Chapter 7

Discussion

Micropropagation, renowned for its capacity to generate vast numbers of identical plant clones, stands as a major achievement in plant biotechnology. This technique has developed into a global industry worth billions of dollars. Initially focused on the mass production of ornamental plants, micropropagation has broadened its application to include a diverse range of crops, such as vegetables, fruits, medicinal and aromatic plants, and trees. This expansion highlights the increasing significance and adaptability of micropropagation within modern agriculture and horticulture (Bajaj, 1986, 1988). Micropropagation is now extensively employed for plants that are difficult to propagate through conventional methods, as well as for the large-scale multiplication of valuable germplasm stocks. This approach serves two main purposes: Firstly, it supports biomass energy production by quickly generating plant materials suitable for sustainable energy solutions. Secondly, it aids in the conservation of critical, elite, and endangered plant species. By rapidly multiplying these species in controlled settings, micropropagation helps protect them from extinction and preserves their genetic diversity, which is crucial for species at risk in natural habitats (Pania *et al*., 2000; Michael *et al*., 2001).

While micropropagation offers numerous benefits, certain limitations have restricted its broader industrial application. One major challenge is its relatively low shoot multiplication rate, which limits efficiency in large-scale production. Additionally, the rising costs of key growth media components pose financial challenges. Another issue is the occasional development of morpho-physiological abnormalities in plants cultured *in vitro*, which can affect their quality and commercial viability. Furthermore, conventional clonal propagation methods remain time-consuming and labor-intensive, reducing their practicality for mass production needs. Due to these limitations, numerous *in vitro* technologies developed for various plant species have largely remained within laboratory confines, with limited success in industrial scaling. These challenges underscore the importance of ongoing research

and innovation in plant tissue culture and micropropagation, aimed at overcoming these barriers to fully harness the technology's potential (Ziv, 2005).

In recent decades, efforts to address the challenges of large-scale plant production and reduce the costs associated with commercial micropropagation have intensified. This focus reflects the need to make plant tissue culture and micropropagation more economically feasible and efficient for industrial use (Andrea-Kodym and Zapata-Arias, 2001). Recent innovations in the field aim to improve micropropagation efficiency, such as employing liquid culture systems, enriching culture environments with CO_2, and enhancing ventilation within culture vessels. These advancements have shown substantial benefits in optimizing the micropropagation process.

This study primarily aimed to develop efficient, reproducible micropropagation protocols for rose by utilizing a liquid culture system and optimized growth conditions. Although traditional *in vitro* methods have been applied to propagate rose, there is a need to refine these protocols to increase the yield of healthy plants while reducing costs. Beyond protocol enhancement, this research also included morpho-physiological analyses to evaluate the quality of the plantlets produced, offering valuable insights into their health and traits. This comprehensive approach seeks to advance rose micropropagation techniques, providing benefits horticulture industries.

7.1 Growth of plant in different growth condition

Liquid culture systems offer clear advantages over traditional agar-based media, with one key benefit being significantly higher multiplication rates. In this study on Rose, the liquid medium was observed to stimulate *in vitro* growth and promote shoot multiplication. The enhanced shoot multiplication and elongation in liquid medium can be attributed to the continuous nutrient availability, as the shoots are consistently surrounded by essential growth nutrients. The liquid culture setup allowed a larger surface area for cells to efficiently absorb nutrients, enabling rapid nutrient exchange at the cell surface through diffusion and movement within the surrounding liquid. This environment also supported enhanced cytokinin uptake, a crucial plant growth hormone, further promoting shoot multiplication and growth

(Sandal *et al*., 2001; Gupta and Timmis, 2005; Mohapatra and Batra, 2017). Similar growth enhancements have been documented in other plant species cultivated in liquid media. For instance, in potato cultures, leaf areas were observed to double, and shoots displayed increased internode length, suggesting that liquid media support overall growth and development in potatoes. Additionally, in *Saccharum officinarum* L., shoots grown in full-strength MS (Murashige and Skoog) liquid medium were significantly longer than those from semi-solid medium, indicating the liquid medium's particular advantage for producing longer, healthier shoots. These findings highlight the effectiveness of liquid culture systems in fostering vigorous plant growth, which can be applied to optimize the cultivation of a range of plant species (Melaku *et al*., 2016). Rapid micropropagation through liquid culture systems has been widely documented, with this method recognized for its capacity to significantly accelerate plant propagation, thereby producing higher yields of healthy plantlets in a shorter period (Gatti *et al*., 2017; Nápoles Borrero *et al*., 2017; Daneshvar, 2019; Vyas *et al*., 2021).

In our research, we identified glass marbles support matrices as the most suitable option for the liquid culture medium. Constructed from locally sourced glass beads, these matrices proved highly effective due to their inert properties, resistance to autoclaving for sterilization, and reusability (Niedz and Marutani-Hert, 2018; Shekhawat *et al*., 2022). Previous studies have demonstrated the cost-effectiveness of using glass beads as an alternative to agar in plant tissue culture, providing further support for this economical approach (Debnath, 2016). Replacing agar with glass beads (marbles) can significantly lower the overall expenses associated with culture medium preparation for Dwarf Cavendish (Souza Costa *et al*., 2020). The successful use of glass beads in the propagation of raspberry and white clove, alongside reported savings of 60% in media costs, demonstrates the practicality and cost-effectiveness of this approach. Furthermore, glass beads have proven satisfactory for maintaining callus and inducing shoot organogenesis, highlighting their versatility and effectiveness in supporting these essential aspects of plant tissue culture (Niedz and Marutani-Hert, 2018) in citrus. The successful substitution of traditional agar with glass beads (marbles), while maintaining the quality of *in vitro* regenerated plantlets of *Typhonium flagelliforme*, represents a significant advancement (Rezali *et al*.,

2017). An important observation was the occurrence of hyperhydricity in most regenerated shoots when cellulose filter paper supports were utilized. This condition, marked by excessive water absorption by plant tissues, can adversely affect plant health and development. Similar findings have been noted in other studies, particularly regarding the use of polystyrene foam, which was found unsuitable for plant growth (Lopez-Guerrero *et al*., 2022). The observation of enhanced growth in a liquid medium supported by filter paper rafts is noteworthy, as it suggests that these rafts improved growth conditions for the cultured plants. However, it is important to highlight that this growth did not exceed that achieved with glass bead-supported liquid medium, indicating that glass beads offer superior support for plant growth. Interestingly, similar results were reported in citrus research by Niedz and Marutani-Hert (2018), which found that filter paper raft-supported liquid medium moderately stimulated overall growth. These parallel findings reinforce the idea that while filter paper rafts can promote growth in certain plant species, they may not be as effective as other support materials, such as glass beads, in all scenarios.

