Chapter 3

Micropropagation in Altered Growth Condition-Morphological Studies

3.1 Experiment on effect of different growth condition on plant growth

Current micropropagation techniques mainly focus on cultivating shoots in a semi-solid culture medium. With the rising need for greater productivity and shorter propagation cycles for economically important plants, the role of these semi-solid systems has become increasingly significant, despite their varying success rates in multiplication yields. The rate of multiplication is crucial for the success of large-scale production projects and is a major determinant of overall production costs (Fehér, 2019). In micropropagation, producing fewer than three times the original number of plant shoots is deemed inefficient. This lower multiplication rate reduces the frequency of transferring plants to new containers, thereby cutting labour costs. Achieving a higher shoot production rate can help mitigate losses due to contamination and challenges in subsequent stages such as rooting, hardening, and acclimatization. Recently, using liquid nutrient solutions has gained popularity as a cost-effective method to enhance shoot multiplication rates (Melaku *et al*., 2016; Mohapatra and Batra, 2017; Royandazagh, 2019; Muhammet, 2022). The liquid medium facilitates increased shoot and root growth by maintaining close contact with plant tissues, which improves the absorption of nutrients and plant hormones, leading to better growth outcomes.

The disadvantages of using liquid media for *in vitro* cultivation, including hypoxia (oxygen deficiency), hyperhydricity (excess moisture), shear stresses (mechanical forces), and the need for advanced equipment, often outweigh the benefits (Hazarika, 2006). To mitigate these issues, alternative methods have been developed. These involve suspending plants above a still liquid using flotation devices like rafts and using culture support materials such as cellulose blocks, paper bridges, or sponges. These approaches create a more suitable environment for plant growth

while avoiding the aforementioned challenges or employing temporary immersion techniques (Carvalho *et al*., 2002). Unlike solid gels, which have limited gas exchange and nutrient absorption due to their few tiny pores, supportive structures enhance plant shoot growth by providing ample air and facilitating nutrient uptake. These support structures are cost-effective as they eliminate the need for expensive gelling agents and reduce cleaning and washing costs. Additionally, they lower the risk of contamination by allowing plants to be transferred to new culture media using sterile liquid alone (Gangopadhyay *et al*., 2002).

Another method to address current challenges involves briefly immersing or growing plant shoots in a liquid solution before returning them to a semi-solid environment. This technique has been explored by researchers such as Madihah Mohd *et al*. (2017), Ruta *et al*. (2020), and Vendrame *et al*. (2023). Various temporary immersion methods have been developed, focusing on ensuring that plants only have short-term contact with the liquid, as discussed by Etienne and Berthouly (2002) and Aragon *et al*. (2010). Thoroughly investigating plant growth in liquid environments and understanding the complex chemical and physical factors that influence regrowth is essential for improving large-scale liquid plant culture (Malik *et al*., 2018). Insights into the roles of ventilation and carbohydrate levels will help optimize conditions for mass production of plant material, development of different plant parts, and ultimately the production of high-quality plants.

Photoautotrophic (Pa) micropropagation is an *in vitro* cultivation method that omits sugar from the growth media. Kozai's 2005 study reported and thoroughly evaluated the numerous benefits of the Pa system compared to the Photomixotrophic (Pm) method (Kozai *et al*., 2005). Enhanced growth and multiplication of plantlets in environments enriched with carbon dioxide have been observed in several plant species (Wu and Lin, 2013; Corrêa *et al*., 2015; Tisarum *et al*., 2018; Santos *et al*., 2020; Mansinhos *et al*., 2022; Pepe *et al*., 2022). Initial research focused on increasing carbon dioxide (CO_2) levels in liquid environments within bioreactors, yielding positive results. However, these studies primarily examined the effects of elevated CO_2 on plants grown in agar-based cultures (Gaspi *et al*., 2013; Vidal and Sánchez, 2019).

The growth of young plants can be hindered by insufficient air circulation in their containers. To address this, using caps with small holes to allow air exchange has proven effective. This approach helps reduce hyperhydricity and promotes better growth in liquid culture (Aguilar *et al*., 2022). Vented closures offer several benefits, as highlighted by Zobayed (2005). These benefits are due to their influence on the internal gas composition, humidity levels, and temperature within the containers. The type of closure and the frequency of air exchange are crucial in shaping these conditions. Vented closures can significantly impact these factors (Gaspi *et al*., 2013; Agrawal *et al*., 2016; Gao *et al*., 2017; Silva *et al*., 2017).

The effectiveness of plant transfer during subculture and the number of plantlets produced in a given space are influenced by the type of container used for growing the plants. Different containers can impact these factors, such as the efficiency of the transfer process and the density of plantlets grown in a specific area. This underscores the importance of selecting the appropriate container for successful plant tissue culture experiments (Silva *et al*., 2017). In controlled lab environments, various container-related factors play a crucial role in the plant growth process. Factors such as the type of container, its capacity, the shape of its neck compared to its base, the size of its openings, and the transparency of its material all significantly impact plant growth during *in vitro* cultivation (Kozai *et al*., 1992; Prakash *et al*., 2004). Various containers have been utilized in plant tissue culture experiments, including disposable food containers that cannot be autoclaved, glass bottles, baby food jars with polypropylene lids, and transparent plastic containers like Magenta™ vessels made from polypropylene, polycarbonate, or polystyrene. These containers facilitate easier handling of cultures due to their wider openings, which can hold more plant explants and streamline the cultivation process.

Additionally, it's worth highlighting that the shape and material of the culture container not only influence light exposure but also significantly impact plant growth, as emphasized in the study by Silva *et al*. (2017). This underscores the importance of carefully selecting the container when conducting plant tissue culture experiments, as it can directly affect the growth dynamics and final outcomes.

Numerous studies indicate the potential of seaweeds to enhance plant growth, sparking interest among researchers to explore growth-promoting substances or

biomolecules present in seaweeds. Subsequent investigations revealed that seaweeds contain various plant growth-promoting hormones, including auxins (such as indole butyric acid (IBA), naphthleacetic acid (NAA), and indoleacetic acid (IAA)), cytokinins (such as 6-benzylaminopurine (BAP), kinetin (Kin), trans-zeatin, and isopentenyladenine (2iP)), and abscisic acid (ABA) (Górka & Wieczorek, 2017). HPLC analysis confirmed the presence of ABA, salicylic acid, IAA, and 2iP phytohormones in species like *Pyropia yezoensis* and *Bangia fuscopurpurea* (Mori *et al*., 2017). Additionally, IAA was detected in *Monostroma oxyspermum* (Gupta *et al*., 2011), while indole-3-pyruvic acid (IPA) was found in *Sargassum tennerrimun*, *Kappaphcus alvarezii*, and *Gracilaria edulis* according to Prasad *et al*. (2010). Likewise, various studies have demonstrated the presence of cytokinins in different seaweeds. For instance, isopentenyladenine riboside (iPR), trans-zeatin riboside (tZR), trans-zeatin (tZ), and other aromatic cytokinins like 5 topolins were identified in species such as *Laminaria japonica*, *Gracilaria edulis*, and *Sargassum tenerrimum* (Stirk *et al*., 2013). Additionally, Gibberellic acid (GA_3) was found in different *Ulva* species, *Monostroma oxyspermum*, and *Gracilaria edulis* (Gupta *et al*., 2011), as well as in *Sargassum tenerrimum* (Prasad *et al*., 2010). Many studies have utilized natural extracts from these algae to prepare liquid biofertilizers for application under in vivo conditions, resulting in enhanced responses in plants, including increased shoot number, root length, and overall growth (Khan *et al*., 2009). Despite the abundance of nutrients and growth-promoting substances, the application of these liquid seaweed extracts under *in vitro* conditions has remained largely unexplored. There are few reports, such as in tomato (Vinoth *et al*., 2012), demonstrating the beneficial role of seaweed extract in promoting better growth and regeneration under *in vitro* conditions.

