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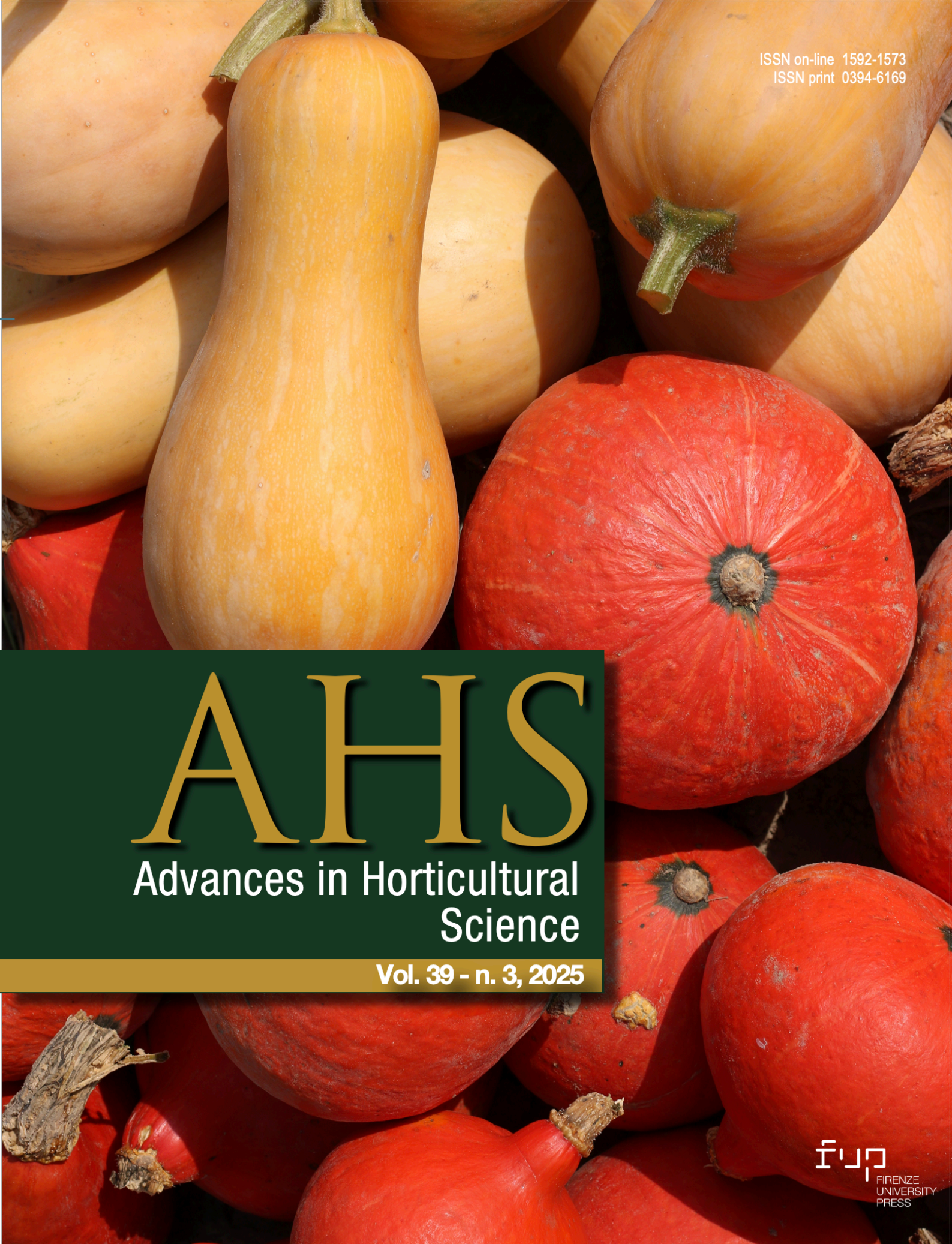

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Digital and multivariate analysis of lettuce seed vigor: Impact of hydropriming on physiological potential

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Key words: Applied statistics, image analysis, physiological variability, seed priming.

Abstract: Digital image analysis has emerged as a highly precise and efficient methodology for assessing the physiological attributes of seeds. This research aimed to assess the morphological and physiological properties of lettuce seeds subjected to hydropriming using multivariate statistical approaches. Two lettuce genotypes, Roxa and Vanda, were evaluated under hydropriming treatments (primed-dry and primed-stored). Seedlings were digitally scanned, and vigor indices were quantified using the Seed Vigor Imaging System (SVIS®). Data were analyzed by multivariate analysis of variance (MANOVA), with tests of normality and homogeneity of covariance ensuring analytical robustness. The primed-dry treatment resulted in minimal improvement in vigor and uniformity, while the primed-stored treatment promoted a partial recovery of these attributes. The Roxa genotype exhibited greater variability in vigor and seedling length, whereas Vanda demonstrated higher uniformity but slightly reduced seedling growth. A strong positive correlation was observed between the vigor index and seedling length, reinforcing the importance of these parameters in seed quality assessment. These findings underscore the utility of digital image analysis combined with multivariate statistical methods for the accurate assessment of seed vigor, thereby improving seed-lot classification and informing decision-making in lettuce production systems.

1. Introduction

Computerized analysis of seed and seedling images has emerged as an

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innovative tool for determining the physiological characteristics of these structures. This technology stands out for its objectivity, specificity in detecting subtle traits, efficiency, and potential for standardization (Rahman and Cho, 2016; Xia *et al*., 2019; Wang *et al.*, 2021; Liu *et al.*, 2023). The use of digital imaging and automated software enables the analysis of a large number of samples in shorter periods, increasing the efficiency and accuracy of evaluations.

Assessing the physiological potential of lettuce seeds through digital image analysis has proven to be a promising approach, particularly as a complement to conventional methods that do not fully reflect seed quality under real field conditions (Waters-Junior and Blanchette, 1983; Marcos-Filho, 1999). The use of the Seed Vigor Imaging System (SVIS®), developed by Sako *et al*. (2001), has been widely adopted to quantify seed vigor in various species, including soybean, corn, melon, sweet corn, castor bean, peanut, okra, common bean, eggplant, tomato, cotton, and sunflower (Hoffmaster *et al.*, 2003; Marcos-Filho *et al*., 2006; Marchi *et al*., 2011; Alvarenga *et al.,* 2013; Caldeira *et al.*, 2014; Gomes-Junior *et al.*, 2014; Rocha *et al.*, 2015). This technology allows for detailed analyses of parameters such as seedling growth uniformity and development, reducing the subjectivity of traditional evaluations. However, to enhance the accuracy of vigor assessment, it is essential to employ multivariate statistical models that enable the simultaneous analysis of multiple interrelated variables.

Multivariate Analysis of Variance (MANOVA) has been used to investigate complex interactions between experimental factors, allowing for the identification of patterns that would be difficult to detect using univariate approaches (Johnson and Wichern, 2002; Nicacio *et al*., 2013). Previous studies have demonstrated that MANOVA is an effective tool for evaluating seed performance under different treatments, ensuring greater robustness in result interpretation (Oliveira *et al*., 2013). This study aimed to analyze the morphological and physiological properties of lettuce seeds subjected to hydropriming using digital imaging of seedlings and a multivariate approach. The study sought to understand the interactions between the physiological attributes of the seeds and the impact of hydropriming on germination potential and early seedling development.

2. Materials and Methods

The research was conducted at the Seed Analysis Laboratories, the 'Professor Silvio Moure Cicero' Image Analysis Laboratory of the Department of Crop Science, and the Department of Math, Chemistry, and Statistics at the Luiz de Queiroz College of Agriculture, University of São Paulo in Piracicaba, SP, Brazil.

Seed material and priming treatment

Lettuce seeds from the genotypes Scarlet Red Crisphead (Roxa) and Vanda Crisphead (Vanda) were used, supplied by Sakata Seed South America Ltd. The selection of the two lettuce genotypes was based on their contrasting physiological and morphological characteristics and on their commercial relevance within Brazilian lettuce production systems. Each genotype was represented by ten seed lots, with germination rates within commercial standards and different vigor levels among the lots. Each seed lot was divided into three treatments: (i) non-primed seeds (control), (ii) hydroprimed dried seeds (dried in an oven at 30°C and 45-55% relative humidity for 96 hours), and (iii) hydroprimed stored seeds (dried and stored in a chamber at 10°C and 30% relative humidity for three months).

Hydropriming was performed using the drum method, utilizing the S-HIDRO® Control equipment, which allowed for the controlled application of water at regular intervals until reaching the required volume for each lot (Kikuti and Marcos-Filho, 2012). The calculation of the required water volume for this method was based on the water imbibition curve, considering the volume needed for each seed lot before primary root protrusion (Caseiro, 2003). At the beginning of each cycle, the electric pump was activated for 1 second, allowing the intake of a water volume between 0.9 and 1.1 ml, adjusted according to the specific needs of each seed lot and accounting for system losses to ensure 100% efficiency. Water application was carried out at one-hour intervals until the total required volume for each seed lot was reached (ranging from 4.00 to 4.31 ml for the Roxa genotype and from 3.67 to 3.87 ml for Vanda). The entire procedure was conducted under laboratory conditions at a constant temperature of 25°C.

Seed vigor assessment using digital image analysis (SVIS® Software)

Four replicates of 25 seeds per lot for each

treatment were arranged in two rows on the upper third of two blotter paper sheets, placed on the lids of transparent plastic boxes (11 × 11 × 3.5 cm). The boxes were covered with transparent plastic bags and incubated in a BOD chamber at 25°C for three days in darkness. To ensure proper seedling development according to natural geotropism, the boxes were positioned at a 70° angle relative to the horizontal plane.

To determine seed vigor, the Seed Vigor Imaging System (SVIS®) (Sako *et al*., 2001) software was used. The seedlings (and ungerminated seeds) from each replicate were transferred onto a blue ethylene-vinyl acetate (EVA) sheet, providing the necessary contrast for system analysis. The seedlings were then scanned using an HP Scanjet 200 scanner, which was inverted and placed inside an aluminum box (60 × 50 × 12 cm), with the resolution set to 300 dpi and connected to a computer. The scanned images were processed using SVIS® software, including manual corrections when necessary to ensure accurate seedling identification (Fig. 1). The seed vigor index is calculated according to the methodology proposed by Sako *et al.* (2001):

$$Vigor\ index = W_G \times Growth \times W_u \times Uniformity$$

$$Seedling\ length = W_G \{W_h \times l_h + W_r \times l_r, 1000\}$$

$$Uniformity\ index = max\ \{1000-(W_{sh} \times S_h + W_{sr} \times S_r + S_{total} + W_{(sr/h)} \times S_{(r/h)}-W_d\ ),0\}$$

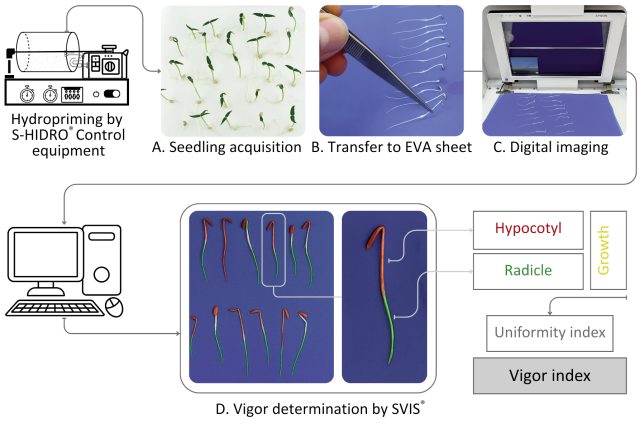

Fig. 1 - Workflow of computerized image analysis of lettuce seedlings, from germination to vigor assessment. Steps include seedling acquisition from the germination test (A), transfer to an EVA sheet (B), digital imaging (C), and vigor determination using the SVIS® software (D). The results include the vigor index (VI) and the uniformity index (UI), both ranging from 0 to 1000 (directly proportional to seedling vigor), as well as the average seedling length, initially measured in pixels and later converted to centimeters.

Vigor results from the combination of two main components: average seedling length and uniformity, both adjusted by weighting factors (*W*) defined by the system, allowing the assignment of greater or lesser relative importance to each characteristic (hypocotyl-to-radicle ratio of 40:60, applied in this research). Growth is estimated from the mean lengths of the hypocotyl (l_h) and radicle (l_r), weighted by their respective coefficients (W_h and W_r), thereby composing the total seedling length. Uniformity, in turn, is calculated based on the standard deviations of hypocotyl length (S_h), radicle length (S_r), total seedling length (S_{total}), and the hypocotyl-to-radicle length ratio ($S_{(r/h)}$), also weighted by their respective coefficients (W_{sh}, W_{sr}, $W_{(sr/h)}$).

Multivariate analysis

Multivariate Analysis of Variance (MANOVA) was used to describe the effects of categorical factors (treatments) on multiple response variables (Huberty and Olejnik, 2006). MANOVA extends ANOVA to the multivariate context, allowing simultaneous testing of multiple dependent variables. In this study, a two-way MANOVA was employed, considering two categorical factors: genotype (Roxa and Vanda) and hydropriming treatment (control, primed dry, primed stored). This factorial approach allows for the evaluation of both the main effects of each factor and their interaction.

The general model for Two-Way MANOVA:

$$X_{lkr} = \mu + \tau_l + \beta_k + \gamma_{lk} + \epsilon_{lkr}$$

X represents the dependent variable, *l* represents the levels of factor 1 (genotype); *k* represents the levels of factor 2 (hydropriming treatment); *r* represents the replications; μ is the overall mean; τ_l representing the interaction effect and β_k representing the treatment or genotype main effects; γ_{lk} is the interaction effect between the factors; represents the random error. The hypothesis tests included:

Interaction effect:

$$H_0: \gamma_{11} = \gamma_{12} = \cdots = \gamma_{gb} = 0 \text{ vs. } H_1: \text{at least one } \gamma_{gb} \neq 0$$

Genotype effect:

$$H_0: \tau_{11} = \tau_{12} = \cdots = \tau_g = 0 \text{ vs. } H_1: \text{at least one } \tau_g \neq 0$$

Hydropriming effect:

$$H_0: \beta_1 = \beta_2 = \beta_3 = 0 \text{ vs. } H_1: \text{at least one } \beta_b \neq 0$$

Statistical significance was determined using Wilks' lambda (Wilks, 1935), Pillai's trace (Hand and Taylor, 1987), Hotelling-Lawley trace (Krzanowsk and

Marriott, 1994; Anderson, 2003), and Roy's largest root (Krzanowski, 2000). When MANOVA indicated significant differences, post hoc univariate ANOVAs were conducted to determine which dependent variables contributed to the observed differences.

Statistical assumptions and data validation

Before conducting MANOVA, the following statistical assumptions were tested: multivariate normality, using the Henze-Zirkler test (Henze and Zirkler, 1990) and homogeneity of covariance matrices using Box's M test (Johnson and Wichern, 2002). In the univariate context, the Anderson-Darling test (Scholz and Stephens, 1987) was used to assess normality. Initial analyses revealed that seedling size did not meet the assumption of normality; therefore, this variable was removed from the final MANOVA model to ensure compliance with statistical assumptions.

Software and data processing

All statistical analyses were performed using the R programming language (R Core Team, 2024), with the packages 'MVN' for normality tests and 'car' for MANOVA. Data visualizations, including boxplots and correlation matrices, were generated using the 'ggplot2' and 'corrplot' packages. Image processing was performed using SVIS® software, ensuring standardization and reproducibility of seed vigor measurements.

3. Results

Descriptive data analysis

The descriptive analysis allowed the identification of the main characteristics of the studied variables. Figure 2 presents boxplots for the three response variables in this research: vigor index, uniformity index, and seedling length. It is observed that the means values of vigor and uniformity are similar between genotypes, while the dispersion of vigor is higher. Seedling length, measured in centimeters, is on a different scale from the other variables and exhibits a lower correlation with them. The Roxa genotype exhibits a higher median and greater variability, suggesting either greater vigor or increased heterogeneity. In contrast, the distribution of the uniformity index between the two genotypes is similar, indicating that growth is uniformly distributed.

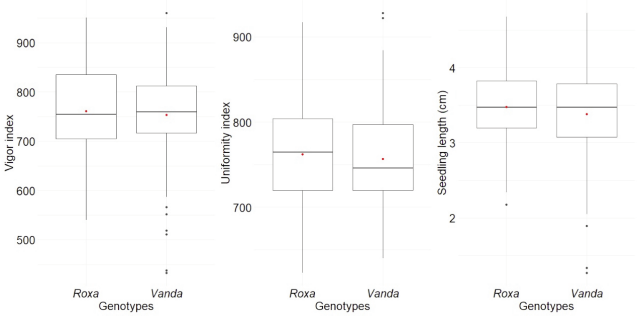

Fig. 2 - Boxplot comparing the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm) between Roxa and Vanda genotypes.

The boxplots for the analyzed variables concerning hydropriming treatments are shown in Figure 3. It is observed that vigor and uniformity vary significantly between treatments. Seeds subjected to the primed dry treatment showed lower means for these variables. Seedling length showed less pronounced differences, with the control treatment presenting the highest mean.

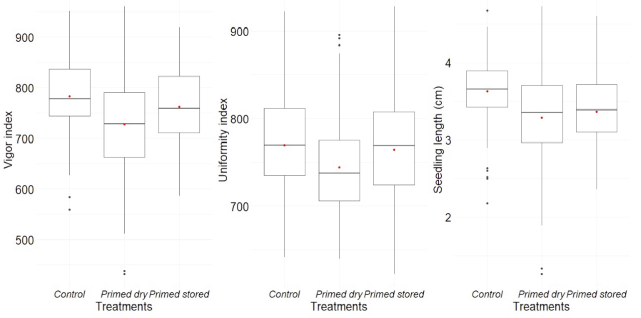

Fig. 3 - Boxplot comparing the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm) across control, primed dry, and primed stored treatments.

The mean values of the variables for each genotype are shown in Table 1, while Table 2 shows the corresponding values for each hydropriming treatment.

Table 1 - Mean values of vigor index, uniformity index, and seedling length for Roxa and Vanda genotypes

Genotype	Vigor index	Uniformity index	Seedling length (cm)
Roxa	761	762	3.47
Vanda	754	757	3.38

Table 2 - Mean values of vigor index, uniformity index, and seedling length for control, primed dry, and primed stored treatments

Hydropriming	Vigor index	Uniformity index	Seedling length (cm)
Control	783	770	3.63
Primed dry	727	745	3.29
Primed stored	762	765	3.37

The distribution, correlation, and dispersion of the vigor, uniformity, and seedling length, their correlations, and dispersion are shown in figure 4. The vigor index is strongly correlated with seedling length (r= 0.903), suggesting that more vigorous seedlings tend to be longer. The correlation between uniformity and length is moderate (r= 0.447), indicating a weaker association between these variables.

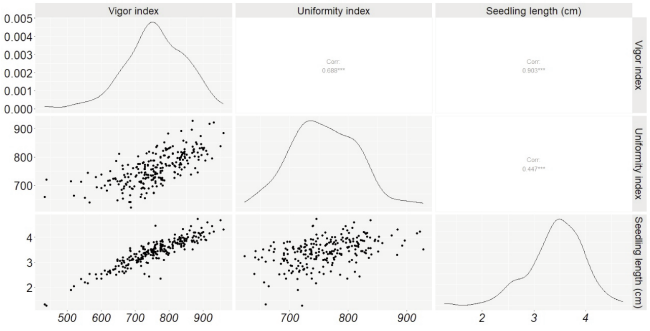

Fig. 4 - Distribution, correlation, and dispersion analysis of the vigor index, uniformity index, and seedling length (cm).

Interaction between genotypes and treatments

The interactions among hydropriming treatments within each genotype are illustrated in figure 5. The primed dry treatment had the least pronounced effect on vigor and uniformity, while primed stored allowed a partial recovery of these parameters. The interaction between genotypes within each treatment is shown in figure 6. Roxa exhibited a better response to the primed stored treatment, while Vanda demonstrated greater sensitivity. Seedling length was more affected in Vanda under the primed stored condition, whereas Roxa showed a tendency toward increased growth.

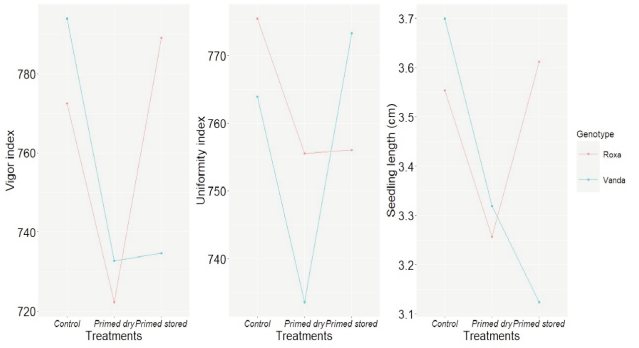

Fig. 5 - Interactions plot of hydropriming treatments within the Roxa and Vanda genotypes for the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm).

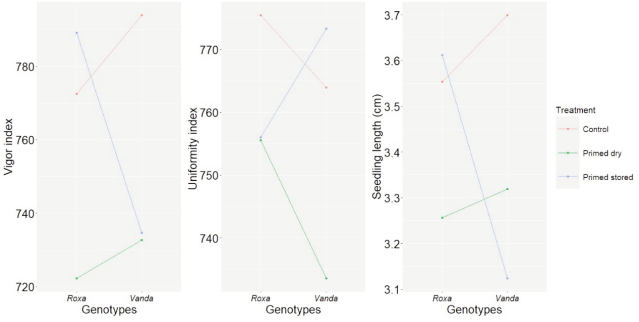

Fig. 6 - Interaction plot between Roxa and Vanda genotypes within each hydropriming treatment for the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm).

Assumptions of MANOVA

The suitability of the data for MANOVA was verified using the Henze-Zirkler and Anderson-Darling tests (Tables 3 and 4). The Henze-Zirkler test indicated that the data did not present multivariate normality due to the length variable. Therefore, this variable was removed for the MANOVA analysis (Table 5). After its removal, multivariate normality was achieved (Table 6). The Box´s M test for equality of covariance matrices confirmed that the data met

Table 3 - Henze-Zirkler test for assessing the multivariate normality of the full dataset

Test	Statistic	p-value	NMV*
Henze-Zirkler	1.84	0.00000005	No

*Not Meeting Validity criteria (normality violated).

Table 4 - Anderson-Darling test assessing the univariate normality of the vigor index, uniformity index, and seedling length variables

Variable	Statistic	p-value	Normality
Vigor	0.6619	0.0830	Yes
Uniformity	0.4255	0.3134	Yes
Seedling length	15.860	0.0004	No

*Not Meeting Validity criteria (normality violated).

Table 5 - Henze-Zirkler test after removal of the seedling length variable, indicating multivariate normality of the remaining variables

Test	Statistic	p-value	NMV*
Henze-Zirkler	0.9217	0.1112	Yes

*Not meeting validity criteria (normality violated).

Table 6 - Anderson-Darling test after removal of the seedling length variable, confirming the normality of the vigor index and uniformity index variables

Variable	Statistic	p-value	Normality
Vigor	0.6619	0.0830	Yes
Uniformity	0.4255	0.3134	Yes

*Not Meeting Validity criteria (normality violated).

Table 7 - Box´s M test for the equality of covariance matrices among the analyzed groups

Statistic	p-value
2.05	0.56

the required assumptions for MANOVA (Table 7). A two-factor MANOVA was performed to evaluate the interaction effect between genotype and hydropriming treatment. The Roy's largest root test indicated no significant differences between genotypes. However, the main effect of hydropriming treatment, as well as the interaction between genotype and treatment, were highly significant (Table 8). These results suggest that hydropriming treatments significantly influenced the analyzed variables, regardless of genotype.

4. Discussion and Conclusions

The findings of this research can be understood in light of the existing literature on seed physiological potential and vigor. Seed vigor is one of the main factors influencing success seedling establishment in the field and early plant development (Black and Bewley, 2000; Marcos-Filho, 2015). The multivariate analysis showed that the Roxa genotype exhibited greater variability in vigor and seedling length, whereas Vanda demonstrated greater uniformity in growth. These results are consistent with studies indicating that different genotypes may exhibit significant variations in their physiological responses (Hampton and Tekrony, 1995; Elias *et al*., 2012; Rahman and Cho, 2016; Cheng *et al*., 2023).

The significant interaction between hydropriming treatments and genotypes supports the hypothesis that the priming response may be cultivar-specific. The primed dry treatment had a weaker effect on vigor and uniformity, as reported in previous studies, which suggest that osmotic stress generated during the process may compromise seed physiological potential (Raj and Raj, 2019; Lewandowska *et al.*, 2020; Pirasteh-Anosheh and Hashemi, 2020; Rhaman *et al.*, 2020 a, 2020 b), particularly during the period immediately following treatment. Conversely, the primed stored treatment exhibited partial recovery of vigor and uniformity parameters, in agreement with studies highlighting the ability of seeds to

Table 8 - MANOVA results for genotype and hydropriming treatment factors, considering the vigor index and uniformity index variables

Source	DF	Roy's Statistic	F Approximation	Num. DF	Den. DF	p-value
Genotype	1	0.002587	0.3014	2	233	0.74
Hydropriming	2	0.0726	85.044	2	234	0.0002721 ***
Interaction	2	0.2100	245.726	2	234	0.00002 ***
Residuals	234					

stabilize after hydropriming when stored under appropriate conditions (Farooq *et al*., 2006, 2010; Huang *et al.*, 2015; Souza *et al.*, 2016; Farooq *et al*., 2021). Furthermore, the current results support research indicating that the effectiveness of hydropriming may vary depending on genotype and environmental conditions (Muhie *et al*., 2024). Recent studies emphasize the importance of evaluating each cultivar separately to determine the most suitable seed treatment method (Cheng *et al.*, 2023; Qiu *et al.*, 2023).

The positive correlation between vigor and seedling length reinforces the relevance of these variables in seed quality assessment. The literature suggests that more vigorous seedlings tend to develop stronger root systems and exhibit higher field emergence rates (Kikuti and Marcos-Filho, 2012; Kikuti and Marcos-Filho, 2013; Alvarenga and Marcos-Filho, 2014; Marcos-Filho, 2015; Rego *et al*., 2023). Prior studies indicate that seed vigor is closely associated with early seedling growth and crop establishment (Marcos-Filho, 2015).

Image analysis has proven to be a promising tool for evaluating seed vigor. Technologies such as the Seed Vigor Imaging System (SVIS®) have demonstrated a high degree of precision in classifying lettuce seed lots and those other crops (Gomes-Junior *et al*., 2009; Rodrigues *et al*., 2020). The use of computer vision and machine learning in seed vigor assessment is increasingly being explored, enabling fast and objective analyses (De Medeiros *et al.*, 2020; Wang *et al.*, 2021; Liu *et al*., 2023; Pang *et al.*, 2023).

The statistical methodology adopted in this research was essential to ensure the robustness of the analyses and the reliability of the results. Initially, the descriptive analysis enabled the identification of trends and patterns in the data, facilitating interpretation. To assess relationships among variables, Pearson´s correlation was applied, revealing a strong association between the vigor index and seedling length. The main statistical method used was Multivariate Analysis of Variance (MANOVA), a widely accepted methodology approach studies involving correlated dependent variables (Oliveira *et al.*, 2013; Din and Hayat, 2021; Baumeister *et al.*, 2024).

MANOVA is particularly suitable when response variables are correlated, allowing for the simultaneous evaluation of the effects of experimental factors (Johnson and Wichern, 2002). To ensure the method's applicability, the Henze-Zirkler test was used to assess multivariate normality, and Box's M test was applied to verify the homogeneity of covariance matrices-a key assumption for valid MANOVA results. The significance of main effects and interactions was assessed using Roy's largest root, which is recommended when effects have a strong impact on data variability (Kose *et al.*, 2018).

The MANOVA results were complemented by univariate analyses, allowing for a more detailed interpretation of the individual factor effects, as suggested by Scholz and Stephens (1987). This combined approach improves precision in identifying significant effects and interactions, thereby enhancing the understanding of genotype responses to hydropriming. However, such statistical procedures also have limitations. In this study, seedling length had to be excluded from the final MANOVA due to the violation of normality assumptions, which restricted the scope of multivariate interpretation.

In conclusion, the Roxa genotype performed better under the primed stored treatment than Vanda. Seedling length was influenced by hydropriming, with primed stored proving unsuitable for Vanda. Therefore, the statistical approach adopted in this study enabled a comprehensive and detailed analysis, enhancing our understanding of the effects of hydropriming on the evaluated genotypes. These findings are crucial for understanding genotypes-treatments interactions and may contributing to the optimization of hydropriming strategies for lettuce seeds. Future studies should consider incorporating a broader range of cultivars, extended storage durations, and the integration of machine learning techniques to improve vigor prediction.

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Effect of auxins on the rooting of the avocado rootstock 'Duke 7'

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Key words: Callus formation, clonal propagation, *Persea americana* Mill., root quality index.

Abstract: Clonal propagation of avocado rootstocks through etiolated shoot rooting represents a key strategy to enhance genetic uniformity, plant health, and productivity in commercial orchards. However, its success largely depends on the rooting phase, where auxins play a critical role. This study evaluated the effect of auxin-based rooting agents (types and concentrations) on root induction and quality in etiolated shoots of the 'Duke 7' rootstock. Five agents (IAA, NAA, IBA, K-IBA, and IBA + NAA combination) were tested at three concentrations (24.6, 34.4, and 44.2 mM) under a completely randomized factorial design (5 × 3) with three replicates per treatment. Morphological variables included rooting percentage, survival rate, root number/length/diameter, secondary root development, callus formation, and root quality index (RQI). Results revealed significant effects of agent type, concentration, and their interaction. NAA (34.4 mM) was the most effective for root number (55.3) and RQI (154.9 cm), albeit with high callus formation and reduced secondary roots. The IBA + NAA combination (34.4 mM) also showed high RQI (140.4 cm), with greater root length and less negative impact on root architecture. IBA alone achieved 100% rooting with moderate root development, balancing efficacy and physiological tolerance. Overall, intermediate concentrations of NAA and IBA + NAA yielded optimal results. These findings can refine clonal propagation protocols for 'Duke 7', with direct applications in commercial nurseries producing high-performance rootstocks.

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1. Introduction

Commercial avocado trees result from the combination of tissues from two distinct plants: A scion that forms the canopy and a rootstock that

provides the root system (Gleeson *et al*., 2016). This propagation technique enables cultivars to achieve early productivity while preserving their phenotypic traits, even when grafted onto juvenile plants (Melnyk, 2017).

In Mexico, commercial avocado nursery production primarily relies on seed-propagated rootstocks derived from native trees exhibiting broad yet poorly characterized genetic diversity (Salazar-García *et al*., 2004 a). This genetic variability leads to heterogeneous tree growth (Medina-Urrutia *et al*., 2017) and increased susceptibility to diseases, pests, and abiotic stressors such as drought, salinity, or nutrient imbalances (Salazar-García *et al*., 2004 a). Furthermore, rootstock type has been documented to directly influence key agronomic parameters including yield, tree size and vigor, as well as fruit quality and postharvest life (Barrientos-Priego, 2017).

The most effective strategy to mitigate issues arising from genetic heterogeneity involves using clonal rootstocks (Salazar-García *et al*., 2004 b; Cohen *et al*., 2023). Although avocado trees grafted onto clonal rootstocks are more expensive than those on seedling rootstocks (Cohen *et al*., 2023), certain Mexican production areas affected by *Phytophthora cinnamomi* Rands (Ochoa-Fuentes *et al*., 2007; Sánchez-González *et al*., 2019), clay soils (Salazar-García *et al*., 2015), alkaline pH, and soil salinity (Medina-Urrutia *et al*., 2017) would significantly benefit from their implementation.

While clonal rootstocks can standardize production, they present inherent technical limitations, particularly during the rooting phase (Ernst, 1999; Gleeson *et al*., 2016). Current commercial methodologies for clonal propagation of avocado species are primarily derived from the technique established by Frolich and Platt (1972). This approach, albeit with potential minor modifications, involves performing air layering on an etiolated shoot, separating it from the mother plant once a root system develops, and grafting the commercial cultivar during the rooting phase when stem diameter permits (Ernst, 1999; Ernst *et al*., 2013).

The formation of adventitious roots in woody species such as avocado is regulated by the balance of growth regulators, with auxin application being one of the most effective strategies to promote rooting (Zhao *et al*., 2022). Synthetic auxins like indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) serve as the primary regulators of adventitious root formation through complex interactions that modulate metabolic, transport, and signaling processes (Lakehal and Bellini, 2019).

Recent studies demonstrate that auxin type and concentration influence rooting quality through mechanisms such as molecular stability, transport, and metabolite conjugation (Damodaran and Strader, 2019; Gomes and Scortecci, 2021). However, current avocado propagation protocols remain based on classical work (Frolich and Platt, 1972), typically limited to IBA application at 7000 mg L^{-1} (Ernst, 1999), without considering the efficacy of other auxin types or concentrations. Furthermore, few recent studies have explored these aspects (Li *et al*., 2024) for standard rootstocks like 'Duke 7'.

In this context, the objective of this study was to evaluate the effect of different auxin types and concentrations on root induction and rooting quality in etiolated shoots of the 'Duke 7' rootstock. This work aims to establish an efficient vegetative propagation protocol to enhance production of clonal avocado rootstocks in Mexico, thereby improving genetic uniformity and resilience of commercial plantations.

2. Materials and Methods

Experimental site and plant material

This study adopted the propagation method proposed by Hofshi (1996) and Ernst (1999), adapted to the environmental conditions of a greenhouse located in Chapingo, Texcoco, State of Mexico (19.4904322, -98.8734917) at 2264 meters above sea level. The research was conducted during 2022 and 2023.

Nurse plants were produced using West Indian avocado seeds (70±17.4 g) from Veracruz, a size determined optimal for etiolated shoot development during rooting (Castro *et al*., 2021). Seeds were sown on November 1, 2022, in 1000 cm^3 polyethylene bags filled with a 1:1:1 (v/v/v) substrate mixture of peat moss, volcanic rock, and perlite. Plants received light irrigation and preventive applications of fungicide (benomyl 1 g L^{-1}) and insecticide (imidacloprid 1 mL L^{-1}) until grafting.

On February 17, 2023, nurse plants were grafted with mature 'Duke 7' buds at 5 cm above substrate level, retaining only two buds per scion. Graft wounds were sealed with polyvinyl acetate resin to prevent desiccation. During graft union formation,

buds were covered with transparent polyethylene bags (5 × 8 cm) which were removed when plants were transferred to the etiolation chamber.

Experimental establishment

Once etiolated shoots reached 25-30 cm in length (Ernst, 1999), treatments were applied. At the base of each shoot, air layering was performed using a cutting blade periodically disinfected with ethanol (70%). A wound of approximately 2 cm in length was caused, to which 100 µL of rooting growth regulator was applied. Each treated shoot was then placed in a 150 cm^3 transparent plastic container filled with coconut coir dust.

The treated plants were maintained in a shaded area within the greenhouse and watered periodically to maintain adequate moisture levels in both the air-layering substrate and the nurse plant's growing medium. All rooting formulations were prepared fresh on the day of treatment application and were not stored for subsequent use, ensuring consistent growth regulator activity and concentration for each experimental unit. During the study period, greenhouse conditions exhibited natural variability, with mean temperatures gradually increasing from 16.3 to 23.7°C while relative humidity fluctuated between 54% and 64% (Fig. 1).

