Chapter 7

Discussion

Micropropagation, which is widely recognized for its ability to create numerous identical plant clones, represents a significant and widely embraced achievement in the field of plant biotechnology. Over time, it has evolved into a thriving global industry worth billions of dollars. Initially, micropropagation was primarily used for the mass production of ornamental plants. However, it has since expanded its scope to encompass a variety of crops, including vegetables and fruits, as well as medicinal, aromatic plants, and trees. This expansion reflects the growing importance and versatility of micropropagation in modern agriculture and horticulture (Bajaj, 1986, 1988). Currently, micropropagation is being applied extensively not only to plants that are challenging to propagate using traditional methods but also for the large-scale multiplication of existing germplasm stocks. This is done for two primary purposes: Firstly, it plays a vital role in the production of biomass energy by rapidly generating plant material that can be used for energy production. This is an environmentally sustainable approach to meet our energy needs. Secondly, micropropagation is instrumental in the conservation of crucial, elite, and endangered plant species. By rapidly multiplying these plants in controlled environments, we can safeguard them from extinction and preserve their genetic diversity. This is particularly important for species that face threats to their survival in the wild. (Pania *et al.,* 2000; Michael *et al.,* 2001).

Despite its numerous advantages, micropropagation also comes with some drawbacks that have hindered its widespread industrial application. One significant limitation is the low rate of shoot multiplication, making it less efficient for large-scale production. Additionally, the increasing cost of the essential ingredients used in the growth media further adds to the challenges. Another concern is the occurrence of morpho-physiological abnormalities in plants grown *in vitro,* which can negatively impact their quality and suitability for commercial purposes. Furthermore, traditional propagation methods for clonal plants are both time-consuming and labor-intensive, making them less practical for mass production. As a result of these constraints, many *in vitro* technologies developed for various plant species have remained confined to laboratory settings and have not been successfully scaled up for industrial use. These

challenges highlight the need for continued research and innovation in the field of plant tissue culture and micropropagation to address these limitations and unlock the full potential of this technology (Ziv, 2005).

In recent decades, there has been a growing focus on addressing issues related to large-scale plant production and finding ways to reduce the costs associated with commercial micropropagation. This heightened interest reflects the importance of making plant tissue culture and micropropagation more economically viable and efficient for industrial applications (Andrea–Kodym and Zapata–Arias, 2001). Innovative approaches have emerged in recent times to enhance the efficiency of micropropagation. These include the utilization of liquid culture systems, creating a CO_2-enriched environment for growing cultures, and enhancing the culture vessel environment through improved ventilation and other methods. These advancements have demonstrated significant benefits in terms of optimizing the micropropagation process.

The primary objective of this study was to develop efficient and consistently reproducible micropropagation protocols for banana *(Musa acuminata)* using a liquid culture system and modified growth conditions. While traditional *in vitro* propagation methods have been employed for these plant species, there was a recognized need for refining these protocols to achieve a higher yield of healthy plants at a more cost-effective rate. In addition to the protocol refinement, this research also included morpho-physiological studies. These studies were conducted to assess the overall quality of the plantlets produced in the culture, providing valuable insights into the health and characteristics of the propagated plants. This comprehensive approach aimed to contribute to the improvement of banana micropropagation techniques, ultimately benefiting the agricultural and horticultural industries.

7.1 Growth of plant in different growth condition

The advantages of a liquid culture system over traditional agar-based solid medium are well-established, with one notable benefit being the higher multiplication rates it offers. In the current study involving *Musa acuminata* it was observed that the liquid medium stimulated *in vitro* growth and shoot multiplication. The increased shoot multiplication and elongation observed in the liquid medium can be attributed to the fact that the shoots were constantly surrounded by nutrients. This arrangement provided a larger surface area for cells to rapidly absorb nutrients and facilitated quick

nutrient replacement at the cell surface through diffusion and movement from the surrounding liquid. Additionally, this environment facilitated improved uptake of cytokinins, which are essential plant growth hormones, contributing to enhanced shoot multiplication and growth. (Sandal *et al.,* 2001; Gupta and Timmis, 2005). (Mohapatra and Batra, 2017). Similar observations of enhanced growth have been reported in other plant species using liquid media. For instance, in potato cultures, it has been noted that leaf areas doubled, and shoots exhibited increased internode length when grown in liquid media. This finding suggests that the liquid medium promoted the overall growth and development of potato plants. Additionally, in the case of *Saccharum officinarum L*, shoots derived from full-strength MS (Murashige and Skoog) liquid medium showed significantly greater length compared to those derived from full-strength MS semi-solid medium. This indicates that the liquid medium was particularly advantageous for the growth of *Saccharum officinarum L*, contributing to longer and healthier shoots. These findings underscore the utility of liquid culture systems in promoting robust plant growth and can be applied to optimize the cultivation of various plant species (Melaku *et al.,* 2016). Rapid micropropagation using a liquid culture system has been documented in numerous cases. This approach has gained recognition for its ability to accelerate the propagation of plants, resulting in increased yields of healthy plantlets in a shorter timeframe (Daneshvar Salaj *et al.,* 2016; Gatti *et al.,* 2017; Nápoles Borrero *et al.,* 2017; Royandazagh, 2019; Vyas *et al.,* 2021).

In our research, when evaluating the most suitable support for the liquid culture medium, we discovered that glass bead support matrices were the optimal choice. These support matrices were constructed using locally sourced glass beads (marbles). They proved to be highly effective due to their inert nature, ability to withstand autoclaving for sterilization, and reusability (Niedz and Marutani-Hert, 2018; Shekhawat *et al.,* 2022). The cost-effectiveness of using glass beads as an alternative to agar in plant tissue culture has been demonstrated in previous studies (Debnath, 2016) work provides evidence of this cost-saving approach. By substituting agar with glass beads, the overall expenses associated with the culture medium preparation can be significantly reduced for Dwarf Cavendish (Souza Costa *et al.,* 2020). The use of glass beads for the propagation of raspberry and white clove, along with the reported 60% savings in media cost, underscores the practicality and cost-

efficiency of this approach. The use of glass beads has proven to be satisfactory for the maintenance of callus and the induction of shoot organogenesis. This finding highlights the versatility and effectiveness of glass beads in supporting these crucial aspects of plant tissue culture (Niedz and Marutani-Hert, 2018) in Citrus. The successful replacement of conventionally used agar with glass beads, without compromising the quality of *in vitro* regenerated plantlets of *Typhonium flagelliforme*, is a significant achievement (Rezali *et al.*, 2017). Hyperhydricity occurring in most regenerated shoots when cellulose filter paper supports were used is a notable observation. This condition, characterized by excessive water uptake by the plant tissues, can negatively impact plant health and development. Similar findings have been reported in other cases, particularly with the use of polystyrene foam, where it was deemed unsuitable for the growth of (Lopez-Guerrero *et al.,* 2022). The observation of stimulated growth in a liquid medium supported with filter paper rafts is noteworthy. This finding indicates that the filter paper rafts contributed to improved growth conditions for the cultured plants. However, it's essential to note that this enhanced growth did not surpass the growth achieved in the glass bead-supported liquid medium, suggesting that glass beads still provided superior support for plant growth. Interestingly, similar results were reported in the case of *citrus* by (Niedz and Marutani-Hert, 2018). Their research also found that filter paper raft-supported liquid medium moderately stimulated overall growth. These parallel findings support the notion that filter paper rafts can enhance growth in certain plant species but may not outperform other support materials like glass beads in all cases.