In plant tissue culture, synthetic MS media are commonly used, containing essential inorganic and organic salts, chelating agents, carbon sources, growth regulators, and water. However, each plant species and even cells within the same plant have specific nutritional requirements. We propose that supplementing the growth medium with natural Liquid Seaweed Extracts (LSE) could potentially replace or reduce the need for synthetic plant growth regulators (PGRs). This not only could lower production costs but also serve as a natural growth enhancer in cultured

conditions. Implementing this innovative approach could significantly decrease production expenses and enhance the commercial viability of the protocol. The objective of this study is to offer a cost-effective method for rose micropropagation.

3.1.1 Materials and methods

3.1.1.1 Experiment on liquid culture system

The purpose of this studies was to find out how various growth medium types affected plant shoot growth and multiplication in an *in vitro* environment. We cut plant shoots from cultures that were growing in a certain shoot multiplication medium in order to do this. Following that, these clusters were put into two different types of media: a liquid medium and a semi-solid medium that included 0.8% agar to give it a gel-like consistency. We provide the plant clusters various supports in the liquid medium. We further placed part of the plant clusters onto the semi-solid medium containing 0.8% agar to study the impact of supporting material on plant development. By using this agar-based medium as our control group, we were able to evaluate and compare the impact of the various media types on plant development. A part of the plant clusters was also transferred onto the semi-solid medium containing 0.8% agar to study the role of supporting material on plant development. We were able to evaluate and compare the impact of the various media types on plant development by using this agar-based medium as our control group. This study also investigated the role of support matrices in the liquid medium, looking at how these matrices affected plant shoot development and multiplication. The results of this study provide important light on the ideal circumstances for plant development when grown *in vitro*. To find a low-cost, environmentally friendly support system that works well with liquid media, we looked at four different types of mechanical support materials in our investigation. Identifying out how effectively these supports work in a liquid media was our aim. The four kinds of mechanicals support we investigated included.

For our studies, we used glass marbles that were 8 to 10 mm in size. We also use cotton, water balls, and tissue paper. Agar at a concentration of 0.8% is used as the semi-solid medium used as the control. For subsequent subcultures was done. Over an interval of 45 to 60 days, we kept a careful eye on a number of growth measurements to evaluate how the plant developed and responded to different environments.

3.1.1.2 Experiment on temporary immersion (TI) in *in vitro* shoot growth

In a controlled laboratory condition, further studies were carried out to determine the effect of temporarily immersing plant shoots in a liquid media on their development and multiplication. To be more precise, we soaked the shoots in a media that contained 45-50 millilitres and held them up with glass beads. The shoots were moved back to a normal semi-solid agar-based media with 0.8% agar content after growing for 30 days in this liquid medium. We kept an eye on their development on this semi-solid media for 30 more days, for a total of 60 days during the experiment. We utilized a control group that was cultured on the conventional semi-solid medium containing 0.8% agar as a point of comparison. Through these experiments, we were able to examine the impact of liquid immersion on plant shoot development and evaluate a variety of growth conditions. The information gathered from these observations will be essential to our thesis as it sheds light on the function of brief immersion in plant tissue culture and how it affects the growth and multiplication of shoots *in vitro*. This understanding advances our knowledge of how to most effectively culture plants in tissue culture to promote growth and productivity, which is crucial for the production of commercial plants.

3.1.1.3 Experiment on CO_2 enrichment

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. In order to investigate more thoroughly how controlled and elevated levels of carbon dioxide (CO_2) affect the growth and multiplication of plant shoots in an *in vitro* environment. We used to seal the bottle in order to keep the environment under control.

To explore the impact of various CO_2 concentrations, we exposed the shoot cultures to different levels of CO_2, including 0.0%, 0.03%, 0.5%, and 2%. To do this, we enclosed the cultures in clear acrylic chambers, each measuring 25 x 50 x 15 cm (length × width × height) and with a volume of 7500 cm^3. The tops of these chamber were sealed with packing tape (Miracle, 5.0 cm width).

We used the procedure given by Solarova *et al*. (1989) to establish a control CO_2 concentration. We used 0.1 M solutions of sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) in a 77/23 (v/v) ratio to maintain a concentration of 0.6 g (CO_2) m^{-3}. Similarly, 3 M solutions of potassium carbonate (K_2CO_3) and bicarbonate ($KHCO_3$) in ratios of 50/50 and 73/27 (v/v) were combined to provide concentrations of 0.0%, 0.03%, 0.5%, and 2%. We filled the acrylic box with a 10.0% potassium hydroxide (KOH) solution in order to provide a CO_2 free environment. To optimize CO_2 diffusion, these solutions were set up in open petri plates within the boxes, and we changed them every 5th day.

We studied the effects of sucrose on plant tissue culture growth by varying the conditions in our experiment. Twenty culture bottles, one for each shoot cluster, were used. Two sets of these cultures were made: one with the media containing sucrose (SCSM, or sucrose-containing semi-solid medium, and SCLM, or sucrose-containing liquid medium), and the other without (SFSM, or sucrose-free semi-solid medium, and SFLM, or sucrose-free liquid medium). We set up different acrylic boxes with these culture bottles to produce different CO_2 concentrations. Different amounts of CO_2 were intended to be provided by these boxes. To provide constant conditions, these sets were kept in a culture room under supervision.

Furthermore, for the control. We used a combination of semisolid media that contained and without sucrose (SCSM, SFSM) and liquid media that contained and without sucrose (SCLM, SFLM). This culture was kept in a growth chamber with natural ventilation. We were able to look at how the concentration of CO_2 and sucrose affected the growth of plant tissue cultures.

3.1.1.4 Experiment on culture vessels environment

Growing shoot cultures in an array of culture vessels was part of our study to find the best container for increasing the rate of shoot multiplication. We used semi-solid and liquid growth mediums for our experiments. The following types of containers were selected for this study:

1. (V1) Regular glass jar with a capacity of 200 ml (height 10.0 cm, mouth diameter 5.5 cm)

2. (V2) Phyta jar with a clear round container and a capacity of 150 ml (Size 67 X 78 mm)
3. (V3) Glass jar with a capacity of 400 ml
4. (V4) Phyta jar with vented lids and a capacity of 150 ml (size 78 X 78 X 95 mm)
5. (V5) Phyta jar with a translucent square container and a capacity of 200 ml (Size 74 X 71 X 134 mm)
6. (V6) Phyta jar with a clear square container and a capacity of 400 ml (Size 74 X 71 X 138 mm)
7. (V7) Conical flasks with a narrow mouth and a capacity of 250 ml (height 12.4 cm, bottom diameter 6.0 cm, neck diameter 2.5 cm)

In order to investigate their effect on shoot multiplication rates in plant tissue cultures, these vessels were carefully selected. Our research attempts to offer important insights into the choice of the best container for maximizing the conditions of plant tissue culture, which are crucial for plant biotechnology thesis.

