Chapter 3

Micropropagation in Altered Growth Condition-Morphological Studies

3.1 Effect of different growth condition on plant growth

The prevailing micropropagation methods primarily involve the expansion of shoots within a semi-solid culture medium. As the demand for increased productivity and shorter propagation periods for economically significant plant material grows, despite the varying degrees of success seen in semi-solid systems in terms of multiplication yields, their significance has risen. The multiplication rate holds a substantial influence over the outcomes of extensive production projects and serves as a key factor in determining the overall production expenses (Fehér, 2019). In the realm of micropropagation, when the number of plant shoots produced is less than three times the original, it's considered not cost-effective. This lower rate means you don't have to transfer the plants to new containers as often, which saves money on labor. Having a higher rate of shoot production can help offset losses caused by contamination and the difficulties in later stages like rooting, hardening, and getting the plants used to their new environment. Lately, using liquid nutrient solutions for growing plants has become a popular and cost-effective way to increase the rate of shoot multiplication. (Daneshvar Melaku *et al.,* 2016; Mohapatra and Batra, 2017; Royandazagh, 2019; Muhammet Dogan, 2022). Increased shoot and root growth is made possible by the liquid medium's ability to stay in close contact with the plant tissue. This property helps the tissue absorb nutrients and plant hormones more effectively, leading to better growth results.

The drawbacks associated with *in vitro* cultivation using liquid media, such as hypoxia (oxygen deficiency), hyperhydricity (excess moisture), shear stresses (mechanical forces), and the necessity for advanced equipment, often outweigh the advantages. (Hazarika, 2006). To address these challenges, alternative approaches have been developed. These strategies involve suspending plants above a quiescent liquid using a flotation device like a raft, and utilizing culture support materials such as cellulose blocks, paper bridges, or sponges. This helps in providing a suitable environment for plant growth while circumventing the difficulties mentioned earlier or temporary immersion (Carvalho *et al.,* 2002; Vidal *et al.,* 2015). In comparison to solid gels, which limit the exchange of

gases and the absorption of nutrients in plant tissue because they have very few tiny holes, these supportive structures actually help plant shoots grow better because they have plenty of air and make it easy for plants to take in nutrients. Using these support structures is also good for saving money because you don't need costly gelling materials, and it reduces the expenses related to cleaning and washing. What's more, using these structures lowers the chances of contamination because you can transfer the plants to new culture media using only sterile liquid (Gangopadhyay *et al.,* 2002).

A different approach to tackle the current difficulties is to briefly soak or grow plant shoots in a liquid solution and then put them back into a partially solid environment. This method has been explored by various researchers, including (Madihah Mohd *et al.,* 2017; Ruta *et al.,* 2020; Vendrame *et al.,* 2023). Several methods for temporary immersion have been developed, with the main focus on ensuring that the plants only have short-term contact with the liquid, as discussed by, Etienne and Bethouly in 2002; Aragon *et al*. 2010. Thorough investigation of how plants grow in a liquid environment and the complex chemical and physical factors that control their regrowth is essential for making liquid plant culture work better on a bigger scale (Malik *et al.,* 2018). Understanding how ventilation and the amount of carbohydrates affect this process will help us create the best conditions for growing lots of plant material, making different plant parts, and producing high-quality plants in the end.

Photoautotrophic (Pa) micropropagation denotes an *in vitro* cultivation approach that excludes the addition of sugar to the growth media. Kozai' 2005 work has encompassed the reporting and comprehensive evaluation of numerous advantages associated with the utilization of the Pa system in contrast to the Pm (Photomixotrophic) method (Kozai *et al.,* 2005). Several plant species have demonstrated enhanced growth and multiplication of plantlets when cultivated in environments enriched with carbon dioxide (Wu and Lin, 2013; Corrêa *et al.,* 2015; Tisarum *et al.,* 2018; Santos *et al.,* 2020; Mansinhos *et al.,* 2022; Pepe *et al.,* 2022)**.** At first, research focused on increasing the amount of carbon dioxide (CO_2) in liquid environments in bioreactors. This showed positive results. However, these studies mainly looked at how adding more CO_2 affected plants growing in agar-based cultures (Gaspi *et al.,* 2013; Vidal and Sánchez, 2019) have been reported.

The growth of young plants can be affected when they don't get enough air in their containers. To solve this, using caps with small holes to let in air has been shown to work

well. This method helps reduce a problem called hyperhydricity and encourages the plants to grow better when they're in a liquid culture (Aguilar *et al.,* 2022)**.** Vented closures offer several benefits, as highlighted by (Zobayed, 2005). These advantages stem from their impact on the gases inside a container, the humidity levels, and the temperature. The choice of closure and how often air exchanges take place play a crucial role in shaping these conditions. Vented closures can significantly influence these factors (Gaspi *et al.,* 2013; Agrawal *et al.,* 2016; H. Gao *et al.,* 2017; S. T. Silva *et al.,* 2017).

The effectiveness of transferring plants during subculture and the number of plantlets produced in a given space are affected by the way we seal the container where the plants are grown. Different types of containers can impact these factors. For instance, the type of culture vessel used can influence how well the transfer process works and how many plantlets you can grow in a particular area. This suggests that choosing the right container is important for successful plant tissue culture experiments (S. T. Silva *et al.,* 2017). During the process of growing plants in a controlled environment like a lab, various factors related to the containers used play a crucial role. These factors include the type of container, how much it can hold inside, the shape of the container's neck compared to its base, whether it has small or large openings, and how transparent the material of the container is. All of these aspects have a significant effect on the growth of plants during *in vitro* cultivation (Kozai *et al.,* 1992). Prakash *et al.* (2004). During the process of conducting plant tissue culture experiments, various types of containers have been used. These containers include disposable food containers that cannot be sterilized using autoclaving, glass bottles, baby food jars with polypropylene lids, and transparent plastic containers like Magenta^{TM} containers made of polypropylene, polycarbonate, or polystyrene. The use of these containers has made it easier to handle the cultures because they have wider openings, which can accommodate more plant explants and simplify the procedure.

Furthermore, it's important to note that the shape and material of the culture container not only affect the amount of light exposure but also impact the overall growth of the plants, as discussed by (S. T. Silva *et al.,* 2017) study. This implies that the choice of container is a crucial factor to consider when conducting plant tissue culture experiments, as it can influence the outcome of the growth dynamics.

There are many reports which o shows that seaweeds have a potency to promote plant growth and these findings leads to attract many researchers to investigate growth promoting substances or biomolecules present in seaweeds. Later on, many workers reported that seaweed have nine different types of plant growth promoting hormones such as auxins (indole butyric acid (IBA), naphthleacetic acid (NAA), indoleacetic acid (IAA)), Cytokinin (6-benzylaminopurine (BAP), kinetin (Kin), trans-zeatin, isopentenyladenine (2iP) and abscisic acid (ABA) (Górka & Wieczorek, 2017). Similarly, based on HPLC data, it was also confirmed that *Pyropia yezoensis* and *Bangia fuscopurpurea* species contain abscisic acid (ABA), Salicylic acid, IAA, 2iP phytohormone (Mori et al., 2017). IAA was also detected from *Monostroma oxyspermum* (Gupta et al., 2011). Prasad *et al.* (2010) reported that *Sargassum tennerrimun* and *Kappaphcus alvarezii* and *Gracilaria edulis* contain indole-3-pyruvic acid (IPA**)**. Similarly, there are other reports which shows cytokinins are also present in different seaweeds. For example, iPR, tZR and tZ and other aromatic cytokinins like 5 topolins were reported in *L. japonica* and in *G. edulis,* and *S. tenerrimum* (Stirk et al., 2013). Similarly Gibberellic acid (GA3) was reported in different *Ulva* species, *M. oxyspermum* and *G. edulis* (Gupta et al., 2011) and also in *S. tenerrimum* (Prasad et al., 2010). In most of the studies, the natural extracts of these algae were used to prepare liquid biofertilizer and apply under *in vivo* conditions. These extracts boost responses in the plant to increase shoot number, root length, and plant growth (Khan et al., 2009). Despite of having so many nutrients and plant growth promoting substances, application of these liquid seaweed extracts under *in vitro* conditions remained unexplored. There are few reports, for example in tomato (Vinoth et al., 2012), where role of seaweed extract was demonstrated in better growth and regeneration under *in vitro* conditions.

The synthetic MS media, which are primarily employed in plant tissue culture, include all necessary inorganic and organic salts, chelating agents, carbon sources, growth regulators, and water. However, the specific nutritional needs vary from individual plant to plant and from cell to cell within the same plant. We hypothesize here that addition of natural Liquid Seaweed Extracts (LSE) in the growth medium, in the form of natural extract, can replace or lower down the requirement of synthetic PGRs and, as a result, it will not only reduce the production cost but also will act as a natural growth enhancer under cultured conditions. This will be an innovative method to decrease the production cost and

make the protocol commercially viable. The current work aims to provide a cost-effective approach to banana micropropagation.

3.1.1 Materials and methods

3.1.1.1 Liquid culture experiment

The experiments conducted in this study aimed to investigate how different types of growth media affect the growth and multiplication of plant shoots in an *in vitro* condition. To do this, we cut the of plant shoots from cultures that were growing in a special shoot multiplication media. These clusters were then placed into two different types of media: one was liquid, and the other was semi-solid media, containing 0.8% agar to give it a gel-like consistency. In the liquid media, we used glass beads with a diameter of 10 mm to provide support for the plant clusters. For the check effect of supporting material in plant growth, we also placed some of the plant clusters onto the semi-solid medium made with 0.8% agar. This agar-based medium served as our control group, allowing us to measure and compare the effects of the different types of media on plant growth. Additionally, this study delved into the role of support matrices within the liquid media, examining how these matrices influenced the growth and multiplication of plant shoots. This investigation provided valuable insights into the conditions that promote optimal plant growth in an *in vitro* condition. In our quest for an affordable and eco-friendly support system that works well with liquid mediums, we conducted a study to examine three distinct mechanical support materials. Our goal was to determine how well these supports can function within a liquid medium. The three types of mechanicals support we evaluated included.