The use of temporary immersion or cultivation in a liquid medium for plant propagation has several advantages, including improved shoot elongation and multiplication, as well as an increase in leaf area. This method has proven effective in promoting growth and development across various plant systems. Temporary immersion culture facilitates a continuous exchange of nutrients and gases around plant tissues, creating an optimal environment for shoot elongation and multiplication. The observed increase in leaf area further indicates the overall health and vitality of the plants (Aragón *et al*., 2014; Esyanti *et al*., 2016; Ruta *et al*., 2020). The observed increase in plant growth during temporary immersion culture can be attributed to several factors. One significant factor is the enhanced aeration provided by the periodic immersion of plant tissues in the liquid medium, which facilitates better oxygen exchange—essential for cellular respiration and overall plant health. Additionally, the renewal of the headspace above the liquid medium during temporary immersion helps to reduce hyperhydricity. Hyperhydricity, characterized by excessive water absorption by plant tissues, can hinder growth. By periodically exposing the plant to air, excess moisture is removed, thereby preventing or alleviating hyperhydricity and fostering more favorable growth conditions (Madihah Mohd *et al*.,

2017; Ruta *et al*., 2020). Furthermore, the combined effect of semi-solid and liquid media plays a crucial role in optimizing plant growth in tissue culture, as this integrated approach provides a balanced environment conducive to plant development.

The enrichment of CO_2 in both semi-solid and liquid media has been shown to significantly enhance *in vitro* shoot growth and multiplication in Rose. This finding highlights the importance of carbon dioxide as a critical factor for promoting healthy plant development during tissue culture. The absence of essential carbon sources, such as CO_2 and sucrose, can result in the gradual deterioration of cultures and their eventual death in both semi-solid and liquid media. This decline in culture health is primarily due to starvation, as both CO_2 and sucrose are vital for plant metabolism and energy production. Without these essential carbon sources, the cultured plants cannot sustain themselves, ultimately leading to their demise (Fujiwara *et al*., 1987; Doi *et al*., 1989). Significant enhancements in *in vitro* growth and multiplication were observed when cultures were grown without sucrose but in a controlled, CO_2-enriched environment. This finding suggests that the plants successfully thrived and grew efficiently through photoautotrophy, utilizing carbon dioxide as their primary carbon source for photosynthesis. Furthermore, cultures receiving additional CO_2 appeared to exhibit luxury consumption, indicating they had access to more carbon dioxide than necessary for optimal growth. This phenomenon may reflect favorable conditions that promote vigorous plant development (Rogers *et al*., 1994). The observation of photoautotrophic growth on a sucrose-free medium is a significant finding, indicating that certain plant cultures can sustain themselves and grow solely through photosynthesis, utilizing carbon dioxide as their primary carbon source. This capability has been recorded in *Physalis angulata* (Santos *et al*., 2020), *Protea cynaroides* L. (Wu and Lin, 2013), and *Lavandula viridis* and *Thymus lotocephalus* (Mansinhos *et al*., 2022). Interestingly, the observation that increased CO_2 concentration beyond the optimal level can lead to reduced growth in some cases is intriguing. This phenomenon may be attributed to a downregulation of photosynthesis in plants exposed to excessive CO_2 enrichment. Over time, plants may exhibit a response known as "acclimation" or "downregulation" of photosynthesis when subjected to elevated CO_2 levels for extended periods. While increased CO_2 initially

boosts photosynthesis, prolonged exposure to high CO_2 concentrations may result in decreased efficiency of the photosynthetic processes (Fernandez *et al*., 2000). The current study demonstrated that the combination of 3.0% sucrose and 0.030 % of CO_2-enriched environment was the most effective for promoting *in vitro* shoot growth and multiplication in the examined plant systems. This combination exhibited a synergistic effect, suggesting that the presence of both sucrose and elevated CO_2 levels positively and complementarily influenced plant development. Similar synergistic effects of combining sucrose and CO_2 on *in vitro* shoot growth have been reported in other plant species, including *Protea cynaroides* L. (Wu and Lin, 2013), *Hevea brasiliensis* (Tisarum *et al*., 2018), and *Pfaffia glomerata* (Corrêa *et al*., 2015). Additionally, another noteworthy observation was that the liquid medium outperformed the semi-solid medium in terms of overall growth under CO_2-enriched conditions. This finding indicates that the use of a liquid culture system, combined with CO_2 enrichment, was particularly effective in promoting robust plant growth, as also observed in *Uniola paniculata* (Valero–Aracama *et al*., 2007).

Our research has demonstrated that the selection of containers for plant tissue culture is crucial for the growth of Rose. The type of vessels used can significantly influence the number of air exchanges occurring through the container's walls, which subsequently affects the internal environment within the vessel. The choice of vessel material and design directly impacts the gas composition inside the container, influencing the exchange of fresh air with the surrounding atmosphere and affecting the levels of carbon dioxide and oxygen available to the growing plant cultures. Furthermore, the type of vessel also plays a role in regulating relative humidity and temperature within the container (Chen and Chen, 2002; Kim, 2002). In terms of shoot multiplication, the best results were achieved using 250 ml flasks. Huang and Chen (2005) suggested that larger vessels with wide openings positively impact the rate of air exchange, leading to improved overall plant growth. Additionally, these spacious vessels promote balanced light transmission, further enhancing the growth process (Manokari *et al*., 2022). Their report indicated that round-shaped baby food jars were optimal for shoot production in *Hemidesmus indicus* (L.). This preference was due to these jars providing an intermediate air-exchange rate that struck a favorable balance for plant growth. Moreover, the round shape of the jars helped

maintain suitable humidity levels, reducing the risk of desiccation and contributing to successful shoot production. The apical explants of *Scrophularia yoshimurae* exhibited their highest *in vitro* performance when cultured in Magenta™ boxes (Lai *et al*., 2005). Studies have documented that larger vessels facilitate a greater rate of shoot multiplication, as seen in both *Gladiolus* (Dantu and Bhojwani, 1992) and lettuce (Tisserat and Silman, 2000). Conversely, conical-shaped vessels resulted in lower air exchange rates, which restricted the overall multiplication rate to a moderate level. Huang and Chen (2005) noted that conical vessels exhibited the lowest air-exchange rates, leading to slower culture growth. However, a study conducted by Joshi *et al*. (2009) found that the size of the containers used for plant tissue culture did not significantly impact the rate of shoot multiplication in *Wrightia tomentosa*. However, Joshi *et al*. (2009) observed an effect on shoot length, noting that in larger vessels with greater capacity, the shoots tended to be more elongated. The maximum shoot elongation was recorded in culture bottles compared to other types of glass containers. This finding suggests that container size can influence the morphology of plant shoots in *Wrightia tomentosa*. While shoot multiplication was not significantly affected by container size, the length of the shoots was influenced, with larger vessels favoring greater elongation.