In each container, we placed a group of five shoots called a shoot cluster. We aided these shoots in multiplying by using a conventional media. This procedure was carried out six times for every kind of container. Following a 45-day period, we meticulously monitored and recorded many growth parameters, such as the amount of shoot multiplication.

3.1.1.5 Experiment on altered gelling agents

In this work, we use several substances in the tissue culture media as gelling agents. *In vitro* plant growth is achieved using agar agar, phyta gel, starch, guar gum, and isabgol, and growth attributes were studied.

3.1.1.6 Experiment on liquid seaweed extract

In our study, we investigated the influence of seaweed extracts on the growth of rose micropropagules. We chose five seaweed species (*Caulerpa racemosa, Ulva lactuca, Sargassum tenerrimum, Sargassum wightii,* and *Gracilaria edulis*) and examined different concentrations of liquid seaweed extract (LSE) ranging from 10% to 50% (v/v). To provide comparison, we included positive and negative controls, consisting of standard MS medium with and without plant growth regulators,

respectively. Each experiment was carried out in 200 ml culture bottles containing 50 ml of medium adjusted to a pH of 5.8. Following autoclaving and solidification, each bottle was inoculated with five aseptic shoots. These bottles were then transferred to a growth room for a period of 45 days. We maintained six replicates per experiment, with three repetitions of each treatment. At the conclusion of the experiment, the micropropagules were assessed based on various growth parameters.

3.1.1.7 Experiment on effects of polyamines

Shoot cultures of bush rose were initiated following the method of Makarov *et al*. (2024). Mature nodal segments were collected from greenhouse-grown plants during 2023, washed, and sterilized using ethanol and sodium hypochlorite. The explants were then inoculated on MS medium with 3.0 mg/L BAP, 0.01 mg/L NAA, 0.8% agar, and 3.0% sucrose, and maintained under controlled conditions (28±2°C, 16-hour light cycle). Various concentrations of polyamines like Spermidine, Putrescine, and Cadaverine (10 mM-50 mM) were added to the medium pre-autoclaving with filter sterilization to assess their effects on shoot multiplication. Cultures were sub-cultured every three weeks over six cycles, totalling 126 days. Growth parameters, biochemical analyses, and antioxidant assays were conducted at the experiment's end, with each treatment replicated three times. The total number of shoots, average shoot length was determined.

3.1.1.8 Materials and methods of *in vitro* rooting

Role of liquid medium on *in vitro* rooting

In order to study the effects of a liquid growth medium on the *in vitro* development of plant roots, we used appropriate-sized (3-4 cm) shoots that had been grown on semi-solid and liquid media in previous studies. Here was our experimental setup. The long shoots that we used were around 3 to 4 cm in length and came from semi-solid and liquid media. The elongated shoots were inoculated into a regular rooting medium, which could have with agar or without agar.

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

Rooted plantlets were placed in culture bottles containing Soiltrite^{TM} that had been moisturised with ¼th strength MS salt solution in order to facilitate *in vitro*

hardening. They were inoculated in the bottles, and after seven days in a culture room they were moved to a greenhouse with a temperature differential of 28±2 °C and an 85% reduction in humidity. Bottle leads were slowly unscrewed. The hardened plantlets were measured for a number of growth attributes including plantlet height, percentage of survival, and number of leaves generated per plant, after 30 days of growth in the greenhouse.

3.1.2 Results

Multiplication

3.1.2.1 Role of liquid Medium

Liquid medium has shown significantly higher growth potential for rose multiplication compared to the control group using a semi-solid medium. The control group, which included only agar media, produced shoots cluster which produced average shoot numbers of 19 and shoot length of 3.00 cm, establishing a baseline for comparison. Using water balls as support material led to slightly high, with a shoot length of 3.04 cm and shoot numbers average of 17.33, compared to the control group. Remarkably, glass marbles (GM) significantly boosted plant growth, producing shoot length of 3.20 cm and average shoot numbers of 21. This highlights GM's effectiveness as a highly efficient supporting material. In contrast, materials such as tissue paper resulted in a slightly lower an average shoot length of 2.99 cm and average of shoot numbers of 16.33, indicating its lesser effectiveness compared to GM and cotton used as support material showed the average shoot length of 2.86 cm and average shoot numbers of 14.33 (Table 3.1).

In conclusion, glass marbles (GM) proved to be the most effective supporting material for enhancing plant growth in liquid culture media, as shown by the highest length of shoots. While water balls also provided a modest improvement, tissue paper and cotton yielded results slightly similar to the control group (Figure 3.2). These findings are crucial for optimizing plant tissue culture conditions and have practical implications for the plant tissue culture industry to improve plant production rates.

3.1.2.2 Effect of TI in *in vitro* shoot growth

Similarly, rose plant growth was significantly improved when shoots were briefly immersed in a liquid media and then allowed to grow on a semi-solid medium (Figure 3.4). The number of shoots and their length increased significantly as a result of this strategy, essentially doubling the values in comparison to the control group. Additionally, this method demonstrated an impressive increase in the rate of multiplication, highlighting its potential to improve rose propagation (Table 3.2).

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

In our experiment, we put rose shoots into SCSM (sucrose contain semisolid medium) to start their growth. After 21 days of growing in the controlled growth room environment, this strategic method produced an astounding almost four-fold increase in shoot numbers (Figure 3.6). Notably, every explant produced in the cells cultured on SCSM showed remarkable shoot production. Interestingly, the growth attributes in terms of both multiplication and elongation exceeded expectations when we compared these results to those obtained from shoot clusters grown on SCLM (sucrose contain liquid medium) under exactly the same growth chamber conditions.

In this work, we investigated how shoot cultures respond to controlled CO_2 environments. Remarkably, cultures grown under ambient air conditions grew at a rate similar to that of cultures grown with additional CO_2. But when we added different CO_2 concentrations (0.03%, 0.5% and 2%), our results took an interesting turn, all growth parameters were generally enhanced in comparison to shoots grown on both SCSM and SCLM in ambient air. Notably, the liquid medium continuously promoted better growth than its semi-solid related under all CO_2-enriched environments. Under regulated conditions, the most favourable response was shown at 0.5% CO_2 concentration, in SCLM, where about more than 20 shoots per cluster were generated with 3% sucrose concentration (Figure 3.7), with an average length of around 3.96 cm (Table 3.4). Nevertheless, there was a noticeable increase in shoot length and number at greater concentrations of 2% CO_2 concentration in SCLM, even if these changes were accompanied with hyperhydricity symptoms. However, 2% CO_2 concentration was shown to be the ideal development condition for SCSM. In a separate environment, cultures grown on sucrose-free shoot multiplication media

(SFSM) showed a substantial drop in all recorded growth parameters when we exposed them to the room's ambient air conditions. Initially inoculated shoot clusters became yellow and found it difficult to continue growing in the presence of ambient CO_2, falling short of the growth seen in SCSM conditions.

