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Morpho-physiological and yield responses of green lettuce to growing media volumes in urban cultivation

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Key words: Lettuce, media, micro-climate, urban farming, volume.

Abstract: Urban agriculture requires efficient production in restricted spaces, making growing medium volume a critical determinant of lettuce performance. This study evaluated the effects of three medium volumes on the morpho-physiological responses and yield of green lettuce (*Lactuca sativa* L.). The experiment was arranged in a randomized complete block design with three polybag sizes, i.e. 15 × 20 cm (V1), 20 × 25 cm (V2), and 25 × 30 cm (V3), each treatment replicated four times, with six plants per replication. The smallest medium volume (V1) increased midday soil temperature by 34°C and leaf temperature by 33°C, while reducing soil moisture by 20% relative to V3, indicating greater heat and water stress. These conditions reduced canopy development, resulting in 61.6% fewer leaves, smaller leaf dimensions (leaf area decreased by 74.8%), and lower yield (-76.7%). In contrast, the largest medium volume (V3) improved the root zone microclimate and plant water status, producing the highest vegetative growth and yield. Leaf area was reliably predicted non-destructively using the linear model (Y = 0.8019x) (where (Y) is leaf area and (x) is leaf length × width), with a coefficient of determination (R^2 = 0.9882). Overall, increasing growing medium volume enhanced root zone conditions, leaf area development and final yield. Small volumes remain usable in urban systems if supported by adequate irrigation to maintain soil moisture.

1. Introduction

Urban agriculture increasingly relies on container-based cultivation (pots, polybags, or modular units), where the binding constraint is often finite root-zone or growing medium volume rather than land area; therefore, performance must be optimized not only per unit area but also per unit cultivation volume (e.g., yield per growing media volume or per unit substrate volume). Land scarcity requires food production systems to adapt to constrained environments such as rooftops, balconies, and vertical structures (Harada and Whitlow, 2020). In a 20 ft shipping container vertical farm (12 m² total footprint; 7 m² growing area), reported harvests reached 55 lettuce plants per week with a mean fresh

mass of 0.118 kg plant^{-1} (±1.85 kg m^{-2} week^{-1}), illustrating how high output depends on the ability of a small, fixed cultivation volume to sustain a stable and productive root zone environment (Carrasco *et al.,* 2024). Root zone conditions are highly consequential for lettuce; for example, a root-zone temperature of 35°C (vs 25°C) was reported to suppress growth, while even relatively small shifts i (± 3°C) can measurably alter productivity, underscoring why container/root-zone limitation is a central design problem in urban systems (Levine *et al.,* 2023). The arrangement of pot size or growing medium volume is a crucial factor because it strongly influences the number of plants that can be cultivated in a limited area. Suboptimal growing medium volume directly affects root development, water retention, nutrient availability, and the overall physiological performance of the plants.

Green lettuce (*Lactuca sativa* L.) is one of the most widely produced leafy vegetables in urban agriculture due to its short cropping cycle, high market demand, and adaptability to container-based production systems (Ria *et al.,* 2025 a). Green lettuce is commonly grown in small media (e.g., net pots) in hydroponic systems (Ezziddine and Liltved, 2021). However, hydroponic systems can entail higher installation and maintenance costs, making conventional substrates in containers an accessible alternative for household and community growers (Nitu *et al.,* 2024). Nevertheless, lettuce growth is highly sensitive to root-zone restriction because its shallow, relatively fine root system depends on an adequately sized substrate volume to buffer water and nutrient supply; when the rooting volume is limited, root exploration and water uptake decline, the root zone dries and warms more rapidly, stomatal conductance and leaf expansion are reduced, and fresh leaf yield decreases (Levine *et al.,* 2023). Reducing growing medium volume can restrict root expansion, reduce water holding capacity, alter water use dynamics, and shift biomass allocation between shoot and root compartments (Holz *et al.,* 2024; Gustiar *et al.,* 2025). Under typical urban practice where growing media volume downsizing is used to increase planting density these constraints can translate into measurable yield penalties and reduced resource use efficiency unless the minimum viable volume and its management requirements are known.

Previous studies have shown that shoot morphology, leaf expansion, root architecture, and physiological traits such as water-use efficiency and photosynthetic capacity are strongly influenced by root-zone restriction (Poorter *et al.,* 2012). However, previous studies have discussed container/root zone restriction in general, but few have quantitatively integrated soil temperature (root-zone micro-climate), leaf area development, and fresh leaf yield to explain how limited growing medium volume constrains lettuce performance in growing media volume urban cultivation. Such research is essential to determine the most appropriate growing media volume, improve resource-use efficiency, and ensure that plant performance remains optimal in small-scale or modular production systems. Therefore, this study aims to evaluate the morpho-physiological responses of green lettuce grown under different growing medium volumes in order to identify the minimum volume that can still maintain optimal growth and yield.

2. Materials and Methods

The experiment was conducted in an open field located in Jakabaring, Palembang, South Sumatra, Indonesia (104°46′44″ E; 3°01′35″ S) from September-November 2025. The site is situated in a lowland tropical climate region. The mean environmental conditions, including temperature, rainfall, and relative humidity during the study period, are presented in figure 1. The plant material used was green lettuce (*Lactuca sativa* L.) cv. Grand Rapids. Prior to sowing, the seeds were soaked in warm water at 60°C, following a previous study, to accelerate germination (Ria *et al.,* 2025 b). After soaking, the seeds were directly sown into seedling trays filled with a mixture of ultisol (loam textured soil) and chicken manure at a 2:1 (v/v) ratio.

At 14 days after planting (DAP), the seedlings were transplanted into polybags of different sizes: 15 × 20 cm (V1≈1.43 L), 20 × 25 cm (V2≈3.18 L), and 25 × 30 cm (V3≈5.97 L). The experiment was arranged in a randomized complete block design (RCBD), in which each treatment was replicated four times, and each replication consisted of six plant samples, resulting in a total of 72 experimental plants. One seedling was planted per polybag. At 7 days after transplanting (DAT), each plant was supplied with a liquid organic fertilizer containing 10.04% organic C, 2.05% (N + P_2O_5 + K_2O), 0.54% organic N, pH 5.44, total Fe 332.21 ppm, Mn 38.8 ppm, B 74.20 ppm, and Mo 2.39 ppm.

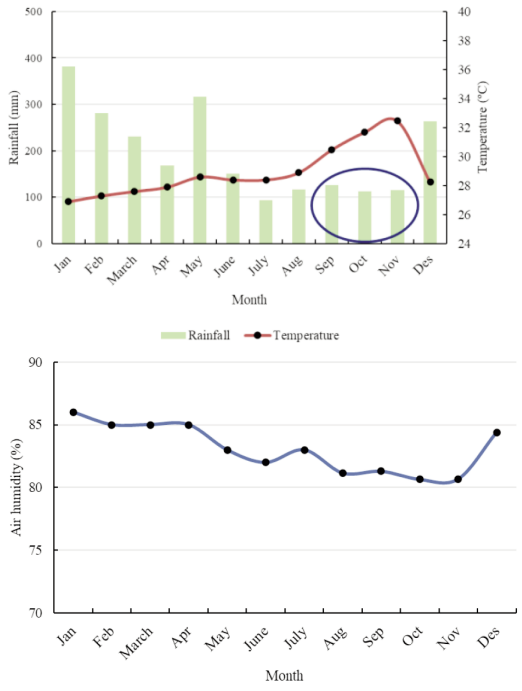


Fig. 1 - Dynamics of temperature, rain fall, and air humidity during the observation period.

Fertilization was conducted once a week in the late afternoon. Each plant received 100 mL of a diluted liquid organic fertilizer solution at a concentration of 2 mL L^{-1}. Plant watering was carried out once daily in the late afternoon.

Data collection

Data collection was carried out from 10 DAT to 35 DAT, and harvesting was conducted at 42 DAT. Air temperature was measured using a digital thermometer. Leaf temperature was measured using a FLIR C3-X thermal camera, with thermal images captured from above the leaf canopy at a distance of ±50 cm from the top of the plants. Soil temperature and soil moisture were measured simultaneously using a digital environmental thermometer and a Lutron PMS-714 soil moisture meter, respectively, at a depth of ±5 cm and a distance of ±7 cm from the plant stem. Measurements were taken on a single day at 2-hour intervals from 08:00 to 16.00.

Growth- and yield-related parameters were also recorded, including canopy area, leaf area, and estimated leaf area. Canopy area was quantified using the Easy Leaf Area application (Easlon and Bloom, 2014), while actual leaf area was measured using LIA32 software from Nagoya University. Leaf area estimation was performed using an exponential regression model based on leaf length and width, and a zero-intercept linear regression model using leaf length × width (cm^2). Plant dry weight was determined after drying plant samples in a forced-air oven at 105°C for 24 hours.

Data analysis

All collected data were analyzed using analysis of variance (ANOVA). For variables showing significant treatment effects, mean separation was performed using the Least Significant Difference (LSD) test at a significance level of $P<0.05$. All statistical analyses were carried out using RStudio software.

3. Results

Root-zone microclimate responses

Measurements of soil temperature and soil moisture indicated that growing medium volume altered the root-zone microclimate (Fig. 2). Across

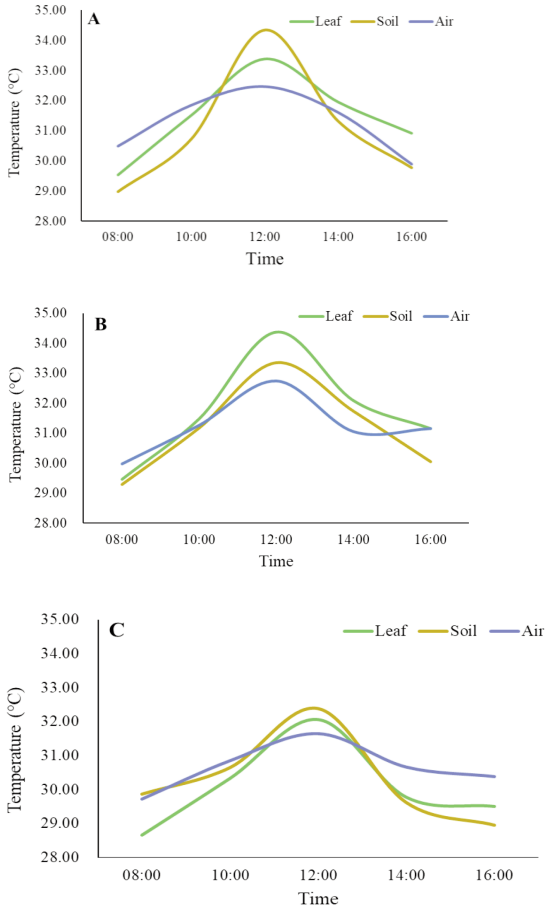


Fig. 2 - Fluctuations in leaf temperature, soil temperature, and air temperature at different volume levels. Small (A), medium (B), and big (C), in green lettuce plants.

treatments, soil temperature showed a clear inverse association with soil moisture: when soil temperature increased from approximately 27°C to 32°C, soil moisture declined (Fig. 3). This pattern suggests that smaller media volumes provide limited buffering capacity against heat gain and water loss, resulting in faster drying of the root zone. In contrast, larger media volumes maintained more stable soil conditions, which likely improved the continuity of water availability for root uptake.

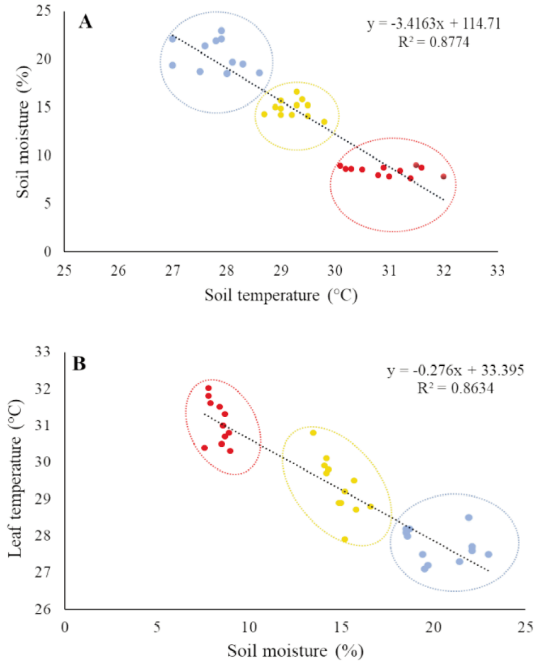


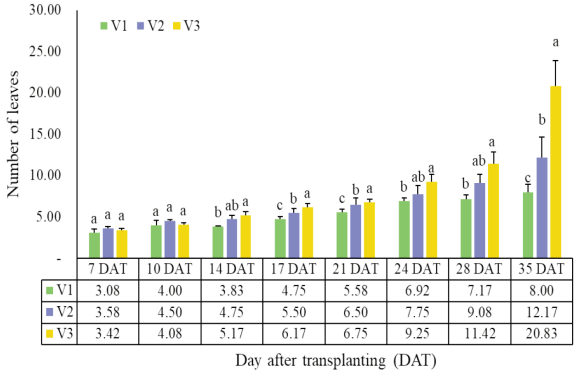
Fig. 3 - Relationship between soil temperature and soil moisture (A); and between soil moisture and leaf temperature (B) in green lettuce plants at different growing media volumes.

Leaf temperature and water relations

Leaf temperature dynamics further reflected differences in root-zone buffering among media volumes. In the morning, air temperature was higher than both leaf and soil temperatures; however, around midday (12:00), leaf and soil temperatures increased more sharply than air temperature, particularly in smaller polybags. This coincided with lower soil moisture and was accompanied by higher leaf temperatures (Fig. 2), indicating tighter coupling between root-zone water status and canopy thermal responses. Elevated leaf temperature can accelerate transpiration and increase the risk of transient tissue dehydration when root water supply is constrained by limited media volume. Consequently, plants grown in smaller polybags are more prone to rapid turgor loss and midday wilting. Conversely, plants grown in larger polybags maintained lower and more stable leaf temperatures, consistent with better root-zone moisture retention and improved capacity to sustain plant water status during peak thermal load.

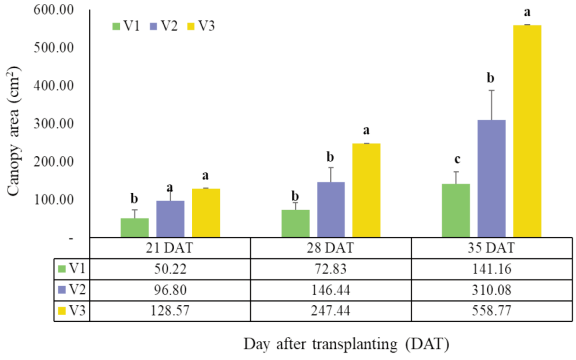
Vegetative growth and canopy development

Growing medium volume strongly influenced vegetative development. Plants cultivated in the largest media volume (V3) consistently produced more leaves than those in V1 and V2, and the differences became increasingly pronounced with plant age (Fig. 4). Canopy development showed a similar response: canopy area increased over time in all treatments, but the rate of canopy expansion was substantially higher in the larger media volumes. The greater canopy area in V3 reflects both higher leaf number and larger leaf size, which likely enhanced light interception and, consequently, photosynthetic potential during vegetative growth (Fig. 5).



	7 DAT	10 DAT	14 DAT	17 DAT	21 DAT	24 DAT	28 DAT	35 DAT
V1	3.08	4.00	3.83	4.75	5.58	6.92	7.17	8.00
V2	3.58	4.50	4.75	5.50	6.50	7.75	9.08	12.17
V3	3.42	4.08	5.17	6.17	6.75	9.25	11.42	20.83

Fig. 4 - Number of leaves of green lettuce plants at different growing media volumes.

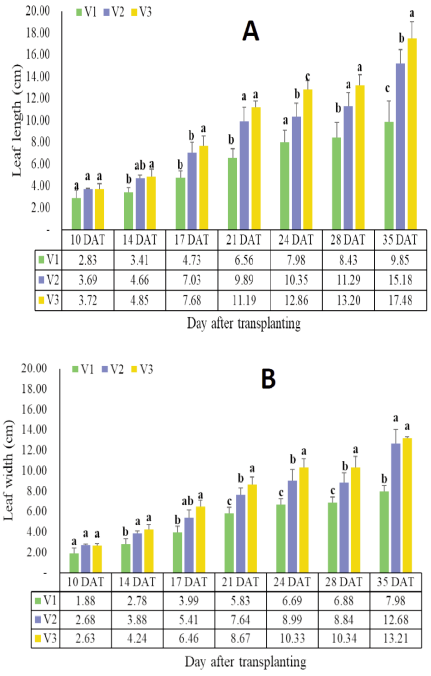


	21 DAT	28 DAT	35 DAT
V1	50.22	72.83	141.16
V2	96.80	146.44	310.08
V3	128.57	247.44	558.77

Fig. 5 - Canopy area of green lettuce at various growing media volumes.

Leaf morphology and area relationships

Leaf length and leaf width increased with days after transplanting (DAT) across all treatments (Fig. 6). Nevertheless, plants grown in V3 exhibited the greatest increase in leaf size throughout the observation period, whereas V1 produced the smallest leaves, consistent with limitations in root space and reduced water retention capacity of the smaller media volume (Fig. 7). The relationships among leaf length, leaf width, and leaf area showed varying R^2 values (Fig. 8). The highest R^2 = 0.9882 value was found in the relationship between leaf length × leaf width and leaf area. The very tight clustering of data points around the regression line



	10 DAT	14 DAT	17 DAT	21 DAT	24 DAT	28 DAT	35 DAT
V1	2.83	3.41	4.73	6.56	7.98	8.43	9.85
V2	3.69	4.66	7.03	9.89	10.35	11.29	15.18
V3	3.72	4.85	7.68	11.19	12.86	13.20	17.48

	10 DAT	14 DAT	17 DAT	21 DAT	24 DAT	28 DAT	35 DAT
V1	1.88	2.78	3.99	5.83	6.69	6.88	7.98
V2	2.68	3.88	5.41	7.64	8.99	8.84	12.68
V3	2.63	4.24	6.46	8.67	10.33	10.34	13.21

Fig. 6 - Growth of leaf length (A) and leaf width (B) of green lettuce at various growing media volumes.

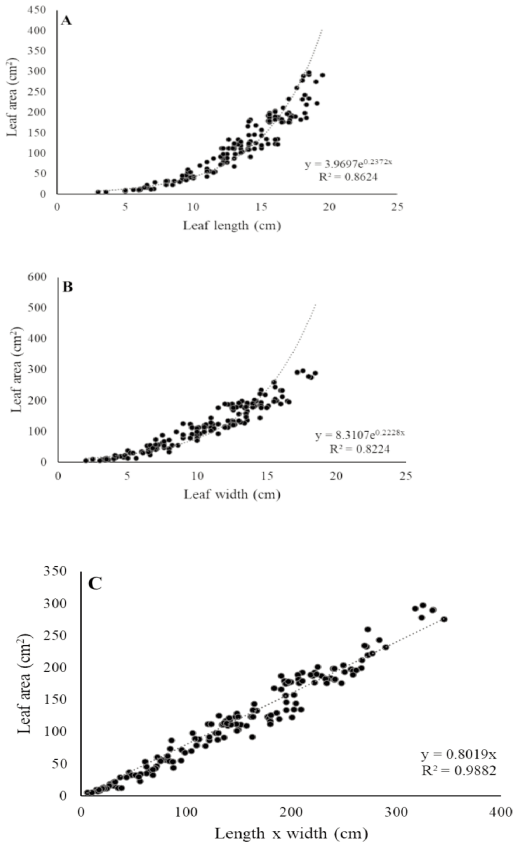


Fig. 8 - Estimation of green lettuce leaf area by relating leaf length and leaf area (A); leaf width and leaf area (B); and leaf length × leaf width and leaf area (C).

confirms that this linear model has very high accuracy across the full range of observed leaf sizes. These results indicate that although leaf length and leaf width individually are already sufficient to estimate leaf area, their combination in the form of length × width produces an estimator that is both more precise and simpler (requiring only a single multiplication constant). Thus, the linear equation based on length × width is highly promising as a



Fig. 7 - Comparison of shoots and roots of green lettuce at different volumes.

practical and non-destructive method.

Yield components and biomass allocation

Increasing growing medium volume significantly improved yield components and leaf morphophysiological traits (Table 1). The largest media volume produced the highest shoot and leaf fresh weight, leaf dry weight, leaf number, and total leaf area, and these values were consistently higher than those obtained in medium and small media volumes. In contrast, leaf water content did not differ significantly among treatments, suggesting that it was relatively insensitive to changes in media volume under the conditions of this study. Overall, the results indicate that enlarging the root zone primarily promoted biomass accumulation and leaf area development rather than altering tissue water content.

Medium volume also affected biomass allocation to stems (Table 2). Larger media volumes were associated with higher stem fresh weight, stem dry weight, and stem length, which are consistent with improved water and nutrient supply supporting photosynthetic production and subsequent allocation of assimilates to structural tissues. The absence of significant differences in stem water content among treatments further indicates that increases in stem mass were driven mainly by dry matter accumulation rather than by greater tissue hydration. Consistent with these patterns, larger media volumes enhanced both the root and shoot growth, producing more balanced and vigorous plant development, whereas limited media volume restricted the expansion of both root system and shoot biomass (Table 3).

Table 1 - Fresh weight, dry weight, and leaf morphophysiological components of green lettuce at various growing media volumes

Treatment	Shoot fresh weight (g)	Number of leaves	Leaf fresh weight (g)	Leaf dry weight (g)	Leaf water content (%)	Total leaf area (cm^2)
V1	36.69 c	12.50 c	30.76 c	2.82 b	90.61 a	1023.12 c
V2	93.12 b	19.11 b	72.43 b	5.42 ab	92.67 a	2558.94 b
V3	170.41 a	28.44 a	131.77 a	9.89 a	92.40 a	3868.95 a
LSD 0.05	42.05	5.78	30.49	7.63	5.48	761.81

Means followed by different letters are significantly different according to the LSD test at the 0.05 significance level.

Table 2 - Fresh weight, dry weight, and stem morphophysiological components of green lettuce at various growing media volumes

Treatment	Stem fresh weight (g)	Stem dry weight (g)	Stem water content (%)	Stem length (cm)
V1	5.93 c	0.56 b	88.48 a	8.69 c
V2	20.69 b	1.13 ab	94.63 a	17.71 b
V3	38.08 a	1.85 a	95.23 a	26.04 a
LSD 0.05	11.47	0.82	11.94	5.63

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05). NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

Table 3 - Fresh weight, dry weight, and root morphophysiological components of green lettuce at various growing media volumes

Treatments	Root length (cm)	Shoot root ratio	Root fresh weight (g)	Root dry weight (g)
V1	7.76 c	6.06 b	7.06 c	0.38 c
V2	18.30 b	8.12 ab	11.49 b	0.84 b
V3	25.00 a	10.65 a	16.99 a	1.67 a
LSD 0.05	6.45	4.52	3.7	0.21

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05).

4. Discussion and Conclusions

Effect of growing media volume on soil microclimate

An increase in soil temperature in small polybags was accompanied by a decrease in soil moisture, as reflected by the negative linear relationship between these two variables. This consistent pattern indicates that restricted media volume accelerates water loss through evaporation and exacerbates fluctuations in substrate moisture in container-grown plants (Witcher *et al.,* 2020). As soil moisture declines, the flow of water from roots to leaves is reduced, while increasing radiation and air temperature continue to drive a steeper water potential gradient between the leaves and the atmosphere. Consequently, the transpiration rate and leaf temperature increase. In small media volumes, the root zone tends to exhibit leaf and soil temperatures above air temperature because of the low heat capacity and low water content, making it prone to overheating (supra-optimal root-zone temperatures) (González-García *et al.,* 2023).

Physiologically, the combination of high leaf temperature and declining soil water availability causes plants to experience simultaneous heat and water stress. Previous studies have shown that the interaction between restricted root volume due to limited media and water stress can reduce stomatal conductance, decrease the photosynthetic rate, and accelerate the decline in turgor pressure, making plants more prone to wilting during the day (Wu *et al.,* 2022; Asargew *et al.,* 2023). This was clearly evident in the smallest media volume treatment (V1), where the high leaf temperature was not compensated by water supply from the roots because of the narrow root zone and limited water content.

In contrast, in large polybags, soil and leaf temperatures tended to be lower and showed smaller fluctuations. Larger media volumes have higher water storage capacity and heat capacity, enabling them to buffer daily temperature changes and maintain soil moisture for longer. Growing media with larger volume or substrates with good water retention capacity can stabilize root-zone temperature, reduce thermal stress, and help maintain leaf turgor in horticultural crops (Irshad *et al*., 2024). In this study, differences in growing media volume primarily acted as a "microclimate buffer" around the roots and canopy, which in turn determined plant water status, stomatal activity, and the physiological performance of green lettuce.

Effect of growing media volume on lettuce morphology

Significant differences in leaf number and canopy area of lettuce indicate that more favorable microclimatic conditions and resource availability occurred in the largest media volume (V3). This promoted leaf meristem activity and prolonged the vegetative growth phase. Root volume restriction reduces the rate of new leaf formation and decreases leaf size as a result of reduced photosynthetic rate and increased growth-inhibiting hormonal signals from stressed roots (Triyono *et al*., 2020; Nasih *et al*., 2025). The results of this study are consistent with those findings, as the V1 treatment (smallest volume) exhibited inhibited shoot growth.

Lettuce leaf length and width increased consistently with days after transplanting; however, the rate of increase was strongly influenced by media volume. Leaf elongation and expansion are highly sensitive to root-zone space and the availability of water and nutrients (Omar *et al*., 2023). The narrow and easily dried root-zone environment in V1 triggered an adaptive response in the form of reduced leaf surface area to minimize water loss through transpiration. In contrast, the wider and moister root zone in V3 enabled leaf cells to maintain turgor, allowing optimal cell expansion and leaf elongation (Li *et al*., 2022).

Effect of growing media volume on yield and biomass of lettuce

Increasing the volume of the growing media had a significant effect on yield components and biomass of lettuce. The V3 treatment consistently produced the highest shoot and leaf fresh weight, leaf dry weight, number of leaves, and total leaf area, whereas V1 gave the lowest values for almost all parameters. Interestingly, leaf water content did not differ markedly among treatments, indicating that differences in fresh weight were primarily determined by increased dry matter accumulation rather than changes in tissue water status. Lettuce and other leafy vegetables tend to increase shoot dry biomass without drastically altering leaf water content (Marutani *et al.*, 2021).

The stem functions as an important "sink" for additional photosynthates produced under larger

media volumes. The improved water and nutrient availability in V3 support a higher photosynthetic rate, thereby increasing carbohydrate supply to storage organs such as the stem (Sarkar *et al*., 2021). The strengthening of stem and shoot sinks under adequate root volume conditions is consistent with the concept that root volume restriction tends to reduce shoot growth and alter root-shoot biomass partitioning in various plant species (Antunes *et al*., 2024).

On the other hand, root system data show that increased media volume not only improved shoot growth but also enhanced root development (Table 3). The V3 treatment produced the greatest root length and root biomass, along with the largest shoot biomass. In contrast, V1 simultaneously restricted both components. The size of the growing media determines the volume of root exploration and, through this, influences the plant's capacity to absorb water and nutrients, which ultimately determines shoot growth and yield (Gallegos *et al.*, 2020). In this study, increasing media volume acted to enhance both source capacity (roots as water and nutrient absorbers) and sink capacity (shoots and stems as photosynthate storage organs) (Table 3).

Leaf area estimation

In this study, the relationships between leaf length, leaf width, and leaf area were measured to estimate leaf area using a non-destructive, coefficient-based approach. Similar studies have been conducted on various leafy vegetables, including Swiss chard (Ria *et al.*, 2023), tatsoi (Kartika *et al*., 2021), and chaya (Gustiar *et al*., 2024). The present results show that the combination of length × width produced the highest coefficient of determination (R^2) with leaf area. The tight clustering of data points around the regression line confirms that this simple linear model based on length × width is a highly precise estimator of leaf area across the full range of leaf sizes observed. These findings are consistent with previous reports showing that the leaf area of lettuce and other leafy vegetables can be accurately predicted using linear regression models based on leaf length and width, with R^2 values above 0.95 (Rahimikhoob *et al*., 2023). Practically, such a model offers advantages because it is non-destructive, easy to apply in the field or greenhouse, and highly useful for monitoring leaf morphological responses to various treatments, including different growing media volumes.

This study shows that increasing growing media volume improves the root zone microclimate (lower soil and leaf temperatures and higher moisture retention), thereby supporting vegetative growth and enhancing yield components. However, the smallest media volume (V1) can still be recommended for urban farming because it is more space-efficient and allows higher planting density per unit area. The use of a minimum media volume is feasible when accompanied by adequate irrigation management particularly more frequent and well-timed watering to maintain substrate moisture and reduce the risk of midday wilting. Since this study was performed under a single environmental condition and involved only one lettuce cultivar, the generalizability of the findings may be limited. Further research under diverse climatic conditions, using different cultivars and irrigation regimes, is therefore necessary to verify the robustness and broader applicability of the results.

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Auxin-induced plantlet formation of *Dendrobium stratiotes* x *Dendrobium lineale* hybrid in *in vitro* culture

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Key words: Crossing, native species, orchid, plant growth regulators.

Abstract: Native species orchids, as a source of genetic diversity, can be used in crossing activities to produce hybrid orchids. *Dendrobium* is a type of orchid in great demand and of high economic value because of its unique flower shape and colour. Developing new varieties through interspecific crossings has produced a *D. stratiotes* × *D. lineale* hybrid that successfully germinated in *in vitro* culture. This experiment was carried out to study the effect of auxin type and concentration on *D. stratiotes* × *D. lineale* hybrid orchid plantlet development in *in vitro* culture, as well as to determine the best type and concentration of auxin to stimulate *D. stratiotes* × *D. lineale* hybrid orchid plantlet development. The results showed that the number of shoots formed and the root length of the *D. stratiotes* × *D. lineale* hybrid were controlled by the interaction between auxin type and its concentration. Adding 15 µM IBA produced the most shoots, 16.33 shoots per explant. In addition, NAA application resulted in the longest shoots (1.38 cm shoot per explants) and most roots (11 roots per explants) at 8 weeks after planting, which were significantly different from those produced by IAA or IBA.

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1. Introduction

Orchids are included in the family Orchidaceae, which is widely distributed worldwide. Until now, Orchidaceae is estimated to encompass 700 to 800 genera and approximately 17,000 to 35,000 species worldwide (Widiarti *et al*., 2023). A genus with a broad distribution is *Dendrobium,* which is one of the orchid genera widely cultivated for its high aesthetic and economic value and for its contribution to efforts to preserve biodiversity. This genus has many variations, including their habitat, shape, size, and flower colour (Apriliyani and Wahidah, 2021). *Dendrobium* is one type of orchid easily adapted to environmental stress, such as lack of water, high light intensity, and winter conditions,

compared to other orchids (Tikendra *et al*., 2018). As a plant with many species that have not been identified in nature, orchids continue to be studied as a source of germplasm diversity.

Orchids have high aesthetic and economic value due to their uniqueness and rarity. The newly produced *D. stratiotes* × *D. lineale* variety has not yet been registered under the Plant Variety Protection (PVP) Act. PVP registration, which is «sui generis» of patents, protects plant varieties developed by plant breeders if they contain new, unique, uniform, and stable elements (Diah and Irianti, 2017). Tini *et al*. (2024) state that Dendrobium stratiotes, which originates from the Sunda Islands, Sulawesi, and Maluku (Halmahera and Morotai), flourishes in lowlands as epiphytes with medium-large sizes, enjoys warm to hot circumstances, and bunches together to form groups. *Dendrobium lineale* is a significant giant orchid species native to Papua that flourishes as an epiphyte or lithophyte in warm-to-hot climates. It is commonly found in riverbeds and coastal areas, up to 800 meters above sea level.

Growth regulators (PGRs) are among the factors that affect *in vitro* hybrid orchid propagation (Saad and Elshahed, 2012). PGRs play a part in controlling explant growth during both the plantlet production and multiplication stages. Auxin is one of the PGRs most commonly used for root induction and multiplication. Although auxin promotes cell division, expansion, and differentiation, its primary function is to induce an explant to form roots (Lavy and Estelle, 2016; Leyser, 2018). Auxins such as naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) are frequently utilized in root induction (Chandler, 2016; Prasetyo *et al*., 2020; Shawky Soliman, 2020; Tamyiz *et al*., 2022). This study aims to test the impact of auxin type and concentration on hybrid orchid progeny (*D. stratiotes* × *D. lineale*). Acclimatization will be more successful in offspring with a more muscular root system.

2. Materials and Methods

Plant materials

The plant materials utilized were microshoots of *D. stratiotes* × *D. lineale*, previously cultured on Murashige-Skoog (MS) medium (Phytotech M519) augmented with 10 µM BAP (Sigma-Aldrich B3408) and 30 g.L^{-1} sucrose, solidified with 8% agar (SNP plant).

Root induction and plantlet formation

Plantlet development of *D. stratiotes* × *D. lineale* was performed on MS medium enriched with 30 g L^{-1} sucrose and solidified with 8 g L^{-1} agar. The effects of IAA (Sigma-Aldrich I2886), IBA (Sigma-Aldrich I5386), and NAA (Sigma-Aldrich N0640) at different concentrations were examined. The research was executed experimentally, employing a split-plot design with three replications. The primary plots represented auxin types, specifically IAA, IBA, and NAA, while the secondary plots indicated auxin concentrations of 0 µM, 5 µM, 10 µM, and 15 µM. The medium was calibrated to pH 5.8. The microshoots were cultivated in a single explant per bottle and incubated at 24°C under continuous illumination for 8 weeks. The observed variable was the production of *D. stratiotes* × *D. lineale* plantlets, with the measured parameters being the number of shoots, leaves, roots, and plant length.

Data analysis

The data were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at a 95% confidence level using DSAASTAT VER 1.514 software.

3. Results

According to the observation results, the hybrid orchid explants *D. stratiotes* × *D. lineale* showed significant development, as evidenced by increased shoot number, leaf number, shoot length, root number, and root length in each treatment. The summary of the analysis of variance (ANOVA) presented in Table 1 shows that the types of auxins significantly affect the shoot length and the number of leaves. Auxin concentration significantly affects the number of shoots but does not significantly affect the number of leaves, shoot length, roots, or root length. Furthermore, the interaction between auxin type and concentration substantially affects the number of shoots and roots. However, it does not significantly affect leaf number, shoot length, or root number.

One of the critical parameters in the formation of *D. stratiotes* × *D. lineale* plantlets is the growth of axillary shoots. The analysis of variance (ANOVA) (Table 1) shows that the number of shoots formed is influenced by the concentration of auxin and the interaction between the type and concentration of auxin. The DMRT results (Fig. 1 and 2) show that the

average number of shoots is highest at a concentration of 15 µM, with 12 shoots per explant. This result is significantly different from all the tested auxin concentrations. The data in figure 1 shows that an increase in auxin concentration led to an increase in the number of shoots formed. The lowest average number of shoots was observed at a concentration of 5 µM, with 4.22 shoots per explant.

Interestingly, the control treatment (0 µM) has a higher average number of shoots per explant (5.11) than the 5 µM treatment. The increase in the number of shoots in media without PGR indicates a high cytokinin content in the explants, which can be caused by using BAP during the multiplication stages. BAP is a cytokinin that can bind to sugar and form a BAP-sugar bond that can be stored longer in plants. The results of this study differ from those of Tikendra *et al.* (2018), who reported that a 10 µM auxin concentration induced the best shoot and root formation in *D. thyrsiflorum* Rchb.f cultures, with an

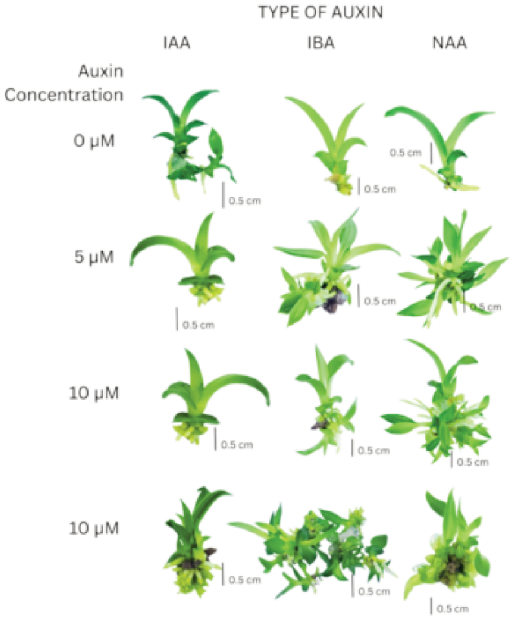


Fig. 2 - The appearance of *D. stratiotes* × *D. lineale* plantlets on media with different types and concentrations of auxin at 8 weeks after planting.

Table 1 - Summary of the analysis of variance (ANOVA) results on the effect of auxin types and concentrations on the parameters of *D. stratiotes* × *D. lineale* hybrid orchid plantlet formation

Source of variance	p value of				
	No. of shoots	No. of leaves	Shoot height	No. of Root	Root length
Auxin type (A)	0.951	0.840	0.040	0.011	0.607
Auxin concentration (K)	0.015	0.291	0.098	0.670	0.050
Interaction A x K	0.046	0.198	0.112	0.332	0.013

p-value <0,01 shows a very significant difference at a 1 % error rate of F-test; p-value < 0,05 shows a very significant difference at 5% error rate of F-test.

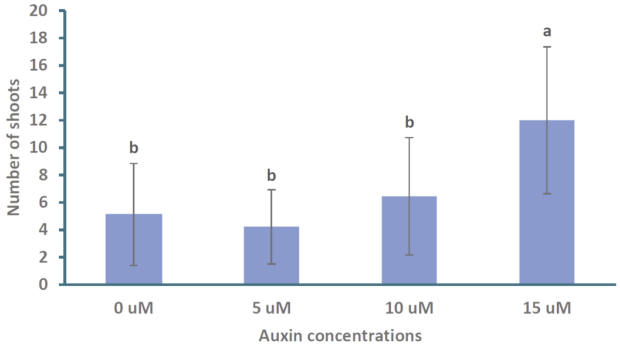


Fig. 1 - The results of DMRT on the average number of shoots of *D. stratiotes* × *D. lineale* hybrid as a response to auxin concentration at 8 weeks after planting. Means marked with different letters are statistically different at DMRT 95%.

average of 3.04 shoots per explant and 6.52 roots per explant. Furthermore, Suryani and Ratnawati (2024) found that the use of auxin (NAA) at a low concentration (0.2 ppm = 1.07 µM) was able to induce the most *Dendrobium* sp. shoots, with an average of 1.44 shoots per explant.

The DMRT test results on the effect of the interaction between auxin types and concentrations on *D. stratiotes* × *D. lineale* plantlet formation (Fig. 3) showed that the 15 µM IBA treatment produced the highest number of shoots, i.e., 16.33 shoots per explant. However, it was not significantly different from those produced by 15 µM IAA and 10 µM NAA, with average shoot counts of 13.67 and 11.33 per explants, respectively. According to Sauer *et al.* (2013), high auxin concentrations can synergize with endogenous cytokinins to increase the number of shoots in culture. Furthermore, Prasetyo *et al.* (2020) state that using the appropriate concentration can accelerate the cell cycle, thereby speeding cell division and differentiation. Barua *et al.* (2022) stated that using 1.5 mg L^{-1} IBA (8.055 µM) supplemented with 0.2% banana extract induced the number of

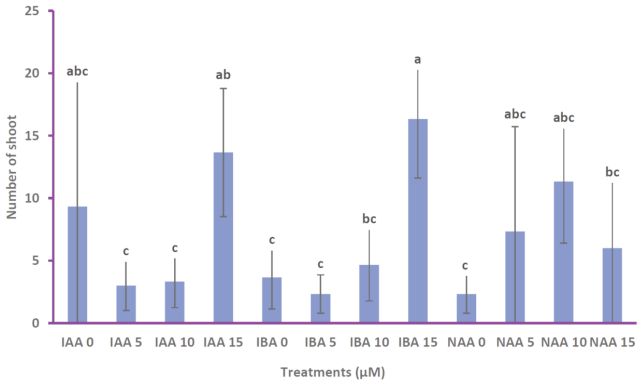


Fig. 3 - The results of DMRT on the average number of shoots of *D. stratiotes* × *D. lineale* hybrid as a response to the interaction between auxin types and concentrations at 8 weeks after planting. Means marked with different letters are statistically different at DMRT 95%.

shoots of *Dendrobium lituiflorum* Lindl. with an average of 14.5 shoots per explant.

Figure 3 shows that the lowest average number of shoots was produced by 0 µM NAA (without PGR/control) and five µM IBA (2.33 shoots per explant). The results of this observation indicate that the addition of auxin to the media can increase the induction of lateral shoots in the hybrid orchid *D. stratiotes* × *D. lineale*. Based on Dobrev *et al.* (2017), auxin can synergize with cytokinin in the formation of axillary shoots by accelerating mitosis.

According to Poothong *et al.* (2018), optimizing nutrient levels in culture media can lead to substantial changes in shoot morphology, including increased shoot length and a greater number of larger, healthier leaves. Putri *et al.* (2024) stated that MS media contains elevated levels of mineral salts and is suitable for most plants, hence optimizing explant growth. According to Müller and Leyser (2011), a high concentration of auxin in the explant caused by exogenous auxin application stimulates ethylene synthesis, thereby inhibiting cell elongation. However, if exogenous auxin is insufficient, growth is suboptimal.

The DMRT result on the effect of auxin types on average shoot length (Fig. 4) showed that NAA yielded the highest average shoot length (1.38 cm). The results obtained differ significantly from those of every other auxin type examined. The media supplemented with IBA showed the shortest shoot length (1.01 cm), followed by the media supplemented with IAA (1.04 cm). All three auxin types tested increased plant length, indicating auxin's role in controlling elongation in explants. Kumar *et al.* (2019) and Saini *et al.* (2013) state that auxin is crucial for cell elongation and enlargement, cell differentiation, and root induction. NAA is a kind of auxin that effectively stimulates root development within a limited concentration range. Tolera (2016) found that NAA and IBA are auxins commonly utilized in the vegetative propagation of sugarcane due to their significant rhizogenic capacity and stability in plant tissue. Furthermore, Kazan (2013) and Pandey *et al*. (2019) state that the auxin hormone signaling stretches cell walls, thereby promoting cell elongation.

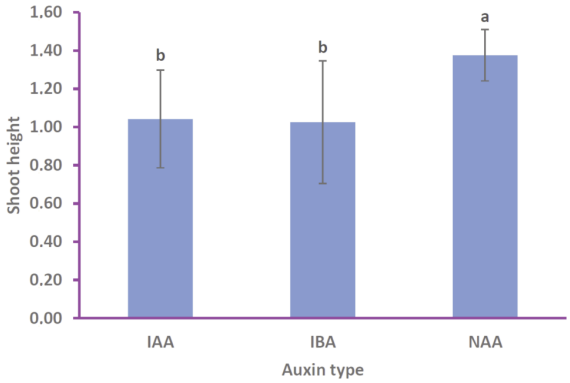


Fig. 4 - The results of DMRT on the average shoot length of *D. stratiotes* × *D. lineale* hybrid as a response to auxin types at 8 weeks after planting. Means marked with different letters are statistically different at DMRT 95%.

Auxin promotes elongation by activating a proton pump at the plasma membrane, secreting H+ ions into the cell wall, thereby lowering pH and facilitating the loosening of cellulose fibers by disrupting their interconnections. Moreover, auxin enhances the expression of expansins, thereby increasing the elasticity of the cell wall. As the cell wall becomes more pliable, an input of water (to be sequestered in the vacuole) results in cellular enlargement (Blakeslee *et al*., 2005; Zhao, 2014; Leyser, 2018).