We used locally made glass beads, which were 8 to 10 millimeters in size, for our experiments. Additionally, we use cellulose filter paper, cotton and pebbles and coconut husk and water ball. For the control, we use a semi-solid medium consisting of agar at a concentration of 0.8% as our control. Each conical vessels received approximately 25 to 30 milliliters of this liquid medium. For subsequent subcultures. We closely monitored various growth parameters over a period of 45 to 60 days to assess the plant's development and response to different conditions.

3.1.1.2 Temporary immersion in *in vitro* shoot growth

Further experiments were conducted to assess the check the impact of immersing plant shoots temporarily in a liquid medium on their growth and multiplication in a controlled laboratory setting. Specifically, we placed the shoots in a liquid medium

containing 25-30 ml of solution and supported them with glass beads. After 30 days of growth in this liquid medium, the shoots were transferred back to a conventional semi-solid agar-based medium with 0.8% agar content. We continued to monitor their growth for an additional 30 days on this semi-solid medium, resulting in a total experimental duration of 60 days. As a reference point, we used a control group that was grown on the standard semi-solid medium with 0.8% agar.

These experiments allowed us to assess various growth parameters and compare the effects of liquid immersion on plant shoot development. the data obtained from these observations will be crucial for our thesis, as they provide insights into the role of temporary immersion in plant tissue culture and its impact on *in vitro* shoot growth and multiplication. this information contributes to our understanding of optimizing plant tissue culture conditions for enhanced growth and productivity, which is of significant importance in the commercial plant production

3.1.1.3 CO_2 enrichment experiment

In our study, we conducted additional experiments to investigate how controlled and elevated levels of carbon dioxide (CO_2) affect the growth and multiplication of plant shoots in a *in vitro* specifically, we placed clusters of shoots onto a standard medium for shoot multiplication in culture vessels containing 50 ml multiplication media with 0.0% (for liquid culture) or 0.8% agar (for Semi solid media) and included or excluded 10, 20 and 30 g/l sucrose and sucrose free media. To maintain a controlled environment, we sealed the bottle with non-absorbent cotton plugs.

To explore the impact of various CO_2 concentrations, we exposed the shoot cultures to different levels of CO_2, including 0.0, 0.6, 10.0, and 40.0 g (CO_2) m^{-3}. We accomplished this by enclosing the cultures within transparent acrylic chambers, each with a volume of 7500 cm_3 (25×50×15 cm; length × width × height). These chambers were sealed at the top and secured with packing tape (Miracle, 5.0 cm width).

To provide a control CO_2 concentration, we followed the method detailed by Solarova *et al*. (1989). For maintaining a concentration of 0.6 g (CO_2) m^{-3}, we used a mixture of 0.1 M solutions of sodium bicarbonate ($nahco_3$) and sodium carbonate (Na_2CO_3) in a 77/23 (v/v) ratio. Similarly, concentrations of 10.0 and 40.0 g (CO_2) m^{-3} were achieved by combining 3M solutions of potassium bicarbonate ($KHCO_3$) and potassium carbonate (K_2CO_3) in ratios of 50/50 and 73/27 (v/v), respectively. To create a CO_2-free atmosphere,

we placed a 10.0% potassium hydroxide (KOH) solution within the acrylic box. These solutions were arranged in open Petri plates within the boxes to maximize CO_2 diffusion, and we replaced these solutions every 5th day.

In our experiment, we set up different conditions to study the impact of sucrose in the growth of plant tissue cultures. We used a total of twenty culture bottles, each containing a shoot cluster. These cultures were divided into two groups: one with sucrose in the medium (SCSM, which stands for sucrose-containing semi-solid medium, and SCLM, sucrose-containing liquid medium) and the other without sucrose (SFSM, sucrose-free semi-solid medium, and SFLM, sucrose-free liquid medium). To create varying CO_2 concentrations, we placed these culture bottles in separate acrylic boxes. These boxes were designed to provide different levels of CO_2. These setups were maintained in a controlled culture room to ensure consistent conditions.

Additionally, for the control. We included both sucrose-containing and sucrose-free semisolid media (SCSM, SFSM) and other set sucrose-containing and sucrose-free liquid media (SCLM, SFLM). This culture was placed in a growth room with ambient air conditions. This experimental design allowed us to investigate the influence of sucrose and CO_2 concentration on the growth of plant tissue cultures.

3.1.1.4 Culture vessels environment experiment

In order to determine the most effective container for enhancing the rate of shoot multiplication, our research involved growing shoot cultures in a variety of culture vessels. We used semi-solid growth mediums for our experiments. The following types of containers were selected for this study:

1. (V1) Neutral glass bottles: 200 ml capacity (height 10.0 cm, mouth diameter 5.5 cm
2. (V2) Conical flasks with a narrow mouth, having a capacity of 250 ml (height 12.4 cm, bottom diameter 6.0 cm, neck diameter 2.5 cm).
3. (V3) Phyta jar with clear round container having capacity 250 ml (Size 67 X 78 mm)
4. (V4) Phyta jars with vented lids, having capacity 350 ml (size 78 X 78 X 95 mm).
5. (V5) Phyta jar with translucent square container having capacity 370 ml (Size 74 X 71 X 134 mm)
6. (V6) Phyta jar with clear square container having capacity 500 ml (Size 74 X 71 X 138 mm)

These vessels were carefully chosen to assess their impact on shoot multiplication rates in plant tissue cultures. Our study aims to provide valuable insights into the selection of the most suitable container for optimizing plant tissue culture conditions, which will be essential for your thesis on plant biotechnology.

In each container, we placed a group of five shoots called a shoot cluster. We used a standard medium to help these shoots multiply. We repeated this process six times for each type of container. After 45 days, we carefully observed and recorded various growth parameters, including how much the shoots had multiplied.

3.1.1.5 Altered gelling agents experiment

In this experiment we use different subtrance as a gelling agent in tissue culture medium. We use agar agar, phyta gel, starch, guar gum, and isabgol for plant growth in *in vitro* condition and study growth parameters.

3.1.1.6 Liquid seaweed extract experiment

In our experiment, we assessed the impact of seaweed extracts on banana micro propagule growth. We selected eight seaweed species (*Caulerpa racemosa, Gracilaria edulis, Caulerpa paspaloides, Ulva Lactuca, Sargassum tenerrimum, Caulerpa sertularioides, Sargassum wightii, and Gracilaria corticata)* and tested various concentrations of liquid seaweed extract (LSE) ranging from 10% to 50% (v/v). We included positive and negative controls (standard MS medium with and without plant growth regulators) for comparison. Each experiment was conducted in 200 ml culture bottles containing 50 ml of medium with a pH of 5.8. After autoclaving and solidification, five aseptically inoculated shoots were placed in each bottle. The bottles were transferred to a growth room for 45 days. We maintained six replicates per experiment, with three repetitions of each treatment. At the experiment's end, micropropagules were evaluated using various growth factors.

3.1.1.7 Materials and methods of *in vitro* rooting

Role of liquid medium on *in vitro* rooting

To investigate how a liquid growth medium affects the development of plant roots *in vitro*, we conducted experiments using shoots of appropriate size (ranging from 3 to 4 cm) that were previously grown on both semi-solid and liquid mediums. Our experimental setup was as follows.

We utilized elongated shoots, each measuring approx. between 3 and 4 cm, which were obtained from both semi-solid and liquid mediums. These shoots were used as

explants for rooting in three different plant species. We inoculated the elongated shoots onto a standard rooting medium, which could either be gelled with agar or without agar.

***In vitro* hardening of shoots rooted on liquid and semi–solid medium**

For *in vitro* hardening, rooted plantlets were transferred to culture bottles containing Soiltrite™ moistened with ¼th strength MS salt solution for *in vitro* hardening. After their inoculation on Soilrite™ in the bottles, they were kept in culture room environment for initial 7 days and then transferred to the greenhouse conditions (28±2 °C temperature and 80–85% reducing humidity gradient). The caps of bottles were opened gradually. After 30 days of their growth in the greenhouse, the hardened plantlets were observed for various growth parameters like percent survival, plantlet height and number of leaves produced per plant.

3.1.2. Results

Multiplication

3.1.2.1 Role of liquid Medium

Liquid medium has demonstrated superior growth potential in terms of *Musa acuminata* multiplication when compared to the control group, which utilized a semi-solid medium (Figure 3.1). In the control group, consisting solely of liquid media without any supporting material, an average of 7 shoots per cluster was observed, serving as the baseline for comparison, with an average shoot length of 5.5 cm (Table 3.2). The incorporation of Tissue paper showed a marginal improvement, resulting in an average of 7.25 shoots per cluster with an average shoot length of 7.35 cm, compared to the control group. Notably, Glass Marbles (GM) exhibited a substantial enhancement in plant growth, yielding an average of 11 shoots per cluster with an average shoot length of 7.5 cm. This underscores the effectiveness of GM as a highly efficient supporting material. Conversely, other materials like Pebbles (PA) yielded a slightly lower average of 6 shoots per cluster with an average shoot length of 6 cm compared to the control group, suggesting its comparative ineffectiveness compared to GM. The utilization of only liquid media without any supporting material resulted in a reduced average of 3.5 shoots per cluster with an average shoot length of 5 cm, underscoring the indispensable role of support structures in facilitating optimal plant growth. However, it is worth noting that coconut husk, while producing results similar to pebbles with an average of 6 shoots per cluster, carries a higher risk of contamination, which should be carefully considered.

In conclusion, Glass Marbles (GM) emerge as the most effective supporting material for promoting plant growth in liquid culture media, as evidenced by the highest number of shoots per cluster and a relatively high average shoot length. Tissue paper also showed modest improvement, while both pebbles (PA) and coconut husk demonstrated results comparable to the control group. The absence of supporting material in liquid media significantly hindered plant growth in terms of both shoot number and length. These findings bear significance in optimizing plant tissue culture conditions and hold practical implications for the plant tissue culture industry to enhance plant production rates (Figure 3.1).

3.1.2.2 Effect of TI in *in vitro* shoot growth

Similarly, employing temporary immersion of shoots in a liquid medium, followed by subsequent growth on semi-solid medium, yielded noteworthy improvements in the overall growth of *Musa acuminata* (Figure 3.2). This technique resulted in a significant increase in both the number of shoots and their length, effectively doubling the values compared to the control group (Table 3.3). Moreover, this approach exhibited a remarkable three-time boost in the multiplication rate, underscoring its potential for enhancing the propagation of *Musa acuminata*.