The use of temporary immersion or culture in a liquid medium for plant cultivation has shown several advantages, including enhanced elongation and multiplication of shoots, as well as an increase in leaf area. This approach has demonstrated its effectiveness in promoting growth and development in various plant systems. Temporary immersion culture provides several benefits for plant growth. It allows for a constant exchange of nutrients and gases around the plant tissues, creating an ideal environment for shoot elongation and multiplication. The increased leaf area observed further supports 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 in temporary immersion culture can be attributed to several factors. One key factor is the improved aeration facilitated by the periodic immersion of plant tissues in the

liquid medium. This process allows for better oxygen exchange, which is essential for cellular respiration and overall plant health. Additionally, the renewal of the headspace above the liquid medium during temporary immersion helps reduce hyperhydricity. Hyperhydricity, characterized by excessive water uptake by plant tissues, can be detrimental to growth. By periodically exposing the plant to the air, excess moisture is removed, preventing or mitigating hyperhydricity and promoting more favorable growth conditions (Madihah Mohd *et al.,* 2017; Ruta *et al.,* 2020). In addition to the benefits of temporary immersion in liquid medium, the combined effect of semi-solid and liquid medium plays a crucial role in promoting optimal plant growth in tissue culture. This combined approach provides a balanced environment for plant development.

The enrichment of CO_2 in both semi-solid and liquid mediums has been found to significantly enhance *in vitro* shoot growth and multiplication in *Musa acuminata*. This observation underscores the importance of carbon dioxide as a crucial factor in promoting healthy plant development during tissue culture. Lack of both carbon sources, namely CO_2 and sucrose, has been shown to lead to a gradual deterioration of cultures and eventual death in semi-solid and liquid mediums. This decline in culture health is primarily attributed to starvation, as both CO_2 and sucrose are essential for plant metabolism and energy production. Without these vital carbon sources, the cultured plants cannot sustain themselves, ultimately leading to their demise (Fujiwara *et al.,* 1987; Doi *et al.,* 1989). Significant improvements in *in vitro* growth and multiplication were observed when cultures were grown without sucrose but in a controlled and CO_2-enriched environment. This finding indicates that the plants were able to thrive and grow efficiently through photoautotrophy, utilizing carbon dioxide as their primary source of carbon for photosynthesis. Moreover, the cultures that received an additional supply of CO_2 appeared to exhibit luxury consumption, suggesting that they had access to more carbon dioxide than required for optimal growth. This phenomenon can be indicative of favorable conditions that support robust plant development (Rogers *et al.,* 1994). The observation of photoautotrophic growth on sucrose-free medium is a significant finding. It demonstrates that certain plant cultures have the capacity to sustain themselves and achieve growth solely through photosynthesis, using carbon dioxide as their primary carbon source was 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). The observation that an increase in CO_2 concentration beyond the optimal level led to decreased growth in some cases is intriguing. This phenomenon may be attributed to a downregulation of photosynthesis in plants subjected to excessive CO_2 enrichment. In some instances, plants can exhibit a response known as "acclimation" or "downregulation" of photosynthesis when exposed to elevated CO_2 levels for extended periods. This means that although increased CO_2 initially promotes photosynthesis, prolonged exposure to high CO_2 concentrations may lead to a reduction in the efficiency of photosynthetic processes (Fernandez *et al.,* 1998). The current study revealed that the combination of 3.0% sucrose and a controlled and enriched CO_2 environment was the most effective for promoting *in vitro* shoot growth and multiplication in the studied plant systems. This combination demonstrated a synergistic effect, indicating that the presence of both sucrose and elevated CO_2 levels had a positive and complementary impact on plant development. Similar synergistic effects of combining sucrose and CO_2 on *in vitro* shoot growth have been observed in other plant species as well in *Protea cynaroides L* (Wu and Lin, 2013), *Hevea brasiliensis* (Tisarum *et al.,* 2018), *Pfaffia glomerata* (Corrêa *et al.,* 2015). In addition to the successful combination of sucrose and CO_2, another noteworthy observation was made: 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, coupled with CO_2 enrichment, was particularly conducive to promoting robust plant growth also observe in *Uniola paniculata* (Valero–Aracama *et al.,* 2007).

Our research has shown that the selection of containers for plant tissue culture plays a pivotal role in the growth of *Musa acuminata*. The type of vessel used can significantly alter the number of air exchanges that occur through the container's walls. This, in turn, has a cascading effect on the internal environment within the vessel. The choice of vessel material and design directly impacts the composition of gases inside the container. It influences how fresh air is exchanged with the surrounding atmosphere and affects the levels of carbon dioxide and oxygen available to the growing plant cultures. Additionally, the relative humidity and temperature within the vessel are also influenced by the vessel type (Chen and Chen, 2002; Kim, 2002). In term of best shoot multiplication was achieved in 250 ml flask. Huang and

Chen (2005) proposed that the use of larger vessels with wide openings had a positive impact on the rate of air exchange, leading to improved overall plant growth. Additionally, these spacious vessels had a shape that promoted balance light transmission, further enhancing the growth process. (Manokari *et al.*, (2022) In their report, it was found that using round-shaped baby food jars was optimal for shoot production in *Hemidesmus indicus (L.)*. This choice was attributed to these jars having an intermediate air-exchange rate, striking a balance that was favorable for plant growth. Additionally, the round shape of the jars helped in maintaining suitable humidity levels, reducing the risk of desiccation and contributing to successful shoot production. The apical explants of *Scrophularia yoshimurae* specie exhibited their highest *in vitro* performance when cultured in Magenta^{TM} boxes (Lai *et al.,* 2005). It has been documented that a greater rate of shoot multiplication occurs in larger vessels for both *Gladiolus* (Dantu and Bhojwani, 1992) and *lettuce* (Tisserat and Silman, 2000). Conversely, the conical shape, in contrast, resulted in a lower rate of air exchange, which, in turn, restricted the overall rate of multiplication to a moderate level. As reported by Huang and Chen (2005), conical vessels exhibited the lowest air-exchange rates, which subsequently led to slower growth of the cultures. In a study conducted by Joshi et all (2009) they found that the size of the containers used for plant tissue culture did not have a significant impact on the rate at which shoots multiplied in *Wrightia tomentosa.* However, they did observe an effect on the length of the shoots. In larger vessels with greater capacity, the shoots tended to be more elongated. In fact, the maximum shoot elongation was noted in culture bottles when compared to other types of glass containers. This suggests that container size can influence the morphology of the plant shoots in this species. It's important to note that while shoot multiplication wasn't significantly affected, the length of the shoots was influenced by container size, with larger vessels favoring greater elongation.

The choice of caps used to seal the containers where we grew *Musa acuminata* plants had an impact on their growth in our laboratory conditions. Specifically, when we used conical containers with non-absorbent cotton plugs as closures, it seemed that these plugs may have hindered the optimal penetration of light to the plant cultures. This finding aligns with the research conducted by Fujiwara and colleagues in 1989. They discovered that both foam rubber plugs and aluminum foil closures were not transparent to light, meaning they didn't allow much light to diffuse into the inner