One of the important parameters in the formation of *D. stratiotes* × *D. lineale* plantlets is the growth of roots. Table 1 shows that the type of auxin treatment influences root formation. The DMRT result on the effect of auxin types on the average number of roots (Fig. 5) showed that NAA yielded the highest number of roots (11.00 roots per explant). However, this result was similar to the IBA treatment, which had an average of 8.33 roots per explant. In contrast, the lowest average number of roots was observed in the IAA-supplemented media, namely 4.33 roots per explant. According to Arli and Noli (2023), NAA 1.5 mg L^{-1} is the best concentration for root formation in *Dendrobium lasianthera* orchids. Furthermore, Lamboro *et al*. (2022) found that 4 mg L^{-1} NAA

induced the greatest rooting in *Arachis hypogaea* cv. Chinese peanut cultivar N3 and Yu-Hua-14 cultures, with an average of 10 roots per explant.

Based on the results of DMRT, the effect of interaction between the type and concentration of auxin on the root length of *D. stratiotes* × *D. lineale* at the age of 8 WAP (Fig. 6) showed that the media supplemented with 10 µM NAA can induce the longest root length with an average of 1.93 cm. The 10 µM NAA treatment showed the best results compared to the media supplemented with IAA and IBA at the same concentration. These results differ from those of Prasetyo *et al*. (2020), who reported that using NAA in sugarcane culture was ineffective at inducing root length; the short root length supports this finding. NAA is included in synthetic auxins used to initiate root formation in plants (Nugroho, 2023). The results of this study differ from those of Bhowmik and Rahman (2020), who stated that *Dendrobium palpebrae* Lindl. cultured in auxin-free media induced the longest roots, averaging 4.82 cm per explant.

Furthermore, Mallik *et al.* (2015) cultured hybrid Dendrobium orchids on media supplemented with 1 mg L^{-1} IAA and 1 mg L^{-1} IBA, which induced a root length of 2.29 cm. The data obtained showed that the shortest average root length was observed in media supplemented with 5 µM IAA. Based on the average root length data obtained, it is assumed that using the right concentration can induce good growth in the formation of hybrid orchid expanses of *D. stratiotes* × *D. lineale* hybrid.

The treatment of auxin types and concentrations on *D. stratiotes* × *D. lineale* hybrid showed that the number of shoots formed and the root length were

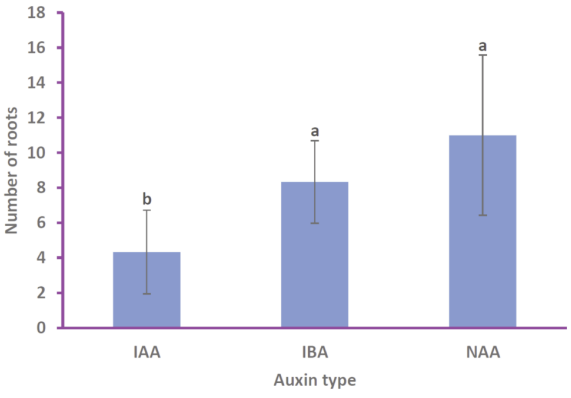


Fig. 5 - The results of DMRT on the average number of roots of *D. stratiotes* × *D. lineale* hybrid as a response to auxin types at 8 weeks after planting. Means marked with different letters are statistically different at DMRT 95%.

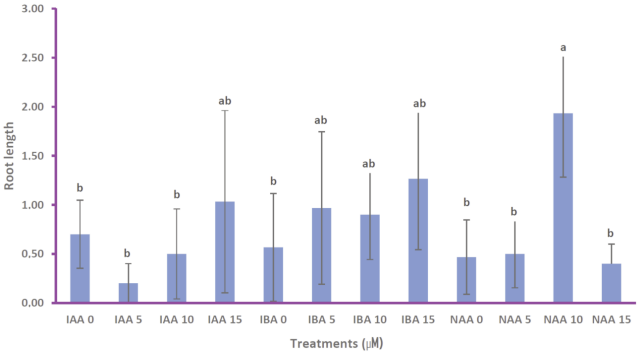


Fig. 6 - The results of DMRT on the average root length of *D. stratiotes* × *D. lineale* hybrid as a response to the interaction between auxin types and concentrations at 8 weeks after planting. Means marked with different letters are statistically different at DMRT 95%.

controlled by the interaction between auxin type and its concentration. The addition of 15 µM IBA produced the most shoots, 16.33 shoots per explant. In addition, NAA application resulted in the longest shoots (1.38 cm shoot per explant) and most roots (11 roots per explant) at 8 weeks after planting, which were significantly different from those produced by IAA or IBA.

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Seed cryopreservation in *Catharanthus roseus* L., impacts on leaf structure, vegetative growth, and biochemical composition of the germinated plantlets

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Key words: Abscisic acid (ABA), alkaloids, desiccation, gibberellic acid (GA_3), seed germination.

Abstract: Cryopreservation offers a valuable approach for the long-term conservation of plant genetic resources, but its influence on seed viability and plant development remains underexplored. This study investigates the physiological and biochemical effects of different pre-cryopreservation drying treatments on *Catharanthus roseus* seeds. Seeds were subjected to silica gel desiccation and oven drying before immersion in liquid nitrogen (-196°C). Results showed that a moderate oven drying period (10 minutes) preserved high seed viability and led to optimal plantlet growth, mirroring untreated controls. In contrast, prolonged silica gel drying (3, and 9 hours) as well as drying for 20 min in oven significantly reduced germination rates, plantlet height, biomass, and levels of carbohydrates and alkaloids. Anatomical evaluations revealed improved vascular and mesophyll structures in plantlet leaves derived from oven-dried seeds, while moderate silica gel exposure increased epidermal thickness, indicating enhanced stress resilience. Hormonal analyses showed higher gibberellic acid (GA_3) and lower abscisic acid (ABA) levels in successfully germinated seeds. In conclusion, the study emphasizes the importance of pre-cryopreservation drying for optimizing plantlet performance and plant development with implications for pharmaceuticals. Thus, this research contributes to refining cryopreservation strategies for sustainable conservation of medicinal plants like *Catharanthus roseus* plants.

1. Introduction

Cryopreservation is a technique used to preserve plant materials by

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ultra-freezing them at temperatures ranging from -80°C to -196°C. It is the most reliable method for long-term genetic resource conservation, provided that species-specific protocols are followed. The fundamental principle behind cryopreservation is to halt all cellular metabolic activities, thereby preventing cell division and significantly reducing metabolism while maintaining viability over time (Benelli, 2021). Unlike conventional freezing methods, which fail to completely arrest metabolic processes and can disrupt the distribution and concentration of essential molecules, cryo-preservation effectively prevents cellular deterioration, thus ensuring successful preservation (Kaviani, 2011).

The success of plant cryopreservation largely depends on the natural ability of seeds to withstand low water content without losing viability (Walters, 2015). Reducing water content before freezing is crucial to prevent ice crystal formation within cells, which could cause structural damage (Kaviani, 2011). In general, water content reduction can be achieved in two ways: (1) using chemical cryoprotectants such as glycerol, sucrose, and dimethyl sulfoxide, which induce osmotic dehydration (Carvalho and Otoni, 2010); or (2) through desiccation, a drying method that removes water without chemical additives, minimizing potential toxicity. While desiccation is effective for seeds and pollen (Ballesteros *et al.*, 2020; Xie *et al.*, 2022), other plant materials like calli, shoot tips, and buds require chemical cryoprotectants for successful dehydration (Jenderek and Reed, 2017; Dinato *et al.*, 2020; Wang *et al.*, 2021). Achieving optimal water content is essential for vitrification, a process in which water transitions into a non-crystalline, supersaturated, amorphous solid state (Coelho *et al.*, 2017).

As seeds serve as one of the primary germplasm sources for ex situ conservation in horticulture and forestry (Li and Pritchard, 2009), establishing seed banks has become essential. The standard preservation method for most plant species involves drying seeds and storing them at -20°C (Wyse and Dickie, 2017). However, some seeds exhibit short viability and lose their ability to germinate over time (Walters *et al.*, 2005; Ballesteros and Pence, 2017; Davies *et al.*, 2018). Additionally, many seeds do not store well or fail to survive at -18 ± 3°C, even if they undergo desiccation (Ballesteros *et al.*, 2020). This highlights the necessity of cryopreservation for long-term seed storage. Moreover, seed cryopreservation plays a crucial role in mitigating the effects of climate change, which can alter germination patterns. It also helps conserve endangered plant species with significant ecological and economic value (Ramanatha Rao and Parthasarathy, 2019; Dar *et al.*, 2020).

The Madagascar periwinkle (*Catharanthus roseus* L. G. Don.), a member of the Apocynaceae family, is one of the most valuable medicinal plants due to its alkaloid content. This species contains vincristine and vinblastine, two potent anticancer compounds used to treat leukemia and Hodgkin's disease (Filippini *et al.*, 2003). Given its pharmaceutical importance, *C. roseus* is highly sought after for medicinal applications. However, natural populations of this plant are limited, necessitating cultivation and conservation efforts through seed preservation. Among the available preservation techniques, cryopreservation remains the most viable method for ensuring the long-term conservation of *C. roseus* genetic resources. While cell suspension cryopreservation has been reported (Fatima *et al*., 2009; Mujib *et al*., 2022), no documented studies exist on seed cryopreservation for this species.

Although *Catharanthus roseus* produces orthodox seeds that can be stored successfully in conventional seed banks at low temperatures (e.g., 5°C), such storage is primarily suitable for medium-term conservation and does not fully prevent gradual physiological and biochemical deterioration over extended periods. Buchwald *et al.* (2007) showed that, despite prolonged viability at 5°C, seed aging and loss of vigour may still occur, potentially affecting subsequent plant development and metabolic performance. Moreover, conventional storage conditions do not adequately safeguard genetic integrity against long-term oxidative damage and subtle cellular alterations (Trusiak *et al.,* 2022; Tan *et al.,* 2025). Seed cryopreservation at ultra-low temperatures (-196°C) offers a distinct advantage by completely arresting metabolic activity, thereby enabling true long-term conservation with minimal risk of genetic or physiological change (Klein, 2003). In the present study, cryopreservation was therefore employed not merely as a storage method, but as a strategic conservation and experimental tool to evaluate its effects on post-germination leaf structure, vegetative growth, and biochemical composition. This approach allows assessment of

whether cryogenic exposure induces developmental or metabolic alterations compared with conventionally stored seeds, providing insights beyond seed viability alone.

Despite extensive studies on seed cryopreservation, the fundamental factors required for developing a standardized protocol remain poorly understood. Limited research has been conducted on the endogenous molecules present in zygotic embryos and germinated plantlets post-cryopreservation. Among these molecules, phytohormones play a vital role in plant growth and development (Shahid *et al.*, 2024). These plant growth regulators (PGRs) are essential for embryogenesis (Rodríguez-Sanz *et al.*, 2014; Pérez-Pérez *et al.*, 2019) and influence the growth patterns of germinated plantlets (Ghosh *et al.*, 2025). However, there is limited information regarding changes in endogenous hormone levels following seed cryopreservation.

This study aims to investigate possible alterations in two key phytohormones, gibberellic acid (GA_3) and abscisic acid (ABA), in plantlets derived from cryopreserved *C. roseus* seeds, given their crucial roles in seed germination and plant development (Abley *et al.*, 2021). The primary objective is to establish an effective, long-term seed cryopreservation protocol. We aim to assess changes in endogenous GA_3 and ABA levels in germinated plantlets. This comparison across different seed drying treatments will help identify key factors influencing the success of seed cryopreservation. In addition, carbohydrate and alkaloid content are analyzed. By addressing the knowledge gaps in seed cryopreservation, this research improves our understanding of the physiological and biochemical response of plantlets derived from cryopreserved seeds. The findings contribute to identifying how different pre-cryopreservation drying treatments influence post-germination performance in Catharanthus roseus, thereby supporting the optimization of seed cryopreservation protocols for conservation and pharmaceutical applications.

2. Materials and Methods

Plant materials

Catharanthus roseus L. G. Don. seeds were collected in March 2024 from plants grown in the Ornamental Horticulture Nursery at Cairo University, Egypt. (30°01’42.6’’ N, 31°12’28.4’’ E). The seeds were then transported to the Laboratory of Ornamental, Medicinal, and Aromatic Plants, Faculty of Agriculture, Cairo University, for cryopreservation experiments.

Seed drying treatments and water content

The seeds were wrapped in aluminum foil envelopes 100 seeds per each and weighed to determine their wet weight. Two drying methods were applied: (1) desiccation using silica gel and (2) oven drying at 70°C. For desiccation, three opened envelopes were incubated in a 7.27 L glass desiccator with 500 g silica gel at room temperature for 3, and 9 hours. For the oven drying, the seeds were incubated at 70°C for 10 and 20 minutes. A total of five drying treatments were performed, along with a control group of fresh seeds. After each drying period, the seed weight was recorded, and the percentage of water content (WC, %) was calculated using the following equation:

$$WC\ (\%) = [(W_1 - W_2) / W_1] \times 100$$

Where: W1 = initial seed weight (wet weight before drying) and W2 = seed weight after drying.

Seed cryopreservation

The seeds, after desiccation, were placed in 3-mL cryogenic tubes and directly immersed in liquid nitrogen at -196°C for 24 hours. Following cryopreservation, the seeds were warmed at room temperature (25±3°C) before germination trials. Six treatments were conducted in a completely randomized design with three replicates of 150 seeds per treatment: (1) Control (unfrozen seeds), (2) Cryopreservation without drying treatment, (3) Cryopreservation after 3 hours of silica gel desiccation, (4) Cryopreservation after 9 hours of silica gel desiccation, (5) Cryopreservation after 10 minutes of oven drying, and (6) Cryopreservation after 20 minutes of oven drying.

Seed germination performance

Following cryopreservation, the seeds were sown in germination trays containing a 2:1:1 growing mixture of coco peat, perlite, and sand whose physical and chemical characteristics were analyzed in the laboratory of Soil, Water and Environment

Research Institute (SWERI), in Agricultural Research Centre (ARC), Giza, Egypt and the results are shown in Table 1. For each treatment, the 450 seeds were evenly distributed among three trays (150 seeds per tray). Each tray was subdivided into three equal quadrants, which were considered sub-replicates, resulting in nine observational units per treatment. Germination percentage and growth parameters were recorded for each quadrant. The experiment was arranged in a randomized complete block design (RCBD) with six cryopreservation treatments and three replicates (trays) per treatment, while quadrants within each tray were treated as subsamples for statistical analysis.

Table 1 - Physical and chemical characteristics of the medium used for *in vivo* germination

Characteristic	Value
PH	6.35
Electron conductivity (EC) (ds/m)	4.54
Total nitrogen (%)	0.82
Total phosphorus (%)	0.05
Total potassium (%)	0.03
C/N ratio	80
Organic carbon (%)	50.32
Bulk density ($g \cdot cm^{-3}$)	0.41

Seed germination percentage and germination speed index

Germination percentage (G%) and germination speed index (GSI) were calculated following De Oliveira *et al.* (2021) as follow:

1. Germination percentage is calculated using this formula:

$$G\ (\%) = (N/A) \times 100$$

where: G (%) = germination percentage, N = number of germinated seeds and A = total number of sowed seeds.

2. Germination speed index (GSI) is calculated using this formula:

$$\mathrm{GSI} = \sum \frac{G_i}{n_i}$$

where: Gi = number of germinated seeds on a given day, and ni = number of days.*Growth parameter measurement*

The plantlets were harvested 60 days after sowing, therefore, the following data parameters on the *C. roseus* were recorded for nine randomly selected plants with three replicates: Plantlet height (cm), plantlet fresh weight (g). In addition, all leaves in each plant were sampled to measure the number of leaf pairs (n). For dry weight determination, plantlets were air-dried for 2 days to prevent immediate heat shock to the plantlet tissue followed by being oven-dried at 70°C overnight, after which their dry weights (g) were measured according to Beecher *et al.* (2013).

Anatomy and light microscopy analysis

Plantlet leaves were collected for anatomical analysis three months post-germination. The leaves were cut into small pieces and fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 3 hours, followed by post-fixation with 2% osmium tetroxide for 4 hours. Dehydration was performed using an ethanol series, and samples were embedded in epoxy resin as described by Hunter (1993). Semithin sections (500 nm) were obtained using a Leica Ultracut UCT Ultramicrotome and stained with toluidine blue. Sections were examined under a Leica optical microscope, and images were captured using the Leica Light Image Analysis System DM 750 at the Cairo University Research Park (CURP), Faculty of Agriculture.

Chemical composition analysis

Carbohydrate content. Samples (0.5 g) of dried plantlets, after removing the roots, were ground and mixed with 1 mL of 5% phenol, 5 mL concentrated sulfuric acid, and deionized water (up to 1000 mL). The mixture was heated in a water bath, followed by the addition of 12 mL NaOH to 15 mL of solution before reheating. After cooling, absorbance was recorded at 490 nm using a UV/VIS spectrophotometer (Jenway, England) against a blank (deionized water). D-glucose served as the standard, and carbohydrate content was determined as described by Dubois *et al.* (1956).

Total alkaloid content. A 100 g plantlet sample was ground and extracted overnight in methanol using a Soxhlet apparatus. The methanol extract was evaporated under vacuum at 45°C and dissolved in diluted HCl. To detect alkaloids, 2-3 drops of Dragendorff's reagent were added; a crystalline precipitate indicated the presence of alkaloids. Dragendorff's reagent with caffeine was used as the standard, and total alkaloid concentration was determined following Ajanal *et al.* (2012).

Endogenous GA_3 and ABA determination. Fresh plantlets were frozen in liquid nitrogen (-196°C for 20 seconds) and stored at -80°C. Samples were ground and extracted in 80% methanol (15 mL/g FW) with butylated hydroxytoluene (BHT) as an antioxidant at 4°C in darkness. The extract was centrifuged at 4000 rpm, and the supernatant was reduced to 10 mL under vacuum at 35°C. An equal volume of pure ethyl acetate was added, and pH was adjusted. The extract was dehydrated using anhydrous sodium sulfate, filtered, and evaporated at 35°C under vacuum. The residue was redissolved in 1 mL absolute methanol. Methylation was performed following Fales and Jaouni (1973) for GA_3 and ABA determination.

Phytohormones were quantified using an ATI Unicam gas-liquid chromatograph (610 Series) with a flame ionization detector, following Vogel (1975). A coiled glass column (1.5 m x 4 mm) was used for fractionation. Phytohormone peaks were identified using exogenous hormone standards, and concentrations were calculated using Microsoft Excel.

Statistical analysis

Data were analyzed using MSTATC software (MSTAT C, 1993). A one-way ANOVA was performed, and Duncan's multiple range test (Snedecor and Cochran, 1967) was used to determine significant differences between means at P≤0.05.

3. Results

Effect of drying methods on seed water content

Desiccation treatments were performed on freshly collected seeds, and the changes in water content percentage following these treatments are presented in figure 1. Fresh seeds without desiccation exhibited the highest water content (71.32%). Desiccation on silica gel for 3 and 9 hours significantly reduced the water content to 50.93% and 48.22%, respectively. Drying in an oven for 10 and 20 minutes further decreased the water content to 40.35% and 35.36%, ranking fourth and fifth, respectively.

Evaluation of seed germination after cryopreservation and drying treatments

Following cryopreservation preceded by different drying pre-treatments, the highest germination percentage (G%) and germination speed index (GSI) were recorded in fresh, non-cryopreserved seeds

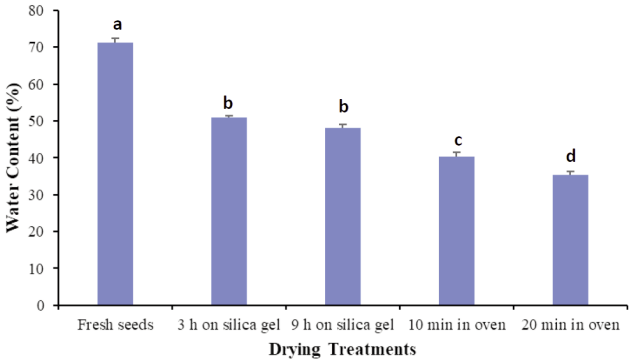


Fig. 1 - Effect of drying treatments on the water content percentage of *Catharanthus roseus* seeds. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test (P≤0.05).

(control) (Fig. 2 and 3). Among cryopreserved seeds, those subjected to 10 minutes of oven drying showed the highest G% and GSI values. No significant difference in G% was observed between the control and seeds cryopreserved after 10 minutes of oven drying, as both treatments belonged to the same statistical group (Fig. 2). In contrast, a significant reduction in G% was detected in all other cryopreservation treatments, including cryopreserved seeds without any drying pre-treatment, which did not differ significantly from seeds desiccated for 3 or 9 h on silica gel or those dried for 20 minutes in the oven (Fig. 2). Regarding GSI, the control exhibited the highest value, followed by seeds cryopreserved after 10 minutes of oven drying (Fig. 3). The lowest GSI values were recorded in cryopreserved seeds without drying and in those

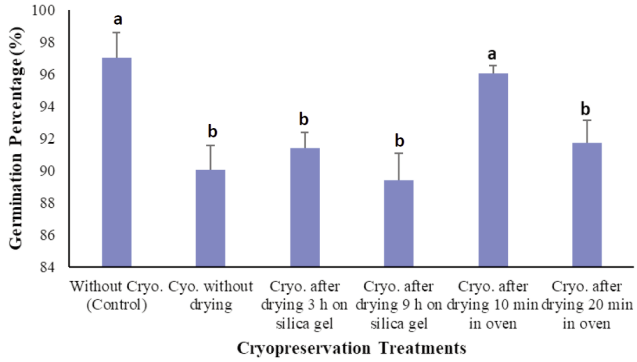


Fig. 2 - Germination percentage of cryopreserved seeds in *Catharanthus roseus* showing the effect of seed drying treatments. Cryo.= Cryopreservation. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test (P≤0.05).

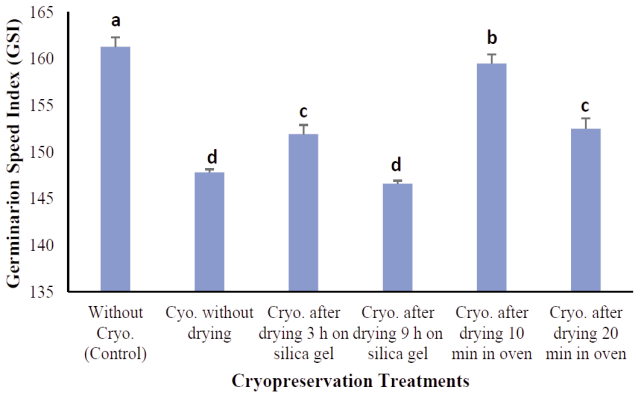


Fig. 3 - Germination speed index (GSI) of cryopreserved seeds in *Catharanthus roseus* showing the effect of seed drying treatments. Cryo.= Cryopreservation. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test (P≤0.05).

desiccated for 9 h on silica gel, indicating delayed germination under insufficient or excessive dehydration prior to cryopreservation. Seeds cryopreserved after 3 h of silica gel desiccation and those dried for 20 minutes in the oven showed intermediate GSI values, differing significantly from both the control and the 10-minute oven-dried treatment (Fig. 3).

Growth parameter evaluation after seed cryopreservation and drying treatments

Seed cryopreservation significantly influenced the growth parameters of *Catharanthus roseus* plantlets depending on the drying treatment applied prior to freezing (Table 2, Fig. 4). Plantlet height was significantly influenced by the drying pre-treatment applied prior to cryopreservation (Table 2). Seeds dried for 10 minutes in the oven before cryopreservation produced plantlets with heights (13.42 cm) comparable to the non-cryopreserved control (13.54 cm), indicating that this treatment effectively preserved post-cryogenic shoot growth. In contrast, cryopreservation without drying resulted in a significant reduction in plantlet height (10.17 cm). Progressive reductions in height were observed with increasing desiccation duration on silica gel, with the 3 h treatment showing an intermediate response, while 9 h of silica gel desiccation and 20 minutes of oven drying led to the lowest plantlet heights. Overall, these results demonstrate that plantlet height is optimized within a narrow dehydration window prior to cryopreservation, and that both insufficient and excessive drying negatively affect shoot elongation. The number of leaf pairs was significantly influenced by the drying pre-treatment applied prior to cryopreservation (Table 2). Seeds dried for 10 minutes in the oven before



Fig. 4 - Development of plantlets obtained from cryopreserved seeds of *Catharanthus roseus*, after two months after sowing the seeds . (A) Without cryopreservation (control), (B) Cryopreservation without drying, (C) Cryopreservation after drying 3 h on silica gel, (D) Cryopreservation after drying 9 h on silica gel, (E) Cryopreservation after drying 10 min in oven, (F) Cryopreservation after drying 20 min in oven.

Table 2 - Effect of seed cryopreservation after different drying treatments on plantlet growth parameters in *Catharanthus roseus*

Seed cryopreservation and drying treatments	Plantlet height (cm)	No. leaf pairs	Fresh weight of plantlet (g)	Dry weight of plantlet (g)
Without Cryo. (Control)	13.54 ± 0.67 a	3.73 ± 0.51 a	0.65 ± 0.05 a	0.40 ± 0.02 a
Cryo. without drying	10.17 ± 0.73 b	2.60 ± 0.61 bc	0.19 ± 0.01 c	0.12 ± 0.01 bc
Cryo. after drying 3 h on silica gel	9.65 ± 0.35 bc	2.93 ± 0.72 abc	0.24 ± 0.02 c	0.15 ± 0.02 b
Cryo. after drying 9 h on silica gel	8.77 ± 0.26 c	2.73 ± 0.76 abc	0.19 ± 0.02 c	0.12 ± 0.01 bc
Cryo. after drying 10 min in oven	13.42 ± 0.48 a	3.70 ± 0.56 a	0.58 ± 0.03 b	0.39 ± 0.02 a
Cryo. after drying 20 min in oven	8.74 ± 0.48 c	3.23 ± 0.15 ab	0.23 ± 0.01 c	0.15 ± 0.01 b

Cryo.= Cryopreservation. No.= Number. Data represent the mean value ± standard deviation for three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test (P≤0.05).

cryopreservation produced plantlets with a leaf pair number (3.70) comparable to the non-cryopreserved control (3.73). Cryopreservation without drying resulted in the lowest number of leaf pairs (2.60). Seeds desiccated on silica gel for 3 or 9 h exhibited intermediate values, while 20 minutes of oven drying maintained relatively high leaf pair numbers, not significantly different from the control. These results indicate that leaf pair formation is a parameter relatively tolerant to cryopreservation stress when seeds are subjected to appropriate pre-drying. Fresh and dry weights of *Catharanthus roseus* plantlets were significantly influenced by cryopreservation and the drying pre-treatments applied prior to freezing (Table 2). The control treatment exhibited the highest fresh (0.65 g) and dry (0.40 g) weights of plantlets. Among plantlets derived from cryopreserved seeds, those originating from seeds dried in the oven for 10 minutes exhibited the highest fresh weight (0.58 g) and a dry weight (0.39 g) comparable to that of the control. In contrast, plantlets derived from cryopreserved seeds without prior drying, as well as those from seeds desiccated on silica gel for 3 or 9 h or oven-dried for 20 minutes, showed markedly reduced fresh and dry weights, with no significant differences observed among most of these treatments. These results indicate that biomass accumulation in plantlets is optimized within a narrow dehydration window prior to cryopreservation, and that fresh weight is more sensitive to cryogenic stress than dry matter accumulation. Overall, the results indicate that short oven drying (10 min) before cryopreservation preserves plantlet growth most effectively, while silica gel drying or lack of drying compromises post-cryopreservation growth performance.

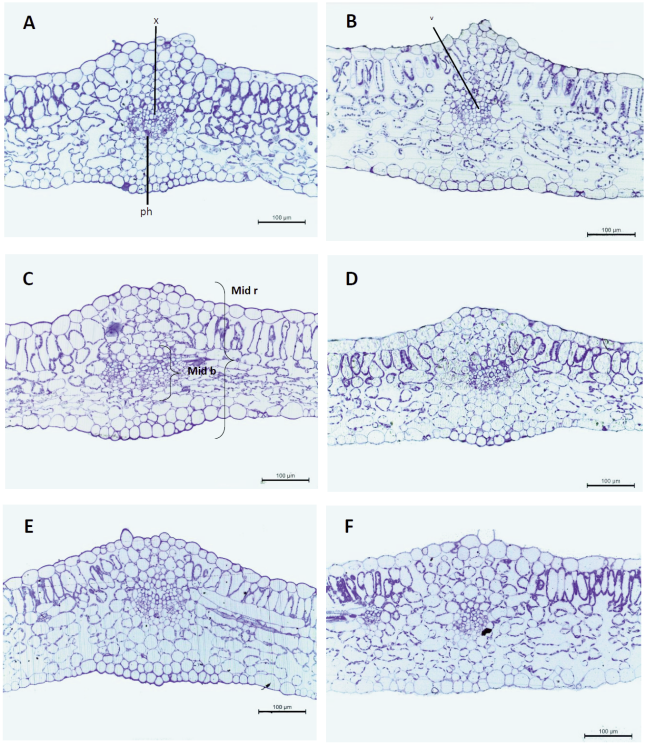


Fig. 5 - Anatomical cross sections of *Catharanthus roseus* leaf harvested from the plantlets aged two months of sowing after cryopreservation (A) Without cryopreservation (control), (B) Cryopreservation without drying, (C) Cryopreservation after drying 3 h on silica gel, (D) Cryopreservation after drying 9 h on silica gel, (E) Cryopreservation after drying 10 min in oven, (F) Cryopreservation after drying 20 min in oven. Mid b: midvein bundle, Mid r: midvein region, ph: phloem, v: vessel, x: xylem. Scale bars = 100 µm.

Leaf anatomy of plantlets germinated from cryopreserved seeds

Anatomical analysis of *Catharanthus roseus* leaves revealed, as shown in the control (Figs. 5 A, 6A), that both upper and lower epidermal layers consisted of a single layer of cells, with stomata present on both surfaces. All examined plantlets exhibited a typical dicotyledonous leaf structure, characterized by an amphistomatic epidermis with stomata present on both adaxial and abaxial surfaces, a differentiated mesophyll consisting of palisade and spongy tissues, and a collateral vascular bundle. The upper epidermal cells were notably larger than the lower ones The mesophyll comprised two compact layers of palisade cells, while the spongy mesophyll consisted of 4-5 layers of parenchyma cells with intercellular spaces and abundant chloroplasts. The vascular bundle was collateral, consisting of internal phloem and external xylem vessels.

Despite this conserved overall organization, clear treatment-dependent variations were observed in tissue thickness, cell size, and mesophyll arrangement (Table 3, Fig. 5 and 6). These variations were particularly evident in lamina thickness, palisade tissue development, spongy mesophyll compactness, epidermal thickness, and midvein bundle dimensions. The comparative ranking of treatments was based on the overall preservation of key anatomical parameters, including midvein thickness, lamina thickness, mesophyll organization (palisade and spongy tissues), epidermal thickness, and dimensions of the main midvein bundle. Control plants (without cryopreservation) exhibited the most

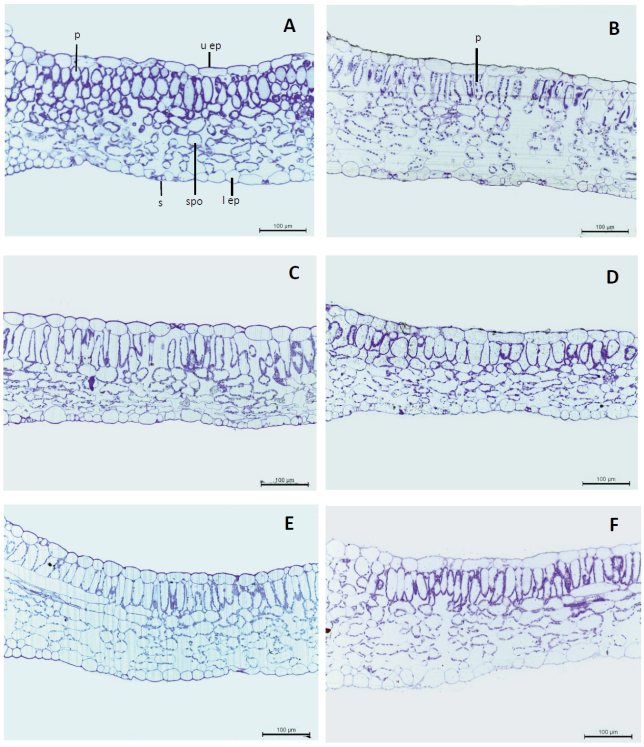


Fig. 6 - Sections of the blade leaf of *Catharanthus roseus*. The leaves are harvested from the plantlets aged two months of seeds sowing after cryopreservation showing the effect of seed drying treatments. (A) Without cryopreservation, (B) Cryopreservation without drying, (C) Cryopreservation after drying 3 h on silica gel, (D) Cryopreservation after drying 9 h on silica gel, (E) Cryopreservation after drying 10 min in oven, (F) Cryopreservation after drying 20 min in oven. l ep: lower epidermis, u ep: upper epidermis, p: plastids, s: stomata, spo: spongy tissue, pal: palisade tissue. Scale bars = 100 µm.

balanced and well-developed leaf anatomy, characterized by uniform mesophyll differentiation and intact vascular tissues (Fig. 5A and 6A). Among cryopreserved treatments, plantlets regenerated from seeds subjected to oven drying for 10 min showed anatomical measurements closest to the control, including slightly higher midvein thickness (+2.08%) and main midvein bundle dimensions (+10.09%) (Table 3, Fig. 5E). This treatment also resulted in modest increases in lamina and spongy tissue thickness (Table 3, Fig. 6E), reflecting structural adjustment rather than anatomical disruption. Similarly, cryopreservation following 3 h desiccation on silica gel maintained well-organized mesophyll tissues and vascular integrity (Fig. 5C and 6C). This treatment was associated with increased thickness of the upper and lower epidermis compared with the control (Table 3), which may indicate adaptive tissue responses to dehydration stress rather than enhanced growth per se. In contrast, prolonged dehydration prior to cryopreservation, particularly drying for 9 h on silica gel, led to marked reductions in midvein thickness, lamina thickness, and palisade tissue thickness (Table 3, Fig. 5D and 6D), suggesting greater anatomical stress. The weakest anatomical development was observed in plantlets regenerated from cryopreserved seeds without prior drying, which showed reduced lamina and palisade tissue thickness and less organized mesophyll structure (Table 3, Fig. 5B and 6B). Overall, moderate drying pre-treatments before cryopreservation (10 min oven drying or 3 h silica gel drying) resulted in better

Table 3 - Measurements (µm) of some histological characters in the transverse sections of *Catharanthus roseus* leaf, showing the effect of seed cryopreservation after different drying treatments

Treatments	Histological aspects						
	Thickness of midvein	Thickness of upper epidermis	Thickness of lower epidermis	Thickness of lamina	Thickness of palisade tissue	Thickness of spongy tissue	Dimensions of main midvein bundle
Without Cryo. (Control)	281.65	18.45	16.95	155.65	71.44	84.20	80.84
Cryo. without drying	286.74	18.33	16.64	147.58	63.93	83.65	80.72
Cryo. after drying 3 h on silica gel	280.67	20.66	19.35	144.81	70.53	74.28	90.06
Cryo. after drying 9 h on silica gel	247.04	20.08	17.76	107.85	38.93	68.92	73.07
Cryo. after drying 10 min in oven	287.52	19.92	19.80	147.48	66.77	80.71	88.70
Cryo. after drying 20 min in oven	281.95	20.02	19.93	143.90	63.53	80.37	76.42

Cryo.= Cryopreservation. Means of three sections from three specimens.

preservation of leaf anatomical integrity, whereas the absence of drying or excessive dehydration adversely affected mesophyll development.

Chemical composition of plantlets after seed cryopreservation and drying treatments

Carbohydrate content. Total carbohydrate content analysis revealed that plantlets derived from cryopreserved seeds dried in the oven for 10 minutes exhibited the highest carbohydrate level (19.32%), second only to the control group (19.59%), with no significant difference between them. In contrast, the remaining treatments showed significant decreases in carbohydrate content. The lowest levels were observed in plantlets derived from cryopreserved seeds without drying, which were not significantly different from those dried for prolonged periods (3 and 9 hours on silica gel, and 20 minutes in the oven) (Table 4).

Alkaloid content. Alkaloid content followed a similar trend to carbohydrate levels. The total alkaloid content in *Catharanthus roseus* plantlets was significantly influenced by the cryopreservation and drying treatments applied to the seeds. The highest alkaloid concentration was observed in the control group (15.39 mg/100 g DW), which did not undergo cryopreservation. Among the cryopreserved groups, plantlets derived from seeds dried for 10 minutes in the oven prior to cryopreservation maintained a comparably high alkaloid content (15.21 mg/100 g DW), showing no significant difference from the control. In contrast, all other drying pre-treatments, including those without drying, dried with silica gel (3 or 9 h), and dried in the oven for 20 minutes, resulted in significantly lower alkaloid levels, ranging from 10.62 to 10.99 mg/100 g DW (Table 4).

Endogenous hormone levels (GA_3 and ABA Content). Endogenous gibberellic acid (GA_3) and abscisic acid (ABA) levels in fresh plantlets of *Catharanthus roseus* were assessed (Fig. 7). The highest GA_3 levels were recorded in the control plantlets (11.25 µg/g FW) and plantlets derived from cryopreserved seeds following 10 minutes of oven drying (10.67 µg/g FW). Lower GA_3 levels were detected in plantlets from seeds cryopreserved after 3 hours of silica gel desiccation (7.86 µg/g FW), 20 minutes of oven drying (7.77 µg/g FW), 9 hours of silica gel desiccation (7.72 µg/g FW), and cryopreservation without drying (7.68 µg/g FW), with no significant differences among these treatments.

Conversely, ABA levels exhibited an inverse trend. The lowest ABA levels were recorded in the control (4.02 µg/g FW) and plantlets derived from

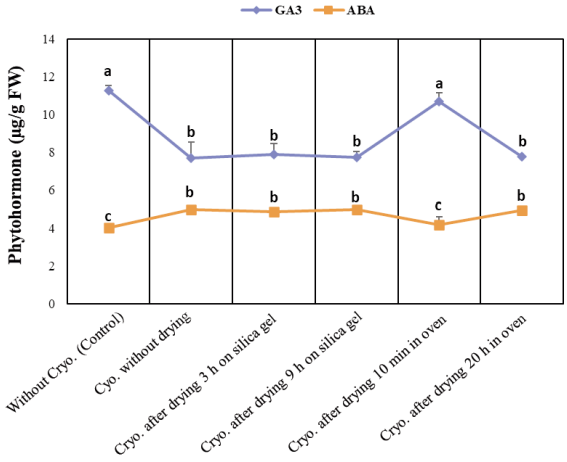


Fig. 7 - The contents of two endogenous hormones, gibberellin A3 (GA3) and Abscisic acid (ABA) for the plantlets obtained from cryopreserved seeds in *Catharanthus roseus*. Cryo.= Cryopreservation. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P \leq 0.05$).

Table 4 - Carbohydrate and total alkaloid contents in the plantlets development from cryopreserved seeds in *Catharanthus roseus*

Seed cryopreservation and drying treatments	Carbohydrate (%)	Total Alkaloids (mg/100 g DW)
Without Cryo. (Control)	19.59 ± 1.67 a	15.39 ± 1.11 a
Cryo. without drying	13.48 ± 1.08 b	10.62 ± 0.33 b
Cryo. after drying 3 h on silica gel	13.96 ± 1.27 b	10.78 ± 0.40 b
Cryo. after drying 9 h on silica gel	13.59 ± 0.67 b	10.67 ± 0.59 b
Cryo. after drying 10 min in oven	19.32 ± 0.68 a	15.21 ± 0.58 a
Cryo. after drying 20 min in oven	14.19 ± 0.90 b	10.99 ± 0.70 b

Cryo.= Cryopreservation. Data represent the mean value ± standard deviation for three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P \leq 0.05$).

cryopreserved seeds after 10 minutes of oven drying (4.19 µg/g FW). This confirms the inverse relationship between endogenous GA_3 and ABA levels in *Catharanthus roseus* plantlets.

4. Discussion and Conclusions

This study investigated the effects of seed cryopreservation on *Catharanthus roseus*, with an emphasis on how various pre-cryopreservation desiccation treatments influence seed germination, vegetative growth, anatomical leaf development, chemical composition, and hormone levels. The findings collectively suggest that the drying method and duration prior to cryopreservation are crucial determinants of post-storage viability and performance.

Drying duration influences seed germination and plantlets vigor after cryopreservation

The study began by evaluating how different drying techniques, namely silica gel desiccation and oven drying influence seed water content. Fresh seeds had the highest water content, indicating their unaltered physiological condition. Silica gel drying reduced seed moisture progressively with time: 3 hours brought it down to 50.93%, and 9 hours to 48.22%. Oven drying, in contrast, resulted in faster moisture reduction: 10 minutes lowered the water content to 40.35%, while 20 minutes further decreased it to 35.36%. These observations support the notion that heat application accelerates desiccation but may introduce stress if overextended (Kumar *et al.*, 2023). The importance of moisture regulation prior to cryopreservation is well-documented, as excessive water can lead to intracellular ice formation, damaging cellular structures (Walters *et al.*, 2010; Campos *et al.,* 2024). Controlled dehydration prior to ultra-low temperature storage is crucial because the amount of residual water determines the likelihood of lethal intracellular ice formation during freezing, especially in intermediate seeds that do not tolerate high residual moisture (da Conceição Moreira *et al.*, 2014; Zeng *et al.*, 2024). In *Catharanthus roseus*, seeds dried on silica gel or in an oven reached lower moisture contents than fresh seeds, and the differences in desiccation rates reflect distinct dehydration mechanisms. Rapid moisture loss via oven drying (~40.35% at 10 min) likely promotes faster removal of free water, which can help avoid ice crystallization during cryostorage (Chetverikova, 2008). However, too aggressive dehydration, as seen with extended oven drying or prolonged silica gel exposure, can itself be a stressor, compromising membrane stability and cellular function (Bharuth and Naidoo, 2020; Luro *et al.,* 2023). This duality between necessary dehydration and over-desiccation has been highlighted in studies on intermediate and partially desiccation-tolerant seeds, where moderately reduced water content enhances survival after cryopreservation, whereas either insufficient drying or excessive dehydration reduces post-thaw viability (Berjak and Pammenter, 2013). Indeed, seeds with very high moisture levels are prone to intracellular freezing injury, whereas seeds that are too dry may suffer increased oxidative stress and reduced metabolic competence for germination (Prada *et al.,* 2015). Thus, in cryopreservation protocols, balancing seed moisture content is essential to minimize both ice formation and dehydration damage, and this balance appears to be reflected in the present study's findings where moderate drying treatments resulted in better post-cryogenic performance compared with the extremes. On the other hand, too much dehydration may compromise membrane stability and enzymatic activity, reducing seed viability (Corbineau, 2024). Thus, balancing moisture content is essential.

Germination trials showed significant differences among drying treatments. Fresh seeds exhibited the highest germination percentage (G%) and germination speed index (GSI). Seeds dried in an oven for 10 minutes showed germination outcomes comparable to the control in terms of G% but with modestly reduced GSI. This suggests that brief oven drying is effective for preserving germinability while mitigating the risks of extended dehydration. These results align with studies emphasizing the need to optimize seed moisture content before cryopreservation. Ballesteros *et al.* (2021) discussed that for both orthodox and intermediate seed species, controlled dehydration to an appropriate moisture range is crucial for effective cryostorage and long-term viability, since seeds with too high or too low water content may not tolerate ultra-low temperature exposure well. Impe *et al.* (2022) also demonstrated in wheat pollen that the rate of drying and cooling interacts to influence post-cryopreservation survival, indicating that both the degree and the kinetics of dehydration are important

determinants of viability after freezing and thawing.