3.1.2.3 Effect of CO_2 Enrichment on *in vitro* plant growth

In our experimental investigation, we initiated the growth of *Musa acuminata* shoots by inoculating them on SCSM (semi-solid medium enriched with sucrose). This strategic approach led to a remarkable approximately four-fold increase in shoot numbers after a 40-day cultivation period within the controlled growth room environment (refer to Figure 3.3). It's noteworthy that the cultures grown on SCSM displayed impressive shoot production, with each explant yielding (details provided in Table 3.4). Interestingly, when we compared these results to those obtained from shoot clusters cultivated on SCLM (liquid medium also enriched with sucrose) within the same growth room conditions, the growth parameters exceeded expectations in terms of both multiplication and elongation.

Our study delved into the responses of shoot cultures when exposed to controlled CO_2 environments. Surprisingly, cultures grown without additional CO_2 exhibited growth comparable to those cultivated under ambient air conditions. However, our findings took an intriguing turn when we introduced various concentrations of CO_2 (0.6, 10.0, and 40.0 g m^{-3}) resulting in a general enhancement of all growth parameters compared to shoots

grown on both SCSM and SCLM in ambient air. It is worth noting that, across all CO_2-enriched conditions, the liquid medium consistently outperformed its semi-solid counterpart in promoting superior growth. The most favourable response was observed at 10.0 g m^{-3} CO_2 in SCLM under controlled conditions, where nearly 20 shoots per cluster were produced (see Figure 3.4), each with an average length of approximately 7.85 cm (Table 3.5). However, at higher concentrations of 40.0 g m^{-3} CO_2 in SCLM, there was a notable increase in both shoot number and length, albeit accompanied by symptoms of hyperhydricity. On the other hand, for SCSM, optimal growth conditions were achieved at 40.0 g m^{-3} CO_2. In a different context, when we exposed cultures to ambient air conditions within the growth room, those grown on SFSM (sucrose-free shoot multiplication medium) exhibited a significant decline in all recorded growth parameters. The initially inoculated shoot clusters turned yellow and struggled to maintain growth under ambient CO_2 concentrations, failing to surpass the growth observed in SCSM conditions.

Furthermore, when we placed shoots in a CO_2-free environment, those grown on SFLM (sucrose-free liquid medium) could only sustain growth for approximately 15 days before experiencing rapid deterioration and eventual demise. Under CO_2-free conditions, shoots on SFLM turned pale yellow within just seven days. However, when exposed to different CO_2 concentrations, shoots on both SFSM and SFLM exhibited sustainable growth. The number of shoots and their lengths increased as CO_2 concentrations rase. Particularly, at a concentration of 10.0 g m^{-3} CO_2 in SFLM, there was a significant increase in shoot length compared to controls grown on SCSM under ambient air conditions. The most remarkable findings emerged at 40.0 g m^{-3} CO_2, where all measured parameters reached their peak values, comparable to those obtained for shoots grown on SCSM in the ambient air environment.

3.1.2.4 Effect of culture vessels environment

In your study, we examined the influence of culture vessel size on the growth of *in vitro* plant shoots, specifically focusing on six different vessel types: 200 mL Glass bottles (V_1), 250 mL flasks (V_2), 250 mL Phyta jar (V_3), 350 mL Phyta jar (V_4), 370 mL Phyta jar (V_5), and 500 mL Phyta jar (V_6) (Figure 3.5).

The number of shoots produced in each culture vessel varied significantly. Among the vessels tested, V_2 flasks exhibited the highest average shoot count at 11.75, (Table 3.6) followed closely by V_3 with an average shoot count of 10. On the other hand, V_2 and V_4

showed lower shoot counts at 7.75 and 8.25, respectively. V_1 and V_5 fell in between, with average shoot counts of 8.75 and 9.5, respectively. Shoot length is another crucial parameter in assessing plant growth. The V_2 exhibited the longest average shoot length, measuring 7 cm. The V3 also demonstrated substantial shoot length at 6.75 cm. In contrast, the V_1, V_3, and V_4 displayed similar shoot lengths, averaging around 4.5 cm. The V_5 exhibited an intermediate shoot length of 6 cm. To assess the overall growth performance of the cultures, we calculated the rate of multiplication fold. This parameter reflects the increase in the number of shoots and shoot length relative to the initial conditions. The V_2 showed the highest multiplication fold rate at 2.92, suggesting robust growth. The V_6 also displayed substantial growth with a multiplication fold rate of 2.33. The V_5 followed closely with a rate of 2.17. The V_1, V_3, and V_4 exhibited lower multiplication fold rates of 1.92, 1.58, and 1.75, respectively. The choice of culture vessel size significantly impacts the *in vitro* growth of plant shoots. V_2 and V_6 demonstrated superior performance in terms of both shoot number and shoot length, as well as the overall multiplication fold rate. These findings suggest that larger culture vessels may be advantageous for promoting plant growth *in vitro*. Further research into the underlying physiological and environmental factors contributing to these differences is warranted to gain a deeper understanding of the observed variations.

3.1.2.5 Effect of different gelling agents

In study we investigated effect of different gelling agents as a cost- e f f e c t i v e micropropagation. In this experiment we found Plant gel (Phyta gel) is best for plant growth in *in vitro* condition is it showed high rate of multiplication compare to other gelling agents and show higher shoot length shows guar gum treated plant as compare to other gelling agents (figure 3.6). Other gelling agents show significantly increase growth of plant compare to control but no much more (in Table no 3.7).

3.1.2.6 Effect of liquid Seaweed extract

In this investigation we studied the effect of different seaweed as a plant growth promoter, because many review showed seaweed contain different type's plant growth promoting substances which promote plant growth. In this study we examined eight different seaweed species in plant growth medium. We tried natural extract of eight different seaweeds (*Caulerpa racemosa, Gracilaria edulis, Caulerpa paspaloides, Ulva lactuca, Sargassum tenerrimum., Caulerpa sertularioides, Sargassum wightii,*

Gracilaria corticata.) To check their role in *in vitro* growth and development of micropropagules and their potential in replacing commercial plant growth regulators (pgrs) in banana micropropagation (Figure 3.7). The *in vitro* developed shoots were allowed to grow on modified MS medium supplemented with different seaweed extracts and regularly subcultured on the same medium throughout the experiment. The growth and parameters were recorded and compared with micropropagules growing in a controlled environment (i.e., MS medium supplemented with/ without standard pgrs). Wefound a significant increase in shoot length (highest in *S. Tenerrimum*, i.e. Double as compared to negative control) (table No. 3.8), shoot number (highest in *G. Edulis*, i.e., threefold as compared to negative control) and fresh weight (*S. Tenerrimum*) of the banana micropropagules when grown under the influence of Liquid Seaweed Extract (LSE). This is comparable to growth observed in standard control conditions (i.e. Positive control). However, addition of LSE of *G. Corticate*, *C. Paspaloides* and *S. Wightii* did not show any noteworthy effect on shoot length, shoot number.

3.1.2.7 Effect on *in vitro* rooting

Effect of liquid medium on *in vitro* rooting

The liquid medium demonstrated a pronounced efficacy in fostering *in vitro* rooting in *Musa acuminata*. Root initiation manifested within a week of inoculation, generally exhibiting an earlier onset by a day or two in comparison to solid medium. Notably, the shoots cultured in the SMLR (Solid Medium to Liquid medium Rooting) exhibited a 100% rooting response, as depicted in Table 3.9. These particular shoots yielded an impressive count of 6.72 fibrous roots, with an average root length of 3.25 cm achieved after a 21-day period. Furthermore, the shoot length experienced a significant increase, reaching 6.0 cm, surpassing the control group cultivated in SMSR (Solid Medium to Solid medium Rooting).

Concurrently, the LMLR (Liquid Medium to Liquid Rooting) cultivated shoots also demonstrated commendable performance in terms of rooting response. These shoots exhibited the production of a maximum number of roots, totaling 5.50, with an average root length of 3.25 cm. remarkable, these shoots attained a maximum height of 7.5 cm and exhibited a lush green coloration, accompanied by a broad leaf lamina, as illustrated in Figure 3.8.

Moreover, shoots that were initially multiplied in liquid medium exhibited the ability to generate roots when subsequently inoculated on semi-solid rooting medium. This

secondary rooting response achieved an average success rate of 90%. However, it is noteworthy that the shoot length and root length in this context were comparable to the control group grown solely on solid medium.

3.2 Studies on leaf surface structure

Analyzing the physical traits of plant cultures is often beneficial. It also involves improving the components in the liquid environment to enhance growth. This helps in creating better methods for micropropagation (Hazarika, 2006). The surface characteristic of leaves grown in a controlled environment have been extensively documented in various research reports. These studies have provided valuable insights into leaf morphology and structure under controlled conditions (Martins *et al.,* 2015; Mani *et al.,* 2021; Shekhawat *et al.,* 2021). Previous studies have highlighted a significant issue in cultured plants: non-functional stomata. Stomata are tiny openings on plant leaves, much like tiny mouths. They play a crucial role in regulating the exchange of gases and water vapor between the plant and the environment. Researchers have used powerful microscopes like SEM (Scanning Electron Microscopy) and light microscopes to examine these stomata. What they discovered was quite interesting. When they compared the stomata of plants grown in *in vitro* to those grown in natural condition like greenhouses or fields, they noticed a distinct difference. Normally, stomata have a typical oval shape with guard cells that are somewhat sunken into the leaf surface. However, in the leaves of plants grown in a lab (*in vitro*), the stomata looked different. Instead of being oval, they were more rounded or even crescent-shaped, and the guard cells appeared quite different from what you'd see in plants grown outside (Zhou *et al.,* 2020; Domblides *et al.,* 2022). When we take micropropagated plants out of their controlled lab environment and place them in the natural condition notice something interesting. These plants tend to have a problem with water regulation, which means they struggle to maintain the right balance of water. This issue seems to be connected to an increase in the frequency of regular-shaped stomata (Wetzstein and Sommer, 1982) and a decrease in the normal structure and movement of these stomata (Hoang *et al.,* 2019; Aliniaeifard *et al.,* 2020; Zein El Din *et al.,* 2020).