space of the containers. This lack of sufficient light exposure could have affected the growth of our *Musa acuminata* plants *in vitro*. In our study, we observed a significant difference in the growth of plants based on the type of closures and vessels used in plant tissue culture. Specifically, we noticed that when closures and vessels were placed in a central position, there was a shading effect that limited the growth of the plants. This means that the plants received less light, which is essential for their growth. However, when we used phyta jar vessels and culture bottles with polypropylene lids, we observed a different outcome. These polypropylene lids allowed a greater amount of light with different wavelengths to penetrate the vessels, resulting in improved plant growth. This suggests that the type of closure and vessel design can have a significant impact on the availability of light to the plants, which in turn affects their growth. The study conducted by Kitaya et al. in 1995 provided evidence that polypropylene caps have the capability to allow the passage of light across a wide range of wavelengths. Additionally, the inclusion of a vent in the polypropylene lid had a notable impact on the exchange of gases within the culture vessels, leading to varying levels of plant growth. According to Hahn and Paek (2001), The use of ventilation within the culture vessel played a pivotal role in preventing the rise of air temperature and relative humidity levels inside the vessel. This, in turn, had a positive effect on the growth of the plants. By maintaining optimal temperature and humidity conditions, the plants were provided with a more conducive environment for their growth was suggest by Hahn and Paek (2001). In other cases, when there was an increase in air exchange within the culture system, it had a notable impact on the photosynthetic activity of the plantlets. Specifically, this increase in 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 take in more carbon dioxide during the daylight hours, which is a crucial component of photosynthesis (Kozai *et al.,* 2005; Xiao *et al.,* 2011; Li *et al.,* 2017; Zarei *et al.,* 2021). There have been reports of improved growth and photosynthesis in *in vitro* plantlets when the number of air exchanges within the culture vessel was increased. In essence, providing more frequent exchanges of air within the vessel had a positive impact on both the growth and photosynthetic activity of the plantlets (Carvalho *et al.,* 2001; Valero-Aracama *et al.,* 2007; Arigita *et al.,* 2010;). Contrary results were documented by Najima et al. in their study involving *C. paniculatus and T. bellerica*. In this case, they found that the

overall growth of the cultures was superior in bottles with unvented caps when compared to those with vented caps. This outcome can be attributed to the fact that vented vessels facilitated a rapid drying of the cultivation medium. Consequently, this quick drying adversely affected the growth of shoot cultures (Nguyen *et al.,* 1999; Zobayed *et al.,* 2001; Mohamed and Alsadon, 2011).

In our current research, we carried out experiments aimed at enhancing the development of roots in *in vitro* grown plant. To achieve this, we employed a liquid medium as part of our methodology. When shoots, which had initially grown in the liquid medium, were later transferred to a specific medium designed for root development, we observed successful root formation in plantlets. In all instances, the most favorable results regarding root growth were observed when the liquid medium, supported by glass beads (without the inclusion of agar), was employed. The utilization of a liquid medium during the *in vitro* rooting process is closely associated with the rapid absorption of growth regulators and essential nutrients present within the medium. This enhances the overall rooting process and contributes to the 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 quest for finding new materials to use in plant tissue culture is an ongoing endeavor in research. This pursuit can be divided into two main facets. The first aspect focuses on achieving cost-effective tissue culture methods, while the second aspect aims to improve root induction and increase the survival rate, especially in the context of micropropagation (Makunga *et al.,* 2006). Glass beads have demonstrated their effectiveness as support matrices for *in vitro* rooting in under study. When utilizing glass beads in conjunction with a liquid medium, several benefits become evident. Firstly, it facilitates optimal root aeration for the plantlets. Additionally, this approach, in combination with the elevated humidity levels within the culture vessels, contributes to the development of plants with robust root systems. This, in turn, reduces the need for extensive hardening procedures, as the plants naturally acquire a stronger foundation for growth.

Filter paper were examined as an alternative support matrix for *in vitro* rooting in plant. While they did support *in vitro* rooting to some extent, their performance was not superior to that of glass beads. Additionally, a notable drawback observed was

that the roots tended to become entangled within the pores of the filter papers when attempting to remove them. This issue with root entanglement during removal is not unique to your study but has also been encountered in previous research. For example, filter paper bridges were used in the cultivation of woody plants such as *Scutellaria***,** *Solanum tuberosum, Wasabi* and, but researchers faced similar challenges, finding it problematic to extract the plant material without causing injury (Tascan *et al.*, 2010; Kaur and Minhas, 2016; Hoang *et al.*, 2019).

In the liquid culture system, we observed several significant advantages in comparison to traditional methods. This approach led to the successful growth of a high number of robust and healthy plants, with improved overall plant quality and an impressive survival rate during the *in vitro* hardening process. One notable 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 described by (Mohapatra and Batra, 2017). This nutrient-rich environment played a crucial role in promoting the development of the plants. For instance, when working with *Scutellaria* shoots using the liquid culture method, observed vigorous growth without the occurrence of hyperhydricity, a common issue in plant tissue culture. This exceptional growth led to a remarkable 100% survival rate when the plants were transferred *ex vitro,* as reported by (Tascan *et al.,* 2010). In sugarcane on a liquid medium yielded similarly impressive results. These microplants exhibited a 100% survival rate after being transferred to a greenhouse, as Recorded by (Nápoles Borrero *et al.,* 2017). This underscores the effectiveness of the liquid culture system in supporting the healthy growth and survival of plants under various conditions.

7.2 Studies on leaf surface structures

Distinct variations in the external appearance of leaves, including features like the texture of the leaf surface, the number of tiny openings called stomata, the shape of these stomata, and the structure of the waxy layer on the leaf's surface, were noticeable when comparing leaves that grew under various conditions and at different stages of development in the *Musa acuminata* plant. Furthermore, changes in the density of stomata and related factors were found to be connected with the specific conditions within the laboratory, such as the amount of light exposure, levels of carbon dioxide (CO_2), the concentration of ethylene gas, humidity levels, and the environment outside of the laboratory setting (Lucchesini *et al.*, 2006).

The most significant structural contrast was the abundance of epicuticular wax found on leaves of plants grown in natural field conditions, as opposed to the minimal wax present on leaves grown in the laboratory during the multiplication (SM and LM leaves) and rooting (SR and LR leaves). In a controlled lab environment, the amount of wax on the surface of *carnation* leaves was found to be significantly lower, just 25% of what was observed on plants cultivated in a greenhouse (Majada *et al.,* 2000). However, when these plants were subjected to a process called *in vitro* hardening, some significant changes occurred. One crucial change was the development of a protective wax layer on the leaves of plant species. Remarkably, within a relatively short period of 6 to 7 weeks after transplantation, the plants that had been micro propagated in the lab reached a wax density similar to that of their counterparts grown in natural field conditions (Dhawan and Bhojwani, 1987).

The stomatal system in leaves that were grown in a controlled laboratory environment (*in vitro*) displayed a typical structure similar to what is commonly observed in other plant species. This similarity in morphology was consistent with our expectations based on prior research findings. These findings suggest that the growth conditions used in our experiment did not significantly alter the fundamental characteristics of the plant's stomatal system. (Aliniaeifard *et al.,* 2020; Joshi *et al.,* 2006) *e.g.* In the context of plant tissue culture experiments, this was recorded distinct alterations in the structure of guard cells. These changes were evident in the round shape of the guard cells, which deviated from their typical form. Additionally, the inner walls of these guard cells appeared thin and exhibited deformations. 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. Secondly, we observed modifications in the arrangement and deposition pattern of cellulose microfibrils within the guard cell walls (Zein El Din *et al.,* 2020).

During the rooting phase of plant development, we observed the emergence of functional stomata in both SR and LR leaves. This marked the beginning of a structural reversal process. Remarkably, the stomata in leaves that were undergoing

in vitro hardening closely resembled those found on leaves grown in their natural environment, in terms of their shape, size, and how frequently they occurred. However, when we transferred the plantlets from their *in vitro* cultures to the greenhouse or the field, we noticed significant alterations in the morphology of the leaves. These changes were particularly noticeable in the characteristics of the stomata (Hazarika, 2006). Such advancements plant tissue culture that effectively control the process of transpiration, which is the loss of water vapor from plant tissues, are of paramount importance for the successful survival of transplanted plants. This phenomenon is vital because it directly impacts the plant's ability to adapt and thrive in different environmental conditions, a key aspect of our research in plant tissue culture (Radochova and Ticha, 2009).

