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# Effectiveness of $KMnO_4$ and activated carbon on the quality and storage properties of mango fruit

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*Key words:* Cat Hoa Loc, fruit quality, *Mangifera indica* L., postharvest, shelf life, vitamin C.

**Abstract: Cat Hoa Loc mango (*Mangifera indica* L.) is a well-known variety in Vietnam because of its distinct taste and aroma. However, it has a short shelf life and can suffer post-harvest losses if not handled and packaged correctly. The demand for fresh Cat Hoa Loc mangoes has been increasing worldwide, and this has led to the development of effective handling methods to extend their shelf life. To address this issue, this study was conducted to evaluate the effectiveness of $KMnO_4$ and activated carbon in preserving the quality and shelf life of Cat Hoa Loc mangoes in Vietnam. The Cat Hoa Loc mango variety in Cao Lanh, Dong Thap province, was chosen for the study. The study used five replications of a completely randomized block design. Six different treatments with $KMnO_4$ and activated carbon (1:1 ratio) were tested, including 0; 4; 8; 12; 16; 20 g/box. Six mangoes were stored in perforated cartons (36x26x9 cm) at room temperature (28-30°C) during the study period. The study evaluated several parameters to assess the quality and shelf life of the mangoes, including weight loss, fruit firmness, browning index, respiration, ethylene release rate, soluble sugar, and vitamin C. The results showed that the quality of the mangoes was extended when treated with 12 g of $KMnO_4$ and activated carbon per box. This treatment resulted in the lowest physiological weight losses, respiration, and ethylene release rate. Furthermore, this treatment showed the highest fruit firmness, soluble sugar, and vitamin C content, as well as the longest shelf life at the end of the storage period.**

## 1. Introduction

Cat Hoa Loc mango (*Mangifera indica* L.) is a popular tropical fruit variety known for its unique flavor, aromatic fragrance, and vibrant color. It is highly valued for its nutritional content, abundant vitamins, and minerals (Athoo *et al.,* 2024). Cat Hoa Loc mangoes have gained

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significant recognition both domestically and internationally and are widely exported to various markets. Despite its popularity and economic importance, Cat Hoa Loc mango faces challenges related to its postharvest shelf life. The limited shelf life poses a considerable threat to its preservation and exportation (Nguyen *et al.,* 2024). To overcome this challenge, researchers and experts have focused on developing postharvest preservation methods to minimize quality deterioration and extend the shelf life of Cat Hoa Loc mangoes. Previous research focused on mango preservation has employed essential oils derived from four aromatic plant species, namely *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica,* and *Rosmarinus officinalis*. The objective has been to impede the proliferation of *Aspergillus niger*, thus prolonging the fruit's storage viability (Javadpour *et al.,* 2018). In addition to essential oils, a spectrum of chemical agents has been extensively utilized for fruit preservation. For instance, potassium phosphite has been employed in the preservation of *Citrus clementina*, while a composite of alginate and *Cyclea barbata* leaf powder has been implemented for guava preservation (Strano *et al.,* 2015; Utama *et al.,* 2022). In the various preservation methods, the use of potassium permanganate ($KMnO_4$) and activated carbon has gained attention. $KMnO_4$ has been utilized in the postharvest management of peaches and mangoes to uphold their quality (Alonso-Salinas *et al*., 2023; Fatima *et al*., 2023). The research findings indicate that the application of 30 g $KMnO_4$ is optimal for preserving and enhancing the color, taste, aroma, firmness, total sugar, pH, and total soluble solids of the fruits, while minimizing weight loss and waste percentage over a 20-day storage period (Fatima *et al*., 2023). Potassium permanganate is a powerful oxidizing agent with antimicrobial properties. When used in postharvest preservation, $KMnO_4$ can effectively inhibit the growth of pathogens and spoilage microorganisms that contribute to the deterioration of fruits (Alonso-Salinas *et al.,* 2023). The antimicrobial action of $KMnO_4$ is attributed to its ability to oxidize the cellular metabolism of microorganisms. $KMnO_4$ can interfere with the vital biochemical processes of microbes, such as respiration and energy production, which are essential for their survival and proliferation. The strong oxidizing properties of $KMnO_4$ can target and oxidize key enzymes and other critical cellular components involved in these metabolic pathways. This oxidative damage can impair the microorganism's ability to carry out normal metabolic functions, ultimately disrupting its ability to function and survive (Rudra *et al.,* 2013). By inhibiting microbial growth, $KMnO_4$ treatment helps to extend the shelf life of Cat Hoa Loc mangoes and maintain their quality during storage and transportation. However, the oxidation of ethylene by $KMnO_4$ requires time, so it is necessary to supplement with some ethylene-adsorbing carriers with porous structures and large surface areas to facilitate the redox reaction. In food preservation, activated carbon has a high capacity for adsorbing ethylene, especially in the form of granular activated carbon. Activated carbonis a highly porous material with a large surface area, which gives it excellent adsorption properties (Roopa *et al*., 2023). When applied in postharvest preservation of fruits, activated carbon acts as a purification agent by adsorbing and removing harmful substances such as ethylene gas, volatile compounds, and toxins (Nooun *et al.,* 2023). Ethylene is a natural plant hormone that accelerates the ripening process in fruits. By adsorbing ethylene, activated carbon helps low the ripening process, thus extending the shelf life. Additionally, activated carbon can also adsorb volatile compounds responsible for off-flavors and odors, thereby preserving the sensory quality of the fruit. Therefore, the objective of this research is to assess the effectiveness of potassium permanganate and activated carbon treatments on various quality parameters of postharvest Cat Hoa Loc mango. By studying the physiological and biochemical changes that occur during the ripening process under the influence of $KMnO_4$ and activated carbon, this research aims to optimize postharvest treatments to improve fruit quality, reduce losses, extend shelf life, and ensure a higher yield of marketable Cat Hoa Loc mangoes. The findings of this study will contribute to a better understanding of the preservation techniques for Cat Hoa Loc mangoes, enhancing their market value and global competitiveness.

## 2. Materials and Methods

*Plant material and experimental design*

The Cat Hoa Loc mangoes were harvested from a commercial orchard located in Cao Lanh City, Dong Thap Province, Vietnam. On May 25, 2023, mango fruits were collected from homogenous plants using

a randomized block pattern. The fruits, with an average weight of about 450 g, were picked precisely 85 days after the fruit set and were carefully hand-picked. After being picked, the mangoes were transported to the University of Science, located in Ho Chi Minh City. A total of 180 mango fruits were used and distributed into five replications using a completely randomized block design. Each replication consisted of six mangoes that were stored in perforated cartons (36 cm x 26 cm x 9 cm). To test the effects of $KMnO_4$ and activated carbon, a bag containing a mixture of the two substances in a 1:1 ratio was placed in each carton. The weight of the mixture ranged from 0, 4, 8, 12, 16, and 20 g per box. The mangoes were stored at a constant temperature of 28-30°C and ambient humidity of 70-80% throughout the study. The research evaluated several parameters, including weight loss, fruit firmness, color, browning index, respiration rate, ethylene release rate, soluble sugar content, vitamin C content, and the shelf life of the mangoes.

*Determination of weight loss and browning index*

Weight loss is determined by recording the initial weight of the fresh sample. After the storage period, the final weight is determined. The percentage of difference between the initial and final weight to initial weight represents the physiological weight loss (Workneh *et al*., 2012). The color of the outer layer of the fruit was determined using the L*a*b* (CIELAB) color space of a digital color meter from Apple Inc. To calculate the browning index (BI), the formula proposed by Ruangchakpet and Sajjaanantakul (2007) was used:

$$[100\ (x - 0.31)]/0.17.$$

In this formula, x is calculated as (a* + 1.75 L*)/(5.645 L* + a* −0.3012b*).

*Determination of fruit firmness, respiration rate, and ethylene release rate*

The fruit firmness was evaluated using a fruit firmness testing device (GY-3, Jiangsu, China) equipped with a cylindrical probe. For the assessment of the fruit's respiration rate in a sealed chamber, a $CO_2$ analyzer with a non-dispersive infrared sensor was utilized (Thang *et al*., 2022). Furthermore, the release of ethylene gas was determined by utilizing an ethylene gas analyzer with an electrochemical sensor (SKY2000-$C_2H_4$, Safegas, China) connected to the same sealed chamber.

*Determination of soluble sugar and vitamin C*

To determine the total sugar content, fresh fruit flesh (1 g) was finely ground and mixed with 10 mL of 96% ethanol. Following this, the mixture was heated in a water bath for 15 min and subjected to centrifugation at 10,000 rpm for 10 min to obtain the supernatant. Then, 1 mL of the extracted solution was combined with 1 mL of a 5% phenol solution and 5 mL of concentrated $H_2SO_4$. The resultant mixture was allowed to react, and the optical density was measured at a wavelength of 490 nm. The total sugar content was then calculated using a sucrose standard curve as a reference (Dubois *et al.,* 1956). To quantify the amount of vitamin C, 1 g of the sample was ground and mixed with 10 mL of a methanol solution. Subsequently, the mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected. Next, 1 mL of the extracted solution was combined with 2 mL of 1% sodium nitroprusside, 1 mL of 1% potassium dichromate, and 1 mL of concentrated sulfuric acid. The resultant mixture underwent a reaction, and the optical density was measured at a wavelength of 564 nm. The content of vitamin C was then determined by comparing it to a corresponding standard curve (Saeed *et al*., 2018).

*Statistical analysis*

The collected data was subjected to an analysis of variance (ANOVA) to determine the significant differences among the means at a 5% probability level. Duncan's Multiple Range Test was then employed using SPSS 20.0 to identify the significant differences. The results were presented as the mean values together with their corresponding standard deviations, and the 'ns' indicates that the differences were not statistically significant.

## 3. Results

*The changes in weight loss and browning index*

The utilization of $KMnO_4$ and activated C in mango preservation has yielded noteworthy results. The experiment revealed that on days 8 and 10, the control group and treated groups of 4, 12, 16, and 20 g/box did not exhibit significant differences in weight loss. However, the treated group with 8 g/box demonstrated a noticeable reduction in weight loss

percentage. Moreover, the treatment with $KMnO_4$ and C significantly enhanced the color changes in the mangoes. The treated fruit manifested a lower browning index than the control group. The flesh of the treated mangoes retained a vibrant, fresh yellow color, while the skin remained a bright green (Figs. 1 and 2).


Fig. 1 - The variations in fruit color among different treatments using $KMnO_4$ and C with various concentrations after a period of 12 days.

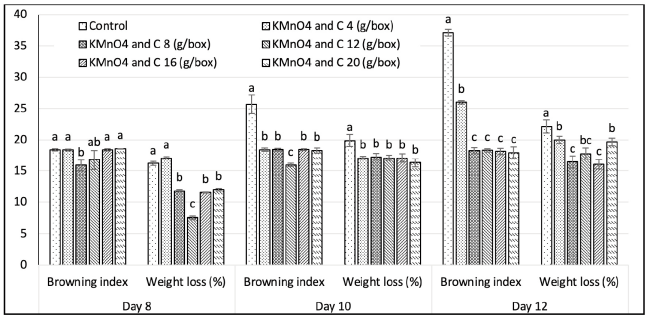


Fig. 2 - The changes in weight loss and browning index during the post-harvest ripening process of mango. Values with different letters are significantly different according to Duncan's test (p=0.05).

*The changes in fruit firmness, respiration rate, and ethylene release rate*

Throughout the course of the analysis period, the fruit's firmness gradually decreased. However, treatments utilizing $KMnO_4$ and activated carbon proved effective in maintaining the fruit's firmness across all three-time points analyzed. Of the treatments tested, $KMnO_4$ and activated carbon at a concentration of 12 g/box delivered the most effective outcomes in preserving fruit firmness. Similarly, the utilization of $KMnO_4$ and activated carbon treatments allowed for the extension of both the ethylene peak and respiration rate. In the control group, respiration intensity and ethylene release were high on the eighth day, decreasing gradually on days 10 and 12. In contrast, the fruits treated with $KMnO_4$ and activated carbon exhibited two peaks of ethylene release and respiration rate, occurring on day 10 (Fig. 3).

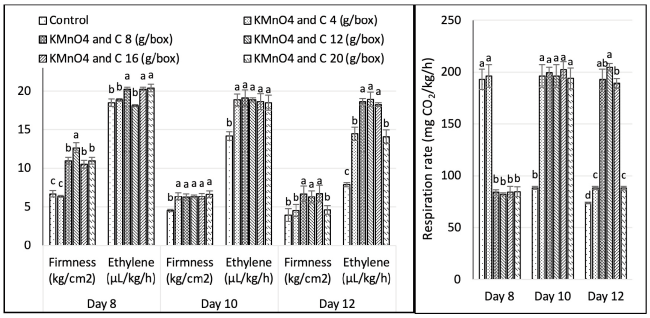


Fig. 3 - The changes in fruit firmness, respiration rate, and ethylene release during the post-harvest ripening process of mango. Values with different letters are significantly different according to Duncan's test (p=0.05).

*The changes in soluble sugar and vitamin C*

Throughout the process of mango ripening, the control group demonstrated a significant increase in total soluble sugar content, while the vitamin C content remained stable. Upon comparison of the control group with the $KMnO_4$ and activated C treatments, it was observed that treatment groups receiving 12, 16, and 20 g/box were instrumental in maintaining the highest level of total soluble sugars on day 12. However, no significant difference was found in vitamin C content between the control group and the $KMnO_4$ and activated C treatments, as noted in figure 4.

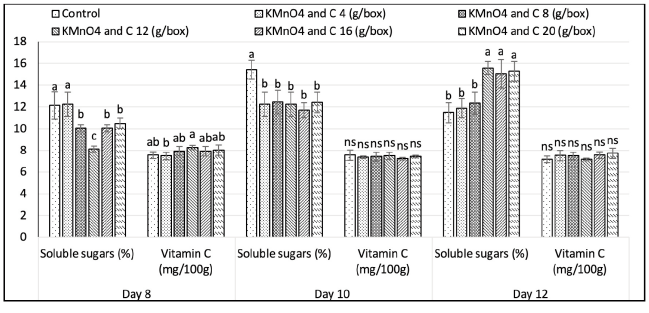


Fig. 4 - The changes in soluble sugar and vitamin C during the post-harvest ripening process of mango. Values with different letters are significantly different according to Duncan's test (p=0.05). NS= not significant.

## 4. Discussion and Conclusions

The preservation of mangoes post-harvest can be extended by utilizing $KMnO_4$ and activated carbon. The treated group, receiving 8 g/box of $KMnO_4$ and activated carbon, demonstrated a significant reduction in weight loss percentage compared to the control, as shown in figure 2. $KMnO_4$ and activated carbon act to preserve the natural color of mangoes by inhibiting the activity of enzymes, such as polyphenol oxidase, responsible for enzymatic browning (Mope *et al.,* 2024). This enzymatic reaction occurs when the fruit's phenolic compounds react with oxygen, resulting in a brownish discoloration. By inhibiting this enzymatic activity, $KMnO_4$ and activated carbon help preserve the fruit's natural color, rendering it visually appealing and marketable for a longer duration. The maintenance of firmness in mangoes is another critical aspect of preservation. $KMnO_4$ and activated carbon inhibit the activity of cell wall-degrading enzymes, such as pectinase and cellulase (Chen *et al*., 2021; Kumar *et al*., 2023). These enzymes break down the cell walls of the fruit, leading to softening and a loss of firmness. By inhibiting these enzymes, $KMnO_4$ and activated carbon help preserve the structural integrity of the fruit and maintain its firm texture over a longer period of time. $KMnO_4$ and activated carbon treatments delay the onset of senescence and over-ripening by extending the ethylene peak and respiration rate. The control group showed a progressive decline in respiration intensity and ethylene release on days 10 and 12, which peaked on the eighth day. In contrast, fruits treated with $KMnO_4$ and activated carbon showed two peaks in the respiration rate and ethylene release on day 10, as shown in figure 3. Ethylene is a natural plant hormone involved in the ripening process. $KMnO_4$ physically absorbs the surrounding ethylene through a porous medium, oxidizing it to produce $CO_2$, manganese oxide, potassium hydroxide, and water (Kumar *et al.,* 2023; Meena *et al*., 2024). Activated carbon, with its porous structure, can adsorb and remove volatile compounds, including those responsible for producing off-flavors. By reducing the presence of these compounds, activated carbon helps maintain the fruit's quality and freshness (Nooun *et al*., 2023; Roopa *et al.,* 2023). By reducing the peak production of ethylene and respiration rate, $KMnO_4$ and activated carbon slow down the ripening process, allowing the fruit to maintain its desirable qualities for a more extended period. Slowing down respiration helps fruits ripen more slowly, and as a result, the carbohydrate metabolism process occurs at a slower rate, allowing fruits to maintain a higher sugar content (Fig. 4). Furthermore, $KMnO_4$ acts as an antimicrobial agent by releasing oxygen and oxidizing organic matter. It helps inhibit the growth of microorganisms on the fruit's surface, reducing the risk of spoilage and extending the shelf life (Alonso-Salinas *et al*., 2023). The treatment regimens of $KMnO_4$ and activated carbon at levels ranging from 12 to 20 g/box each exhibited significant efficacy. However, the most optimal treatment for enhancing fruit quality and extending postharvest shelf-life was observed at the 12 g/box dosage. At this level, minimal weight losses were recorded, and essential fruit attributes such as firmness, soluble sugar, and vitamin C content were well-preserved. Notably, elevating the concentration of the $KMnO_4$ and activated carbon mixture beyond 12 g/box did not yield additional benefits in terms of prolonging storage life. Conversely, it resulted in escalated costs due to the higher treatment dosage.

The current study elucidates the physiological changes that occur during the ripening of mango fruit and the efficacy of $KMnO_4$ and activated carbon treatment in this regard. $KMnO_4$ and activated carbon (12 g/box) treatment can effectively delay the ethylene climacteric, which is responsible for the rapid deterioration of fruits. This delay in ethylene production and subsequent ripening processes significantly extends the storage period, consequently enhancing the flexibility in handling and distributing mango fruits for farmers and distributors alike. The treatment also aids in preserving the firmness and weight of the fruit, ultimately enhancing the nutritional quality of the fruit through increased sugar content and vitamin C. These outcomes enable consumers to enjoy mango fruits that have an extended shelf life and improved nutritional value.

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# Assessment of genetic parameters and heritability of *Dendrobium* species section *Spatulata* native to Indonesia

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*Key word*: Diversity, morphological traits, *Orchidaceae*, plant breeding.

**Abstract: Being one of the most abundant genera of orchids, *Dendrobium* presents a valuable genetic resource for hybridization programs. Morphological characterization and assessment of genetic parameters plays a crucial role in establishing genetic connections among orchid species within the same genus. The study aims to discern the morphological traits of five Indonesian *Dendrobium* species, intending to evaluate their potential as candidates for crossbreeding programs. The materials used in this study were *D. antennatum, D. discolor, D. stratiotes, D. lineale, D. gouldii,* and *D. sylvanum*. This research examines 21 quantitative traits and 21 qualitative morphological traits of the studied *Dendrobium*. The findings reveal variations in characters related to flowers, leaves, and pseudobulbs. The analysis of genetic parameters indicates the presence of genetic diversity in traits such as flower stalk length, length of inflorescence, flower series length, flower length, flower width, dorsal sepal width, lateral sepal width, petal length, petal width, labellum length, labellum width, and the number of florets. All observed traits demonstrate high heritability. So, the characters that have high genetic variability and heritability are valuable in selection criteria for plant breeding.**

## 1. Introduction

Indonesia possesses abundant biodiversity, encompassing a variety of orchids. Out of the approximately 30,000 orchid species worldwide, around 5,000 are present in Indonesia, distributed across diverse regions, with certain species being endemic to the country (Puspitaningtyas, 2020). *Dendrobium* as the most plentiful genus of orchids globally, boasting approximately 1,600 species and holding the title of the largest genus within the Orchidaceae family. The collective number of hybrids, derived from both natural variations and crossbreeding, exceeds 20,000 and encompasses single and multi-genera varieties (Hartati *et al*., 2021). Indonesia also known for hosting a significant variety of *Dendrobium*

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orchids, contributing to the overall diversity of orchid species in the region (Rahayu and Yusri, 2022).

Schuiteman (2012) has identified 20 sections of *Dendrobium*, and one of these is the *Spatulata* section. The *Spatulata* species within the section display significant diversity in flower characters, encompassing variations in colors, labellum shapes and colors, horn shapes and colors, as well as the duration of flower bloom shelf life. Therefore, it is essential to assess the genetic variation of these species to offer fundamental genetic insights and facilitate genetic enhancements within the *Spatulata* orchid section (Purwantoro *et al.*, 2023). To discern the variations among species, it is essential to employ a characterization method. Utilizing morphological features such as leaves, stems, tubers, fruits, and roots for characterization is anticipated to facilitate the identification and understanding of the specific utility of these characterized plants (De *et al*., 2015). Observing morphological characters is visually straightforward, allowing for a swift assessment of their diversity in comparison to other traits. Furthermore, employing morphological characterization proves valuable in evaluating the relationships among orchids, which is crucial for conservation initiatives and enhances the practicality of plant genetic resources (Vo *et al*., 2015). While the assessment of genetic relationships through morphological characters can be significantly affected by environmental factors, it remains essential to thoroughly characterize these traits. This thorough characterization is necessary to ease the utilization of germplasm by breeders (Aloysius *et al*., 2017).

The characterization of plants plays a crucial role in evaluating the genetic proximity between orchid species within the same genus, influencing the effectiveness of plant crossbreeding. A more intimate genetic connection enhances the likelihood of successful crosses. This characterization serves not only to craft plant descriptions but also to ascertain the genetic relationships among different species (Mursyidin *et al*., 2021). In genetics studies of quantitative traits, the primary role of heritability lies in its predictive function, indicating the dependability of phenotypic value as a predictor of breeding value (Ponzi *et al*., 2018). Genetic variability in plant breeding refers to the range of genetic differences or variations that exist among individuals within a population of plants. It is a key concept in plant breeding because this variability is the raw material that plant breeders work with to develop new and improved crop varieties (Yani *et al*., 2018). By assessing genetic variability, plant breeders can identify traits that exhibit variation within a population. This information is crucial for selecting traits that are desirable and heritable, meaning they can be passed on to future generations (Wirasti and Purwantoro, 2018). Traits with high heritability are more likely to respond positively to selection, making them prime candidates for improvement through breeding (George *et al*., 2020). The objective of this research is to characterize the *Dendrobium* section *Spatulata* orchid from Indonesia and investigate its genetic diversity and heritability as part of a plant breeding initiative.

## 2. Materials and Methods

*Experimental location*

The research was carried out in Banjarsari village, Sumbang District, Banyumas Regency, Central Java Province, Indonesia 7.3576° S, 109.2445° E. The research was carried out from March to June 2023.

*Experimental materials*

The characterization was conducted on the following *Dendrobium* orchids: *D. antennatum, D. discolor, D. stratiotes, D. lineale, D. gouldii*, and *D. sylvanum.* These plants were sourced from farmers and orchid collectors in Indonesia, and they represent mature specimens in the flowering stage. A total of three plants per species were analyzed. The tools used are digital calipers, rulers, digital single-lens reflex (DSLR) cameras, and writing instruments.

*Cultivation methods*

*Dendrobium* was cultivated in a screen house with 50% shade. Air temperature at daytime temperature around 27-32°C and nighttime temperature around 22-25°C, and humidity levels between 50-80%. Plants are watered once a day or according to plant needs. If the media humidity is still high, the plants can be watered every two days. The medium used for cultivation is charcoal. Fertilization is carried out once a week with NPK 20:20:20 fertilizer with follicular application. Regularly inspect *Dendrobium* orchids for pests. Additionally, monitor the orchids for signs of fungal or bacterial diseases and take necessary precautions to prevent spread.

*Characterization procedure*

The characterization procedure is based on the

Orchid Characterization Guidelines published by the Indonesian Ornamental Plant Research Institute which was adapted from the *Dendrobium* Characterization Guidelines by the International Union for the Protection of New Varieties of Plants (UPOV). Variables observed and analyzed were nature of stem, leaf shape, apexes of leaf, apexes of dorsal sepal, apex of lateral sepal, apex of petal, petal curvature, lip shape, lip margin, dorsal sepal shape, lateral sepal shape, petal shape, dorsal sepal cross section, lateral sepal cross section, petal cross section, lip overlapping of basal part, lip shape of lateral lobe, lip shape of apical lobe, lip type of curving, lip shape of eye, color of anther cap; plant height (cm), pseudobulb diameter (cm), internode length (cm), leaf length (cm), leaf width (cm), leaf area ($cm^2$), peduncle-ovary length (cm), length of inflorescence (cm), length of flower arrangement (cm), leaf thickness (mm), flower length (cm), flower width (cm), dorsal sepal length (cm), dorsal sepal width (cm), lateral sepal length (cm), lateral sepal width (cm), petal length (cm), petal width (cm), labellum length (cm), labellum width (cm), number of flowers per spike. The tools used are digital calipers, rulers, digital single-lens reflex (DSLR) cameras, and writing instruments.

*Statistical analysis*

To evaluate the performance of observed traits, conducted a variance analysis. The estimation of genetic parameters such as Coefficient of Genetic Varibility (CGV), Coefficient of Phenotypic Variability (CPV) and Heritability followed Jambormias (2014) steps.

## 3. Results

*Dendrobium species origin*

*Dendrobium antennatum*is native to Papua (Indonesia) (Table 1, Fig. 1). These orchids thrive on tall tree branches within coastal forests, mangrove swamps, and rainforests, typically below 1200 meters in elevation.

*Dendrobium discolor* var. *Tanimbar* is native to the Tanimbar Islands. It thrives in warm to hot climates, growing both as an epiphyte and a lithophyte. It can be found in various habitats, including mangrove forests along the coast, behind sand dunes where it may experience salt spray, as well as on cliffs and rock faces up to an elevation of 550 meters.

*Dendrobium stratiotes* is native to the Moluccas (including Halmahera and Morotai), the Sunda Islands, and Sulawesi, this orchid thrives at lower elevations. It typically grows as a medium to large-sized epiphyte, preferring warm to hot conditions, often forming clustered groups.

*Dendrobium lineale* is native to Papua, this orchid species grows as a large to giant-sized plant, thriving in warm to hot climates as either an epiphyte or

Table 1 - *Dendrobium* species used in the study

| Name of germplasm | Source of germplasm |
|---|---|
| *Dendrobium antennatum* | Papua |
| *Dendrobium discolor* var. Tanimbar | Tanimbar Island, Maluku |
| *Dendrobium stratiotes* | Western Papua, the Moluccas (Halmaheira and Morotai), the Sunda Islands and Sulawesi |
| *Dendrobium lineale* | Papua |
| *Dendrobium gouldii* | Papua |
| *Dendrobium sylvanum* | Papua, New Guinea |

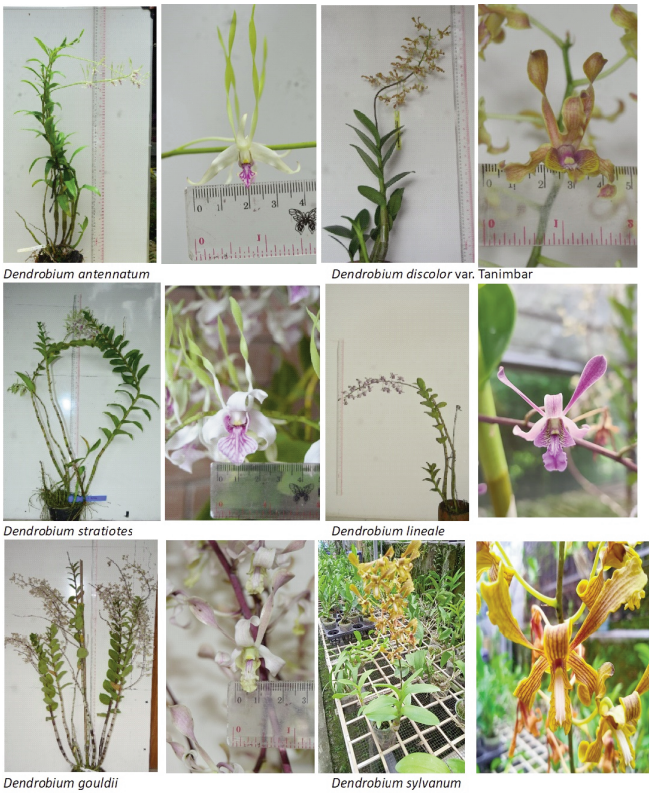


Fig. 1 - Six species of *Dendrobium* section *Spatulata* native to Indonesia studied.

lithophyte. It is typically found at elevations of up to 800 meters, often near streams and coastal areas.

*Dendrobium gouldii* is native to Papua. This orchid species grows as a large to giant-sized plant, thriving in warm to hot climates as either an epiphyte or lithophyte. It can be found in riverine forests, coastal forests, swamp forests, beaches, and plantations, typically at altitudes ranging from sea level to 700 meters.

*Dendrobium sylvanum* is native to Papua New Guinea in lowland areas, this orchid species grows as a large to giant-sized epiphyte, thriving in warm climates.

*Morphological characters*

Most diversity was found in the characters of leaves (leaf shape, apexes of leaf), pseudobulb (nature of stem), flowers (apexes of dorsal sepal, apex of lateral sepal, apex of petal, petal curvature, lip shape, lip margin, dorsal sepal cross-section, lateral sepal cross-section, petal cross-section, lip overlapping of basal part, lip shape of lateral lobe, lip shape of apical lobe, lip type of curving, and lip shape of eye) (Table 2).

*Flowers*

In this study, all the flower parts of *Dendrobium* that were observed had diverse characters, even though they were still in one section, namely the *Spatulata* section. The section *Spatulata* includes several species known for their characteristic flattened or spatula-shaped lip petals. This diversity shows differences in the shape of the petals, dorsal sepals, lateral sepals and labellum.

The Spatulata section's characters are the dorsal, lateral sepal and petal shapes were categorized as narrow elliptic. However, there is diversity in petal curvature, namely, *D. antennatum* and *D. statiotes* exhibit a spiral pattern. Conversely, in *D. discolor, D. gouldii,* and *D. sylvanum,* the curvature is deflexed, while in *D. lineale*, it remains straight.