The choice of caps used to seal the containers for growing Rose plants significantly impacted their growth under our laboratory conditions. Specifically, using conical containers with non-absorbent cotton plugs as closures appeared to hinder optimal light penetration to the plant cultures. This finding is consistent with research conducted by Fujiwara *et al*. (1989), which revealed that both foam rubber plugs and aluminum foil closures were not transparent to light, resulting in minimal light diffusion into the inner space of the containers. The lack of sufficient light exposure could have negatively impacted the growth of our Rose plants *in vitro*. In our study, we observed significant differences in plant growth based on the type of closures and vessels used in tissue culture. Specifically, when closures and vessels were positioned centrally, a shading effect limited the plants' light access, which is essential for their growth. However, when we utilized phyta jar vessels and culture bottles with polypropylene lids, the outcome was markedly different. These polypropylene lids permitted a greater amount of light, with varying wavelengths, to

penetrate the vessels, leading to improved plant growth. This suggests that the type of closure and vessel design significantly impacts light availability for the plants, subsequently affecting their growth. A study by Kitaya *et al*. (1995) demonstrated that polypropylene caps allow light to pass through across a wide range of wavelengths. Additionally, the inclusion of a vent in the polypropylene lid significantly improved gas exchange within the culture vessels, resulting in varying levels of plant growth. According to Hahn and Paek (2001), incorporating ventilation within the culture vessel is crucial for preventing elevated air temperatures and relative humidity levels. This optimization of temperature and humidity creates a more favorable environment for plant growth, as suggested by Hahn and Paek (2001). In certain instances, an increase in air exchange within the culture system significantly impacted the photosynthetic activity of the plantlets. Specifically, enhanced air exchange led to a higher rate of CO_2 uptake by the plants during the photoperiod. In simpler terms, improved ventilation allowed the plants to absorb more carbon dioxide during daylight hours, which is essential for photosynthesis (Kozai *et al*., 2005; Xiao *et al*., 2011; Li *et al*., 2017; Zarei *et al*., 2021). Reports indicate that increasing the number of air exchanges within the culture vessel improves both growth and photosynthesis in *in vitro* plantlets. Essentially, more frequent air exchanges positively influence the growth and photosynthetic activity of the plantlets (Carvalho *et al*., 2001; Valero-Aracama *et al*., 2007; Arigita *et al*., 2010). Contrary findings were documented involving *C. paniculatus* and *T. bellerica*. They discovered that the overall growth of the cultures was superior in bottles with unvented caps compared to those with vented caps. This outcome can be attributed to the rapid drying of the cultivation medium in vented vessels. The quick drying adversely affected the growth of shoot cultures (Nguyen *et al*., 1999; Zobayed *et al*., 2001; Mohamed and Alsadon, 2011).

In our study, we conducted experiments aimed at enhancing root development in *in vitro* grown plants using a liquid medium. When shoots, initially grown in the liquid medium, were later transferred to a specific medium designed for root development, we observed successful root formation in the plantlets. The most favorable results regarding root growth were consistently achieved with the liquid medium supported by glass marbles (without agar). Utilizing a liquid medium during the *in vitro* rooting process is closely associated with the rapid absorption of growth

regulators and essential nutrients, significantly enhancing the rooting process. This approach resulted in an increased number of roots, greater average root and shoot lengths, a higher average leaf count, and an elevated percentage of successful rooting (Makunga *et al*., 2006; Hung *et al*., 2016; Jagiełło-Kubiec *et al*., 2021).

The ongoing quest for new materials in plant tissue culture encompasses two primary objectives: achieving cost-effective methods and enhancing root induction while increasing survival rates, particularly in micropropagation (Makunga *et al*., 2006). In our study, glass marbles have shown effectiveness as support matrices for *in vitro* rooting. Utilizing glass marbles in conjunction with a liquid medium offers several benefits. Firstly, it promotes optimal root aeration for the plantlets, which is crucial for healthy root development. Additionally, the elevated humidity levels within the culture vessels complement this aeration, fostering the growth of robust root systems. As a result, this approach reduces the necessity for extensive hardening procedures, allowing the plants to establish a stronger foundation for growth naturally.

In our research, tissue paper was evaluated as an alternative support matrix for *in vitro* rooting in plants. While tissue paper did facilitate some rooting, its performance was not on par with that of glass marbles. A significant drawback observed was the tendency of the roots to become entangled within the pores of the filter paper, complicating their removal. This issue of root entanglement is not isolated to our study; it has been noted in previous research as well. For instance, when filter paper bridges were used in the cultivation of woody plants such as *Scutellaria*, *Solanum tuberosum*, and *Wasabi*, researchers encountered similar challenges, finding it difficult to extract the plant material without inflicting damage (Tascan *et al*., 2010; Kaur and Minhas, 2016; Hoang *et al*., 2019). This highlights the limitations of using tissue paper as a support matrix in plant tissue culture compared to more effective alternatives like glass beads.

In our research using a liquid culture system, we observed several significant advantages compared to traditional methods. This innovative approach resulted in the successful growth of a high number of robust and healthy plants, characterized by improved overall quality and an impressive survival rate during the *in vitro* hardening process. A key factor contributing to this success was the enhanced accumulation of carbohydrates and organic nitrogen in the shoots of plants that were multiplied and

rooted in the liquid medium, as noted by Mohapatra and Batra (2017). The presence of these vital nutrients played a crucial role in supporting plant growth, leading to healthier plantlets that were better equipped to thrive during the subsequent hardening phase. This underscores the efficacy of liquid culture systems in promoting superior plant development and survival in tissue culture applications. The nutrient-rich environment provided by the liquid culture system played a crucial role in promoting plant development. For instance, when working with *Scutellaria* shoots using this method, we observed vigorous growth without the occurrence of hyperhydricity, which is a common issue in plant tissue culture. This exceptional growth led to an impressive 100% survival rate when the plants were transferred *ex vitro*, as reported by Tascan *et al*. (2010). Similarly, research on sugarcane in a liquid medium yielded remarkable result, with microplants also achieving a 100% survival rate after being transferred to a greenhouse, as recorded by Nápoles Borrero *et al*. (2017). These findings underscore the effectiveness of the liquid culture system in supporting the healthy growth and survival of various plant species under diverse conditions. The ability of this system to enhance growth while minimizing issues such as hyperhydricity highlights its potential as a preferred method in plant tissue culture.