At a microscopic level, we observed that the tiny hair-like structures called "trichomes" were visibly shorter and had a blunted appearance in leaves that were grown in a controlled laboratory environment (*in vitro*) compared to leaves from plants grown outdoors in natural conditions (field-grown leaves). Furthermore, we noticed that trichomes tended to cluster together more consistently on leaves during the "*in vitro* hardening" stage of plant development, as opposed to the "rooting" stage. In the case of date plants, we observed a variation in the number of these epidermal hairs in different growth conditions. Specifically, the number of epidermal hairs was relatively low in leaves that developed *in vitro*, increased in leaves that formed after the plants were transplanted into different environments, and reached the highest count in plants that were cultivated in greenhouses or grown directly in the field (Zein El Din *et al.,* 2020). The reduced variety and limited spread trichomes on the *in vitro* grown compared to those grown naturally in teak trees are linked to greater water loss in the *in vitro* plants. This increased water loss ultimately results in a lower chance of survival for these plants after they are transplanted (Zein El Din *et al.,* 2020). The decreased presence of trichomes can be attributed, in part, to a deficiency in cell specialization in plants that have undergone micropropagation. This means that when plants are reproduced using micropropagation techniques, they may not develop trichomes as effectively as their naturally grown counterparts (Monja-Mio *et al.,* 2021).

The composition of the growth medium also had an impact on the characteristics of leaf surfaces, although the influence was somewhat limited.

Notably, the alterations were most conspicuous in terms of the size and occurrence of stomata on the leaves. The substantial enlargement of stomata observed in leaves obtained from a liquid medium could likely be attributed to the greater surface area of the leaves in this condition. Additionally, it is worth noting that the variations in leaf surface structures were more prominent in the context of our plant tissue culture experiment. This suggests that the choice of culture medium can play a significant role in shaping the physical attributes of the leaves, particularly with respect to stomatal characteristics (Aliniaeifard *et al.,* 2020). The extent of deformities in the LM (leaf margins) was notably higher. This could potentially be attributed, at least in part, to the presence of excessive moisture in the liquid growth medium. This excess moisture may have contributed to the observed malformation in the leaves (de La Vina *et al.,* 2001; Dutta Gupta and Prasad, 2010). In the plant grown in control condition some researcher discovered a higher occurrence of actively functioning stomata in LR leaves. This observation suggests the beginning of a process known as stomatal reversal. Notably, when comparing the stomatal characteristics, LH leaves exhibited superior attributes compared to SH leaves. This phenomenon highlights the intricate relationship between environmental factors, such as radiation and humidity, and the physiological responses of plant leaves, specifically in the context of stomatal behavior. (Hazarika, 2006). In both the semi-solid and liquid systems within *Gladiolus*, we observed that the stomatal behavior appeared to be within the expected range of normalcy. This suggests that the experimental conditions did not significantly impact the functioning of stomata in *Gladiolus* plants (Dutta Gupta and Prasad, 2010).

In the study conducted by Yang and Yeh in 2008, it was found that *Calathea* plants grown in a liquid medium, specifically through a temporary immersion technique, showed better stomatal characteristics when compared to those grown in a semi-solid medium. This suggests that the choice of growth medium can significantly influence the development of stomata in *Calathea* plants. Our findings indicate that the adjustments needed for micro propagated plants to adapt well to their new environment primarily started during root differentiation. These changes were particularly noticeable in the liquid rooting leaves (LR). This implies that plants cultivated in a liquid medium might undergo a faster and more effective hardening process, leading to a greater chance of survival when they are transferred to a *in vivo*

or field condition. This outcome suggests that liquid culture can be advantageous for plant acclimatization and overall success in transplantation.

In our study, we examined the stem, leaves, and roots of *Musa acuminata* plants that were cultivated on both semi-solid and liquid growth mediums. Our aim was to discern any structural variations between these two growth conditions. When we analyzed cross sections of the aerial stem, we found that the anatomical structure remained quite similar for plants grown in both types of media. This suggests that there were no apparent signs of hyperhydricity in response to the liquid growth medium. Furthermore, the leaves of the *in vitro*-cultured plants displayed some distinct characteristics. They appeared thinner compared to those grown under natural conditions, which aligns with findings from previous studies (de Souza *et al.,* 2021; Jagiełło-Kubiec *et al.,* 2021). The palisade layer in the leaves was notably underdeveloped.

Our investigation also revealed differences in leaf thickness between plants cultivated in a temporary immersion system and those on a semi-solid medium. Specifically, the chlorenchyma, responsible for photosynthesis, was thicker in the temporary immersion system. This observation highlights the impact of *in vitro* culture systems on leaf morphology. The development of photosynthetic tissues plays a pivotal role in a plant's ability to adapt and thrive, as indicated by previous research (de Souza *et al.,* 2021)

7.3 Studies on chlorophyll fluorescence

7.3.1 Photosynthetic efficiency during different stages of growth

In the current study, the assessment of photosynthetic efficiency was conducted through the measurement of chlorophyll a fluorescence in *Musa acuminata*. Various chlorophyll fluorescence parameters, including F0, Fm, Fv/Fm, ΦPS2, ETR, qP, and qN, were investigated across different stages of plant growth and on diverse types of media.

The fluorescence parameters exhibited fluctuations in both field-grown plants and *in vitro* cultures, depending on the specific growth phase. Notably, during the *in vitro* multiplication phase in both shoot meristem (SM) and leaf meristem (LM), significant variations in F0 and Fm values were observed. These fluctuations suggested a disruption in light absorption, possibly attributed to the breakdown in the chlorophyll antenna complex, a phenomenon documented in previous studies. (Batista

et al., 2018; Santos *et al.,* 2020). The Fv/Fm ratio serves as a metric indicative of the maximum efficiency in capturing excitation energy by 'open' photosystem II reaction centers. A reduction in this parameter stands as a sensitive and early indicator signaling that a plant is experiencing alterations in its environmental milieu. *In vitro*-cultured ginger plantlets exhibited a lack of photosynthetic activity, a phenomenon commonly observed in micropropagated plants due to their exposure to low light intensity conditions (Cai *et al.,* 2010). The reduction in the quantum yield of photosystem II (ΦPS2) under light-induced conditions can be attributed to the diminished efficiency in energy capture by open photosystem II (PS2) reaction centers. (Cai *et al.,* 2010; Osorio *et al.,* 2010).

As ΦPS2 represents the quantum yield of photosystem II (PSII) photochemistry, it is commonly employed for the computation of linear electron transport rate (ETR), thereby determining the overarching photosynthetic capacity (Genty *et al.,* 1989). The observation of a markedly reduced ETR in leaves cultivated *in vitro* signifies a correspondingly diminished photosynthetic capacity within our plant systems. This decline in ETR is frequently associated with the imposition of low light intensity conditions. Results similar to us were obtained in micro propagated *Zinziber officinale* (Guan *et al.,* 2008) and *Rhododendron ponticum* (Osorio *et al.,* 2010).

qP quenching reflects the capacity of reaction centers to compete for chlorophyll excitation states and is connected with the redox state of Q_A-the primary electron acceptor after PS 2 (Georgieva *et al.,* 1996; Maxwell and Johnson, 2000). The qP values obtained from our experiments indicated a normal functional activity of reaction centers. No discernible distinctions were observed between the leaves of mature plants grown in the field and the *in vitro* leaves of shoot cultures cultivated on either type of medium. Our findings align with prior research on tobacco, where no significant variations in photochemical quenching of fluorescence were noted between leaves from field-grown plants and those from *in vitro* cultures (Habibi and Purohit, 2019b). This observation further implies that the lower photosynthetic capacity under *in vitro* conditions in all our plant systems can be attributed to alterations in pigment ultrastructure and likely improper functioning of photosynthetic enzymes, rather than a malfunction of the photosynthetic apparatus itself (Sáez *et al.,* 2016).

