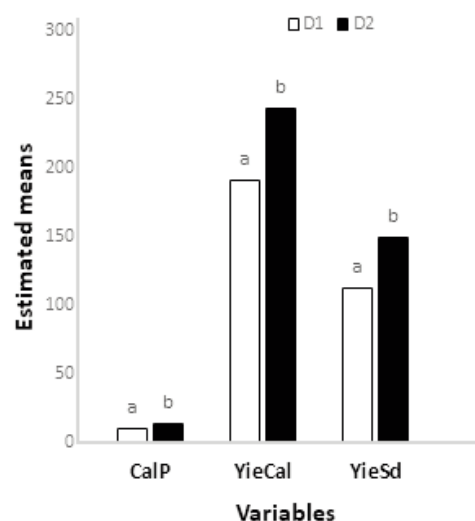


Fig. 10 - Effect of plant density on physiological parameters. PlanH= plant height ($p=0.139$); DiaSt= stem diameter ($p=0.117$); LOGCAL= calyx length ($p<0.0001$); WiCal= calyx width ($p<0.0001$); NBR= number of branches ($p=0.004$); G= genotype; D= sowing density.

Interactions between different factors

The analysis of variance revealed both significant and non-significant interactions between genotypes and fertilization regimes across the studied parameters (Table 4). Significant interactions were observed for flowering traits (FBF50, FFLO, and FLO50), maturity (MAT50), plant height (PlanH), stem diameter (DiaSt), number of branches per plant (NBR), number of bolls, and grain yield, indicating that the response to fertilization varied among genotypes and, conversely, that genotype performance depended on the fertilization regime. In contrast, calyx yield and calyx dimensions (length and width) did not show significant interactions.

Concerning genotype $\times$ sowing density interaction, significant interactions between genotypes and sowing densities were detected for all the parameters analyzed (Table 5), demonstrating that genotype responses were strongly influenced by plant density and vice versa.

As for fertilization regime $\times$ sowing density interaction, the interaction between fertilization regime and sowing density resulted in both

Table 4 - Analysis of variance of the genotype × fertilization regime interaction

Parameters	Source	DoF	Sum of squares	Mean square	Frequency	Pr > F
FBF50	Gen X Fert	35	6005.12	171.57	32.70	< 0.0001
FFLO	Gen X Fert	35	7706.04	220.17	37.64	< 0.0001
FLO50	Gen X Fert	35	11052.15	315.78	23.31	< 0.0001
MAT50	Gen X Fert	35	11135.43	318.15	84.09	< 0.0001
PlanH	Gen X Fert	35	17225.73	492.16	3.57	< 0.0001
DiaSt	Gen X Fert	35	383.68	10.97	3.16	< 0.0001
LOGCAL	Gen X Fert	35	1769.19	50.55	0.82	0.753
WiCal	Gen X Fert	35	548.78	15.68	0.77	0.820
NBR	Gen X Fert	35	1792.78	51.22	2.71	< 0.0001
CalP	Gen X Fert	35	1929.29	55.12	2.37	0.000
YieCal	Gen X Fert	35	720263.63	20578.96	1.42	0.073
YieSd	Gen X Fert	35	368666.82	10533.34	2.30	0.000

FBF50= days to 50% flower bud formation; FFLO= days to first flowering; FLO50= days to 50% flowering; MAT50= days to 50% calyx maturity; PlanH= plant height; DiaSt= stem diameter; LOGCAL= calyx length; WiCal= calyx width; NBR= number of branches per plant; CalP= number of calyx per plant; YieCal= dry calyx yield; YieSd= seed yield.

Table 5 - Analysis of variance of the genotype × sowing density interaction

Parameters	Source	DoF	Sum of squares	Mean square	Frequency	Pr > F
FBF50	G X D	11	5856.90	532.45	99.40	< 0.0001
FFLO	G X D	11	7547.93	686.17	115.58	< 0.0001
FLO50	G X D	11	10515.37	955.94	65.54	< 0.0001
MAT50	G X D	11	11084.87	1007.71	281.01	< 0.0001
PlanH	G X D	11	8228.68	748.06	4.51	< 0.0001
DiaSt	G X D	11	99.22	9.02	2.02	0.028
LOGCAL	G X D	11	1994.32	181.30	3.40	0.000
WiCal	G X D	11	758.76	68.98	4.067	< 0.0001
NBR	G X D	11	1044.64	94.97	4.67	< 0.0001
CalP	G X D	11	1483.35	134.85	5.93	< 0.0001
YieCal	G X D	11	312840.40	28440.04	1.93	0.038
YieSd	G X D	11	193779.50	17616.32	3.60	0.000

FBF50= days to 50% flower bud formation; FFLO= days to first flowering; FLO50= days to 50% flowering; MAT50= days to 50% calyx maturity; PlanH= plant height; DiaSt= stem diameter; LOGCAL= calyx length; WiCal= calyx width; NBR= number of branches per plant; CalP= number of calyx per plant; YieCal= dry calyx yield; YieSd= seed yield.

significant and non-significant effects on the evaluated traits (Table 6). Significant interactions were found for plant height (PlanH), stem diameter (DiaSt), number of branches per plant (NBR), number of calyx per plant (CalP), calyx length (LOGCAL), calyx width (WiCal), calyx yield (YieCal), and seed yield (YieSd). Conversely, phenological traits related to the crop cycle - namely flowering (FBF50, FFLO, FLO50) and maturity (MAT50) - were not significantly affected by this interaction.

Susceptibility of genotypes to jassid attack

The symptoms observed with jassid attacks on the different genotypes of *H. sabdariffa* are essentially yellowing of the leaves from the edges (Fig. 11a), inward rolling of the leaves (Fig. 11a), production of small leaves (Fig. 11b), stunted growth (Fig. 11b) and reddening of the leaves (Fig. 11c). The severity of leafhopper attacks on roselle varied from 2.5 for genotype HSE1 to 2.91 for genotype HSE6 (Table 7). These results show a uniformity of jassid attacks,

Table 6 - Analysis of variance of the fertilization regime × sowing density interaction

Parameters	Source	DoF	Sum of squares	Mean square	Frequency	Pr > F
FBF50	Fert X D	11	49.57	4.51	0.13	1.000
FFLO	Fert X D	11	57.37	5.21	0.12	1.000
FLO50	Fert X D	11	203.81	18.53	0.28	0.988
MAT50	Fert X D	11	99.54	9.05	0.16	0.999
PlanH	Fert X D	11	10380.25	943.66	6.08	< 0.0001
DiaSt	Fert X D	11	320.24	29.11	8.63	< 0.0001
LogCal	Fert X D	11	1992.87	181.17	3.40	0.000
WiCal	Fert X D	11	587.67	53.42	3.00	0.001
NBR	Fert X D	11	1076.85	97.89	4.85	< 0.0001
CalP	Fert X D	11	1560.98	141.91	6.35	< 0.0001
YieCal	Fert X D	11	626126.15	56920.56	4.30	< 0.0001
YieSd	Fert X D	11	230782.33	20980.21	4.45	< 0.0001

FBF50= days to 50% flower bud formation; FFLO= days to first flowering; FLO50= days to 50% flowering; MAT50= days to 50% calyx maturity; PlanH= plant height; DiaSt= stem diameter; LOGCAL= calyx length; WiCal= calyx width; NBR= number of branches per plant; CalP= number of calyx per plant; YieCal= dry calyx yield; YieSd= seed yield.



Fig. 11 - Symptoms of jassid attacks on roselle. a) yellowing of leaves from the edges and inward rolling of leaves; b) stunted growth and production of small leaves; c) red-vening of leaves.

Table 7 - Severity of jassid attacks on roselle

Genotypes	Severity index
HSE6	2.91
HSE4	2.87
HSE2	2.86
HSE3	2.83
HSE5	2.77
HSE1	2.49

with damage rates ranging from 26 to 50 % of leaves damaged.

4. Discussion and Conclusions

A number of qualitative and quantitative variables enabled clear discrimination among the six roselle genotypes. These genotypes were distinguished by

the colouring of their aerial organs (red and green), the shape of their leaves, their calyxes and their seeds. Similar discriminating traits have been reported by Konaté (2024) and Ouangraoua (2023) for *Hibiscus sabdariffa* accessions. This diversity of color and shape could be due to the ploidy of the species, as it is an allotetraploid plant (Salehi *et al.*, 2022). According to Combes Gavalda (2015), allotetraploid plants have great genetic diversity, due to their origin from interspecific hybridization followed by chromosome doubling. This process results in the coexistence of two distinct but homologous genomes within the same organism. The presence of duplicated genes (homeologs) increases allelic variation and allows for gene redundancy, subfunctionalization, or neofunctionalization. Moreover, differential regulation of these genes contributes to phenotypic plasticity, enabling allotetraploid plants to express multiple phenotypes and enhancing their adaptive potential under varying environmental conditions. In addition, the species' allogamy rate, although insignificant (1%), can contribute to cross-fertilization, resulting in natural genetic mixing. This great variability in colour reflects the plant's synthesis of anthocyanins, which is a drought tolerance factor for the plant (Palé *et al.*, 2004). Analysis of quantitative characteristics showed a highly significant difference at the 5% threshold for grain yield, number of branches and bolls, flowering and ripening periods, height, stem diameter and boll width and length, indicating significant agro-morphological variability within genotypes. Such

diversity provides opportunities for growers to select cultivars according to production objectives and consumer preferences (Ouangraoua, 2023). In addition to the variability, several negative and positive correlations were observed between certain variables. Negative correlations between flowering and ripening times and the number of bolls indicate that late-flowering and late-ripening genotypes produce lower yields and smaller bolls. On the other hand, the positive correlations observed between the period of flower bud formation and flowering, maturity, calyx length, number of bolls and yield suggested that short-cycle genotypes are more productive. These patterns can be partly explained by rainfall distribution. Early-flowering genotypes benefited from adequate rainfall, ensuring proper boll filling, whereas late-flowering genotypes suffered from water deficit due to the early cessation of rains. Given the short rainy season in Burkina Faso (approximately three months), long-cycle genotypes are particularly vulnerable to water stress, which negatively affects growth and productivity. Positive correlations between the number of branches and plant height, stem diameter, number of bolls and yield further indicate that vigorous plants are generally more productive. This could be explained by the fact that photosynthetic activity would be important because of the high leaf production linked to height and branching. These results are similar to those of Ouangraoua *et al.* (2021) and Ognalaga *et al.* (2017), who pointed out that height could be used as a production index in *H. Sabdariffa*. Genotypes HSE4 and HSE5 are characterized by high height and good calyx yield and can therefore be considered the most productive. As *H. sabdariffa* is grown for its leaves, seeds and calyxes, these correlations can be exploited for a plant improvement programme. Cluster analysis based on morphological variability structured the genotypes into four distinct groups differing in cycle-duration, morphology and yield. The HSE6 genotype with a white calyx had the shortest cycle (maximum three months). According to Bâ (2023) white calyx genotypes have a shorter cycle than red calyx genotypes. For leaf production, this genotype could be important for selection as it has very rapid vegetative growth. In contrast, genotype HSE1 had the longest cycle (at least 4 months). Naeem and Defries (2009) reported that varieties with a cycle longer than three months are likely to disappear because the shortening of the rainy season does not favour their production and adoption by

growers. The yields of such varieties can be improved by shortening their growth cycle or by adjusting the sowing dates. Analysis of variance showed that cropping treatments did not significantly affect phenological parameters, indicating that the crop cycle is mainly genotype-dependent, although it may be influenced by environmental factors such as photoperiod. *H. sabdariffa* is a short-day plant, flowering when night length exceeds day length. Thus, early sowing extends its cycle and late sowing shortens it. According to Hien (2012), *H. sabdariffa* flowering depends not only on the variety but also on environmental conditions and sowing periods. Physiological and agronomic performance varied from one treatment to another compared with the control. The agro-morphological performances obtained with the different doses of droppings were superior to those of the control, highlighting its nutrient richness. However, the combination with a legume such as voandzou without fertilizer showed very poor performance, lower than the control, likely due to competition for water and nutrients. Our results are in line with those of Ognalaga *et al.* (2017), who report that the yields of *H. sabdariffa* are reduced in intercropping systems, particularly when associated with cereals. The best yields were obtained with poultry manure at a dose of 6000kg/ha, i.e. 6t/ha. Comparable results were reported by Haruna *et al.* (2011), who recommended 5000 kg/ha of poultry manure supplemented with mineral fertilizer. Optimal performance was also achieved at a sowing density of 0.4×0.6 m. As *H. sabdariffa* is a bushy plant (it branches strongly), it needs a very airy sowing density to encourage growth in height and thickness (Sié *et al.*, 2009). The yields obtained from the different genotypes were too low for all treatments, likely due to soil fertility constraints, late sowing and water stress caused by early rainfall cessation in October. Hien (2012) reported that sowing in August considerably reduces plant size and therefore yield. These results could also be explained by the different sowing densities, which may not be very conducive to good plant growth. According to the FAO (2021), the optimum density for this crop is 50 cm x 100 cm, although this also depends on the soil and climate conditions in the area. Finally, all genotypes were subjected to significant jassid pressure, with damage affecting 26-50% of leaves. This level of attack can lead to a significant drop in grain yield and biomass. According to Sawadogo *et al.* (2023), jassids are sweaty biters

that attack leaves by sucking sap and causing desiccation, stunting and complete leaf drop, with a consequent reduction in photosynthesis and yields. This highlights the need to integrate pest resistance into breeding programs and to promote integrated pest management, including cultivation practices and the use of tolerant varieties.

The results of this study provided a better understanding of the variability of six roselle genotypes and the influence of agricultural practices on their agro-morphological performance, despite the biotic pressure from jassid attacks. Agro-morphological characterization revealed that the genotypes differed in terms of the color and shape of the leaves, calyxes, seeds, flowers and stems. They differed on the basis of phenolic (flowering and ripening periods), physiological (stem and calyx size and thickness) and agronomic (seed and calyx yields) characteristics. Depending on the length of the growing cycle, some genotypes were very early-maturing (HSE6) whereas others showed a longer growth cycle (HSE1). Genotypes HSE4 and HSE5 emerged as the most productive. This study clearly showed that cultivation practices, particularly density and fertilization, had a significant impact on the performance of the genotypes. Indeed, sowing at a density of 0.4x0.6 m and fertilising with poultry droppings at a dose of 6000 kg/ha led to the best agro-morphological performance. Intercropping without fertilization proved detrimental to good plant growth. Assessment of the damage caused by jassids on the six genotypes of *Hibiscus sabdariffa* revealed a uniform level of attack, with damage rates varying between 26 and 50%. Despite the application of fertilizers, the genotypes showed low productivity overall, making it impossible to achieve the high calyx and grain yields expected. Effective pest management, particularly targeting jassids, is therefore necessary to ensure good plant growth and yield. Further studies are recommended to evaluate the impact of jassids on *Hibiscus* performance and to broaden the range of genotypes tested in order to identify and promote resistant genotypes.

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A plant physiological approach to reduce greenhouse energy consumption

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Key words: Greenhouse energy reduction, low-temperature acclimation, plant growth regulators, salicylic acid, tomato transplant quality.

Abstract: The economic and environmental pressures associated with greenhouse heating for warm-season vegetable transplant production have prompted a search for biologically driven, cost-effective alternatives. This study investigates the potential of exogenously applied salicylic acid (SA), a phenolic phytohormone known for its stress-mitigating properties, to reduce energy consumption by enhancing seedling cold tolerance. Tomato seedlings (*Solanum lycopersicum* cv. Rutgers) were subjected to three concentrations of foliar-applied SA (0, 75, and 150 mg/L) and grown under two temperature regimes 16°C and 25°C, in growth chambers. Transplant morphological parameters, including shoot and root biomass, stem thickness, leaf development, and root system architecture, were analyzed over a 49-day growth cycle. At 16°C, application of 75 mg/L SA significantly enhanced seedling vigor, with shoot fresh weight, stem diameter, and leaf area comparable to those grown at standard commercial conditions of 25°C without SA. SA-treated seedlings exhibited enhanced root biomass and surface area, indicating improved cold tolerance. Importantly, energy modeling indicated that reducing the heating setpoint from 25°C to 16°C could lower heating energy requirements by approximately 45%, a savings enabled by the physiological benefits of SA. These findings underscore the dual role of SA as both a growth regulator and an abiotic stress mitigator, suggesting that targeted SA application could transform transplant production by reducing reliance on costly supplemental heating. This represents a novel integration of plant physiological intervention into sustainable energy management practices in horticulture.

1. Introduction

The growing demand for high-quality, affordable fresh produce has emphasized the need for more efficient production management strategies. One of the most critical sectors supporting this demand is the greenhouse transplant production industry, which supplies seedlings for both protected and open-field cultivation systems (Javanmardi and

Moradiani, 2017). Temperature is a primary factor that affects plant development rate (Hatfield and Prueger, 2015). Plants are considered cold-sensitive if they die or suffer severe damage at temperatures between 0 and 15°C, while cold-tolerant plants are still able to grow near the freezing point (Janda *et al.*, 2007).

For warm-season vegetable crops, primarily fruit-bearing species, field transplanting typically occurs after the last spring frost. Consequently, transplant production often begins when ambient temperatures remain well below the optimal range for these crops. During colder seasons, especially on winter nights, maintaining optimal internal greenhouse temperatures with supplemental heating is essential to ensure high-quality, healthy seedling growth and development.

To achieve a high-quality crop, greenhouses are heated when the average daily temperature falls below a specific threshold, depending on the crop species (Nishad and Krupa, 2022). This requirement significantly increases heating costs, especially in light of rising global energy prices. Greenhouse heating is one of the most energy-intensive aspects of protected cultivation, especially in colder climates. Energy expenses can account for 30-80% of total production costs, varying by location (Esen and Yuksel, 2013; Sun *et al.*, 2022). The annual natural gas consumption as the main energy source for greenhouse heating typically falls between 25 and 40 m³ per square meter, with an average cost of approximately 7.8 US\$ per square meter (de Zwart *et al.*, 2019; Stanghellini *et al.*, 2024). These high energy demands have forced the industry to adopt innovative technologies such as passive solar heating (Xu *et al.*, 2022), solar thermal collectors (Prieto *et al.*, 2023), geothermal systems (Luo *et al.*, 2023), heat pumps (Asgari *et al.*, 2025), and phase change materials (Zhu *et al.*, 2023), all of which offer more eco-friendly and cost-effective alternatives for maintaining optimal temperatures in greenhouse transplant production.

Salicylic acid (SA), a phenolic phytohormone, has emerged as a multifaceted regulator in plant biology, mediating both biotic and abiotic stress responses while influencing key developmental processes (Thilakarathne *et al.*, 2025). Its role in cold stress tolerance and growth regulation has been extensively documented by Saleem *et al.* (2021), underscoring its critical function in plant resilience

and performance across diverse environments (Safari and Majidi, 2025).

Cold stress severely impairs plant metabolic functions, leading to membrane damage, destabilization of photosystem II, and oxidative stress due to the accumulation of reactive oxygen species (ROS) (Feng *et al.*, 2025). SA mitigates the effects of cold stress through a complex regulatory network (Hayat *et al.*, 2013; Saleem *et al.*, 2021). In tomatoes, SA enhances cold tolerance by preserving membrane integrity under chilling conditions (Aghdam *et al.*, 2014).

Beyond stress mitigation, SA exerts a significant influence on plant morphogenesis, including regulating seed germination, vegetative growth, root architecture, flowering, and fruit development through its interplay with other hormones, such as auxin and abscisic acid (Rivas-San Vicente and Plasencia, 2011; Li *et al.*, 2022). In tomatoes, SA application, either as a foliar spray or seed priming, has been shown to enhance seedling vigor, root and shoot biomass, and nutrient uptake under both optimal and stressful conditions (Javanmardi and Akbari, 2016). These growth-promoting effects are particularly evident under suboptimal temperatures or water-limited conditions, suggesting that SA plays a dual role as both a developmental regulator and a stress mitigator.

Tomato (*Solanum lycopersicum* L.) is one of the world's most important vegetable crops, with approximately 5.3 million hectares cultivated and an average annual production of about 190 million metric tons for fresh and processed markets (FAOSTAT, 2026). High-quality tomato transplants are essential across all production systems, as uniform and vigorous seedlings improve establishment, enable more predictable harvest timing, and support higher, more consistent yields (Leskovar, 2020).

Energy conservation in controlled horticultural systems has gained global attention following the Kyoto Protocol, which established binding commitments to reduce greenhouse gas (GHG) emissions (Protocol, 1997). Greenhouses, in particular, are energy-intensive facilities, with heating alone accounting for over 50% of total energy consumption (Djevic and Dimitrijevic, 2009; Zhang *et al.*, 2022). As such, implementing energy-saving strategies has become essential not only to reduce operational costs but also to meet climate mitigation goals without compromising crop

productivity. While many studies have explored structural improvements and operational optimization (Zhang *et al.*, 2022), to the best of our knowledge, none have used plant physiology-based approaches, such as hormonal signaling, to reduce energy input requirements during transplant production without affecting quality or economic performance.

Given the background on heating requirements, SA, its effects on plant growth and development, and its role in mitigating cold stress, we hypothesized that SA application during transplant production could reduce greenhouse heating requirements by increasing seedling tolerance to low temperatures. The main goal of this study was to evaluate SA as a low-cost, plant physiology-based strategy to reduce greenhouse energy use while maintaining transplant quality, thereby improving the economic and environmental sustainability of late-season greenhouse transplant production systems.

2. Materials and Methods

Plant material and experimental design

Tomato seeds (*Solanum lycopersicum* cv. Rutgers) (BURPEE®, PA, USA) were used for this experiment. Seeds were sown in 72-cell plug trays, each cell containing 75 mL of PRO-MIX® BX growing media. For the first few days after seed sowing and until seed emergence, the trays were kept in a growth chamber at $25 \pm 2^\circ\text{C}$ and $70 \pm 5\%$ relative humidity in the dark. Starting cotyledon expansion, the light was then applied using Philips®, 20-watt, 2ft fluorescent Plant and Aquarium Grow Light Bulbs. Light intensity was set to an average instantaneous photosynthetic photon flux (PPF) of approximately $400 \mu\text{mol}/\text{m}^2/\text{s}$ at the plant surface inside the chamber (16/8 h light/dark periods), as reported by Javanmardi *et al.* (2013). The temperature was maintained at $25 \pm 2^\circ\text{C}$ until the first true leaves appeared (one week). On day 14, after seed sowing, all plants were foliar sprayed with SA solutions (0, 75, and 150 mg/L) in a manner that allowed the solution to drip from the leaves. The second SA application was performed on day 28 after sowing. The seedlings were then divided into two groups, each containing treated seedlings with SA solutions (0, 75, and 150 mg/L). The first group was maintained in a growth chamber at $16 \pm 2^\circ\text{C}$, $70 \pm 5\%$ relative humidity, and 16/8 h light/dark

conditions. The second group was maintained in another identical growth chamber, but at $25 \pm 2^\circ\text{C}$, $70 \pm 5\%$ relative humidity, and 16/8 h light/dark conditions (considered as a greenhouse condition for commercial transplant production). These conditions were kept for 3 weeks until the end of the experiment. The third SA application was performed on day 42 after sowing.

Measurements

All transplants were harvested for measurements on day 49 after sowing. Shoot length (cm), leaf number, leaf area (cm^2) using Petiole2® leaf area meter app, stem thickness at 2 cm above the substrate surface (mm) using digital caliper, shoot fresh weight (g), root characteristics including root fresh weight (g), root length, root surface area, root average diameter, root volume, root tips no. (using EPSON PERFECTION V750 Pro, STD 4800 Scanner, Calibrated for image analysis with WinRHIZO™) were determined. Recommended chemical fertilization for tomato transplant production was applied during the experiment, as described by Javanmardi and Moradiani (2017).

To estimate the greenhouse heat requirement (Q) for a specific temperature setup, the standard formula (Eq.1) for greenhouse heating load was used (Wang and Zhang, 2021):

$$Q = U \times A \times \Delta T \times t \quad (1)$$

where Q is the required heating energy in Watt-hours (Wh), U is the thermal transmittance of the greenhouse coverings ($\text{W}/\text{m}^2\cdot\text{K}$), A is the greenhouse exposed surface area to the outside (m^2), ΔT is the difference between the desired indoor temperature and the outdoor air temperature ($T_{\text{in}} - T_{\text{out}}$) in $^\circ\text{C}$, and t is the duration (hours).

Statistical analysis

The experiment was arranged in a completely randomized design (CRD), with six treatments (three SA concentrations at two temperatures). Each treatment consisted of 9 plants as replicates. The data were checked for normality and homoscedasticity before ANOVA. One-way analyses of variance for data analyses and the Least Significant Differences (LSD) test for mean comparison were performed using JMP® 13.0.0 (SAS Institute, Inc., Cary, NC) statistical software.

3. Results and Discussions

Analysis of variance (ANOVA) indicated that salicylic acid (SA) exerted a significant effect on tomato seedling growth, markedly enhancing shoot traits, including shoot growth and length, leaf number, leaf area, stem thickness, and shoot fresh weight, across both low- and high-temperature regimes, demonstrating a consistent promotion of above-ground development. SA also significantly improved several root architectural and biomass parameters, such as root fresh weight, length, surface area, volume, and fork number; however, root average diameter and the number of root tips were not significantly affected, indicating that SA selectively influences root traits at this stage of seedling development (Table 1).

Shoot characteristics: Influence of SA at 16°C and 25°C

Shoot length. At 25°C, shoot length increased significantly with SA, particularly at 75 mg/L (18.22 cm), which was superior to both the control (16.55 cm) and the 150 mg/L treatment (16.27 cm). This

suggests that SA promotes elongation more effectively under higher temperatures, likely by enhancing cell division and expansion. However, at 16°C, shoot length remained statistically unchanged across SA treatments, indicating that shoot elongation under cold stress is relatively insensitive to SA (Table 2).

In the transplant industry, the shorter, well-rooted, and compact seedlings are often favored over tall, leggy ones. They are sturdier, easier to handle, and less prone to damage during transport or transplanting, helping reduce transplant shock and improve field establishment (Huber *et al.*, 2021). Research by Kozai (2005) and Kubota *et al.* (2008) highlights that high-quality transplants are uniform, compact, and possess strong biomass traits that not only support healthy growth but also align well with automation systems. Unlike stretched seedlings, which are more vulnerable to stress, compact seedlings are more resilient and dependable from the greenhouse to the field. It has been stated that exogenous SA application often causes dwarf plant phenotypes, which is interpreted as being an SA-induced shift of plant resources from growth to

Table 1 - ANOVA for tomato seedlings' shoot and root characteristics, treated with salicylic acid (SA) and grown in the growth chambers at 16°C and 25°C

Source	DF	Shoot length	Leaf No.	Leaf area	Stem thickness	Shoot FW	Root FW	Root length	Root surface area	Root average diameter	Root volume	Root tips no.
Treatment	5	132.92 **	4.96 **	7171.09 **	1.66 **	14.47 **	2.29 **	25690.80	34336.10	2.32 NS	724.09 **	1729056 NS
Error	48	0.559	0.27	428.72	0.18	0.40	0.06	4219.2	5856.5	1.34	153.53	725068
Total	53											

Mean values followed by ** show statistical significance ($p \leq 0.01$).

Table 2 - Mean comparison of tomato seedlings' shoot characteristics, treated with salicylic acid (SA) and grown in the growth chambers at 16°C and 25°C

Treatments	SA (mg/L)	Shoot length (cm)	Leaf No.	Leaf area (cm ²)	Stem thickness (mm)	Shoot FW (g)
Low temperature (16°C)	0	10.22±0.25 c	5.22±0.15 d	75.26±4.54 c	4.53±0.17 b	3.41±0.28 c
	75	10.31±0.35 c	6.00±0.17 bc	107.96±6.36 b	5.16±0.06 a	4.51±0.18 b
	150	9.81±0.20 c	5.33±0.24 d	85.24±5.38 c	4.52±0.14 b	3.73±0.25 c
High temperature (25°C)	0	16.55±0.19 b	6.33±0.17 b	120.33±9.97 b	5.16±0.20 a	4.57±0.14 b
	75	18.22±0.22 a	7.22±0.15 a	154.49±7.57 a	5.23±0.11 a	6.99±0.15 a
	150	16.27±0.23 b	5.67±0.17 cd	118.61±5.93 b	4.22±0.15 b	5.08±0.23 b

Values represent mean ± Std Err.

In each column, the same letters show no significant differences using the Least Significant Difference (LSD) test.

defense (Bagautdinova *et al.*, 2022).

Leaf number

SA positively influenced leaf production at both temperatures, although the response was temperature-dependent. At a low temperature of 16°C, 75 mg/L SA significantly improved leaf number (6.00) compared to the control (5.22). The same impact was observed at 25°C, where 75 mg/L SA yielded the highest leaf number (7.22), exceeding that of the control (6.33). However, the difference between 75 mg/L SA at 16°C and control plants under 25°C was not significant (Table 2). The results indicate that although SA more effectively stimulates the initiation of leaf formation under optimal temperature conditions, the application of 75 mg/L SA at lower temperatures can also result in the production of transplants with a similar number of leaves compared to those grown under optimal conditions without SA. This aligns with SA's role in modulating hormonal pathways involved in leaf primordia formation, even under low temperatures.

Several studies underscore the beneficial role of SA in maintaining or even enhancing leaf development in tomatoes under chilling stress. SA was found to preserve leaf number and shoot elongation under cold stress by stabilizing PSII (Photosystem II) via HSP21 (Heat Shock Protein 21) and promoting the accumulation of antioxidants, as well as activating ethylene-regulated transcription factors that promote photoprotection and ascorbate production (Saleem *et al.*, 2021). Gharib and Hegazi (2010) observed that SA pretreatment at 10^{-4} M significantly increased the number of leaves and shoot length in bean seedlings under chilling (15°C), suggesting similar benefits in tomatoes, particularly at early growth stages. Similar results were observed in tomato seedlings, where SA priming improved leaf area and leaf number under low-temperature stress, in part by boosting antioxidant enzyme activity and stabilizing the hormonal balance (GA_3 /ABA ratio) (Galviz *et al.*, 2021). The increase in the number of leaves can be attributed to several biochemical and molecular actions of SA: 1) enhanced cell division and expansion, particularly in the shoot apical meristem, contributing to increased leaf primordia initiation (Li *et al.*, 2022), 2) modulation of phytohormones through elevating auxin (IAA) and gibberellin (GA_3) levels while reducing abscisic acid (ABA), which create a hormonal environment conducive to leaf proliferation (Gharib and Hegazi, 2010), 3)

antioxidant defense activation, by enhancing enzymes like SOD, CAT, and APX, SA reduces oxidative damage under chilling, allowing normal meristem activity and leaf initiation to continue (Saleem *et al.*, 2021), and 4) interaction with ethylene pathway, through regulation of SIEIL transcription factors, SA maintains photosystem activity and cellular redox homeostasis, indirectly supporting sustained leaf development (Saleem *et al.*, 2021).

Leaf area

Leaf area, a key indicator of photosynthetic potential, responded well to SA at both temperatures. At 16°C, the control showed reduced expansion (75.26 cm²), while 75 mg/L SA boosted it to 107.96 cm², a 43% increase. At 25°C, the largest leaf area (154.49 cm²) was again achieved with 75 mg/L SA (Table 2). The consistent effectiveness of 75 mg/L at both temperatures suggests this dose optimally activates pathways related to cell expansion and chloroplast development. However, the difference between 75 mg/L SA at 16°C and control plants under 25°C was not significant (Table 2). The results indicate that although SA more effectively stimulates the leaf expansion under optimal temperature conditions, the application of 75 mg/L SA at lower temperatures can also result in the production of transplants with a similar leaf area compared to those grown under optimal conditions without SA.

SA plays a central role in sustaining or enhancing leaf area in tomatoes under stress conditions through a range of mechanisms involving hormonal signaling, antioxidant defense, and cellular metabolism. Although chilling stress reduces leaf expansion in tomato plants by inhibiting cell division and elongation, the exogenous application of SA has been shown to ameliorate these effects. Saleem *et al.* (2021) found significantly higher leaf area retention in SA-treated tomato plants exposed to cold compared to untreated controls. This was linked to SA-induced upregulation of protective heat shock proteins (HSP21) and enhanced ascorbate production, which maintained photosystem II integrity and protected chloroplast structures essential for cell expansion (Saleem *et al.*, 2021). Further supporting this, Gharib and Hegazi (2010) showed that SA pretreatment (10^{-4} M) improved leaf area in common bean (*Phaseolus vulgaris*) under 15°C stress by modulating phytohormones such as

gibberellic acid and indoleacetic acid, suggesting a similar effect could be expected in tomato due to comparable physiological stress responses.

Optimal concentrations of exogenous SA stimulate root and shoot elongation, enhance leaf area, and improve photosynthetic efficiency, whereas above-optimal levels can inhibit growth, likely due to the induction of oxidative stress (Chakma *et al.*, 2021; Bagautdinova *et al.*, 2022).

The improvement in leaf area under SA treatment is closely linked to enhanced antioxidant enzyme activity (e.g., superoxide dismutase, catalase), osmoprotectant accumulation (e.g., proline, soluble sugars), and hormonal balance that favors cell expansion. Moreover, SA appears to mediate these effects via complex crosstalk with auxins and gibberellins, thereby promoting sustained mitotic activity in leaf meristem regions even under stress (Li *et al.*, 2022).

Stem thickness

Stem diameter increased significantly with SA, particularly at 75 mg/L, under both conditions. Under the low temperature of 16°C, SA improved stem robustness from 4.53 mm (control) to 5.16 mm. Interestingly, no further increase was observed at 25°C since the 0, 75, and even 150 mg/L SA treatments showed similar values (~5.16-5.23 mm), suggesting a threshold of responsiveness under non-stress conditions. The difference between 75 mg/L SA at 16°C, control plants, and 75 mg/L SA under 25°C was not significant (Table 2). The results indicate that the application of 75 mg/L SA at lower temperatures can result in the production of transplants with a similar stem thickness compared to those grown under optimal conditions without SA.

A greater stem diameter of seedlings exhibits superior transplant resilience and higher yield potential. A robust stem facilitates the development of a well-structured vascular system, ensuring efficient transport of water, nutrients, and photoassimilates to expanding tissues (Chakma *et al.*, 2021). In stress environments, such as under drought or salinity, stem diameter often serves as a reliable morphological marker for plant health and stress adaptation. A thicker stem enhances the plant's ability to support larger leaf areas and fruit loads while reducing the risk of lodging or breakage during handling and environmental extremes. It has been demonstrated that foliar application of SA at 150 mg/L significantly increased stem diameter in

grape tomato seedlings under moderate drought conditions (Chakma *et al.*, 2021), accompanied by improvements in root biomass, water-use efficiency, and yield. Similarly, 1.0 mM SA preserved stem thickness in eggplants exposed to salinity stress, suggesting its relevance for tomatoes under similar conditions (Sousa *et al.*, 2024). In peppers, SA-enhanced stem girth has been linked to improved hormonal balance, turgor maintenance, and reduced electrolyte leakage under deficit irrigation, suggesting that SA-mediated mechanisms promoting stem growth may be applicable broadly across solanaceous crops (Ghahremani *et al.*, 2023).

It has been demonstrated that the foliar application of 150 mg/L SA (~1.08 mM) significantly increased stem diameter compared to untreated controls under moderate drought stress (75% FC) (Chakma *et al.*, 2021). Ghahremani *et al.* (2023) demonstrated that foliar spraying of pepper plants with 1.5 mM SA under deficit irrigation significantly enhanced stem diameter compared to untreated plants. The increase in stem thickness was associated with improved water-use efficiency, reduced electrolyte leakage, and higher chlorophyll content. These physiological improvements help maintain cell turgor and vascular integrity, leading to sturdier stems under water stress.

Sousa *et al.* (2024) showed that 1.0 mM SA maintained the stem diameter of eggplant seedlings near control values (non-saline, untreated), at moderate salinity (2.5 dS m⁻¹). The increase was attributed to SA-enhanced antioxidant activity, osmolyte accumulation, and maintenance of relative water content, all of which support vascular growth under stress.

Shoot fresh weight

SA at 75 mg/L concentration increased shoot FW compared to 0 and 150 mg/L SA, irrespective of the ambient temperature. The increase was 32 and 53 percent higher compared to the 0 mg/L SA, at 16 and 25°C, respectively. Notably, the difference between 75 mg/L SA at 16°C and control plants under 25°C was not significant (Table 2). The results indicate that although 75 mg/L SA effectively increased shoot fresh weight under optimal temperature conditions, the application of 75 mg/L SA at lower temperatures can also result in the production of transplants with a similar shoot fresh weight compared to those grown under optimal conditions without SA. This aligns with SA's role in modulating hormonal pathways involved

in biomass production, even under low temperatures.

The elevated biomass at 75 mg/L SA may reflect enhanced resource assimilation and partitioning toward shoot tissues, driven by SA-induced metabolic upregulation. In a study by Chakma *et al.* (2021), foliar application of SA at 150 mg/L under moderate drought conditions significantly increased shoot fresh weight in grape tomato seedlings compared to untreated controls. Similarly, Abd-Elkader *et al.* (2022) investigated the effects of acetylsalicylic acid (ASA) at 178 mM on tomato plants under low-temperature stress. They revealed that ASA application, especially when combined with potassium silicate, significantly enhanced shoot fresh weight and other agronomic traits, suggesting a synergistic effect in mitigating chilling injury.

Ghahremani *et al.* (2023) showed that 1.5 mM SA significantly improved shoot fresh weight compared to untreated sweet pepper plants under drought stress. They attributed the increase in biomass to enhanced water-use efficiency and reduced oxidative damage, highlighting SA's role in stress amelioration. Additionally, Mohammadi *et al.* (2023) reported that the combined application of gypsum and SA under salinity stress conditions resulted in a notable increase in shoot fresh weight in pepper plants. This combination treatment improved physiological traits and stress tolerance, emphasizing the potential of SA in integrated stress management strategies. In eggplant, Sousa *et al.* (2024) demonstrated that the exogenous application of SA at concentrations ranging from 0 to 1.5 mM under saline irrigation conditions effectively maintained shoot fresh weight by enhancing antioxidant activity and osmolyte accumulation. Furthermore, a study by Zhang *et al.*

(2023) indicated that SA application improved shoot fresh weight in eggplant seedlings subjected to low-temperature stress. The treatment enhanced photosynthetic efficiency and reduced electrolyte leakage, contributing to better growth performance under chilling conditions.

Root characteristics: Influence of SA at 16°C and 25°C

Root fresh weight. At 16°C, 75 mg/L SA increased root fresh weight (FW) from 0.71 g to 1.11 g (a 56% increase), underscoring its stimulatory role even under cold-induced growth suppression. However, SA at 150 mg/L level reduced this benefit. At 25°C, 75 mg/L SA nearly doubled the root fresh weight compared to the control (2.06 g vs. 0.98 g), while 150 mg/L SA still performed well (1.47 g) (Table 3). The difference between root fresh weight produced under low temperature and 75 mg/L SA was not significant when compared with 0 mg/L SA at 25°C. These findings suggest a dose-dependent enhancement in root biomass, peaking at 75 mg/L SA under both temperature regimes.