7.2 Studies on leaf surface structures

Distinct variations in the external appearance of leaves, including characteristics such as leaf surface texture, the number of stomata (tiny openings), their shape, and the structure of the waxy cuticle, were observed in Rose plants grown under different conditions and developmental stages. These variations reflect how environmental factors can influence leaf morphology. Moreover, changes in stomatal density and related attributes were correlated with specific laboratory conditions, including light exposure, carbon dioxide (CO_2) levels, ethylene gas concentration, humidity, and the external environment. As noted by Lucchesini *et al*. (2006), these factors can significantly impact leaf structure and function. For instance, increased light intensity may enhance stomatal development to facilitate greater gas exchange for photosynthesis, while higher humidity levels could influence stomatal closure to reduce water loss. Understanding these relationships is crucial for optimizing growth conditions and improving the overall health of Rose plants in tissue culture. The most significant structural contrast observed was the abundance of epicuticular wax on the

leaves of plants grown in natural field conditions compared to the minimal wax present on leaves cultivated in laboratory conditions during multiplication. This difference highlights the impact of environmental factors on leaf structure. Notably, when these plants underwent *in vitro* hardening, significant changes were observed. One crucial change was the development of a protective wax layer on the leaves of the micropropagated plant species. Remarkably, within just 6 to 7 weeks after transplantation, the plants that had been micropropagated in the laboratory exhibited a wax density comparable to that of their counterparts grown in natural field conditions, as reported by Dhawan and Bhojwani (1987). This rapid development of epicuticular wax is essential, as it enhances the plants' resistance to environmental stresses and improves their overall adaptability upon being transferred to field conditions.

The stomatal system in leaves grown in a controlled laboratory environment (*in vitro*) exhibited a typical structure akin to that commonly observed in other plant species. This morphological similarity aligned with our expectations based on previous research findings, indicating that the growth conditions employed in our experiment did not significantly alter the fundamental characteristics of the plant's stomatal system (Joshi *et al*., 2006). However, distinct alterations in the structure of guard cells were noted in the context of our plant tissue culture experiments. These changes were particularly evident in the round shape of the guard cells, which deviated from their typical morphology. Additionally, the inner walls of these guard cells appeared thin and exhibited noticeable deformations. Such modifications in guard cell structure could potentially impact the stomatal function, influencing gas exchange and overall plant physiology, especially under *in vitro* conditions. This underscores the importance of monitoring structural changes in guard cells during tissue culture, as they can have significant implications for plant growth and adaptability. These modifications were particularly noticeable around the wide-open pore of the guard cells. Notably, we observed the deposition of wax within these cells, further contributing to their altered appearance. These structural alterations in the guard cells were not isolated phenomena; they were often accompanied by two significant factors. Firstly, there was a loss of elasticity in the guard cell walls, which impeded their ability to maintain their usual shape and function. This loss of elasticity could hinder the guard cells' responsiveness to environmental changes, ultimately

affecting stomatal opening and closing mechanisms essential for gas exchange. Secondly, we observed modifications in the arrangement and deposition pattern of cellulose microfibrils within the guard cell walls (Zein El Din *et al*., 2020). These changes in microfibril orientation could influence the mechanical properties of the guard cells, impacting their overall performance in regulating stomatal dynamics. Together, these factors highlight the complexity of guard cell morphology and function under *in vitro* conditions, emphasizing the need for further investigation into how these alterations affect plant physiology and adaptability.

During the rooting phase of plant development, we observed the emergence of functional stomata in both semi-solid rooted (SR) and liquid-rooted (LR) leaves. This marked the beginning of a structural reversal process, where the stomatal features began to align more closely with those found in naturally grown plants. Remarkably, the stomata in leaves undergoing *in vitro* hardening closely resembled those on leaves grown in their natural environment regarding their shape, size, and frequency. However, upon transferring the plantlets from *in vitro* cultures to the greenhouse or field, we noted significant alterations in leaf morphology, particularly in stomatal characteristics (Hazarika, 2006). Such advancements in plant tissue culture that effectively control transpiration the loss of water vapor from plant tissues are crucial for the successful survival of transplanted plants. This phenomenon is vital because it directly impacts the plant's ability to adapt and thrive in varying environmental conditions, a key focus of our research in plant tissue culture (Radochova and Ticha, 2009). These observations underline the importance of monitoring stomatal development and function during the transition from *in vitro* to *ex vitro* conditions. Understanding how these structural changes influence water regulation will be critical for optimizing micropropagation protocols and improving plant survival rates in natural settings.

At a microscopic level, we observed that the tiny hair-like structures called "trichomes" appeared visibly shorter and had a blunted appearance in leaves grown in a controlled laboratory environment (*in vitro*) compared to those from plants cultivated outdoors in natural conditions (field-grown leaves). This difference in trichome morphology suggests that environmental factors play a significant role in their development and characteristics. Additionally, we noted that trichomes tended to

cluster together more consistently during the "*in vitro* hardening" stage of plant development compared to the "rooting" stage. This clustering may indicate a response to the controlled conditions of tissue culture, potentially impacting the plant's ability to regulate transpiration and other physiological processes. In our observations of date plants, we identified variations in the number of these epidermal hairs under different growth conditions. Specifically, leaves that developed *in vitro* exhibited a relatively low number of epidermal hairs, which increased after the plants were transplanted into different environments. The highest count of trichomes was recorded in plants cultivated in greenhouses or directly in the field (Zein El Din *et al*., 2020). These findings highlight the dynamic nature of trichome development in response to environmental conditions and emphasize the importance of transitioning plants from *in vitro* culture to *ex vitro* environments to enhance their physiological traits and overall adaptability. Understanding these changes can inform strategies for improving plant growth and resilience in various cultivation practices. The reduced variety and limited distribution of trichomes on *in vitro*-grown teak trees, compared to those grown naturally, are linked to increased water loss in the *in vitro* plants. This heightened water loss ultimately results in a lower chance of survival for these plants after transplantation (Zein El Din *et al*., 2020). The diminished presence of trichomes can be attributed, in part, to a deficiency in cell specialization in plants that have undergone micropropagation. In the context of micropropagation techniques, plants may not develop trichomes as effectively as their naturally grown counterparts (Monja-Mio *et al*., 2021). Trichomes play a crucial role in protecting plants from desiccation by reducing water loss through transpiration. Their absence or reduced number in micropropagated plants can compromise their ability to adapt to environmental stresses, ultimately affecting their survival rates post-transplantation. This highlights the importance of optimizing micropropagation protocols to promote cell specialization and trichome development, thereby enhancing the resilience of *in vitro*-grown plants when they are transferred to natural conditions. Understanding the factors influencing trichome formation could lead to improved strategies for increasing the success rates of transplanted plants and ensuring their adaptation to diverse environments.