Quenching, denoted as qN, is intricately linked to the establishment of a proton gradient. It serves as a defensive mechanism for Photosystem II (PS II) against photoinhibition, as previously elucidated by Osorio et al. in 2010. This parameter quantifies the dissipation of light energy in the form of heat or excess excitation energy, as expounded by Maxwell and Johnson in 2000. Our experimental findings reveal minimal losses of light energy through non-radiative channels, attributable to the exceedingly low light intensity provided for optimal plant growth conditions. In the context of our study, *Rhododendron ponticum* plants subjected to micropropagation and acclimated under low light conditions exhibited notably lower qN values in comparison to those cultivated under high light conditions (Osorio *et al.,* 2010). This observation underscores the impact of light intensity on the photoprotective mechanisms of PS II, with implications for the photosynthetic performance of micro propagated *Rhododendron ponticum*.

In the course of the rooting phase, a restoration of photosynthetic competence in *in vitro* leaves was discerned, as evidenced by the recuperation of the Fv/Fm parameter across all three plant species under investigation. Guan et al. (2008) previously established that the recovery of Fv/Fm serves as an indicator for the restoration of photosynthetic competence in *in vitro* leaves. The findings align with the observations made by (Sharma *et al.,* 2020), who suggested that diminished photosynthetic capability may be linked to the presence of sucrose in the growth medium. Consequently, in the current study, the reduction in sucrose concentration within the rooting medium resulted in an enhancement of various fluorescence parameters compared to the *in vitro* multiplication phase. This adjustment in sucrose concentration, as highlighted by (Joshi *et al.,* 2010), contributed to the development of a more proficient photosynthetic apparatus in *in vitro* rooted shoots. The augmented functionality became apparent through enhanced measurements of chlorophyll fluorescence at the culmination of the rooting phase. The elevated photosynthetic competence observed during acclimation is a consistent trait across diverse plant species cultivated in a controlled *in vitro* environment (Chaari-Rkhis *et al.,* 2015; Pan *et al.,* 2019). The complete recovery of F_v/F_m in *Rhododendron pointicum* during hardening and acclimation points to scenario of PS 2 down regulation or dynamic photoinhibition (Osorio *et al.,* 2010). These results are in accordance with those reported in the literature (Faisal and Anis, 2010). On the contrary, a decline in F_v/F_m,

caused by down–regulation of photosynthesis or photoinhibition during the early days after transfer, was reported in studies with micro propagated *Arabidopsis* (H. Zhang *et al.,* 2008) *olive* (Chaari-Rkhis *et al.,* 2015) and Tomato (Pan *et al.,* 2019). These findings are consistent with our experimental outcomes, wherein the Fv/Fm parameters exhibited a moderate reduction. Upon the immediate transplantation of the plant specimens, a notable decline in Fv/Fm was discerned on the third day, suggesting that the plants experienced stress in response to alterations in environmental conditions. A parallel phenomenon of plant acclimation under similar circumstances has been previously documented in scientific literature. (Schreiber and Klughammer, 2021).

In *Musa acuminata,* the fluorescence parameters assessed in leaves obtained from a liquid medium exhibited superior results compared to those from semi-solid medium, suggesting a more robust photosynthetic system in the former. Consequently, the *in vitro* environments supporting the growth of our shoot cultures imposed subtle and transient stress, with neither medium type, particularly the liquid medium, inducing prolonged severe stress on the plants. This observation underscores the resilience of the *in vitro* shoot cultures to the experimental conditions, reinforcing the viability of the selected culture mediums in maintaining a favorable physiological state in *Musa acuminata.*

7.3.2 Photosynthetic efficiency during CO_2 enrichment

The photosynthetic potential was evaluated by supplementing the cultures with exogenous CO_2. Distinct responses were observed in relation to all fluorescence parameters under these experimental conditions. Assessments of Fv/Fm, ϕPS2, and ETR yielded an indirect measure of the photosynthetic efficacy of the cultures. Elevated values indicated enhanced photosynthetic efficiency, while lower values corresponded to diminished efficiency in the cultures.

In plant systems cultivated on a medium enriched with sucrose, the introduction of elevated carbon dioxide (CO_2) levels has a substantial impact on various fluorescence parameters, notably affecting Fv/Fm and ΦPS2 in shoot cultures. *In vitro* multiplying cultures originating from SCSM and SCLM exhibit a significant enhancement in maximum photochemical yield under conditions characterized by elevated CO_2 levels. The photochemical efficiency experiences a progressive decline with an increase in carbon dioxide concentrations. Moreover, a noteworthy reduction

in photochemical efficiency is observed in SCSM supplemented with the maximum CO_2 concentration, i.e., 40.0 g m^{-3}. This observation aligns with the hypothesis that excess sugars contribute to the downregulation of photosynthesis. (Gago *et al.,* 2022; Park *et al.,* 2018; Sáez *et al.,* 2016). Down regulation of photosynthesis was observed when sugars were fed to suspension cultured cells (Arigita *et al.,* 2010) or *Pfaffia glomerata* leaves (Saldanha *et al.,* 2013), or when the balance between the production and the consumption of carbohydrates was disturbed (Koch, 1996). Sugar in the medium has been reported to reduce the Rubisco activity, and thus the photosynthetic efficiency of *in vitro* plantlets (Saldanha *et al.,* 2013).

Cultures of *Musa acuminata* grown under CO_2–free and sucrose–free conditions demonstrated extremely poor photosynthetic capability as indicated by their extremely low F_v/F_m, ϕPS2 and ETR. The experimental results obtained by (Vahdati *et al.,* 2017) and (Manokari *et al.,* 2022b) suggested that insufficient CO_2 supply into the vessel limits the photosynthesis.

The augmented concentration of carbon dioxide (CO_2) in the absence of sucrose within the growth medium significantly enhanced the chlorophyll fluorescence parameters. The photoautotrophic cultivation of plantlets in a medium devoid of saccharides facilitates the maturation of a fully operational photosynthetic apparatus. Typically, such plantlets necessitate an increased CO_2 concentration and elevated irradiance compared to the conventional cultivation conditions (Morini and Melai, 2003; Kozai *et al.,* 2005; Martins *et al.,* 2015; Aldrey *et al.,* 2018; Santos *et al.,* 2020). Our findings align with the aforementioned assertion. In, the highest values for the maximum quantum yield of photosystem II (Fv/Fm) and the effective quantum yield of photosystem II (ΦPS2) were attained at concentrations of 10.0 or 40.0 gm^{-3} of carbon dioxide (CO2) in both less sucrose containing Solid medium and Liquid Medium. The augmentation in photosynthetic rate has been documented across diverse crop species by multiple researchers in response to elevated CO_2 concentrations (Morini and Melai, 2003; Rahman and Alsadon, 2007; Aldrey *et al.,* 2018).

The photoautotrophic cultivation of cultures in a liquid medium was effectively showcased in the plant systems under scrutiny in this investigation. The modulation of sucrose presence or absence in the liquid medium exhibited analogous

impacts on photosynthetic parameters, mirroring observations made in the semi-solid medium. These findings substantiate that the liquid medium employed in this study did not impart any discernible influence on hyperhydricity within the examined plant systems.