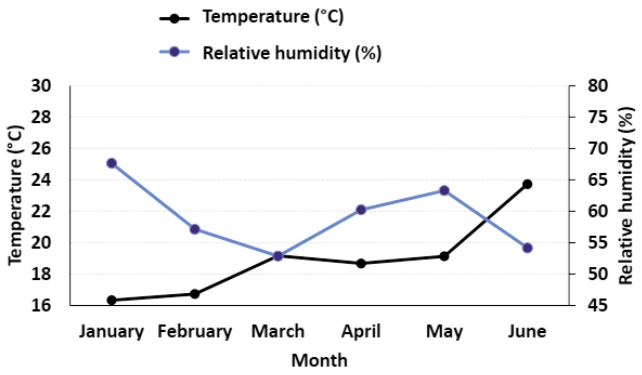

Fig. 1 - Average monthly ambient temperature and relative humidity variation during the experimental period.

Experimental design

The study employed a completely randomized factorial design (5 × 3) with three replications per treatment. Each experimental unit consisted of three air-layered plants. The first factor represented auxin type with five levels, while the second factor comprised three concentrations: 24.6, 34.4, and 44.2 mM (Table 1). This design enabled systematic evaluation of 15 treatment combinations under standardized conditions.

For each treatment, 20 mL of solution was prepared using distinct protocols according to auxin type. The potassium salt of indole-3-butyric acid (K-IBA) and commercial IBA+NAA formulation (Dip'N Grow®) were prepared exclusively with distilled water. For other auxins (IAA, NAA, IBA), a two-step dissolution was employed: initial solubilization in 96% ethanol (5 mL for 24.6 mM, 7 mL for 34.4 mM, or 9 mL for 44.2 mM) followed by volume completion with distilled water. This methodology ensured complete auxin solubility while maintaining precise target concentrations across all experimental treatments.

Evaluated variables

Eighty days after treatment application, the air-layered shoots were carefully separated from nurse plants and the substrate adhered to the roots was removed through water immersion with intermittent manual agitation. Quantitative assessments included: shoot survival (percentage of viable shoots), rooting success (proportion of shoots that emitted at least one root of 0. 5 cm in length or more), root number (mean per rooted shoot), root dimensions (length and diameter of five primary roots measured with Mitutoyo® digital caliper, ±0.01 mm precision), secondary root development (percentage of shoots with secondary roots), callus formation (percentage of shoots showing callus at wound site), and the Root Quality Index (RQI) calculated as the product of mean

Table 1 - Experimental treatments: auxin types and concentrations evaluated in the rooting of 'Duke 7' avocado rootstock etiolated shoots

Treatment Rooting agent (auxin type)	Concentrations (mM)		
Indole-3-acetic acid (IAA, Sigma®)	24.6	34.4	44.2
1-naphthaleneacetic acid (NAA, Sigma®)	24.6	34.4	44.2
Indole-3-butyric acid (IBA, Sigma®)	24.6	34.4	44.2
Potassium salt of indole-3-butyric acid (K-IBA, Sigma®)	24.6	34.4	44.2
Combination of IBA + NAA (Dip'N Grow®)	24.6	34.4	44.2

root number and average length (cm). This comprehensive evaluation protocol enabled systematic comparison of treatment effects on both root initiation and development.

Statistical analysis

For statistical analysis, mean values per experimental unit were used. The data were processed using the GLM procedure in SAS® statistical software (SAS OnDemand for Academics, version 3.1.0; SAS, 2021). Variables expressed as proportions or percentages were subjected to arcsine square root transformation of the original decimal fraction values to meet assumptions of normality and homogeneity of variances.

Following analysis of variance (ANOVA), when significant differences (P<0.05) were detected for treatment effects or their interactions, means were compared using Tukey's test. Data values were back-transformed to their original units for presentation and interpretation.

The statistical model applied was as follows:

$$Y_{ij} = \mu + A_i + C_j + AC_{ij} + \varepsilon_{ij}$$

where: Y_{ij} is the value of the variable evaluated with the i-th rooting agent and the j-th concentration; μ is the overall mean; A_i is the fixed effect of the i-th rooting agent; C_j is the fixed effect of the i-th concentration; AC_{ij} is the fixed effect of the interaction of the i-th rooting agent with the j-th concentration; and ε_{ij} is the experimental error.

3. Results and Discussion

The experimental results demonstrate the significant influence of auxin type and concentration on root development in etiolated shoots of avocado rootstock 'Duke 7'. Our findings reveal distinct morphological responses to different auxin treatments, with particular combinations showing optimal performance in root initiation and development. These outcomes are analyzed through three critical lenses: Their physiological implications for adventitious root formation, practical applications for commercial clonal propagation systems, and comparative relevance to established literature in avocado propagation. The data presentation focuses on treatment efficacy across measured parameters including rooting percentage, root architecture features, and callus formation patterns, providing a comprehensive evaluation of auxin effects on this economically important rootstock cultivar.

Analysis of variance

Analysis of variance revealed significant differences (P<0.05) among treatments for most evaluated variables, confirming the influence of auxin type and concentration on adventitious root development in etiolated avocado shoots. Specifically, the rooting agent type showed highly significant effects on root quality index (P<0.001), root number (P<0.001), root length (P<0.01), and callus formation (P<0.01). Concentration significantly affected only root number (P<0.01), while the Rooting agent × Concentration interaction was significant for survival rate, rooting percentage, root length and diameter, and secondary root presence (P<0.05) (Tables 2 and 3).

These findings demonstrate that both the composition of the rooting agent and its concentration distinctly influence various aspects of the rooting process, and that their interaction can substantially modify the morphological response of etiolated shoots.

Effect of the rooting agent

The rooting agent type exerted a decisive

Table 2 - Mean squares obtained in the analysis of variance to evaluate the effect of five rooting agents and three concentrations on variables related to the rooting of etiolated shoots of avocado rootstock 'Duke 7'

Variation source	DF	SSP	Rooting		PCP
			Percentage	Quality index	
Rooting agent (A)	4	0.135 NS	0.133 NS	89156.6 ***	0.987 **
Concentration (C)	2	0.067 NS	0.046 NS	8474.8 NS	0.488 NS
A × C	8	0.269 *	0.135 *	12153.5 NS	0.183 NS
Experimental error	30	0.253	0.057	40686.9	0.230

DF= Degrees of freedom; SSP= Shoot survival percentage; PCP= Percentage of callus presence; NS= Not significant (P>0.05); * Significant (P<0.05); **Significant (P<0.01); ***Highly significant (P<0.001); ×= Interaction between factors.

Table 3 - Mean squares obtained in the analysis of variance to evaluate the effect of five rooting agents and three concentrations on variables related to the rooting of etiolated shoots of avocado rootstock 'Duke 7'

Variation source	DF	Roots number	Length	Diameter	SRP
Rooting agent (A)	4	2545.16 ***	1.154 **	0.106 NS	2.133 ***
Concentration (C)	2	675.16 **	0.018 NS	0.012 NS	0.020 NS
A × C	8	128.08 NS	0.460 *	0.283 *	0.431 *
Experimental error	30	133.93	0.120	0.123	0.144

DF= Degrees of freedom; SRP= Secondary roots presence; NS= Not significant (P>0.05); * Significant (P<0.05); ** Significant (P<0.01); ***Highly significant (P<0.001); ×: Interaction between factors.

influence on multiple root development parameters in etiolated 'Duke 7' shoots. Although all treatments achieved rooting rates exceeding 94% (Table 4), significant variations were observed in three critical aspects: 1) post-treatment survival rates, 2) root system quality (including architecture and developmental patterns), and 3) callus formation intensity at wound sites. These differential responses highlight the importance of precise auxin selection in clonal propagation protocols, where optimal root system architecture must be balanced with minimal callus interference for successful transplant establishment.

Among the evaluated rooting agents, 1-naphthaleneacetic acid (NAA) promoted the highest root number (55.3) and root quality index (154.9 cm) (Table 4), confirming its efficacy as a potent inducer of adventitious root formation. This response likely stems from NAA's enhanced stability in plant tissues, reduced susceptibility to enzymatic degradation, and prolonged persistence at the application site (da Costa *et al*., 2013; Raggi *et al*., 2020). However, this treatment also reduced shoot survival to 98.1% (Table 4), suggesting phytotoxic effects potentially linked to ethanol solvent use, and the heightened sensitivity of etiolated tissues to elevated auxin concentrations (Amri, 2010; Grossmann, 2009; Ludwig-Müller, 2020). These findings underscore the need to balance rooting efficacy with tissue tolerance, particularly for etiolated shoots whose cell walls exhibit modified xyloglucan and pectin composition. Such alterations increase tissue flexibility but also enhance susceptibility to apoplastic pH imbalances when critical auxin thresholds are exceeded (Duman *et al*., 2020; Wang *et al*., 2025).

In contrast, indole-3-acetic acid (IAA) and indole-3-butyric acid potassium salt (K-IBA) exhibited

Table 4 - Average values by type of rooting agent for the variables: percentage of shoot survival, percentage of rooting, rooting quality index, and percentage of callus presence in the rooting of etiolated shoots of the avocado rootstock 'Duke 7'

Rooting agent	SSP (%)	Rooting		PCP (%)
		Percentage	Quality index (cm)	
IAA	100.0 a[z]	94.2 a	40.1 c	68.86 ab
NAA	98.1 b	96.0 a	154.9 a	87.89 a
IBA	100.0 a	100.0 a	100.2 b	13.74 b
K-IBA	100.0 a	94.2 a	34.0 c	68.86 ab
IBA + NAA	100.0 a	100.0 a	98.3 b	83.56 a
CV (%)	5.94	16.44	43.07	50.88
HLSD	1.57	10.20	50.36	37.09
Average	99.93	98.46	85.51	65.33

[z] Average values in the same column followed by different letters indicate statistical differences (Tukey, P<0.05). IAA= indole-3-acetic acid; IBA= indole-3-butyric acid; NAA= 1-naphthalene acetic acid; K-IBA= indole-3-butyric acid potassium salt; SSP= Shoot survival percentage; PCP= Percentage of callus presence; CV= Coefficient of variation; HLSD= Honest least significant difference.

maximum survival rates (100%) but produced limited root formation (14.9 and 14.4 roots, respectively; Table 5) and significantly lower root quality indices (Table 4). This reduced efficacy likely stems from IAA's inherent instability, being rapidly degraded by peroxidase enzymes and light exposure (Roussos, 2023; Yun *et al*., 2023). Furthermore, IAA readily forms biologically inactive conjugates with amino acids and sugars, substantially reducing its bioavailability and root-promoting activity (Pincelli-Souza *et al*., 2024).

While potassium indole-3-butyric acid potassium salt (K-IBA) offers greater stability than IAA and eliminates the need for organic solvents in solution preparation (Lesmes-Vesga *et al*., 2021), its efficacy as a rooting inducer appears constrained by distinct physiological transport limitations (Yang *et al*., 2022). In its ionic form, K-IBA demonstrates restricted apoplastic diffusion, significantly impeding passive transport to target cells near the application site in etiolated shoots. Effective mobilization instead requires active transport mechanisms (Roussos, 2023) mediated by specialized carrier proteins, including AUX1/LAX family influx transporters and PIN-FORMED (PIN) and ABCB efflux transporters, which collectively regulate auxin distribution across cellular membranes (Hammes *et al*., 2021). Crucially, K-IBA must undergo conversion to its non-ionic (protonated) form to cross plasma membranes and subsequently trigger adventitious root formation (Pincelli-Souza *et al*., 2024), adding a metabolic conversion step that may delay or limit its biological activity compared to more mobile auxin forms.

In contrast, both IBA and the commercial IBA + NAA combination (Dip'N Grow®) demonstrated an optimal balance between shoot survival and root quality (Table 4), establishing them as viable candidates for clonal avocado propagation protocols. These rooting agents produced consistent, reliable responses particularly valuable for nurseries requiring both high rooting success and preservation of etiolated shoot viability. The observed performance suggests these formulations effectively navigate the critical compromise between root induction efficacy and minimal phytotoxicity, a decisive advantage for commercial scale production of 'Duke 7' rootstock.

The evaluation revealed significantly higher callus formation with NAA (87.9%) and IBA+NAA (68.9%) treatments compared to IBA alone (13.7%) (Table 4), consistent with previous reports of synthetic auxins promoting unorganized tissue proliferation (Zhai and Xu, 2021). While callus formation may initially facilitate root primordia initiation, excessive development can negatively impact rooting success through three primary mechanisms: physical obstruction of emerging roots, disruption of normal root system architecture, and competition for essential metabolic resources that would otherwise support root growth (Chen *et al*., 2020).

The current study revealed that callus development was solely influenced by the type of rooting agent applied, with no consistent correlation observed between callus presence and root quantity. While NAA treatment produced both the highest callus formation (87.89%) and root number (55.29), IBA which generated minimal callus (13.74%) still induced intermediate root formation (34.04) (Tables

Table 5 - Mean values by rooting agent type for the variables number, length, and diameter of roots, and presence of secondary roots in the rooting of etiolated shoots of the avocado rootstock 'Duke 7'

Rooting agent	Roots			SRP (%)
	Number	Length (cm)	Diameter (mm)	
IAA	14.92 c [(z)]	2.29 b	1.49 a	80.7 a
NAA	55.29 a	2.83 ab	1.59 a	39.8 b
IBA	34.07 b	2.90 a	1.53 a	95.8 a
K-IBA	14.40 c	2.27 b	1.60 a	58.7 a
IBA + NAA	32.11 b	3.03 a	1.77 a	58.7 a
CV (%)	38.37	16.77	22.06	44.17
HLSD	15.82	0.61	0.48	24.59
Average	30.16	2.67	1.59	57.33

[(z)] Average values in the same column followed by different letters indicate statistical differences (Tukey, $P<0.05$). IAA= indole-3-acetic acid; IBA= indole-3-butyric acid; NAA= 1-naphthalene acetic acid; K-IBA= indole-3-butyric acid potassium salt; SRP= Secondary roots presence; CV= Coefficient of variation; HLSD= Honest least significant difference.

4 and 5).

Contrasting with findings in woody *Eucalyptus* species where adventitious roots originate from callus tissue (Fett-Neto *et al*., 2001; Zhang *et al*., 2022), our observations demonstrated direct root emergence from stem tissue above the auxin application site, without visible callus involvement (Fig. 2). This response suggests etiolated 'Duke 7' shoots maintain an intrinsic capacity for direct rhizogenesis, a phenomenon previously documented in other avocado rootstocks like 'VC801' (Duman *et al*., 2020).

Morphological analysis revealed significant suppression of secondary root growth following NAA application (Fig. 2), likely due to auxin-induced temporal inhibition of lateral root development in primary root tissues. This phenomenon aligns with observations in *Arabidopsis thaliana* (Biswas *et al*., 2019), where supraoptimal auxin levels negatively affect lateral root formation through disruption of polar auxin transport in pericycle cells, cell cycle arrest in lateral root primordia, and downregulation of lateral root-promoting genes such as ARF7 and ARF19. While NAA effectively stimulates primary root formation in avocado rootstock 'Duke 7', our results indicate that higher concentrations may delay optimal root system development by inhibiting secondary branching. This architectural limitation presents key practical challenges: extended production timelines due to delayed shoot separation from nurse plants, and potential requirement for additional agronomic interventions (e.g., supplemental growth regulator treatments) to promote secondary root growth before transplanting. These findings suggest that while NAA remains a potent rooting agent, commercial nurseries should carefully evaluate the trade-off between rapid root initiation and subsequent root system complexity when selecting auxin formulations for clonal propagation.

The findings of this study offer valuable guidance for optimizing clonal propagation of avocado rootstocks in commercial settings. For nurseries prioritizing root quantity, NAA emerges as the most effective option despite its tendency to reduce secondary root development. IBA presents a balanced alternative, producing intermediate root numbers (34.0) while maintaining excellent shoot survival rates (100%), making it particularly suitable for operations where plant viability is paramount. The commercial IBA + NAA formulation (Dip'N Grow®) provides a practical ready-to-use solution that combines the benefits of both auxins while simplifying nursery workflows. Importantly, the results demonstrate that IAA and K-IBA are unsuitable for large-scale propagation due to their limited root induction capacity (14.9 and 14.4 roots respectively) and inherent biochemical instability. These evidence-based recommendations allow propagation specialists to select auxin treatments

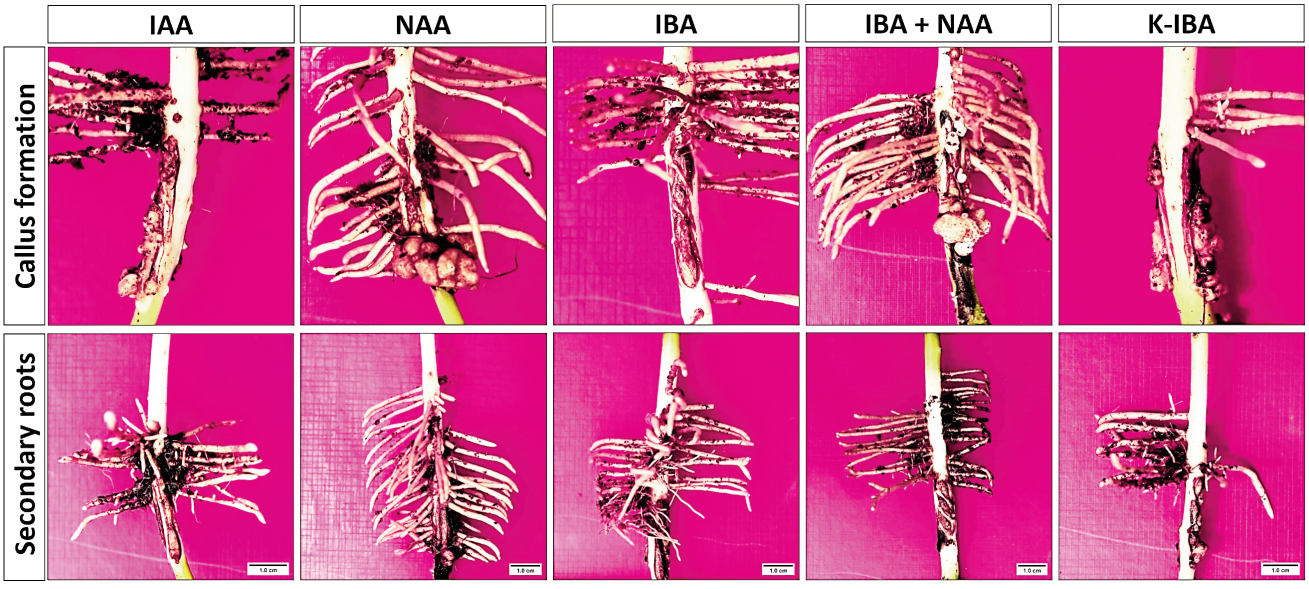

Fig. 2 - Influence of five rooting agents on callus formation and secondary root development in the rooting of etiolated shoots of the avocado rootstock 'Duke 7'. IAA= indole-3-acetic acid; IBA= indole-3-butyric acid; NAA= 1-naphthalene acetic acid; K-IBA= indole-3-butyric acid.

based on their specific production requirements, whether the priority is maximizing root biomass, ensuring transplant success, or streamlining operational efficiency.

These findings represent a significant advancement over traditional IBA-only protocols (Ernst, 1999), which typically employ high concentrations (34.4 mM). The demonstrated benefits of auxin diversification align with recent studies in other woody species like apple (*Malus* spp.) and mulberry (*Morus alba*), where combined auxin treatments have outperformed IBA in promoting adventitious root formation (Sourati *et al*., 2022; Tahir *et al.*, 2022; Wang *et al*., 2024).

The research confirms that auxin selection critically impacts not just rooting efficiency but also root system morphology, both determining factors for successful clonal propagation of avocado rootstocks. These morphological qualities ultimately influence field establishment and long-term productivity of grafted trees.

Effect of the concentration

The study revealed significant auxin concentration effects on key root development, particularly root number (Table 6). A clear dose-dependent response was observed, with progressive increases in root formation corresponding to higher auxin concentrations: from 24.6 roots per shoot at 24.6 mM to 37.6 roots at 44.2 mM (Table 6). This pattern aligns with classical auxin response curves reported in the literature (Nissen, 1985), where rooting typically improves with increasing auxin concentrations up to an optimal threshold, beyond which phytotoxic effects suppress root initiation (Nissen, 1985; Sahoo *et al*., 2021).

Historically, avocado clonal propagation has employed varying concentrations of auxins, particularly indole-3-butyric acid (IBA) as the most commonly used rooting agent. Reported applications range from 500 to 10,000 mg L^{-1}, equivalent to approximately 2.5-51.3 mM (Rogel-Castellanos *et al*., 2000; Mindêllo-Neto *et al*., 2006; Li *et al*., 2024). Significant cultivar-specific differences emerge from these protocols: In 'Duke 7', IBA application at 2,500 mg L^{-1} achieved 56.6% rooting success (Li *et al*., 2024), while 'Fuerte' required five-fold lower concentrations (500 mg L^{-1}) to reach 47.5% efficacy (Mindêllo-Neto *et al*., 2006). Notably, the rootstock 'Dusa' demonstrated striking physiological-state dependence, with 82% rooting in etiolated shoots versus less than 10% in non-etiolated tissue at 2,500 mg·L^{-1} (Li *et al*., 2024), highlighting the critical importance of the plant material's physiological condition in propagation success. These collective findings underscore the dual influence of genetic factors and tissue physiology in determining optimal auxin protocols for different avocado cultivars.

These historical precedents contrast sharply with the findings of the current study, where the tested concentration range (24.6 to 44.2 mM) achieved rooting success rates exceeding 95% while maintaining shoot survival rates in most treatments (Table 3). Furthermore, the results demonstrate that precise concentration adjustments can simultaneously maximize rooting efficiency and improve root system quality without inducing the severe adverse effects (particularly phytotoxicity) typically observed when exceeding optimal NAA concentration (Yan *et al*., 2014). This refined

Table 6 - Mean values by rooting hormone concentration for the variables number, length, and diameter of roots and presence of secondary roots in the rooting of etiolated shoots of the avocado rootstock 'Duke 7'

Concentration	Roots			SRP (%)
	Number	Length (cm)	Diameter (mm)	
24.6 mM	24.62 b [(z)]	2.65	1.56	61.50 a
34.4 mM	28.24 ab	2.65	1.60	55.23 a
44.2 mM	37.62 a	2.71	1.62	55.23 a
CV (%)	38.37	16.77	22.06	44.17
HLSD	10.42	0.40	0.32	11.22
Average	30.16	2.67	1.59	57.33

[(z)] Average values in the same column followed by different letters indicate statistical differences (Tukey, P<0.05). SRP= Secondary roots presence; CV= Coefficient of variation; HLSD= Honest least significant difference.

approach represents a significant improvement over traditional protocols, as it achieves near-universal rooting success while avoiding the compensatory trade-offs between root quantity and plant viability that characterize many existing methods. The study specifically identified 34.4 mM as the most balanced concentration for commercial applications, combining high rooting percentages (98.1%) with excellent root architecture development and minimal callus formation (Tables 6 and 7).

The clonal propagation of 'Duke 7' rootstock has presented particular difficulties. Previous studies reported limited success, with only 26% rooting after 180 days when using non-etiolated shoots treated with Dip'N Grow® at 3,000 mg L^{-1} (approximately 15.2 mM equivalent) (Salazar-García *et al*., 2004 b). Alternative approaches using IBA-saturated wood chips (10,000 mg L^{-1}) on etiolated shoots improved rooting to 60% (Escobedo and Escobedo, 2011). In marked contrast, the current study demonstrates that optimized auxin selection and concentration in etiolated shoots can achieve 100% rooting efficiency with superior morphological quality, results that substantially surpass all previously reported values for this challenging rootstock. This breakthrough reflects both the importance of physiological preconditioning (etiolation) and precise auxin formulation in overcoming the historical propagation barriers for 'Duke 7'.

From a physiological perspective, the enhanced efficacy observed at higher auxin concentrations may stem from increased hormone availability at the application site, promoting activation of key genes involved in cellular differentiation (such as WOX11/12, *ARF*, and *LBD*) that are essential for adventitious root formation (Lakehal and Bellini, 2019; Li *et al*., 2024). However, this concentration-dependent effect was not uniform across all measured parameters. Callus formation, root length and diameter, and secondary root development showed no significant differences between concentrations (Tables 6 and 7), suggesting that structural root system quality may be modulated by additional factors beyond concentration alone, including auxin type, formulation characteristics, and local hormonal interactions (Druege *et al*., 2016; Lakehal and Bellini, 2019). These differential responses highlight the complex regulatory networks governing root organogenesis, where concentration primarily drives root initiation while other factors determine subsequent root architecture development.

Interactive effects of rooting agent type and concentration

The significant interaction between auxin type and concentration across multiple key rooting variables demonstrates that the morphogenic response of etiolated 'Duke 7' shoots depends not merely on the auxin type or applied dose in isolation, but rather on their specific combination (Tables 1 and 2). This interaction was particularly pronounced for critical parameters including: survival rate, rooting percentage, root length and diameter, and secondary root presence (Fig. 3). The non-additive effects reveal complex phytohormonal regulation where certain auxin-concentration combinations synergistically enhance rhizogenesis while others exhibit antagonistic relationships, suggesting tissue-specific saturation thresholds for different auxin

Table 7 - Mean values by rooting agent concentration for the variables: Survival percentage, rooting percentage, quality index, and callus presence percentage in the rooting of etiolated shoots of the avocado rootstock 'Duke 7'

Concentration	SSP (%)	Rooting percentage	Quality Index (cm)	PCP (%)
24.6 mM	100.00 a [(z)]	98.91 a	71.74 a	75.00 a
34.4 mM	100.00 a	99.33 a	80.56 a	74.66 a
44.2 mM	99.30 a	96.55 a	104.24 a	58.42 a
CV (%)	5.94	16.44	43.07	50.88
HLSD	0.68	4.51	33.15	17.46
Average	99.93	98.46	85.51	65.33

[(z)] Average values in the same column followed by different letters indicate statistical differences (Tukey, P<0.05). SSP= Shoot survival percentage; PCP= Percentage of callus presence; CV= Coefficient of variation; HLSD= Honest least significant difference.

formulations. These findings necessitate a dual-parameter optimization approach for clonal propagation protocols, as neither factor alone sufficiently predicts rooting performance.

In contrast to other treatments, increasing IAA concentrations showed a positive correlation with rooting percentage, improving from 66.6% at 24.6 mM to 88.8% at 34.4 mM, and reaching 100% at 44.2 mM. For both IBA and the IBA + NAA combination (Dip'N Grow®) maintained consistent 100% rooting across all three tested concentrations, indicating a broad efficacy window for these formulations. NAA exhibited optimal performance at 24.6 mM and 34.4 mM (100% rooting), but efficacy declined to 77.7% at 44.2 mM, likely reflecting phytotoxic effects at higher doses. Notably, K-IBA performed best at the lowest concentration (100% at 24.6 mM), with progressively reduced rooting at higher levels (88.8% at 34.4 mM and 66.6% at 44.2 mM). These results clearly demonstrate the significant impact of the auxin type × concentration interaction on the rhizogenic response of etiolated avocado shoots (Fig. 3).

Previous studies have demonstrated that the transport and physiological activity of auxins can vary significantly depending on their structure and molecular form (Korasick *et al.*, 2013). For example, IBA undergoes conversion to both IAA and IBA conjugates during plant tissue transport, resulting in prolonged, multiphasic rooting promotion (Damodaran and Strader, 2019). In contrast, externally applied IAA tends to remain in its free form during transport, making it more susceptible to enzymatic inactivation and oxidative degradation (Hayashi *et al*., 2021). This difference may explain why IBA shows higher efficacy at lower concentrations, while IAA requires higher doses to induce comparable rooting responses.

NAA exhibits superior chemical stability compared to other auxins, allowing prolonged activity in plant tissues (da Costa *et al*., 2017). This stability stems from its synthetic molecular structure and likely involves specialized transporters that facilitate its movement and accumulation at target sites (Yang *et al*., 2006; Napier, 2021). Studies report that NAA resists rapid degradation or conjugation in plant tissues, enhancing its capacity to induce abundant root formation (Nissen and Sutter, 1990; Gomes and Scortecci, 2021) (Fig. 4). However, at 44.2 mM, NAA application resulted in the highest root numbers but reduced rooting percentage (77.8%) and suppressed secondary root development (Fig. 4). These findings

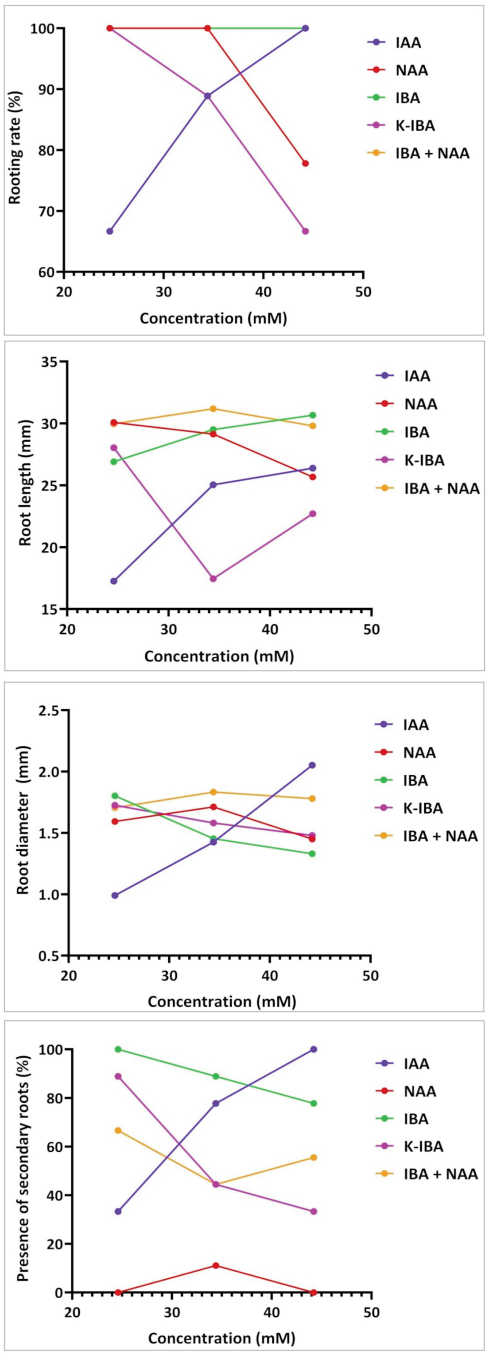

Fig. 3 - Interaction of auxin type and concentration on rooting of etiolated shoots of avocado rootstock 'Duke 7'. IAA: indole-3-acetic acid, NAA= 1-naphthalene acetic acid, IBA= indole-3-butyric acid, K-IBA= indole-3-butyric acid potassium salt, IBA + NAA= indole-3-butyric acid + 1-naphthalene acetic acid (Dip'N Grow®).

suggest that while certain formulations effectively promote primary root formation, they may also cause unintended effects like lateral root inhibition, potentially due to localized hormonal imbalance at the rooting site (Lakehal and Bellini, 2019; Bhalerao *et al*., 2002).

Fig. 4 - Rooting of etiolated shoots of 'Duke 7' rootstock with the application of IAA (indole-3-acetic acid), NAA (1-naphthalene acetic acid), IBA (indole-3-butyric acid), K-IBA (indole-3-butyric acid potassium salt) and IBA + NAA (indole-3-butyric acid + 1-naphthalene acetic acid) at three concentrations. ANR= Average number of roots; Scale bar: 1.0 cm.

The interaction between auxin type and concentration significantly influenced primary root length. The greatest average length (31.19 mm) was achieved with the IBA + NAA combination (Dip'N Grow®) at 34.4 mM, suggesting a synergistic effect favoring root elongation. In contrast, the shortest roots formed with K-IBA at 34.4 mM (17.46 mm) and IAA at 24.6 mM (17.22 mm), indicating reduced efficacy in promoting cellular elongation under these specific conditions. For the remaining treatments, no statistically significant differences were observed, with an average length of 27.83 mm, suggesting a more uniform response across these combinations (Fig. 3).

Root diameter was similarly affected by concentration, particularly in the case of IAA. At 24.6 mM, IAA produced thinner roots (0.97 mm), while at 34.4 mM, it generated thicker roots (2.05 mm), demonstrating a concentration-dependent response in radial root expansion. The other auxins, regardless of concentration, produced roots of intermediate diameter, averaging 1.60 mm with no statistical differences between treatments, indicating more stable growth patterns in terms of root thickness (Fig. 3).

The rooting agent type and concentration interaction exerted a clear effect on secondary root formation. NAA consistently restricted lateral root development across all three tested concentrations (Fig. 4), likely due to its potent growth regulator activity and potential induction of apical dominance or excessive local accumulation in basal tissues (Aloni *et al*., 2006). Recent studies indicate that synthetic auxins like NAA exhibit enhanced stability and tissue persistence, creating strong but localized hormonal signaling that may suppress lateral root initiation through downregulation of Lateral Organ Boundaries Domain (LBD) transcription factors critical for adventitious rooting, and prolonged activation of AUX/IAA repressor proteins that inhibit auxin-dependent gene expression by blocking ARF transcription factors (Lakehal and Bellini, 2019; Jing and Strader, 2019). This dual regulation at the genetic level explains NAA's capacity to simultaneously promote primary root growth while inhibiting secondary root formation.

In contrast, IAA demonstrated a positive and progressive effect on secondary root formation as concentration increased, likely attributable to its lower stability. This auxin undergoes rapid metabolic conversion or conjugation, preventing excessive accumulation while enabling dynamic tissue transport, characteristics that facilitate lateral root differentiation (Casanova-Sáez *et al*., 2021; Zhang *et al*., 2023).

These findings underscore the importance of developing tailored propagation protocols that carefully consider both auxin type and optimal concentration for specific plant materials. For commercial nurseries where consistency and productivity are paramount, pre-mixed formulations such as IBA+NAA (Dip'N Grow®) may serve as practical solutions, though they require precise concentration adjustments to prevent phytotoxic effects in sensitive etiolated tissues. Critical implementation considerations include establishing appropriate concentration thresholds for different auxin combinations, accounting for tissue-specific sensitivity variations, and carefully balancing the trade-offs between root quantity and overall root system quality. The research demonstrates that successful clonal propagation depends on this multifaceted optimization approach rather than relying on standardized auxin applications.

The significant interaction between auxin type and concentration conclusively refutes the concept of a "universal concentration" suitable for all rootstocks or growing conditions. This research instead provides empirical evidence supporting the development of customized propagation protocols through careful refinement of auxin formulations, adjustments based on the physiological state of plant material, and optimization of multiple interdependent parameters.

These insights prove particularly valuable for enhancing clonal propagation of difficult-to-root rootstocks such as 'Duke 7', where well-balanced root system architecture critically determines subsequent field performance. The data-driven methodology established in this study could be effectively adapted to improve propagation protocols for other commercially significant avocado cultivars, potentially revolutionizing nursery production standards through science-based precision agriculture approaches.

4. Conclusions

The results demonstrated that auxin type and concentration significantly and differentially influenced adventitious root induction in etiolated shoots of 'Duke 7' avocado rootstock. These effects were evident in both rooting percentages and the morphological quality of the root system.

NAA promoted the highest root number and quality index, but also induced substantial callus formation and reduced secondary root development, suggesting potential phytotoxicity at elevated concentrations. In contrast, IAA showed a more balanced dose-dependent response and enhanced secondary root growth, while IBA and its commercial formulation with NAA (IBA + NAA) provided stable and reliable performance.

Auxin concentration modulated rooting efficiency, with dose-dependent physiological responses being

most pronounced for IAA and K-IBA. The tested concentration range (24.6-44.2 mM) proved effective for rooting (>95%) while maintaining shoot survival in most treatments.