The composition of the growth medium significantly influenced the characteristics of leaf surfaces, particularly in terms of stomatal size and occurrence. The substantial enlargement of stomata observed in leaves obtained from a liquid medium may be attributed to the increased surface area of the leaves in this condition. This suggests that the liquid medium facilitated enhanced leaf expansion and development, allowing for larger stomatal openings. Additionally, the variations in leaf surface structures were more pronounced within the context of our plant tissue culture experiment, indicating that the choice of culture medium can play a critical role in shaping the physical attributes of the leaves. Specifically, the differences in stomatal characteristics highlight the importance of selecting an appropriate culture medium to optimize plant growth and development *in vitro*. These findings align with previous research indicating that the environment in which plants are cultured, including the type of growth medium, can have substantial effects on leaf morphology and physiological traits (Aliniaeifard *et al*., 2020). Understanding these relationships can aid in refining tissue culture practices to improve the quality and adaptability of plants produced through micropropagation. The observed deformities in the leaf margins (LM) were notably higher in the plant cultures grown in a liquid medium, which can likely be attributed to the excessive moisture present in this environment. This surplus moisture may have contributed to the malformation of the leaves, as noted by previous research (Dutta Gupta and Prasad, 2010). In contrast, plants grown under controlled conditions exhibited a higher occurrence of actively functioning stomata in their lower leaves (LR). This finding suggests the initiation of a process known as stomatal reversal, where the stomata begin to regain functionality, allowing for improved gas exchange and potentially enhancing photosynthetic efficiency. The differences in stomatal activity between the two growth conditions highlight the impact of environmental factors on leaf morphology and function, indicating that managing moisture levels in tissue culture systems is crucial for maintaining optimal plant health and development. Notably, when comparing the stomatal characteristics of leaves, lower leaves exhibited superior attributes compared to upper leaves. This phenomenon underscores the intricate relationship between environmental factors, such as radiation and humidity, and the physiological responses of plant leaves, particularly regarding stomatal behavior (Hazarika, 2006). In our experiments with *Gladiolus*, both the semi-solid and liquid culture systems displayed stomatal behavior

that fell within the expected range of normalcy. This observation indicates that the experimental conditions employed did not significantly affect the functioning of stomata in *Gladiolus* plants (Dutta Gupta and Prasad, 2010). This consistency in stomatal function is critical for maintaining optimal gas exchange and photosynthesis, suggesting that *Gladiolus* is relatively resilient to the variations in culture media used in tissue propagation.

In the study conducted by Yang and Yeh in 2008, it was found that *Calathea* plants grown in a liquid medium using a temporary immersion technique exhibited better stomatal characteristics compared to those grown in a semi-solid medium. This highlights the significant impact that the choice of growth medium can have on the development of stomata in *Calathea* plants. Our findings align with this, indicating that the adjustments necessary for micropropagated plants to adapt to their new environment primarily commence during root differentiation. This process was particularly pronounced in the liquid rooting leaves (LR), suggesting that plants cultivated in a liquid medium might experience a faster and more effective hardening process. Consequently, this leads to a greater likelihood of survival when these plants are transferred to in vivo or field conditions. Overall, this outcome underscores the potential advantages of using liquid culture for enhancing plant acclimatization and increasing the overall success of transplantation. By facilitating better stomatal development and root differentiation, liquid culture systems may provide a more favorable environment for plants transitioning from tissue culture to natural growing conditions.

In our study, we examined the stem, leaves, and roots of Rose plants cultivated on both semi-solid and liquid growth media to discern structural variations between these two growth conditions. Upon analyzing cross-sections of the aerial stem, we found that the anatomical structure remained quite similar for plants grown in both types of media. This similarity suggests that there were no apparent signs of hyperhydricity in response to the liquid growth medium. Additionally, the leaves of the *in vitro*-cultured plants exhibited distinct characteristics. They appeared thinner compared to those grown under natural conditions, which is consistent with findings from previous studies (de Souza *et al*., 2021; Jagiełło-Kubiec *et al*., 2021). Notably, the palisade layer in these leaves was underdeveloped, indicating that the growth

medium may have influenced leaf development and morphology. This underdevelopment could potentially affect the photosynthetic efficiency of the plants, highlighting the importance of optimizing growth conditions to promote robust leaf structures in tissue-cultured plants. These observations emphasize the need for careful consideration of growth media when aiming to achieve optimal structural and functional characteristics in Rose and other plant species in tissue culture.

Our investigation revealed notable differences in leaf thickness between Rose plants cultivated in a temporary immersion system and those grown on a semi-solid medium. Specifically, the chlorenchyma-the tissue responsible for photosynthesis was found to be thicker in plants grown in the temporary immersion system. This observation underscores the significant impact of *in vitro* culture systems on leaf morphology. The development of photosynthetic tissues is crucial for a plant's ability to adapt and thrive in its environment, as indicated by previous research (de Souza *et al*., 2021). Thicker chlorenchyma in the temporary immersion system suggests enhanced photosynthetic capacity, which may contribute to improved growth and overall plant health. These findings emphasize the importance of selecting appropriate culture systems to optimize leaf structure and function, ultimately supporting better acclimatization and survival rates for micropropagated plants when transferred to field conditions.

7.3 Studies on water relations

7.3.1 Water loss studies

The amount of water loss in Rose differed depending on several factors, including the type of plant, its developmental stage, and the growth medium utilized for cultivation. This indicates that variations in plant type, growth stages, and selected growth media significantly affect the rate of water loss in Rose.

The highest level of water loss in Rose occurred during the *in vitro* multiplication phase. Key factors contributing to this increased water loss include insufficient deposition of protective epicuticular wax on the leaf surfaces of plants grown *in vitro*, impaired stomatal function, and inadequate cuticle development. These issues significantly hinder the success of transplantation efforts (Sajeevan *et al*., 2017; Zein El Din *et al*., 2020). During the *in vitro* hardening phase, exposing the

plants to higher light levels, along with a gradual reduction in relative humidity, has been found to effectively reduce excessive water loss from the leaves. This process aids in restoring stomatal function, helping to prevent leaf wilting and enhancing the overall survival of Rose plantlets after transplantation. The observed decrease in water loss is associated with an increase in the deposition of cuticular wax on the leaves (Hazarika, 2006). This finding aligns with previous studies on water relations in micropropagated plants, including *Wrightia tomentosa* (Joshi *et al*., 2006), *Vitis vinifera* (Salomon *et al*., 2014), *Morus indica* (Sajeevan *et al*., 2017), and date palm (Zein El Din *et al*., 2020). However, Revathi *et al*. (2019) found that the amount of epicuticular wax alone was not a reliable predictor of the survival of micropropagated plantlets during acclimatization in greenhouses. The distinction between photosynthetically competent and non-competent species suggests a potential link between increased transpiration losses and the *in vitro* hardening process of *Musa acuminata*.