7.4 Studies on water relations

7.4.1 Water loss studies

The percentage of water loss in *Musa acuminata* varied based on factors such as the type of plant, its growth stage, and the specific growth medium used for cultivation. This observation suggests that different plant types, various growth stages, and the choice of growth medium play significant roles in influencing the rate of water loss in *Musa acuminata*.

The highest degree of water loss was observed during the *in vitro* multiplication stage in *Musa acuminata*. Inadequate deposition of protective epicuticular wax on the leaf surface of *in vitro* cultivated plants, impaired stomatal functionality, and suboptimal cuticle development have been identified as pivotal factors contributing to an exacerbated water loss phenomenon. This condition significantly compromises the success of transplantation endeavors (Sajeevan *et al.,* 2017; Zein El Din *et al.,* 2020). In the process of *in vitro* hardening, exposure to elevated irradiance, coupled with a systematic reduction in relative humidity, has been observed to effectively mitigate the occurrence of excessive transportational water loss in leaves. This phenomenon contributes to the restoration of stomatal function, thereby preventing leaf wilting and promoting the overall survival of *Musa acuminata* plantlets post-transplantation. The observed reduction in the rate of water loss correlates with a concurrent augmentation in the deposition of cuticular wax on the leaves (Hazarika, 2006). These results are in accordance with other water relation reports on micro propagated plants, such as *Wrightia tomentosa* (Joshi *et al.,* 2006), *Vitis vinifera* (Salomon *et al.,* 2014), *Morus indica* L. (Sajeevan *et al.,* 2017), Date palm (Zein El Din *et al.,* 2020). (Revathi *et al.,* 2019) however, noted that the quantity of epicuticular wax alone was not a good predictor of survival of micro propagated plantlets in the greenhouse during acclimatization. Cultured plants are divisible in photosynthetic non–competent and competent specie. This hypothesis posits a correlation between heightened transpirational losses and the *in vitro* hardening process of *Musa acuminata*.

The selection of the growth substrate exhibited negligible influence on the overall transpirational rate in our investigation. These outcomes are in consonance with our antecedent investigations into plant attributes, wherein we discerned comparable instances of stomatal irregularities and the accumulation of cuticular waxes on the foliar epidermis of plants cultivated in diverse culture media. Consequently, the extent of transpirational water loss in leaf specimens derived from cultures cultivated in both semi-solid and liquid substrates exhibited analogous patterns. In the investigation conducted by (Z. Zhang *et al.*, 2021), noteworthy observations were documented wherein shoot meristems were subjected to inoculation with paclobutrazol in a liquid medium and subsequently maintained within an environment characterized by high humidity levels, approximately 98%. The outcome revealed a distinctive phenomenon, whereby the resultant plants originating from these treated shoot tips exhibited a reduced stature compared to their conventional counter parts. Subsequently, an augmented presence of epicuticular wax was discerned on their surface. Lastly, upon transplantation to a conventional agricultural setting, these plants exhibited diminished wilting compared to their counterparts. Notably, congruent findings were reported by a distinct cohort of researchers, as elucidated by (Markovic *et al.*, 2020), who conducted analogous investigations involving *Fritillaria meleagris*. The cultivation of these plants transpired within a liquid medium containing 9% sucrose along with supplementary plant hormones.

7.4.2 Biomass accumulation and water content.

In our current research, we have found a clear connection between the rate at which plant cells multiply and the amount of fresh and dry weight they accumulate in their cultures. When we grew plants in controlled environments in the lab (*in vitro*), we observed that the highest amounts of both fresh and dry weight were recorded when the rate of shoot multiplication was at its peak. This suggests that the growth and multiplication of plant cells are closely linked to the overall weight of the plant tissue in these specific culture conditions. This finding highlights the importance of shoot multiplication as a key factor influencing plant growth and development *in vitro*.

In our current study, we observed that the use of a liquid medium had a positive impact on the growth of plant systems we investigated. This positive effect was evident in the increase in both fresh and dry weight of the plants. The higher biomass

accumulation we observed can be attributed to several factors. Firstly, there was an increase in the number of shoots, which contributed to greater biomass. Additionally, there was an increase in the number of leaves and the overall leaf area of the plants. This increase in leaf number and area likely contributed to enhanced photosynthesis and, subsequently, greater biomass production. Furthermore, the presence of cytokinins in the liquid medium played a crucial role in promoting plant growth. The plants readily absorbed these cytokinins from the liquid medium, leading to increased shoot elongation, which further contributed to the overall biomass accumulation. (Gupta and Timmis, 2005). The mean weight of *Pogostemon erectus* when cultured in a liquid medium was found to be higher than the weight of shoot clusters that developed in a solid medium. This suggests that the growth conditions in the liquid medium may be more favorable for the initial development of these plant structures (Muhammet Dogan, 2022) Similar results were also obtained for papaya (Gatambia *et al.,* 2016), *Saccharum officinarum L* (Melaku *et al.,* 2016). Among the various support materials we examined, we found that the highest increase in both fresh and dry plant weight occurred when we used a liquid medium supported by glass beads for all three plant species under study. Glass beads possess a substantial surface area, which fosters an effective interaction between the explant and the liquid medium. This advantageous feature of glass beads enables the efficient absorption of nutrients from the medium, resulting in enhanced plant growth. In the case of sugarcane propagation, that plantlets cultivated on glass beads not only exhibited greater growth in terms of offshoots, leaves, and storage runners but also showed remarkable improvements compared to other support matrices (Nápoles Borrero *et al.,* 2017). The increased moisture content in the plant cultures grown in a liquid medium may be explained by the elevated relative humidity and abundant water supply provided to these cultures, as noted in the study by Casanova et al. in 2008. It's important to highlight that despite these conditions, there were no observed signs or symptoms of excessive water-induced stress or hyperhydricity in the plants under investigation.

In our current research, we looked at how immersing plant cultures partially in a liquid medium affected their growth. This process had some interesting effects: it caused the plants to accumulate more fresh and dry weight, and it also increased the amount of water inside the plants. During literature we found that after 42 days of growth, plants like *Caralluma edulis* (Maciej Serda *et al.,* 2019) and *Juglans nigra L.*

(Stevens and Pijut, 2018) showed increased fresh and dry weight. This was likely because they experienced the combined effects of both liquid and solid growth medium.

Increasing the carbon dioxide concentration along with the addition of sucrose had a positive impact on the growth of *Musa acuminata* plants. When we compared the growth of these plant in semi-solid and liquid mediums, we found that the liquid medium was more effective in promoting the accumulation of biomass. Furthermore, the plants grown in the liquid medium had a higher water content percentage.

In the case of *Vitis vinifera L* plants, providing them with fully photoautotrophic conditions led to the best results in terms of both fresh and dry weight. This was observed in a study conducted by (Zhao *et al.*, 2019). Photoautotrophic conditions, it has been scientifically demonstrated that the growth of numerous plant species is significantly enhanced. This growth improvement is particularly noticeable when we measure both the fresh weight, in different 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), *Pfaffia glomerata* (Louback *et al.,* 2021), *Lippia dulcis* (Rocha *et al.,* 2022). Plantlet growth has been demonstrated to significantly improve when exposed to higher photosynthetic photon flux (PPF) levels, as reported by Kozai et al. in 1990. Additionally, an elevated concentration of carbon dioxide (CO_2), as highlighted by Kozai in 1991, has also been found to enhance plantlet growth. The rise in water levels within the SCLM cultures occurred because of the elevated humidity inside the culture containers, primarily caused by the liquid medium, as mentioned by Casanova et al. in 2008. However, when sucrose was removed from the medium, it did not promote the growth of fresh and dry plant masses. This is consistent with the findings of Arigita *et al*. (2010) with kiwi explants (*Actidinia deliciosa* Chev. Liang and Ferguson "Hayward") and Valero–Aracama *et al.* (2007) in sea oats (*Uniola paniculata*) under similar culture conditions. The rationale for this phenomenon lies in the fact that certain plant cultures initially rely on a carbon source present in the growth medium before they can fully utilize carbon dioxide (CO_2) from the surrounding air as their primary carbon source. It's important to note that sucrose plays a significant role in significantly boosting the growth of young plants. This enhancement in plantlet biomass is a crucial aspect of our research.