Seeds dried on silica gel for 3 and 9 hours showed slight declines in both G% and GSI, which may reflect marginally greater dehydration stress compared to the short oven drying. The broader literature on seed dehydration highlights physiological constraints: seeds that lose too much water may enter a state where cellular membranes are destabilized and metabolic processes are impaired, delaying germination. For example, Smolikova *et al.* (2020) reviewed mechanisms of seed desiccation tolerance, emphasizing that dehydration is a complex physiological transition involving modulation of membrane lipids and stress protective proteins; when dehydration exceeds the tolerance threshold, seed vigor and germination rates often decline. Similarly, Feng *et al.* (2017) reported that drought and dehydration stresses in plant tissues lead to altered redox balance and membrane integrity changes, which in seeds can slow or reduce germination potential, especially under severe water deficits. Although these studies focus on stress responses broadly rather than cryopreservation per se, they provide mechanistic insight into why extended dehydration may impede germination by perturbing normal physiological recovery after rehydration. Saha *et al.* (2022) further emphasize that drought-type stress responses, such as increased oxidative stress and perturbations in metabolite balance, can reduce germination performance, which is conceptually relevant here since extensive drying mimics aspects of drought stress at the cellular level. Likewise, reviews of seed deterioration processes (Choudhury and Bordolui, 2023) describe how cumulative physiological damage from prolonged water loss and associated oxidative damage contributes to reduced seed vigor and slower or incomplete germination.

Taken together, these findings suggest that moderate dehydration before cryopreservation, such as brief oven drying, can reduce free water sufficiently to minimize cryo-injury without imposing substantial physiological stress. In contrast, extended dehydration, as with prolonged silica gel exposure, may overstep the tolerance range of seed tissues, leading to subtle developmental or metabolic limitations that manifest as reduced germination performance. This highlights the need to optimize dehydration protocols to balance cryoprotective dehydration with the preservation of physiological seed integrity.

Effects of seed cryopreservation on vegetative growth of the germinated plantlets

Vegetative growth analysis of the resulting plantlets revealed that seed cryopreservation influenced height, biomass, and leaf number. Predictably, control plants exhibited superior growth. Similar to our findings, previous work has shown that seed cryopreservation may preserve viability but alter subsequent plant development. In hybrid orchid seeds, cryopreservation did not reduce germination but led to slower long-term protocorm growth, possibly due to sublethal cellular injury caused by freezing stress (Popova *et al.,* 2003). This supports the idea that post-cryopreservation reductions in plantlet height and biomass observed in the present study may reflect physiological stress effects rather than loss of viability.

However, seeds oven-dried for 10 minutes before cryopreservation produced plantlets that closely matched the control group in height and biomass, again underscoring the efficacy of short-duration heat drying in preserving physiological integrity (Pammenter and Berjak, 2014; Zeng *et al.*, 2024). Notably, seeds cryopreserved without prior drying also showed survival and successful germination, despite having water content comparable to the control. Although vegetative growth parameters of these plantlets were generally reduced relative to the control and optimally dried treatments, their ability to regenerate suggests that C. roseus seeds possess a certain tolerance to cryogenic exposure even at higher moisture levels. This response may be attributed to intrinsic protective mechanisms, such as cellular compartmentalization of water or partial desiccation tolerance, which can mitigate lethal ice formation under specific conditions. However, the reduced lamina and palisade tissue thickness observed in these plantlets indicates that the absence of drying prior to cryopreservation imposed sub-lethal stress that was reflected in subsequent vegetative development. Therefore, while cryopreservation without drying did not completely inhibit survival, moderate pre-drying remains important for ensuring optimal post-cryogenic growth and anatomical integrity.

Our results suggest that oven drying may remove sufficient moisture without triggering the cellular damage seen in prolonged desiccation and drying. These results align with earlier studies indicating that

while cryopreservation may not impair seed germination per se, it can reduce growth parameters, likely due to the depletion of physiological reserves (Cejas *et al.*, 2012; Ren *et al.*, 2023). The observed variability in growth performance among the different drying treatments indicates that Catharanthus roseus seeds exhibit differential tolerance to pre-cryopreservation stress levels. Short-duration oven drying appears to maintain cellular and metabolic stability, whereas excessive or insufficient dehydration imposes stress that is later expressed as reduced vegetative growth. This response reflects the species-specific capacity of seeds to cope with dehydration-rehydration cycles, a trait known to vary among genotypes and to influence post-cryogenic recovery.

Anatomical Leaf structure in C. roseus plantlets after seeds cryopreservation

The anatomical analysis of leaves from different treatment groups revealed significant differences in structural traits , the overall leaf organization was conserved across all treatments, characterized by a typical amphistomatic epidermis and a dicotyledonous mesophyll composed of palisade and spongy tissues. This structural consistency indicates that cryopreservation and drying treatments did not induce fundamental alterations in leaf patterning or tissue identity. This study adds new insights, as previous anatomical evaluations focused on seed or embryo structure rather than post-cryopreservation plantlets (Schvambach *et al.*, 2022; Vargas Andriolli *et al.*, 2023).

Nevertheless, quantitative variations in tissue thickness and mesophyll organization were evident and treatment-dependent. Control plants exhibited the most balanced anatomical development, with well-differentiated mesophyll layers and intact vascular bundles. Among cryopreserved treatments, oven drying for 10 min resulted in anatomical measurements closest to the control, including increased midvein thickness and larger main vascular bundle dimensions. These changes likely represent compensatory structural adjustments that enhance vascular transport and mechanical support rather than anatomical disruption. Seeds dried on silica gel for 3 h also maintained relatively well-organized mesophyll tissues, although increased epidermal thickness was observed. Such epidermal thickening has been associated with adaptive responses to dehydration stress, potentially enhancing protective functions without necessarily promoting enhanced growth. In contrast, prolonged desiccation (9 h silica gel drying) caused marked reductions in lamina, palisade, and midvein thickness, reflecting impaired cell expansion and mesophyll differentiation. These anatomical limitations are generally associated with reduced photosynthetic capacity and weaker vegetative growth observed in these treatments (Terashima *et al.*, 2011; Flexas *et al.*, 2012; Clarke *et al*., 2021).

Plantlets regenerated from cryopreserved seeds without drying showed the weakest mesophyll development, particularly reduced palisade thickness and lamina depth. This supports the notion that insufficient dehydration prior to freezing permits intracellular ice formation, which, although not lethal, compromises subsequent tissue differentiation and leaf functional anatomy. These traits are linked to enhanced photosynthetic capacity and water-use efficiency (Peguero-Pina *et al.*, 2017; Agustí and Blázquez, 2020). Enhanced vascular bundle development was also observed in these groups, potentially facilitating better nutrient transport. In contrast, plantlets from undried, cryopreserved seeds exhibited the weakest anatomical characteristics, suggesting that inadequate pre-treatment compromises structural integrity (da Conceição Moreira *et al.*, 2014).

Seed cryopreservation and carbohydrate and alkaloid composition in C. roseus plantlets

Carbohydrate analysis indicated that the drying method significantly affects sugar retention. Control and 10-minute oven-dried seeds had the highest carbohydrate levels, with no significant difference between them. These findings suggest that short oven drying prevents enzymatic degradation and preserves carbohydrate reserves necessary for early growth (Pammenter and Berjak, 2014). By contrast, plantlets from cryopreserved seeds without drying, 3-, and 9- hour silica gel-dried seeds, and drying the seeds for 20 min in the oven had the lowest carbohydrate contents, likely due to prolonged stress-induced degradation. Since carbohydrates play a vital role in protecting cellular structures during freezing (Hell *et al.*, 2019; Ballesteros *et al.*, 2020), maintaining their levels is essential for post-germination success.

The study also examined alkaloid concentrations,

a key indicator of medicinal quality in *C. roseus*. Results showed that alkaloid content mirrored the carbohydrate trends. The highest levels were recorded in the control and 10-minute oven-dried samples, suggesting that these treatments effectively preserved secondary metabolites (Kumar *et al.*, 2025). Conversely, extended drying reduced alkaloid levels, likely due to oxidative degradation. These results parallel findings in cryo-derived plants where specific stressors trigger secondary metabolite accumulation, including vinblastine and vincristine, which are the essential alkaloid compounds in *C. roseus* (Mujib *et al.*, 2022; Terletskaya *et al.*, 2024).

GA_3 and ABA alterations in cryopreserved C. roseus seeds

The findings of this study highlight the effects of different drying treatments on endogenous gibberellic acid (GA_3) and abscisic acid (ABA) levels in *Catharanthus roseus* plantlets. The results indicate a significant interplay between these two phytohormones, which are known to regulate seed dormancy, germination, and plantlet development.

Gibberellic acid (GA_3) plays a crucial role in promoting seed germination, stem elongation, and other growth processes in plants (Yamaguchi, 2008). In the present study, the highest GA_3 levels were recorded in control and in plantlets from seeds cryopreserved after 10 minutes of oven drying. This suggests that short drying prior to cryopreservation maintains a hormonal balance that supports plantlet growth. Indeed, GA_3 levels were significantly lower in plantlets from seeds subjected to prolonged drying treatments. The decline in GA_3 levels with extended drying could be attributed to decrease in the plantlet growth parameters, which might impair biosynthesis or enhance degradation of this hormone (Muniandi *et al.,* 2018).

Abscisic acid (ABA) is known for its role in seed dormancy induction and stress responses (Cutler *et al.*, 2010). In contrast to GA_3, ABA levels were lowest in control and in plantlets from cryopreserved seeds after 10 minutes of oven drying. However, higher ABA level was observed in plantlets from cryopreserved seeds after silica gel desiccation, suggesting that prolonged drying before cryopreservation induces stress, leading to an upregulation of ABA biosynthesis. This aligns with previous studies demonstrating that ABA accumulation is a key response to dehydration stress in seeds (Finkelstein, 2013).

The inverse correlation between GA_3 and ABA levels observed in this study is well-documented in plant physiology. ABA acts as an antagonist to GA_3 in regulating seed dormancy and germination, where higher ABA levels promote dormancy, while increased GA_3 levels facilitate germination and early plantlet growth (Kumar *et al.*, 2022). Our findings confirm that prolonged drying before cryopreservation disrupts this balance, leading to reduced GA_3 and increased ABA levels, which could negatively affect plantlet vigor post-germination.

The study underscores the significance of drying treatments in seed preservation and subsequent plantlet performance. While moderate oven drying (10 minutes) before cryopreservation-maintained seed viability and growth potential, prolonged desiccation negatively impacted germination, growth, and biochemical properties. These findings contribute to optimizing seed conservation protocols, ensuring enhanced viability and sustainable plant propagation strategies. The study highlights the importance of pre-cryopreservation drying techniques in preserving and even enhancing leaf anatomical features post-germination. Brief oven drying (10 minutes) before cryopreservation led to improved vascular bundle, mesophyll, and lamina thickness, while moderate silica gel desiccation (3 hours) increased epidermal thickness, potentially enhancing stress tolerance. In contrast, the weakest anatomical development was observed in plantlets from cryopreserved seeds without drying, reinforcing the necessity of proper moisture management before freezing.

In summary, seed cryopreservation in *Catharanthus roseus* induces physiological changes that influence both vegetative growth and secondary metabolite accumulation. While initial growth rates may be impacted, the overall plant vigor and metabolite production demonstrate promising adaptations. These findings contribute to our understanding of plant responses to cryo-preservation and provide valuable insights for future applications in biotechnology and pharmaceutical research.

Future research should investigate how different drying treatments impact seed membrane integrity, metabolic stability, and long-term viability in cryostorage. Also, it is needed to investigate the molecular mechanisms underlying alkaloid

degradation during desiccation and cryopreservation, as well as the potential impacts on seed viability and germination success.

The results of this study highlight the potential of seed cryopreservation not only as a conservation tool but also as a means to enhance bioactive compound production in medicinal plants. The increase in alkaloid content suggests that cryopreservation could be strategically utilized in pharmaceutical industries to optimize metabolite yield. Further research should focus on optimizing cryopreservation protocols to balance plant viability with enhanced bioactive compound synthesis. Additionally, long-term field studies are needed to assess the agronomic performance and stability of cryopreserved-derived plants.

Future research should explore the physiological mechanisms behind the results obtained of anatomical analyses, such as the role of stress-related genes and proteins in maintaining cellular integrity during freezing and thawing. Additionally, further studies could investigate how these anatomical differences correlate with long-term plant health, biomass accumulation, and secondary metabolite production. Finally, localization of the studied phytohormones should be deepened to find the distribution of the endogenous hormones as a result of seed cryopreservation.

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AHS
Advances in Horticultural Science

Enhancement of chitosan coating performance by hot water treatment: Effects on stability, nutritional quality, and antioxidant retention during storage in litchi cv. China-3

OPEN ACCESS

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Key words: Antioxidants, β-carotene, edible coating, hot water treatment, *Litchi chinensis* Sonn., postharvest quality, shelf life.

Abstract: Litchi (*Litchi chinensis* Sonn.) is a high-value tropical fruit, yet it suffers from rapid postharvest deterioration. This research appraised the combined effects of chitosan (CHT) coatings and hot water (HW) treatments on the shelf life, physicochemical quality, and antioxidant retention of litchi cv. China-3. Fruits were treated with 0.5% or 1% CHT, alone or following HW immersion (50°C for 5 minutes), and stored at ambient temperature. The combination of CHT with HW significantly reduced weight loss, decay, and browning index, while extending shelf life 35.71% longer than untreated fruits. This treatment preserved firmness, attenuated the decline in titratable acidity (TA), and minimized increases in total soluble solids (TSS). After 9 Days of Storage (DAS) at ambient temperature, fruits treated with 0.5% CHT + HW maintained higher levels of β-carotene (6.84 µg/100 g), total sugar (TS) (21.27%), ascorbic acid (46.99 mg/100 g), phenolics (12.60 mg/g), and flavonoids (3.13 mg/g) compared to untreated fruits, indicating a beneficial effect on the nutritional quality of the fruits. The antioxidant activity (DPPH* scavenging activity) was also significantly higher (45.04%) in treated fruits compared to the control (29.75%). Furthermore, compared to the control, the treatment delayed colour degradation, preserved lightness (L*=22.14), and achieved superior sensory ratings in flavour, sweetness, appearance, and overall acceptability. These findings suggest that combining 0.5% CHT with HW is an eco-friendly postharvest strategy to enhance storage stability, retain nutritional and sensory qualities in litchi.

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Author contributions
M.J. Hadika and R.B. Anwar designed and conducted the experiment, analyzed the data, and prepared the draft. S.R. Joy assisted in data collection and contributed to manuscript preparation. M.H. Rahman provided supervision. M.S. Islam and F. Akther contributed to revising the manuscript. As part of the study team, each author read and approved the manuscript.

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1. Introduction

Litchi (*Litchi chinensis* Sonn.) is a climatic fruit, a member of the family Sapindaceae, which is universally cultivated in tropical and subtropical regions. Its attractive, brilliant red peel with soft, juicy, sweet pulp and heightened levels of bioactive compounds resulted in an increasing consumer demand globally. Additionally, it is an excellent source of

minerals, vitamins, polysaccharides, and polyphenols (Flavan-3-ol compounds, phenols, terpenes, steroids, and flavonoids) (Castillo-Olvera *et al.*, 2025). These bioactive components can decrease cholesterol, scavenge oxygen pollutants, and reduce the hazards associated with smallpox, dyspepsia, and cardiovascular disease (Gao *et al.*, 2017) and also promote hunger and nourish the brain.

However, litchi is a highly perishable fruit with a short shelf life and lacks postharvest processing technologies; barely 10% of the entire yield is processed (Kumar *et al.*, 2020). The fruit's hot-season maturation leads to rapid water loss due to turgor pressure in the pericarp cells, causing tissue collapse and a dull, brown peel color within 24 to 48 hours after harvest at ambient temperature. The primary causes of pericarp browning and rapid degradation are the oxidation of phenolic substances, the quick breakdown of the anthocyanin color, and increased enzyme activity of PPO and POD (Jiang *et al*., 2005). Fumigation with sulfur dioxide (SO_2) is commonly used to prevent litchi pericarp browning. However, it poses health risks to humans, as it triggers allergies in individuals with asthma (Huang *et al.*, 2024). Thus, there is a pressing demand for a postharvest solution that is safe to consume, environmentally sustainable, and cost-effective.

The earliest and most basic method of postharvest decay management is HW treatment, which prevents fruit quality loss and post-harvest pathogens (Khalil *et al*., 2022). This antibacterial action results from heat-induced damage to the cell membranes and proteins of pathogenic microbes, which denatures enzymes, disrupts structural proteins, and ultimately causes cell lysis and death (Brunda *et al*., 2025). HW treatment showed a promising result by reducing the disease incidence of mango, peach, and banana fruit (Kim *et al.*, 2007; Huan *et al.*, 2017; Ajenifujah-Solebo *et al*., 2019). Additionally, this treatment accelerated fruit ripening, skin browning, and softening. Therefore, we must discover a novel treatment that can slow down the ripening process, prevent postharvest deterioration, and mitigate the adverse effects of HW treatment.

Edible coatings represent a promising innovation in the field of postharvest technology. They are safe, biodegradable, and capable of enhancing the shelf life of whole and fresh-cut fruits, offering a hopeful outlook for the future of litchi fruit preservation (Prieto-Santiago *et al*., 2025). It controls respiratory behavior and acts as a physical barrier, reducing moisture loss and oxidation (Araújo *et al*., 2024). Among these polysaccharides, CHT stands out due to its high molecular weight, natural origin from chitin, and significant promise in postharvest technology. Its inherent antibacterial qualities, film-forming ability, antioxidant capacity, non-toxicity, and biocompatibility make it a confident choice for improving the postharvest quality of fruit (Martins *et al.*, 2024). This polysaccharide has been used to prolong storage life, reduce post-harvest decay, and decrease ethylene production of mangoes (Parvin *et al.*, 2023), litchi (Kumar *et al*., 2020), and papaya (Ali *et al*., 2011) fruit.

Even though edible coatings and HW have been thoroughly studied recently, the combined impact of these treatments, particularly on litchi fruits, has not gotten as much attention. However, to the author's best knowledge, limited information is available on the interactive effects of different concentrations and combinations of CHT and HW treatments on pericarp browning, biochemical attributes, and shelf life of litchi under practical storage conditions. In response to this important gap, this study aimed to determine the best combination of HW and CHT concentrations to develop an edible coating. This coating aimed to prolong the overall quality and shelf life of litchi while fulfilling consumer dietary expectations and ensuring the treatment's safety by excluding any harmful chemical residues.

2. Materials and Methods

Plant materials, design, and treatments

This experiment was carried out using a completely randomized design (CRD) with five treatments and three replications at the Horticultural Laboratory of Hajee Mohammad Danesh Science and Technology University in Dinajpur. Five treatments were T_0= Control (only water), T_1= 0.5% CHT, T_2= 1% CHT, T_3= 0.5% CHT+ HW, T_4= 1% CHT + HW. Commercially matured China-3 litchi fruits were collected directly from the farmer's orchards early in the morning based on their uniformity in size, shape, and color. Then, they were washed in purified water, air dried, and promptly transferred to the laboratory within one hour. To determine the physical and chemical changes at 0, 3, 6, and 9 Days of Storage (DAS), twenty fruits were randomly selected from each replication per treatment and analyzed.

Preparation and application of film

The film was prepared following the methodology outlined by Muthmainnah *et al.* (2019). CHT powder (CAS #:9012-76-4, Laboratory Grade, Merck KGaA, Germany) was solubilized within 1% acetic acid (CAS#:64-19-7, ACS Grade, Merck KGaA, Germany) solution at two different concentrations of 0.5% and 1%. The solution was stirred (at 1500±20 rpm, MS-H380-Pro, DLAB, USA) for 60±2 minutes at 40±1°C and filtered through a cotton cloth to avoid any undissolved impurities. The solution was then cooled, and 0.5% glycerol (CAS#: 56-81-5, Molecular Biology Reagent Grade, Sigma-Aldrich) was added as a plasticizer. Fresh, clean water was heated to 50±2°C, and measured by a scientific thermometer (ASTM 110°C Mercury Thermometer). Moreover, fruits were treated through immersion in 0.5% and 1% CHT solutions for 5±1 minutes. For combined treatment, fruits were first immersed in HW for 5±1 minutes at 50±2°C, then air-dried, and dipped in the respective CHT solution (0.5% or 1%). Only water was applied to the non-treated litchi fruit. After that, treated fruits were stored at ambient conditions (27±2°C and 80-85% RH).

Determination of physiological weight loss, decay percentage, and shelf life

Weight loss measurements were taken using a digital balance (Model: ND EKI-620, Korea, SD ± 0.001 g). Shelf-life and decay percentage were determined following Akhter *et al*. (2025) with minor modifications, using standardized visual assessment of fruit appearance. Fruits were considered to have reached the end of their shelf life once they became unsuitable for consumption or commercial sale based on predefined quality criteria, including visible decay and excessive browning (>25 browning). Decay was defined based on visible qualitative symptoms, including fungal growth, water-soaked lesions, tissue softening, skin breakdown, and/or off-odor development.

Fruit firmness, total soluble solids, ascorbic acid, titratable acidity, total sugar, and ß-carotene

Fruit firmness was determined using a texture analyzer (FR-5120 Lutron Made in Taiwan, SD:± 5°) with a 5/16-inch plunger, as stated by Magness and Taylor (1925). TSS were determined using a digital refractometer (Hanna Digital Refractometer, Model HI96801, SD: ±0.2% Brix). The measurement was recorded as %Brix at room temperature, according to AOAC (1980).

Ascorbic acid content was determined according to Ranganna *et al*. (1997). Briefly, 1g of pulp tissue was mixed with 50 mL of 3% (w/v) metaphosphoric acid in a pestle and mortar. The mixture was then filtered using Whatman No. 41 filter paper. Following that, 1 mL of the filtrate was mixed with 10 mL of the 2,6-dichlorophenol dye solution in a Falcon tube and vortexed. A spectrophotometer (Shimadzu Europe UV mini-1240) was used to measure the absorbance at a wavelength of 518 nm and reported as mg/100 g.

The TA was quantified using the method of AOAC (1980). The obtained litchi pulp extract (10 mL) was titrated using 0.1 N NaOH and 1% phenolphthalein until a bright pink endpoint observed.

The TS content was measured using the method described by Lane and Eynon (1923). A 50 mL sample was dispensed into a 100 mL volumetric flask, then 5 mL (1:1) HCL was added, heated at 70°C to 80°C for 30±2 minutes, and neutralized by adding sodium carbonate. After cooling, 100 mL of distilled water was added to adjust the volume and calculated through the following equation:

$$\text{Total sugar (\%)} = \text{Fehling factor} \times \text{Dilution} \times 100/\text{Weight of sample} \times \text{Titrate}$$

Subsequently, β-carotene was determined by Ultra Performance Liquid Chromatography (UPLC) at a temperature of 4°C, which was modified from Ahamad *et al.* (2007). In brief, 5 g of material was homogenized in 30 mL of acetone, BHT (1%) was added, and the mixture was filtered and washed twice. After discarding the residue, the filtrate was dried over anhydrous sodium sulfate (20 g).

Total phenolic compounds, total flavonoid content, antioxidant activity

The phenolic compounds were quantified spectrophotometrically as described by Nur Sazwi *et al.* (2013). 1 mL diluted extract (1 g pulp with 10 ml methanol) was combined with 5 mL Folin Ciocalteu reagent (Sigma-Aldrich, Laboratory grade, MDL number: MFCD00132625) (10%, v/v) and 4 mL Na_2CO_3 (7.5%, v/v), then agitated at ambient temperature for 2 hours and quantified using a spectrophotometer (Shimadzu, UVmini-1240) at a wavelength of 765 nm. 1 mL blank was also determined. A calibration curve was generated using gallic acid standard (R^2 = 0.9997), and total phenolic

content was expressed as mg GAE/g DW.

Litchi pulp extract was diluted in methanol (10 mL) and mixed with 3 mL of 5% (w/v) $NaNO_2$ and distilled water (40 mL) to determine total flavonoid content as described by Asem *et al.* (2020). Following a 5 min reaction period, 3 mL of 10% (w/v) $AlCl_3$ was added, and after an additional 5 min, 20 mL NaOH was added. Then the mixture was incubated at ambient temperature for 15 min, and measured with a spectrophotometer (Shimadzu, UVmini-1240) at a wavelength of 415 nm. In the same manner, a blank specimen was made and measured. A calibration curve was established using quercetin as the standard, with a coefficient of measurement (*R*) of 0.9999, and the results were presented as mg/100 g.

The antioxidant activity, as DPPH scavenging activity, was quantified spectrophotometrically (517 nm, Shimadzu, UVmini-1240) as outlined by Do *et al.* (2014). In brief, 1 mL of diluted extract (1g pulp with 10ml methanol) was incubated with 2 mL of 0.004% DPPH for 30 min.

Measurement of color

Hunter color (L*, a*, b*) was measured using a biobased chromameter (BCM110, China). The L*, a*, b* values represent lightness to darkness, greenness to redness, and blueness to yellowness, respectively.

Sensory evaluation

Sensory analysis was performed at 9 DAS by five trained panelists (aged 25-60 years, three men and two women) (Sortino *et al*., 2017). Prior to the evaluation, the panelists received one week of training focused on litchi fruit attributes, including appearance, flavour, sweetness and overall impression. Each panelist independently evaluated three randomly presented, blind-coded samples per treatment using a structured questionnaire. All assessments were performed under ambient conditions, with water to rinse mouth rinsing between samples. Scores were recorded on a nine-point hedonic scale (1 - Dislike immensely, 2 - Dislike very much, 3 - Dislike moderately, 4 - Dislike slightly, 5 - Neither like nor dislike, 6 - Like slightly, 7 - Like moderately, 8 - Like very much, and 9 - Like immensely) (Nasrin *et al*., 2025).

Browning index

At 3-day intervals, the browning index of both coated and uncoated litchi fruits was assessed by qualitative analysis based on visual estimation (Kumar *et al*., 2013). Randomly, ten fruits were selected from each replication and marked using a 6-point visual scale. The six-scale point included: 0 - No browning, 1 - less than 5% browning, 2 - less than 25% browning, 3 - 25-50% browning, 4 - 50-75% browning, 5 - more than 75% browning (poor quality). The browning index was determined as ∑ (browning scale score x percentage of corresponding fruits within each class).

Statistical analysis

The statistical analysis was done using Statistix 10.0 (Analytical Software Inc., Tallahassee, FL, USA, 2010) software. Means and standard deviation were calculated as per the standard procedure. The statistical differences between treatments were evaluated using Tukey's test at a significance threshold of $p<0.05$.

3. Results

Weight loss, decay percentage, and shelf life

CHT and HW treatments significantly impacted the weight loss of litchi fruit during storage. The weight loss was reduced compared to the control with the application of CHT alongside HW (Fig. 1). On 6 DAS, the combination of 0.5% CHT with HW treatment resulted in the lowest weight loss at 13.03%, while the control fruits exhibited the highest weight loss (20.80%). At 9 DAS, application of 0.5% CHT with HW effectively reduced weight loss, resulting in a 1.5-fold decrease in comparison to the control.

According to figure 1, the longer the fruits were stored, the faster they deteriorated. No fruit deterioration was observed in all treatments up to 5 DAS. At 6 DAS, the control and 1% CHT-treated fruit showed a significant decay rate of 20.5% and 2.03%, respectively. Furthermore, after 9 DAS, the control exhibited a maximum decay rate (60%). In contrast, 0.5% CHT+HW and 1% CHT+HW treated litchi fruits showed similar decay rates (1.83% and 2.00%, respectively), which were significantly lower than 0.5% and 1% CHT (5.33% and 10.00%, respectively) treated litchi fruits. Similarly, 0.5% CHT+HW showed the maximum shelf life (14 days), which was 35.71% greater than the control (9 days), as presented in figure 1. Furthermore, our current findings showed that shelf-life was improved by 34.11%, 32.48%, and 27.07% for T_4, T_1, and T_2 treatments, respectively, in

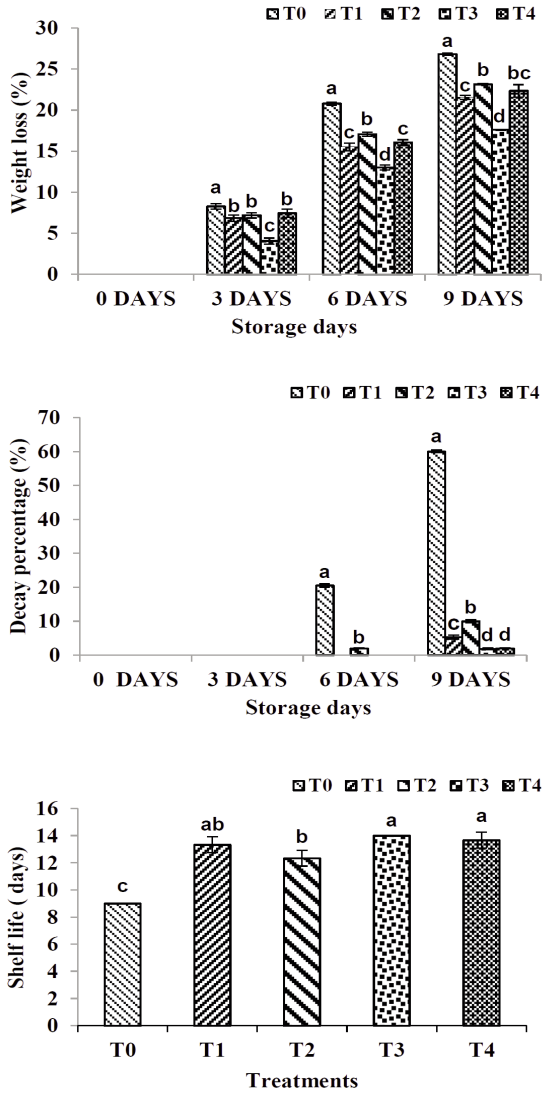


Fig. 1 - Effect of chitosan and hot water treatments on weight loss, decay percentage, and shelf life of litchi fruit during storage. Values denoted by distinct letters exhibit significant differences among the treatments within same storage day ($p<0.05$). Data are expressed as mean ± standard deviation (SD), illustrated by vertical bars. T0= control; T1= 0.5% chitosan; T2= 1% chitosan; T3= 0.5% chitosan + hot water; T4= 1% chitosan + hot water.

comparison to the control.

Firmness, total soluble solids, titratable acidity, total sugar, and content of ascorbic acid

CHT and HW treatments significantly influenced fruit firmness during storage (Table 1). At 3 DAS, no significant differences were observed among the treated and untreated fruits ($P>0.05$), whereas a significant decline in firmness was observed at 6 DAS ($P<0.05$). However, from the initial to the end of the storage days, the firmness of the sample coated with 0.5% CHT + HW remained high (from 1.43 to 1.48 kg/cm²); meanwhile, the control group experienced a more substantial loss (from 1.45 to 1.09 kg/cm²).

TSS content gradually increased with the advancement of the storage duration, reaching the maximum value (17.07%) in the control, which is statistically similar with T1 (15.70%) and T2 (15.89%) treated fruits, while the minimum was (15.47%) in T_3 treatment at the ninth day of storage (Table 1). On the initial day, TSS content showed no significant variation among treatments. At 3 DAS, the TSS content was 3% higher in control fruits than in T_3-treated fruits, although the difference was not significant. In contrast, at 6 and 9 DAS, control fruits showed significantly higher TSS (12% and 9%, respectively) compared with the T_3 treatment. At 9 DAS, TSS content in the control was 9.37%, 8.51%, 6.95%, and 8.06% higher than in T_3, T_4, T_2, and T_1, respectively.

As presented in Table 1, TA in both coated and uncoated fruits exhibited a steady decline during ripening at room temperature, with a more pronounced decrease in the untreated fruits. At the initial (0) day, a significantly similar TA level was observed in all treated and untreated fruit. Moreover, the control group showed the lowest levels of 0.22%, 0.15%, and 0.13% while treated with 0.5% CHT, and HW exhibited the highest TA levels of 0.24%, 0.18%, and 0.17%, respectively, at 3, 6, and 9 DAS. Additionally, at the endpoint of storage, the second-highest TA level was in T2, which is statistically non-significant compared to T_3. The TS content in both coated and uncoated samples increased gradually during postharvest storage, while ascorbic acid levels declined (Table 1). The highest TS content was recorded at 21.27% in the 0.5% CHT combined with HW, and the lowest was 15.80% in the control at 9 DAS. By the end of storage, the TS content rose by 25.70% in the 0.5% CHT + HW treatment compared to the control. Additionally, 0.5% CHT +HW-treated fruits exhibited the lowest degradation of ascorbic acid, decreasing from 70.97 to 46.99 mg/100 g, while the untreated group showed the maximum deterioration, dropping from 70.99 to 31.65 mg/100 g during storage.

Beta-carotene, total phenol, flavonoid content, and antioxidant activity

A significant difference was observed across all

Table 1 - Effects of chitosan and hot water treatment on firmness, total soluble solid (TSS), titratable acidity (TA), total sugar (TS), and ascorbic acid content of litchi fruits during storage

Treatments	Storage periods (days)			
	0	3	6	9
Fruit firmness (kg/cm²)				
T0	1.45±0.01 a	1.49±0.09 a	1.21±0.03 c	1.09±0.04 c
T1	1.46±0.14 a	1.54±0.21 a	1.39±0.06 b	1.33±0.01 b
T2	1.47±0.17 a	1.58±0.09 a	1.37±0.03 b	1.32±0.02 b
T3	1.43±0.04 a	1.62±0.21 a	1.52±0.04 a	1.48±0.01 a
T4	1.41±0.17 a	1.59±0.12 a	1.41±0.03 b	1.39±0.04 b
CV (%)	8.56	10.01	2.74	2.16
LSD	0.33	0.42	0.10	0.07
TSS (% Brix)				
T0	14.59±0.57 a	15.07±1.09 a	16.57±0.50 a	17.07±0.78 a
T1	14.23±1.15 a	14.61±0.18 a	15.12±0.22 b	15.70±0.33 ab
T2	14.40±0.20 a	14.62±0.53 a	15.40±0.22 b	15.89±0.48 ab
T3	14.35±0.57 a	14.59±0.11 a	14.64±0.50 b	15.47±0.52 b
T4	14.40±0.43 a	14.89±0.28 a	15.09±0.58 b	15.62±0.42 b
CV (%)	3.42	2.47	2.82	3.31
LSD	1.32	0.98	1.16	1.42
Titratable acidity (%)				
T0	0.253±0.005 a	0.22±0.01 b	0.15±0.01 b	0.133±0.005 b
T1	0.250±0.001 a	0.236±0.001 ab	0.176±0.005 a	0.15±0.01 ab
T2	0.256±0.005 a	0.240±0.005 ab	0.183±0.005 a	0.16±0.01 a
T3	0.253±0.005 a	0.243±0.005 a	0.180±0.001 a	0.170±0.001 a
T4	0.253±0.005 a	0.243±0.005 a	0.176±0.025 c	0.157±0.015 ab
CV (%)	2.04	3.27	4.45	6.27
LSD	0.01	0.02	0.02	0.03
Total sugar (%)				
T0	12.33±0.34 a	13.61±0.09 b	14.83±0.14 c	15.80±0.27 c
T1	12.34±0.17 a	14.49±0.19 a	16.85±0.28 b	18.36±0.29 b
T2	12.24±0.16 a	13.64±0.34 b	17.10±0.11 b	17.99±0.36 b
T3	12.22±0.08 a	14.55±0.23 a	18.21±0.54 a	21.27±0.34 a
T4	12.29±0.08 a	14.47±0.06 a	17.50±0.40 ab	18.46±0.21 b
CV (%)	1.92	1.81	2.44	1.99
LSD	0.64	0.69	1.11	0.98
Ascor bic Acid (mg/100 g)				
T0	70.99±0.19 a	59.96±0.90 b	48.24±0.37 c	31.65±0.78 d
T1	71.05±0.90 a	63.69±0.91 a	53.65±0.15 b	42.81±0.18 c
T2	71.82±0.95 a	61.58±0.35 b	54.64±0.41 ab	41.64±0.50 c
T3	70.98±0.38 a	64.64±0.56 a	57.20±1.97 a	46.99±0.33 a
T4	71.05±0.90 a	64.31±0.59 a	55.07±1.34 ab	44.19±0.24 b
CV (%)	1.03	1.11	2.40	1.11
LSD	1.98	1.88	3.47	1.24

Values denoted by distinct letters exhibit significant differences among the treatments within same storage day ($p<0.05$). Data are expressed as mean ± standard deviation (SD). T0 = control; T1 = 0.5% chitosan; T2 = 1% chitosan; T3 = 0.5% chitosan + hot water; T4 = 1% chitosan + hot water.

treatments during the period from 3 to 9 DAS (Fig. 2). Initially, all treatments showed similar carotenoid levels, with no significant differences among them. However, during 3 to 9 DAS, both coated and uncoated fruits experienced an increase in carotenoid content. The highest carotenoid content observed was 6.84 µg/100 g in the 0.5% CHT+HW treatment, which increased by almost 1.49-fold, while the untreated fruit had the lowest level at 5.29 µg/100g, experiencing a 1.14-fold increase from the

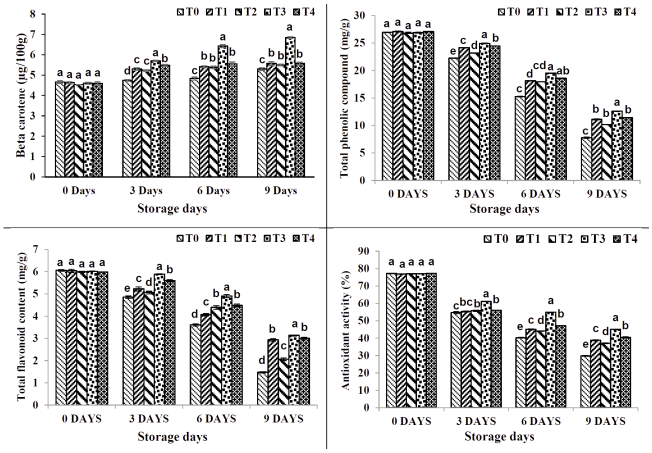


Fig. 2 - Effects of chitosan and hot water treatment on beta-carotene, total phenol, flavonoid content, and DPPH* scavenging activity of litchi fruit during storage. Values denoted by distinct letters exhibit significant differences among the treatments within same storage day ($p<0.05$). Data are expressed as mean ± standard deviation (SD), illustrated by vertical bars. T0= control; T1= 0.5% chitosan; T2= 1% chitosan;, T3= 0.5% chitosan + hot water;

beginning of the study. A comparable upward pattern was also observed for T_1, T_2, and T_4, which increased by nearly 1.2 times from the beginning to the last day of storage (9).

Figure 2 demonstrates that the total phenolic and flavonoid content in litchi decreased consistently throughout the storage period for both treated and untreated fruits. Based on the data, it can be observed that throughout the storage period (0-9 DAS) the combined 0.5% CHT + HW treatment maintained the highest total phenol content among all treatments. The application of 0.5% CHT with HW showed twice the flavonoid content (3.13 mg/g) compared to the control (1.47 mg/g) at 9 DAS. Correspondingly, DPPH* scavenging (antioxidant) activity gradually decreased during the storage period (Fig. 2). The combined application of 0.5% CHT with HW showed the highest DPPH* scavenging activity (77.0% to 45.04%), whereas the control displayed the lowest, ranging from 77.18% to 29.75% at 9 DAS. The results indicated that T_3 (0.5% CHT + HW) treated fruit produced 33.94% more antioxidant content in comparison to the untreated fruits on 9 DAS.

Color (a, b*, L*)*

Color is a crucial characteristic of fruit, indicating freshness and influencing consumer acceptance. Our study showed a significant increase in the a* (green-to-red transition) and b* (yellow to blue), while the L* (lightness) of the fruits decreased during storage (Fig. 3). A significant difference was observed between all coated treatments (T1-T4) and control (T0). In contrast, differences among coated treatments (T1-T4) showed no significant differences for a* and L* value, while b* value varied significantly. Fruits coated with 0.5% CHT + HW exhibited the lowest increasing rate of the a* value, achieving 9.45 to 10.41, denoting a slow color transformation in comparison to the uncoated (9.85 to 14.25) fruits. The b* value was higher in untreated

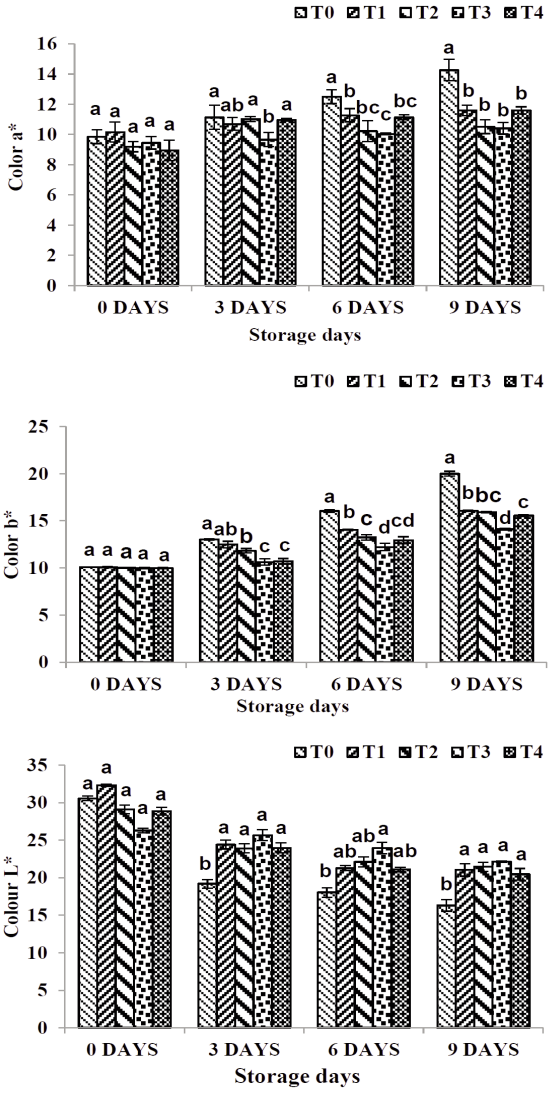


Fig. 3 - Effects of chitosan and hot water treatment on color a*, b*, and L* of litchi fruit during storage. Values denoted by distinct letters exhibit significant differences among the treatments within same storage day ($p<0.05$). Data are expressed as mean ± standard deviation (SD), illustrated by vertical bars. T0= control; T1= 0.5% chitosan; T2= 1% chitosan;, T3= 0.5% chitosan + hot water; T4= 1% chitosan + hot water.

fruits (20.01) compared to the T_3 (14.09) treatment at 9 DAS. Regarding the treatment of 0.5% CHT + HW, higher lightness (L*) values were preserved. On the 9th day, the 0.5% CHT + HW group exhibited a maximum L* value (22.14), while the untreated group recorded the minimum (16.31).

Browning index (scale)

The browning index of litchi fruits, which measures the enzymatic browning that occurs when phenolic substances interact with enzymes, leading to the formation of brown color (Zhang *et al.*, 2015). According to figure 4, the browning index significantly increased over time across all samples (treated and untreated), while a significant. The browning index of untreated fruits gradually increased. At 3, 6, and 9 DAS, maximum browning index (3.63, 4.40, and 5) was found in the control, while the minimum (0.1 and 2.93) was observed in T_3-treated fruits.

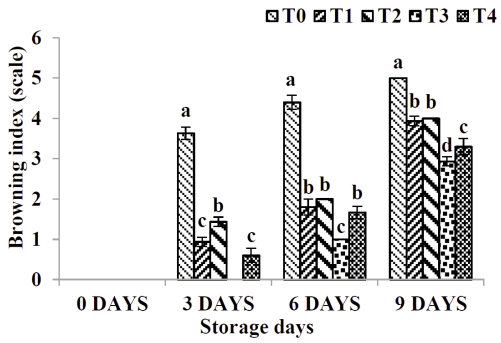


Fig. 4 - Effects of chitosan and hot water treatment on the browning index of litchi fruit during storage. Values denoted by distinct superscript letters exhibit significant differences among the treatments within same storage day ($p<0.05$). Data are expressed as mean ± standard deviation (SD), illustrated by vertical bars. T0= control; T1= 0.5% chitosan; T2= 1% chitosan;, T3= 0.5% chitosan + hot water; T4= 1% chitosan + hot water.

Sensory evaluation

The treated fruits demonstrated a significant improvement in sensory evaluations compared with the untreated fruits. (Fig. 5). Overall, after 9 DAS, fruits treated with 0.5% CHT + HW attained superior scores in flavour (8.00), sweetness (8.33), appearance (6.67), and overall acceptability (7.67), markedly outperforming the untreated fruits, which registered scores of 5.33, 6.00, 4.33, and 4.33, correspondingly.