A clear auxin type × concentration interaction was observed, emphasizing the need for genotype (and physiological state) specific optimization of both factors. The responses documented are not universal and must be considered when designing propagation protocols for avocado rootstocks.

The combination of etiolated shoots with properly selected and dosed auxins achieved 100% rooting in certain treatments, surpassing results from traditional protocols. These findings represent significant progress toward standardizing and optimizing clonal propagation of 'Duke 7' for commercial production.

This study provides experimental evidence for developing more efficient, reproducible, and economically viable protocols applicable in commercial nurseries. By improving the availability of clonal avocado rootstocks, these advances will enhance the sustainability and competitiveness of avocado production systems. The optimized protocols specifically address three industry needs: consistent rooting success, superior root system architecture, and scalable production methods. Future research should explore applications to other commercially important rootstock varieties while maintaining the precision agriculture approach demonstrated here.

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Comparative evaluation of *Aloe vera* and chitosan edible coatings on shelf life and quality of strawberries during cold storage

OPEN ACCESS

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Abstract: Strawberries (*Fragaria × ananassa* Duch.) are nutrient-rich specialty fruits with a short shelf life due to microbial spoilage, softening, darkening, and moisture loss. This study aimed to investigate the effectiveness of edible coatings in extending shelf life and maintaining fruit quality. Freshly ripened, randomly selected strawberries were coated with 1.5% chitosan, 1.5% chitosan+1% $CaCl_2$, *Aloe vera* gel (AVG), and AVG+1% $CaCl_2$, along with an uncoated control. Each treatment was replicated 3 times with 25 samples per replicate, followed by air drying. The coated strawberries were stored in sterilized polypropylene containers under standard refrigerated conditions (4±1°C; 50±5% relative humidity) for 9 days. The application of edible coatings significantly ($p<0.05$) reduced respiration rates (by 25 to 34%) and microbial load (by 41 to 62%), helping to preserve fruit color, moisture content, ascorbic acid, firmness, and overall acceptability. The effect was more pronounced in strawberries coated with AVG and AVG+1% $CaCl_2$ coatings on strawberries throughout storage period. Uncoated strawberries had an acceptability score of 4.0, while all coated fruits scored above 5, showing a significant improvement by 20 to 37%. Strawberries treated with AVG, with or without $CaCl_2$, maintained the highest acceptability score of 5.5, outperforming all other coatings. These findings suggest that *Aloe vera*-based coatings are particularly effective in extending the shelf life and preserving the quality of strawberries during refrigerated storage.

1. Introduction

Strawberries (*Fragaria × ananassa* Duch.) are among the most widely

consumed specialty fruits worldwide, prized for their vibrant color, distinct flavor, sweet-tart taste, and high nutritional value. The global demand for strawberries continues to rise with worldwide production reaching 40.8 million tons in 2020 (FAO, 2022). Their versatility culinary applications - from fresh consumption to processed products such as jams, jellies, beverages, dairy items, and flavored drinks - makes strawberries one of the most widely used and adaptable crops in the world (Wise *et al*., 2024).

Rich in vitamins, minerals, flavonoids, anthocyanins, proteins, and phenolic compounds (Temiz and Ozdemir, 2021), strawberries offer numerous health benefits. However, their delicate texture and high respiration rate significantly reduce their shelf life. As a result, strawberries are highly susceptible to bruising, moisture loss, discoloration, microbial spoilage, and softening (Nasrin *et al.,* 2017).

To maintain fruit quality, strawberries should be rapidly chilled and stored at low temperatures (0-4°C) immediately after harvest. However, even with proper cold storage, their shelf life typically remains limited to less than five days (Shankar *et al.,* 2021). To further reduce postharvest losses and extend shelf life, several complementary strategies have been investigated, including active packaging, modified atmosphere packaging, and the use of edible coatings (Zhang *et al.,* 2022).

Among the emerging postharvest preservation techniques, edible coatings have attracted considerable attention in the fruit and vegetable industry for their proven effectiveness in preserving quality and prolong shelf life (Sousa Cesar de Albuquerque *et al.,* 2024). These coatings act as physico-chemical barriers, protecting against microbial contamination and water loss while preserving texture, color, flavor, and volatile compounds. Additionally, they help reduce respiration and transpiration rates, thereby delaying senescence (Nasrin *et al.,* 2023). Polysaccharide-based coatings are particularly valued for their excellent film-forming ability, mechanical strength, and selective permeability - especially in regulating oxygen exchange (Rios *et al.,* 2022).

Aloe vera gel (AVG) is an increasingly recognized edible coating material, primarily due to its high polysaccharide and soluble sugar content, which aids in preserving fruit quality by regulating water and oxygen exchange. This regulation helps lower respiration rates and maintain the fruit's texture, moisture content, color, taste, and firmness (Sogvar *et al.,* 2016; Nicolau-Lapena *et al.,* 2021). AVG is colorless, tasteless, and does not alter the sensory attributes of coated fruits (Hasan *et al.,* 2021). Moreover, it contains more than 200 bioactive compounds with antioxidant, antiviral, and antibacterial properties (Nguyen *et al.,* 2020). Beyond extending shelf life, AVG coatings also offer the potential to enhance the functional qualities of fruits through the incorporation of additional bioactive ingredients.

Due to excellent film-forming and antimicrobial properties, AVG is an effective edible coating that can be applied alone or in combination with other ingredients to extend the shelf life of a wide variety of fruits and vegetables, including lime (Pimsorn *et al.,* 2022), pistachio (Valverde *et al.,* 2005), apples (Ergun and Satici*,* 2012), guava (Shabir *et al.,* 2021), strawberries (Hassan *et al.,* 2022), and tomato (Chrysargyris *et al.,* 2016). Chitosan-based coatings have also demonstrated efficacy in reducing fungal decay, delay ripening and senescence, and maintaining the postharvest quality of fresh produce such as tomatoes, strawberries, and cherry tomatoes (Zheng *et al.,* 2024).

Similarly, calcium chloride ($CaCl_2$) applications have demonstrated benefits such as improved fruit firmness, enhanced antioxidant activity, reduced disease incidence, and mitigation of physiological disorders like internal browning and senescence (Nguyen *et al.,* 2020). However, there is limited information on the combined effects of AVG or chitosan with $CaCl_2$ on the postharvest quality of strawberries.

This study tests the hypothesis that AVG, either alone or in combination with $CaCl_2$, will enhance the postharvest quality and shelf life of strawberries more effectively than conventional chitosan-based coatings. By comparing key quality parameters such as firmness, microbial stability, moisture retention, and sensory acceptability, the study aims to determine the most effective edible coating strategy for maintaining strawberry freshness during storage.

2. Materials and Methods

Plant material

Freshly ripened strawberries (var. BARI Strawberry 3) were randomly harvested from the

field plots of the Fruit Research Farm at the Horticulture Research Center (HRC), Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh.

A total of 375 strawberries were selected based on uniform size, absence of microbial infection or physical damage, and having more than 80% red surface area. *Aloe Vera* leaves were sourced from the Flower Research Farm of HRC at BARI, Gazipur.

Edible coating formulations

High molecular weight chitosan-652 was procured from Mahtani® Chitosan Pvt. Ltd., India was used to prepare a 1.5% chitosan solution, by dissolved 1.5 g of chitosan in 75 mL of distilled water, followed by the addition of 2 mL of reagent-grade acetic acid. The mixture was warmed to 55±2°C and stirred continuously to ensure homogeneity. A 2M NaOH solution was then added to adjust the pH to 5.6. Sterilized distilled water was subsequently added to bring the final volume to 100 mL (Jiang and Li, 2001). To prepare the 1.5% chitosan+1% $CaCl_2$ solution formulation, 1 g of $CaCl_2$ was added to the 1.5% chitosan solution and thoroughly stirred.

Mature *Aloe Vera* leaves were washed with a 25% aqueous chlorine solution. The outer layer was removed to extract the clear, water-based gel (lymph), which was ground and filtered to remove fibers. The gel was pasteurized at 70°C for 45 min and then cooled to room temperature (~25 °C). To stabilize the pH at ~4.0, ascorbic acid (2.0 g L^{-1}) and citric acid (4.5 g L^{-1}) were added. To enhance viscosity and coating performance, 1% sodium carboxymethyl cellulose (CMC), a natural cellulose-based gelling agent, was incorporated and thoroughly mixed. The solution was stored in an opaque glass container to prevent oxidation (Nasrin *et al.,* 2017). For another formulation, 1 g of $CaCl_2$ was added to the AVG solution and thoroughly mixed to obtain the AVG+1% $CaCl_2$ solution.

Experiment and coating application

The experiment was conducted using a completely randomized design (CRD) under controlled laboratory conditions, with 75 strawberries assigned to each coating treatment. Five coating treatments were evaluated: (1) control (no coating), (2) 1.5% chitosan solution, (3) 1.5% chitosan + 1% $CaCl_2$ solution, (4) AVG, and (5) AVG + 1% $CaCl_2$ solution. Each treatment was replicated three times, with 25 randomly selected fresh strawberries per replicate.

Strawberries were immersed in the respective coating solutions for one minute, then air-dried using a high-speed fan. Following drying, the fruits were stored in clear polypropylene containers at 4±1°C and 50±5% relative humidity in an incubator. Selected chemical, physical, and sensory attributes were assessed at harvest (day 0) and after 3, 6, and 9 days of storage.

Chemical and physical properties of strawberries

Respiration rate

For respiration rate measurement, 10 fruits from each replication were used. Throughout the storage period, respiration rates were recorded at designated intervals. Fruits from each replication were placed in a 1000 mL airtight jar sealed with septa and incubated at 20±2°C for two hours. After incubation, a 1 mL gas sample was extracted from the headspace of the jar using a syringe and analyzed with a CO_2/O_2 gas analyzer (Quantek® Instruments, Model 902D, USA). The concentration of CO_2 produced within the jar was recorded. Respiration rate was then calculated using the total gas volume of the jar, the weight and volume of the strawberries, and the incubation time. Results were expressed as mL CO_2 kg^{-1} h^{-1} (Nasrin *et al.,* 2020).

Firmness

Three fruits from each replication were used to evaluate strawberry firmness using a Fruit Texture Analyzer (GUSS®, Model GS25, SA). A stainless-steel flat-headed probe with an 8 mm diameter penetrated the fruit at a speed of 5 mm s^{-1}. Firmness was defined as the maximum force required to penetrate the fruit tissue. Following zero-force contact between the probe and the horizontally positioned strawberry, the equatorial region of each fruit was tested at two evenly spaced points, with 3 mm penetration depth. Data analysis was based on the maximum force recorded during probe movement, with results expressed in Newtons (N).

Weight (moisture) loss

Strawberry weight loss (moisture content) was measured using 10 fruits from each replication at the start of the experiment - immediately after the surface coating was applied and dried - and subsequently at three-day intervals throughout the storage period. Weight loss was computed by using

the following equation and then displaying the result as a percentage.

Weight loss (%) = (Initial fruit weight -Final fruit weight at indicated period)/(Initial fruit weight) x100

External fruit color

Surface color of strawberries was measured on five fruits from each replication using a Chroma Meter (Model CR-400, Minolta Corp., Japan) based on the CIE Lab* system. Here, L* denotes lightness, while a* and b* values were used to calculate Chroma (c) and hue angle (h°). The instrument was calibrated with the provided white tile before measurement. Multiple readings were taken from different areas of each fruit.

After measuring respiration rate, weight loss, and color, the fruits were returned to refrigeration to continue the experiments and assess shelf life.

Ascorbic acid, titratable acidity, and sugar

Ascorbic acid, titratable acidity, total sugar, and reducing sugar concentrations were analyzed following AOAC (1994) standard methods. Total soluble solids (TSS) were measured using a refractometer, and the pH of strawberry juice was determined using a pH meter (HANNA® Instruments, pH-211; Microprocessor pH Meter, Italy).

Ascorbic acid concentration in strawberry juice was determined using a titration method with 2,6-dichlorophenolindophenol (DCPIP) dye. In this method, ascorbic acid in an alkaline medium reduces the blue-colored DCPIP dye to a colorless form. The dye solution was first standardized against known concentrations of ascorbic acid to determine the dye factor. For analysis, strawberry juice was diluted with 3% metaphosphoric acid and then titrated with the DCPIP solution until a persistent pink endpoint lasting 15 seconds was observed.

Dye factor= 0.5/(Titrate volume)

Ascorbic acid (mg/100g) = (Titre x Dye factor x Volume x 100)/(Aliquot of extract taken x Weight of sample)

Titratable acidity was determined by blending 10 g of strawberries with 100 mL of distilled water, followed by filtration of the mixture. Three to four drops of phenolphthalein indicator were then added, and the filtrate was titrated with 0.1 M NaOH. The titratable acidity was calculated using the following formula:

Titratable acidity (%) = [(titre vol.× normality of NaOH × vol.made up × eq.wt.of acid)/(aliquot of sample × vol. of sample × 1000)] x100

Total soluble solids were measured using a hand-held refractometer (Atago® MASTER-53α, Japan) and expressed in °Brix. A small volume of strawberry juice was placed on the prism surface of the refractometer, and the TSS value in °Brix was recorded directly from the instrument's display

Microbiological analysis of strawberries

A 10 g sample of fresh strawberries were thoroughly combined with 90 mL of sterilized 0.9% NaCl solution. The homogenized sample was included in 1 ml to the corresponding dilutions (10^{-1} to 10^{-6}) applying a 0.9% sodium chloride solution. Total bacterial count (TBC) was measured using nutrient agar (DifcoTM, USA, H 7.0-7.4), while molds as well as yeast were counted using potato dextrose agar (PDA, HiMedia, India). The media was made as directed by the manufacturer. Inoculated nutrient agar media plates underwent incubation for 24 to 28 hours at 37°C, while plates with PDA were kept for 5 days at room temperature (26 ± 2°C). Plates showing colonies were examined after incubation. TBC was calculated by multiplying the dilution factor by the average number of colonies in a given dilution. Colony forming units per gram (cfu/g) were used to represent the microorganisms present in the samples (Mahfuza *et al.,* 2016).

Sensory quality

Sensory quality of fresh strawberries was assessed by 15 trained panelists (aged 25-50 years, both male and female). The panel evaluated the samples based on color, flavor, texture, and overall acceptability. Prior to the evaluation, panelists underwent pre-training focused on strawberry appearance, aroma, and taste. For each treatment, three samples were evaluated per panelist in a randomized order. Samples were blindly labeled using random three-digit codes to minimize bias. To cleanse their palate between samples, panelists rinsed their mouths with plain water.

Evaluations were conducted using a 9-point hedonic scale ranging from 1 to 9, where: 1 = dislike extremely, 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 extremely (Nasrin and Anal, 2015). A score of 5 ("neither like nor dislike") was

used as the cutoff point for consumer acceptability.

Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) based on a CRD to assess the effects of different coating treatments and storage time on various quality parameters of strawberries. The ANOVA was conducted using SAS® software (version 9.2, 2010), with coating treatment considered a fixed effect and time treated as a random effect to account for temporal variation. The model evaluated the main effects and interactions on dependent variables such as firmness, color, weight loss, TSS, acidity, pH, total sugar, reducing sugar, flavor, texture, and overall acceptability. The Least Significant Difference (LSD) test was used to compare treatment means, with significance set at $p \leq 0.05$ unless otherwise noted. Graphical representations and regression analyses were performed using SigmaPlot® software.

3. Results

Respiration rate

The initial respiration rate of strawberries was 63 mL kg^{-1} h^{-1}, which was significantly and non-linearly reduced by nearly half when coated with either AVG alone ($y = 61.6 - 9.61*X + 0.79*X^2$) or AVG with 1% $CaCl_2$ ($y = 61.7 - 9.83*X + 0.79*X^2$), accounting 93 and 94% of the variation in respiration rates during storage, respectively (Fig. 1). In uncoated control strawberries, respiration rate began increasing significantly from the 3rd day, while in coated samples, it started to rise slightly only after the 6th day ($y = 33 + 30.3^{(-0.56*X) + 2.35*X}$). By the 9th day, the highest respiration rate (54.3 mL kg^{-1} h^{-1}) was observed in the uncoated control strawberries, whereas the lowest (35.6 mL kg^{-1} h^{-1}) was recorded in strawberries coated with AVG+1% $CaCl_2$. However, no significant differences were found among the coated treatments.

Fruit firmness

The initial firmness of the strawberries was 2.42 N, which declined significantly and linearly over time; however, the rate of softening varied among the coating treatments (Fig. 2). Strawberries coated with AVG+1% $CaCl_2$ retained the highest firmness ($y = 2.47 - 0.05*X$) explaining 86% of the variability in firmness. These strawberries lost only 20.2% of their initial firmness by day 9. In contrast, uncoated control strawberries exhibited the greatest decline in firmness ($y = 2.31 - 0.12*X$), showing a 47% reduction over the same period.

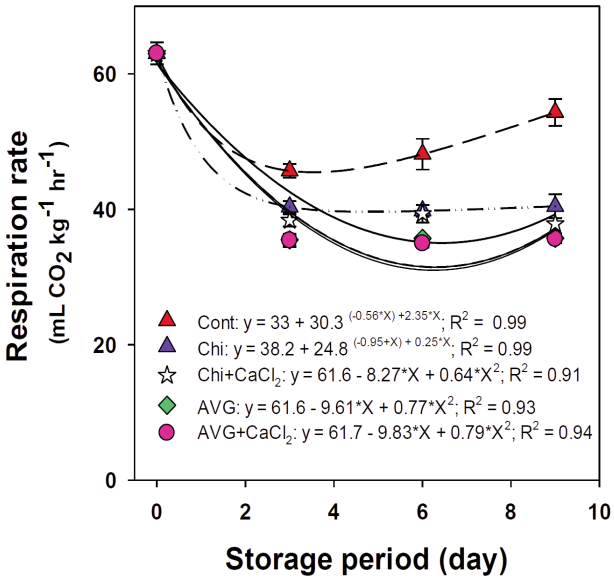

Fig. 1 - Effects of various edible coatings on respiration rates (mL CO_2 kg^{-1} h^{-1}) of strawberries during storage at 4±1°C Control= Uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$= 1.5% chitosan +1% $CaCl_2$ coated, AVG= *Aloe vera* gel coated, AVG+$CaCl_2$= AVG+1% $CaCl_2$ coated. Data presented with standard error of mean.

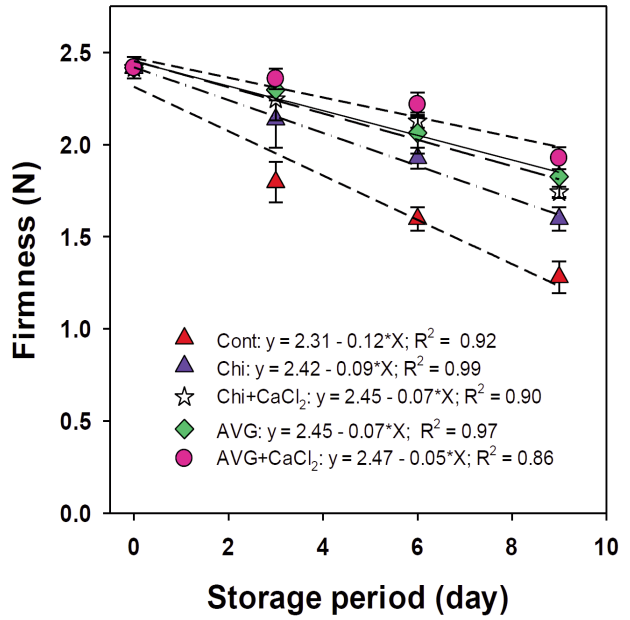

Fig. 2 - Effects of various edible coatings on firmness (N) of strawberries during storage at 4±1°C [Control= Uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$= 1.5% chitosan + 1% $CaCl_2$ coated, AVG= *Aloe vera* gel coated, AVG+$CaCl_2$= AVG+1% $CaCl_2$ coated. Data presented with standard error of mean.

Weight (moisture) loss

Uncoated control strawberries exhibited the highest linear weight (moisture) loss during storage (y = 0.08 + 0.97*X) compared to coated strawberries (Fig. 3). Coating with 1.5% chitosan, either with (y = 0.30 + 0.64*X) or without 1% $CaCl_2$ (y = 0.20 + 0.67*X) significantly reduced weight loss to approximately ~ 6%. In contrast, strawberries coated with AVG, with (y = 0.17 + 0.55*X) or without 1% $CaCl_2$ (y = 0.13 + 0.53*X), exhibited the lowest weight loss—around 5%—relative to the uncoated control.

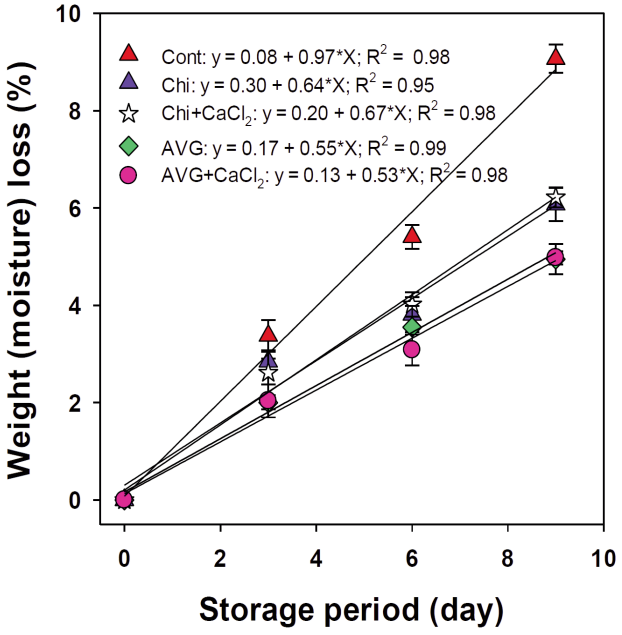

Fig. 3 - Effects of various edible coatings on weight (moisture, %) loss of strawberries during storage at 4±1°C. Control= Uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$= 1.5% chitosan +1% $CaCl_2$ coated, AVG= *Aloe vera* gel coated, AVG+$CaCl_2$ = AVG+1% $CaCl_2$ coated. Data presented with standard error of mean.

External fruit colour

Throughout storage, uncoated control strawberries appeared darker than coated ones (Fig. 4a). Among coatings, AVG-treated strawberries exhibited a brighter red color than those with chitosan. The addition of 1% $CaCl_2$ improved lightness in both coatings. By day nine, the lightness (L*) value declined significantly and linearly in uncoated control fruits (y = 40 – 1.28X), with a 29.5% reduction, compared to 14.7% for chitosan + $CaCl_2$ (y = 40.4 – 0.59X) and 11.7% for AVG + $CaCl_2$ (y = 40.8 – 0.53*X), explaining 99% of color variation.

The hue angle (h°) of uncoated control

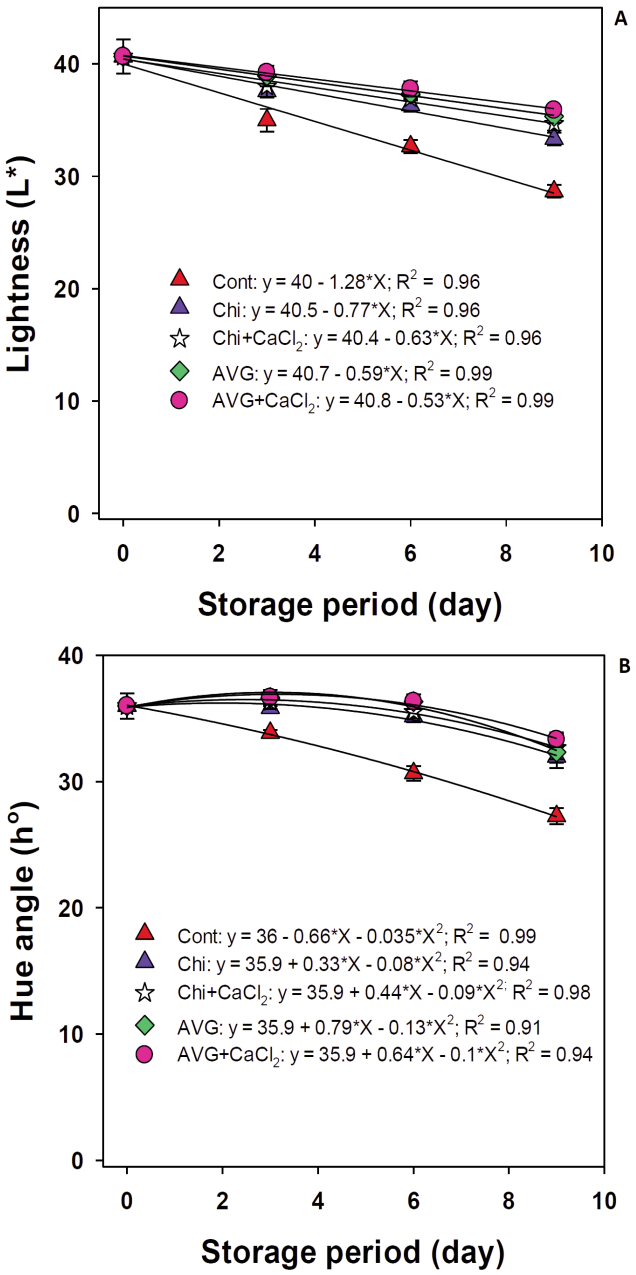

Fig. 4 - a) Effects of various edible coatings on external fruit colour evolution (lightness, L*) of strawberries stored at 4±1°C. b) Effects of various edible coatings on external fruit colour evolution (hue angle, ho) of strawberries stored at 4±1°C Control= uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$= 1.5% chitosan +1% $CaCl_2$ coated, AVG= *Aloe vera* gel coated, AVG+$CaCl_2$= AVG+1%$CaCl_2$ coated. Data presented with standard error of mean.

strawberries began decreasing after day two (y = 36 – 0.66X – 0.035X²), dropping by 24.2% by day six (Fig. 4b). In contrast, coated fruits maintained a stable hue angle until day six, followed by a slight decline. On day nine, the lowest hue angle (27.3) was recorded in the uncoated control, while the highest

(33.3) was observed in AVG+$CaCl_2$-coated strawberries ($y = 35.9 + 0.64X - 0.1X^2$).

Ascorbic acid, titratable acidity, and sugar

At the beginning of storage, the ascorbic acid, titratable acidity, and pH of strawberries were 47.8 mg 100 g^{-1}, 0.86%, and 3.94, respectively (Table 1). By the ninth day, the ascorbic acid concentration in control fruits had significantly decreased to 35.6 mg/100 g. In contrast, strawberries coated with AVG+1% $CaCl_2$ maintained the highest ascorbic acid concentration at 44.9 mg 100 g^{-1}, which was significantly higher than that of both the control and other coated treatments. Although titratable acidity declined and pH slightly increased by day nine, these changes were not statistically significant among the different coating treatments (Table 1).

Regarding sugar content, initial values for TSS, total sugars, and reducing sugars in fresh strawberries were 7.5°Brix, 5.4%, and 4%, respectively (Table 1). All these values increased by day nine. However, there were no significant differences in total and reducing sugar contents among the treatments. The highest TSS (9.4°Brix) was observed in the control (uncoated) strawberries, whereas significantly lower TSS values (8.3°Brix) were recorded in strawberries coated with AVG, either alone or combined with $CaCl_2$. There were no significant differences in TSS among the coated fruits.

Microbiological analysis

Although coating treatments initially caused a non-significant reduction in total bacterial count (TBC), microbial loads increased non-linearly over time from a baseline of 2.11 log CFU g^{-1} (Fig. 5). Strawberries coated with AVG, with ($y = 2.08 – 0.64X$

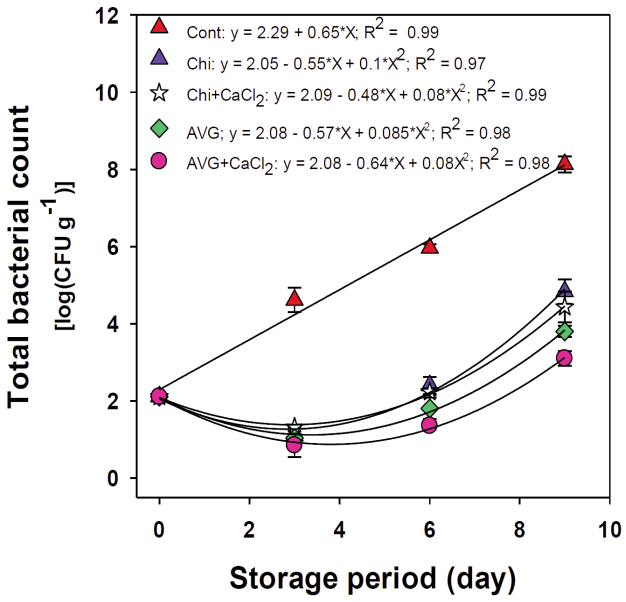

Fig. 5 - Effects of various edible coatings on total bacterial count (log CFU g^{-1}) of strawberries during storage at 4±1°C Control= uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$= 1.5% chitosan +1% $CaCl_2$ coated, AVG= *Aloe vera* coated, AVG+$CaCl_2$= AVG+1% $CaCl_2$ coated. Data presented with standard error of mean.

Table 1 - Effect of various edible coatings on biochemical properties (ascorbic acid, acidity, pH, total soluble solids, total sugar, and reducing sugar) of strawberries stored in the refrigerator (at 4±1°C) over a nine-day period

Coating treatments	Day	Ascorbic acid (%)	Acidity (%)	pH	TSS (%)	Total sugar (%)	Reducing sugar (%)
Control	0	47.8	0.86	3.94	7.5	5.4	4.0
	9	35.6	0.73	3.99	9.4	5.6	4.2
Chi	0	47.8	0.86	3.94	7.5	5.4	4.0
	9	41.6	0.74	3.97	8.6	5.5	4.1
Chi+$CaCl_2$	0	47.8	0.86	3.94	7.5	5.4	4.0
	9	41.5	0.77	3.97	8.5	5.5	4.1
AVG	0	47.8	0.86	3.94	7.5	5.4	4.0
	9	42.1	0.75	3.96	8.3	5.5	4.0
AVG+$CaCl_2$	0	47.8	0.86	3.94	7.5	5.4	4.0
	9	44.9	0.77	3.95	8.3	5.4	4.0
$LSD_{p\leq0.05}$							
Coating		2.66	0.02 NS	0.02 NS	0.28	0.23 NS	0.12 NS
Time		1.68	0.01	0.01	0.18	0.14 NS	0.08
Coating x time	1.20	0.01 NS	0.01 NS	0.13	0.10 NS	0.05 NS	

Control= Uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$ = 1.5% chitosan +1% $CaCl_2$ coated, AVG= *Aloe vera* gel (AVG) coated, AVG+$CaCl_2$= Aloe vera gel (AVG)+1% $CaCl_2$ coated. TSS=Total soluble solids. Ns= Not significant.

+ 0.08X²) or without 1% $CaCl_2$ (y = 2.08 – 0.57X + 0.085X²), showed significantly lower TBC compared to the uncoated control (y = 2.29 + 0.65*X). By the end of storage, TBCs were 3.1 and 3.8 log CFU g^{-1} for AVG+$CaCl_2$ and AVG coatings, respectively, versus 8.13 log CFU g^{-1} for the control. AVG alone reduced microbial load by 4.33 log CFU g^{-1} (~53%), while the addition of $CaCl_2$ provided an incremental reduction of 0.7 log CFU g^{-1} (~9%). Notably, all coated strawberries, except the control, remained below the permissible microbial contamination limit throughout storage.

Sensory quality and its relationship with other properties

Throughout the storage period, all fruits regardless of coatings exhibited variations in sensory properties (Table 2 and Fig. 6). By the 7th day of storage, the uncoated control strawberries exhibited lower sensory scores, with color (4.1), flavor (4.3), texture (4.3), and overall acceptability (4.0), falling below the consumer acceptability threshold. In

Table 2 - Effects of various edible coatings on sensory quality of strawberries (color, flavor, texture and overall acceptability) during storage in the refrigerator (at 4±1°C) over a nine-day period

Coating	Day	Color	Flavor	Texture	Overall acceptability
Control	7	4.1	4.3	4.3	4.0
	9	3.0	3.1	2.6	2.5
Chi	7	5.5	5.3	5.8	5.5
	9	4.1	4.2	4.4	4.1
Chi+$CaCl_2$	7	5.5	5.0	5.9	5.8
	9	4.3	4.6	4.5	4.4
AVG	7	7.2	6.9	7.0	7.3
	9	5.8	5.9	5.1	5.5
AVG+$CaCl_2$	7	7.6	6.9	7.3	6.9
	9	5.6	5.5	5.5	5.5
$LSD_{p\leq0.05}$					
Coating		0.46	0.40	0.43	0.41
Time		0.29	0.25	0.27	0.26
Coating x time	0.19 NS	0.17 NS	0.18 NS	0.17 NS	

Control= Uncoated, Chi= 1.5% chitosan coated, Chi+$CaCl_2$= 1.5% chitosan +1% $CaCl_2$ coated, AVG= *Aloe vera* gel coated, AVG+$CaCl_2$= AVG+1% $CaCl_2$ coated. Ns= Not significant.

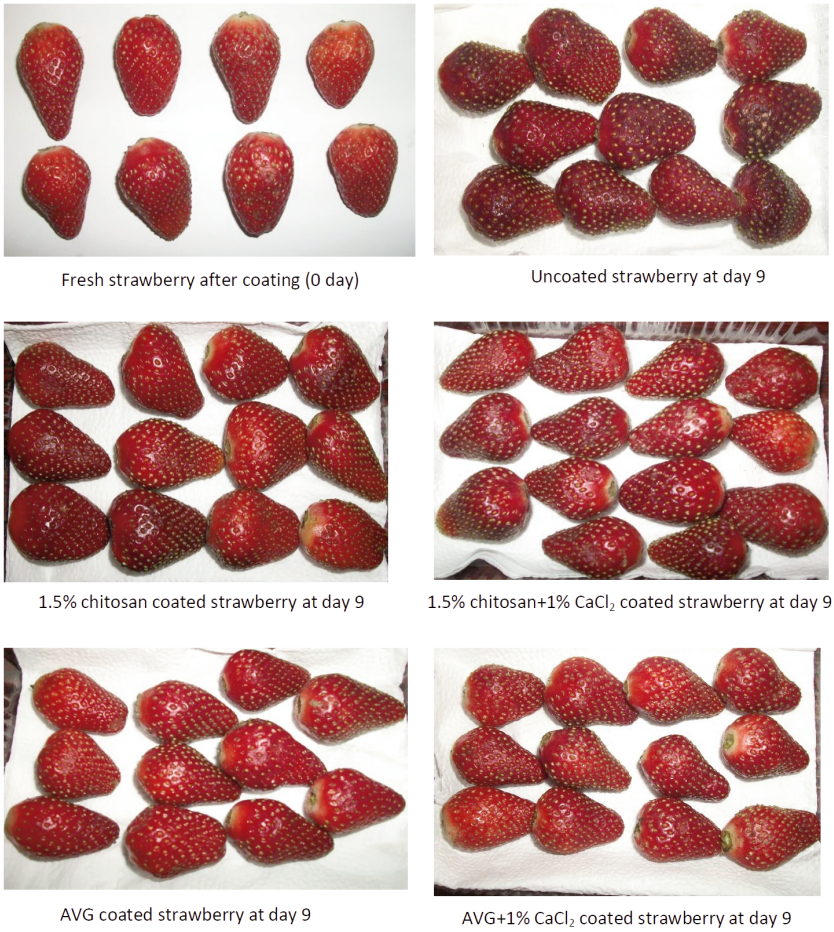

Fig. 6 - Visual appearance of post edible-coated strawberry treatments (control=uncoated, Chi= 1.5% chitosan, Chi+$CaCl_2$= 1.5% chitosan +1% $CaCl_2$, AVG= *Aloe vera* gel, AVG+$CaCl_2$= AVG+1% $CaCl_2$) for 9 days refrigerated storage at 4±1°C.

contrast, all coated strawberries-maintained scores above 5. Notably, strawberries coated with AVG, with or without $CaCl_2$, achieved higher sensory scores ranging from 6.9 to 7.6 and retained an average overall acceptability of 5.5 even by the 9th day of storage. Meanwhile, the sensory scores of strawberries coated with chitosan, with or without $CaCl_2$, declined below acceptable levels by the 9th day, with overall acceptability dropping below 4.5, once again falling beneath the consumer acceptance threshold.