The choice of growth substrate had minimal impact on the overall rate of transpiration in our study. These findings are consistent with our previous investigations into plant characteristics, where we observed similar stomatal irregularities and the accumulation of cuticular waxes on the leaf surfaces of plants grown in various culture media. As a result, the transpirational water loss in leaf samples from cultures grown in both semi-solid and liquid substrates displayed comparable patterns. In a study by Zhang *et al*. (2021), significant observations were made when shoot meristems were inoculated with paclobutrazol in a liquid medium and subsequently maintained in a high humidity environment of approximately 98%. The results demonstrated a unique phenomenon in which the plants derived from the treated shoot tips were shorter than their traditionally cultivated counterparts. Additionally, an increased accumulation of epicuticular wax was observed on their surfaces. Upon being transplanted into a conventional agricultural environment, these plants displayed less wilting compared to those grown under standard conditions. Similar findings were reported by a different group of researchers, as detailed by Markovic *et al*. (2020), who conducted comparable studies on *Fritillaria meleagris*. These plants were cultivated in a liquid medium containing 9% sucrose and additional plant hormones.

7.3.2 Biomass accumulation and water content

Our current study has identified a distinct relationship between the rate of plant cell multiplication and the accumulation of both fresh and dry weight in cultured plants. In our controlled laboratory environments (*in vitro*), we observed that the maximum levels of fresh and dry weight coincided with the peak rates of shoot multiplication. This indicates that the growth and division of plant cells are closely associated with the overall weight of the plant tissue under these specific culture conditions. These findings underscore the significance of shoot multiplication as a crucial factor impacting plant growth and development *in vitro*.

In our current research, we found that employing a liquid medium and polyamines positively influenced the growth of the plant systems we examined. This beneficial effect was reflected in the increased fresh and dry weight of the plants. The higher biomass accumulation can be attributed to several factors. Firstly, there was an increase in the number of shoots, which contributed significantly to the overall biomass. Additionally, the number of leaves and the total leaf area also expanded. This rise in both leaf number and area likely enhanced photosynthesis, leading to increased biomass production. Additionally, the presence of cytokinins in the liquid medium significantly contributed to promoting plant growth. The plants effectively absorbed these cytokinins, resulting in increased shoot elongation and further enhancing overall biomass accumulation (Gupta and Timmis, 2005). In the study, the average weight of *Pogostemon erectus* cultured in a liquid medium was higher compared to shoot clusters developed in a solid medium. This indicates that the growth conditions in the liquid medium may be more conducive to the initial development of these plant structures (Muhammet Dogan, 2022). Similar findings have also been reported for papaya (Gatambia *et al*., 2016) and *Saccharum officinarum* L. (Melaku *et al*., 2016). Among the various support materials we evaluated, we observed that the greatest increases in both fresh and dry plant weight occurred when using a liquid medium supplemented with glass beads across all three plant species examined. The substantial surface area of glass marbles facilitates effective interaction between the explant and the liquid medium, allowing for efficient nutrient absorption, which enhances plant growth. Specifically, in sugarcane propagation, plantlets grown on glass beads not only demonstrated increased growth

in terms of offshoots, leaves, and storage runners but also showed significant improvements compared to other support matrices (Nápoles Borrero *et al*., 2017). The elevated moisture content in the plant cultures cultivated in a liquid medium can be attributed to the higher relative humidity and abundant water supply provided to these cultures, as noted by Casanova *et al*. in 2008. Importantly, despite these favourable conditions, no signs or symptoms of excessive water-induced stress or hyperhydricity were observed in the plants under investigation.

In our study, we investigated the effects of partially immersing plant cultures in a liquid medium on their growth. This method resulted in notable outcomes, including an increase in both fresh and dry weight, as well as an enhancement in the water content of the plants. Literature reviews revealed that after 42 days of growth, plants such as *Caralluma edulis* (Parihar and Dwivedi, 2019) and *Juglans nigra* L. (Stevens and Pijut, 2018) also exhibited increases in fresh and dry weight. These improvements were likely due to the synergistic effects of both liquid and solid growth media.

Enhancing carbon dioxide concentration and incorporating sucrose positively influenced the growth of Rose plants. Our comparisons between plants grown in semi-solid and liquid media indicated that the liquid medium was more effective in facilitating biomass accumulation. Additionally, plants cultivated in the liquid medium demonstrated a higher percentage of water content.

In the case of *Vitis vinifera* L plants, providing fully photoautotrophic conditions resulted in optimal outcomes for both fresh and dry weight, as noted in a study by Zhao *et al*. (2019). Research has shown that many plant species experience significant growth enhancements under photoautotrophic conditions. This improvement is particularly evident when measuring fresh weight across various plants, including *Protea cynaroides* L (Wu and Lin, 2013), *Pfaffia glomerata* (Corrêa *et al*., 2015), *Lippia alba* (Batista *et al*., 2017), *Hevea brasiliensis* (Tisarum *et al*., 2018), *Fragaria x ananassa* (Kepenek, 2019), and *Lippia dulcis* (Rocha *et al*., 2022). Research has shown that plantlet growth significantly improves when exposed to higher levels of photosynthetic photon flux (PPF), as noted by Kozai *et al*. in 1990. Furthermore, increased concentrations of carbon dioxide (CO_2) have been found to enhance plantlet growth, as highlighted by Kozai in 1991. The increase in water levels

within the semi-solid culture medium (SCLM) cultures can be attributed to the elevated humidity inside the culture containers, primarily due to the liquid medium, as discussed by Casanova *et al*. in 2008. However, the removal of sucrose from the medium did not lead to improvements in the growth of fresh and dry plant biomass. This observation aligns with the findings of Arigita *et al*. (2010), who studied kiwi explants (*Actinidia deliciosa* Chev. Liang and Ferguson "Hayward"), and Valero-Aracama *et al*. (2007) with sea oats (*Uniola paniculata*) under comparable culture conditions. This phenomenon can be explained by the fact that certain plant cultures depend on the carbon source provided in the growth medium before they can efficiently utilize carbon dioxide (CO_2) from the surrounding air as their primary source of carbon. Notably, sucrose is instrumental in significantly enhancing the growth of young plants. This increase in plantlet biomass is a key focus of our study. In our study, we found that a decrease in water content in the plant cultures indicated that they were not excessively hydrated (non-hyperhydric). Notably, even in the absence of sucrose, the liquid growth medium showed superior performance compared to the semi-solid medium in various physiological aspects. The best growth and quality of *Coffea arabusta* (Afreen *et al*., 2002) and *Eucalyptus* plantlets (Businge *et al*., 2017) were observed when they were cultured in a sugar-free liquid medium supplemented with carbon dioxide (CO_2).