Bagautdinova *et al.* (2022) cited several reports that SA promotes fresh and dry weights (through increased adventitious rooting) in a dose-dependent manner, where low concentrations enhance root formation, while higher levels (above 100-800 µM) inhibit it.

Root length. Root elongation was particularly stimulated by 75 mg/L SA at 16°C (401.22 cm), significantly higher than the control (251.01 cm). This was substantially higher than the root length produced under optimal temperatures of 25°C and 0 mg/L SA (Table 3). At 25°C, the effect was less pronounced, with 150 mg/L SA yielding the longest roots (352.22 cm), while 75 mg/L SA showed a

Table 3 - Mean comparison of tomato seedlings' root characteristics, treated with salicylic acid (SA) and grown in the growth chambers at 16°C and 25°C

Treatments	SA (mg/L)	Root FW (g)	Root length (cm)	Root surface area (cm ²)	Root volume (cm ³)
Low temperature (16°C)	0	0.71±0.07 e	251.01±42.91 c	209.46±23.55 cd	12.37±1.57 c
	75	1.11±0.09 c	401.22±23.39 a	274.06±9.98 bc	15.28±1.20 c
	150	0.81±0.06 de	298.07±16.12 bc	277.10±20.59 bc	22.85±2.75 bc
High temperature (25°C)	0	0.98±0.05 cd	219.25±10.21 c	149.08±10.43 d	18.36±1.53 c
	75	2.06±0.12 a	309.65±25.18 bc	386.57±67.57 a	41.38±12.89 a
	150	1.47±0.09 b	352.22±27.16 ab	365.60±71.83 a	33.08±11.90 ab

Values represent mean ± Std Err.

In each column, the same letters show no significant differences using the Least Significant Difference (LSD) test.

moderate enhancement. This suggests that while SA enhances root elongation under cold stress, higher doses may be required under warm conditions to sustain meristem activity.

Bagautdinova *et al.* (2022) cited several reports on the concentration-dependent effect of SA on the radicle/primary root length in dicots, where low SA concentrations stimulated and higher ones retarded root growth.

Root surface area. SA treatments significantly improved surface area at both temperatures, most notably at 25°C, where 75 and 150 mg/L SA increased root surface area to 386.57 and 365.60 cm², respectively, compared to 149.08 cm² in the control. The effect was also notable at 16°C, where SA boosted surface area by 30-32% relative to the untreated seedlings. This expanded root interface likely improves water and nutrient uptake capacity, contributing to overall seedling vigor.

Root volume. Data in Table 3 show that SA treatment significantly enhanced root volume, particularly at 25 °C, where 75 mg/L SA more than doubled the root volume (41.38 cm³ vs. 18.36 cm³ in the control). This substantial increase likely reflects enhanced lateral root proliferation and thickening. At 16 °C, SA had a moderate effect, with 150 mg/L SA increasing root volume from 12.37 to 22.85 cm³, though the variation was less pronounced than at the higher temperature. Although root volume tended to increase with higher SA concentrations at both 16 °C and 25°C, the volume produced with 75 mg/L SA at 16°C was statistically similar to that produced at 25°C without SA.

In tomatoes, SA was shown to stabilize membranes, enhance antioxidant activity (via enzymes such as SOD, CAT, and APX), and aid in maintaining photosynthetic capacity, thereby indirectly supporting better root function and growth under chilling conditions (Galviz *et al.*, 2021). Seed treatment or exogenous application of SA enhanced early root elongation under cold by reducing reactive oxygen species (ROS) damage and modulating stress-responsive gene expression (Saleem *et al.*, 2021). In eggplant, SA at 1.0 mM mitigated the inhibitory effects of salt-induced osmotic stress, which shares physiological mechanisms with cold-induced water stress. This treatment helped maintain relative water content and reduced electrolyte leakage, indirectly sustaining root growth and overall seedling vigor (Sousa *et al.*, 2024).

SA, through interaction with auxin, shapes root architecture, enhancing or inhibiting elongation depending on concentration, developmental conditions, and stress, while also bolstering antioxidant enzyme activity and protecting roots from oxidative stress (Bagautdinova *et al.*, 2022). Additionally, SA signaling, via NPR1, MAPKs, and ROS-Ca²⁺ pathways, enhances stress perception and adaptive responses, promoting root resilience and growth (Saleem *et al.*, 2021). These integrative roles highlight SA's dynamic influence on root development under various environmental scenarios.

Energy savings from greenhouse temperature setpoint reduction after salicylic acid application

We investigated the role of SA as a plant physiology-based approach to reduce energy inputs during transplant development at lower temperatures without compromising seedling quality. Remarkably, transplants treated with 75 mg/L SA and maintained at 16°C developed morphological traits statistically comparable to those grown at 25°C without SA. Traits such as stem length, stem diameter, leaf number, leaf area, shoot and root fresh weights, and root volume did not differ significantly ($p>0.05$) between these two groups. This suggests that exogenous SA application can counterbalance the physiological slowdown typically induced by suboptimal temperatures. Thus, SA-mediated temperature compensation presents a promising strategy for lowering greenhouse heating requirements without compromising transplant quality.

To explore the real-world implications of these findings, we modeled potential energy savings associated with reducing the greenhouse setpoint temperature from 25°C to 16°C. This scenario is especially relevant during winter transplant production, where seedlings are typically started indoors 3-12 weeks (depending on crop species) before the last expected frost date. In many temperate regions, outdoor temperatures during this period can fall well below the optimal 18-25°C range for seedling development, necessitating supplemental heating.

One common approach to winter greenhouse heating is to maintain the air temperature at a crop-specific target level. We used the standard formula for greenhouse heating load (Eq. 1) to estimate the heat (Q) required to achieve a specific temperature

in a greenhouse. Assuming an average outdoor winter temperature of 5°C, the temperature differentials were 20°C and 11°C for the 25°C and 16°C setpoints, respectively. We compared two scenarios of inside greenhouse temperature under otherwise identical conditions.

The relative energy savings can thus be estimated as:

$$\frac{Q_{25}-Q_{16}}{Q_{25}} = A$$

While all other variables in Eq.1 are constant (U, A, and t), the relative energy savings can thus be estimated based on the ΔT temperatures as:

$$\frac{(25-5)-(16-5)}{(25-5)} = 0.45 = 45\%$$

This indicates that reducing the heating setpoint from 25°C to 16°C has the potential to reduce heating energy demand by approximately 45%, assuming insulation, greenhouse design, and heating duration remain constant. It is essential to note that while some cool-season crops or vegetative seedlings may tolerate lower air temperatures with minimal developmental penalties, fruiting warm-season vegetables, such as tomatoes, are more thermosensitive (Galviz *et al.*, 2021).

Our findings demonstrate that while suboptimal temperatures can hinder plant growth and development, the strategic application of SA effectively mitigates these limitations. Our finding is consistent with other empirical studies, which report reductions of 30–50% in heating energy when dynamic setpoint strategies are employed (Chakma *et al.*, 2021; Zhang *et al.*, 2022).

By integrating SA treatments with reduced heating setpoints, growers can maintain plant quality and profitability while capitalizing on the energy-saving benefits of lower air temperatures. This approach offers a practical, climate-resilient pathway to more energy-efficient greenhouse operations.

4. Conclusions

This study reveals that exogenous application of SA, particularly at 75 mg/L, significantly enhances the morphological and physiological performance of tomato seedlings under suboptimal temperature conditions (16°C), achieving traits comparable to those grown under optimal standard greenhouse

temperatures (25°C). SA improved shoot and root biomass, stem robustness, and leaf area while maintaining membrane integrity, hallmarks of improved cold tolerance.

From an energy efficiency perspective, these physiological improvements enabled a potential reduction in greenhouse heating setpoints by 9°C, which modeling indicated could lead to a 45% decrease in heating energy demand. This biologically driven strategy not only mitigates the adverse effects of cold stress but also offers a cost-effective, low-input alternative to conventional thermal energy inputs during critical transplant growth stages.

Incorporating SA treatments into commercial transplant production thus represents a promising avenue for reconciling high-quality seedling development with sustainable greenhouse operations. Future work should focus on applying SA to other crops and scaling this approach under variable greenhouse environments to validate its broader applicability and economic benefits.

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Nutrient chemistry in coconut-mineral mixed substrates for soilless culture

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Key words: Carbon slag, perlite, pumice, soluble and exchangeable fractions, vermiculite, zeolite.

Abstract: The use of substrate mixtures in soilless culture systems requires a comprehensive understanding of their chemical behavior, particularly regarding their capacity to supply or retain nutrients. This study evaluated the soluble and exchangeable fractions of coconut coir substrate mixed at five different proportions (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) with five mineral materials: coal slag, perlite, pumice, vermiculite, and zeolite. A total of 25 treatments were assessed under greenhouse conditions using carnation cuttings (*Dianthus caryophyllus* cv. Pomodoro) as a plant bioindicator. Results showed that vermiculite mixtures exhibited the highest cation exchange capacity (CEC) and significant sodium, manganese, and iron concentrations, while zeolite showed elevated levels of calcium and sodium in the exchangeable phase. Root development was positively correlated with soluble potassium, sodium, chloride, and sulfate levels. Treatments with equal proportions of vermiculite or pumice and coconut substrate (50:50) achieved the best rooting performance. These results highlight the importance of considering both soluble and exchangeable nutrient fractions to support substrate selection and fertigation management decisions in sustainable soilless crop production.

1. Introduction

Although substrate research has primarily focused on individual material characterization, synergistic effects in mixtures remain underexplored. The characterization of these mixtures is critical, as their properties determine a substrate's capacity to store and release water and nutrients, facilitate aeration, and influence root development (Gruda, 2019), with potential for substantial variability. Specifically, studies on the chemical properties of substrate materials have aimed to: (1) quantify nutritive element concentrations, (2) verify elevated levels of potentially

phytotoxic elements (e.g., sodium - Na), and (3) assess heavy metal presence (Block *et al.*, 2008). These evaluations are typically conducted prior to plant growth applications, with analyses performed on unaltered, homogeneous solid phases. However, during plant growth, organic compounds may be secreted by plants or generated through cumulative organic matter decomposition processes (Silber, 2008). Consequently, newly formed solid surfaces become heterogeneous, and the mixture's chemical properties may significantly diverge from those observed during initial characterization of individual materials (Silber and Raviv, 1996).

Materials used as soilless cultivation substrates may be of organic or mineral origin. Compared to organic materials, mineral-based substrates exhibit higher stability, retaining their physical and chemical properties over longer periods, which is why their use is widespread, particularly in Europe. In Colombia, their adoption has not been extensive due to their high cost and limited availability (Monsalve *et al.*, 2021). Rice husks are the most used substrate material in Colombia, but in recent years, they have also been utilized in other sectors, such as concrete production, posing a threat to their future availability as agricultural substrates (Monsalve *et al.*, 2021). This has forced many farmers to seek viable alternatives to replace or supplement this material. In this regard, Monsalve *et al.* (2021) chemically characterized a wide range of organic and mineral materials with potential use in soilless cultivation, two organic substrates other than rice husks (coconut and oil palm-derived materials) were identified as particularly suitable, together with five mineral materials: coal slag, perlite, pumice, vermiculite, and zeolite. These materials, except oil palm, were therefore selected for further evaluation in the present study. Coconut fiber, obtained from the mesocarp or outer husk of the coconut fruit (*Cocos nucifera*), specifically from the fibrous layer located between the hard inner shell and the outer layer of the coconut (Satyanarayana *et al.*, 1982; Abad *et al.*, 2002), is widely used due to its favorable structure and availability. Physically, coconut fiber has a tubular structure with concentric microfibrils, a low density (around 0.69 g/cm³), high water retention capacity, good aeration, and good hydraulic conductivity which promotes root development in plants (Asiah *et al.*, 2004; Abad *et al.*, 2005; San Andres *et al.*, 2023). Moreover coconut substrate has revealed to be successfully used for consecutive

monoculture of tomato given that the reuse did not induce phytotoxicity for the plants (Capodilupo *et al.*, 2015). Chemically, the fiber is mainly composed of cellulose, hemicellulose, and lignin, which give it strength and durability; it also contains small amounts of waxes, fats, and minerals such as silica and metal oxides (Brígida *et al.*, 2010).

Coal slag is a byproduct of coal combustion in thermoelectric plants and steel furnaces (Serna, 2012). It has been successfully used in crops such as tomatoes (Peña *et al.*, 2013) and carnations (Quintero *et al.*, 2012), both alone and in mixtures. Although considered inert, it may exhibit phosphorus fixation or excess boron (Martínez and Roca, 2011). Perlite is a thermally expanded volcanic aluminum silicate that forms a porous, lightweight, and sterile structure. With its low density, high water retention capacity, and excellent aeration properties, it is widely used as an inorganic substrate in soilless cultivation. These physical and chemical characteristics make perlite highly favorable for optimal root development and overall plant growth (Doğan and Alkan, 2003; Markoska *et al.*, 2018 a, 2018 b). Perlite is chemically inert, meaning it does not react with fertilizers or other compounds present in the substrate, and it maintains its stable composition over time. In addition, its high porosity and low density promote water retention and aeration, while its chemical inertness helps prevent salt accumulation, as it does not release soluble ions into the medium (Markoska *et al.*, 2018 a, 2018 b; Reka *et al.*, 2019). Silber *et al.* (2012) demonstrated that its Zn adsorption capacity depends on pH, ionic strength, and temperature. In hydroponic systems and in the production of vegetables such as lettuce and cucumber, perlite has been shown to promote crop development and quality. Moreover, compared to soil cultivation, perlite-based soilless cultivation of lettuce showed microbiological and qualitative performance better than those grown in soil (Pace *et al.*, 2018). However, when used as the sole substrate, it may require careful management of fertilization and irrigation to prevent nutrient leaching (Gul *et al.*, 2007; Stoian *et al.*, 2022; Yang *et al.*, 2023). Pumice is a pyroclastic volcanic material (Huang *et al.*, 2025), extremely lightweight (Hosniyeh *et al.*, 2024), primarily composed of SiO₂, Al₂O₃, Fe₂O₃, CaO, Na₂O, K₂O, and H₂O (Boodt *et al.*, 1974). Vermiculite, a thermally expanded mica, exhibits numerous significant physical and chemical properties, including developed porous structures, high water

retention, low density, and elevated cation exchange capacity due to its mineral structure (Hao *et al.*, 2023). Its use is recommended as an alternative for mixing with other substrates (Schneider *et al.*, 2018). Zeolites, particularly clinoptilolite, are crystalline aluminosilicates with high porosity and cation exchange capacity, allowing them to retain and gradually release nutrients (Głąb *et al.*, 2023), as confirmed by Eshghi *et al.* (2014) who found that it absorbs and slowly releases nitrogen to the culture medium.

Due to their large surface area, high water retention, and high cation exchange capacity, the application of zeolite and vermiculite is highly relevant as inorganic soil amendments in agriculture (Jarosz *et al.*, 2022; Głąb *et al.*, 2023).

Some countries, including Colombia, lack specific regulations for the use of substrates in horticultural crop production, international standards have been proposed for the most relevant chemical properties. Table 1 summarizes the recommended optimal levels. Nevertheless, the substrates used throughout the world differ significantly as to their makeup, while attempting to adhere to a specific set of principles. These principles are quite complex, relating to physical and chemical factors of solids, liquids, and gases in the root zone of the plant (Raviv *et al.*, 2019).

Given the diversity in chemical behavior of these materials, it is important to understand not only their individual properties but also how they interact when combined in substrate mixtures. Therefore, the objective of this study is to characterize the soluble and exchangeable fractions of the organic coconut substrate and the mineral materials coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE), mixed in different organic-mineral proportions, using carnation (*Dianthus caryophyllus* L.) as a plant of interest. Carnation was selected due to its high sensitivity of early root development to substrate chemical properties, its widespread use in commercial soilless cultivation systems, and its relevance as a bioindicator species for evaluating substrate agronomic suitability during the rooting stage.

2. Materials and Methods

Treatments, design, and sample preparation

The study was conducted at the facilities of Flores

Aurora S.A.S., located in the municipality of Suesca (Cundinamarca, Colombia), at coordinates 05°03.740' N and 73°50.201' E, an elevation of 2,584 meters above sea level, and an average ambient temperature of 14°C. The experiment was established in a chapel-type greenhouse adapted to function as a humidity chamber, maintaining relative humidity levels above 80% and an average internal temperature of 30°C. Five mineral materials [coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE)] were evaluated in mixtures with coconut substrate (CS) at five CS mineral ratios: 100-0, 75-25, 50-50, 25-75, and 0-100. All chemical and plant-related evaluations were performed once, at the end of the rooting period (24 days after establishment). A completely randomized design (CRD) was applied in a 5×5 factorial arrangement with two factors: substrate type (five mineral materials) and proportion (five mixing levels), resulting in 25 treatments and 75 experimental units, with three replicates per treatment.

Each experimental unit consisted of a plastic tray (0.6 × 0.3 × 0.15 m; surface area = 0.18 m²; volume = 0.03 m³), containing 104 carnation cuttings.

Prior to mixture preparation, the coconut coir substrate was pre-treated by the manufacturer through washing with potable tap water for a period ranging between 30 and 60 minutes. This washing process aimed to reduce the concentration of soluble salts, particularly sodium and chloride, which are commonly elevated in coconut-based substrates.

Exchangeable, soluble, and available phase characterization

For the exchangeable phase, K⁺, Ca²⁺, Mg²⁺, and Na⁺ were extracted with 1 M ammonium acetate (pH 7) and analyzed via absorption atomic spectroscopy AAS. Cation exchange capacity (CEC) was determined by replacing NH₄⁺ with NaCl, followed by volumetric titration. Soluble-phase parameters included electrical conductivity (EC, measured at 25°C), pH (potentiometric method), phosphate (PO₄³⁻) using the stannous chloride colorimetric method, sulfate (SO₄²⁻) with the barium chloride method, chloride (Cl⁻) by silver nitrate titration, and K⁺, Ca²⁺, Mg²⁺, and Na⁺ using (AAS). Total N content was determined by high-temperature combustion using an elemental analyzer, P by colorimetric titration with molybdate and ammonium vanadate. Available Cu²⁺, Fe²⁺, Mn²⁺, and Zn²⁺ were extracted using DTPA and analyzed by AAS.

Plant growth suitability

Carnation cuttings (*Dianthus caryophyllus*, cv. Pomodoro) were used as a bioindicator of substrate suitability for plant growth. All mineral components were sieved to a particle size ≤ 2 mm prior to planting. The rooting phase lasted 24 days, which corresponded to the entire duration of the experiment, from cutting establishment to final chemical and plant evaluations, during which no fertilization was applied. This plant was selected due to the rapid growth cycle of the species, its relevance in Colombian soilless cultivation systems, and the high sensitivity of root development to substrate properties. This approach enabled the evaluation of both the rooting efficiency and substrate-plant interaction across a wide range of substrate mixtures.

Rooting percentage was evaluated by removing 10 cuttings per experimental unit and counting those showing visible root emergence and physical anchorage to the substrate. Each rooted cutting was assigned a value of 10%, so that the presence of roots in all 10 cuttings corresponded to 100% rooting. This approach was selected as a practical and standardized indicator of rooting success, commonly used in commercial carnation propagation to rapidly assess substrate suitability. Root dry weight was determined after drying the roots at 70°C for 48 h. Root formation and biomass accumulation were considered the main quality indicators for cuttings, based on grower-established standards for transplant readiness.

Statistical analysis

A correlation matrix was used to explore associations between chemical parameters and plant growth viability. In addition, a principal component analysis (PCA) was conducted as an exploratory multivariate approach to assess overall relationships among chemical variables and to identify potential collinearity patterns within the dataset. Treatment differences were analyzed using one-way ANOVA and Tukey’s HSD test. Prior to ANOVA, normality and homogeneity of variances were tested using the Shapiro-Wilk and Bartlett tests, respectively. All statistical analyses were conducted using R (version 4.4.3) (R Core Team, 2025), with the mvoutlier (Filzmoser and Gschwandtner, 2021), Hmisc (Harrell, 2025), and agricolae (de Mendiburu, 2023) packages.

3. Results and Discussion

Soluble phase

The soluble-phase chemical properties of the evaluated substrates and mixtures revealed marked differences associated with mineral type and mixing proportion, while remaining within agronomically acceptable ranges for early plant establishment. Both pH and electrical conductivity (EC) values fell within the recommended limits for soilless substrates (Table 1), indicating no immediate chemical constraints for root development. Among individual mineral substrates, vermiculite (VE) exhibited the highest pH (7.37), whereas pumice (PM) showed the highest EC (0.26 dS m⁻¹) (Fig. 1). At the treatment level, VE25 reached the maximum pH (7.5), while PM50 presented the highest EC (0.30 dS m⁻¹) (Table 2).

Table 1 - Recommended optimal levels for the chemical characteristics of substrates used in soilless culture

Parameter	Value	Source
pH	5.5 a 7.0	Ansorena, 1994; Sonneveld and Voogt, 2009
EC (dS m ⁻¹)	<2.0	Ansorena, 1994; Nelson, 2012
CEC (cmol _c kg ⁻¹)	>20	
N- NH ₄ (mg L ⁻¹)	<30	Sonneveld and Voogt, 2009
N-NO ₃ (mg L ⁻¹)	<200	Sonneveld and Voogt, 2009
P (mg L ⁻¹)	<30	Nelson, 2012
PO ₄ (mg L ⁻¹)	<92	Nelson, 2012
K (mg L ⁻¹)	<250	Sonneveld and Voogt, 2009
Ca (mg L ⁻¹)	<200	Nelson, 2012
Mg (mg L ⁻¹)	<70	Raviv and Lieth, 2008
Na (mg L ⁻¹)	<100	Abad <i>et al.</i> , 2002; Sonneveld and Voogt, 2009
Cl (mg L ⁻¹)	<5	Sonneveld and Voogt, 2009
SO ₄ (mg L ⁻¹)	<70	Nelson, 2012

Although statistically significant differences in pH were observed among mixtures, none reached values considered detrimental to plant growth. In soilless systems, maintaining pH within a slightly acidic to circumneutral range (≈ 5.5 -7.0) minimizes the risk of nutrient deficiencies or toxicities, while even small variations within this interval can influence nutrient solubility and ionic equilibria in the root zone (Huang *et al.*, 2025). The moderate EC values observed across all treatments contrast with reports describing coconut-based substrates as inherently saline (Abad *et al.*, 2001; Machado and Bui, 2021) and can be attributed to the pre-washing of the coconut coir.

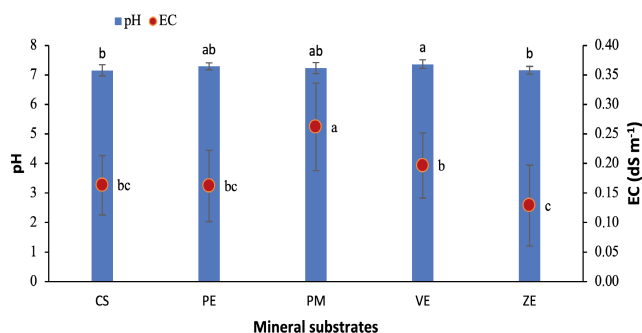


Fig. 1 - Comparison of pH and electrical conductivity (EC) of the evaluated substrates and substrate mixtures. Data correspond to all coconut-mineral (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) mixtures (namely, coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE) evaluated at the end of the rooting period (24 days). Bars represent mean values (n= 15), and error bars indicate the standard deviation. Different letters indicate significant differences according to Tukey's test (P<0.05).

This pretreatment effectively reduced the concentration of readily soluble salts, confirming its relevance for ensuring the suitability of coconut fiber in nursery and propagation systems (Gbollie *et al.*, 2021). Similar EC stability under non-fertigated conditions was previously reported by Monsalve *et al.* (2021) for the same materials prior to plant establishment.

Soluble cation concentrations exhibited a clear dominance of sodium (Na^+) over potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) across all mineral substrates. Vermiculite showed the highest Na^+ concentration (336 mg L^{-1}), exceeding recommended thresholds (Table 1), followed by pumice (216 mg L^{-1}) (Fig. 2). This pattern reflects the combined influence of the intrinsic Na content of coconut coir and the contribution of specific mineral components. Vermiculite also exhibited the highest soluble K^+ (61.9 mg L^{-1}) and Mg^{2+} (35.1 mg L^{-1}), while

Table 2 - pH, electrical conductivity (EC), and soluble nutrient concentrations (K, Ca, Mg, Na, PO_4^{3-} , SO_4^{2-} , Cl) of the evaluated treatments

Treatment	Soluble phase								
	pH	EC dS m ⁻¹	K mg L ⁻¹	Ca mg L ⁻¹	Mg mg L ⁻¹	Na mg L ⁻¹	PO_4^{3-} mg L ⁻¹	SO_4^{2-} mg L ⁻¹	Cl mg L ⁻¹
CS0	7.3 ab	0.3 abc	42.3 abcdefg	4.3 f	2.8 d	148.6 defgh	16.7 c	72.5 defghi	117.5 fg
CS25	7.3 ab	0.1 cde	28.5 cdefg	8.2 ef	4.3 d	164.9 defg	49.4 a	102.9 cdefg	172.3 ef
CS50	7 b	0.2 bcde	14.3 defg	30.2 cdef	12.6 d	137.3 defgh	29.3 b	141.1 bc	178.3 ef
CS75	7 b	0.1 cde	12.4 defg	36.2 abcd	12.8 d	110.9 efgh	16.6 c	135.5 bcde	169.9 ef
CS100	7.2 ab	0.1 cde	9 efg	57.4 ab	12.6 d	84.6 fgh	9.5 cdef	80.8 cdefghi	132.8 fg
PE0	7.3 ab	0.3 abc	42.3 abcdefg	4.3 f	2.8 d	148.6 defgh	16.7 c	72.5 defghi	117.5 fg
PE25	7.3 ab	0.1 cde	32.3 bcdefg	6.9 ef	3.5 d	162.1 defg	8.9 cdef	117.7 cdef	180.7 ef
PE50	7.3 ab	0.2 bcde	46.9 abcdef	6.9 ef	5.5 d	217.4 cdef	13.7 cde	130.7 bcde	244.9 de
PE75	7.4 ab	0.1 cde	14.6 defg	17.7 cdef	9.7 d	127.6 efgh	4.4 cdef	68.7 efghi	157.9 ef
PE100	7.3 ab	0.1 de	10.1 efg	38.8 abc	8.5 d	66.6 gh	0.4 f	39.1 ghi	104.1 fg
PM0	7.3 ab	0.3 abc	42.3 abcdefg	4.3 f	2.8 d	148.6 defgh	16.7 c	72.5 defghi	117.5 fg
PM25	7.3 ab	0.3 ab	77.1 ab	15.5 cdef	6 d	270 bcd	11.6 cdef	82 cdefghi	348.1 bcd
PM50	7.1 b	0.3 a	72.5 abc	61.1 a	14.8 d	344 bc	15.7 cd	84.7 cdefgh	328.6 bcd
PM75	7.1 ab	0.3 abc	49.1 abcde	19.4 cdef	14.3 d	247.3 bcde	7 cdef	198.7 ab	268.8 cde
PM100	7.4 ab	0.2 abcde	6.7 efg	32.1 bcde	8 d	67.6 gh	2.7 ef	19.7 hi	101.1 fg
VE0	7.3 ab	0.3 abc	42.3 abcdefg	4.3 f	2.8 d	148.6 defgh	16.7 c	72.5 defghi	117.5 fg
VE25	7.5 a	0.2 bcde	84.6 a	12.1 def	12.2 d	310.2 bc	14.7 cde	133.1 bcde	357.4 bc
VE50	7.3 ab	0.2 abcd	63 abc	22.7 cdef	33.2 c	525 a	33 b	216.1 a	415.4 b
VE75	7.3 ab	0.2 bcde	57.6 abcd	26.3 cdef	51.7 b	330.5 bc	9.5 cdef	139.1 bcd	412.4 b
VE100	7.4 ab	0.2 abcde	62.1 abc	26 cdef	75.7 a	364.5 b	6.2 cdef	75.6 cdefghi	560.7 a
ZE0	7.3 ab	0.3 abc	42.3 abcdefg	4.3 f	2.8 d	148.6 defgh	16.7 c	72.5 defghi	117.5 fg
ZE25	7.1 ab	0.1 cde	6.9 efg	30.3 cdef	13.7 d	107.7 fgh	3.7 def	57.6 fghi	179.1 ef
ZE50	7.2 ab	0.1 de	3.1 fg	25.7 cdef	8.5 d	93.1 fgh	4 def	84.9 cdefgh	113.7 fg
ZE75	7.1 ab	0.1 de	2 fg	21.7 cdef	6.5 d	85.6 fgh	2.3 ef	76.1 cdefghi	94.5 fg
ZE100	7.0 b	0.1 e	0.7 g	11.2 cdef	3.4 d	22.7 h	0.3 f	15.9 i	27.9 g

Data correspond to all coconut-mineral mixtures evaluated at the end of the rooting period (24 days). Values represent mean values (n = 3). Different letters within each column indicate significant differences among treatments according to Tukey's test (P<0.05).

no significant differences were detected for Ca^{2+} among mineral substrates (Fig. 2).

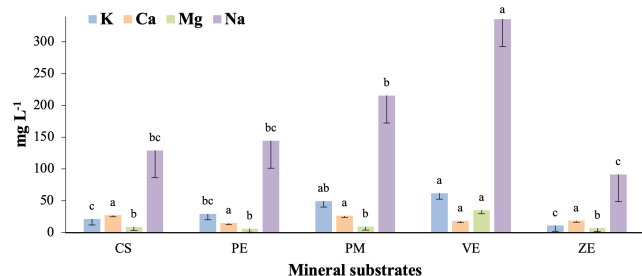


Fig. 2 - Comparison of soluble K^+ , Ca^{2+} , Mg^{2+} , and Na^+ concentrations in the evaluated substrates and substrate mixtures. Data correspond to all coconut-mineral (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) mixtures (namely, coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE) evaluated at the end of the rooting period (24 days). Bars represent mean values ($n = 15$), and error bars indicate the standard deviation. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

At the mixture level, the soluble ionic composition was strongly modulated by both substrate type and proportion. Treatment VE50 displayed the highest Na^+ concentration (525 mg L^{-1}), whereas VE25, PM50, and VE100 reached the maximum levels of K^+ (84.6 mg L^{-1}), Ca^{2+} (61.1 mg L^{-1}), and Mg^{2+} (75.7 mg L^{-1}), respectively (Table 2). These results indicate that increasing the proportion of high-CEC minerals such as vermiculite enhances the capacity of the substrate to retain and subsequently release soluble cations into the soil solution, thereby modifying the ionic environment encountered by roots during the rooting phase. Regarding anions, chloride (Cl^-) was the dominant soluble species in several substrates, with particularly high concentrations in vermiculite (372.7 mg L^{-1}) and pumice (232.9 mg L^{-1}) (Fig. 3). Coal slag exhibited the highest phosphate (PO_4^{3-}) concentration (24.3 mg L^{-1}), whereas vermiculite showed the highest sulfate (SO_4^{2-}) levels (127.3 mg L^{-1}). At the treatment scale, VE100 reached the maximum Cl^- concentration (560.7 mg L^{-1}), CS25 exhibited the highest PO_4^{3-} content (49.4 mg L^{-1}), and VE50 presented the highest SO_4^{2-} concentration (216.1 mg L^{-1}) (Table 2).

Despite the elevated concentrations of Na^+ and Cl^- in several mixtures (particularly those containing vermiculite) the EC values remained within acceptable limits for soilless cultivation. This apparent decoupling between individual ion concentrations and bulk EC highlights the importance of evaluating specific ionic composition rather than

relying solely on EC as an indicator of salinity risk. The high variability observed for Cl^- (Fig. 3) likely reflects the heterogeneous chemical nature of organic-mineral mixtures, where differences in mineralogy, particle size distribution, and water-holding capacity influence ion mobility and leaching dynamics.

In coconut coir-mineral systems, the soluble-phase composition is governed by the interaction between substrate chemistry and irrigation water, allowing highly mobile ions such as Cl^- to accumulate or be displaced depending on water management practices (Knaus *et al.*, 2025; Malík and Tlustoš, 2025). Vermiculite, characterized by high porosity, fine texture, and strong water retention, can promote localized accumulation of mobile anions within the rhizosphere, generating spatial heterogeneity in ion distribution. Comparable behavior has been documented in soil systems, where Cl^- profiles are strongly influenced by texture and water fluxes rather than uniform fertilizer inputs (Li *et al.*, 2025; Svensson *et al.*, 2023).

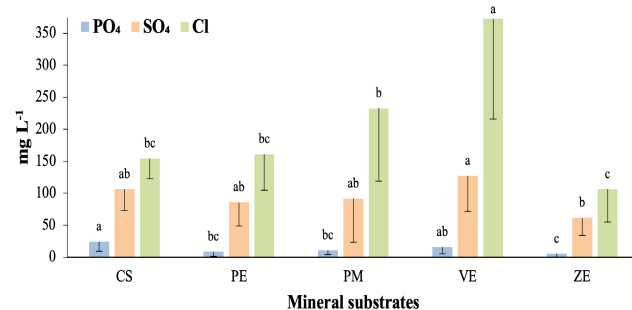


Fig. 3 - Comparison of the PO_4^{3-} , SO_4^{2-} , and Cl^- concentrations in the evaluated substrates and substrate mixtures. Data correspond to all coconut-mineral (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) mixtures (namely, coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE) evaluated at the end of the rooting period (24 days). Bars represent mean values ($n = 15$), and error bars indicate the standard deviation. Different letters indicate significant differences among treatments according to Tukey's test ($P < 0.05$).

While the Cl^- content in vermiculite substantially exceeded optimal limits (Table 1) and this material also contained high levels of Na^+ and SO_4^{2-} , these did not impact EC, which, as noted earlier, remained within acceptable ranges for all mixtures. The high variability observed in this anion (Fig. 3) may be due to the intrinsic chemical heterogeneity of the materials of mineral and organic origin, where differential leaching during irrigation can cause marked fluctuations in the root zone. In soilless

cultivation systems using coconut coir-vermiculite mixtures, the substrate functions as a reservoir of mobile nutrients, whose final ionic composition is determined by the combined effects of irrigation water chemistry and the intrinsic chemical properties of the substrate, allowing salts to accumulate or be released depending on water management practices (Knaus *et al.*, 2025; Malík and Tlustoš, 2025). Vermiculite, characterized by a high water- and nutrient-holding capacity, fine texture, and elevated porosity, promotes the formation of local gradients in the substrate solution, which can facilitate the localized accumulation of highly mobile ions such as Cl^- within the rhizosphere (Knaus *et al.*, 2025; Malík and Tlustoš, 2025). Field experiments in potato production have shown that Cl^- leaching is strongly influenced by soil texture (loam vs. sandy loam) and by precipitation or irrigation patterns, resulting in highly variable vertical Cl^- profiles even under identical applications of chloride-based fertilizers (Li *et al.*, 2025). Similarly, landscape-scale studies across moisture gradients have documented pronounced spatial heterogeneity of Cl^- in soils and associated vegetation, driven primarily by local hydrology and profile position rather than uniform chloride inputs (Svensson *et al.*, 2023).

Overall, the soluble-phase results demonstrate that coconut-mineral mixtures exhibit distinct ionic signatures that depend on mineral identity and mixing ratio. These differences are agronomically relevant, as they directly affect the chemical environment experienced by roots during the early establishment phase and must be considered when designing fertigation strategies to avoid ionic imbalances or unintended nutrient accumulation.

Exchangeable phase

Among the mineral substrates, vermiculite (VE) showed the highest average CEC ($76.1 \text{ cmol}_c \text{ kg}^{-1}$) as well as the highest Mg content ($54.3 \text{ cmol}_+ \text{ kg}^{-1}$) in the exchangeable phase. Zeolite (ZE) exhibited the highest concentrations of Ca ($43.2 \text{ cmol}_c \text{ kg}^{-1}$) and Na ($32.2 \text{ cmol}_c \text{ kg}^{-1}$). No significant differences were observed in K concentration among the evaluated mineral substrates (Fig. 4).

The 100% coconut coir substrate (treatments: CS0, PE0, PM0, VE0, and ZE0) displayed the highest CEC ($103.3 \text{ cmol}_c \text{ kg}^{-1}$), followed by treatments combined with vermiculite (VE25, VE50, VE100, and VE75) with values of 84.9, 68.5, 66.8, and $57 \text{ cmol}_c \text{ kg}^{-1}$, respectively (Table 3).

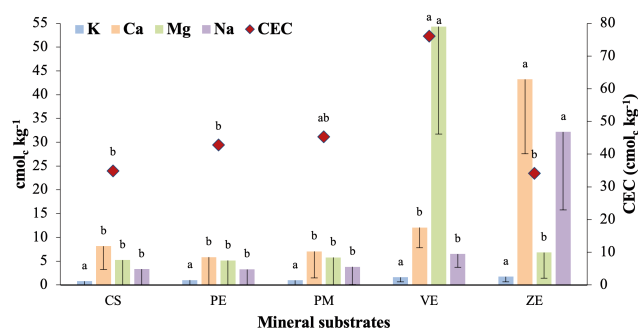


Fig. 4 - Comparison of cation exchange capacity (CEC) and exchangeable K^+ , Ca^{2+} , Mg^{2+} , and Na^+ in the evaluated substrates and substrate mixtures. Data correspond to all coconut-mineral (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) mixtures (namely, coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE) evaluated at the end of the rooting period (24 days). Bars represent mean values ($n=15$), and error bars indicate the standard deviation. Different letters indicate significant differences among treatments according to Tukey's test ($P<0.05$).