Moreover, shoots developed on sucrose-free liquid medium (SFLM) could only continue to develop for around 15 days before rapidly deteriorating and dying when we put them in a CO_2-free environment. In just seven days, shoots on SFLM in CO_2-free conditions became light yellow. However, shoots on both SFSM and SFLM showed continued growth when exposed to varying CO_2 concentrations. When the concentration of CO_2 rose, both the number and length of the shoots increased. Specifically, as compared to controls grown on SCSM in ambient air, there was a substantial increase in shoot length on SFLM at a concentration of 2% CO_2 concentration. The most notable results were found at 2% CO_2 concentration, at which point all parameters studied increased and were similar to those of shoots grown on SCSM in ambient air (Table 3.3).

3.1.2.4 Effect of culture vessels environment

Six totally different vessel types and regular glass jar were the subject of our study, which looked at the effect of culture vessel size on the growth of *in vitro* plant shoots in semi-solid and liquid medium and different support material were used for liquid medium (glass marbles, water balls, tissue papers, tissue paper and cotton). Culture vessels were as followed: (V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml.

The number of shoots and length of shoots produced in each culture vessel varied considerably in semi-solid medium (Figure 3.3). Among the vessels tested, the V3 glass jar with a capacity of 400 ml exhibited the highest average shoot count in a semi-solid medium, reaching 31 (Table 3.5). This was followed by V7, which had an average of 27 shoots. In contrast, V2 and V4 showed lower shoot counts, with 11 and

16 respectively. V1, V5, and V6 fell in between, with average shoot counts of 19, 19, and 23, respectively. Shoot length is another essential parameter for assessing plant growth. V7 had the longest average shoot length at 4.0 cm, followed by V6 with an average shoot length of 3.56 cm. In contrast, V2 and V5 showed lower shoot lengths of 1.6 cm and 1.5 cm, respectively. V1, V3, and V4 fell in between, with average shoot lengths of 3 cm, 2.55 cm, and 2.03 cm, respectively. The size of the culture vessel significantly affects the *in vitro* growth of plant shoots. V3, V6, and V7 exhibited superior performance in both shoot number and shoot length, as well as in overall multiplication rates.

The number of shoots and length of shoots produced in each culture vessel varied considerably in liquid medium with glass marbles as the support material (Figure 3.1). Among the vessels tested, the V3 glass jar with a 400 ml capacity exhibited the highest average shoot count in a liquid medium, reaching 33 (Table 3.6). This was followed by V7, which had an average of 29 shoots. In contrast, V2 and V4 showed lower shoot counts, with 13 and 18, respectively. V1, V5, and V6 fell in between, with average shoot counts of 21, 21, and 25, respectively. Regarding shoot length, V7 had the longest average at 4.2 cm, followed by V6 with an average of 3.7 cm. In contrast, V2 and V5 had lower shoot lengths of 1.8 cm and 1.7 cm, respectively. V1, V3, and V4 fell in between, with average shoot lengths of 3.2 cm, 2.73 cm, and 2.23 cm, respectively. Overall, V3, V6, and V7 exhibited superior performance in both shoot number and shoot length, as well as in overall multiplication rates.

The number of shoots and length of shoots produced in each culture vessel varied considerably in liquid medium with water balls as the support material (Figure 3.1). Among the vessels tested, the V3 glass jar with a 400 ml capacity exhibited the highest average shoot count in a liquid medium, reaching 29.66 (Table 3.7). This was followed by V7, which had an average of 27.66 shoots. In contrast, V2 and V5 showed lower shoot counts, with 12.33 and 10.66, respectively. V1, V4, and V6 fell in between, with average shoot counts of 17.33, 17, and 24, respectively. Regarding shoot length, V7 had the longest average at 4.02 cm, followed by V6 with an average of 3.7 cm. In contrast, V2 and V5 had lower shoot lengths of 1.66 cm and 1.37 cm, respectively. V1, V3, and V4 fell in between, with average shoot lengths of 2.86 cm,

2.66 cm, and 1.94 cm, respectively. Overall, V3, V6, and V7 exhibited superior performance in both shoot number and shoot length, as well as in overall multiplication rates.

The number of shoots and length of shoots produced in each culture vessel varied considerably in liquid medium with tissue paper as the support material (Figure 3.1). Among the vessels tested, the V3 glass jar with a 400 ml capacity exhibited the highest average shoot count in a liquid medium, reaching 28.66 (Table 3.8). This was followed by V7, which had an average of 26.66 shoots. In contrast, V2 and V5 showed lower shoot counts, with 11.33 and 9.66, respectively. V1, V4, and V6 fell in between, with average shoot counts of 16.33, 16, and 23, respectively. Regarding shoot length, V7 had the longest average at 3.97 cm, followed by V6 with an average of 3.67 cm. In contrast, V2 and V5 had lower shoot lengths of 1.79 cm and 1.50 cm, respectively. V1, V3, and V4 fell in between, with average shoot lengths of 2.99 cm, 2.76 cm, and 2.07 cm, respectively. Overall, V3, V6, and V7 exhibited superior performance in both shoot number and shoot length, as well as in overall multiplication rates.

The number of shoots and length of shoots produced in each culture vessel varied considerably in liquid medium with cotton as the support material (Figure 3.1). Among the vessels tested, the V3 glass jar with a 400 ml capacity exhibited the highest average shoot count in a liquid medium, reaching 26.66 (Table 3.9). This was followed by V7, which had an average of 24.66 shoots. In contrast, V2 and V5 showed lower shoot counts, with 9.33 and 7.66, respectively. V1, V4, and V6 fell in between, with average shoot counts of 14.33, 14, and 21, respectively. Regarding shoot length, V7 had the longest average at 3.84 cm, followed by V6 with an average of 3.54 cm. In contrast, V2 and V5 had lower shoot lengths of 1.66 cm and 1.37 cm, respectively. V1, V3, and V4 fell in between, with average shoot lengths of 2.89 cm, 2.66 cm, and 1.94 cm, respectively. Overall, V3, V6, and V7 exhibited superior performance in both shoot number and shoot length, as well as in overall multiplication rates.

These results indicate that larger culture vessels might be more beneficial for promoting plant growth *in vitro*. Further investigation into the physiological and

environmental factors behind these differences is necessary to better understand the observed variations.

3.1.2.5 Effect of different gelling agents

In our study, we looked at the effects of several gelling agents as an affordable method of micropropagation. In this study, we observed that agar is the most effective for plant growth *in vitro* conditions since it demonstrated a larger shoot length in agar treated plants and a faster rate of multiplication when compared to other gelling agents (Figure 3.5). Other gelling agents exhibit a considerable but not significant increase in plant growth when compared to the control (Table 3.10).

3.1.2.6 Effect of liquid seaweed extract

In this investigation, we examined the effects of various seaweeds as plant growth promoters. Previous reviews have indicated that seaweeds contain different types of plant growth-promoting substances that enhance plant growth. In this study, we evaluated five different seaweed species in plant growth medium by using natural extracts from *Caulerpa racemosa, Ulva lactuca, Sargassum tenerrimum, Sargassum wightii,* and *Gracilaria edulis*. The aim was to assess their role in the *in vitro* growth and development of micropropagules and their potential to replace commercial plant growth regulators (PGRs) in rose micropropagation (Figure 3.8). The *in vitro* developed shoots were cultivated on a modified MS medium supplemented with various seaweed extracts and were regularly subcultured on the same medium throughout the experiment. Growth parameters were recorded and compared with micropropagules grown in a controlled environment (i.e., MS medium with or without standard PGRs). We found a significant increase in shoot length (highest in *S. Tenerrimum i.e.* with average of 4.25 cm) (Table 3.11), shoot numbers (highest in *S. Tenerrimum i.e.* with average of 35). However, addition of LSE of *Caulerpa racemosa, Ulva lactuca, Sargassum wightii,* and *Gracilaria edulis* did not show any noteworthy effect on shoot length, shoot number compare to positive control.