The diversity in the labellum lies in the lip shape of the lateral lobe, namely in *D. antennatum, D. sylvanum*, and *D. lineale* it is triangular, while in *D. discolor* it is broadly trapezoidal, and in *D. gouldii* and *D. stratiotes* it is ovate. Variability is also found in the lip shape of the apical lobe, namely in *D. antennatum, D. stratiotes, D. discolor, D. gouldii* it is rhombic, while in *D. lineale* it is reniform, and in *D.*

Table 2 - Qualitative characters of *Dendrobium* studied

| No. | Characters | *D. antennatum* | *D. discolor* | *D. stratiotes* | *D. lineale* | *D. gouldii* | *D. sylvanum* |
|---|---|---|---|---|---|---|---|
| 1 | Nature of stem | Cane cylindric | Cane cylindric | Cane | Cane cylindric | Cane cylindric | Cane cylindric |
| 2 | Leaf shape | Lenceolate | Lanceolate | Lanceolate | Elliptic | Ovate | Lanceolate |
| 3 | Apexes of leaf | Acute | Obtuse | Acute | Acute | Acute | Obtuse |
| 4 | Apexes of dorsal sepal | Acuminate | Obtuse | Acuminate | Acute | Obtuse | Obtuse |
| 5 | Apex of lateral sepal | Acuminate | Obtuse | Acuminate | Acute | Obtuse | Obtuse |
| 6 | Apex of petal | Acuminate | Obtuse | Acuminate | Acute | Obtuse | Obtuse |
| 7 | Petal curvature | Spiral | Deflexed | Spiral | Straight | Deflexed | Deflexed |
| 8 | Lip shape | Ovate | Undulate | Ovate | Ovate | Oblong | Obovate |
| 9 | Lip margin | Entire | Undulate | Entire | Undulate | Undulate | Undulate |
| 10 | Dorsal sepal shape | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic |
| 11 | Lateral sepal shape | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic |
| 12 | Petal shape | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic | Narrow elliptic |
| 13 | Dorsal sepal cross section | Narrow elliptic | Moderately convex | Strongly concave | Strongly concave | Strongly concave | Moderately convex |
| 14 | Lateral sepal cross section | Narrow elliptic | Moderately convex | Strongly concave | Strongly concave | Strongly concave | Flat |
| 15 | Petal cross section | Narrow elliptic | Straight | Strongly concave | Strongly concave | Strongly concave | Moderately concave |
| 16 | Lip overlapping of basal part | Present | Absent | Present | Present | Absent | Absent |
| 17 | Lip shape of lateral lobe | Tringular | Broad trapezoid | Ovate | Tringular | Ovate | Triangular |
| 18 | Lip shape of apical lobe | Rhombic | Rhombic | Rhombic | Reniform | Rhombic | Elliptic |
| 19 | Lip type of curving | Type I | Type 1 | Type 1 | Type I | Type VI | Type IV |
| 20 | Lip shape of eye | Type I | Type 1 | Type 1 | Type I | Type II | Type I |
| 21 | Color of anther cap | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow |

*sylvanum*it is elliptic (Table 2).

There was diversity in the quantitative flower parameters observed (Table 3). The length of the flowers in *D. sylvanum* is smaller compared to other species, measuring only 2.36 cm, but the width of the flowers reaches 5.00 cm. This indicates that the petals and sepals extend outward. This flower type is also similar to *D. gouldii*, characterized by a length of 3.43 cm and a width of 5.26 cm. *D. antennatum* and *D. stratiotes* have petal shapes resembling antlers, resulting in a longer length than width. Besides that, in *D. antennatum*, the length is 4.75 cm, and the width is 2.15 cm. For *D. discolor* and *D. lineale*, the proportions of flower length and width are more or less the same. The flower size of *Dendrobium* studied showed that *D. stratiotes* had the largest flower length, namely 9.33 cm compared to other species. This is related to the longer petals on *D stratiotes*, namely 6.23 cm. Besides that, in *D. discolor*, the length is 4.50 cm, and the width is 4.00 cm. In *D. lineale*, the length is 5.15 cm, and the width is 6.16 cm.

*Leaves*

Leaves characterization included leaf shape, apexes of leaf, leaf length (cm), leaf width (cm), and leaf area ($cm^2$). The variability in the leaf shape of *Dendrobium* was *D. antennatum, D. discolor, D. stratiotes,* and *D. sylvanum*, being lanceolate. In contrast, *D. lineale* has an elliptic shape, and *D. gouldii* has an ovate shape. Variability in the apexes of leaves in *D. antennatum, D. stratiotes, D. linelae, D. gouldii* is acute, *D. sylvanum* and *D. discolor* are obtuse. Variability in leaf length is not wide, namely around 10.93 - 13.67 cm. The narrowest leaf width is *D. antennatum*, which is 2.50 cm, while the widest is *D. discolor*, which is 4.13 cm. The difference in leaf width affects the leaf area, the largest leaf on *D. discolor* is 47.30 $cm^2$, while the smallest leaf is *D. antennatum*, namely 25.13 $cm^2$.

*Pseudo bulb*

There is variation in the height among the observed *Dendrobium* species. Specifically, in *D. stratiotes* and *D. gouldii*, the height exceeds 100 cm, reaching 108.50 cm and 156 cm, respectively. In contrast, *D. lineale* reaches a height of 90 cm, while *D. antennatum* and *D. discolo*r have heights of 51.33 cm and 57.33 cm. The lowest height is recorded in *D. sylvanum* at 34.60 cm. Additionally, the length of pseudo bulb internodes varies across different

Table 3 - Quantitative characters of *Dendrobium* species studied

| No. | Characters | *D. antennatum* | *D. discolor* | *D. stratiotes* | *D. lineale* | *D. gouldii* | *D. sylvanum* |
|---|---|---|---|---|---|---|---|
| 1 | Plant height (cm) | 51.33 | 57.33 | 108.5 | 90.00 | 156.00 | 34.6 |
| 2 | Pseudobulb diameter (cm) | 38.3 | 43.00 | 45.00 | 25.2 | 21.67 | 22.5 |
| 3 | Internode length (cm) | 8.1 | 12.80 | 11.6 | 4.13 | 5.13 | 4.4 |
| 4 | Leaf length (cm) | 12.2 | 13.6 | 13.26 | 10.93 | 13.67 | 12.00 |
| 5 | Leaf width (cm) | 2.5 | 4.13 | 2.96 | 3.83 | 2.83 | 3.5 |
| 6 | Leaf area ($cm^2$) | 25.13 | 47.3 | 34.2 | 36.00 | 35.96 | 33.6 |
| 7 | Peduncle-ovary length (cm) | 2.66 | 2.2 | 3.4 | 2.06 | 2.4 | 1.6 |
| 8 | Length of inflorescence (cm) | 12.00 | 32.00 | 20.00 | 58.00 | 50.00 | 20.00 |
| 9 | Length of flower arrangement (cm) | 5.00 | 26.00 | 14.5 | 48.00 | 41.00 | 14.00 |
| 10 | Leaf thickness (mm) | 2.3 | 1.03 | 1.7 | 1.43 | 1.9 | 1.2 |
| 11 | Flower length (cm) | 4.75 | 4.5 | 9.33 | 5.15 | 3.43 | 2.36 |
| 12 | Flower width (cm) | 2.15 | 4.00 | 2.7 | 6.16 | 5.26 | 5.00 |
| 13 | Dorsal sepal length (cm) | 2.00 | 2.5 | 1.98 | 2.1 | 1.6 | 1.56 |
| 14 | Dorsal sepal width (cm) | 0.4 | 0.7 | 1.00 | 1.3 | 0.83 | 0.6 |
| 15 | Lateral sepal length (cm) | 1.85 | 2.5 | 2.6 | 2.9 | 1.9 | 2.36 |
| 16 | Lateral sepal width (cm) | 0.65 | 0.6 | 0.93 | 1.16 | 0.56 | 0.63 |
| 17 | Petal length (cm) | 3.8 | 4.3 | 6.23 | 4.4 | 3.00 | 2.93 |
| 18 | Petal width (cm) | 0.15 | 0.7 | 0.35 | 0.8 | 1.06 | 0.8 |
| 19 | Labellum length (cm) | 1.65 | 2.5 | 3.7 | 2.7 | 2.23 | 2.26 |
| 20 | Labellum width (cm) | 1.05 | 1.00 | 0.96 | 1.5 | 0.7 | 0.63 |
| 21 | Number of flowers per spike | 6.00 | 13.00 | 8.3 | 25.00 | 19.67 | 12.00 |

*Dendrobium* species. The lengths of *D. discolor* and *D. stratiotes* measure 12.80 cm and 11.60 cm, respectively. *D. antennatum* exhibits a length of 8.10 cm, while the shortest internodes are observed in *D. lineale*, *D. gouldii*, and *D. sylvanum* at 4.13 cm, 5.13 cm, and 4.40 cm, respectively. This suggests that plant height does not necessarily corellate to longer pseudobulb internodes in *Dendrobium* species. Besides that, the diameter of the pseudobulb varies among different *Dendrobium* species. In *D. antennatum*, *D. discolor*, and *D. stratiotes*, the pseudobulb diameter reaches 38.30 cm, 43.00 cm, and 45.00 cm, respectively. Meanwhile, for *D. lineale*, *D. gouldii*, and *D. sylvanum*, it is around 25.20 cm, 21.67 cm, and 22.50 cm.

*Genetic parameters*

The analysis of variance revealed a significant effect of *Dendrobium* species on all observed traits. A low coefficient of variation indicates that the variation in the data from the average is relatively small compared to the average value (Table 3). This early finding suggested the presence of genetic diversity within the *Dendrobium* species. The variance attributed to genotypes was highly significant for all the studied characteristics, indicating that the selected genotypes were genetically different (Table 4).

Genetic parameter estimation was conducted to verify the presence of genetic variability within the *Dendrobium* section *Spatulata* were observed. The high values of broad-sense heritability (Hbs) and genotypic coefficient of variation (GCV) suggest a substantial genetic influence on phenotypic variability. The genetic variability coefficient (Table 5) showed that the flowering characters, namely flower stalk length (24.86), length of inflorescence (56.43), flower series length (74.40), flower length (47.91), flower width (36.08), dorsal sepal width (38.99), lateral sepal width (31.29), petal length (29.39), petal width (51.72), labellum length (27.12), labellum width (31.38), number of florets (50.10) are included in the high genetic variability category. Other vegetative characters are leaf width (45.43), leaf thickness (28.24), leaf area (51.77), plant height (57.66), stem diameter (42.72) also characterized by high genetic variability. Besides that, the length of dorsal sepal (17.63) and lateral sepal (17.07) was categorized as moderate genetic variability, whereas the length of leaf (8.43) was categorized as low genetic variability.

Table 4 - Analysis of variance (mean square) for quantitative characters in *Dendrobium* species studied

| Characters | Mean square | | | CV (%) |
|---|---|---|---|---|
| | Replication | Genotype | Error | |
| Peduncle-ovary length (cm) | 0.035555556 | 1.116888889 | 0.058222222 | 10.10063 |
| Length of inflorescence (cm) | 1.791666667 | 989.3 | 0.691666667 | 2.585478 |
| Length of flower arrangement (cm) | 0.1666666667 | 885.125 | 0.166666667 | 1.768585 |
| Leaf length (cm) | 0.157222222 | 3.527555556 | 0.136555556 | 2.930227 |
| Leaf width (cm) | 0.093888889 | 16.32588889 | 0.041888889 | 3.991354 |
| Leaf thickness (mm) | 0.002222222 | 0.659222222 | 0.050888889 | 14.14823 |
| Leaf area (cm2) | 21.86166667 | 2476.621333 | 11.763 | 6.194563 |
| Plant height (cm) | 141.5555556 | 6649.747222 | 57.32222222 | 9.313237 |
| Pseudobulb diameter (mm) | 15.73388889 | 130.8982222 | 2.509888889 | 10.34715 |
| Internode length (cm) | 0.035 | 0.569 | 0.055 | 5.350284 |
| Flower length (cm) | 0.223888889 | 17.07922222 | 0.392222222 | 12.72345 |
| Flower width (cm) | 0.292638889 | 7.282138889 | 0.345305556 | 13.94501 |
| Dorsal sepal length (cm) | 0.000688889 | 0.358688889 | 0.000555556 | 1.203245 |
| Dorsal sepal width (cm) | 0.010555556 | 0.300555556 | 0.004555556 | 8.378672 |
| Lateral sepal length (cm) | 0.005138889 | 0.504138889 | 0.020138889 | 6.03166 |
| Lateral sepal width (cm) | 0.00125 | 0.171916667 | 0.002916667 | 7.121693 |
| Petal length (cm) | 0.010555556 | 4.404888889 | 0.025222222 | 3.863069 |
| Petal width (cm) | 0.001116667 | 0.335383333 | 0.00145 | 5.9037 |
| Labellum length (cm) | 0.000416667 | 1.396583333 | 0.00775 | 3.509664 |
| Labellum width (cm) | 0.000416667 | 0.28458333 | 0.00375 | 6.280743 |
| Number of flowers per spike | 8.166666667 | 152.5333333 | 4.9 | 15.81139 |

All traits observed in this study had high heritability (Table 6). If genetic factors rather than environmental influences primarily determine a trait, it is more likely to have high heritability. Traits controlled by a few genes with large effects, known as major genes, are often highly heritable.

Table 5 - Coefficient of genetic variability (CGV), and coefficient of phenotypic variability in the *Dendrobium* species studied

| Characters | Range | Mean | CGV | Category | CPV | Category |
|---|---|---|---|---|---|---|
| Peduncle-ovary length (cm) | 1.60 - 3.40 | 2.38 | 24.86 | High | 26.84 | High |
| Length of inflorescence (cm) | 12.50 - 59.00 | 32.17 | 56.43 | High | 56.49 | High |
| Length of flower arrangement (cm) | 5.00 - 48.00 | 23.08 | 74.4 | High | 74.42 | High |
| Leaf length (cm) | 10.80 - 14.00 | 12.61 | 8.43 | Low | 8.92 | Low |
| Leaf width (cm) | 2.40 - 9.00 | 5.12 | 45.43 | High | 45.6 | High |
| Leaf thickness (cm) | 0.80 - 2.40 | 1.58 | 28.24 | High | 31.58 | High |
| Leaf area ($cm^2$) | 23.70 - 112.40 | 55.37 | 51.77 | High | 52.14 | High |
| Plant height (cm) | 24.60 - 156.00 | 81.29 | 57.66 | High | 58.41 | High |
| Pseudobulb diameter (cm) | 7.30 - 28.60 | 15.31 | 42.72 | High | 43.96 | High |
| Internode length (cm) | 3.50 - 5.40 | 4.38 | 9.44 | Low | 10.85 | Moderate |
| Flower length (cm) | 2.30 - 10.00 | 4.92 | 47.91 | High | 49.57 | High |
| Flower width (cm) | 2.00 - 7.50 | 4.21 | 36.08 | High | 38.68 | High |
| Dorsal sepal length (cm) | 1.60 - 2.50 | 1.95 | 17.63 | Moderate | 17.67 | Moderate |
| Dorsal sepal width (cm) | 0.30 - 1.30 | 0.8 | 38.99 | High | 39.88 | High |
| Lateral sepal length (cm) | 1.60 - 3.00 | 2.35 | 17.07 | Moderate | 18.1 | Moderate |
| Lateral sepal width (cm) | 0.50 - 1.20 | 0.75 | 31.29 | High | 32.09 | High |
| Petal length (cm) | 2.80 - 6.30 | 4.11 | 29.39 | High | 29.64 | High |
| Petal width (cm) | 0.10 - 1.10 | 0.64 | 51.72 | High | 52.06 | High |
| Labellum length (cm) | 1.50 - 3.80 | 2.5 | 27.12 | High | 27.35 | High |
| Labellum width (cm) | 0.60 - 1.60 | 0.97 | 31.38 | High | 32 | High |
| Number of flowers per spike | 6.00 - 25.00 | 14.00 | 50.1 | High | 52.54 | High |

Table 6 - Heritability of in the *Dendrobium* species studied

| Characters | σ2e | σ2*g* | σ2f | H2bs | Category |
|---|---|---|---|---|---|
| Peduncle-ovary length (cm) | 0.0582 | 0.3528 | 0.4111 | 85.83 | High |
| Length of inflorescence (cm) | 0.6916 | 329.53 | 330.22 | 99.79 | High |
| Length of flower arrangement (cm) | 0.1667 | 294.98 | 295.15 | 99.94 | High |
| Leaf length (cm) | 0.1365 | 11.303 | 12.668 | 89.22 | High |
| Leaf width (cm) | 0.0418 | 5.428 | 54.698 | 99.23 | High |
| Leaf thickness (cm) | 0.05 | 0.202 | 0.253 | 79.93 | High |
| Leaf area (cm2) | 11.763 | 821.61 | 833.38 | 98.58 | High |
| Plant height (cm) | 57.32 | 2197.475 | 2254.797 | 97.45 | High |
| Pseudobulb diameter (cm) | 2.509 | 42.796 | 45.306 | 94.46 | High |
| Internode length (cm) | 0.055 | 0.1713 | 0.2263 | 75.69 | High |
| Flower length (cm) | 0.392 | 5.562 | 5.954 | 93.41 | High |
| Flower width (cm) | 0.3453 | 23.122 | 26.575 | 87 | High |
| Dorsal sepal length (cm) | 0.0005 | 0.1193 | 0.1199 | 99.53 | High |
| Dorsal sepal width (cm) | 0.0045 | 0.0986 | 0.1032 | 95.58 | High |
| Lateral sepal length (cm) | 0.02 | 0.161 | 0.181 | 88.9 | High |
| Lateral sepal width (cm) | 0.0029 | 0.056 | 0.059 | 95.07 | High |
| Petal length (cm) | 0.0252 | 14.598 | 14.851 | 98.3 | High |
| Petal width (cm) | 0.00145 | 0.11311 | 0.11276 | 98.71 | High |
| Labellum length (cm) | 0.00775 | 0.4629 | 0.4706 | 98.35 | High |
| Labellum width (cm) | 0.00375 | 0.0936 | 0.0973 | 96.14 | High |
| Number of flowers per spike | 4.9 | 49.21 | 54.11 | 90.94 | High |

## 4. Discussion and Conclusions

*Morphological characteristics*

In the context of orchids, flowers typically consist of three outer floral parts known as sepals and three inner floral parts called petals. The petals are often more colorful and visually striking than the sepals. They play a crucial role in attracting pollinators, such as insects, and contribute to the overall aesthetic appeal of the orchid flower. The arrangement and characteristics of petals are important features used in the identification and classification of orchid species (Dirks-Mulder *et al.*, 2017)

In addition to attracting pollinators, orchid petals, along with other floral parts, may also have specialized structures or markings that aid in the orchid's reproductive process, such as facilitating the transfer of pollen. Orchids are known for their intricate and diverse floral structures, and the characteristics of their petals contribute significantly to their overall beauty and ecological function (Li *et al.,* 2021). The labellum is a specialized petal in orchids that stands out from the other floral parts due to its distinct shape, size, and often elaborate structure. It is the modified third petal of the orchid flower, differentiating from the two lateral petals and three sepals (Dalayap *et al.*, 2011). The diversity in the part of *Dendrobium* flower studied, can be a source of diversity in plant breedingaimed at increasing the aesthetic value of hybrid *Dendrobium* orchids.

Studying leaf shape contributes to understanding the phenotypic variation within a species. This information is essential for describing the diversity and range of characteristics exhibited by plants. Considerable genetic diversity exists in both the size and shape of leaves among different species and populations within the same species. This diversity was influenced by robust heredity, carrying both genetic and environmental information that contributes to variations (Ren *et al*., 2020).

Pseudo bulb is a modified form of stem in several types of orchid plants. A pseudobulb in orchids is a specialized, swollen, or bulbous structure that serves as a storage organ for water and nutrients. This structure is a key adaptation to various environmental conditions, particularly in epiphytic and lithophytic orchids (Zhang *et al*., 2018). The pseudobulbs on *Dendrobium* orchids belong to the homoblastic type, characterized by two or more internodes of the same or varying lengths (Ng and Hew, 2000). Furthermore, the nature of stem (pseudobulbs) can vary significantly among different *Dendrobium* species. *D. antennatum*, *D. discolor*, *D. lineale*, *D. gouldii*, and *D. sylvanum* typically exhibit cylindrical canes. In contrast, D. stratiotes stands out with its cane-shaped stem, representing a distinctive morphological trait within the genus.

The size of the pseudobulb indicates the large carbohydrate reserves in the organ. According to Ng and Hew (2000), carbohydrate reserves in orchid pseudobulbs are an important part in the initiation of new seedling growth. The large size of the pseudobulb also functions to support the growth of new shoot and flower development.

*Genetic parameters*

Genetic diversity plays an important role in the development of *Dendrobium* breedings with high economic value. It serves as the main germplasm in plant breeding. Greater genetic diversity increases the potential for enhancing plants in accordance with the desired traits. This variability offers ample opportunities for plant breeders to choose superior genotypes for improving crops (Swarup *et al*., 2021).

High genetic diversity in plants reflects substantial genetic variation, signifying numerous genetic differences between individual plants. This diversity holds promising potential for more effective plant breeding endeavors. Moniruzzaman *et al.* (2012) supports this, emphasizing the critical role of high genetic diversity in breeding programs, particularly in the development of new crop varieties featuring improved traits like exotic flower diversity. Genetic variation offers breeders a wide genetic reservoir, enabling the selection and crossbreeding of individuals to produce desirable hybrids.

In this study, high CGV values indicate that genetic factors have a significant influence on the observed traits. On the other hand, it is also known that there is a difference in value between CPV and CGV for each lower trait. These findings suggest that the environment has comparatively minor influence on the observed traits. This is in accordance with research of Malek *et al*. (2014) stated that narrow distinction between CPV and CGV in the majority of traits suggests a minimum impact of environmental factors on the manifestation of these traits, increasing the probability of achieving significant selection gains.

The observation results showed that all the observed characters showed high broad sense

heritability (Table 6). This suggests that the variability in these traits is primarily influenced by genetic factors rather than environmental factors (Swarup *et al.*, 2021). In the research of Singh *et al*. (2018) on *Dendrobium* orchid, heritability estimates were identified for characteristics such as plant height, leaf count per shoot, quantity of aerial roots, length and thickness of aerial roots, shoot thickness, internodal length, leaf length, and leaf area. Heritability values represent the degree of genetic impact on a trait (Hadi *et al.*, 2019). Heritability of quantitative traits are influenced by multiple genes as well as environmental factors. If a trait is primarily determined by genetic factors rather than environmental influences, it is more likely to have high heritability. Traits controlled by a few genes with large effects, known as major genes, are often highly heritable. The potential for improvement through direct selection was indicated by high heritability with high genetic variability flower stalk length, length of inflorescence, flower arrangement length, flower length, flower width, dorsal sepal width, lateral sepal width, petal length, petal width, labellum length, labellum width, number of florets. When heritability is high in plants, it indicates that a significant genetic variation is a primary factor in the observed traits. High heritability typically indicates that the majority of phenotypic variation in a trait can be attributed to genetic factors, and this is frequently associated with the influence of additive genes (Amien *et al.*, 2021). Additive genes contribute cumulatively to the phenotypic expression of a trait, and when these genes are predominant, heritability tends to be high (Beavis *et al.*, 2021). Therefore, high heritability is commonly regarded as an indication that genetic factors, especially additive genes, exert a substantial influence on the plant's traits (Karavolias *et al*., 2020). High GCV and Hbs values increase the chances of obtaining *Dendrobium* orchids have superior characters that can be inherited in their phylogeny. Genetic variability plays a crucial role in selecting parents for hybridization and breeding initiatives effectively (Mazid *et al*., 2013; Mai *et al*., 2021).

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# Effects of foliar application of two forms of selenium on anatomic features of *Thymus daenensis* Celak

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All relevant data are within the paper and its Supporting Information files.

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**Abstract: To investigate selenium effects on anatomic features of *Thymus daenensis,* three concentrations of bulk and nanoparticles of the element (2, 4 and 8 ppm) along with distilled water, as a control, were sprayed on foliar of young seedlings of the plant species for six times with 2 weeks intervals. Transverse sections of stems, roots and leaves of the treated plants were manually produced and stained by Carmine and Methylene blue. Average of 5 readings was recorded for each treatment on several anatomic traits. Statistical analysis of the data revealed that the effects of selenium treatments were significantly different, at 1% of probability, on all of the studied anatomic characters. The first level of nanoparticles showed the most positive effects on several root characteristics. The highest level of selenium nanoparticles (8 ppm) showed the most positive effects on stem diameter and stem vascular cylinder diameter. The last two levels of nanoparticles had similar and the largest effect on stem epidermis thickness. The lowest level of bulk selenium (2 ppm) showed the most increment on leaf characteristics. Application of a low concentration of selenium by foliar spraying on young plants of the species is suggested for more vegetative growth and higher photosynthetic efficiency.**

## 1. Introduction

*Thymus daenensis* Celak related to Lamiaceae family is endemic to western part of Iran, known as a medicinal herb, with high concentration of thymol, and high antimicrobial activities on human pathogenic strains (Zarshenas and Krenn, 2015). The species is regarded as a valuable spice

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plant, growing mainly on Zagros mountain chain of the country. It is a perennial shrub which was traditionally used as a tonic or herbal medicine to treat gastrointestinal disorders, headache and cold.

Numerous studies indicated that environmental factors such as nutrient deficiency, highly affect biological properties of medicinal plants, including their anatomic features. On the other hand, anatomic studies have found a vast application domain, such as identifying fragmentary plant organs as well as providing characters of taxonomic significance, in medicinal plants. This technique could be used as a proper tool to identify the exact effects of environmental factors such as fertilizers and chemical substances on plant ultrastructure. In other words, the anatomical features are supposed to be good indicators of environmental fluctuation effects. Also in areas with high levels of toxic elements, a sensitive plant species such as *Thymus daenensis* have potential to be used as indicator of the environmental conditions.

Selenium is rarely found in its elemental form in our living environment, it is found in several other forms such as selenide, selenite and selenate oxidation status in soil and water (Gomez-Ariza *et al.,* 1998). However, a suitable level of selenium plays a significant role in plants, animal and human health in direct or indirect ways. So that in selenium deficient areas there may be a health risk for plants, animal and human population.

Selenium is identified as affecting plant growth by causing physiological, morphological and anatomical alterations in plant tissues. Some researches indicated that alterations depend on the concentration of the applied nanoparticle treatment (Tymoszuk and Kulus, 2020). In fact, as a part of selenium role in plant, antioxidant enzymes are activated, minerals are balanced, chlorophyll is also increased, and the plant would be more resistant to various stresses (Raina *et al.,* 2021). Selenium also increases plant resistance against oxidative stress, which is caused by free oxygen radicals. It delays senescence of higher plants, increases their tolerance to UV-induced oxidative stress, and promotes seedling growth. It is also shown that selenium regulates water status of plants under drought conditions (Kuznetsov *et al*., 2003). Although utilizing proper concentration of selenium have positive effects such as increasing crop yield, but adverse effects of some nanoparticles have also been reported on growth characteristics of some plant species (Singla *et al.,* 2019; Gao *et al.,* 2023). Plants can absorb chemical substances in nanoparticle form, better than other forms. Achievements of nanofertilizers are mainly due to very small particles that are easily absorbed by plant root system. It is also due to their large surface area that increases the plant sorption effectiveness by three folds (El-Saadony *et al.,* 2021).

Selenium used to be considered as a toxic element for human being. Whereas, its role has been changed during the past 40 years. It contributes to healthy growth, as well as reducing the toxicity of elements such as mercury. Maximum limit of selenium intake for human being is suggested to be 400 µg/day, over which negative effects of the element are expected (Arthur, 1991). However, daily selenium intake of 50-200 µg/day is recommended to overcome deficient effects of the element (Navarro-Alarcon and Lopez-Martinez, 2000). Although selenium may be available at toxic level in some areas, which is harmful for grazing livestock, but as a result of soil acidity, it is not available enough in some areas, resulting to low selenium intake in human at the final food chain. In other words, suitable level of selenium supplementation seems to be beneficial for plants, animals and human being. In areas with selenium deficit in soil, fertilizers are recommended to be supplemented with some selenium forms such as sodium selenite.

This research was conducted mainly to investigate the possible effects of exogenous application of bulk and nanoparticles of selenium on anatomic structure of *Thymus daenensis*, as well as finding a safe dosage of the element to optimize foliar application of selenium for the species.

## 2. Materials and Methods

*Seed germination and plant establishment*

A trial was performed at Research Institute of Forests and Rangelands of Iran, to study influence of selenium on growth and anatomic structures of a medicinal plant species named *Thymus daenensis* Celak. Seeds of the species were first washed with tap water and soaked in distilled water for two days. Then they were first scattered on wet filter paper and kept at 4°C for 72 hours.Then they were sown in planting trays. The new grown seedlings were transplanted to plastic pots containing peat moss growing media and kept in a greenhouse.

*Treatment application*

By adaptation of the seedlings to the greenhouse conditions, the pots were arranged in five replications with three pots per each plot. Exogenous application of selenium treatments was conducted on the plantlets, as follows: three concentrations of selenium nanoparticles (2, 4, and 8 ppm of NanoSe), and sodium selenate as the bulk form (2, 4, and 8 ppm of SoSe), along with distilled water as a control for the experiment, were applied on the plantlets by 6 times foliar spraying with intervals of two weeks, starting at four leave stage of the plantlets based on a completely randomized design, with five replications. The concentrations of selenium treatments were determined based on a preliminary experiment in the greenhouse.

*Microscopic sample preparation*

Three days after the last application of the treatments, several leaf, stem and root samples were randomly taken from each plot and fixed in a mixture of ethanol 96% and glycerin (1:1). Transverse sections of the fixed samples of stems, roots and leaves were performed manually to produce slices in about 20 µm thickness and washed by distilled water. To distain the samples, they were socked in 10% sodium hypochlorite solution for 10 minutes and washed thoroughly by distilled water. For staining the sections, a combination of Carmine (for 20 min) and Methylene Blue (for 30 sec.) were used to make the tissues with wooden skeleton, in green, and tissues with cellulosic skeleton in pink (Sotoodehnia-Korani *et al.,* 2020). Then the sections were washed by distilled water again. The process was completed with the samples assembled on glass slides. The slides were observed under an Olympus microscope coupled with digital camera. Five readings and measurements were recorded per each experimental unit. Several anatomic characteristics were recorded as indicated in Table 1.

*Statistical analysis*

The mean values of the data recorded on the anatomic features of the experimental units, affected by the seven different selenium treatments, were subjected to analysis of variance, after testing the homogeneity of their error variance using SAS software. In other words, regarding five replications and seven treatments of the experiment, 35 mean values were analyzed for each anatomic characteristic, based on a completely randomized design. Duncan multiple range test was used to classify the treatment means on the studied characteristics.

Table 1 - Results of analysis of variation on the data recorded on the effects of seven different selenium treatments on anatomic characteristics of roots, stems and leaves of *Thymus daenensis*

| Source of variation | Treatment [z] | Error [y] |
|---|---|---|
| Traits | (DF=6) | (DF=28) |
| Root diameter | 17078.5 ** | 479.6 |
| Root vascular cylinder diameter | 7580.9 ** | 453.9 |
| Root epidermis thickness | 748.6 ** | 119.4 |
| Stem diameter | 36951.9 ** | 5871.4 |
| Stem vascular cylinder diameter | 4679.7 ** | 681.2 |
| Stem epidermis thickness | 3138.7 ** | 305.3 |
| Stem xylem diameter | 2.8 ** | 0.07 |
| Leaf thickness | 8944.2 ** | 666.6 |
| Leaf upper cortex thickness | 58.0 ** | 8.8 |
| Leaf main vascular thickness | 1372.0 ** | 56.0 |
| Leaf lower cortex thickness | 63.1 ** | 11.8 |
| Leaf spongy mesophyll thickness | 141.5 ** | 5.8 |
| Leaf palisade mesophyll thickness | 104.1 ** | 6.7 |
| Leaf upper epidermis thickness | 2.5 ** | 0.5 |
| Leaf lower epidermis thickness | 1.2 ** | 0.2 |
| Leaf number of vascular bundles | 3.0 ** | 8.8 |

[z] **= Significant at 1% level of probability.
[y] Seven treatments and five replications, resulted in 35 data values for each trait.

## 3. Results and Discussion

The statistical analysis of the data revealed that the selenium treatments had significant effects on all of the studied anatomic characteristics at 1% of probability (Table 1). Classified means of the studied characteristics are presented in Table 2.