The results indicate that many evaluated mixtures contain soluble and exchangeable cation concentrations that must be considered in fertigation programs, particularly those with a high cation exchange capacity ($\text{CEC}>20 \text{ cmol}_c \text{ kg}^{-1}$), such as coconut coir-vermiculite mixtures, which promote favorable cation adsorption conditions in the medium to long term. Coconut coir exhibits a relatively high CEC ($\approx 32\text{--}96 \text{ meq } 100 \text{ g}^{-1}$) and the ability to adsorb and gradually release Ca^{2+} , Mg^{2+} , K^+ , and Na^+ , providing temporal nutrient buffering (Huang *et al.*, 2025; Malík and Tlustoš, 2025), while vermiculite, a 2:1 phyllosilicate with a very high CEC and permanent charges, can retain K^+ , Ca^{2+} , and Mg^{2+} within its interlayer spaces and gradually release them (Huang *et al.*, 2025; Malík and Tlustoš, 2025). In mixed substrates, materials with elevated CEC enable essential cations such as K^+ , Ca^{2+} , Mg^{2+} , and NH_4^+ to be temporarily retained in the exchange complex and subsequently released into the substrate solution for plant uptake, rather than being rapidly lost through leaching (Monsalve *et al.*, 2021). Consequently, a high CEC in soilless substrates reduces ionic concentration fluctuations, increases the temporary storage capacity for NH_4^+ and K^+ , and improves fertilizer use efficiency (Monsalve *et al.*, 2021; Xu *et al.*, 2021), while systems with higher effective CEC exhibit lower cation leaching and greater buffering capacity during leaching events by acting as reversible sinks and sources of exchangeable cations (Guarnieri *et al.*, 2021; Ng *et al.*, 2022). However, this

Table 3 - Cation exchange capacity (CEC) and exchangeable K⁺, Ca²⁺, Mg²⁺, and Na⁺ of the evaluated treatments. Data correspond to all coconut-mineral mixtures evaluated at the end of the rooting period (24 days)

Treatment	Exchangeable phase				
	CEC cmolc kg ⁻¹	K cmolc kg ⁻¹	Ca cmolc kg ⁻¹	Mg cmolc kg ⁻¹	Na cmolc kg ⁻¹
CS0	103.3 a	2.9 a	16.7 d	17.2 d	10.7 d
CS25	36 fgh	0.6 b	8.9 fgh	5.3 e	3.4 g
CS50	16.6 i	0.2 b	6.6 ghij	2.1 e	1.2 h
CS75	10.5 i	0.2 b	4.7 hijkl	0.9 e	0.8 h
CS100	8.2 i	0.1 b	3.9 hijkl	0.7 e	0.6 h
PE0	103.3 a	2.9 a	16.7 d	17.2 d	10.7 d
PE25	39.8 efg	0.8 b	4.6 hijkl	4.1 e	2.5 gh
PE50	37.1 fgh	0.7 b	5.1 hijkl	3.5 e	2.2 gh
PE75	21.2 hi	0.4 b	2.1 jkl	0.8 e	0.6 h
PE100	12.7 i	0.3 b	0.8 l	0.2 e	0.5 h
PM0	103.3 a	2.9 a	16.7 d	17.2 d	10.7 d
PM25	50.1 def	1 ab	7.2 fghi	5.6 e	3.9 g
PM50	36.5 fgh	0.4 b	6.4 hijk	4.3 e	2.2 gh
PM75	21.2 hi	0.4 b	3.3 ijkl	1.6 e	1.4 h
PM100	15.2 i	0.2 b	1.6 kl	0.3 e	0.9 h
VE0	103.3 a	2.9 a	16.7 d	17.2 d	10.7 d
VE25	84.9 b	1.9 ab	14.7 de	46.7 c	8.2 e
VE50	68.5 bc	1.4 ab	12 def	65.4 b	6.1 f
VE75	57 cde	1 ab	11.4 efg	80.6 a	4.2 fg
VE100	66.8 cd	0.9 b	5.4 hijkl	61.7 b	3.5 g
ZE0	103.3 a	2.9 a	16.7 d	17.2 d	10.7 d
ZE25	22 ghi	2.8 a	46.8 b	5.4 e	39.2 b
ZE50	19.6 hi	1.3 ab	54.6 a	4.3 e	46.9 a
ZE75	15.6 i	0.9 b	59.5 a	4.1 e	48.3 a
ZE100	10.2 i	0.8 b	38.5 c	3.1 e	15.8 c

Data correspond to all coconut-mineral mixtures evaluated at the end of the rooting period (24 days). Values represent mean values (n = 3). Different letters within each column indicate significant differences among treatments according to Tukey's test (P<0.05).

buffering effect is conditional on the saturation state of the exchange complex and the cation composition of the nutrient solution, as coconut coir may initially sequester Ca²⁺ and Mg²⁺ if not properly pre-saturated, thereby altering ionic equilibria (Malík and Tlustoš, 2025).

Na levels in the mixtures exceeded recommended thresholds (Table 2), consistent with Jenagathan (2008), who reported that coconut-based materials exhibit the highest Na concentrations. Excess Na⁺ is a persistent challenge in organic substrates of coastal origin, as it competes with K⁺ for root absorption sites; this ionic imbalance alters osmoregulation and cell turgor, restricting tissue expansion and compromising both plant growth and photosynthetic efficiency (Munns *et al.*, 2020; Balasubramaniam *et al.*, 2023). Elevated Na was particularly notable in vermiculite-containing mixtures, where Na concentrations increased proportionally with

vermiculite content (Table 3), suggesting an additive effect between the Na contributions from coconut substrate and vermiculite. Vermiculite is a 2:1 phyllosilicate characterized by a high cation exchange capacity (CEC, ~ 80-150 meq/100 g) and a significant ability to adsorb cations within its interlaminal structure (De Jesus Andrade *et al.*, 2025). This allows the mineral to sequester and concentrate monovalent cations, such as Na⁺, from the substrate solution, releasing or re-adsorbing them in response to shifts in chemical equilibrium (Yan *et al.*, 2024; Andrade *et al.*, 2025). In systems influenced by NaCl, Na⁺ effectively displaces Ca²⁺ from the interlaminal spaces, leading to a substantial increase in the Na₂O content within the vermiculite matrix (Feng *et al.*, 2020).

When blended with coconut coir—which inherently contributes high levels of exchangeable Na⁺ and K⁺ due to its coastal origin and post-harvest processing (Heller and Nunez, 2022; Malík and Tlustoš, 2025)

vermiculite functions as a “cationic sponge”. This mineral component can fix a portion of the Na^+ released by the coir while simultaneously releasing its own Na^+ if the mineral was previously sodified by process water or fertilization (Feng *et al.*, 2020; Yan *et al.*, 2024; Andrade *et al.*, 2025). Consequently, characterization studies of soilless media confirm that the addition of expandable minerals (such as vermiculite or zeolites) to coir-based substrates markedly enhances cation retention, thereby modifying the ionic profile of the substrate solution available for plant uptake (Petmuenwai *et al.*, 2024; Yan *et al.*, 2024).

Available and total nutrients

Coal slag (CS) showed the highest phosphorus concentration (187.5 mg kg^{-1}), with a highly significant difference compared to other mineral substrates (Fig. 5). Consistent with the high native phosphorus content of coal slag [0.4% (Monsalve *et al.*, 2021)], which was the highest among all tested materials. Similarly, CS-based treatments (CS100,

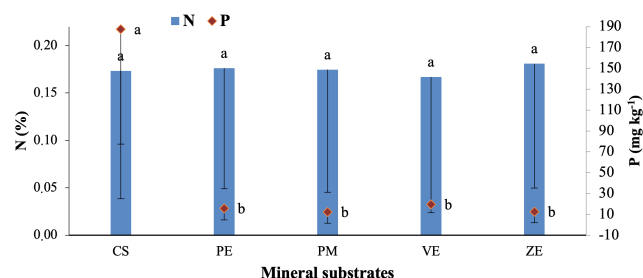


Fig. 5 - Total N and P content in the evaluated substrates and substrate mixtures. Data correspond to all coconut-mineral (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) mixtures (namely, coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE) evaluated at the end of the rooting period (24 days). Bars represent mean values ($n=15$), and error bars indicate the standard deviation. Different letters indicate significant differences among treatments according to Tukey's test ($P<0.05$).

CS75, CS50, and CS25) showed the highest phosphorus concentrations, with values of 311.8 , 265.7 , 215.6 , and 112.4 mg kg^{-1} , respectively (Table 4). This consistent enrichment across both the pure

Table 4 - Total nitrogen (N), phosphorus (P), and available micronutrient concentrations (Fe, Mn, Cu, and Zn) of the evaluated treatments

Treatment	Total		Available micronutrients			
	N %	P mg kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Cu mg kg^{-1}	Zn mg kg^{-1}
CS0	0.4 a	32.1 d	25.6 bc	45.6 b	4.8 efghi	17.3 a
CS25	0.3 b	112.4 c	33.4 b	24.6 cde	8.2 cdefghi	20.1 a
CS50	0.2 bc	215.6 b	26.3 bc	12.5 cdef	18.8 bc	15.3 ab
CS75	0.1 ef	265.7 ab	22.6 cd	9.4 def	20.1 ab	13.2 abcde
CS100	0 ef	311.8 a	17.3 cde	5 ef	30.6 a	7 cdefgh
PE0	0.4 a	32.1 d	25.6 bc	45.6 b	4.8 efghi	17.3 a
PE25	0.3 b	9.6 d	6.8 efghi	17.6 cdef	5.8 efghi	14.2 abcd
PE50	0.2 c	17.9 d	9.8 efghi	20.2 cdef	7.4 defghi	8.4 bcdefg
PE75	0.1 ef	13.5 d	9.5 efghi	12.3 cdef	12.2 bcdefgh	4.2 fgh
PE100	0 ef	6 d	1.8 i	2.1 f	18.1 bcd	1.7 fgh
PM0	0.4 a	32.1 d	25.6 bc	45.6 b	4.8 efghi	17.3 a
PM25	0.3 b	8.9 d	13 defgh	26.1 bcd	4.3 fghi	5.6 efgh
PM50	0.2 cd	8.3 d	14.7 def	20 cdef	8.6 cdefghi	8.6 bcdef
PM75	0.1 de	6 d	6.1 fghi	10.3 cdef	13.4 bcdefg	6.5 defgh
PM100	0 ef	6 d	2.4 hi	2.7 f	15.7 bcde	1.3 fgh
VE0	0.4 a	32.1 d	25.6 bc	45.6 b	4.8 efghi	17.3 a
VE25	0.2 bc	20.9 d	50.4 a	80.1 a	7.1 defghi	14.7 abc
VE50	0.2 cd	14.5 d	55.5 a	100.7 a	11.6 bcdefgh	8.2 bcdefgh
VE75	0.1 ef	12.5 d	52.2 a	97.2 a	13.7 bcdef	4.6 fgh
VE100	0 ef	17.4 d	13.6 defg	30.2 bc	13.3 bcdefg	5 fgh
ZE0	0.4 a	32.1 d	25.6 bc	45.6 b	4.8 efghi	17.3 a
ZE25	0.3 b	6.8 d	6.3 fghi	13.5 cdef	2.4 ghi	2.9 fgh
ZE50	0.2 bc	7.6 d	3.2 ghi	11.5 cdef	1.2 hi	1.4 fgh
ZE75	0.1 ef	6.3 d	2.4 hi	7.1 def	0.3 i	0.3 gh
ZE100	0 f	10.5 d	2.1 i	1.8 f	0 i	0.3 h

Data correspond to all coconut-mineral mixtures evaluated at the end of the rooting period (24 days). Values represent mean values ($n=3$). Different letters within each column indicate significant differences among treatments according to Tukey's test ($P<0.05$).

mineral material and its mixtures indicates that CS contributes substantial native P to the substrate system, which becomes relevant when evaluating both nutrient supply potential and risks of over-fertilization under standard fertigation regimes. This finding is particularly relevant within a circular economy framework, as coal slag functions not only as a physical support but also as a slow-release source of native P for recycled industrial by-products used as substrate components (Jupp *et al.*, 2020; Yu *et al.*, 2022).

No significant differences in nitrogen concentration were observed among substrates (Fig. 5), but differences were detected among treatments. The highest nitrogen concentration (0.4%) was found in the 100% coconut substrate (Treatments: CS0, PEO, PM0, VE0, and ZE0) (Table 4), consistent with its organic nature (Monsalve *et al.*, 2021). This pattern is expected because the N contribution from inorganic substrates through native nutrient release is typically minimal during crop cycles. As Silber (2008) explains, with few exceptions (such as when using phosphate rock), this contribution is negligible. Organic substrates, however (particularly compost) contain substantial nitrogen quantities, making their concentration in these growth media potentially significant. In this context, coconut coir-based treatments provide an intrinsic N baseline that may influence early-stage nutrient conditions even in the absence of fertigation.

Among micronutrients, Mn stood out for its high concentration in vermiculite-containing mixtures (70.8 mg kg^{-1}) (Fig. 6), increasing proportionally with vermiculite content. This trend was reflected in its high standard deviation (Fig. 6). These findings correspond with vermiculite's naturally high Mn concentration - the highest among evaluated materials [390.2 mg kg^{-1} (Monsalve *et al.*, 2021)], which notably exceeded optimal levels (Table 1). According to Kebabi *et al.* (2017), vermiculite exhibits a high adsorption capacity for manganese (Mn), which could explain the elevated concentrations of this element. This behavior is consistent with the characteristic cation retention properties of vermiculite, which have been exploited for the removal of metals such as manganese from various compounds. This substrate also contained the highest iron concentration (39.5 mg kg^{-1}), compared to other materials (Fig. 6), consistent with both its natural concentration (Monsalve *et al.*, 2021) and chemical structure. This behavior aligns with

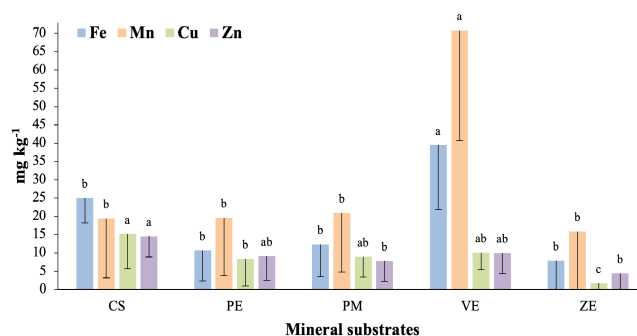


Fig. 6 - Available Fe, Mn, Cu, and Zn concentrations in the evaluated substrates and substrate mixtures. Data correspond to all coconut-mineral (100-0%, 75-25%, 50-50%, 25-75%, and 0-100%) mixtures (namely, coal slag (CS), perlite (PE), pumice (PM), vermiculite (VE), and zeolite (ZE)) evaluated at the end of the rooting period (24 days). Bars represent mean values ($n = 15$), and error bars indicate the standard deviation. Different letters indicate significant differences among treatments according to Tukey's test ($P < 0.05$).

vermiculite's characteristic cation retention properties, which is further utilized for metal removal (e.g., Mn) from various compounds (Kebabi *et al.*, 2017). CS accumulated the highest amounts of copper (15.2 mg kg^{-1}) and zinc (15 mg kg^{-1}). Among treatments, those containing VE (VE100, VE75, VE50, and VE25) consistently showed the highest Fe and Mn concentrations, while CS-based treatments (CS25, CS50, CS75, and CS100) exhibited the maximum Cu and Zn levels (Table 4). These results reveal a clear mineral "signature" in the available micronutrient fraction, which complements what was observed previously for exchangeable bases and reinforces the importance of mineral selection when designing coconut-mineral mixtures.

As with exchangeable bases, N, P, and micronutrient concentrations must be considered in substrate fertilization programs. Failure to account for the native nutrient content of soilless substrates may result in excessive nutrient inputs when standard fertigation programs are applied indiscriminately. Elevated concentrations of soluble and exchangeable ions, such as Na^+ , Cl^- , or NO_3^- , can lead to ionic toxicity or nutrient imbalances in plants. In addition, surplus nutrients not retained or absorbed by the crop may be leached from the root zone, increasing the risk of nutrient losses to drainage water and subsequent environmental contamination. Therefore, accurate characterization of substrate nutrient fractions is essential to adjust fertigation strategies, improve nutrient use

efficiency, and minimize both agronomic and environmental risks.

To further explore potential multicollinearity among the evaluated chemical variables, a principal component analysis (PCA) was conducted as an exploratory tool (data not shown). The analysis did not reveal a dominant principal component structuring the dataset, as more than 70% of the total variance was only explained after the fourth principal component (PC4), indicating a complex multivariate system without a clear dimensional reduction. This result reflects the heterogeneous chemical behavior of the coconut-mineral mixtures, where soluble, exchangeable, and available nutrient fractions respond differently depending on substrate composition and proportion. The only consistent association identified was between exchangeable Na^+ and Ca^{2+} , which is mechanistically consistent with cation exchange processes and competitive interactions within high-CEC substrates. This relationship supports the interpretation that substrate mixtures with elevated exchange capacity can simultaneously retain multiple base cations, modifying the ionic balance of the substrate solution available for plant uptake.

Plant growth suitability

Plant growth suitability was evaluated through two key parameters: rooting percentage and root dry weight (RDW). All treatments and substrates achieved 100% rooting success, with no variability among treatments; therefore, no statistical differences were detected, and the data are not shown. Results indicate all substrates would be suitable for plant growth, as they exhibited 100% survival rates even without nutrient solution application, consistent with conventional management practices for indicator plants (carnation cuttings). However, significant differences were observed in root dry mass accumulation (RDW), with vermiculite and perlite-containing substrates showing particularly notable results (Table 5). Statistical differences were observed among the evaluated treatments. The highest RDW value was recorded for VE (1.92 g), followed by PE (1.88 g), PM (1.7 g), and CS (1.65 g). ZE showed the statistically lowest weight (1.61 g). Overall, treatment VE50 (2.41 g) demonstrated the best performance, followed by PM50 (2.21 g) and VE100 (2.09 g) (Table 5). The study site owner reported that carnation cuttings with root dry weights exceeding 1.0 g are considered adequate

for field transplantation. This suggests that, except for PM100, all mixtures are appropriate for use as substrates in such production systems. Additional factors, including economic and environmental considerations, should be evaluated to determine the most viable mixture.

Pearson correlation analysis revealed highly significant relationships between root dry matter accumulation and soluble concentrations of K, Na, SO_4^{2-} , and Cl^- (Table 6).

The positive correlations observed between root dry weight and soluble Na^+ and Cl^- concentrations in the present study (Table 6) may appear counterintuitive, given the well-documented negative effects of salinity on plant growth. However, these results are consistent with previous

Table 5 - Root dry weight (RDW) of carnation cuttings grown in the evaluated substrate mixtures

Treatment	RDW (g)
CS0	1.67 fgh
CS25	1.69 fg
CS50	1.77 defg
CS75	1.73 efg
CS100	1.38 hi
PE0	1.67 fgh
PE25	1.88 cdef
PE50	1.91 cdef
PE75	2.06 bcd
PE100	1.88 cdef
PM0	1.67 fgh
PM25	1.78 defg
PM50	2.21 ab
PM75	2.00 bcde
PM100	0.83 j
VE0	1.67 fgh
VE25	1.68 fg
VE50	2.41 a
VE75	1.75 efg
VE100	2.09 bc
ZE0	1.67 fgh
ZE25	1.77 defg
ZE50	1.51 ghi
ZE75	1.75 efg
ZE100	1.34 i

Data correspond to all coconut-mineral mixtures evaluated at the end of the rooting period (24 days). Values represent mean values (n= 3). Different letters within each column indicate significant differences among treatments according to Tukey's test ($P < 0.05$).

Table 6 - Pearson correlation matrix between root dry weight (RDW) and the evaluated chemical variables (n = 75)

Phase	Parameter	RDW
Soluble	pH	-0.02
	EC	0.22
	K ⁺	0.43 ***
	Ca ²⁺	0.06
	Mg ²⁺	0.36 **
	Na ⁺	0.63 ***
	PO ₄ ³⁻	0.21
	SO ₄ ²⁻	0.55 ***
	Cl ⁻	0.55 ***
Exchangeable	CIC	0.14
	K ⁺	-0.04
	Ca ²⁺	-0.18
	Mg ²⁺	0.31 **
	Na ⁺	-0.15
Total	N	0.08
	P	-0.19
Available micronutrients	Fe	0.24 *
	Mn	0.31 **
	Cu	0.31 **
	Zn	0.09

* = P<0.05), ** = P<0.01, and *** = P<0.001.

physiological evidence indicating that root biomass in carnation is less sensitive to salinity stress than shoot growth. Kwon *et al.* (2019) demonstrated that increasing NaCl concentrations (up to 200 mM) caused severe reductions in plant height ($\approx$ 68.3%), stem dry weight, flower weight, and leaf area ($\approx$ 57.6%), while root dry weight was not significantly affected, even under high salinity. As a consequence, NaCl stress reduced the shoot-to-root ratio, mainly due to stronger suppression of shoot growth rather than root development.

In the study of Kwon *et al.* (2019), sodium concentrations above 2,299 mg L⁻¹ significantly affected shoot-related traits, whereas root and leaf dry weights remained comparatively stable, suggesting a degree of root tolerance to elevated Na⁺ and Cl⁻ levels. This differential sensitivity supports the interpretation of our results, where mixtures exhibiting higher soluble Na⁺ and Cl⁻ concentrations (particularly VE50 and PM50), which also showed the highest root dry weight, did not impair early root development. Instead, moderate salinity in these substrates may have promoted osmotic adjustment and preferential allocation of assimilates to roots,

allowing carnation cuttings to maintain or even enhance root biomass during the rooting phase.

Correlation analysis suggests that the accumulation of K, Na, SO₄, and Cl is associated with enhanced root development (Table 6), which is consistent with the performance of vermiculite-containing mixtures that exhibited the highest concentrations of soluble K, SO₄, and Cl. Overall, mixtures combining equal proportions of vermiculite and pumice with coconut substrate showed the best performance, in agreement with reports indicating improved growth and yield in raspberry plants when pumice is combined with coconut fiber (Zhao *et al.*, 2024) and superior root development and net photosynthetic rates in wasabi seedlings grown in vermiculite (Hoang *et al.*, 2019). Although Na⁺ and Cl⁻ are commonly regarded as stress-inducing ions, evidence from ornamental potted plants exposed to moderate salinity (NaCl 4.5-7.5 dS m⁻¹) indicates that osmotic adjustment can be achieved through the accumulation of Na⁺ and Cl⁻ together with organic osmolytes, allowing tolerant species to maintain acceptable growth despite increased ion levels (García-Caparrós *et al.*, 2016; García-Caparrós and Lao, 2018). Similarly, studies in *Callistemon citrinus* and *Viburnum lucidum* under moderate NaCl conditions have shown that vacuolar compartmentalization of Na⁺ and Cl⁻ constitutes a tolerance mechanism, whereby these ions act as low-cost osmotic solutes while cytosolic homeostasis is preserved through organic osmolytes and K⁺ (Cirillo *et al.*, 2019).

Therefore, the positive Na⁺ and Cl⁻ root growth relationships observed here should not be interpreted as an absence of salinity risk, but rather as an indication that, at the concentrations and exposure duration evaluated, these ions may contribute to short-term osmotic regulation without limiting root formation. This reinforces the importance of distinguishing between early rooting responses and long-term plant performance, as well as the need for careful fertigation management when using high-CEC substrates such as vermiculite-based mixtures.

4. Conclusions

This study demonstrates that mixing coconut substrates with mineral substrates substantially modifies the chemical environment of soilless

growing media, with clear implications for nutrient availability, retention, and early root development. Among the evaluated materials, vermiculite and pumice-based mixtures, particularly at equal proportions (50:50), showed the most favorable balance between cation exchange capacity, soluble nutrient availability, and root dry weight, indicating a high agronomic suitability for carnation propagation.

The results highlight the importance of considering both soluble and exchangeable nutrient fractions when designing fertigation programs for soilless systems. Substrates with high cation exchange capacity, such as coconut-vermiculite mixtures, can temporarily retain nutrients and buffer ionic fluctuations, potentially improving fertilizer use efficiency. However, the elevated concentrations of Na^+ and Cl^- observed in some mixtures underline the need for careful fertigation management, including appropriate substrate washing, monitoring of irrigation water quality, and the possible use of calcium-based amendments to mitigate ionic imbalances.

Coal slag mixtures contributed significant amounts of native phosphorus, suggesting a potential role of this material as a supplementary P source within circular economy strategies. Zeolite-containing mixtures enhanced Ca and Na retention, confirming their capacity to modify the cationic composition of the substrate exchange complex. Overall, the combination of organic and mineral components proved advantageous, as mixing different mineral substrates with coconut coir allowed complementary chemical properties to interact, resulting in improved substrate performance compared with single-material systems.

This study was intentionally focused on the chemical characterization of substrate mixtures and their agronomic viability, using rooting percentage and root dry weight as indicators of suitability. A limitation of the work is that plant physiological responses were assessed only at the end of the rooting period, without time-series measurements or detailed analyses of plant ion content and root architecture. These aspects should be addressed in future studies to better capture dynamic substrate-plant interactions and long-term crop performance.

In practical terms, the findings provide growers with quantitative information to support substrate selection and fertigation adjustment, particularly when using coconut-mineral mixtures under commercial soilless cultivation. Future research

should evaluate the long-term behavior of these mixtures under fertigation, their economic feasibility, and their environmental performance under commercial production conditions.

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Influence of biostimulants and indole-3-butyric acid on seasonal rooting performance, growth, quality and biochemical dynamics of difficult-to-root *Bougainvillea* 'Thimma'

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Key words: *Bougainvillea peruviana*, cutting, hardwood, indole-3-butyric acid, peroxidase, polyphenol oxidase.

Abstract: Achieving higher percentage and uniform rooting in difficult-to-root variegated *Bougainvillea* cultivars remain a challenge for nurserymen. In this study, 20 cm hardwood cuttings of *Bougainvillea peruviana* 'Thimma' were treated with different biostimulants including seaweed extract, coconut water, willow bark extract, Saridex powder and indole-3-butyric acid (IBA) at concentrations ranging from 500 to 6000 mg L⁻¹ during rainy and spring seasons. The cuttings were placed into sand beds under partial shade in completely randomized design in three replications. Results indicated that cuttings treated with IBA at 4000 mg L⁻¹ during the rainy season yielded promising outcomes, with earliest root initiation (33.8 days), highest survival rate (80.7%), and maximum rooting (70.0%). Coconut water-treated cuttings also showed comparable results, with a survival rate of 60.0% and a rooting percentage of 53.3% compared to control. Biochemical analysis further revealed seasonal variation across treatments, with total soluble sugars ranging from 60 to 220 mg g⁻¹, proteins from 20 to 45 mg g⁻¹, and polyphenol oxidase activity from 25 to 130 µ ml⁻¹. The findings indicate that treating *Bougainvillea* 'Thimma' cuttings with IBA at 4000 mg L⁻¹ during the rainy season enhances rooting and survival percentage, improves plant quality, and increases propagation efficiency.

1. Introduction

Bougainvillea commonly known as 'Glory of the Tropics' is a popular hardy perennial, widely used in landscaping as a shrub, climber, ground cover, hedge and as a potted specimen (Shrestha *et al.*, 2023). The

Bougainvillea genus belongs to the *Nyctaginaceae* family comprising 14 species and is a native of South America (Datta *et al.*, 2021). The species of horticultural importance include *B. spectabilis* Willd., *B. glabra* Choisy, and *B. peruviana* Bonpl. (Datta, 2022). The shrub is appreciated for its vibrant and colourful bracts that add varying vibrant hues to the surroundings and is propagated vegetatively (Asif *et al.*, 2024).

Bougainvillea is multiplied through asexual means through hardwood cuttings, layering, and budding. Hardwood cuttings of *Bougainvillea* 'Sweet Heart', 'Dr. HB Singh' 'Red September', 'Thimma', 'Mahatma Gandhi' and 'Manila Red' are difficult-to-root and require optimal conditions for rooting (Kumar *et al.*, 2021). Stem cuttings are widely adopted for multiplication but often yield inconsistent rooting (authors personal observation). The rooting and survival percentage of cuttings is not high enough without use of rooting hormones (Singh *et al.*, 2021; Yoon *et al.*, 2021; Shrestha *et al.*, 2023). Successful outcome of rooting of cuttings depends on several endogenous factors (Hartmann *et al.*, 2013) such as variety, genetic traits, cutting type, anatomical structure, nutrient and physiological status (Khan *et al.*, 2022), along with exogenous factors including humidity, light, season, timing and growth substances such as biostimulants (Tripathi *et al.*, 2022). Auxin derivatives (Pereira *et al.*, 2021) and dilution carriers (Mofokeng *et al.*, 2020) have been known to significantly enhance root and shoot development.

The difficulty in propagation arises from the plant's limited ability to form adventitious roots (ARs), which is crucial for its perpetuation (Ma *et al.*, 2024). Variegated *Bougainvillea* cultivars are harder to root because they have reduced photosynthetic capacity due to leaf variegation, involve complex tissue patterns and are relatively sensitive to rooting environment. Further, complications related to root development in cuttings often lead to significant commercial losses (Schäfer *et al.*, 2022; El Malahi *et al.*, 2024). To enhance rooting, growers commonly use exogenous auxins of both organic and chemical origin (Gomes and Scortecci, 2021; Li and Yuan, 2025). The biostimulants of organic origin such as coconut water have emerged as viable options to improve rooting success in cuttings. Auxin (IBA) is well-known for enhancing rooting of cuttings (Yoon *et al.*, 2021) by enhancing carbohydrate (CHO) accumulation as energy source for ARs formation, while endogenous peroxidase activity may hinder

root initiation (Lee *et al.*, 2025). Studies reveal that easy-to-root cultivars of *Bougainvillea* had higher contents of CHOs and lower Nitrogen (N) compared to those that are difficult-to-root (Huang *et al.*, 2022). In easy-to-root cultivars, the carbohydrate (CHO) content ranged from 24.0% to 40.6% and nitrogen (N) content varied between 2.2% and 2.5%, whereas in difficult-to-root cultivars such as 'Refulgens', 'Thimma' and 'Mahatma Gandhi', CHO and N contents ranged from 21% to 38.0% and 1.93% to 3.9%, respectively (Pratiksha *et al.*, 2017).

Research on the rooting of hard-to-root *Bougainvillea peruviana* Bonpl. cv. Thimma is limited, especially regarding seasonal effects and the influence of biostimulants. This study hypothesized that treating cuttings of *Bougainvillea* cv. Thimma with biostimulants and IBA across different seasons would enhance rooting. Therefore the study was planned with an objective to evaluate the effects of biostimulants and IBA on rooting, growth, quality, and biochemical traits of hard-to-root cuttings of *Bougainvillea* during the rainy and spring seasons.

2. Material and Methods

Experimental site

The experiment was conducted at research farm of department of Floriculture and Landscaping, Punjab Agricultural University, Ludhiana, Punjab, India. The site is located at 30.9°N latitude, 75.85°E longitude, and elevated at 267 m a.s.l. The site experiences a humid subtropical to hot semi-arid climate with four distinct seasons. The soil is characterized as sandy loam, moderately fertile, slightly alkaline (pH 8.2), and had relatively low water-holding capacity.

Plant material

A 20 cm long approximately pencil-thick hardwood cuttings of *Bougainvillea* cv. Thimma comprising 6 to 7 nodes were excised from healthy stock plants during end-August 2023 (rainy season) and mid-March 2024 (spring season). A smooth slanting cut (45°) was given one-cm above distal node and another circular cut was given one-cm below the node at proximal end (Fig. 1 a). The cuttings were immersed in sodium hypochlorite (0.5%) for surface sterilization for 5 min. Thereafter, the cuttings were washed in deionized water and were subjected to different rooting treatments.

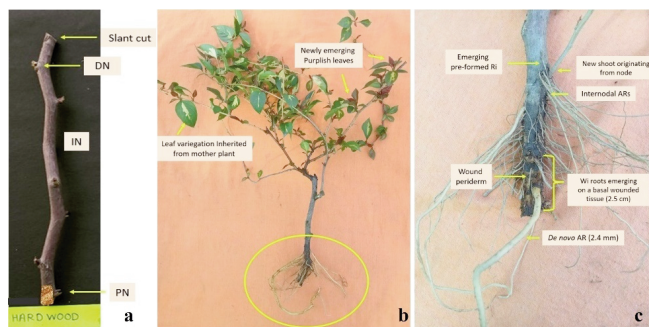


Fig. 1 - Hardwood cutting of bougainvillea (a); Rooted cutting of *Bougainvillea* var 'Thimma' 180 days after sticking of cuttings in rooting media (b); Emergence of multiple ARs originating in response to wounding (Wi roots) and from performed root initials (Ri) at basal stem tissue; mass of undifferentiated cells (callus) can also be seen over the wound tissue (c). DN= Distal node; PN= Proximal node; IN= Internode.

Treatment details

The treatments were broadly categorized under 'biostimulants', that contain a complex mixture of bioactive compounds, known to promote rooting indirectly by enhancing multiple physiological pathways. The auxin (IBA) treatments are hereby referred to as 'true PGR', directly influencing the plant's rooting hormonal pathways. Different treatments comprised 24h basal (1.5 cm) dip of cuttings in distilled water (dw; control), coconut water (100%), seaweed extract (100 ml L⁻¹ water), willow bark extract (500 mL L⁻¹ water) and Saridex powder (type hard-to-root; 60 g powder mixed in 50 ml water to make a thick paste). Immature green coconuts were drilled through the eye and water was collected in a clean container. Seaweed extract (28% w/w) was procured from Indian farmers fertiliser cooperative limited (IFFCO) traded as 'Sagarika' that contained a concentrated liquid extract from red and brown seaweed sap. A mature loose bark of willow tree (*Salix babylonica* L.) was extracted with knife. The bark was cleaned of any debris and dipped in once hot-boiled water and kept overnight. Next day, the decoction was sieved and collected in a clean container for treating the cuttings. The auxin treatments comprised Indole-3-butyric acid (IBA) i.e.: 500, 1000, 2000, 4000 and 6000 mg L⁻¹ water for a holding period of 15, 7.5, 3.5, 1.5 and 0.5 min respectively. The laboratory grade active auxin-IBA (98% pure) with molecular weight 203.24 g mol⁻¹ was procured from Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India. A stock solution of IBA (6000 mg L⁻¹) was prepared first by dissolving 6 g IBA in 20

ml ethanol (70%) ensuring the solution is clear of any precipitates. Thereafter, the volume was made to 1000 ml with DW and thereafter, this solution was preserved in an amber colored bottle labeled appropriately. The stock solution was further diluted with DW to make up the volume to 200 ml for formulating other concentrations (working solution) of IBA (i.e.: 500, 1000, 2000 and 4000 mg L⁻¹). The working solution for each of the IBA concentration was 200 ml.

Experimental procedure and growth measurements

Each treatment was replicated three times with 10 cuttings per replication, totaling 30 cuttings per treatment. Cuttings were planted in coarse sand (2 mm particle size), which was kept moist throughout the rooting phase. The phenological trait namely days to leaf sprouting was recorded with the emergence of first leaf from nodes from the date of sticking of cuttings. To assess root primordia initiation (2 mm outgrowth), six cuttings per treatment (two per replication) were examined bi-weekly, beginning 30 days after planting. Observations on survival percentage, rooting percentage, mean root count, and maximum root length were recorded after 180 days of sticking cuttings in rooting media. Growth traits including number of leaves, stem collar diameter, plant height and longest shoot length were measured. Quality attributes such as sturdiness quotient (SQ), Dickson's quality index (DQI) (Dickson *et al.*, 1960), and shoot-root ratio were also calculated to evaluate the vigour of rooted cuttings as per the following equation:

$$\text{Sturdiness Quotient (SQ)} = \frac{\text{Height of plant (cm)}}{\text{Collar diameter (mm)}}$$

$$\text{DQI} = \frac{\text{Total plant dry weight (g)}}{\left(\frac{\text{Height (cm)}}{\text{Collar diameter (mm)}} \right) + \left(\frac{\text{Shoot dry weight (g)}}{\text{Root dry weight (g)}} \right)}$$

The shoot:root ratio was derived from the respective dry weights of the aboveground and belowground biomass of *Bougainvillea*, expressed as a shoot to root (S:R) ratio.

Anatomical observation

To study the changes in cell de-differentiation and phases for root primordia formation, hand sectioning of basal 0.5 to 1 mm thick tissue of cut end was done with a sharp razor blade. The tissue was excised 30 days after cuttings were planted in coarse sand

media. The freshly cut tissue sections (L.S.) were gently washed with dw to remove any dirt or debris. The cut sections were stained with a 1% Aniline blue dye to temporarily stain the cells revealing division and differentiation during induction of root primordia. After staining, the sections were placed on clean glass slides over which a drop of glycerol was poured as a mounting medium to prevent the sample from drying prior to gently covering with a coverslip, avoiding air bubbles. The anatomical observations were viewed under AXL binocular compound microscope (Labovision) at 40X magnification to observe the cellular layers and root primordia formation. For scaling, the images were processed in ImageJ software (Schneider *et al.*, 2012) following calibration using line tool for measuring a known reference length. The scale bar was set by entering the known distance and units. The scale bars were added with custom adjustments for font size, position of bar and colour as needed. The images were then saved with the embedded scale bars for presentation.

Biochemical analysis

The biochemical analyses were performed on rooted cuttings (n=6) at 180 days post-planting of cuttings. From each replication per treatment, three healthy cuttings were gently removed from the rooting medium with their roots intact for biochemical determination as per established protocols. Total soluble sugars (TSS) were estimated by the phenol-sulphuric acid method (Nielsen, 2024). One gram of root tissue was extracted twice with 10 mL of 80% ethanol in a boiling water bath (20 min), then centrifuged at 5,000 rpm for 5 min. Lead ions were removed using lead acetate and sodium oxalate. A glucose standard curve (100 to 1,000 µg) was prepared. For each standard/sample, 5% phenol, dw (0.1-0.9 mL), and 5 mL concentrated H₂SO₄ were added, and absorbance was recorded at 490 nm and was expressed in mg g⁻¹ of tissue.

Total protein content was determined using the Lowry method as described by Lowry *et al.* (1951). Root tissue (0.5 g) was homogenized in 5 mL phosphate buffer and the extract was centrifuged at 8,000 rpm for 20 min to clarify the solution. Protein was precipitated from the supernatant by adding 1 mL of 20 % trichloroacetic acid (TCA) and incubating for 30 min, followed by centrifugation. The resulting pellet was washed with acetone, re-dissolved in buffer, and the protein content was quantified

colorimetrically. Absorbance was measured at 660 nm in spectrophotometer (Infitek SP IV722G 721G 100 Single beam Visible, 325-1100 nm), and concentrations were calculated against a standard curve prepared from known protein standards and expressed as mg L⁻¹.

Polyphenol oxidase (PPO) activity was determined following a standard protocol (Esterbauer *et al.*, 1977). One gram of root tissue was homogenized in 10 mL phosphate buffer, centrifuged at 10,000 rpm at 4°C for 20 min, and the crude extract was retained. Thereafter, 0.2 mL of extract was mixed with 2.5 mL of catechol substrate and incubated at 37°C for 30 min. PPO activity (Unit ml⁻¹ or U ml⁻¹) was determined by measuring the absorbance change per minute at 410 nm wavelength in spectrophotometer.

Peroxidase activity was assayed using fresh root tissue (0.5 g), which was homogenized in 5 mL of chilled 0.1 M phosphate buffer (pH 6.8) and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was used as the enzyme extract. POD activity was determined by the o-dianisidine method: the reaction mixture contained 2.4 mL phosphate buffer, 0.1 mL o-dianisidine (0.1 %), 0.1 mL hydrogen peroxide (0.2%), and 0.4 mL enzyme extract. The increase in absorbance at 460 nm was recorded over 2 to 3 min to monitor the oxidation of o-dianisidine by peroxidase. Enzyme activity was expressed (Unit g⁻¹ Fresh weight or U g⁻¹ FW) based on the rate of change in absorbance per unit time.

Statistical analysis

Descriptive statistics, including means ± standard error (SE), were calculated using Microsoft Excel (Office Home & Student, version 2501). Treatment effects were evaluated by two-way analysis of variance (ANOVA) using the general linear model in SPSS software (version 27, IBM). Homogeneity of variance and other ANOVA assumptions were verified prior to analysis. Significant effects were further examined by Tukey's honest significant difference (HSD) post-hoc test to compare treatment means at a significance threshold of *p*= 0.05.