One major reason behind the highwater loss and less successful transplantation of *in vitro* produced plants is the absence of a protective layer known as epicuticular wax on the leaf surfaces of *in vitro* grown plants is a significant factor contributing to increased water loss and decreased success in transplanting these plants. This wax serves as a natural

shield, playing a crucial role in minimizing water loss and enhancing the overall ability of the plant to withstand challenging conditions (Sajeevan *et al.,* 2017). In comparison to the leaves of plantlets cultivated *in vitro*, the leaves of transplanted and field-grown plants exhibit a fully developed cuticle. This means that the protective layer on their leaves is more mature and robust, which is an important adaptation for plants facing the complexities of the natural environment (Manokari, Badhepuri, *et al.,* 2022).

In the micro propagated plants, scientists believed that a major reason they lose a lot of water and high transpiration rate is because their leaf epidermal layers, isn't spread out properly, and things like tiny hairs called trichomes don't developed well. Think of these epidermal layers like a protective coat for the plant this can make these plants more prone to drying out (Brutti *et al.,* 2002). Trichomes are like little attachments on the outer layer of a plant, and they come in many different shapes and sizes. These tiny structures serve various functions. They influence how much heat the plant loses, how much sunlight it can soak in, and even the layer of air around the leaves. One of their crucial roles is controlling how much water the plant loses during transpiration. Trichomes can slow down this water loss. Additionally, they can act as storage units of some secondary metabolites (Uzelac *et al.,* 2021). Some trichomes also work as a defense system, protecting the plant from harmful invaders like pathogens and hungry herbivores (Abdalla *et al.,* 2021; Tarfeen *et al.,* 2022)**.**

To the understanding of how trachoma's contribute to the process of hardening and acclimation in *Aerva lanata* plants, a comprehensive investigation was carried out by (Priyadharshini, *et al.,* 2022). This study delved into several aspects of trachoma, including their shape, variety, how they are spread out, and their density, comparing teak plants grown in an *in vitro* and those grown naturally in their native environment.

3.2.1 Materials and Methods

3.2.1.1 Light Microscopy

For stomata experiments using light microscopy, epidermal strips were peeled from leaves grown on both systems (liquid and semi–solid) at various stages of micropropagation, including *in vitro* shoot multiplication (M), *in vitro* rooting (R), *in vitro* hardening (A1), and mature plant (F) (liquid and semi–solid). Preparation samples for microscopy examination was carried out according to the method described by Capellades *et al.* (1990).

To study stomata, develop and change during the various stages of micropropagation in different growth condition we use a light microscope, we took tiny pieces of the leaf's outer layer, called epidermal strips. These strips were collected from leaves grown in both liquid and semi-solid media at different stages of micropropagation. These stages included when the plant was just starting to grow shoots *in vitro* (Multiplications stage; M), when it was developing roots *in vitro* (Rooting stage; R), Acclimatization (Hardening stage A1), and when it had fully matured into a plant (F) in both liquid and semi-solid medium. For the study change in stomata we followed a procedure described by Capellades and colleagues in 1990. Upon initiating the unsealing procedure of the cultivation vessel, the adaxial and abaxial surfaces of leaves were coated with a transparent nail polish. Subsequently, upon thorough drying, the applied nail polish was meticulously removed utilizing a transparent adhesive tape (Wonder, 2.0 cm). The resulting epidermal imprints were meticulously transposed onto microscopic slides for subsequent analysis. Three distinct leaf imprints were procured at each experimental phase, and stomatal assessments were conducted at three discrete loci on each imprint. Various morphological parameters, including the number of stomata (NS), the number of epidermal cells (NE), stomatal size, stomatal density, and stomatal index, were quantified from leaf imprints employing 40X magnification through light microscopy (Olympus, India).

Stomatal size assessment was conducted utilizing an Ocular Micrometer (Erma, Japan) that underwent pre-calibration with a stage micrometer. The determination of stomatal density and stomatal index was executed through the application of the ensuing formulas:

Stomatal density (SD) = NS/A

NS – Number of stomata in the microscopic field

A – Area of the microscopic field

Stomata index (SI) = NS × 100 / NS + NE

NS – Number of stomata in the microscopic field

NE – Number of epidermal cells in the microscopic field

3.2.1.2 Scanning Electron Microscopy

In our SEM (Scanning Electron Microscope) experiments, we collected leaves from growing shoots at different stages of *in vitro* growth and under various culture conditions. These leaves were then promptly preserved using a solution that included 2%

glutaraldehyde and 2% freshly prepared formaldehyde. After preserving them, we let the leaves sit overnight at a chilly 4°C. Following this preservation process, we took these prepared samples to a scanning electron microscope.

Microscopy Facility at Department of Biotechnology Junagadh Agriculture University, Junagadh. The Leaf scans were carried out in Zeiss, SEM. Both abaxial and adaxial surfaces of leaf of plants from all various stage of micropropagation as well as from field growing plants were scanned at resolutions ranging between 250–2500X. The stomatal characteristics, epicuticular waxes and trichomes were observed for further analysis.

3.2.2 Results

In the both experiment we found wide different in stomata morphology**.** In the conducted investigation, both light microscopy and scanning electron microscopy were employed to examine leaf surfaces, revealing significant variations in stomatal frequency, stomatal size, epicuticular wax formation, and functional characteristics of stomata in the leaves of micro shoots cultivated on agar–gelled semi–solid and liquid medium across various plant species.

Under light microscopic analysis, *Musa acuminata* exhibited stomata on both leaf surfaces, with a characteristic monocot pattern of distribution in field-grown leaves, featuring dumbbell-shaped guard cells. Notably, stomata were more densely distributed on the abaxial surface compared to the adaxial surface. The stomata on both surfaces were similar in size and fully functional.

Micrographs of leaves from *in vitro* cultures grown on liquid and semi–solid medium presented distinct stomatal features compared to their field-grown counterparts. In these cultures, stomata were present on both surfaces, with larger stomata observed in leaves from liquid medium, albeit at a lower frequency than semi–solid medium leaves. Stomatal density decreased during the rooting phase for both surfaces in subsequent stages (SR and LR leaves). Stomatal size notably increased in SR leaves compared to SM leaves. However, no significant difference in stomatal size was observed between LM and LR leaves. Stomatal frequency exhibited a remarkable increase from 15 mm^{-2} in the adaxial surface of SR leaves to 60 mm^{-2} in the lower surface of SH leaves, while LH leaves showed a moderate level of increment.

Scanned microscopic of *Musa acuminata* at various stages of micropropagation from SM and LM medium revealed the deposition of amorphous epicuticular wax on both leaf surfaces. Wax deposition was limited in LM and SM leaves, commencing during the rooting stage and persisting through the hardening phase. In SH and LH leaves, epidermal cells and stomata were somewhat sunken in the waxy layer.

Further scanning electron microscopy analysis of stomatal structure with respect to their functional status exhibited considerable differences between field-grown and *in vitro* developed leaves. Stomata on adaxial and abaxial surfaces of field-grown plants were fully functional, characterized by thick-walled guard cells and a narrow pore opening. Conversely, stomata in *in vitro* developed leaves were highly distorted and surrounded by thin-walled guard cells, with a higher degree of deformity in LM leaves. Distortion levels decreased as leaves progressed through subsequent phases of rooting and hardening (Figure 3.9).

3.3 Histological studies

In recent years, there have been numerous studies focusing on the anatomical differences of plantlets grown in a controlled condition (*in vitro*), especially their leaf shapes and structures. These studies have highlighted a significant issue when these *in vitro* gown plants are moved to a natural environment. They often experience transplant shock because their inner leaf tissues, called mesophyll, aren't well-developed, and their leaf vessels, known as vasculature, are not properly formed during grown in control condition. This transplant shock can be a problem because it makes it challenging for these plants to adapt and thrive when they're taken out of the lab and placed in more natural condition (Mani *et al.,* 2021). Comparatively to plants grown in a greenhouse, *in vitro* plants' leaves have a significantly higher volume of mesophyll air gaps and a palisade layer that is thinner and generally less developed.

When compare the leaves of *in vitro*-grown plants to those grown in a greenhouse, (Chen *et al.*, 2020) was notice some significant differences. *In vitro* plants have leaves with much more empty space inside, called mesophyll air gaps. Additionally, their palisade layer, which is a critical part of the leaf, is thinner than and not as well-developed as that of greenhouse-grown plants.

This study also has been carried out in other plants like *Acca sellowiana* (Caetano *et al.,* 2022), *Aechmea bromeliifolia* (E. C. e Silva *et al.,* 2020), Aloe vera (Manokari *et al.,* 2021), Sweet pepper (Y. Gao *et al.,* 2022), Potato (Chen *et al.,* 2020).

Moreover, it's not just the leaves that show these differences. The stems of *in vitro*-grown plantlets are slenderer and have less of the supportive tissue compared to plants that have grown in the field or natural conditions.

3.3.1 Materials and methods

To prepare the plant samples for observation, we followed a series of steps: Fresh plant material was carefully cut into thin sections by hand. These sections were then soaked in a solution of sodium hypochlorite (NaOCl) at a concentration of 1.0%. Sodium hypochlorite is a bleaching agent commonly used for cleaning and disinfection. After the bleaching step, the sections were thoroughly rinsed with distilled water to remove any residue. Next, the sections were placed in a solution of acetic acid at a concentration of 1.0% for a brief period, typically 1 to 5 minutes. Again, they were rinsed well with distilled water to ensure all traces of the acid were removed. To aid in the visualization of the plant sections, they were stained with safranin, an aqueous dye. In some cases, a counter-stain called fast green was applied as needed. The prepared sections were then viewed under a light microscope after being fixed in 30% glycerin. Glycerin helps preserve the plant sections for observation. For more detailed observations, a phase contrast microscope, specifically the Eclipse 50i by Nikon, was used to capture images of the sections.

3.3.2 Results

The cross-sectional analyses of *Musa acuminata* leaves revealed morphological features consistent with those typical of monocotyledonous leaves. In a semi-solid medium, both surfaces of the leaf sections were enveloped by upper and lower epidermal layers. The mesophyll sandwiched between these epidermal layers exhibited a notable abundance of chloroplasts. The vascular system comprised conjoint, collateral, and closed vascular bundles intricately embedded within the spongy mesophyll. Notably, the distinct bundle sheath surrounding the vascular bundles was accurately discerned. The foliar structures derived from the aqueous medium displayed analogous cellular arrangements in transverse section, with the notable distinction of less advanced vascular bundles and an increased chlorophyll content.