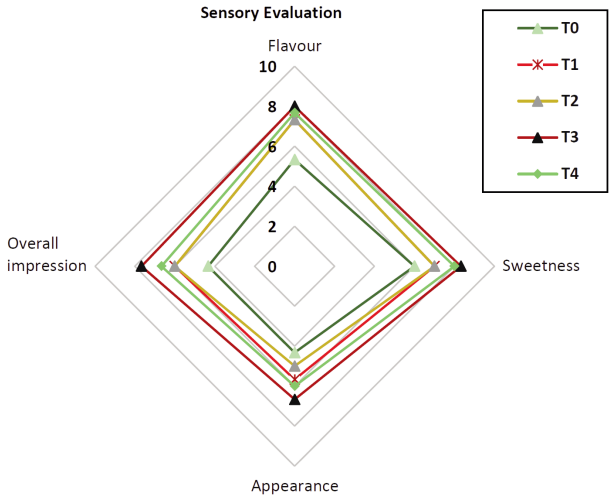


Fig. 5 - Effects of chitosan and hot water treatment on the sensory characteristics of litchi fruits at 9 DAS. T0 = control; T1 = 0.5% chitosan; T2 = 1% chitosan; T3 = 0.5% chitosan + hot water; T4 = 1% chitosan + hot water.

Furthermore, fruits treated with 0.5% and 1% CHT alone showed significantly higher flavour (7.33) and sweetness (7.00) compared to the control (T_0); however, their appearance and overall impression scores were lower than the corresponding combined treatments (0.5% and 1% CHT + HW). Notably, fruit flavour scores were statistically similar between samples treated with CHT alone and those receiving the combined CHT + HW treatments, whereas sweetness, appearance, and overall impression scores were significantly higher in the combined treatments (T_3 and T_4), indicating their superior overall sensory performance.

4. Discussion and Conclusions

Extending the shelf life of litchi fruit is crucial for minimizing post-harvest losses. In the present study, CHT treatment reduced weight loss compared to the control, with the combined application CHT and HW showing the greatest weight loss (Fig. 1). Notably, the combination of 0.5% CHT + HW and 0.5% CHT alone reduced weight loss by approximately 1.5-fold and 1.2-fold compared to the control. This reduction can be attributed to the formation of a semipermeable coating on the fruit's surface by CHT, which limits transpiration and O_2 and CO_2 exchange, thereby reducing moisture loss (Saberi Riseh *et al.*, 2024).

Moreover, short-duration HW treatment enhanced cuticle integrity, decreasing respiration and minimizing transpiration, making this combination more effective (Brunda *et al.*, 2025). Similar findings were observed in CHT and HW treated mango fruit (Khalil *et al.*, 2022). Furthermore, prolonged storage increases susceptibility to browning and drying, which is a key indicator of litchi fruit quality. Our results indicate reduced decay percentage and extended shelf life, suggesting a potential reduction in disease-related deterioration. CHT demonstrates notable antibacterial and antifungal properties that effectively inhibit the growth of harmful microbes by forming a continuous, thick, and flexible semi-permeable layer on the coated surface (Wang *et al.*, 2021). In addition, HW can either directly inhibit pathogen growth or spore germination or boost the fruit's defense against diseases by triggering a defense response (Ferreira Da Silva *et al.*, 2020). Moreover, coating with CHT and HW enhanced the storage life of litchi (Fig. 1), likely due to reduced weight loss and lower fruit decay, which helped maintain quality during storage. The reduction in respiration rate leads to low ethylene production, thereby improving shelf life (Kumarihami *et al.*, 2021). Similar trends have been reported by Nguyen *et al.* (2020), Khalil *et al.* (2022), and Zhou *et al.* (2020), where the combination of CHT and HW improved antimicrobial activity and shelf life.

Fruit firmness is an important quality attribute reflecting structural and metabolic changes during storage. Litchi fruits subjected to CHT and HW treatments exhibited significantly higher firmness towards the end of shelf-life (day 6-9) compared to the control (Table 1), indicating improved maintenance of tissue integrity. This effect can be attributed to the ability of CHT coatings to form a protective barrier and inhibit cell-wall-degrading enzymes, thereby preserving structural components of the fruit. In addition, such treatments may regulate carbohydrate metabolism and ethylene activity, contributing to the delayed softening and enhanced firmness (Dai *et al.*, 2025). Similar effects of coating treatments in maintaining fruit firmness during storage have been reported for fig and apple fruits (Shao *et al.*, 2012; Kaur *et al.*, 2024). Changes in TSS during storage reflect ongoing metabolic processes, including sugar accumulation and utilization (Xu *et al.*, 2018). TSS increased with storage for all treatments (Table 1) and indicated conversion of polysaccharides and organic acids to soluble sugars, as a result of ripening (Zhang *et al.*, 2018). The higher TSS in control fruits at later stages is an outcome of water loss induced acceleration in respiration and metabolic activity. On the other hand, the TSS increased more slowly in fruits treated with chitosan, probably due to the semi-permeable coating affecting the O_2/CO_2 balance, thereby suppressing respiration, ethylene production and hydrolysis of carbohydrates (Gholami *et al.*, 2017; Zhou *et al.*, 2025). Also, coated fruits showed reduced transpiration which further limited the solute concentration that resulted in delayed ripening and lower TSS in total (Xing *et al.*, 2020). Similar trends were reported for chitosan-coated strawberries (Bertolo *et al.*, 2025) and mangoes (Akhter *et al.*, 2025), where coatings delayed ripening and maintained lower TSS throughout storage.

TS content gradually increased in coated samples compared to the control (Table 1). TS is an important indicator of fruit ripening, influenced by ethylene and associated enzymatic activities (Farcuh *et al.*, 2018). Furthermore, this increase in TS can also be attributed to the breakdown of polysaccharides and the conversion of starch into simpler sugars during ripening (Kumar *et al.*, 2020) processes that the hydrolytic action of heat could further facilitate. Additionally, mild heat treatment may enhance enzymatic hydrolysis of polysaccharides, contributing to sugar accumulation (Lu *et al.*, 2018). Similar trends in sugar dynamics have been reported in coated papaya and mango fruits (Ban *et al.*, 2015; Khalil *et al.*, 2022).

Ascorbic acid is a key component in quality assurance during postharvest storage and influences fruit ripening and stress tolerance in secondary metabolites (Zheng *et al.*, 2022). Our results showed that ascorbic acid content decreased with storage time; however, fruits treated with CHT combined with HW retained higher levels of ascorbic acid than the untreated control (Table 1). The decrease in ascorbic acid content appears to be primarily associated with the oxidative degradation during storage. The improved retention of ascorbic acid in treated fruits may be more appropriately explained by the barrier function of CHT coating, which reduces oxygen permeability and associated oxidative reactions (Mohamed *et al.*, 2020). In addition, HW treatment has been reported to have no direct adverse effect on ascorbic acid content while helping

to maintain overall postharvest fruit quality (Brunda *et al*., 2025).

Fruits' ascorbic acid and phenolic levels are closely related to their antioxidant action (Njus *et al*., 2020). The total phenolic content decreased in all treatments during storage while fruits treated with CHT+HW consistently maintained higher levels compared to the control (Fig. 2). This may be attributed to the inhibitory effect of CHT coating on polyphenol oxidase (PPO) (Zhang and Quantick, 1997), a primary enzyme that is responsible for oxidative degradation of phenolics (Tilley *et al.*, 2023). The decline in phenolic content in the control group could be due to vacuolar disruption and increased oxidative stress under ambient conditions (Xu and Wang, 2025). Similarly, flavonoid content declined over time in all samples (Fig. 2). However, CHT+HW-treated fruits retained nearly double the flavonoid levels compared to the controls at 9 DAS. Flavonoids, a key antioxidant synthesized via the phenylpropanoid pathway, are essential for delaying fruit senescence and protecting against reactive oxygen species (ROS) (Chen *et al*., 2022). The higher flavonoid content retention in CHT-HW treated fruits is likely due to HW treatment modulating physiological responses and reducing microbial load, while CHT coating acts as a semi-permeable barrier that limits oxygen exposure, reduces oxidative degradation, and preserves cellular integrity during storage (Wang *et al.*, 2023; Brunda *et al.*, 2025; Dai *et al.*, 2025). Moreover, in 'Sanhua' plum fruit, the combined application of CHT and heat treatment resulted in higher levels and improved retention of total phenolics and flavonoids compared to the untreated controls (Chang *et al.*, 2019).

Antioxidant activity, as measured by DPPH radical scavenging capacity, decreased progressively during storage in all samples (Fig. 2). At 9 DAS, the combined treatment maintained approximately 33.94% higher antioxidant activity than the control and about 15-20% higher than individual treatments. This trend corresponds with the preservation of phenolic compounds, flavonoids, and ascorbic acid, which collectively contribute to antioxidant capacity. The higher antioxidant activity in treated fruits can therefore be attributed to reduced oxidative degradation and better retention of antioxidant compounds under modified atmospheric conditions created by the coating (Ummarat *et al*., 2011; Huan *et al.*, 2017). These findings largely corroborate those of previous studies, which have shown that CHT application retains higher levels of antioxidant activity during ripening in mango (Akhter *et al.*, 2025). β-carotene content increased during storage in all treatments (Fig. 2), likely due to ripening-associated carotenoid biosynthesis and enhanced metabolic activity during fruit maturation (Wisutiamonkul *et al*., 2015; Jahan *et al*., 2025). The CHT and HW treatments may have modulated the ripening process and reduced oxidative degradation by limiting oxygen availability and moisture loss. Since carotenoids are highly susceptible to oxidation during storage (Salinas-Roca *et al.*, 2018), the barrier properties of chitosan likely contributed to better retention of β-carotene, as previously reported in coated papaya and mango fruits (Akhter *et al.*, 2025).

Our findings suggest that the changes in L*, a*, and b* values occur more slowly in treated fruits (Fig. 3). The L* (lightness and darkness) value decreased, while values for a* (ranging from greenness to redness) and b* (from blueness to yellowness) rose. Additionally, the browning index of the pericarp has significantly risen in control fruits; however, this increase is more pronounced in uncoated fruit compared to coated fruit (Fig. 4). According to Sun *et al.* (2020), variations in overall phenolic levels and the activity of PPO significantly impact fruit browning during storage. Similarly, Medeiros *et al.* (2012) stated that CHT formed a semipermeable layer on top of fruit peel and changed the environment by raising CO_2 and decreasing O_2, which prevented the generation of ethylene, postponed ripening, decreased color changes, and maintained chlorophyll content. Additionally, HW may have also stimulated the metabolic pathways involved in pigment and phenolic compound synthesis, resulting in improved color retention by delaying ethylene production and lowering respiration rates. These treatments helped to maintain the desired pulp and peel color for a longer period in dragon (Nguyen *et al*., 2020) fruit during storage.

Sensory evaluation revealed that treated fruits maintained higher scores for flavor, sweetness, appearance, and overall acceptability compared to the control (Fig. 5). These results indicate that the treatments were effective in preserving desirable sensory attributes during storage rather than merely preventing deterioration. Higher sensory scores in treated fruits were associated with improved firmness, reduced browning, higher L* values, and

better retention of sugars and antioxidants. Similar trends have been reported in coated papaya, mango, and grape fruits (Vilaplana *et al.*, 2020; Khalil *et al.*, 2022; Tavasolinasab *et al.*, 2023).

This study demonstrates that the combined application of 0.5% CHT and HW treatment effectively enhances postharvest stability and extends storage life of litchi cv. China-3 by limiting weight loss, pericarp browning, and decay development. The treatment further preserves fruit quality attributes, including firmness, TSS, β-carotene, titratable acidity, ascorbic acid, and antioxidant-related metabolites (phenolics and flavonoids), indicating a comprehensive delay in senescence and oxidative deterioration. Beyond its biological efficacy, this cost-effective and scalable approach requires minimal technological modification, facilitating its integration into existing postharvest systems and supporting commercial adoption to reduce losses and improve supply chain efficiency. Its implementation may further improve postharvest performance and sustain market acceptability.

However, validation under commercial conditions and across diverse cultivars is required to enable large-scale industrial application.

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Innovative applications of flamingo explants and TDZ in *Digitalis purpurea* L. organogenesis

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Abstract: *Digitalis purpurea* L. is a valuable medicinal and ornamental species, but its conventional propagation is constrained by low seed germination and high genetic variability. The present study aimed to develop an efficient and reproducible *in vitro* regeneration system for *D. purpurea* through both direct and indirect organogenesis by optimizing explant type and plant growth regulator (PGR) combinations. Among the explants evaluated, hypocotyl-cotyledon (flamingo) explants exhibited the highest morphogenic competence, whereas leaf and root explants showed no regenerative response under any treatment. In direct organogenesis, MS medium supplemented with 0.25 mg L^{-1} thidiazuron (TDZ) was the most effective, producing the maximum shoot length (6.3 mm) and shoot number (8.6 shoots per explant). Indirect organogenesis was strongly influenced by auxin type, with callus induction reaching 100% in flamingo explants cultured on media containing 0.5-1 mg L^{-1} NAA, while 2,4-D was markedly less effective. Shoot regeneration from callus was significantly enhanced by cytokinin-polyamine interactions, with the highest shoot proliferation (up to 28 shoots per callus) and superior shoot elongation obtained on MS medium supplemented with 0.25-0.5 mg L^{-1} BAP in combination with 50 mg L^{-1} putrescine. Root induction was optimized on half-strength MS medium containing IBA, where 0.25 mg L^{-1} IBA resulted in the highest rooting frequency (91%), maximum root number, and well-developed roots. Overall, this study highlights the critical role of explant selection and tailored PGR-polyamine combinations in establishing a high-efficiency micropropagation protocol for *D. purpurea*, providing a reliable platform for large-scale propagation and future biotechnological applications.

1. Introduction

Digitalis purpurea L. (foxglove) is a commercially significant biennial

plant, valued both as an ornamental and a vital medicinal herb. It is the primary source of cardiac glycosides, such as digoxin and digitoxin, which are crucial in the treatment of heart conditions (Bourgaud *et al*., 2001). These compounds inhibit the Na^+/K^+-ATPase pump, increasing myocardial contraction (Gavidia and Pérez-Bermúdez, 1997; Bourgaud *et al*., 2001). However, their narrow therapeutic index demands precise dosage, highlighting the need for standardized cultivation to ensure consistent metabolite levels (Gavidia and Pérez-Bermúdez, 1997).

Conventional propagation of *D. purpurea* via seeds faces significant challenges, including low natural germination rates (≤2%) and high genetic variability in seed-grown populations, which complicate the production of uniform plant material (Mastenbroek, 1985). These limitations hinder reliable cultivation for both pharmaceutical and horticultural purposes. Moreover, inconsistent seed-based propagation constrains large-scale commercial production, especially where uniformity in bioactive compound profiles is critical for regulatory compliance.

Plant tissue culture techniques offer a robust solution by enabling the rapid, large-scale production of genetically uniform plants under controlled conditions (Abbaszadeh *et al*., 2023). For medicinal species, efficient *in vitro* regeneration systems are essential for mass propagation, germplasm conservation, and future genetic improvement. While somatic embryogenesis is a powerful method for such goals (Quiroz-Figueroa *et al*., 2006), successful protocol development is highly genotype-dependent (Blasco *et al*., 2013). In the case of *D. purpurea*, existing studies primarily emphasize callogenesis and secondary metabolite accumulation, resulting in a noticeable research gap regarding dependable and reproducible whole-plant regeneration systems. Research on *in vitro* regeneration of *D. purpurea* remains limited, with studies often focusing on secondary metabolite production in callus cultures (Jan *et al*., 2023) rather than efficient whole-plant regeneration pathways like organogenesis.

Given the global expansion of the medicinal plant industry and the emerging demand for standardized, high-quality raw material, establishing a robust regeneration platform for *Digitalis* spp. has become particularly timely. Given Iran's long history and significant role in medicinal plant cultivation within the Middle East, developing advanced biotechnological protocols for key species is of strategic importance. A reproducible, genotype-independent regeneration approach would not only support commercial propagation but also facilitate downstream applications such as metabolic engineering, conservation programs, and genetic transformation aimed at enhancing cardiac glycoside biosynthesis.

To address the propagation constraints of *D. purpurea*, this study was designed to establish a reliable *in vitro* regeneration protocol. Specifically, we focused on direct organogenesis as a rapid and mutation-minimizing pathway for regenerating true-to-type plantlets. The present study aims to optimize an *in vitro* regeneration protocol for *D. purpurea* through direct organogenesis, with the following specific objectives: (a) to determine the optimal combination of PGRs and culture medium for inducing direct shoot organogenesis from seed-derived explants, and (b) to identify the most effective culture conditions for subsequent shoot elongation and root development to produce complete plantlets.

2. Materials and Methods

Plant material

Cultivated seeds of *D. purpurea* L. were collected from the Agricultural Sciences and Natural Resources University of Khuzestan (31°59' N, 48°88' E; 14 m above sea level) during early spring. Seeds were collected within a single growing season and were used immediately for experimental purposes. A voucher specimen (No. KHAU993) was prepared and deposited in the Herbarium of the Agricultural Sciences and Natural Resources University of Khuzestan for future reference.

Decontamination

Mature seeds were washed under running tap water for 20 min with the addition of one drop of liquid detergent. Surface sterilization was carried out by immersion in 70% (v/v) ethanol for 15 s, followed by treatment with 5% (v/v) sodium hypochlorite solution (commercial bleach, Clorox®) supplemented with one drop of Tween-20 for 5 min. Sterilized seeds were rinsed six times with sterile distilled water under aseptic conditions and cultured on a germination medium consisting of half-strength

Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 30 g L^{-1} sucrose, and 8 g L^{-1} agar. The pH was adjusted to 5.7 prior to autoclaving. Cultures were maintained in 100 mL glass jars containing 25 mL of medium. Contamination frequency during seed germination was less than 2%.

Explant preparation

Four explant types were used: hypocotyls with one attached cotyledon (commonly referred to as "flamingo bill" explants due to their characteristic shape), leaves, nodes, and roots. Explants were excised aseptically from *in vitro*-germinated seedlings aged 10-15 days. The variation in seedling age reflected natural differences in germination timing. Explants were cut immediately prior to culture and transferred to media without delay. Leaf and node explants were approximately 7 mm in length, whereas root and hypocotyl-cotyledon explants were approximately 10 mm long. Slight variation in explant size was unavoidable due to the curved morphology of certain tissues and manual excision. All explants were cultured on full-strength MS basal medium containing MS salts and vitamins, 30 g L^{-1} sucrose, and 8 g L^{-1} agar, with pH adjusted to 5.7 before autoclaving.

Direct organogenesis

For direct organogenesis, explants were cultured on MS basal medium supplemented with different concentrations of 6-benzylaminopurine (BAP) or thidiazuron (TDZ), as detailed in Table 1. A plant growth regulator (PGR)-free MS medium served as the control. Media were dispensed into 100 mL glass jars (25 mL per jar) and autoclaved at 121°C for 15 min. Cultures were maintained in a growth chamber at 25 ± 1°C under a 16 h photoperiod provided by cool-white fluorescent lamps with a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1}. Each treatment consisted of three biological replicates, with 10 explants per replicate (n= 30). After six weeks of culture, regeneration responses were evaluated by recording (1) the percentage of explants producing shoots, (2) the mean number of shoots per responsive explant, and (3) the mean length (cm) of the longest shoot. The experiment followed a factorial design with explant type and PGR concentration as experimental factors.

Table 1 - Composition of media for direct shoot organogenesis

Treatment code	BAP (mg L^{-1})	TDZ (mg L^{-1})	Basal medium
D1 (Control)	0.00	0.00	MS
D2	0.25	0.00	MS
D3	0.50	0.00	MS
D4	1.00	0.00	MS
D5	0.00	0.25	MS
D6	0.00	0.50	MS
D7	0.00	1.00	MS

Indirect organogenesis via callus culture

Callus induction. For callus induction, explants were cultured on MS basal medium supplemented with various combinations of α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and BAP, as shown in Table 2. A PGR-free MS medium was used as the control. Cultures were maintained at 25 ± 1°C in continuous darkness (24 h). Each treatment consisted of three biological replicates, with 10 explants per replicate. After six weeks, callus induction frequency (%) and mean callus fresh weight (mg) per responsive explant were recorded. This stage followed a factorial experimental design with explant type and PGR treatment as factors.

Table 2 - PGR combinations for callus induction media

Treatment code	NAA (mg L^{-1})	2,4-D (mg L^{-1})	BAP (mg L^{-1})	Basal medium
C1 (Control)	0.0	0.0	0.0	MS
C2	0.5	0.0	0.0	MS
C3	1.0	0.0	0.0	MS
C4	0.5	0.0	0.5	MS
C5	1.0	0.0	0.5	MS
C6	0.0	0.5	0.0	MS
C7	0.0	1.0	0.0	MS
C8	0.0	0.5	0.5	MS

Shoot regeneration from callus. Friable and actively proliferating callus obtained from the most effective induction treatment was selected for shoot regeneration experiments. Approximately 100 mg of callus was transferred to MS basal medium supplemented with different combinations of BAP, NAA, and putrescine, as detailed in Table 3. Cultures were maintained under the same environmental conditions as described for direct organogenesis. The experiment followed a completely randomized one-

Table 3 - PGR combinations for shoot regeneration media from callus

Treatment code	BAP (mg L^{-1})	NAA (mg L^{-1})	Putrescine (mg L^{-1})	Basal medium
SR1	0.25	0.00	0.00	MS
SR2	0.50	0.00	0.00	MS
SR3	0.25	0.10	0.00	MS
SR4	0.50	0.10	0.00	MS
SR5	0.25	0.00	50.00	MS
SR6	0.50	0.00	50.00	MS

factor design based solely on PGR treatments. After six weeks, shoot regeneration efficiency was evaluated by recording (1) the percentage of callus clumps producing shoots, (2) the mean number of shoots per regenerating callus, and (3) the mean length (cm) of the longest shoot. No subculturing was performed during the regeneration period due to the absence of tissue browning or growth limitation.

Root induction

Shoots regenerated through both direct and indirect organogenesis (1.5-2.0 cm in length) were excised and transferred to rooting media. The basal medium consisted of half-strength MS salts and vitamins, 15 g L^{-1} sucrose, and 8 g L^{-1} agar (pH 5.7). Media were supplemented with indole-3-butyric acid (IBA) or NAA at the concentrations listed in Table 4. A PGR-free medium served as the control. Cultures were maintained under the same growth chamber conditions as described previously. Each treatment consisted of three biological replicates with 10 shoots per replicate (n = 30). After six weeks, rooting frequency (%), mean number of roots per plantlet, and mean root length (cm) were recorded.

Acclimatization and greenhouse transfer

Rooted plantlets were gently removed from the culture vessels, and residual agar was washed off with sterile distilled water. Plantlets were transplanted individually into sterile plastic pots (9 cm diameter × 8 cm height) containing an autoclaved substrate mixture of cocopeat and perlite (1:1, v/v). Pots were transferred to a greenhouse maintained at 25±2°C under a 16/8 h light/dark photoperiod, relative humidity of 85±5%, and a PPFD of approximately 55 µmol m^{-2} s^{-1}. For the first two weeks, plantlets were covered with transparent polyethylene bags to maintain high humidity and were irrigated every four days with one-quarter-strength MS salt solution. The covers were gradually perforated and removed over the following two weeks. Acclimatization was conducted as a standard non-comparative step to complete the regeneration cycle. Survival rates were recorded 60 days after transfer.

Table 4 - Auxin treatments for root induction media

Treatment code	NAA (mg L^{-1})	IBA (mg L^{-1})	Basal Medium
R1 (Control)	0.00	0.00	½ MS
R 2	0.25	0.00	½ MS
R3	0.50	0.00	½ MS
R4	0.00	0.25	½ MS
R5	0.00	0.50	½ MS

Experimental design and statistical analysis

All experiments were conducted using a completely randomized design. Each treatment consisted of three independent biological replicates, with 10 explants, callus clumps, or shoots per replicate. Percentage data were arcsine square-root transformed prior to analysis. Data were subjected to analysis of variance (ANOVA). Factorial ANOVA was applied where appropriate (direct organogenesis and callus induction stages), while one-way ANOVA was used for subsequent experiments involving a single factor. Mean comparisons were performed using the Least Significant Difference (LSD) test at $p<0.05$, which is commonly applied in plant tissue culture studies. Statistical analyses were conducted using SPSS software (version 16.0), and results are presented as mean ± standard error (SE).

3. Results

Direct organogenesis

The results for both shoot length and number from direct organogenesis reveal consistent trends across explant types and cytokinin treatments. The flamingo (hypocotyl-cotyledon) explant demonstrated the highest regenerative capacity. For shoot length, the optimal treatment was D5, yielding 6.3 mm. This same treatment also produced the greatest number of shoots (8.6 shoots) in the flamingo explant. Shoot number generally increased with D3 (6.3 shoots) and peaked with TDZ before declining at the highest concentration (D7). Node explants showed a moderate response, with maximum shoot length (4.3 mm) and number (4.3

shoots) also achieved with D5. In stark contrast, leaf and root explants exhibited no regeneration (0 value for all parameters) under any treatment, including the hormone-free control. This confirms the absolute necessity of exogenous cytokinins and the critical explant-specific competence for direct organogenesis in *D. purpurea* (Fig. 1).

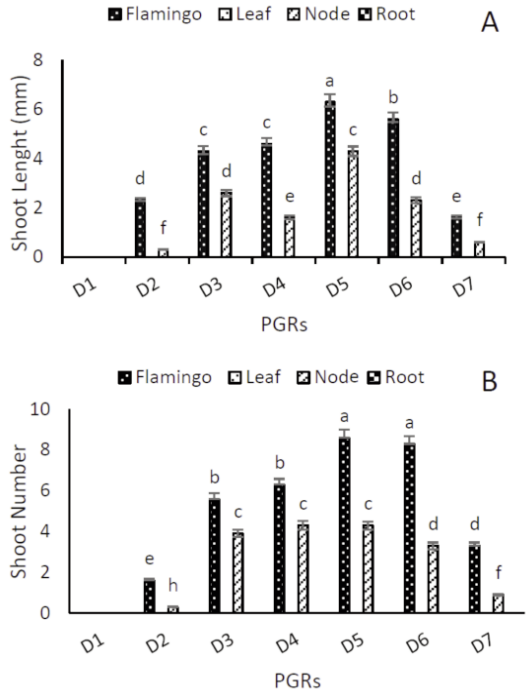


Fig. 1 - Effect of explant types and PGRs on shoot length (A) and number (B) in direct organogenesis of D. purpurea L. after 6 weeks. Columns within each graph marked with the same letter are not significantly different according to the LSD test (P<0.05), with LSD values of 0.167 and 0.598 for shoot length and number, respectively.

Indirect organogenesis

The callus induction data reveal distinct responses based on explant type and PGR treatment. The flamingo explant was consistently superior, achieving 100% callus formation with NAA (C2 and C3) and its combinations with BAP (C4 and C5). This high induction percentage corresponded with the greatest callus fresh weight (4.6 g) on medium containing 0.5 mg L^{-1} NAA alone (C2). For the flamingo explant, the addition of BAP to auxin treatments generally reduced callus weight, and the use of 2,4-D, either alone (C6 and C7) or with BAP (C8), resulted in lower induction percentages and markedly reduced biomass. Root explants showed the second-highest competency for callogenesis, with induction reaching 80% using NAA. However, the resultant callus weight was moderate (~2.3 g). In contrast, leaf and node explants exhibited low responsiveness across most treatments, with callus induction rarely exceeding 25% and producing minimal biomass. For all explant types, the control treatment (C1) resulted in little to no callus formation, underscoring the essential role of exogenous auxins. Notably, the synthetic auxin 2,4-D was consistently less effective than NAA in promoting robust callus growth across all explants tested (Fig. 2).

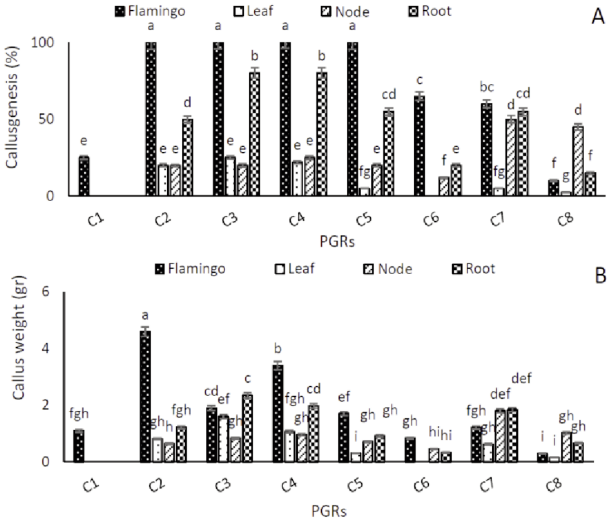


Fig. 2 - Effect of explant types and PGRs on callusgenesis (A) and callus weight (B) in indirect organogenesis of D. purpurea L. after 6 weeks. Columns within each graph marked with the same letter are not significantly different according to the LSD test (P<0.05), with LSD values of 0.167 and 5.981 for callusgenesis and callus weight, respectively.

Shoot regeneration was significantly influenced by the type and combination of PGRs in the regeneration medium (Fig. 3). Media supplemented with BAP alone (SR1 and SR2) resulted in a relatively low to moderate number of regenerated shoots. The lowest shoot number (3 shoots per callus) was observed on MS medium containing 0.25 mg L^{-1} BAP (SR1), whereas increasing the BAP concentration to 0.5 mg L^{-1} (SR2) markedly enhanced shoot proliferation, yielding an average of 12 shoots per callus. The addition of a low concentration of NAA (0.1 mg L^{-1}) to BAP-containing media further improved shoot regeneration (SR3 and SR4). Calli cultured on MS medium supplemented with 0.25 mg L^{-1} BAP plus 0.1 mg L^{-1} NAA (SR3) produced an average of 12 shoots per callus, while increasing BAP to 0.5 mg L^{-1} in combination with NAA (SR4) resulted in a higher regeneration response, with up to 14

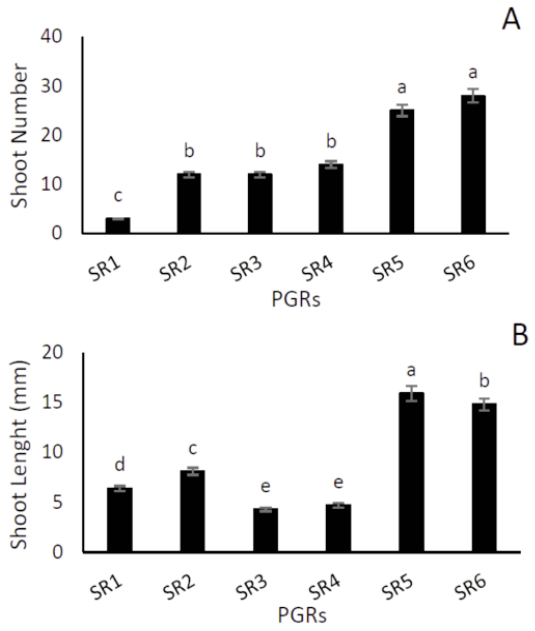


Fig. 3 - Effect of PGRs on number (A) and length (B) formed shoot in indirect organogenesis of *D. purpurea* L. after 6 weeks. Columns within each graph marked with the same letter are not significantly different according to the LSD test (P<0.05), with LSD values of 2.61 and 1.38 for shoot number and length weight, respectively.

shoots per callus. The highest shoot regeneration efficiency was achieved in media supplemented with putrescine in combination with BAP (SR5 and SR6). The inclusion of 50 mg L^{-1} putrescine significantly enhanced shoot proliferation, producing 25 and 28 shoots per callus when combined with 0.25 and 0.5 mg L^{-1} BAP, respectively (SR5 and SR6). These treatments were significantly superior to all other PGR combinations tested (Fig. 3A). Shoot elongation exhibited a pattern distinct from shoot number. Media containing BAP alone promoted moderate shoot elongation, with mean shoot lengths of 6.4 and 8.1 mm at 0.25 and 0.5 mg L^{-1} BAP, respectively (SR1 and SR2). In contrast, the addition of NAA to BAP reduced shoot elongation, resulting in shorter shoots (4.3-4.7 mm), despite increased shoot numbers. The longest shoots were obtained on putrescine-containing media. Calli cultured on MS medium supplemented with 0.25 mg L^{-1} BAP plus 50 mg L^{-1} putrescine (SR5) produced shoots with a mean length of 15.9 mm, while a slightly shorter but comparable shoot length (14.8 mm) was recorded for the combination of 0.5 mg L^{-1} BAP and 50 mg L^{-1} putrescine (SR6). These results indicate a strong promotive effect of putrescine on shoot elongation as well as shoot proliferation during indirect organogenesis.

Root induction

Root induction of regenerated shoots was significantly affected by both the type and concentration of auxin applied to the rooting medium (Fig. 4). Shoots cultured on auxin-free half-strength MS medium (control) exhibited a low rooting response, with only 30% of shoots forming roots. In addition, root development in the control treatment (R1) was weak, producing an average of

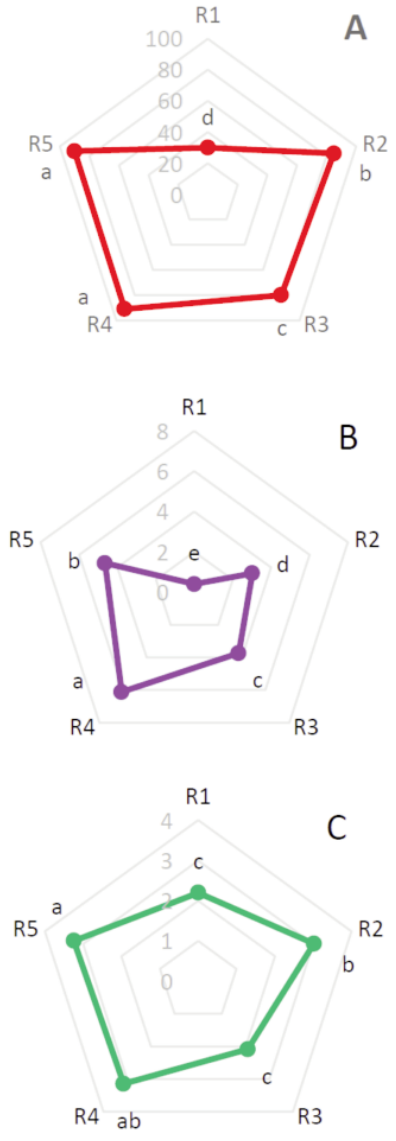


Fig. 4 - Effect of PGRs on the percentage (A), number (B), and length (C) of roots formed in root genesis of D. purpurea L. after 6 weeks. In each graph, points marked with the same letter are not significantly different according to the LSD test (P<0.05), with LSD values of 6.61, 0.37 and 0.33 for percentage, number and length, respectively.

0.4 roots per shoot with a mean root length of 2.2 cm. The inclusion of NAA in the rooting medium markedly enhanced rooting efficiency. At a concentration of 0.25 mg L^{-1} NAA (R2), rooting frequency increased to 85%, accompanied by the formation of an average of 3.02 roots per shoot with a mean length of 3.02 cm. Increasing NAA concentration to 0.5 mg L^{-1} (R3) resulted in a slightly lower rooting percentage (80%) but a higher number of roots per shoot (3.74), although root elongation was reduced, yielding shorter roots with a mean length of 2.09 cm. IBA proved to be more effective than NAA in promoting both root induction and root development. The highest rooting frequency (91%) and the greatest number of roots per shoot (6.12) were obtained on medium supplemented with 0.25 mg L^{-1} IBA (R4). Shoots cultured under this treatment also developed well-elongated roots with a mean length of 3.15 cm. A comparable rooting frequency (90%) was observed at 0.5 mg L^{-1} IBA (R5); however, the number of roots per shoot decreased to 4.65, while the longest mean root length (3.24 cm) was recorded at this concentration. Overall, among the auxins tested, IBA at 0.25 mg L^{-1} was the most effective treatment for inducing a high rooting frequency combined with superior root number and adequate root elongation, indicating its suitability for efficient root induction in regenerated *D. purpurea* plantlets. In addition, a strong positive correlation was observed between rooting frequency and the number of roots per shoot (r = 0.89), indicating that treatments enhancing root induction also promoted root proliferation. Root length showed moderate positive correlations with rooting percentage (r = 0.67) and root number (r = 0.65), suggesting partially independent regulation of root initiation and elongation (Fig. 5).

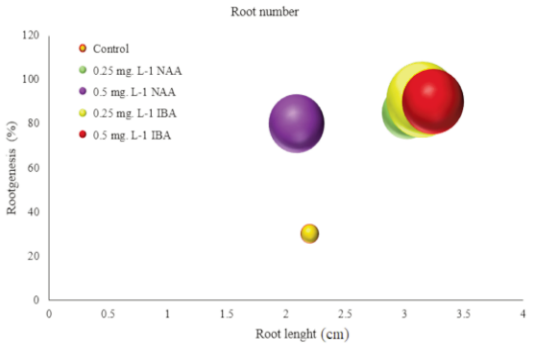


Fig. 5 - Analysis of root formation in *D. purpurea* L.: Correlation of percentage, number, and length of roots.

4. Discussion and Conclusions

The present study demonstrates that direct shoot organogenesis in *D. purpurea* is strictly dependent on both explant type and the presence of exogenous cytokinins. Among the explants tested, flamingo (hypocotyl-cotyledon) explants showed the highest regeneration capacity, whereas leaf and root explants failed to respond under all treatments, including hormone-free media. This confirms that organogenic competence in *D. purpurea* is highly tissue-specific. The superior response of flamingo explants may be attributed to their juvenile developmental stage and higher cellular plasticity, which has been associated with increased responsiveness to plant growth regulators (Guo *et al*., 2017).

Low concentrations of TDZ were particularly effective, with 0.25 mg L^{-1} TDZ (D5) producing the greatest shoot number and shoot length, especially in flamingo explants. This result supports previous reports emphasizing the importance of optimizing cytokinin concentration to avoid inhibitory effects at higher levels (Kairuz *et al*., 2021). TDZ is known to exhibit both cytokinin- and auxin-like activity, influencing endogenous hormone balance and promoting shoot induction at low concentrations (Novikova and Zaytseva, 2018). In contrast, higher TDZ levels can suppress organized shoot development. Although leaf explants of *D. purpurea* have been reported to regenerate shoots under specific hormonal conditions (Bansal *et al*., 2024), their lack of response in the present study suggests that additional optimization of PGR composition or explant physiological status is required. These findings highlight that medium composition and explant selection are critical determinants of direct organogenesis, as previously reported for *D. purpurea* (Kairuz *et al*., 2021).

Callus induction responses differed markedly among explant types and auxin treatments. Flamingo explants consistently exhibited the highest callus induction frequency and biomass, achieving 100% induction on media containing 0.5-1 mg L^{-1} NAA, either alone (C2 and C3) or in combination with BAP (C4 and C5). This strong callogenic response indicates a high capacity for cellular dedifferentiation, which is known to be promoted by auxin-mediated signaling and stress-related pathways (Ikeuchi *et al*., 2013; Feher, 2005; Jimenez, 2005). In contrast, node, leaf, and root explants showed limited callus formation,

reflecting inherent physiological and biochemical differences among tissues (Guo *et al*., 2017).

Among the auxins tested, NAA was more effective than 2,4-D in promoting both callus induction and biomass accumulation. Although 2,4-D is commonly used for callogenesis (Isah, 2016; Abbaszadeh *et al*., 2023), its lower efficiency in this study suggests that it may not support sustained callus growth in *D. purpurea*. Similar variability in auxin effectiveness has been reported across species, including cashew, mango, and woody plants (Nadgauda and Gogate, 2005; Ara *et al*., 2005; Klimek-Chodacka *et al*., 2020). The synergistic effect observed between NAA and BAP further supports the established role of auxin-cytokinin balance in regulating cell division and callus development (Novikova and Zaytseva, 2018).

Shoot regeneration from calli was significantly enhanced by cytokinin-polyamine combinations. The highest shoot numbers and longest shoots were obtained on media containing BAP in combination with putrescine, confirming a strong synergistic effect. Polyamines are known to regulate cell division, stabilize membranes, and modulate ion transport, thereby supporting both shoot initiation and elongation (Hagos and Gebremdhin, 2015; Taheri *et al*., 2021). Similar promotive effects of polyamines on shoot regeneration have been reported in Bacopa monnieri and several horticultural species (Dey *et al*., 2019; NowakowsKa *et al*., 2022). In contrast, the addition of NAA to BAP increased shoot number but reduced shoot elongation, reflecting the antagonistic interaction between auxins and cytokinins during shoot growth (Müller and Leyser, 2011; Abbaszadeh *et al*., 2023). These results highlight the importance of carefully balancing PGR ratios to optimize both shoot proliferation and elongation.

Root induction was strongly influenced by auxin type and concentration, with IBA outperforming NAA in all rooting parameters. The highest rooting frequency, root number, and satisfactory root elongation were achieved with 0.25 mg L^{-1} IBA (R4). The superior performance of IBA has been attributed to its metabolic stability, resistance to degradation, and efficient conversion to IAA within plant tissues (Nakhooda *et al*., 2012; Marquez *et al*., 2016; Fattorini *et al*., 2017). Similar findings have been reported in various plant species, including Panax ginseng, where IBA was more effective than NAA in promoting lateral root development (Damodaran and Strader, 2019). The weak rooting response observed in auxin-free media confirms that exogenous auxins are essential for rhizogenesis, consistent with previous reports emphasizing the importance of auxin-cytokinin balance in root formation (Nakhooda *et al*., 2012; Depaepe *et al*., 2024). The positive correlations observed between rooting percentage, root number, and root length further indicate coordinated regulation of root initiation and development.

In conclusion, this study establishes an efficient and reproducible *in vitro* regeneration system for *D. purpurea* based on both direct and indirect organogenesis. Regeneration efficiency was strongly explant-dependent, with flamingo (hypocotyl-cotyledon) explants consistently exhibiting the highest morphogenic competence across all stages. Low concentrations of TDZ (0.25 mg L^{-1}, D5) were optimal for direct shoot induction, highlighting the importance of precise cytokinin regulation. For indirect organogenesis, NAA was the most effective auxin for callus induction, while the combination of BAP and putrescine markedly enhanced shoot regeneration and elongation, demonstrating the synergistic role of cytokinins and polyamines. Root induction was most efficiently achieved using low concentrations of IBA, which produced high rooting frequencies and well-developed root systems (Fig. 6). Despite the effectiveness of the established protocols, this study was limited to a restricted range of explant types and PGR concentrations, and endogenous hormone dynamics were not investigated. Future studies integrating broader hormonal regimes and molecular or physiological analyses would provide deeper insight into the mechanisms governing explant-specific responses. Nevertheless, the regeneration system described here offers a practical and reliable framework for the large-scale micropropagation of *D. purpurea*. Given the medicinal and economic importance of this species, the optimized protocols presented in this study can support sustainable propagation, conservation strategies, and future biotechnological applications.