Pearson correlation analysis of strawberry chemical, physical, and sensory attributes revealed that increased moisture loss was significantly and strongly correlated with reductions in firmness and pH, and moderately associated with decreases in reducing sugars, color, flavor, texture, and overall acceptability (Table 3). Firmness exhibited significant positive correlations with pH, reducing sugars, color, flavor, texture, and overall acceptability. Similarly, color showed significant positive correlations with flavor, texture, and overall acceptability, and these relationships were reciprocal. Total soluble solids (TSS) did not show significant correlations with any measured strawberry properties. In contrast, increasing ascorbic acid (vitamin C) content was positively correlated with total sugar concentration but negatively correlated with color, flavor, texture, and overall acceptability. Titratable acidity was significantly negatively correlated with both total and reducing sugar concentrations.

4. Discussion and Conclusions

Respiration rate

A significant reduction in respiration rates of strawberries was due to AVG coatings with or without $CaCl_2$ was beneficial as the elevated respiration rates accelerate carbohydrate depletion, leading to faster deterioration of fruit quality and reduced shelf life (Nasrin *et al.,* 2022). AVG coatings, especially when combined with $CaCl_2$, can significantly influence fruit gas exchange possibly causing saturation effects (Valverde *et al*., 2005). $CaCl_2$ enhances the structural stability of AVG and introduces Ca ions that may interact with *Aloe vera* polysaccharides and phenolics, altering viscosity, film properties, and antimicrobial activity (Shabir *et al.*, 2021). These interactions results in decreased respiration, ethylene production, transpiration, mechanical damage, and microbial growth (Blancas-Benitez *et al.,* 2022). Shafique *et al.* (2023) reported that strawberries coated with 20% AVG showed a two-fold reduction in respiration rate at five days of shelf life, when compared to control ones. Furthermore, Eshghi *et al.* (2014) observed significantly lower respiration rates in strawberries coated with nano-chitosan compared to uncoated control ones.

Fruit firmness

As anticipated, strawberries coated with AVG exhibited the highest firmness, primarily due to the

Table 3 - Pearson correlations among strawberry chemical, physical, and sensory properties

	Firmness	Respirative rate	Total soluble solids	pH	Ascorbic acid	Titrable acid	Total sugar	Reducing sugar	Color	Flavor	Texture	Acceptability
Moisture	-0.95 ***	-0.51 *	0.23 NS	-0.88 ***	0.25 NS	0.63 **	-0.45 *	-0.69 **	-0.48 *	-0.46 *	-0.53 *	-0.50 *
Firmness		0.32 NS	-0.29 NS	0.85 ***	-0.48 *	-0.48 *	0.24 NS	0.67 **	0.58 *	0.56 *	0.63 **	0.61 **
Resp			0.21 NS	0.49 *	0.27 NS	-0.66 **	0.60 *	0.30 NS	0.01 NS	-0.06 NS	0.06 NS	0.07 NS
TSS				-0.23 NS	0.26 NS	-0.09 NS	0.12 NS	0.11 NS	-0.26 NS	-0.23 NS	-0.29 NS	-0.21 NS
pH					-0.25 NS	-0.53 *	0.31 NS	0.66 **	0.25 NS	0.21 NS	0.33 NS	0.28 NS
Aacid						-0.3 2 NS	0.63 **	-0.2 2NS	-0.67 **	-0.68 **	-0.64 **	-0.68 **
Tacid							-0.87 ***	-0.64 **	0.17 NS	0.19 NS	0.12 NS	0.16 NS
Tsugar								0.35 NS	-0.36 NS	-0.36 NS	-0.31 NS	-0.34 NS
Rsugar									0.15 NS	0.16 NS	0.19 NS	0.19 NS
Color										0.99 ***	0.97 ***	0.98 ***
Flavor											0.94 ***	0.97 ***
Texture												0.98 ***

*, **, and *** indicate significant correlations at p<0.05, 0.01, and 0.001 levels, respectively.
NS = Not significant.

preservation of polysaccharide structures that enhance cell wall strength. Fruit softening typically occurs during development, ripening, and senescence, driven by water loss and the degradation of structural components through respiration. However, AVG helps mitigate these effects by forming a protective physical barrier that reduces water evaporation and maintains the integrity of the fruit's dry matter content, thereby slowing the softening process and extending postharvest quality (Nasrin *et al*., 2023).

The AVG + 1% $CaCl_2$ coating was particularly effective in reducing fruit softening by inhibiting enzyme activity, especially polygalacturonase, which breaks down cell walls during ripening. Additionally, their synergistic effects limit oxygen uptake, moisture loss, microbial growth, and metabolic activity, thereby delaying ripening and preserving firmness in strawberries (Rehman *et al*., 2022).

Calcium contributes further by strengthening cell walls and enhancing tissue firmness through pectin formation (White and Broadley, 2003). Additionally, a 1.5% chitosan coating - with or without $CaCl_2$ - has been shown to better maintain strawberry firmness than a 1% $CaCl_2$ dip over a 4-day storage period at 20°C (Hernandez-Munoz *et al*., 2006).

Weight (moisture) loss

Weight (moisture) loss in strawberries increases susceptibility to shrinkage, skin wounding, softening, deterioration, and color darkening. The extent of moisture loss is largely influenced by the gradient in atmospheric pressure between the internal fruit tissues and the surrounding environment. Coating treatments, such as AVG and chitosan, effectively reduce this gradient by forming semi-permeable barriers that minimize transpiration, regulate gas exchange, and retain cellular moisture. The hygroscopic nature of AVG further enhances its ability to prevent water transfer between the fruit and its surroundings (Valverde *et al*., 2005).

Studies have reported that AVG coatings significantly reduce water vapor transmission rate and moisture loss in perishable fruits compared to untreated controls (Olivas and Barbosa-Cánovas, 2005). Likewise, Hassan *et al.* (2022) reported that uncoated strawberries exhibited the highest weight loss (11.9%), while those coated with 40% AVG + 1% lemongrass essential oil showed the lowest (6.1%), followed by 20% AVG + 1% lemongrass essential oil (7.6%) after 16 days of storage at 5 ± 1°C. Similarly, Nguyen *et al.* (2020) demonstrated that a coating of 0.2% nano-chitosan combined with 3% $CaCl_2$ was more effective in reducing weight loss than nano-chitosan alone or uncoated controls. This improvement is likely due to calcium's role in modifying tissue gas diffusion rates and affecting respiratory metabolism.

Overall, the reduced moisture loss in coated strawberries helps maintain fruit weight, limits respiration, delay senescence, and enhance postharvest shelf life by slowing physiological processes associated with deterioration.

External fruit colour

Coating fruits with AVG creates a modified environment that helps prevent chlorophyll degradation and carotenoid pigment synthesis, thereby delaying ripening (Ergun and Satici, 2012). Similarly, chitosan coatings have been shown to slow respiration and reduce ethylene production in papaya, resulting in delayed ripening and senescence, along with reduced softening and color changes (Ali *et al.*, 2011). In strawberries, uncoated fruits showed approximately a 29% reduction in lightness, whereas fruits coated with 1%, 1.5%, and 2% chitosan exhibited lightness losses of around 16%, 11%, and 10%, respectively. Enzymatic browning, primarily driven by the oxidation of polyphenolic compounds catalyzed by polyphenol oxidase (PPO), remains a major postharvest issue in strawberries (Kebriti *et al.*, 2025). However, strawberries coated with AVG showed only a 4% loss in lightness, likely due to the inhibition of PPO activity (Nasrin *et al.*, 2017). This suppression of enzymatic browning helps preserve the fruit's color and overall quality during storage.

Moreover, strawberries coated with AVG alone or in combination with lemongrass essential oil retained their lightness more effectively and maintained visual quality up to 16 days at 5°C (Hassan *et al.*, 2022). Treatments combining 40% AVG + 1% lemongrass essential oil and 20% AVG + 1% lemongrass essential oil also preserved higher hue angle values than the control during eight days of storage at 5°C. Additionally, Amal *et al.* (2010) found that strawberries coated with soy or gluten films incorporating thymol, as well as soy-based coatings containing $CaCl_2$, exhibited brighter red coloration and higher chroma values compared to uncoated fruits.

Ascorbic acid, titratable acidity, and sugar

The reduced loss of ascorbic acid (vitamin C) in coated strawberries is attributed to the protective effect of the edible coating, which limits oxygen exposure and slows respiration, thereby minimizing oxidative degradation (Ayranci and Tunc, 2004). Additionally, $CaCl_2$ has been shown to inhibit antioxidant loss, including that of ascorbic acid (Luna-Guzman and Barrett, 2000). Our findings align with those of Muzzaffar *et al*. (2016), who reported initial values for ascorbic acid, TSS, acidity, total sugars, and reducing sugars in fresh strawberries as 38.6 mg 100 g^{-1}, 8 °Brix, 1.3%, 5.2%, and 4.3%, respectively.

Similarly, Shabir *et al*. (2021) observed that during storage, fruit pH and TSS increased, while ascorbic acid and titratable acidity decreased. These changes were attributed to moisture loss through respiration and transpiration, starch breakdown, and the synthesis of pectin and free acids. Hassan *et al*. (2022) further confirmed that pH levels gradually increased and titratable acidity declined due to the oxidation of organic acids during ripening.

Lower TSS in AVG-coated strawberries result from both reduced concentration effects and slowed metabolic changes. AVG coatings form a semi-permeable barrier that minimizes water loss, limiting the passive concentration of sugars typically seen in uncoated fruits during storage (Olivas and Barbosa-Cánovas, 2005; Nasrin *et al*., 2017). This barrier helps maintain fruit moisture, preventing the artificial TSS increase caused by dehydration. Simultaneously, AVG reduces respiration rates by restricting oxygen diffusion, thereby slowing ripening and metabolic activity (Valverde *et al.*, 2005; Rehman *et al.*, 2022). This delay in ripening impedes the enzymatic breakdown of complex carbohydrates into simple sugars by inhibiting enzymes like amylases and polygalacturonases (Hassan *et al.*, 2022; Nicolau-Lapena *et al.*, 2021). Studies in guava and other fruits support this, showing that coated fruits accumulate sugars more slowly than uncoated controls (Ali *et al*., 2011; Rehman *et al*., 2022). Therefore, AVG-coated strawberries are expected to exhibit stable sugar concentrations over storage, avoiding the TSS spikes measured in uncoated fruit due to dehydration and accelerated metabolism.

Microbiological analysis

The reduction in total bacterial counts on of AVG and chitosan coated strawberries, with or without the addition of $CaCl_2$ is largely attributed to the complementary antimicrobial actions. AVG's efficacy stems from its bioactive compounds - primarily aloin and aloe-emodin - which inhibit fungal spore germination and suppress the growth of yeasts and molds (Huang and Yan, 2023). Moreover, AVG forms a semi-permeable barrier that reduces moisture loss, thereby limiting the water availability essential for microbial proliferation. $CaCl_2$ complements this effect by lowering cellular pH and water activity within the fruit's microenvironment, indirectly inhibiting microbial survival and possibly interfering with spore germination. Though $CaCl_2$ has limited direct action against spore-forming bacteria, it alters environmental conditions unfavorable for their growth. Coatings combining AVG with antimicrobial agents, such as lemongrass oil or $CaCl_2$, have demonstrated enhanced microbial suppression (Hassan *et al.*, 2022). In this study, AVG alone reduced microbial loads by ≈53%, while the addition of $CaCl_2$ resulted in an additional reduction of ≈9%. These results confirm that AVG is the primary antimicrobial agent, with $CaCl_2$ providing modest but synergistic enhancement, offering an effective strategy against postharvest spoilage, especially from yeasts, molds, and some bacteria.

Sensory quality and its relationship with other properties

Throughout the storage period, all fruits exhibited a decline in sensory properties, likely due to factors such as endogenous enzyme activity, skin darkening, and moisture loss (Badawy *et al.,* 2017). Our research aligns with the findings of Shabir *et al.* (2021), who reported that refrigerated fruits such as guavas coated with 10% AVG and 2% $CaCl_2$ achieved the highest overall acceptability score (6.18), compared to a score of 5.18 for uncoated fruits. Similarly, Pimsorn *et al.* (2022) observed that lime coated with AVG exhibited superior sensory and visual qualities, reduced weight loss, improved firmness, elevated ascorbic acid concentration and acidity, delayed increases in TSS, reduced decay, and extended shelf life compared to uncoated counterparts. Chrysargyris *et al.* (2016) found that tomatoes coated with 10% and 15% AVG and stored at 11 °C and 90% relative humidity maintained their overall quality by reducing ripening indices and ethylene production. Amiri *et al.* (2022) demonstrated that a coating formulation containing AVG, 2% $CaCl_2$, and 5% nano-encapsulated

catechin significantly enhanced the quality and shelf life of refrigerated strawberries.

Pearson correlation analysis revealed that moisture loss was a key factor in strawberry quality decline, strongly negatively correlated with firmness and pH, and moderately with sugars, color, flavor, texture, and acceptability. Firmness showed strong positive correlations with nearly all quality traits, making it a reliable quality indicator. Color was also positively linked with key sensory attributes. Total soluble solids showed no significant associations, indicating stability during storage. Ascorbic acid correlated positively with total sugars but negatively with sensory traits, suggesting stress-related changes. Titratable acidity was negatively associated with sugars, reflecting ripening processes. Moisture retention and firmness are shown vital for shelf life.

This study demonstrated that uncoated strawberries retained acceptable quality for only 6 days, whereas those coated with 1.5% chitosan extended shelf life to 8 days. Notably, strawberries coated with AVG, with or without $CaCl_2$, maintained freshness for over 9 days, exhibiting superior firmness, color, moisture, and flavor, and texture retention, with no visible decay. Although the addition of $CaCl_2$ slightly enhanced firmness, moisture retention, and microbial suppression, these enhancements were not statistically significant. Interestingly, AVG alone provided sensory quality comparable to the AVG+$CaCl_2$ combination. These findings underscore AVG's strong potential as a natural, eco-friendly coating for extending strawberry shelf life and preserving nutritional quality, offering a sustainable alternative to synthetic packaging. Further research is recommended to investigate the phytochemical and metabolomic profiles, essential nutrient content, and cost-effectiveness to support broader commercial application.

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Effect of boron priming on germination traits of shallot (*Allium ascalonicum* L.)

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Abstract: Shallot (*Allium ascalonicum* L.) is a vital horticultural commodity in Indonesia, valued for its culinary and medicinal properties. Seed priming, especially with boron (B), represents a promising approach to enhance germination performance. This study investigated the effects of various boron concentrations on the germination performance of shallot seeds. The experiment was conducted under screen house conditions at the Faculty of Agriculture, Hasanuddin University, in May 2024, using a randomized block design with 11 boron concentrations (0-100 mg L^{-1}) and three replications. Priming with 100 mg L^{-1} resulted in the best performance across several germination parameters, including as germination percentage (GP), germination speed index (GSI), the time required for 10% of seeds to germinate (T10), the time required for 50% of seeds to germinate (T50), mean germination time (MGT), mean germination rate (MGR), and germination speed coefficient (GSC). Regression analysis showed a strong positive linear relationship between boron concentration and both final germination percentage (r^2 = 0.84) and germination speed index (r^2 = 0.76). These findings suggest that boron priming, especially at 100 mg L^{-1}, significantly enhances germination performance of shallot seeds and may as an effective method for improving seedling vigor.

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1. Introduction

Shallot (*Allium ascalonicum* L.) is a horticultural commodity extensively cultivated by farmers across various regions in Indonesia. In addition to its widespread use as a culinary spice, shallots contain numerous bioactive compounds with potential medicinal properties, such as quercetin, flavonoids, saponins, tannins, glycosides, polyphenols, and alkaloids (Devika *et al*., 2021). The ongoing development of shallot cultivation is driven by its various advantages, including meeting national consumption needs, providing a source of income for farmers, and its potential to contribute to foreign exchange earnings. These attributes position shallots as a strategically important commodity with high economic value.

Shallot production in Indonesia reached 1,985,233 tons in 2023, an increase from 1,982,360 tons in 2022 but a decrease from 2,004,590 tons in 2021 (Central Bureau of Statistics of Indonesia, 2024). Shallot production still experiences fluctuations, often resulting in imbalances between supply and demand in the market. National demand for shallot has been increasing at a rate of approximately 5.0-6.67% annually (Shrestha *et al*., 2019). Therefore, efforts to boost production must continue, including improving the quality of planting materials by transitioning to true shallot seed (TSS). However, challenges are often encountered during the seedling process when cultivating shallots from TSS. The nursery process, which typically lasts 30-45 days before planting, extends the time required for crop production and delays harvest (Nciizah *et al*., 2020). Additionally, shallot seeds are highly susceptible to losings viability due to various factors during storage, leading to reduced germination ability and speed (Simatupang and Pangaribuan, 2022). Germination is a crucial stage in the plant life cycle that determines the success of subsequent growth stages (Atabakia *et al*., 2022).

Seed priming is an effective method for producing high-quality seedlings (Tanjung *et al*., 2021). This technique can improve initial seed germination and seedling growth (Shimizu *et al*., 2023), accelerate germination, and enhance germination performance even under extreme environmental conditions and poor soils (Chookhampa *et al*., 2023). Several factors influence seed priming, including water potential, priming agents, duration, temperature, seed vigor, and storage conditions (Farooq *et al*., 2011; Faisal *et al*., 2023). Among these, the choice of priming agent is crucial for the success of seed priming. Micronutrients, such as boron (B) can be used as priming agents to enhance plant productivity and quality (Mansouri *et al*., 2022). Boron priming enhances seed vigor and promotes better growth and seedling development compared to untreated seeds (Chakraborty and Dwivedi, 2022). As a priming agent, boron contributes to improving and stabilizing cell wall structure and has a positive impact on reducing disease severity (Pangestuti *et al*., 2021).

Research by Rehman *et al.* (2022) reported that seed priming with 0.01% boron significantly accelerated seedling emergence and the time to reach 50% seedling emergence, improved germination index, leaf number, fresh and dry root weight, chlorophyll content, various yield parameters, and increased carbohydrate, protein, and fiber content in mung bean plants (*Vigna radiata* L.). Research by Nciizah *et al*. (2020) also revealed that seed priming with 0.01% boron reduced the number of days to seedling emergence by 94% and increased seedling fresh and dry weight, as well as the chlorophyll index by 29%, 47%, and 58%, respectively, compared to the control in maize plants (*Zea mays* L.). Mansouri *et al*. (2022) found that seed priming with boron enhanced chlorophyll and carotenoid pigment content and increased the levels of enzymatic and non-enzymatic antioxidants such as anthocyanin, superoxide dismutase, and peroxidase in wheat seeds. Based on the above reviews, this study aims to determine the effect of different boron concentrations as a priming agent on the germination performance of shallot seeds.

2. Materials and Methods

Research location and experimental design

This research was conducted under screen house conditions at the Experimental Farm of the Faculty of Agriculture, Hasanuddin University, in May 2024. The study was designed as a randomized block experiment with a single treatment factor. The treatment factor was boron concentration, which included 11 levels: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg L^{-1}. Each treatment level was replicated three times, resulting in a total of 33 experimental units.

Priming and germination assay

The seed variety used in this study was 'Maserati'.

The seeds were primed with a boron solution following the specified treatment protocols. They were then placed in plastic jars containing the solution at a seed-to-solution ratio of 1:5, with an aerator added to ensure an adequate oxygen supply. The priming process lasted for 20 hours at room temperature (Ghassemi-Golezani *et al*., 2010; Farahzety *et al*., 02023; Katriani *et al.*, 2023; Faried *et al*., 2024). Afterward, the seeds were removed and air-dried at room temperature for 48 hours before further use.

Germination was evaluated using a soil and compost growth medium in a 1:1 (v/v) ratio. The medium was thoroughly mixed and placed into transparent plastic boxes with drainage holes at the bottom. Each plastic box had a volume of 500 cm^3 and was filled with 500 g of the growth medium. The primed seeds were then planted by creating furrows 1 cm deep. Each plastic box contained 50 shallot seeds. After sowing, the seeds were lightly irrigated twice daily (morning and evening) with approximately 30 mL of water per application to maintain adequate soil moisture under the warm ambient conditions of the experiment. Watering volumes were carefully controlled to prevent waterlogging while providing optimal conditions for germination. The seedling emergence was observed daily until day 14.

Data collection and analysis

The parameters evaluated in this study included final germination percentage (FGP) (%), germination speed index (GSI), the time required for 10% of seeds to germinate (T10) (d), the time required for 50% of seeds to germinate (T50) (d), mean germination time (MGT) (d), mean germination rate (MGR), variance of germination time (VGT), coefficient of variation of germination time (CVGT), germination synchrony (GS), and germination speed coefficient (GSC) (Table 1). These ten parameters were calculated using observational data on the number of seeds germinating daily. Seeds were considered to have germinated when the plumule emerged above the surface of soil.

An analysis of variance (ANOVA) was conducted to test significant differences among treatments. When significant effects were found, Duncan's Multiple Range test was applied to separate means. Additionally, regression analysis was conducted to assess the relationship between boron concentration and the germination parameters. Calculations for the parameters were performed using the Seedcalc package, while data analysis and visualization were conducted using the ggplot2 and AgroR packages in RStudio version 4.2.1 (Wickham, 2016; Shimizu *et al*., 2023; R Core Team, 2024).

3. Results

Germination parameters

The germination parameters of shallot seeds exhibited a significant response to increasing boron concentrations (Table 2 and Table 3). The FGP rose from 80.67% at 0 mg L^{-1} to 96.00% at 100 mg L^{-1}, reflecting a 15.33% increase, which highlights boron's positive influence on germination efficiency. Similarly, GSI improved by 63.79%, from 6.96 to 11.40, indicating enhanced metabolic activity. T10 decreased from 4.03 days to 2.40 days, showing a 40.45% reduction, while T50 decreased by 33.39%, from 6.08 days to 4.05 days. MGT also improved, dropping by 28.25% from 6.30 days to 4.52 days, reflecting faster and more uniform germination under higher boron concentrations.

The application of boron significantly influenced several germination parameters of shallot seeds. MGR and GSC increased significantly with higher boron concentrations, with the highest values observed at 100 mg L^{-1}, indicating faster germination compared to the control. Although VGT and GS were not significantly affected ($p > 0.05$), lower VGT values at 20, 50, 80, and 100 mg L^{-1} suggest more uniform germination, while GS tended to improve at 20, 50, and 80 mg L^{-1}. The CVGT was significantly reduced at 20 and 50 mg L^{-1}, reflecting more consistent germination timing. Overall, boron concentrations between 50 and 100 mg L^{-1} were most effective in enhancing germination performance by improving speed, rate, and uniformity.

Regression analysis

The impact of boron concentration on various germination parameters of shallot seeds is depicted in the series of linear regression analyses (Table 4, Fig. 1). The analysis demonstrates that boron application significantly enhances several key germination parameters. For instance, FGP shows a strong positive correlation with boron concentration ($R^2 = 0.84$), indicating that higher boron levels lead to a substantial increase in germination efficiency. This suggests that boron plays a vital role in breaking seed dormancy and supporting metabolic processes essential for successful germination.

Similarly, the GSI exhibited a positive trend (R^2 =

Table 1 - Overview of the measured parameters related to seed germination

No	Parameters	Formula
1	Final germination percentage (FGP)	FGP = (n/N) x 100 n is the number of seeds germinated, and N is the total number of seed.
2	Germination Speed Index (GSI)	GSI = $\sum_{i=1}^{n} (\frac{ni}{ti})$ n is the number of seeds germinated on each day of daily counting up to the last count, and t is the number of days after the beginning of the test in each count.
3	T10 Germination (T10)	Days at which 10% cumulative germination is reached.
4	T50 Germination (T50)	$T50 = \frac{ti + \left\{\left[\frac{N}{\frac{100}{10}} - ni\right]\right\}(tj - ti)}{(nj - ni)}$ N is the final number of seeds germinated, and n_i and n_j are the total number of seeds germinated in adjacent counts in time t_i and t_j, respectively, when $n_i < \frac{N+1}{2} < n_j$
5	Mean Germination Time (MGT)	MGT = $\sum_{i=1}^{k} (\text{niti}) / \sum_{i=1}^{k} \text{ni}$ n_i is the number of seeds germinated per day (not the accumulated number, but the number corresponding to the i-th observation), and t_i is the time since the beginning of the germination test up to the i-th observation.
6	Mean Germination Rate (MGR)	MGR = CoVg / 100 = 1/t t is the mean germination time, and CoVg is the germination speed coefficient.
7	Variance of Germination Time (VGT)	$VGT = \sum_{i=1}^{k} ni\ (ti - t)^2 / (\sum_{i=1}^{k} ni - 1)$ t is the mean germination time, t_i is the time between the beginning of the experiment and the i-th observation (day or hour), n_i is the number of seeds germinated in time i, and k is the last count of the germination test.
8	Coefficient of Variation of Germination Time (CVGT)	CVGT= (S_t / t) 100 S_t is standard deviation of the germination time, and t is mean germination time.
9	Germination Synchrony (GS)	Sinc = ∑*Cni*, 2 / N Cni, 2 = ni (ni – 1) / 2 N = ∑*ni* (∑*ni* -1) / 2 Cni is the combination of the seeds germinated in time i, two by two, and ni is the number of seeds germinated in time i.
10	Germination Speed Coefficient (GSC)	$CVG = (\sum_{i=1}^{k} fi / \sum_{i=1}^{k} fi\ xi)\ 100$ f_i is the number of newly germinated seeds on day i, and x_i is the number of days from sowing.

0.76), indicating that higher boron concentrations accelerated germination rate. The reductions in T10 (R^2 = 0.43) and T50 (R^2 = 0.47) further supported this, as boron shortened the time needed to reach early and intermediate germination stages. MGT showed a negative correlation (R^2 = 0.45), reflecting a decrease

Table 2 - Germination performance parameters of shallot seeds under different boron concentrations

Boron concentration (mg L^{-1})	Final germination percentage (%) [x]	Germination speed index [x]	Gemination time T10 (d) [x]	Gemination time T50 (d) [x]	Mean germination time (d) [x]
0	80.67 d	7.26 de	3.52 bc	5.19 bc	5.92 ab
10	82.00 d	7.42 de	3.39 bcd	5.57 ab	5.86 abc
20	82.00 d	8.10 d	3.34 bcd	4.94 bcd	5.22 bcdef
30	82.00 d	6.96 e	3.66 ab	6.08 a	6.3 0a
40	82.00 d	8.10 d	3.18 cde	4.67 cde	5.36 bcde
50	86.00 c	7.95 de	4.03 a	5.00 bcd	5.5 9bcd
60	88.67 c	9.65 bc	2.84 efg	4.34 de	4.89 def
70	88.67 c	9.40 c	2.82 efg	4.23 de	5.21 bcdef
80	92.67 b	10.52 ab	2.67 fg	4.23 de	4.65 ef
90	95.33 a	9.99 bc	2.95 def	4.41 cde	5.14 cdef
100	96.00 a	11.40 a	2.40 g	4.05 e	4.52 f
p-value	<0.01	<0.01	<0.01	<0.01	<0.01

[x] Different letters within the same column indicate statistically significant differences according to Duncan's Multiple Range Test.

Table 3 - Metabolic and synchronization parameters of shallot seed germination under different boron concentrations

Boron concentration (mg L^{-1})	Mean germination rate [x]	Variance of germination time	Coefficient of variation of germination	Germination synchrony	Germination speed coefficient [x]
0	0.169 de	2.38	25.04 bc	0.24	16.95 de
10	0.170 de	2.11	24.73 bc	0.25	17.05 de
20	0.191 bcd	0.87	17.91 c	0.34	19.12 bcd
30	0.158 e	2.57	25.31 bc	0.22	15.88 e
40	0.187 cd	2.09	24.81 bc	0.25	18.79 cd
50	0.179 cde	1.04	17.42 c	0.39	17.98 cde
60	0.204 abc	1.74	26.31 abc	0.30	20.48 abc
70	0.192 bcd	3.05	32.00 ab	0.24	19.26 bcd
80	0.215 ab	1.06	22.27 bc	0.33	21.59 ab
90	0.194 bcd	4.07	36.78 a	0.29	19.48 bcd
100	0.220 a	1.64	27.81 abc	0.28	22.08 a
p-value	<0.01	>0.05	<0.01	>0.05	<0.01

[x] Different letters within the same column indicate statistically significant differences according to Duncan's Multiple Range Test.

in total seedling emergence time, while MGR increased (R^2 = 0.47), highlighting improved metabolic efficiency and faster development. In contrast, VGT (R^2 = 0.01), CVGT (R^2 = 0.12), and GS (R^2 = 0.03) all revealed weak or negligible correlations, suggesting that boron had limited effects on uniformity, consistency, and synchronization of germination.

Conversely, GSC demonstrated a moderate positive correlation (R^2 = 0.47), emphasizing boron's role in enhancing the speed of germination completion. Overall, these findings suggested that boron effectively improved germination performance, particularly in terms of percentage and speed, while having minimal impact on uniformity and synchronization.

Correlation analysis

The correlation matrix illustrates the relationships between different germination parameters of shallot seeds under varying boron concentrations. GP showed a strong positive correlation with GSI (r =

Table 4 - Regression analysis of parameters

No.	Paramaters	Equation	R^2
1	Final germination percentage (%)	Y = 78.6 + 0.165	0.84
2	Germination speed index	Y = 6.77 + 0.040	0.76
3	Time to 10% seed germinated	Y = 3.69 + 0.010	0.43
4	Time to 50% seed germinated	Y = 5.55 - 0.015	0.47
5	Mean germination time	Y = 5.99 – 0.013	0.45
6	Mean germination rate	Y = 0.166 + 0.000466	0.47
7	Variance of germination time	Y = 1.82 + 0.00482	0.01
8	Coefficient of variation of germination time	Y = 21.4 + 0.0819	0.12
9	Germination synchrony	Y = 0.27 + 0.000365	0.03
10	Germination speed coefficient	Y = 16.6 + 0.0466	0.47

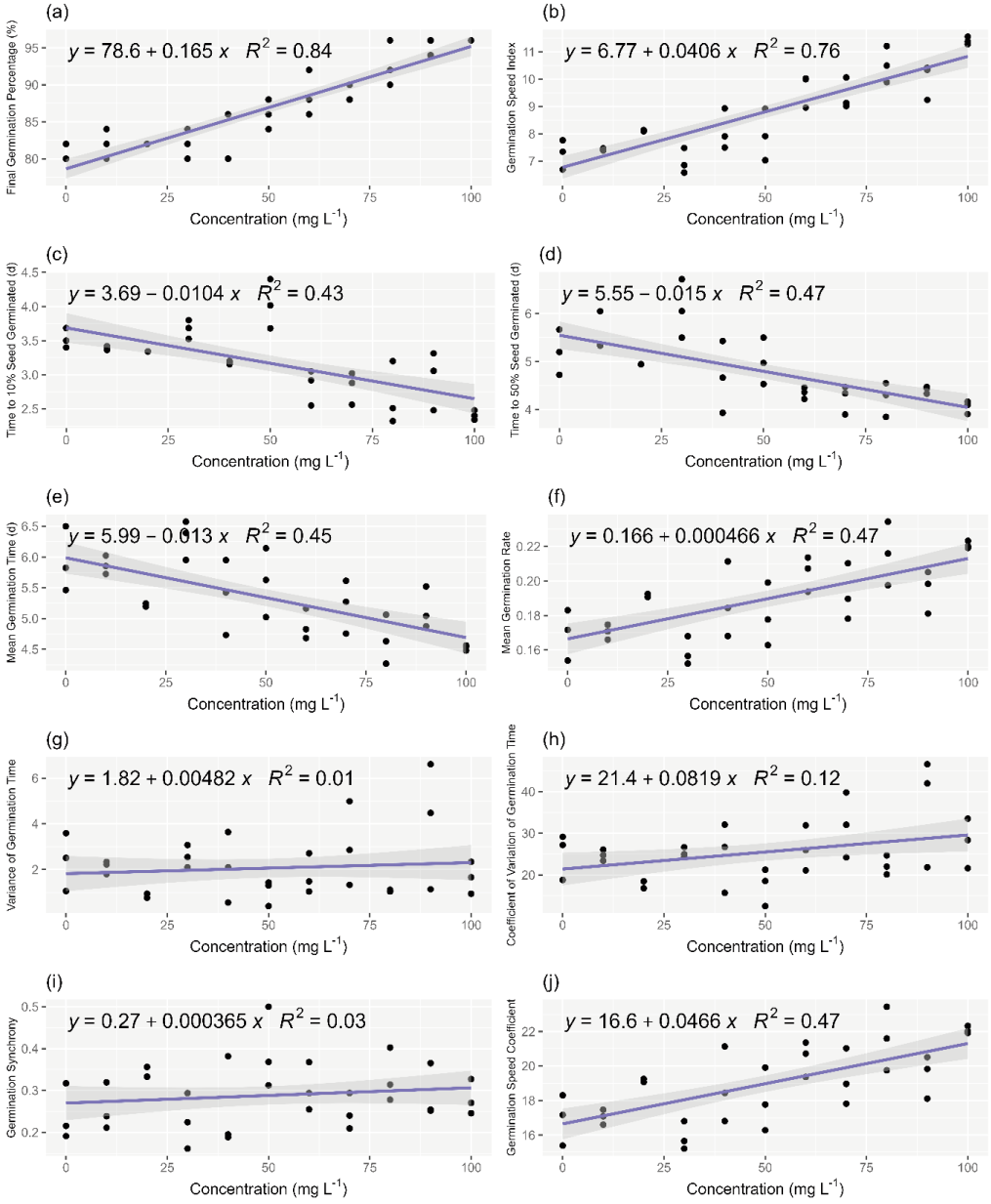

Fig. 1 - Regression analysis of each germination trait to boron concentration.

0.90), suggesting that higher germination percentages are associated with faster germination rates. However, GP negatively correlates with T10 (r = -0.64), T50 (r = -0.69), and MGT (r = -0.71), indicating that increased boron concentration reduces the time needed for germination (Fig. 2).

GSI also exhibited significant positive correlations with MGR (r = 0.93) and VGT) (r = 0.93), showing that faster germination is linked to higher consistency in seed performance. Conversely, GSI has a strong negative correlation with MGT (r = -0.91), emphasizing that a higher speed index leads to shorter germination times. T10, T50, and MGT parameters displayed high intercorrelations, with T10 and T50 showing r = 0.78 and T50 and MGT showing r = 0.87. Parameters related to uniformity, such as VGT and GS, showed weaker and mostly non-significant correlations with the other considered parameters.