The root anatomical analysis of *Musa acuminata* indicated a disruption in the outermost layer, the epiblema, when cultivated in a semi-solid medium. Nevertheless, the cortex displayed robust development, characterized by parenchymatous tissue with notable intercellular spaces. Notably, no clear differentiation between the endodermis and pericycle was discernible. The vascular bundles were densely concentrated within the pith, composed primarily of parenchymatous cells.

In contrast, the liquid rooting (LR) demonstrated a superior degree of organization. The cross-sectional view revealed a circular root structure enveloped by a continuous and distinct epidermis, albeit with slightly hypertrophied epidermal cells. Additionally, occasional root hairs were evident in the cross section. The cortical cells maintained their integrity, featuring small intercellular spaces between them. A subtle demarcation between the endodermis and pericycle was observable. Furthermore, a limited number of initially developed vascular bundles were dispersed throughout the stele.

Table 3.1 Effect of liquid culture medium on *in vitro* growth and shoot multiplication in banana shoot clusters (Observations were recorded after 45days).

Medium	No. of shoots per cluster ± SD	Rate of multiplication (in folds) ± SD	Length of shoots (cm) ± SD
SM	7 ± 0.81	2.33 ± 0.27	3.0 ± 0.81
LM	5.5 ± 0.57	1.83 ± 0.19	3.5 ± 0.57
SEM	8.882312	11.31371	16.4122
CD 5%	0.288675	0.117851	0.338502
CD 1%	0.998948	0.407819	1.17137
CV	1.513551	0.617905	1.774796

SM= Semi–solid medium; LM= Liquid medium, SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation; SD - Standard Deviation.

Table 3.2 Effect of support systems in liquid culture medium on *in vitro* growth and shoot multiplication in banana shoot clusters (Observations were recorded after 45 days).

Medium	No. of shoots per cluster	Rate of multiplication (in folds)	Length of shoots (cm)
GM	9.250 a	3.083 a	7.250 a
Tissue paper	8.750 a	2.917 ab	6.500 ab
PA	8.250 ab	2.750 ab	5.000 bc
Liquid	5.500 c	1.833 c	4.500 c
Control	7.000 b	2.333 bc	5.000 bc
SEM	8.664392	9.677419	13.51792
CD 5%	0.322749	0.111803	0.381881
CD 1%	0.972869	0.32982	1.151114

GM= Glass marble support; PA= pebbles; Control = Semi Solid Medium; SEM - Standard Error Mean; CD - Critical Difference; SD - Standard Deviation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.3 Effect of temporary immersion in liquid medium on *in vitro* shoot growth and multiplication in banana shoot clusters (Observations were recorded after 45 days).

Medium	No. of shoots per cluster ± SD	Rate of multiplication (in folds) ± SD	Length of shoots (cm)±SD
SM	7 ± 0.81	2.33 ± 0.27	3 ± 0.81
TI	9.5 ± 0.57	3.16 ±0.19	4.75 ±0.5
SEM	6.448498	8.570991	15.14479
CD 5%	0.270031	0.117851	0.444878
CD 1%	0.93443	0.407819	1.539482
CV	1.415797	0.617905	2.332539

SM= Semi–solid medium; TI= Temporary Immersion; SD - Standard Deviation; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.4 Effect of CO_2 enrichment with and without sucrose on *in vitro* shoot growth and multiplication in banana, shoot clusters grown on semi–solid medium (Observations were recorded after 45 days).

	Sucrose %	CO_2 Conc. (g m^{-3})	Number of shoots per cluster	Rate of Multiplication (in folds)	Shoot Length
SFSM	0	0	0.000 j	0.000 j	0.000 n
		0.6	4.000 i	1.333 i	0.933 m
		10	7.000 g	2.333 g	1.333 jk
		40	6.667 g	2.222 g	1.217 kl
		GR	3.333 i	1.111 i	1.100 lm
SCSM	1	0	4.167 i	1.389 i	1.467 ij
		0.6	5.333 h	1.778 h	1.583 i
		10	7.000 g	2.333 g	1.883 h
		40	8.500 f	2.833 f	2.350 g
	2	0	7.167 g	2.389 g	2.767 f
		0.6	8.167 f	2.722 f	3.333 e
		10	11.333 c	3.778 c	4.250 c
		40	10.333 d	3.444 d	4.400 bc
	3	0	9.333 e	3.111 e	3.617 d
		0.6	11.167 c	3.722 c	4.500 b
		10	14.833 a	4.944 a	5.550 a
		40	13.667 b	4.556 b	4.483 b
		GR	9.833 de	3.278 de	4.250 c
		SEM	9.056568	9.056568	6.62396
		CD 5%	0.291336	0.097112	0.07364
		CD 1%	0.818532	0.272844	0.206897
		CV	1.084234	0.361411	0.274058

SCSM= Sucrose containing semi–solid medium; medium; SFSM= Sucrose free semi–solid medium. SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.5 Effect of CO_2 enrichment with and without sucrose on *in vitro* shoot growth and multiplication in banana, shoot clusters grown on Liquide medium (Observations were recorded after 45 days).

	Sucrose %	CO_2 conc. (g m^{-3})	Number of shoots per cluster	Rate of Multiplication (in folds)	Shoot Length
SFLM	0	0	0.000 h	0h	0.000 g
		0.6	6.000 fg	2fg	1.733 ef
		10	9.000 def	3def	2.333 e
		40	9.333 def	3.11def	2.100 e
		GR	5.000 g	1.67g	1.333 f
SCLM	1	0	5.000 g	1.667g	1.867 ef
		0.6	7.000 efg	2.33efg	2.100 e
		10	9.000 def	3def	2.433 e
		40	10.000 cde	3.3cde	3.200 d
	2	0	9.000 def	3def	3.800 d
		0.6	10.000 cde	3.33cde	4.733 bc
		10	13.000 bc	4.33bc	5.233 bc
		40	12.000 bcd	4bcd	5.067 bc
	3	0	11.000 cd	3.67cd	4.533 c
		0.6	13.000 bc	4.33bc	5.233 bc
		10	17.000 a	5.67a	6.833 a
		40	15.000 ab	5ab	5.367 b
		GR	12.000 bcd	4bcd	5.033 bc
		SEM	9.856568	9.556568	6.562396
		CD 5%	0.491336	0.077112	0.08364
		CD 1%	0.918532	0.572844	0.106897
		CV	1.084234	0.361411	0.274058

SCLM= Sucrose containing liquid medium; SFLM= Sucrose free liquid medium; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.6 Effect of vessel type on *in vitro* shoot growth and multiplication in banana, shoot clusters grown on liquid medium (Observations were recorded after 45 days).

Vessel Type	No. of shoots per cluster	Rate of multiplication (in folds)	Length of shoots (cm)
C	7.000 b	3.725 b	3.725 b
V_1	10.750 a	7.500 a	7.500 a
V_2	8.000 b	4.200 b	4.200 b
V_3	8.250 b	4.100 b	4.100 b
V_4	9.500 a	4.350 b	4.350 b
V_5	10.667 a	7.167 a	7.167 a
SEM	8.881169	8.881169	8.757337
CD 5%	0.399653	0.133218	0.226231
CD 1%	1.187429	0.39581	0.672167
CV	1.626878	0.542293	0.920926

(C) Neutral glass bottles: 200 ml capacity (height 10.0 cm, mouth diameter 5.5 cm, (V2) Conical flasks with a narrow mouth, having a capacity of 250 ml (height 12.4 cm, bottom diameter 6.0 cm, neck diameter 2.5 cm), (V3) Phyta jar with clear round container having capacity 250 ml (Size 67 X 78 mm), (V4) Phyta jars with vented lids, having capacity 350 ml (size 78 X 78 X 95 mm).,(V5) Phyta jar with translucent square container having capacity 370 ml (Size 74 X 71 X 134 mm),(V6) Phyta jar with clear square container having capacity 500 ml (Size 74 X 71 X 138 mm). SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.7 Effect of Gelling agents on *in vitro* shoot growth and multiplication in banana, shoot clusters grown on liquid medium (Observations were recorded after 45 days).

Medium	No. of shoots per cluster	Rate of multiplication	Length of shoots (cm)
Agar	8.000 ab	2.667 ab	3.000 b
Phyta Gel	9.000 a	3.000 a	3.750 ab
Guar gum	8.500 a	2.417 b	4.000 ab
Isabgol	7.000 b	2.333 b	4.750 a
starch	7.250 b	2.833 a	3.500 ab
SEM	8.664392	9.677419	13.51792
CD 5%	0.322749	0.111803	0.381881
CD 1%	0.972869	0.32982	1.151114
CV	1.344984	0.449888	1.591407

SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.8 Effect of LSE on *in vitro* shoot growth and multiplication in banana, shoot clusters grown on semi solid medium (Observations were recorded after 45 days).

Liquid Seaweed Extract (LSE)	Concentration of LSE (% v/v)	No. of shoots per cluster	Rate of multiplication (in folds)	Length of shoots (cm)
Caulerpa racemosa	10	7.00 f	1.33 f	1.900 j
	30	7.60 e	1.53 e	2.700 g
	50	8.80 d	1.93 d	3.530 d
Gracilaria corticata	10	6.00 h	1.00 h	2.800 f
	30	6.80 g	1.27 g	2.850 f
	50	6.60 g	1.20 g	2.870 f
Caulerpa paspaloides	10	6.60 g	1.20 g	2.800 f
	30	7.80 e	1.60 e	2.370 h
	50	5.20 i	0.73 i	2.770 g
Ulva lactuca	10	6.40 g	1.13 g	2.670 g
	30	7.00 f	1.33 f	2.530 h
	50	6.00 h	1.00 h	2.770 g
Sargassum tenerrimum	10	7.40 e	1.47 e	3.270 e
	30	8.20 d	1.73 d	4.370 b
	50	11.0 b	2.67 b	4.970 a
Caulerpa sertularioides	10	9.60 c	2.20 c	2.470 h
	30	7.20 f	1.40 f	3.470 e
	50	6.40 g	1.13 g	3.800 c
Sargassum wightii	10	8.20 d	1.73 d	1.600 k
	30	9.60 c	2.20 c	2.300 i
	50	6.20 h	1.07 h	1.370 k
Gracilaria edulis	10	9.20 c	2.07 c	2.800 f
	30	11.2 b	2.73 b	3.870 c
MS medium with standard PGR		11.00 a		4.500 b
MS medium without standard PGR		3.300 i		2.300 i
CV		15.35		11.90
SEM		0.40		0.17
CD 5%		1.12	0.50	0.50

SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test.