3.1.2.7 Effects of polyamines

In this study, incorporating different concentrations of polyamines (PAs) into the standard rose multiplication medium produced varied responses. Adding PAs to the MS medium before autoclaving resulted in significant differences in growth

parameters, including shoot length, shoot number which were notably increase. For comparison, rose cultures at the multiplication stage grown on the standard MS medium supplemented with the recommended plant growth regulators (PGRs) were used as controls. At a low concentration of polyamines (10 mM), fewer shoots and shorter shoot lengths were observed. As PA concentrations increased to 30 mM, both shoot number and length showed a proportional increase, exceeding those of the control plantlets. However, at 50 mM, there was a decline in both shoot number and length compared to the 30 mM concentration (Table 3.13).

3.1.2.8 Effect on *in vitro* rooting

Effect of liquid medium on *in vitro* rooting

The liquid medium significantly enhanced *in vitro* rooting in Rose. Root initiation occurred within a week of inoculation, generally a day or two earlier than with solid medium (Figure 3.9). Notably, the shoots cultured in the SMLR (Solid Medium to Liquid medium Rooting) showed a 100% rooting response, as indicated in (Table 3.12). These particular roots, with an average root length of 3.37 cm achieved after a 21-day period. Moreover, the shoot length exhibited a substantial increase, reaching 3.16cm, surpassing the control group cultivated in SMSR (Solid Medium to Solid medium Rooting). The LMLR (Liquid Medium to Liquid Rooting) cultivated shoots also showed impressive performance in terms of rooting response. These shoots produced a maximum of 7.33 roots, with an average root length of 4.50 cm. Notably, these shoots reached a maximum height of 4.14 cm. Additionally, shoots that were initially multiplied in liquid medium demonstrated the capability to produce roots when later transferred to a semi-solid rooting medium. This secondary rooting response achieved an average success rate of 77.35%. However, the shoot and root lengths in this scenario were similar to those of the control group grown exclusively on solid medium.

3.2 Morphology of studies on leaf surface structure

Examining the physical characteristics of plant cultures is often advantageous, especially when refining components within the liquid environment to enhance growth. This endeavour contributes to the development of improved micropropagation methods (Hazarika, 2006). Extensive research has documented the

surface features of leaves cultivated under controlled conditions. These investigations have yielded valuable insights into leaf morphology and structure (Martins *et al*., 2015; Mani *et al*., 2021; Shekhawat *et al*., 2021). Previous studies have underscored a notable issue observed in cultured plants: the presence of non-functional stomata. Stomata, akin to tiny mouths, are minuscule openings found on plant leaves. Stomata play a vital role in regulating the exchange of gases and water vapor between plants and their surroundings. Researchers have employed advanced microscopy techniques such as Scanning Electron Microscopy (SEM) and light microscopy to scrutinize these stomata. Their findings were intriguing: a notable contrast was observed between the stomata of plants cultivated *in vitro* and those grown under natural conditions like greenhouses or fields. Typically, stomata exhibit an oval shape with guard cells slightly recessed into the leaf surface. In leaves of plants cultivated in a laboratory setting (*in vitro*), the stomata displayed distinctive features. Instead of the typical oval shape, they appeared more rounded or even crescent-shaped, with guard cells exhibiting variations from those observed in plants grown outdoors (Zhou *et al*., 2020; Domblides *et al*., 2022). Upon transitioning micropropagated plants from controlled lab conditions to natural environments, an intriguing observation emerges. These plants often encounter challenges in water regulation, struggling to maintain optimal water balance. This phenomenon appears correlated with an increase in the occurrence of irregularly-shaped stomata (Wetzstein and Sommer, 1982) and a disruption in the normal structure and function of these stomata (Hoang *et al*., 2019; Zein El Din *et al*., 2020). The absence of a protective layer called epicuticular wax on the leaf surfaces of *in vitro*-grown plants is a significant factor contributing to high water loss and less successful transplantation. This natural wax acts as a shield, playing a crucial role in minimizing water loss and enhancing the plant's ability to withstand challenging conditions (Sajeevan *et al*., 2017). In contrast to the leaves of plantlets cultivated *in vitro,* the leaves of transplanted and field-grown plants exhibit a fully developed cuticle. This indicates that the protective layer on their leaves is more mature and robust, which is an essential adaptation for plants facing the complexities of the natural environment (Manokari, *et al*., 2022).

In micropropagated plants, scientists attribute their high-water loss and transpiration rate to the improper spread of their leaf epidermal layers, which leads to

underdeveloped structures such as trichomes. These epidermal layers serve as a protective coat for the plant, making them more susceptible to drying out (Brutti *et al*., 2002). Trichomes are small appendages on the outer surface of plants, coming in various shapes and sizes, and serving multiple functions. They regulate heat loss, light absorption, and the microenvironment around the leaves, crucially controlling water loss during transpiration. Trichomes can mitigate water loss and act as storage for secondary metabolites (Uzelac *et al*., 2021). Additionally, some trichomes function as a defense mechanism, safeguarding the plant against pathogens and herbivores (Abdalla *et al*., 2021; Tarfeen *et al*., 2022).

In order to comprehend the role of trichomes in the hardening and acclimation process of *Aerva lanata* plants, a thorough investigation was conducted by Priyadharshini *et al*. (2022). This study examined various aspects of trichomes, such as their morphology, diversity, distribution, and density, comparing plants grown *in vitro* with those grown in their natural habitat.

3.2.1 Materials and methods

3.2.1.1 Scanning electron microscopy

We collected leaves from growing shoots at various phases of *in vitro* growth. and under different environmental conditions for our SEM (Scanning Electron Microscope) studies. Following that, these leaves were quickly preserved using a solution containing 2% glutaraldehyde and 2% freshly made formaldehyde. We preserved the leaves and then left them to sit at a freezing 4°C for the whole night. We used a scanning electron microscope to examine these generated samples after the preservation procedure.

Microscopy Facility, Junagadh Agriculture University, Junagadh, Department of Biotechnology. The Zeiss SEM was used to perform the Leaf scans. Plants from field-grown plants and all stages of micropropagation had their abaxial and adaxial leaf surfaces scanned at resolutions ranging from 250X to 4kX. For additional testing, the trichomes, epicuticular waxes, and stomatal characteristics were noted.

3.2.2 Results

In the SEM experiment, we observed significant differences in stomata morphology. Scanning electron microscopy was used to examine leaf surfaces, revealing notable variations in stomatal frequency, stomatal size, epicuticular wax formation, and the functional characteristics of stomata in the leaves of micro shoots grown on agar-gelled semi-solid and liquid media. Microscopic examination of Rose at various stages of micropropagation from both SM and LM media revealed the deposition of amorphous epicuticular wax on both leaf surfaces. Wax deposition was limited in leaves from both LM and SM media, beginning during the rooting stage and continuing through the hardening phase. During the hardening stage in plants from semi-solid and liquid media, epidermal cells and stomata were somewhat sunken into the waxy layer.