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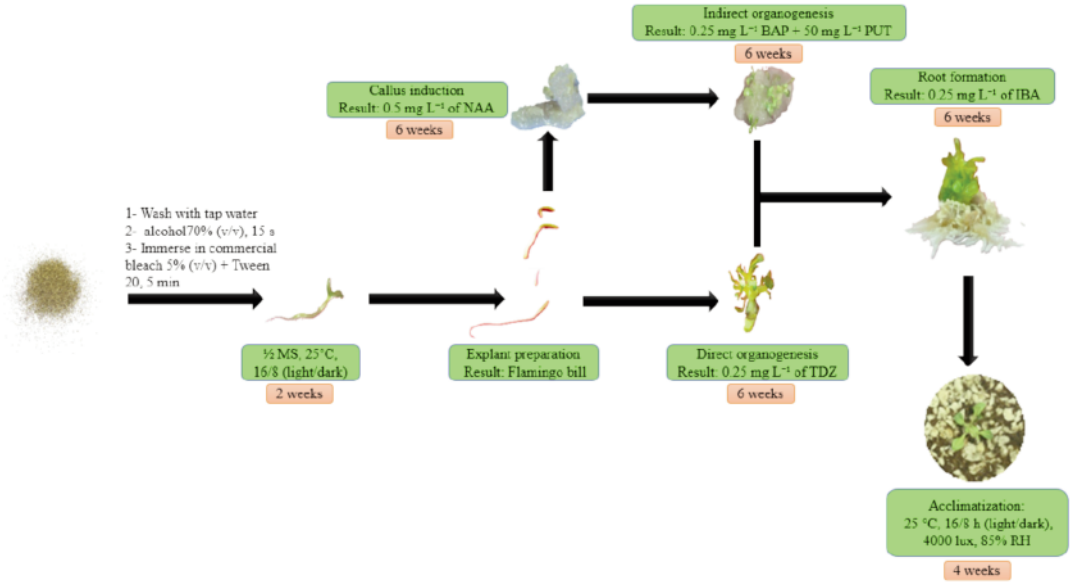


Fig. 6 - Direct and indirect organogenesis techniques for foxglove plant regeneration and optimal media culture and time for each step.

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Fruit morphological and biochemical diversity in five Algerian pomegranate (*Punica granatum* L.) genotypes

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Key words: Fruit quality traits, genotypes, physicochemical characteristics, *Punica granatum* L., seed traits.

Abstract: Pomegranate (*Punica granatum* L.) is an ancient fruit linked to several cultures worldwide. It ranks as the second most valuable fruit crop in the Algerian Messaad region, after apricots, in terms of cultivated area, and is widely consumed in traditional dishes and juices, while being recognized for its therapeutic properties as a herbal remedy. Despite its importance, this fruit remains poorly studied in the region, with limited research addressing its productivity and characteristics. Local pomegranate genotypes possess distinct qualities that make them suitable for fresh consumption or processing. Therefore, evaluating local genotypes is essential to understand their physicochemical characteristics, nutritional composition, and potential contribution to the development of pomegranate cultivation and local agriculture. To achieve this goal, five pomegranate genotypes ('Hamraye', 'Snin Laâloudj', 'El Karess', 'Tounsi', and 'Khadraye') were evaluated based on laboratory analysis and the UPOV script to compare 27 fruit and seed parameters, including the physical characteristics of fruits, arils and seeds, and chemical properties of the juice, during two consecutive seasons (2023 and 2024). The studied genotypes revealed remarkable morphological and biochemical characteristics. The results showed that 'Snin Laâloudj' and 'Tounsi' had high sweetness (TS: 14.35 and 15.8 g 100 mL^{-1}), with a significant juice yield of 77.37 and 79.96%, respectively, together with a significant chemical composition, especially for 'Snin Laâloudj'. 'El Karess' is a unique cultivar with its sour-sweet taste (pH= 2.85), a significant juice yield of 71.28%, and a larger fruit and grain shape. However, 'Khadraye' and 'Hamraye' had the lowest values among the 27 studied variables. These findings demonstrate that local genotypes can provide valuable insights into the potential industrial use of 'Elkares' and the nutritional applications of other genotypes.

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1. Introduction

Pomegranate (*Punica granatum* L.) is an old and beloved plant and fruit. Recent studies have confirmed the traditional use of pomegranates as a medicinal remedy, as each part of this plant contains various chemical compounds (Holland *et al*., 2009). It is also acknowledged as a vital commodity that is incredibly helpful in the human diet; its nutritional, therapeutic, and ornamental aspects have been known since antiquity (Liu *et al*., 2025).

Wild pomegranates grow across Transcaucasia and Central Asia, from Iran and Turkmenistan to northern India (Chandra *et al.,* 2010; Shilpa *et al.*, 2024). As a result, it is considered to be indigenous to these regions. Since the pomegranate tree is extremely adaptable to a broad range of climatic and soil conditions, it has been planted in several tropical and subtropical environments (Yilmaz *et al*., 2021). However, pomegranate is also widely cultivated in temperate regions and considered one of the best suitable areas for growing pomegranate like the Mediterranean climate (Beghè *et al*., 2019). Interestingly, most of its cultivation is confined to the northern hemisphere, and the highest fruit quality is produced in arid lands (Rao, 2016). The plant is highly tolerant and resilient to poor soils, brackish water, and high altitudes (Rao, 2016). This makes the pomegranate tree a rustic species typical of temperate climates and is considered one of the most significant fruits of the restricted regions because of its low maintenance cost, significant yields, high preserving quality, and capacity to flourish with minimal moisture (Day and Wilkins, 2011; Ferrara *et al*., 2021). Although this species is not very demanding and has the potential to expand and diversify fruit production, it receives less attention and is considered a neglected crop despite its economic, agronomic, and medicinal importance (Singh *et al*., 2022).

Despite being assumed to have a restricted genetic base, pomegranates have a wide range of germplasm available globally, indicating great genetic diversity (Özgüven *et al*., 2010). Although numerous countries have developed collections to conserve genetic material (Bartual *et al.,* 2025), the pomegranate remains a relatively understudied species on a national scale, despite its potential to improve and diversify fruit output in numerous parts of the world. In terms of genetic resources, there are many undetermined genotypes of pomegranates characterized by diverse physicochemical qualities. In Algeria, pomegranate cultivation is important, where the cultivated area was estimated at 91578 ha to 93868 ha based on yield and production data (Meziane *et al*., 2016). Although, the plantations are limited in Algeria. There are many genotypes of pomegranates, with very different qualities. Several genotypes are reported in small gardens in Kabylie such as Lahlou, and El-mouze, while numerous regions cultivate about 14 distinct genotypes, which are currently under production processes, such as Molla, Blanca, and Si Hueso (FAO, 2006). The fruit is popular for fresh consumption owing to its flavour and is widely grown, with the country's production contributing considerably to its economy. Moreover, studies are currently underway to evaluate the genetic diversity of Algerian pomegranate genotypes and their potential applications. However, in the Djelfa region, and more precisely in the commune of Messaad (Lahouel *et al*., 2024). Several unstudied genotypes are highly appreciated in terms of taste quality, with an average yield ranging from 7.83 t ha^{-1} to 8.42 t ha^{-1} (Meziane *et al*., 2016). Furthermore, the physical and chemical evaluation of pomegranates is crucial as it directly influences product morphology and fruit composition and is significantly affected by cultivar type (Asadi-Gharneh *et al*., 2017). This evaluation and determination of different genetic resources also help identify genetic diversity and provides an opportunity for selection and fruit improvement programs.

Several prospecting visits were conducted to identify the pomegranate genotypes cultivated in the Messaad region, leading to the identification of five poorly documented local genotypes: 'Hamraye' (also known as 'Hamraoui'), 'Snin Laâloudj', 'El Karess', 'Tounsi', and 'Khadraye' ('Khadraoui'). These genotypes are traditionally grown in smallholder orchards and are locally consumed either fresh or as processed products; however, their physicochemical traits remain largely uncharacterized. Although the region possesses considerable pomegranate diversity, systematic scientific data on the fruit quality characteristics of these genotypes are limited to a few studies, except for the recent work of Lahouel *et al*. (2024) that include 3 out of our 5 chosen genotypes. while the initial study provided foundational insights, several genotypes remain unexplored, which this study could address through extending previous research by examining 2 more genotypes, and confirming the earlier conclusions as

well. On the other hand, missing study create a gap that limits their conservation, valorisation, and potential inclusion in breeding programs. Despite their local importance, a few comprehensive evaluations has been conducted to characterize the biochemical and morphologic attributes of these Messsad's genotypes in the same study. Consequently, their comparative nutritional potential, quality, and suitability for fresh consumption or industrial processing remain insufficient. Hence, in this study it was hypothesized that these traditional genotypes exhibit significant variation in their physicochemical and biochemical fruit qualities, reflecting the underlying genetic diversity shaped by local agro-ecological conditions. Such variability may offer valuable traits for consumer preference targeting, breeding, and agro-industrial development of these species. Therefore, this study aims to: (1) characterize the physicochemical properties (e.g. fruit morphology, juice quality, and biochemical composition) of the five selected genotypes; (2) compare their quality profiles to identify distinctive traits of nutritional, agronomic, or industrial relevance; and (3) provide baseline data needed for genetic improvement programs, enhancement of local market value, and conservation strategies. By focusing on five little-known Messaad cultivars, this study offers original insights into the underexplored pomegranate diversity of the region and contributes valuable information to guide future food science, agricultural and industrial applications.

2. Materials and Methods

Site description

The study was conducted in the locality of Fayd Saaid in Messaad, south of Algiers. It is situated at coordinates 34°12'41'' N and 3°32'18'' E at an altitude of 798 m above sea level. The soil at the study site has a sandy-clayey-silty texture with a pH of 7.79. The average annual rainfall registered during the period from 2004 to 2024 was 314.95 mm for a mean rainfall duration of 68 days per year, and the average temperatures reached a maximum of 37 °C in July and a minimum of 2.8°C in January.

Planting materials and field conditions

The plant material evaluated consisted of five local genotypes Hamraye (HMR), Snin Laâloudj (SLL), El Karess (EKS), Tounsi (TNS) and Khadraye (KDR) planted in an orchard of 3150 m^2, representing a total of 200 trees (40 trees for each genotype), planted together in 1995 at a spacing of 4.5 × 3.5 m. This spacing is not favourable for the vegetative growth of the species, which leads to difficulty in various cultivation operations (pruning, soil work, treatments, fertilization, and harvesting). Therefore, maintenance pruning (tree thinning) was performed once a year, considering the excess foliage generated by the tree to promote regular development. The orchard is irrigated at a yearly rate of 2500 m^3 ha^{-1} by a system of channels distributed from May to mid-October, a period where rainfall is less frequent as a complementary application based on the availability of pluviometry and replace the amount of water that crops need to replace water lost through evapotranspiration as highlighted by Volschenk (2020) which allows us to provide a mean value of 49.3 L for each tree per day based on plant requirement in a semi-arid condition (Meshram *et al*., 2011). Fertilization was limited to organic manure in the summer period, and genotypes were distributed randomly in the same orchard, undergoing the same horticultural management practices according to recommendations for two main reasons: the traditional practice in the area and to improve soil quality characterized by a pH above 7 and low organic matter content.

Fruit sampling process

The optimal harvest time was determined based on visual and touching inspection, using a combination of objective, pre-defined criteria used locally including: skin colour, skin texture, fruit shape, calyx condition, easy fruit detachment and the acoustic response to tapping, all correlated against a pre-established calendar date range from first to mid-October 2023 and 2024, and then data from both years were pooled. Thus, following Williams *et al*. (2002), each tree was considered an experimental unit. Sampling was systematic, targeting 25 fully ripe fruits from multiple cardinal directions and vertical canopy heights (outer, mid, and inner canopy) on five representative trees to account for biological variability, we avoided fruits with visible damage, those that were most exposed to the sun, and those that were at the bottom of the tree. Regarding arils parameters, 50 arils per fruit were systematically taken from the top, middle, and bottom sections of the fruit and from different radial positions, then

mean was applied. Fruits were harvested manually at commercial maturity based on peel color development, fruit size, and total soluble solids stability. For each genotype, fruits were collected from five randomly selected trees, ensuring uniform exposure and excluding damaged or sunburned fruits. Twenty-five fruits per genotype per season were analysed. For seed and aril traits, measurements were performed on arils and seeds extracted from each fruit.

Fruits and seeds physical evaluation

The purpose of these measurements was conducted at the Biochemistry Laboratory (Faculty of Sciences, Yahia Fares University of Medea) to determine the size and dimensions of the collected fruits to highlight a distinction of the studied genotypes based on the guiding principles for conducting the examination of the distinctness and homogeneity provided by the UPOV script available online (UPOV, 2013). A total of nine parameters were evaluated using 25 fruits: Fruit weight in g (FW), average number of arils per fruit (NAF), average length of fruits (FL) in mm, average width of fruits (FT) in mm, average thickness of the peel (PT) in mm, weigh of peel and husk after separation of the edible part per fruit (PHW) in g, total weight of arils in g for each fruit (TAW), average width of the calyx (CT) in mm and average length of the calyx (CL) in mm. In addition to 6 physical properties of arils and seeds including: average length of arils (AL) in mm, average width of arils (AT) in mm, average seed weight (SW) in g, average width of seeds (ST) in mm, average length of seeds (SL) in mm and average weight of arils (AW) in grams.

Fruits biochemical analyses

To determine the main chemical components, we also used laboratory testing protocols, such as the volume of juice in millilitres 100 g^{-1} of arils (JY). Juice was extracted from the arils using a manual press and filtered through a nylon mesh, followed by the measurement of fruit water percentage (FWP) and dry matter weight (DMW) in grams. The dry matter rate (DMR) was then estimated as a percentage (g per 100 g of fresh matter). The samples were kept at 4°C in the dark for later analysis of total soluble solids content (TSS) using a digital refractometer (Atago N1, Tokyo, Japan), and the results were reported as °Brix at 20°C. The pH was measured using a digital pH meter (Metrohm model 601) at 21°C. The titratable acidity (TA) was determined by titrating 10 mL of juice with 0.1 N NaOH solution to pH 8.1 and expressed as grams of citric acid per 100 mL of juice (Tyl and Sadler, 2017). The maturity index (MI) was generated based on the ratio of TSS to TA, as suggested by Martínez *et al*. (2006). Vitamin C or ascorbic acid concentration (AA) was determined in triplicate using a colorimetric method with some modifications as described by Arendse *et al.* (2014), expressed as mg 100 ml^{-1}. The total protein (TP) content in dry pomegranate pulp was determined in g 100 mL^{-1} according to the Folin phenol reagent method described by Meziane *et al.* (2016). The total sugar content (TS) was calculated in g 100 mL^{-1} using the method described by Dubois *et al.* (1956). Juice pulp (JP) was estimated in grams of pulp per 100 mL of pure juice according to Barkhatov and Elisseev (1979).

Statistical analysis

Data were analyzed statistically through analysis of variance (One-Way ANOVA) using R software (version 4.1.1). The homoscedasticity was verified with the test of pairwise Mean comparisons were performed using Fisher's least significant difference test at α =0.05. Principal component analysis (PCA) was performed to explore relationships among variables and to reduce data dimensionality. Prior to analysis, data were standardized to eliminate scale effects (mean= 0, SD= 1), PCA was conducted on the correlation matrix, and principal components were retained based on cumulative explained variance, with the selected components accounting for at least 70% of the total variance. Finally, clustering was performed using the Euclidean distance and Ward's method of linkage.

3. Results

Pomological-based characterization of pomegranate genotypes

For all the physical variables of the fruits, the results showed strong fluctuations in values with respect to the studied genotypes, whose fruits have diverse shapes and dimensions. However, it should be noted that there is no significant difference between the recorded means of the peel thickness ($p>0.05$). Accordingly, the statistical test showed homogeneity of variance, and the physical characterization of the fruits according to the

analysis of variance showed a significant difference for all physical parameters (p-value less than 0.05), except for peel thickness (PT), where it was not significant (p-value > 5%) (Table 1). Based on means comparisons, the average FW varied between a minimum of 156.40 g for HMR genotype (group "a") and a maximum value of 249.11 g for EKS (group "b"), reflecting a marked genetic influence on fruit size. The highest PHW were recorded in EKS (99.46 g), whereas the lowest were observed in SLL (66.64 g). However, the TAW ranged from the lowest value of 84.35 g, HMR to the highest mean of 150.44 g EKS in this study. The comparison test also revealed notable differences in fruit dimensions: in term of fruit length genotype TNS recorded the highest mean value (67.12 mm), whereas HMR exhibited the lowest value (57.82), whereas regarding the fruit width, HMR belonged to the highest significance group, while SLL was classified in the lowest group. ranging from 66.0 mm to 75.36 mm respectively. The CL results showed that several genotypes shared the highest statistical group, including KDR, SLL and TNS, while EKS was grouped among the lowest. While the calyx diameter the highest mean value was observed in genotype SLL, while the lowest was recorded in genotype EKS and KDR, with values ranging between 15.64 mm and 18.24 mm.

Seed and aril-based characterization of pomegranate genotypes

The physical variables of the seeds presented average outputs with remarkable variations among the studied genotypes, and their seeds were of different shapes and sizes. Statistically, the analyses of variance showed a significant difference among the seed variables of the genotypes (p-value < 5%) (Table 2). The average AW of the examined genotypes ranged from 0.41 g for EKS to 0.32 g for HMR and KDR. Moreover, the average SW is an indicator of its quality. Hence, our investigated cultivars, EKS and KDR, have the highest SW with 0.045 g and 0.042 g (group "b"), respectively, while

Table 1 - Physical characterization of the five genotypes fruits associated with their means and standard deviation

Parameters	Genotypes					p-value
	El Karess	Hamraye	Khadraye	Snin Laâloudj	Tounsi	
Peel thickness (mm)	2.42±0.095 a	2.24±0.082 a	2.12±0.082 a	2.34±0.087 a	2.18±0.079 a	0.09355 NS
Fruit weight (g)	249.12±20.16 b	156.40±9.12 a	195.65±15.76 ab	197.16±16.94 ab	209.51±13.82 ab	0.001887 **
Fruit length (mm)	66.02±1.92 b	57.82±1.27 a	63.29±1.23 ab	64.64±1.52 b	67.12±1.15 b	0.0001286 ***
Fruit width (mm)	74.68±2.22 b	66.02±1.41 a	69.91±1.49 ab	75.36±1.83 b	72.20±1.74 ab	0.001568 **
Calyx length (mm)	18.53±0.52 b	16.19±0.69 ab	14.86±0.61 a	14.24±0.64 a	15.89±0.74 a	0.0009214 ***
Calyx width (mm)	15.11±0.53 a	17.12±0.60 ab	15.64±0.44 a	18.24±0.46 b	16.42±0.71 ab	0.001089 **
Number of arils per fruit	364.32±18.80 bc	230.14±10.04 a	417.84±16.38 cd	295.28±21.32 ab	469.16±25.31 d	5.019e-15 ***
Total aril weight per fruit (g)	150.44±11.93 b	84.35±4.97 a	129.47±11.40 b	130.52±10.61 b	138.09±8.89 b	0.000103 ***
Peel (husk) weight per fruit (g)	99.46±10.07 b	72.05±6.76 ab	70.58±8.71 ab	66.64±7.62 a	71.42±5.85 ab	0.02947 *

Values represent mean ± standard deviation (SD). Different letters indicate significant differences among genotypes for each trait : ***Very highly significant, **Highly significant, *Significant, ns: non-significant (p-value > 0.05). Statistical analysis was determined using One-Way ANOVA.

Table 2 - Physical characterization of the five genotypes seeds associated with their means and standard deviation

Parameters	Genotypes					p-value
	El Karess	Hamraye	Khadraye	Snin Laâloudj	Tounsi	
Seed weight (g)	0.045±0.003 b	0.03±0.001 a	0.042±0.002 b	0.026±0.0013 a	0.028±0.0014 a	2.49e-12 ***
Seed length (mm)	8.65±0.20 c	5.97±0.13 a	6.76±0.088 b	6.56±0.199 ab	6.16±0.117 a	< 2.2e-16 ***
Seed width (mm)	3.15±0.11 b	2.71±0.073 a	2.78±0.102 a	2.97±0.065 ab	2.97±0.098 ab	0.00754 **
Aril weight (g)	0.41±0.018 b	0.32±0.011 a	0.33±0.013 a	0.395±0.013 b	0.386±0.012 b	0.000005014 ***
Aril length (mm)	12.42±0.28 b	10.52±0.19 a	10.47±0.201 a	11.28±0.176 a	11.34±0.236 a	0.0000000094
Aril width (mm)	7.53±0.12 ab	7.43±0.17 ab	7.30±0.207 a	8.11±0.152 b	7.91±0.227 ab	0.008921 **

Values represent mean ± standard deviation (SD). Different letters indicate significant differences among genotypes for each trait : ***Very highly significant, **Highly significant, *Significant, ns: non-significant (p-value > 0.05). Statistical analysis was determined using One-Way ANOVA.

the TNS, SLL and HMR genotypes possess the lowest SW with 0.02 g, 0.026 g and 0.03 g respectively (group “a”). The KDR and HMR genotypes had the smallest AL (10.47 mm and 10.52 mm, respectively), whereas the EKS genotype had the largest AL (12.42 mm), The test revealed that EKS (group “b”) significantly outperformed all other genotypes that showed the poorest performance (group “a”). Furthermore, the ANOVA test showed a significant difference for the seed dimensions of the EKS genotype (length 8.65 mm, width 3.15 mm) was significantly higher than the remaining genotypes, while genotype HMR (length 6.16 mm, width 2.71 mm) was significantly lower.

Biochemical-based characterization of pomegranate genotypes

A large variability was observed in the chemical parameters of the fruits, with remarkable deviations in the recorded means. The test showed homogeneity of variance, and all the biochemical parameters showed highly significant variability between the studied genotypes, as indicated by the analysis of variance (p-value < 5%) (Table 3).

Multivariate approach to characterize pomegranate genotypes

Principal component analysis allowed us to establish a link between the treated physicochemical parameters. The first two axes (also known as the first plan) presented a total inertia rate of 75.27% (Fig. 1). Thus, the first group of variables was positively correlated and explained a strong relationship between SL, CL, SW, PHW, and DMW, which characterized the unique EKS genotype. This genotype also registered a higher value of the second group, especially for the first dimension (47.91%) for JP, NAF, total protein content, FL, FT, AW, DMR, TAW, ST, AL, FW, and PT. Both KDR and HMR individual are distributed in this group (Fig. 1 and 2). Moreover, a strong positive correlation was observed

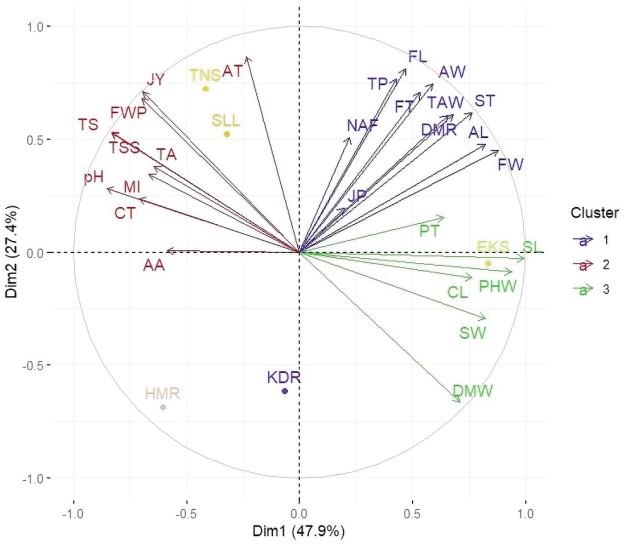


Fig. 1 - Dimension reduction plot showing the distribution of variables and individuals on the first component, which explains 75.27% of the total variance; using Principal Component Analysis (PCA).

Table 3 - Chemical characteristics of the five genotypes fruits associated with their means and standard deviation

Parameters	Genotypes					p-value
	El Karess	Hamraye	Khadraye	Snin Laâloudj	Tounsi	
Juice yield (%)	71.28±0.48 a	74.91±0.89 b	73.27±0.46 ab	77.37±0.58 c	79.96±0.62 d	< 2.2e-16 ***
Fruit water percentage ((%)	79.79±0.302 a	81.47±0.501 ab	80.87±0.23 ab	82.23±0.49 bc	83.85±0.59 c	0.000000047 ***
dry matter weight (g)	20.20±0.303 c	18.44±0.499 b	19.12±0.236 bc	17.77±0.49 ab	16.15±0.59 a	0.000000048 ***
pH	2.85±0.036 a	3.89±0.041 c	3.44±0.023 b	4.35±0.049 d	3.81±0.029 c	< 2.2e-16 ***
Titratable acidity (%)	0.34±0.005 a	0.38±0.005 b	0.34±0.007 a	0.41±0.005 c	0.37±0.004 b	< 2.2e-16 ***
Dry matter rate per fresh matter (%)	0.34±0.007 e	0.24±0.004 b	0.22±0.005 a	0.27±0.005 c	0.30±0.005 d	< 2.2e-16 ***
Total protein (g)	1.29±0.034 c	0.72±0.030 a	1.01±0.029 ab	0.99±0.029 ab	1.49±0.04 d	< 2.2e-16 ***
Juice pulp (g)	1.41±0.0072c	1.19±0.006 a	1.64±0.008 e	1.28±0.014 b	1.55±0.011 d	< 2.2e-16 ***
Total soluble solids (°Brix)	11.67±0.178 a	14.97±0.087 c	13.56±0.089 b	15.40±0.081 c	17.05±0.097 d	< 2.2e-16 ***
Maturity index	34.41±0.546 a	40.19±0.59 c	39.76±0.72 c	37.17±0.45 b	45.87±0.43 d	< 2.2e-16 ***
Vitamin C (mg)	8.60±0.098 a	11.67±0.073 c	13.70±0.081 d	9.55±0.052 b	13.99±0.08 d	< 2.2e-16 ***
Total sugar (g)	10.79±0.164 a	13.87±0.082 c	12.55±0.090 b	14.35±0.105 d	15.80±0.091 e	< 2.2e-16 ***

Values represent mean ± standard deviation (SD). Different letters indicate significant differences among genotypes for each trait : ***Very highly significant, **Highly significant, *Significant, NS: non-significant (p-value > 0.05). Statistical analysis was determined using One-Way ANOVA.

between AA, CT, maturity index, pH, TA, TSS, TS, FWP, JY, AT, which characterized the TNS and SLL genotypes as a third group (Fig. 1 and 2). The latter were negatively correlated with the former (Fig. 1).

4. Discussion and Conclusions

Physical assessment is considered an important trait for distinguishing cultivars (Radunić *et al*., 2024) and is easy to evaluate, offering a better description and genetic diversity comparison. Physical analysis revealed remarkable differences in the characteristics of Algerian pomegranate genotypes. These valuable yet overlooked fruits come from a rich genetic pool.

In this work, the PT was the only variable that revealed no significant difference among cultivars; conversely, fruit weight showed significant differences between cultivars. EKS pomegranate is the heaviest, while HMR weighs the least. These results are comparable to those reported by Beghè *et al*. (2024) for some Italian cultivars and Meziane *et al*. (2016) for some Algerian cultivars, confirming that fruit weight is strongly cultivar-dependent. Based on the classification proposed by Martínez-Nicolás *et al*. (2006) for Spanish cultivars, all studied genotypes can be classified as medium to large fruits (150-350 g). However, the inner thick spongy white tissue and the outer smooth skin layer constitute the peel (da Silva *et al*., 2013). When measured, it indicates the proportion of inedible part per fruit (PHW), which differs from one cultivar to another, giving a juice transformation disadvantage to cultivars such as EKS but offering an opportunity to provide a particularly high peel natural source of nutritional, therapeutic, and industrial compounds, as highlighted by Kanatt *et al*. (2010). This increased interest in peel thickness, even if consumers generally prefer pomegranate fruit with a thin peel because they have reduced fruit waste and are easy to peel (Radunić *et al*., 2015), indicates potential health benefits.

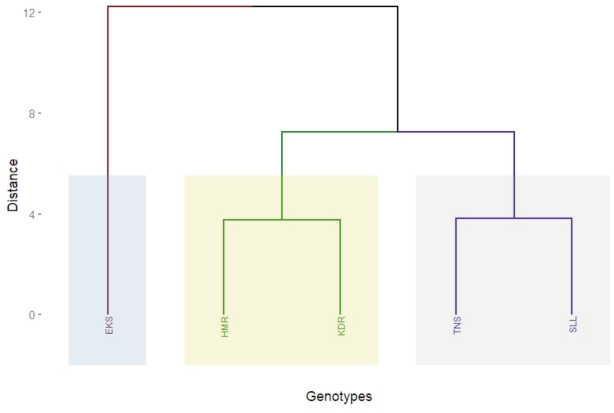


Fig. 2 - Dendrogram showing three clusters based on the distances among the pomegranate genotypes studied using the Euclidean distance and Ward's method of linkage.

The length and width attributes of the fruits measure the fruit size and express variable values (Wetzstein *et al*., 2013), which exhibit considerable phenotypic diversity in fruit size among the five genotypes. This revealed that TNS and KDR were more spherical (oblate spheroid) and the remaining three genotypes were more elongated (ellipsoid), similar to the mass modelling provided by Khoshnam *et al*. (2007) confirming that fruit shape varies according to genotype and has implications for the market and industrial processing. Accordingly, most cultivars have round shapes, but our genotypes have an angular form, except that SLL presents a distinct smooth shape. Similarly, HMR, TNS, and KDR have a rounded calyx, whereas genotypes such as EKS and SLL possess an elongated calyx. These results are in line with those reported for other pomegranate genotypes by Fawole and Opara (2013) and Shi (2025).

The arils are the edible portion of pomegranate fruit, containing the juice, the pulp and the seeds. Thus, knowing these characteristics enable producers and processors to promote this fruit better (Meziane *et al*., 2016). The number of arils per fruit increased in accessions HMR and TNS respectively. These results reflect the combined influence of genetic and environmental factors and are comparable to those reported by Hmid (2013), while remaining consistent with Radunić *et al*. (2015) and Rajkumar *et al.* (2020), highlighting the role of genotype and fertilization in aril yield. In addition, several studies have shown that pollen sources can also modify the number, size, and weight of arils (Gharaghani *et al*., 2017). Furthermore, certain physical properties of the fruit, such as mass and volume, can indicate the potential number of arils (Çelik and Ercisli, 2009). Moreover, the aril weight was higher for EKS, SLL, and TNS genotypes and weak for the other two accessions, similar to seed sizes, which may influence consumer preference and suitability for industrial processing (Chandra *et al*., 2013). None of the genotypes were

seedless; as a result, larger seed weights were associated with EKS and KDR accessions. However, the other genotypes are more appreciated for direct consumption due to low SW. This could be a result of genotype characteristics and/or cross-pollination, which often causes increased fruit set and fruit and AW, all of which improve the yield and quality of this fruit crop (Derin and Sinan, 2001), as our genotypes were planted in the same orchard. In addition, the same author recommends planting at least two different cultivars of pomegranates in an orchard to encourage pollination and optimal fruit set.

Analysis of the essential components of five overlooked Algerian pomegranate genotypes revealed clear patterns in their fruit quality. Pomegranate juice is a rich source of dietary nutrients and bioactive compounds with potential therapeutic effects (Hegazi *et al*., 2021). In addition to medicinal quality, the juice yield varied from genotypes to another, with the TNS genotype exhibiting the highest yield. This is higher than the yields reported by Lahouel *et al*. (2024) and by other genotypes studied in the wet area of Boufarik (Meziane *et al.*, 2016) and is comparable to the values reported by Radunić *et al.* (2015) and Zaouay and Mars (2011). High juice yield is a critical factor in the juice industry, as it directly impacts the economic viability of processing operations (Taylor, 2004). The differences in juice yield among the genotypes can be attributed to genetic factors and growing conditions (Li *et al*., 2015). Genotypes with higher juice yields, such as TNS and SLL, are particularly valuable for industrial applications, where maximizing the volume of juice extracted is essential. This is associated with the FWP, dry matter weight and ratio (DMW, DMR), and the TSS, since water is the essential component of juice and the dry matter reflects the juice concentration that could be affected by water availability. As mentioned by Adiba *et al*. (2022), water stress could not only affect juice yield but also the quality of juice. Accordingly, the water content per fruit of genotypes was inferior in EKS and superior in TNS. This characteristic depends on climatic factors and the irrigation regime during critical phases. The results are comparable to those reported by Hmid (2013) for other pomegranate genotypes, confirming the influence of genetic and environmental factors on juice yield and quality.

The pH levels varied significantly among the genotypes. SLL exhibited the highest pH, while EKS, a local variety known for its acidic taste showed an acidic profile. Moreover, these pH results were generally lower than those reported by Assadi *et al*. (2019), except for SLL genotype. Similarly, compared with the findings of Lahouel *et al.* (2024) in the Messaad region, lower pH values were recorded HMR, KDR, and TNS, whereas Snin Laâloudj displayed comparable values in both studies. Furthermore, the pH values for EKS genotype were consistent with those reported by Elfazazi *et al.* (2022) for other genotype such as Acco l genotype. These differences may result from intra-cultivar variation, fruit maturity, and environmental or cultural factors. In addition to the impact of genotype, temperature and storage conditions can affect juice pH (Darvishi, 2013). Consequently, the titratable acidity also differed significantly among cultivars; SLL had the highest acidity, while EKS showed the lowest, which is consistent with the values reported by Lahouel *et al.* (2024). These acidity levels are comparable to those reported for Spanish genotypes and Tunisian cultivars (Zaouay *et al.,* 2012; Alcaraz-Mármol *et al*., 2017). Although Assadi *et al*. (2019) reported higher acidity value. However, excessively high acidity may require adjustments during processing to achieve the desired flavour profile.

Regarding protein, pulp, sugar, and AA contents, the TNS and SLL genotypes had higher values than the other genotypes, demonstrating the nutritional importance of this genotype, alongside a higher maturity index, which is a vital indicator of fruit quality and important in maintaining high total component content (Fawole and Opara, 2013). The values observed are close to those of the Kashmir genotypes (Wani *et al.*, 2012) and consistent with Lahouel *et al.* (2024).

The total soluble solids (TSS) content of juice increases with maturity (Mohamed *et al*., 2015; Kumar and Gill, 2017). Our results suggest that the TNS genotype had the highest Brix value, while the EKS genotype had the lowest. The values for SLL and HMR were similar, in agreement with Lahouel *et al.* (2024) and Beghè *et al.* (2024) for other genotypes. Furthermore, regional climatic conditions, such as temperature and humidity, have an important impact on determining TSS levels, with Mediterranean regions create greater TSS values than in desert climate (Schwartz *et al.*, 2009). Accordingly, Mphahlele (2016) found that genotype-specific variables had the greatest effect on the sugar profiles

of pomegranates.

In terms of vitamin C content, the TNS genotype juice had the highest concentration, followed by KDR and HMR. The EKS genotype had the lowest AA content. AA levels were greater than those reported by Tehranifar *et al*. (2010) and Ferrara *et al*. (2011). Previous studies show that high concentrations of AA are associated with strong antioxidant potential (Ramezanian *et al*., 2010). Variability in soil conditions may affect the chemical composition of pomegranates (Hassen *et al.*, 2021).

According to the data, the protein content varied by genotype, with the highest value expressed by TNS. The protein content of the juice was comparable to the values reported by Hmid (2013) and Meziane *et al*. (2016). As demonstrated by Hassen *et al*. (2021), soil type significantly affects protein accumulation in harvested fruits.

Pomegranate juice pulp is a vital dietary component. It contains substantial sugars and minerals such as potassium, magnesium, and calcium, which support various physiological functions and enhance the nutritional value of the fruit (Elfalleh *et al*., 2011). The finding in this study reports that the KDR and TNS genotypes had the highest pulp content in juice, while the HMR genotype had the lowest value. Although the extraction process may impact pulp concentration.

According to SaeiAhagh *et al*. (2015), increased aril weight and volume exert higher internal pressure inside fruits, which can lead to cracks inside the peel and consequently affect the pulp yield. In addition, fruit shape and skin thickness play a crucial role in cracking susceptibility. Weather conditions, such as temperature and humidity, significantly affect the rate of fruit cracking, which can reduce the composition and quality characteristics of the yield (Singh *et al.*, 2020). Different pollen sources can lead to variations in fruit set rates and overall fruit quality, thereby affecting pulp yield (Gharaghani *et al*., 2017). Pre- and post-harvest handling: Practices such as irrigation, fertilization, and post-harvest treatments can enhance the bioactive compounds in the pulp, thereby improving its quality (Mphahlele, 2016). However, while these elements typically increase pulp output, extreme environmental stress or poor agricultural techniques might result in inferior fruit quality and pulp levels, which emphasizes the importance of balanced cropping strategies.

Principal Component Analysis (PCA) identified an evident pattern in the various characteristics. Therefore, we chose to proceed using the first two dimensions, revealing several key relationships, and a variable correlation exists between the three previously mentioned groups. Variable distribution analysis revealed significant relationships between several physicochemical parameters. For instance, a positive correlation was observed between each group; CL was highly associated with PHW, and genotypes holding those parameters possessed long SL that weighed more SW, resulting in a higher dry matter (DMW). This is slightly negative for the second group, especially in the second dimension, which is in harmony with the findings of Zarei *et al.* (2011), where ripe fruit had the maximum amount of aril and juice, but the lowest percentage of peel and seed. However, the TAW is more affected by the NAF, ST, AW, and AL. In addition, JY is associated with fruit size (FL and FT), which results in a higher FW, DMR, TP, and JP. Moreover, the PT was situated between these groups, suggesting its contribution to both groups. The present results oscillated around the mean values reported by Dhineshkumar *et al.* (2015) for average fruit mass, length, and width. Consequently, increasing the overall aril weight resulted in higher juice production (Radunić *et al*., 2024), which is consistent with the findings of the current study.

A negative correlation appeared between the first two groups and the third group, showing a high interaction among the remaining eight variables, including FWP, which contains a higher TSS, AA, total sugar content, juice pH, and titratable acidity, all of which offer an interesting maturity index (MI) and are characterized by a large CT and AT. Similarly, fruit and aril size decreased, with reduced total soluble solids and acidity, when exposed to low nitrogen fertilization (Lyu *et al*., 2020), , their results indicated that soluble solids content, juice pH, total sugars, and AA levels in the juice were abundant. These findings provide insights into the changes in the physical and chemical properties of pomegranate fruits during maturation (Erkan and Kader, 2011).

Consequently, KDR and HMR genotypes hold lower values for these variables, and EKS is considered a better fruit for industrial purposes, producing more JY and a lower pH that could be adjusted within the transformation process and a thick peel that may hold significant importance in medicine purposes. Moreover, TNS and SLL contain smaller seeds which give them an advantage in the market and are more appreciated in direct

consumption as fresh fruit.

This study demonstrated significant physico-chemical variability among five Algerian pomegranate genotypes cultivated in the Messaad region, reflecting clear genotype-dependent differences in fruit quality traits. TNS and SLL were particularly suitable for fresh consumption due to their higher sweetness, whereas EKS showed strong potential for industrial processing owing to its larger fruit size and thicker peel. Similarities were observed between KDR and HMR, while EKS exhibited a distinct physico-chemical profile. Although the results are based on a limited number of genotypes and growing seasons, they provide valuable insight into the characterization and valorization of local pomegranate genetic resources. Further multisite and multiyear studies are recommended to strengthen the understanding of genotype environment interactions and to support their optimized use for fresh consumption and processing.

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Foliar titanium, Mn-graphene oxide, and molybdenum nanoparticles mitigate salt stress in *Pelargonium graveolens* L.

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Abstract: The present study aimed to evaluate the ameliorative effects of foliar-applied nanoparticles on growth, nutrient uptake, essential oil content, and physiological responses of *Pelargonium graveolens* under salt stress. A factorial experiment was conducted based on a completely randomized design with 3 replications. Plants were subjected to three salinity levels (0, 75, and 150 mM sodium chloride) and foliar spray of TiO_2 (2 mg L^{-1}), Mn oxide-graphene oxide (10 mg L^{-1}), molybdenum trioxide nanoparticles (0.5 µM), or no spray (control). Salt stress significantly reduced biomass, nutrient content (N, P, Ca), and adversely enhanced malondialdehyde content. The highest biomass and nutrients content were observed under no-saline conditions + all nanoparticle treatments, and under 75 mM NaCl + Mn oxide-GO and MoO_3 sprays. Essential oil content was the highest at 0 mM and 150 mM NaCl + MoO_3, and at 75 mM NaCl + all three foliar treatments. MDA levels peaked at 44 (75 mM NaCl) and 49 nmol g^{-1} FW (150 mM NaCl) without foliar sprays. In conclusion, *P. graveolens* tolerated salinity of up to 75 mM NaCl when supplemented with foliar Mn oxide-GO and MoO_3 nanoparticles. These treatments mitigated salinity-induced oxidative damages, improved nutrient status and enhanced essential oil yield, highlighting their potential as stress-alleviating agents under saline conditions.

1. Introduction

Salt stress is a prevalent environmental challenge that impairs plant growth, reduces yield, and threatens the sustainability of agricultural systems in many regions. High salt concentrations in the root zone induce water stress due to osmotic imbalance and cause toxic ion accumulation, and disrupt ionic homeostasis, normal metabolism, lipid peroxidation, and photosynthesis (Parihar *et al*., 2015; Deshmukh and Khare, 2017; Liu *et al*., 2020; Liu *et al*., 2024 a).

In *Pelargonium graveolens* (rose-scented geranium), an economically important aromatic-medicinal plant from the Geraniaceae family (Gevrenova *et al*., 2024), salt stress reduced growth, biomass, chlorophyll

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content, nutrient uptake, essential oil yield, and altered oil composition (Vojodi Mehrabani *et al*., 2022). The essential oil of this plant is rich in citronellol, geraniol, linalool, isomenthone, geranyl acetate, and citronellyl acetate, and is widely used in pharmaceutical, perfumery, and food industries (Gevrenova *et al*., 2024; Balabanova *et al.,* 2025). Iran hosts suitable microclimates for the cultivation of this valued crop, but salt stress owing to climate change in the arid and semi-arid areas severely limits the sustainable production of this plant (Akbary and Sayad, 2021; Vojodi Mehrabani *et al.*, 2022).

Plants counteract salinity damage through mechanisms such as osmotic adjustment, reactive oxygen species (ROS) scavenging, photosynthetic adaptation, hormonal regulation, ionic homeostasis, and activation of stress signaling pathways (e.g., CDPK cascades, SOS pathway, ABA signaling, and gene networks) (Refahi and Shahsavar, 2017; Li, 2024; Wang *et al*., 2024 a).

Nanotechnology has emerged as a promising tool in agriculture over the past decade to mitigate abiotic stresses, including salinity. Nanoparticles (<100 nm) offer high surface area, reactivity, and unique properties, influencing plant morphology, physiology, and biochemistry in a concentration, size, type, application method, and species-dependent manners (Etesami *et al.,* 2021; Zulfiqar and Ashraf, 2021). At optimal levels, they enhance stress tolerance by modulating hormonal balance, antioxidant enzyme activity, ionic balance, gene expression, and other defense systems.

Titanium nanoparticles (metal oxide semiconductor nanocrystals) have wide applications in air purification, skin care products, and reduce the effects of environmental stresses in agriculture (Alabdallah *et al*., 2024; Jadhav *et al*., 2025). TiO_2 nanoparticles improve the growth and development of plants due to their chemical stability and non-toxic nature at low concentration (Nafchi *et al*., 2024). Foliar spraying of titanium nanoparticles reduced malondialdehyde content and improved the growth and yield of chamomile (Ezzatmand *et al*., 2024), Echinacea (Behnam *et al*., 2021) and *Solidago canadensis* L. (Nafchi *et al*., 2024) under salt stress.

Manganese is an essential micronutrient in plants, which is involved in the activity of photosynthetic enzymes, carbohydrate and nitrogen metabolism, fatty acid synthesis, and prevention of cell membrane damage (Ye *et al*., 2020). The chemical properties of manganese nanoparticles are changing when exposed to water or NaCl, which results in their affinity for proteins and variations in the N-H bond in several macromolecules (Ye *et al*., 2020; Liu *et al*., 2024 b). In pepper plants, salt stress inhibited root growth and caused changes in the pattern of proteins and lignins. Also, a decrease in the content of manganese, sodium, potassium, and calcium was observed in plants under salt stress. The application of $MnSO_4$ under such conditions improveed the aforementioned traits in several plants and showed how nano-priming with manganese caused compositional changes and molecular interactions between key biomolecules (Ye *et al.,* 2020).

Graphene oxide (GO) is a biocompatible nanomaterial, promotes germinations, root development, photosynthesis, and antioxidant capacity in plants (Guo *et al*., 2021; Zhang *et al*., 2021; Yang *et al*., 2022). Under salt stress (100 mM NaCl), foliar spraying of quantum dot-graphene oxide increased phenolic content, while combined application with titanium nanoparticles enhanced flavonoid accumulation in pepper (Hassan-pouraghdam *et al*., 2025).