The lowest level of nanoparticles (2 ppm of NanoSe) promoted root growth by increasing root diameter (595 µm), root epidermis (75.5 µm) and root vascular cylinder diameter (38 µm) (Table 2). Therefore, addition of 2 ppm NanoSe promoted the root growth and its vascular cylinder increment. This is in accordance with the results of Domokos-Szabolcsy *et al.* (2012). Root sections formed concentric cylinders and deformation was not observed by the studied treatment levels. Meanwhile, root diameter was negatively affected by the treatments, so that the smallest diameter

Table 2 - Classification of the anatomic characteristics of *Thymus daenensis*, affected by seven different concentrations of sodium selenate as the bulk selenium (SoSe), and nanoparticles of selenium (NanoSe)

| Treatments [z] | Control [y] | 2 mg/L of SoSe | 2 mg/L of NanoSe | 4 mg/L of SoSe | 4 mg/L of NanoSe | 8 mg/L of SoSe | 8 mg/L of NanoSe |
|---|---|---|---|---|---|---|---|
| Traits | | | | | | | |
| Root diameter (µm) | 520.2 b | 480.0 c | 595.0 a | 518.7 b | 450.1 d | 465.4 cd | 415.8 e |
| Root vascular cylinder diameter (µm) | 286.6 de | 326.4 b | 381.6 a | 316.5 bc | 295.8 cd | 275.7 de | 263.8 e |
| Root epidermis thickness (µm) | 36.3 c | 52.2 b | 75.5 a | 60.3 b | 65.3 ab | 51.8 b | 56.8 b |
| Stem diameter (µm) | 734.0 b | 618.6 c | 662.3 bc | 685.2 bc | 747.2 b | 711.3 bc | 888.9 a |
| Stem vascular cylinder diameter (µm) | 323.2 ab | 258.7 c | 294.4 b | 321.9 ab | 290.0 bc | 304.0 b | 355.4 a |
| Stem epidermis thickness (µm) | 112.8 bc | 91.8 cd | 99.3 cd | 75.8 d | 148.3 a | 87.8 d | 127.0 ab |
| Stem xylem diameter (µm) | 8.1 a | 6.1 d | 7.7 b | 6.4 cd | 6.7 c | 6.8 c | 7.6 b |
| Leaf thickness (µm) | 259.8 c | 260.5 c | 293.0 bc | 268.1 c | 319.5 b | 327.7 b | 373.1 a |
| Leaf upper cortex thickness (µm) | 30.7 a | 22.4 c | 23.1 bc | 23.6 bc | 29.5 a | 26.8 ab | 23.1 bc |
| Leaf main vascular thickness (µm) | 138.7 d | 170.4 b | 155.1 c | 185.5 a | 147.7 cd | 174.6 b | 171.0 b |
| Leaf lower cortex thickness (µm) | 27.8 b | 32.5 a | 24.2 bc | 24.2 bc | 22.3 c | 25.6 bc | 22.7 c |
| Leaf spongy mesophyll thickness (µm) | 100.6 c | 108.9 a | 106.1 ab | 103.7 bc | 94.2 d | 97.0 d | 106.0 ab |
| Leaf palisade mesophyll thickness (µm) | 90.6 c | 98.1 a | 92.5 bc | 87.5 de | 89.3 cd | 84.8 e | 95.3 ab |
| Leaf upper epidermis thickness (µm) | 6.0 ab | 6.1 a | 5.0 bc | 5.6 ab | 5.0 bc | 4.2 c | 6.0 ab |
| Leaf lower epidermis thickness (µm) | 4.2 bc | 4.9 a | 3.7 c | 4.4 ab | 4.1 bc | 3.5 d | 4.5 ab |
| Leaf number of vascular bundles | 5.0 a | 5.0 a | 4.0 b | 5.0 a | 3.0 c | 5.0 a | 4.2 ab |

[z] Mean of each trait and treatment, resulted from 5 data values obtained by five replicates.
[y] Means with similar letters in the same raw are not significantly different at 5% level of probability.

belonged to the highest level (8 ppm) of both NanoSe and SoSe (Table 2). Therefore, it may be concluded that the application of a proper quantity of selenium, in either form of NanoSe or SoSe, would improve organogenesis and root growth of *Thymus daenensis.* Zsiros *et al.* (2019) had the same conclusion on tobacco. However, high concentrations of the element would not be recommended. Zayed *et al.* (1998) concluded that plants accumulate more selenium in their shoots and leaves than in their root tissues. Regarding their conclusion roots of the plant species studied in this research could have been less affected by the treatments than its stems and leaves. Regarding the effects of the treatments on appearance of the root transverse sections, all the treatments had similar effects, so that all of the sections of the different treatments looked circular (Fig. 1).

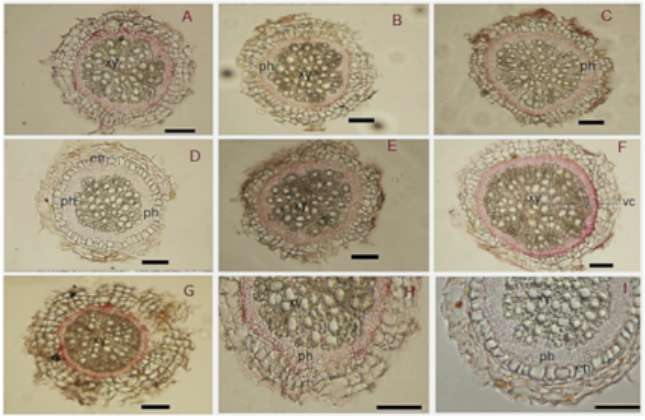


Fig. 1 - Transvers sections of roots of *Thymus daenensis* affected by seven different selenium treatments. A) 8 ppm of sodium selenate (SoSe), B) 4 ppm of SoSe, C) 2 ppm of SoSe, D) 8 ppm of nanoparticles of selenium (NanoSe), E) 4 ppm of NanoSe, F) 2 ppm of NanoSe, G) control, H) 8 ppm of SoSe, I) 8 ppm of NanoSe, e: epiderm, en= endodermis, ph= phloem, vc= vascular cylinder, xy= xylem, Bars = 50 µm.

Transverse sections of the stems were not completely quadrangular for the studied treatments (Fig. 2). Meanwhile, there were several fluctuations due to the treatments effects. Two to four layers of collenchyma cells were located at the corners of the stems. Five to six layers of roundish parenchyma cells formed the cortex of the stems. Two opposite sides of the quadrangular stems were convex (Fig. 2A),

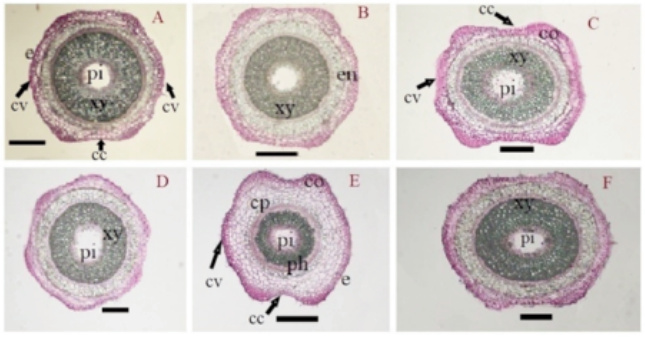


Fig. 2 - Transvers sections of the stems of *Thymus daenensis* affected by seven different selenium treatments. A) Control, B) 4 ppm of sodium selenate (SoSe), C) 2 ppm of SoSe, D) 8 ppm of nanoparticles of selenium (NanoSe), E) 4 ppm of NanoSe, F) 2 ppm of NanoSe, cc= concave, co= collenchyma, cp= cortex parenchyma, cv= convex, e= epiderm, en= endodermis, pi= pith, ph= phloem, xy= xylem, Bars = 100 µm.

whereas, the other two sides were concave. The mentioned cavity was deeper in the selenium treatments (Fig. 2C and 2E). Epidermis, exodermis and endodermis were all single layer. Epidermis with one layer of cells covered by cuticle and various trichomes, were similar for the studied treatments. Within the range of studied concentrations of selenium nanoparticles (NanoSe), the largest stem xylem diameter was obtained by control of the experiment (no selenium in both forms). The highest level of the nanoparticles showed the most effects on stem diameter (888.9 µm) (Table 2) and stem vascular cylinder diameter (355.4 µm). Four ppm concentration of selenium nanoparticles had the largest effect on stem epidermis thickness (148.3 µm) (Table 2). According to Aly *et al.* (2023) there is a significant correlation between nutrients content, growth parameters and nanoparticles concentrations. They came to this conclusion that some nanoparticles were more efficient by low concentrations, whereas, others were more efficient by higher concentrations. We came to this conclusion that even in a single nanofertilizer, some part of the plant species, lower concentration of selenium was more effective (leaf mesophyll) (Table 2), whereas, in other parts of the same plant, higher concentration of the element was more effective (stem diameter and leaf thickness). This study confirmed that an appropriate concentration of NanoSe enhanced the vascular cylinder and epidermis of both roots and stems of the plant species.

Two ppm of bulk selenium showed the largest increments on leaf characteristics such as leaf upper and lower epidermis thickness, leaf spongy and palisade mesophyll thickness (Table 2). Leaf main vascular thickness was mainly affected by 4 ppm concentration of bulk selenium treatment (Fig. 3). Whereas, the highest level of nanoparticles (8 ppm) showed the same trend but weaker effects on majority of the mentioned leaf characters (Table 2). Epidermis of the leaves, along with cuticle, provide protection for plants, thickening of epidermis on both upper and lower epidermis of the leaves

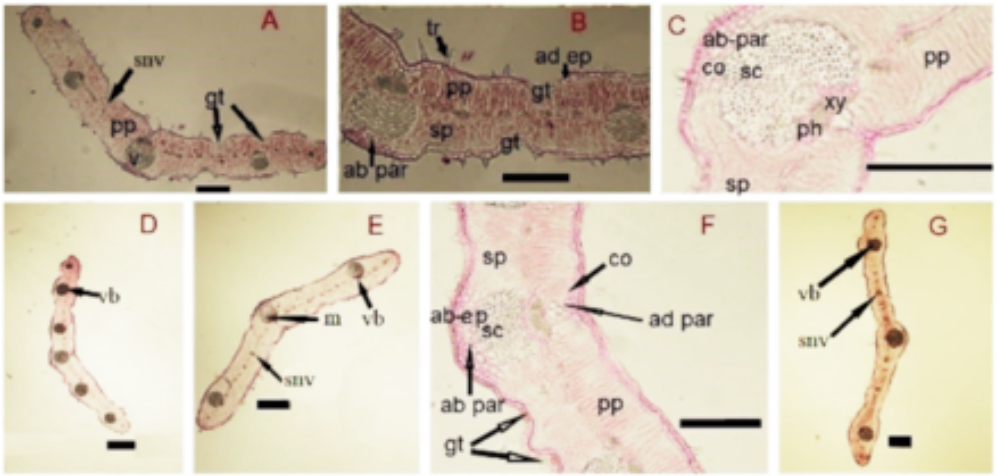


Fig. 3 - Transvers sections of the leaves of *Thymus daenensis* affected by seven different selenium treatments. A, B and C) 8 ppm of sodium selenite (SoSe), D) 2 ppm of SoSe, E) 4 ppm of NanoSe, F) 2 ppm of SoSe, G) control, ab-ep= abaxial epidermis, ab-par= abaxialparechyma, ad-ep= adaxial epidermis, ad-par= adaxial parenchyma, co= collenchyma, gt= glandular trichome, m: main middle vein, ph: phloem, pp= palisade parenchyma, sc= sclerenchyma, sp= spongy parenchyma, snv= small netted vein, tr= trichome, vb= vascular bundle, xy= xylem, Bars = 100 µm.

generated by selenate sodium (SoSe) may alleviate the negative and diverse effects of biotic and abiotic stresses. In fact, both forms of selenium, increased leaves upper and lower epidermis thickness, thereby leaf heat load is reduced, causing leaf internal temperature and resultantly transpiration rate to be reduced. Application of nanoparticles of other elements such as silicates in $K_2SiO_3$ and $CaSiO_3$ forms also showed positive results on banana, by higher stomatal density and smaller stomatal size (Asmar *et al.,* 2015). Both upper and lower epidermises may act as a barrier to excessive transpiration, therefore, the thicker epidermis observed on selenium containing treatments, can better protect the plant leaves against water loss as well as protecting the plant tissues against biotic and abiotic stresses. Abbas-Azimi *et al.* (2020) also came to this conclusion that some plant species change their anatomic features to combat dehydration. Application of either form of selenium has thicken both mesophyll tissue layers (spongy and palisade parenchyma). It can be noticed that the leaves under either form of selenium treatments, have wider palisade and spongy mesophyll compared to untreated leaves. Since mesophyll tissues are the main container of chloroplasts, thickness increment of the mesophyll layers would lead to increment of chlorophyll in the plant tissues, which in turn leads to improvement of photosynthetic efficiency. Smrkolj *et al.* (2006) also showed that selenium induced higher respiratory potential in the leaves of foliar treated potato plants.

NanoSe also negatively affected the number of vascular bundles, compared to the control of the experiment. Whereas, the three levels of bulk selenium had the same effect as that of the control. It means selenate sodium (SoSe) treatment does not have significant effect on the number of vascular bundles. It should be noticed that according to Yang *et al.* (2022), selenium content is higher in actively plant tissues and younger leaves, but higher buildup of the element occurs in aging leaves. Therefore, selenium toxic effects may be seen in older leaves, whereas its deficient symptoms appear on new leaves. Therefore, the exact toxic effects of the used dosage of the element could be examined on the older leaves of the plants under study. However, this study suggested that the leaf anatomic characteristics of *Thymus daenensis* are highly affected by different concentrations of both forms of selenium.

Different plant species respond differently to selenium fertilization. Plants are able to transform selenite into organic selenium by foliar spraying or fertilization. Meanwhile, plant species have different capabilities to absorb and maintain selenium in their tissues. Most plant species contain 1-2 mg/kg of selenium, but there are some species with ability to accumulate a larger amount of the element (Juhaszne Toth and Csapo, 2018). For instance, *Brassicaceae* and *Fabaceae* families are able to accumulate much more selenium in a kilogram of dry matter than other plant species (Ellis and Salt, 2003). These species may be used for soil purification purposes in toxic soils.

In this study, the highest level of selenium did not favor several studied characteristics such as root diameter, root vascular cylinder diameter, root epidermis thickness and leaf lower and upper cortex thickness; accordingly, the species under study seems to be sensitive to the large quantities of selenium.

According to Tymoszuk and Kulus (2020), treating plants with nanoparticles, particularly high level of the particles, may even cause genetic variation, depending on their size, type and concentration. Therefore, using the right concentration of the element is the first step to be taken. This was done in this research by a preliminary experiment to select the appropriate concentrations as the treatments of the experiment.

The interaction between plant cells and nanoparticles may also cause positive or negative morpho-physiological alterations, depending on several factors such as size, shape, concentration, surface covering and mode of nanoparticle application as well as plant age, genotype, and developmental phase (Rajput *et al.,* 2021).

However, utilizing appropriate form and concentration of selenium by foliar spraying results in anatomical and structural changes of *Thymus daenensis,* that seems beneficial to the species. Moderate concentrations of selenium acts highly positive in relation to different anatomic characteristics of the foliar parts of the plant species. Root and stem epidermis thickness and vascular cylinder diameter, are favored by foliar addition of selenium in moderate concentrations of either nanoparticle or bulk forms. Spongy and palisade mesophyll thickness of leaves of the plant species are also favored by the foliar spraying of selenium especially when 2 ppm of nanoparticles of the element or 8 ppm of bulk selenium is used. The lower

level of nanoparticles and higher examined level of bulk selenium also showed their positive effects on increasing upper and lower leaf epidermis thickness as well as the number of leaf vascular bundles. Therefore, foliar spraying on the young plants of the species by a moderate concentration of selenium, within the range of studied concentrations of the element is suggested for more vegetative growth and higher photosynthetic efficiency.

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# Morphological and molecular characterization of some Chrysanthemum (*Dendranthema grandiflora*) cultivars

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*Key words:* Flower yield traits, gas exchange, pigments, SSR markers, vegetative growth traits.



**Abstract: The diversity and genetic relationships among seven commercial chrysanthemum cultivars were analyzed using morphological and molecular markers. Vegetative growth, flowering, flower yield, and flower quality parameters were evaluated to assess genetic variability across the cultivars. Cultivars Crystal Red, Kodiack, and Crystal White exhibited superior vegetative growth, while Abrun, Crystal Red, and Kodiack displayed better flowering characteristics, particularly in terms of the number of inflorescences per plant and mass of colored flowering. Crystal White, Coca Bleach, and Crystal Red cultivars demonstrated the highest inflorescence stalk length, while cvs. Crystal Red, Crystal Yellow, Crystal Pink, and Kodiack Yellow recorded the maximum number of ray floret inflorescences. Other quality parameters such as inflorescence diameter and ray floret length were found to be optimal in Kodiack, Crystal White, Crystal Pink, Coca Bleach, and Crystal Yellow cultivars. Simple Sequence Repeat (SSR) markers were employed to distinguish and identify standard-type chrysanthemum cultivars, utilizing twelve SSR markers from the chrysanthemum SSR database. The results suggest that these SSR markers hold promise for identifying additional chrysanthemum cultivar types and assessing genetic relationships among them. Association studies combining morphological and molecular data offer a valuable approach to identifying informative markers for plant breeding purposes.**

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**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

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The authors declare no conflict of interests.

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## 1. Introduction

Chrysanthemum (mums) (*Dendranthema grandiflora* Tzvelev, formally, *Chrysanthemum morifolium* Ramat.) is one of the most important ornamental crops grown worldwide. It belongs to the family Compositae (Asteraceae) and has been commonly cultivated in gardens for more than 2500 years (Bose *et al*., 2003). It is produced on a large scale as a cut flower or as a potted plant due to its commercial significance (Van Der

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Ploeg and Heuvelink, 2006).

Analyzing genetic variability in chrysanthemum is essential for breeding programs as it can provide data on genetic relationships among different genotypes of this genus. Among the available strategies for assessing genetic variability, molecular markers are the most widely applicable, as they are best suited for understanding the genome and can be used for genetic variability characterization, paternity testing, elucidating genetic relationships between genotypes, developing methods for maintaining genetic variability in germplasm banks, and identifying genes or combinations of features related to key biological and agronomic traits (Hayden *et al*., 2010).

Another approach to studying genetic variability is the analysis of morphological and phenotypic characters, as these methods are relatively simple to perform. However, analysis based solely on morphological features may not be conclusive due to the limited number of characters and the strong influence of plant development stage and environmental factors. Morphophenological characterization does not replace molecular analyses but may complement both characterization and genetic variability studies and cultivar development (Fufa *et al*., 2005). In contrast, molecular markers based on DNA sequence polymorphisms are unaffected by environmental factors and exhibit high rates of polymorphism.

While morphological markers reflect variation in the coding regions of the genome, DNA-based molecular markers represent variations occurring in various regions of the genome, including coding and non-coding regions. Thus, molecular markers provide a rapid and reliable method to estimate genetic relationships between genotypes (Tatikonda *et al*., 2009). Molecular characterization has been widely used to quantify genetic variability among different accessions comprising germplasm banks (Glaszmann *et al*., 2010), enabling researchers to elucidate the genetic structure and diversity in a wide range of plant species (Kilian *et al*., 2007; Leišová *et al*., 2007).

Various methods have been employed to evaluate genetic diversity, with morphological character measurement being a commonly used index due to its simplicity in quantifying genetic variation while simultaneously assessing genotype performance under normal growing conditions (Fu *et al*., 2008). However, investigating morphological traits is labor-intensive, and the phenotypic plasticity of plants poses challenges due to environmental variation (Van Beuningen and Busch, 1997). In contrast, molecular markers offer several advantages over morphological measurement for assessing genetic diversity.

Assessing genetic variability is crucial in breeding programs, with molecular markers providing a direct means to access genome sequences and enabling the isolation of genetic differences from environmental influences (Ferrão *et al*., 2007). Simple Sequence Repeats (SSR) markers have shown potential in assessing genetic diversity among chrysanthemum species, cultivars, and germplasm bank collections, as well as determining geographical origin, level of domestication, dispersal history, species and cultivar identification, and genealogy (Lopez-Gartner *et al*., 2009; Hong *et al*., 2013; Hong *et al*., 2016).

SSR markers offer several advantages over other markers such as RAPD, AFLP, SRAP, and ISSR, including co-dominance, multi-allelic nature, abundance, and wide distribution across the genome, making them easy to score (Powell *et al*., 1996; Feng *et al*., 2016). Various SSR databases have been constructed and utilized for purposes such as cultivar identification, seed purity tests, and determining parent-offspring relationships in crops like citrus and pear (Kim and Nou, 2016; Nguyen *et al*., 2019). Recent studies have also employed SSR markers for variety identification in chrysanthemum and other crops (Caramante *et al*., 2011; Zhang *et al*., 2014).
Chrysanthemum, particularly standard-type cultivars with long, sturdy stems and large flowers, is a commercially significant crop, valued highly as cut flowers and for flower arrangements. Therefore, accurate genetic identification and fingerprinting of these cultivars are crucial for safeguarding breeders' intellectual property rights (Manjulatha *et al*., 2020). The promising potential of SSR markers in assessing chrysanthemum diversity has prompted this research endeavor.

The objectives of this study were to: (a) compare morphological analysis and molecular markers (SSR) of seven commercial chrysanthemum cultivars and provide molecular data to assess genetic relationships among accessions, and (b) declare the genetic diversity among cultivars.

## 2. Materials and Methods

*Plant material and experimental site*

Seven commercial chrysanthemum (*Dendran-*

*thema grandiflora* Tzvelev) cultivars were selected for morphological and molecular characterization: C1 - Crystal Pink (Violet), C2 - Crystal White (White), C3 - Crystal Yellow (Yellow), C4 - Crystal Red (Dark Purple Red), C5 - Kodiack (Yellow), C6 - Coca Bleach (Brown Red), and C7 - Abrun (Violet) (Table 1 and Fig. 1).

18°C, with the ventilators opening at 22°C. Short days (10 hrs light) and daytime relative humidity were set at 70%, and the area was maintained shaded with black polythene sheets.

Once the cuttings were established, they were decapitated (pinched) above the 3$^{rd}$ - 4$^{th}$ leaf from the base to encourage the production of lateral shoots.

Table 1 - List of chrysanthemum cultivars used in this study and their inflorescence colors

| No. | Code | Cultivars | Royal Horticultural Society color chart No. | Inflorescence color |
|---|---|---|---|---|
| 1 | C1 | Crystal pink | 77D | Violet, light center |
| 2 | C2 | Crystal white | N155D | White, yellow center |
| 3 | C3 | Crystal yellow | 5C | Yellow |
| 4 | C4 | Crystal red | 53A | Dark purple red |
| 5 | C5 | Kodiack | 4B | Yellow |
| 6 | C6 | Coca bleach | 179A | Brown red, yellow green center |
| 7 | C7 | Abrun | N78A | Dark violet, yellow green center |


Fig. 1 - Standard-type chrysanthemum cultivars used in this study.

The investigation was conducted in a greenhouse at the nursery of the Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia, during the period 2019-2020.

*Planting method and experimental design*

Uniform rooted cuttings of the seven chrysanthemum cultivars, each measuring 7 cm in height with 5-6 true leaves, were selected. Nine rooted cuttings per cultivar were then planted on February 2$^{nd}$, 2019 (first growing season), and February 4$^{th}$, 2020 (second growing season), by placing them in 6-inch diameter plastic pots (one cutting per pot) filled with a mixture of peat and perlite growing media (2:1 by volume). The minimum day and night temperatures in the greenhouse were

The pinching procedure ensured that the apical meristems of all plants started active growth at the same time under the same conditions, increasing the uniformity of flowering (Cockshull, 1976).

A chemical growth retardant, B-Nine (Crompton Uniroyal Chemical Co., Washington, DC, USA), was applied as an aqueous solution with concentrations of 2000 ppm via foliar spray until runoff. This treatment was repeated three times at one-week intervals, starting three weeks after planting on February 23rd, March 2nd, and March 9th, respectively. A slow-release fertilizer, Osmocote (The Scotts Co., Marysville, OH, USA), was applied at the rate of 140 mg Kg$^{-1}$ soil of media, which contained Nitrogen (N), Phosphorus ($P_2O_5$), and Potassium ($K_2O$), in the ratios of 17:11:10, respectively (El-Nashar, 2013).

The experiment was conducted using seven chrysanthemum cultivars in a Completely Randomized Design (CRD) with three replications. Each cultivar represented one treatment, and each replication included three plants of a cultivar.

*Morphological characteristics*

Plant characterization took place in late April, and the morphological evaluation was carried out when the plants were in full bloom. Observations regarding the color of the flowers were recorded with the assistance of the Royal Horticultural Society (RHS) color chart (RHS, 1966; Dorling, 2008). All cultivars were observed and divided into two parts: the vegetative parts and the inflorescence parts. The number of characters that differed from each other was scored to determine the distinctiveness and uniformity of the plant under investigation. The data from all plants were compared to identify any variations between cultivars.

Plant development and growth were recorded per pot/plant unit during both the 2019 and 2020 growing seasons by measuring the following parameters: plant height (cm) (PH), number of branches (NL), number of leaves (NL), leaf area ($cm^2$) (LA), using a leaf area meter (Li-Cor, Lincoln, 404, NE), shoot fresh and dry weights (g) (SFW and SDW), root fresh and dry weights (g) (RFW and RDW), root length (cm) (RL), leaf area of one leaf 10 cm from plant height ($cm^2$) (LA10), leaf width (cm) (LW), and leaf length (cm) (LL). Additionally, flower production per pot/plant unit was monitored, taking into account the following traits: number of inflorescences (flower yield) (NI), inflorescence diameter (cm) (ID), total inflorescences fresh and dry weights (g) (IFW and IDW), inflorescence stalk length (cm) (ISL), number of inflorescences per branch (NIB), number of ray florets per inflorescence (NRFI), length of ray florets per inflorescence (cm) (LRFI), and fresh and dry weights of a single inflorescence (g) (SFWI and SDWI) using a precision balance (KERN, 440-47N, Balingen, Southern Germany). Fresh weight was carefully recorded after removing the plants (88 days from planting). Dry weight trait was determined after drying the plant material in a dry oven for 48h at 70°C until the weight became constant.

*Photosynthetic pigments*

Extraction of photosynthetic pigments (chlorophyll (Chl.) A and B) from leaves were implemented using N, N- dimethylformamide (DMF) method. Chls A, B, Chls A+ B concentrations in µmol $L^{-1}$ and Chls ratio were then estimated utilizing the equations of Porra *et al*. (1989) as follows:

$$\text{Chl A} = 13.43\ w^{663.8} - 3.47\ w^{646.8}$$

$$\text{Chl B} = 22.90\ w^{646.8} - 5.38\ w^{663.8}$$

$$\text{Total chlorophyll (Chl A+B)} = 19.43\ w^{663.8} - 8.05\ w^{646.8}$$

Chls a and b concentrations were estimated spectrophotometrically using UV Spectrophotometer (Pharmacia Biotech Ultrospec 2000).

Moreover carotenoid = (1000 $w^{480}$ – 0.89 Chl A – 52.02 Chl B)/245 (Wellburn, 1994; Vicaş *et al*. 2010) and anthocyanin = $w^{530}$ - 0.25 $w^{657}$ were taken into account (Mancinelli, 1994).

*Gas exchange*

The assessment of the leaves' gas exchange was conducted using a portable photosynthesis system, known as the Li-COR 6400, manufactured by LI-COR Inc., based in Lincoln, U.S.A. The evaluation of net photosynthetic rate ($P_n$), stomatal conductance of water ($g_s$), transpiration rate ($E$), and the intercellular $CO_2$ concentration ($C_i$) was carried out on fully expanded fourth leaves between 10:20 and 11:30 am on a sunny day with a humidity level of approximately 60±5%. The measurements were taken at an ambient temperature of 27°C and under a photosynthetic photon flux density (PPFD) of about 720 µmol $m^{-2}$ $s^{-1}$. The $CO_2$ concentrations were compared to the reference levels present in the growth chamber.

*DNA extraction*

Fresh young leaf tissues were collected from all chrysanthemum cultivars, then frozen in liquid nitrogen in a mortar, and stored at -80°C. The genomic DNA was extracted utilizing the DNeasy Plant Mini Kit, manufactured by QIAGEN in Germany. Subsequently, the quality of the DNA was assessed through electrophoresis on a 1% agarose gel, while the DNA concentration was determined using Quick Drop, a product of Molecular Devices in the United States. The DNA was then appropriately diluted to a concentration of 25 ng $\mu L^{-1}$ and employed for SSR analysis.

*SSR analysis*

Seven standard-type chrysanthemum cultivars were classified and identified using a total of twelve SSR markers. Detailed information about these

markers is provided in Table 2. Each SSR marker was amplified separately in a reaction volume of 25 µL. This reaction volume included 8 µmol of each forward (5' FAM labelled) and reverse primer, 50 ng of total genomic DNA, 2 mM $MgCl_2$, 10 mM tris-HCl (pH = 7.5), 50 mM KCl, and 0.3 U/µL of Taq DNA polymerase in 1 X PCR supplied buffer. The PCR reaction took place in a thermocycler (Applied Biosystems, Veriti, C.A., U.S.A.) following these cyclic parameters: one cycle of 3 min at 94°C, 45 cycles of about 1 min at 94°C, 1 min at 50 to 60°C, 2 min at 72°C, and a final extension for 10 min at 72°C. The PCR product was then analyzed by checking 2 µL of it on a 2% agarose gel electrophoresis. To detect the product, DNA Loading STAR (Dyne Bio, S. Korea) was used. Images were captured and photographed following the application of ethidium bromide stain on a gel deposition apparatus.

*Data analysis*

Concerning morphological and physiological analyses, the average and standard deviation values were calculated by One-way Analysis of Variance (ANOVA) using the statistical analysis software computer program (SAS Institute Inc., Cary, NC). The Least Significant Difference (LSD) procedure was used to determine significant differences among the means of cultivars at the 0.05 significance level (Steel *et al*., 1997).

Regarding genetic data analyses, Power Marker was used to calculate the total number of alleles, genetic diversity, heterozygosity, allele frequency, and polymorphism information content (PIC) for each SSR locus (Liu and Muse, 2005). The SSR amplification bands were assigned a score of 0 for absence and 1 for presence. A Simple Matching similarity index was utilized to calculate the similarity of the qualitative data. The genetic similarity data were subjected to cluster analysis using the unweighted pair group method of arithmetic averages (UPGMA), and a dendrogram was generated using DendroUPGMA software. Principal Component Analysis (PCA) was conducted using the software PAST (version 3.14).

## 3. Results

*Plant vegetative growth*

Significant differences were observed among the various cultivars in terms of plant height, number of leaves per plant, leaf area, and leaf length in both seasons (Table 3). During the first season, the mean plant height ranged from 18.03 to 23.76 cm, while in the second season, it ranged from 17.98 to 23.03 cm. The cultivar Crystal Red recorded the highest plant height, followed by Coca Bleach, while Crystal Pink exhibited the shortest plant height, followed by 'brun' (Table 3).