3. Results

Leaf sprouting and rooting characteristics

The interaction between treatment and season for leaf sprouting was not significant (*p*= 0.236),

indicating that the response to biostimulant and IBA treatments was consistent across seasons and therefore, treatment means can be interpreted independently of season (Table 1). Among treatments, IBA 1000 mg L⁻¹ recorded significantly earlier (1.3 days) leaf sprouting relative to control. The biostimulant treatments and IBA above 1000 mg L⁻¹ showed delayed sprouting and were found comparable to control. Rainy-season cuttings sprouted leaves approximately five days earlier compared to those planted in spring.

A highly significant T×S interaction ($p=0.000$) was observed for days to root initiation, demonstrating that treatment effects depended strongly on season, where roots emerged relatively 6 days earlier during the rainy season than in spring. This seasonal advantage was more pronounced under IBA treatments, particularly IBA 2000 to 4000 mg L⁻¹, which showed relatively earlier root initiation, during the rainy season (Table 1). In contrast, biostimulant treatments (seaweed extract, coconut water, willow bark extract) including saridex powder and control exhibited higher seasonal responsiveness, with cuttings taking over 50 days to emerge roots during spring.

The interaction between treatment and season was also found significant ($p=0.007$), for root

elongation, indicating a differential response to treatments across seasons. IBA (4000 and 6000 mg L⁻¹) produced substantially longer roots (over 20.0 cm) during the rainy season, which got reduced (over 24% to 30.0%) during spring (Table 1). Biostimulant treatments showed moderate root elongation with limited seasonal variation, except for cuttings treated with willow bark extract that had almost 5-times shorter root length in spring relative to rainy season. Higher IBA dose (up to 4000 mg L⁻¹) were more effective in root elongation in rainy season.

A highly significant T×S interaction was also recorded for root number ($p=0.000$). The mean root count for IBA-treated cuttings was higher compared to those treated with biostimulants during rainy season.

The biostimulant treatments including control showed minimal seasonal plasticity, yielding consistently lower mean root count during both the seasons, except for coconut water treated cuttings that had 1.5-times higher root count in rainy season cuttings relative to spring (Table 1).

Survival and rooting percentage

The T×S interaction for mean survival and rooting percentage was found non-significant ($p=0.214$), indicating treatment means can be interpreted

Table 1 - Effect of biostimulants and Indole-3-butyric acid on days to leaf sprout and rooting characteristics of *Bougainvillea* cuttings planted during rainy and spring season

Treatment (T)	Leaf sprout (days)			Root initiation * (days)			Maximum root length (cm)			Root count (No.)		
	Season (S)		Mean	Season (S)		Mean	Season (S)		Mean	Season (S)		Mean
	Rainy	Spring		Rainy	Spring		Rainy	Spring		Rainy	Spring	
Control (dw)	8.7	14.3	11.5 a	47.7	53.7	50.7	4.3	0.2	2.3	2.7	0.3	1.5
Seaweed extract	8.3	13.7	11.0 abc	44.7	51.7	48.2	7.0	7.0	7.0	2.7	6.3	4.5
Coconut water	8.7	14.3	11.5 a	44.7	49.7	47.2	12.2	9.7	10.9	10.0	6.3	8.2
Willow bark extract	8.3	13.0	10.7 abc	46.0	51.7	48.8	8.5	1.7	5.1	2.7	2.7	2.7
Saridex powder	9.0	13.7	11.4 ab	45.7	52.3	49.0	7.8	4.3	6.0	7.0	2.3	4.7
IBA 500 mg L ⁻¹	8.3	12.3	10.3 bc	40.3	47.3	43.8	11.7	8.0	9.8	8.3	5.0	6.7
IBA 1000 mg L ⁻¹	7.7	12.7	10.2 c	41.3	45.3	43.3	13.7	11.0	12.4	11.7	3.7	7.7
IBA 2000 mg L ⁻¹	8.7	13.7	11.2 abc	38.7	43.7	41.2	16.1	12.0	14.1	16.3	7.7	12.0
IBA 4000 mg L ⁻¹	8.7	13.7	11.2 abc	37.3	46.7	40.0	21.0	16.0	18.5	22.3	14.0	18.2
IBA 6000 mg L ⁻¹	8.7	14.0	11.4 ab	38.3	46.7	42.5	20.4	14.0	17.2	21.3	9.0	15.2
Mean	8.5	13.5		42.5	48.5		12.3	8.4		10.5	5.7	
Sig. of fixed factors	T (0.000); S (0.000)			T (0.000); S (0.000)			T (0.000); S (0.000)			T (0.000); S (0.000)		
	TXS (0.236)			TXS (0.000)			TXS (0.007)			TXS (0.000)		

Significant difference ($p\leq 0.05$) between rooting treatment and seasonal mean values were computed using Tukey's mean separation test and are indicated by different alphabetical letters for varying treatments for days to leaf sprout. Post-hoc multiple comparison tests were not conducted for season because there were fewer than three groups in this factor. The means for above observations were computed from $n=9$ cuttings; except for days to root initiation ($n=6$).

* Any root initials that were >2 mm in length and easily visible with a naked eye.

independently of seasons. The average data of rainy and spring seasons across different biostimulant and IBA treatments revealed significant differences (Fig. 2). The highest mean per cent survival was recorded in cuttings that were treated with IBA (4000 mg L⁻¹), which was over 1.5-fold higher compared to the untreated cuttings (control). At the same IBA level, the cuttings recorded highest rooting percentage, which was nearly 2.5-fold higher compared to the untreated lot. The percentage of rooting declined at highest IBA level, however the reduction was found non-significant compared to IBA (4000 mg L⁻¹). The rooting percentage of coconut water treated cuttings was also found comparable to that observed in IBA treated cuttings at any level, but differed significantly compared to other biostimulant treatments (Fig. 2 a). The per cent survival and rooting of cuttings was significantly higher (1.5 and 0.5-times respectively) in rainy season, compared to those planted during spring (Fig. 2 b).

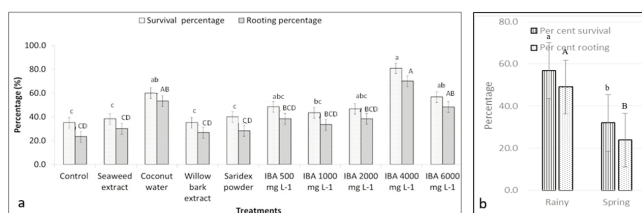


Fig. 2 - Effect of different rooting treatments (a) and seasons (b) on survival and rooting percentage of hardwood cuttings of *Bougainvillea* cv. Thimma. The mean values in column graph (a) represent average of rainy and spring seasons. Columns designated with same case alphabets did not reveal significant differences among the treatment means computed at $p \leq 0.05$ level of significance using Tukey's post hoc test.

Dry weight and shoot-root ratios

The interaction among rooting treatments and seasons for root and shoot dry weight, including S:R ratio was found non-significant. The root dry weight revealed mean variation of 0.68 to 2.12 g across the treatments (Fig. 3 a). The IBA-treated cuttings revealed higher mean root dry weight compared to other treatments, and was found comparable to coconut water, except for IBA at 4000 and 6000 mg L⁻¹. The cuttings treated with IBA at 6000 mg L⁻¹ had highest root dry weight, however was found non-significant compared to IBA at 2000 and 4000 mg L⁻¹. Coconut water treated cuttings also had nearly twice the dry weight of roots weighed compared to control and variation was found non-

significant for IBA-treated cuttings up to 2000 mg L⁻¹. The dry weight of shoots was found highest in cuttings dip treated with IBA at 4000 mg L⁻¹ and at higher dose (6000 mg L⁻¹), the dry weight of shoots decreased (12.4%) with non-significant variation (Fig. 3 a). Non-significant variation was observed in dry weight of shoots in cuttings that were dip treated with IBA (500 to 1000 mg L⁻¹) and were found comparable with cuttings treated with coconut water. Seasons had a significant effect on dry weight of shoot and roots. The shoot and root dry weight was measured 1.87-times greater in rainy-season cuttings compared to spring (Fig. 3 b).

The S:R increased with increase in concentration of IBA, however, the ratio decreased significantly (16.3%) at highest IBA dose compared to the S:R ratio of cuttings that were treated with IBA 4000 mg L⁻¹ (Fig. 3 a). The coconut water treated cuttings had a comparable S:R ratio with seaweed extract and IBA treated cuttings (500 to 2000 mg L⁻¹), whereas non-significant variation was observed for lineale ratio of cuttings across the seasons (Fig. 3 b).

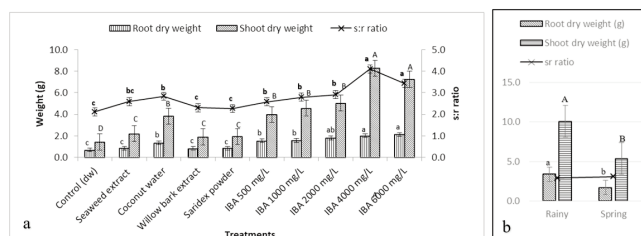


Fig. 3 - Effect of different rooting treatments (a) and seasons (b) on root dry weight (g), shoot dry weight (g) and shoot-root ratio of hardwood cuttings of *Bougainvillea* cv. Thimma. The mean values in column graph (a) represent average of rainy and spring seasons. Destructive analysis of cuttings (n=9) was done to assess the dry weight of shoots and roots. Columns and line markers designated with same case alphabets are not significantly different as computed by Tukey means separation test at the 0.05 level of confidence. Error bars represent $\pm$ SE.

Shoot growth characteristics

The effect of seasons and interaction effects among TxS for mean leaf number was found non-significant, indicating observed variation in leaf number was due to rooting treatments. The cuttings treated with IBA at 4000 mg L⁻¹ produced nearly 5-times more leaf count and 4-times longer shoots than the cuttings raised under control (Table 2). The mean leaf count among other IBA concentrations didn't differ significantly, except 500 mg L⁻¹, which had notably fewer leaves. IBA at 500 mg L⁻¹ had

significantly lower number of leaves compared to cuttings treated with the coconut water. No significant differences in collar diameter were found due to treatment, season, or their interaction (Table 2).

A significant treatment $\times$ season interaction ($p \leq 0.05$) was observed for both plant height ($p=0.000$) and shoot length ($p=0.002$), highlighting the inconsistency of applied rooting treatments for these traits across the seasons. The height of plants was nearly one-third greater during the rainy season planted cuttings compared to those planted during spring, however, the magnitude of seasonal differences varied among treatments. All the treatments revealed taller plants during rainy season, except for the cuttings that were treated with willow bark extract, where the spring planted cuttings had taller plants (Table 2). The shoots length in rainy season planted cuttings was nearly 20.0% longer, compared to those planted during spring season. Except for the cuttings treated with seaweed extract and coconut water, the length of shoots was found higher for the cuttings planted during rainy season (Table 2).

Quality characteristics

Non-significant TxS interaction was observed for the quality characteristics of cuttings, for SQ and DQI indicating the observed variation was due to rooting

media treatments and that was found consistent across the seasons. The SQ of rooted cuttings ranged 3.85 to 1.53 with the highest SQ computed in cuttings that were dip-treated with IBA at 2000 mg L⁻¹, that was found non-significant compared to cuttings dip treated with IBA 4000 mg L⁻¹ (Fig. 4 a). Non-significant differences in mean SQ values were found in organic-based biostimulants including saridex powder, however, coconut water treated cuttings had significantly highest SQ value, that was comparable to IBA at 4000 mg L⁻¹ and to other biostimulants, including control (Fig. 4 a).

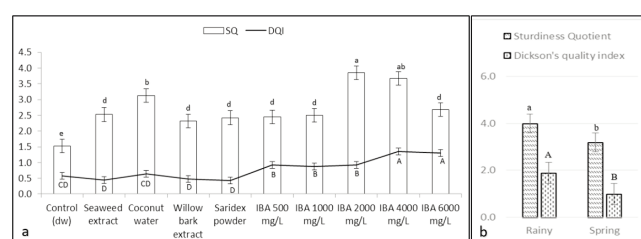


Fig. 4 - Effect of different rooting treatments (a) and seasons (b) for Sturdiness Quotient and Dickson quality index of hardwood cuttings of *Bougainvillea* cv. Thimma. Columns and line markers designated with same case alphabets are not significantly different as computed by Tukey means separation test at the 0.05 level of confidence. Data refer to mean values ($n = 10$), error bars represent $\pm$ SE.

Table 2 - Effect of biostimulants and Indole-3-butyric acid on vegetative characteristics of *Bougainvillea* cuttings planted during rainy and spring season

Treatment (T)	Leaves (No.)			Collar diameter (mm)			Height (cm)			Shoot length (cm)		
	Season (S)			Season (S)			Season (S)			Season (S)		
	Rainy	Spring	Mean	Rainy	Spring	Mean	Rainy	Spring	Mean	Rainy	Spring	Mean
Control	5.3	1.0	3.2 e	9.3	8.0	8.7	20.7	8.7	14.7	9.2	1.3	5.2
Seaweed extract	8.0	7.0	7.5 de	9.2	8.5	8.9	36.7	21.7	29.2	8.8	12.8	10.8
Coconut water	11.0	13.0	12.0 bcd	8.9	8.6	8.8	43.3	33.7	38.5	9.3	15.1	12.2
Willow bark extract	5.0	5.0	5.0 e	8.3	8.7	8.5	21.3	24.7	23.0	6.4	5.0	5.7
Saridex powder	7.7	4.0	5.9 e	9.0	8.4	8.7	26.0	22.0	24.0	8.8	4.2	6.5
IBA 500 mg L ⁻¹	8.3	5.0	6.7 e	8.0	8.5	8.3	34.0	15.0	24.5	7.8	3.6	5.7
IBA 1000 mg L ⁻¹	9.7	8.7	9.2 cde	8.9	8.1	8.5	32.0	27.7	29.8	12.2	9.2	10.7
IBA 2000 mg L ⁻¹	12.3	14.3	13.3 bc	8.1	8.6	8.4	39.0	32.2	35.6	13.9	11.4	12.6
IBA 4000 mg L ⁻¹	17.7	20.0	18.9 a	8.6	8.7	8.7	51.2	41.0	46.1	22.7	20.7	21.7
IBA 6000 mg L ⁻¹	17.0	15.7	16.3 ab	9.8	8.5	9.2	49.3	40.3	44.8	22.3	17.1	19.7
Mean	10.2	9.4		8.8	8.5		35.3	26.7		12.1	10.0	
Sig. of fixed factors	T (0.000); S (0.473)			T (0.899); S (0.157)			T (0.000); S (0.000)			T (0.000); S (0.005);		
	TXS (0.465)			TXS (0.640)			TXS (0.000)			TXS (0.002)		

Significant difference ($p \leq 0.05$) between rooting treatment and seasonal mean values were computed using Tukey's mean separation test and are indicated by different alphabetical letters for varying treatments for leaf number. Post-hoc multiple comparison tests were not conducted for Season because there were fewer than three groups in this factor. The means for above observations were computed from $n = 9$ cuttings.

The DQI ranged 1.35 to 0.43 with significantly higher value recorded in cuttings dip treated with IBA at 4000 mg L⁻¹ which was found comparable with IBA at 6000 mg L⁻¹. The DQI of IBA 4000 mg L⁻¹ treated cuttings was 3.13 times higher compared to control. Non-significant differences were observed for DQI values for cuttings treated with biostimulants of organic origin, that were relatively on the lower side compared to those cuttings dip-treated with IBA (Fig. 4 a). Quality indices were also elevated in rainy-season cuttings, with significantly higher SQ and DQI during rainy season, relative to spring (Fig. 4b).

Histology of root primordia development during AR formation

Histological analysis of lateral section of basal stem tissue of hardwood cuttings showed a periderm layer followed by the cortex, comprising 2 to 6 layers of collenchyma cells (Fig. 5). The collateral vascular bundles were found circular in arrangement. The central portion of stem contained a parenchymatous pith and secondary xylem. Study identified four stages in AR formation in *Bougainvillea* stem cuttings. In essence, the IBA treatment had a marked effect on cellular activity in the cambial and phloem regions of the stem. The IBA treated tissues had a more condensed active cell growth during AR formation, while the untreated stem tissues didn't reveal profusely dividing cells. The formation of root primordia (Rp) occurred 25th DAP (Fig. 5) with IBA treatments 4000mg L⁻¹. These primordia grew perpendicularly to the cutting's main axis, displacing the phloem tissue. The Rp expanded owing to rapid cell division along with the development of tracheal elements (newly formed vascular tissue).

Biochemical modulation during AR formation

The biochemical assessment showed pronounced seasonal and treatment effects on TSS in *Bougainvillea* cuttings. The TSS increased from 60 mg g⁻¹ in spring (control group) to 220 mg g⁻¹ in rainy-season (6000 mg L⁻¹ IBA), with mild increase through untreated control to saridex powder and sharp rises at IBA treatments i.e. 500 mg L⁻¹ to IBA 6000mg L⁻¹. The highest TSS accumulation appeared in IBA at 4000 mg L⁻¹ followed by 6000 mg L⁻¹ (Fig. 6 A). The protein content ranged from 20 mg g⁻¹ in the control lot to 45 mg g⁻¹ in IBA treated cuttings at 6000 mg L⁻¹. While protein levels remained relatively stable across control to IBA treatments up to 2000 mg L⁻¹, thereafter a substantial increase was observed in

cuttings treated with higher IBA levels, indicating that these biostimulants applications may have stimulated greater synthesis of structural or enzymatic proteins. Notably, the rainy season consistently outperformed the spring season for both TSS and protein content (Fig. 6 B).

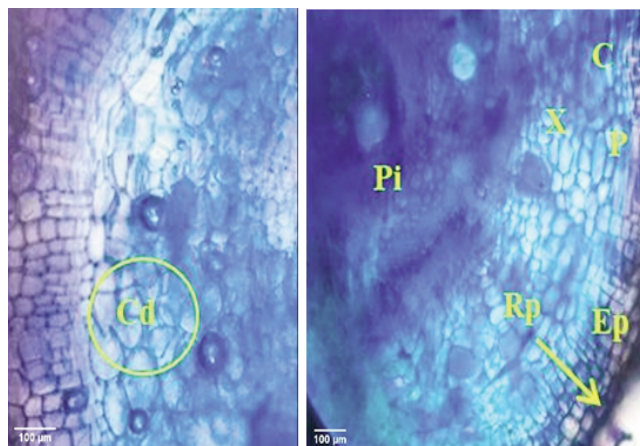


Fig. 5 - The anatomical features of lateral section of basal 0.5 to 1 mm of tissue of *Bougainvillea* cutting treated with IBA 4000 mg L⁻¹. The sections were excised 30 days after the hard-to-root cuttings were stuck in media for rooting. The observations were made under AXL binocular compound microscope, (Labovision) at 40X magnification. Cd= cell division (encircled). The picture on left shows the advent of induction phase of AR formation with the increase of cell number and cell size in cambial zone. The LS section (on right) reveals different cellular layers and initiation of root primordia. Rp= root primordia, Ep= Epidermis, Pi= pith, X= xylem, Ph= phloem, C= cortex.

The PPO activity ranged from 25 to 130 U ml⁻¹, with significantly higher values recorded during the rainy season compared to the spring season across all treatments. Among the treatments, IBA at 4000 mg L⁻¹ exhibited the highest PPO activity (130 U ml⁻¹ in the rainy season), closely followed by IBA at 6000 mg L⁻¹. In contrast, the lowest activity (25 to 30 U ml⁻¹) was observed in cuttings under control and seaweed extract, particularly during the spring season in all biostimulant treatments, except coconut water which differed significantly from other biostimulants. IBA at 4000 mg L⁻¹ and IBA at 6000 mg L⁻¹ were found most effective in enhancing PPO enzyme levels (Fig. 6 C).

POD activity in *Bougainvillea* cuttings ranged between 0.180 and 0.455 U g⁻¹ FW. The highest activity (0.455 U g⁻¹ FW) occurred in spring under control conditions, closely followed by the rainy season. Similarly, cuttings treated with Saridex powder exhibited elevated POD activity. Conversely,

the lowest activity was observed with IBA treatments, particularly at 6000 mg L⁻¹, followed by 4000 mg L⁻¹ during the rainy season (0.180 U g⁻¹ FW). Overall, seasonal variation was evident, with spring cuttings generally recording higher POD activity than those grown during the rainy season (Fig. 6 D).

4. Discussion and Conclusions

The results demonstrate that both biostimulants and varying concentrations of IBA influenced rooting success, survival, vegetative growth, and biochemical responses in the hard-to-root *Bougainvillea* var. 'Thimma'. Significant treatment × season interactions were observed for several traits, including days to root initiation, root length, root count, plant height, and shoot length. Consequently, the effectiveness of specific rooting treatments and optimal seasons for these traits should be interpreted in a season-specific context. Our findings suggest that application of coconut water and auxin (IBA) treatments at suitable concentration can enhance overall cutting performance of hard-to-root *Bougainvillea*. Coconut water is a rich source of endogenous growth regulators (auxins, cytokinins, gibberellins) and supportive nutrients that promote cell division, callus formation, and AR initiation and growth (Yong et al., 2009; Druge et al., 2019). These bioactive

compounds are known to elicit stress-like signalling and metabolic changes, including up-regulation of antioxidant and defense pathways that involve PPO and POD enzymes (Ali et al., 2021; Hasanuzzaman et al., 2021).

In our study, the IBA effectively promotes rooting in hard-to-root *Bougainvillea* cv. Thimma. The IBA concentrations between 3000 to 4000 mg L⁻¹ have been reported to induce early root initiation, enhance rooting percentage, root number, and root length in *Bougainvillea* (Maurya and Bharti, 2025). These effects resulted from a stimulated callus formation and improved cambial differentiation (Ma et al., 2024). The application of IBA also boosts CHO hydrolysis and metabolite build up, aiding root growth (Zhang et al., 2024; Zhou et al., 2025). Higher IBA levels correlate with increased root fresh and dry weight, indicating enhanced biomass (Singh et al., 2023). At optimum dose, IBA accelerates earlier leaf sprouting, higher leaf count, and mobilizes stored carbohydrates resulting in greater shoot growth in cuttings. Studies reveal that IBA treated cuttings develop more roots that increase surface area for nutrient and moisture absorption. Increased leaf area supports metabolism, while auxin-driven secondary growth promotes cell division in lateral meristems, improving plant vigor. IBA, particularly at higher concentrations (4000 mg L⁻¹), is most effective for enhancing rooting of *Bougainvillea* 'Thimma'

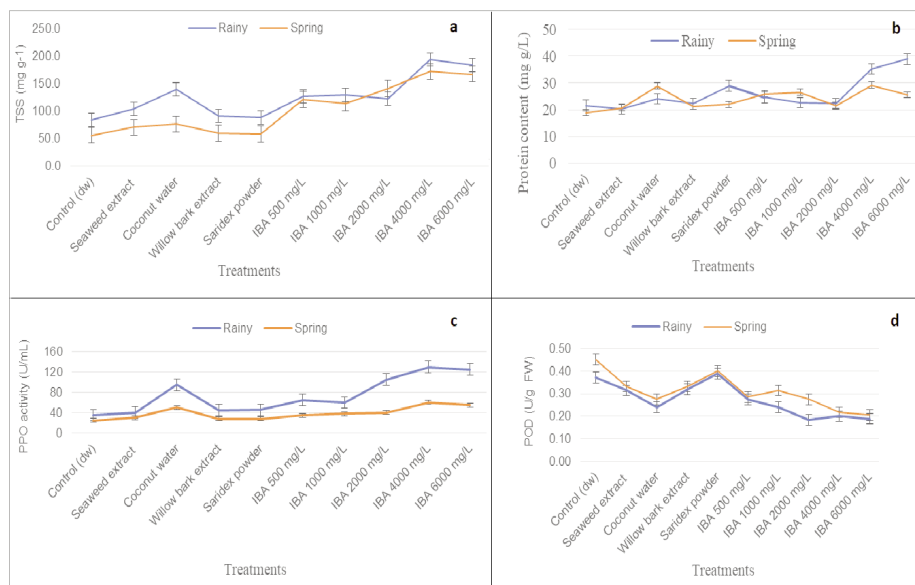


Fig. 6 - Seasonal variation in the TSS (mg g⁻¹) content (a); protein (mg g⁻¹) content (b); PPO (U mL⁻¹) (c); POD (U g⁻¹ FW) (d) in response to different biostimulant treatments and Indole-3-butyric acid concentration at basal stem tissue of hardwood cuttings of *B. peruviana* cv. Thimma during adventitious root formation. Line markers designated with dissimilar capital and small letter alphabets represents significant differences to separate means across treatments in rainy and spring season, respectively. The observations for cuttings (n=6) were recorded 180 days after sticking of cuttings in media. Error bars represent ±se.

cuttings raised during the rainy season (Zhou *et al.*, 2025).

The histology of rooting also presented an interesting variation in terms of the origin of ARs. The ARs may arise either directly from pericycle, parenchyma, or cambium cells, or indirectly via wound-induced callus formation (Huang *et al.*, 2022; Singh *et al.*, 2023). However, sometimes excessive callus can sometimes impede proper root development by creating a barrier between the developing root and the vascular system of the cutting (Irvani *et al.*, 2010; Joel *et al.*, 2023). In *Bougainvillea* AR induction was clearly associated with callus formation where parenchyma cells regained meristematic potential and differentiated into root primordia, thereby classifying it as a callus-type rooting process (Huang *et al.*, 2022). The AR formation is believed to be induced by activation of dormant pre-formed root initials (Mhimdi and Pérez-Pérez, 2020), suggesting auxin as a core regulator of AR formation by modulating physiological and molecular processes during rooting (Liu *et al.*, 2025).

The quality indices, SQ and DQI of rooted cuttings are critical metrics for evaluating the potential for survival and subsequent growth of cuttings post-transplanting (Wilson and Jacobs, 2006). High SQ values (more than 6.0) indicate tall, slender seedlings, while favorable ratios (less than 6.0) reflect stress tolerance and resource efficiency. The DQI value nearly to 2.0 or higher signifies superior seedling quality and establishment potential (Dickson *et al.*, 1960). An optimum shoot-to-root ratio in hardwood seedlings indicate sturdier growth, with ARs arising from diverse tissues (Warrier *et al.*, 2022).

Antioxidant enzymes like POD and PPO are vital not only for plant defence but also for regulating AR initiation and development (Sato *et al.*, 1993; Meng *et al.*, 2019). The PPO, in particular, plays a crucial role in cell division, tissue differentiation, and the formation of root primordia. It also supports the synthesis of IAA-phenolic complexes, which enhance AR formation (El Malahi *et al.*, 2024; Lee *et al.*, 2025). In our study, PPO activity steadily increased with auxin treatment indicating its continuous involvement throughout the rooting process (Warrier *et al.*, 2022). On the other hand, POD is important for lignin production; however, excessive lignin can restrict root formation by reducing cell wall flexibility, limiting callus formation, and hindering root primordia development (Meng *et al.*, 2019; Wang *et al.*, 2022). Higher IBA concentrations can suppress

POD activity during rooting, lowering lignin buildup and enhancing cell wall plasticity, thus facilitating better root development (Pütter and Becker, 1983). Soluble proteins in cuttings are vital for enzyme activity, cell wall formation, and cell signalling during AR development. A brief increase in protein content during root initiation is likely due to active protein synthesis driven by auxin signalling (Wang *et al.*, 2022). Later, during root elongation, protein levels drop again as resources shift to root development. Similar findings were revealed in *Altingia gracilipes*, suggesting that IBA promoted rooting by enhancing protein synthesis and also highlighting the synergistic effect of seasonal conditions and hormonal treatments in enhancing protein content.

Rainy season substantially influenced PPO activity relative to spring season, and higher IBA concentrations had minimal impact on enhancing POD activity. Humidity and water availability during the rainy season can reduce accumulation and oxidation of phenolic compounds usually produced in response to water deficit (Cho *et al.*, 2011). In addition, studies have shown that higher PPO activity in rainy season rapidly oxidise phenols or convert these to relatively bound forms during humid weather, resulting in better rooting compared to spring planted cuttings that may reveal relatively have higher phenolic content that interfere with rooting and stability of auxin (Thakur and Kapila, 2017).

Hardwood cuttings of *Bougainvillea peruviana* Bonpl. cv. Thimma treated with 4,000 mg L⁻¹ IBA exhibited markedly enhanced rooting, growth, and quality characteristics compared with untreated material, achieving 80.7% survival and 70.0% rooting along with favorable sturdiness quotient (SQ), Dickson quality index (DQI), and shoot:root ratios. These findings corroborate earlier reports that appropriate IBA concentrations substantially improve rooting performance in *Bougainvillea* cuttings. Cuttings treated with coconut water similarly demonstrated improved survival and rooting, suggesting that coconut water may be a viable, low-cost alternative for the small-scale propagation of difficult-to-root cultivars. Additionally, the rainy season provided the most conducive environmental conditions for successful establishment of hardwood cuttings, indicating the importance of seasonal timing on rooting response. Collectively, these results offer practical insights for nursery practitioners aiming to improve rooting efficiency and survival in the

challenging propagation of *Bougainvillea* cv. 'Thimma', especially where large-scale multiplication is desired.

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Field-based assessment of sowing date-induced temperature stress on growth, sensitivity, and yield of four cucurbit species

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Key words: Bottle gourd, cucumber, composite cucurbit growth index (CCGI), *Cucumis sativus*, growing degree days, *Lagenaria siceraria*, *Luffa acutangula*, *Luffa cylindrica*, oxidative stress, ridge gourd, sensitivity index (SI), sponge gourd.

Abstract: Climate-induced thermal stress is a major constraint limiting cucurbit productivity in subtropical agro-ecosystems. This study quantified the impact of sowing date-induced temperature variations (-0.49°C to +1.9°C) across three regimes - optimal (SD1), late (SD2), and very late (SD3) - on phenological, physiological, biochemical, and yield traits of four Cucurbitaceae species (ridge gourd, sponge gourd, bottle gourd, cucumber) over two seasons (summer-spring and kharif) in field experiments at IARI, New Delhi, India. Climatic suitability analysis indicated that summer-spring crops performed best within a temperature deviation of 0 to 0.95°C deviation, while kharif crops showed optimal performance near 0 to -0.2°C. The composite cucurbit growth index (CCGI) and sensitivity index (SI) revealed species-specific thermal responses, with sponge gourd showing maximum sensitivity during summer-spring and ridge gourd during kharif. Mid-season sowing (SD2) favored greater growth stability, as indicated by higher CCGI (0.466 in summer-spring and 0.388 in kharif). Overall, summer-spring provided more stable productivity (CCGI 0.4325; SI -0.0163) than kharif (CCGI 0.3458; SI -0.0899). These results highlight the utility of integrated indices for assessing crop resilience and identify mid-season sowing as an effective strategy to sustain cucurbit productivity under increasing climate variability.

1. Introduction

Global warming, characterized by a persistent rise in atmospheric temperatures, poses a substantial threat to agricultural sustainability by increasing climatic variability and the frequency of extreme weather events (Zandalinas *et al.*, 2017; IPCC, 2019; Seneviratne *et al.*, 2021). Temperature, one of the key environmental parameters altered by climate change, is particularly critical, as it governs plant morphological, anatomical, physiological, and biochemical functions, especially during

sensitive phenophases such as flowering and fruit development (Rehman *et al.*, 2015). In India, anticipated temperature rises could reduce overall agricultural productivity by 4-8%, with worst-case projections estimating up to 25% yield loss by 2050 (Pingali *et al.*, 2019). Horticulture plays a pivotal role in India's food and nutritional security, contributing 30.4% to agricultural GDP while occupying just 13.1% of the total cropped area (Datta, 2013; Ayyogari *et al.*, 2014). In 2021-2022, vegetable production reached 204.63 million tons over 11.28 million hectares (Press Information Bureau, 2022). Among horticultural crops, the Cucurbitaceae family holds particular importance, accounting for approximately 18% of India's total vegetable output (Nagendran *et al.*, 2017). Globally, cucurbits such as watermelon, cucumber, melon, pumpkin, and squash are cultivated across diverse agroecological zones (<http://faostat.fao.org>). In addition to their dietary relevance, cucurbits are valued for medicinal, ceremonial, and industrial applications (Rahman *et al.*, 2008). Despite their broad adaptability and utility, cucurbits are acutely sensitive to climate-induced abiotic stresses, particularly elevated temperatures, water scarcity, and soil acidity, which together can cause productivity losses of up to 10% (Parvathi *et al.*, 2022; Smiti *et al.*, 2025). Most cucurbits thrive in warm-season environments, with optimal growth between 20°C and 30°C. However, temperatures exceeding 32°C disrupt physiological and reproductive functions, manifesting as reduced chlorophyll content, skewed male-to-female flower ratios, oxidative stress from reactive oxygen species (ROS), and impaired fruit development (Verma *et al.*, 2016; Chandra *et al.*, 2023). One of the most widely accepted methods to quantify crop growth under variable thermal conditions is the Growing Degree Days (GDD) approach. GDD calculates the cumulative heat units required for a crop to complete its development stages, offering a reliable metric to model phenology in response to changing temperature regimes (Yang *et al.*, 1995; McMaster and Wilhelm, 1997). Under thermal stress, cucurbits often experience shortened vegetative periods, early senescence, and declines in biomass and yield (Tao *et al.*, 2013; Figueiredo *et al.*, 2015; Wang *et al.*, 2016). These responses are closely linked to temperature-driven disturbances in cellular redox homeostasis, which lead to elevated oxidative damage and compromised resilience (Bisbis *et al.*, 2019; Shah *et al.*, 2024). Given these challenges, the present study

focuses on four cucurbit species: cucumber (*Cucumis sativus*), bottle gourd (*Lagenaria siceraria*), sponge gourd (*Luffa cylindrica*), and ridge gourd (*Luffa acutangula*). The selection was made with the intention of balancing research gaps within the Cucurbitaceae family. Cucumber is the most extensively studied cucurbit under abiotic stress conditions, accounting for nearly 45% of relevant literature (Smiti *et al.*, 2025). On the other end of the spectrum, ridge gourd and sponge gourd remain among the least explored species, despite their substantial agronomic value in tropical and subtropical agro-ecosystems (Karmakar *et al.*, 2013; Sindhu *et al.*, 2021; Dutta *et al.*, 2024). Their inclusion aims to fill critical gaps and broaden the taxonomic scope of cucurbit stress physiology. Bottle gourd, occupying a middle ground in terms of research representation, serves as an ideal intermediary species to evaluate stress response variability. In the context of climate adaptation, adjusting sowing dates is one of the most effective and low-cost agronomic strategies available. Aligning the crop's growth stages with more favorable temperature, rainfall, and humidity conditions can significantly reduce exposure to environmental stress and optimize resource utilization (Qureshi *et al.*, 2007; Kabesh *et al.*, 2009). Extensive research in cereals such as wheat, maize, and rice has demonstrated that aligning phenology with favorable thermal regimes improves resilience and yield stability (Abbas *et al.*, 2017; Huang *et al.*, 2018; Mourtzinis *et al.*, 2019). Comparable research in cucurbits, however, remains limited. Existing studies are often restricted to a single species - commonly cucumber or muskmelon - and emphasize routine yield and physiological traits under delayed sowing (Sawale *et al.*, 2022; Dhakal *et al.*, 2023). Under-utilized but economically valuable cucurbits such as ridge gourd and sponge gourd remain underrepresented, and their sowing responses to temperature shifts are poorly characterized. Moreover, no study to date has attempted to integrate phenological, physiological, biochemical, reproductive, and yield traits into a unified framework for ranking cucurbit resilience across seasons. This lack of comparative and integrative evidence restricts the development of robust, species-specific sowing recommendations for cucurbit growers facing climate variability. The objectives of this study were as follows: 1. Investigate the effects of different sowing dates on phenological development and yield attributes of four cucurbit species e.g., Ridge gourd,

bottle gourd, sponge gourd, and cucumber. 2. To assess their physiological and biochemical responses under stressful conditions. 3. To identify the most tolerant and sensitive cucurbit and most favorable sowing window through integrative indices. The novelty of this research lies in its comparative and integrative approach. Unlike previous cucurbit studies that typically focus on a single species or trait under delayed sowing, this work simultaneously evaluates four cucurbit species across two contrasting seasons, capturing species- and season-specific responses. Furthermore, by developing and applying the composite cucurbit growth index (CCGI) and sensitivity index (SI), the study moves beyond routine phenological and yield observations to provide an integrative decision-support framework. This framework allows the ranking of species and sowing windows for climate resilience, with particular emphasis on under-studied crops such as ridge gourd and sponge gourd. To our knowledge, this is the first attempt to combine multivariate physiological, biochemical, and yield traits into composite indices to guide cucurbit sowing strategies under climate variability.

2. Materials and Methods

Plant material, experimental site and condition, and experimental layout

Seeds of four cucurbit species were obtained from the National seed bank, Indian Agricultural Research Institute, New Delhi (Table 1, Fig. 1 a-p).

The field experiment was carried out during the optimal growing season at the Research Farm of the Indian Agricultural Research Institute (IARI), New Delhi, located at Latitude 28°38'23" N, Longitude 77°09'27" E, at an altitude of 228.61 m above sea level in the subtropical region of Northern Plain Zone of India. The soil pH in the experimental field ranged between 7.5 and 8.5. Soil organic carbon content (0-



Fig. 1 - Habit, Male flower, Female flower, Mature fruit of Ridge gourd (a-d), Bottle gourd (e-h), Sponge gourd (i-l), Cucumber (m-p).

15 cm) was <0.5%, available nitrogen was <280 kg ha⁻¹, available phosphorus ranged between 25 and 50 kg ha⁻¹, available potassium was >280 kg ha⁻¹. Fields were typically prepared with 2 ploughings, incorporating organic matter, such as farmyard manure (FYM). A recommended dose of 10 kg of FYM per pit, along with 100 g of NPK (6:12:12) as basal dose and N at 10 g per pit applied 30 days before sowing, was used. Cucurbits require consistent moisture for optimum growth and productivity; hence drip irrigation was applied at 5-7 day intervals, based on soil moisture. Cucurbit pests (red pumpkin beetle and leaf miner) were managed with insecticide (Ridomil Gold) spray (2 mL L⁻¹, 1 spray per week), while diseases like powdery mildew and downy mildew were typically controlled using fungicides such as Mancozeb.

A split-split plot design was adopted, with season (summer-spring and kharif) as the main plot factor, crop species (Ridge Gourd, Bottle Gourd, Sponge Gourd, and Cucumber) as the sub-plot factor, and temperature stress imposed through three sowing dates as the sub-sub-plot factor. The sowing dates were defined as normal/optimal (SD1), late (SD2; 12-day delay), and very late (SD3; 24-day delay). SD1 corresponded to 16 February and 23 July for the summer-spring and kharif seasons, respectively; SD2 to 28 February and 4 August; and SD3 to 12 March and 16 August (Table 2). Four seeds were sown per mud pit, spaced 50 cm apart on ridges (34-40 m long, 10-15 cm high). Crop species and sowing dates were

Table 1 - Selected cucurbits for the experiment

Cucurbit	Scientific name	Variety
Ridge gourd	<i>Luffa acutangula</i>	Pusa swarna
Bottle gourd	<i>Lagenaria siceraria</i>	Pusa santushti
Sponge gourd	<i>Luffa cylindrica</i>	Pusa sneha
Cucumber	<i>Cucumis sativus</i>	Swarna sheetal

Table 2 - Sowing dates and the average meteorological conditions during the experimental period (sowing date-harvesting date)

Season	Sowing date (SD)	T _{max} (°C)	T _{min} (°C)	T _{avg} (°C)	T. stress (°C)	Rainfall (mm)	Relative humidity (%)	Bright sunshine (hrs.)	Evaporation (mm)
Summer-spring	SD1	36.76	21.19	28.95	Reference	1.80	54.8%	5.72	54.75
Summer-spring	SD2	37.75	22.13	29.9	0.95	1.83	53.8%	5.92	53.80
Summer-spring	SD3	38.67	23.08	30.85	1.9	2.00	52.9%	6.15	52.90
Kharif	SD1	32.56	22.37	27.47	Reference	4.91	73.5%	3.44	73.51
Kharif	SD2	32.48	22.07	27.27	-0.2	4.59	73.1%	3.43	73.14
Kharif	SD3	32.34	21.63	26.98	-0.49	4.48	73.2%	3.37	73.16

randomized independently within each season. All comparisons were made using SD1 as the control i.e., recommended sowing dates for optimum productivity.