Further scanning electron microscopy analysis of stomatal structure and function revealed significant differences between field-grown leaves and those developed *in vitro*. Stomata on the adaxial and abaxial surfaces of field-grown plants were fully functional, with thick-walled guard cells and narrow pore openings. In contrast, stomata in *in vitro* developed leaves were highly distorted, surrounded by thin-walled guard cells, and showed a higher degree of deformity in LM leaves. Distortion levels decreased as the leaves advanced through the rooting and hardening phases (Figure 3.12 and 3.13).

3.3 Histological studies

In recent years, numerous studies have focused on the anatomical disparities of plantlets cultivated in controlled environments (*in vitro*), particularly regarding their leaf shapes and structures. These investigations have underscored a notable challenge encountered when transitioning these *in vitro*-grown plants to natural settings. Upon transplantation, they frequently undergo transplant shock due to underdeveloped inner leaf tissues, known as mesophyll, and improperly formed leaf vasculature during their growth in controlled conditions. This transplant shock presents a hurdle for these plants to adapt and thrive upon removal from the lab and exposure to more natural conditions (Mani *et al*., 2021). In comparison to plants grown in greenhouse settings, *in vitro* plants' leaves exhibit significantly higher

volumes of mesophyll air gaps and thinner, generally less developed palisade layers. Upon comparison between the leaves of *in vitro*-grown plants and those cultivated in a greenhouse, significant differences were observed (Chen *et al*., 2020). *In vitro* plants display leaves with considerably more empty space inside, known as mesophyll air gaps. Furthermore, their palisade layer, a crucial component of the leaf, is thinner and less developed compared to that of greenhouse-grown plants.

This study has also been conducted in other plants such as *Acca sellowiana* (Caetano *et al*., 2022), *Aechmea bromeliifolia* (Silva *et al*., 2020), Aloe vera (Manokari *et al*., 2021), Sweet pepper (Gao *et al*., 2022), and Potato (Chen *et al*., 2020). Furthermore, it's not only the leaves that exhibit these differences. The stems of *in vitro*-grown plantlets are also slimmer and contain less supportive tissue compared to plants grown in natural conditions or in the field.

3.3.1 Materials and methods

In order to ready the plant samples for examination, we conducted the following procedure: Initially, we delicately sliced fresh plant material into thin sections manually. Subsequently, these sections were immersed in a 1.0% solution of sodium hypochlorite (NaOCl). Sodium hypochlorite, a bleaching agent frequently employed for sanitation and cleansing purposes, was utilized in this step. Following the bleaching process, the sections underwent a comprehensive rinse with distilled water to eliminate any remaining residue. Subsequently, they were immersed in a 1.0% solution of acetic acid for a short duration, usually lasting between 1 to 5 minutes. Once more, thorough rinsing with distilled water was carried out to guarantee the complete removal of any residual acid. To enhance the clarity of the plant sections, safranin, an aqueous dye, was used for staining. Additionally, when necessary, a counter-stain known as fast green was applied. Following preparation, the sections were fixed in 30% glycerine to aid preservation for observation. These treated sections were subsequently examined under a light microscope. For more intricate observations, particularly utilizing phase contrast, images of the sections were captured using the Nikon Eclipse 50i microscope.

3.3.2 Results

The cross-sectional analyses of Rose leaves revealed morphological features characteristic of monocotyledonous leaves. In a semi-solid medium, the leaf sections were enveloped by upper and lower epidermal layers. The mesophyll, situated between these layers, exhibited a significant abundance of chloroplasts. The vascular system consisted of conjoint, collateral, and closed vascular bundles intricately embedded within the spongy mesophyll. Notably, the distinct bundle sheath surrounding the vascular bundles was clearly discernible. Leaves derived from the liquid medium displayed similar cellular arrangements in transverse sections, with the key differences being less developed vascular bundles and an increased chlorophyll content.

The root anatomical analysis of Rose revealed a disruption in the outermost layer, the epiblema, when grown in a semi-solid medium. However, the cortex showed robust development, characterized by parenchymatous tissue with significant intercellular spaces. Notably, there was no clear differentiation between the endodermis and pericycle. The vascular bundles were densely concentrated within the pith, primarily composed of parenchymatous cells.

In contrast, the liquid rooting (LR) method exhibited a superior degree of organization. The cross-sectional view revealed a circular root structure surrounded by a continuous and distinct epidermis, though the epidermal cells were slightly hypertrophied. The cortical cells retained their integrity, featuring small intercellular spaces between them. A subtle distinction between the endodermis and pericycle was observable. Additionally, a limited number of initially developed vascular bundles were dispersed throughout the stele (Figure 3.11).

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

Media	Shoot fresh weight (gm) ± SD	Shoot dry weight (gm) ± SD	Shoot no. ± SD	Shoot length (cm) ± SD
SM	3.42 ± 0.15	0.35 ± 0.02	22.33 ± 2.08	2.13 ± 0.01
LM	4.03 ± 0.13	0.39 ± 0.01	32.33 ± 2.52	4.14 ± 0.02
CV	3.750	3.950	8.449	0.504
SEM	0.081	0.008	1.333	0.009
CD 5%	0.317	0.033	5.235	0.036
CD 1%	0.525	0.055	8.682	0.059

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 temporary immersion in liquid medium on *in vitro* shoot growth and multiplication in rose (Observations were recorded after 45 days)

Media	Shoot fresh weight (gm) ± SD	Shoot dry weight (gm) ± SD	Shoot no. ± SD	Shoot length (cm) ± SD
SM	3.42 ± 0.15	0.35 ± 0.02	22.33 ± 2.08	2.13 ± 0.01
TI	4.04 ± 0.13	0.40 ± 0.01	32.33 ± 2.52	4.14 ± 0.02
CV	3.745	3.897	8.449	0.504
SEM	0.081	0.008	1.333	0.009
CD 5%	0.317	0.033	5.235	0.036
CD 1%	0.525	0.055	8.682	0.059

SM= Semi–solid medium; TI= Temporary Immersion; SD - Standard Deviation; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation

Table 3.3 Effect of CO_2 enrichment with and without sucrose on *in vitro* shoot growth and multiplication in rose, grown on semi-solid medium (Observations were recorded after 21 days)

	Sugar Concentration (%)	**CO_2 Concentration (%)**	**Shoot no.**	**Shoot length (cm)**
SFSM	**0**	0.000	0.000 i	0.000 n
		0.030	1.000 hi	0.537 l
		0.500	2.000 fgh	0.733 j
		2.000	2.000 fgh	1.193 f
		GR	1.333 ghi	1.040 h
SCSM	**1**	0.000	1.333 ghi	0.427 m
		0.030	1.667 gh	0.547 l
		0.500	1.667 gh	0.747 j
		2.000	2.333 fgh	1.143 g
		GR	2.000 fgh	1.043 h
	2	0.000	2.000 fgh	0.523 l
		0.030	2.667 fg	0.612 k
		0.500	3.333 ef	0.823 i
		2.000	4.333 de	1.925 d
		GR	5.000 cd	1.252 e
	3	0.000	6.000 bc	2.903 c
		0.030	8.000 a	3.106 b
		0.500	8.000 a	3.140 a
		2.000	7.000 ab	3.109 b
		GR	6.000 bc	3.108 b
		CV	23.522	1.224
		SEM	0.459	0.010
		CD 5%	1.313	0.028
		CD 1%	1.757	0.038