Table 3.9 Effect of liquid medium on *in vitro* rooting in banana (Observations were recorded after 21 days).

Medium	Rooting response %	No. of roots	Length of roots (cm)	Length of shoots (cm)
SMSR	80.0	6.62	2.4	4.5
SMLR	100.0	8.32	3.25	6.0
LMLR	100.0	12.77	3.25	7.5
LMSR	90.0	13.50	2.2	5.5
SEM		0.6922	0.06124	0.303
CD 5%		2.042	0.1806	0.8938
CD 1%		2.788	0.2466	1.22
CV		10.5699	13.33	13.8074

SMSR= semi–solid to semi–solid medium; SMLR = semi–solid to liquid medium; LMLR= liquid to liquid medium; LMSR= liquid to semi–solid medium; SD - Standard Deviation; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test

Table 3.10 Stomatal characteristics of abaxial and adaxial leaf surfaces of banana during different stages of micropropagation.

Media type and Stage of Growth	Leaf Surface	Stomatal frequency (mm^{-2})	Stomatal index (%)	Stomata size (µm)	
				Length	Width
SM	AD	79.25	28.71	13.82	7.25
	AB	116.28	32.64	13.76	6.77
LM	AD	73.12	18.27	16.12	6.35
	AB	100.51	31.75	16.36	6.77
SR	AD	11.55	18.47	18.87	6.07
	AB	15.7	15.67	20.3	6.77
LR	AD	38.12	13.11	16.67	7.71
	AB	66.77	21.27	17.12	6.77
SH	AD	41.52	17.95	20.02	9.87
	AB	60.82	29.2	21.62	10.97
LH	AD	52.92	21.9	19.7	8.45
	AB	78.51	27.6	21.32	6.77
Field	AD	46.01	31.31	24.9	10.48
	AB	92.2	42.1	25.1	12.02
	SEM	1.594	1.206	1.19	0.7731
	CD 5%	5.186	3.493	3.447	2.24
	CD 1%	7.405	4.715	4.653	3.023
	CV	4.7142	7.6796	9.7320	12.8040

SM=Multiplication stage on semi–solid medium; LM=Multiplication stage on liquid medium; SR=Rooting stage on semi–solid medium; LR=Rooting stage on liquid medium; SH=Hardened shoots from semi–solid medium; LH=Hardened shoots from liquid medium; AD=Adaxial surface; AB=Abaxial surface. SD -Standard Deviation; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation.

Figure: 3.1 Effect of different supporting material on *in vitro* growth of the *Musa acuminata* on liquid medium. **(A)** (a) Control SS medium, (b) Tissue paper, (c) Glass marble, (d) Pebbles, (e) Liquid medium without support. **(B)** Growth of plantlets on liquid medium with glass marble support, **(C)** Growth on pebbles.

Figure: 3.2 Temporary immersion system designed in Plant Biotechnology Laboratory to study *in vitro* growth of *Musa acuminata*

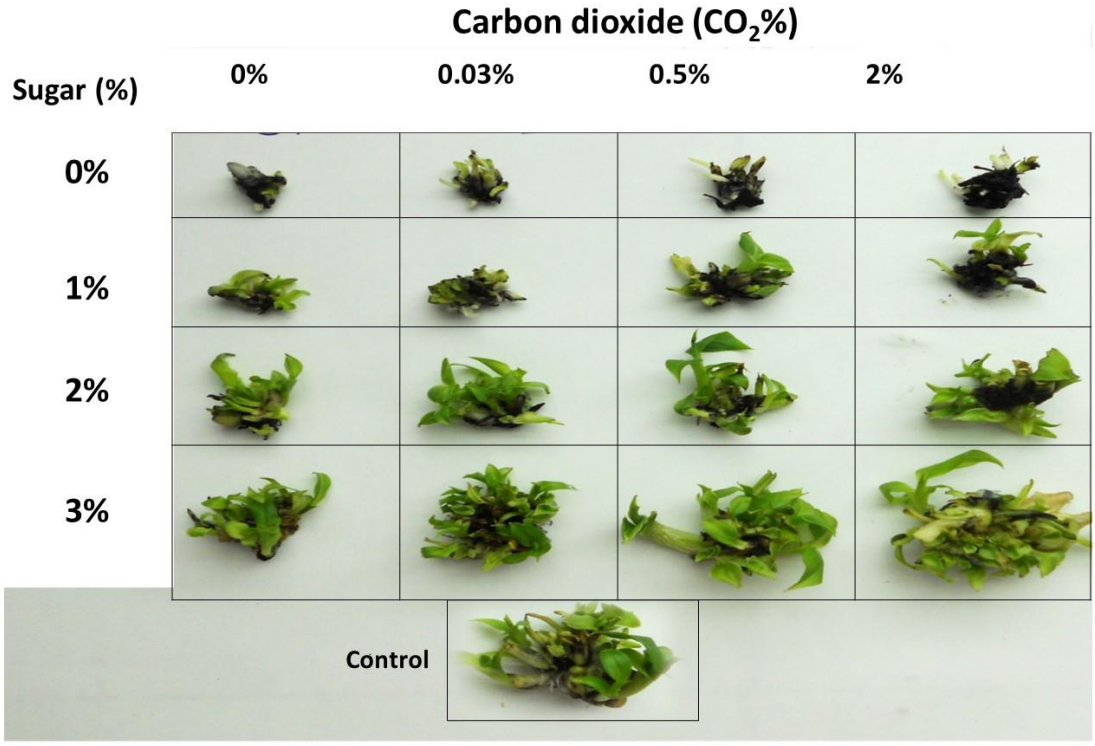


Figure: 3.3 Effect of CO_2 enrichment on shoot multiplication of *Musa acuminata* plant grown on sucrose free semi solid medium (SFSM), Sucrose containing medium (SCSM)

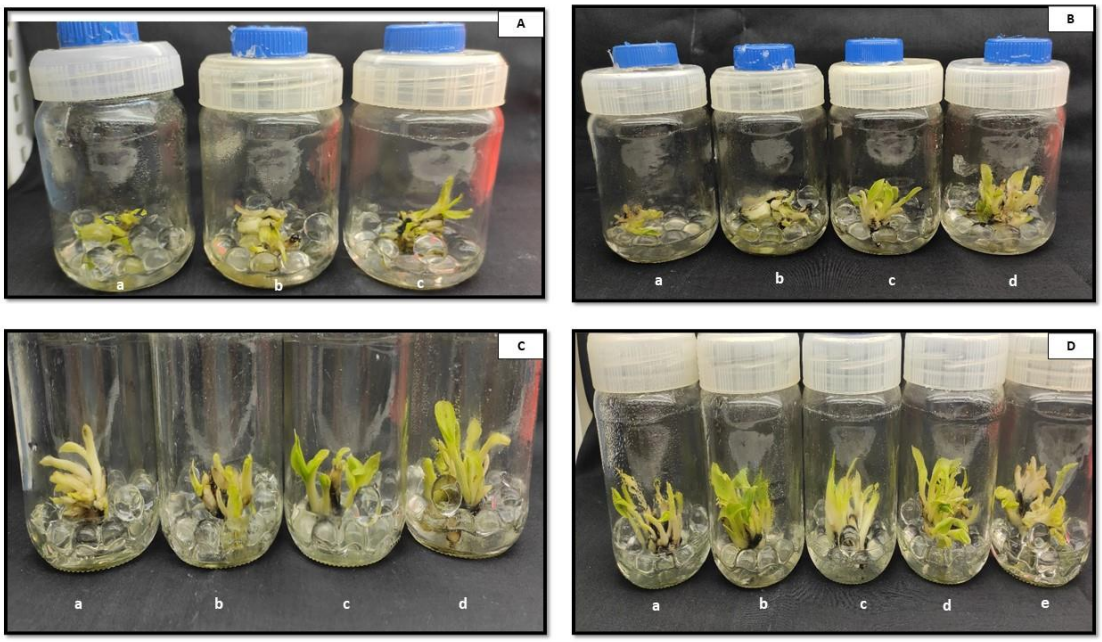


Figure: 3.4 Effect of CO_2 enrichment on shoot multiplication of *Musa acuminata* plant grown on Sucrose Free Liquid Medium (SFLM), Sucrose containing medium (SCSM). **(A)** growth of plant in SFLM with (a) 0.03% CO_2; (b) 0.5% CO_2; and (c) 2% CO_2; **(B)** growth of plant in 1% SCLM with (a) 0% CO_2; (b) 0.03% CO_2; (c) 0.5% CO_2; and (d) 2% CO_2 ; **(C)** growth of plant in 2% SCLM with (a) 0% CO_2; (b) 0.03% CO_2; (c) 0.5% CO_2; and (d) 2% CO_2 ; **(D)** growth of plant in 3% SCLM with (a) 0% CO_2; (b) 0.03% CO_2; (c) 0.5% CO_2; and (d) 2% CO_2.