The mean number of branches per plant and leaf width was not significantly affected by the compared cultivars in both seasons. The number of leaves per plant ranged from 27.33 to 33.07 for cv. Coca Bleach and 52.02 to 60.00 for cv. Crystal Red respectively. 'Abrun' exhibited the lowest leaf area (231.39 and 230.02 $cm^2$) in both seasons, while the largest leaf area was detected for cv. Crystal Red (390.53 and

Table 2 - List of twelve SSR primers that were screened to distinguish the seven standard-type chrysanthemum cultivars

| S. No. | SSR Primers | Primer sequence | |
|---|---|---|---|
| | | Forward | Reverse |
| 1 | Xcfd1 | 5' ACCAAAGAACTTGCCTGGTG 3' | 5' AAGCCTGACCTAGCCCAAAT 3' |
| 2 | Xgwm205 | ' CGACCCGGTTCACTTCAG 3'5 | 5' AGTCGCCGTTGTATAGTGCC 3' |
| 3 | Xgwm133 | 5' ATCTAAACAAGACGGCGGTG 3' | 5' ATCTGTGACAACCGGTGAGA 3' |
| 4 | Xcfd9 | 5' TTGCACGCACCTAAACTCTG 3' | 5' CAAGTGTGAGCGTCGG 3' |
| 5 | Xcfd46 | 5' TGGTGGTATAGTCGTTGGAGC 3' | 5' CCACACACACACACCATCAA 3' |
| 6 | Xgwm181 | 'TCATTGGTAATGAGGAGAGA 3'5 | 5' GAACCATTCATGTGCATGTC 3' |
| 7 | Xcfd49 | 5' TGAGTTCTTCTGGTGAGGCA 3' | 5' GAATCGGTTCACAAGGGAAA 3' |
| 8 | Xgwm174 | 5' GGGTTCCTATCTGGTAAATCCC 3' | 5' GACACACATGTTCCTGCCAC 3' |
| 9 | Xcfd18 | 5' CATCCAACAGCACCAAGAGA 3' | 5' GCTACTACTATTTCATTGCGACCA 3' |
| 10 | Xcfd183 | 5'ACTTGCACTTGCTATACTTACGAA3' | 5' GTGTGTCGGTGTGTGGAAAG 3' |
| 11 | Xgwm210 | 5' TGCATCAAGAATAGTGTGGAAG 3' | 5' TGAGAGGAAGGCTCACACCT 3' |
| 12 | Xcfd66 | 5' AGGTCTTGGTGGTTTTGGTG 3' | 5' TTTTCACATGCCCACAGTTG 3' |

Table 3 - Plant height, number of branches, number of leaves, leaf area, leaf width and leaf length of the seven studied chrysanthemum cultivars

| Cultivars | Vegetative growth character | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plant heigh (cm) | | Branches (No.) | | Leaves (No.) | | Leaf area ($cm^2$) | | Leaf width (cm) | | Leaf length (cm) | |
| | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 |
| Crystal pink | 18.03 c | 17.98 b | 3.73 a | 3.37 a | 37.67 bc | 42.07 | 308.80 | 256.10 c | 4.71 a | 4.80 a | 6.90 bc | 6.80 d |
| Crystal white | 20.77 bc | 20.83 ab | 4.37 a | 4.73 a | 42.01 ab | 42.03 | 380.88 a | 377.17 | 4.43 a | 5.11 a | 8.20 ab | 8.83 ab |
| Crystal yellow | 18.23 c | 20.23 ab | 3.74 a | 3.73 a | 38.67 bc | 44.34 bc | 244.65 b | 289.96 | 3.91 a | 4.60 a | 6.71 c | 7.43 bcd |
| Crystal red | 24.43 a | 23.03 a | 4.36 a | 4.77 a | 52.02 a | 60.00 a | 390.53 a | 455.31 a | 5.23 a | 4.97 a | 9.30 a | 9.23 a |
| Kodiack | 22.43 ab | 20.27 ab | 3.43 a | 4.03 a | 49.01 ab | 36.67 cd | 380.24 a | 313.16 | 4.67 a | 4.96 a | 6.86 bc | 7.10 cd |
| Coca bleach | 23.76 a | 22.93 a | 3.07 a | 2.76 a | 27.33 c | 33.07 d | 252.00 b | 270.12 | 4.43 a | 4.73 a | 7.1 bc | 8.66 abc |
| Abrun | 19.01 c | 18.04 b | 3.70 a | 3.37 a | 47.32 ab | 49.77 b | 231.39 b | 230.02 c | 4.56 a | 4.23 a | 7.5 bc | 8.01 a-d |

Values in each column followed by the different letter(s) are significantly different at $P \leq 0.05$. Least Significant Difference.

455.31 $cm^2$) in both seasons. 'Crystal Red' recorded the maximum leaf area followed by cv. Crystal White, while the least leaf area was recorded in 'Abrun' followed by 'Coca Bleach'. Leaf length ranged from 6.90 to 6.80 cm for 'Crystal Pink' and 9.30 to 9.23 cm for 'Crystal Red' respectively (Table 3).

The mean values of the leaf area of one leaf 10 cm from the plant height did show significant differences among plant cultivars in both seasons (Table 4 and Fig. 2). 'Crystal Pink' exhibited the lowest leaf area (9.34 and 13.10 $cm^2$) in both seasons, while the largest leaf area was detected in 'Crystal Red' (19.99 and 20.46 $cm^2$) in both seasons. The mean values of the compared cultivars did not show any significant

Table 4 - Leaf area, shoots fresh weight, shoots dry weight, root fresh, root dry weight and root length of the seven studied chrysanthemum cultivars

| Cultivars | Vegetative growth characters | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LA10 ($cm^2$) | | SFW (g) | | SDW (g) | | RFW (g) | | RDW (g) | | Root length (cm) | |
| | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 |
| Crystal pink | 9.34 e | 14.78 bc | 17.17 a | 16.27 a | 1.72 a | 1.67 a | 4.21 b | 4.95 b | 0.74 a | 1.02 a | 22.43 a | 22.63 a |
| Crystal white | 17.98 ab | 19.91 a | 19.80 a | 18.92 a | 2.15 a | 2.02 a | 5.17 b | 5.50 b | 1.39 a | 1.06 a | 22.14 a | 21.60 a |
| Crystal yellow | 11.13 de | 13.75 bc | 16.67 a | 19.77 a | 1.74 a | 2.61 a | 9.94 a | 8.94 a | 1.71 a | 2.13 a | 24.63 a | 26.20 a |
| Crystal red | 19.99 a | 20.46 a | 18.53 a | 22.65 a | 2.15 a | 2.46 a | 5.42 b | 5.80 b | 1.21 a | 1.45 a | 22.40 a | 24.67 a |
| Kodiack | 15.52 bc | 18.49 ab | 17.87 a | 14.76 a | 2.04 a | 1.88 a | 4.11 b | 4.48 b | 0.84 a | 0.99 a | 24.11 a | 25.80 a |
| Coca bleach | 14.71 | 17.67 | 15.17 a | 20.31 a | 1.75 a | 2.40 a | 3.65 b | 4.46 b | 0.82 a | 1.14 a | 24.13 a | 19.41 a |
| Abrun | 12.01 | 13.10 c | 17.41 a | 18.16 a | 2.24 a | 2.71 a | 6.33 b | 6.25 b | 1.31 a | 1.45 a | 18.53 a | 20.04 a |

LA10= Leaf area measured 10 cm from plant height; SFW= Shoot fresh weight per pot unit; SDW= Shoot dry weight per pot unit; RFW= Root fresh weight per pot unit; RDW= Root dry weight per pot unit.
Values in each column followed by the different letter(s) are significantly different at P≤0.05 (Least Significant Difference).

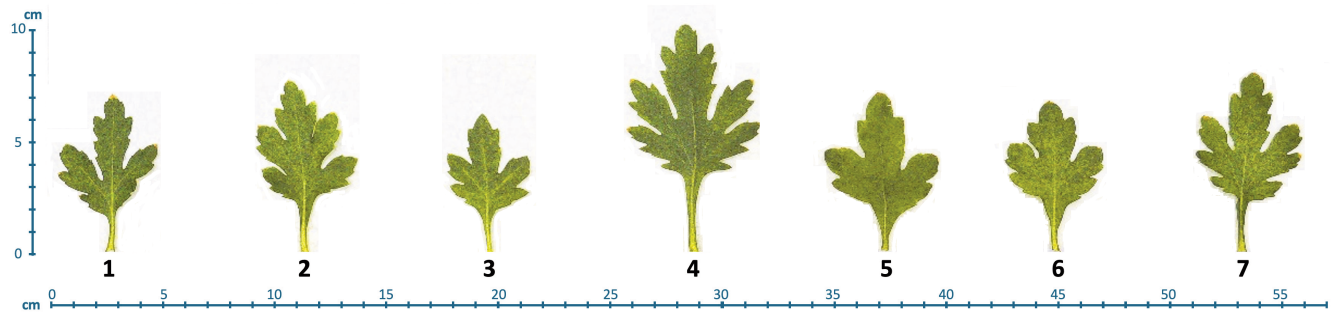


Fig. 2 - Leaf morphology of the studied chrysanthemum cultivars. 1) 'Crystal pink', 2) 'Crystal white', 3) 'Crystal yellow', 4) 'Crystal red', 5) 'Kodiack yellow', 6) 'Coca bleach', 7) 'Abrun'. Refer to Table 1 cultivars

differences in plant shoot fresh and dry weights per plant in both seasons (Table 3). Regarding the cultivar's effect on root characteristics in both seasons, the highest root fresh weight was recorded for cv. Coca Bleach (3.65 and 4.46 g), whereas the lowest root fresh weight (9.94 and 8.94 g) was detected for cv. Crystal Yellow. The mean values of the compared cultivars did not show any significant differences in root dry weight and root length per plant in both seasons.

*Flower characteristics*

The plants comparison cultivars had highly significant effects on number of inflorescence per plant, inflorescence diameter and inflorescence stalk length in both seasons. In the first season, the mean number of inflorescence per plant varied from 10.07 to 29.40, while the mean number height varied from 11.06 to 26.71 in the second season. The cv. Abrun recorded maximum number of inflorescence per plant followed by Crystal red and Crystal pink cultivars recorded the least height followed by cv. Crystal white (Table 5 and Fig. 3). The inflorescence diameter ranged from cv. Abrun (6.07 and 6.13 cm) to cv. Kodiack (9.93 and 10.47 cm), respectively (Fig. 3). A highest inflorescence stalk length was recorded at cv. Crystal white (6.37 and 6.77 cm), whereas the shortest inflorescence stalk length (2.77 and 2.43 cm) was detected at the cv. Crystal Yellow.

Insignificant differences were detected between the first and second seasons in fresh inflorescence weight. Significant differences in chrysanthemum inflorescence dry mass per plant were detected in the second season. The lower value resulted in an increase in inflorescence dry weight with cv. Crystal Pink (1.36 g). On the other hand, the highest value of inflorescence dry weight was observed with cv. Abrun (2.86 g). No significant differences were detected among the first season in chrysanthemum inflorescence dry weight per plant (Table 5).

The compared cultivars had highly significant effects on the number of inflorescences per branch, number of ray florets per inflorescence, ray floret length, and one inflorescence fresh weight in both seasons. In the first season, the mean number of

Table 5 - Number of inflorescences, inflorescence diameter, Inflorescence stalk length, inflorescences fresh weight and inflorescences dry weight of the seven studied chrysanthemum cultivars

| Cultivars | Flower characteristics | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Inflorescences (No.) | | Inflorescences diameter (cm) | | Inflorescence stalk length (cm) | | Inflorescences fresh weight (g) | | Inflorescences dry weight (g) | |
| | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 |
| Crystal pink | 10.07 c | 11.06 b | 9.33 a | 9.37 ab | 5.20 abc | 3.90 cd | 25.36 a | 23.04 a | 1.58 a | 1.36 c |
| Crystal white | 11.03 c | 13.46 b | 9.01 a | 9.97 ab | 6.37 a | 6.77 a | 20.00 a | 23.27 a | 1.85 a | 1.89 bc |
| Crystal yellow | 12.13 bc | 12.40 b | 7.23 bc | 7.70 c | 2.77 d | 2.43 d | 23.04 a | 28.22 a | 1.75 a | 2.65 ab |
| Crystal red | 24.07 a | 22.03 a | 6.17 c | 6.13 d | 4.46 bcd | 4.23 bc | 17.18 a | 26.47 a | 1.53 a | 3.01 a |
| Kodiack | 14.77 bc | 12.73 b | 9.93 a | 10.47 a | 3.63 cd | 2.96 cd | 20.70 a | 21.10 a | 2.01 a | 2.03 abc |
| Coca bleach | 17.03 b | 14.77 b | 8.60 ab | 8.87 bc | 6.23 ab | 5.57 ab | 14.03 a | 22.24 a | 1.11 a | 2.05 abc |
| Abrun | 29.40 a | 26.71 a | 6.07 c | 6.13 d | 4.1 cd | 4.40 bc | 19.10 a | 23.76 a | 2.20 a | 2.86 ab |

Values in each column followed by the different letter(s) are significantly different at P≤0.05 (Least Significant Difference).

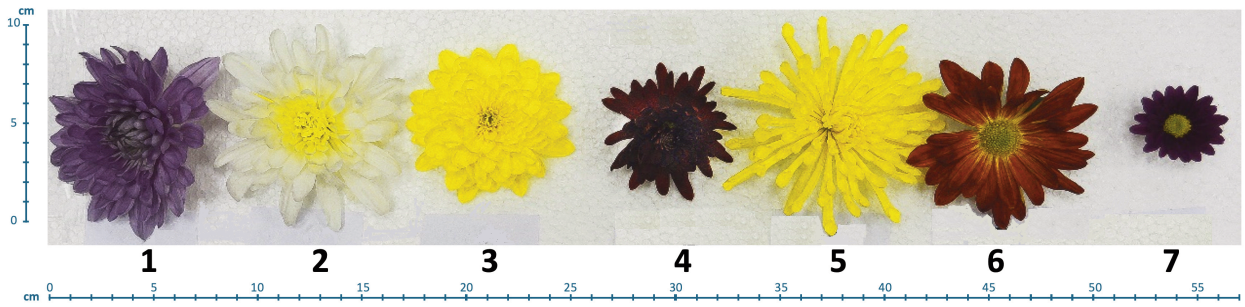


Fig. 3 - Standard-type inflorescences of chrysanthemum cultivars used in this study. 1) Crystal pink, 2) Crystal white, 3) Crystal yellow, 4) Crystal red, 5) Kodiack yellow, 6) Coca bleach, 7) Abrun. Refer to Table 1 cultivars.

inflorescences per branch varied from 4.37 to 9.73, while the mean height varied from 3.80 to 8.73 in the second season. The cultivar Crystal Red recorded the maximum number of inflorescences per branch followed by cv. Abrun; however, Crystal Yellow cv. recorded the least height followed by cv. Kodiack (Table 5). The number of ray florets per inflorescence ranged from cv. Coca Bleach (32.33 and 28.30) to cv. Crystal Red (121.00 and 126.66) in both seasons, respectively. The ray floret length ranged from cv. Abrun (2.03 and 2.30 cm) to cv. Kodiack (4.13 and 4.83 cm). The one inflorescence fresh weight ranged from (0.92 and 0.95 g) in cv. Abrun to (5.94 and 4.26 g) in cv. Crystal Pink On the other hand, one inflorescence dry weight, as affected by the compared cultivars, showed negative effects in both seasons (Table 6).

*Photosynthetic pigments*

The mean values of leaf chlorophyll contents are presented in figure 4A, while figure 4B shows the mean values of carotenoid and anthocyanin contents in leaves. The levels of chl a and chl b showed negligible effects, but significant changes were observed in total chlorophyll and the chl a/b ratio. Moreover, there were significant differences in carotenoid and anthocyanin contents. 'Crystal Pink' showed an increase in total chlorophyll content, while cv. Kodiack exhibited a significant reduction in total chlorophyll content compared to all other cultivars. In the present study, the levels of carotenoid and anthocyanin in the compared cultivars increased in cv. Crystal Pink, whereas cv. Kodiack had lower levels of carotenoid and anthocyanin.

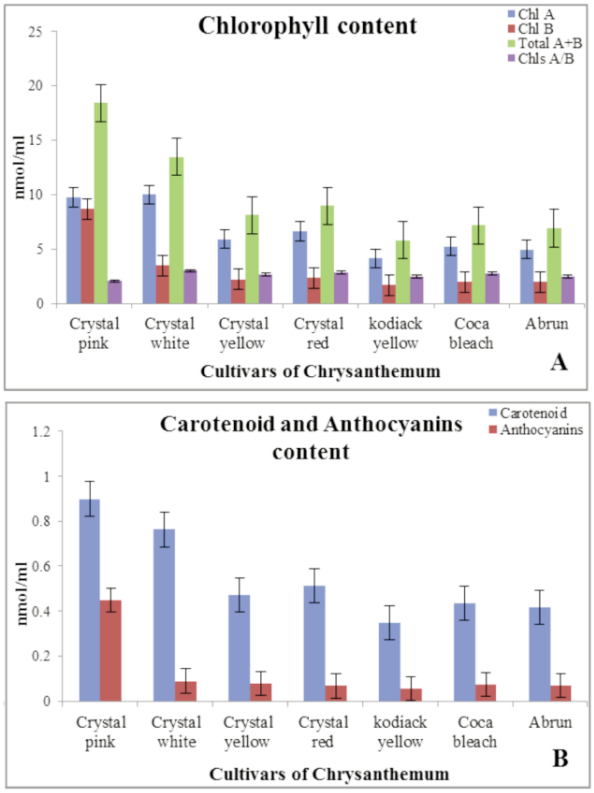


Fig. 4 - Leaf pigments of the chrysanthemum cultivars under study A) Chlorophyll (Chl) A, B, total and A/B Chls content; B) Carotenoid and anthocyanins content. Means are given with standard error.

*Gas exchange*

Gas exchange parameters [net photosynthetic rate ($P_n$), stomatal conductance ($g_s$), transpiration ($E$), and intercellular $CO_2$ concentration ($C_i$)] of the seven chrysanthemum cultivars under study showed significant variation and are depicted in figure 5.

Table 6 - Number of inflorescences per branch, number of ray floret per inflorescences, ray floret length, one inflorescence fresh weight and one inflorescence dry weight of the seven studied chrysanthemum cultivars

| Cultivars | Flower characteristic | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NIB | | NRFI | | LRFI (cm) | | SFWI (g) | | SDW (g) | |
| | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 |
| Crystal pink | 4.47 c | 4.70 bc | 108.67 ab | 115.00 ab | 3.87 ab | 4.30 ab | 5.94 a | 4.26 a | 0.40 a | 0.38 a |
| Crystal white | 6.36 bc | 6.73 ab | 84.65 c | 91.33 c | 4.06 a | 3.93 bc | 3.32 b | 3.41 b | 0.32 a | 0.35 a |
| Crystal yellow | 4.37 c | 3.80 c | 114.68 a | 106.32 bc | 3.20 b | 3.47 c | 2.79 c | 3.36 b | 0.47 a | 0.35 a |
| Crystal red | 9.73 a | 8.73 a | 121.00 a | 126.66 a | 2.43 c | 2.83 d | 2.01 d | 1.89 c | 0.23 a | 0.24 a |
| Kodiack | 4.40 c | 4.03 c | 98.64 b | 100.67 bc | 4.13 a | 4.83 a | 2.08 d | 3.83 ab | 0.21 a | 0.34 a |
| Coca bleach | 6.03 bc | 5.66 bc | 32.33 d | 28.30 d | 3.53 ab | 3.93 bc | 2.16 d | 1.69 cd | 0.26 a | 0.17 a |
| Abrun | 8.13 ab | 8.41 a | 33.32 d | 30.34 d | 2.30 c | 2.03 e | 0.92 e | 0.95 d | 0.23 a | 0.25 a |

NIB= Number of inflorescences per branch; NRFI= Number of ray florets per inflorescence; LRFI= Length of ray florets per inflorescence; SFWI= Fresh weight of a single inflorescence; SDWI= Dry weights of a single inflorescence.
Values in each column followed by the different letter(s) are significantly different at P≤0.05 (Least Significant Difference).

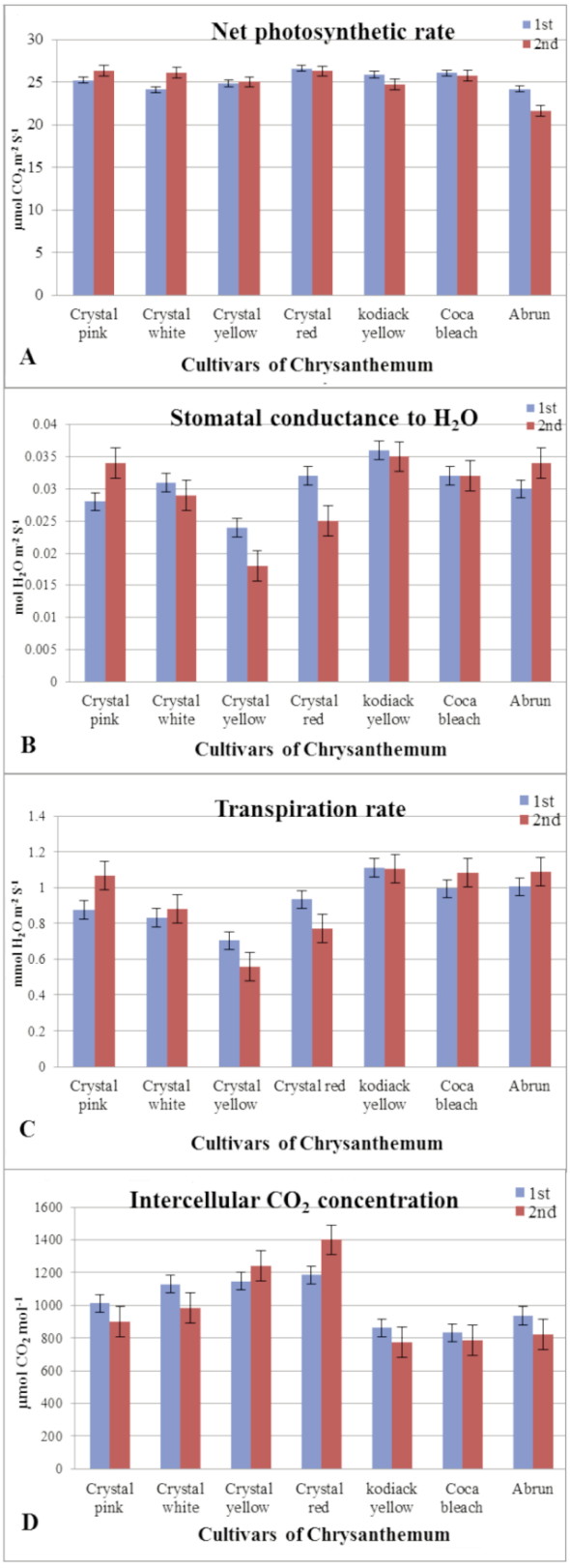


Fig. 5 - Gas exchange parameters of the chrysanthemum cultivars under study (a) Net photosynthesis rate, (b) Stomatal conductance to $H_2O$, (c) Transpiration rate, and (d) Intercellular $CO_2$ concentration). Means are given with standard error.

Cultivar 'Crystal Red' had the highest $P_n$, while 'Abrun' had the lowest $P_n$ compared to other cultivars (Fig. 5A). 'Crystal Yellow' exhibited the lowest $g_s$ and $E$, whereas 'Kodiack Yellow' had the highest values under the given conditions (Figs. 5B and C). $C_i$ increased in response to 'Crystal Yellow', while it decreased in 'Kodiack Yellow', compared to other cultivars (Fig. 5D). Cultivars showed significant differences in net photosynthetic activities; however, these variations were only evident under controlled conditions.

*SSR analysis*

PCR amplification of DNA using 12 primers for SSR analysis resulted in a total of 40 amplified bands, all of which were polymorphic bands with a 100% polymorphism rate (Table 7; Figs. 6A and B). These results also demonstrated the presence of uniquely amplified bands in the genomic DNA of the seven chrysanthemum cultivars, which were used as molecular markers to identify each of these seven different chrysanthemum cultivars. The number of amplified bands varied from two in primers Xgwm205, Xgwm133, Xgwm181, and Xgwm210, three in primers Xcfd49, Xcfd18, and Xcfd66, four in primers Xcfd9, Xcfd46, and Xgwm174, and five in primer Xcfd1, with a total of 40 bands and DNA lengths ranging from 100 to 600 bp. Additionally, six bands were found in primer Xcfd183, with DNA lengths ranging from 900 to 1000 bp.

The results obtained from the phylogenetic tree, based on twelve SSR primers as displayed in figure 7, indicated that the seven different chrysanthemum cultivars were separated into two main clusters. Cluster A consisted of the C1 (Crystal Pink) and C5 (Kodiack Yellow) cultivars, whereas cluster B was further divided into two sub-clusters. Sub-cluster B1 contained only the C2 (Crystal White) cv., while sub-cluster B2 was also divided into two sub-clusters. The first sub-cluster included the C6 (Coca Bleach) and C7 (Abrun) cultivars, forming a closely related group, whereas the second sub-cluster consisted of the C3 (Crystal Yellow) and C4 (Crystal Red) cultivars. The similarity matrix indicated a range of values from 0.18 to 0.39, with Crystal White standing out as a distinct cultivar among the seven cultivars analyzed.

## 4. Discussion and Conclusions

Vegetative growth parameters such as plant

Table 7 - Total numbers of amplified fragment and polymorphic fragments generated by PCR using SSR primers

| S. No. | Primer name | Total number of bands | Monomorphic bands | Polymorphic bands | Unique bands | Percent of polymorphism % |
|---|---|---|---|---|---|---|
| 1 | Xcfd1 | 5 | 0 | 5 | 0 | 100 |
| 2 | Xgwm205 | 2 | 0 | 2 | 0 | 100 |
| 3 | Xgwm133 | 2 | 0 | 2 | 0 | 100 |
| 4 | Xcfd9 | 4 | 0 | 4 | 0 | 100 |
| 5 | Xcfd46 | 4 | 0 | 4 | 0 | 100 |
| 6 | Xgwm181 | 2 | 0 | 2 | 0 | 100 |
| 7 | Xcfd49 | 3 | 0 | 3 | 0 | 100 |
| 8 | Xgwm174 | 4 | 0 | 4 | 0 | 100 |
| 9 | Xcfd18 | 3 | 0 | 3 | 0 | 100 |
| 10 | Xcfd183 | 6 | 0 | 6 | (1) 900-1000 bp C6 | 100 |
| 11 | Xgwm210 | 2 | 0 | 2 | 0 | 100 |
| 12 | Xcfd66 | 3 | 0 | 3 | 0 | 100 |
| Total | | 40 | 0 | 40 | 1 | 100 |

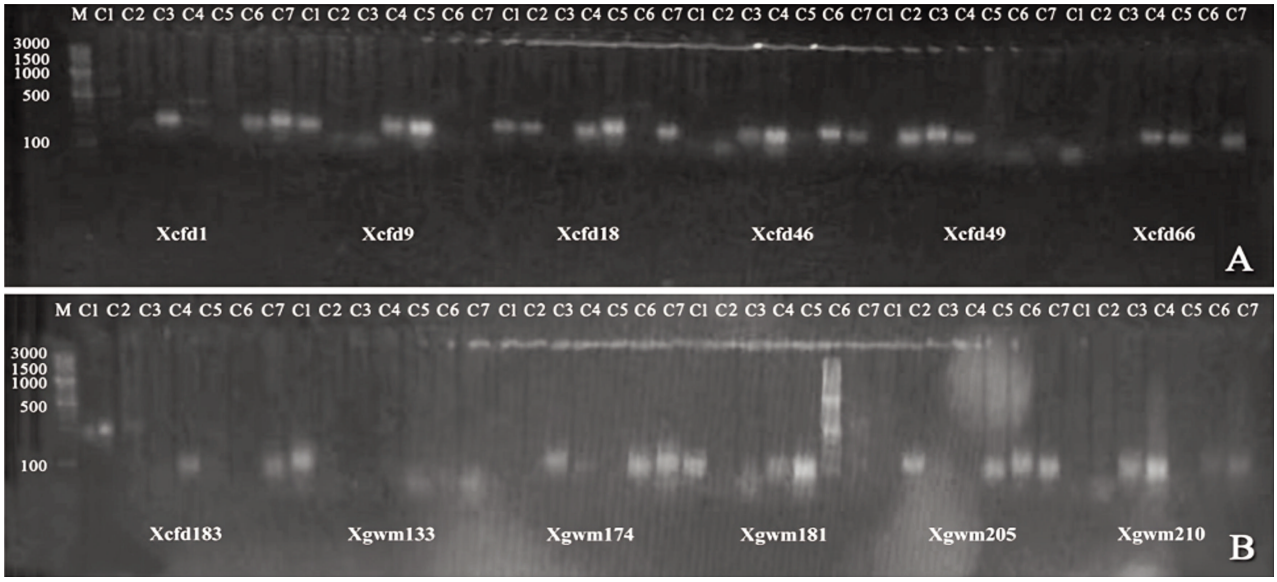


Fig. 6 - Banding of SSR patterns of seven chrysanthemum cultivars using twelve selected random primers, C1 - Crystal pink, C2 - Crystal white, C3 - Crystal yellow, C4 - Crystal red, C5 - Kodiack yellow, C6 - Coca bleach, C7 - Abrun. A) first six primers, and B) second six primers of the list primers used in this study.

height, number of branches, number of leaves per plant, leaf area, and dry weight accumulation play a crucial role in determining the overall crop yield. In this study, cultivars Crystal Red, Coca Bleach, Kodiack, and Crystal White exhibited vigorous growth, while Abrun and Crystal Yellow cultivars displayed medium growth, and Crystal Pink was characterized by dwarfism, recording the shortest plant height. The observed variations in plant height among the cultivars may be attributed to a combination of genetic factors, environmental conditions during growth, and plant management practices (Sirohi and Behera, 2000; Gharge *et al*., 2009). The increased number of leaves per plant in these cultivars was associated with higher plant height and number of branches per plant. Similar results were reported by Tarannum and Naik (2014) and Prasanth *et al*. (2020). These variations in growth characteristics may contribute to higher leaf area and ultimately increased dry weight production per plant

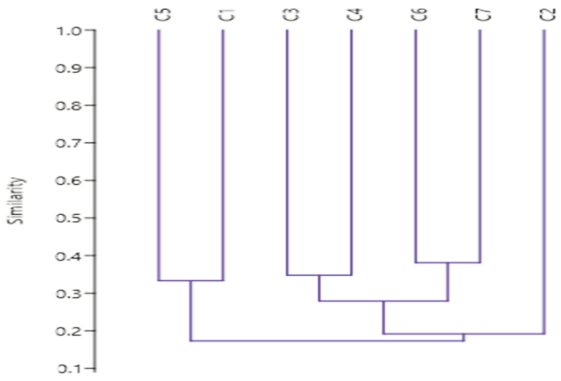


Fig. 7 - Dendrogram of relationship between seven chrysanthemum cultivars using Jaccard's (1908) index for SSR primers. C1 - 'Crystal pink', C2 - 'Crystal white', C3 - 'Crystal yellow', C4 - 'Crystal red', C5 - 'Kodiack yellow', C6 - 'Coca bleach', C7 - 'Abrun'.

in superior cultivars. These findings align with the conclusions of Barigidad *et al*. (1992) and Yoon-Jung *et al*. (2013) in chrysanthemum. The differences in growth characteristics between genotypes may be attributed to their inherent genetic traits, as all plants were subjected to similar practices under the same environmental conditions; Baskaran *et al*. (2016) also reported comparable findings.