Meteorological conditions during the experimental period

Observations of maximum temperature (T_{max}; °C), minimum temperature (T_{min}; °C), average temperature (T_{avg}; °C), and rainfall (mm) (Figs. 2 a, b and 3 a, b) as well as relative humidity (%), bright sunshine hours (hrs), evaporation (mm) were obtained for the entire experimental period from the meteorological monitoring database system, Division of Agricultural Physics, New Delhi (Table 2, Table S1, supplementary materials - SM). The temperature stress (T. stress) associated with each sowing date was calculated using the following formula:

$$[T_{avg} \cdot (\text{Sowing-Harvesting}) - T_{avg} \cdot (\text{Sowing-Harvesting})]$$

Optimal sowing].

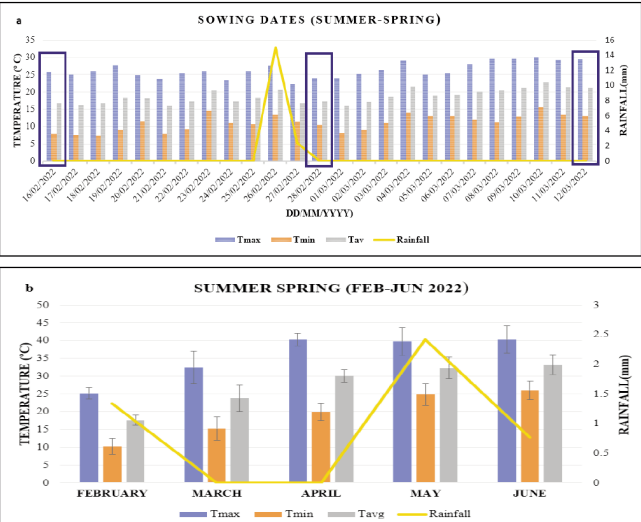


Fig. 2 - Temperature and rainfall profile during the (a) sowing period in summer-spring season (b) complete experimental period in summer-spring season.

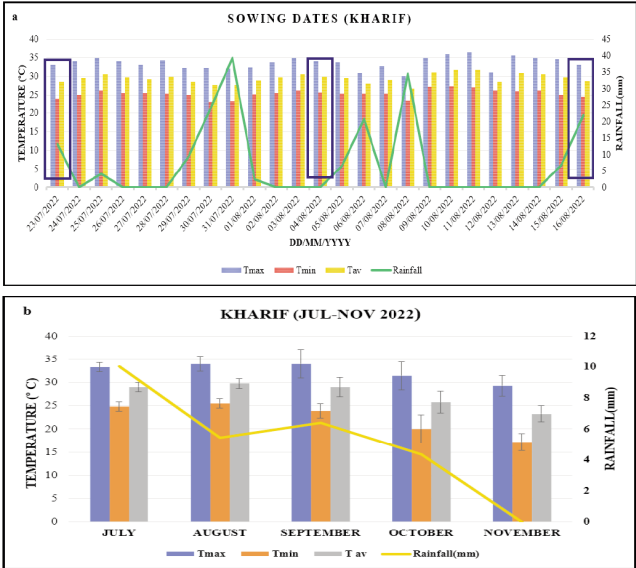


Fig. 3 - Temperature and rainfall profile during the (a) sowing period in kharif season (b) complete experimental period in kharif season.

Phenology and growing degree days (GDD)

The following five major phenological stages of each selected cucurbits were observed weekly during the experimental period: (i) emergence (Stage 1); (ii) complete unfolding of cotyledons (Stage 2); (iii) appearance of first flower on the plant (Stage 3); (iv) appearance of first fruit on the plant (Stage 4); (v) maturation stage (Stage 5); The number of days required to reach each phenological stage and their respective growing degree day (GDD) requirements were calculated using the following formulae:

$$GDD = \sum(T_{max} + T_{min})/2 - T_b$$
 (Eq. 1)

T_{avg} = Average shift in temperature across the season
N= Number of days in the growing season
Where, GDD - growing degree days, T_{min} - daily minimum air temperature (°C), T_{max} - daily maximum air temperature (°C), Tb - base temperature below

which the crop cannot grow ($^{\circ}\text{C}$) with ridge gourd, cucumber having Tb of 15.5°C and bottle gourd, sponge gourd having Tb of 15.5°C (Doijode, 2012).

Physiological and biochemical parameters

Leaf samples were collected 50 days after sowing (DAS) to assess the physiological attributes namely, relative water content (RWC), total chlorophyll (T.CHL) content and the biochemical attributes namely, lipid peroxidation which is measured by malonaldehyde (MDA) content, antioxidant enzymes e.g., superoxide dismutase (SOD), catalase (CAT), peroxidase (POX).

The method outlined by Rajagopal *et al.*, 1977 was used to calculate the RWC using the formula:

$$\text{RWC} = (\text{FW}-\text{DW})/(\text{TW}-\text{DW}) \times 100$$

where FW=sample fresh weight, DW=sample dry weight, and TW=sample turgid weight. The total chlorophyll content was determined by the DMSO method as described by Hiscox and Israelstam (1979). For determination of leaf extract pH, 5 g of fresh leaf tissue were homogenized in 50 mL of deionized water and measured using a pH meter. MDA content was determined as per the method prescribed by Heath and Packer (1968). SOD activity was determined by the method given by Dhindsa *et al.* (1982). The catalase (CAT) enzyme activity was assayed by method given by Aebi (1984). POX activity was assayed as an increase in optical density due to the oxidation of guaiacol to tetra-guaiacol as described by Castillo *et al.* (1984).

Reproductive and biomass parameters

The number of male and female flowers per plant was recorded weekly between 45-50 days after sowing (DAS) and the male-to-female flower ratio (M:F) was calculated. Total plant biomass (TB) was determined by measuring the fresh weight of aboveground and belowground parts at harvest.

Yield and yield indices

Mature fruits were collected from each plant and then yield traits (number of fruits per plant (Y) and the weight of fruits per plant (FW) were measured prior to final plant harvest. To quantify the effects of temperature stress resulting from shift in sowing dates, the following yield indices were used:

(i) the tolerance index (TOL), defined as the difference between yield under normal and stress

conditions; (ii) the mean productivity index (MP), defined as the average yield under normal (Y_p) and stress conditions (Y_s); (iii) the yield stability index (YSI), calculated as the ratio between the yield under stress and normal conditions. Higher values of MP, YSI, along with lower values of TOL, indicate greater tolerance to temperature stress.

$$\text{TOL} = Y_p - Y_s \quad (\text{Eq. 2})$$

Mean productivity (MP) (Rosielles and Hamblin, 1981) was calculated as follows:

$$\text{MP} = (Y_p + Y_s)/2 \quad (\text{Eq. 3})$$

Yield stability index (YSI) (Bouslama and Schapaugh, 1984) was calculated as:

$$\text{YSI} = Y_s/Y_p \quad (\text{Eq. 4})$$

where Y_p and Y_s are the yield performance under normal and temperature stress conditions, respectively.

Statistical analysis and index development

Data from each sowing date were subjected to analysis of variance (ANOVA) at a significance level of $p = 0.05$, using MINITAB 19 software. Principal Component Analysis (PCA) was performed on all the selected variables in MINITAB 19 ensuring standardization of the variables. Temperature stress and season were excluded as variables but used only as labels for grouping observations. CCGI values were calculated across replicates for each temperature stress-season combination and ranked to identify optimal sowing strategies. Principal components (PCs) with eigenvalues greater than 1 were chosen, as they explain the variance at least as much as an original standardized variable according to Kaiser's rule. Parameters with loading values within 10% of the maximum loading within each PC were chosen for the minimum data set MDS. When more than one variable was retained within a PC, the correlation sum was examined. The variable with the highest correlation sum was chosen as the indicator parameter (Rezaei *et al.*, 2006). Normalization of selected MDS indicator parameters was done using the linear scoring (Andrews *et al.*, 2002).

$$\text{Weightage of each PC was calculated (Wi)} = \frac{\text{Individual variance}}{\text{Total Variance}}$$

Normalization followed by Scoring (S_i) was done by

using the following formula:

Indicator score (More is better): $(X - X_{\min}) / (X_{\max} - X_{\min})$

Indicator score (Less is better): $(1 - (X - X_{\min}) / (X_{\max} - X_{\min}))$

$Si = (X - X_{\min}) / (X_{\max} - X_{\min})$ or $(1 - (X - X_{\min}) / (X_{\max} - X_{\min}))$

Composite Cucurbit growth Index = $\sum Wi \times Si$

Sensitivity Index compares the composite scores across different sowing conditions:

Sensitivity Index = $\frac{\text{Composite score (Late/very late sowing)} - \text{Composite score (Optimal sowing)}}{\text{Composite score (Optimal sowing)}}$

3. Results

Meteorological conditions

During the summer-spring season (February-June), $T_{\max}$ ranged from 25.2°C to 40.4°C, $T_{\min}$ from 10.1°C to 26.0°C, and T_{avg} from 18.0°C to 33.1°C (Fig. 2). In the kharif season (July-October) $T_{\max}$ range was 31.5-33.3°C, $T_{\min}$ range was 20.0-24.8°C, and T_{avg} range was 26-29.0°C (Fig. 3). Table 2 summarizes the meteorological parameters for the three sowing regimes, while Table S1 presents the monthly meteorological variations. The elevated temperature peaks recorded during these periods led to visible heat injury symptoms across the investigated crops, including leaf scorching, floral burning, and fruit deformation (Fig. 4).

Phenological growth stages and growing degree day requirements (GDD)

Delayed sowing significantly shortened the

duration of all major phenological stages across cucurbit crops in both experimental seasons, with the strongest effects observed under very late sowing. The reduction was consistently greater during the summer-spring season, indicating higher sensitivity to elevated temperature conditions. Early growth stages were most responsive; emergence and cotyledon unfolding durations declined by more than 50% in several crops under very late sowing, whereas reductions in later stages such as flowering, fruit initiation, and harvesting were generally below 20% (Tables S2 and S3). Despite shorter phenological duration, crops accumulated significantly higher thermal units under delayed sowing. In the summer-spring season, GDD requirement for emergence increased by more than 900% in some crops, while increases during flowering, fruit initiation, and harvesting remained comparatively modest ($\leq 21\%$). During the kharif season, GDD accumulation also increased under delayed sowing, though the magnitude was lower than in summer-spring, reflecting seasonal moderation of heat stress (Tables S4 and S5).

Physiological and biochemical parameters

Delayed sowing caused pronounced and statistically significant changes in plant water status, photosynthetic pigments, and oxidative stress responses, with distinct crop- and season-specific patterns (Table 3). RWC declined substantially in ridge gourd and cucumber, with maximum reductions exceeding 60% under very late sowing during summer-spring, indicating severe dehydration stress. In contrast, bottle gourd maintained higher RWC, showing significant increases of up to ~48% under late sowing, suggesting superior water status regulation. T.CHL content declined significantly under delayed sowing in most crops, particularly during summer-spring. In ridge gourd and sponge gourd, T.CHL reduction exceeded 50% under very late sowing, whereas bottle gourd showed a significant increase in T.CHL content (approximately 55-62%) during kharif, indicating enhanced photosynthetic stability under moderate stress. Oxidative damage, as indicated by lipid peroxidation (MDA content), increased sharply under delayed sowing. During summer-spring, MDA levels increased by more than 7000% in ridge gourd, confirming severe membrane damage under high thermal stress. In contrast, cucumber exhibited comparatively smaller or



Fig. 4 - (a) Burnt leaves of Ridge gourd (b) Burnt leaf Cucumber (c) Yellow leaves of sponge gourd (d) Burnt flower of bottle gourd (e) Scald on bottle gourd fruit (f) Scald on sponge gourd fruit (g) Deformed cucumber fruit due to increased temperature stress

Table 3 - Three-way ANOVA (p values <0.05) for physiological, biochemical, reproductive, biomass, and yield attributes

Source of variation	DF	RWC (%)	T. CHL (mg/g)	MDA (nmol/cm)	CAT (Units/g)	POX (Units/g)	SOD (Units/g)	M: F	Yield (Fruit/plant)	FW (g)	TB (g)
Season	1	***	***	NS	NS	***	NS	NS	NS	NS	**
Temperature Stress (TS)	2	***	***	***	***	NS	NS	NS	***	***	**
Crop	3	***	***	***	NS	***	***	***	***	NS	***
Season × TS	2	***	***	***	**	***	***	NS	NS	NS	NS
Season × Crop	3	***	***	***	*	***	NS	*	NS	NS	**
TS × Crop	6	***	***	***	NS	**	NS	NS	***	*	**
Season × TS × Crop	6	***	***	***	NS	***	***	*	*	NS	NS
Error	48										
Total	71										

Where, NS= p>0.05, * = p≤0.05, **= p ≤ 0.01, *** = p≤0.001.

declining changes in MDA, suggesting greater oxidative tolerance. Antioxidant enzyme activities responded strongly to sowing delays. CAT activity increased significantly under very late sowing, particularly in ridge gourd, where it increased by more than 600% during summer-spring. POX activity also increased across most crops and seasons, whereas SOD showed variable responses, with marked stimulation under late sowing but partial suppression under very late sowing in some crops, especially during kharif (Fig. S1 a-f; Fig. S2 a-f).

Reproductive and biomass parameters

Delayed sowing significantly altered reproductive traits and biomass accumulation (Table 3). In the summer-spring season, the M:F increased by more than 100% in ridge gourd and bottle gourd under very late sowing, indicating a stress-induced shift toward male flower dominance. Sponge gourd exhibited a contrasting response under severe sowing delay, showing a reduction in male dominance. During kharif, reproductive responses were more variable and crop-specific. TB declined significantly under delayed sowing in ridge gourd and cucumber across both seasons, with reductions exceeding 70% under very late sowing, particularly during summer-spring. Bottle gourd showed partial tolerance, with a ~59% increase in TB under late sowing, but declined under very late sowing. In contrast, sponge gourd accumulated substantially higher TB during kharif, showing increases exceeding 600% under delayed sowing, suggesting strong vegetative compensation under favorable moisture conditions (Fig. S1 g-h; Fig. S2 g-h). Mean values and variability are presented in Table S6.

Yield parameters and yield indices

Y and FW were significantly influenced by sowing date, crop species, and their interactions with season (Table 3), confirming the strong effect of temperature stress induced by delayed sowing. Yield responses differed markedly between the summer-spring and kharif seasons. During the summer-spring season, Y generally declined under delayed sowing, with very late sowing causing the strongest reductions. Ridge gourd showed severe yield penalties, with Y declining by up to 100%, while sponge gourd also exhibited substantial reductions. Bottle gourd displayed partial tolerance under late sowing, with Y increases of about 50%, but declined under very late sowing. Cucumber maintained Y under late sowing but it declined sharply under very late sowing. FW followed a similar pattern: under late sowing, some crops showed compensatory increases (up to ~115%), whereas very late sowing resulted in consistent reductions across all cucurbits (approximately 20-80%). In the kharif season, Y was comparatively more stable under late sowing. Ridge gourd and bottle gourd showed moderate Y increases (approximately 12-66%) under late sowing, whereas very late sowing caused strong Y losses, particularly in cucumber (up to ~75%). Sponge gourd maintained relatively stable Y across all the sowing windows. FW responses mirrored these trends, with increases under late sowing in ridge gourd and bottle gourd, but consistent declines under very late sowing. Yield indices further highlighted seasonal differences in stress tolerance. In the summer-spring season, very late sowing was associated with high tolerance index (TOL) values and low yield stability index (YSI), as observed in ridge gourd (YSI ≈ 0.0) and bottle gourd

(YSI $\approx$ 0.5), indicating poor yield stability under severe stress. In contrast, during the kharif season, several crops exhibited negative TOL values and higher YSI (>1.0 -1.6) under late sowing, reflecting improved tolerance under milder temperatures ($T_{avg} \approx 27^\circ\text{C}$). Nevertheless, very late sowing remained detrimental across seasons, particularly for cucumber (YSI $\approx$ 0.25 in kharif). Overall, high mean productivity (MP) and YSI values combined with low or negative TOL consistently identified crops with greater tolerance to temperature stress, with summer-spring conditions amplifying yield penalties, while the kharif season allowed better yield buffering. Figure S1 i-j and figure S2 i-j represent heat maps for yield parameters of each crop in summer-spring and kharif seasons respectively. Table S6 and Table S7 represent the mean and standard error values of yield parameters and yield index values respectively.

Correlation

The two correlograms, representing season 1 (summer-spring) and season 2 (kharif), illustrate the correlation between physiological and yield-related parameters (e.g., RWC, T.CHL, MDA, CAT, POX, SOD, TB, M: F, Y, and FW) and environmental factors, including temperature stress, with correlation coefficients ranging from -0.5 (strong negative) to 0.5 (strong positive). In summer-spring (Season 1), T. stress, ranging from 0.95°C to 1.9°C above the reference, showed a significant negative correlation with RWC (-0.54) and FW (-0.36), indicating that elevated temperature stress reduced water retention and fruit size. Conversely, positive correlations with CAT (0.52) and POX (0.56) suggested that temperature stress enhances antioxidant enzyme activity, aiding in mitigating oxidative damage. Temperature stress also showed a negative correlation with Y (-0.29) (Fig. 5 a). In kharif (Season 2), T. stress was negative (-0.49°C to -0.2°C below the reference, with T_{avg} of 26.98 - 27.47°C), correlating negatively with CAT (-0.59) and MDA (-0.45), indicating reduced antioxidant enzyme activity and MDA under cooler conditions. Positive correlations with TB (0.22), Y (0.34) and FW (0.29) indicate that the cooler and wetter kharif environment supported vegetative growth and yield, despite oxidative challenges (Fig. 5 b).

Composite cucurbit growth index and sensitivity index

The principal component analysis (PCA) reduced the variability among the measured traits into a

smaller number of independent dimensions, with the first four principal components explaining 63.0% of the total variance. The first two components (PC1 = 19.5%, PC2 = 18.2%) together accounted for 37.7% of the overall variation and were thus used for biplot visualization. PC1 was largely defined by a contrast between productivity-related traits such as FW, Y, and TB, which loaded negatively, and stress-associated traits, including MDA, CAT, and RWC, which loaded positively. This pattern indicates that PC1 represents a trade-off between stress response and productivity. PC2 separated samples based on positive associations with Y, RWC, and SOD, in contrast to negative associations with CAT and POX. This PCA loading plot highlights the relationships among yield, physiological, and stress-related traits under different conditions. Y, FW, T.CHL, RWC, and SOD clustered closely and loaded positively on PC2, showing that these traits are positively associated and represent key indicators of productivity and stress tolerance. In contrast MDA was positioned away from Y, suggesting that higher stress damage is negatively linked with productivity. Antioxidant enzymes such as CAT and POX also loaded in the

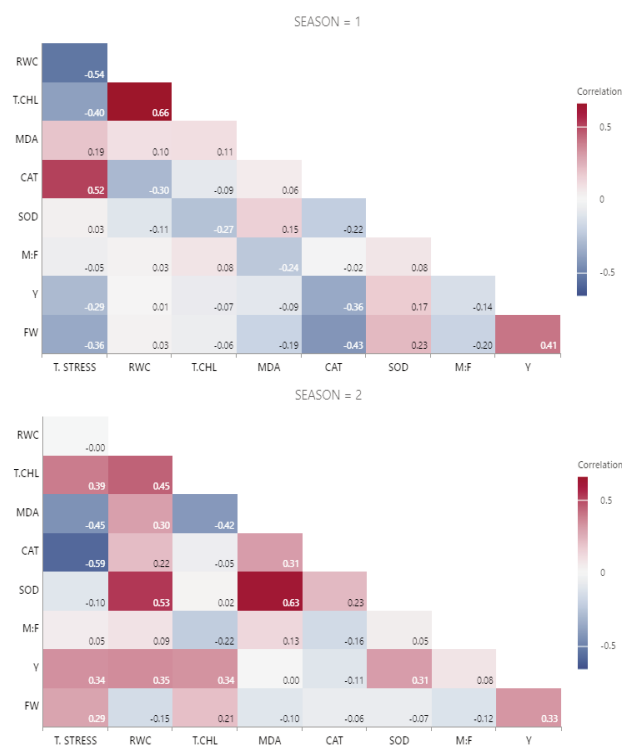


Fig. 5 - Correlogram representing the relationship between sowing date (SD) and the observed parameters of the selected cucurbits during (a) season 1 (summer-spring) and (b) season 2 (kharif).

opposite direction to Y, indicating that although their activity increases under stress, they do not directly contribute to enhanced productivity. TB loaded separately, reflecting variability not strongly connected to yield-related traits. Overall, the analysis reveals that yield performance is best explained by high RWC, T.CHL, SOD activity, and FW, whereas high MDA and antioxidant enzyme activity reflect stress responses that are less favorable for Y (Fig. 6). The composite cucurbit growth index (CCGI) was developed to quantitatively evaluate the optimality of sowing dates for ridge gourd, bottle gourd, sponge gourd, and cucumber cultivation under T. stress conditions. It integrates key phenological, physiological, and biochemical parameters into a single score to assess plant performance across different temporal planting windows. The following equation was used to calculate the CCGI values:

$$0.202 \times [\text{MDA (nmol/cm)}] + \text{TB (g)} + \text{FW (g)} + 0.188 \times [\text{RWC (\%)} + (\text{SOD (Unit/g)}) + 0.144 \times [\text{T.CHL (mg/g)}] + 0.117 \times [\text{M:F}].$$

A higher mean index value indicates more favorable composite physiological and yield performances, while the sensitivity index quantifies crop response to delayed sowing, with more negative value indicating increased sensitivity, and positive values denoting tolerance. The PCA-derived mean index values, reflecting plant health and growth parameters, showed distinct performance trends for the four crops across three sowing dates in summer-spring and kharif seasons. Table 4 and 5 compare the performance of four cucurbit crops across two seasons, summer-spring and Kharif, using average sensitivity index values (indicating sensitivity to

Table 4 - Crop-wise sensitivity index (SI) to sowing date shifts

Crop species	Average SI	
	Summer-spring	Kharif
Ridge gourd	-0.021	-0.337
Bottle gourd	-0.106	0.027
Sponge gourd	-0.172	-0.085
Cucumber	0.135	0.037

Table 5 - CCGI response to sowing date-induced temperature deviations

Sowing date	Temperature shift from optimum sowing date (°C)		Average CCGI	
	Summer-Spring	Kharif	Summer-Spring	Kharif
1 (Optimum)	0.0	0.0	0.448	0.368
2	0.95	-0.20	0.466	0.388
3	1.90	-0.49	0.382	0.273

sowing date shifts relative to a baseline) and average CCGI values (reflecting overall growth performance). In summer-spring, sponge gourd was the most sensitive crop with an average sensitivity index of -0.172, indicating significant growth reduction under delayed sowing, while cucumber was the most tolerant at 0.135, showing improved performance. In kharif season, ridge gourd exhibited the highest sensitivity (-0.337), while cucumber again showed tolerance (0.037). For identifying the most suitable sowing date corresponding to the optimal temperature regime, sowing date 2 consistently produced the highest average CCGI in both seasons (0.466 for summer-spring, 0.388 for kharif), making it the most suitable regime for sowing of cucurbits, while sowing date 3 resulted in the worst CCGI values (0.382 for summer-spring and 0.273 for kharif, respectively). Seasonal comparison revealed that summer-spring was the more favorable season, with a higher average CCGI (0.4325 vs. 0.3458) and a less negative average sensitivity index (-0.0163 vs. -0.0899), indicating superior and more stable growth performance across crops and sowing dates compared to kharif, where crops generally exhibit greater sensitivity to sowing date shifts (Table 6, Table S8). In summer-spring, cucumber proved to be the most tolerant crop, followed by ridge gourd and

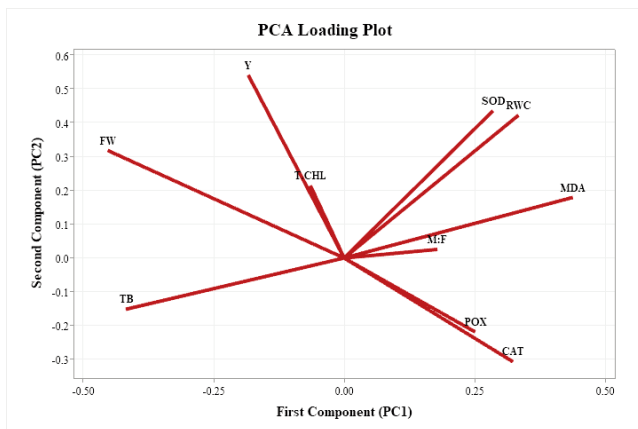


Fig. 6 - PCA loading plot representing the spread of parameters across two major principal components (PCs).

Table 6 - Overall seasonal performance

Season	Average CCGI	Average SI
Summer–spring	0.4325	-0.0163
Kharif	0.3458	-0.0899

bottle gourd, with sponge gourd being the most sensitive. In the kharif season, cucumber again demonstrated the highest tolerance, followed closely by bottle gourd, while ridge gourd was the most sensitive. Climatic suitability analysis reveals that summer-spring crops perform best within a 0°C to +0.95°C shift from the optimal sowing date, while in kharif, the ideal conditions occur under a temperature shift ranging from 0°C and -0.2°C. Overall, summer-spring conditions provided higher climatic growth potential and reduced sensitivity to sowing date shifts compared to kharif, making them more favorable for cultivation.

4. Discussion and Conclusions

Alterations in crop phenology serve as a critical indicator of climate change impacts and are a potential adaptation strategy aimed at yield maximization (Wolkovich *et al.*, 2012; Muleke *et al.*, 2022). Consistent with prior research (Phelan *et al.*, 2018; Liu *et al.*, 2021), our study demonstrates that sowing time profoundly influences plant phenology (Parya *et al.*, 2010). Delayed sowing generally shortens the vegetative growth phase, likely due to higher minimum temperatures accelerating developmental processes (Guo *et al.*, 2023). The shortening of critical phenological phases, which regulate photoperiod sensitivity and crop productivity, may explain the poor performance under delayed sowing (Ferrise *et al.*, 2010). Delayed sowing increases the possibility of crops being exposed to high temperatures during the fruit formation stage, which is harmful to leaf photosynthesis, flowering, and final yield formation. (Garg *et al.*, 2013). High values of GDD under late sowing can be mainly attributed to the higher daily and cumulative temperatures (Aslam *et al.*, 2017). Increased accumulation of heat over time accelerates the phenology and introduces trade-offs in plants, such as reduced capacity of vegetative structures to acquire resources and disruptions in source-sink

relationships, limiting assimilate allocation to reproductive organs (Jeffrey *et al.*, 2025; Wingler and Soualiou, 2025). RWC is a well-established marker of plant water status and stress resilience, reflecting both osmotic adjustment and cellular hydration (Rajagopal *et al.*, 1977; Hasanuzzaman *et al.*, 2021). Declines in RWC under delayed sowing indicate compromised protoplasmic stability and a reduced capacity to sustain photosynthesis during temperature stress (Raja *et al.*, 2020). Variability among cucurbits suggests species-specific plasticity: some maintain RWC through osmotic adjustment and stomatal regulation, while others show sharp declines linked to membrane instability and oxidative injury (Hasanuzzaman *et al.*, 2013). High humidity conditions may further reduce effective RWC by limiting transpiration and weakening osmotic gradients (Hasanuzzaman *et al.*, 2013). Chlorophyll content provides an equally critical indicator of stress adaptation, as pigment degradation directly compromises photosynthetic efficiency. High temperature and excessive rainfall accelerate chlorophyllase activity, destabilize thylakoid membranes, and suppress pigment biosynthesis (Camejo *et al.*, 2005; Zhou *et al.*, 2017). In some species, moderate stress associated with intermediate sowing dates triggers acclimatory adjustments such as altered membrane lipid composition or protective protein synthesis, sustaining or temporarily increasing chlorophyll levels (Tang *et al.*, 2018; Zhao *et al.*, 2020). Under prolonged stress, however, electron transport disruption and inhibition of biosynthetic enzymes dominate, leading to chlorophyll decline (Purnama *et al.*, 2018). These dynamics reinforce that RWC and chlorophyll are interlinked determinants of stress tolerance, sensitive to both thermal load and moisture regimes (Amin *et al.*, 2024). Delayed sowing intensified oxidative stress, as evidenced by enhanced production of reactive oxygen species (ROS) such as singlet oxygen, superoxide radicals, hydrogen peroxide, and hydroxyl radicals (Liu *et al.*, 2020; El-Remaly, 2023). Excess ROS destabilize membranes, oxidize proteins, and induce lipid peroxidation, reflected in elevated malondialdehyde (MDA) accumulation (Hasanuzzaman *et al.*, 2020). Increases in MDA observed under high temperature stress are consistent with oxidative damage reported across crops including chickpea, turfgrass, and cucumber (Wilson *et al.*, 2014; Verma *et al.*, 2016; Nagar *et al.*, 2021). Plants counteract ROS damage

primarily via enzymatic defense systems. Catalase (CAT) decomposes hydrogen peroxide but its activity often fluctuates: declines have been attributed to high temperature-induced inhibition of new protein synthesis (Dat *et al.*, 1998), while recovery under prolonged stress indicates compensatory scavenging activity (Almeselmani *et al.*, 2006; Mustafa *et al.*, 2021). Peroxidase (POX) also scavenges hydrogen peroxide and is often linked with hormonal regulation, showing induction under moderate stress but suppression when stress overwhelms cellular capacity (Prerostova *et al.*, 2020; Aydogan *et al.*, 2021). Superoxide dismutase (SOD) catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen, with increased activity generally reflecting stress adaptation (Gosavi *et al.*, 2014; Raja *et al.*, 2020). Declines in SOD observed under severe stress suggest enzyme inactivation or insufficient induction (Zhao *et al.*, 2018; Zhou *et al.*, 2017). Temperature stress strongly influenced reproductive allocation, as reflected in altered male-to-female flower ratios. Elevated temperatures during delayed sowing increased the proportion of male flowers, a response mediated by ethylene biosynthesis, hormonal signaling, and photoperiod sensitivity (Daba *et al.*, 2016; Gao *et al.*, 2021; Liu *et al.*, 2021). While this strategy conserves resources, it reduces fruit set and yield potential, consistent with previous findings in cucumber under temperature stress (Schultheis *et al.*, 2016). Male gametophyte formation is generally more sensitive to stress than female reproductive development (Lohani *et al.*, 2020). This explains why male flower abundance increased under moderate stress, but collapsed under extreme late sowing in some species, reflecting disrupted pollen development. Seasonal variability also contributed, with water deficits and high evaporative demand compounding reproductive imbalance under kharif conditions (Sawan, 2018). Biomass accumulation, a central indicator of resource capture and energy storage, was constrained under delayed sowing by shortened vegetative phases that reduced light interception and temperature utilization (Westoby *et al.*, 2002; Liu *et al.*, 2016; Chandra *et al.*, 2023). Ridge gourd and cucumber showed marked biomass declines, reflecting chlorophyll loss, photosystem II damage, and redox imbalance (Hasanuzzaman *et al.*, 2013). Sponge gourd, however, maintained stability in kharif, potentially linked to temperature-induced

acclimation involving abscisic acid accumulation, apoplastic hydrogen peroxide, RBOH transcripts, and HSP70 expression (Li *et al.*, 2014). Bottle gourd exhibited a hormetic response, gaining biomass under moderate stress through the activation of basic helix-loop-helix (bHLH) transcription factor families that regulate stress adaptation (Wang *et al.*, 2024), but declining sharply under extreme stress and rainfall-induced root damage (Agathokleous *et al.*, 2019; Manghwar *et al.*, 2024). Ridge gourd was consistently sensitive, in agreement with earlier findings of reduced flower initiation and fruit set under delayed sowing (Singh *et al.*, 2020). Bottle gourd and cucumber displayed partial resilience, with yield maintenance under moderate delays but sharp losses under severe stress. Sponge gourd exhibited recovery under very late Summer-spring sowing, suggesting delayed acclimation. Fruit weight trends confirmed that moderate delays occasionally aligned fruiting with favorable microclimates (Jaksunarno and Akali Sema, 2001; Kishor *et al.*, 2010; Singh *et al.*, 2020), but very late sowing universally reduced fruit size across species due to limited assimilate supply (Ball *et al.*, 2000). Multivariate analyses clarified how physiological and reproductive traits interact under stress. PCA and correlogram results revealed strong positive associations among RWC, chlorophyll stability, and biomass, consistent with their central role in sustaining productivity under stress (Kalaji *et al.*, 2018; Hamawi *et al.*, 2023). In contrast, oxidative stress markers such as MDA clustered inversely with yield parameters, underscoring lipid peroxidation as a key determinant of growth penalties (Hasanuzzaman *et al.*, 2020). Antioxidant enzymes exhibited variable associations, with moderate increases linked to effective ROS detoxification and tolerance, but extreme induction indicating overcompensation and oxidative injury (Dat *et al.*, 1998; Zhao *et al.*, 2018; Mustafa *et al.*, 2021). Reproductive shifts, particularly altered male-to-female flower ratios, were also aligned with stress markers, pointing to an integrated regulatory network involving ethylene biosynthesis, ROS signaling via RBOH, and heat shock responses (Li *et al.*, 2014; Lohani *et al.*, 2020; Gao *et al.*, 2021). The Composite Cucurbit Growth Index (CCGI) integrates physiological, biochemical, reproductive, and yield traits into a single cumulative measure, while the sensitivity index (SI) identifies sowing windows that optimize resilience under variable environments.

Together with PCA, these indices revealed a fundamental trade-off: productivity-related traits such as yield, fruit weight, chlorophyll content, superoxide dismutase activity, and relative water content declined as stress indicators like MDA, catalase, and peroxidase increased. This underscores that enhanced antioxidant activity alone does not guarantee yield stability; resilience must be defined by the combined capacity to tolerate stress and sustain productivity. In summer-spring, sponge gourd was most vulnerable, showing sharp growth reductions with delayed sowing, whereas cucumber-maintained tolerance, reflecting its ability to preserve chlorophyll levels and water status under heat stress. In kharif, ridge gourd emerged as the most sensitive due to its narrow thermal and water tolerance, while cucumber again demonstrated resilience. Across both seasons, sowing date 2 (12-day delay) consistently achieved the highest CCGI values, confirming it as the most favorable window for balancing acclimation with yield stability. Seasonal comparisons further indicated that summer-spring conditions supported higher resilience and growth potential than kharif, where excessive rainfall and humidity amplified sensitivity to temperature stress due to shifts in sowing dates. These results validate the utility of CCGI and SI as multidimensional tools that integrate stress physiology with agronomic outcomes, enabling practical identification of optimal sowing schedules. Early planting under cooler conditions (<30°C) promotes assimilate distribution, delays senescence, and boosts yield potential (Gomez-Macpherson and Richards, 1995; Hatfield and Prueger, 2015). By contrast, delayed sowing intensifies oxidative stress, disturbs reproductive balance, and reduces stability, highlighting the need for season-specific adjustments (mid-February for summer-spring, late July for kharif). Complementary measures such as adopting high temperature-tolerant cultivars, and improving irrigation management can further alleviate stress, aligning with IPCC (2024) recommendations that prioritize sowing shifts as a low-cost adaptation strategy (Zhang *et al.*, 2019; Pathak *et al.*, 2022). By comparing four cucurbit species, this study shows that resilience is crop- and season-dependent, with cucumber consistently tolerant and ridge gourd consistently vulnerable. Incorporating under-researched cucurbits such as ridge gourd and sponge

gourd extends physiological insights beyond well-studied crops like cucumber. Most importantly, the integration of diverse traits into CCGI and SI establishes a quantitative, decision-support framework that transcends routine agronomy, offering a robust, data-driven approach to climate-resilient cucurbit cultivation.

This study demonstrates that sowing date-induced temperature shifts strongly regulate cucurbit growth, physiology, and yield through the shortening of phenological cycles, altered reproductive allocation, and intensified oxidative stress. Species responses diverged, varied with ridge gourd consistently showing high sensitivity, cucumber maintaining resilience, and bottle gourd and sponge gourd exhibiting intermediate plasticity depending on season. The integration of multivariate traits into the Composite Cucurbit Growth Index (CCGI) and Sensitivity Index (SI) provided a robust framework for quantifying crop resilience. These indices consistently identified mid-season sowing as the most favorable strategy, while very late sowing proved universally detrimental. Seasonal comparisons further confirmed summer-spring as a more stable production window than kharif. By moving beyond routine agronomic evaluation and adopting integrative indices, this study establishes sowing window optimization as a low-cost, data-driven adaptation strategy for cucurbit production under climate variability. The framework presented here offers practical guidance for farmers and policymakers while highlighting the need for future studies incorporating broader genetic diversity and multi-location validation to enhance the generalizability of these findings.

This study, conducted at a single location over one year, limits extrapolation across agro-climatic zones and seasons. Evaluation was restricted to four cucurbit species with fixed genotypes, leaving genotype × environment interactions unaddressed. Additionally, the focus on temperature stress through sowing shifts did not account for interactions with other abiotic and biotic factors. Future research should involve multi-location, multi-year trials with a broader genetic base, complemented by molecular and transcriptomic analyses to elucidate stress tolerance mechanisms. Integrating crop simulation models with weather-based advisories and assessing combined agronomic interventions will further enhance climate-resilient cucurbit production.

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Field-based assessment of sowing date-induced temperature stress on growth, sensitivity, and yield of four cucurbit species



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Abstract: Climate-induced thermal stress is a major constraint limiting cucurbit productivity in subtropical agro-ecosystems. This study quantified the impact of sowing date-induced temperature variations (-0.49°C to +1.9°C) across three regimes - optimal (SD1), late (SD2), and very late (SD3) - on phenological, physiological, biochemical, and yield traits of four Cucurbitaceae species (ridge gourd, sponge gourd, bottle gourd, cucumber) over two seasons (summer-spring and kharif) in field experiments at IARI, New Delhi, India. Climatic suitability analysis indicated that summer-spring crops performed best within a temperature deviation of 0 to 0.95°C deviation, while kharif crops showed optimal performance near 0 to -0.2°C. The composite cucurbit growth index (CCGI) and sensitivity index (SI) revealed species-specific thermal responses, with sponge gourd showing maximum sensitivity during summer-spring and ridge gourd during kharif. Mid-season sowing (SD2) favored greater growth stability, as indicated by higher CCGI (0.466 in summer-spring and 0.388 in kharif). Overall, summer-spring provided more stable productivity (CCGI 0.4325; SI -0.0163) than kharif (CCGI 0.3458; SI -0.0899). These results highlight the utility of integrated indices for assessing crop resilience and identify mid-season sowing as an effective strategy to sustain cucurbit productivity under increasing climate variability.