Molybdenum is one of the essential microelements needed for plant growth (Haque, 1987). The different forms of molybdenum oxide have led to their widespread applications in various industries, making molybdenum a valuable metal for research and commercial applications (De Castro *et al*., 2017). Molybdenum homeostasis helps the adaptation of plants to the extreme environments and increases plant tolerance to stress conditions (Kaiser *et al*., 2005; Huang *et al*., 2022).

The progressive salinity in Iran's arid and semi-arid regions exacerbates threats to food/feed security and even restricts crop production (Akbary and Sayad, 2021). There is a critical need to explore novel, nano-enabled approaches tailored to salt-sensitive essential oil crops like *P. graveolens*. The present study addresses this gap by evaluating the effects of TiO_2, Mn oxide-graphene oxide, and MoO_3 nanoparticles on biomass and key physiological traits of fragrant geranium under salt stress. These nanoparticles were selected for their reported abilities to enhance antioxidant defenses, maintain ionic balance, regulate ROS, and enhance essential oil content at appropriate concentrations. The findings are expected to provide practical insights for pioneer farmers and extension services in salinity-affected areas, potentially improving sustainable cultivation and economic viability of this high-demanded crop.

2. Materials and Methods

To study the effects of NaCl salinity (0, 75, and 150 mM) and foliar spraying of titanium oxide (2 mg L^{-1}) (Ezzatmand *et al*., 2024), Mn oxide-graphene oxide (Mn_2O_3: graphene oxide 1:1) (10 mg L^{-1}) (El-Beltagi *et al*., 2024), and MoO_3 (0.5 µM) (Mabiya *et al*., 2023) on *Pelargonium graveolens*, a factorial design was conducted based on CRD with three replications at the Research Greenhouse of Azarbaijan Shahid Madani University, Tabriz, Iran. All nanoparticles used in this experiment were supplied by the US-Nano Company (US Research Nano Materials, Texas, USA). The homogeneous (in size) *P. graveolens* seedlings (10-12 cm) were planted in 5-liter pots in soil (one plant per pot). Each pot was filled with approximately 4.8-5.0 kg of air-dried silty loam soil (pH: 7, EC 1.2 ds m^{-1}, total N content: 0.8%, available P content: 10.5 mg kg^{-1} and K content 180 mg Kg^{-1}). Light intensity was about 460 µmol m^{-2} $s^{-1,}$ and the temperature regime was 25°C and 19°C day and night. After transferring the plants to pots, they were irrigated with unsalted water for two weeks. Irrigation was performed manually by applying a fixed volume of 250 mL of the respective solution (unsalted water during the first two weeks, and thereafter the designated saline solution ± foliar treatments) to the soil surface of each pot using a graduated cylinder/measuring cup. When plants reached 6-7 leaflets, salinity treatment was applied [0 (2.1 mS cm^{-1}), 75 (9.0 mS cm^{-1}), and 150 mM (15 mS cm^{-1})] for 9 weeks. Salt stress started from 25 mM and reached the final concentration by adding 25 mM to the salt concentration every three days over 15 days. One week after salinity treatment, plants were sprayed with distilled water (control), titanium oxide, manganese oxide-graphene oxide, and MoO_3. Two weeks later, the plants were resprayed with the same nanoparticles. To avoid salt deposition in the pot surfaces, the pots were regularly washed with tap water once every week. Seven weeks after the second foliar spraying, the plants were harvested for evaluation of physiological, biochemical and morphological traits. The pots were arranged as a factorial experiment based on CRD layout across the greenhouse benches, with spacing of 75 cm between rows and 40 cm within rows. The experiment consisted of 12 treatments with three replications, and each experimental unit had three pots (108 individual pots). Treatments were fully randomized to the positions using random numbers to minimize any potential positional or environmental gradients.

Dry weight (Biomass)

At the end of the experiment, plants were harvested and separated into roots and aerial parts. Samples were oven-dried at 32°C until reaching the constant weight (approximately 72-96 h), and dry biomass of roots and aerial parts was measured using a digital analytical balance (BB141, Boeco, Germany; precision 0.001 g). Other growth parameters such as plant height, leaf length and width, and petioles length were measured prior to harvest using a ruler.

Essential oil extraction

Essential oil was extracted from 20 g of dried aerial parts (primarily leaves, stems and flowers) of *P. graveolens* using hydrodistillation during 4h by a Clevenger-type apparatus. The aerial parts, selected because they are the primary site of essential oil biosynthesis and accumulation in rose-scented geranium, yielding the highest concentrations of key volatile oil components (Al-Mijalli *et al*., 2022).

Minerals analysis

Dry leaf samples (0.4 g) were acid-digested according to the methods described by AOAC (1990). The flame photometric method quantifies sodium and potassium content in the leaf tissue (Corning, 410, England), and the content of Ca and Mg was quantified using atomic absorption spectroscopy (Shimadzu, AA6300, Japan). Nitrogen content was measured by the Kjeldahl method, and phosphorus was determined with the vanadate molybdate method (Honarjoo *et al*., 2013).

Chlorophyll index

Portable chlorophyll meter (SPAD 502 Plus Chlorophyll Meter, Japan) was used to determine chlorophyll index.

Total soluble solids (TSS) content

A hand refractometer (Erma, Tokyo, Japan) quantified TSS in *P. graveolens* leaves. The data were reported as °Brix.

Proline content

The amount of 0.3 g of fresh leaf tissue was homogenized in 5 mL of 3% sulfosalicylic acid. Then centrifuged for 7 min at 6000 rpm. One mL of supernatant was incubated with 1 mL acid ninhydrin

and 1 mL glacial acetic acid. Samples were incubated for 30 seconds at 100°C in a water bath and then in an ice bath for 30 min. The red phase formed above the sample was used for the determination of proline content at 520 nm (Fedina *et al*., 2006).

Lipid peroxidation and hydrogen peroxide (H_2O_2) content

The amount of 0.3 g of *P. graveolens* leaf tissue was homogenized in 0.1% trichloroacetic acid. The extract was centrifuged for 15 min at 12000 × *g*. The reaction mixture of 0.5 mL of the extract and 1.5 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid was incubated at 95°C for 30 min. The samples were then cooled in an ice bath. The absorbance was determined at 520 nm and corrected for nonspecific absorbance at 600 nm. The malondialdehyde content was determined using the extinction coefficient of 155 mM cm^{-1} (Sahu and Sabat, 2011).

The amount of 0.3 g of leaf tissue was rubbed off in liquid N_2. The samples were ground in 0.1% ice-cold trichloroacetic acid and centrifuged for 15 min at 12000 × *g*. 0.5 mL of the supernatant was mixed with 0.5 mL of potassium phosphate buffer (10 mM) at pH 7.5 and 1 mL of potassium iodide (1M). The H_2O_2 content was evaluated using standards of H_2O_2. The absorbance of samples was measured at 390 nm (Alexieva *et al*., 2001).

Catalase activity

0.5 g of *P. graveolens* leaf samples were homogenized with 0.1 M cold potassium phosphate buffer with 0.5 mM EDTA. 0.05 mL of supernatant was added to 1.5 mL of 0.1 mM phosphate buffer with pH 7 and 1.45 mL of double-distilled water. The reaction was started by adding 0.5 mL of 75 mM hydrogen peroxide, and a decrease in absorption was recorded at 240 nm (1 min) (Luhova *et al*., 2003).

Data analysis

A two way analysis of variance (ANOVA) (Y = μ + Si + Nj + S*N{ij} + ε) was performed by MSTATC ver 2.1 software, and the significant differences among the means were evaluated with the Least Significant Difference (LSD) test at p≤0.05.

3. Results

Growth traits

Root and aerial parts dry weight was influenced by the interaction effects of salinity + foliar spray. The top amounts of root and aerial parts dry weight were recorded in $NaCl_0$ × foliar spray + all three nanoparticles and $NaCl_{75\ mM}$ + foliar spray with Mn oxide-graphene oxide, and MoO_3 (Table 1). Independent effects of salinity and foliar spray

Table 1 - Mean comparison for the interaction effects of varying concentrations of sodium chloride stress and foliar spray (spray with distilled water, Mn oxide-graphene oxide, MoO_3 and TiO_2 nanoparticles) on biomass, essential oil content, and chlorophyll index of *P. graveolens*

NaCl (mM)	Nanoparticle foliar spray	Aerial part dry weight (g pot^{-1})	Root dry weight (g pot^{-1})	Chlorophyll index (Spad)	Essential oil content (%)
0	Without spray	14 e	4.6 b	29 b	0.11 f
0	Mn oxide-graphene oxide (10 mg L^{-1})	26 a	5.3 a	37 a	0.15 e
0	MoO_3 (0.5 μM)	24 a	5.4 a	39 a	0.21 a
0	Titanium oxide (2 mg L^{-1})	21 ab	5.3 a	36 a	0.17 d
75	Without spray	13.5 f	4.4 b	21 d	0.19 c
75	Mn oxide-graphene oxide (10 mg L^{-1})	24.7 a	5.1 a	35 a	0.25 a
75	MoO_3 (0.5 μM)	23.1 a	5.3 a	36 a	0.24 a
75	Titanium oxide (2 mg L^{-1})	19.6 b	4.8 b	31 ab	0.22 a
150	Without spray	11.1 f	2.9 d	19 e	0.18 c
150	Mn oxide-graphene oxide (10 mg L^{-1})	16.9 c	3.6 c	23 d	0.20 b
150	MoO_3 (0.5 μM)	17.4 c	3.4 c	26 c	0.21 a
150	Titanium oxide (2 mg L^{-1})	15.3 d	3.7 c	24 c	0.19 c
Source of variance					
Salinity (S)		*	**	**	**
Foliar (F)		**	**	**	**
S × F		**	**	*	*
CV (%)		6.4	8.5	4.3	5.2

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05). NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

influenced plant height, leaf length, leaf width, and petiole length of the plant. The highest values for plant plant height (41.0 g and 39.9 g), leaf width (4.9 cm and 4.7 cm), and leaf length (5.2 cm and 5.1 cm) were recorded in the non-saline (control) and the 75 mM NaCl treatment, respectively. By increasing the salt stress to 150 mM, petiole length of the plants decreased by 13% compared to the control. The highest petiole length was observed in no-saline conditions (Table 2). Foliar spray with Mn oxide-graphene oxide and MoO_3 NP significantly improved plant height, leaf length, leaf width, and petiole length (Table 3).

Chlorophyll index

The interaction effects of salinity and foliar treatments affected chlorophyll index. Chlorophyll index was increased for all foliar sprayings × $NaCl_{0 \text{ and } 75 \text{ mM}}$. The lowest data of chlorophyll index was observed in $NaCl_{150 \text{ mM}}$ × without foliar treatment (Table 1).

Essential oil content

The interaction effects of salt stress and foliar treatments affected the essential oil content of plants. Based on the results, the highest essential oil content was observed under no salinity × MoO_3, $NaCl_{75 \text{ mM}}$ × all three nanoparticle foliar spray, and $NaCl_{150 \text{ mM}}$ × MoO_3 foliar spray (Table 1).

Malondialdehyde content

Malondialdehyde content was influenced by the interaction effects of experimental treatments (Table 4). The highest malondialdehyde content was observed in $NaCl_{75 \text{ and } 150 \text{ mM}}$ × without foliar spray, which indicated an increase of 42% and 58% compared to the control, respectively. In all salinity levels, foliar spray with nanoparticles positively declined the malondialdehyde content of plants (Fig. 1).

Hydrogen peroxide content

H_2O_2 content was influenced by the independent effects of salt stress and foliar treatments (Table 4). The highest H_2O_2 content was observed in 75 (1.95 µmol g^{-1} FW) and 150 mM (2.25 µmol g^{-1} FW) salt stress. The lowest content of H_2O_2 was recorded under no salinity conditions (Fig. 2A). All three foliar

Table 2 - Mean comparison for the effects of salt stress on growth characteristics of *P. graveolens*

Salinity (mM)	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)
0	41 a	5.2 a	4.9 a	6.1 a
75	39.9 ab	5.1 a	4.7 a	5.5 b
150	26.4 b	4.2 b	3.8 b	5.3 b
Source of variance				
Salinity (S)	*	*	**	*
Foliar (F)	**	*	*	*
S × F	NS	NS	NS	NS
C.V. (%)	10	12.1	8.9	10.4

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05). NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

Table 3 - Mean comparison for the effects of foliar spray (spray with distilled water, Mn oxide-graphene oxide, MoO_3 and TiO_2 nanoparticles) on growth traits of *P. graveolens*

Nanoparticle foliar spray	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)
Without spray	34 c	4.7 b	3.8 c	5.2 b
Mn oxide-graphene oxide (10 mg L^{-1})	39 a	5.4 a	4.3 a	5.9 b
MoO_3 (0.5 µM)	37 ab	5.6 a	4.7 a	5.6 ab
Titanium oxide (2 mg L^{-1})	35.5 b	4.8 b	4.0 b	5.1 b

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05).

Table 4 - ANOVA for the effect of salinity and foliar spraying (no foliar spray, Mn oxide-graphene oxide, MoO_3, titanium oxide NPs) on malondialdehyde, H_2O_2 content, and catalase activity of *P. graveolens*

Source of variance	df	Malondialdehyde content	H_2O_2 content	Catalase activity
Salinity (S)	2	**	**	**
Foliar (F)	3	*	**	NS
S×F	6	*	NS	NS
CV (%)		8.7	10.1	9.1

NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

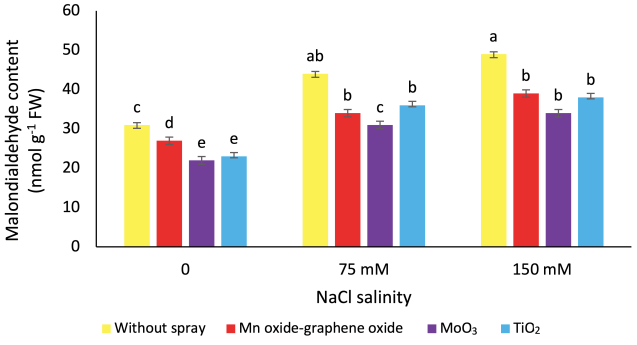


Fig. 1 - Mean comparison for the interaction effects of varying concentrations of sodium chloride stress and foliar spray on malondialdehyde content of *P. graveolens*. Means with different letters on bars are statistically different according to the LSD test (p≤0.05).

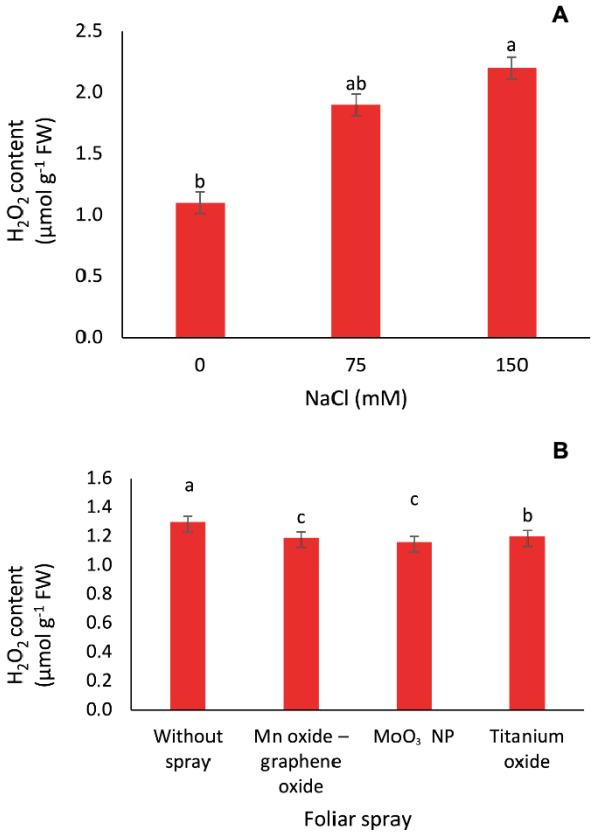


Fig. 2 - Means comparisons for the effect of NaCl salinity (A) and foliar spray (B) on H_2O_2 content of P. graveolens. Means with different letters on bars are statistically different according to the LSD test (p ≤ 0.05).

treatments reduced H_2O_2 content of the plant, but the lowest H_2O_2 content was recorded in Mn oxide-graphene oxide and MoO_3 foliar spraying (Fig. 2B).

Proline and soluble solids content

The variance analysis showed that the total soluble solids and proline content were affected by the interaction effects of experimental treatments. The top content of proline and total soluble solids was recorded under $NaCl_{75 \text{ and } 150 \text{ mM}}$ × all three nanoparticles foliar spray (Table 5).

Catalase activity

The highest catalase activity was recorded under 75 (40.2 units mg^{-1} protein) and 150 mM (42 units mg^{-1} protein) salinity conditions, which showed a 21% and 26% enhancement compared to control (Fig. 3).

Nitrogen, phosphorus, and potassium content

The interaction effects of salinity and foliar treatments affected the nitrogen and phosphorus content of the plants. The highest phosphorus and nitrogen content was recorded in $NaCl_0$ × foliar spray with all three nanoparticles and $NaCl_{75 \text{ mM}}$ × foliar spray with Mn oxide-graphene oxide, and MoO_3 nanoparticles (Table 6). The potassium content was responsive to the independent effect of salinity and foliar spray (Table 7). Potassium content significantly decreased with enhancing salinity to 75 and 150 mM

Table 5 - Mean comparison for the interaction effects of varying concentrations of sodium chloride stress and foliar spray (spray with distilled water, Mn oxide-graphene oxide, MoO_3 and TiO_2 nanoparticles) on total soluble solids, and proline content of *P. graveolens*

NaCl (mM)	Nanoparticle foliar spray	Total soluble solids content	Proline content (µg g-1 FW)
0	Without spray	1.3 d	29 e
0	Mn oxide-graphene oxide (10 mg L^{-1})	1.5 d	31.2 d
0	MoO_3 (0.5 µM)	2.1 bc	33 d
0	Titanium oxide (2 mg L^{-1})	1.7 c	32.8 d
75	Without spray	1.9 c	39 b
75	Mn oxide-graphene oxide (10 mg L^{-1})	2.5 ab	45 a
75	MoO_3 (0.5 µM)	2.6 a	46.2 a
75	Titanium oxide (2 mg L^{-1})	2.5 ab	41.3 ab
150	Without spray	2.2 b	39 c
150	Mn oxide-graphene oxide (10 mg L^{-1})	2.6 a	41.2 ab
150	MoO_3 (0.5 µM)	2.7 a	43.8 ab
150	Titanium oxide (2 mg L^{-1})	2.5 ab	43.2 ab
Source of variance			
Salinity (S)		**	NS
Foliar (F)		NS	**
S×F		*	*
CV (%)		11	7.9

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05). NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

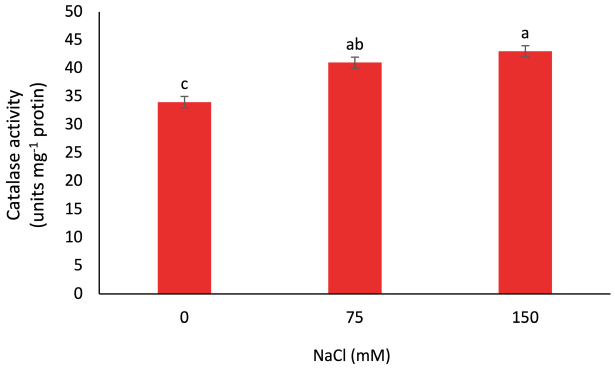


Fig. 3 - Means comparisons for the effect of NaCl salinity on the catalase activity of *P. graveolens*. Means with different letters on bars are statistically different according to the LSD test (p≤0.05).

compared to the control (Fig. 4A). Foliar spray with Mn oxide-graphene oxide, and MoO_3 nanoparticles enhanced potassium content compared to control and titanium oxide foliar spray (Fig. 4B).

Calcium and magnesium content

The magnesium and calcium content was affected by the interaction of salinity and foliar spray (Table 6). Based on the results, the highest content of calcium and magnesium was observed in $NaCl_0$ × all three nanoparticle foliar sprays and $NaCl_{75\ mM}$ × Mn oxide-graphene oxide, MoO_3 nanoparticles treatment. With a salinity of 150 mM × without the foliar spray, the magnesium and calcium content decreased by 40% and 33% compared to the control. The results showed that $NaCl_{150\ mM}$ × foliar spray with all nanoparticles had positive effects on calcium and magnesium content compared to $NaCl_{150\ mM}$ × without foliar spray (Table 6).

Sodium content and sodium-to-potassium ratio

Sodium content was influenced by the interaction effects of salinity and foliar treatments (Table 6). The sodium-to-potassium ratio was responsive to the independent effects of salinity and foliar treatment (Table 7). Salt stress led to a significant increase in the Na^+/K^+ ratio, with the peak value at 150 mM NaCl, corresponding to 49% and 16% increase relative to the control and 75 mM NaCl, respectively (Fig. 4C). The least sodium-to-potassium content was recorded under the foliar spray with Mn oxide-graphene oxide, MoO_3 nanoparticles treatment which shows the positive effects of foliar spray on reducing the Na/K ratio (Fig 4D). Foliar treatment with nanoparticles reduced Na content in 75 and 150

Table 6 - Mean comparison for the interaction effects of varying concentrations of sodium chloride stress and foliar spray (spray with distilled water, Mn oxide-graphene oxide, MoO_3 and TiO_2 nanoparticles) on the elemental content of *P. graveolens*

NaCl (mM)	Foliar spray	Nitrogen content (g kg^{-1} DW)	Phosphorous content (g kg^{-1} DW)	Sodium content (g kg^{-1} DW)	Magnesium content (g kg^{-1} DW)	Ca content (g Kg^{-1} DW)
0	Without spray	8.1 e	2.2 d	1.9 e	1.0 b	2.7 b
0	Mn oxide-graphene oxide (10 mg L^{-1})	13.9 ab	3.4 a	1.5 f	1.5 a	3.2 a
0	MoO_3 (0.5 µM)	15.1 a	3.6 a	1.2 g	1.4 a	3.6 a
0	Titanium oxide (2 mg L^{-1})	12.8 ab	3.5 a	1.1 g	1.4 a	3.3 a
75	Without spray	7.2 f	2.4 c	8.1 b	0.9 c	2.1 bc
75	Mn oxide-graphene oxide (10 mg L^{-1})	12.9 ab	3.1 ab	5.3 d	1.4 a	2.9 ab
75	MoO_3 (0.5 µM)	12.6 ab	3.2 ab	5.1 d	1.2 ab	3.1 a
75	Titanium oxide (2 mg L^{-1})	11.2 b	2.9 b	5.6 d	1.1 b	2.8 b
150	Without spray	4.9 g	1.9 e	10.2 a	0.6 e	1.8 c
150	Mn oxide-graphene oxide (10 mg L^{-1})	8.9 e	2.3 c	8.6 b	0.7 d	2.2 bc
150	MoO_3 (0.5 µM)	10.3 c	2.6 b	7.1 c	0.9 c	2.5 b
150	Titanium oxide (2 mg L^{-1})	9.8 d	2.1 d	7.4 c	0.6 e	2.4 b
Source of variance						
Salinity (S)		**	**	*	**	*
Foliar (F)		*	*	*	**	*
S × F		**	**	*	*	*
CV (%)		7.9	4.9	5.8	7.7	9.7

Means with different letters within the columns are statistically different according to the LSD test (p≤0.05). NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

Table 7 - ANOVA for the effect of varying concentration of sodium chloride and foliar spraying (no foliar spray, Mn oxide-graphene oxide, MoO_3, titanium oxide NPs) on the potassium content and sodium/ potassium ratio of *P. graveolens*

Source of variance	df	Potassium content	Sodium/ potassium ratio
Salinity (S)	2	**	**
Foliar (F)	3	*	*
S×F	6	NS	NS
CV (%)		12	10.1

NS, non-significant, * significant difference at P≤5%, and ** significant difference at P≤1%. CV= Coefficient of variation.

mM salt stress. The highest Na content was recorded in NaCl $_{150\ mM}$ × without foliar spray, which showed 81% increase compared to the control (Table 6).

4. Discussion and Conclusions

Salt stress is a major abiotic factor that disrupts plant nutrition and physiology, leading to toxic ions accumulation (e.g., Na^+ and Cl^-), reactive oxygen species over-production, reduced photosynthesis, membrane peroxidation and osmolytes accumulation, as well as alterations in cell structures (Zeeshan *et al.*, 2020). In this study, the highest root and aerial parts dry weight were observed under no-saline conditions (NaCl 0 mM) + foliar spraying of all three nanoparticles, and under moderate salinity (NaCl 75 mM) + Mn oxide-graphene oxide and MoO_3 nanoparticles foliar use (Table 1). These findings indicate that foliar-applied nanoparticles, particularly Mn oxide-graphene oxide and MoO_3, effectively mitigated the adverse effects of moderate salinity on biomass accumulation, likely by enhancing nutrient availability, improving chlorophyll index, and reducing oxidative damage. High Na^+ accumulation disrupts K^+ uptake, leading to reduced cell division and expansion (Zeeshan *et al*., 2020). In the present study, 150 mM NaCl severely reduced biomass and growth traits (plant height, leaf dimensions, petiole length), consistent with Na^+ toxicity and altered Na^+/K^+ ratios, which impair metabolic processes.

Nanofertilizers are in common use to reduce the effects of environmental stresses on plants (Abou Seeda *et al.*, 2020). In our study, foliar spraying with

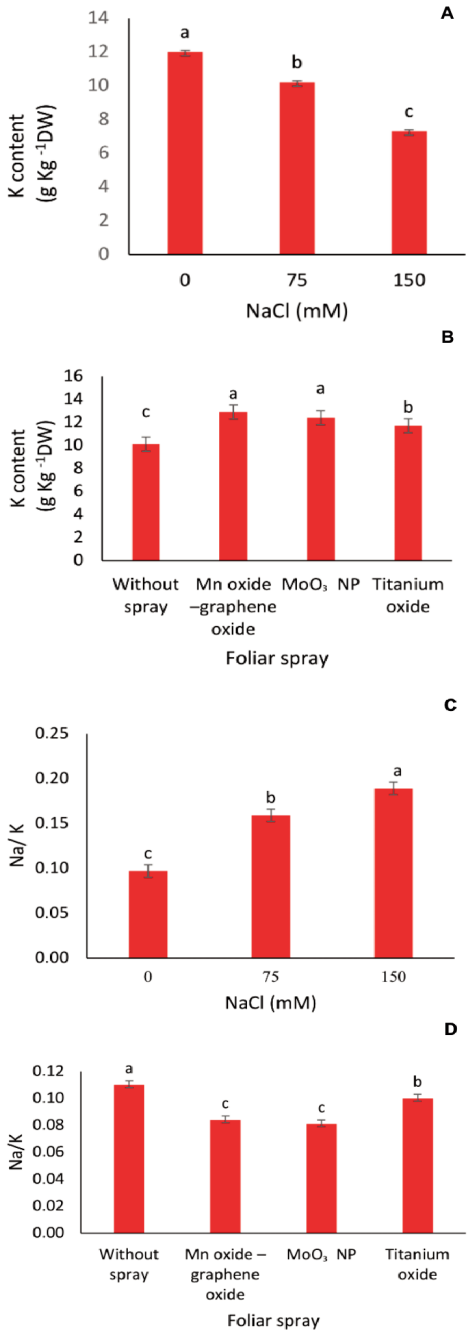


Fig. 4 - Means comparisons for the effect of NaCl salinity (A and C) and foliar spray (B and D) on K content and Na/K ratio of *P. graveolens*. Different letters on bars are statistically different according to the LSD test (p≤0.05).

Mn oxide-graphene oxide and MoO_3 nanoparticles significantly improved these growth parameters particularly under 0 and 75 mM NaCl (Table 4), suggesting that these treatments counteract salinity-induced inhibition of cell division and expansion, possibly through enhanced ionic homeostasis, elevated enzymatic activities, and promoted carbohydrate metabolism. Moreover, this improvement may stem from Mn's role in carbohydrate metabolism, phenolics and lignin biosynthesis, cell wall strengthening, and activation of enzymes in the shikimic acid and phenylpropanoid pathways (Yano and Yachandra, 2014; Eskandari and Sharifnabi, 2020). Graphene oxide likely contributes by promoting cell division, stomatal conductance, photosynthesis, and nutrient uptake (Zhang *et al*., 2021), as observed in related studies where graphene oxide mitigated salinity by enhancing photosynthetic efficiency and ion homeostasis in pepper plants (Hassanpouraghdam *et al*., 2025). Treatment of wheatgrass (Babenko *et al*., 2015) and Chinese cabbage (Zhang *et al*., 2014) with molybdenum, and foliar spraying of mungbean with manganese (Shahi and Srivastava, 2016) improved plant yield and growth traits by reducing the effects of free radicals and increasing the activity of antioxidant enzymes under salt stress. MoO_3 nanoparticles positively influenced growth, potentially via improved nitrogen metabolism and nitrate assimilation, which support overall plant vigor under moderate stress (Kaiser *et al*., 2005). The positive effect of nanoparticles on plant growth and physiological traits seems to be related to the polycationic nature of nanoparticles or the regulation of ionic metabolism in the plant, which enhance plant tolerance to stress conditions (Hnilickova *et al*., 2021). The results of the present study also confirmed the above studies and indicated the effectiveness of the nanoparticles used in controlling salt stress.

Chlorophyll index was affected by the interaction of salinity × foliar spray (Table 1). The lowest chlorophyll index was recorded under 150 mM NaCl without foliar spray, while applications of all nanoparticles increased the pigments content under 0 and 75 mM NaCl. This suggests that nanoparticles protect photosynthetic pigments from salinity-induced degradation, possibly by reducing ROS-mediated damage to the oxygen-evolving complexes in PSII and via maintaining membrane stability (Lima Neto *et al.,* 2014; Zhang *et al*., 2019; Nafchi *et al*., 2024). Mn plays a crucial role in chlorophyll biosynthesis and electron transfer in photosynthesis (Eskandari and Sharifnabi, 2020), while graphene oxide enhances light energy conversion and PSII performance (Hassanpouraghdam *et al*., 2022; Haydar *et al*., 2023). These mechanisms likely explain

the sustained chlorophyll index up to 75 mM NaCl, supporting better carbon assimilation and biomass production under moderate stress environments. Under salt stress, foliar spraying of titanium to eggplant (Khalid *et al*., 2024) and bean (Abdel Latef *et al.,* 2018), declined stress effects by reducing hydrogen peroxide content, improving photosynthesis, and increasing biomass. Foliar spraying of molybdenum under salt stress improved the chlorophyll content of sorghum (Mabiya *et al*., 2023) and Chinese cabbage (Zhang *et al*., 2014). At higher salinity (150 mM) levels, the protective effects on growth and chlorophyll index were less pronounced, indicating a threshold beyond which nanoparticle mitigation is limited, though they still conferred some tolerance.

Overall, foliar spraying with Mn oxide-graphene oxide and MoO_3 nanoparticles effectively ameliorated salt stress up to 75 mM NaCl by improving growth traits, biomass, and chlorophyll index. These benefits align with nanofertilizers' role in enhancing stress tolerance through improved nutrient delivery, antioxidant support, and photosynthetic protection, offering a promising strategy for crop production in saline environments (Eskandari and Sharifnabi, 2020; Mabiya *et al*., 2023). Further studies with focusing on molecular mechanisms, such as gene expression and ionomics, could refine detailed application protocols.

Salt stress induces profound ultrastructural changes in plants, including thickening of cuticles and epidermis, as well as damage or disintegration of nuclei, chloroplasts, and mitochondria (Zeeshan *et al.*, 2020), which collectively impair primary metabolism and redirect resources toward secondary metabolites biosynthesis. In the present study, these stress-induced alterations likely contributed to variations in essential oil content, where moderate salinity (75 mM NaCl) combined with nanoparticle foliar sprays, and severe salinity (150 mM NaCl) + MoO_3, yielded the highest essential oil content compared to control (Table 1). This aligns with the established role of abiotic stress in modulating secondary metabolism, as evidenced by increased β-citronellol (24%) and citronellyl acetate (18.5%) in geranium under 50 mM NaCl + selenium foliar spraying (Vojodi Mehrabani *et al*., 2022). The observed reductions in essential oil biosynthesis under unstressed or extreme stress conditions without adequate nanoparticle support may stem from diminished cytokinin biosynthesis in roots and its translocation to shoots (Charles *et al*., 1990), alongside ionic imbalances and disrupted enzymatic activities that curtail terpenoid metabolism (Deshmukh and Khare, 2017).

The intensity and duration of salt stress, coupled with plant species-specific responses, further dictate essential oil accumulation (Deshmukh and Khare, 2017), as seen in our results where nanoparticles use mitigated these effects. Specifically, the hydrophilic groups (-COOH and -OH) on nanoparticle surfaces, such as those in quantum dots, enhance water uptake and alleviate osmotic stress (Tounekti *et al*., 2008), potentially explaining the elevated essential oil content under 75 mM NaCl with all nanoparticles and under 150 mM NaCl with MoO_3 (Table 1). This is corroborated by studies on basil, where glycine betaine-functionalized graphene oxide under salinity increased essential oil, photosynthetic pigments, proline, and phenols while reducing malondialdehyde and hydrogen peroxide (Ganjavi *et al.*, 2021). MoO_3 nanoparticles likely exert their beneficial effects through activation of key molybdenum-dependent enzymes, including nitrate reductase, aldehyde oxidase, xanthine dehydrogenase, and sulfite oxidase, which bolster metabolic and physiological resilience (Kaiser *et al*., 2005). Overall, the synergy between salt stress and nanoparticle foliar spraying in this study particularly MoO_3 under non-saline and high-salinity conditions, and all nanoparticles at moderate salinity, appears to promote essential oil accumulation by improving nutrient uptake, lowering oxidative damage (reduced malondialdehyde content), and enhancing chlorophyll index (Table 1).

Plants have evolved multifaceted adaptive mechanisms to counteract salt stress, encompassing osmotic adjustment, reactive oxygen species (ROS) scavenging, photosynthetic modulation, phytohormone signaling, and ion homeostasis regulation (Liu *et al.*, 2020; Wang *et al*., 2024 b). Salinity elevates cellular osmotic pressure through excessive Na^+ and Cl^- uptake and promotes osmotic regulation via biosynthesis of compatible solutes to maintain cytosolic homeostasis (Munns, 2002). Both enzymatic and non-enzymatic antioxidants mitigate ROS-induced damage under such conditions. Stress signaling activates the related gene expression for antioxidant enzymes biosynthesis (Ye *et al*., 2020), while non-enzymatic antioxidants, including soluble amino acids (proline) and sugars, scavenge hydroxyl

and peroxyl radicals, stabilize membranes, and reduce redox imbalances (Deshmukh and Khare, 2017). Proline, in particular, scavenges free radicals, lowers redox potential, preserves cellular water status, and stabilizes macromolecules. Otherwise, its rapid catabolism under stress promotes ATP production and mitochondrial repair (Lehmann *et al*., 2010; Parihar *et al*., 2015). In the present study, salt stress markedly upregulated catalase activity at both 75 mM and 150 mM NaCl (Fig. 3), consistent with the role of CAT as a pivotal enzyme in decomposing hydrogen peroxide (H_2O_2) into water and molecular oxygen, thereby mitigating oxidative damage to cellular components. This response aligns with observations in various species where moderate to high salinity induces CAT activity as part of the enzymatic antioxidant defense (e.g., in *Brassica oleracea* with Mn foliar spraying under 150-300 mM NaCl) (Altintaş *et al*., 2024). The observed improvements (21-26%) likely reflect an adaptive acclimation to elevated H_2O_2 generation from salinity-induced ionic and osmotic perturbations.

Foliar nanoparticle applications, including MoO_3, further potentiated this antioxidant response, as evidenced by reduced H_2O_2 and malondialdehyde contents in treated plants. MoO_3 nanoparticles likely contribute via activation of molybdenum-dependent enzymes (e.g., nitrate reductase, aldehyde oxidase) that support metabolic resilience and indirectly bolster antioxidant systems (Kaiser *et al*., 2005). Molybdenum enhances antioxidant defense under abiotic stresses, as seen in sorghum where Mo application improved proline content and salt tolerance (Mabiya *et al*., 2023). Analogous effects occur with other nanoparticles; for instance, graphene oxide spraying elevated CAT activity in basil under salt stress (Vojodi Mehrabani *et al.,* 2023).

The synergy between salt stress and nanoparticle treatments in this experiment empowered enzymatic (including CAT) and non-enzymatic antioxidant biosynthesis, leading to decreased H_2O_2 and MDA content. This is congruent with broader evidence that nanoparticles act as stress ameliorants by scavenging ROS directly or modulating gene expression and enzyme activities, thereby enhancing physiological tolerance in medicinal and crop plants.

Plant tolerance to salt stress is largely determined by the ability to maintain ion homeostasis, particularly by restricting toxic Na^+ accumulation while preserving essential nutrient uptake such as N, P, K, Ca, and Mg (Karimi and Hasanpour, 2014; Kumari *et al*., 2022). In the present study, salinity levels of 75 and 150 mM NaCl, significantly disrupted nutrient uptake in the absence of foliar treatments, leading to reduced N, P, K, Ca, and Mg content, elevated Na^+ content, and a marked increase in Na^+/K^+ ratio (Tables 6, Fig. 4). These changes align with well-documented salinity-induced ionic imbalances, where excess Na^+ competes with K^+ and Ca^{2+} uptake, disrupts membrane stability, and impairs metabolic processes (Liu *et al*., 2024 b; Wang *et al.,* 2024 a).

Foliar spraying of nanoparticles (NPs) particularly Mn oxide-graphene oxide and MoO_3 effectively mitigated these adverse effects, with the most pronounced benefits observed at 75 mM NaCl and, to a lesser extent, at 150 mM NaCl. The interaction between salinity and foliar treatments significantly influenced N, P, Ca, and Mg content (Table 6), while K content responded to both main effects (Table 7). The highest N and P levels were recorded under control (0 mM NaCl) with all three NPs and under 75 mM NaCl + Mn oxide-graphene oxide or MoO_3 (Table 6). Similarly, Ca and Mg contents were maximized in non-stressed conditions + NPs and at 75 mM NaCl + Mn oxide-graphene oxide and MoO_3 (Table 6). These improvements suggest that foliar NPs enhance nutrient acquisition and translocation under moderate salinity, possibly by improving root uptake efficiency, membrane integrity, or promoted translocation pathways.

Potassium content decreased progressively with enhancing salinity (Fig. 4A), consistent with sodium-induced K^+ efflux and competition at uptake sites (Guo *et al*., 2015). However, foliar sprays with Mn oxide-graphene oxide and MoO_3 significantly elevated K content compared to the unsprayed control and TiO_2 treatment (Fig. 4B). This selective enhancement likely contributed to the reduced Na^+/K^+ ratio observed under these treatments (Fig. 4D), as the lowest ratios occurred with Mn oxide-graphene oxide and MoO_3 applications. The Na^+ content itself was lowered by all NPs at 75 and 150 mM NaCl. In contrast, the highest Na^+ accumulation was recorded in unsprayed plants exposed to 150 mM NaCl (81% increase compared to control) (Table 6). These findings indicate that NPs promote ion exclusion or compartmentalization, helping maintain cytosolic K^+ homeostasis essential for enzyme activation, osmotic adjustment, and stomatal

regulation under stress (Cavusoglu *et al.*, 2018; Liu *et al*., 2024 a).

The superior performance of Mn oxide-graphene oxide and MoO_3 NPs, especially at 75 mM NaCl, over TiO_2 may stem from their specific elemental roles. Mn is critical for nitrogen metabolism and antioxidant enzyme activity, potentially aiding nitrogen uptake and reducing oxidative disruption to nutrient transporters (Pradhan *et al.,* 2014; Ye *et al.*, 2019). In contrast, graphene oxide likely improves water retention and nutrient delivery via its high surface area and hydration properties, facilitating better ionic balance under stress (Lian *et al*., 2018; Vojodi Mehrabani *et al*., 2023). MoO_3 NPs, provide molybdenum, support nitrate reduction and N assimilation, which in turn promote proline synthesis and further enhance K^+, Ca^{2+}, and P uptake (El-sayed *et al.,* 2017; Divyanshu *et al.*, 2019). At higher salinity (150 mM), NPs still conferred benefits over unsprayed plants (e.g., higher Ca and Mg), though less effectively, suggesting a threshold where severe ionic toxicity limits mitigation.

Calcium, a vital element for membrane stability and stress signaling (Chen *et al*., 2015), increased under NP treatments up to 75 mM NaCl, potentially explaining improved tolerance by preserving membrane function and reducing Na^+ influx. Overall, foliar NPs appear to counteract salinity by enhancing essential nutrient status, reducing Na^+ accumulation, and optimizing Na^+/K^+ ratios, with Mn oxide-graphene oxide and MoO_3 showing particular promise for moderate stress levels.

These results explain previous findings on NP-mediated salinity alleviation by demonstrating differential efficacy among NPs and salinity × treatment interactions specific to nutrient dynamics. Future studies could explore underlying molecular mechanisms, such as expression of ion transporters (e.g., HKT, NHX, SOS1) or antioxidant genes, to confirm direct causation (Tester and Davenport, 2003; Yin *et al*., 2019; Wang *et al*., 2024 a). Limitations include the lack of mechanistic data beyond ion contents; however, the observed patterns strongly support a role for these NPs in improving ion homeostasis and nutrient efficiency under salinity.

Manganese is an essential micronutrient for plants that plays an important role in the growth, phytochemical, and metabolic activities of the plant. The use of manganese nanoparticles improves nitrogen metabolism in plants by increasing the activity of enzymes involved in nitrogen metabolism (Pradhan *et al*., 2014). The application of manganese under salt stress reduces the content of free radicals by decreasing the sodium ions availability in the cell, regulating osmotic pressure, and improving water status of the plant (Ye *et al*., 2019). Foliar spraying of manganese under salt stress conditions reduced the root sodium content, sodium to potassium and sodium to calcium ratios in *Brassica oleracea* L., which was due to the downstream regulation of the mTERFs gene (Altintaş *et al.*, 2024). Foliar spraying of graphene oxide elevated the calcium and magnesium content in basil (Vojodi Mehrabani *et al*., 2023). One of the reasons for the increased nutrient uptake under graphene oxide application treatment may be related to the ability of graphene oxide nanoparticles to absorb and release water (Lian *et al*., 2018), which maintains water relations under stress conditions and creates ionic triazones in plants (Zarifi *et al*., 2025). Considering the above and the role of the elements used in foliar spraying in plant metabolic and enzymatic activities, it can be concluded that in the present study, nanoparticles, by improving the physiological activities of several elements, have helped the plant to absorb other nutrients under stress conditions.

In this study, *P. graveolens* plants exhibited moderate tolerance to salt stress by maintaining acceptable growth, physiological performance, and essential oil production up to 75 mM NaCl. At this salinity level, key growth traits including root and shoot dry weights, plant height, leaf length and width, petiole length, and chlorophyll index remained comparable to the non-saline control or showed only minor reductions. Essential oil content was preserved or slightly increased, particularly under nanoparticle foliar treatments. Nutrient homeostasis (N, P, K, Ca, and Mg) was effectively maintained, with the highest concentrations recorded under no-saline conditions and 75 mM NaCl when supplemented with nanoparticles. By contrast, severe salinity (150 mM NaCl) caused substantial negative effects, including marked reductions in root and shoot dry weight, petiole length, chlorophyll index, and nutrient uptake. These impairments were accompanied by severe ionic imbalance, as evidenced by elevated Na^+ content and a substantially higher Na^+/K^+ ratio. Oxidative damage was intensified by the fact that malondialdehyde content increased by 42% and 58%

at 75 and 150 mM NaCl without foliar spraying, respectively. Foliar spraying of Mn oxide-graphene oxide and MoO_3 effectively alleviated salinity-induced damage across all stress levels. These treatments improved physiological and biochemical traits, elemental content and Na^+/K^+ ratio. Notably, essential oil content was maintained or even increased under 150 mM NaCl, especially with MoO_3. Overall, *P. graveolens* can be cultivated under moderate salinity (≤75 mM NaCl) with minimal yield penalty, while foliar spraying of Mn oxide-graphene oxide and MoO_3 nanoparticles offers an effective and sustainable strategy to mitigate severe salt stress via enhancing physiological resilience, improving nutrient efficiency, and maintaining or even increasing essential oil yield in this high-valued medicinal crop.