Flower yield is a crucial factor in determining the suitability of specific genotypes for commercial cultivation, which directly affects the cost of cultivation. The maximum number of inflorescences per plant was recorded in the cultivars Abrun and Crystal Red, while Crystal Pink and Crystal White cultivars exhibited the lowest numbers. The study revealed that larger leaf area, more number of leaves and branches per plant, along with increased dry weight accumulation, resulted in higher photosynthetic activity, contributing to the production of more and larger flowers. These results are consistent with the findings of Tarannum and Naik (2014), Reddy *et al*. (2016), Palai *et al*. (2018), Singh *et al*. (2019), and Prasanth *et al*. (2020).

Flower stalk length is a critical quality characteristic that influences the quality of chrysanthemum cut flowers and extends their post-harvest life. The variation in stalk length among genotypes may be attributed to inherent genetic factors and growing environmental conditions, as reported by Dalal *et al*. (2009) and Tarannum and Naik (2014).

Flower diameter, being a genetically controlled trait, was found to be superior in cultivars Kodiack Yellow, Crystal Pink, Crystal White, and Coca Bleach, possibly due to the presence of more petals per inflorescence. However, Abrun cultivar produced smaller-sized flowers, which may be attributed to the fewer number of ray florets in its flower buds. The variation in inflorescence size could be attributed to the genetic makeup of the genotypes (Reddy *et al*., 2016; Neelam *et al*., 2018; Prabhu *et al*., 2018). Cultivar Crystal Red exhibited superiority in terms of inflorescence dry weight, followed by Abrun, while it was lower in cultivar Crystal Pink. Variations in inflorescence weight could be expected among different cultivars due to differences in genetic structure (Gharge *et al*., 2009). Carbohydrates serve as an energy source for growing buds, inflorescence opening, and longevity, ultimately resulting in strong and long inflorescence stalks and large-sized buds or inflorescences. These variations may be attributed to varietal characteristics, as reported by Halvey and Mayak (1979). Similar variations have been observed in chrysanthemum and carnation by several researchers, such as Sirohi and Behera (2000), Singh and Sangama (2003), and Uddin *et al*. (2015).

The yield and growth of any flower crop are influenced by various factors, including environment, season, and varieties. Among these factors, varieties play a significant role in the evolution of any flower crop, particularly in selecting varieties with high inflorescence production. Therefore, the selection of appropriate varieties is crucial for successful floriculture cultivation (Palai and Rout, 2011).

*Photosynthetic pigments*

The values of chlorophyll, carotenoid, and anthocyanin contents in the leaves of chrysanthemum cultivars are presented. Results clearly distinguish cultivars with high pigment content (Crystal Pink, Crystal White, Crystal Red, and Crystal Yellow) from those with low pigment content (Kodiack Yellow, Coca Bleach, and Abrun).

Photosynthesis in plants relies on capturing light energy using the pigment chlorophyll (Blankenship, 2014). Differences in chlorophyll a, b, carotenoid, and anthocyanin contents are indicators of damage to the photosynthetic apparatus, stress, or senescence and affect the normal course of plant biological processes (Filimon *et al*., 2016). Having a higher amount of chlorophyll could lead to increased light absorption, which is advantageous for photosynthesis in Rosa hybrida (Terfa *et al*., 2013).

The genotype of a plant affects pigment

accumulation by influencing the morphology and anatomy of the leaves (Hopkins and Hüner, 2009). Leaf area has been identified as a factor that can limit the photosynthetic capacity of plants, as reported by Petrie *et al*. (2000). However, it is important to note that the intensity of net photosynthesis ($P_n$) is not necessarily correlated with chlorophyll content, with differences potentially arising from variations in intracellular spaces and gaseous conductivity (Patakas *et al*., 2003). Chlorophyll loss is often linked to environmental stress, and changes in the Chlorophyll/Carotenoid ratio can serve as an indicator of stress in plants (Netto *et al*., 2005). The specific cultivar of a plant can also impact the accumulation of photosynthetic pigments by influencing the morphology and anatomy of the leaves, including factors like mesophyll thickness, area, and perimeter (Salem-Fnayou *et al*., 2011).

*Gas exchange*

Light affects not only the photosynthetic rates but also the stomatal function. Previous research has focused on studying the impact of long-term acclimation to specific wavelength light on stomatal morphology, density, and opening rates, as demonstrated by studies conducted by Wang *et al*. (2016) and Zheng and Van Labeke (2018). Numerous studies have also shown that light has the ability to induce stomatal opening, as observed in research conducted by Shimazaki *et al*. (2007). Stomatal conductance ($g_s$) is influenced by the density of stomata on the leaf surface as well as how wide the stomata are open. When plants have abundant water, high $g_s$ levels can lead to more transpiration, resulting in reduced leaf water content. Closing stomata can help maintain leaf water content by reducing transpiration. In the case of Crystal Yellow cv. leaves, the low stomatal conductance and leaf transpiration can mainly be attributed to a decrease in stomatal conductance compared to other cultivars.

The impact of different types of light on the process of photosynthesis and transpiration in chrysanthemums at various stages of growth is uncertain. Scientists conducted experiments to measure the exchange of $CO_2$ and $H_2O$ in the leaves and entire plants of chrysanthemums under long-day and short-day conditions. It was observed that all light sources effectively stimulated leaf photosynthesis, regardless of whether it was a long or short day (Leonardos *et al*., 2019).

*Molecular analysis*

Chrysanthemum cultivars pose challenges in terms of genetic backgrounds and similar morphological features, making it difficult to distinguish among them. Various molecular markers like sequence-related amplified polymorphism (SRAP) (Fei *et al*., 2011), inter simple sequence repeats (ISSR) (Shao *et al*., 2010), and simple sequence repeats (SSR) (Chang *et al*., 2018) have been employed to identify and classify chrysanthemum cultivars. Between these markers, SSRs offer advantages such as co-dominance, high variability, and reproducibility. SSR markers have also been utilized in the construction of molecular maps, analysis of genetic diversity, and assessment of intellectual property rights in different plants (Feng *et al*., 2016; Mekapogu *et al*., 2020). Previous studies have employed SSRs for genetic analysis of chrysanthemum and related genera (Chang *et al*., 2018). This investigation introduces a method for identifying standard-type seven cultivars in chrysanthemum.

Previous research has already established a database consisting of SSR markers that can be used to identify different cultivars of chrysanthemum. In two separate studies conducted by Shim *et al*. (2015) and Olejnik *et al*. (2021), a total of 28 SSR markers from the chrysanthemum DNA profile database were utilized to analyze the genetic relationship among a vast number of chrysanthemum cultivars, specifically 147 and 97, respectively. However, it is worth noting that very few studies have delved into the potential use of SSR markers for distinguishing standard-type chrysanthemum cultivars, as highlighted by Han *et al*. (2018) and Thakur *et al*. (2023).

In the current study, a set of twelve SSR markers was employed to distinguish and classify seven different standard-type chrysanthemum cultivars. It was determined that out of the twelve SSR markers utilized, there was noticeable genetic variation observed in the seven standard-type cultivars.

The evaluation of genetic relationships between populations serves as the foundation for both selective breeding and cultivar identification. By analyzing the SSR data and constructing a UPGMA-based dendrogram, it was observed that the tested chrysanthemum cultivars could be divided into two main groups. The similarity matrix indicated a range of values from 0.18 to 0.39, with Crystal White standing out as a distinct cultivar. Among the seven

cultivars analyzed (Crystal Pink, Crystal White, Crystal Yellow, Crystal Red, Kodiack Yellow, Coca Bleach, and Abrun) which formed cluster I, Coca Bleach and Abrun cultivars showed a moderate level of distinction and displayed complete genetic similarity. This suggests that there is a relatively low genetic diversity between these cultivars and that they may have been developed from a limited genetic background. In this study, it was found that Kodiack Yellow and Crystal White cultivars exhibited genetic divergence, which aligns with the findings of Shim *et al*. (2015), Olejnik *et al*. (2021), and Thakur *et al*. (2023), who also observed a distant relationship between Kodiack Yellow and Crystal White cultivars.

SSR genetic diversity does not necessarily match morphological differences. However, in the present investigation, to a certain extent, the clustering of genotypes in the sub-clusters seemed to correspond with a few phenotypic traits. Crystal pink and Kodiack, with the same shape of inflorescences (spoon-type) and without disk florets; the leaves are three-lobed, and both have an average inflorescence diameter, root fresh weight per pot unit, length of ray florets per inflorescence, and number of inflorescences per branch, formed sister relationships within cluster A. Within the second sub-cluster B2, two genotypes with the same shape of inflorescences (reflex-type); the leaves are five-lobed, and both have a light fresh weight of a single inflorescence, length of ray florets per inflorescence, number of ray florets per inflorescence, and inflorescence diameter (Crystal yellow and Crystal red), formed the sister relationships. Coca bleach and Abrun have the same shape of inflorescences (single/sami-double-type) and disk florets color (yellow-green center), and both have a low number of ray florets per inflorescence, leaf area, and leaf length, forming sister relationships within the first sub-cluster B1. The genotype Crystal white remained as a single cultivar in a separate sub-cluster within the major (B), which is the only white color cultivar in the group (B). Inflorescence was the type of irregular-type chrysanthemums. Buldewo *et al*. (2012) performed clustering based on spathe color in *Anthurium andraeanum* to group phenotypic traits/colors. Dai *et al*. (2012) used SSR markers to classify chrysanthemum germplasm based on inflorescence. The economic uses of various cultivars were observed to be linked with different clusters within the primary groups. In a study by Minano *et al.* (2009), chrysanthemum cultivars were grouped according to their inflorescence type and cultural characteristics.

The use of twelve SSR primers resulted in 100% polymorphism in seven chrysanthemum cultivars, demonstrating a remarkably high level of diversity. This confirms the effectiveness of these SSR markers in analyzing the genetic characteristics of chrysanthemum cultivars (Mekapogu *et al*., 2020; Olejnik *et al*., 2021).

Chrysanthemums possess various traits, including diverse flower shapes and colors, plant sizes, forms, and flowering periods, which are extensively utilized in landscaping. The study identified notable distinctions among the different cultivars. This research reveals that our method of using SSR markers is effective in assessing the genetic connections between closely related chrysanthemum cultivars and distinguishing between them. These microsatellites can be employed for certifying protected varieties and conducting pedigree analysis.

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# Pruning date and hydrogen cyanamide effects on growth and yield of grapevine var. Cabernet Sauvignon

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**Abstract: Harvesting the grapes before the monsoon season is crucial to ensure the quality of berry and bunches. This study aims to identify the optimal window for pruning and hydrogen cyanamide (HC) application to prepone the berry harvesting. The experiment was conducted in randomized complete block design with five treatments and four replications. Treatments were five different pruning dates in 2021: Jan. 17, Jan. 24, Jan. 31, Feb. 7 and Feb. 14, followed by 5% HC application one week after pruning. Annual growth stages of grapevine were recorded by using modified E-L growth stage; reproductive attributes recorded during flowering; and vine yield and berry quality attributes recorded at harvesting. The earlier pruning resulted earlier budburst compared to late pruning. Vines prunefed after Feb. 7 had <50% budburst, while vines pruned before Feb. 7 reached 50% budburst, exhibiting no differences in number of days needed to achieve it. Jan. 17 pruning had the highest budburst (%) and bud fruitfulness (%), where pruning on Feb. 7 had lower values. Average bunch weight did not differ while berry quality attributes differed between treatments for the same day harvest. The negative responses in late pruned and HC treated vines, potentially attributed to the phytotoxic effect of HC on tender buds near the natural time of dormancy break. The early pruning and subsequent application of HC triggered earlier budburst, and advances flowering and harvesting of berries in grapevine. This research demonstrated a potential techniques for advancing harvesting time (2-3 weeks) in grapevine.**

## 1. Introduction

Grape (*Vitis vinifera* L.) is a non-climacteric berry fruit of the deciduous woody vines belonging to the family Vitaceae, indigenous to Eurasia (This *et al.*, 2006). Grapes can be consumed in fresh or processed products like juice, wine, vinegar, jelly, jam, grape seed oil, and raisins. Cabernet Sauvignon is a widely cultivated grape variety for wine due to its small berries with a higher concentration of tannin and coloring pigment (Robinson *et al.*, 2012). Grape cultivation in Nepal was started about 70

years ago but there was no significant expansion of viticulture farms (Dahal *et al.*, 2017). Currently, few commercial vineyards are producing mainly wine grapes while table grapes are in small quantities. The estimated production of Nepal was 76 t from 20 ha area with a productivity of 8.5 t $ha^{-1}$ (Atreya *et al.*, 2015) but the demand of grape has been increasing manifolds in recent years (Acharya *et al.*, 2023). It shows there is a huge demand for grapes along with bottlenecks of Nepalese viticulture. The main challenge is harvesting time coincides with the rainy season causing a high risk of fungal diseases and insect infestation during ripening resulting in inferior quality berries and bunch (Shrestha, 1998). Such problems are also common in humid subtropical areas in other region too.

Grapevine dormant buds starts the resumption of annual growth cycle after winter in most of the subtropical and temperate climates. Chilling requirement and breaking dormancy are crucial affecting temperate fruit trees when grown in tropical climates (Botelho and Müller, 2007). Various chemicals, such as mineral oil (Black, 1936), Dinitro-ortho-cresol (Erez and Sur, 1981), Thiourea (Blommaert, 1965), Garlic extract (Botelho and Müller, 2007), and HC have been used to break dormancy in grapevine. Among these chemicals, HC has great efficiency in bud breaking (Nir and Levee, 1993) as well as enhances uniform and rapid bud breaking (McColl, 1986; Halaly *et al.*, 2008). However, the effect of HC depends upon the time and concentration used. Hydrogen cyanamide breaks endo-dormancy by respiratory disturbance, hormonal signaling, and oxidative stress (Liang *et al.,* 2019). This research was purposed to advance the natural budburst period, which might lead to bunches being harvested before monsoon. To harvest the crop before monsoon, breaking of bud dormancy earlier than natural time is required. In the warmer climates, pruning followed by HC are employed to induce budburst. Aiming to prepone natural budburst and to allow the berry harvesting before the heavy rainfall or monsoon.

## 2. Materials and Methods

The experiment was conducted from January 17 to July 04, 2021, at the commercial vineyard (27°44' N, 85°6' E) in Dhading, on a south-facing plot with a gentle slope at an altitude of 800 meters above mean sea level. The grapevine cv. Cabernet Sauvignon (6 years old) grafted on 5C rootstock, was selected for the research. Five pruning dates followed by HC application treatments was arranged on a Randomized Complete Block Design with five replications considering a vine as replication (Table 1).

Table 1 - Description of treatment details and coding of treatment practiced in a commercial vineyard, Dhading, Nepal, 2021

| Treatments | Pruning date | 5% HC application date | Treatment code |
|---|---|---|---|
| T1 | January 17 | January 24 | J17J24 |
| T2 | January 24 | January 31 | J24J31 |
| T3 | January 31 | February 07 | J31F07 |
| T4 | February 07 | February 14 | F07F14 |
| T5 | February 14 | February 21 | F14F21 |

To differentiate treatments, vines within replication were tied with different colored ribbons. One-year-old vines were spur-pruned, leaving three basal buds per spur. Ten spurs from each vine were selected and tagged with different colored threads. Buds in spur were marked as 1, 2, and 3 from the basal to distal. Hence, 30 buds were marked per vine. Phenological observations using the modified Eichhorn and Lorenz (E-L) grapevine growth stages scale began on February 16, 2021, and carried out in every four days interval until April 26, 2021. The growth stages, reproductive attributes during flowering, vine yield, and berry quality attributes were recorded at harvest. Development parameters such as budburst, fruitfulness in total buds, and fruitfulness in burst buds were calculated using the following formulas:

Budburst (%) = Number of burst buds/Total buds x 100

Observed fruitfulness (%) = Number of buds with inflorescence(s)/ Total buds x 100

Fruitfulness in burst buds (%) = Number of buds with inflorescence(s) / Total burst buds x 100

All bunches of each treatment were harvested on the same day, July 4, 2021 to ensure that the minimum standard quality berries and bunches harvested before the monsoon arrives. Quantitative attributes were measured from randomly selected 10 bunches. The qualitative attributes (Total soluble solid and Total titratable acidity) were assessed by randomly selected 10 berries from each selected bunch (Dahal *et al.*, 2019). Data recorded from the field were

entered, tabulated and analyzed using MS Excel 12 and GENESTAT version 18.1.

## 3. Results

*Phenological observations*

*Annual growth stages of grapevine.* Considerable variation in average E-L growth stages among different treatments were observed throughout the experimental period as shown in Table 2. Lower values were recorded in later treated vines (F07F14 and F14F21) while early treated (J17J24 and J24J31) vines had higher values of E-L stages. At the last date of observation (26th April), the average E-L stage of J17J24 and F07F14 were 19.69±6.27 and 3.21±2.98, respectively.

*Number of days to budburst.* The number of days to first budburst differed significantly between treatments while treatments did not significantly differ in days to 50% budburst (Table 3). Budburst was earlier

Table 3 - Effect of pruning date followed by HC application on number of days to 1st and 50% budburst, Dhading, Nepal, 2021

| Treatments | Days to the first budburst | Days to 50% budburst |
|---|---|---|
| J17J24 | 0.8 ±0.8 a | 16.8 ±2.8 |
| J24J31 | 8.0 ±1.26 ab | 17.2 ±0.8 |
| J31F07 | 10.4 ±1.6 b | 19.0±1.03 |
| F07F14 | 26.4 ±3.7 c | NA |
| F14F21 | 24.8 ±4.08 c | NA |
| Grand mean | 14.1 | 17.67 |
| LSD | 7.93 | NA |
| F-probability | <0.001 | NA |
| CV% | 42.0 | 19.1 |

Mean with the same letter(s) within the column do not differ significantly by DMRT at 5%. Values are μ±SE where μ = Mean and SE = Standard error. LSD=Least Significance Difference. CV = Coefficient of variance. NA= Not applicable.

Table 2 - Average E-L stage of grapevine buds in different dates of pruning and HC application, Dhading, Nepal, 2021

| Observation day | Average E-L growth stage in treatments | | | | | Statistical analysis | |
|---|---|---|---|---|---|---|---|
| | J17J24 | J24J31 | J31F07 | F07F14 | F14F21 | Grand mean | LSD (α =5) |
| D0 | 1.39±0.38 c | 1.07±0.11 b | 1.03±0.07 b | 1.01±0.04 a | 1.01±0.04 a | 1.10 | 0.105 ** |
| D4 | 1.73±0.52 d | 1.33±0.24 c | 1.16±0.16 b | 1.01±0.04 a | 1.01±0.04 a | 1.25 | 0.101 ** |
| D8 | 2.23±0.8 d | 1.71±0.45 c | 1.31±0.26 b | 1.02±0.06 a | 1.01±0.05 a | 1.47 | 0.279 ** |
| D12 | 3.26±1.17 d | 2.39±0.76 c | 1.73±0.38 b | 1.05±0.10 a | 1.03±0.07 a | 1.89 | 0.514 ** |
| D16 | 4.97±1.69 d | 3.76±1.4 c | 2.71±0.88 b | 1.1±0.18 a | 1.09±0.15 a | 2.72 | 0.836 ** |
| D20 | 6.11±1.92 c | 5.08±1.84 c | 4.14±1.55 b | 1.17±0.33 a | 1.17±0.24 a | 3.53 | 1.044 ** |
| D24 | 7.74±2.34 c | 6.57±2.29 bc | 5.65±2.11 b | 1.27±0.46 a | 1.43±0.45 a | 4.53 | 1.392 ** |
| D28 | 8.7±2.55 c | 7.25±2.48 bc | 6.08±2.24 b | 1.39±0.61 a | 1.78±0.73 a | 5.07 | 1.527 ** |
| D32 | 9.35±2.76 c | 8.05±2.73 bc | 6.86±2.52 b | 1.51±0.77 a | 2.14±0.95 a | 5.61 | 1.735 ** |
| D36 | 10.85±4.75 c | 8.9±3.02 b | 8.11±2.91 b | 1.71±1.04 a | 2.85±1.39 a | 6.48 | 1.895 ** |
| D40 | 11.16±3.26 c | 9.93±3.38 bc | 8.48±3.02 b | 1.91±1.29 a | 3.36±1.71 a | 6.97 | 2.161 ** |
| D46 | 12.43±3.63 c | 11.1±3.78 bc | 9.69±3.46 b | 2.12±1.54 a | 4.25±2.27 a | 7.92 | 2.510 ** |
| D49 | 13.79±4.04 c | 11.98±4.13 bc | 10.58±3.93 b | 2.31±1.8 a | 4.87±2.62a | 8.13 | 3.017 ** |
| D51 | 15.1±4.56 b | 12.76±4.53 b | 12.57±4.52 b | 2.50±2.03 a | 5.49±3.02 a | 9.70 | 3.525 ** |
| D55 | 16.09±5 b | 13.82±5.02 b | 13.61±5.02 b | 2.69±2.28 a | 6.21±3.49 a | 10.48 | 3.725 ** |
| D59 | 16.9±5.28 b | 14.72±5.32 b | 14.63±5.35 b | 2.87±2.5 a | 6.97±3.97 a | 11.21 | 4.140 ** |
| D65 | 18.33±5.74 b | 16.26±5.92 b | 16.00±5.86 b | 3.05±2.75 a | 7.83±4.52 a | 12.25 | 5.081 ** |
| D69 | 19.69±6.27 b | 17.65±6.46 b | 17.53±6.46 b | 3.21±2.98 a | 8.62±5.06 a | 13.29 | 5.679 ** |

D= Date on phenological observation was done. D0 = 16th Feb.; D4 = 20th Feb.; D8 = 24th Feb.; D12 =28th Feb.; D16 =4th Mar.; D20 = 8th Mar.; D24 = 12th Mar.; D28 = 16th Mar.; Day32= 20th Mar.; D36 =24th Mar.; D40 = 28th Mar.; D46 = 3rd Apr.; D49 =6th Apr.; D51= 8th Apr.; D55 = 12th Apr.; D59 = 16th Apr.; D65 = 22nd Apr.; D69= 26th Apr.; Values are μ ± SE where μ = Mean stage; LSD = Least Significance Difference; ** highly significant at α =5%; NS = Not significant.

in the early pruned vine as compared to late pruned grapevines. Early pruned (Jan. 17) grapevine burst their first bud in 0.8±0.8 days while late pruned vines (Feb. 7) took 26.4±3.7 days after HC application.

*Growth and development observations*

*Budburst percentage.* The overall budburst was less than 50% for all treatments. Significant differences between treatments were found in different observation dates after treatment application. Early pruned and HC treated vines (J17J24, J24J31, and J31F07) were not only early in budburst, but they also had higher budburst (%) compared to late pruned and HC treated vines. Late pruned and HC treated vines had lower budburst (%) even around 10% as shown in figure 1. HC application close to natural budburst time damages the buds and buds did not sprout (<4 E-L growth stage), hence late pruned and HC treated (F07F14 and F14F21) vines did not reach the 50% budburst.

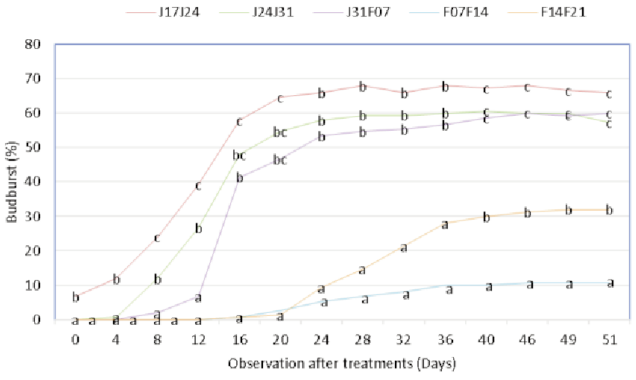


Fig. 1 - Effect of different date of pruning followed by HC application on budburst (%). Means are separated with different letter(s) for the respective day of observation using Duncan's multiple range test at 5%.

*Observed fruitfulness in total buds.* The overall flowering of all treatments was less than 40%, however, significant difference was observed between treatments on different dates of observations (Fig. 2). Flowering (%) variation among treatments follows a similar trend to that of budburst percentage as shown in figure 1 and figure 2. Late pruned and HC treated vines had lower flowering (%) and delayed in flowering. Vine took 40-49 days to flower after budburst. Late pruned and HC treated vines started to flower after 49 days while earlier treated vines started flowering after 40 days.

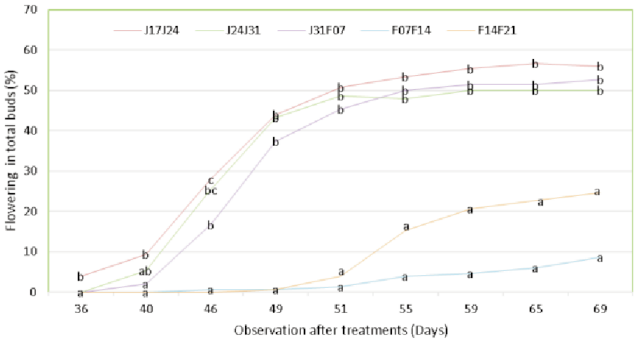


Fig. 2 - Effect of different timing of pruning followed by HC application on observed fruitfulness. Means are separated with different letter(s) for the respective day of observation using Duncan's multiple range test at 5%.

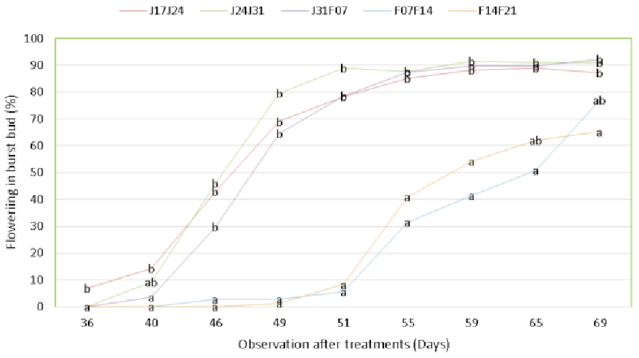


Fig. 3 - Effect of different timing of pruning followed by HC application on observed fruitfulness. Means are separated with different letter(s) for the respective day of observation using Duncan's multiple range test at 5%.

*Fruitfulness in burst buds.* Flowering percentage in burst bud was insignificant between treatments from the last date of bud observation (Fig. 3). On April 26, the flowering (%) observed in the burst bud was 82.6% with the death of average 10% buds and the remaining bud did not reach the flowering stage. Treatments J17J24, J24J31, J31F07, F07F14, and F14F21 had bud death percentage as 14.28%, 13.41%, 6.98%, 6.25%, and 4% of total burst buds, respectively.

*Yield attributes.* The TSS and TA of berries were significantly different between treatments (Table 4). Early pruned and HC treated vines produced berries with higher TSS and lower TTA. TSS and TTA of J17J24, J24J31, J31F07, and F07F14 were statistically similar to each other while F14F21 had a higher TTA and lower TSS value. The average bunch weight was similar for all treatments (Table 4).

Table 4 - Effect of pruning date followed by HC application on yield attributes of grapevine, Dhading, Nepal, 2021

| Treatments | Total soluble solid (°B) | TTA (g/L tartaric acid) | Average bunch weight |
|---|---|---|---|
| J17J24 | 18.96±0.55 b | 9.93±0.81 a | 69.56±14.31 |
| J24J31 | 19.26±0.59 b | 9.93±0.32 a | 62.86±8.81 |
| J31F07 | 18.84±0.89 b | 10.27±0.94 a | 70.06±6.98 |
| F07F14 | 17.98±0.43 b | 10.87±0.57 a | 74.92±12.04 |
| F14F21 | 16.08±0.71 a | 12.72±0.3 b | 70.05±6.70 |
| Grand mean | 18.22 | 10.75 | 69.5 |
| LSD (5% level) | 1,829 | 1,537 | na |
| F-probability | 0.013 ** | 0.00 ** | 0.96 ns |
| CV (%) | 7.5 | 10.7 | 35.50 |

Mean with the same letter(s) within the column do not differ significantly by DMRT at 5%. Values are μ±SE where μ = Mean and SE = Standard error. LSD=Least Significance Difference. CV = Coefficient of variance. NA= Not applicable.

## 4. Discussion and Conclusions

This study showed the growth and phenological stages were significantly influenced by the date of pruning followed by 5% HC application in grapevine cv. Cabernet Sauvignon. Budburst depends on warm or forcing conditions in some cultivars after endo-dormancy (Keller and Tarara, 2010). The general threshold temperature for shoot development is 10°C. To some extent, the budburst date corresponds to the cumulated temperatures above this threshold (Lebon *et al.*, 2004). Martin and Dunn (2000) found that HC did not significantly affect the times of the onset of budburst, 60% budburst, anthesis or veraison, or fruit maturity at harvest, but interacted significantly with later pruning to delay fruit maturity. Martin and Dunn (2000) reported that six-week differences in pruning time resulted in 5 days differences in budburst time in the cultivar Cabernet Sauvignon in Melbourne, Australia. HC treatment prepone budburst of vine due to increased accumulation of $H_2O_2$, soluble sugar/starch ratio, IAA, and cytoplasmic protein-tyrosine kinase concentration with decreased ABA concentration (Liang *et al.*, 2019). Delayed winter pruning postponed 10-11 days for budburst which reported a possible solution to prevent spring freeze damage (Persico *et al.,* 2021).

In late pruned and HC treated vine, dramatic decrease in budburst was observed. Low budburst is potentially linked to phytotoxic effects caused by late application of HC. As the natural budburst time approaches, buds are succulent and vulnerable to the toxic effect of HC. George and Nissen (1988), George *et al.* (1988), and Shulman *et al.* (1983) also reported the drying out of young shoots due to too early or late application of HC. Early burst bud may dry out as succulent and young shoots have to face the frost. In most fruit trees, the biggest effect of HC reported when applied few weeks before the natural budburst (Pontikis, 1989). While the late application (F14F21) may not have an impact because the chemical resistance reduces quickly after being released from endo-dormancy (Snir, 1988; Klinac *et al.*, 1991). Using two different HC application dates (mid-Dec. and mid-Jan.), Or *et al.* (1999) reported that there was no discernible difference in the budburst (%). In both dates, the budburst was found to be 50% after four weeks and an additional 20% within the following two weeks. Cabernet Sauvignon has a shorter duration of budburst to flowering period as compared to Merlot and Cabernet Franc in Bordeaux, France (Leeuwan *et al.*, 2004). Further they reported that the low yield was typically associated with low budburst rates, while HC influence on grapevine yield has been attributed to its impact on budburst. A high level of budburst would result in an increased shoot number and, therefore, a high yield (Or *et al.*, 1999). The phytotoxic effect of HC acting upon naturally burst tender buds has been reported since its early application (Shulman *et al.*, 1983).

Lower budburst and flowering in late pruned and HC treated vines result in lower fruitfulness. Bud fruitfulness depends upon climatic variables mainly sunshine and daily maximum temperature between 82°F and 90°F and water availability (Williams, 2000). Unfavorable climatic conditions hasten diseases and insect infestation such as anthracnose, Downy mildew, mealy bugs, thrips, and leaf hoppers that result in burst bud mortality (Somkuwar *et al.*, 2021).