Table S1 - Monthly meteorological parameters (Mean $\pm$ SD) in the respective seasons

Month	Tmax (°C)	Tmin (°C)	Avg. Temp (°C)	Rainfall (mm)	Humidity (%)	BSS (hrs)	Evaporation (mm)
<i>Summer spring season</i>							
February	25.2 $\pm$ 1.6	10.1 $\pm$ 2.3	17.7 $\pm$ 1.4	1.3 $\pm$ 4.2	88.8 $\pm$ 2.9	7.3 $\pm$ 2.4	3.3 $\pm$ 0.6
March	32.4 $\pm$ 4.5	15.3 $\pm$ 3.3	23.8 $\pm$ 3.7	0.0 $\pm$ 0.0	88.8 $\pm$ 1.7	7.4 $\pm$ 1.3	3.3 $\pm$ 1.0
April	40.2 $\pm$ 1.7	19.9 $\pm$ 2.4	30.0 $\pm$ 1.8	0.0 $\pm$ 0.0	87.6 $\pm$ 1.8	7.4 $\pm$ 2.2	3.3 $\pm$ 0.8
May	39.7 $\pm$ 3.8	24.8 $\pm$ 3.1	32.3 $\pm$ 3.0	2.4 $\pm$ 7.2	87.0 $\pm$ 2.3	7.3 $\pm$ 2.5	3.3 $\pm$ 1.3
June	40.4 $\pm$ 3.9	26.0 $\pm$ 2.6	33.2 $\pm$ 2.8	0.8 $\pm$ 3.6	86.7 $\pm$ 2.6	7.4 $\pm$ 2.5	3.2 $\pm$ 0.5
<i>Kharif season</i>							
July	33.3 $\pm$ 1.1	24.8 $\pm$ 1.0	29.0 $\pm$ 1.0	10.0 $\pm$ 13.7	87.4 $\pm$ 6.5	3.8 $\pm$ 2.8	3.5 $\pm$ 0.5
August	34.0 $\pm$ 1.5	25.5 $\pm$ 1.0	29.7 $\pm$ 1.1	5.4 $\pm$ 10.2	80.7 $\pm$ 6.3	5.5 $\pm$ 3.6	3.8 $\pm$ 0.7
September	34.0 $\pm$ 3.1	23.9 $\pm$ 1.5	29.0 $\pm$ 2.1	6.4 $\pm$ 6.4	84.4 $\pm$ 7.6	6.0 $\pm$ 3.8	4.0 $\pm$ 4.0
October	31.5 $\pm$ 3.0	20.0 $\pm$ 3.0	25.7 $\pm$ 2.4	4.4 $\pm$ 17.6	88.5 $\pm$ 7.2	6.1 $\pm$ 3.0	3.5 $\pm$ 1.0
November	29.3 $\pm$ 2.2	17.2 $\pm$ 1.8	23.3 $\pm$ 1.7	0.0 $\pm$ 0.0	90.1 $\pm$ 1.9	4.1 $\pm$ 2.9	1.8 $\pm$ 0.4

Table S2 - Phenological days (time required to reach the mentioned stages) during summer-spring season

Summer-spring season						
Crop	Sowing date	Emergence	Cotyledons completely unfolded	First flower open on main stem	First fruit on main stem	Harvested
Ridge Gourd	SD1	13 $\pm$ 1	21 $\pm$ 1	60.33 $\pm$ 0.58	87.67 $\pm$ 1.53	125 $\pm$ 0
	SD2	8 $\pm$ 1	12.67 $\pm$ 1.53	52.67 $\pm$ 1.53	79.33 $\pm$ 4.04	121 $\pm$ 0
	SD3	6 $\pm$ 1	10.67 $\pm$ 0.58	46.33 $\pm$ 2.52	72.33 $\pm$ 1.53	117 $\pm$ 0
Bottle Gourd	SD1	6.7 $\pm$ 0.6	13.3 $\pm$ 1.2	70.7 $\pm$ 2.5	82.7 $\pm$ 1.5	110.0 $\pm$ 0.0
	SD2	5.3 $\pm$ 0.6	10.3 $\pm$ 1.5	66.7 $\pm$ 4.7	79.0 $\pm$ 4.0	107.0 $\pm$ 0.0
	SD3	5.0 $\pm$ 1.0	7.7 $\pm$ 0.6	60.3 $\pm$ 3.2	69.7 $\pm$ 1.5	98.0 $\pm$ 0.0
Sponge Gourd	SD1	5.3 $\pm$ 0.6	14.3 $\pm$ 2.1	113.3 $\pm$ 1.2	136.0 $\pm$ 1.7	144.0 $\pm$ 0.0
	SD2	4.7 $\pm$ 0.6	10.3 $\pm$ 2.1	112 $\pm$ 2.0	132.7 $\pm$ 2.9	140.0 $\pm$ 0.0
	SD3	3.7 $\pm$ 0.6	7.7 $\pm$ 1.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	130.0 $\pm$ 0.0
Cucumber	SD1	5.7 $\pm$ 0.6	13.0 $\pm$ 1.0	47.3 $\pm$ 1.5	84.7 $\pm$ 4.5	110.0 $\pm$ 0.0
	SD2	5.3 $\pm$ 0.6	9.3 $\pm$ 1.5	45.3 $\pm$ 2.5	82.0 $\pm$ 2.0	105.0 $\pm$ 0.0
	SD3	4.0 $\pm$ 0.0	6.7 $\pm$ 0.6	42.0 $\pm$ 5.3	79.0 $\pm$ 4.6	102.0 $\pm$ 0.0

Table S3 - Phenological days (time required to reach the mentioned stages) during Kharif season

Kharif season						
Crop	Sowing date	Emergence	Cotyledons completely unfolded	First flower open on main stem	First fruit on main stem	Harvested
Ridge Gourd	SD1	15 $\pm$ 1	24.67 $\pm$ 1.53	58.67 $\pm$ 1.53	87.33 $\pm$ 1.53	125 $\pm$ 0
	SD2	11 $\pm$ 1	14 $\pm$ 1	54 $\pm$ 1	82 $\pm$ 1	115 $\pm$ 0
	SD3	9 $\pm$ 1	10.67 $\pm$ 0.58	48 $\pm$ 1	75.33 $\pm$ 1.53	107 $\pm$ 0
Bottle Gourd	SD1	9.0 $\pm$ 1.0	16.0 $\pm$ 1.0	68.0 $\pm$ 1.0	86.0 $\pm$ 1.0	116.0 $\pm$ 0.0
	SD2	7.0 $\pm$ 1.0	15.0 $\pm$ 1.0	67.0 $\pm$ 2.0	81.0 $\pm$ 1.0	113.0 $\pm$ 0.0
	SD3	4.0 $\pm$ 1.0	11.0 $\pm$ 1.0	67.0 $\pm$ 1.0	77.0 $\pm$ 1.0	108.0 $\pm$ 0.0
Sponge Gourd	SD1	7.0 $\pm$ 1.0	16.0 $\pm$ 1.0	69.0 $\pm$ 1.0	83.0 $\pm$ 1.0	116.0 $\pm$ 0.0
	SD2	5.0 $\pm$ 1.0	15.0 $\pm$ 1.0	67.0 $\pm$ 1.0	81.0 $\pm$ 1.0	113.0 $\pm$ 0.0
	SD3	4.0 $\pm$ 1.0	11.0 $\pm$ 1.0	65.0 $\pm$ 1.0	81.0 $\pm$ 1.0	108.0 $\pm$ 0.0
Cucumber	SD1	9.0 $\pm$ 1.0	16.0 $\pm$ 1.0	68.0 $\pm$ 1.0	92.3 $\pm$ 1.5	116.0 $\pm$ 0.0
	SD2	7.0 $\pm$ 1.0	15.0 $\pm$ 1.0	67.0 $\pm$ 1.0	78.0 $\pm$ 1.0	113.0 $\pm$ 0.0
	SD3	4.0 $\pm$ 1.0	11.0 $\pm$ 1.0	63.0 $\pm$ 2.6	76.0 $\pm$ 1.0	108.0 $\pm$ 0.0

Table S4 - Growing degree day (GDD) values of cucurbits at each developmental stage during summer-spring season

Crop	Sowing date	Emergence	Cotyledons completely unfolded	First flower open on main stem	First fruit on main stem	Harvested
Ridge Gourd	SD1	26.65 ± 1.53	56.33 ± 5.28	522.42 ± 8.80	965.80 ± 30.08	1595.40 ± 0.00
	SD2	51.32 ± 4.68	78.90 ± 9.20	584.30 ± 21.47	1035.62 ± 77.97	1764.50 ± 0.00
	SD3	129.37 ± 11.67	189.92 ± 10.35	670.05 ± 41.06	1120.88 ± 16.21	1773.20 ± 4.35
Bottle Gourd	SD1	47±4.2	101.3±7.6	1064.05±54.6	1326.3±35.1	1945.1±0.0
	SD2	125.3±6.6	176.1±17.6	1219.8±101.8	1506.6±99.8	2152.7±0.0
	SD3	260.8±16.1	305.8±9.2	1343.1±76.5	1572.8±37.7	2222.4±0.0
Sponge Gourd	SD1	38.2±3.4	109±14.9	1996±51.2	2538.2±33.7	2715.1±0.0
	SD2	118.6±5.0	176.5±22.8	2284.3±20.7	2704.9±61.3	2862.3±0.0
	SD3	239.8±8.3	306.7±19.8	0.0±0.0	0.0±0.0	2905.4±0.0
Cucumber	SD1	9.0±0.2	27.8±1.2	333.1±18.6	814.8±73.5	1340.1±0.0
	SD2	38.2±3.5	38.2±3.5	458.1±37.5	1068.8±37.3	1460.1±0.0
	SD3	96.1±0.0	125.9±7.4	589.2±79.8	1299.6±80.9	1621.9±0.0

Table S5 - Growing degree day (GDD) values of cucurbits at each developmental stage during kharif season

Crop	Sowing date	Emergence	Cotyledons completely unfolded	First flower open on main stem	First fruit on main stem	Harvested
Ridge Gourd	SD1	26.65 ± 1.53	56.33 ± 5.28	522.42 ± 8.80	965.80 ± 30.08	1595.40 ± 0.00
	SD2	51.32 ± 4.68	78.90 ± 9.20	584.30 ± 21.47	1035.62 ± 77.97	1764.50 ± 0.00
	SD3	129.37 ± 11.67	189.92 ± 10.35	670.05 ± 41.06	1120.88 ± 16.21	1773.20 ± 4.35
Bottle Gourd	SD1	47±4.2	101.3±7.6	1064.05±54.6	1326.3±35.1	1945.1±0.0
	SD2	125.3±6.6	176.1±17.6	1219.8±101.8	1506.6±99.8	2152.7±0.0
	SD3	260.8±16.1	305.8±9.2	1343.1±76.5	1572.8±37.7	2222.4±0.0
Sponge Gourd	SD1	38.2±3.4	109±14.9	1996±51.2	2538.2±33.7	2715.1±0.0
	SD2	118.6±5.0	176.5±22.8	2284.3±20.7	2704.9±61.3	2862.3±0.0
	SD3	239.8±8.3	306.7±19.8	0.0±0.0	0.0±0.0	2905.4±0.0
Cucumber	SD1	9.0±0.2	27.8±1.2	333.1±18.6	814.8±73.5	1340.1±0.0
	SD2	38.2±3.5	38.2±3.5	458.1±37.5	1068.8±37.3	1460.1±0.0
	SD3	96.1±0.0	125.9±7.4	589.2±79.8	1299.6±80.9	1621.9±0.0

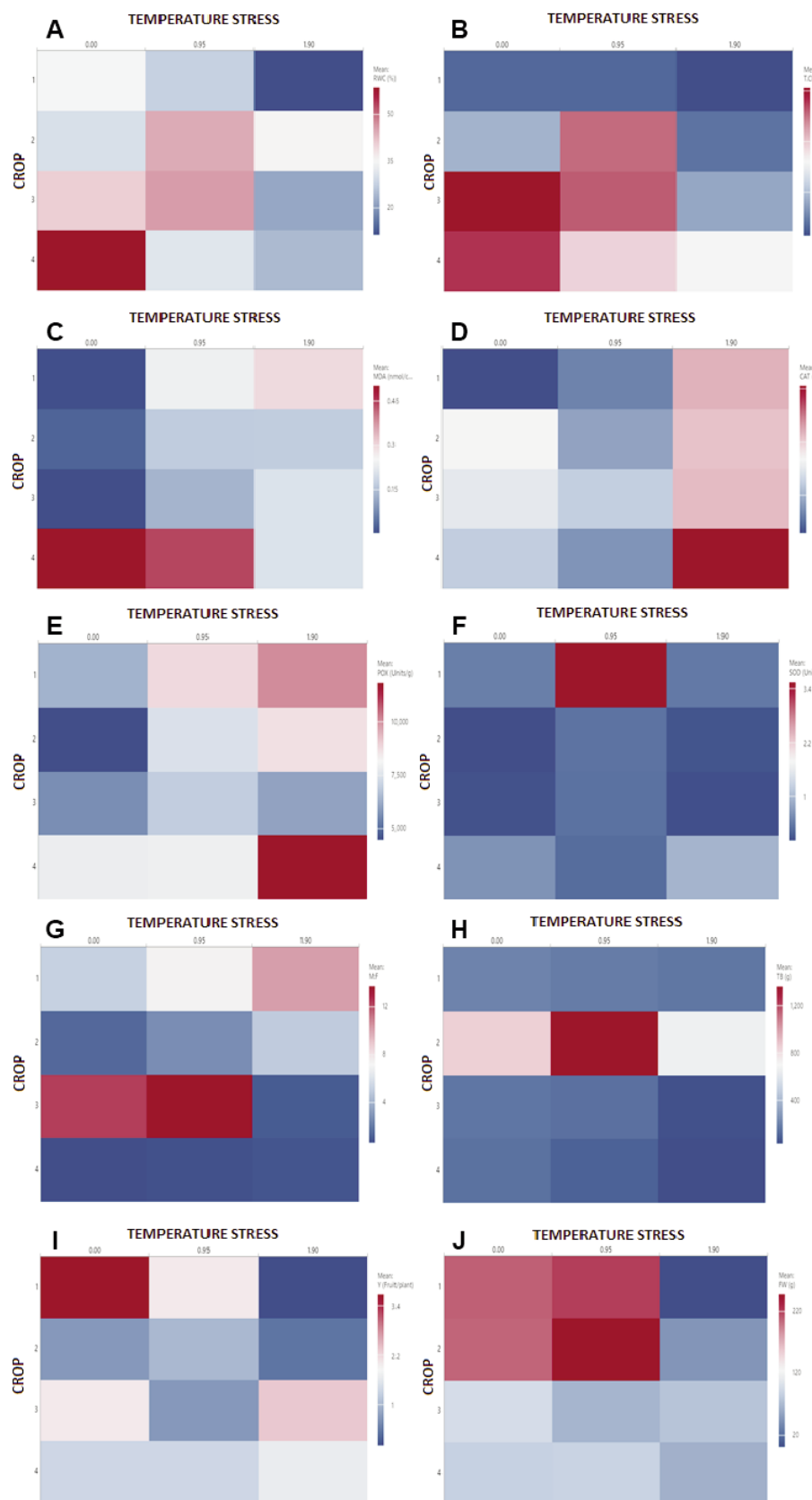


Fig. S1 - Heatmap of (a) Relative water content (RWC; %) (b) Total chlorophyll content (T.Chl; mg/g) (c) Catalase (CAT) activity (d) Lipid peroxidation (MDA; nmol/cm) (e) Peroxidase (POX) activity (mmol tetra-guaiacol/min/ g FW) (f) Superoxide Dismutase (SOD) activity (g) Male: Female flower ratio (M: F) (h) Total biomass (TB; g) (i) Yield (Y; Fruit/plant) (j) Fruit weight (FW; gm) in summer-spring

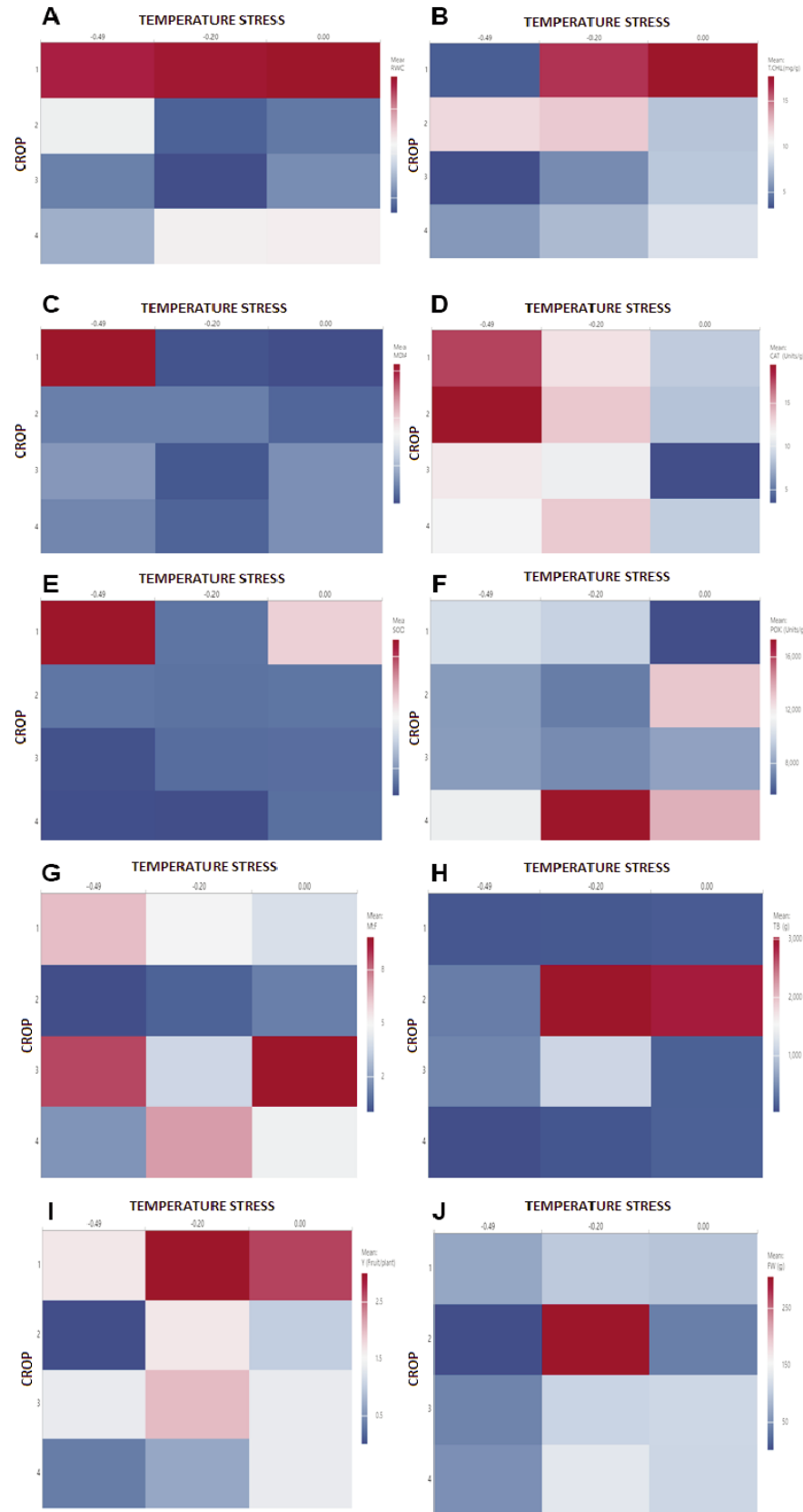


Fig. S2 - Heatmap of (a) Relative water content (RWC; %) (b) Total Chlorophyll Content (T.CHL; mg/g) (c) Catalase (CAT) activity (d) Lipid peroxidation (MDA; nmol/cm) (e) Peroxidase (POX) activity (mmol tetra-guaiacol /min/ g FW) (f) Superoxide Dismutase (SOD) activity (g) Male: Female flower ratio (M: F) (h) Total biomass (TB; g) (i) Yield (Y; Fruit/plant) (j) Fruit weight (FW; gm) in kharif

Table S6 - Physiological, reproductive and yield parameters of cucurbits across

Season	SD	Crop	Relative water content (%)	MDA (nmol/cm)	Catalase (units/g)	Peroxidase (units/g)	Superoxidase dismutase (units/g)	Total Chlorophyll (mg/g)	Male to female flower ratio	Fruit/plant	Fruit weight (g)	Total biomass (g)
Summer spring	1	Ridge Gourd	34.8 ± 2.5	0.004 ± 0.001	2.31 ± 1.6	6309.8 ± 1744	0.41 ± 0.3	3.04 ± 0.1	5 ± 0.78	4 ± 0.57	208.3 ± 16	202 ± 9.9
Summer spring	2	Ridge Gourd	27.4 ± 3.6	0.238 ± 0.06	5.09 ± 1.1	8822.7 ± 3114	3.53 ± 0.1	3.02 ± 0.1	7 ± 1	2 ± 1	221.7 ± 89	177 ± 10
Summer spring	3	Ridge Gourd	11.2 ± 6.2	0.297 ± 0.1	17.47 ± 5.1	10081.8 ± 344	0.37 ± 0.3	1.40 ± 0.08	10 ± 2.2	0 ± 0	0 ± 0	159 ± 6
Summer spring	1	Bottle Gourd	30.03 ± 5.34	0.03 ± 0.01	13.53 ± 3.39	4442.36 ± 910.48	0.01 ± 0.00	8.84 ± 0.86	1.37 ± 0.29	0.67 ± 0.33	206.44 ± 146.69	856.59 ± 19.67
Summer spring	2	Bottle Gourd	44.47 ± 8.57	0.17 ± 0.02	6.93 ± 1.74	7426.07 ± 585.93	0.31 ± 0.02	25.44 ± 2.09	2.60 ± 1.01	1.00 ± 0.58	247.33 ± 123.78	1362.66 ± 302.29
Summer spring	3	Bottle Gourd	35.42 ± 8.47	0.17 ± 0.00	16.70 ± 4.30	8660.40 ± 346.57	0.05 ± 0.02	3.95 ± 0.28	4.87 ± 2.13	0.33 ± 0.33	42.73 ± 42.73	658.83 ± 17.21
Summer spring	1	Sponge Gourd	40.67 ± 3.17	0.00 ± 0.00	11.93 ± 1.85	5585.21 ± 237.17	0.03 ± 0.02	30.57 ± 0.18	12.33 ± 3.84	2.00 ± 0.00	95.44 ± 1.28	156.16 ± 22.52
Summer spring	2	Sponge Gourd	45.97 ± 1.73	0.13 ± 0.03	9.75 ± 1.41	6890.98 ± 630.81	0.30 ± 0.01	26.17 ± 2.49	13.67 ± 3.53	0.67 ± 0.33	64.45 ± 32.23	138.60 ± 23.57
Summer spring	3	Sponge Gourd	21.70 ± 2.06	0.21 ± 0.12	17.06 ± 4.01	5984.65 ± 656.76	0.02 ± 0.01	7.80 ± 0.12	1.00 ± 1.00	2.33 ± 0.88	75.67 ± 11.57	40.24 ± 6.59
Summer spring	1	Cucumber	58.53 ± 4.29	0.50 ± 0.15	9.64 ± 1.99	7816.73 ± 671.19	0.59 ± 0.10	28.00 ± 1.31	0.60 ± 0.60	1.33 ± 0.33	85.00 ± 14.57	139.84 ± 12.58
Summer spring	2	Cucumber	31.13 ± 2.30	0.44 ± 0.04	6.06 ± 2.26	7912.59 ± 320.50	0.24 ± 0.04	19.14 ± 0.68	0.67 ± 0.67	1.33 ± 0.67	86.65 ± 43.57	89.68 ± 3.47
Summer spring	3	Cucumber	24.40 ± 2.69	0.21 ± 0.12	24.42 ± 6.31	11823.62 ± 594.58	0.92 ± 0.12	15.84 ± 0.92	0.80 ± 0.80	1.67 ± 0.33	61.33 ± 9.82	32.73 ± 5.52
Kharif	1	Ridge Gourd	88.18 ± 2.2	0.056 ± 0.005	8.67 ± 2.8	5591.8 ± 2356	1.73 ± 0.38	17.7 ± 5.9	4 ± 0.6	3 ± 0.5	93 ± 6	122 ± 10
Kharif	2	Ridge Gourd	86.96 ± 2.4	0.065 ± 0.01	12.56 ± 1.5	9634.1 ± 1755	0.26 ± 0.05	16.4 ± 2.2	5 ± 0.2	3 ± 1.0	96.8 ± 3.8	105 ± 6.2
Kharif	3	Ridge Gourd	83.97 ± 0.5	0.943 ± 0.03	17.69 ± 1.9	10148.5 ± 2693	2.84 ± 2.1	3.7 ± 2.0	7 ± 1.5	2 ± 0.5	66.7 ± 25.6	95 ± 2.5
Kharif	1	Bottle Gourd	19.27 ± 6.44	0.10 ± 0.02	8.23 ± 1.13	13079.26 ± 356.18	0.26 ± 0.01	7.62 ± 0.32	1.17 ± 0.67	1.00 ± 0.00	35.43 ± 5.30	2897.56 ± 1227.52
Kharif	2	Bottle Gourd	14.83 ± 3.07	0.16 ± 0.01	13.67 ± 1.28	6902.57 ± 309.35	0.24 ± 0.01	12.37 ± 0.62	0.44 ± 0.42	1.67 ± 0.00	304.29 ± 9.60	3023.33 ± 35.07
Kharif	3	Bottle Gourd	47.17 ± 2.98	0.16 ± 0.00	19.46 ± 2.12	7787.91 ± 1172.70	0.27 ± 0.00	11.85 ± 0.65	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	372.07 ± 65.77
Kharif	1	Sponge Gourd	23.30 ± 8.34	0.20 ± 0.05	3.42 ± 0.86	7995.93 ± 218.43	0.20 ± 0.01	7.73 ± 0.40	9.83 ± 2.13	1.33 ± 0.33	111.32 ± 36.25	149.87 ± 68.79
Kharif	2	Sponge Gourd	11.60 ± 3.07	0.07 ± 0.01	10.84 ± 1.28	7313.60 ± 309.35	0.20 ± 0.01	5.29 ± 0.62	3.57 ± 0.42	2.00 ± 0.00	107.67 ± 9.60	1112.00 ± 35.07
Kharif	3	Sponge Gourd	20.80 ± 7.93	0.22 ± 0.04	12.25 ± 3.81	7813.28 ± 406.17	0.03 ± 0.01	3.18 ± 0.69	8.67 ± 2.60	1.33 ± 0.67	39.15 ± 19.64	427.68 ± 117.64
Kharif	1	Cucumber	52.80 ± 3.26	0.20 ± 0.05	8.72 ± 1.25	13652.57 ± 121.16	0.21 ± 0.00	9.02 ± 0.22	4.64 ± 4.19	1.33 ± 0.33	110.00 ± 15.95	152.88 ± 63.98
Kharif	2	Cucumber	51.83 ± 2.74	0.10 ± 0.03	13.57 ± 0.94	17302.01 ± 1272.17	0.01 ± 0.00	7.22 ± 0.47	7.22 ± 6.41	0.67 ± 0.33	130.65 ± 15.37	84.67 ± 5.96
Kharif	3	Cucumber	30.23 ± 3.20	0.18 ± 0.00	11.28 ± 3.27	10974.00 ± 803.68	0.02 ± 0.01	5.77 ± 0.69	1.67 ± 1.67	0.33 ± 0.33	48.17 ± 24.55	34.34 ± 5.41

Table S7 - Yield Indices of cucurbits across different sowing dates

Season	SD	Crop	TOL = $Y_p - Y_s$	(YSI) = Y_s/Y_p	MP
1	1	1	0	0	0
1	2	1	1.666666667	0.5454545455	2.833333333
1	3	1	3.666666667	0	1
2	1	1	0	0	0
2	2	1	-0.3333333333	1.125	2.833333333
2	3	1	1	0.625	2.166666667
1	1	2	0	0	0
1	2	2	-0.3333333333	1.5	0.7886751346
1	3	2	0.3333333333	0.5	0.5
2	1	2	0	0	0
2	2	2	-0.6666666667	1.666666667	1.333333333
2	3	2	1	0	0.5
1	1	3	0	0	0
1	2	3	1.3333	0.33335	1.33335
1	3	3	-0.3333333333	1.166666667	2.166666667
2	1	3	0	0	0
2	2	3	-0.6666666667	1.5	1.666666667
2	3	3	-3.333333333e-05	1.000025	1.33335
1	1	4	0	0	0
1	2	4	-3.333333333e-05	1.000025	1.33335
1	3	4	-0.3333333333	1.25	1.5
2	1	4	0	0	0
2	2	4	0.6666333333	0.500025	1.000016667
2	3	4	0.9999333333	0.25005	0.8333666667

Table S8 - Cumulative cucurbit growth Index mean values and sensitivity index values

Season	Crop	Sowing date	Mean index value $\pm$ SD	Sensitivity index	Average SI (Late sowing)
Summer-spring	1 (RG)	1	0.4576 $\pm$ 0.0131	0	-0.02140895394
Summer-spring	1 (RG)	2	0.5465 $\pm$ 0.0440	0.1943001083	
Summer-spring	1 (RG)	3	0.3491 $\pm$ 0.0215	-0.2371180162	
Summer-spring	2 (BG)	1	0.4430 $\pm$ 0.0945	0	-0.1064775902
Summer-spring	2 (BG)	2	0.4714 $\pm$ 0.0930	0.06412520113	
Summer-spring	2 (BG)	3	0.3203 $\pm$ 0.0663	-0.2770803815	
Summer-spring	3 (SG)	1	0.5149 $\pm$ 0.0134	0	-0.1727891155
Summer-spring	3 (SG)	2	0.4530 $\pm$ 0.0170	-0.1200692052	
Summer-spring	3 (SG)	3	0.3988 $\pm$ 0.0437	-0.2255090258	
Summer-spring	4 (CUC)	1	0.3774 $\pm$ 0.0767	0	0.1354899498
Summer-spring	4 (CUC)	2	0.3959 $\pm$ 0.0154	0.04883999009	
Summer-spring	4 (CUC)	3	0.4613 $\pm$ 0.0471	0.2221399095	
Kharif	1 (RG)	1	0.4022 $\pm$ 0.0429	0	-0.3379965887
Kharif	1 (RG)	2	0.3419 $\pm$ 0.0171	-0.1497972652	
Kharif	1 (RG)	3	0.1905 $\pm$ 0.0780	-0.5261959122	
Kharif	2 (BG)	1	0.3003 $\pm$ 0.1137	0	0.02706475008
Kharif	2 (BG)	2	0.4263 $\pm$ 0.0303	0.4196478811	
Kharif	2 (BG)	3	0.1905 $\pm$ 0.0127	-0.365518381	
Kharif	3 (SG)	1	0.4147 $\pm$ 0.0644	0	-0.08576993242
Kharif	3 (SG)	2	0.4125 $\pm$ 0.0082	-0.005211396563	
Kharif	3 (SG)	3	0.3457 $\pm$ 0.0397	-0.1663284683	
Kharif	4 (CUC)	1	0.3559 $\pm$ 0.0068	0	0.03716063261
Kharif	4 (CUC)	2	0.3727 $\pm$ 0.0255	0.04746390605	
Kharif	4 (CUC)	3	0.3654 $\pm$ 0.0194	0.02685735917	



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Bok choy (*Brassica rapa* L.) growth and yield in a floating system on freshwater swamps with various substrate composition and number of wicks

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Key words: Capillary, composition, floating, planting media, water content.

Abstract: Bok choy (*Brassica rapa* L.) is commonly cultivated using a floating system, a sustainable agricultural method suitable for flooded areas. This study aimed to evaluate the growth and yield of Bok choy grown using different substrate compositions and numbers of wicks. This study implemented following Randomized Block Design (RBD) with two factors. The first factor was the substrate composition: topsoil (M1), soil + cow manure + sand (4:2:1) (M2), and soil + cow manure + rice husk biochar (4:2:1) (M3). The second factor was the number of wicks: two (S2), three (S3), and four (S4). The results demonstrated that substrate composition and the number of wicks significantly influenced water mobilization, soil moisture, and plant performance. Substrates containing manure with sand (M2) or rice husk biochar (M3) showed superior growth and yield compared to topsoil (M1). Additionally, using three wicks (S3) optimized water absorption, resulting in the best plant growth. The combination of soil + cow manure + rice husk biochar (M3) with three wicks (S3) produced the highest growth and yield of Bok choy in floating systems within freshwater swamp conditions. These findings provide practical applications for improving sustainable Bok choy cultivation in swampy and flood-prone areas.

1. Introduction

Population growth and the continuous reduction of arable land have

raised serious concerns regarding global food security. Technologies continues to be evolved in efforts to enhance sustainable productivity through the utilization of marginal land (Gustiar *et al.*, 2025; Lakitan *et al.*, 2025). Freshwater swamps are suboptimal lands that hold great potential to be utilized as agricultural land. In Indonesia, approximately 1.8 million ha of swamp land have been developed, consisting of 1,452,569 ha of tidal marshes and 347,431 ha of non-tidal marshes. Qualitative descriptive and quantitative assessments conducted in Central Kalimantan, Indonesia, indicated that swamp lands cover around 33.4 million hectares, with approximately 9.5 million hectares having potential for agricultural use (Fahmid *et al.*, 2022). Despite this potential, agricultural utilization of swamp lands is constrained by seasonal flooding and prolonged waterlogging, which limit the application of conventional cultivation systems. Floating cultivation has emerged as a promising alternative to overcome these constraints. The floating vegetable cultivation technique is a vegetable production system on swampy land that uses rafts as a solution during flooding or inundation (Siaga and Lakitan, 2021). This system aligns with the principles of sustainable agriculture by enabling the use of marginal lands, reducing pressure on fertile soils, maintaining ecosystem balance, and supporting long-term food security. One major advantage of floating cultivation is that plants do not require manual irrigation, as moisture is continuously supplied through capillary movement from the underlying water body (Jaya *et al.*, 2021; Susilawati *et al.*, 2024). Efficient water management without intensive watering contributes to water conservation and enhances system sustainability, particularly under increasingly unpredictable climate conditions.

Brassica rapa L. (Bok choy) is a leafy vegetable with a relatively short growth cycle and high nutritional value, making it suitable for cultivation in adaptive production systems. Bok choy has shown adaptability to diverse growing environments, including soilless and floating cultivation systems. Previous studies reported that plants grown under floating cultivation exhibited higher leaf numbers and better leaf and petiole development, particularly in tatsoi and green mustard (Siaga and Lakitan, 2021; Ria *et al.*, 2025). Bok choy is also among the most widely consumed leafy vegetables, with steadily increasing market demand, making it economically attractive for cultivation in marginal environments

(Muslimah *et al.*, 2024). Bok choy contains nutrients including protein, fat, carbohydrates, calcium, phosphorus, magnesium (Mg), iron (Fe), and vitamins A, B, and C (Veazie *et al.*, 2020).

The increasing demand for vegetables driven by population growth necessitates the expansion of cultivation areas beyond conventional agricultural land. Utilization of freshwater swamps offers a strategic option to meet this demand (Hidayatulah *et al.*, 2022). However, swamp agriculture faces dual challenges of drought during dry seasons and flooding during rainy seasons. Floating cultivation systems have been shown to produce yields comparable to, or even higher than, conventional land-based cultivation under such conditions (Lakitan *et al.*, 2018). Cultivation on water surfaces, either using terrestrial or aquatic crops, has therefore been proposed as a sustainable agricultural strategy for tropical regions with extensive flooded areas (Radulovich *et al.*, 2015).

Floating vegetable cultivation is conducted by positioning substrate pots so that their bases are in direct contact with the water surface, allowing continuous water supply through capillary movement. Therefore, the raft load must be carefully adjusted to ensure sufficient submergence for water contact without compromising buoyancy and structural stability (Jaya *et al.*, 2021). In practice, maintaining a stable raft position and uniform water contact is challenging due to fluctuations in water level and changes in plant biomass. However, maintaining uniform and stable water contact under field conditions is challenging. A practical solution is the use of floating rafts combined with a hydroponic approach employing a wick irrigation system. Wick irrigation is considered a simple, low-cost, and sustainable technique capable of maintaining continuous substrate moisture through capillary action (Junejo *et al.*, 2022). In this system, water is transported from the water body to the planting medium via wicks, and substrate moisture is influenced by factors such as substrate composition, wick length, wick diameter, and the number of wicks used (Oh *et al.*, 2007). Despite its importance, information on the optimal number of wicks for floating vegetable cultivation remains limited. While increasing the number of wicks may enhance water supply, excessive wicks may reduce system efficiency and potentially impair root aeration.

In addition to wick number, substrate composition plays a critical role in regulating capillary

movement, water retention, and aeration. Modifications in soil physical properties can significantly influence capillarity, with biochar addition reported to increase capillary pore space and water-holding capacity (Šimanský *et al.*, 2022). Rice husk biochar is known for its high porosity and cation exchange capacity, which may improve water retention and nutrient availability, while sand contributes to improved drainage and aeration. The planting medium is therefore a key factor determining growth and yield performance of Bok choy under floating cultivation (Rizal *et al.*, 2023). Incorporation of manure further supports nutrient supply, particularly nitrogen, phosphorus, and potassium, which are essential for vegetative growth (Bartley *et al.*, 2022). The soil-based growing medium alone does not fully meet plant nutrient requirements; therefore, manure is added as an organic nutrient source to improve nutrient availability. However, the interactive effects between substrate composition and wick number on the growth and yield of Bok choy under floating cultivation in freshwater swamps remain poorly understood. Therefore, this study aimed to experimentally evaluate the effects of different substrate compositions and wick numbers on growth and yield parameters of Bok choy cultivated under floating conditions in freshwater swamps.

2. Materials and Methods

This study was conducted at the Field Laboratory of Freshwater Swamp, Faculty of Agriculture, Sriwijaya University, South Sumatra. The implementation period of the study was from May to August 2024.