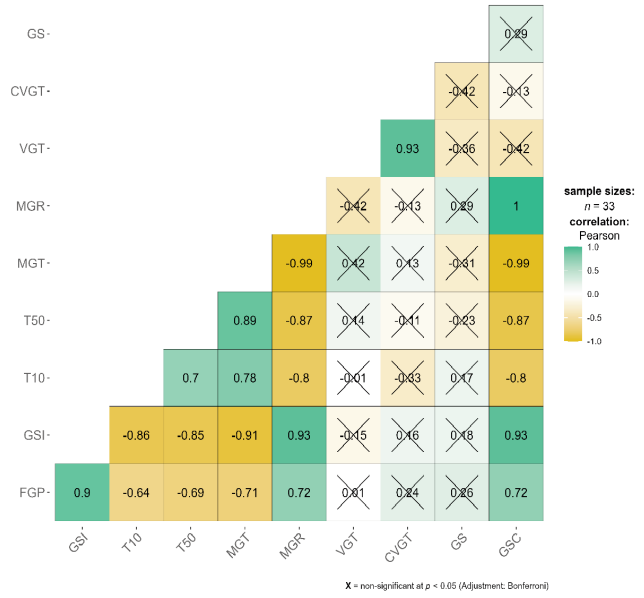

Fig. 2 - Correlation analysis among parameters.

4. Discussion and Conclusions

Seed priming is a crucial technique in agriculture aimed at enhancing seed germination and seedling establishment under various stress conditions. By promoting pre-germination metabolic activity through controlled hydration and drying, seed priming can significantly improve germination rates and seedling vigor, helping plants to overcome abiotic stresses such as salinity and drought (Chatterjee *et al*., 2018; Hameed *et al*., 2025; MacDonald and Mohan, 2025). The positive impacts of seed priming are not limited to increasing germination and vigor, but also extend to improving crop yields and quality, making it an essential practice for sustainable agriculture (Gour *et al*., 2023; Jarrar *et al*., 2024; Zhang *et al*., 2025).

The significant improvement in shallot seed germination parameters in response to increasing boron concentrations highlights boron's essential role in seed physiology. The germination percentage increased by 15.33%, from 80.67% at 0 mg L^{-1} to 96.00% at 100 mg L^{-1}, demonstrating boron's positive impact on germination efficiency. This improvement is likely due to biochemical and physiological changes induced by priming, which enhance water uptake, activate metabolic pathways, and initiate early growth processes. Bajwa *et al.* (2018) similarly found that priming wheat seeds increased alpha-amylase activity, facilitating carbohydrate breakdown and providing energy for embryo development. A comparable enzymatic activation may contribute to the enhanced germination observed in shallot seeds.

Boron also accelerated germination times, as indicated by the 40.45% reduction in time to 10% germination, a 33.39% decrease in time to 50% germination, and a 28.25% reduction in mean germination time. The shortest durations were recorded at 2.40 days (T10), 4.05 days (T50), and 4.52 days (MGT), all significantly lower than the control. These findings align with previous research by Rehman *et al*. (2022), which demonstrated that boron priming accelerates the germination process. However, it is important to consider that at certain concentrations, boron can become toxic to plants (Hussain *et al*., 2011; Atique-ur-Rehman *et al*., 2020).

Additionally, boron priming enhanced the germination speed index. At 100 mg L^{-1}, the highest GSI (11.40) was recorded, whereas the control treatment had the lowest value (6.96). This suggests that boron plays a crucial role in expediting germination, likely due to its involvement in cell wall formation, membrane integrity, and metabolic regulation. Similar results were reported by Farooq *et al.* (2011), who found that boron priming significantly increased the germination index of rice seeds. Kaya and Ergin (2023), in a study conducted on *Carthamus tinctorius* seeds, also observed a linear increase in germination index with higher boron concentrations, reinforcing boron's beneficial effects on seed germination.

Interestingly, the coefficient variation of germination time increased by 105.46%, reflecting improved

synchronization of the germination process. This suggests that boron not only accelerates germination but also promotes more uniform seedling establishment. However, the lack of significant differences in germination synchrony and the stability of the germination speed coefficient indicate that boron's impact is more pronounced in accelerating germination rather than reducing variability. Chookhampa *et al.* (2023) also reported that seed priming on *Andrographis paniculata*, *Sesamum indicum*, and *Abelmoschus esculentus* improves the germination performance, leading to more synchronized germination. The limited effect of boron on germination uniformity may be influenced by inherent genetic factors and environmental conditions.

Beyond improving germination rates and speed, priming has also been linked to enhanced seedling vigor and quality (Biradar *et al*., 2023; Faried *et al*., 2023). Overall, this study demonstrates that increasing boron concentrations positively influence germination percentage, speed index, and synchronization, while also accelerating the overall germination process. These findings underscore boron's potential as a seed priming agent to enhance germination efficiency and metabolic activity in shallot seeds. However, the effects observed are indeed influenced by multiple factors. The success of seed priming depends on the choice of priming agent, its concentration, the duration of priming, and the specific response of the plant species or variety (Siyar *et al.*, 2020; Corbineau *et al*., 2023; Fu *et al*., 2024; Jarrar *et al*., 2024). Moreover, priming cannot overcome issues arising from genetic factors; seeds lacking in genetic purity, will result in poorly growing plants (Reed *et al*., 2022).

Priming with 100 mg L^{-1} produced the most favorable outcomes on several germination parameters, such as final germination percentage, germination speed index, time required for 10% of seeds to germinate, time required for 50% of seeds to germinate, mean germination time, mean germination rate, and germination speed coefficient. Regression analysis showed a strong positive linear relationship between boron concentration and final germination percentage ($r^2 = 0.84$) as well as germination speed index ($r^2 = 0.76$). The study concludes that boron priming, particularly at 100 mg L^{-1}, significantly improves germination performance in shallot seeds, highlighting its potential to enhance seedling vigor and productivity. The results of this research offer valuable insights for both shallot researchers and farmers.

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Improving drought tolerance of *Leucophyllum frutescens* through the application of paclobutrazol and cycocel plant growth regulators

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Abstract: *Leucophyllum frutescens* is an evergreen shrub renowned for its drought tolerance. Paclobutrazol (PBZ) and cycocel (CCC) growth retardants were applied as foliar spray on *L. frutescens* plants cultivated in pots to assay the possibility of increasing these plants' tolerance to water stress while maintaining high quality. The experiment was accomplished under full sun in the open field of the Nursery of Ornamental Plants and Landscape Gardening Res. Dept., Hort. Res. Inst., ARC, Egypt during the 2022 and 2023 seasons. Different concentrations of PBZ at 50, 100 and 150 ppm and CCC at 1000, 2000 and 3000 ppm were combined with 100, 75 and 50% pot capacity (P.C.) irrigation water levels, and some morphological, chemical and tolerance indices were examined. The obtained results showed a great reduction in all studied parameters except for proline content due to reduced irrigation levels. All growth retardants applied increased the values over control except for plant height and fresh weight of 10 flowers. Regarding the interaction treatments, the highest concentration of PBZ (150 ppm) and CCC (3000) produced the highest values in most cases when combined with both 75 and 50% irrigation water levels. Such treatments increased the number of main and lateral branches/plant, fresh and dry weights of vegetative growth and roots and root length, while mediated values were obtained for chemical constituents. Water use efficiency, relative stress index and stress tolerance index are also, greatly enhanced by such treatments. Cycocel at 3000 ppm could be recommended to treat *Leucophyllum frutescens* plants cultivated in 30 cm pots and subjected to only 50% irrigation water level as this treatment demonstrated good performance with high stress tolerance index to drought.

1. Introduction

Leucophyllum frutescens (syn., *Leucophyllum texanum*) is native to Southwestern Texas and northeastern Mexico so it is known as Texas

Ranger or Texas-sage. It belongs to the family *Scrophulariaceae* and is considered the largest species in the genus *Leucophyllum*. The plant is an evergreen shrub with silver-grey foliage that reaches 2.0 meters in height and 2.5 meters in spread. The foliage is soft to the touch, and densely clustered along spreading branches. The leaves are small measuring 2.50 × 1.25 cm and broaden at the tip. The flowers are bell-shaped and range from white to pink to purple with up to 2.5 cm long. The plants bloom well in summer when the temperature and humidity are high. Texas Ranger is a popular choice for hedges, specimens, visual screening and wind control and could certainly be used as an accent plant (Mielke, 1993). *Leucophyllum frutescens* has recently been cultivated extensively in Egypt due to its reputation for drought and salinity tolerance, although more research is needed to substantiate this. Younis *et al.* (2017) found that, compared to other landscape plants, *L. frutescens* shows strong potential as it requires minimal water. In this context, Ashour and El-Attar (2017) concluded that *L. frutescens* can be irrigated with tap water every 10 days, and saline water at 2000 ppm can be used every 4 days without an obvious reduction in plant growth and quality.

Water insufficiency is a serious challenge affecting many countries worldwide. To address this issue the strategy of minimizing water use could be adopted, particularly in areas where it is not essential, such as the irrigation of green open spaces and landscape plants. Deister (2013) reported that, in Egypt, open and privately owned areas consume very high amounts of irrigation water (4.76-7.14 l/m²/day and 7.14 -11.9 l/m²/day, respectively) compared with other countries. To reduce the water consumption for open areas and landscape plants, selecting species with low water requirements or increasing the tolerance of the cultured species by different available means is required.

Research has demonstrated that plant growth regulators (PGRs), particularly growth-retarding types, can significantly improve drought tolerance in various plant species through multiple physiological mechanisms. These compounds help plants withstand water deficit conditions by: (1) strengthening antioxidant defenses against oxidative stress caused by drought (Abbasi *et al.*, 2015; Singh *et al*., 2016); (2) controlling stomatal function (Marshall *et al*., 1991; Dehghanzadeh and Adavi, 2023); (3) facilitating osmotic adjustment via accumulation of protective compounds like proline (Waqas *et al.,* 2017) and (4) improving water acquisition through enhanced root growth relative to shoot biomass (Fletcher *et al*., 2000).

Paclobutrazol (PBZ) inhibits gibberellin biosynthesis and subsequently limits stem elongation (Tesfahun, 2018). PBZ has been shown to help safeguard various crops against a range of environmental stresses, such as drought. Paclobutrazol (PBZ) has proven effective for drought mitigation in plants across multiple studies: *Robinia pseudoacacia* (Thakur *et al*., 2000); hybrid poplar and birch (ZhongZhu and XiangWei, 2006); *Lonicera implexa* (Navarro *et al.*, 2008); *Bougainvillea* spp. (Ting *et al*., 2014); *Odontonema strictum* (Rezazadeh *et al*., 2016); *Petunia* (Hatamifar and Samani, 2017); *Sequoia sempervirens* (Shu-ming *et al*., 2020); and *Salvia officinalis* (Maghsoudi *et al*., 2023).

Cycocel (CCC), a plant growth regulator valued for its low toxicity and broad crop effectiveness, can be absorbed by both roots and leaves. Cycocel is used to restrict plant growth while enhancing the harvest index through increased enzymatic activity, leaf thickness, pigment levels, and assimilation efficiency. Spraying cotton with 500 ppm of CCC resulted in higher chlorophyll and relative water content, along with improved nutrient uptake and a reduction in leaf size (Dhopte and Lall, 1987). Drought-protective effects of CCC have been documented in: sunflower (Kumari and Bharti, 1992); groundnut (Mathew and Pandey, 2006); barley (Sharif *et al*., 2007; Afkari and Ghaffari, 2018); Japanese mint (Mathur and Farooqi, 2009); olive (Memari *et al*., 2011; Akbari *et al*., 2015); safflower (Partovian *et al*., 2013); basil (Estakhroeih and Babaei, 2016); chickpea (Safari and Azadikhah, 2021); and wheat (Dehghanzadeh and Adavi, 2023).

This study hypothesized that foliar application of paclobutrazol (PBZ) and cycocel (CCC) at optimized concentrations would enhance water stress tolerance in potted *Leucophyllum frutescens*, while preserving morphological and physiological quality markers. This improvement is anticipated to be evident across varying irrigation regimes, with PBZ and CCC mitigating drought-induced damage through growth regulation and osmotic adjustment mechanisms.

2. Materials and Methods

The experiment was accomplished on *Leucophyllum frutescens* (Berland.) I.M. Johnst. shrubs in the open field under full sun at the Nursery

of Ornamental Plants and Landscape Gardening Res. Dept., Hort. Res. Inst., ARC, Egypt, during the spring to autumn of 2022 and 2023 seasons to assay the possibility of increasing these plants' tolerance to water stress by applying foliar spraying with paclobutrazol (PBZ) and Cycocel (CCC) at different concentrations under different irrigation water levels.

Plant material

In the first week of March each season, well-developed healthy transplants of *L. frutescens* (30-35 cm height and 3 main branches) were acquired from a private nursery at Shibin El Qanater, Qalyubia governorate, Egypt. The transplants were planted in 30-diameter plastic pots (1 plant/pot) filled with 5.5 kg loamy soil. The analysis of the experiment's growing medium and meteorological parameters are shown in Tables 1 and 2. For complete establishment, the plants were left for about one month at the nursery to supply them with the original water amount and required maintenance.

Experimental layout

A factorial experiment with two factors in a randomized complete block design (RCBD) as reported by Gomez and Gomez (1984) was utilized to lay out this pot experiment. Three irrigation levels at 100, 75 and 50% of pot capacity were represented as factor A, while factor B included seven foliar spray treatments, three concentrations of paclobutrazol (PBZ: 50, 100, and 150 ppm), three concentrations of cycocel (CCC: 1000, 2000, and 3000 ppm), and an untreated control (0 ppm PBZ or CCC). Consequently, the present experiment contained 21 treatments (3 irrigation levels × 7 foliar spraying with growth retardants), each treatment was replicated three times, and each replicate contained 5 pots.

Irrigation water treatments

Different water irrigation levels were applied one month after transplantation (on the first week of April each season) as a percentage (100, 75, 50%) of pot capacity (the equivalent of field capacity for soils in pots). The weighing method described by Brown

Table 1 - Physical and chemical analyses of the used soil

Soil type	Particle size distribution (%)			S.P. (%)	E.C. (dS/m)	pH	Cations (meq/l)				Anions (meq/l)		
	Sand	Silt	Clay				Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO_3	Cl^{-}	SO_4^{--}
Loamy	48.0	35.5	16.5	44.0	1.36	8.28	3.5	2.5	6.63	0.65	0.5	7.5	5.28

S.P. = Saturation percentage; E.C. = Electrical conductivity.

Table 2 - Some meteorological parameters at Giza governorate during the experiment period of 2022 and 2023 seasons

Months	2022						2023					
	Temperature (°C)			R.H. (%)	W.S. (m/s)	Precip. (mm/d)	Temperature (°C)			R.H. (%)	W.S. (m/s)	Precip. (mm/d)
	Avg.	Max.	Min.				Avg.	Max.	Min.			
March	13.39	28.98	2.40	59.98	3.02	1.30	17.46	32.65	6.97	54.11	2.67	0.19
April	21.82	40.00	8.51	44.97	3.20	0.00	21.03	36.58	8.64	45.51	2.88	0.10
May	25.06	41.25	11.75	39.75	3.37	0.00	25.08	38.39	11.69	42.51	3.43	0.03
June	29.24	44.30	17.36	42.11	3.24	0.00	29.30	42.56	18.42	39.58	3.50	0.04
July	29.91	40.61	19.26	42.45	3.02	0.06	31.52	43.89	20.49	40.54	3.23	0.00
August	30.32	41.90	21.25	46.09	3.06	0.06	30.86	43.53	20.66	44.56	3.21	0.00
Septemb	28.43	39.48	18.87	49.15	3.12	0.13	29.62	43.48	19.82	45.29	2.87	0.00
October	23.80	39.56	15.76	57.68	2.72	0.18	25.10	35.81	17.19	58.10	2.59	1.71

RH= Relative humidity at 2 meters; WS= Wind speed at 2 meters; Precip.: Precipitation.
Power Data Access Viewer Program of NASA was utilized to collect daily data, and then the data were averaged monthly to fit the experiment period (https://power.larc.nasa.gov).

(2002) was adopted to determine water pot capacity. The plants were irrigated three times a week. The amount of water applied/pot/irrigation and the total water applied/pot/season (6 months/season) is shown in Table 3.

Table 3 - Amount of water applied for each pot (l/pot)

Irrigation level (% of pot capacity)	For each irrigation (l/pot)	Total water applied each season (l/pot)
100%	0.820	59.040
75%	0.615	44.280
50%	0.410	29.520

Foliar spraying with growth retardants

Both paclobutrazol (PBZ) and cycocel (CCC) were dissolved in distilled water to present solutions with the applied concentrations (50, 100 and 150 ppm for PBZ and 1000, 2000 and 3000 ppm for CCC). In this study, plants that did not receive foliar spray application of growth retardants served as the control treatment. Plants received six foliar applications of PBZ or CCC (at specified concentrations) at monthly intervals, with each treatment applied to runoff (defined as the first appearance of solution droplets from leaf margins, indicating full surface coverage and absorption saturation). The first application was done in the first week of April each season. Paclobutrazol ($C_{15}H_{20}ClN_3O$, MW: 293.80) and cycocel (chlormequat chloride; $C_5H_{13}Cl_2N$, MW: 158.10) were obtained from a local company in Egypt.

Data recorded

At the beginning of October each season the following data and measurements were done:

Vegetative growth and flowers. Plant height (cm), number of main branches/plant (emerged on main stems), number of lateral branches/plant (emerged on main branches), vegetative growth fresh and dry weights (g) and fresh weight of 10 flowers (g).

Root system parameters. At the end of this study, plants were extracted by tilting the pots and tapping the bases until the root balls released. Roots were cleaned through sequential shaking, low-pressure washing, and soft brushing to remove all soil while preserving root architecture. The following parameters were recorded on the clean extracted roots: root system length (cm) and fresh and dry weights of the root system (g).

Chemical composition analysis. Chlorophylls a+b (mg/g f.w.) and carotenoids (mg/g f.w.) in fresh leaves according to Wellburn and Lichtenthaler (1984), total carbohydrates (%) in dry leaves according to Herbert *et al.* (1971), proline (mg/g d.w.) in dry leaves according to Bates *et al.* (1973). These determinations were done in the second season only.

Water use efficiency and stress indices. Water use efficiency (WUE) g/l according to Karkanis *et al.* (2011), relative stress index (RSI) according to Fischer and Wood (1979), and Stress tolerance index (STI) according to Fernandez (1993). The following equations were adopted based on the aerial parts dry mass of plants subjected to different treatments:

$$WUE\ (g/l) = D/A.$$
$$RSI = (Dp/Ds)/(Xs/Xp)$$
$$STI = (Dp \times Ds)/Xp^2$$

Where D: dry mass of aerial parts (g) whether under normal or water stress conditions, A: amount of water applied per pot (l) during the experiment, Dp: dry mass of aerial parts of plants of each treatment under normal water conditions, Ds: dry mass of aerial parts of plants of each treatment under water stress conditions, Xp: mean dry mass of aerial parts for all plants under normal conditions and Xs: mean dry mass of aerial parts for all plants under stress conditions.

Statistical analysis

MSTAT Computer Program (MSTAT, 1989) was used to statistically analyze the obtained data by applying an ANOVA test for the two-factor experiment in RCBD (Gomez and Gomez, 1984). The means were compared using Duncan's Multiple Range Test as described by (Duncan, 1955).

3. Results

Vegetative growth and flower parameters:

Different irrigation water levels showed a significant influence (Tables 4 and 5). Reducing water levels gradually caused a reduction in all studied vegetative growth and flowers. The lowest values were obtained by the lowest irrigation water level for plant height (86.00 and 77.05 cm), number of main branches/plant (12.86 and 15.62), number of lateral

Table 4 - Effect of water irrigation levels and two plant growth retardants on plant height (cm), number of main branches/plant and number of lateral branches/plant of Leucophyllum frutescens during two seasons of 2022 and 2023

Treatments	Plant height (cm)		Number of main branches/plant		Number of lateral branches/plant	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Water irrigation levels						
100% P.C.	117.30 a	114.60 a	16.00 a	19.24 a	47.90 a	52.90 a
75% P.C.	100.30 b	90.50 b	14.33 b	17.00 b	41.29 b	45.81 b
50% P.C.	86.00 c	77.05 c	12.86 c	15.62 c	33.05 c	37.48 c
Growth retardants						
Control	115.90 a	110.30 a	6.78 f	8.67 f	19.67 f	20.89 e
PBZ 50 ppm	117.80 a	100.70 b	10.33 e	13.22 e	30.33 d	31.78 d
PBZ 100 ppm	102.30 c	94.33 c	15.44 c	16.67 c	45.67 c	52.44 c
PBZ 150 ppm	94.44 d	91.89 d	19.67 a	23.22 a	54.33 b	61.33 b
CCC 1000 ppm	108.40 b	90.00 e	12.33 d	14.67 d	26.67 e	31.56 d
CCC 2000 ppm	88.22 e	86.78 f	16.78 b	20.78 b	47.11 c	51.22 c
CCC 3000 ppm	81.33 f	84.33 g	19.44 a	23.78 a	61.44 a	68.56 a

P.C. = Pot capacity.
Means within a season followed by the same letter are not significantly different (p>0.05) according to Duncan (1955).

Table 5 - Effect of water irrigation levels and two plant growth retardants on vegetative growth fresh weight (g), vegetative growth dry weight (g) and fresh weight of 10 flowers (g) of *Leucophyllum frutescens* during two seasons of 2022 and 2023

Treatments	Vegetative growth fresh weight		Vegetative growth dry weight (g)		Fresh weight of 10 flowers (g)	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Water irrigation levels						
100% P.C.	194.10 a	206.10 a	50.83 a	58.36 a	1.36 a	1.51 a
75% P.C.	159.70 b	172.80 b	42.64 b	47.08 b	1.24 b	1.35 b
50% P.C.	131.50 c	139.20 c	32.09 c	35.83 c	1.08 c	1.19 c
Growth retardants						
Control	99.98 e	109.20 f	24.78 f	27.04 g	1.08 d	1.22 d
PBZ 50 ppm	125.40 d	138.00 e	33.19 d	38.54 e	1.09 d	1.23 d
PBZ 100 ppm	166.40 c	179.50 d	44.02 c	51.66 d	1.22 c	1.31 c
PBZ 150 ppm	204.10 ab	213.70 b	50.28 b	58.11 b	1.30 b	1.42 ab
CCC 1000 ppm	127.40 d	138.60 e	29.97 e	34.35 f	1.28 b	1.37 bc
CCC 2000 ppm	199.80 b	208.80 c	49.83 b	55.58 c	1.28 b	1.40 b
CCC 3000 ppm	209.40 a	221.20 a	60.93 a	64.33 a	1.35 a	1.48 a

P.C. = Pot capacity.
Means within a season followed by the same letter are not significantly different (p>0.05) according to Duncan (1955).

branches/plant (33.05 and 37.48), vegetative growth fresh weight (131.50 and 139.20 g), vegetative growth dry weight (32.09 and 35.83 g) and fresh weight of 10 flowers (1.08 and 1.19 g) in both seasons, respectively.

Foliar spraying with paclobutrazol and cycocel at various concentrations generally reduced plant height, except for paclobutrazol at 50 ppm, which unexpectedly insignificantly increased plant height in the first season (117.80 cm) compared to the control untreated plants (115.90 cm). Notably, cycocel at 3000 ppm resulted in the lowest plant height values for both seasons, measuring 83.33 and 84.33 cm, respectively. Regarding other vegetative growth characteristics, cycocel at 3000 ppm, followed by paclobutrazol at 150 ppm, resulted in the highest

values. However, an exception occurred with paclobutrazol at 150 ppm, which produced the highest number of main branches per plant (19.67) in the first season only, surpassing cycocel at 3000 ppm (19.44 main branches/plant). The highest values obtained by foliar spraying with cycocel at 3000 ppm were 23.78 for number of main branches/plant (in the second season only), then 61.44 and 68.56 for number of lateral branches/plant, 209.40 and 221.20 g for vegetative growth fresh weight, 60.93 and 64.33 g for vegetative growth dry weight and 1.35 and 1.48 g for fresh weight of 10 flowers in both seasons, respectively (Tables 4 and 5).

Regarding the interaction between irrigation water levels and plant growth retardants, there was a great influence on vegetative growth and flowering traits (Tables 6 and 7). The different treatments greatly influenced plant height. The tallest plants were obtained either by the control (without foliar spraying with growth retardants) or with paclobutrazol at 50 ppm, when both of them were combined with the 100% irrigation water level. In the first season, these two combined treatments resulted in plant heights of 140.30 and 136.70 cm, respectively, while in the second season, the heights were 150.30 and 127.80 cm, respectively. The shortest plants were obtained by cycocel at 2000 ppm (71.00 and 71.50 cm in both seasons, respectively) and cycocel at 3000 ppm (72.00 and 96.17 cm in both seasons, respectively) when combined with the lowest water level (50%). Despite plant height, cycocel at 3000 ppm resulted in the highest values on the other characteristics when combined with the irrigation water level of 100%

Table 6 - Effect of interaction between water irrigation levels and two plant growth retardants on plant height (cm), number of main branches/plant and number of lateral branches/plant of *Leucophyllum frutescens* during two seasons of 2022 and 2023

Growth retardants	Water irrigation levels					
	100% P.C.	75% P.C.	50% P.C.	100% P.C.	75% P.C.	50% P.C.
	1st season			2nd season		
Plant height (cm)						
Control	140.30 a	115.70 cd	91.63 hi	150.30 a	98.17 f	82.33 kl
PBZ 50 ppm	136.70 a	114.00 cd	102.70 f	127.80 b	93.67 g	80.67 lm
PBZ 100 ppm	117.70 bc	100.30 fg	89.00 h-j	110.50 c	92.00 gh	80.50 lm
PBZ 150 ppm	110.00 de	93.00 hi	80.33 k	106.00 d	90.50 hi	79.17 m
CCC 1000 ppm	123.00 b	107.00 ef	95.33 gh	105.00 d	89.00 ij	76.00 n
CCC 2000 ppm	105.30 ef	88.33 ij	71.00 l	102.20 e	86.67 j	71.50 o
CCC 3000 ppm	88.00 ij	84.00 jk	72.00 l	100.30 ef	83.50 k	69.17 o
Number of main branches/plant						
Control	7.67 k	6.67 k	6.00 k	9.33 j	9.00 jk	7.67 k
PBZ 50 ppm	14.00 gh	9.67 j	7.33 k	16.00 gh	12.33 i	11.33 i
PBZ 100 ppm	17.67 de	15.33 fg	13.33 h	20.67 ef	14.67 h	14.67 h
PBZ 150 ppm	20.33 a	19.67 a-c	19.00 a-d	24.33 ab	23.33 b-d	22.00 de
CCC 1000 ppm	13.67 gh	12.33 hi	11.00 ij	16.67 g	16.00 gh	11.33 i
CCC 2000 ppm	18.33 b-e	16.67 ef	15.33 fg	22.67 cd	20.00 f	19.67 f
CCC 3000 ppm	20.33 a	20.00 ab	18.00 c-e	25.00 a	23.67 a-c	22.67 cd
Number of lateral branches/plant						
Control	29.33 kl	18.00 o	11.67 p	27.33 hi	23.00 i	12.33 j
PBZ 50 ppm	39.00 hi	33.00 jk	19.00 no	40.33 fg	33.67 gh	21.33 i
PBZ 100 ppm	57.00 cd	45.33 g	34.67 ij	64.33 bc	50.00 d	43.00 ef
PBZ 150 ppm	62.33 ab	58.00 b-d	42.67 gh	69.67 b	64.67 bc	49.67 de
CCC 1000 ppm	29.67 j-l	27.00 lm	23.33 mn	34.67 g	33.67 gh	26.33 i
CCC 2000 ppm	50.67 ef	46.00 fg	44.67 g	53.33 d	52.67 d	47.67 de
CCC 3000 ppm	67.33 a	61.67 bc	55.33 de	80.67 a	63.00 bc	62.00 c

P.C. = Pot capacity.
Within each season, values sharing the same letter across water levels and growth retardant treatments indicate no significant difference

Table 7 - Effect of interaction between water irrigation levels and two plant growth retardants on vegetative growth fresh weight (g), vegetative growth dry weight (g) and fresh weight of 10 flowers (g) of *Leucophyllum frutescens* during two seasons of 2022 and 2023

Growth retardants	Water irrigation levels					
	100% P.C.	75% P.C.	50% P.C.	100% P.C.	75% P.C.	50% P.C.
	1st season			2nd season		
Vegetative growth fresh weight (g)						
Control	134.40 g	100.90 h	64.70 j	141.60 h	112.20 i	73.72 k
PBZ 50 ppm	165.50 f	130.70 g	80.14 i	180.40 f	139.40 h	94.35 j
PBZ 100 ppm	196.00 cd	172.20 f	131.20 g	221.20 cd	174.80 f	142.70 h
PBZ 150 ppm	234.60 b	205.60 c	172.10 f	250.30 b	228.40 c	162.50 g
CCC 1000 ppm	128.30 g	127.20 g	126.70 g	141.00 h	137.40 h	137.30 h
CCC 2000 ppm	241.70 b	187.80 de	169.90 f	242.70 b	202.00 e	181.90 f
CCC 3000 ppm	258.50 a	193.90 cd	175.80 ef	265.80 a	215.60 d	182.30 f
Vegetative growth dry weight (g)						
Control	32.10 i	22.63 lm	19.62 m	34.21 ij	24.36 l	22.54 l
PBZ 50 ppm	50.73 ef	29.95 ij	18.90 m	58.40 d	32.68 ij	24.55 kl
PBZ 100 ppm	52.53 d-f	48.37 f	31.15 ij	66.15 bc	53.11 ef	35.74 i
PBZ 150 ppm	57.62 c	56.00 cd	37.22 h	68.35 b	64.89 bc	41.08 h
CCC 1000 ppm	37.90 h	27.07 jk	24.94 kl	43.39 h	31.63 j	28.03 k
CCC 2000 ppm	55.05 c-e	51.97 d-f	42.48 g	62.78 c	56.63 de	47.32 g
CCC 3000 ppm	69.90 a	62.53 b	50.35 f	75.24 a	66.22 bc	51.52 f
Fresh weight of 10 flowers (g)						
Control	1.28 e-g	1.07 l	0.89 m	1.48 bc	1.18 ij	0.99 k
PBZ 50 ppm	1.25 f-h	1.10 kl	0.93 m	1.39 c-f	1.23 g-i	1.07 jk
PBZ 100 ppm	1.33 de	1.21 g-i	1.13 j-l	1.41 b-e	1.32 d-h	1.21 hi
PBZ 150 ppm	1.36 b-d	1.34 c-e	1.19 h-j	1.53 ab	1.43 b-e	1.30 e-i
CCC 1000 ppm	1.39 b-d	1.25 f-h	1.19 h-j	1.52 bc	1.35 d-g	1.25 g-i
CCC 2000 ppm	1.42 b	1.32 d-f	1.08 kl	1.54 ab	1.44 b-d	1.22 g-i
CCC 3000 ppm	1.50 a	1.41 bc	1.15 i-k	1.66 a	1.52 b	1.27 f-i

P.C. = Pot capacity.
Within each season, values sharing the same letter across water levels and growth retardant treatments indicate no significant difference ($p>0.05$) according to Duncan's multiple range test (Duncan, 1955).

P.C., this was followed significantly or insignificantly with other treatments e.g. cycocel at 3000 ppm + 75% water level, paclobutrazol at 150 ppm + 100% water level and paclobutrazol at 150 ppm + 75% water level. In this regard, cycocel at 3000 ppm + 75% water level resulted in intermediate values 20.00 and 23.67 for number of main branches/plant, 61.67 and 63.00 for number of lateral branches/ plant, 193.90 and 215.60 g for vegetative growth fresh weight, 62.53 and 66.22 g for vegetative growth dry weight and 1.41 and 1.52 g for fresh weight of 10 flowers in both seasons, respectively. The lowest values (except for plant height) were obtained by the lowest irrigation water level (50%) when the plants were deprived of growth retardants or sprayed with paclobutrazol at 50 ppm.

Although plants treated with the highest concentrations of PBZ (150 ppm) and CCC (3000 ppm) under the lowest irrigation level (50%) did not achieve the highest values for vegetative growth and flower parameters, these values were still relatively high compared to plants exposed to the three irrigation levels without foliar application of PBZ and CCC, except for plant height and the fresh weight of 10 flowers.

Root system parameters

Table 8 showed that both irrigation levels at 100 and 75% resulted in the longest roots without significant differences between them (48.88 and 55.79 cm for 100% and 47.74 and 55.91 cm for 75%, in both seasons, respectively). the highest water level (100%) produced the highest fresh weight of root system (47.40 and 54.83 g) and dry weight of root

Table 8 - Effect of water irrigation levels and two plant growth retardants on root system length (cm), root system fresh weight (g) and root system dry weight (g) of *Leucophyllum frutescens* during two seasons of 2022 and 2023

Treatments	Root system length (cm)		Root system fresh weight (g)		Root system dry weight (g)	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Water irrigation levels						
100% P.C.	48.88 a	55.79 a	47.40 a	54.83 a	16.30 a	17.77 a
75% P.C.	47.74 a	55.91 a	42.58 b	47.56 b	14.74 b	16.01 b
50% P.C.	43.49 b	49.18 b	36.85 c	43.13 c	12.02 c	13.45 c
Growth retardants						
Control	33.71 e	42.40 d	23.70 f	29.77 f	7.92 f	10.06 d
PBZ 50 ppm	39.69 d	44.57 d	30.68 e	36.00 e	9.28 e	10.13 d
PBZ 100 ppm	46.21 c	53.02 c	38.93 d	45.43 c	13.00 c	13.98 c
PBZ 150 ppm	53.97 b	60.00 b	55.21 b	59.08 b	18.17 b	19.59 b
CCC 1000 ppm	38.99 d	43.32 d	32.19 e	38.73 d	10.88 d	12.47 c
CCC 2000 ppm	51.61 b	58.19 b	52.77 c	57.56 b	17.73 b	19.44 b
CCC 3000 ppm	62.74 a	73.89 a	62.47 a	72.98 a	23.52 a	24.55 a

P.C. = Pot capacity.
Means within a season followed by the same letter are not significantly different (p>0.05) according to Duncan (1955).

system (16.30 and 17.77 g) in both seasons, respectively. the lowest irrigation water level (50%) the lowest values were recorded.

As shown in Table 8, cycocel at 3000 ppm followed significantly by paclobutrazol at 150 ppm produced the highest values in terms of root system characteristics (62.74 and 73.89 cm for root system length, 62.47 and 72.98 g for fresh weight of root system and 23.52 and 24.55 g for dry weight of root system, in the first and second seasons, respectively). Control plants (deprived of plant growth retardants) recorded the lowest values for all studied root system characteristics.

Data presented in Table 9 cleared that there were no significant differences between foliar spraying with cycocel at 3000 ppm when combined with the highest irrigation water level (100%) and the medium one (75%) in case of root length (63.53 and 62.70 cm in the first season and 74.23 and 74.17 cm in the second one for these two treatments, respectively), fresh weight of root system (65.95 and 62.69 g in the first season for these two treatments, respectively) and dry weight of root system (24.08 and 23.81 g in the first season, 25.69 and 25.62 g in the second season for these two treatments, respectively), while a significant difference was observed between these two combined treatments in the second season only in the case of fresh weight of root system (76.30 and 71.90 g, for cycocel at 3000 ppm + 100% water level and cycocel at 3000 ppm + 75% water level, respectively). Although plants treated with the highest concentrations of PBZ (150 ppm) and CCC (3000 ppm) under the lowest irrigation level (50%) did not achieve the highest values for root system parameters, these values were still relatively high compared to plants exposed to the three irrigation levels without foliar application of PBZ and CCC.