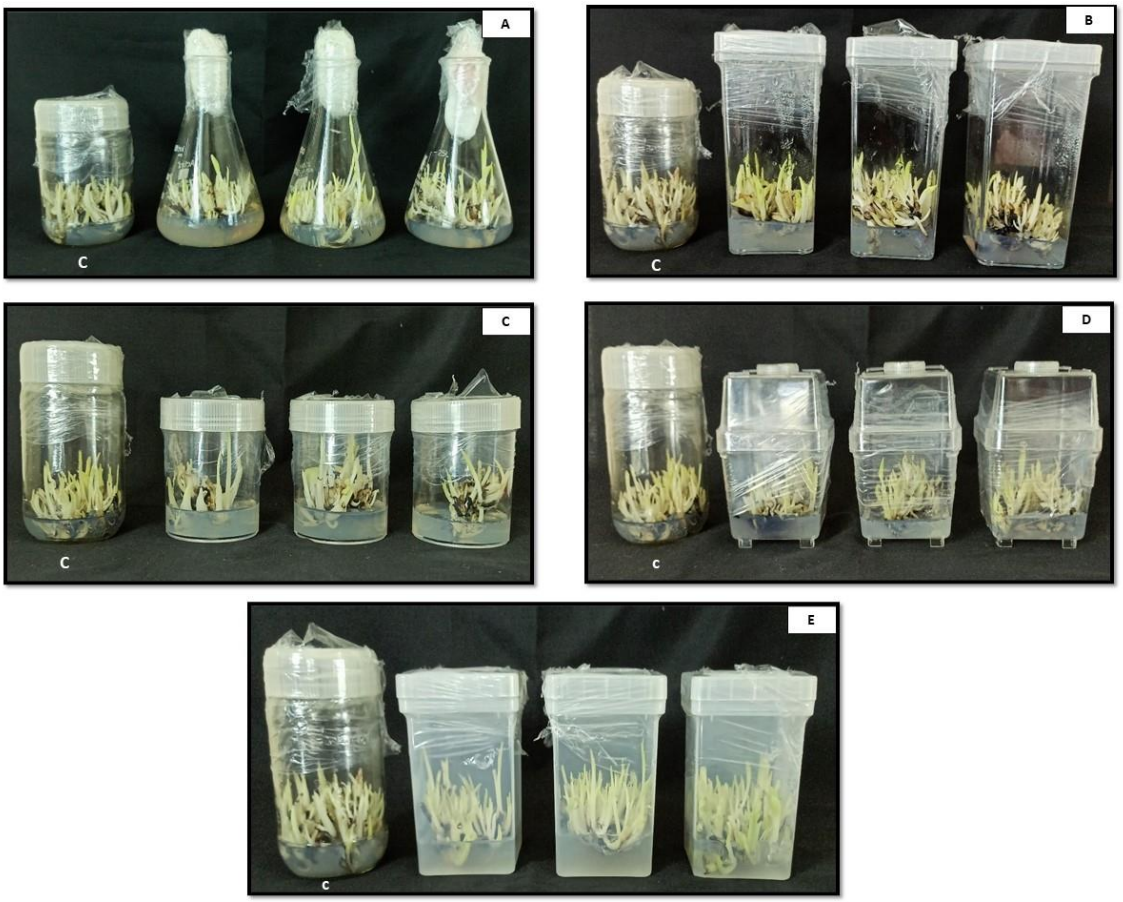


Figure: 3.5 Effect of different culture vessels on *in vitro* plant growth in multiplication stage of *Musa acuminata.* **(A)** Conical flasks with a narrow mouth, having a capacity of 250 ml (height 12.4 cm, bottom diameter 6.0 cm, neck diameter 2.5 cm), **(B)** Phyta jar with clear square container having capacity 500 ml (Size 74 X 71 X 138 mm), **(C)** Phyta jar with clear round container having capacity 250 ml (Size 67 X 78 mm), **(D)** Phyta jars with vented lids, having capacity 350 ml (size 78 X 78 X 95 mm).,**(E)** Phyta jar with translucent square container having capacity 370 ml (Size 74 X 71 X 134 mm). '**c' – control,** neutral glass bottles: 200 ml capacity (height 10.0 cm, mouth diameter 5.5 cm,

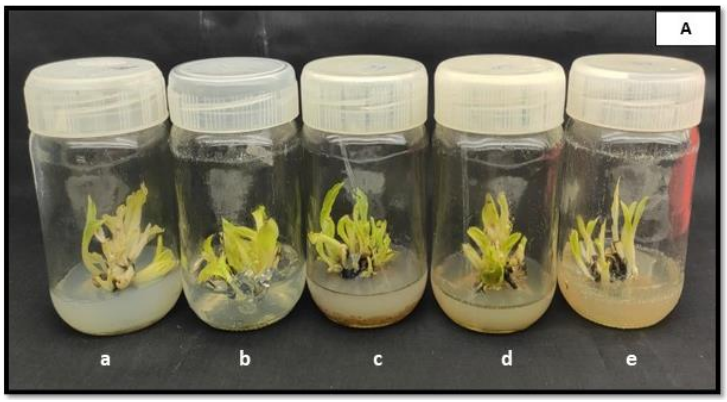


Figure: 3.6 Effect of different gelling agents on *in vitro* plant growth in multiplication stage of *Musa acuminata.* (a) Agar Containing SS Medium, (b) Phyta gel, **(c)** Isabgol + agar, (d) Guar gum + agar, and (e) Starch +agar

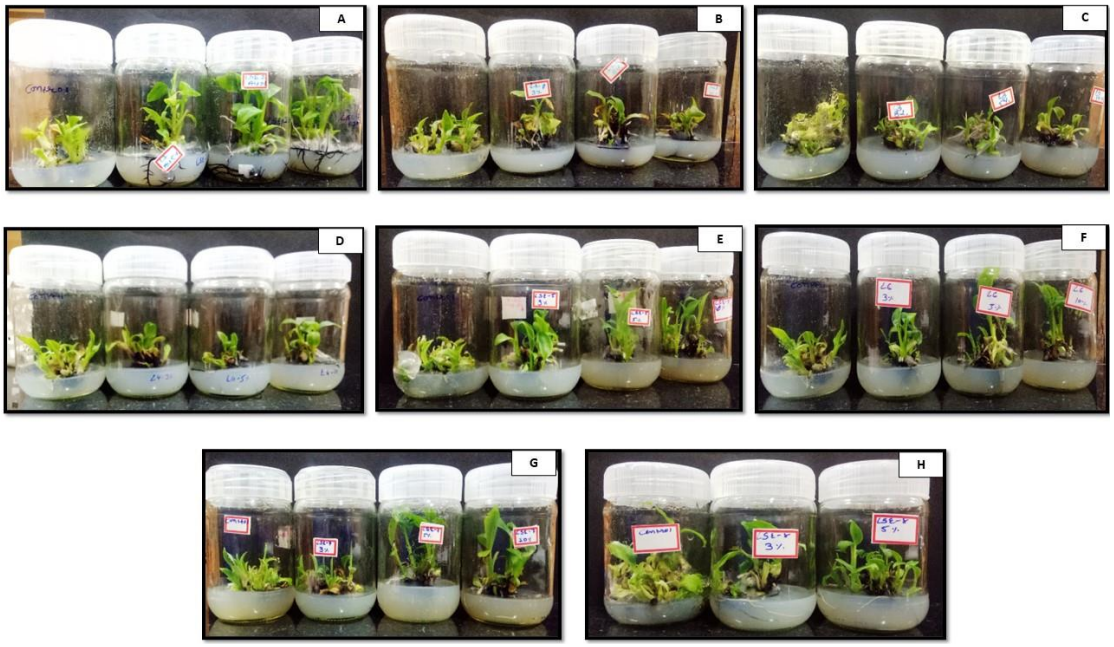


Figure 3.7 Effect of different Liquid Seaweed Extract (LSE) on *in vitro* growth at multiplication stage of *Musa acuminata.* LSE used - **(A)** *Caulerpa recemosa,* **(B)** *Gracilaria Corticata,* **(C)** *Culerpa Paspaloides,* **(D)** *Ulva lactuca,* **(E)** *Saegassum tenerrimum*, **(F)** *Culerpa sertularioids,* **(G)** *Sargassum wightii* & **(H)** *Glacilaria edulis.*

Figure: 3.8(A) Effect of Semi solid and Liquid Medium on *in vitro* rooting of *Musa acuminata*. **(A)** rooting stage of micropropagules - (a) semi solid medium, (b) Liquid Medium. **(B)** rooting stage of micropropagules - (a) liquid medium, (b) semi solid Medium

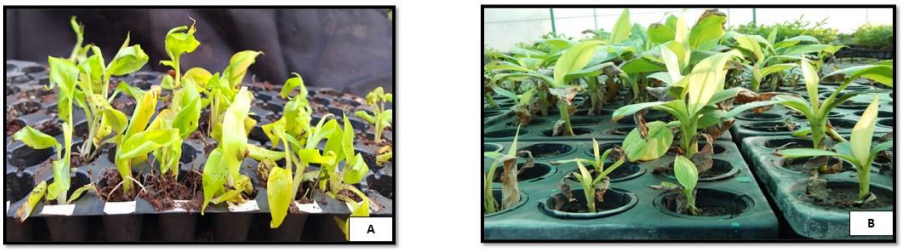


Figure: 3.8(B) Acclimatization of on *in vitro* grown *Musa acuminata* plant. **(A)** Primary hardening; (B) Secondary hardening.

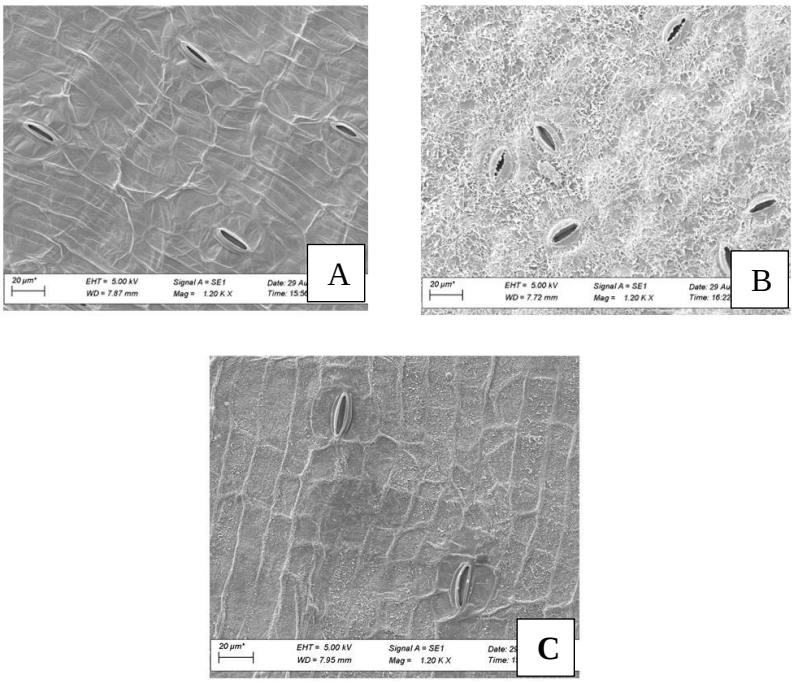
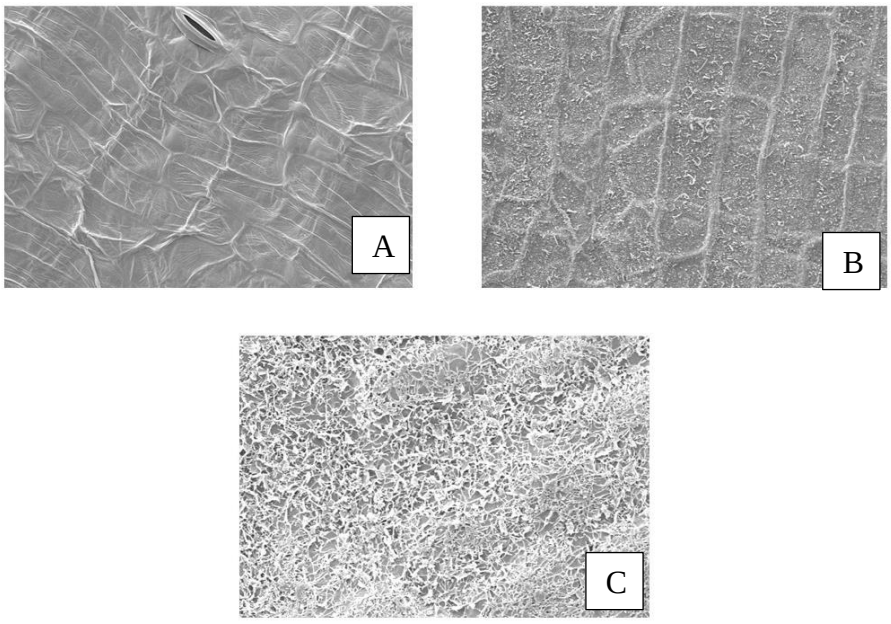