Experimental design and planting material

This study was conducted using a factorial randomized block design (FRBD) with two treatment factors. The first factor was substrate composition, consisting of M1 = soil, M2 = soil : cow manure : sand (4:2:1, v/v), and M3 = soil : cow manure : rice husk biochar (4:2:1, v/v). The second factor was the number of wicks, comprising S2 = two wicks, S3 = three wicks, and S4 = four wicks (Fig. 1A). Each treatment combination was replicated three times, and each experimental unit consisted of three plants, resulting in a total of 81 plants. Bok choy (*Brassica rapa* L.) var Nauli F1 were used in this experiment. Seedlings were grown in a nursery until reaching a

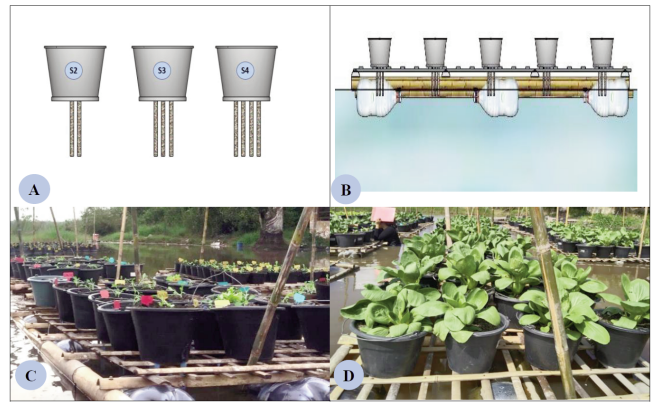


Fig. 1 - Treatment of the number of wicks (A), the appearance of the floating raft design (B), and the study of floating Bok choy cultivation activities (C, D).

uniform size prior to transplanting. Uniform seedlings were transplanted into plastic pots with a diameter of 20 cm, each filled with planting growing medium according to the assigned substrate treatment.

Plants were arranged on floating rafts measuring 2.0×1.2 m, constructed from bamboo and supported by nine recycled 19^L mineral water gallons to maintain buoyancy. Pot spacing on the raft was arranged uniformly to avoid shading effects. Each pot was connected to the underlying freshwater through flannel cloth wicks, which acted as capillary channels supplying water from the swamp to the planting growing medium. The dimensions of each wick were kept uniform across treatments to ensure consistency in water supply, with only the number of wicks varying according to treatment.

Variables and statistical analysis

The parameters observed included the planting media condition, especially humidity. Growth parameters were leaf length, width, area, and number. Leaf morphology was measured by SPAD values using Minolta 504, canopy area and leaf area using the Eazyleaf area meter device, stem diameter, root length, and plant height. Data on bok choy growth and yield were analyzed using two-way analysis of variance (ANOVA) to evaluate the effects of substrate composition, number of wicks, and their interaction. Statistical analyses were performed using R Studio software. When significant differences were detected, mean comparisons were conducted using LSD test at a 5% significance level.

Preparation of floating cultivation raft

The floating raft was made of 200 x 120 cm bamboo with the upper and lower parts separated

and modified to avoid direct contact with the water in the freshwater swamps using 9 plastic gallons as floats. The design of the raft used and the study activities of the floating Bok choy is shown in figure 1. Floating cultivation was carried out using a wick system hydroponic cultivation approach where the planting media derive a water supply without direct contact with the surface of the freshwater swamps where the water depth ranged from approximately 50 to 100 cm under tropical conditions. Flannel cloth was used as a connector for water with substrate and capillary capacity, the distance between the base of the pot and the water surface was ± 10 cm.

Agroclimatic conditions at experimental site

During the study activities, rain only occurred once, at the beginning. Temperature and humidity conditions were quite fluctuating, with the highest air temperature not exceeding 30°C, and the highest humidity not exceeding 20%. Agroclimatology conditions during the study activities are shown in figure 2.

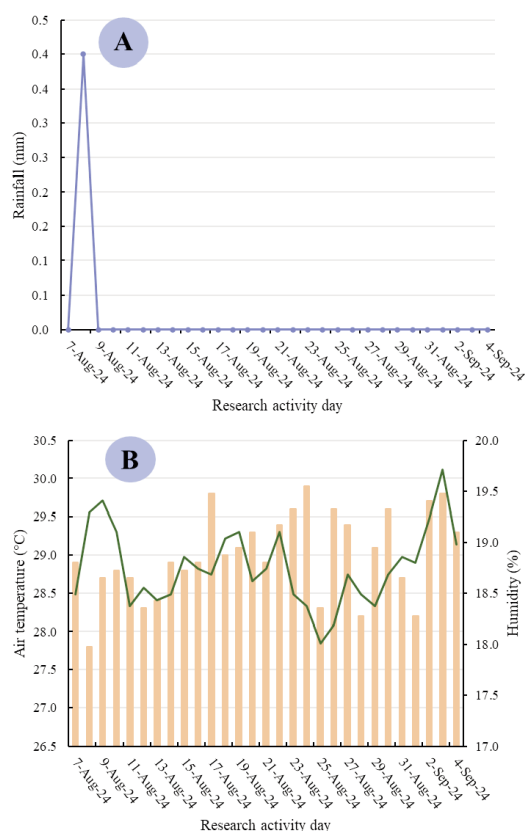


Fig. 2 - Rainfall (A), temperature, and humidity (B) patterns at experimental sites during study period (August to September, 2024). Data source: Meteorological, Climatological, and Geophysical Agency/BMKG.

3. Results and Discussion

Environment conditions and soil moisture dynamics

Freshwater swamps are characterized by alternating conditions of drought, fire, and flooding (Kurniawan, 2024). This study was conducted in a deep freshwater swamp during the dry season, when the land remained inundated despite very low rainfall. Deep freshwater swamps are land inundated throughout the year (Ratmini and Herwenita, 2021) where almost all plant cultivation activities cannot be carried out. Conducting the experiment during the dry season minimized rainfall interference, allowing the effects of substrate composition and wick number on water distribution within the floating system to be evaluated more clearly. Under these conditions, plant growth was primarily influenced by the ability of the substrate-wick system to regulate water availability rather than by external environmental fluctuations.

Substrate composition and wick number significantly influenced soil moisture content in the floating cultivation system. The highest moisture content was recorded in the M3 substrate (soil:cow manure:rice husk biochar, 4:2:1), whereas the lowest moisture content occurred in M1 (soil only). Increasing the number of wicks resulted in higher substrate moisture, indicating enhanced capillary water supply. A significant interaction between substrate composition and wick number was observed, with the highest moisture level occurring in the M3S4 treatment (Fig. 3).

Substrate composition plays a crucial role in determining bulk density, texture, and water-holding capacity. The incorporation of organic matter, rice husk biochar, and sand altered the physical and hydraulic properties of the planting media, thereby improving its ability to absorb and retain water. A mixture of mineral soil and organic matter can cause changes in the physical and hydraulic properties (Wallach, 2019). This explains the higher moisture observed in M2 and M3 compared with M1, which relied solely on mineral soil. In particular, rice husk biochar has been shown to increase porosity, improve aeration for root respiration, and stabilize soil moisture by retaining water in micropores (Singh *et al.*, 2018). These properties support sustained water availability and microbial activity within the root zone.

Regarding wick number, treatments S3 (three wicks) and S4 (four wicks) consistently exhibited

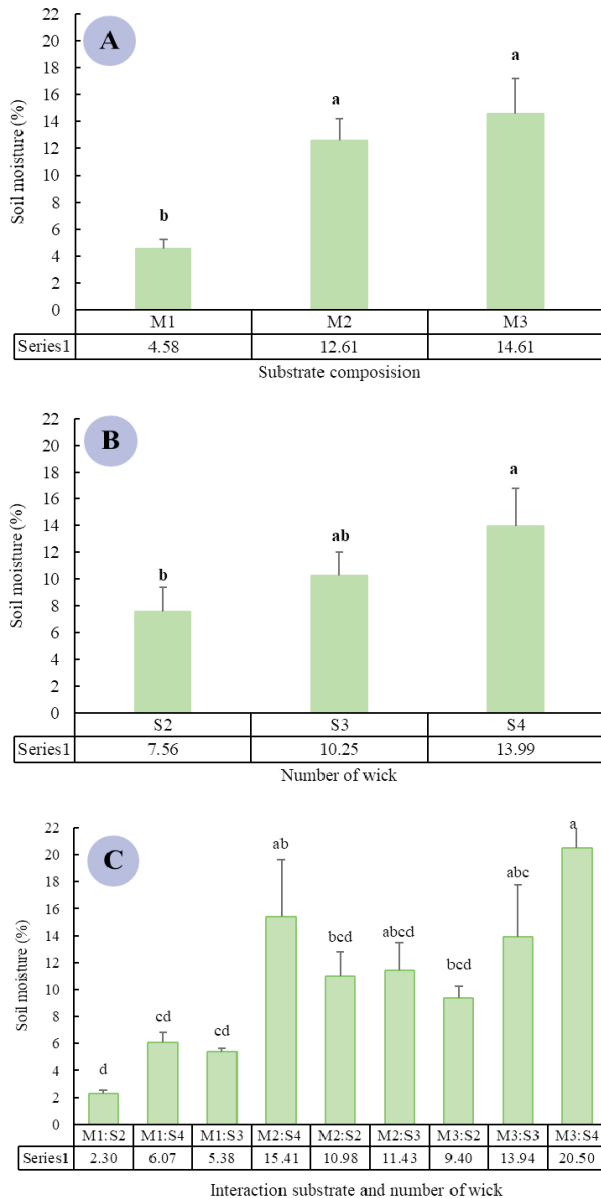


Fig. 3 - Soil moisture conditions under different planting substrate compositions (A), wick numbers (B), and combinations of substrate composition and wick number (C) in floating cultivation.

higher substrate moisture than S2 (two wicks). Increasing wick number increases the number of capillary pathways, thereby enhancing water transport from the swamp to the root zone (Ansar *et al.*, 2019). Wick irrigation functions as a subsurface capillary irrigation system, where water movement is driven by capillary forces rather than gravity (He *et al.*, 2024). However, although S4 produced the highest moisture content, excessive moisture may reduce root-zone aeration, suggesting the existence of an optimal wick number rather than a linear response. Similar studies on subsurface irrigation

systems have reported benefits such as reduced evaporation, nutrient conservation, and improved plant quality, while excessive wetness may increase the risk of oxygen limitation in the root zone (Felipe and Bareng, 2022).

Growth rate of Bok choy (*Brassica rapa* L.)

In general, leaf length, width, and area increased progressively with plant age, particularly from leaf expansion until 12 days after full leaf opening. Substrate composition significantly affected the rate of leaf enlargement. In the M1 treatment, leaf growth tended to stagnate after the fifth day, whereas in M2 and M3, leaf length, width, and area continued to increase until the twelfth day with relatively similar growth rates (Fig. 4). At 25 days after planting (DAP), plants grown in M1 exhibited smaller leaves (length 6.5 cm, width 5.0 cm, leaf area 15 cm²) compared with those grown in M2 and M3. The slower leaf growth in M1 was associated with suboptimal physical and nutritional properties of the soil-only substrate. Although M1 exhibited relatively high moisture levels, the absence of organic amendments likely limited nutrient availability and

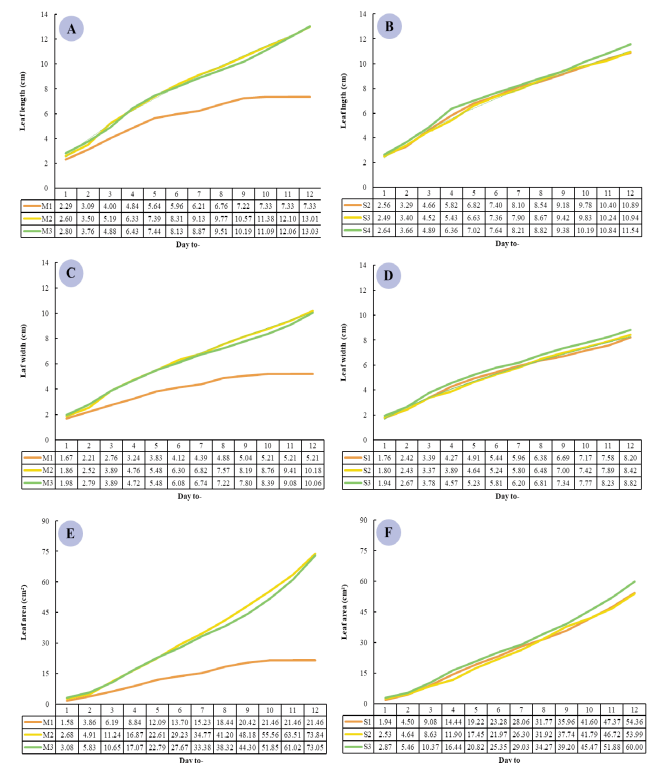


Fig. 4 - Effects of substrate composition (left) and number of wicks (right) on the daily rate of increase in leaf length (A, B), leaf width (C, D), and leaf area (E, F) of Bok choy grown under floating cultivation. Leaf traits were measured repeatedly over a 12-day cultivation period.

root development. In contrast, substrates M2 and M3, which contained cow manure, provided improved nutrient supply and better physical conditions for root growth, thereby supporting sustained leaf expansion.

The number of wicks also influenced leaf growth. The largest leaf dimensions were observed in S4, with leaf length of approximately 11 cm, width of 9 cm, and leaf area of 63 cm². However, leaf growth in S3 was statistically comparable to S4 and not markedly different from S2, indicating that increasing wick number beyond three did not proportionally enhance leaf expansion. This suggests that three wicks may provide sufficient water supply for optimal leaf growth without causing excessive moisture accumulation.

M2 and M3 treatments exhibited faster and more sustained leaf growth than M1, reflecting the combined effects of improved substrate structure and nutrient availability. The presence of organic matter in M2 and M3 likely enhanced soil pH balance, nutrient availability, and microbial activity, which are essential for vegetative growth (Ho *et al.*, 2022). Manure contributes essential nutrients and increases soil organic matter, thereby stimulating plant growth, whereas mineral soil alone is often insufficient to meet the nutritional requirements of Bok choy (Augustien and Suhardjono, 2016).

Substrate composition and wick number influenced the temporal pattern of leaf production in Bok choy plants. During the early growth stage (up to 13 DAP), the highest leaf number was observed in the M2 substrate, whereas at 16 DAP and towards the end of the experiment, plants grown in M3 exhibited the greatest number of leaves. In contrast, M1 consistently produced the lowest leaf number from 10 DAP until harvest, indicating suboptimal growing conditions in the soil-only substrate (Fig. 5 A).

The number of wicks did not significantly affect leaf number during the early growth phase (≤13 DAP). However, from 16 DAP onwards, treatments S3 (three wicks) and S4 (four wicks) produced a greater number of leaves than S2 (two wicks). Although S4 recorded the highest leaf number at harvest, it was not statistically different from S3, suggesting that increasing wick number beyond three provided no additional advantage for leaf initiation (Fig. 5 B).

The delayed response of leaf number to substrate and wick treatments indicates that early vegetative growth was not limited by water availability. At the initial growth stage, the root system was still

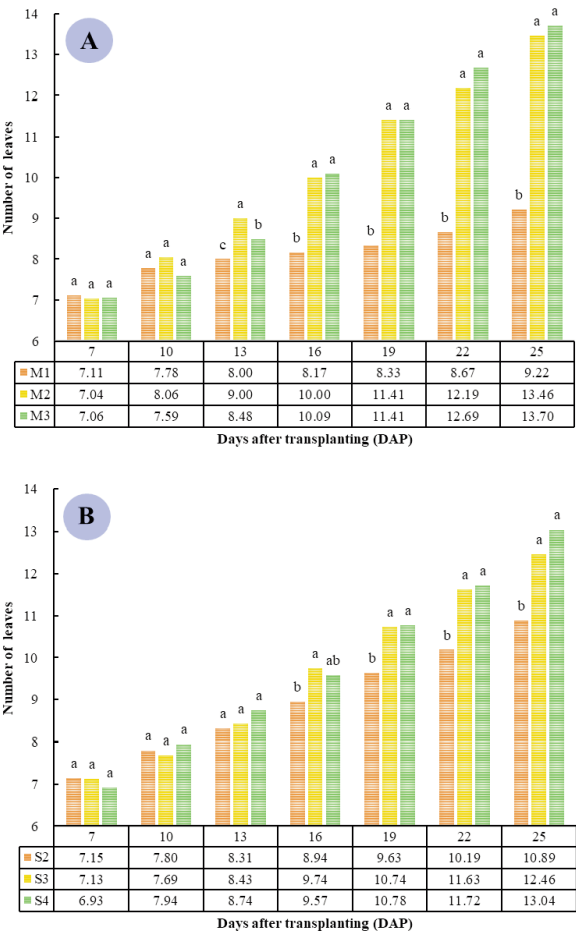


Fig. 5 - The leaf growth rate in different substrate composition (A) and number of wicks (B).

underdeveloped, limiting the plant’s capacity to utilize increased water supply from additional wicks. Consequently, differences in leaf number became apparent only after root establishment, when plants could more effectively exploit substrate moisture and nutrient availability.

These findings are consistent with Maludin *et al.* (2020), who reported that significant differences in Bok choy growth parameters emerged only after 15 DAP, reflecting the transition from establishment to active vegetative growth. The superior performance of M3 at later stages may be attributed to the improved physical structure and water-holding capacity provided by rice husk biochar, which supports sustained leaf initiation under continuous moisture supply. Meanwhile, the comparable performance of S3 and S4 suggests the existence of an optimal wick number, beyond which additional water supply does not proportionally enhance leaf production and may risk excessive moisture in the root zone.

Morphology and yield of Bok choy

In general, substrate composition affects the observed SPAD and plant morphological variables, including, canopy area, total leaf area, stem diameter, root length, and plant height. Substrate treatment using only soil (M1) had the smallest value for all morphological variables. The highest value was found in M3, but not significantly different from M2. This is presumably because the addition of manure supplies the nutrient needs of Bok choy plants, thereby making plants grow better. The morphology of the plant is presented in figure 6. The number of wicks also affected plant morphological variables where the smallest value was found in the pot prepared using two wicks (S2). The largest value was found in plants with four wicks (S4) but not significantly different from plants using three wicks (S3). In the interaction of substrate composition treatment and the number of wicks, the highest value was found in the M2S3 combination, while the lowest was recorded in the M1S2 treatment. The overall data are presented in Table 1.

Changes in plant morphology also affect the variables of plant yield in the form of fresh and dry

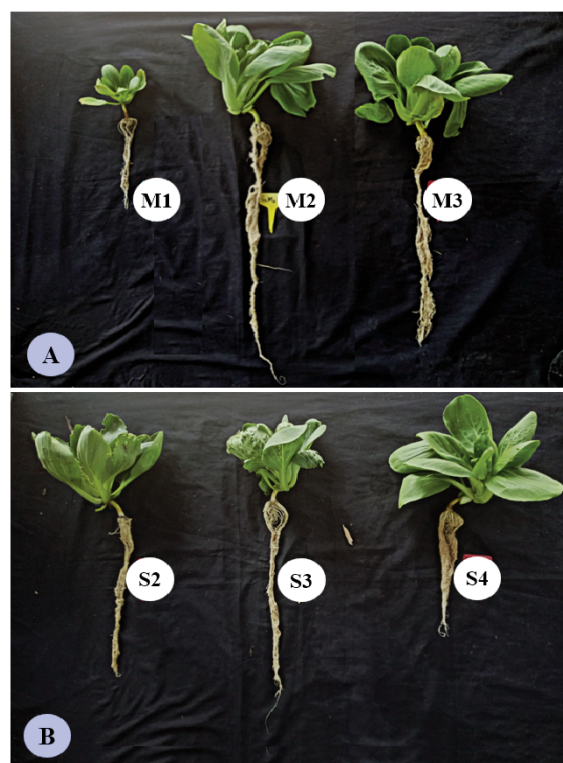


Fig. 6 - Bok choy plants grown under various substrates (A) using 3 types of wicks (B).

Table 1 - Effect of substrate composition and number of wicks on the morphology of Bok choy plants in floating cultivation

Description	SPAD value	Canopy area (cm ²)	Total leaf area (cm ²)	Stem diameter (mm)	Root length (cm)	Plant height (cm)
Substrate composition						
M1	38.26 b	126.28 b	130.94 b	3.96 b	28.83 b	3.79 a
M2	49.61 a	615.07 a	1056.52 a	7.80 a	45.81 a	3.85 a
M3	52.55 a	516.46 a	1079.88 a	8.14 a	43.77 a	4.07 a
LSD0.05	3.52	215.45	328.21	1.33	10.48	0.56
Number of wicks						
S2	45.57 a	257.79 b	520.10 b	5.51 b	35.52 b	3.97 a
S3	47.08 a	486.10 a	849.30 a	7.25 a	41.39 a	3.79 a
S4	47.77 a	513.93 a	897.94 a	7.14 a	41.51 a	3.95 a
LSD0.05	3.52	215.45	328.21	1.33	10.48	0.56
Interaction substrate composition and number of wicks						
M1S2	33.57 d	99.70 d	93.53 c	3.35 d	26.83 c	3.77 a
M1S3	38.23 cd	106.77 d	138.56 c	4.00 cd	27.65 c	3.68 a
M1S4	42.98 bc	172.38 d	160.73 c	4.53 cd	32.02 bc	3.92 a
M2S2	50.20 a	349.32 bcd	574.78 bc	6.15 bc	35.98 abc	3.83 a
M2S3	50.88 a	785.87 a	1275.45 a	9.02 a	48.47 ab	4.18 a
M2S4	47.75 ab	710.03 ab	1319.33 a	8.23 ab	52.98 a	3.53 a
M3S2	52.95 a	324.35 cd	892.00 ab	7.02 ab	43.73 abc	4.31 a
M3S3	52.13 a	565.67 abc	1133.88 ab	8.73 a	48.07 ab	3.50 a
M3S4	52.57 a	659.37 abc	1213.75 a	8.67 a	39.51 abc	4.41 a
LSD0.05	6.10	373.17	568.49	2.31	18.14	0.97

The means followed by different letters within each column indicate significant difference at the LSD 0.05.

leaf, stem, and root weights. The smallest planting substrate composition treatment was found in M1, and the highest weight was recorded in M3, but not significantly different from M2. The greatest wick treatment was found in S4, which was significantly different from S2 but not S3. Complete data are shown in Table 2.

In general, the best substrate treatments were found in M2 and M3 while the best wick treatment was found in the use of three (S3) or four wicks (S4). The interaction of substrate composition and number wicks had a very significant effect on fresh and dry weight variables. The highest fresh leaf weight was found in M2S3 at 139.07 g, the highest dry leaf weight as well as fresh and dry stem weight were found in M3S4, and the highest root weight was recorded in M2S4.

The effect of M2 was not significantly different from M3 but differed from M1 in terms of plant morphology and yield variables. This is the effect of administering manure and rice husk biochar or sand which enhance the biochemical and physical properties of the soil. Changes in the biochemical and physical properties of the soil produce better growth,

which will have implications for improving plant biomass. Manure is a material with high levels of micro and macronutrients. Furthermore, the administration of cow manure to the planting media has been proven to increase microbial composition, soil enzyme activity, and soil carbon (Ozlu *et al.*, 2019). The use of rice husk charcoal or biochar as a soil conditioner has long been recognized in agriculture to improve soil productivity. Given that biochar has a high CEC, it can bind soil cations crucial for growth. Furthermore, the role of biochar in the soil is to keep moisture and improve fertility. The benefits of administering rice husk biochar to the soil include enhancing air and water circulation as well as providing nutrients to evoke growth (Setyawan, 2023).

4. Conclusions

In conclusion, the highest growth and yield performance of Bok choy was found in the substrate with soil, cow manure and sand - the ratio 4:2:1 (M2). In general, the floating cultivation system

Table 2 - Effect of substrate composition and number of wicks on the weight of Bok choy plant biomass in floating cultivation

Description	Fresh leaf weight (g)	Dry leaf weight (g)	Fresh stem weight (g)	Dry stem weight (g)	Fresh root weight (g)	Dry root weight (g)
Substrate composition						
M1	10.15 b	1.49 b	0.99 b	0.13 b	3.27 b	0.31 b
M2	101.09 a	9.56 a	2.55 a	0.32 a	13.22 a	1.35 a
M3	103.67 a	9.88 a	3.08 a	0.36 a	15.31 a	1.26 a
LSD0.05	29.79	2.42	0.65	0.08	4.92	0.45
Number of wicks						
S2	45.79 b	4.42 b	1.69 b	0.23 a	6.20 b	0.67 b
S3	84.75 a	7.68 a	2.47 a	0.27 a	12.12 a	0.98 ab
S4	84.37 a	8.82 a	2.47 a	0.30 a	13.48 a	1.27 a
LSD0.05	29.79	2.42	0.65	0.08	4.92	0.45
Interaction substrate composition and number of wicks						
M1S2	6.38 d	1.07 de	0.80 c	0.10 e	1.55 c	0.19 d
M1S3	9.31 d	1.01 e	0.94 c	0.12 de	3.57 bc	0.22 d
M1S4	14.76 d	2.38 de	1.25 c	0.17 cde	4.70 bc	0.51 cd
M2S2	50.20 cd	5.25 cd	1.66 bc	0.24 bcd	5.98 bc	0.78 bcd
M2S3	139.07 a	12.06 a	3.38 a	0.41 a	15.61 a	1.53 ab
M2S4	113.99 ab	11.38 a	2.62 ab	0.31 ab	18.08 a	1.75 a
M3S2	80.78 bc	6.97 bc	2.62 ab	0.36 ab	11.09 ab	1.04 abc
M3S3	105.87 ab	9.95 ab	3.08 a	0.30 abc	17.19 a	1.20 abc
M3S4	124.36 ab	12.70 a	3.54 a	0.42 a	17.65 a	1.54 ab
LSD0.05	51.59	4.20	1.13	0.14	8.52	0.78

The means followed by different letters within each column indicate significant difference at the LSD 0.05.

required at least three wicks (S3) to achieve satisfactory growth and yield across most substrate compositions, whereas two wicks (S2) were less effective. However, for the M2 substrate, two wicks (S2) were sufficient to provide adequate water and nutrient supply, resulting in the best performance among all treatment combinations (M2S2).

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Heat and light induced physiological disorders in apples: Sunburn, mechanisms, and management strategies

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Key words: Climate change, fruit surface temperature, heat stress, oxidative stress, photoselective net, sunburn.

Abstract: Global climate change has intensified heat and light stress in apple (*Malus domestica* Borkh.) orchards, markedly increasing the incidence of sunburn and related physiological disorders. Sunburn represents a complex stress response triggered when fruit surface temperature (FST) exceeds critical thresholds, leading to oxidative imbalance and structural damage. This review provides an integrated synthesis of sunburn typology - sunburn browning, sunburn necrosis, and photo-oxidative sunburn - and explores underlying cellular and biochemical mechanisms, including reactive oxygen species (ROS) accumulation, membrane peroxidation, and pigment degradation. Evidence indicates that FST values between 46-52°C are decisive in the onset of photo-oxidative injury and necrotic lesions, compromising fruit integrity and storage potential. Secondary disorders such as watercore, lenticel breakdown, and bitter pit are discussed as consequential outcomes of thermal stress. Mitigation strategies are critically evaluated, highlighting the efficacy of photoselective nets, kaolin-based particle films, and evaporative cooling, which collectively reduce sunburn incidence by up to 60%. Emerging technologies, including sensor-based early warning systems and precision agriculture tools, offer novel perspectives for proactive management. Conclusively, sustaining fruit quality under increasingly variable climatic conditions requires a multidimensional approach that integrates microclimate regulation with physiological optimization.

1. Introduction

Apple (*Malus domestica* Borkh.) is one of the most widely cultivated fruit species worldwide, occupying more than 4.6 million hectares with an annual production exceeding 97 million tons, and it represents a crop of high nutritional and economic importance (FAO, 2023). Despite continuous improvements in orchard management and cultivar development, the increasing frequency and intensity of heat and radiation events associated with climate change have markedly

intensified the incidence of sunburn and related physiological disorders in apple production systems. In regions characterized by Mediterranean-type climates, FST frequently reaches 45-52°C during summer, a threshold range that substantially elevates sunburn risk and severity (Schrader *et al.*, 2003; Reig *et al.*, 2020). This damage is not limited to visual peel injury but also predisposes fruit to secondary storage disorders such as sunburn necrosis, Fuji stain, and lenticel breakdown.

Sunburn is fundamentally the result of the combined action of thermal stress and photo-oxidative damage. When FST rises approximately 10-15°C above ambient air temperature, cellular membranes destabilize, proteins denature, and reactive oxygen species (ROS) accumulate, triggering oxidative imbalance within peel tissues (Morales-Quintana *et al.*, 2020). Concomitant degradation of photosynthetic and photoprotective pigments - including chlorophylls, carotenoids, and anthocyanins - further weakens photoprotection and leads to irreversible color loss (Olivares-Soto and Bastías, 2020). Based on physiological injury patterns, sunburn is classified into three major forms: sunburn browning (SB), sunburn necrosis (SN), and photo-oxidative sunburn (PS), which develop at increasing combinations of radiation load and thermal stress (Racsko and Schrader, 2012).

Field studies conducted across multiple seasons in neotropical orchards have demonstrated that FST is the primary operational variable integrating incoming radiation, air temperature, and orchard water status in determining sunburn onset and progression. Under photoselective netting systems, FST distributions shift downward and become more homogeneous compared with non-netted canopies, thereby reducing the occurrence of extreme thermal events (Severino *et al.*, 2021). Notably, sunburn progression has been shown to correlate more strongly with soil water balance (SWB) than with daily maximum air temperature, highlighting irrigation and water relations as critical co-determinants of risk alongside thermal load (Severino *et al.*, 2020).

The microclimate surrounding individual fruits is further shaped by canopy structure, fruit position, exposure duration, air humidity, wind velocity, and plant water status (Kalcsits *et al.*, 2017). Water stress, in particular, reduces epidermal thickness and increases thermal conductivity of the peel, thereby enhancing cellular susceptibility to heat injury

(Yaqoobi *et al.*, 2024). These interacting factors explain why substantial variability in sunburn severity is commonly observed even within the same orchard block.

Recent advances in sensing technologies now permit more precise quantification of sunburn risk at both the tree and harvest scales. Sensor-based diagnostics, including visible-near-infrared reflectance indices such as the Plant Senescence Reflectance Index (PSRI) and whole-fruit hyperspectral imaging (HSI), provide reliable tools for assessing sun exposure, pigment transitions, and early sunburn development, thereby complementing FST-based management approaches (Severino *et al.*, 2020; Mogollón *et al.*, 2024). At the biochemical level, cultivar-specific δ -absorbance signatures and associated heat-stress metabolites scale with browning severity, strengthening mechanistic links between thermal load, pigment degradation, and oxidative status (Ross *et al.*, 2023). Moreover, rootstock-dependent differences in damage-free yield under sustained heat stress underscore the importance of genotype $\times$ microclimate $\times$ water relations interactions in determining orchard resilience (Xu *et al.*, 2022).

Collectively, these findings establish sunburn as a complex physiological disorder driven by tightly coupled environmental, biochemical, and genetic processes. Effective mitigation therefore requires an integrated strategy that combines microclimate modification, physiological monitoring, and targeted orchard management. In addition, recent molecular evidence indicates that transcriptional control of the procyanidin pathway (e.g., MdGATA15/MdMYBR9 $\rightarrow$ MdANR) contributes to peel resilience under combined heat-light stress, providing a mechanistic bridge from gene regulation to field-level tolerance (Feng *et al.*, 2025; Fu *et al.*, 2025). This review synthesizes current knowledge on the mechanisms of sunburn development, its cellular and biochemical consequences, associated postharvest disorders, and modern mitigation strategies, with the objective of guiding the development of more resilient apple production systems under a warming climate.

2. Types and Mechanisms of Sunburn

Definition and formation process of sunburn

Sunburn in apples is defined as a physiological disorder that occurs in epidermal and subepidermal

cells as a result of direct exposure of the fruit surface to solar radiation. This disorder arises from the synergistic effect of heat and radiation and is generally characterized by symptoms such as discoloration of the fruit surface, tissue degradation, skin cracking, and necrosis. The severity of sunburn is evaluated as a result of the interaction between environmental factors (air temperature, solar radiation, relative humidity, wind speed) and the physiological characteristics of the fruit (water content, pigment composition, epidermal thickness). According to Schrader *et al.* (2003) and Racsko and Schrader (2012), sunburn occurs when FST exceeds 45°C and is classified into three different forms (Fig. 1): Sunburn Browning (SB), Sunburn Necrosis (SN), and Photo-oxidative Sunburn (PS).

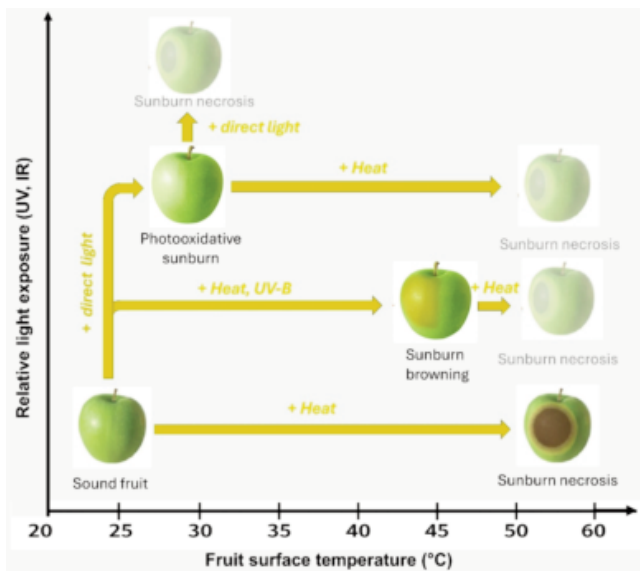


Fig. 1 - Conditions for the development of three sunburn types in apple fruit. Sunburn necrosis (SN), Sunburn browning (SB), and Photo-oxidative sunburn (PS). SN results from thermal cell death at fruit surface temperatures (FST) near 52°C. SB occurs under combined exposure to high temperature (45-49°C) and UV-B radiation without cell mortality. PS develops under visible light when non-acclimated, shade-grown fruit are suddenly exposed to sun-light and can occur at FST below 31°C. SB and PS may progress to SN with increasing FST or prolonged solar exposure. Excessive heat and UV-B are prerequisites for SB and SN; SB and SN do not revert to PS. (Adapted from Racsko and Schrader, 2012).

Sunburn browning (SB)

Sunburn browning is defined as the most common form of sunburn in apples and is induced by both an increase in FST and exposure to UV-B radiation (Racsko and Schrader, 2012). SB generally occurs under conditions where FST remains in the range of

46-49°C for approximately 60 minutes accompanied by UV-B radiation (Schrader *et al.*, 2003). At the onset of this form, cell death was not observed; however, yellowing, bronzing, and browning were reported in the epidermal tissue as a result of chlorophyll degradation and phenolic oxidation (Felicetti and Schrader, 2009 a, b). Pigment analyses revealed a decrease in anthocyanin and chlorophyll concentrations, while phenolic compounds accumulated; this was interpreted as an indicator of oxidative stress response (Morales-Quintana *et al.*, 2020). Moving beyond visual symptoms, recent studies on 'Buckeye Gala' apples provide a more detailed quantitative picture. It has been observed that as sunburn browning (SB) becomes more severe, the fruit develops a thicker cuticle and the sun-exposed side becomes notably firmer. On a metabolic level, while anthocyanin levels in the peel drop, there is a significant buildup of tartaric esters and total phenolics in the flesh underneath (Ross *et al.*, 2023). Researchers are now using specific measurements like skin absorbance ($\delta A_{670-720}$) to better track how fruit reacts to long-lasting heat waves (Ross *et al.*, 2023). Additionally, in regions with neotropical climates, monitoring tools such as chlorophyll fluorescence and reflectance indices have become essential for detecting the early stages of this damage (Severino *et al.*, 2020). The incidence of SB was reported to be higher under conditions dominated by hot and dry winds, especially in fruits located on the southwest side of the tree. Microscopic examinations revealed shrinkage in epidermal cells, cuticular cracks, and stomatal deformations (Reig *et al.*, 2020). Differences in susceptibility among cultivars were observed; 'Honeycrisp' and 'Cameo' were found to be more sensitive, while 'Pink Lady®' was more tolerant (Schmidt, 2018). Among management strategies, photoselective nets and kaolin applications were found to significantly reduce the incidence of SB (Kalcsits *et al.*, 2017; Reig *et al.*, 2020).

On peak heat-stress days, the use of 50% black shading nets has been shown to eliminate FST values exceeding approximately 46°C, significantly reduce within-canopy thermal variability, and shift harvest distributions toward the mild-damage category. Nevertheless, these thermal advantages should be carefully weighed against associated capital expenditures (CAPEX), including net infrastructure, support systems, and installation costs, as well as operating expenditures (OPEX), such as seasonal

handling and maintenance, cleaning and re-application, water and energy consumption, and labor training requirements. Moreover, potential impacts on fruit coloration and market quality should be explicitly considered when implementing shading technologies in commercial orchard systems (Severino *et al.*, 2021).

Sunburn necrosis (SN)

Sunburn necrosis is the most severe form of sunburn in apples and is entirely heat-induced, not requiring UV radiation. It has been determined that when FST temporarily exceeds approximately 52°C, plasma membranes in epidermal and subepidermal cells deteriorate and irreversible cell death occurs (Schrader *et al.*, 2003; Racsko and Schrader, 2012). In this case, dark brown to black necrotic spots form on the fruit surface, and tissue collapse and skin cracking are observed due to the release of lysosomal enzymes (Glenn *et al.*, 2002). Sunburn necrosis generally occurs more frequently in regions dominated by hot and dry winds, especially in fruits located on the southwest side of the tree. At the cellular level, increased activities of phenolic oxidase and peroxidase, elevated lipid peroxidation, and increased membrane permeability leading to ion leakage have been identified; this accelerates water loss and causes wrinkling of the skin (Reig *et al.*, 2020; Morales-Quintana *et al.*, 2020). Microscopic examinations revealed epidermal shrinkage, cuticular cracks, and stomatal deformations. The incidence of SN increases under conditions of high air temperature and low humidity, and FST can be 10–15°C higher than air temperature (Schrader *et al.*, 2003). Photosensitive nets and evaporative cooling applications were found to reduce FST by 2–4°C and significantly decrease SN risk (Kalcsits *et al.*, 2017; Reig *et al.*, 2020).

Photo-oxidative sunburn (PS)

Photo-oxidative Sunburn (PS) has been reported to occur when fruits that have developed in shade are suddenly exposed to full sunlight, particularly to high levels of photosynthetically active radiation (PAR) (Racsko and Schrader, 2012). This type of damage can occur even at low fruit surface temperatures (30–40°C) and is characterized by pale, bleached spots on the surface, with severity ranging from mild photo-oxidative lesions to advanced necrosis as illustrated in figure 2. The incidence of PS is higher in fruits exposed after branch breakage,

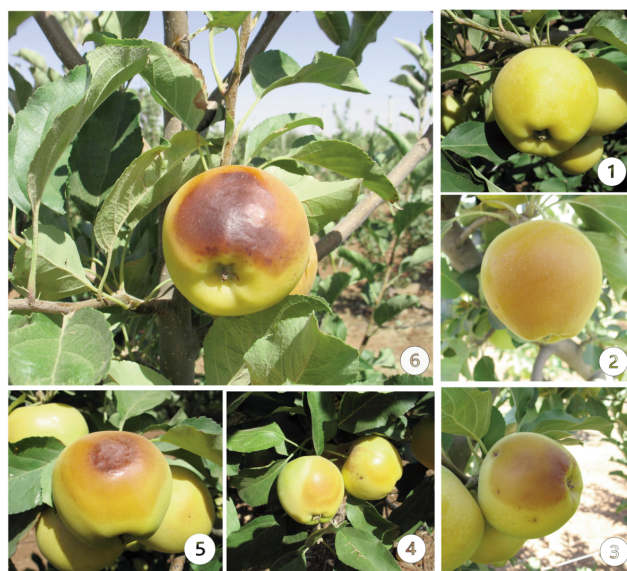


Fig. 2 - Types and severity levels of sunburn in apples: (1) Photo-oxidative sunburn; (2-4) Sunburn browning progressing from mild to severe; (5-6) Sunburn necrosis.

thinning, summer pruning, or postharvest exposure. Mechanistically, high photon flux density suppresses Photosystem II (PSII) in chloroplasts, resulting in increased accumulation of reactive oxygen species (ROS) (Felicetti and Schrader, 2009a). ROS accumulation leads to lipid peroxidation, protein denaturation, and nucleic acid damage, forming the basis of photo-oxidative stress. Furthermore, low anthocyanin and flavonoid contents play an important role in the development of PS; the deficiency of these pigments reduces photoprotective capacity and intensifies oxidative damage (Morales-Quintana *et al.*, 2020). Microscopic examinations revealed shrinkage in epidermal cells, cuticular cracks, and stomatal deformations (Reig *et al.*, 2020).