Chemical composition analysis

The highest level of irrigation water (100%) as shown in Table 10 resulted in the highest values in case of chlorophylls a+b (1.88 mg/g f.w.), carotenoids (0.51 mg/g f.w.) and total carbohydrates (27.14%) and the lowest value of proline (0.97 mg/g d.w.). The highest proline content was obtained by water irrigation level of 50% (1.70 mg/g d.w.).

The two growth retardants also had a significant, though varying, impact on the biochemical variable evaluated in this study (Table 10). Cycocel at 3000 ppm seems to be more effective than other treatments on chlorophylls a+b, total carbohydrates and proline as produced the highest values (1.98 mg/g f.w., 26.75% and 1.62 mg/g d.w., respectively). The same treatment recorded the lowest carotenoids content (0.41 mg/g f.w.). Control plants deprived of spraying with growth retardants produced the highest value for carotenoids and the lowest values in terms of chlorophylls a+b (1.38 mg/g f.w.), total carbohydrates (21.30%) and proline (1.08 mg/g d.w.).

Regarding the combined treatment effects on chlorophyll (a + b) content, the highest concentration (2.18 mg/g fresh weight) was

Table 9 - Effect of interaction between water irrigation levels and two plant growth retardants on root system length (cm), root system fresh weight (g) and root system dry weight (g) of *Leucophyllum frutescens* during two seasons of 2022 and 2023

Growth retardants	Water irrigation levels					
	100% P.C.	75% P.C.	50% P.C.	100% P.C.	75% P.C.	50% P.C.
	1st season			2nd season		
Root system length (cm)						
Control	38.63 h-j	35.43 j	27.07 k	44.50 ij	42.13 i-k	40.57 j-l
PBZ 50 ppm	42.67 gh	41.90 g-i	34.50 j	49.97 fg	45.33 hi	38.40 kl
PBZ 100 ppm	47.63 d-f	47.00 ef	44.00 fg	53.17 ef	55.00 e	50.90 fg
PBZ 150 ppm	55.97 b	56.03 b	49.90 c-e	59.27 cd	65.20 b	55.53 de
CCC 1000 ppm	41.93 g-i	37.93 ij	37.10 j	48.80 gh	44.37 ij	36.80 l
CCC 2000 ppm	51.80 b-d	53.17 bc	49.87 c-e	60.57 c	65.20 b	48.80 gh
CCC 3000 ppm	63.53 a	62.70 a	62.00 a	74.23 a	74.17 a	73.27 a
Root system fresh weight (g)						
Control	27.17 j	26.11 j	17.82 k	33.35 hi	33.23 hi	22.73 j
PBZ 50 ppm	39.21 gh	26.71 j	26.11 j	43.72 f	33.23 hi	31.04 i
PBZ 100 ppm	43.55 f	41.73 fg	31.51 i	52.07 e	43.85 f	40.37 fg
PBZ 150 ppm	63.80 a	56.27 cd	45.55 f	69.30 b	56.93 d	51.00 e
CCC 1000 ppm	36.60 h	31.81 i	28.15 ij	43.83 f	36.58 gh	35.77 h
CCC 2000 ppm	55.53 cd	52.70 de	50.07 e	65.23 c	57.17 d	50.28 e
CCC 3000 ppm	65.95 a	62.69 ab	58.77 bc	76.30 a	71.90 b	70.73 b
Root system dry weight (g)						
Control	9.04 jk	8.09 kl	6.62 l	11.58 g-i	9.56 h-j	9.05 ij
PBZ 50 ppm	13.33 gh	8.04 kl	6.45 l	14.05 fg	8.42 j	7.91 j
PBZ 100 ppm	15.68 f	15.17 fg	8.16 kl	17.16 de	16.39 ef	8.39 j
PBZ 150 ppm	20.63 bc	19.11 cd	14.75 fg	22.09 b	20.23 bc	16.44 ef
CCC 1000 ppm	12.40 hi	10.88 ij	9.35 jk	13.48 g	12.25 gh	11.66 g-i
CCC 2000 ppm	18.93 cd	18.11 de	16.15 ef	20.35 bc	19.63 b-d	18.34 c-e
CCC 3000 ppm	24.08 a	23.81 a	22.67 ab	25.69 a	25.62 a	22.35 b

P.C. = Pot capacity.
Within each season, values sharing the same letter across water levels and growth retardant treatments indicate no significant difference ($p>0.05$) according to Duncan's multiple range test (Duncan, 1955).

Table 10 - Effect of water irrigation levels and two plant growth retardants on chemical composition analysis of *Leucophyllum frutescens* during the second season 2023

Treatments	Chlorophylls a+b (mg/g f.w.)	Carotenoids (mg/g f.w.)	Total carbohydrates (%)	Proline (mg/g d.w.)
Water irrigation levels				
100% P.C.	1.88 a	0.51 a	27.14 a	0.97 c
75% P.C.	1.74 b	0.39 c	23.82 b	1.24 b
50% P.C.	1.52 c	0.49 b	19.15 c	1.70 a
Growth retardants				
Control	1.38 g	0.51 a	21.30 e	1.08 g
PBZ 50 ppm	1.58 f	0.49 b	22.00 d	1.13 f
PBZ 100 ppm	1.68 e	0.49 b	22.82 c	1.26 d
PBZ 150 ppm	1.80 c	0.46 c	24.21 b	1.39 c
CCC 1000 ppm	1.73 d	0.45 c	22.58 cd	1.24 e
CCC 2000 ppm	1.85 b	0.44 d	23.92 b	1.42 b
CCC 3000 ppm	1.98 a	0.41 e	26.75 a	1.62 a

P.C. = Pot capacity.
Means followed by the same letter are not significantly different ($p>0.05$) according to Duncan (1955).

observed under the 3000 ppm cycocel treatment with 100% irrigation. This was followed by the 2000 ppm cycocel + 100% water level treatment (2.02 mg/g fresh weight). Total chlorophyll concentrations were relatively lower with both paclobutrazol at 150 ppm + water level at 100% and cycocel at 3000 ppm + water level at 75%, without significant differences between them. These four treatments also yielded the highest total carbohydrate contents: cycocel at 3000 ppm + 100% water level (31.74%), cycocel at 2000 ppm + 100% water level (27.42%), paclobutrazol at 150 ppm + 100% water level (28.14%), and cycocel 3000 ppm + 75% water level (26.37%). The highest carotenoid concentration (0.58 mg/g f.w.) was observed in control plants at 50% irrigation, with progressively lower values under paclobutrazol treatments at 100 ppm (0.57 mg/g f.w.) and 150 ppm (0.56 mg/g f.w.) combined with full irrigation (100% water level).

Data presented in Table 11 demonstrated that proline was enhanced by cycocel at 3000 ppm when combined with the lowest water level (50%) as the highest value was recorded (2.01 mg/g d.w.). The lowest chlorophylls a+b (1.23 mg/g f.w.), carotenoids (0.32 mg/g f.w.), total carbohydrates (17.21%) and proline (0.82) were obtained by control + 50% water level, cycocel at 3000 ppm + 75% water level, paclobutrazol at 50 ppm + 50% water level and control + 100% water level, respectively.

WUE and stress indices

Although the lowest water level (50%) produced the lowest dry mass, as shown in figure 1 and Table 12 this was accompanied by the highest water use efficiency (WUE) obtained (1.09 and 1.21 g/l for both seasons, respectively). On the other hand, cycocel at 3000 ppm enhanced WUE in both seasons as the highest values (1.43 and 1.51 g/l, respectively) were obtained. In the case of the combined treatments, the superiority was observed by cycocel at 3000 ppm + 50% irrigation water level (1.71 and 1.75 g/l in both seasons, respectively), this was followed significantly by cycocel at 2000 ppm + 50% water level (1.44 and 1.60 g/l) then cycocel at 3000 ppm + 75% water level (1.41 and 1.50 g/l) in both seasons respectively.

Water stress caused by reducing irrigation water levels was measured by the relative stress index (RSI) and presented in figure 2. At 150 ppm, paclobutrazol reduced water stress effects most effectively under 75% irrigation, showing the lowest RSI values (1.23 and 1.31 in both seasons, respectively), meaning that these plants treated with this treatment experienced minimal water stress and are in good condition. This was followed by paclobutrazol at 100 ppm, cycocel at 2000 ppm and 3000 ppm resulting in low RSI values and mitigated water stress at 75% level compared with 50% level. It is worth mentioning that the highest water stress experienced by plants was observed with the application of PBZ at 50 ppm

Table 11 - Effect of interaction between water irrigation levels and two plant growth retardants on some chemical constituents of *Leucophyllum frutescens* during the second season of 2023

Growth retardants	Water irrigation levels		
	100% P.C.	75% P.C.	50% P.C.
Chlorophylls a+b (mg/g			
Control	1.57 jk	1.33 m	1.23 n
PBZ 50 ppm	1.71 hi	1.66 i	1.38 m
PBZ 100 ppm	1.79 ef	1.73 gh	1.53 kl
PBZ 150 ppm	1.97 bc	1.85 d	1.59 j
CCC 1000 ppm	1.94 c	1.78 efg	1.48 l
CCC 2000 ppm	2.02 b	1.83 de	1.69 hi
CCC 3000 ppm	2.18 a	1.99 bc	1.77 fg
Carotenoids (mg/g			
Control	0.43 f	0.53 b	0.58 a
PBZ 50 ppm	0.51 cd	0.42 f	0.53 b
PBZ 100 ppm	0.57 a	0.39 g	0.50 d
PBZ 150 ppm	0.56 a	0.35 h	0.46 e
CCC 1000 ppm	0.46 e	0.38 g	0.51 cd
CCC 2000 ppm	0.50 d	0.36 h	0.46 e
CCC 3000 ppm	0.52 bc	0.32 i	0.38 g
Total carbohydrates			
Control	23.70 f	22.13 h	18.06 kl
PBZ 50 ppm	25.61 e	23.17 f-h	17.21 l
PBZ 100 ppm	27.11 b-d	23.20 fg	18.15 kl
PBZ 150 ppm	28.14 b	25.40 e	19.10 jk
CCC 1000 ppm	26.25 de	22.29 gh	19.21 ij
CCC 2000 ppm	27.42 bc	24.16 f	20.18 i
CCC 3000 ppm	31.74 a	26.37 c-e	22.13 h
Proline (mg/g d.w.)			
Control	0.82 q	1.08 m	1.33 g
PBZ 50 ppm	0.84 p	1.14 jk	1.40 f
PBZ 100 ppm	0.90 o	1.15 j	1.73 d
PBZ 150 ppm	0.96 n	1.31 h	1.91 b
CCC 1000 ppm	0.89 o	1.10 l	1.73 d
CCC 2000 ppm	1.13 k	1.32 gh	1.80 c
CCC 3000 ppm	1.29 i	1.56 e	2.01 a

P.C. = Pot capacity.
Values sharing the same letter across water levels and growth retardant treatments indicate no significant difference (p>0.05) according to Duncan's multiple range test (Duncan, 1955).

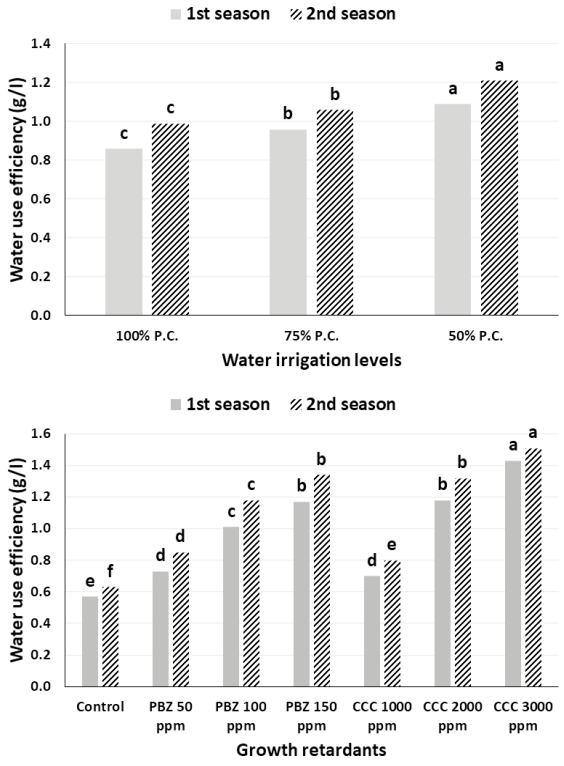

Fig. 1 - Effect of water irrigation levels and two plant growth retardants on water use efficiency (g/l) of *Leucophyllum frutescens* during two seasons of 2022 and 2023. Means within a season followed by the same letter are not significantly different (p > 0.05) according to Duncan (1955).

under an irrigation level of 50%, as the highest RSI levels were recorded (4.24 and 3.87 in both seasons, respectively).

Under moderate and low irrigation water levels, the stress tolerance index was calculated to determine how far the applied plant growth retardants can enhance the plants under these stress conditions (Fig. 2). Cycocel at 3000 ppm significantly enhanced water stress tolerance, particularly under 75% and 50% irrigation levels. This treatment yielded the highest values in both seasons, as recorded 1.70 and 1.36 (season 1) and 1.47 and 1.14 (season 2),

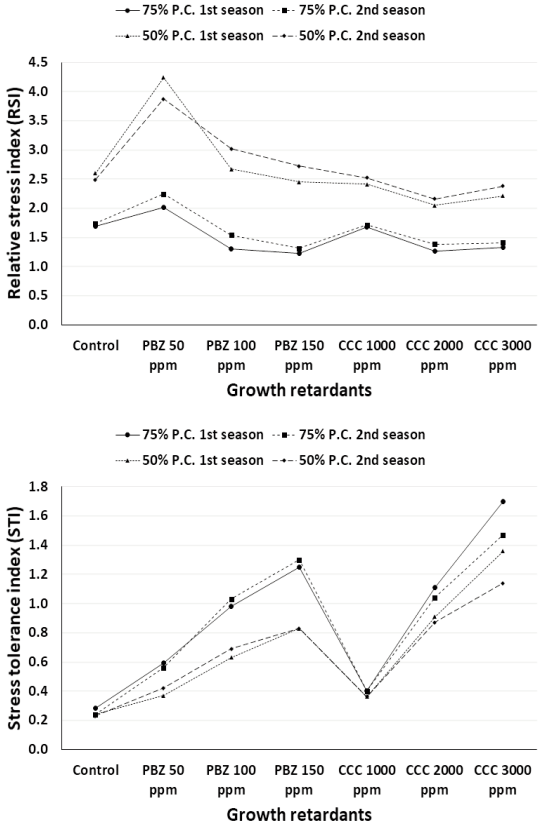

Fig. 2 - Effect of water irrigation levels and two plant growth retardants on relative stress index (RSI) and stress tolerance index (STI) of *Leucophyllum frutescens* during two seasons of 2022 and 2023.

Table 12 - Effect of interaction between water irrigation levels and two plant growth retardants on water use efficiency (g/l) of *Leucophyllum frutescens* during two seasons of 2022 and 2023

Growth retardant	Water irrigation levels					
	100% P.C.	75% P.C.	50% P.C.	100% P.C.	75% P.C.	50% P.C.
	1st season			2nd season		
Control	0.54 jk	0.51 k	0.66 i	0.58 m	0.55 m	0.76 kl
PBZ 50 ppm	0.86 h	0.68 i	0.64 i	0.99 ij	0.74 l	0.83 k
PBZ 100 ppm	0.89 gh	1.09 de	1.06 ef	1.12 gh	1.20 e-g	1.21 ef
PBZ 150 ppm	0.98 fg	1.26 c	1.26 c	1.16 fg	1.47 cd	1.39 d
CCC 1000 ppm	0.64 i	0.61 ij	0.85 h	0.74 l	0.71 l	0.95 j
CCC 2000 ppm	0.93 gh	1.18 cd	1.44 b	1.06 hi	1.28 e	1.60 b
CCC 3000 ppm	1.18 cd	1.41 b	1.71 a	1.27 e	1.50 c	1.75 a

P.C. = Pot capacity.
Within each season, values sharing the same letter across water levels and growth retardant treatments indicate no significant difference (p>0.05) according to Duncan's multiple range test (Duncan, 1955).

respectively. However, paclobutrazol at 150 ppm enhanced the tolerance to water stress at 75% level (1.25 and 1.30, in the first and second seasons, respectively).

4. Discussion and Conclusions

The findings of the present study revealed a marked reduction in all assessed parameters at 50% irrigation level, except proline content and water use efficiency (WUE), both of which increased under severe water deficit. Application of paclobutrazol (PBZ) and cycocel (CCC) significantly enhanced drought tolerance in *Leucophyllum frutescens* under these conditions, with the highest concentrations of each compound producing the most pronounced effects. Although both growth regulators led to a significant reduction in plant height, this effect was accompanied by notable increases in the number of branches and the fresh and dry biomass of shoots and roots, suggesting an overall improvement in plant vigor. Furthermore, CCC demonstrated greater efficacy than PBZ in enhancing growth-related parameters, indicating its superior potential for mitigating the adverse effects of severe water limitation.

Regarding the effect of irrigation levels, the results obtained were consistent with similar trials i.e. Ashour and El-Attar (2017) who reported that water deficiency induced by prolonged irrigation intervals reduced survival percentage, plant height, number of branches/plant, root length, and fresh and dry weights of vegetative growth and roots of *Leucophyllum frutescens*. Multiple studies demonstrate consistent physiological responses to water deficit across diverse plant species. Research by Noor El-Deen (2020) on *Duranta erecta* L. 'Golden Edge', Noor El-Deen *et al*. (2018) on *Zinnia elegans*, and Khalil *et al*. (2012) on *Jatropha curcas* L. revealed a common pattern where reduced irrigation significantly decreased most growth parameters (plant height, biomass), while simultaneously increasing proline accumulation - a key osmoprotectant. Similar findings were reported by Amin (2013) in *Pinus radiate* and *Robinia pseudoacacia* transplants, where extended irrigation intervals reduced shoot and root biomass.

Closing stomata, inefficient carbon fixation process, photosynthesis rate reduction and decreasing formation and expansion of leaf tissues are the main disorders of water deficit (Pallardy, 2008). On the other hand, Dhopte and Ramteke (2017) divided the effects of drought into physiological (desiccation of protoplasm which causes inhibition of cell division, elongation and differentiation as well as reduction of water absorption and dehydration, stopping stem and leaf elongation) and biochemical (proteolysis and protein synthesis retardation, nucleic acid synthesis failure, effect on enzyme synthesis, cytokinin levels are low, toxic products accumulation like ammonia-a main cause of drought injury, starch synthesis inhibition and ABA level in roots are increased) effects. Under drought conditions, the excessive production of reactive oxygen species (ROS) and the generation of radical-scavenging compounds like ascorbate and glutathione further intensify the negative effects (Deotale, 2017). ROS-generated compounds cause oxidative damage to plant cells by restraining membrane attributes (Singh *et al.*, 2016).

In this study, WUE was calculated as the ratio of aerial biomass (shoot dry weight) to total water applied during the experimental period under each level of water. The values obtained showed that WUE in drought-stressed plants (50%), relative to 100% PC-grown plants, was improved. This result suggests that the reduction in biomass (due to water deficit) was proportionally smaller than the 50% reduction in water application, so the value was high in this case.

Our study demonstrated that the highest concentrations of both PBZ and CCC significantly improved drought tolerance in *Leucophyllum frutescens* under severe water deficit (50% irrigation level). Regarding PBZ, our results agreed with Thakur *et al.* (2000) on *Robinia pseudoacacia* seedlings, who found that paclobutrazol improved water relations, enhanced drought tolerance, and increased proline and soluble sugar contents. Also, this fits ZhongZhu and XiangWei (2006) on two-year-old seedlings of *Populus alba × Populus berolinensis*, *Ulmus pumila* and *Betula platyphylla*, who reported an increase of water use efficiency. In the same line Navarro *et al.* (2008) on *Lonicera implexa* seedlings, found that PBZ was significant for leaf water potential and leaf osmotic potential, and demonstrated that PBZ attenuates the effects of deficit irrigation. Hatamifar and Samani (2017) found that application of paclobutrazol on *Petunia × grandiflora* 'Bravo Blue' increased the number of flowers, aerial parts dry weight and number of lateral branches, and decreased the plant height, root dry weight and

amount of carotenoids. The above-mentioned findings were confirmed in *Bougainvillea spp.* (Ting *et al*., 2014), later observed in *Odontonema strictum* (Rezazadeh *et al*., 2016), *Sequoia sempervirens* (Shuming *et al*., 2020), and most recently in *Salvia officinalis* (Maghsoudi *et al*., 2023).

The principal role of paclobutrazol (PBZ), as a triazole compound, is the reduction in plant height by inhibiting gibberellin biosynthesis by blocking the oxidative steps from ent-kaurene to ent-kaurenoic acid, a key step in the gibberellin production pathway (Rademacher, 1997). This leads to decreased levels of active gibberellins and subsequently limits stem elongation (Tesfahun, 2018). This could explain our results, which showed a reduction in the height of plants treated with PBZ.

Our study showed that application of paclobutrazol (PBZ) and cycocel (CCC) significantly reduced plant height by inhibiting gibberellin biosynthesis; they concurrently increased the number of branches and enhanced fresh and dry biomass of leaves and shoots. These results were in agreement with Youssef and Abd El-Aal (2013), who showed that PBZ at 50 ppm or CCC at 1000 ppm increased the number of branches/plant as well as leaves and roots fresh and dry weights of *Tabernaemontana coronaria* compared to control untreated plants.

These enhancements in branching and biomass likely result from hormonal modulation: PBZ suppresses gibberellin synthesis and shifts the isoprenoid pathway toward elevated cytokinin levels and abscisic acid accumulation, thereby reducing stem elongation while promoting lateral and root growth. CCC similarly inhibits gibberellin action, diminishes apical dominance, and encourages lateral bud outgrowth, collectively leading to increased branching and biomass (Rademacher, 2000).

On the other hand, Singh *et al.* (2016) enumerated the positive role of PBZ-treated plants grown under drought conditions. These plants possess a superior defense mechanism against free radicals, allowing them to neutralize harmful oxygen species. PBZ treatment enhances stress resistance by boosting the activity of certain antioxidant enzymes. It also protects plants from oxidative damage by either increasing antioxidant levels or reducing the activity of oxidative enzymes. Furthermore, PBZ treatment stimulates the production of abscisic acid and phytol, hormones that promote plant growth and health. Fletcher *et al*. (2000) added that PBZ induces a large elastic module in the cell walls of treated plants, which in turn maintains turgor and thus increases the plant tolerance to drought stress.

The elevated proline levels likely result from biosynthesis enzymes ornithine aminotransferase (OAT) and pyrroline-5-carboxylate reductase (P5CR) combined with suppressed activity of proline oxidase, the key catabolic enzyme (Debnath, 2008). Paclobutrazol seems to increase proline content by increasing the enzyme activity of OAT, which is involved in the biosynthesis of proline via ornithine (precursors for L-proline biosynthesis) pathway (Zhang *et al.*, 2024).

PBZ blocks the oxidation of ent-kaurene to ent-kaurenoic acid by inhibiting cytochrome P450-dependent oxygenases. This inhibition reduces gibberellin (GA) production, causing precursors in the terpenoid pathway to accumulate. These accumulated precursors, such as farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), are redirected toward ABA biosynthesis, leading to higher ABA levels. PBZ also inhibits the activity of ABA 8'-hydroxylase, an enzyme responsible for ABA degradation, further increasing ABA concentrations in plant tissues (Soumya *et al*., 2017).

PBZ can also control stomata movements under drought stress. Jack pine seedlings treated with paclobutrazol before a drought showed improved drought tolerance. This was evident in their quicker closure of leaf stomata to conserve water, better water content within the plant, and higher survival rates compared to untreated seedlings (Marshall *et al*., 1991). Additionally, MingEr and SanYu (1999) reported the role of paclobutrazol treatment in decreasing the leaf transpiration rate of *Satsuma mandarin*, and prorogue its reduction during drought stress. Abbasi *et al.* (2015) showed that paclobutrazol minimizes the negative effects of drought stress with evidence of enhancing chlorophyll content and antioxidant enzymes such as alternative oxidase (APX), catalase (CAT) and glutathione peroxidase (GPX) that reduces hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content.

As an anti-gibberellin agent, cycocel inhibits the conversion of geranyl geranyl pyrophosphate (GGPP) into ent-kaurene by blocking ent-kaurene synthetases in the mevalonic acid pathway. This results in lower gibberellin levels, ultimately decreasing plant height (Rademacher, 1997).

Applying cycocel (CCC) in plants enhances root growth, water potential, and stomatal resistance in leaves, ultimately improving water-use efficiency by stimulating root activity and reducing transpiration (Deotale, 2017). CCC is considered highly effective in mitigating the negative impacts of various abiotic and biotic stresses on crops. CCC plays a key role in the signal transduction pathways of plants under environmental challenges, including drought stress. Khondoker *et al.* (2019) reported the positive role of CCC on the growth and flowering of tuberose subjected to drought stress. In this regard, exogenous application of CCC is believed to influence nutrient uptake and transport, regulate stomatal function, and enhance growth, photosynthesis, chlorophyll synthesis, and transpiration processes (Dehghanzadeh and Adavi, 2023). The interoperation of the positive role of PBZ under drought stress could be applied in the case of cycocel, particularly further enhancing the activities of the key antioxidant enzymes and accumulation of osmolytes, and decreasing the levels of H_2O_2 (as harmful ROS compounds) and malondialdehyde in drought-stressed plants (Dehghanzadeh and Adavi, 2023). As mentioned in the case of the effect of PBZ on ABA synthesis, the accumulated precursors induced by the CCC block of gibberellin synthesis are redirected toward ABA biosynthesis, leading to higher ABA levels.

It is noteworthy that, based on the obtained results, CCC generally exhibited greater effectiveness than PBZ. This may be attributed to differences in absorption pathways: paclobutrazol is usually applied either as a foliar spray or through soil drenching, and when applied to foliage, its uptake through mature leaves is relatively limited, with absorption likely occurring via the stem or from droplets reaching the soil. In contrast, cycocel is readily absorbed by both leaves and roots (Barrett and Bartuska, 1988; Rademacher, 2000; Maghsoudi *et al*., 2023).

Although plants treated with the highest concentrations of PBZ (150 ppm) and CCC (3000 ppm) under the lowest irrigation level (50%) did not achieve the best values for most morphological characteristics, WUE and STI, these values were still relatively high compared to plants exposed to the three irrigation levels without foliar application of PBZ and CCC, except for plant height and the fresh weight of 10 flowers. Cycocel at 3000 ppm outperformed PBZ at 150 ppm in this aspect, proving more effective in improving plant growth parameters, enhancing water use efficiency (WUE), and increasing tolerance to reduced water supply levels down to 50%. So, it is recommended to apply foliar spraying with Cycocel at 3000 ppm + irrigation water level at 50% P.C. of *Leucophyllum frutescens* plants cultivated in 30 cm pots, as this treatment demonstrated good performance with a high-stress tolerance index to drought.

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Review paper

Insight to the conventional and biotechnological approaches in tomato on potato grafting (Pomato): A review

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Key words: Chimeric, grafting, signalling, somatic hybridization, stresses, TomTato.

Abstract: Pomato is the result of a combination of a tomato scion and a potato rootstock. This grafted combination is also acknowledged as a horticultural magic plant, a recombinant double harvest plant, or a chimeric double harvest plant. This type of plant could meet the need for proper vertical resource usage in the future, as urbanization is increasing rapidly and agricultural land is now becoming rare and expensive. Moreover, even though changes in the environment are the most substantial real limitation in vegetable production, this category of graft could be employed as an alternative strategy. Few reports on using somatic interbreeding to establish tomato-potato fusion hybrids have been available since the early 1900s. This strategy can be used again in the future to save time and convenience during labor-intensive procedures, as this plant can indeed be established through any other technique, instead of trying to make grafting and somatic combination the only reasonable alternatives. The grafted union of tomato and potato has indeed positively influenced output, reliability, hormone levels, signaling pathways, and mechanisms. This paper has been reviewed to gather all information available on the tomato plant to date, since there has been some experimentation over the past few decades.

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1. Introduction

Vegetable grafting has turned out to be a promising technique to boost the productivity of vegetable crops, particularly those belonging to the Solanaceae and Cucurbitaceae families. This technique is most commonly associated with sustaining the effects of intensive crop cultivation in several countries (Lee *et al.*, 2010; Thakur *et al.*, 2024). Beginning publicly in the early 1960s, tomato grafting became an

indispensable technique for tomato cultivation worldwide (Lee and Oda, 2003). The primary rationale for tomato grafting was to use this approach instead of methyl bromide to control soil-borne pathogen incidence under protected conditions (Rivard *et al.*, 2010; McAvoy *et al.,* 2012; Rana *et al.,* 2024). Grafting is currently being used on a large scale to combat abiotic stresses, alike low and high temperature stresses, heavy metals, salinity and water stress, as well as to improve quality and yield (Venema *et al.,* 1999; Abdelmageed and Gruda, 2009; Flores *et al.*, 2010; Turhan *et al.*, 2011; Colla *et al.*, 2013; Bhatt *et al.,* 2015; Kumar *et al.*, 2015; Wudu and Kassaw, 2022). Because of this, the major purpose of adopting grafting techniques in tomatoes is to boost output while preserving nutritional value and minimizing the harmful effects of environmental conditions. The success rate of the grafted plant is determined by a variety of factors, but genomic considerations may be found to be the most important among all others because they confirm the interoperability percentage between the scion and rootstock (Thakur *et al.*, 2022). Various studies have verified that grafting can have either positive or negative effects in terms of performance and yield (Huh *et al.*, 2003; Yetisir *et al.*, 2003; Davis *et al.*, 2008 a). Furthermore, conflicting changes in the transplanted union may occur as a result of a sensitive transplanted union, physical incompatibility, grafted seedling collapse, and toxic compound accumulation (Davis *et al.*, 2008 b; Villyáni *et al.*, 2024). However, graft compatibility is more likely if the scion and rootstock are taxonomically close to each other (Wang, 2011; Farooque *et al.*, 2024). Numerous reports have predicted that grafting can be performed intraspecifically and interspecifically among crops in solanaceous crops (Petran and Hoover, 2014; Chaudhari *et al.,* 2016). Tube and cleft grafting are the furthermost common techniques used in tomato grafting (Lee and Oda, 2003; Thakur *et al.*, 2024), but amongst these two, tube grafting is found to be the most utilized technique by accelerated farmers worldwide (Hanna, 2012; Vu *et al.,* 2015). Tube grafting is known to produce strong, raised grafts because it confirms a robust vascular attachment amid the union of scion and rootstock (Bausher, 2013; Rana *et al.,* 2024). Successful adaptation, which is the process of rehabilitation and densification of transplanted seedlings before polytunnel or field seeding, is another factor that guarantees successful graft establishment (Lee and Oda, 2003). Some of the countries, such as China, South Korea, Japan, Spain, Italy, France, Canada, Turkey, the USA, Mexico, India, and other countries (Israel, Netherlands, Egypt, and Brazil) are the major contributors in the vegetable grafting market (Nawaz *et al.,* 2017).

Oscar Soderholm presented the basic concept of conceptualizing the tomato onto a potato, i.e., Tomapotato, in 1930. The Max Planck Institute for Developmental Biology in Tübingen, Germany, initially conceived this grafted pairing in 1977; nevertheless, the resulting grafts failed to produce fruit and tubers. Moreover, the Institute of Plant Biotechnology Research in Koln, Germany, made a successful attempt at tomato/potato grafts in 1977, which produced fruits and tubers on the plant mixture (Reinhard, 2008; Bahadur *et al.,* 2020; Thakur *et al.,* 2022, Thakur *et al.,* 2024). Peres *et al.* (2005) studied the combination by grafting tomato on top of a potato plant: A method for studying leaf-derived signaling on tuberization. The study revealed that tomato and potato seedlings were crossbred when they were 20 days old to maintain nursery stability and yield. Tomato and potato crossbreds can yield up to 5 kg of tomato and 0.5 kg of potato, which is comparable to the yield of the control plant. Kiambu Prison made another attempt, according to another piece of literature cited by Lubbock Online News in 2002. In 2013, a UK-based horticultural mail company, Thompson and Morgan, sold grafts of the "TomTato" plant. The next year, New Zealand's Incredible Edible Nursery announced a grafted plant called Double UP Potato Tom (Gillies, 2013; Kumar *et al.*, 2015). BARI and BADC are currently producing pomato plants for production purposes (Nusrat, 2014). In India, CSK, HPKV Palampur began research on the tomato plant in 2015 under protected conditions, while ICAR-IIVR Varanasi made a successful attempt in open field conditions for the first time in 2013 (Kumar *et al.*, 2015; Bahadur *et al.*, 2020). Several successful reports have been documented by various researchers until 2021 that claim the Pomato plant's success story is not only one of comparable yield production but also matches the required quality attributes. Besides this, it can also help in combating biotic and abiotic stresses.

Pomato is a chimeric plant and the result of hetero-grafting, in which a tomato scion is grafted onto a potato rootstock from two different species. As a result, a plant combination is developed that may produce potato tubers below ground and

tomato fruits on stems on the same plant (Albacete *et al.,* 2015). Because crossbreeding cannot be used to develop pomato grafts, grafting is the only viable and possible method (Arefin *et al.,* 2019). Pomato plant yields, tomato fruits per plant about 2.72 kg, while potato tuber yield per plant was 211 g, with the number of fruits and tubers obtained from a double harvest plant estimated to be 35 fruits and 4 to 5 tubers (Islam *et al.,* 2019). Based on the dry matter and mineral distribution process of tomato and potato plant products, nitrogen and phosphorus are distributed along with, but to a lesser extent than, dry matter. Meanwhile, potassium distribution was found to be more liberal than dry matter, whereas magnesium and calcium showed a different pattern than N, P, K, and dry matter content (Bünemann and Grassia, 1973). The combination of the tomato hybrid Sweet Million F1 and red potato variety Memphis had a significantly lower response for the degree of fruit binding than the control in a behavioral study of the tomato plant (Giosanu *et al.,* 2020). Conferring to the report of Negi *et al.* (2016), the combination of GS-600 grafted on Kufri Himalini using the cleft grafting technique resulted in the highest survival rate and grafting success rate. The production of tomato fruits and potato tubers from the pomato plant can also be affected by the scion age; the scion was 25 days old (Arefin *et al.,* 2019). When compared to individual tomato and potato crop returns of 1:1.93 and 1:0.26, respectively, the pomato plant ensures higher returns of about 1:2.12 (Negi *et al.,* 2017). Because tomato is a double-harvest crop, the demand for fertilizer can also double. There is no standard fertilizer application recommendation for the tomato plant. To know the fertilizer's application doses, experimentation was conducted on the tomato plant by Kumar *et al.* (2021). They suggested using the necessary amount of fertilizer as well as fertigation twice a week to meet the grafted plant's nutrient demand. The recommended dose was 75% RDF with fertigation (19:19:19) 4.56 g m^2 and 100% RDF with fertigation (19:19:19) 4.56 g/m^2 (19:19:19); 6.84 g/m2 (Kumar *et al.,* 2021; Thakur *et al.,* 2024). Furthermore, the plastids of tomato and potato belong to the same family and contain a single species of DNA, the size and density of which (156 kbp and 1.697 g/cm3) are comparable to those of higher plants' mtDNA. So, from the standpoint of utilizing vertical space, the primary need for developing this type of dual-crop potato plant is one of the most feasible ways to utilize the balcony and backyard space. As the population grows, the effects of urbanization, industrialization, and industrialization on agricultural lands will exacerbate, making agricultural lands more scarce. So, to fulfill the need for food availability, these kinds of combinations can benefit more and more in the future. In another case study conducted by Villyáni *et al.* (2024) studied the influence of tomato grafted onto potato tubers on skin colour and the metabolome of the produce. The outcome of the study confirmed the significant variation in the studied quality traits, skin colour, and metabolome of the harvested produce. Meanwhile, out of the one hundred twelve identified metabolites of the tubers, all three cultivars showed a consistent trend in the amounts of twelve chemicals. Each cultivar showed a rise in tuber starch content relative to the self-grafted control, except 'White Lady' and 'Hópehely,' where protein levels were found to be unchanged. The formerly oval tubers become more circular. There was a correlation between the enhanced anthocyanin content of 'Hópehely' and 'Désirée' tuber skins and the elevation of *StAN1* expression, which was caused by the tomato scion. This research shows that tomato scion significantly affects potato tuber quality measures.