The selection of culture containers had a significant effect on the accumulation of both fresh and dry plant matter. We found that plants flourished in containers with both round and square shapes, leading to a notable increase in their fresh and dry weight. This enhancement can be attributed to improved gas exchange facilitated by the larger openings and dimensions of the culture containers (Chen and Chen, 2002). The air exchange within the cap significantly contributed to the accumulation of fresh and dry mass in the plant shoot cultures. This improvement resulted from the increased availability of oxygen and the removal of excess moisture, creating a conducive environment for plant growth. Furthermore, the enhanced ventilation promoted efficient gas exchange, supporting the metabolic processes essential for plant development. Similar trends in fresh and dry weight accumulation have been reported in various plant species, including *Wrightia tomentosa* (Joshi *et al*., 2009), *Capsicum annuum* (Mohamed and Alsadon, 2011), *Scrophularia yoshimurae*

(Welander *et al*., 2014), and sugarcane (Neto *et al*., 2020). However, it is important to note that some studies have reported different outcomes (Zobayed *et al*., 2001b; Islam *et al*., 2005).

In our study biomass was increased when plants were grown under polyamines concentration compared to control. Polyamines interact with phytohormones, functioning as plant growth regulators, hormonal secondary messengers, and sources of carbon and nitrogen in cultured tissues (Sivanandhan *et al*., 2011). Vasudevan *et al*. (2008) suggested that polyamine application could significantly improve regeneration and differentiation in *Cucumis sativus* L. A similar trend was observed for total biomass (fresh and dry weight).

7.4 Carbonic anhydrase activity

The research investigated the activity of the carbonic anhydrase (CA) enzyme in Rose plants cultured in a controlled laboratory environment and compared it to those grown outdoors. The findings indicated that the CA enzyme exhibited the highest activity in shoots cultivated in a liquid medium. Previous studies have measured carbonic anhydrase activity in various plant species, including *Carludovica palmata* (Minchala-Buestán *et al*., 2023), *Eurycoma longifolia* (Madihah Mohd *et al*., 2017), *Eucalyptus* (Businge *et al*., 2017), and *Dianthus caryophyllus* (Ahmadian *et al*., 2017). Yanyou *et al*. (2006) provided insights into the relationship between the Relative Growth Rate (RGR) of plantlets and their CA activities, noting a significant linear correlation. They found that as CA activity increases, the net photosynthetic rate also rises, resulting in enhanced growth rates. Based on Wu's findings, we hypothesized that the accelerated growth of shoots in liquid medium is associated with a marked increase in CA activity, indicating a potential positive relationship between shoot growth and CA activity.

7.5 Studies on biochemical investigation

In our recent study, we investigated Rose, concentrating on the changes in biochemical factors such as metabolites and enzymes throughout different stages of *in vitro* cultivation. We observed significant differences in biochemical traits among cultures grown in different-gelled medium, semi-solid medium, different polyamines concentration and liquid medium. Additionally, it is important to emphasize that these

variations in biochemical parameters are vital for comprehending how plants grow and develop under various culture conditions.

During the *in vitro* rooting phase of Rose, the total carbohydrate content reached its lowest point. Van Huylenbroeck and Riek (2005) explained that carbohydrates are primarily directed toward developing root structures during this process, leading to a decline in carbohydrate levels. This decrease is a natural response as the plant reallocates resources to support root growth, which is essential for establishing a robust root system. In the liquid medium and different polyamines concentration experiments we observed high carbohydrate accumulation, likely due to the less negative high potential present in these plants. It is crucial to recognize that both inorganic and organic components in the culture medium act as nutrients and also influence plant cell growth through their osmotic properties, as noted by Chen and Ziv in 2003. This indicates that the composition of the medium significantly affects how plants respond to stress and their carbohydrate accumulation.

A decrease in superoxide dismutase (SOD) and peroxidase (POD) activity was observed in Rose as the plants moved from the multiplication phase to the hardening phase. Notably, the liquid medium employed for cultivation did not appear to induce oxidative stress in these plants. This absence of stress was reflected in the steady increase of POD activity throughout all growth phases, indicating that the liquid medium offered a stable environment conducive to the growth of Rose, without significant increases in oxidative stress. POD activity was significantly enhanced with increasing concentrations of polyamines (PAs). Different concentrations of PAs resulted in increased POD activity compared to the control. Similar results were observed with superoxide dismutase (SOD) activity. Polyamines play a complex role in plant oxidative stress, as they can enhance the function of the enzymatic antioxidant defense system, aiding in the efficient regulation of oxidative stress in plants exposed to environmental challenges (Wang *et al*., 2020). Exogenously applied spermidine (Spd) increased levels of Spd and spermine (Spm) while reducing putrescine (Put) levels in cucumber roots under hypoxic stress. This effect was attributed to enhanced enzymatic antioxidant activity, greater reactive oxygen species (ROS) detoxification, ultimately improving stress resistance (Wu *et al*., 2018). However, polyamines can also produce ROS due to their catabolism, which generates

strong oxidizers such as hydrogen peroxide (H_2O_2) and acrolein, potentially leading to cellular breakdown under stress. At the same time, H_2O_2 acts as a signalling molecule in the stress signal transduction pathway and triggers an antioxidant defense response. Therefore, polyamines appear to be regulators of redox homeostasis, exhibiting a dual role in plant oxidative stress (Shao *et al*., 2022).

In our study, liquid medium and polyamines reduced phenolic content in plantlets compared to the semi-solid medium and control without polyamines. Plants grown *in vitro* often demonstrate reduced photosynthetic efficiency (Martins *et al*., 2015). This phenomenon is attributed to lower chlorophyll concentrations, decreased Rubisco activity, and abnormalities in chloroplast structure (Habibi and Purohit, 2019a). Biochemical analyses of *Saccharum* (Taku *et al*., 2020), *Agave potatorum* Zucc. (Correa-Hernández *et al*., 2022), and *Saccharum* spp. (Sorcia-Morales *et al*., 2021) have shown that these species typically have lower chlorophyll levels during the initial stages of multiplication. However, the total chlorophyll content in cultures grown in liquid medium and polyamines different concentration experiments was found to be higher compared to those in agar medium. These findings suggest that cultures in liquid medium and polyamines exhibit enhanced photosynthetic competence.