In shade-grown fruit suddenly exposed, visible-NIR skin spectroscopy and δ -absorbance changes provide early optical markers of PS severity; low anthocyanin/flavonoid content reduces photoprotection, consistent with cultivar-resolved metabolite patterns (Ross *et al.*, 2023).

Factors affecting sunburn

It has been determined that both environmental and plant-related factors play a role in the occurrence of sunburn. Among environmental factors, temperature, solar radiation intensity, wind speed, relative humidity, and water stress are

reported as the most important (Racsko and Schrader, 2012). Plant-related factors are associated with fruit position on the tree, peel structure, color pigments, cuticular thickness, and antioxidant capacity. In trees with open canopy structure and sparse pruning, fruit surfaces are exposed to more sunlight, thus increasing sunburn incidence (Felicetti and Schrader, 2009 b). Fruit surface color is also an important factor; dark-colored fruits (e.g., 'Fuji') absorb more radiation, thereby increasing FST (Felicetti and Schrader, 2008; Torres *et al.*, 2013). The interaction of these factors is considered the main mechanism determining the incidence and severity of sunburn. Recent multi-season field observations further demonstrate that sunburn dynamics are more closely associated with shortwave radiation balance (SWB) than with maximum air temperature (T_{max}), exhibiting moderate to strong interannual coupling (-0.37 to -0.70, depending on season). Moreover, these analyses indicate that rootstock-dependent differences in canopy vigor and plant water relations play a critical role in modulating damage-free yield under prolonged heat stress conditions (Severino *et al.*, 2020; Xu *et al.*, 2022).

Fruit surface temperature (FST) and threshold

FST has been defined as the most critical parameter in sunburn formation. Schrader *et al.* (2003) reported that sunburn in apples begins at 46°C and that necrotic lesions develop at temperatures above 52°C. Factors determining FST include ambient temperature, radiation intensity, wind speed, fruit angle, and surface albedo.

Especially under conditions of low wind speed and low air humidity, the fruit surface cannot provide convective cooling, and heat accumulation increases rapidly. This situation causes large temperature differences even under microclimatic conditions. Glenn *et al.* (2002) reported that kaolin particle films increased solar reflectance on the fruit surface, reduced FST by an average of 4-5°C, and decreased sunburn incidence by 40%.

The biophysical basis of sunburn is the disruption of oxidative balance at the cellular level due to heat and light stress. The development process is shaped by pigment degradation, membrane damage, ROS accumulation, and changes in enzymatic activity. Management of FST is possible through both microclimate adjustments and the use of photoselective materials.

Recent side-by-side field trials further support the

central role of FST management, demonstrating that shading net systems markedly reduce the proportion of fruit experiencing injurious thermal exposure (e.g., 0% of observations exceeding 46°C under 50% black netting, compared with 35-43% in non-netted or particle-film-treated plots). In contrast, particle film applications exhibited season-dependent effectiveness and practical limitations, including reduced coverage persistence and the need for removal during packing operations, particularly under humid summer conditions (Severino *et al.*, 2021).

Therefore, effective control of sunburn requires integrated use of practices that reduce fruit surface temperature, increase reflectance, and balance plant physiology (e.g., kaolin, netting systems, evaporative cooling).

3. Molecular and Physiological Mechanisms of Sunburn

Sunburn in apples is a multilayered stress response that occurs at the cellular and molecular level due to the simultaneous effect of heat and radiation. The increase of FST up to 50°C disrupts the stability of cell membranes, leading to oxidative imbalance, metabolic dysfunction, and structural deformation. This process is shaped by the interaction of interconnected biochemical pathways, including excessive production of reactive oxygen species (ROS), weakening of the antioxidant defense system, pigment degradation, activation of heat shock proteins, and impairment of cuticle-cell wall integrity (Racsko and Schrader, 2012; Morales-Quintana *et al.*, 2020).

Recent advances in quantitative spectroscopy have further strengthened this mechanistic framework by demonstrating that the severity of browning is closely associated with characteristic shifts in δ absorbance and the accumulation of heat-stress-related metabolites, thereby providing a functional diagnostic link between thermal load, pigment dynamics, and cellular ROS status (Ross *et al.*, 2023).

Moreover, recent molecular findings suggest that the fruit's ability to defend itself depends heavily on how it regulates the flavonoid and procyanidin (PA) biosynthetic pathways. Specifically, the accumulation of PAs and chlorogenic acid acts as a natural protective barrier against heat and light stress, a

process largely controlled by the key regulatory gene MdANR (Feng *et al.*, 2025).

Oxidative stress and cellular damage process

High light and temperature conditions disrupt the electron transport systems in organelles such as chloroplasts, mitochondria, and peroxisomes, causing excessive ROS formation (Schrader *et al.*, 2003). Reactive molecules such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot$) interact with membrane lipids, proteins, and nucleic acids, weakening cellular integrity (Torres *et al.*, 2013; Morales-Quintana *et al.*, 2020).

In this process, the increase in malondialdehyde (MDA) accumulation indicates that membrane permeability has deteriorated and ion leakage has begun. Mitochondrial dysfunction leads to disruptions in energy production, causing the collapse of cellular metabolism (Yaqoobi *et al.*, 2024). ROS accumulation also plays a role in cellular signaling, altering the activity of stress response genes. Thus, sunburn transforms from a local tissue reaction into a systemic stress response throughout the fruit (Willsea *et al.*, 2023).

These organelle level effects align with field scale observations where shortwave radiation balance (SWB) modulates the pace of sunburn progression more than T_{max} , underscoring water relations as a central axis for risk reduction (Severino *et al.*, 2020).

Weakening of the antioxidant defense system

Plants have developed an advanced antioxidant defense system to limit the harmful effects of ROS. However, under sunburn conditions, the efficiency of this system decreases. Significant reductions are observed in the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), while the activities of phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) increase (Ranjan *et al.*, 2024).

This enzymatic imbalance accelerates the oxidation of phenolic compounds, causing browning of the peel and reduction in tissue firmness. The weakening of oxidative defense facilitates ROS accumulation, leading to further damage to membrane lipids, increased water loss, and disruption of ion balance. This cascade of events constitutes the fundamental biochemical processes determining the progression of sunburn.

In parallel with these enzymatic changes, the

activation of procyanidin (PA) biosynthetic pathways provides an additional biochemical buffering mechanism. Enhanced PA accumulation contributes to improved ROS scavenging capacity and stabilization of cellular membranes, thereby functionally complementing the classical enzymatic antioxidant network under sunburn stress conditions (Feng *et al.*, 2025; Fu *et al.*, 2025).

Pigment degradation and photo-oxidative damage

The color changes observed in sunburn result from the degradation of photosynthetic and photoprotective pigments. High temperature and radiation cause the breakdown of chlorophyll, oxidation of carotenoids, and suppression of anthocyanin synthesis (Olivares-Soto and Bastías, 2020). These events have been associated with inhibition of electron transport in Photosystem II (PSII) (Racsco and Schrader, 2012).

In cultivars such as ‘Fuji’ and ‘Gala’, a decrease in the expression of genes responsible for anthocyanin synthesis, such as *chalcone synthase (CHS)* and *UDP-glucose:flavonoid 3-O-glucosyltransferase (UGT)*, has been detected (Willsea *et al.*, 2023). Pigment loss not only causes color changes but also reduces the fruit’s capacity to reflect light energy and prevent photo-oxidative damage, as illustrated by sunburn symptoms on the red cultivar ‘Mondial Gala’ in figure 3.

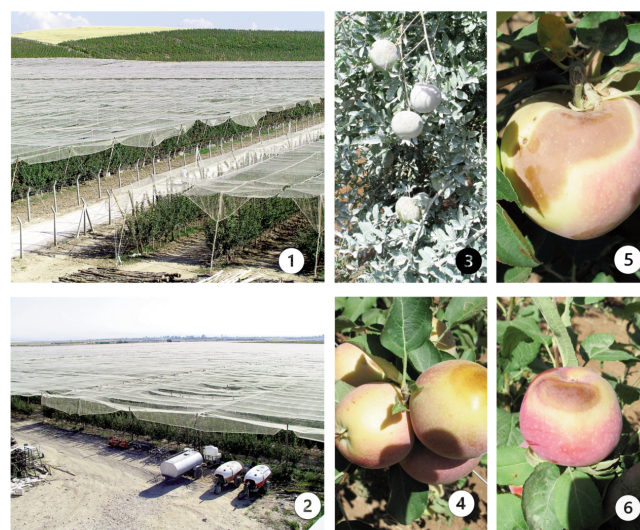


Fig. 3 - Sunburn management and damage types in fruit crops: (1-2) Shade nets used in apple orchards to prevent sunburn, hail, and bird damage; (3) Kaolin application in pomegranate to reduce sunburn; (4-6) Different sunburn types on ‘Mondial Gala’ apple cultivar.

In parallel with these pigment-related changes, cultivar-specific δ absorbance patterns show a clear relationship with pigment loss and the severity of browning, allowing reliable on-tree assessment of sunburn intensity and supporting the design of targeted mitigation strategies (Ross *et al.*, 2023).

Heat shock proteins and cellular protection mechanisms

Excessive heat disrupts the natural structure of proteins in the cell, causing loss of function. In response, the plant synthesizes heat shock proteins (HSPs) as a protective mechanism. Particularly, families such as HSP70, HSP90, and small HSPs (sHSPs) support protein stability by enabling the refolding of denatured proteins (Morales-Quintana *et al.*, 2020; Ranjan *et al.*, 2024). These proteins also contribute to maintaining membrane integrity by regulating lipid composition.

However, when FST exceeds 52°C, the protective effect of HSPs becomes insufficient, resulting in protein aggregation, cytoplasmic precipitation, and irreversible cellular damage (Schrader *et al.*, 2003). This stage represents the critical threshold at which sunburn physiologically progresses to the irreversible necrotic phase.

Consistent with field observations, the protective capacity of HSP-mediated responses collapses once FST exceeds approximately 52°C. Under these conditions, no durable external chemical interventions have yet been shown to sufficiently enhance HSP expression or antioxidant gene activity to extend this intrinsic tolerance limit, thereby underscoring the importance of genetic and molecular approaches for improving heat resilience in apple fruit (Morales-Quintana *et al.*, 2020; Severino *et al.*, 2021).

Gene expression and epigenetic regulation

Sunburn causes profound changes not only in cellular metabolism but also in gene regulation. Transcriptome analyses show that under high-temperature conditions, the expression of antioxidant genes (APX1, CAT1) decreases, while the activity of ethylene-responsive factor (ERF) genes increases (Morales-Quintana *et al.*, 2020).

Additionally, epigenetic regulation mediated by microRNAs (miRNAs) is also effective in this process. Changes in the levels of miRNAs such as miR398, miR156, and miR172 have been reported to influence

the functional control of SOD and HSP genes (Ranjan *et al.*, 2024). Although these regulations support short-term adaptation, they cannot provide complete protection under prolonged sunburn stress.

Recent functional studies have provided deeper insight into this regulatory network. It has been demonstrated that the transcription factor MdGATA15 directly activates the MdANR promoter, thereby enhancing procyanidin biosynthesis and mitigating photo-thermal damage under combined high light and temperature stress; both overexpression and transient expression assays have confirmed reduced visible sunburn symptoms and increased accumulation of PA-associated metabolites (Fu *et al.*, 2025). In parallel, MdMYBR9 has been shown to directly upregulate MdANR, conferring anti-sunburn responses in both fruit peel and callus tissues. Dual-luciferase reporter assays, yeast one-hybrid analysis, and electrophoretic mobility shift assays collectively verify promoter binding and transcriptional activation, positioning the MdMYBR9-MdANR-PA regulatory module as a central adaptive mechanism in apple fruit under thermal stress (Feng *et al.*, 2025).

Changes in cell wall and cuticle structure

Sunburn causes significant structural changes in the cuticle and cell wall, which are the primary defense barriers of the fruit peel. High temperature reduces the proportion of wax components in the cuticle, increasing permeability; this accelerates water loss and oxidative damage (Olivares-Soto and Bastías, 2020). In addition, the observed increase in the activity of pectin methylesterase (PME) and polygalacturonase (PG) enzymes leads to the breakdown of pectin chains and a reduction in cell wall strength (Ranjan *et al.*, 2024).

These structural deteriorations make the fruit more susceptible to oxidative stress and penetration by secondary fungal pathogens (e.g., *Alternaria alternata*, *Botrytis cinerea*) (Racsko and Schrader, 2012; Morales-Quintana *et al.*, 2020). Therefore, these impairments in the cell wall and cuticle are considered the morphological reflections of the physiological collapse observed in the advanced stages of sunburn (Racsko and Schrader, 2012).

Recent evidence further indicates that time-sensitive nutritional management interacts closely with cell wall and cuticle dynamics. In particular, mid-May foliar applications of CaCl₂ (1%) combined with

boron (2 g L^{-1}) have been shown to significantly reduce sunburn incidence while improving fruit firmness and size. In contrast, similar treatments applied in mid-June exhibit limited effectiveness, highlighting a narrow phenological window during which calcium-mediated stabilization of cell wall and cuticle structures can be achieved (Khadivi and Hosseini, 2024).

4. Associated Physiological Disorders

Although sunburn in apples appears to be a superficial problem primarily observed in the epidermis, it actually triggers a multidimensional stress response that deeply affects the physiological balance of the fruit, often accompanied by disorders such as lenticel breakdown, bitter pit, and watercore (Fig. 4). High temperature and intense light exposure increase epidermal permeability, accelerate ROS accumulation, and weaken cell membrane stability, making the fruit more susceptible to various secondary disorders. Therefore, in orchards where sunburn occurs, the likelihood of secondary disorders such as sunburn necrosis, Fuji stain, bitter pit, watercore, and lenticel blotch pit, which significantly reduce quality increases. Indeed, Racsko and Schrader (2012) and Reig *et al.* (2020) reported that the incidence of secondary disorders associated with sunburn can reach up to 30%. This indicates that

sunburn is not limited to damage on the peel but initiates a chain reaction that threatens the metabolic integrity of fruit tissue.

Thermal regulation through fruit surface cooling and canopy shading contributes not only to the mitigation of external sunburn symptoms but also to the maintenance of internal physiological homeostasis. By constraining fruit temperature within non-injurious limits, these practices preserve vascular functionality and stabilize the translocation of calcium and carbohydrates. Such stabilization reduces the risk of localized mineral deficiencies and carbohydrate imbalances that predispose fruit to secondary disorders, including bitter pit and watercore.

Secondary disorders associated with sunburn

In fruits affected by sunburn, structural weakness is observed in cell membranes, cuticle, and epidermal tissue; this increases the impact of oxidative stress and paves the way for the emergence of secondary damage. Increased epidermal permeability accelerates water loss, disrupts ion balance, and makes tissues more vulnerable to storage stress, especially due to the oxidation of phenolic compounds. Reig *et al.* (2020) reported that the incidence of post-storage disorders significantly increased in fruits exhibiting SN-type sunburn and that the occurrence of skin scald reached high levels. Racsko and Schrader (2012) stated that fruits with sunburn showed significant increases in both postharvest respiration rate and oxidative stress indicators. These findings confirm that sunburn acts as an “initiating physiopathological trigger” for secondary disorders.

Sunburn scald

Sunburn scald is an important oxidative stress disorder that occurs during cold storage in fruits exposed to sunburn. It is particularly evident in cultivars such as ‘Golden Delicious’, ‘Granny Smith’, and ‘Honeycrisp’. During sunburn, various terpenoid compounds, especially α -farnesene, accumulate in epidermal tissues. Under storage conditions, these compounds undergo oxidative polymerization, converting into conjugated trienol hydroperoxides, and these reactions cause dark brown to black discoloration of the peel (Morales-Quintana *et al.*, 2020). Reig *et al.* (2020) reported that the scald rate in fruits with SN-type sunburn could reach up to 70%, while in shaded fruits this rate remained around only



Fig. 4 - Heat- and light-related physiological disorders in apples: (1) Lenticel breakdown in ‘Honeycrisp’ apple [adapted from Lora and Faruh, 2025]; (2) Bitter pit; (3) Moderate lenticel breakdown in ‘Gala’ apple [adapted from Lora and Faruh, 2025]; (4) Internal browning; (5) Lenticel blotch pit [adapted from Dar, 2024]; (6) Watercore.

15%. These findings reveal that sunburn plays a critical role in the development of scald after storage.

Recent postharvest studies indicate that HSI enables the early identification of fruit lots with elevated scald risk at harvest, allowing susceptible fruit to be diverted from long-term storage and thereby reducing the overall incidence of storage-related losses (Mogollón *et al.*, 2024). In parallel, the application of selected plant essential oils, including thyme and lavender, has been reported to suppress postharvest decay and contribute to the maintenance of quality in heat-stressed fruit during storage (Al-Hassanavi *et al.*, 2023).

Preventing sunburn scald requires avoiding sudden changes in storage temperature, reducing oxidative stress, and limiting α -farnesene oxidation. In this context, diphenylamine applications, ethylene inhibitors such as 1-MCP, and controlled atmosphere conditions are recommended as effective strategies to reduce the risk of scald.

Fuji stain

Fuji stain is a disorder observed particularly in the 'Fuji' cultivar and is characterized by irregular brown spots on the peel. Its development is mainly determined by high FST, low relative humidity, and intense light exposure (Torres *et al.*, 2013). On sun-exposed surfaces, FST exceeding 45°C, increased epidermal water loss, and the formation of microcracks disrupt pigment stability; suppression of anthocyanin synthesis and increased phenolic oxidase activity result in rough spots on the peel.

Glenn *et al.* (2002) demonstrated that kaolin application could reduce the incidence of Fuji stain by up to 50%, and this effect was associated with both heat reduction and UV reflection. In addition, integrated microclimate management involving photoselective net systems, evaporative cooling, and antioxidant support is recommended to limit the development of this disorder. The severity of Fuji stain has been reported to increase particularly during periods when FST rises rapidly within a short time; therefore, controlling temperature fluctuations is of critical importance.

Bitter pit

Bitter pit is an important physiological disorder associated with calcium (Ca^{2+}) deficiency and appears as localized necrotic depressions on the peel surface. The higher incidence of bitter pit in fruits affected by sunburn indicates that sunburn negatively affects

Ca^{2+} transport (Racsko and Schrader, 2012). Increased transpiration, epidermal water loss, and disruption of membrane integrity adversely affect xylem flow, reducing calcium (Ca^{2+}) uptake. Ca^{2+} deficiency significantly weakens membrane stability and accelerates electrolyte leakage. De Freitas *et al.* (2015) showed that Ca^{2+} deficiency severely compromises cell membrane stability and is a key determinant in bitter pit formation.

Preventing bitter pit involves foliar calcium applications (e.g., CaCl_2), implementing balanced irrigation programs, and reducing excessive fruit load. Additionally, reducing heat stress, which negatively affects calcium transport, can indirectly prevent the increased risk of bitter pit caused by sunburn.

Watercore

Watercore is a disorder that creates a transparent, water-soaked appearance in the fruit flesh and develops before harvest, especially during hot and sunny periods. Its formation is closely related to imbalances in sorbitol transport and sugar metabolism. Liu *et al.* (2022) reported that under high-temperature conditions, the transfer of sorbitol from phloem to xylem was disrupted, resulting in fluid accumulation in intercellular spaces.

The disorder progresses through local increases in osmotic pressure, rapid conversion of starch to sugar, and deterioration of cell membrane permeability. Chen *et al.* (2024) reported that the incidence of watercore increased up to 35% after a heatwave and that this increase was directly associated with a 4-5°C rise in FST. The development of watercore has been reported to be more intense in late-maturing cultivars such as 'Fuji' and 'Ambrosia™' (Morales-Quintana *et al.*, 2020; Reig *et al.*, 2020).

In advanced stages, watercore can turn into more serious physiological disorders such as internal browning and core breakdown. Additionally, imbalances in Ca, K, and Mg increase the risk of watercore; particularly excess K and Mg create an antagonistic effect by inhibiting Ca transport (Uçgun *et al.*, 2014). Therefore, microclimate regulation, balanced irrigation, and early harvest practices in sensitive cultivars are recommended during hot periods.

Lenticel blotch pit

Lenticel blotch pit is a disorder characterized by small, brown, and superficial depressions on the peel

and has a higher incidence especially in orchards where sunburn is common. Schrader *et al.* (2003; 2011) reported that lenticels on surfaces directly exposed to sunlight dry out and become clogged, which disrupts gas exchange and makes epidermal cells more susceptible to oxidative stress. High relative humidity, low air circulation, and peel softening due to moisture can increase the severity of the disorder (Lora and Farquh, 2025).

Nutrient imbalances are also important determinants. Dar (2024) reported that local calcium deficiency increased the incidence of lenticel blotch pit in 'Fuji' apples. Additionally, Blakey (2018) stated that excess K and Mg inhibited Ca uptake, weakening peel tissue. In cultivars with high susceptibility such as 'Gala', 'Fuji', 'Honeycrisp', 'Braeburn', 'Ambrosia', and 'Golden Delicious', the large lenticel structure and thin epidermis lead to more frequent occurrence of this disorder (Lora and Farquh, 2025).

5. Management Strategies (Physical, Chemical, Cultural)

Physical management strategies

Shading and photosensitive net systems. Shading and photosensitive net systems are among the most effective methods for reducing sunburn in apple orchards. Glenn and Puterka (2010) reported that photosensitive nets providing 20% shading reduced FST by an average of 4-5°C and decreased sunburn incidence by 40-60%. Photosensitive nets (Fig. 3) not only reduce light intensity but also optimize photosynthetically active radiation (PAR) through wavelength selectivity. Shahak *et al.* (2009) compared the effects of nets of different colors and found that white nets reduced FST the most, while red nets had less impact on fruit color development. These findings indicate that net color is critical for both temperature control and color formation. Shading systems also help balance the microclimate by reducing wind speed and evaporation; however, excessive shading may limit photosynthetic activity, so net density should be determined at an optimum level. In conclusion, shading and photosensitive nets provide an effective solution against sunburn, but they should be applied considering cultivar-specific light requirements and net characteristics.

Across multiple seasons, the use of black nets with 50% shading was shown to substantially narrow FST distributions and eliminate peak-day readings

exceeding 46°C, thereby shifting harvest outcomes toward lower sunburn severity classes. Nevertheless, the agronomic benefits of this thermal regulation must be carefully balanced against capital and operational costs (CAPEX/OPEX), ongoing maintenance requirements, and potential impacts on fruit coloration, underscoring the importance of site-specific calibration of shading systems (Severino *et al.*, 2021).

Evaporative cooling systems. Evaporative cooling (EC) is considered one of the most effective active methods for controlling sunburn, especially in hot and dry climates. In these systems, both ambient temperature and FST are reduced through the evaporation of water sprayed onto the fruit surface. Schrader *et al.* (2003) reported that overhead irrigation reduced FST by an average of 8–10°C on hot days and significantly decreased sunburn incidence. Felicetti and Schrader (2008) stated that EC could reduce FST by 3-7°C. Goodwin (2017) reported in a study conducted under Australian conditions that EC could lower FST by as much as 15-20°C. Reig *et al.* (2020) revealed that the system reduced FST from 47°C to 42°C and decreased necrotic sunburn by 70%. These findings show that EC provides critical protection during extreme heat periods.

Evaporative cooling not only provides thermal protection but also improves uniform color development and peel quality. However, water quality and spraying frequency should be carefully managed; excessive humidity may trigger the development of fungal diseases (Racsko and Schrader, 2012). Therefore, modern applications prefer pulsing irrigation (e.g., 15 minutes on - 15 minutes off cycles), and irrigation is initiated when FST approaches the damage threshold, usually when air temperature exceeds 32°C (Goodwin, 2017). In new-generation systems, micro-sprinkler nozzles and automatic humidity sensors are used to increase energy and water efficiency. Furthermore, combining EC with lipophilic film coatings (Raynox™) has been reported as one of the most successful management strategies, reducing sunburn incidence by up to 95% (Schrader *et al.*, 2011; Schmidt, 2018).

Today, EC systems are integrated with precision agriculture technologies to become more efficient. Sensor-based early warning systems monitor FST and meteorological data, ensuring that evaporative cooling is automatically triggered at the right time (Wittich, 2021; Ranjan *et al.*, 2024). Additionally,

when used together with photosensitive nets and reflective film coatings, synergistic protection targeting both light and heat stress is achieved (Afonso *et al.*, 2025).

Integration of EC with HSI- and PSRI-based early warning systems has been shown to improve cooling precision and water-use efficiency, while structured pulsing regimes further reduce disease pressure; however, successful adoption depends on proper sensor calibration, operator training, and local water resource constraints (Severino *et al.*, 2020; Mogollón *et al.*, 2024).

These integrated approaches are among the modern strategies recommended to achieve the lowest risk level against sunburn.

Chemical management strategies

Particle film coatings (Kaolin, Surround WP). Kaolin ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$)-based particle films form a thin white layer on the fruit surface, reflecting part of the sunlight and reducing FST Glenn *et al.* (2002) reported that FST decreased by an average of 4-6°C in apples treated with kaolin and that sunburn incidence dropped by up to 60%. Similarly, Glenn *et al.* (2002) stated that kaolin-based films reduced FST by 4-5°C and decreased sunburn by 40-60%. This effect is mainly due to kaolin's high reflectance capacity, which removes part of the solar radiation (Fig. 3).

Kaolin applications not only provide thermal protection but also create a physical barrier against pests and reduce water loss from the fruit surface (Fig. 3). Olivares-Soto and Bastías (2020) reported that kaolin application in 'Fuji' and 'Gala' cultivars reduced FST by an average of 4.8°C, limited ROS accumulation, and increased antioxidant enzyme activities (SOD, CAT, APX). These findings reveal that kaolin also plays an important role in reducing oxidative stress. Application usually begins when fruit diameter reaches 3-4 cm and is repeated at intervals of 3-4 weeks.

Some studies have reported that kaolin application may cause slight fading in fruit color but positively affects storage quality (Reig *et al.*, 2020). Schmidt (2018) emphasized that commercial formulations (Surround® WP, ScreenDuo®) could reduce UV-B absorption by up to 40%, but they create limitations such as washing requirements and color changes from a marketing perspective. Additionally, Schrader *et al.* (2011) revealed that

lipophilic film coatings (RAYNOX™) provide protection by reflecting UV-B radiation and, when combined with evaporative cooling, can reduce sunburn by up to 95%. In new-generation formulations, mixtures of kaolin, calcite, and silica have been used to increase both reflectance and water retention capacity.

In conclusion, kaolin and particle film applications offer an effective strategy against sunburn by reducing both thermal load and oxidative stress; however, application intensity, cultivar-specific color requirements, and postharvest cleaning costs should be considered.

Under humid neotropical summer conditions, repeated applications, limited coverage persistence, and removal requirements during packing can constrain long-term effectiveness. Comparative field trials have further reported inferior sunburn control relative to netting systems in certain seasons, highlighting important practical limitations and cost-related trade-offs associated with particle film use (Severino *et al.*, 2021).

Oil-based coatings (RAYNOX™, Vapor Gard). Oil-based film coatings (e.g., RAYNOX™, Vapor Gard) provide protection by reflecting UV-B radiation and, when combined with evaporative cooling, have been reported as one of the most effective methods for preventing sunburn. Schrader *et al.* (2003) revealed that this combination could reduce sunburn incidence by up to 95%.

Schmidt (2018) emphasized that lipophilic film coatings offer marketing advantages but that application costs and the risk of color changes should be considered. In conclusion, chemical management strategies alone are not sufficient; when integrated with physical methods such as photosensitive nets and evaporative cooling, they provide the most effective protection against sunburn.

Anti-transpirants and other film coating materials. Among chemical protectants used against sunburn, anti-transpirants and film coating materials play an important role. These substances form a semi-permeable layer on the fruit surface, reducing evaporation and preventing excessive increases in FST. Anti-transpirants are compounds that limit water loss by reducing permeability on leaf and fruit surfaces. For example, products such as di-1-p-menthene (Vapor Gard®) and pinolene (Nu-Film 17®) form a thin layer that reflects light on the surface, preventing excessive increases in FST (Felicetti and

Schrader, 2009 b). However, depending on application intensity, changes in fruit color and reductions in respiration rate may occur. Racsko and Schrader (2012) stated that these substances could sometimes increase FST instead of reducing it; therefore, they should only be used under high humidity conditions and for temporary protection. Additionally, coatings containing calcium carbonate (CaCO_3) have been reported to not only reduce FST but also enhance peel mechanical strength through improved calcium availability. Beyond CaCO_3 -based formulations, recent studies further indicate that combined mineral nutrition exerts synergistic protective effects against sunburn. In particular, foliar application of 1% calcium together with 2 g L⁻¹ boron in mid-May was shown to be highly effective in reducing SB incidence in 'Golden Delicious' apples. This early-season nutritional intervention not only mitigates heat-related injury but also improves key quality attributes, including fruit firmness and total soluble solids, underscoring that nutrient-based preventive strategies are most effective when implemented during the initial stages of fruit development (Khadivi and Hosseini, 2024).

Antioxidants and plant activators. Antioxidant applications are an important strategy for reducing oxidative stress associated with sunburn. Antioxidants such as ascorbate, α -tocopherol, glutathione, and phenolic compounds protect cell membrane integrity by increasing the capacity to scavenge reactive oxygen species (ROS) and alleviate the effects of sunburn. Morales-Quintana *et al.* (2020) reported that applications based on ascorbic acid and tocopherol provided internal physiological protection and reduced oxidative stress levels. The effect of these compounds becomes evident particularly when applied during fruit development and before storage. However, such applications should be carefully dosed based on leaf analyses; otherwise, there is a risk of growth retardation. Signal molecules known as plant activators, such as salicylic acid, jasmonic acid, and brassinosteroids, increase stress tolerance by stimulating cellular defense systems. Ranjan *et al.* (2024) stated that these compounds triggered the expression of heat shock proteins (HSPs) and antioxidant genes against sunburn. This mechanism strengthens the plant's adaptation capacity under photo-oxidative stress conditions. Furthermore, it has been reported that the application of these activators strengthens the

antioxidant defense system on the fruit surface and reduces ROS accumulation (Olivares-Soto and Bastías, 2020).

Recent studies have shown that the combined use of antioxidant supplements and plant activators creates a synergistic effect. Afonso *et al.* (2025) reported that this combination could reduce sunburn incidence by 40-70% and preserve fruit quality. These findings reveal that chemical management strategies should focus not only on surface protection but also on strengthening plant physiology.

Although plant activators can transiently induce antioxidant and HSP-related defense pathways, no durable upregulation sufficient to extend HSP-based tolerance beyond the critical threshold of FST $\geq 52^\circ\text{C}$ has been demonstrated under field conditions. This limitation highlights the need to prioritize genetic and molecular strategies for achieving sustained improvements in heat tolerance (Morales-Quintana *et al.*, 2020).

Preventive mineral nutrition (Ca-B). Preventive mineral nutrition has recently emerged as an important complementary strategy for mitigating sunburn through the stabilization of cell wall and membrane structures. In this context, foliar application of calcium chloride (CaCl_2 , 1%) in combination with boron (2 g L⁻¹), when applied in mid-May - coinciding with the transition from cell division to the onset of fruit enlargement - has been shown to significantly reduce sunburn incidence while improving key quality attributes such as fruit firmness and size. In contrast, identical treatments applied in mid-June were found to be largely ineffective, demonstrating the existence of a narrow phenological window during which calcium-mediated structural reinforcement of cell walls and membranes can be achieved. These findings emphasize that the efficacy of mineral-based preventive strategies depends not only on nutrient composition but also on precise timing relative to fruit developmental stage (Khadivi and Hosseini, 2024).

Cultural management strategies

Irrigation Management. Balanced irrigation is one of the most critical cultural factors that reduce the incidence of sunburn. In water-stressed fruits, epidermal thickness decreases, which increases thermal conductivity and raises fruit surface temperature, thereby increasing the risk of sunburn. Regular and controlled irrigation helps maintain

stable FST and preserve cellular turgor (Kalcsits *et al.*, 2017).

For optimum water management, soil moisture sensors, tensiometers, or plant-based water potential measurement systems can be used. However, excessive irrigation may cause cuticular thinning and the formation of microcracks on the fruit surface, creating a predisposition to fungal infections. Therefore, irrigation programs should be dynamically planned considering evapotranspiration data and climatic conditions.

Pruning and canopy management. Canopy structure is one of the main factors determining the degree of direct exposure of the fruit surface to sunlight. Excessive thinning pruning that reduces leaf density too much causes fruits to lose protective shade and increases the risk of sunburn. Balanced pruning maintains the leaf-to-fruit ratio, optimizes light distribution, and sustains photosynthetic efficiency.

During fruit thinning, fruits on sun-exposed parts should be protected, and care should be taken not to excessively open the leaf cover. The timing of pruning is also critically important; late pruning performed in summer can increase the risk of photo-oxidative stress and sunburn in fruits suddenly exposed (Racsko and Schrader, 2012). In modern applications, canopy architecture can be optimized using light simulation models.

Cultivar selection and harvest time. The susceptibility of cultivars to sunburn varies depending on peel pigment structure, epidermal thickness, and cuticular characteristics. Dark red cultivars ('Fuji', 'Red Delicious') are more sensitive due to higher light absorption, whereas light-colored cultivars ('Golden Delicious') are more tolerant.

Determining the correct harvest time plays a critical role in reducing the effects of sunburn on quality. With delayed ripening, cuticular thickness increases, but surface pigments become more sensitive to oxidative stress. Therefore, the optimum harvest time for each cultivar should be determined considering both color development and sunburn risk (Serra *et al.*, 2020).

Beyond cultivar choice, increasing evidence indicates that rootstock selection plays a decisive role in determining damage-free yield under heat stress conditions. In 'Ambrosia' apples, rootstock-dependent differences in tree vigor and canopy architecture markedly influence the fruit microclimate. Trees grafted onto more resilient

rootstocks (e.g., Geneva 935) have been shown to maintain higher yield efficiency and superior fruit quality following sustained heat events compared with more susceptible rootstocks, underscoring rootstock choice as a fundamental cultural strategy for climate-resilient apple production (Xu *et al.*, 2022).

Integrated approaches

A single method is not sufficient to prevent sunburn; physical, chemical, and cultural methods must be applied in an integrated manner. For example, the use of photoselective nets together with kaolin application can reduce FST by 6-8°C; when supported by irrigation and evaporative cooling strategies, sunburn incidence can be reduced by up to 70% (Glenn *et al.*, 2002; Afonso *et al.*, 2025).

Today, sensor systems supported by artificial intelligence enable real-time monitoring of fruit surface temperature, radiation intensity, and humidity, optimizing the timing of preventive measures. Thus, both yield and quality losses are minimized. Integrated approaches stand out as a critical strategy for sustainable production.

However, the adoption of integrated management strategies must be carefully evaluated in terms of economic feasibility, operational complexity, and long-term sustainability. Capital and operational expenditures (CAPEX/OPEX), system maintenance requirements, and potential trade-offs related to fruit coloration should be critically assessed under site-specific conditions to ensure durable field performance (Severino *et al.*, 2021).

A pragmatic risk-management framework further supports tiered triage at harvest: visibly sunburned fruit are removed at intake, HSI-classified high-risk lots are redirected to early markets, and low-risk fruit are reserved for long-term storage. This approach integrates microclimate control (netting and evaporative cooling), time-sensitive mineral nutrition (Ca-B), and sensor-guided decision support, thereby optimizing both risk reduction and resource efficiency (Mogollón *et al.*, 2024; Khadivi and Hosseini, 2024).

6. Conclusion and Future Perspectives

Rising temperatures and increasing radiation intensity associated with global climate change are driving more frequent and severe sunburn and heat-light-induced physiological disorders in apple

production. This stress is not merely a superficial quality defect but profoundly disrupts fruit metabolism, storage potential, and commercial value.

At the physiological level, sunburn develops when critical thresholds of FST are exceeded, triggering oxidative imbalance characterized by excessive reactive oxygen species (ROS) accumulation, pigment degradation, membrane peroxidation, and weakening of the antioxidant defense system. The three major forms of sunburn - sunburn browning, sunburn necrosis, and photo-oxidative sunburn - manifest across a gradient of thermal and radiative stress, reflecting distinct but interconnected injury pathways.

Recent advances demonstrate that precise monitoring and management of FST are central to effective sunburn control. Sensor-based measurement platforms, artificial intelligence-supported modeling, and early-warning systems now provide producers with the ability to anticipate risk and deploy interventions with greater temporal precision. When physical (photosensitive nets, kaolin, evaporative cooling), chemical (anti-transpirants, antioxidants), and cultural practices (balanced irrigation, canopy management, cultivar and rootstock selection) are integrated, substantial reductions in sunburn incidence can be achieved, although performance remains strongly dependent on local climate and cultivar-specific responses.

Looking forward, the limits of current physical and chemical interventions are increasingly defined by the intrinsic biological tolerance of the fruit. Extending cellular tolerance beyond critical thermal thresholds (e.g., $FST \geq 52^{\circ}C$) will likely require advances in genetic and molecular strategies. Identification of sunburn-tolerant genotypes and the targeted use of genes associated with heat shock proteins, antioxidant systems, and photoprotective pigments represent promising directions for breeding climate-resilient cultivars.

In parallel, the continued expansion of precision agriculture will enable real-time integration of FST sensing, radiation and humidity monitoring, on-tree spectral diagnostics, and at-harvest hyperspectral triage. Future research should resolve genotype $\times$ environment $\times$ management interactions by coupling these sensing platforms with cultivar/rootstock variability and by systematically evaluating time-critical nutritional windows (e.g., early-season Ca-B

applications) across diverse climatic regions. Such efforts must also explicitly quantify trade-offs between thermal protection, color development, postharvest performance, and phytosanitary outcomes.

In conclusion, sustainable mitigation of sunburn and its associated physiological disorders requires a holistic production framework that unites plant physiology, microclimate regulation, genetic improvement, and advanced agricultural technologies. The success of future apple production systems will depend on their capacity to translate this integrated knowledge into resilient, climate-adaptive management strategies that preserve both yield and fruit quality.

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