2. Developmental procedure of pomato plants using the vegetable grafting method

Grafting tomato scion onto potato rootstock resulted in pomato plants. Firstly, for the preparation of scion tomato seeds were grown in protrays filled with a commercial mixture under protected cultivation. Potato tubers were cut into two pieces and stored for at least 7 days at 18°C with 80% RH before developing scion seedlings. Grafting was done when the potato plant reached a graftable size of 15-20 cm (Thakur *et al.,* 2022). For successful graft union establishment, the polyhouse should be kept at the optimal temperature and humidity. Grafting vegetables is a cost-effective method for developing viable tomato grafts. For a strong union between the scion and rootstocks, there is a need for skilled workers and optimum climate conditions (Rana *et al.,* 2024). The detailed procedure for graft development is shown in figures 1 and 2. Detailed instructions for the development of grafts were provided by Kumar *et al.* (2015), Negi *et al.* (2016), Arefin *et al.* (2019), Islam *et al.* (2019) and Kumar *et al.* (2021). Pomato

grafting, which unites a tomato plant (aerial portion) with a potato plant (subterranean portion), often employs a grafting clip or silicone tube to tightly bind the grafted stems throughout the healing phase (Thakur *et al.*, 2024). An acute grafting knife is important for executing precise, congruent incisions on both the scion (tomato) and rootstock (potato) stems, often using a "splice" or "cleft" grafting method. Complementary equipment, such as grafting tape or a film, could be employed to secure and safeguard the graft union, hence reducing transpiration and infection. A humidity structure or grafting chamber maintains optimal humidity and temperature, facilitating graft union development. These instruments facilitate alignment, sterility, and healing, which are essential for the effective cultivation of pomato plants (Thakur *et al.,* 2024). Farooque *et al.* (2024) successfully grafted two different varieties of tomato, Red cherry tomato and BARI Tomato-15, onto potato rootstock, namely Diment. the results confirmed that the success rate of grafted plants was 94 percent, and also improved

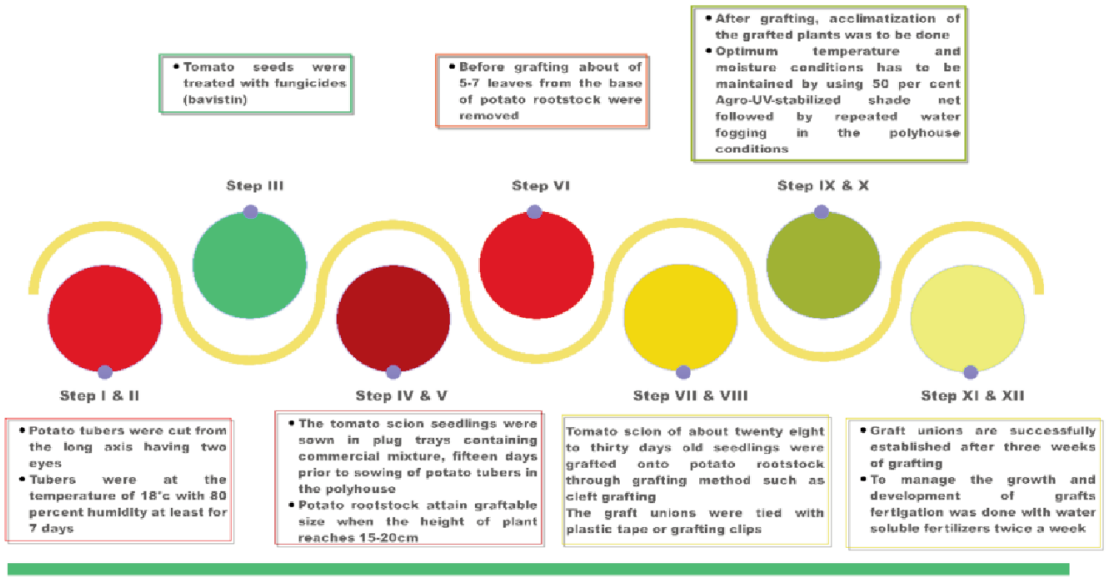

Fig. 1 - Sequential procedure for the successful pomato graft development.

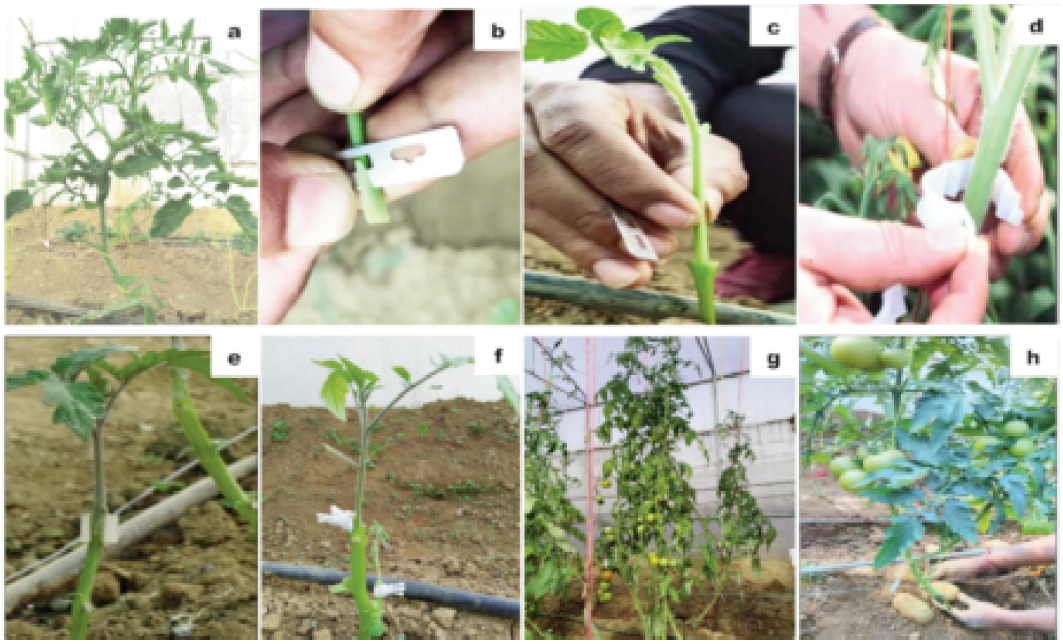

Fig. 2 - Pomato development a) Selection of scion; b) Scion preparation; c: Grafting; d,e,f) Clipping; g) Sucession of graft; h) Yielding (Source: Personal communication).

the growth and yield attributes of harvested produce, i.e., tomato and potato. Meanwhile, another researcher had confirmed that the grafting success rate among the tomato variety "Mersa" grafted onto the potato rootstock variety "Belete" was 64 percent (Wudu and Kassaw, 2022).

3. Differential responses of tomato/potato grafting on fruiting, quality, and abiotic stress

Yield improvement

The pomato plant's proliferation and production characteristics were significantly improved. (Singh *et al.,* 2020). The application of GS-600 to Kufri Himalini improved tuber yield (rootstock) and fruit yield (Scion) per grafted plant (Negi *et al.,* 2016). There is a significant improvement in growth and yield attributes in grafted tomato plants between BARI tomato-11 (scion) and two potato rootstocks, namely Asterix and Cardinal (Arefin *et al.,* 2019) (Table 1). Moreover, among grafting techniques in pomato plants, cleft and tongue grating showed better results in terms of yield-related aspects such as tuber weight, tubers per plant, tuber yield/plant and meter square, and in tomato for least days to first flowering and harvest, longest harvest duration, fruit length, width, and plant height (Kumar *et al.,* 2016). In some graftings, due to excessive uptake of nitrogen in grafted plants, there was accelerated vegetative growth, resulting in delayed maturity, tuberization, and a reduction in potato tuber quality (Zebarth and Rosen, 2007). Also, it is observed that key qualities to growth and yield are affected by fertigation treatments and fertilizer effects. Treatments affected the no. of shoots per plant, length of the harvest, the time it took to reach graftable shoots, the time it took for the plant to flower, and yield attributes (Kumar *et al.,* 2021). From the above-discussed finding, it has been concluded that the tuber and fruit yield obtained from tomato and potato grafted plants produced a comparable yield as solely grown potato and tomato yield per plant, which could assure a fair means of production and be economically beneficial (Table 2). Meanwhile, in the year 2024, one of the researchers confirmed that the pomato plant can produce more yield than of individual plant of tomato and potato. Apart from this, a single graft union of pomato, from the aerial part of tomato, had the 103.87 g of average fruit weight, and fruit yield per plant, i.e., 4.11 kg (Thakur *et al.,* 2022). From the same plant, potato had the average tuber weight of 175.83 g, where the tuber yield per plant was 908.11 g (Thakur *et al.,* 2024). Takeuchi *et al.* (2022) made a reverse combination of potato onto tomato to breed potato cultivars through the conventional method. This combination has been made to resolve the flower initiation and seed setting issue in potatoes because the tuber and flowering will occur simultaneously. The outcomes of the results suggested that the fruit formation rate of potatoes was increased by up to 19% in potato/tomato grafts than of non-grafted potato plants with 1.1% success. So, this procedure can ease in development of null sergeant progenies by crossing the mutant lines of potatoes.

Quality focuses on nutritional content

Numerous contradictory reports have been published concerning the quality of fruits (Rouphael *et al.,* 2010). Limited research work has been done on tomato/potato plant quality analysis because the material is scarce for this aspect. A tomato cultivar grafted on four different potato cultivars showed an increase in vitamin C, TSS, and soluble sugars in tomato fruits, while in potatoes, only the reduced sugar content had been leveled up (Zhang and Guo, 2018). Among the various grafting techniques, tongue grafting was found to be significantly beneficial in tomato grafts for aspects of potato starch content, pericarp thickness, TSS, and tomato ascorbic acid content. Maximum TSS was observed in potatoes during cleft grafting (Kumar *et al.,* 2016). However, a different grafted-splice method combination of Yuvraj scion onto K. Jyoti and K. Pukhraj had a significant effect on the biochemical compositions of tomato plant products. So, as per the assurance of the results, the pomato plant might be used as an alternative strategy to get the best quality product along with a double harvest (Diwan and Sharma, 2021). Furthermore, the quality aspects related to the fruits and tubers of the pomato plant influenced it in a positive direction (Panahandeh *et al.,* 2020). Table 2 lists a recent achievement in the quality aspects of the tomato harvest. The quality aspects of pomato are questionable among growers and consumers. As discussed above, quality attributes such as TSS, ascorbic acid, total sugars, and soluble sugars of pomato products have been enhanced along with other yield traits. This assures

Table 1 - Recent achievements in terms of yield, quality and tolerance of pomato plant

Origin of scion and rootstock	Targeted traits	References
GS-600 onto Kufri Himalini	Tuber yield/plant and marketable fruit yield/plant	Negi *et al.,* 2016
ZY988 onto LS6, QS9, HZ88 and DTS1	Tomato: vitamin C, TSS, soluble sugars, fruit number and size; Potato: tuber sprouting, reducing sugar	Zhang and Guo, 2018
BARI tomato-11 onto Asterix and Cardinal	Morphological traits: Plant height, branches, leaves, and clusters Yielding: Fruits per plant, size of tuber, fruit weight, and yield per plant;	Arefin *et al.*, 2019
Avinash-2 onto Kufri Jyoti	Fruiting attributes nd tuber yield/plant	Islam *et al.,* 2019
Moneymaker × San Marzano (F1) onto Agria	Chlorophyll index, total soluble solids, dry weight, and titratable acidity	Panahandeh *et al.,* 2020
Moneymaker × San Marzano (F1) onto Agria	Foliage fresh, fruit weight, and plant height,	Panahandeh *et al.,* 2020
Kufri Pukhraj onto Pusa Ruby and Kufri Bahar onto Cherry Tomato	Number of fruits per plant, average fruit weight, days to harvest (tubers), tuber yield per plant	Singh *et al.,* 2020
Cherry tomato onto potato	Tomato-fruit yield and potato-tuber yield (0.5-0.8 kg per plant)	Bahadur *et al.,* 2020
Lakshmi, Aviral, Yuvraj onto Kufri Jyoti and K. Pukhraj	Ascorbic acid content, acidity %, total sugar% %, reducing sugar %, and non-reducing sugar %.	Diwan and Sharma, 2021
Palam Tomato Hybrid-1 onto Kufri Himalini	Morphological traits like height, number of tubers per plant, tuber yield, tuber weight, marketable fruits, and fruit yield per plant, and tomato fruit weight	Kumar *et al.,* 2021
Ikram to Charlotte (CV)	Salinity tolerance	Parthasarathi *et al.,* 2021
Marsa (Tomato scion) grafted onto Belete (potato rootstock)	Improved number of fruits per plant (18), average fruit weight (53 g), fruit yield per plant (954 g), number of tubers per plant (4-5), tuber yield per plant (219.27 g)	Wudu and Kassaw, 2022
Avtar on Kufri Pukhraj	Highest fruit yield per plant	Thakur *et al.*, 2022
Avtar onto Kufri Jyoti	Minimum days to first flowering, days to first harvest	Thakur *et al.,* 2022
Avtar (F1 hybrid) onto Kufri Pukhraj	Maximum average fruit weight, fruit yield per square meter, and fruit yield quintal per hectare, tuber yield per plant and quintal per hectare, tuber equivalent yield, B: C ratio,	Thakur *et al.,* 2024
Red cherry tomato and BARI Tomato-15 grafted onto Diamant	Maximum plant growth, tomato and potato yield per plant	Farooque *et al.,* 2024
Potato scion Qingshu No. 9 grafted onto tomato rootstock Zhongyan 988	Improved pollen viability up to 15-20 percent, identified 13 DEGs (differentially expressed genes associated with gametophyte, pollen development, protein processing, and carbohydrate metabolism.	Zhang *et al.* 2024

Table 2 - Germination performance parameters of shallot seeds under different boron concentrations

S. No.	Aim	Methodology	Characterization of materials	Results obtained	References
1	Rejuvenation and genomic count determination in potato and tomato somatic hybrids by iso-electric focusing of RuBPcase subunits	Protoplast Fusion	Incubation of protoplasts in the existence of Polyethylene glycol containing a high concentration of Ca2+ ions	RuBPcase can be utilized to show that the plants are hybrids due to the change in chromosomal number from 48 to 50 in three hybrids and near to 70 in the fourth hybrid.	Melchers and Sacristan, 1978
2	Chilling resistance	Protoplast Fusion	An Aminco DW-2a dual-wavelength spectrophotometer monitored callus transfer and cytochrome reduction.	All four tomato-potato hybrids had intermediate chilling resistance between tomato and potato.	Smillie *et al.,* 1979
3	Ribulose bisphosphate carboxylase small subunit somatic hybrid peptide mapping	Protoplast Fusion	Ribulose bisphosphate carboxylase isoelectric focusing pattern as a nuclear and protoplast genome phenotypic marker.	Four somatic hybrids had ribulose bisphosphate carboxylase oligomers with tomato and potato genomes. Potato-tomato somatic hybrids have functioning tomato DNA and tyrosine-containing	Poulsen *et al.,* 1980
4	Restriction endonuclease analysis of fused hybrid plastids DNA, potato, and tomato	Callus Fusion	12 somatic inter-generic hybrid progenies of dihaploid potato and tomato	Each species carries ptDNA of tomato and potato at a 0.1 to 3 % level of detection	Schiller *et al.,* 1982
5	Steroidal Glyco-alkaloids analysis in tomato and potato somatic hybrids	Protoplast Fusion (pomato with the plastid of potato and topato with the plastid of tomato)	Protoplasts from dihaploid potato liquid cultures and tomato mesophyll fusion	Somatic hybrids had 98 percent potato alkaloid, while tubers had 60–70% tomatine.	Roddick and Melchers, 1985
6	Somatic hybrid identification by pollen, anther protein of somatic hybrids of potato and tomato plants	Protoplast fusion	Extraction of proteins from pollen and anthers of somatic hybrids. Pollen Viability Test by Fluoro-chromatic reaction. Protein estimation by the Lowry method Protein extraction by Isoelectric focusing in polyacrylamide gel	For hybrid identification by pollen and anther proteins	Chen and Ninnemann, 1990
7	Chloroplast and mitochondrial DNA triploid and tetraploid tomato-potato somatic hybrids	Protoplast fusion	Southern Blotting technique with four mtDNA-specific probes	DNA reduction. The 18S + 5S rRNA genes in tomato and potato mt DNAs may be connected to coxII genes.	Wolters *et al.,* 1991
9	GUS activity, total genomic DNA content, and chloroplast type, shoot regeneration potential, expression of potato iso-enzymes, and relative genomic composition	Asymmetric somatic hybridization protoplast fusion	Flurometric β-glucuronidase assay, Flow cytometric analysis, DNA isolation, DNA probes, Southern blot, and dot blot analysis	No viable plants were obtained; calli were highly polyploid, and hybrids expressed GUS activity	Schoenmakers *et al.,* 1994
10	Mitotic and meiotic irregularities in somatic hybrids	Protoplast fusion, Root tip meristems culture	Genomic in situ hybridization, cytological analyses for chromosome counts, and karyotype analysis	Exclusively sterile pollens	Wolters *et al.,* 1994
11	Regeneration, alien chromosome identification through RFLP, and GISH	Protoplast fusion	Cytological technique for meiosis study, fertility, crossability, and embryo rescue technique, starch composition determination	All fusion hybrids were sterile, the hexaploids produced stainable pollen and berries with badly developed seeds	Jacobsen *et al.,* 1992

that the quality of pomato products can be improved through grafting; satisfactory results were documented, which match the needs of growers and consumers.

Salinity tolerance

According to various case studies, the potato genome is vital for improving the yield and quality of tubers by the tomato/potato graft (Sue *et al.,* 2010; Arefin *et al.,* 2019). However, the graft of transmissible Ribonucleic acid from the inverse combination of potato onto tomato articulated the phenotype of the scion (Kudo and Harada, 2007). Salinity tolerance was tested in the tomato scion cv. Ikram on the potato cv. Charlotte plant. The results showed that using potato rootstock as a substitute approach to assure irrigation water salinity tolerance and boost the quality and quantity of fruits and tubers harvested from the grafted plant may be useful. The improved characteristics include higher total dry mass, varied root characteristics, equal mineral distribution across the entire plant, and higher water productivity than non-grafted plants (Parthasarathi *et al.,* 2021). Table 2 gives an illustration of salinity tolerance. Only a few attempts have been made recently by researchers to manage abiotic stress, namely using a graft combination of tomato and potato plants. With the studies mentioned above, it is evident that abiotic stresses like salinity, temperature, and mineral stresses can be managed by utilizing tolerant and resistant rootstocks.

4. Developmental procedure of hybrid fusion through somatic hybridization

For the isolation of plant protoplasts, 50 ml of a three- to four-day-old suspension culture was centrifuged at 600 rpm for five minutes. The pallets were then resuspended in 30 ml of AM media. After centrifuging the filtrate suspension, the protoplast pellets were re-suspended in BM media and centrifuged at 600 rpm for five minutes. The centrifuged protoplast pellets were resuspended again in BM media. Using a nylon filter and a centrifuge set at 1000 rpm for 10 minutes, the protoplasts were separated from cell debris. The protoplast was rinsed in AS media (1 fold), excluding enzymes, and in BS media (2 folds). Before electro-fusion, a protoplast mixture (1:1) was made, and 0.4 millilitre of the combination was added to a fusion chamber before electrodes were affixed to a glass petri dish. An alternating current field was useful to the allied protoplasts, followed by a reduction in the AC field to zero. The protoplasts were cultured in TM2G media at a density of 2.5 x 10^5 protoplasts per ml. The details of the procedure were explained by Roddick and Melchers (1985), Schoenmakers *et al.* (1994), and Wolters *et al.* (1995).

Effect of somatic hybridization on various traits of Pomato fusion hybrid

A few scientists initiated the research work for developing the tomato plant via protoplast fusion. In 1955, a potato + tomato fusion hybrid was developed by researchers through protoplast fusion (Jacobsen *et al.,* 1992). A first attempt had been made to develop a fusion hybrid plant by Melchers and Sacristan in 1978. Roddick and Melchers (1985) carried out the research on the creation of somatic fusion hybrids of tomato and potato, with topato having tomato plastids and pomato comprising potato plastids. Smillie *et al.* (1979) investigated chilling resistance in various somatic fusions of tomato and potato and discovered that the chilling resilience of tomato-potato somatic hybrids was intermediate between that of tomato and potato. These somatic fusions may be beneficial for relocating genes for chilling resistance to cultivated tomatoes, to know the potential and confines of asymmetric somatic hybridization among tomato and potato plants. The two mutation products of the nitrate reductase-deficient mutant of tomato are isolated and assessed, and they might be used as selectable markers in potato somatic hybrid fusion assessment. The outcome showed that several hybrid fusion plants were developed, but plant regeneration did not take place, as mentioned by Schoenmakers (1993). Wolters *et al.* (1995) demonstrated that mtDNA segregation occurs independently in somatic fusions from chloroplast DNA (cpDNA). It is concluded that the mtDNA of both tomato and potato may contain the coxII gene, which is closely associated with the 18S and 5S rRNA genes. A protein of pollen and other sources is used for inter-specific assessment, while Chen and Ninnemann (1990) confirmed the presence of an intermediate protein band pattern in somatic hybrids of tomato and potato. Besides this, a report by Schoenmakers *et al.* (1994) mentioned that an excessive variation had been seen among all the

fusion hybrids for all studied traits, while the expression of GUS activity was also reported in somatic fusion. In another case study, the chromosome count had been analyzed at the mitotic and meiotic stages of division in 107 somatic hybrids of tomato + potato. Although about 79% of fusion combinations are found to be aneuploid with the absence of one or two chromosomes, among the five studied hybrids, 46 chromosomes were found in hybrid K2H2-IC, with the highest percentage. Even though all the microspores degenerated with immediate effect after the tetrad stage, this resulted in pollen sterility (Poulsen *et al.,* 1980; Wolters *et al.,* 1991; Wolters *et al.*, 1994). Table 1 summarises attempts made in the early 1900s to develop fusion hybrid lines through somatic hybridization. To reduce labor and maintenance costs, there is an urgent need to focus on developing the tomato onto potato plants via somatic hybridization. Tissue culture is the only method through which these grafts can be developed. Because seeds can't be obtained from a pomato plant due to a lack of skilled labor and knowledge about the procedure of grafting, growers cannot perform this at the field level. Somatic hybrids can resolve this issue of plant material availability at the market level.

5. Signaling mechanism involved in Pomato

Potato tuberization can be affected by photomorphogenesis and hormones, while the tomato scion does not succeed in producing these substances, which leads to converting stolen into tubers in an established strong source-sink relationship. The source-sink relationship is important in potato tuberization because cytokinin affects tuberization and is thought to be a major contributor (Roitsch and Ehneb, 2000). Thus, the involvement of genes can also contribute to altering the tuberization success, as the over-expression of the "*Knox*" gene can diminish the level of gibberellic acid and level up the cytokinin production that ultimately leads to augmented tuberization (Sakamoto *et al.,* 2001; Frugis *et al.,* 1999; Rosin *et al.,* 2003). In recent case studies, results revealed that the "*ipt* gene" exhibits a better ability for potato tuber formation (Galis *et al.,* 1995). As a result, the diverse range of homoerotic tomato mutants can be used as an effective means of targeting the substances involved in the conversion of stolon into tuber formation, which forms a strong sink. However, the elimination of *PHYB* through a gene, i.e., anti-sense *PHYB,* resulted in tuber formation in short and long-day conditions, while the over-expression of this gene improved the inhibition effect of long days on tuber formation (Aksenova *et al.,* 2002). Kudo and Harda (2007) carried out experiments on hetero-grafting using potato and tomato as scion and rootstock, respectively. The combination had been tested to ensure that the RNA molecule responsible for altering the leaf shape could function transversely in grafted seedlings. The study found no significant changes in the potato scion as a result of RNA molecule transmission in the grafted plant. But this can be utilized in further cultivar development programmes in vegetable crops. However, in another case study, Nielsen and Stitt (2001) confirmed that a sufficient amount of chimeric transcript can be supplied from the rootstock without leaves to modify the leaves of the scion. Even though the PFP enzyme works well in young leaves, it is distributed evenly throughout the plant. Meanwhile, this *PFP* transcript can also actively participate in the root system. So, this methodology has now been used in vegetable production to combat the various biotic and abiotic stresses. Several reports confirm the utility of *PFP* transcripts in the grafted plant by altering the characters of the scion (Ohata, 1991; Taller *et al.,* 1999) and may lead to the transportation of gene transcripts as per the report of Liu (2006). A recent investigation initiated on the tomato plant revealed that potato rootstock expresses merely a negligible phenotypic alteration in tomato scion, but has shown a minute impact on differential expression genes. On the other hand, in the meantime, tomato scion has shown a strong impact on the rootstock of potatoes, leading to the expression of thousands of differential genes, some of which are concerned with hormone pathways and their signaling (Zhang *et al.,* 2019). Due to the presence of two dissimilar hormone-regulated signaling systems, the yield aspect of the pomato plant would be challenged in numerous tomato-potato combinations (Peres *et al.,* 2005). In the meantime, changes in hormone signaling, either for fruit setting or tuberization, may occur in pomato plants, leading to a downplaying of the antagonistic interactions of hormones such as gibberellic acid and cytokinins (Yasinok *et al.,* 2009). Approximately 209 genes related to the synthesis of starch and sucrose were identified as being upregulated in the tomato scion. The tomato scion had less of an effect on the

development of the signaling substance involved in tuberization, but it did initiate the development of stolons and an aerial stem portion during germination. Based on RNA-sequencing, upregulated and downregulated genes were identified based on a count of 1529 and 1329, respectively, amongst *St-SW* and *St-R*. A few of them took part in hormone signaling transduction via St DELLA (receptor) and StGID 1 (protein) (Zhang *et al.,* 2019). The graft union establishment is affected by the signaling mechanisms of the scion and rootstock. Further research is required to improve plant survival rates and to understand the actual mechanism of the available hormonal pathway between the scion and rootstock combination. This type of investigation has the potential to broaden the research area in grafted vegetable plants. This can also ensure the quality aspects that are in dispute.

6. Movement of small RNA molecules in grafted plants

The transfer of genetic information in the form of small RNAs is a critical problem that has received a lot of attention in the literature over the last decade. So, to accomplish this, grafting has been widely utilised in plant physiology and biology to discover mobile molecules that include small RNA, mRNA, and proteins that influence significant facets of plant growth and development. In higher plants, the phloem transports amino acids, carbohydrates, proteins, vital nutrients, and certain RNA molecules (Wu *et al.*, 2006). Grafting has been employed in several experiments to demonstrate that messenger RNA molecules move throughout plants via the phloem (Turnbull and Lopez-Cobollo, 2013). It has been suggested that phloem sap includes real, tiny regulatory RNAs because small RNAs match multiple potential target genes (Yoo *et al.*, 2004). Yoo *et al.* (2004) also found the same in the phloem sap of cucumber, pumpkin, and castor beans, where they found the existence of an endogenous population of small RNA species with 18 to 25 nucleotides. Ruiz-Medrano *et al.* (1999) confirmed the presence of several mRNAs in the cDNA clones obtained from the phloem sap of pumpkin. Recently, it was proposed that FT (flowering locus T) mRNAs, which are synthesised in the leaves and transported by the phloem to the shoot apex, interact with another transcription factor to form the flowering locus T protein (Biazquez, 2005). As a result, in reaction to particular environmental factors, the long-distance RNA translocation mechanism seems to influence the development of the entire plant. When Me-like potato leaves appeared to have been induced by the transcript transported through the graft junction from the Me tomato rootstock, Kudo and Harda (2007) demonstrated the presence of chimeric transcripts. PFP promoter activity was seen in the lower sections of the plant, notably in the root system, in addition to the identification of PFP transcripts in the sap from the cut surface. Genetic material has recently been reported to be horizontally transmitted between the two grafted partners, either as DNA or plastids, by Stagemann and Bock (2009). Later in 2012, Stagemann and his coworkers verified that *N. benthamiana* had successfully received the chloroplast DNA of *N. tabacum* (a chloroplast transgenic strain) through a grafting junction. Small RNAs, such as phosphorus deficiency-induced miRNAs, have been demonstrated to transfer from shoot to root systems through the use of micrografting studies (Pan *et al.*, 2008). According to Bhogale *et al.* (2014), additional miRNAs like miR156, miR172, and miR395 may transfer from the scion to the rootstock. Since it is thought that sRNAs move through the phloem and plasmodesmata, it has been demonstrated that sRNA mobility within grafted plants is more effective when sRNAs are produced in the scion and move towards the rootstock rather than vice versa (Melnyk *et al.*, 2011). Tomato graft unions were developed using non-transgenic scions and transgenic rootstocks (silenced fatty acid desaturase gene). The silencing of the fatty acid desaturase gene (LeFAD7) and the presence of siRNA in grafted scions suggest that both were transferred to the scion via genetically engineered rootstock. It is conceivable that siRNAs generated from the rootstock might similarly go in that direction among these compounds and highlight the considerable phenotypic alterations seen in the scion. In addition, it has been demonstrated that the tomato scion receives viral resistance from the rootstock. The findings of an experiment conducted by Spano *et al.* (2015) demonstrated that when resistant tomato rootstock varieties with higher RNA interference were grafted onto resistant tomato scion varieties that accumulate less viral RNA, the grafted plants displayed the expression of crucial RNAi mechanism genes. The roots of resistant grafted plants showed upregulation of genes such as

Agronaute (AGO) and RDR. It has also been proven that self-grafted plants showed stronger RNAi silencing, and even grafting itself can provoke the activation of resistance mechanisms.

7. Future prospects

Vegetable grafting provides a new perspective to broaden the research area, increase yield, and improve quality attributes associated with vegetable crops. This can be used as a backup method to ensure food availability. Because variety development takes eight to ten years, grafting can compensate for production losses and aid in the resistance to biotic and abiotic stresses during this time. However, the basic requirement for grafting is the development of resistant or tolerant rootstock material on the ground level. This is a farmer-friendly technique because it is environmentally friendly. The pomato, also known as the "horticultural wonder plant," is the best example of vegetable grafting. Two products can be obtained from a single plant, namely tomato fruit and potato tubers. More research on pomato plants is required because only a few reports have been documented to date. The quality aspects of this plant product are always in doubt, which could be a major hurdle that must be overcome so that farmers can also grow these profitable plants with high-quality produce. Aside from the manual grafting of tomato plants, researchers should concentrate on using biotechnological techniques to create tomato/potato fusion hybrids, such as somatic hybridization. So, because the major downside of developing a pomato plant is that it cannot be grown from seeds, the only way to develop these grafts is through vegetable grafting. To modernize the development process and ensure the availability of grafts on the market, these grafts must be developed using the tissue culture technique.

8. Conclusions

The primary goal of developing pomato plants is to ensure maximum space utilization and combat environmental challenges. A few inconsistent findings on the fixable effects of graft union on horticultural and quality aspects have also been reported. Pomato plants can be grown in two ways: through grafting or somatic hybridization. Several attempts were made in the 1900s to create hybrid mutants through somatic hybridization. Later, manual grafting was used to develop tomatoes onto potato grafts. Now that only grafting is used on commercial-level tomato plants, there is an urgent need to focus on developing a hybrid tomato/potato graft. To be aware of the utility and benefits of this dual-harvest plant, researchers must focus on the start of additional research work. Many aspects of its biochemical and physiological properties have yet to be studied, particularly to make this grafted plant commercially available to growers. This grafted plant may also be considered an alternative way to produce clean potato seed tubers, especially in hydroponics and aeroponics systems, and in the future, it might be proven to be one of the approaches worth considering. So, there is a need to work on this aspect to get more benefits in terms of quality and quantity. However, several successful reports regarding the tomato/potato plant were documented, so the possible information has been reviewed to learn more about the successful experimentation.

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Review paper

Achillea millefolium L.: A comprehensive review of its phytochemistry and pharmacological properties

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Key words: Antioxidant activity, essential oils, ethnopharmacology, flavonoids, wound healing.

Abstract: *Achillea millefolium* L., commonly known as yarrow, is a perennial herb traditionally used in various cultures for its therapeutic properties. Belonging to the Asteraceae family, it has gained attention for its rich phytochemical composition, including flavonoids, terpenoids, sesquiterpene lactones, phenolic acids, and essential oils. These bioactive compounds contribute to a wide range of pharmacological activities such as anti-inflammatory, antimicrobial, antioxidant, antispasmodic, and wound-healing effects. Ethnobotanical use of yarrow includes treatment of gastrointestinal disorders, skin injuries, menstrual irregularities, and respiratory infections. Modern preclinical studies have substantiated many of these traditional claims, although clinical validation remains limited. Additionally, its essential oil components show potential in cosmetic and food industries due to their preservative and aromatic properties. Despite its therapeutic promise, concerns related to allergic reactions and toxicity at high doses, particularly from thujone-containing oils, warrant further safety assessment. This review aims to consolidate current scientific knowledge on *A. millefolium*, highlighting its pharmacological relevance, phytochemical diversity, and future potential in evidence-based herbal medicine and natural product development.

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1. Introduction

Achillea millefolium L., commonly known as yarrow, is a herbaceous perennial species belonging to the Asteraceae family, historically recognized for its extensive use in traditional medicine across Europe, Asia, and North America. References to its medicinal applications date back to ancient Greece, where the warrior Achilles was said to use the

plant to treat battlefield wounds-hence its genus name *Achillea* (Applequist and Moerman, 2011). Traditionally, yarrow has been employed to treat a wide array of conditions including gastrointestinal disturbances, menstrual irregularities, respiratory infections, wounds, and skin inflammations (Falk *et al*., 1975). These uses have prompted growing scientific interest, especially as research has begun to validate many of its ethnomedicinal claims through the lens of modern pharmacology. Phytochemical investigations reveal that *A. millefolium* is rich in secondary metabolites such as flavonoids (e.g., apigenin, luteolin), phenolic acids (e.g., caffeic and chlorogenic acids), sesquiterpene lactones (e.g., achillicin), and essential oils including monoterpenes (e.g., camphor, 1,8-cineole) (Falconieri *et al*., 2011; Judzentiene, 2016). These constituents have demonstrated diverse biological activities in vitro and in vivo, including antioxidant, anti-inflammatory, antimicrobial, antispasmodic, wound-healing, and neuroprotective effects (Montanari *et al*., 1998; Amirghofran and Karimi, 2002; Keser *et al*., 2013; Dias *et al*., 2013). However, while these preclinical studies provide a strong pharmacological basis for traditional claims, the translation of these findings into clinical practice remains inconsistent and underdeveloped.