In our research, we observed that when the water content in the plant cultures decreased, it was a sign that the cultures were not excessively hydrated (non-hyperhydric). Interestingly, even without sucrose in the medium, the liquid growth medium outperformed the semi-solid medium in terms of various physiological aspect. The most favorable growth and quality of *Coffea arabusta* (Afreen *et al.*, 2002), *Eucalyptus* (Businge *et al.*, 2017), plantlets were achieved when they were cultured in a sugar-free liquid medium enriched with carbon dioxide (CO_2).

The choice of culture container significantly impacted the accumulation of fresh and dry plant matter. We observed that plants thrived in containers with both round and square shapes. In these containers, we noticed a substantial increase in the fresh and dry weight of the plants. This improvement can be attributed to the enhanced exchange of gases, which was facilitated by the wider openings and size of the culture containers (Chen and Chen, 2002). Air exchange within the cap significantly enhanced the accumulation of both fresh and dry mass in the shoot cultures of the plants. This improvement in mass accumulation can be attributed to the increased availability of oxygen and removal of excess moisture, creating a favorable environment for plant growth. Additionally, the enhanced ventilation facilitated efficient gas exchange, aiding in the metabolic processes crucial for plant development. This phenomena in terms of fresh and dry weight accumulation was reported in a number of plant species such as *Wrightia tomentosa* (Joshi *et al.*, 2009), *Capsicum annuum* (Mohamed and Alsadon, 2011), *Scrophularia yoshimurae* (Welander *et al.*, 2014), Sugarcane (Neto *et al.*, 2020), In contrast to these findings, it is worth noting that in some instances, different outcomes have been observed as well. (Zobayed *et al.*, 2001b; Islam *et al.*, 2005).

7.5 Carbonic anhydrase activity

The study assessed the activity of the carbonic anhydrase (CA) enzyme in cultured *Musa acuminata* plants in a controlled laboratory setting and compared it to *Musa acuminata* plants grown in their natural outdoor environment. The research revealed that the CA enzyme showed the highest level of activity in shoots that were cultivated in a liquid medium. Wu et all., (2006) determined the carbonic anhydrase activity in leaves of *Carludovica palmata* (Minchala-Buestán *et al.*, 2023), *Eurycoma longifolia* (Madihah Mohd *et al.*, 2017), *Eucalyptus* (Businge *et al.*, 2017), *Dianthus caryophyllus* (Ahmadian *et al.*, 2017). Yanyou *et al.*,(2006) proposed a valuable

insight regarding the connection between the Relative Growth Rate (RGR) of plantlets and their Carbonic Anhydrase (CA) activities. He emphasized that this relationship follows a noteworthy linear pattern. Additionally, he explained that as the carbonic anhydrase activity of a plantlet increases, its net photosynthetic rate also rises, leading to a faster growth rate. Building upon Wu's research, we formulated a hypothesis. We conjectured that the rapid growth of shoots in a liquid medium is accompanied by a notable increase in CA activity within these cultures. This suggests that there may be a positive correlation between shoot growth and CA activity.

7.6 Studies on biochemical investigation

In our current research, we examined *Musa acuminata*, specifically focusing on the variations in biochemical factors, including metabolites and enzymes, at various stages of *in vitro* cultivation. We found notable alterations in biochemical characteristics when comparing cultures grown on agar-gelled medium, semi-solid medium, and liquid medium. Furthermore, it is essential to highlight that these variations in biochemical parameters play a crucial role in understanding the growth and development of plants under different culture conditions.

During the phase of *in vitro* rooting in *Musa acuminata*, the total amount of carbohydrates reached its minimum level. Van Huylenbroeck and Riek (2005) elucidated that during the rooting process, carbohydrates are primarily transported to the developing root structures, causing a reduction in the carbohydrate levels during this phase. This decrease in carbohydrate levels is a natural response as the plant allocates resources towards root growth. This allocation of carbohydrates to the developing roots is crucial for the successful establishment of the root system. In the liquid medium, lower carbohydrate accumulation. This difference was attributed to the less negative water potential of the liquid medium in plants. It's important to note that the components present in the culture medium, both inorganic and organic, not only serve as nutrients but also impact the growth of plant cells due to their osmotic properties, as previously suggested by Chen and Ziv in 2003. This suggests that the composition of the medium plays a crucial role in influencing plant responses to stress conditions and carbohydrate accumulation.

A decline in SOD (Superoxide Dismutase) activity was noticed in *Musa acuminata* as the plants transitioned from the multiplication phase to the hardening phase. Interestingly, the liquid medium used for cultivation did not seem to cause

oxidative stress in *Musa acuminata.* This lack of stress was evident in the consistent reduction in SOD activity across all growth phases. This suggests that the liquid medium provided a stable environment for *Musa acuminata* growth, with no notable increase in oxidative stress. Stress responses in plants frequently result in the heightened generation of reactive oxygen species (ROS), including superoxide anion radical ($O2^{-\circ}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO°). These ROS compounds are known to induce oxidative stress within plant cells. The phrase 'oxidative stress' denotes a significant disparity between ROS production and their elimination (Halliwell, 1997). To elaborate further, when plants encounter stressful conditions, their cells often produce higher levels of ROS. These molecules, like superoxide anion radical, hydrogen peroxide, and hydroxyl radical, are highly reactive and can cause damage to various cellular components, including DNA, proteins, and lipids. This oxidative stress can lead to a cascade of harmful effects, ultimately affecting the overall health and growth of the plant. García-Caparrós *et al.*,(2021) showed that the activity of an enzyme called Superoxide Dismutase (SOD) in plants was influenced by the nutrients in the growth medium and the type of plant tissue used. They found that the levels of reactive oxygen species (ROS), which are harmful molecules in plants, could rise due to different environmental conditions like light exposure, lack of water (drought), extreme temperatures, high salt levels (salinity), and the presence of heavy metals. This suggests that the plant's defense mechanism against ROS can be influenced by what it's grown in and the conditions it's exposed to.

An investigation into the accumulation of proline revealed that when plants were exposed to osmotic stress caused by a liquid medium, they produced more proline throughout all stages of growth. Proline is a common substance that plants build up as a response to salt stress (Parida and Das, 2005; Nedjimi *et al.,* 2006) and as part of their defense mechanism. However, when there was no osmotic stress from the liquid medium, this accumulation only occurred during the hardening phase (Molinari *et al.,* 2007). The build-up of proline in the plant tissue, which reduces the water potential of the cells, allows young plants to absorb more water (Shetty, 2004). Additionally, it's worth noting that proline plays a crucial role in helping plants cope with stressful conditions, particularly those related to salt and drought. This amino

acid acts as an osmos protectant, aiding in the maintenance of cell turgor and preventing water loss, ultimately contributing to the plant's resilience in challenging environments.