Vine productivity is a distinguishing feature of a variety that fluctuates depending on several parameters, such as rootstock, and vine management. The significance of pruning in the grapevine is relatively consistent (Rives, 2000). Martin and Dunn (2000) found that the earlier pruned (7 July) vines matured earlier than the later pruned (17 August) vines, and the mean TSS of the berry was 0.91°B lower for the later pruned vines. Dhakal (2021) found insignificant difference between the average bunch weight of vine pruned at different times followed by HC application

in Kirtipur, Nepal.

This research demonstrated the advancing budburst date through pruning and HC application ultimately advances the harvesting time. Thus, it can be a potential and viable strategy to address the challenges of monsoon coinciding with harvesting time in grapevine growing areas. This study demonstrated that shoot pruning during second fortnight of January followed by 5% HC application advances 2-3 weeks in harvesting of grapevine without compromising in minimum acceptable berry quality. Particularly, in the subtropical conditions of Nepal, it is recommended to apply HC before Feb. 7th to optimize its effects with 5% HC application.

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# Efficacy of active modified atmosphere packaging containing thymol on fortification of antioxidant capacity and reducing the microbial contamination of pomegranate fresh arils

OPEN ACCESS

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**Abstract: Pomegranate arils pose a significant challenge when it comes to preserving their nutritional value and preventing microbial contamination. This study aimed to explore the impact of thymol fumigation and active modified atmosphere packaging (MAP) on enzymatic activity and microbial contamination prevention in pomegranate arils. The results indicated that arils stored in a high $O_2$ atmosphere ($HO_2A$) with thymol had notably different catalase (CAT) and peroxidase (POD) activity levels compared to other treatments. These arils exhibited the highest CAT activity and the lowest POD activity. The highest phenylalanine ammonia-lyase (PAL) activity was observed in arils stored in $HO_2A$ with thymol, although it was not significantly different from those stored in a high $CO_2$ atmosphere ($HCO_2A$) with thymol ($P<0.05$). Arils stored in a low oxygen atmosphere ($LO_2A$) and $HCO_2A$ with thymol showed the highest polyphenol oxidase (PPO) activity levels, while arils in $HO_2A$ with thymol had the lowest. The $HO_2A$ with thymol treatment resulted in the lowest presence of psychrophilic bacteria, although it was not significantly different from arils stored in $LO_2A$ with thymol ($P<0.01$). Based on cluster analysis results, $HO_2A$ with thymol, $LO_2A$ with thymol, and $HCO_2A$ with thymol could be considered the most effective treatments for extending the storage life of packaged pomegranate arils.**

## 1. Introduction

To increase the shelf life of fresh-cut fruits, it is essential to slow down biochemical changes, enzymatic degradation, and microbiological deterio-

ration (Kumar *et al.*, 2020). Fruit respiration, which affects metabolic processes, is the main cause of the majority of the physiological changes (Saltveit, 2019), and decreased respiration indirectly slows down ATP-dependent metabolic activities (Wang *et al.*, 2019).

Stress produced after cutting or processing fruit activates numerous defense mechanisms involved in the production and/or degradation of antioxidant compounds in the fruits (Belay *et al.*, 2019 a). Damage by reactive oxygen species (ROS) or phenolic monomer polymerization during storage could be a factor for the decrease in antioxidant activity (Piretti *et al.*, 1996). It is typical to correlate phenolic compounds' nutritive advantages to their antioxidant activity (Karaat and Serce, 2020). Phenolic molecules have a critical function in minimizing or preventing lipid oxidation as well as scavenging oxygen free radicals and they are extremely sensitive to environmental and biological stresses (Gang *et al.*, 2007). The antioxidant activity is affected by redox characteristics of phenolic compounds as well as their capacity as reducing agents, hydrogen ion donors, singlet oxygen quenchers, or metal ion scavengers (Romadanova *et al.*, 2021).

Due to their nature or environmental factors, pathogenic microbes can survive during the food's shelf life (Caleb *et al.*, 2012). So far, few studies on the impact of modified atmospheres containing essential oil on the quality of pomegranate arils (Banda *et al.*, 2015). According to the researchers' results, passive modified atmosphere packaging (MAP) of pomegranate arils cv. Wonderful effectively preserved overall acceptability (El-Eryan *et al.*, 2020). MAP is effective in preserving bioactive compounds while inhibiting the growth of aerobic microorganisms (Ranjbar and Ramezanian, 2022). In a different study, active MAP increased the quality characteristics such as anthocyanin, vitamin C, and the shelf life of the pomegranate arils (Moradinezhad *et al.*, 2020). When pomegranate arils are stored, their chemical, sensory, and quality characteristics can be impacted by the high non-reducing sugars provided by the active modified atmosphere (Patanè *et al.*, 2019). A gaseous mixture of 2-5% $O_2$ and 10-20% $CO_2$ is advisable during storage pomegranates (Irtwange, 2006). Decreased oxygen slows senescence, ethylene synthesis, and respiration (Pareek *et al.*, 2015). On the other hand, fresh-cut pomegranate quality has been preserved by the application of high $O_2$ concentrations (Guo *et al.*, 2019). Super atmospheric $O_2$ concentration effectively inhibited microbial growth by preventing anaerobic respiration on minimally processed pomegranate arils (cv. Wonderful) (Belay *et al.*, 2017). According to Belay *et al.* (2019 b), $O_2$ had the biggest impact on color, organic acid, the development of decay, and alcoholic volatile organic compounds. In addition to antimicrobial effects, it has been shown that high concentrations of $CO_2$ also control overall quality, such as color, texture firmness and volatile organic compounds (aldehydes, ketones, monoterpenes) of the cv. Wonderful. Since the products are free of chemical residues, they are regarded as organic products and have increased their commercial value (Li *et al.*, 2018; Li *et al.*, 2020). However, in certain circumstances, the modified atmosphere is insufficient to assure product quality and safety (Adiletta *et al.*, 2017). The active packaging was used for this purpose (Serrano *et al.*, 2008). Essential oils are an interesting selection of active components used in antimicrobial packaging (Almenar *et al.*, 2006). On the other hand, the lipophilic characteristics of essential oil slow down oxidative reactions by limiting gas release and respiration rate (Ranjbar *et al.*, 2024).

Carvacrol, cinnamaldehyde, citral, p-cymen, eugenol, limonene, menthol, and thymol, are a few active compounds with antibacterial functions that the United States has registered as food flavorings (Mari *et al.*, 2016). Thymol, also known by its chemical names 2-isopropyl-5-methylphenol and 5-methyl-2-isopropyl-5-methylphenol, is a non-toxic food additive (FDA, 2020) and has antifungal and antimicrobial properties (Reyes-Jurado *et al.*, 2020; Ranjbar *et al.*, 2022). The majority of bio-active additives, especially phenolics such as thymol, carvacrol, and tocopherol, function as antioxidants (Maqsoudlou *et al.*, 2020). The increase in antioxidant capacity caused by the components essential oil has a significant impact on the resistance to pathogens and slows down physiological deterioration. *Aspergillus flavus*, *Candida albicans*, and *Botrytis cinerea* cannot grow in the modified atmosphere, which preserves the bioactive compounds of the fruit (Li *et al.*, 2012). During an investigation, thymol was more effective in preventing strawberry fruit rot compared to eugenol and menthol (Wang *et al.*, 2007).

Since there is no scientific report on the combined effect of MAP and volatile organic compounds on pomegranate arils, this study was conducted to determine the best atmospheric composition, either alone or in combination with thymol, to maintain bioactive characteristics, and antioxidant activity and

extend the shelf life of pomegranate arils performed.

## 2. Materials and Methods

*Fruit selection, storage and treatments applied*

The pomegranates (cv. Rabbab) were harvested from Neyriz orchards (1605 m above sea level, 29°11'55.68" N 54°19'40.08" E), after reaching the mature stage (TSS/TA ≥16). The fruits were transferred to a postharvest lab at Shiraz University. A selection of fruits was made uniform in shape, color, and size. This was followed by disinfecting them in sodium hypochlorite (1%) for five minutes before washing them in distilled water. Pomegranate arils were manually plucked out of peels and mixed before packaging. Fifty g of arils were included in each unit of replication. They were packed in polyethylene + polyester (PE+PES) transparent, having dimensions of 150 × 250 mm, thickness of 90 microns, $CO_2$ transmission rate of 45-50 g/m$^2$/ day/bar, $O_2$ transmission rate of 60-70 g/m$^2$/ day/bar, and water vapor transmission rate of 45 g/m$^2$/ day/bar with three replicates. Then, four atmospheric compositions including 21% $O_2$ + 0.03% $CO_2$ + 78% $N_2$ (Passive- MAP), 5% $O_2$ + 5% $CO_2$ + 90% $N_2$ [Low $O_2$ atmosphere ($LO_2A$)], 70% $O_2$ + 10% $CO_2$ + 20% $N_2$ [High $O_2$ atmosphere ($HO_2A$)], 5% $O_2$ + 20% $CO_2$ ,75% $N_2$ [High $CO_2$ atmosphere ($HCO_2A$)] were selected to store the pomegranate arils. The packaging was performed using a vacuum packing machine (Dz-400 Wenzhou Zhonghuan Packaging Machine Co., Ltd, China) which was connected to a gas mixer. These packages were divided into two groups, with and without thymol (50 mg/L). The thymol applied (Purity ≥ 99%, CAS number 89-83-8) was purchased from Sigma-Aldrich Company.

After storing the samples (5±1°C, 92±3% RH), they were measured for variables every five days.

*Total phenols content (TPC)*

The Folin-Ciocalteu reagent was applied for measuring the TPC (Meyers *et al.*, 2003). Briefly, 100 µL of fruit juice was diluted with distilled water (1:25 ratio). Then, 100 µL sodium carbonate (2%) was added. After 3 minutes, Folin-Ciocalteu reagent (20 µL, 50%) was included and the sample remained for 30 minutes. Sample absorption was measured (750 nm) by a spectrophotometer (Epoch Biotech, Germany). The TPC concentration was reported as gallic acid (g/L fruit juice) (SM Fig. 1S).

*Total anthocyanins content (TAC)*

The anthocyanin concentration was determined by the pH differential method. Briefly, the aril sample extract was mixed with KCl buffer (0.025 M, pH 1.0) and NaOAc buffer (0.4 M, pH 4.5), separately. The absorbance was measured at 510 and 700 nm and the data were reported as mg cyanidin-3-glucoside per liter of fruit juice. For the calculation of TAC, the absorbance value (A) entered Equation. 1:

$$A= (A_{510} - A_{700})\, pH_{1.0} - (A_{510} - A_{700})\, pH_{4.5} \qquad Eq.\ 1$$

TAC based on the concentration of cyanidin-3-glucoside was calculated using Equation 2 (Lako *et al.*, 2007):

$$TAC\ (mg/L) = (A \times MW \times DF \times 1000 / \varepsilon \qquad Eq.\ 1$$

Where A represents absorbance value, MW represents cyanidin-3- glucoside molecular weight (449.2), dilution-factor (DF) (5), and ε (26,900) stands for the molar absorptive coefficient of cyanidin-3-glucoside.

*Extraction of enzymatic extract*

The amount of 500 mg of homogenized pomegranate arils in 50 mM potassium phosphate buffer (pH 7.2) containing 1% polyvinylpyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 32869 × *g* for 15 min at 4°C. Every step of the enzyme extraction process was carried out on ice. Enzymatic tests for catalase, peroxidase, polyphenol oxidase, and total soluble protein were conducted using the supernatant.

*Catalase (CAT) activity*. For the assay, a mixture consisting of 50 mM potassium phosphate buffer (pH 7.2), 30 mM hydrogen peroxide and crude extract was prepared and its absorbance measured at 240 nm using a spectrophotometer (UV-visible spectrophotometer, Dynamic Halo VIS-20 single beam, UK). Enzyme activity was described as the decrease in absorbance over time per U/mg protein by measuring the rate of conversion of hydrogen peroxide into water and oxygen molecules (Sun *et al.*, 2013).

*Peroxidase (POD) activity*. For the assay, a mixture of 50 mM potassium phosphate buffer (pH 7.2), hydrogen peroxide (% 1), guaiacol (4%) and crude extract was prepared and its absorbance measured at 470 nm using a spectrophotometer (UV-visible spectrophotometer, Dynamica Halo VIS-20 single beam, UK). The enzyme activity was expressed as delta

absorbance after 1 min reaction at 470 nm per U/mg protein (Sun *et al.*, 2013).

*Polyphenol oxidase (PPO) activity.* For the assay, a mixture consisting of 50 mM potassium phosphate buffer (pH 7), 0.02 M pyrocatechol solution, and the crude extract was prepared and its absorbance was measured at 420 nm using a spectrophotometer (UV-visible spectrophotometer, Dynamica Halo VIS-20 single beam, UK). The enzymatic activity was expressed as U/mg of protein (Silva and Koblitz, 2010).

*Phenylalanine ammonia-lyase (PAL) activity*. Extracts prepared from 500 mg homogenized pomegranate arils in 50 mM of sodium borate buffer (pH 8.8), 5 mM $\beta$-mercapto-ethanol, and 1% PVP buffer, followed by centrifugation at 28341×*g* at 4 °C for 20 min and the supernatant was used for enzyme assays. For assay, a mixture consisting of sodium borate buffer (pH 8.8), and 20 mM L-phenylalanine, and crude extract was incubated at 37°C for 60 min. The reaction was stopped by addition of 6 mol/L HCl. The absorbance of the samples before and after incubation at 290 nm was measured by spectrophotometer (UV-visible spectrophotometer, Dynamica Halo VIS-20 single beam, UK). The enzymatic activity was expressed as per U/mg of protein (Liu *et al.*, 2016). Total soluble protein was measured using the Bradford (1976) method. One mL of Bradford reagent with 100 µL enzymatic extract was mixed completely and its absorption measured at 595 nm. Protein content was estimated using calibration curve of bovine serum albumin (BSA) (SM Fig. 2S) (Bradford, 1976).

*Hydrogen peroxide ($H_2O_2$) content*. To measure the $H_2O_2$ content, 500 mg of pomegranate arils were homogenized with 5 mL of trichloroacetic acid (TCA) (1% w/v) and was centrifuged at 24149×*g* for 15 min. Then, the supernatant was mixed with 10 mM potassium phosphate buffer (pH 7) and 1 mM potassium iodide, and its absorbance at 390 nm was detected using a microplate spectrophotometer (Microplate spectrophotometer, Epoch Biotech, Germany). The standard curve of different concentrations of $H_2O_2$ was used to calculate the $H_2O_2$ content and was expressed as mmol/L fruit juice (Nukuntornprakit *et al.*, 2015).

*Determination of microbial contamination*

A stomacher was used for one minute to homogenize 10 g of pomegranate arils with 90 mL of physiological solution (0.9%). Dilutions (0.01, 0.001, and 0.1) were made using physiological solutions. For both aerobic mesophilic and psychrophilic bacteria, microbial culture was carried out on plate count agar medium (PCA). For mold and yeast, it was carried out on yeast extract glucose chloramphenicol agar (YGC Agar). Every step was performed in a sterile environment using two duplicates of every dilution. Molds and yeasts were incubated at 25±1°C for five days (ISO, 2008), aerobic mesophilic bacteria at 37±1°C for 48 hours, and psychrophilic bacteria at 6.5±1°C for five days (NP-4405, 2002). Log CFU per gram of pomegranate arils was used to calculate the number of microbial colonies.

*Sensory quality*

Overall acceptance test (flavor, color, and texture) carried out by 10 trained panelists provided hedonic evaluations (Test aimed at measuring the overall hedonic perception of a product by consumers). Quality scores defined based on 5= highest quality score, 3= limit of acceptance and 1= poorest quality value (Watts *et al.*, 1989).

*Statistical analysis*

The experiment was conducted as a three-factor factorial design, including different atmosphere compositions (21% $O_2$ + 0.03% $CO_2$ + 78% $N_2$, 5% $O_2$ + 5% $CO_2$ + 90% $N_2$, 70% $O_2$ + 10% $CO_2$ + 20% $N_2$ and 5% $O_2$ + 20% $CO_2$ +75% $N_2$), concentrations of thymol (0 and 50 mg/L), and storage period (0, 5, 10, 15, 20 and 25) arranged according to a completely randomized design (CRD) based on a completely randomized design (CRD) , having three replicates. SAS software enabled the analysis of variance (Two-Way ANOVA). Mean values were evaluated for significant differences by Duncan's multiple range test ($P \leq 0.05$). Principal component analysis (PCA) was performed using the factoMineR ver. 2.4 package to explain the relationship between the different measured parameters. Cluster analysis was performed using the factoextra package for data-mining and grouping treatments which were more similar to each other.

## 3. Results

*TPC and TAC*

The results showed statistical significance in the main effects and reciprocal effects of two and three-fold treatments on TPC ($P<0.01$) (Table 1). On the 15$^{th}$ day of storage, when all treated arils were consumable, the highest TPC (796.13 mg GAE/L)

occurred in arils packaged under the $HO_2A$ containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. TPC in arils packaged with $HO_2A$ containing thymol was 25.04%, 21.51%, and 6.55% more than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. TPC in arils packaged with $HO_2A$ containing thymol was 28.55% more than $HO_2A$ without thymol (Fig. 1A).

The results showed statistical significance main effects and reciprocal effects of two and three-fold treatments on TAC (P<0.01) (Table 1). On the 15th day of storage, when all treated arils were consumable, the highest TAC (154.53 mg/L) occurred in arils packaged with $HO_2A$ containing thymol, although it had no statistical significance (P<0.01) compared to arils packaged with $HCO_2A$ containing thymol. TAC in arils packaged with $HO_2A$ containing thymol was 17.69%, 9.75%, and 7.15% higher than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. TAC in arils packaged with $HO_2A$ containing thymol was 15.93% more than $HO_2A$ without thymol (Fig. 1B).

Table 1 - Results of variance analysis for the effect of MAP, Thymol and storage time on the TPC and TAC of pomegranate aril

| Source of variations | Degrees of Freedom (df) | Mean of squares | |
|---|---|---|---|
| | | TPC | TAC |
| Storage time (S) | 5 | 2715771.76 ** | 46557.58 ** |
| MAP | 3 | 13991.19 ** | 1542.59 ** |
| Thymol (T) | 1 | 757373.01 ** | 63881.39 ** |
| S × MAP | 15 | 1661.14 ** | 166.41 ** |
| S × T | 5 | 95210.57 ** | 12444.42 ** |
| MAP × T | 3 | 2183.04 ** | 125.56 ** |
| MAP × T × S | 15 | 2365.83 ** | 122.26 ** |
| Error | 72 | 274.21 | 27.98 |
| C.V. (%) | — | 2.01 | 4.14 |

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

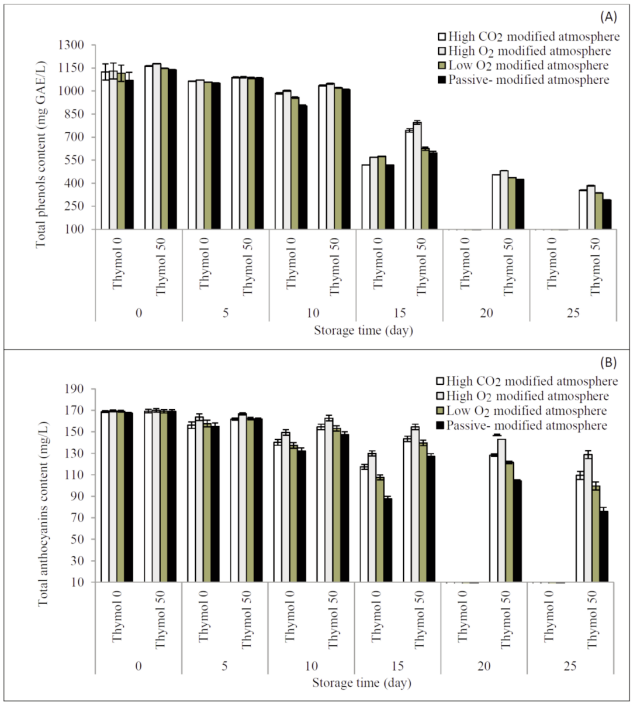


Fig. 1 - Interaction effects of modified atmosphere, thymol and storage time on TPC (A) and TAC (B) of pomegranate arils. Data are the mean ± SE (n=3). Vertical bars represent the standard errors of the means. Duncan's multiple

*CAT, POD, PPO and PAL activity*

Statistical significance was observed in the main effects and reciprocal effects of two and three-fold treatments on CAT activity (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were con-

Table 2 - Results of variance analysis for the effect of MAP, Thymol and storage time on the antioxidant enzymes activity and $H_2O_2$ content of pomegranate aril

| Source of variations | Degrees of freedom (df) | Mean of squares | | | | |
|---|---|---|---|---|---|---|
| | | CAT | POD | PPO | PAL | $H_2O_2$content |
| Storage time | 5 | 33495.00 ** | 1205.78 ** | 326.27 ** | 1129.05 ** | 195.40 ** |
| MAP | 3 | 2166.53 ** | 238.75 ** | 112.78 ** | 124.03 ** | 8.56 ** |
| Thymol | 1 | 16966.43 ** | 2809.24 ** | 737.47 ** | 471.44 ** | 308.61 ** |
| Storage time× MAP | 15 | 149.33 ** | 59.42 ** | 11.14 ** | 12.95 ** | 1.16 ** |
| Storage time× Thymol | 5 | 1171.73 ** | 1209.52 ** | 574.24 ** | 52.62 ** | 361.15 ** |
| MAP× Thymol | 3 | 217.47 ** | 105.69 ** | 13.10 ** | 10.22 * | 0.34 ns |
| MAP× Thymol × Storage time | 15 | 58.90 ** | 48.00 ** | 8.75 ** | 6.56 * | 1.68 ** |
| Error | 72 | 24.00 | 1.74 | 2.30 | 3.60 | 0.24 |
| C.V. (%) | — | 6.11 | 6.09 | 10.37 | 10.10 | 3.64 |

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

sumable, the highest activity (89.16 U/mg protein) occurred in arils packaged under the $HO_2A$ containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. CAT activity in arils packaged with $HO_2A$ containing thymol was 40.28%, 23.71%, and 16.53% higher than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. CAT activity in arils packaged with $HO_2A$ containing thymol was 31.85% more than $HO_2A$ without thymol (Fig. 2A).

Statistical significance was observed in the main effects and reciprocal effects of two and three-fold treatments on POD activity (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were consumable, the lowest activity (25.00 U/mg protein) occurred in arils packaged under the $HO_2A$ containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. POD activity in arils packaged with $HO_2A$ containing thymol was 33%, 11%, and 11% lower than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. POD activity in arils packaged with $HO_2A$ containing thymol was 20.92% lower than $HO_2A$ without thymol (Fig. 2B).

Statistical significance was observed in the main effects and reciprocal effects of two and three-fold treatments on PPO activity (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were consumable, the lowest activity (13.90 U/mg protein) occurred in arils packaged under the $HO_2A$ containing thymol, although it had no statistical significance (P< 0.01) compared to arils packaged with $LO_2A$ and $HCO_2A$ containing thymol. PPO activity in arils packaged with $HO_2A$ containing thymol was 51.65%, 18.92%, and 15.82% lower than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. PPO activity in arils packaged with $HO_2A$ containing thymol was 32.73% lower than $HO_2A$ without thymol (Fig. 2C).

Statistical significance was observed in the main effects and reciprocal effects of two-fold (except for the modified atmosphere × thymol interaction effect) on PAL activity (P<0.01), whereas the reciprocal effects of three-fold treatments were significant at P< 0.05 (Table 2). On the 15th day of storage, when all treated arils were consumable, the highest activity (23.41 U/mg protein) occurred in arils packaged under the $HO_2A$ containing thymol, although it had no statistical significance (P< 0.05) compared to those packaged with $HCO_2A$ containing thymol. PAL activity in arils packaged with $HO_2A$ containing thymol was 35.36%, 27.29%, and 13.24% higher than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. The PAL activity in arils packaged with $HO_2A$ containing thymol was 18.45% higher than $HO_2A$ without thymol (Fig. 2D).

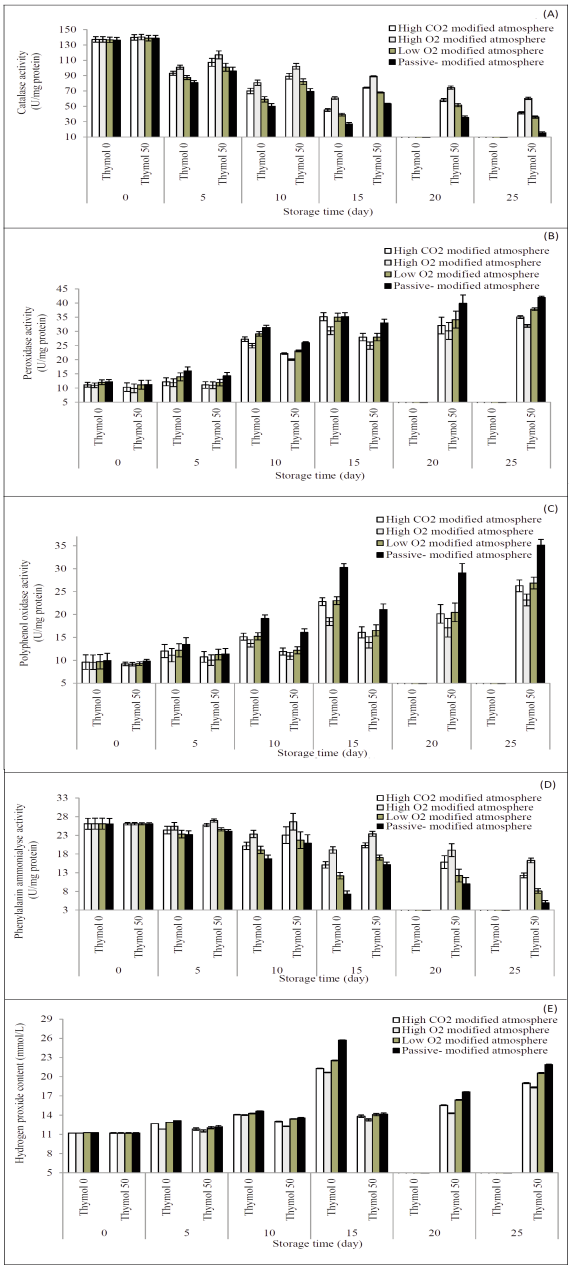


Fig. 2 - Interaction effects of modified atmosphere, thymol and storage time on CAT activity (A), POD activity (B), PPO activity (C), PAL activity (D) and $H_2O_2$ content (E) of pomegranate arils. Data are the mean ± SE (n=3). Vertical bars represent the standard errors of the means. Duncan's multiple range test (P<0.01). Interaction effects of modified atmosphere, thymol and storage time on PAL activity (D) of pomegranate arils. Data are the mean ± SE (n=3). Duncan's multiple range test (P<0.05).

*$H_2O_2$ content*

Significant effects were observed in both the main effects and reciprocal effects of two-fold treatments (except for modified atmosphere × thymol which was not significant), and reciprocal effects of three-fold on $H_2O_2$ content ($P<0.01$) (Table 2). On the 15th day of storage, when all treated arils were consumable, the least amount of $H_2O_2$ (13.25 mmol/L) occurred in arils packaged under the $HO_2A$ containing thymol, although it had no statistical significance ($P<0.01$) compared to those packaged with passive MAP, $LO_2A$, and $HCO_2A$ containing thymol. $H_2O_2$ level in arils packaged with $HO_2A$ containing thymol was 6.86%, 6.26%, and 4.37% lower than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. $H_2O_2$ level in arils packaged with $HO_2A$ containing thymol was 55.92% lower than $HO_2A$ without thymol (Fig. 2E).

*Microbial contamination*

Significant effects were observed in both the main effects and reciprocal effects of two-fold treatment (except for modified atmosphere × storage time and modified atmosphere × thymol, which were not significant) on aerobic mesophilic bacteria at $P<0.05$ and $P<0.01$, respectively (Table 3). On the 15th day of storage, when all treated arils were consumable, the lowest number of aerobic mesophilic bacteria (1.20 Log CFU/g) occurred in arils packaged under the $HO_2A$ containing thymol, there was no significant difference between arils packaged in $HO_2A$, $LO_2A$, and $HCO_2A$ containing thymol simultaneously ($P<0.05$). The number of aerobic mesophilic bacteria in arils packaged with $HO_2A$ containing thymol was 21.36%, 17.5%, and 12.5% lower than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. The number of aerobic mesophilic bacteria in arils packaged with $HO_2A$ containing thymol was 12.5% lower than $HO_2A$ without thymol (Fig. 3A).

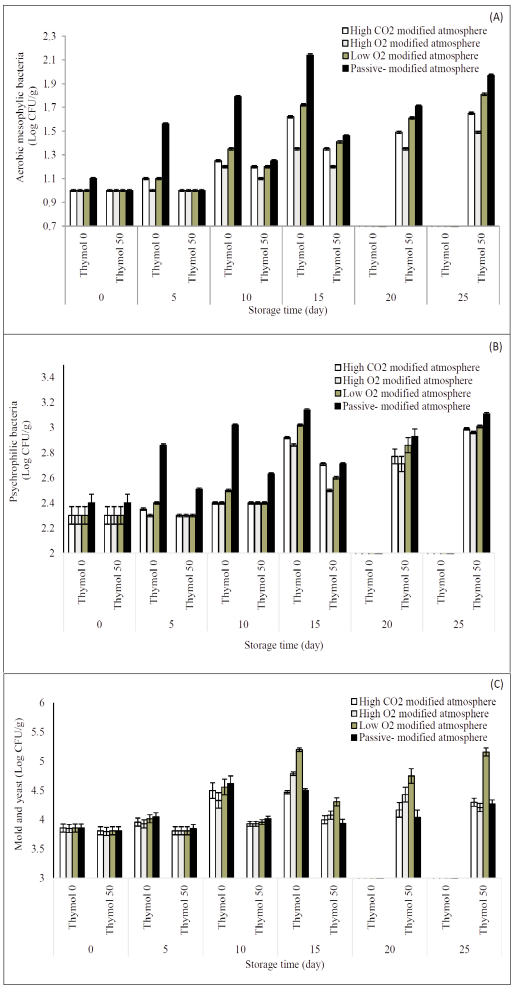


Fig. 3 - Interaction effects of modified atmosphere, thymol and storage time on aerobic mesophilic bacteria (A) of pomegranate arils. Data are the mean ± SE (n=3). Duncan's multiple range test ($P<0.05$). Interaction effects of modified atmosphere, thymol and storage time on psychrophilic bacteria (B) and mold and yeast (C) of pomegranate arils. Data are the mean ± SE (n=3). Vertical bars represent the standard errors of the means.