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 3.4 Effect of CO_2 enrichment with and without sucrose on *in vitro* shoot growth and multiplication in rose, grown on liquid medium (Observations were recorded after 21 days)

	Sugar Concentration (%)	**CO_2 Concentration (%)**	**Shoot no.**	**Shoot length (cm)**
SFLM	**0**	0.000	0.000 k	0.000 j
		0.030	1.000 jk	0.567 i
		0.500	2.000 hij	0.767 hi
		2.000	2.000 hij	1.100 g
		GR	1.333 ijk	0.900 gh
SCLM	**1**	0.000	2.333 ghij	0.600 i
		0.030	2.667 ghi	0.700 hi
		0.500	2.667 ghi	0.800 hi
		2.000	3.333 fgh	1.100 g
		GR	3.000 fgh	0.933 gh
	2	0.000	3.000 fgh	1.800 f
		0.030	3.667 fg	2.167 e
		0.500	4.333 ef	2.633 d
		2.000	5.333 de	3.167 c
		GR	6.000 cd	2.600 d
	3	0.000	7.000 bc	4.700 a
		0.030	9.000 a	3.900 b
		0.500	9.000 a	3.967 b
		2.000	8.000 ab	3.800 b
		GR	7.000 bc	3.767 b
		CV	19.254	8.121
		SEM	0.459	0.094
		CD 5%	1.313	0.268
		CD 1%	1.757	0.358

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 3.5 Effect of different vessel's type on *in vitro* shoot growth and multiplication in rose grown on semi-solid medium (Observations were recorded after 45 days)

Vessel's type	Shoot no.	Shoot length (cm)
V1	19.000 d	3.000 c
V2	11.000 f	1.600 f
V3	31.000 a	2.533 d
V4	16.000 e	2.033 e
V5	19.000 d	1.500 f
V6	23.000 c	3.567 b
V7	27.000 b	4.000 a
CV	4.795	4.813
SEM	0.577	0.072
CD 5%	1.751	0.220
CD 1%	2.431	0.305

(V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml. 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 different vessel's type with use of glass marble as support material on *in vitro* shoot growth and multiplication in rose grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Shoot no.	Shoot length (cm)
V1	21.000 d	3.200 c
V2	13.000 f	1.800 f
V3	33.000 a	2.733 d
V4	18.000 e	2.233 e
V5	21.000 d	1.700 f
V6	25.000 c	3.767 b
V7	29.000 b	4.200 a
CV	4.375	4.469
SEM	0.577	0.072
CD 5%	1.751	0.220
CD 1%	2.431	0.305

(V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml. 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 different vessel's type with use of water balls as support material on *in vitro* shoot growth and multiplication in rose grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Shoot no.	Shoot length (cm)
V1	17.333 c	3.047 c
V2	12.333 d	1.843 f
V3	29.667 a	2.843 d
V4	17.000 c	2.123 e
V5	10.667 d	1.550 g
V6	24.000 b	3.727 b
V7	27.667 a	4.027 a
CV	10.566	0.884
SEM	1.208	0.014
CD 5%	3.665	0.042
CD 1%	5.087	0.059

(V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml. 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 different vessel's type with use of tissue paper as support material on *in vitro* shoot growth and multiplication in rose grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Shoot no.	Shoot length (cm)
V1	16.333 c	2.997 c
V2	11.333 d	1.793 f
V3	28.667 a	2.793 d
V4	16.000 c	2.073 e
V5	9.667 d	1.500 g
V6	23.000 b	3.677 b
V7	26.667 a	3.977 a
CV	11.128	0.901
SEM	1.208	0.014
CD 5%	3.665	0.042
CD 1%	5.087	0.059

(V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml. 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 different vessel's type with use of cotton as support material on *in vitro* shoot growth and multiplication in rose grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Shoot no.	Shoot length (cm)
V1	14.333 c	2.867 c
V2	9.333 d	1.663 f
V3	26.667 a	2.663 d
V4	14.000 c	1.943 e
V5	7.667 d	1.370 g
V6	21.000 b	3.547 b
V7	24.667 a	3.847 a
CV	12.452	0.946
SEM	1.208	0.014
CD 5%	3.665	0.042
CD 1%	5.087	0.059

(V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml. 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 Effect of different gelling agents on *in vitro* shoot growth and multiplication in rose, grown on medium (Observations were recorded after 45 days)

Media	Shoot no.	Shoot length (cm)
Agar	8.667 a	3.397 a
Phyta gel	6.667 ab	3.293 b
Guar gum	8.000 ab	3.177 c
Isobgol	6.000 bc	2.810 d
Starch	4.000 c	2.807 d
CV	18.574	1.176
SEM	0.715	0.021
CD 5%	2.253	0.066
CD 1%	3.204	0.094

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 3.11 Effect of LSE on *in vitro* shoot growth and multiplication in rose, grown on semi-solid medium (Observations were recorded after 45 days)

Liquid Seaweed Extract (LSE)	**Concentration of LSE (% v/v)**	**Shoot no.**	**Shoot length (cm)**
Caulerpa racemosa	10	25.000 cdef	3.250 m
	30	27.000 bcd	3.340 j
	50	24.000 def	3.290 l
Ulva lactuca	10	22.000 f	3.260 m
	30	28.000 bc	3.310 k
	50	23.000 ef	3.290 l
Sargassum tenerrimum	10	22.000 f	3.610 e
	30	25.000 cdef	3.590 f
	50	35.000 a	4.250 a
Sargassum wightii	10	23.000 ef	3.720 d
	30	28.000 bc	4.090 b
	50	27.000 bcd	3.810 c
Gracilaria edulis	10	29.000 b	3.220 n
	30	34.000 a	3.460 h
	50	26.000 bcde	3.360 i
MS medium with standard PGR		32.667 a	3.530 g
MS medium without standard PGR		15.000 g	2.920 o
CV		6.564	0.287
SEM		0.993	0.006
CD 5%		2.855	0.017

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 3.12 Effect of liquid medium on *in vitro* rooting in rose (Observations were recorded after 21 days)

Media	Root no. ± SD	Root length (cm) ± SD	Shoot length (cm) ± SD	Rooting response (%)
SMSR	2.67 ± 1.53	2.73 ± 0.35	2.13 ± 0.01	61.66
SMLR	4.33 ± 2.08	3.37 ± 0.15	3.16 ± 0.02	100
LMLR	7.33 ± 0.58	4.50 ± 0.20	4.14 ± 0.02	100
LMSR	5.67 ± 0.58	4.00 ± 0.10	4.06 ± 0.02	77.35
CV	27.080	6.075	0.499	
SEM	0.782	0.128	0.010	
CD 5%	2.549	0.417	0.032	
CD 1%	3.710	0.607	0.046	

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

Table 3.13 Effect of different polyamines concentration on *in vitro* growth of rose micropropagation (Observations were recorded after 21 days)