Plants cultivated *in vitro* exhibit diminished photosynthetic efficacy (Martins *et al.,* 2015), The observed phenomenon is ascribed to diminished chlorophyll concentration, a decline in Rubisco activity, and malformation of chloroplasts (Habibi and Purohit, 2019a). Biochemical studies on *Saccharum* (Taku *et al.,* 2020), *Agave potatorum Zucc*. (Correa-Hernández *et al.,* 2022) and *Saccharum spp*. (Sorcia-Morales *et al.,* 2021) have indicated that these plants have much less chlorophyll contents during initial stages of multiplication. However, total chlorophyll, recorded for such cultures were higher in comparison with cultures growing on agar medium. These observations are indicative of better photosynthetic competence of cultures in liquid medium.

7.7 Studies on molecular evaluation

In recent years, propelled by the emergence of recombinant DNA technology, molecular markers have found extensive application in diverse research endeavors. These include the assessment of genetic fidelity in micro propagated plants, the characterization of plant genetic resources, and the elucidation of genome mapping and tagging. The preference for DNA-based markers stems from the inherent stability of DNA, rendering it resistant to developmental, physiological, or environmental fluctuations.

Molecular markers have been employed for discerning genetic variations or validating genetic integrity in the course of micropropagation (González-Benito *et al.,* 2020). Within the realm of polymerase chain reaction (PCR)-derived markers, the random amplified polymorphic DNA (RAPD) technique is acknowledged for its efficiency and cost-effectiveness. The methodology necessitates only a minimal quantity of DNA in the order of nanograms for rapid polymorphism analysis. It obviates the necessity for prior acquaintance with the DNA sequence and precludes the utilization of radioactivity in the process (Williams *et al.,* 1990). Modifications in the RAPD (Random Amplified Polymorphic DNA) pattern can arise due to the alteration in primer annealing, induced by point mutations, or through the insertion, deletion of sequences, or transposition elements (Amiteye, 2021). Random Amplified

Polymorphic DNA (RAPD) markers have been utilized for the assessment of clonal fidelity in micro propagated plants (Biswas and Kumar, 2023).

In the current investigation, polymerase chain reaction (PCR)-based methodologies, specifically Random Amplified Polymorphic DNA (RAPD) analysis, were employed to assess the genetic fidelity of three distinct accessions of *Musa acuminata*. The selection of these methodologies was predicated upon their inherent simplicity and facile execution. Employing two distinct categories of markers, each amplifying disparate genomic regions, affords a heightened capacity for the scrutiny of genetic stability and variation within the propagated plantlets (Palombi and Damiano, 2002). RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat) markers have been efficaciously employed to discern genetic affinities or disparities within micro propagated specimens across diverse plant species. (Thakur *et al.,* 2021; Biswas and Kumar, 2023)

In the current investigation, RAPD markers were estimated more appropriate in contrast to ISSR for assessing the clonal fidelity of *Musa acuminate*. The amplifications, eventually executed using RAPD markers, were chosen due to their superior performance in the generation of amplification products.

RAPD polymorphism arises either due to a nucleotide base alteration leading to a modification in the primer-binding site or as a result of an insertion or deletion event within the amplified genomic region (Babu *et al.,* 2021). The identification of genetic variation within a given plant population is commonly ascertained through the discernment of the presence or absence of an amplification product originating from a singular genomic locus. The outcomes of these amplifications may exhibit polymorphism and serve as genetic markers (Farahani *et al.,* 2022). The absence of result reproducibility across diverse laboratory surroundings has emerged as a principal apprehension in employing RAPD markers for assessing the genetic uniformity of cultivars and somatic clones. Upon optimization of experimental conditions, it was noted that Random Amplified Polymorphic DNA (RAPD) analysis consistently produced reproducible outcomes. Reliable data were acquired by meticulously standardizing the appropriate protocols, ensuring precision in the experimental procedures. The efficacy of Random Amplified Polymorphic DNA (RAPD) markers for molecular analysis of plants regenerated *in vitro* has been

extensively substantiated in existing literature (Gautam and Bhattacharya, 2021; Abdalla *et al.,* 2021).

In the current investigation, the genetic stability of micro-clones was assessed using Random Amplified Polymorphic DNA (RAPD) markers under various culture conditions of *Musa acuminata.*

9 primers were selected based on their proficiency in yielding a maximal number of distinct and robust bands. Each primer exhibited a distinctive amplification pattern. The remaining primers either failed to yield any bands or produced faint and challenging-to-score bands. Consequently, these excluded primers were not incorporated into subsequent experimental procedures.

In *Musa acuminata*, the duration of plantlet maintenance within the culture medium exhibited no discernible impact on the genetic stability of micro-clones. Notably, no alterations were observed in the genetic profiles across distinct culture passages.

The lack of polymorphic variations observed in the current investigation aligns with the findings obtained through Random Amplified Polymorphic DNA (RAPD) analysis conducted on micro propagated specimens of *Salvia Hispanica* (James *et al.,* 2007). Similarly, (Gautam and Bhattacharya, 2021) Used Random Amplified Polymorphic DNA (RAPD) profiling to investigate the genetic stability of *in vitro*-cultured *Crocus Sativus*.

Substantial heterogeneity has been reported in plantlets cultivated through tissue culture methodologies employing these molecular markers. (Verma *et al.,* 2021) have documented a polymorphic variation of 23.2% within a cohort of 10 micro propagated plants belonging to the apple rootstock MM106, which were regenerated via axillary branching. (Lin *et al.,* 2022) have evaluated the genetic fidelity of micro propagated *Ananas comosus* plantlets employing Random Amplified Polymorphic DNA (RAPD) markers. Analysis with 44 randomly selected primers disclosed a 2.8% polymorphic pattern among the regenerated plants. This polymorphism is attributed to the supplementation of the culture medium with a high concentration of 6-benzylaminopurine (BAP). It has been posited that the duration of *in vitro* cultivation may potentially induce soma clonal variation (Mehta *et al.,* 2011). Diverse elements influencing genetic diversity encompass the employed micropropagation

methodology, genotype variations, the composition of the growth medium, and growth conditions (James *et al.,* 2007).

In the investigation of *Musa acuminata*, the molecular examination employing Random Amplified Polymorphic DNA (RAPD) analysis revealed a paucity of genetic diversity among the cultured plantlets. This finding contrasts with observations from cytological and biochemical analyses. The marginal variations identified by RAPD markers in this context may be attributed to subtle genetic rearrangements occurring during prolonged cultivation under *in vitro* conditions (Verma *et al.,* 2021)

The incapacity of Random Amplified Polymorphic DNA (RAPD) analysis to elucidate discernible polymorphic patterns linked to soma clonal variation was similarly observed in *coffer arabica* (Bobadilla Landey *et al.,* 2015), Chickpea (Alghamdi *et al.,* 2021), and *Ananas comosus* (Lin *et al.,* 2022). The ineffectiveness observed in this study has been ascribed by certain researchers to the limited genomic coverage provided by Random Amplified Polymorphic DNA (RAPD) markers. It is conceivable that the quantity of employed primers in our current investigation might be inadequate for achieving saturation of the *Musa acuminata* genome. Given that RAPD markers predominantly exhibit dominance in their expression, it is further plausible that mutations affecting solely one of the two alleles in a diploid dominant homozygote at a specific locus could elude detection (Das *et al.,* 2021). While RAPD analysis serves as a valuable tool for genetic discrimination, the lack of RAPD polymorphism in micro propagated plants does not guarantee genetic stability. This is attributed to the potential occurrence of undetected morphological and chromosomal variations. In the extended cultivation period of three years for *Camelia assamica*, cytological examinations revealed the existence of tetraploid and aneuploid cells. Notably, despite the observed cytological variations, the genetic stability of these cultures persisted when assessed using Random Amplified Polymorphic DNA (RAPD) analysis. This implies that the plantlets regenerated from prolonged cultures exhibited a proclivity to selectively counteract soma clonal variations (Bajpai and Chaturvedi, 2021).