Table 3 - Results of variance analysis for the effect of MAP, Thymol and storage time on the microbial load of pomegranate aril

| Source of variations | Degrees of Freedom (df) | Mean of squares | | |
|---|---|---|---|---|
| | | Aerobic mesophilic bacteria | Psychrophilic bacteria | Mold and yeast |
| Storage time | 5 | 20.40 ** | 0.78 ** | 7.71 ** |
| MAP | 3 | 0.33 ** | 0.56 ** | 0.32 ** |
| Thymol | 1 | 50.39 ** | 0.05 * | 21.67 ** |
| Storage time× MAP | 15 | 0.02 * | 0.49 ** | 0.04 ** |
| Storage time× Thymol | 5 | 11.79 ** | 0.38 ** | 13.24 ** |
| MAP× Thymol | 3 | 0.03 NS | 0.92 ** | 0.03 ** |
| MAP× Thymol × Storage time | 15 | 0.03 * | 0.45 ** | 0.04 ** |
| Error | 72 | 0.01 | 0.01 | 0.006 |
| C.V. (%) | — | 8.05 | 4.11 | 2.09 |

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

We observed statistical significance in the main effects and reciprocal effects of two and three-fold treatments on psychrophilic bacteria (P<0.01) (except for the effects of thymol, which were significant at P<0.05) (Table 3). On the 15th day of storage, when all treated arils were consumable, the lowest number (2.50 Log CFU/g) occurred in arils packaged under the $HO_2A$ containing thymol, although it had no statistical significance (P<0.01) compared to those packaged with $LO_2A$ containing thymol. The number of psychrophilic bacteria in arils packaged with $HO_2A$ containing thymol was 8.14%, 4%, and 8.4% lower than passive MAP, $LO_2A$, and $HCO_2A$ containing thymol, respectively. Thymol reduced the number of psychrophilic bacteria in arils packaged in $HO_2A$ by 14.4% compared to $HO_2A$ without thymol (Fig. 3B).

We observed statistical significance in the main effects and reciprocal effects of two and three-fold treatments on mold and yeast (P<0.01) (Table 3). On the 15th day of storage, when all treated arils were consumable, the lowest number (3.94 Log CFU/g) occurred in arils packaged under the passive MAP containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. The number of mold and yeast in arils packaged with passive MAP containing thymol was 3.43%, 8.58%, and 1.5% lower than $HO_2A$, $LO_2A$, and $HCO_2A$ containing thymol, respectively. Thymol reduced the number of mold and yeast in arils packaged in passive MAP containing thymol by 12.4% compared to passive MAP without thymol (Fig. 3C).

*Overall acceptance*

Acceptability had a similar pattern and decreased during cold storage. The variance results indicated that with the exception of the interaction effect of the modified atmosphere × thymol and the three-fold interaction effects, which was not significant, both the main effect and the two-fold interaction effects on acceptability were significant (P<0.01) (Table 4). The highest quality score (4.50) was recorded in arils packaged in $HO_2A$ containing thymol and the lowest quality score (2) was recorded in arils packaged in passive MAP without thymol on the fifteenth day of storage, when all treatments were edible, with significant (P<0.01) differences from the other treatments at the same time. Acceptability in arils packaged in $HO_2A$ containing thymol was 26%, 17%, and 6% higher than passive MAP, $LO_2A$, $HCO_2A$ containing thymol, respectively. Acceptability in arils packaged in $HO_2A$ containing thymol was 10% more than in $HO_2A$ without thymol (Fig. 4).

Table 4 - Results of variance analysis for the effect of MAP, Thymol and storage time on the acceptability of pomegranate aril

| Source of variations | Degrees of Freedom (df) | Mean of squares |
|---|---|---|
| | | Acceptability |
| Storage time (S) | 5 | 32.59 ** |
| MAP | 3 | 16.91 ** |
| Thymol (T) | 1 | 5.77 ** |
| S × MAP | 15 | 2.11 ** |
| S × T | 3 | 2.22 ** |
| MAP × T | 3 | 0.21 NS |
| MAP × T × S | 9 | 0.15 NS |
| Error | 360 | 0.45 |
| CV (%) | — | 16.54 |

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

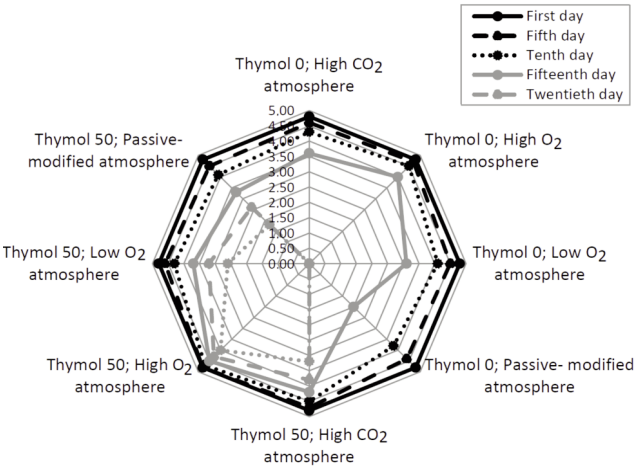


Fig. 4 - Mean comparison effects of treatment at 5°C on ready-to-eat pomegranate arils acceptability during storage.

Using a correlation matrix designed from measured characteristics across various treatments PCA was performed. With 94.71% of the variance covered, the two principal components (PC1 and PC2) are shown in figure 5. Only 3.91% of the total variance was explained by PC2, whereas PC1 was estimated to account for the maximum amount at 90.80%. As shown in figure 5, there was a close correlation between the contents of TPC and TAC as well as the activities of CAT and PAL. These attributes were apparently associated with passive MAP, $LO_2A$, $HO_2A$, $HCO_2A$ containing thymol and $HO_2A$ without thymol. Increases in the bioactive compounds and antioxidant activity were related to the enhancement of shelf life of ready-to-eat pomegranate arils. A close relationship existed among POD activity, PPO activity, $H_2O_2$ content, number of aerobic mesophilic

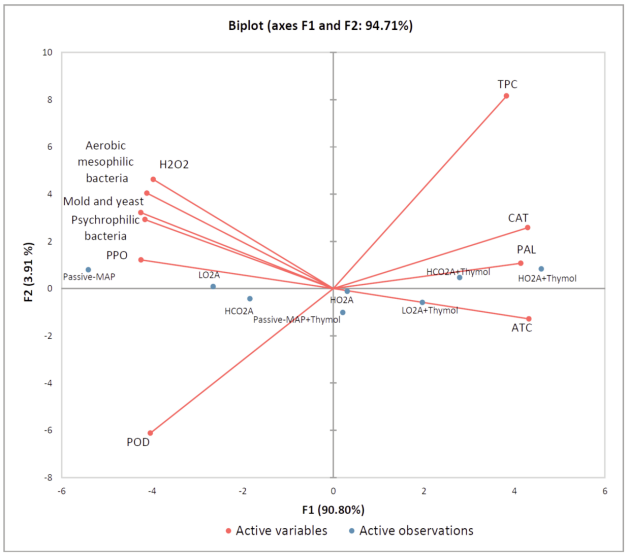


Fig. 5 - PCA of measured attributes of ready-to-eat pomegranate arils with different treatments.

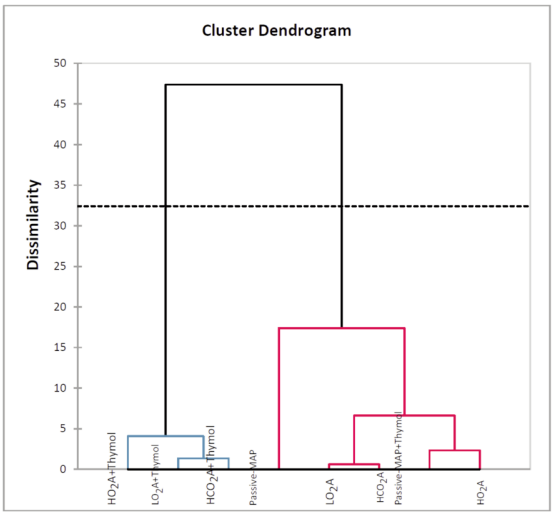


Fig. 6 - Dendrogram of 8 modified atmospheres based on evaluated traits by ward method.

bacteria, psychrophilic bacteria and yeast and mold. These attributes were apparently associated with passive MAP, $LO_2A$, $HCO_2A$ without thymol. Increases in POD and PPO activity levels, $H_2O_2$ content and microorganism's contamination was negatively related to the shelf life of pomegranate arils (Fig. 5).

Overall, PCA analysis showed that the relative variables were affected by MAP, although, the effects of passive MAP containing thymol, modified atmospheres containing thymol and $HO_2A$ without thymol were more, because these treatments showed a close relationship with bioactive compounds and antioxidant systems. The results of PCA and biplot diagram were consistent with the grouping obtained from cluster analysis. Cluster analysis divided the treatments into two main groups in terms of similarity of the evaluated traits. Treatments of $LO_2A$ containing thymol, $HO_2A$ containing thymol, and $HCO_2A$ containing thymol were in one group, and the rest of the treatments were in another group. According to the results of groupings and the importance of traits in postharvest storage, the $LO_2A$, $HO_2A$, and $HCO_2A$ containing thymol could be considered the best atmospheric composition (Fig. 6).

## 4. Discussion and Conclusions

Changes in postharvest storage conditions can lead to abiotic stress and the synthesis or accumulation of polyphenols (Senica *et al.*, 2018). HO2A influences the metabolism of secondary compounds and results in the synthesis or accumulation of phenolic compounds (Zheng *et al.*, 2007). The findings of our research were consistent with those reported by Zheng and colleagues, who observed a higher TPC in high $O_2$-treated Chinese bayberries from day 6 until the end of storage (Zheng *et al.*, 2008). Myrtle fruits exposed to 60-80% $O_2$ showed higher TPC and quality characteristics compared to fruit stored in passive MAP, indicating the positive impact of $HO_2A$ on fresh produce (Fadda *et al.*, 2017). Storing strawberries at low temperatures and in $HO_2A$ (90 kPa) maintained the phenolic content, improved the antioxidant capacity, and enhanced the quality of the fruit for up to 20 days (Van de Velde *et al.*, 2019 a, b). Our results suggest that $HO_2A$ preserved the polyphenol content and cellular integrity by reducing the levels of superoxide and $H_2O_2$ (Yang *et al.*, 2020). The phenolic compounds of pomegranate cv. wonderful packed in $HO_2A$ were found to be higher than those in passive MAP, according to our results (Belay *et al.*, 2017). Additionally, cinnamaldehyde was shown to maintain higher levels of phenolic compounds in ready-to-eat pomegranate arils (Ranjbar and Ramezanian, 2022). Thymol seems to act as a signaling molecule that increases the TPC by generating a signal similar to mild stress.

Under modified atmospheric conditions, the stability of anthocyanins is higher due to lower oxidation (Banda *et al.*, 2015). Moradinezhad *et al.* (2020) found that pomegranates packaged in $HO_2A$ had a higher anthocyanin content by the end of the storage

time. Storage of blackberries, sweet cherries, cherries and strawberries in the modified atmosphere compared to the normal atmosphere increased in anthocyanin content (Dziedzic *et al.*, 2020). The production of anthocyanin in blood orange fruits stored at $HO_2A$ (70%) is a known physiological response to oxidative stress (Baenas *et al.*, 2014). Similar changes in anthocyanin accumulation and PAL activity indicate that the anthocyanin biosynthesis pathway is controlled by PAL through the supply of cinnamic acid (Dziedzic *et al.*, 2020). The decrease in total anthocyanin during storage is due to hydrolytic reactions that convert anthocyanin glycosides to chalcones, which are degraded to phenolic acid aldehydes (Aguilera *et al.*, 2016). Essential oils reduce the reactivity of anthocyanins with $O_2$ by saturating the inner space of the package.

SOD, CAT and POD are responsible for inhibition of free radicals related to low temperature stress (Ahmad, 2014). Ayhan and Esturk, 2009 found that an increase in antioxidant activity was reported in pomegranate arils cv. Hicaznar stored under $HO_2A$ (70 kPa). At $HO_2A$, the activity of $H_2O_2$ inhibitors, including CAT and SOD was higher (Liu and Wang, 2012). Our results suggest that CAT activity is related to the content of phenolic acids (Liu *et al.*, 2021) and similar results have been obtained for kiwifruit (Liu *et al.*, 2019) and dragon fruit (Pasko *et al.*, 2021).

Low-temperature stress in pomegranate fruit is linked to the production of oxygen free radicals such as superoxide and $H_2O_2$. Therefore, to prevent oxidative stress damage at low temperatures, alternative respiratory systems or the inhibition and decomposition of toxic substances are necessary (Fung *et al.*, 2004). Essential oil, known for its high antioxidant properties, appears to be effective in delaying the lipid peroxidation process and inhibiting oxygen free radicals (Rodriguez-Garcia *et al.*, 2016). Current results, using the electron spin resonance and oxygen radical absorbance capacity (ORAC) assays, have shown that thymol has the ability to increase enzymatic and non-enzymatic antioxidants to inhibit oxygen free radical production in fruit tissue (Wang *et al.*, 2007). Consistent with our findings, the activity of antioxidant enzymes increased in mangos packaged with MAP containing thymol (Perumal *et al.*, 2017). Additionally, cinnamaldehyde increased antioxidant capacity and delayed the reduction in nutritional quality of citrus fruit (Gao *et al.*, 2018). Both carvacrol and anethole increased SOD and CAT activity in raspberries (Jin *et al.*, 2012), and grapefruit extract increased catalase activity in grapes (Xu *et al.*, 2019). It appears that essential oils stimulate the antioxidant mechanism or the production of secondary metabolites and increase antioxidant capacity.

POD is a crucial enzyme in fruit tissue browning that utilizes $H_2O_2$ as a catalyst for the oxidation of phenolic compounds (Singh *et al.*, 2018). It accelerates the breakdown of phenols when PPO is present (Richard-Forget and Gauillard, 1997). Our findings indicate that reducing $H_2O_2$ levels to increase access to high $O_2$ helps maintain polyphenolic content, cell integrity, and decreases POD activity (Yang *et al.*, 2020). $HO_2A$ also delays the peak activity of POD by preventing oxidative stress (Wang *et al.*, 2020).

It has been reported that higher activity of POD and PPO in melon is related to metabolic activity and accelerated respiration rate (Menon and Ramana Rao, 2012). The production of oxygen free radicals and cell membrane damage lead to the reaction of phenolic compounds and PPO, which leads to tissue browning (Mishra *et al.*, 2012). High levels of $O_2$ could reduce browning and inhibit PPO and POD activity which was in accordance with previous research (Li *et al.*, 2014). One of the main purposes of using essential oils is to delay the activity of PPO enzyme and prevent browning (Marandi *et al.*, 2010).

Microorganisms cause damage to the structure of the cell membrane, leading to the proximity of phenolic compounds and the enzyme PPO, resulting in browning. Essential oils can delay the activity of PPO and prevent the browning of fruit tissue by reducing microorganisms and membrane damage (Marandi *et al.*, 2010). PPO activity is inhibited at low pH (Hithamani *et al.*, 2018), and essential oils can reduce the activity of PPO by lowering the pH. Additionally, the antioxidant activity of essential oils can decrease the decomposition rate of pigments and prevent the browning of fruits caused by PPO activity (Serrano *et al.*, 2005). Treatment of grapefruit with grapefruit extract has been shown to prevent the increase of PPO (Xu *et al.*, 2009).

PAL activity leads to an increase in the synthesis of polyphenolic phytoalexins, which results in a decrease in the oxidation of phenolic substrates by reducing the activity of PPO (Galani *et al.*, 2017). The high ratio of PAL to PPO leads to the accumulation of phenols and increased activity of the antioxidant system, resulting in less accumulation of ROS. This also helps maintain membrane integrity by preventing the peroxidation of unsaturated fatty acids, ultimately reducing pomegranate browning (Martinez-Espla *et*

*al.*, 2018). Therefore, $HO_2A$ is effective in reducing the enzymatic browning of arils during storage by boosting the activity of the antioxidant system and increasing the PAL to PPO activity ratio (Martinez-Espla *et al.*, 2018). Our findings suggest that thyme essential oil enhances the activity of the PAL enzyme in avocado fruit (Assis *et al.*, 2001).

The low amount of $H_2O_2$ in fruits packed in $HO_2A$ is related to the mechanism of $H_2O_2$ inhibitory enzymes and non-enzymatic antioxidant. Research shows that the activity of $H_2O_2$ inhibitory enzymes is higher at high $O_2$ concentrations (Liu and Wang, 2012). Mitochondrial dysfunction due to the accumulation of ROS is the leading causes of senescence (Qin *et al.*, 2009). In the normal atmosphere, anthocyanins and phenol decreases due to increased activity of PPO, POD and accumulation of $H_2O_2$ (Luo *et al.*, 2017).

According to our results, the lowest number of aerobic mesophilic bacteria was observed in $HO_2A$ and $HCO_2A$, whereas the highest number was in the normal atmosphere (Moradinezhad *et al.*, 2020). Since the growth of anaerobic microorganisms occurs at very low $O_2$ levels and the growth of aerobic microorganisms happens at atmospheric $O_2$ concentrations (around 21 kPa), $HO_2A$ inhibits both aerobic and anaerobic microorganisms. The inhibitory effect of $HO_2A$ on aerobic mesophilic bacteria is linked to the toxicity of high $O_2$ concentrations (Tomas-Callejas *et al.*, 2011), which can cause damage to DNA and nucleoproteins in microorganisms (Moradas-Ferreira *et al.*, 1996). Additionally, the reduction of microbial load in $HO_2A$ is attributed to ROS produced at a partial pressure of $O_2$ (Zhang *et al.*, 2013), which damages the antioxidant system of microorganisms. Our results also show a decrease in the number of aerobic mesophilic bacteria in minimally processed pomegranates cv. Hicaznar under $HO_2A$ (70 kPa) (Ayhan and Esturk, 2009).

According to our results, $HO_2A$ reduced the psychrophilic bacteria in melon slices (Oms-Oliu *et al.*, 2008). In products prone to mold growth, high oxygen has a strong inhibitory effect on mold growth (Rojas-Grau *et al.*, 2009). Our findings indicate that modified atmospheric packaging of pomegranate arils has reduced the number of molds and yeasts at 5°C (Ayhan and Esturk, 2009). $HCO_2A$ is effective in inhibiting aerobic microorganisms, especially gram-negative bacteria and molds, but is not very effective in inhibiting yeasts (Al-Ati and Hotchkiss, 2002). Inhibition of mold growth at a 10% $CO_2$ concentration has been reported, but no fungicidal effect was observed (Poubol and Izumi, 2005).

Thymol is a natural volatile monoterpenoid phenol and the main active ingredient in the oil extracted from the species *Thymus vulgaris* L. The antimicrobial activity of essential oils is attributed to their high monoterpenes content, which have antibacterial and antifungal properties (Bouaziz *et al.*, 2009). Our research revealed that cinnamaldehyde in arils stored in a modified atmosphere significantly reduced microbial agents (Ranjbar and Ramezanian, 2022). Generally, essential oils are more effective at low pH levels. At low pH, the hydrophobic nature of essential oils increases, allowing them to easily dissolve in cell membrane lipids and cause the leakage of cell contents (Burt, 2004).

Storage at $HO_2A$ prevents enzymatic browning and flavor changes due to control of anaerobic conditions (López-Gálvez *et al.*, 2015). Our findings suggest that hot air treatment and a modified atmosphere containing pure $O_2$ and pure $CO_2$ on pomegranate arils show that the modified atmosphere containing 80% $O_2$ + 20% $N_2$ and the heat treatment at 45 °C, compared to a modified atmosphere containing 20% $CO_2$ + 80% $N_2$ and the heat treatment at 55 °C, had a better effect on physicochemical properties and pomegranate quality (Maghoumi *et al.*, 2013). Additionally, the quality of cherries (Wang *et al.*, 2014) and blood oranges (Molinu *et al.*, 2016) was affected by $HO_2A$. On the other hand, the antioxidant properties of strawberry fruit were improved by $HO_2A$ (Odriozola-Serrano *et al.*, 2010), which aligns with our findings. Our results also indicate that thyme oil has a positive effect on the quality and overall acceptance of organic bananas (Vilaplana *et al.*, 2018). The main advantage of essential oils is their strong antioxidant properties that prevent changes in taste due to the release of free radicals (Dorman and Deans, 2000).

According to our results, the combined application of thymol and eugenol in the passive MAP of cherries had no organoleptic effect (Serrano *et al.*, 2005).

The results of this research demonstrated that MAP, especially MAP with a high $O_2$ concentration, is a valuable technique for maintaining the nutritional quality, and antioxidant activity, and controlling the microbial load of pomegranate arils within the acceptable range for commercial purposes. A synergistic effect was found when using $HO_2A$, which contains thymol, on the qualitative characteristics of ready-to-eat pomegranate arils. This includes pre-

serving phenolic compounds, antioxidant enzymatic activity, delaying enzymatic browning, and maintaining visual appearance. Additionally, the application of $HO_2A$ packaging is effective in preserving bioactive compounds that help maintain fruit quality, appearance, taste, and health-promoting properties.

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*Review paper*

# Future prospects and challenges in developing saline-tolerant banana

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*Key words*: Banana, breeding, genetic diversity, saline-stress, transgenesis.

**Abstract: Banana (*Musa* spp.) is an essential fruit with high economic value worldwide. One of the main environmental constraints in banana cultivation is abiotic stress on marginal land caused by salinity stress. This salinity stress can significantly reduce banana productivity and even cause plant death. Global climate change due to warming and reduced rainfall has increased the number of agricultural areas affected by salinity. This review article will describe current knowledge of biodiversity and various biological responses when banana plants are exposed to saline stress. This article also mentions the challenges in the banana breeding program with the current development of plant breeding and genetic engineering technology that could be used to create saline-tolerant banana accessions, its prospects, and the societal controversy underlying this technology. Thus, the information presented in this article could be a reference in the saline-tolerant banana breeding program.**

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## 1. Introduction

Bananas (*Musa* spp.) are ancient monocotyledonous plants grown in nearly 120 countries in tropical and subtropical regions worldwide. Moreover, banana is also a staple food and a major source of nutrition for more than 500 million people (Wang *et al.*, 2019). After rice, maize, and wheat, bananas are among the world's most important food crops and the second most popular fruit. The worldwide production of bananas (*Musa* spp.) totaled 113.9 million metric tons in 2017 (Evans *et al.*, 2020), highlighting its importance as a substantial crop in global diets and commerce. Banana has a significant market share globally due to their broad appeal (De Langhe *et al.*, 2009; Wang *et al.*, 2021). It is rich in protein, vitamins, and minerals and is a premium export commodity in some countries (Aquil *et al.*, 2012; Ji *et al.*, 2021). Banana consumption worldwide continues to increase year by year. Consequently, the area of banana cultivation will expand as well.

In order to support the growth of the increasing demand for bananas, marginal land has emerged as a viable option for agricultural land development for planting bananas (Kusdianti *et al.*, 2016). However,

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marginal land has relatively poor agricultural yield potential (Dikayani *et al.*, 2017). This land is widely accessible, but its agricultural potential remains largely untapped for agricultural activities. As an illustration, in Indonesia, coastal agricultural areas often face salinity issues attributed to rising sea levels resulting from climate change. Approximately 12.020 million hectares, or 6.20% of Indonesia's total land area, are estimated to be prone to salinity near the coast (Karolinoerita and Annisa, 2020).

Salinity is among the most critical abiotic stressors affecting plant development and productivity worldwide. Salinity is a prominent concern among the abiotic stressors affecting farming (Rao *et al.*, 2016). Salinity induces two types of stress: direct osmotic stress and delayed ion toxicity stress. They will affect plant development directly and indirectly. Osmotic stress occurs when excessive salt concentration disrupts osmotic balance, causing a physiological drought and thus inhibiting plant water uptake (Endris and Mohammad, 2007; Manojkumar *et al.*, 2022).

Salinity problems can arise in coastline areas, dry environments on saline soil and from using low-quality irrigation water. Furthermore, an area with higher water evaporation rates in agricultural lands than rainfall also has a significant amount of land at risk of salinity stress. Salinity stress can cause reduced growth, fruit yield, and quality, which can have significant economic impacts on banana producers. Excessive salinity also increases salt content in the roots, reducing potassium absorption, slowing development, delaying flowering, and reducing crop production (Rao *et al.*, 2016). Developing banana plants with improved salinity tolerance is crucial to ensure food security, especially in regions prone to stress.

Fortunately, some plant accessions possess natural tolerance to environmental stressors, enabling them to withstand adverse conditions. This adaptation relies on the activation of intricate molecular networks responsible for stress detection, signal transduction, metabolite production, and the expression of stress-related genes. Additionally, it is crucial to consider the interplay between signaling molecules to understand the synergistic or antagonistic interactions that influence plant responses to abiotic stressors. We can better understand plant performance under stress conditions by identifying the genes involved in stress tolerance, including those responsible for cellular protection and stress response regulation. This comprehensive understanding is vital for developing effective strategies to enhance plant tolerance to salinity (Nguyen *et al.*, 2018).

To our knowledge, no national or global-scale banana breeding efforts have yet been executed to generate salt-tolerant banana cultivars successfully deployed for utilization by general farmers and the banana cultivation sector. This review explores the importance of banana biodiversity, genetic engineering technology advancements, challenges, and opportunities to improve banana salinity tolerance. It also highlights crucial aspects of banana breeding, identifies the unknown physiological and molecular processes underlying saline stress adaptation, evaluates the current status of banana genetic transformation technology, and discusses the potential application of newly discovered genome editing technology in banana breeding in saline stress adaptation.

## 2. Saline stress response in plants and bananas

Soil water salinity generally restricts plant development by affecting the soil solution's osmotic potential. It hinders plant water uptake and retards plant growth, which is commonly referred to as water deficit due to salinity. Moreover, excess salt entering the plant system through transpiration force can produce ion toxicity and cell damage in plant organs, further reducing plant development (Greenway and Munns, 1980). At the cellular level, high salt concentration could also deactivate plants' main cellular activities (Cuartero *et al.*, 2006; Anusuya and Soorianathasundaram, 2014). Because the osmotic pressure in the soil is higher than in plant cells, excessive soil salt levels can also trigger cell plasmolysis in plants. In addition, plant growth caused by excessive salinity has the following characteristics: (1) plants with low water potential, (2) $Na^+$ and $Cl^-$ ion toxicity, and (3) nutrient imbalance in plants (Dikayani *et al.*, 2017). Next, salinity will also affect plant physiology, including $CO_2$ uptake, protein synthesis, respiration, and phytohormone cycles (Mengel and Kirkby, 1987; Ravi *et al.*, 2013; Rao *et al.*, 2016).

Firstly, saline stress reduced water availability, higher respiration rates, mineral distribution changes, membrane instability, and inability to maintain turgor pressure are some factors that

contribute to poor plant performance due to saline stress (Cuartero *et al.*, 2006; Anusuya and Soorianathasundaram, 2014). Secondly, ionic stress causes the accumulation of *sodium chloride* (*NaCl*) ions in plant cells and tissues. Ionic stress could result in toxicity and physiological disturbances. Thus, salinity has an inhibitory effect on plant development due to the excess of $Na^+$ and $Cl^-$ ions toxicity towards water uptake as well (Cherian and Reddy, 2003; Sharif *et al.*, 2018; Verma *et al.*, 2019; Manojkumar *et al.*, 2022).

Generally, saline-tolerant plants can show one or a combination of processes, such as plant organ robustness, salt excretion, and osmotic adjustment, by producing solutes that can counteract ion accumulation in the tonoplast with more unsaturated fatty acids in the membrane composition (Mengel and Kirkby, 1987). Additionally, plants perform tolerance-related responses such as protein stabilization, osmotic protection, reactive oxygen species (*ROS*) detoxification, and ion homeostasis to adapt to a saline environment, enhancing plant durability (Jacob *et al.*, 2017; Nguyen *et al.*, 2018). Furthermore, protein stabilization compounds for osmotic adjustment, such as Proline (*Pro*), Trehalose (*Tre*), and other soluble sugars, are excellent osmoprotectants that can reduce osmotic damage caused by salt pressure (Türkan and Demiral, 2009; Deinlein *et al.*, 2014).

On the other hand, during saline stress, *peroxidase (APX), superoxide dismutase (SOD), peroxidase family protein (POD), thioredoxin family protein, and glutathione S-transferase family protein (GST)* were identified as differentially expressed genes (DEGs) in a plant. Transcription factors (TFs) can function as gene activators, repressors, or both (Ji *et al.*, 2021). On top of that, saline tolerance is a complex characteristic involving multiple genes and various physiological and biochemical adaptive pathways (Cuartero *et al.*, 2006; Anusuya and Soorianathasundaram, 2014). It also implies that there are genetic and metabolic mechanisms that plants can use to adapt to saline stress and enhance their ability to better adapt to these environmental conditions.

Bananas are especially vulnerable to saline stress, as high salinity levels can significantly reduce banana yields (Dikayani. *et al.*, 2017). Saline stress injuries manifest as leaf edge necrosis and then spread to the centre of the banana leaf. Moreover, salinity stress also causes yellowing and decreased chlorophyll content and stability, followed by leaf drying, significantly affecting plant development. The impact of salt is then significantly reducing stem thickness and prolonged flowering period of vulnerable cultivars. Due to lower photosynthesis activity in these leaves, harvest yields are reduced due to decreased banana finger weight which also produces low-quality commercial bunches and fails to develop the characteristic finger (fruit) physical characteristics of fruit, such as length, circumference, fruit flesh, skin weight, volume, and density (Israeli *et al.*, 1986; Ravi *et al.*, 2013; Ravi *et al.*, 2014; Rao *et al.*, 2016; Ravi and Vaganan, 2016). Thus, salinity reduces banana production significantly. Further research on the genetic mechanisms responsible for adaptation to saline environments is expected to facilitate breeding of high-yielding banana cultivars that can thrive in saline environments and meet market demand.


Fig. 1 - From left to right, pictures A to D show the conditions of the banana seedling phase with a plant height of ±40 cm exposed to salinity through hydroponic treatment. Pictures E to H show the conditions of banana leaves exposed to a saline environment. Starting from before the salt application treatment until the banana plant eventually dies. The plant wilts and dries; it starts from the older leaves to the younger ones, gradually from the leaf edges until eventually drying out completely in three months (author personal collection, unpublished).

## 3. Breeding for saline tolerant banana

*Genetic diversity for salinity adaptation*

The potential tolerance sources should be identified before breeding stress tolerance bananas. Thus, screening cultivars is the main approach to identifying genetic resources for plant breeding programs in the future (Anusuya and

Soorianathasundaram, 2014). Genetic resources for environmental saline stress tolerance could be derived at least from (1) the natural gene pool of banana accessions and their close relatives already present in the wild, (2) by promoting natural mutations in existing banana accessions using cis-genesis methods, and (3) by incorporating genes from entirely different species using genetic engineering approaches (transgenesis).

Several types of banana accessions are known to be more tolerant of environmental stress than other accessions. These accessions have several genes related to their useful agronomic traits, including height, leaf angle, root spread, and resistance to abiotic and biotic stresses (Heslop-Harrison and Schwarzacher, 2007). The B genome of bananas, for example, has long been the target of breeding programs because of the strong tolerance of bananas to abiotic stress (Tripathi *et al.*, 2019). Therefore, genotyping and phenotyping exploration of the B banana genome, both accessions of cultivars and their wild relatives, should be considered as a way to find novel metabolisms or genes that can play a significant role in increasing the tolerance of banana cultivars to saline environments.