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Foliar applications of ferulic acid and salicylic acid improve growth, bioactive compounds, and fruit quality in tomato

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Abstract: Ferulic acid (FA) and salicylic acid (SA) are phenolic compounds with recognized potential as metabolic elicitors in plants. This study evaluated the effects of exogenous application of FA and SA (0.5 mmol L^{-1}), applied individually or in combination, on the morphophysiological and biochemical responses of tomato cv. Santa Clara. The experiment was conducted in a greenhouse using plants cultivated in pots. Morphophysiological evaluations included growth and yield parameters, while biochemical analyses comprised photosynthetic pigments, soluble sugars, and phenolic compounds in the fruits. FA increased chlorophyll content and soluble sugars, whereas SA enhanced the accumulation of polyphenols and flavonoids. The combined application of FA+SA led to greater increases in leaf area, plant height, fruit number, fruit diameter, and biomass production, particularly root dry mass. In addition, the combined treatment improved the accumulation of bioactive compounds associated with fruit nutritional quality. Overall, the results indicate that FA and SA, particularly when applied together, represent a promising strategy to enhance tomato growth, productivity, and fruit quality under protected cultivation conditions.

1. Introduction

Tomato, belonging to the Solanaceae family, is one of the most widely cultivated and consumed crops worldwide. China is among the largest producers, while Brazil ranks among the top ten (CONAB, 2025). Beyond its economic importance, tomatoes stand out for their nutritional value, being rich in vitamins (A and ascorbic acid), phenolic acids (*p*-coumaric, ferulic acid, and caffeic acid), flavonoids (rutin, quercetin, naringenin, and kaempferol), and carotenoids (lycopene, α-carotene, and β-carotene) (Chaudhary *et al.,* 2018). Additionally, they provide essential minerals, sugars, amino acids, and fiber, underscoring their nutritional value (Shah *et al.,* 2021).

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Despite their nutritional and economic importance, tomato crops face several challenges, particularly biotic and abiotic stresses. Climate variability, including rising temperatures, water scarcity, and extreme rainfall, can adversely affect yield and fruit quality (Ahmad *et al.,* 2023). These factors directly affect essential physiological processes, such as plant photosynthesis and cellular respiration, thereby compromising plant development and fruit quality (Bacelar *et al.,* 2024). Additionally, these climate changes can increase the incidence of fungal and bacterial diseases, exacerbating productivity losses (Singh *et al.,* 2023).

To face these challenges, new agricultural strategies have been explored. The use of elicitors has gained prominence as a sustainable tool in agriculture, enabling increased yield and improved crop quality (Gorni *et al.,* 2023; Silveira *et al.,* 2025). Compounds such as ferulic acid (FA) and salicylic acid (SA), recognized as elicitors, have been widely studied for their roles in modulating plant physiological and biochemical responses, enhancing tolerance to environmental stresses, and improving agronomic traits (Gorni *et al.,* 2021 a; Linić *et al.,* 2021).

Regarding elicitors that act on secondary metabolism, it is well established that SA is a phenolic compound abundantly present in plants and is involved in various physiological processes of the plant, functioning both as a growth regulator and as an abiotic elicitor, capable of enhancing the synthesis of secondary compounds of commercial interest (Gorni and Pacheco, 2016; Gorni *et al.,* 2020). SA acts to promote plant tolerance to high and low temperatures (Jalili *et al.,* 2023), in the induction of flowering (El-Hady *et al.,* 2021), in the stimulation of root development, in the closure of stomata, and in the reduction of transpiration (Saleem and Fariduddin, 2024) and in the regulation of the synthesis of chlorophylls, carotenoids, photosynthesis, transpiration, and stomatal conductance (Ramos Melo *et al.,* 2023; Saleem and Fariduddin, 2024). SA increases the activation of enzymes involved in the biosynthesis of certain classes of secondary metabolites (Ali, 2021; Gorni *et al*., 2021 b).

FA is a phenolic acid commonly found in plant cell walls, providing structural support and protection against environmental stresses (Khan *et al.,* 2024) and exerts different effects on plant morphological, physiological, and biochemical processes (He and Ding, 2020; Fiorito *et al.,* 2024), including in promoting plant growth and development (Fiorito *et al.,* 2024), increasing photosynthetic efficiency (Linić *et al.,* 2021), and in addition to being associated with defense processes (Khan *et al.,* 2024). In addition, FA is a molecule that interacts with different phytohormones such as jasmonic acid (JA) and SA, which are key regulators of plant defense responses (Niharika *et al.,* 2021), and this elicitor has been used in applications as a stimulating agent to produce several secondary metabolites (Gorni *et al.,* 2021 b; Fiorito *et al.,* 2024).

FA and SA applications in tomato plants have demonstrated potential to stimulate physiological responses and improve fruit quality (Shu *et al.*, 2022; Fiorito *et al.*, 2024). Previous studies at concentrations of 1 mmol L^{-1} reported positive effects, including increases in biomass accumulation, chlorophyll content, and secondary metabolite biosynthesis (Gorni *et al.,* 2021 a; Shu *et al.*, 2022; Gorni *et al.*, 2023; Fiorito *et al.*, 2024). However, higher concentrations of phenolic elicitors may also induce oxidative stress or phytotoxic effects, potentially limiting plant performance and productivity. Despite the growing interest in the use of phenolic compounds in horticultural crops, studies evaluating the combined application of FA and SA at lower concentrations remain limited, particularly under greenhouse cultivation conditions for tomato plants

In this context, using 0.5 mmol L^{-1} for both FA and SA represents a more balanced and potentially safer strategy to enhance plant metabolism while minimizing adverse effects associated with excessive elicitor concentrations. Furthermore, investigating the interaction between these compounds at reduced doses may provide important insights into their combined effects on plant growth, fruit production, and biochemical quality.

We hypothesized that the combined application of FA and SA at low concentrations would improve morphophysiological traits, yield, and the accumulation of bioactive compounds in tomato plants, without causing metabolic stress. Therefore, this study aimed to evaluate the effects of exogenous application of FA and SA, individually or in combination, at 0.5 mmol L^{-1} on growth, productivity, photosynthetic pigments, soluble sugars, and phenolic compounds in tomato plants cultivated under protected conditions.

2. Materials and Methods

Experimental conditions and plant cultivation

The experiment was conducted in 18-liter pots in a greenhouse at the Escola Superior de Agronomia de Paraguaçu Paulista (ESAPP), Paraguaçu Paulista, São Paulo, Brazil, from March 2020 to July 2020. The soil, classified as a Latosol with a sandy texture, was collected from the field and passed through a 5-mm mesh sieve prior to use. Its chemical analysis revealed the following characteristics as shown in Table 1. The soil was corrected with 15 g of dolomitic limestone per pot 30 days before sowing. Subsequently, each pot was fertilized with 0.6 g N, 6.3 g P_2O_5, 1.8 g K_2O, 22.5 mg B, 27 mg Cu, and 45 mg Zn, according to the recommendations in Bulletin 100 (Cantarella *et al.,* 2022).

Four seeds of the tomato cultivar Santa Clara were sown per pot. This cultivar was selected because it is widely cultivated in Brazil, is well adapted to greenhouse conditions, and exhibits uniform plant growth and fruit production, making it suitable for studies of plant physiology and fruit quality (Oliveira *et al.*, 2021). After germination, seedlings were thinned to ensure uniformity, leaving one plant per pot. Irrigation was provided twice daily using a sprinkler system. During this period, the average maximum and minimum temperatures were 26.2 and 16.9°C, respectively, and the mean relative humidity of 70.2%. The average solar radiation was 15.9 MJ m^{-2} day^{-1}, characterizing the microclimatic conditions during the experiment.

Experimental design and treatments

The experiment was arranged in a completely randomized design with four treatments and five replicates per treatment, with each replicate consisting of one plant.

Twenty days after planting, treatments were applied. Foliar applications of FA and SA at a dose of 0.5 mmol L^{-1}, both individually and in combination, were applied to plants using a 2 L pressurized hand sprayer, with the nozzle adjusted for a fan spray, aiming to ensure uniform distribution of the solutions on the leaf surface. The spray volume was standardized to 10 mL per plant across all treatments, and applications were carried out until runoff for three consecutive days. The spray solution contained 50 µL L^{-1} of the non-ionic surfactant Agral® as an adjuvant to improve leaf coverage and absorption. Agral® is composed of nonyl phenoxy poly(ethyleneoxy) ethanol (200 g L^{-1}) and inert ingredients (824 g L^{-1}). Control plants received only distilled water plus surfactant under the same application conditions. Each treatment consisted of five independent replications, maintaining the same conditions of application and experimental management.

Leaves and roots were collected at the final harvest from all plants within each treatment (n= 5 plants per treatment). Fruits were harvested at full maturity throughout the experimental period, and all fruits from the five plants in each treatment were collected for evaluation.

Chlorophyll and soluble sugar determination

Leaf chlorophyll extraction was performed following the method described by Lichtenthaler (1987); 0.2 g of leaf discs (5 mm) was added in 80% acetone (v/v). For quantification of total soluble sugars, 0.5 g of fresh tissue was macerated in 80% ethanol and centrifuged at 5000 rpm for 10 min. The supernatant was mixed in phenol-sulfuric solution following the Dubois *et al.* (1956) method, using glucose as the standard.

Determination of total polyphenols and flavonoids

Tomato juice was used as the sample matrix for polyphenol and flavonoid analyses. Initially, 50 g of fresh tomato fruits were washed with distilled water,

Table 1 - Chemical and granulometric characterization of the soil used in the pot experiment, including texture (sand, silt, and clay fractions) and other physical attributes

pH $CaCl_2$	P (resin) mg/dm³	O.M.S. g/kg	K^+ mmol/dm³	Ca^{2+} mmol/dm³	Mg^{2+} mmol/dm³	H+Al mmol/dm³	BS mmol/dm³	CEC mmol/dm³	V %
4.4	6.0	6.0	0.8	7.0	2.0	20.0	12.5	30.0	33.0
Particle size distribution (g/kg)									
			Sand		Silt	Clay			
			751		69	180			

weighed, and homogenized using a Philips blender operating at 3000 rpm for 2 min. The homogenized material was then filtered through a 2-mm mesh sieve to obtain a uniform sample for subsequent analyses.

The concentration of total polyphenols was determined using the Folin-Ciocalteu colorimetric method, as described by Stagos *et al.* (2012). Gallic acid served as the calibration standard, and results were expressed as micrograms of gallic acid equivalents per milliliter (μg mL^{-1}).

Flavonoid content was estimated following the procedure described by Yao *et al.* (2013). Rutin was used as the reference compound, and the results were expressed in micrograms of rutin equivalents per milliliter (μg mL^{-1}).

Carotenoid analysis

Lycopene, β-carotene, and lutein were extracted from fresh tomato samples using an extractor solution containing hexane, acetone, and ethanol (2:1:1, v/v/v) (Sadler *et al.,* 1990). The quantification was based on the equations proposed by Craft and Soares (1992), using molar extinction coefficients of 172,000 L mol^{-1} cm^{-1}, 135,300 L mol^{-1} cm^{-1}, and 145,100 L mol^{-1} cm^{-1}, respectively.

Growth and allometric measurements

To evaluate the effects of FA and SA on plant development, several growth parameters were measured: leaf area, number of leaves and fruits per plant, plant height, shoot and root dry weight, and average fruit weight. Leaf area was analyzed using ImageJ® software (Powerful Image Analysis). The number of leaves and fruits, plant height, shoots and roots dry weight were recorded after drying the plant material in a forced-air oven at 70°C until reaching constant weight.

Allometric traits were calculated using the following equations: root-shoot ratio (RDW/SDW), specific leaf area (LA/SDW), leaf area ratio (LA/TDW), and leaf mass ratio (SDW/TDW). In these formulas, RDW represents root dry weight, SDW is shoot dry weight, LA corresponds to leaf area, and TDW refers to total dry weight.

Statistical analysis

The experiment was carried out in a completely randomized design with four treatments and five replicates per treatment. Data were analyzed using two-way ANOVA to evaluate the individual effects of FA and SA, as well as the interaction between both factors (FA × SA). Before the analyses, the assumptions of normality and homogeneity of variances were verified using the Shapiro-Wilk and Levene tests, respectively. When significant differences were observed, means were compared using Tukey's test at the 5% significance level ($p < 0.05$). Statistical analyses were performed using SISVAR (Ferreira, 2019), and the results are presented as means ± standard error. For the multivariate analyses, data were normalized and auto-scaled prior to principal component analysis (PCA) and Pearson correlation analysis, which were conducted using MetaboAnalyst (Pang *et al.,* 2024). These analyses were used to better understand the relationships among variables and the overall response patterns of the treatments.

3. Results

Ferulic acid (FA) application increased the concentrations of chlorophyll *a* (153.2%) and total chlorophyll (29.3%) compared to the control (Fig. 1 a and 1 c). The FA+SA combination increased

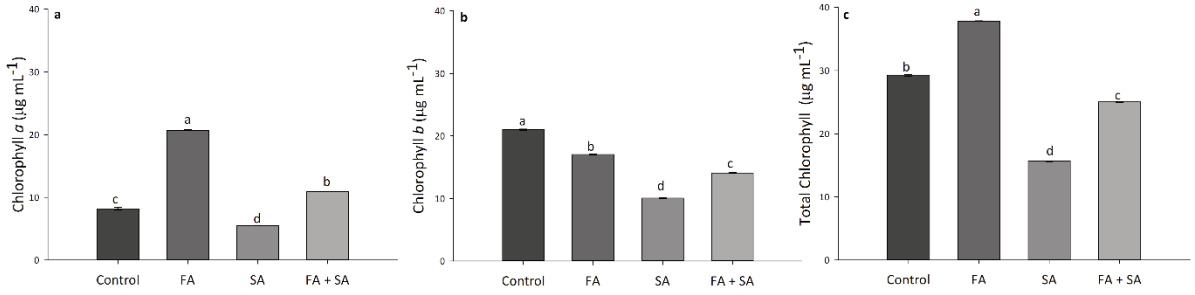


Fig. 1 - Chlorophyll a (a), chlorophyll b (b), and total chlorophyll (c) concentrations in Solanum lycopersicum plants treated with ferulic acid (FA, 0.5 mmol L^{-1}), salicylic acid (SA, 0.5 mmol L^{-1}), and the combination of FA and SA (0.5 mmol L^{-1}). Different letters indicate significant differences by Tukey's test ($p \leq 0.01$). Vertical bars represent the standard error.

chlorophyll *a* concentration by 33.2% compared with untreated plants (Fig. 1 a). However, chlorophyll *b* levels were reduced in all treatments relative to the control (Fig. 1 b).

Salicylic acid (SA) application increased polyphenol (61.1%) and flavonoid (26.0%) concentrations relative to untreated plants (Fig. 2 e, f). On the other hand, the application of FA showed significant increases in the concentration of total soluble sugars (147.3%), lycopene (78.9%), β-carotene (78.8%), lutein (78.7%), polyphenols (91.6%), and flavonoids (85.7%), respectively, compared to the control (Fig. 2 a-f). However, the FA+SA combination increased the concentrations of soluble sugars (152.2%), polyphenols (37.3%), and flavonoids (76.5%) relative to untreated plants (Fig. 2 a, 2 e, 2 f).

Growth parameters increased with both the isolated application of FA and SA and with the combination of elicitors. Increases of 18.7% (FA), 28.9% (SA), and 46.8% (FA+SA) were observed in the shoot dry weight, 68.9% (FA), 66.6% (SA), and 76.7% (FA+SA) in the root dry weight and 26.5% (FA), 34.8% (SA), and 51.5% (FA+SA) in the total dry weight, respectively in relation to the control (Fig. 3 a, 3 b, 3 c). However, fruit weight at SA application increased by 8.5%, and the FA+SA combination increased by 4.9%, compared with untreated plants (Fig. 3 d).

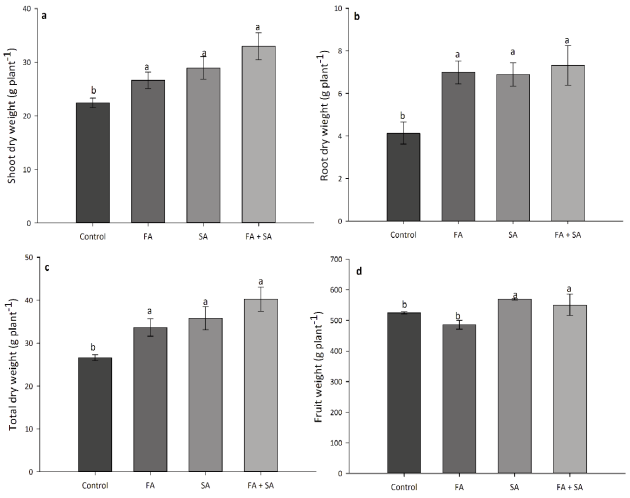


Fig. 3 - Shoot dry weight (a), root dry weight (b), total dry weight (c), and fruit weight (d) in Solanum lycopersicum plants treated with ferulic acid (FA, 0.5 mmol L^{-1}), salicylic acid (SA, 0.5 mmol L^{-1}), and the combination of FA and SA (0.5 mmol L^{-1}). Different letters indicate significant differences by Tukey's test ($p<0.05$). Vertical bars represent the standard error.

Increases in leaf area, leaf number, fruit size, and fruit diameter were observed in plants treated with FA of 82.1%, 8.2%, 39.6%, and 44.1%, respectively, compared to untreated plants (Fig. 4 a, 4 b, 4 e, and 4 f). Plants treated with SA showed increases in leaf area (90.3%), plant height (12%), fruit size (32.3%),

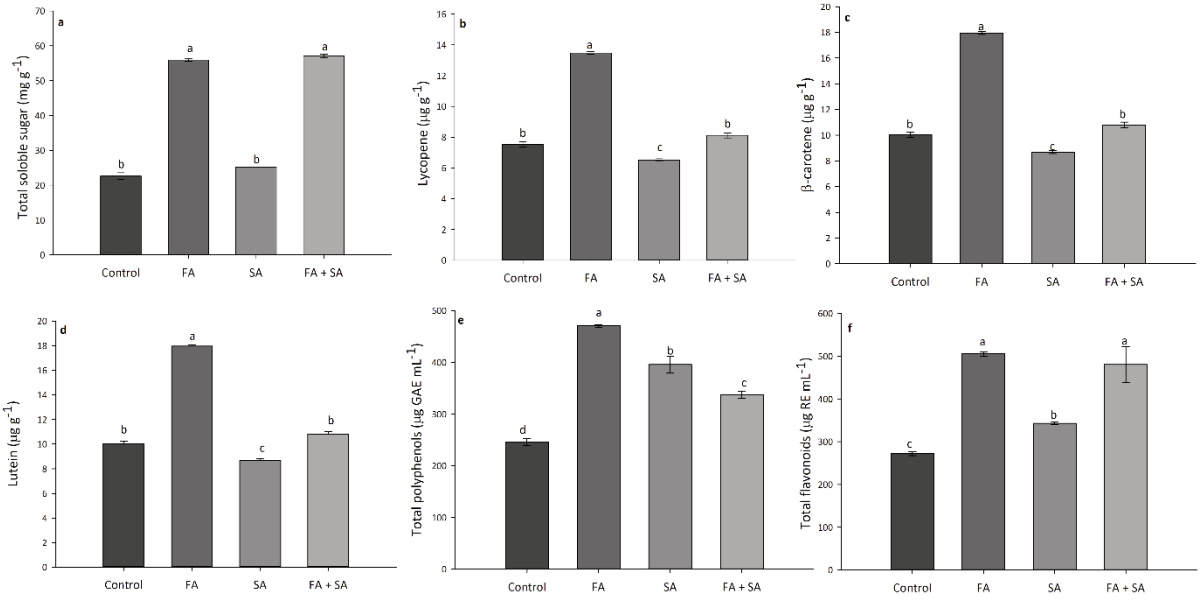


Fig. 2 - Total soluble sugar (a), lycopene (b), β-carotene (c), lutein (d), total polyphenols (e), and total flavonoids (f) concentrations in Solanum lycopersicum plants treated with ferulic acid (FA, 0.5 mmol L^{-1}), salicylic acid (SA, 0.5 mmol L^{-1}), and the combination of FA and SA (0.5 mmol L^{-1}). Different letters indicate significant differences by Tukey's test ($p<0.05$). Vertical bars represent the standard error.

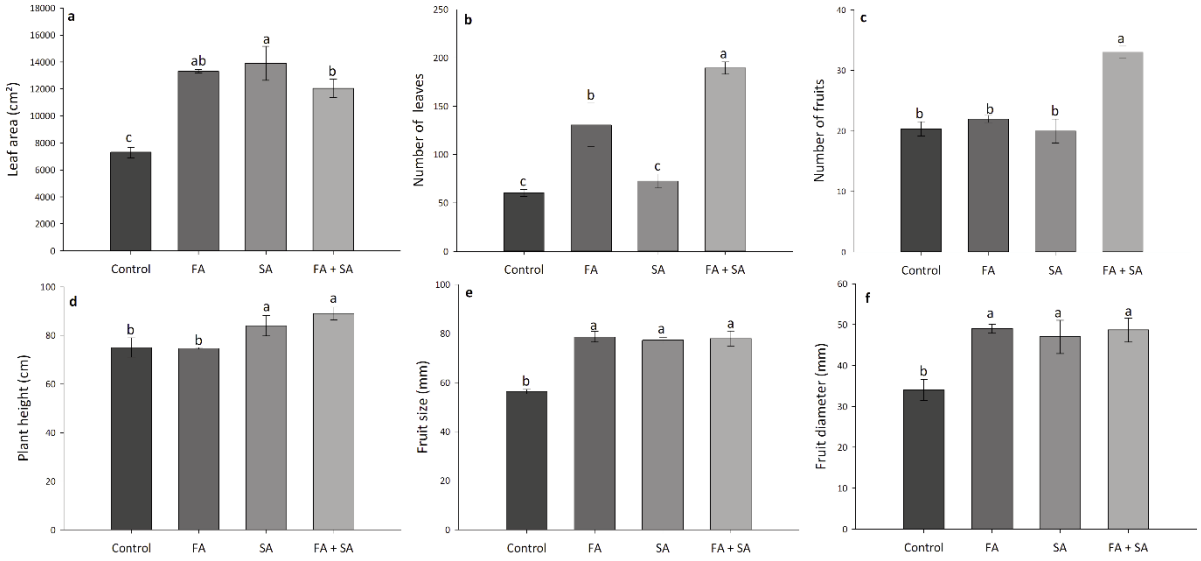


Fig. 4 - Leaf area (a), number of leaves (b), number of fruits (c), plant height (d), fruit size (e), and fruit diameter (f) in Solanum lycopersicum plants treated with ferulic acid (FA, 0.5 mmol L^{-1}), salicylic acid (SA, 0.5 mmol L^{-1}), and the combination of FA and SA (0.5 mmol L^{-1}). Different letters indicate significant differences by Tukey's test ($p< 0.05$). Vertical bars represent the standard error.

and fruit diameter (38.2%), respectively, compared to the control (Fig. 4 a, 4 d, 4 e, 4 f). The combination of FA+SA showed increases in leaf area (64.9%), number of leaves (213.8%), number of fruits (62.3%), plant height (18.7%), fruit size (38.5%), and fruit diameter (43.1%), respectively, compared to the control (Fig. 4 a-f).

FA and SA treatments significantly increased allometric traits in tomatoes (Fig. 5). The highest values were observed in the isolated and combined application of FA and SA, which resulted in an increase of 41.9%, 56% and 44.1% in the root: shoot ratio (Fig. 5 a), 68.7%, 48.4% and 18.5% in the leaf area ratio (Fig. 5 b) and 78.9%, 60.4% and 24.4% in the specific leaf area (Fig. 5 c), respectively in comparison to the untreated plants. However, the leaf mass ratio of the treated plants did not differ significantly from that of the control plants (Fig. 5 d).

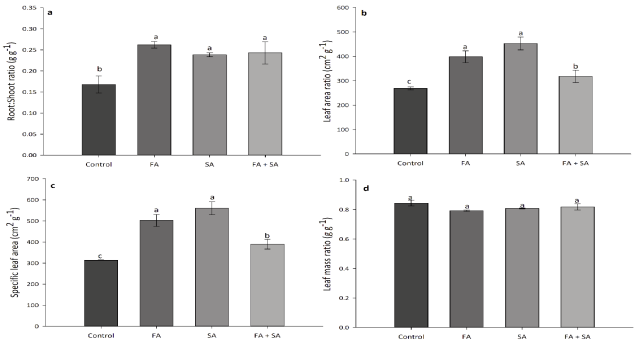


Fig. 5 - Root: shoot ratio (a), leaf area ratio (b), specific leaf area (c), and leaf mass ratio (d) in *Solanum lycopersicum* plants treated with ferulic acid (FA, 0.5 mmol L^{-1}), salicylic acid (SA, 0.5 mmol L^{-1}), and the combination of FA and SA (0.5 mmol L^{-1}). Different letters indicate significant differences by Tukey's test ($p<0.05$). Vertical bars represent the standard error.

In Pearson's correlations of *S. lycopersicum* under FA and SA elicitation (Fig. 6), the glow matrix highlights how the elicitors FA, SA, and their combination (FA+SA) influence different aspects of plant metabolism and development, revealing potential underlying physiological mechanisms. Under the FA application, significant associations were observed among growth, physiological, and biochemical variables (Fig. 6 a). FrD and FrS were associated with RDW, LA, and TDW ($p<0.01$), suggesting that improvements in plant growth were accompanied by better fruit development. FLV, TSS, LYC, BETA, and chlorophyll-related traits also showed significant associations ($p<0.001$), indicating that FA influenced both photosynthetic activity and antioxidant metabolism. Chl a and Chl t were significantly associated with carotenoid accumulation ($p<0.001$), reinforcing the relationship between pigment biosynthesis and fruit quality. In contrast, FrW showed few significant associations with the evaluated variables, suggesting a more isolated response compared with the other production traits.

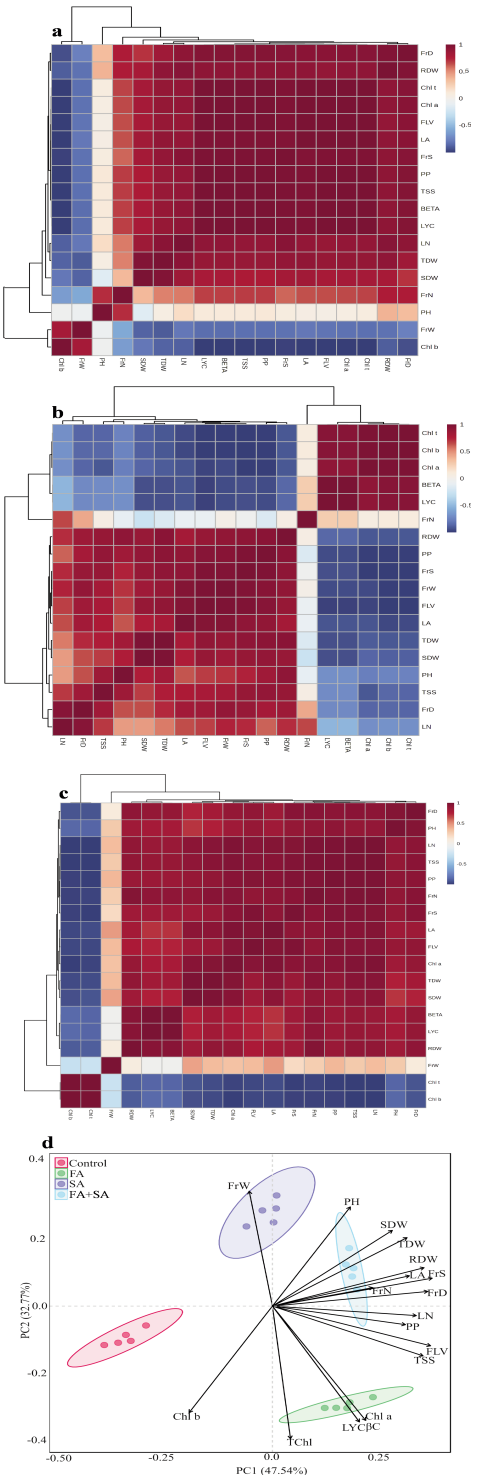


Fig. 6 - Pearson's correlations between the variables studied: (a) correlation related to FA; (b) correlation with SA; (c) joint correlation of FA+SA, and (d) principal component analysis (PCA) between treatments. Chl a (chlorophyll a), Chl b (chlorophyll b), TChl (total chlorophyll), TSS (total soluble sugar), FLV (flavonoids), PP (polyphenols), LYC (lycopene), BETA (β-carotene), SDW (shoot dry weight), RDW (root dry weight), TDW (total dry weight), FrW (fruit weight), LA (leaf area), LN (number of leaves), FrN (number of fruits), PH (plant height), FrS (fruit size), FrD (fruit diameter).

Under SA treatment (Fig. 6 b), chlorophyll variables were significantly associated with growth and biochemical traits, including FLV, TSS, RDW, and fruit-related variables ($p<0.01$). LYC and BETA were also associated with chlorophyll contents ($p<0.001$), indicating coordinated changes between photosynthetic pigments and antioxidant compounds. FrS and FrW were associated with FLV, LA, and TDW ($p<0.01$), suggesting that SA application promoted simultaneous responses in plant growth and fruit quality. PH and TSS also showed significant associations with productive traits ($p<0.05$).

For the combined FA+SA treatment (Fig. 6 c), strong associations were observed among growth, productivity, and biochemical variables. FrD, FrN, FrS, and TSS were significantly associated with chlorophyll-related traits, FLV, and biomass accumulation variables ($p<0.001$), indicating an integrated response of plant metabolism under the combined application. LYC and BETA were associated with chlorophyll content, RDW, and TDW ($p<0.01$), suggesting a close relationship between antioxidant metabolism and plant growth. In contrast, FrW did not show significant associations with most variables, indicating a distinct response pattern relative to the other production parameters.

When the two compounds were applied simultaneously, the correlation patterns reflected a combination of the effects observed in the individual treatments. There is a strong correlation between antioxidant compounds and growth parameters, suggesting a combined effect between FA and SA. It is observed that the correlation between biomass and antioxidant compounds is more balanced than in individual treatments, indicating that the plant may be balancing investment in defense and growth.

PCA was performed to explore the multivariate patterns among morphophysiological traits and the FA, SA, and FA+SA treatments (Fig. 6 d). The variables were distributed along two principal components that together explained 80.31% of the total variance (PC1: 47.54%; PC2: 32.77%). The control treatment was positioned in the lower-left quadrant, in close association with Chl b, indicating that untreated plants were characterized mainly by basal levels of photosynthetic pigments, without an expressive increase in biomass accumulation or fruit-related traits. Plants treated with SA were located in the upper-left quadrant and positioned near FrW, suggesting a greater contribution of this variable to the SA treatment profile and indicating enhanced

fruit production. In contrast, the FA treatment was grouped in the lower-right quadrant and positioned close to Chl a, TChl, LYC, and βC, indicating that these variables contributed strongly to the FA-associated response pattern, especially regarding pigment accumulation and fruit quality-related traits. The combined treatment (FA+SA) was distributed in the upper-right quadrant and was associated with variables related to growth and productivity, including PH, FrN, SDW, RDW, TDW, LA, FrS, and FrD. This distribution suggests that the combined application elicited broader morphophysiological responses associated with vegetative growth and reproductive performance.

4. Discussion and Conclusions

One of the most pronounced responses observed was the increase in photosynthetic pigments, particularly chlorophyll a and total chlorophyll, mainly in plants treated with FA and FA+SA (Fig. 1 a and c). Increased chlorophyll content is commonly associated with greater photosynthetic efficiency and improved carbon assimilation, which directly supports vegetative growth and reproductive development. Similar responses were reported by Linić *et al.* (2021), who observed improved photosynthetic performance in Chinese cabbage treated with SA and FA under stress conditions. In addition, FA has been associated with the maintenance of photosynthetic apparatus stability and protection against reactive oxygen species (Fiorito *et al.*, 2024; Khan *et al.*, 2024). Previous studies on tomato plants treated with 1 mmol L^{-1} FA and SA also reported increases in chlorophyll accumulation (Gorni *et al*., 2021 a). However, the present study demonstrates that similar physiological responses can be achieved using a lower concentration (0.5 mmol L^{-1}), suggesting that reduced doses are sufficient to stimulate pigment biosynthesis without imposing high metabolic costs. This finding is relevant because higher elicitor concentrations may overstimulate defense pathways and compromise plant growth under some conditions (Queiroz *et al.*, 2023).

The increase in chlorophyll content was accompanied by higher accumulation of soluble sugars in plants treated with FA and FA+SA (Fig. 2 a). This relationship is expected because chlorophyll accumulation improves light capture and photosynthetic activity, increasing carbon fixation and carbohydrate production (Fiorito *et al.*, 2024; Khan *et al.*, 2024). Similar increases in chlorophyll and carbohydrate contents after FA and SA application were also observed by Linić *et al.* (2021) and Gorni *et al*. (2021 b). Nevertheless, our results suggest that FA-containing treatments promoted a more balanced relationship between photosynthetic metabolism and biomass production, reinforcing the idea that FA may act directly on primary metabolism rather than exclusively on stress signaling pathways.

Also evident was the accumulation of carotenoids such as lycopene, β-carotene, and lutein, as well as flavonoids and polyphenols (Fig. 2 b-f). These compounds are associated with antioxidant activity and fruit nutritional quality, and their accumulation indicates activation of secondary metabolism. Similar responses have been reported in tomato fruits treated with phenolic elicitors (Gorni *et al.*, 2021 a; Shah *et al.*, 2021). However, the stronger responses observed in FA-containing treatments suggest that FA may have a more direct role in modulating the phenylpropanoid pathway and carbon allocation toward antioxidant biosynthesis. As a hydroxycinnamic acid derivative, FA participates in cell wall metabolism and can influence enzymes associated with phenolic synthesis (Antonopoulou *et al.*, 2022). This mechanism may explain the simultaneous increase in carotenoids, flavonoids, and biomass accumulation observed in the present study.

Gorni *et al.* (2021 a) and Linić *et al.* (2021) previously demonstrated positive effects of SA and FA on phenolic metabolism in tomato plants. However, most previous studies evaluated the isolated action of these compounds or used higher concentrations. In the present study, the combined application at 0.5 mmol L^{-1} led to simultaneous increases in photosynthetic pigments, bioactive compounds, and growth-related traits, suggesting that lower doses may be sufficient to induce coordinated physiological responses. Rather than suggesting a purely additive effect, the results indicate that FA and SA may act through complementary metabolic pathways. SA is primarily associated with defense signaling and regulation of oxidative stress, whereas FA appears to be more closely linked to carbon metabolism, cell expansion, and fruit development.

In physiological terms, SA acts as an important

signaling molecule involved in abiotic stress responses and antioxidant metabolism, favoring the synthesis of flavonoids and phenolic compounds (Ali, 2021; Ramos-Sotelo and Figueroa-Pérez, 2023). Its action also includes regulation of photosynthetic activity and protection of the photosynthetic apparatus (Aires *et al.*, 2022; Saleem and Fariduddin, 2024). SA-mediated signaling is strongly associated with ROS homeostasis and activation of defense-related pathways (Pacheco and Gorni, 2021). However, activation of defense metabolism often requires high energetic investment, which can reduce carbon allocation to growth and reproduction (Watts *et al.*, 2023). This may explain why SA-treated plants showed weaker associations with biomass and fruiting variables compared with FA-treated plants.

In contrast, FA appears to act more directly on primary metabolism and developmental processes. FA has been associated with cell division, tissue elongation, and fruit development (Niharika *et al.*, 2021; Antonopoulou *et al.*, 2022). In addition, its participation in cell wall biosynthesis and enzymatic regulation may contribute to greater biomass accumulation and improved reproductive development. Unlike SA, whose effects are more closely linked to defense signaling, FA was associated more strongly with vegetative growth and fruit-related parameters, suggesting distinct physiological roles for the two elicitors.

The correlation analyses reinforced these differences between elicitors (Fig. 6 a-c). SA was predominantly associated with antioxidant protection and stress-related responses, whereas FA was more strongly associated with biomass accumulation and reproductive development. These results support the physiological trade-off between defense and growth proposed by Watts *et al.* (2023). Interestingly, the combined FA+SA treatment promoted a broader response pattern, with simultaneous gains in growth, pigment accumulation, soluble solids, and antioxidant compounds, suggesting a more integrated metabolic adjustment.

PCA further supported this interpretation by grouping FA+SA-treated plants in a region associated with multiple agronomic and biochemical variables (Fig. 6 d). This distribution indicates that the combined application promoted a more balanced interaction between primary and secondary metabolism, allowing plants to maintain growth while activating protective mechanisms. Similar integrated responses between growth and defense metabolism have been discussed by Pang *et al.* (2024) and Fiorito *et al.* (2024) in studies involving phenolic signaling compounds.

Finally, the dose used in the present study (0.5 mmol L^{-1}) proved effective, with no visible phytotoxic effects. Compared with the previous study using 1 mmol L^{-1} (Gorni *et al.*, 2021 a), the lower concentration promoted comparable increases in photosynthetic pigments and antioxidant-related compounds while maintaining balanced growth and productivity responses.

These findings reinforce the importance of dose adjustment in elicitor-based strategies, as excessive stimulation of defense pathways may impair plant metabolism and carbon allocation (Queiroz *et al.*, 2023). Overall, the results indicate that low-dose applications of FA and SA represent a promising approach to improving both the agronomic performance and nutritional quality of tomato fruits.

In conclusion, this study demonstrates that the combined application of ferulic acid (FA) and salicylic acid (SA) at 0.5 mmol L^{-1} promoted simultaneous improvements in plant growth and in the accumulation of nutritionally important compounds in tomato fruits. These findings suggest that the combined use of these phenolic compounds can effectively enhance both yield-related and quality-related traits in tomato plants. Overall, FA and SA may represent a promising and sustainable agronomic strategy to improve fruit quality and plant performance, although further studies under different environmental conditions and cultivation systems are needed to confirm the consistency and applicability of these effects.

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Functional foods and sensory analysis: Evidence from a magnesium-enrichment radish study

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Key words: Food quality, magnesium biofortification, organoleptic assessment, radish, sensory analysis.

Abstract: Evidence from the literature highlights a strong consumer preference and/or purchase intention for fortified food products due to their health benefits. They prefer enriched food products with an increase of micronutrients and a better nutritional profile, specific for their needs. Yet sensory appeal remains a key driver to their final acceptance and at the same time, there are few evidence in literature regarding organoleptic aspect of horticultural fortified. Investigating sensory profiles is therefore critical to developing enriched food products that meet both health expectations and hedonic preferences. This paper aims to evaluate organoleptic aspects of a radish plant enriched with magnesium, and a combination of magnesium and zeolite solution, using a trained panel of tasters. Data collected were analyzed using the Co-efficient of Variation, Analysis of Variance (ANOVA), and Tukey's Honestly Significant Difference (HSD) tests. Treatment samples with magnesium solutions increased magnesium concentrations relative to the control sample. The estimated increase is 27.2% in swollen root and 58.4% in leaf. In fact, the treatment significantly altered the sensory profiles of the radish plant. Both magnesium and magnesium and zeolite samples significantly exhibited lower bitterness and crunchiness intensity while showing an increase intensity of spiciness. Notably, the treatment samples were perceived as spicier but less bitter than the control sample. Sensory profiles of functional foods are altered by incorporation of nutrients and foliar treatments, ultimately having the ability to affect the hedonic preferences of potential consumers. The study produces new data on the topic of sustainable product innovation in the agri-food industry, delineating new opportunities for future research. Finally, it addresses the gap in the literature on fortified vegetable sensory evaluation. A further hedonic analysis with a larger number of panelists along with surveys assessing consumer willingness to consume and purchase these products are needed.

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1. Introduction

Consumers in recent years, driven by increased life expectancy and

improved quality of life, have begun to understand the nexus between food composition and well-being status. They are becoming more aware of the consequences related to purchasing decisions and the food choices they make (Smeding *et al*., 2023; Yang *et al*., 2023). This has led to the adoption of eating habits based on targeted and tailor-made diets with reference to effective nutritional needs of each individual. A growing cohort of consumers are also considering socially responsible consumption style such as waste reduction, reuse, recycling, and responsible agricultural practices to avoid or minimize environmental impacts (Prendergast and Tsang, 2019; Hosta and Zabkar, 2021).

Therefore, the need to transition towards more sustainable diets has become a shared priority for both consumers and the economic market. Sustainability is no longer seen as just an environmental issue, but a complex commitment that intertwines perceived quality, global concerns, product innovation, and ethical behavior.

Among new trends is an increase in the consumption of functional food products with high nutritional content. These products are developed by incorporating nutritional substances with the ability to induce a specific physiological benefit (Dotto and Chacha, 2020).

Nowadays, the interest in integrating diets with fortified foods, also proves to be a great opportunity for the food industry, as small and medium-sized businesses can find greater profitability through the commercialization of innovative and functional products as well as increase their competitiveness (Hermundsdottir and Aspelund, 2021). In fact, many authors state that the global size of the functional foods market is increasing, covering various food categories (i.e bakery and dairy products, meat, fish, eggs, fats and oils) with a compound annual growth rate of 6.9% and is expected to rise to 597.1 billion dollars by 2032 (Martirosyan *et al*., 2022; Mudondo *et al*., 2025;). It is expected that this increase of the functional foods market is linked to a greater importance given to health and quality food products which generates an alternative to medical formulations. Indeed, the literature presents a very large number of contributions aimed at analyzing consumer attitudes towards innovative products, use of novel ingredients, and implementation of sustainable production processes (Nazzaro *et al*., 2024). Recent functional food formulations involve the addition of nutrients to improve a specific functionality (e.g., digestion, learning ability), reduction or replacement of an ingredient to reduce a source of health risk (e.g., "lactose-free" and "gluten-free"), or an increase of the bioavailability of a component normally present in the food. These formulations include a wide range of compounds such as bioactive peptides, phenolic compounds, carotenoids, lipids, vitamins, dietary fibers, fatty acids, minerals, prebiotics, and probiotics (Yao *et al*., 2020).

Among the foods subject to enrichment, fruit and vegetables are the most studied categories, as they are sources of polyphenols and other compounds with antioxidant action which are necessary to prevent the excessive accumulation of free radicals (Tadesse and Emire, 2020).

However, the sensory profile of these horticultural product is still being fully explored, especially in relation to their various functional applications.

The creation of innovative functional foods involves all facets of the food supply chain, including cultivation using sustainable methods and technologies. This allows, novel product development, and revitalize underutilized and genetically diverse varieties, and support waste management (Lee *et al*., 2024; Onawo and Egboduku, 2025).

Furthermore, not all food innovations are accepted by consumers, especially in terms of organoleptic characteristics, and survive in markets. Consequently, it is important to understand the extent consumers embrace these new products form new eating habits and overcome limits related to food techno-neophobia (Zamparo *et al.*, 2023).

Consumer behavior theory

According to Kotler and Keller (2016) "consumer buying behavior is the study of the ways of buying in order to satisfy their needs", and is determined by the cultural, personal, social, and psychological factors (Qazzafi, 2020).

The food quality is a key factor in their decision-making process and plays an important role to determinate the perception of a specific product; and it is influenced by various characteristics. The values sought and expected by consumers have an impact on the desirable aspects of quality and the way in which product/service attributes are perceived and evaluated (Wang *et al.*, 2020).

Food quality can also be considered as a

multidimensional construct which is characterized by four essential dimensions that are interconnected: the hedonic properties of food, convenience, health, and the production process (Sadilek, 2019).

The perception of food quality has an objective and a subjective aspect; the first regard nutraceutical composition and food safety, while the second aspect refers to process attributes including origin, as well as other organoleptic attributes such as taste. For consumers to form a quality judgement of a particular food product, they must draw from any quality cues available or information stimuli and can be predictive of the desired attribute and ascertained through the senses prior to consumption (e.g., food certificates or organic production) (Steenkamp, 1990).