Figure: 3.9 SEM analysis of leaf adaxial surface of *in vitro* gown plant *Musa acuminata* in **(A)** semi solid, and **(B)** liquid medium, **(C)** field grown plant (leaves adaxial surface)



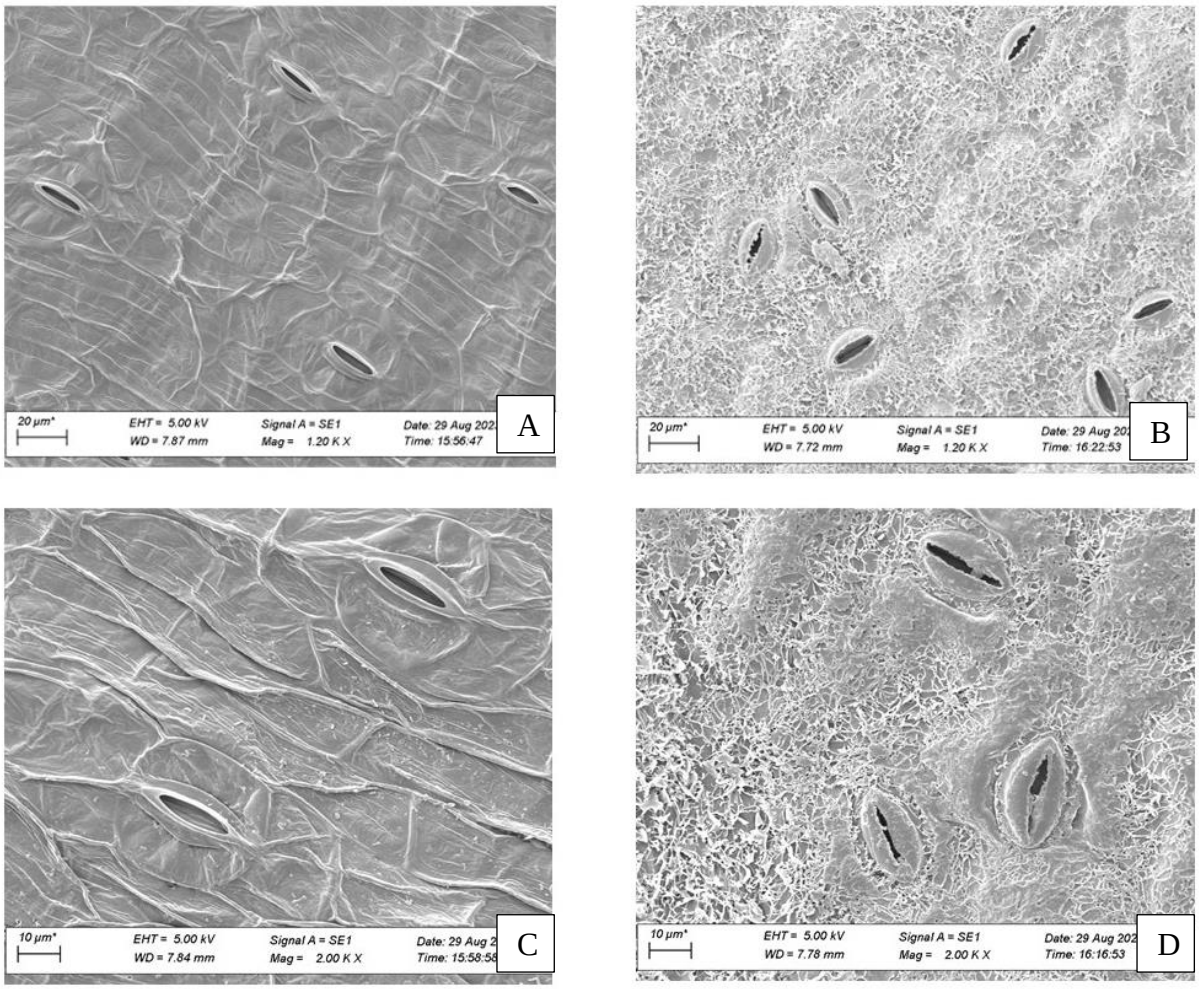


Figure 3.11 SEM analysis at higher magnification of leaf adaxial surface of *in vitro* gown plant *Musa acuminata* **(A)** *in vitro* grown plant 1.2kX, **(B)** field grown plant 1.2k X, **(C)** *in vitro* grown plant 2kX, **(D)** field grown plant 2.0 k X

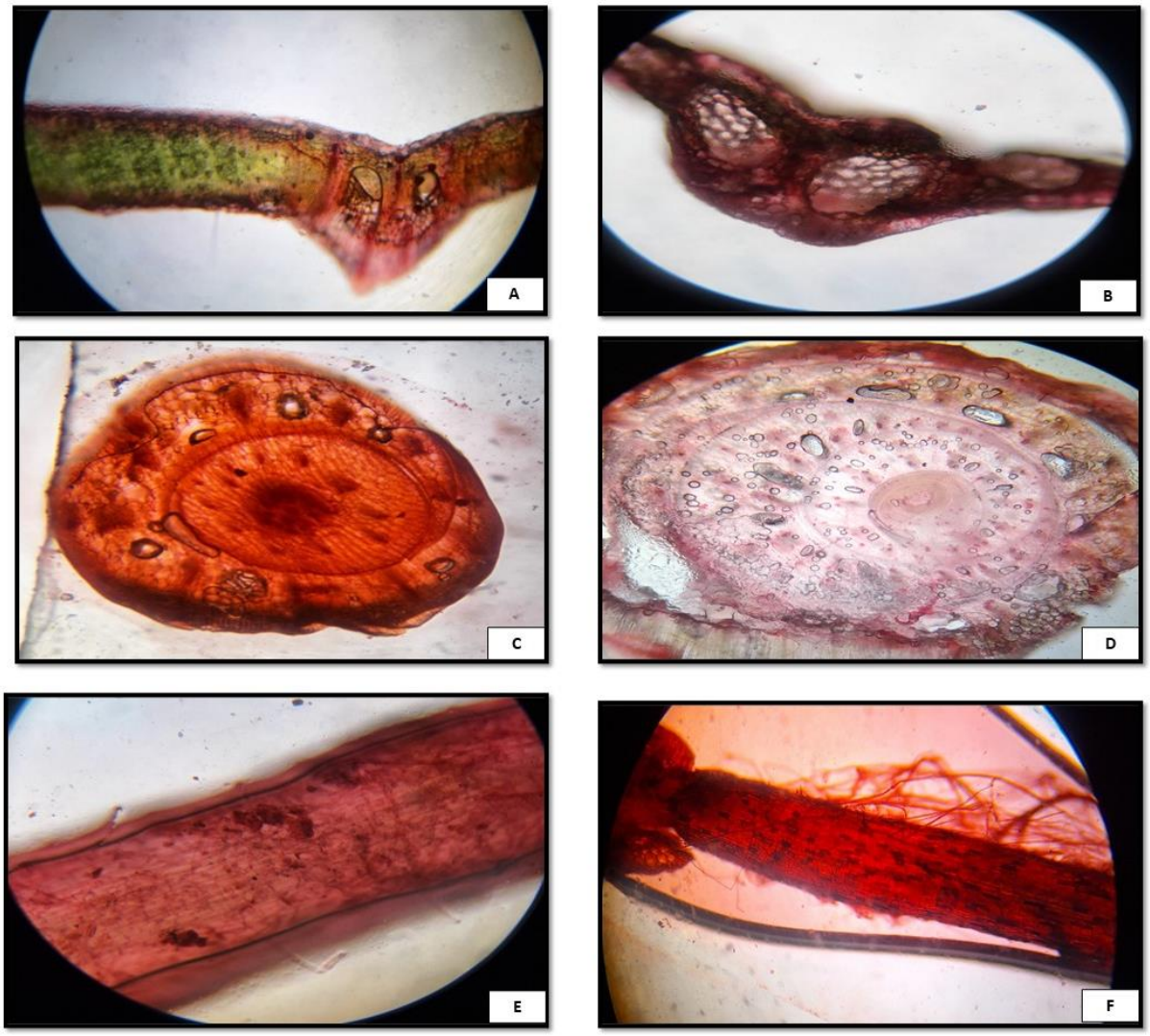


Figure 3.12 Transverse section through shoot, root and leaf of *Musa acuminata* during *in vitro* growth (multiplication stage) on semi solid and liquid medium: **(A)** leaf TS grown on semi solid medium; (B) leaf TS grown on liquid medium; (C) stem TS grown on semi solid medium: (D) stem TS on liquid medium; (E) magnifying view of rooting on semi solid medium; and (F) liquid medium.