7.6 Studies on molecular evaluation of genetic fidelity

In recent years, the advent of recombinant DNA technology has led to the widespread use of molecular markers in various research fields. These markers are utilized for multiple purposes, including evaluating genetic fidelity in micropropagated plants, characterizing plant genetic resources, and conducting genome mapping and tagging. The choice of DNA-based markers is primarily due to the stability of DNA, which makes it less susceptible to changes caused by developmental, physiological, or environmental factors.

Molecular markers have been utilized to identify genetic variations and confirm genetic integrity during the micropropagation process (González-Benito *et al*., 2020). Among the polymerase chain reaction (PCR)-derived markers, the random amplified polymorphic DNA (RAPD) technique is recognized for its efficiency and affordability. This method requires only a small amount of DNA, typically in the

nanogram range, for quick analysis of polymorphisms. Additionally, it does not require prior knowledge of the DNA sequence and eliminates the need for radioactivity in the procedure (Williams *et al*., 1990). Changes in the RAPD (Random Amplified Polymorphic DNA) pattern can occur due to variations in primer annealing, which may be caused by point mutations or by the insertion or deletion of sequences, as well as transposable elements (Amiteye, 2021). RAPD markers have been employed to evaluate clonal fidelity in micropropagated plants (Biswas and Kumar, 2023).

In this study, we utilized polymerase chain reaction (PCR)-based techniques, specifically Random Amplified Polymorphic DNA (RAPD) analysis, to evaluate the genetic fidelity of three different accessions of Rose. These methods were chosen for their simplicity and ease of use. By employing two different types of markers that amplify distinct genomic regions, we enhanced our ability to investigate genetic stability and variation within the propagated plantlets (Palombi and Damiano, 2002). Both RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat) markers have proven effective in identifying genetic relationships or differences among micropropagated specimens across various plant species (Thakur *et al*., 2021; Biswas and Kumar, 2023).

In this study, RAPD markers were found to be more suitable than ISSR markers for evaluating the clonal fidelity of Rose. The amplifications carried out using RAPD markers were selected due to their superior efficiency in generating amplification products.

RAPD polymorphism can occur due to changes in nucleotide bases that alter the primer-binding site or as a result of insertion or deletion events within the amplified genomic region (Babu *et al*., 2021). Genetic variation within a plant population is typically identified by observing the presence or absence of amplification products derived from a specific genomic locus. These amplification results can exhibit polymorphism and serve as genetic markers (Farahani *et al*., 2022). However, a significant concern regarding the use of RAPD markers for evaluating the genetic uniformity of cultivars and somatic clones is the lack of reproducibility across different laboratory environments. After optimizing the experimental conditions, it was observed that Random Amplified Polymorphic DNA (RAPD) analysis yielded

consistently reproducible results. Reliable data were obtained by carefully standardizing the appropriate protocols, which ensured accuracy in the experimental procedures. The effectiveness of RAPD markers for the molecular analysis of plants regenerated *in vitro* has been well-documented in the literature (Gautam and Bhattacharya, 2021; Abdalla *et al*., 2021).

In this study, the genetic stability of micro-clones was evaluated using Random Amplified Polymorphic DNA (RAPD) markers across different culture conditions of Rose.

Eight primers were chosen for their effectiveness in generating a maximum number of clear and distinct bands. Each primer displayed a unique amplification pattern. The other primers either did not produce any bands or yielded faint, difficult-to-score bands. As a result, these excluded primers were not included in the subsequent experimental processes.

In Rose, the length of time that plantlets were maintained in the culture medium had no noticeable effect on the genetic stability of micro-clones. Importantly, no changes were detected in the genetic profiles across different culture passages.

The absence of polymorphic variations noted in this study is consistent with the results from Random Amplified Polymorphic DNA (RAPD) analyses performed on micropropagated specimens of *Salvia hispanica* (James *et al*., 2007). Similarly, Gautam and Bhattacharya (2021) utilized RAPD profiling to examine the genetic stability of *in vitro*-cultured *Crocus sativus*.

Significant heterogeneity has been observed in plantlets produced through tissue culture techniques utilizing molecular markers. Verma *et al*. (2021) reported a polymorphic variation of 23.2% among a group of 10 micropropagated apple rootstock MM106 plants regenerated via axillary branching. In a separate study, Lin *et al*. (2022) assessed the genetic fidelity of micropropagated *Ananas comosus* plantlets using Random Amplified Polymorphic DNA (RAPD) markers. Their analysis with 44 randomly selected primers revealed a polymorphic pattern of 2.8% among the regenerated plants, which was attributed to the addition of a high concentration of 6-benzylaminopurine (BAP) in the culture medium. It has been suggested that the length of *in vitro* cultivation may lead to somaclonal variation (Mehta *et al*., 2011).

Several factors that can influence genetic diversity include the specific micropropagation techniques used, variations among genotypes, the composition of the growth medium, and the overall growth conditions (James *et al*., 2007).

In the study of Rose, the molecular analysis using Random Amplified Polymorphic DNA (RAPD) revealed limited genetic diversity among the cultured plantlets. This result stands in contrast to findings from cytological and biochemical analyses. The slight variations detected by RAPD markers may be due to minor genetic rearrangements that occur during extended *in vitro* cultivation (Verma *et al*., 2021).

The inability of Random Amplified Polymorphic DNA (RAPD) analysis to reveal clear polymorphic patterns related to somaclonal variation has also been noted in studies involving *Coffea arabica* (Bobadilla Landey *et al*., 2015), chickpea (*Cicer arietinum*) (Alghamdi *et al*., 2021), and *Ananas comosus* (Lin *et al*., 2022). Some researchers attribute this limitation to the RAPD markers relatively narrow genomic scope. It is possible that the number of primers used in this study may not have been sufficient to fully cover the Rose genome. Additionally, because RAPD markers are generally dominant, mutations affecting only one allele in a diploid dominant homozygote could go undetected (Das *et al*., 2021). Although RAPD analysis is a useful tool for identifying genetic differences, the absence of detectable RAPD polymorphism in micropropagated plants does not necessarily confirm genetic stability. This limitation arises because morphological and chromosomal changes can remain undetected. For instance, a study on *Camellia assamica* over a prolonged three-year cultivation period showed the presence of tetraploid and aneuploid cells through cytological analysis. However, despite these cellular variations, RAPD analysis indicated consistent genetic stability. This suggests that plantlets derived from long-term cultures may possess mechanisms to selectively counteract somaclonal variations (Bajpai and Chaturvedi, 2021).