In fact, consumers are willing to pay a premium price for goods considered to be ethically superior, respectful of health, environmentally friendly, and innovative with symbolic and communicative values rather than material values (Valaskova *et al*., 2018).

Despite safety control associated with innovative food products, consumers tend to have difficulty accepting and purchasing them, when unfamiliar technologies are used in production. (Bánáti, 2011). This skepticism leads to consumers boycotting newly developed products which is often caused by lack of information and knowledge availability regarding production methods (Krystallis *et al*., 2007). The trade-off between risks, perceived quality and benefits is the major factor that determine consumer acceptance or rejection of novel food (Sajdakowska *et al*., 2018).

The aim of this study is to evaluate the sensory profile of magnesium-enriched radish obtained through a sustainable cultivation approach based on targeted foliar fertilization, using a trained sensory panel.

2. Materials and Methods

Sensory analysis

Sensory analysis is part of food science and has the primary aim of providing an 'objective' measure of the sensory quality of food items. It consists of a set of techniques and conditions that measure and evaluate, using one or more of the human senses, certain characteristics of specific products. Sensory analyses are performed by a panel of selected and trained tasters who have completed training courses and passed examinations in accordance with Regulation (EC) No. 640/2008 and other national and international laws, enabling them to carry out sensory analysis of a product under controlled conditions.

Through the use of a specific tasting score sheet the tasters record the intensity of a descriptor, in a graduate scale from 0 (no intensity) to 10 (maximum intensity), with which the items on the sheet are perceived in their evaluation of the food product. The taster then gives a final judgement on the basis of the individual scores given to each attribute, determining the overall sensory quality of the product (Meilgaard *et al*., 1999; Alvarez and Blanco, 2000). The attributes measured address one or more of the following aspects: visual, auditory, somesthetic, kinesthetic, olfactory, chemesthetic, and gustatory (Lawless and Heymann, 2010; Lawless and Civille, 2013). The panelists undergo specific training depending on the attributes to be analyzed and they learn how to use a pre-established sensory language used during evaluations. Each attribute has a description and a specific reference to allow the taster to identify and measure the sensory attribute (Drake *et al*., 2023).

Case study

The selected species was radish *(Raphanus raphanistrum* subsp. *sativus* (L.) Domin*)*, belonging to the *Brassicaceae (Cruciferae)* family, a vegetable plant with a wide range of distribution, in particular in the Mediterranean area. Radish includes only the species *Raphanus raphanistrum*, of which there are multiple subspecies and varieties in different colors. The plant is monocarpic and produces a single edible storage organ per year. It has a stem with an erect floral axis with few leaves and branched at the top. The root is of the thickened taproot type and has the ability to accumulate different quantities of nutrients within it, which will then be used during the subsequent development of the flower and enlarged hypocotyl-root complex.

This species was chosen for its widespread use, short growing cycle, and ability to absorb at different level (swollen root , roots and leaves) various types of molecules. It is also of great agronomic and commercial interest, in particular the edible storage organ. Its color is normally bright red with a variable shape (round, globular, semi-long, long). From a nutritional point of view, 100 grams of product contain 3.45 g of carbohydrates, a moderate content

of potassium, flavonoid (24.4 of QE/g), total phenols (95.8 of GAE/g), low concentrations in magnesium (0.016 mg) (Khan *et al*., 2022).

The radish plant was cultivated with seeds purchased from the local retailer and placed in polystyrene containers. The experimental design consists of 3 theses of which 1 is a control and 2 treatment combinations, with 3 replicates for each thesis, for a total of 9 samples.

During the growth phase, which occurred over a period of six weeks, the radish samples were treated twice with a foliar fertirrigation application of only 2% magnesium sulfate (sample 2), with the addition of microgranular zeolite (chabazite species) (sample 3), according to manufacturer's recommendations and studies conducted in the literature.

The magnesium was obtained from pharmacies, and the zeolite was directly purchased from the producer (Fitokem srl, Imperia, Italy). Zeolites are aluminosilicates (AlO_4 and SiO_4) naturally found in sedimentary rocks, that can be used as soil ameliorants with the effect to facilitate the improvement of physical and chemical properties, as well as alleviate heavy metal toxicity reduce nutrient leaching and their use leads to cultivation and water use efficiency (Cataldo *et al.,* 2021; Mondal *et al*., 2021).

Two foliar treatments were carried out, approximately a couple of weeks apart, one during the intermediate phase of vegetative development and the last one shortly before harvesting, when the leaves were at the peak of growth in weight and size. Given their rapid growth cycle, which can reach up to 20 days in well-adapted conditions, they were harvested in mid-October when the edible storage organ were at peak growth. After that, the samples were treated for subsequent instrumental, and organoleptic analyses was performed. Concentration analyses were performed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The Mg concentration was expressed as mg kg^{-1} dw.

Given the limited literature available on the sensory profiles of horticultural crops, an ad hoc sensory profile model was built based on the descriptors of similar crops, to evaluate whether the various samples presented distinctions in sensory terms.

For the sensory analysis, the three different samples (control, treatment with only Mg, and treatment with zeolite and Mg,) were cleaned, trimmed, prepared in uniform portions, and served at room temperature to avoid altering the characteristics of the product (Table 1).

They were then tasted in a single session in the tasting room, by a panel made up of 10 trained tasters (officially recognised by Italian Ministry of Agricultural, Food and Forestry Policies), that operates permanently in according to the criteria set out in specific regulations.

At the beginning of the analysis, the group was given a brief introduction on the objectives of the test in question and a description of the novel evaluation sheet. In fact, regarding to the specific attributes for radish, a few evidences exist in literature; for these reasons a wide number of hypothetical attributes were added.

Table 1 - Treatments and experimental approach

Sample	Treatment
1	Control
2	Solution Mg Sulfate 2%
3	Zeo + Mg

Sample 1 is a control; Sample 2 was treated with Magnesium; Sample 3 was treated with a combination of Magnesium and zeolite solution.

The three samples were presented in a casual way to the panelists on small plates, and numbered with progressive values (number 1, number 2 and number 3) without indicating the treatment administered in a randomized design to eliminate any effect of the order of service. The panelists were asked to taste the samples and fill in the sheet immediately following the codes written on the cards. They evaluated the color, flavour, taste, and consistency attributes of the samples to be analyzed. The flavour was evaluated for intensity of fruitiness and herbaceousness, sweetness, acidity, and bitterness. The consistency was described and analyzed in terms of compactness and juiciness. Subsequently, the results of the attributes selected, were summarized during a round table discussion (open panel), where other perceived aromatic scents (floral, woody, citrus, etc.), previously not considered, were identified. The results of the sensory evaluations were estimated using the coefficient of variation (CVr). It is a dispersion index that allows the evaluation of the dispersion of data around the mean

value, with a dimensionless quantity. According to the regulations governing sensory tests, an evaluation whose CVr% is less than 20 can be considered reliable. All the values obtained from the panel test are within the CVr limit.

These values were then recorded in an excel file and the averages were represented with the characteristic spider web graph.

For a robust analysis, we applied the Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) tests. To assess the appropriateness of using one-way ANOVA, additional analyses were conducted to evaluate the potential influence of panelist effects on the sensory evaluation results. A two-way ANOVA including panelist as a factor was performed for each of the nine sensory attributes. The panelist effect was not statistically significant for any attribute (all $p>0.05$), indicating that differences among panelists did not contribute meaningfully to the observed variation in sensory scores. The findings suggest that observed differences were primarily attributable to treatment effects rather than individual panelist differences.

Furthermore, mixed-effects models treating panelists as random effects were fitted for each sensory attribute. The results were consistent with those obtained from the one-way ANOVA. The intraclass correlation coefficients (ICCs) were negligible across all sensory attributes (all ICCs < 0.001; approximately zero), indicating minimal within-panelist dependence and virtually no clustering of observations within panelists.

In addition, all evaluations were conducted under standardized conditions by trained panelists, and the order of sample presentation was randomized to minimize potential carryover, learning, and fatigue effects. Collectively, these findings indicate that panelist-related variability had a negligible influence on the sensory evaluation outcomes and support the use of the one-way ANOVA.

3. Results and Discussion

Two different treatments - only Magnesium (S2) and a combination of Zeolite and Magnesium (S3) were conducted with respect to the control (S1). However, for these two treatments both the leaves and enlarged hypocotyl-root complex of the plant were analyzed in order to verify whether the Magnesium (Mg) translocated and accumulated preferentially. Instrumental analysis revealed that the leaves absorbed a greater amount of Mg than the control, as predicted by foliar fertigation. At the same time, the treatments (S2 and S3) increased magnesium concentrations compared to the control (S1), both in the swollen roots and leaves. In particular, the treatment with only Mg solution (S2) increased the Mg concentration about 27.2% in swollen root and 58.4 in leaf respectively (Table 2).

From the sensory analysis, compared to the control (S1), the treatments on the radish samples seem to have given the product more balanced and harmonious characteristics and an overall positive opinion from the tasters. The treatments affected both the structure, especially the crunchiness, and the flavor, with spicy notes prevailing over bitterness. The spicy sensation refers to the pungent scent, characterized by burning or tingling sensations, which are caused by chemical molecules (such as capsaicin in chili peppers or oleocanthal in EVOO) with an "irritant or stimulating" action.

Table 2 - Concentration of Mg (mg kg^{-1}) in radish

Sample	Swollen root conc. Mg (mg kg^{-1})	Leaves conc. Mg (mg kg^{-1})
Control	72.31	315.41
Mg treatment	99.34 (+27.2%)	758.55 (+58.4%)
Zeo + Mg treatment	87.63 (+17.5%)	728.67 (+56.7%)

Value concentrations of magnesium in the homogenized samples under different treatment, along with the percentage variation relative to the control.

The results from the organoleptic evaluation were statistically analyzed with ANOVA and Tukey's Honestly Significant Difference (HSD) test (Table 3).

The ANOVA test was used to determine whether at least one sample mean of radish plant sensory attribute differs significantly from the others. The results support the rejection of the null hypothesis, indicating that at least one sample mean of all attributes differ significantly across the Control (S1), Mg treatment (S2), and Zeo+Mg treatment (S3) samples, with the exception of savory attribute. This suggests that tasters' evaluations of the attributes, on average, varied across the three samples, except for savory attribute, where no significant difference was observed.

Table 3 - Comparison of sensory attributes among the three samples

Attribute	Control (S1)	Mg treatment (S2)	Zeo+Mg treatment (S3)	ANOVA F-value	P-value
Herbaceous	2.95 ± 0.33 a	2.26 ± 0.31 c	3.99 ± 0.38 b	64.84	0.0000 *
Vegetal notes	6.46 ± 0.27 a	3.05 ± 0.34 c	4.02 ± 0.38 b	273.73	0.0000 *
Bitter	3.66 ± 0.41 a	2.50 ± 0.48 c	2.09 ± 0.33 b	38.64	0.0000 *
Savory	4.52 ± 0.40 a	4.15 ±0.57 a	4.19 ± 0.39 a	2.47	0.1038
Crunchiness	5.62 ± 0.42 a	3.61 ± 0.37 b	3.61 ± 0.37 b	90.14	0.0000 *
Succulence	6.01 ± 0.40 a	4.11 ± 0.38 c	2.62 ± 0.44 b	171.92	0.0000 *
Texture	5.36 ± 0.39 a	3.56 ± 0.35 c	3.00 ± 0.25 b	133.81	0.0000 *
Spicy	2.83 ± 0.41 a	5.42 ± 0.29 b	4.46 ± 0.42 b	120.51	0.0000 *
Fruity	5.77 ± 0.57 a	2.98 ± 0.35 c	3.20 ± 0.34 b	127.97	0.0000 *

Values are presented as mean ± standard deviation. Means within a row with different letters differ significantly according to Tukey's HSD test ($p<0.05$). * denote 5% significance level.

Prior to conducting the Tukey HSD test, a test of homogeneity of attributes across samples was conducted using Levene's, Brown-Forsythe, and Brown-Forsythe tests. This was necessary to determine the appropriate post-hoc procedure to follow. Results revealing equal variances are consistent with the application of Tukey HSD while unequal variances are consistent with Games-Howell test. The results revealed that all p-values of attributes across the three samples were greater than 0.05, thus we failed to reject the null hypothesis of equal variances. Consequently, we applied the Tukey's HSD test that allows post hoc comparisons among samples and identifies the specific pairs of samples that differ significantly.

The Tukey's HSD test revealed significant differences between all pairs of samples - S2 vs S1; S3 vs S1; and S3 vs S2 - across all attributes, except for savory attribute. S3 vs S1 exhibited significantly higher herbaceous values than S2 vs S1 (mean difference = 1.04, 95% CI [0.66, 1.42]).

In fact, relative to the control sample (S1), S2 revealed a significant reduction of herbaceous intensity (Fig. 1). The finding suggests that the sample treated with Zeo + magnesium exhibited higher herbaceous intensity than the control sample. This could possibly be explained by an enhanced accumulation of compounds (i.e. aldehydes) associated with herbaceous sensory notes by the sample treated with magnesium, and consequently they may have developed features perceived by panelists.

This result can be compared with Gautam *et al*. (2025), where the effect of biologically synthesized magnesium oxide nanoparticles on radish was evaluated. The study reported that magnesium treatments may influence carbohydrate, flavonoid, phenolic, and tannin contents in radish; however, these parameters were not assessed in the present study.

Relative to S1, the treated samples (S2 and S3) exhibited lower vegetal notes intensity. The mean difference for S2 vs S1, and S3 vs S2 are -3.41 and -2.44 respectively. This suggests that in terms of vegetal notes such as aromas, flavors or scent, the treatment of Radish plant with magnesium sulfate or microgranular zeolite (chabazite species) may have little influence on consumer perception and preferences relative to no treatment.

In terms of bitterness, a comparison between S2 and S1 showed a mean difference of −1.16 (95% CI [-1.6193, -0.7007]), while that of S3 and S1 showed a mean difference of −1.57 (95% CI [-2.0293, -1.1107]).

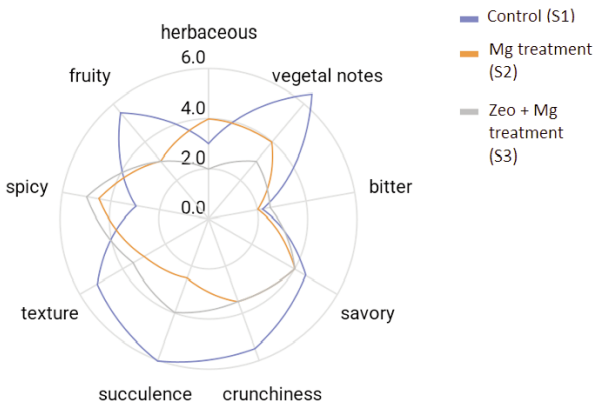


Fig. 1 - Sensory profile of radish. The Mg treatments have influenced the aromatic profile, in particular, in gustatory and tactile aspects.

These findings indicate that both treated samples (S2 and S3) were perceived as significantly less bitter than the control sample (S1). The larger reduction in bitterness observed for S3 suggests that the combination of zeolite and magnesium treatment exhibit a greater decrease in bitterness intensity compared with both magnesium-only treatment and the control. The results can be explained by a decrease of tannins compounds, that may influence flavor, color, and nutrient absorption, and at the same time, they are subject to variation due to factors such as ripeness and food processing methods, as suggested by previous studies (Cosme *et al*., 2025; Gautam *et al*., 2025).

Similarly, S2 and S3 were perceived as significantly less crunchy than the control sample (S1). The mean difference between S2 and S1, and S3 and S1 is -2.01 respectively. This suggests that the treatment samples exhibited the same degree of reduction in crunchiness.

Furthermore, we observed that the treatment sample S2 and S3 statistically decreased the degree of succulence relative to the control sample (S1), among the tasters (mean difference = -1.9, 95% CI [-2.3543, -1.4456]; -3.39, 95% CI [-3.8444, -2.9356]). This suggests that magnesium and Zeo+Mg treatment may be unfavorable in enhancing the characteristics associated with juiciness or moisture retention, ultimately resulting in a less succulent sensory experience for consumers.

The finding can be attributed to micro-zeolite capacity to improve water retention and nutrient availability in horticultural systems. According to Contreras-Martínez *et al.* (2026), however, its influence on plant physiological responses may vary depending on plant species and application rate.

Regarding the texture attribute, we found that both treatment samples (S2 and S3) significantly reduce the intensity of texture relative to the control sample (S1). Although both exhibit a reduction in texture, S2 showed greater reduction in texture than S3, implying a significant difference in treatment samples.

Moreover, the results indicate that sample S2 and S3 were perceived as significantly spicier than S1 (mean difference = 2.59, 95% CI [2.1718, 3.0082]; 1.63, 95% CI [1.2118, 2.0482]). The higher perception of spiciness observed in treated samples may be associated with changes in the concentration of pungent compounds, such as isothiocyanates, although no chemical characterization was performed to confirm this hypothesis. The finding is consistent with Gunjal *et al.* (2026), who applied foliar treatments and highlighted an enhancement of antioxidant activities compounds (i.e. total phenolics, flavonoids, chlorophyll, carotenoids, anthocyanins), to indicate a strong association among growth and biochemical composition, under controlled foliar nutrition.

Finally, the analysis showed that the treated samples (S2 and S3) exhibited lower degree of fruitiness relative to the control sample (S1). The mean difference is -2.79, 95% CI (-3.2708, -2.3092) and -2.57, 95% CI (-3.0508, -2.0891) respectively.

These results demonstrated first, that the aromatic profile can be modified by dosage of nutrients and foliar treatments applied, and second that the zeolites can regulate and drive the reaction mechanisms and the catalytic activity with effect on texture and structure (Thangaraj and Lee, 2025).

Finally, the identification and quantification of the compounds responsible for the organoleptic aspect can influence the consumption of the product.

Neophobic individuals tend to have unfavorable perceptions and lower expectations regarding the taste of new food products, such as functional foods, thus they resist trying or consuming them (Yaşa Özeltürkay *et al*., 2025). Previous research has suggested that the acceptance of these foods depend on the type of base product that serves as the vehicle for the specific ingredient and to their commercial and market value (Lu *et al*., 2020).

Furthermore, for the enrichment food consumption, the positive perceptions of health benefits related to the fortifier increased consumers' willingness to buy such innovative products, while under sensory profile the attributes considered more relevant were foods' taste and texture (Embling *et al*., 2024).

However, the type of food produced, processing and the physio-chemical properties of the active ingredients (for example solubility, chemical interactions and stability) could influence the effective functionality of nutraceutical foods and, consequently, their health benefits may not be recognized by the human body. Furthermore, some nutraceuticals can negatively influence the chemical and sensory properties over time, or the shelf life of foods, and can lead to undesirable effects in the case of interactions with other food components.

Therefore, it is essential to focus information about the functionality of these foods, emphasize the health and environmental benefits of enrichment foods, linked to sustainable agriculture practices (Russo *et al*., 2020).

In fact, from a circular economy perspective, the use of active compounds from agriculture waste and by-products as a source of nutritional added-value compounds can be crucial for functional food production and to sustainable development. For example, organic waste from vinary chains, thanks to their high content of polyphenols, has attracted increasing interest to improve their nutritional value, as well as to enhance sensory properties, by increasing the acidity, bitterness, and purple color attributes (Almanza-Oliveros *et al*., 2024). Nonetheless, it is important to evaluate the food matrix, including texture of the original product and how it can be incorporated in the design of a new product to obtain a tasteful and balanced flavor without affecting the appearance and appeal of the product (Theagarajan *et al*., 2019). Finally, according to Gunjal *et al*. (2026), these findings demonstrate the potential of Magnesium targeted foliar sprays to optimize the nutritional and sensory quality of radish plant.

4. Conclusions

Nowadays consumers show a positive attitude towards innovative foods and their positive effects on human health, in particular, when they are informed of the benefits linked to the intake of the functional element.

The present study demonstrated that foliar Mg biofortification effectively increased Mg accumulation in radish tissues and significantly modified the sensory profile of the product. The treatments altered several sensory attributes, reducing bitterness and increasing perceived spiciness, while also affecting texture-related properties such as crunchiness and succulence.

Therefore, nutrient-based biofortification strategies can influence not only the nutritional composition but also the organoleptic characteristics of horticultural products.

Further studies involving larger consumer panels, hedonic tests, and additional nutritional and phytochemical analyses are required to assess the market potential and functional value of biofortified vegetables.

Nonetheless, the study enriches the current literature on enrichement foods, in particular, on sensory assessment of horticultural products obtained with a fertirrigation foliar. It provides practical knowledge on the fortification process of an enriched food, and through sensory analysis potential consumer acceptance of the product is revealed. Moreover, the study outlines practical and theoretical implications on sustainable consumption.

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Review paper

Pre and postharvest determinants of quality in hydroponically grown lettuce. A review

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Key words: Hydroponics, lettuce, postharvest quality, shelf life.

Abstract: Hydroponic lettuce production has increased rapidly due to its efficient use of water, nutrients, and space, as well as its ability to consistently produce high-quality lettuce. However, hydroponically grown lettuce remains highly perishable, and maintaining quality after harvest is a significant challenge. This review summarizes the key pre and postharvest factors affecting the quality of hydroponically grown lettuce, including appearance, texture, flavor, nutritional composition, and shelf life. Preharvest factors such as nutrient management, water quality, environmental conditions, cultivar selection, and plant growth regulators influence structural and physiological characteristics at harvest. These characteristics interact with postharvest conditions, including temperature, relative humidity, handling practices, ethylene exposure, and microbial activity, to determine storage performance. Compared with soil-grown lettuce, hydroponically grown lettuce may differ in hydration, although postharvest recommendations are often generalized from conventional systems. This review highlights existing knowledge gaps and emphasizes the need for integrated, hydroponic-specific strategies to improve quality retention and shelf life.

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1. Introduction

Hydroponic lettuce production has experienced rapid growth due to its efficient use of water, nutrients, and space, making it a crucial system for meeting the growing demand for fresh and high-quality leafy vegetables (Debangshi, 2021). Lettuce (*Lactuca sativa L.*) is valued for its crisp texture, appealing appearance, and nutritional benefits; however, it remains one of the most perishable vegetables (Adhikari, 2025). Lettuce undergoes rapid physiological changes after harvest, such as wilting, browning, tissue softening, and nutrient degradation due to transpiration and respiration, which reduce its shelf life and marketability (Yang *et al*., 2024). Therefore, understanding the factors that influence postharvest quality is essential for maintaining freshness and reducing losses throughout the supply chain. Hydroponic production can enhance lettuce quality by providing optimal nutrient availability, optimum growth

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conditions, and reduced soilborne contamination (Jan *et al*., 2020). Preharvest factors such as nutrient solution composition, environmental stress, and water quality, combined with postharvest factors including temperature, humidity, ethylene exposure, and handling practices, strongly influence visual quality, texture, flavor, and nutrient stability (Damerum *et al*., 2020). However, research on harvest and postharvest behavior of hydroponic lettuce remains limited compared to that of soil-grown lettuce. While several factors affecting lettuce quality have been investigated, gaps remain in understanding their combined effects under hydroponic production systems. This review summarizes current knowledge on the quality of hydroponic lettuce and factors affecting postharvest performance, identifies key research gaps, and outlines future directions to improve the quality of hydroponically grown lettuce.

2. Methodology

This narrative review was synthesized based on peer-reviewed literature retrieved from Google Scholar, ScienceDirect, and Web of Science, covering studies published between 2000 and 2025. Keywords related to hydroponic lettuce, postharvest quality, shelf life, and controlled environment agriculture were used. Studies focusing on hydroponically grown lettuce were prioritized. Soil-grown lettuce was included only when hydroponic-specific evidence was limited and used for comparative interpretation. Non-peer-reviewed articles and studies unrelated to the review focus were excluded. The selected literature was qualitatively analyzed to identify key factors influencing the quality and shelf life of hydroponically grown lettuce.

3. Key postharvest quality attributes of lettuce

Quality refers to the characteristics or attributes that determine the value and suitability of food for human consumption (Kader, 1983). In lettuce, quality is defined by key attributes including appearance, texture, flavor, and nutritional composition, which are essential for freshness, marketability, and consumer acceptance (Kader, 2002 b; Ahmed *et al*., 2024). These quality attributes are influenced by cultivar selection, preharvest growing conditions, harvesting practices, and postharvest handling, and together they serve as important indicators of shelf life and overall product stability (Damerum *et al*., 2020).

Appearance

Visual appearance is a primary determinant of consumer acceptance of lettuce and strongly influences purchase decisions. Freshness is commonly associated with a vibrant green color and leaves free of blemishes, discoloration, or decay. Mechanical damage during harvesting and processing, including shredding, tearing, and bruising, negatively affects visual quality and often results in immediate consumer rejection (Rico *et al*., 2007). Visual quality of romaine lettuce is frequently assessed using a 5-to-9-point rating scale developed by Kader *et al.* (1973), which integrates parameters such as firmness, decay, discoloration, wilting, and other visible defects. In addition to physical injury, factors such as microbial contamination and wound-induced responses can further compromise visual quality. Chlorophyll degradation is a significant contributor to yellowing leaves and a decline in green color intensity, resulting in reduced visual appeal during storage (Toivonen and Brummell, 2008). Color plays a critical role in food selection, preference, and overall consumer satisfaction, influencing sensory perception and acceptance (Clydesdale, 1993). During storage, biochemical processes such as enzymatic browning and pigment degradation alter lettuce coloration, thereby accelerating quality deterioration (Ihl *et al*., 2003; Rico *et al*., 2007). Conversely, controlled light exposure during storage has been shown to maintain or enhance chlorophyll content, thereby improving visual quality and delaying senescence in lettuce leaves (Kasım and Kasım, 2017). However, storage under continuous illumination may raise product temperature, accelerating respiration and water loss, resulting in a negative impact on lettuce's shelf life. Even a slight increase in storage temperature can substantially elevate respiration rate due to the temperature sensitivity of plant metabolic processes. Although LED lighting systems generate less radiant heat than conventional light sources and have relatively cool surfaces (Sena *et al*., 2024), localized heat accumulation may still occur in enclosed systems. Perera *et al*. (2022) reported that LED lighting is an excellent alternative for preserving fresh vegetables; however, it must be integrated with adequate

cooling and air circulation to prevent unintended thermal stress. Therefore, illuminated storage should be engineered carefully to balance chlorophyll retention and metabolic stability. This highlights a critical trade-off between maintaining visual quality and physiological stability, underscoring the need for integrated light-temperature management strategies. Most studies focus on evaluating visual quality using a scoring system developed for soil-grown lettuce, which may not fully record the changes specific to hydroponically grown lettuce.

Texture

Leaf texture, defined by firmness and juiciness, is a critical component of postharvest quality and strongly influences consumer preference. Firmness is primarily determined by leaf anatomical characteristics, including cell wall thickness, cell size, cell-to-cell adhesion, and turgor pressure (Toivonen and Brummell, 2008). Due to its high surface area, lettuce is particularly sensitive to water loss, facilitating rapid moisture exchange and accelerating dehydration during storage (WUR, 2025). Excessive water loss during storage disrupts cellular integrity, leading to tissue softening and a decline in textural quality (Lee and Chandra, 2018). Additionally, factors such as initial leaf firmness, storage temperature, and mechanical stress during handling and transportation significantly contribute to texture degradation and moisture loss (Zhang *et al*., 2023; Shezi *et al*., 2024). Conversely, elevated calcium concentrations in plant tissues have been shown to strengthen cell walls and delay softening, thereby helping to maintain textural quality during postharvest storage (Kader, 2002 b). Calcium plays a critical role in cell wall structure by interacting with pectin to form calcium pectate, which stabilizes the cell wall and middle lamella (Sahin *et al*., 2025). Calcium uptake and distribution within plants are largely driven by tissue transpiration and the rate of Ca2+ absorption (McLaughlin and Wimmer, 1999; Yang and Jie, 2005). High humidity in a greenhouse, especially during winter, can reduce transpiration, limiting calcium transportation to rapidly growing tissue and increasing the risk of calcium deficiency symptoms. Calcium deficiency primarily affects young leaves, leading to disorders such as tip burn in lettuce, which appear as brown necrotic lesions along the edges of younger leaves. Similarly, calcium deficiency reduces water and nutrient absorption, leading to a loss of turgor pressure and a softer leaf texture (Sahin *et al.*, 2025). Although several studies link texture degradation to water loss and calcium availability, the influence of hydroponic nutrient management and root-zone conditions on the stability of postharvest texture remains less well understood.

Flavor

Flavor is a complex sensory attribute resulting from the integration of taste, aroma, texture, and temperature, and it may also be influenced by the visual appearance of food (Touzani and Sclafani, 2017). Although flavor is a critical component of overall product quality and consumer satisfaction, it often receives less attention than visual attributes and can deteriorate before visible signs of spoilage become apparent (Damerum *et al*., 2020). In lettuce, flavor is largely influenced by the balance of sugars and bitter compounds. Glucose contributes to sweetness and positively affects consumer preference, whereas bitterness negatively impacts acceptability (Chadwick *et al*., 2016). During storage, ongoing respiratory metabolism degrades sugars and organic acids, resulting in a gradual decline in flavor quality over the shelf life period (Saltveit, 2015).

Nutritional content

Lettuce is widely consumed due to its low-calorie content and favorable nutritional profile, which contribute to a healthy diet (Kim *et al*., 2016). The principal nutritional components of fresh lettuce are summarized in Table 1.

These values may vary depending on cultivar, growing conditions, and environmental factors. In addition to primary nutrients, lettuce contains a diverse range of bioactive compounds, including polyphenols such as chicoric acid and chlorogenic acid, as well as anthocyanins, quercetin, and kaempferol derivatives (Nicolle *et al*., 2004; Llorach *et al*., 2008; Damerum *et al*., 2015). These phytonutrients have been linked to various health benefits, including a reduced risk of cancer, obesity, and cardiovascular and neurological disorders (Martin, 2013; Cory *et al.*, 2018). Rather than acting solely as direct antioxidants, many phytochemicals enhance endogenous antioxidant defense systems through cellular signaling pathways. For example, quercetin has been shown to activate the Sestrin-2-AMPK-mTOR signaling pathway *in vitro* models, thereby contributing to the suppression of colon cancer cell growth (Kim *et al*., 2013; Bjørklund and

Table 1 - Nutritional composition of fresh lettuce (per 100 g fresh weight)

Component category	Nutrient	Amount (per 100 g FW)
Moisture	Water	94.3 g
	Carbohydrates	4.06 g
Macronutrients	Protein	0.98 g
	Lipids	0.30 g
	Potassium	260 mg
Minerals	Calcium	28 mg
	Phosphorus	23 mg
	Magnesium	12 mg
	Vitamin A	8710 IU
	β-carotene	5230 µg
	Lutein + Zeaxanthin	23,120 µg
Vitamins and bioactive compounds	Folate	136 µg
	Vitamin K	102 µg
	Vitamin E (α-tocopherol)	0.14 mg

Values for moisture, macronutrients, and minerals are available at USDA-ARS, 2022; values for vitamins and bioactive compounds at USDA-ARS, 2019.

Chirumbolo, 2017). From a nutritional perspective, consuming raw lettuce is preferable, as thermal processing, such as boiling, can significantly reduce levels of heat-sensitive phytochemicals, including chlorogenic acid (Clifford, 2000). Although the nutritional composition at harvest has been widely documented, relatively few integrative studies have examined nutrient retention and postharvest stability, especially in a hydroponic system under commercial storage conditions. Therefore, further research is needed to determine how hydroponic nutrient management, light regimes, and storage conditions interact to influence the stability of vitamins and phytochemicals throughout the postharvest supply chain.

4. Factors affecting postharvest quality of hydroponically grown lettuce

The postharvest quality of lettuce is influenced by both preharvest and postharvest factors from production to consumption. As pre and postharvest factors operate in an interconnected manner, a system-based perspective is important to understand quality development. Figure 1 presents a conceptual model linking preharvest practices to structural and physiological status at harvest, postharvest processes, and ultimate quality outcomes.

Preharvest factors

Production systems, such as hydroponics and soil-grown methods, can impact the postharvest quality of lettuce. Hydroponically grown lettuce can influence hydration and structural integrity due to the constant availability of water and nutrients; however, it has been reported to be more sensitive to weight loss, which decreases its shelf life compared to lettuce grown in soil (Manzocco *et al.*, 2011). This may be due to the higher initial moisture content in the leaves, which makes them more prone to moisture loss during storage. However, hydroponic lettuce, when harvested with its root, helps to maintain freshness during storage (Suo *et al*., 2021). Furthermore, Pace *et al*. (2018) reported that soilless-grown multi-leaf lettuce exhibited improved microbial stability and lower ammonium accumulation during storage than soil-grown lettuce. Water quality plays a crucial role in a hydroponic system as it can directly impact crop growth, nutrient uptake, and overall crop health. Poor water quality may be contaminated with microbes, which can cross-contaminate edible lettuce leaves during production, leading to spoilage or disease outbreaks (Dankwa *et al*., 2019). Optimizing the yield and nutritional quality of lettuce in a hydroponic system can be achieved through effective nutrient management. Excessive nitrogen (N) application leads to higher nitrate accumulation in lettuce tissue,

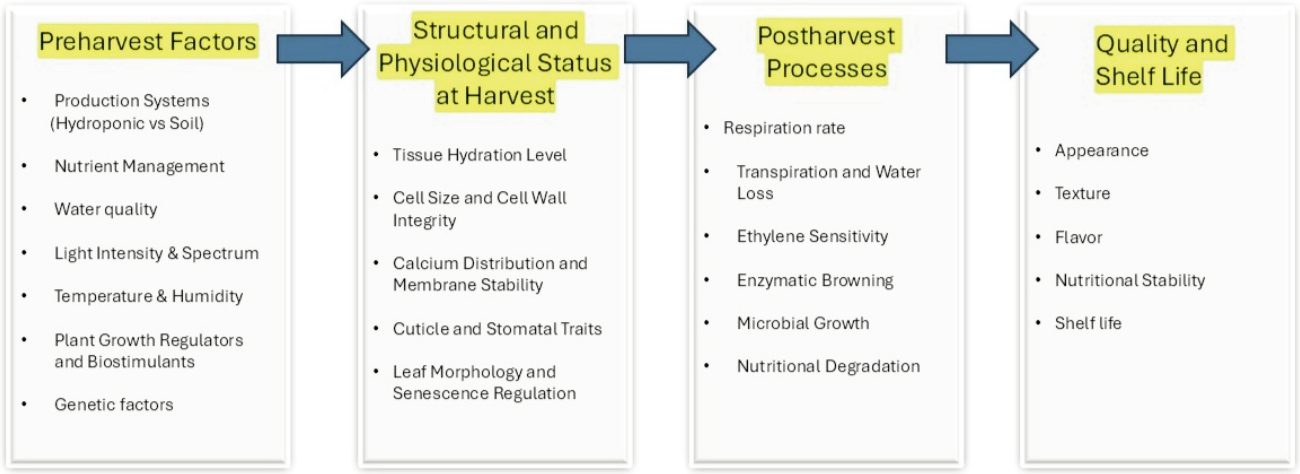


Fig. 1 - Conceptual framework illustrates how preharvest factors in hydroponic production systems influence structural and physiological characteristics at harvest, which regulate postharvest processes and ultimately determine quality attributes and shelf life of lettuce.

resulting in storage disorders, increased susceptibility to physiological damage, and a greater risk of postharvest decay (Hoque *et al.*, 2010). Nevertheless, replacing the nutrient solution with water 72 hours before harvest reduces nitrate accumulation by 63% in leafy greens, thereby improving lettuce quality (Ruangrak *et al*., 2023). Low level of nitrate (NO_3) application during production can increase soluble sugar and Vitamin C (Resh, 2016; Guo *et al.*, 2019). Moreover, the application of calcium chloride ($CaCl_2$), known for preventing calcium deficiencies, also enhances chlorophyll levels during storage (Perucka *et al*., 2014)**.** Therefore, a balanced nutrient formula is recommended to maintain the yield, nutritional value, and extended shelf life by ensuring proper nutrient uptake (Bar-Yosef *et al*., 2009).

Plant growth regulators and biostimulants applied during production can influence the postharvest quality and shelf life of hydroponically grown lettuce. For instance, the application of gibberellic acid (GA_3) at a concentration of 10^{-6} mol L^{-1} in the nutrient solution has been shown to delay senescence, reduce nitrate accumulation, and maintain overall quality in lettuce during storage (Miceli *et al*., 2019). As a plant growth regulator, GA_3 plays a crucial role in regulating physiological processes associated with leaf expansion and senescence, thereby indirectly influencing postharvest performance. In addition to plant growth regulators, biostimulants derived from natural sources have gained increasing attention for their role in improving lettuce quality. For example, the application of the seaweed-based bio-stimulant *Ecklonia maxima* in nutrient solutions has been reported to enhance yield and quality attributes at harvest, including biomass accumulation, leaf expansion, stomatal conductance, water-use efficiency, and nitrogen-use efficiency (Miceli *et al*., 2021). These improvements in plant physiological status at harvest may contribute to enhanced postharvest quality and shelf life. Genetic factors are also crucial for postharvest and shelf life. Research by Chase *et al.* (2024) found that genetic markers, such as senescence-associated genes (SAGs) and transcription factors (e.g., WRKY, NAC, JAZ), linked to leaf morphology can prolong lettuce shelf life. Modifications to the lettuce cell wall improved texture and reduced water loss, both of which are essential for extending shelf life. For example, the addition of silicon (84 mg L^{-1}) to nutrient solutions during the growing period enhances growth and development, reduces water loss, and consequently results in greater firmness during storage at 8±2°C for 16 days (Mattiuz *et al*., 2015). Neves *et al.* (2020) reported similar findings when silicon was applied directly to leaves in the form of calcium silicate at 4 mg L^{-1}, promotes growth and development and reduces postharvest weight loss. Harvesting time is another crucial factor that can impact postharvest quality. Harvesting too early or too late can result in suboptimal quality, leading to increased postharvest

loss (Alemu and Oanh, 2024). Environmental conditions, such as air temperature, affect the growth of hydroponic lettuce during production (Holmes *et al*., 2019; Liu *et al*., 2020). Temperatures above 28°C induce heat stress, leading to bolting, increased leaf latex, and bitter leaves (Holmes *et al*., 2019; Liu *et al*., 2020). Under controlled-environment conditions, higher light intensities (270 and 470 µmol m^{-2} s^{-1}) applied during 6-7 days before harvest significantly increase carbohydrates, vitamin C, and shelf life, especially at 469 µmol m^{-2} s^{-1} (Min *et al*., 2021). The study used red-white LEDs, with only light intensity varied rather than the spectrum. Therefore, the improvements were due to the higher light intensity rather than specific red, blue, or green wavelengths. Overall, while individual preharvest factors have been studied, their combined effects on postharvest quality are poorly understood.

Postharvest factors

Low temperatures are crucial for maintaining lettuce postharvest quality, as they reduce respiration (Watada and Qi, 1999). High temperatures increase respiration, which involves breaking down complex molecules, such as starch, sugars, and organic acids, into simpler molecules (CO_2 and H_2O), producing energy and intermediates essential for cellular maintenance (Watada and Qi, 1999; Kader, 2002 a). Similarly, relative humidity (RH) is another critical factor for maintaining postharvest quality during storage. Relative humidity affects the transpiration rate by altering the moisture gradient between plant tissue and the surrounding air (Saltveit, 2016). Therefore, maintaining an optimal relative humidity level (95-98%) is necessary to minimize moisture loss and extend shelf life (Agüero *et al*., 2011). Transpiration occurs when moisture is lost through stomatal openings, resulting in wilting and a decline in texture, pigment, and quality during storage (Kramer and Boyer, 1995; Agüero *et al*., 2011; Mansfield, 2012).

Using an appropriate harvesting tool is crucial to reducing mechanical damage. Harvesting lettuce with sharp-edge blades can reduce physical damage, while harvesting with blunt blades can increase cellular damage, loss of cell sap, and accelerate browning (Bolin *et al*., 1977).

Ethylene exposure during storage triggers enzymatic discoloration, tissue deterioration, wilting, and senescence, while elevated carbon dioxide (CO_2) levels can lead to brown staining, ultimately reducing the quality and shelf life of lettuce (Damerum *et al.*, 2020). Microorganisms, including pathogenic and saprophytic bacteria or fungi, are primary contributors to causing postharvest quality loss, as their growth alters lettuce's color, texture, and taste, making it unmarketable (Lugtenberg *et al.*, 2017; Myszka *et al*., 2017). High temperatures favor bacterial growth. For example, the Shiga toxin-producing strain *E. coli* O157:H7 grows faster at 12°C than at 5°C (Luo *et al*., 2010). Microbial metabolism increases respiration rates, leading to moisture loss, spoilage, and quality degradation during storage (Simko and Hayes, 2018). Lettuce surface-to-volume ratio, tissue damage, and chemical composition may further influence microbial proliferation (Kramer and Boyer, 1995; Mansfield, 2012). Hydroponically grown lettuce differs in hydration and nutrient status, which may alter postharvest handling responses. Most postharvest handling techniques are based on soil-grown lettuce, underscoring the need for further research on the storage and handling of hydroponically grown lettuce.

5. Discussion and Conclusions

The studies examined in this review suggest that postharvest quality in hydroponically grown lettuce is largely influenced by conditions established before harvest. Although storage temperature and relative humidity remain critical, structural and physiological traits developed during cultivation appear to determine postharvest responses. The continuous supply of water and nutrients in hydroponic systems promotes rapid vegetative growth and high tissue hydration (Manzocco *et al*., 2011), resulting in desirable visual freshness and crisp texture at harvest. However, accelerated cell expansion under these conditions may reduce structural reinforcement, potentially producing tissues that are highly hydrated (Toivonen and Brummell, 2008). Following harvest, these tissues may be more susceptible to moisture loss if storage conditions are inadequate. This mechanism may help explain postharvest weight loss in hydroponically grown lettuce compared with soil-grown systems, although direct comparative evidence remains limited.

Nutrient management plays an important role in determining structural stability. Nitrogen enhances

vegetative growth, yet excessive availability may increase nitrate accumulation and alter tissue composition, which can influence postharvest storage behavior (Hoque *et al*., 2010; Guo *et al*., 2019). In contrast, adequate calcium supports cell wall integrity and membrane stability, contributing to firmness and delayed senescence (Sahin *et al*., 2025). These contrasting roles highlight a potential trade-off between maximizing vegetative growth and enhancing postharvest durability. Optimizing hydroponic production, therefore, requires balancing nitrogen-driven productivity with an adequate supply of calcium to maintain structural resilience. Preharvest light management also appears to influence storage performance. Increased light exposure before harvest can stimulate carbohydrate and antioxidant accumulation (Min *et al*., 2021), which may improve tolerance to oxidative stress during storage. However, higher metabolic activity may simultaneously elevate respiration rates, particularly if accompanied by temperature increases (Kader and Saltveit, 2002). These observations indicate that improving a single quality trait does not always lead to longer shelf life. Another important consideration is that most existing postharvest recommendations were developed using a conventional production system (Kader, 2002 a). Given the differences in hydration status and nutrient supply under hydroponic cultivation, it is reasonable to question whether conventional postharvest recommendations are fully transferable. Hydroponic lettuce may require more precise humidity control and rapid cooling to minimize water loss and physiological deterioration. Overall, the available evidence supports a more integrated perspective in which preharvest management decisions shape structural characteristics, metabolic status, and stress responses, ultimately determining postharvest behavior. Future research should focus on coordinated preharvest-to-postharvest studies that evaluate how production strategies can be adjusted to enhance resilience during storage, rather than treating cultivation and storage as independent phases.

This review demonstrates that lettuce quality is influenced by both preharvest factors, including production systems, nutrient management, environmental conditions, and the use of plant growth regulators and biostimulants, as well as postharvest factors such as temperature, relative humidity, handling practices, ethylene exposure, and microbial activity. Although extensive research on lettuce exists, information specific to hydroponically grown systems remains limited and fragmented. Most studies focus on individual factors, and postharvest recommendations are often based on soil-grown lettuce, even though hydroponic lettuce differs in tissue hydration. Future studies should formulate and test clear hypotheses on how hydroponic nutrient management, light regimes, and environmental controls influence structural traits at harvest and, ultimately, determine postharvest stability and shelf life.

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