Previously, research has been carried out on the tolerance level to saline stress on various genotypes of banana cultivars. Various banana cultivars such as cv. Tongat (AA), cv. Ney Poovan (AB), cv. H 212 (AB), cv. Grand Naine (AAA), cv. Karpooravalli (ABB), cv. H 96/7 (ABB), cv. Poovan (AAB), cv. Rasthali (AAB), cv. Chakkaiya (ABB), cv. FHIA-1 (AAAB), cv. FHIA-23 (AAAA), and cv. FHIA-17 (AAAA) which were tested with four salt concentrations, namely 0 mM NaCl NaCl, 150 mM NaCl, and 200 mM NaCl, in a research by Anusuya and Soorianathasundaram (2014). The experiment demonstrated significant variability in the tolerance response of bananas to salinity stress, whereas the banana accessions 'FHIA-1' and 'Grand Naine' are very sensitive to the saline treatment given. Meanwhile, the banana accessions 'Ney Poovan', 'Poovan' and 'Karpooravalli' appeared to be more tolerant compared to other banana cultivars in the study (Anusuya and Soorianathasundaram, 2014). This finding suggests that while bananas are generally susceptible to saline stress, unique molecular processes in the different banana accessions can be utilized in breeding programs to develop new, saline-tolerant banana cultivars. In short, genetic diversity is crucial for successful plant breeding (Nandariyah *et al.*, 2020) and in producing saline-tolerant bananas.

In addition, Rao *et al.* (2016) found that among the tested genotypes, Ney Poovan, Poovan, and Karpooravalli can be classified as tolerant bananas based on their capacity to develop and maintain tolerable physiological conditions even under concentrated saline stress levels of 200 mM. However, saline stress affected fruit growth in vulnerable banana cultivars, particularly in the Cavendish group but not in the tolerant Saba genotype. Mature Saba bananas grown in affected areas produced normal bunches (Rao *et al.*, 2016; Ravi and Vaganan, 2016).

On the other hand, Southeast Asia is known as the origin of bananas (Wang *et al.*, 2021). This location also has the highest diversity of wild *Musa* species *(Musa acuminata AA* and *Musa balbisiana BB)* found today, as well as the earliest domestication (Simmonds, 1962; Rao *et al.*, 2016). However, testing and recording the saline tolerance traits of various cultivars and endemic wild banana accessions in Southeast Asia are not widely reported. Research on the tolerance level of salinity stress in banana cultivars and wild species is expected to be used to identify banana accessions as donor genes for the salinity stress tolerance trait in bananas that have not been documented previously.

Essentially, the most significant cultivated banana type in the world is triploid, resulting from inter- or intraspecific hybridization of two wild diploid species, *Musa acuminate* (genome A) and *Musa balbisiana* (genome B) (Wang *et al.*, 2019). As a result, most banana cultivars are sterile, and their genetic diversity is limited, making traditional breeding schemes challenging to implement for this plant. Based on this, although candidate genes for saline tolerance in bananas have been identified, there will be further challenges in transferring these genes of interest through conventional breeding methods to existing industrial and consumer banana accessions. On the opposite, since the genetic engineering method avoids reproductive barriers and promotes the production of superior strains based on large cultivars, this may be a solid alternative to traditional banana breeding (Heslop-Harrison and Schwarzacher, 2007). Considering these conditions, the genetic engineering approach could be a reasonable option to produce saline-tolerant banana accessions for consumption and industry.

An additional issue with cultivated banana plants is that they are propagated vegetatively, leading to a

decrease in genetic variation (López *et al.*, 2017; Hasim *et al*., 2021). However, improving plant traits can be achieved by increasing genetic diversity, including inducing mutations. Induced mutations can enhance the genetics of vegetatively propagated plants (Ghag and Ganapathi, 2017; Nandariyah *et al.*, 2020). Gamma radiation technology can be an option to increase the genetic diversity of bananas. Gamma radiation mutagen is frequently used as a mutagenic agent to induce mutations (Indrayanti *et al.*, 2012). Gamma rays are commonly used in mutation breeding because they can interact with atoms or molecules in cells with water to form free radicals. These radicals can damage or alter key components in plant cells, affecting plants' morphology, anatomy, biochemistry, and physiological differentiation (Datta *et al.*, 2018). Gamma radiation is high-energy electromagnetic radiation produced by radioactive isotopes or nuclear reactors. When applied to plant tissue, it can affect the gene structure, increasing genetic variability in irradiated plants (Astutik, 2009; Khumaida *et al.*, 2015; Maharani *et al.*, 2015).

In another research, Nandariyah *et al.* (2020) developed a gamma radiation protocol to generate genetic variability in bananas, which is one of the options that can be used. Observations of the number of fruits per bunch on each cv. Raja Bulu banana plant that received gamma radiation treatment from the first to the third bunch showed significant differences. The study showed that gamma radiation treatment had a more significant effect on the average number of fruits per bunch, bunch weight, and fruit weight than the control. According to Datta *et al.* (2018), gamma radiation with a dose of 10 Gy changed the appearance of morphological varieties in the generative phase of the M1V1 Raja Bulubanana (Nandariyah *et al.*, 2020). In their study, Nandariyah *et al.* (2020) successfully produced a new genotype of a banana with superior traits compared to its parent.

However, this method also poses challenges that need to be considered. One of them is that gamma radiation can cause random mutations. It means that this method will require laborious phenotyping selection which could take considerable time to separate the desired mutant plants from the undesired ones. The random nature of this method also means that there is a possibility that the survived mutants from the experiment may ultimately fail to produce a better cultivar than their parent.

*Screening methods for salinity stress tolerance in bananas*

Screening for salinity tolerance plants has been conducted using various methodological approaches, including controlled artificial conditions such as *in vitro* culture, pot planting conditions, hydroponic media, greenhouse cultivation, and direct field planting. However, the latter approach is relatively challenging to control due to high environmental variability and difficulty fully controlling field conditions.

Tissue culture techniques have rapidly assessed the saline tolerance traits of different tested banana accessions on an *in vitro* scale. It provides a controlled environment where the effects of salinity can be evaluated by exposing banana explants or tissue cultures to different salt concentrations. Currently, *in vitro* culture of bananas has been developed using various explants, such as shoot tips (Matheka *et al.*, 2019), immature male flowers (Jalil *et al.*, 2003), apical meristems (Novak *et al.*, 1989), bulbs (May *et al.*, 1995), flower tops (Liu *et al.*, 2017), and Embryogenic Cell Suspension (ECS) (Vuylsteke and De Langhe, 1985; Ságі *et al.*, 1995; Côte *et al.*, 1996; Cote *et al.*, 2000). In addition, the development of *in vitro Musa acuminata* L. is likely influenced by salt. The treatment in cv. Barangan with *NaCl* in 200 mM for plant height character was lower than the control. The height of banana plantlets decreased after the cultivation age in the 4$^{th}$ week. This study also showed that *NaCl* treatment could simulate saline tolerance in banana plants *in vitro* (Dikayani *et al.*, 2017). This strategy can yield reliable data in a short period as salinity can significantly impact the growth of banana plantlets.

The *in vitro* test approach allows for evaluating various specific physiological and biochemical responses to salinity stress, including changes in ion accumulation, osmotic adjustment, and antioxidant enzyme activity. Morphologically, the impact of salinity is characterized by a brown coloration, particularly in the leaves, and dark brown coloration in the roots. This is attributed to the high concentration of $Na^+$ ions, which hinders the plant's uptake of $K^+$ ions. Normal plant cells typically contain a higher concentration of $K^+$ than $Na^+$. Potassium ions are crucial in maintaining osmotic pressure in cells, regulating stomatal opening and closing, protein

synthesis, and functioning as enzymes such as pyruvate kinase. Therefore, low $K^+$ concentration in cells leads to chlorosis and necrosis (Munns and Termaat, 1986; Hasegawa *et al.*, 2000; Mahajan and Tuteja, 2005; Dikayani *et al.*, 2017).

In a different research study using the *in vitro* assay technique, Kusdianti *et al.* (2016) found that the activity of banana plantlets induced proline accumulation after exposure to saline stress. This study also found that saline stress affected the gene expression pattern *of Heat Shock Protein 81-2 (HSP 81-2)* and *delta-1-pyrroline-5-carboxylate synthase (P5CS1)*. In this experiment, shoot meristem *Musa acuminata* cv. Barangan was cultured *in vitro* with 25, 50, 75, and 100 mM *NaCl* (Kusdianti *et al.*, 2016). Thus, *in vitro* testing of saline-tolerant banana traits can be considered one of the most powerful research methods for screening saline tolerance cultivars. This method enables testing many samples in a relatively short time.

On the other hand, hydroponic methods also offer another advantageous platform for studying saline stress in bananas. By growing banana plants in nutrient solutions with controlled salt concentrations, researchers can investigate the effects of different salinity levels on plant growth, physiological parameters, and gene expression profiles. Thus, hydroponic-based screening enables the identification of key genes and pathways involved in saline tolerance, providing valuable insights into the molecular mechanisms underlying plant responses to saline stress. Moreover, evaluating various banana genotypes under hydroponic conditions allows identifying potential candidates with more specific salinity tolerance levels.

However, while *in vitro* and hydroponic testing offers controlled conditions, it must be complemented by direct testing in saline-affected fields to ensure accuracy. This is because both tests are conducted in controlled environments, simplifying the real-world complexity of abiotic-biotic interactions in a field. Thus, field experiments with actual cultivation conditions are still essential to evaluate banana plants' performance under saline stress accurately. Nevertheless, the inherent environmental variability in field settings, including temperature fluctuations, rainfall patterns, and soil heterogeneity, poses challenges in obtaining consistent data. Factors such as temperature fluctuations, rainfall patterns, and soil heterogeneity contribute to the complexity of field trials, making it difficult to assess the true genetic potential and performance of banana genotypes under saline stress.

Based on the aforementioned points, screening banana genotypes for salinity tolerance necessitates a combination of controlled artificial conditions and realistic evaluations in field settings. *In vitro* culture and hydroponic systems provide controlled environments where physiological and molecular responses to saline stress can be studied in detail. On the other hand, field evaluations offer insights into the performance of banana plants under actual cultivation conditions. Ultimately, the breeding outcomes of bananas will be grown by farmers in agricultural fields. Therefore, in the end, banana accessions selected through rapid *in vitro* testing still need to be validated through multi-location field trials. Integrating these approaches, robust experimental design, and statistical analysis will contribute to developing saline-tolerant banana varieties through combined plant breeding programs and precise molecular analyses.

## 4. Saline-tolerant genes in Banana

*Potential saline-tolerant genes in banana*

Various genes in multiple plant species have been discovered to enhance plant tolerance to abiotic stress. These include the over production of enzymes involved in metabolite synthesis, membrane lipid biosynthesis, antioxidant defense, protective proteins, and transporters (Yang *et al.*, 2018; Zheng *et al.*, 2018). Genes like *PROLINE DEHYDROGENASE (ProDH)* and *1-pyrroline-5-carboxylate synthetase (P5CS)* regulate proline synthesis and contribute to stress response (Villao *et al.*, 2021). Up-regulation of *OsbZIP23* (Dey *et al.*, 2016) and *OsNAC6* (Nakashima *et al.*, 2007) in transgenic rice increases salt and drought tolerance, while *SNAC1* and *SNAC2* enhance cold, salt, and drought tolerance (An *et al.*, 2018). The expression of protein kinases such as *SnRK2, CIPK*, and *CDPK* has also been shown to improve salt, cold, and drought tolerancein various crops (Saijo *et al.*, 2000; Boudsocq and Lauriere, 2005; Xiang *et al.*, 2007; Zhang *et al.*, 2016; Fedorowicz-Strońska *et al.*, 2017). The *Mitogen-Activated Protein Kinase (MAPK)* cascade (Boudsocq and Lauriere, 2005) and *Protein Phosphatase 1a (OsPP1a)* (Liao *et al.*, 2016) are involved in stress response pathways. Identifying key regulators and utilizing them for stress acclimation is

an important research focus. Such findings can be valuable references for identifying potential gene donors in breeding programs to enhance abiotic stress tolerance in bananas.

Various attempts have also already been made to make bananas more tolerant of saline stress. Among them is to look for genes that play a significant role in making plants able to tolerate the effects of environmental salinity. The search for this target gene can be carried out using the RNA-seq method, as done by Ji *et al.* (2021). They use the RNA-seq approach to compare transcriptome changes in banana roots exposed to salt (60 mM *NaCl*) with those grown under normal conditions. After 12 and 24 hours of salt treatment, 1466 and 2089 DEGs were found, consisting of 542 up-regulated and 924 down-regulated genes, 507 up-regulated and 1582 down-regulated genes, respectively. Stress signalling transduction, reactive oxygen species (*ROS*) scavenging, osmoregulation, and TFs are all elements of the saline stress response. A total of 3,355 DEGs were classified into 40 functional groupings, with biological processes (15 subcategories), cellular components (10 subcategories), and molecular functions (7 subcategories) being the most common (Ji *et al.*, 2021).

In that research, *HSP 81-2* and *P5CS1* genes were expressed at varying levels in all banana plantlets. With 75 mMNaCl treatment, *HSP 81-2* was most abundant in the shoots and roots of the plants. Similarly, this treatment resulted in the most significant accumulation of proline. The expression of the *HSP 81-2* gene was greater than the *P5CS1* gene in all roots and shoots given NaCl treatment. They concluded that *Musa acuminata* cv.*Barangan* has a defense mechanism against salt (Kusdianti *et al.,* 2016). Proline accumulation by plantlets given NaCl and expression of the *HSP 81-2* and *P5CS1* genes was found to vary among all plantlets, and maximum *HSP 81-2* was expressed by the shoots and roots of plants given 75 mMNaCl treatment (Kusdianti *et al.*, 2016). In general, gene findings using genetic factors and molecular methods are rapidly growing research activity and widely used in the past decade. This method allows for discovering various genes that contribute to a specific trait. Thus, understanding genetic and molecular mechanisms underlying the trait can be more comprehensive.

*Confirming the potential saline-tolerant gene*

Our current understanding of saline stress perception, signaling pathways, and downstream

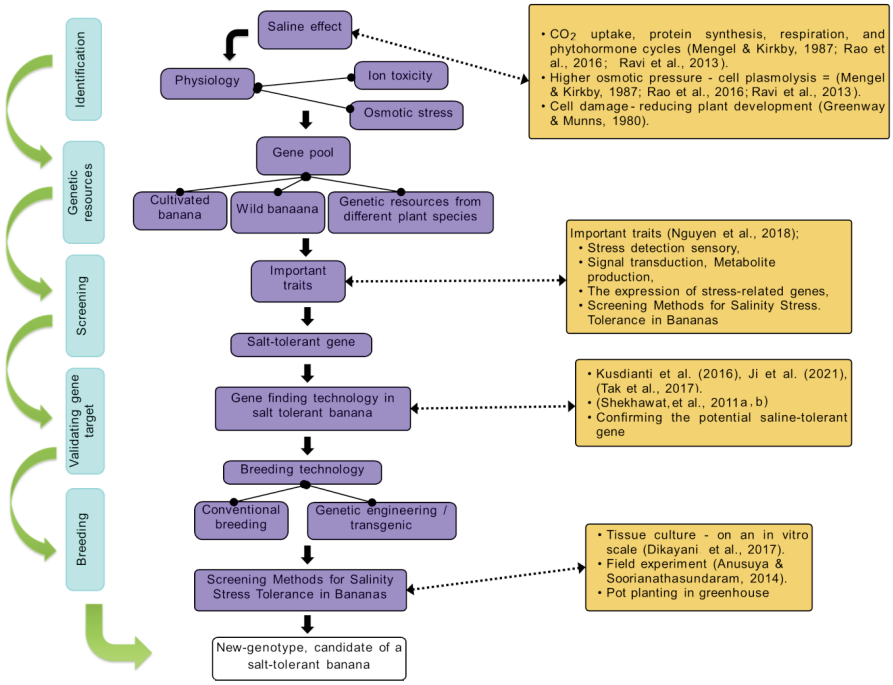


Fig. 2 - Phases of breeding salt-tolerant banana plants. These phases initiates with identifying the negative effects of saline stress on banana plants. The next steps entail exploring genetic diversity for salt stress tolerance within the existing gene pool and developing an appropriate breeding scheme. These steps aim to produce new banana genotype as potential candidates for salt-resistant banana cultivar.

factors is primarily based on research on the model plant *Arabidopsis thaliana*. It has yet to be widely applied to other crops. To effectively enhance saline tolerance in different plant species, it is crucial to identify and understand the specific growth adaptations to salt that benefit each species. Even in *Arabidopsis*, saline stress's early cellular perception and signaling are still obscure. During saline stress, intracellular $Ca_2^+$ spikes and wave signals seem to be a convergent sensory mechanism through which the cell perceives high salinity. It triggers a signaling pathway involving the Salt Overly Sensitive (*SOS*) proteins, which regulate sodium concentration in the cytosol. The *SOS* pathway comprises *SOS1, SOS2*, and *SOS3* and is induced by saline stress. Upon binding with $Ca_2^+$, *SOS2* is activated and forms a complex with *SOS3*, which then phosphorylates the $H^+$/cation antiporter *SOS1*, excluding sodium from the cell. This mechanism has been reviewed in detail in 2020 (van Zelm *et al.,* 2020).

Functional genetic studies aimed at elucidating the function of banana genes in saline stress are even more scarce. Table 1 lists known validated factors in the body of current literature. Two transcription factors from the banana cv. Karibale Monthan (ABB), *Musa NAC04* (Tak *et al.*, 2017), and *Musa WRKY71* (Shekhawat *et al.*, 2011 a) have been identified to be involved in the control of the saline stress response. *WRKY71* expression is elevated by many stressors, such as cold, dehydration, salt, and other hormones, while *Musa NAC04* is activated by salt treatment. *NAC04* or *WRKY71* overexpression in transgenic bananas has been associated with increased saline tolerance. It is indicated by sustained chlorophyll content, lower oxidative damage, and a better Fv/Fm ratio than wild-type (Tak *et al.*, 2017). Moreover, the induction of putative gene expression related to abiotic stress responses in *WRKY71* overexpression lines upon salt treatment suggests that *WRKY71* is involved in the up-regulation of down stream stress response genes in bananas (Shekhawat *et al.*, 2011 a).

Aquaporins are among the other factors that have been validated to be involved in banana saline tolerance. Aquaporins are water channels that promote symplastic water movement. The banana aquaporin gene, *MaTIP1;2,* is known to be expressed in the root, stem, and leaves and is strongly upregulated by salt and drought conditions. Transgenic *Arabidopsis* overexpressing *MaPIP1;1* is more tolerant to saline stress. Similarly, over expression of *MusaPIP2;6* in transgenic bananas resulted in higher saline tolerance than that of the wild type. A protection mechanism from osmotic shock through up-regulation of Banana Dehydrin1 was suggested in a study by Shekhawat *et al.* (Shekhawat *et al.*, 2011 b). *Banana Dehydrin1*, a type of Late Embryo Abundance (LEA) protein encoded by the *MusaDHN-1* gene, was induced by drought, cold, and high salt (Shekhawat *et al.*, 2011 b). Over expression of *MusaDHN1* leads to enhanced banana tolerance via accumulation of proline and reduced malondialdehyde levels upon drought and saline stress (Shekhawat *et al.*, 2011 b).

Nevertheless, various genetic profiling studies recorded in bananas are generally held on cultivated bananas. Exploration and research of genetic profiling of saline-tolerant traits in wild banana relatives in their center of diversity in Southeast Asia can be expected to provide information and potential tolerance traits that are not yet known. This is partly due to wild bananas undergoing more complex adaptation processes in nature than those cultivated on agricultural land. This diversity of traits is expected to enrich the genetic and molecular mechanism toolbox that can be applied to cultivated bananas. Therefore, it can provide new possibilities in breeding programs to produce cultivated bananas tolerant to saline environments.

*The prospect of genetic transformation technology in Banana breeding*

Natural selection and conventional banana crossing have been the most commonly used breeding methods worldwide. This is based on considerations of its simplicity and not requiring sophisticated instruments in their implementation. However, conventional breeding is based on natural genetic variation, and an extensive backcross program is required to add desirable characteristics to elite plants. This has led to the use of this method in banana breeding being considered laborious and time-consuming. Moreover, the availability of favorable alleles or genetic variation in nature is limited to exploit this approach (Manshardt, 2004).

Meanwhile, breeding through random mutagenesis (physical, chemical, or biological mutations) can result in mutations of many traits and undesirable changes. Breeding these mutations should also be followed by screening huge and time-

Table 1 - List of genes with validated functions related to saline stress

| Gene | Protein | Role | Origin oganism | Expression nduced by | Expression pattern | Functional genetic testing plant | Phenotypes | Molecular mode of action | Publication(s) |
|---|---|---|---|---|---|---|---|---|---|
| *MaROP5g* | Rho-like GTPases | Signaling | Fenjiao and Baxi Jiao banana | Mainly salt, but also cold and drought | Protein localized at the plasma membrane | Overexpressed in *A. thaliana* | Longer primary roots and increased survival rates in response to salt stress | The increased salt toleranceconferred by *MaROP5g*gene might be related to reduced membrane injury and the increased cytosolic *K+/Na+* ratio and *Ca2+* concentration in the transgenic plants | (Miao *et al.*, 2018) |
| *MusaDHN-1* | Novel Banana SK3-type dehy-drin, highly hydrophilic pro-teins (LEA family protein)? | Protecting cells from osmotic shock | Banana cv. Karibale Monthan | Drought, salinity, cold, oxidative and heavy metal stress, as well as by treatment with signalling molecules like abscisic acid, ethylene and methyl jasmonate | Leaves | Overexpressed in banana cv. Rastali | Improved toleranceto drought and salt-stress treatments in both i*n vitro* and *ex-vitro* assays | Enhanced accumulation of proline and reduced malondialdehyde levels in drought and salt-stressed | (Shekhawat *et al.*, 2011 b) |
| *MaTIP1;2 promoter* | Aquaporin | Water channels in symplastic pathways | *Musa acuminata* L. AAA group cv. Brazilian | Drought and salt | Roots, stems, leaves, flowers and fruits. Highest in leaves | Tested in *A. thaliana* | Reporter responds to salt and drought stress | Reporter responds | (Song *et al.*, 2018) |
| *MusaNAC042* | Signaling | Transcription factor | cv. Karibale Monthan | Induced by salt | Leaves | Transgenic *Musa acuminate* L. cv. Rasthali | Transgenic plants retained higher levels of total chlorophyll and lower levels of MDA content in response to salt stress. Transgenic plants have higher proline content and better Fv/Fm ratio | Modulating the expression of *CBF/DREB, LEA,* and *WRKY* | (Tak *et al.*, 2017) |
| *MusaPIP2;6* | Aquaporin | Water channels in symplastic pathways | cv. Karibale Monthan | Induced by salt and drought | Leaves | Overexpressed and using inducible construct transgenic banana (cv. Rasthali) | Transgenic plant has higher salt tolerance | better Fv/Fm, lower MDA | (Sreedharan *et al.*, 2015) |
| *MaPIP2;7* | Aquaporin | Water channel protein function in symplastic pathways | *Musa acuminata* L. AAA group, cv. Brazilian | Up-regulated after osmotic (mannitol), cold, and salt treatments | No data | Gongjiao Banana (*Musa acuminata* L. AA group, cv. Mas) | Overexpression of *MaPIP2-7* in bananas enhanced toleranceto drought, cold, and salt | lower levels of MDA and ion leakage, but higher contents of chlorophyll, proline, soluble sugar, and abscisic acid (ABA) compared with wild-type (WT) plants under stress and recovery conditions | (Xu *et al.*, 2020) |
| *MaPIP1;1* | Aquaporin | Water channel in symplastic pathways | *Musa acuminata* L. AAA group, cv. Brazilian | Induced by salt and drought | Root. Localized in plasma membrane | *Arabidopsis thaliana* | Overexpression of *MaPIP1;1* in Arabidopsis resulted in increased primary root elongation, root hair numbers and survival rates com-pared to WT under salt or drought conditions | Reduced membrane injury and high cytosolic $K^+/Na^+$ ratio | (Xu *et al.*, 2014, 2021) |
| *MusaWRKY71* | Transcription factor | | *Musa acuminata* L. cv. Karibale Monthan (ABB) | Up-regulated by cold, dehydration, salt, ABA, $H_2O_2$, ethylene, salicylic acid and methyl jasmonate | Localized in nucleus | *Musa acuminata* L. cv. Rasthali suspension culture cells | Overexpressed line exhibited upregulation of putative genes related to abiotic stress responses | Transcriptional upregulation of stress response genes | (Shekhawat *et al.*, 2011 a) |

consuming populations to identify mutants with the desired traits (McCallum *et al.*, 2000). Breeding mutations usually have a shallow frequency (0.1% of total mutations). In contrast, crossbreeding using markers is often very expensive, and linking markers to the desired trait is sometimes very difficult and time-consuming. This method will also produce products requiring complex regulatory processes, time-consuming requirements, and expensive safety analyses (Lusser *et al.*, 2012). Moreover, most Banana consumption cultivars are triploid. Thus, the sterility and polyploidy of bananas greatly inhibit conventional or molecular breeding from providing a practical and effective way for genetic improvement (Tenkouano *et al.*, 2011). In order to overcome this problem, transgenic bananas achieved through genetic engineering techniques might be a reasonable way to produce bananas with saline tolerance traits. In addition, genetic transformation technology also allows the addition or insertion of genes from groups of living things that are phylogenetically distant or sexually incompatible.

In the modern genetic engineering approach using agrobacterium plasmid, tissue culture methods are generally needed in the implementation stage. Usually, *in vitro* bananas could be genetically modified using particle bombardment or *Agrobacterium*-mediated transformation (May *et al.*, 1995; Sági *et al.*, 1995; Wang *et al.*, 2021). It is also worth noting that the approach using *Agrobacterium tumefaciens* is one of the most widely used due to its ease of use and ability to be applied to various plant tissues (Villao *et al.*, 2021). In addition, regeneration in the genetic modification of bananas is often carried out by organogenesis based on meristematic tissue or somatic embryogenesis based on ECS (Novak *et al.*, 1989; May *et al.*, 1995). Because of its rapid regeneration rate, meristematic tissue from shoot tips and flower buds are usually used as explants for cloning and genetic transformation (May *et al.*, 1995; Liu *et al.*, 2017; Wang *et al.*, 2021). Additionally, scientists commonly prefer to use plant materials for the genetic transformation process using the Agrobacterium-mediated transformation technique in somatic embryos, callus, and protoplast cultures (Villao *et al.*, 2021).

*Development of saline-tolerance banana through transgenesis and gene editing*

Perhaps the most known successful transgenesis in bananas so far is transgenic Cavendish banana using *Agrobacterium tumefaciens* from Dale *et al.* (2017). Normally, Cavendish bananas are highly susceptible to TR4, a destructive fungal disease that poses a significant threat to plantations worldwide. Currently, there is no known effective resistant replacement for Cavendish banana. The researchers conducted a 3-year field trial using transgenic banana lines transformed with genes *RGA2* and *Ced9* in this experiment. Both lines remained disease-free, indicating resistance to TR4. This study demonstrates how transgenic and gene editing approaches can practically enhance Banana cultivars to overcome environmental challenges through single gene transformation (Dale *et al.*, 2017).

In addition, just recently, in 2021, one experiment by Villao *et al.* (2021) successfully developed a genetic transformation protocol from banana apical meristem for the cv. Williams (genotype AAA). This protocol was based on the co-cultivation of explants (whole *in vitro* plants or meristematic tissue divided in two from *in vitro* plants) with *Agrobacterium tumefaciens* separately. As an illustration of the timeline required to implement this protocol, Villao *et al*. also mentioned that after embryogenic cell suspension transformation mediated by Agrobacterium, it would take six to twelve months to obtain transgenic banana plants *in vitro* (Villao *et al.,* 2021). In addition, the maturation of embryogenic cell suspensions can take eight to twelve months (Tenkouano *et al.*, 2011). The technique designed in the Dale *et al*. and Villao *et al.* experiments, mentioned above, could aid in genetically transforming various banana cultivars. That approach could also be considered to produce transgenic banana tolerant.

Recently, genome editing in plant breeding has been on the rise due to the development of CRISPR/Cas9 technology. This is partly because the CRISPR/Cas9 system allows for various methods of genome editing, depending on the repair route and the availability of repair templates (Bortesi and Fischer, 2015), contributing to its precision. As a result, with the open-access publication of the banana genome and the development of viable genetic transformation methods, gene editing systems such as host-induced gene silencing (HIGS) and CRISPR have been increasingly used to mutate specific genes, resulting in the creation of mutant plants without the need for foreign gene insertion (Tripathi *et al.*, 2019; Wang and Chen, 2020; Wang *et al.*, 2021). Hence, CRISPR technology has emerged as

a promising option in genetic transformation programs to produce banana cultivars that are tolerantto saline environments. By considering this factor, the development of genetic engineering technology for producing saline-tolerantbanana cultivars can progress more smoothly than before.

Although there have not been many massive genetic transformation programs to produce banana cultivars that can tolerate and thrive in saline environments, scientists have successfully created saline-tolerantcultivars of other crops using genetic transformation. The information from these studies is becoming more relevant as various molecular mechanisms involved in the process are also known to occur in banana tissues. This information can help study genetic transformation techniques and their potential application in banana cultivars in the near future.

## 5. Concluding remarks and future prospect

Bananas are popular worldwide, but abiotic stress factors often limit their production. One such stressor is salinity, exacerbated by global warming, particularly in tropical regions like Southeast Asia with extensive coastlines. As the center of banana diversity, Southeast Asia has abundant genetic variability in wild and local bananas. This biodiversity richness can be utilized further to address the various challenges in banana cultivation. Additionally, countries experiencing higher water evaporation rates in agricultural lands than rainfall face significant risks from salinity stress.

However, developing elite cultivars tolerant to salinity stress presents a unique challenge, mainly since elite cultivar bananas are typically seedless and triploid or parthenocarpic. Various breeding and genetic engineering techniques can be employed to overcome these challenges. By using these techniques, it is possible to produce elite banana cultivars that are more tolerant to saline stress, have high yields, and are preferred by consumers in a shorter period. Therefore, continued efforts towards developing improved banana cultivars for salinity tolerance are necessary for the sustainable production of this vital crop.

The following are some essential topics related to banana breeding for saline tolerance that may be of significant research interest in producing an elite cultivar of saline-tolerant banana. Firstly, utilizing wild banana genetic resources alone or combined with mutation can enhance genetic variability for screening saline tolerance. Additionally, translational studies in model plants and omics approaches like genomics, transcriptomics, and metabolomics can uncover biological response and tolerance genes in bananas. Investigating the physiological and biochemical mechanisms underlying saline tolerance, such as ion transporters, osmoprotectants, and antioxidant enzymes, provides insights into the molecular basis of saline tolerance in bananas. Furthermore, improving micropropagation, embryogeniccell induction, and regeneration techniques facilitate genetic transformation and gene editing in diverse elite banana cultivars. Additionally, marker-assisted selection strategies integrated with molecular markers linked to saline tolerance traits can expedite the screening and selection of saline-tolerant genotypes. Finally, integrating traditional breeding approaches with biotechnological tools like marker-assisted backcrossing and genome editing can accelerate breeding progress and the development of this field.

As for the safety concerns surrounding the use of genetically modified crops, including transgenic bananas, this will still be a controversial topic for the foreseeable future. It is crucial for all stakeholders, including scientists and governments, to apply the precautionary principle when dealing with GMO products and technology. The emergence of gene editing approaches such as CRISPR/Cas9 offers an alternative approach to conventional transgenesis, which may change the acceptance of genetically modified products in the coming years.

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