A critical issue is the disconnect between laboratory evidence and human trials. Many clinical studies on yarrow suffer from limitations such as small sample sizes, poor extract characterization, variable dosages, and lack of placebo control. For example, clinical trials investigating its efficacy in dysmenorrhea or functional gastrointestinal disorders have shown promise but lack the methodological rigor required for medical validation (Madisch *et al*., 2004; Benedek *et al*., 2005). Moreover, the high variability in phytochemical content across different ecotypes and growing conditions complicates standardization, which is essential for reproducibility and regulatory approval (Georgieva *et al*., 2015).

Underexplored but promising therapeutic areas include neuroprotection, metabolic regulation, and dermatological applications. Animal studies have highlighted GABAergic modulation and acetylcholinesterase inhibition by flavonoids, suggesting anxiolytic and cognitive-supportive effects, yet robust human data are absent (Falk *et al*., 1975; Ivancheva *et al*., 2002). Similarly, studies in diabetic rats show hypoglycemic and lipid-lowering effects (Judzentiene and Mockute, 2010), warranting further investigation in metabolic syndrome and type 2 diabetes.

In addition to its pharmacological significance, *A. millefolium*'s ecological resilience and adaptability to various climates offer potential for sustainable cultivation and industrial use. Recent efforts in optimizing cultivation conditions, such as soil composition, irrigation regimes, and genetic selection, show promise in enhancing biomass yield and phytochemical uniformity (Stojanović *et al*., 2005; Benedek *et al*., 2007). Incorporating these agronomic insights into large-scale production models could support supply chain consistency for pharmaceutical and nutraceutical development.

Furthermore, to ensure safe integration into therapeutic systems, clearer safety profiles and potential interactions must be addressed. While generally considered safe, compounds like thujone and sesquiterpene lactones require careful dosing and labeling. Regulatory frameworks, including EMA and Commission E monographs, support traditional use but demand further toxicological evaluation, especially for high-dose or long-term applications.

This review aims to synthesize current scientific knowledge on *A. millefolium*, emphasizing standardized extraction methods, quantification of bioactive constituents, clinical applicability, and sustainability. By bridging traditional use with scientific rigor, *A. millefolium* can emerge as a credible, multifunctional agent in herbal therapeutics and commercial natural product development.

2. Taxonomy and botanical description

Recognized both in traditional medicine and botany, *Achillea millefolium* L. (yarrow) is a taxonomically diverse member of the Asteraceae family, one of the largest plant families globally with over 1,600 genera and 23,000 species (Khan and Gilani, 2011) (Fig. 1). The genus *Achillea* comprises over 100 species, primarily distributed across Europe, temperate Asia, and North America. Among these, *A. millefolium* is the most widely known and studied due to its medicinal and ecological importance (Pires *et al*., 2009). Botanically, *A. millefolium* is a rhizomatous, herbaceous perennial that typically grows between 30 and 90 cm in height. It features a highly branched, erect stem covered with fine hairs (Babaei *et al*., 2007). The leaves are alternate,

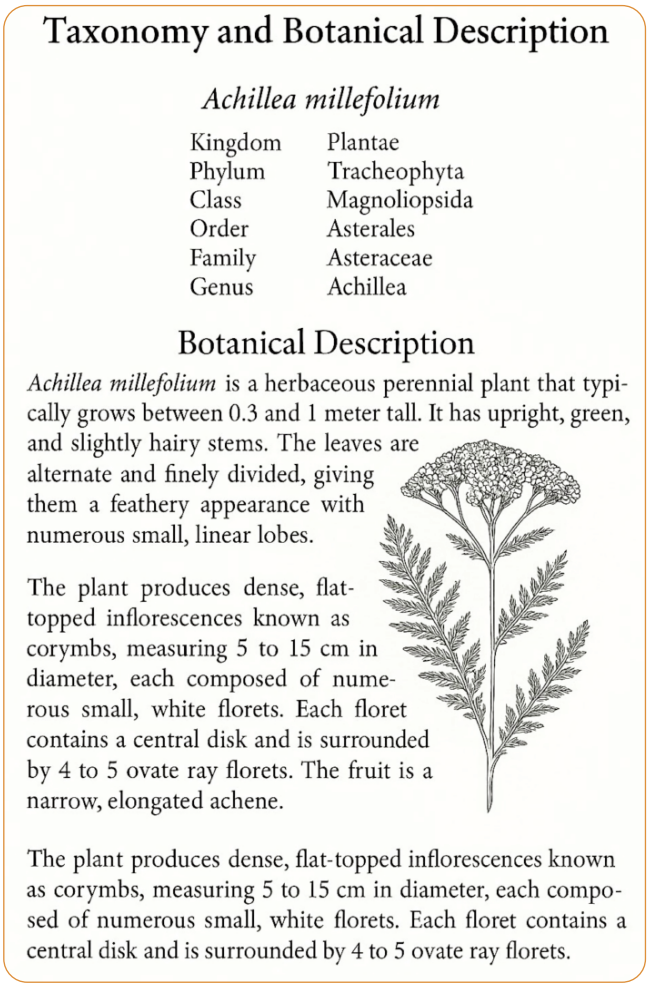

Fig. 1 - *Achillea millefolium*: Taxonomy and botanical description.

bipinnate to tripinnate, finely dissected, and resemble feathers-hence the species epithet *millefolium*, meaning "thousand leaves" in Latin (Li *et al*., 2011). These leaves emit a strong, characteristic aroma when crushed, attributed to its volatile oil content (Jaimand *et al*., 2006).

The inflorescence is a compound corymb composed of numerous small capitula, each containing both central disc florets and peripheral ray florets. Flower colors range from white and pale pink to purple, depending on the variety and environmental conditions (Applequist and Moerman, 2011). The plant flowers from late spring to early fall and is an important nectar source for pollinators.

Achillea millefolium exhibits considerable morphological and genetic variability, which has led to taxonomic complexities and ongoing debate regarding its classification into subspecies and ecotypes. Molecular studies using DNA barcoding and ISSR markers have revealed significant intraspecific diversity, especially among populations in Europe and North America (Dalsenter *et al*., 2004; Benedek *et al*., 2007).

Ecologically, *A. millefolium* thrives in a variety of habitats, including meadows, grasslands, roadsides, and disturbed soils. It is tolerant to drought and poor soils, making it a resilient species in both wild and cultivated environments (Stojanović *et al*., 2005**)**. Its robust root system and allelopathic properties also make it useful in soil stabilization and ecological restoration projects (Lazarevic *et al*., 2010).

3. Phytochemical composition

Achillea millefolium L. is a chemically rich plant whose therapeutic properties are largely attributed to its diverse array of bioactive secondary metabolites (Table 1) (Georgieva *et al*., 2015). These include flavonoids, terpenoids, phenolic acids, sesquiterpene lactones, coumarins, tannins, and alkaloids, among others. Both the aerial parts and essential oils of the plant have been extensively studied, revealing a complex chemical profile that varies depending on geographical origin, harvesting time, plant part, and extraction method (Usmanghani *et al*., 1997; Falconieri *et al*., 2011; Keser *et al*., 2013).

Flavonoids are one of the major phytochemical classes in *A. millefolium*, known for their antioxidant, anti-inflammatory, and vasoprotective effects (Dall'Acqua *et al*., 2011**)**. Common flavonoids identified include: apigenin; luteolin; quercetin; kaempferol; rutin.

These compounds are present mainly as glycosides and aglycones in the leaves and inflorescences. Luteolin and apigenin have demonstrated anti-inflammatory activity via inhibition of cytokine release and COX-2 expression (Lopes *et al*., 2005).

Phenolic acids are potent antioxidants that contribute to free radical scavenging and metal ion chelation. In *A. millefolium*, the following are commonly found: caffeic acid; chlorogenic acid; ferulic acid; p-Coumaric acid. Chlorogenic acid, in particular, is known for its hepatoprotective and anti-diabetic effects (Judzentiene and Mockute, 2010).

The essential oil composition of *A. millefolium*

varies widely depending on chemotype but typically includes: monoterpenes: camphor, 1,8-cineole (eucalyptol), α- and β-pinene, sabinene; sesquiterpenes: β-caryophyllene, chamazulene, germacrene D; thujone (α- and β-thujone): A controversial component due to its potential neurotoxicity at high concentrations.

These volatile compounds confer antimicrobial, spasmolytic, and carminative properties (Goldberg *et al*., 1969). The oil is typically blue due to the presence of chamazulene, formed from matricin during distillation (Dalsenter *et al*., 2004).

Found primarily in the aerial parts, sesquiterpene lactones such as achillicin and millefolide are known for their anti-inflammatory and cytotoxic properties. These compounds act by inhibiting NF-κB signaling pathways and interfering with cell proliferation (Applequist and Moerman, 2011).

Coumarins such as umbelliferone and scopoletin have been reported in small amounts and are recognized for their anti-coagulant and anti-microbial effects. Tannins contribute to the plant's astringent and wound-healing activities, useful in topical formulations for cuts and abrasions.

Although present in lower concentrations, alkaloids such as stachydrine and betonicine have been detected and may require caution, particularly during pregnancy.

Polysaccharides and sterols, such as β-sitosterol, have also been isolated from *A. millefolium*, contributing to its immunomodulatory and cholesterol-lowering properties (Lazarevic *et al*., 2010).

4. Pharmacological activities

Achillea millefolium L. exhibits a broad spectrum of pharmacological effects, many of which are consistent with its extensive traditional use in herbal medicine. Modern phytopharmacological studies have confirmed numerous biological activities, largely attributed to its rich phytochemical composition, including flavonoids, terpenoids, phenolic acids, sesquiterpene lactones, and essential oils (Fig. 2) (Kristoffersen *et al*., 2022**)**. The following sections summarize key pharmacological properties with supporting scientific evidence.

Anti-inflammatory activity

The anti-inflammatory effects of *A. millefolium* are among its most studied properties. Extracts, particularly those rich in flavonoids (e.g., apigenin, luteolin), and sesquiterpene lactones, have

Table 1 - Phytochemical composition of *Achillea millefolium*

Phytochemical class	Main compounds	Location in plant	Biological Activities
Flavonoids	Apigenin, Luteolin, Rutin, Quercetin, Kaempferol	Leaves, flowers	Antioxidant, anti-inflammatory, spasmolytic, vasoprotective
Phenolic acids	Caffeic acid, Chlorogenic acid, Ferulic acid	Leaves, stems, flowers	Antioxidant, hepatoprotective, antimicrobial
Sesquiterpene lactones	Achillicin, Millefolide, Proazulene derivatives	Aerial parts (mainly flowers)	Anti-inflammatory, cytotoxic, antimicrobial
Essential oils	Camphor, 1,8-Cineole, Thujone, Borneol, Chamazulene	Flowers, leaves	Antimicrobial, anti-inflammatory, carminative
Tannins	Hydrolyzable and condensed tannins	Whole plant	Astringent, wound healing, antimicrobial
Coumarins	Umbelliferone, Scopoletin	Stems, flowers	Anticoagulant, anti-inflammatory
Alkaloids	Stachydrine, Betonicine	Leaves, roots (in trace)	Uterotonic, cardiotonic (possible)
Sterols	β-sitosterol	Whole plant	Anti-inflammatory, cholesterol-lowering
Polysaccharides	Arabinogalactans, Pectins	Aerial parts	Immunomodulatory, healing support

demonstrated significant inhibitory effects on pro-inflammatory mediators such as prostaglandins, cytokines (e.g., TNF-α, IL-6), and nitric oxide (NO) (Amirghofran and Karimi, 2002).

Mechanism of action: inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes; suppression of NF-κB and MAPK signaling pathways; reduction in leukocyte infiltration and edema in animal models (Falconieri *et al*., 2011).

These findings support its traditional use for inflammatory conditions such as arthritis, menstrual cramps, and gastrointestinal inflammation.

Antimicrobial and antiviral effects

Essential oils and extracts from *A. millefolium* show broad-spectrum antimicrobial activity against bacteria, fungi, and some viruses. Volatile constituents like camphor, 1,8-cineole, and α-thujone are largely responsible for these effects (Baretta *et al*., 2012).

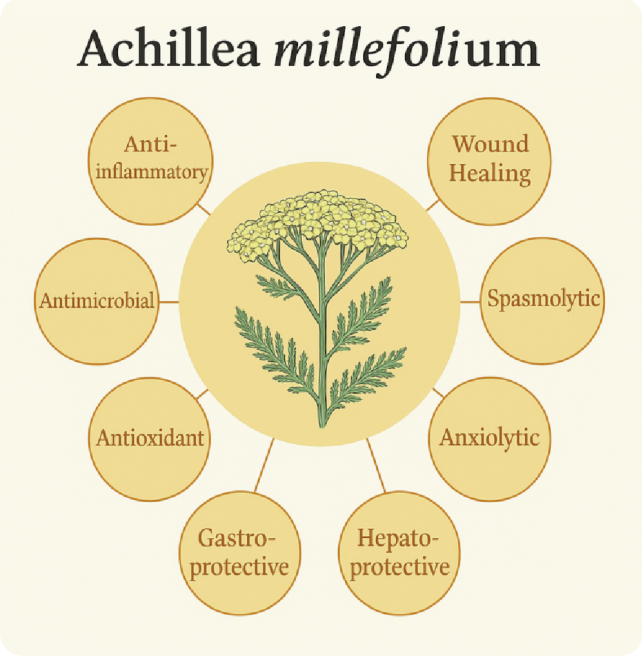

Fig. 2 - Graphical abstract of the pharmacological activities of *Achillea millefolium*. This infographic illustrates the primary pharmacological properties of *Achillea millefolium* (yarrow), supported by preclinical and limited clinical evidence. The central botanical drawing is surrounded by key bioactivities, including anti-inflammatory, antimicrobial, antioxidant, gastroprotective, hepatoprotective, wound healing, spasmolytic, and anxiolytic effects. These therapeutic actions are attributed to the plant's diverse phytochemical constituents, such as flavonoids, sesquiterpene lactones, and essential oils.

Reported effects: inhibition of *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Helicobacter pylori* (Falconieri *et al*., 2011; Gharibi *et al*., 2011); suppression of biofilm formation and quorum sensing (Guo *et al*., 2008); moderate activity against herpes simplex virus *in vitro* (Falk *et al*., 1975).

These properties may support the external and internal use of yarrow for infections, wounds, and gastrointestinal disturbances.

Antioxidant activity

Achillea millefolium has potent antioxidant properties due to its high content of polyphenolic compounds, including caffeic acid, chlorogenic acid, flavonoids, and chamazulene (Candan *et al*., 2003).

Effects observed: strong free radical scavenging (DPPH, ABTS assays); chelation of metal ions (e.g., Fe^{2+}); protection against oxidative damage in lipid and protein models (Keser *et al*., 2013; Judzentiene, 2016).

Antioxidant activity is believed to underlie many of its protective effects in cardiovascular, neurodegenerative, and metabolic disorders.

Wound healing and hemostatic effects

Historically known as “soldier’s woundwort,” yarrow has long been applied to cuts and wounds (Anne *et al*., 2006). Preclinical studies confirm its effectiveness in accelerating wound closure and improving tissue regeneration (Falconieri *et al*., 2011).

Mechanisms involved: stimulation of fibroblast proliferation and collagen synthesis; anti-inflammatory and antimicrobial protection of the wound site; local vasoconstriction and astringent action facilitating hemostasis (Borrelli *et al*., 2012).

A topical gel containing *A. millefolium* extract significantly improved healing in full-thickness wounds in rats, supporting its use in dermatological applications (Applequist and Moerman, 2011).

Gastroprotective and antispasmodic activity

Achillea millefolium is traditionally used to treat dyspepsia, ulcers, and irritable bowel syndrome. Studies in animal models demonstrate: reduction in gastric ulceration and acidity; relaxation of intestinal smooth muscle (antispasmodic); inhibition of *H. pylori* and pro-inflammatory cytokines in gastric tissues (Gharibi *et al*., 2011).

These actions are linked to flavonoids and

essential oil components that modulate gut motility and inflammation.

Metabolic and antidiabetic effects

Recent studies suggest that *A. millefolium* may have antidiabetic and lipid-lowering potential. Judzentiene and Mockute (2010) reported that ethanolic extracts: lowered blood glucose and total cholesterol in diabetic rats; enhanced antioxidant enzyme activity (SOD, CAT); increased collagen synthesis and improved skin morphology.

Such findings indicate potential for *A. millefolium* in metabolic syndrome and diabetes-related complications.

Neuroprotective and anxiolytic effects

Preliminary studies indicate that *Achillea millefolium* may exert beneficial effects on the central nervous system. Its mild anxiolytic and sedative properties, observed in animal models, are primarily attributed to the presence of flavonoids and monoterpenes (Falk *et al*., 1975). Additionally, the plant has demonstrated acetylcholinesterase inhibitory activity, suggesting potential for cognitive enhancement and neuroprotection (Keser *et al*., 2013). These findings highlight the plant's promise as a candidate for further investigation in the context of neurodegenerative diseases and stress-related disorders.

Anticancer activity

Some sesquiterpene lactones and flavonoids from *A. millefolium* have shown cytotoxic activity against cancer cell lines, including breast, colon, and leukemia cells (Amirghofran and Karimi, 2002).

Mechanisms may involve: induction of apoptosis; cell cycle arrest; anti-angiogenic effects.

However, clinical relevance is yet to be established.

5. Toxicity and safety

While *Achillea millefolium* is widely regarded as a safe medicinal plant in traditional medicine and herbal formulations, its long-term safety and toxicity profile warrant careful examination, particularly due to certain bioactive constituents such as thujone, sesquiterpene lactones, and potential allergenic compounds (Table 2) (Kazemi, 2015). Understanding the safety of yarrow is essential for its proper therapeutic use and formulation in nutraceuticals, cosmeceuticals, and pharmaceuticals.

General safety and traditional use

Yarrow has a long history of traditional use, including as a tea, tincture, topical poultice, and essential oil. It is included in several national pharmacopeias (e.g., British Herbal Pharmacopoeia, European Pharmacopoeia) for its wound-healing and digestive properties. At traditional dosages, it is generally well tolerated with low acute toxicity (Falk *et al*., 1975).

Thujone content and neurotoxicity risk

One of the main safety concerns associated with *A. millefolium* is the presence of α-thujone and β-thujone, monoterpene ketones found in the plant's essential oils (Farasati Far *et al*., 2023). These compounds are known to be neurotoxic in high

Table 2 - Toxicity data summary

Aspect	Evidence summary
Acute toxicity	Low; LD_{50} > 2,000 mg/kg in rodents (oral extracts)
Chronic toxicity	Limited data; high doses may stress liver/kidney function
Allergenicity	Moderate risk in sensitive individuals (sesquiterpene lactones)
Thujone neurotoxicity	Present in essential oils; not recommended for internal use in concentrated form
Teratogenicity	Not well studied; avoided during pregnancy due to uterine stimulation
Drug interactions	Possible with anticoagulants, sedatives, NSAIDs

doses, capable of causing: seizures; restlessness and hallucinations; convulsions in extreme cases.

Thujone acts as a GABA_A receptor antagonist, reducing inhibitory neurotransmission in the central nervous system. The European Food Safety Authority (EFSA) has established a maximum daily intake level of 0.1 mg/kg body weight/day for thujone (EFSA, 2012). Although thujone levels in commercial yarrow preparations are generally below this threshold, concentrated essential oils may exceed it if improperly dosed or ingested (Dalsenter *et al*., 2004).

Allergic reactions and contact dermatitis

As a member of the Asteraceae family, yarrow contains sesquiterpene lactones, which are known sensitizers and can cause: allergic contact dermatitis; photosensitivity reactions; skin rashes and itching, especially with topical use. Individuals allergic to other Asteraceae species (e.g., chamomile, ragweed, daisies) are more likely to react adversely to yarrow (Lazarevic *et al*., 2010). Patch testing is advisable before widespread dermal application.

Reproductive and pregnancy concerns

Although traditionally used to regulate menstruation, *Achillea millefolium* has emmenagogue and uterotonic properties, which may stimulate uterine contractions (Hajhashemi *et al*., 2016). Animal studies have shown that high doses can potentially influence reproductive hormones or embryo implantation (Amirghofran and Karimi, 2002). Therefore: not recommended during pregnancy, especially the first trimester; avoid use in women with heavy menstruation or bleeding disorders. Safety during lactation has not been adequately studied, and caution is advised.

Hepatic and renal effects

Limited evidence suggests that chronic high-dose exposure may exert stress on liver enzymes and renal markers in animals. However, these effects are dose-dependent and typically associated with non-standardized or excessive use of extracts or essential oils (Gharibi *et al*., 2011). No hepatotoxicity has been reported in traditional doses or short-term clinical use.

Drug interactions

Yarrow contains compounds that may interact with conventional medications (Ghavami *et al*., 2010), such as: anticoagulants (due to coumarins and flavonoids) - increased risk of bleeding; sedatives or CNS depressants - possible additive effects; NSAIDs or corticosteroids - overlapping anti-inflammatory actions may enhance effects or side effects. Patients on medication should consult a healthcare provider before using *A. millefolium*.

Clinical and regulatory observations

The German Commission E monograph considers *Achillea millefolium* safe when used for dyspepsia and loss of appetite, with minor side effects such as photosensitivity reported rarely.

The European Medicines Agency (EMA) includes it in the list of herbal substances for traditional use, acknowledging its safety in specified indications and dosages (Gervais, 1977).

No major adverse effects were reported in small-scale clinical trials involving topical or oral use (Applequist and Moerman, 2011).

6. Clinical evidence and applications

Despite its long-standing use in traditional medicine and promising preclinical findings, clinical evidence for *Achillea millefolium* (yarrow) remains limited but steadily growing. Most human data come from small-scale trials, ethnopharmacological surveys, and herbal combination studies, rather than large randomized controlled trials (RCTs) (Fierascu *et al*., 2015**)**. Nevertheless, these studies offer valuable insights into the plant's therapeutic potential across various systems, especially in gastrointestinal health, wound healing, gynecological care, and cosmeceutical applications (Fig. 3).

Gastrointestinal disorders

The most well-supported clinical applications of *A. millefolium* involve its spasmolytic, carminative, and anti-inflammatory properties in the treatment of functional dyspepsia, gastritis, and irritable bowel syndrome (IBS) (Eghdami and Sadeghi, 2010).

In a randomized clinical trial, a combination herbal formulation containing *A. millefolium*, *Mentha piperita*, and *Matricaria chamomilla* significantly reduced symptoms of indigestion, bloating, and abdominal pain in patients with functional dyspepsia (Madisch *et al*., 2004). These effects are attributed to the plant's ability to relax smooth muscle, reduce gastric inflammation, and modulate gut motility.

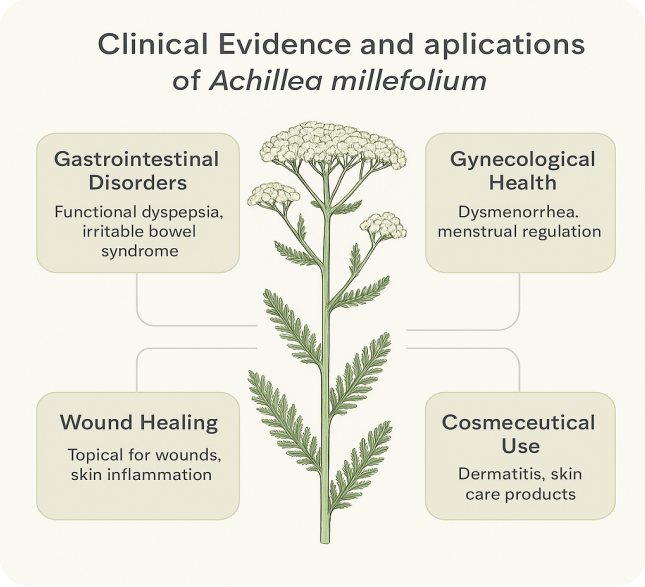

Fig. 3 - Graphical abstract of the clinical evidence and applications of *Achillea millefolium.* This infographic summarizes the primary clinical applications of *Achillea millefolium* supported by traditional use and emerging scientific evidence. Highlighted therapeutic areas include gastrointestinal disorders (e.g., functional dyspepsia, irritable bowel syndrome), gynecological health (e.g., dysmenorrhea, menstrual regulation), wound healing (topical use for skin inflammation), and cosmeceutical applications (e.g., treatment of dermatitis and inclusion in skincare formulations). These uses reflect the plant's anti-inflammatory, spasmolytic, and tissue-repairing properties.

Wound healing and dermatology

Topical applications of *A. millefolium* have been investigated for accelerating wound healing, particularly due to its astringent, antimicrobial, and anti-inflammatory properties (Tozyo *et al*., 1994). In a clinical case series, a yarrow-based cream applied to superficial skin wounds and minor cuts resulted in faster re-epithelialization and lower infection rates compared to standard treatment (Csupor *et al*., 2009). Another clinical study reported that a topical gel containing yarrow extract improved skin hydration and reduced inflammation in patients with mild eczema (Applequist and Moerman, 2011). These outcomes are consistent with earlier in vivo findings demonstrating increased fibroblast activity, collagen deposition, and wound closure speed (Konyalioglu and Karamenderes, 2005).

Gynecological health

Traditionally used for regulating menstruation and relieving menstrual cramps, yarrow has also shown promise in early clinical studies (De Souza *et al*., 2011).

A randomized trial in Iran found that *A. millefolium* extract capsules reduced dysmenorrhea pain intensity and duration in adolescent girls compared to placebo, likely due to the plant's antispasmodic and anti-inflammatory constituents (Benedek *et al*., 2005). Additionally, herbal vaginal washes containing yarrow are used in some clinical settings for vaginitis and vaginal irritation, with anecdotal evidence of symptom relief and microbial balance restoration.

Cosmeceutical and topical use

Achillea millefolium is increasingly featured in cosmetic and dermatological formulations due to its anti-inflammatory, antioxidant, and astringent properties (Farooq *et al*., 2012). Clinical-grade formulations such as creams, lotions, serums, and cleansing gels are used for: acne and oily skin regulation; soothing inflamed or sensitive skin; enhancing skin tone and elasticity.

Preliminary human-use studies report good tolerability and efficacy, though large-scale dermatological trials are still lacking (Falk *et al*., 1975).

Metabolic and cardiovascular effects (Emerging Area)

While human data remain sparse, animal studies showing antidiabetic, antihyperlipidemic, and antioxidant effects of *A. millefolium* have encouraged nutraceutical exploration (Gadgoli and Mishra, 2007**)**.

A pilot human study using a multi-herbal formula containing yarrow in type 2 diabetic patients showed modest improvements in fasting glucose and lipid profile, though it was not placebo-controlled (Judzentiene and Mockute, 2010). The presence of flavonoids and phenolic acids may contribute to insulin sensitization and lipid-lowering effects, indicating future potential pending more rigorous trials.

Traditional and integrative medical use

Yarrow is officially recognized in several European herbal monographs (De Santanna *et al*., 2009**)**, including those from: German Commission E, which approves it for gastrointestinal spasms and minor inflammation; European Medicines Agency (EMA), which lists it as a traditional herbal remedy for dyspeptic complaints and minor skin inflammations

It is also included in integrative and naturopathic protocols, often combined with other herbs like peppermint, calendula, or chamomile for synergistic effects.

7. Industrial and cosmetic applications

Achillea millefolium L. has garnered increasing industrial interest due to its rich phytochemical profile, which includes volatile oils, flavonoids, phenolic acids, and sesquiterpene lactones (Costescu *et al*., 2014**)**. These constituents contribute to the plant's antimicrobial, anti-inflammatory, antioxidant, and astringent properties—traits that are highly valued in the cosmetic, pharmaceutical, nutraceutical, and agricultural industries. Yarrow is used in a wide range of commercial products from topical skin formulations to botanical extracts in eco-friendly agriculture (Candan *et al*., 2003**)**.

Cosmetic industry applications

The skin-soothing and antimicrobial qualities of yarrow make it a valuable ingredient in the cosmetic and personal care industries (Boskovic *et al*., 2005). It is commonly incorporated into products designed for sensitive, inflamed, or acne-prone skin, owing to its natural anti-inflammatory and wound-healing activities.

Common Cosmetic Uses: facial cleansers and toners: Used for its astringent action that tightens pores and reduces oiliness; moisturizers and serums: Yarrow extracts are included to reduce redness, irritation, and signs of inflammation in sensitive or damaged skin; anti-aging formulations: Its antioxidant compounds (e.g., flavonoids, phenolic acids) combat oxidative stress and may protect against premature aging; aftershave and soothing balms: Yarrow's cooling and antimicrobial effects aid in reducing razor burn and skin irritation; scalp and hair care: Infusions or extracts are used in shampoos to treat dandruff and soothe itchy or inflamed scalps.

In a 2021 study, topical application of a yarrow-containing cream led to significant improvements in skin hydration, elasticity, and reduction of inflammatory skin symptoms, such as in mild cases of eczema and dermatitis (Applequist and Moerman, 2011).

Pharmaceutical and herbal formulations

Industrially, yarrow is processed into standardized extracts, tinctures, capsules, and teas that are distributed through herbal supplement markets. It is especially popular in European phytotherapy and is listed in the European Pharmacopoeia (David *et al*., 2010**)**.

Key pharmaceutical uses include: digestive aids: as an ingredient in herbal bitters, often in combination with gentian, peppermint, and fennel, for bloating, dyspepsia, and poor appetite; topical antiseptic agents: creams and gels containing yarrow extract are used for minor wounds, burns, and abrasions, owing to its antibacterial and wound-healing properties; women's health: included in herbal combinations for menstrual regulation and pain relief. Industrial formulations often standardize yarrow preparations to their flavonoid or essential oil content, ensuring consistent therapeutic outcomes.

Nutraceuticals and functional foods

With growing consumer interest in natural health products, yarrow is emerging in the functional food and beverage sector (Bimbiraite *et al*., 2008), especially in: Herbal teas and infusions: Dried aerial parts of the plant are used in herbal blends marketed for digestion, relaxation, and detox; Functional beverages: Combined with other herbs to create health-promoting drinks that claim anti-inflammatory or digestive benefits; Botanical dietary supplements: In capsule or powder form, yarrow is marketed for gastrointestinal support and immune modulation.

A recent trend in Europe includes the incorporation of yarrow extracts into fortified waters and botanical sodas, emphasizing their antioxidant potential and natural origin (Keser *et al*., 2013).

Essential oil and fragrance industry

Yarrow's essential oil, obtained through steam distillation of the flowering tops, is used in (Cavalcanti *et al*., 2006**)**: aromatherapy: for stress relief, minor wound care, and muscle relaxation; perfume and fragrance formulations: due to its herbal, sweet, and slightly camphoraceous scent; massage oils: blended with carrier oils to relieve muscle tension and improve circulation.

Its striking blue color, due to chamazulene formed during distillation, enhances its visual and marketing appeal in natural product lines.

Ecological and agricultural applications

Yarrow also plays a role in sustainable agriculture

and ecological industries (Benetis *et al*., 2008) natural pest repellent: Volatile oils act as a mild insect deterrent; pollinator support: Its extended flowering season attracts bees and beneficial insects, enhancing crop pollination; soil stabilization and phytoremediation: Its deep roots help prevent erosion and assist in soil recovery in degraded environments (Lazarevic *et al*., 2010).

Yarrow is increasingly used in organic farming as a companion plant and in biodynamic preparations to improve soil vitality and compost activity.

Industrial processing considerations

For commercial applications, the plant is typically harvested during full bloom, when phytochemical content is at its peak. It is processed into: dried herb (cut or powdered); aqueous, ethanol, or CO_2 extracts; steam-distilled essential oil; freeze-dried powders for encapsulation or food formulation

Standardization and quality control are critical for maintaining efficacy and ensuring regulatory compliance, particularly for exports in the EU and North American markets (Niu, 2020).

8. Discussion and Conclusions

Despite the wealth of traditional knowledge and preclinical data supporting the pharmacological efficacy of *Achillea millefolium*, its integration into modern evidence-based medicine is constrained by several gaps (Akram, 2013).

1. Clinical vs. Preclinical Gap: While many laboratory and animal studies demonstrate significant pharmacological activities, translation into clinical application is weak. Most clinical studies suffer from small sample sizes, lack of placebo-controlled designs, and inconsistent dosing protocols. For instance, while yarrow showed strong spasmolytic and anti-inflammatory activity in models of irritable bowel syndrome and gastritis, clinical trials validating these effects are limited and methodologically fragile (Madisch *et al*., 2004; Eghdami and Sadeghi, 2010)
2. Phytochemical Variability and Standardization: One major challenge in assessing efficacy across studies is the phytochemical variability of *A. millefolium.* Composition can fluctuate based on environmental conditions, harvesting time, and extraction method (Georgieva *et al*., 2015). This affects reproducibility of results and complicates formulation standardization, especially when active constituents such as flavonoids or thujone-containing essential oils vary widely in content. Future studies should prioritize well-characterized, standardized extracts with quantification of key bioactives.
3. Conflicting and Inconclusive Evidence: In several areas, research findings either conflict or remain inconclusive. For example, while some studies show notable anxiolytic or cognitive-enhancing effects, others do not observe such benefits, likely due to variability in extract types and dosing. A comparative analysis of different extracts and their phytochemical profiles may clarify these discrepancies.
4. Underexplored Therapeutic Areas: Neuroprotective and metabolic effects of *A. millefolium* represent two of the most promising yet underinvestigated fields. While GABA modulation and acetylcholinesterase inhibition have been observed (Falk *et al*., 1975; Keser *et al*., 2013), no human studies have evaluated its impact on anxiety or cognitive decline. Similarly, animal models show antidiabetic and lipid-lowering potential, but these effects remain untested in robust clinical trials (Judzentiene and Mockute, 2010).
5. Ecological Resilience and Industrial Supply: *Achillea millefolium*'s ecological adaptability, including drought tolerance and allelopathic behavior, enhances its suitability for sustainable cultivation (Lazarevic *et al*., 2010). This resilience makes it a strong candidate for commercial exploitation in phytopharmaceutical and cosmeceutical industries. However, standardized cultivation practices and genetic profiling are essential to maintain consistent therapeutic quality.
6. Recommendations for Future Research: conduct large-scale, placebo-controlled clinical trials using chemically standardized yarrow extracts; include pharmacokinetic and toxicological assessments in long-term human studies; investigate phytochemical variability across ecotypes to link specific bioactive profiles with therapeutic efficacy; explore integrative applications in neuro-degeneration, metabolic syndrome, and dermatology; clearly delineate areas where findings converge, conflict, or remain uncertain.

Achillea millefolium L. is a botanically and pharmacologically significant species with deep roots in traditional medicine and growing relevance in modern therapeutic, cosmetic, and industrial applications. Its rich phytochemical composition-including flavonoids, sesquiterpene lactones, phenolic acids, and essential oils-forms the basis for

a broad spectrum of biological activities, notably antioxidant, anti-inflammatory, antimicrobial, antispasmodic, and wound-healing effects. These pharmacological actions validate many of the plant's historical uses and support its continued inclusion in herbal pharmacopeias and integrative medicine. However, several challenges remain. Clinical data are still sparse and often lack methodological rigor, emphasizing the need for larger, well-controlled trials using standardized extracts. Toxicological assessments indicate that while the plant is generally safe in traditional doses, components like thujone and sesquiterpene lactones necessitate cautious use, particularly in essential oil formulations and during pregnancy. Additionally, the taxonomic complexity and phytochemical variability across populations underscore the importance of genetic characterization and quality control in both research and industry. Looking forward, *Achillea millefolium* presents exciting opportunities for new drug development, phytocosmetics, and evidence-based herbal therapies. Bridging the gap between traditional knowledge and scientific validation will be key to fully unlocking the plant's therapeutic and commercial potential. Collaborative research, regulatory standardization, and interdisciplinary innovation are essential to ensure the safe, effective, and sustainable use of this valuable medicinal herb.

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