Polyamines	Polyamines concentration (mM)	No. of shoots (mean)	Length of shoots (cm)
Control	0	8.667 a	3.407 a
Spermidine	10	13.667 b	3.453 bc
	30	18.000 c	3.573 d
	50	12.667 b	3.493 c
Putrescine	10	11.667 b	3.463 bc
	30	18.333 c	3.547 d
	50	13.000 b	3.433 ab
Cadaverine	10	11.333 b	3.463 bc
	30	17.333 c	3.493 c
	50	12.667 b	3.397 a

Means in the same columns followed by different letters are significantly different (P≤0.05) according to Duncan's Multiple Range Test

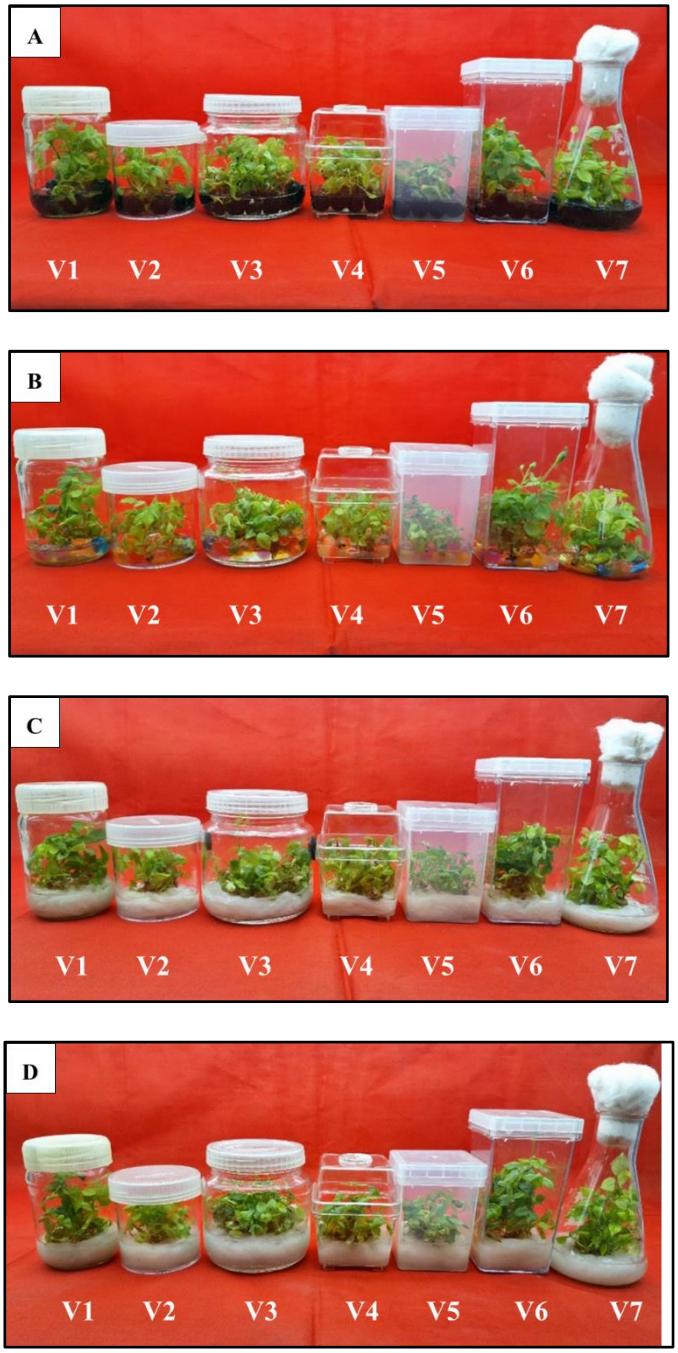

Figure 3.1 Effect of different culture vessels on *in vitro* growth of Rose on liquid medium was studied. The culture vessels included: (V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml

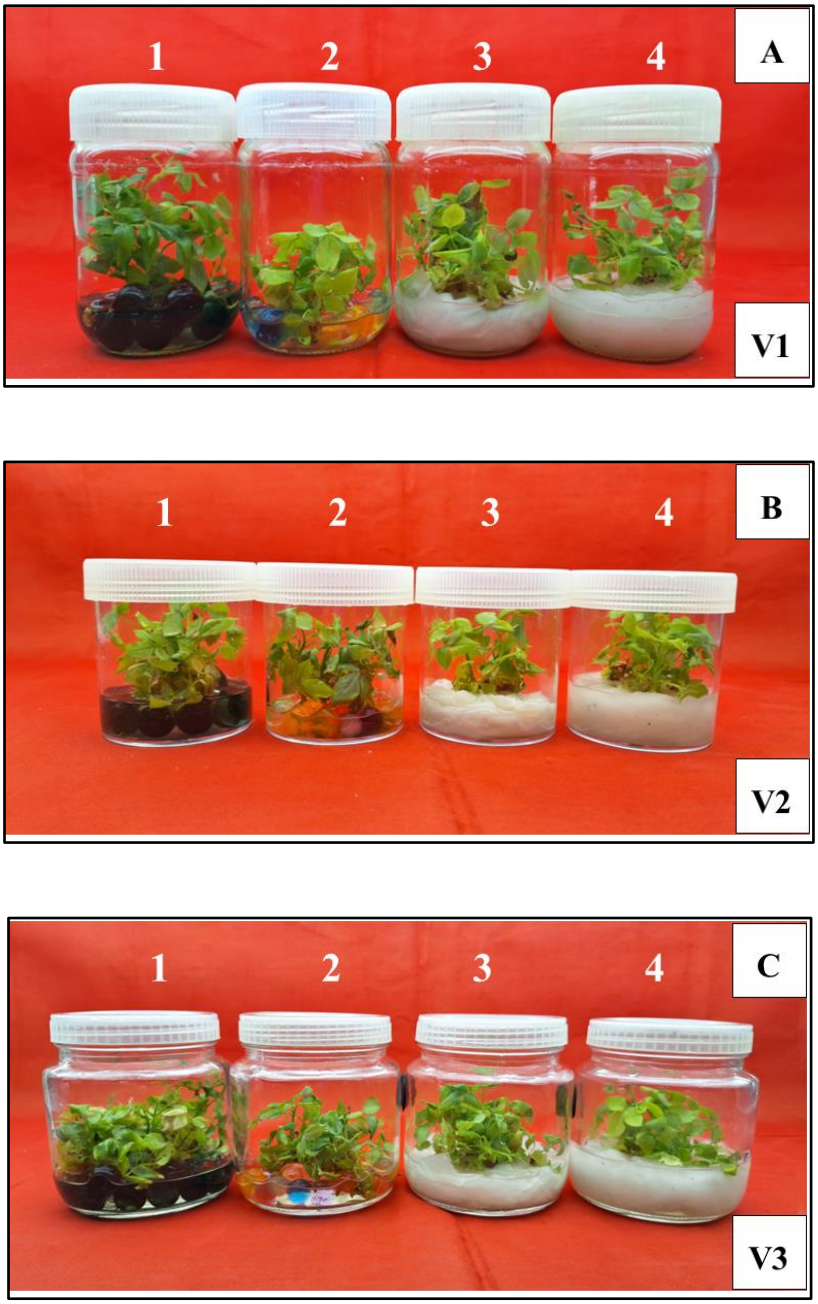

Figure 3.2(a) Effect of various supporting materials on *in vitro* growth of Rose on liquid medium was studied (1) Glass marbles, (2) Water balls, (3) Tissue paper and (4) Cotton. The culture vessels included: (V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml

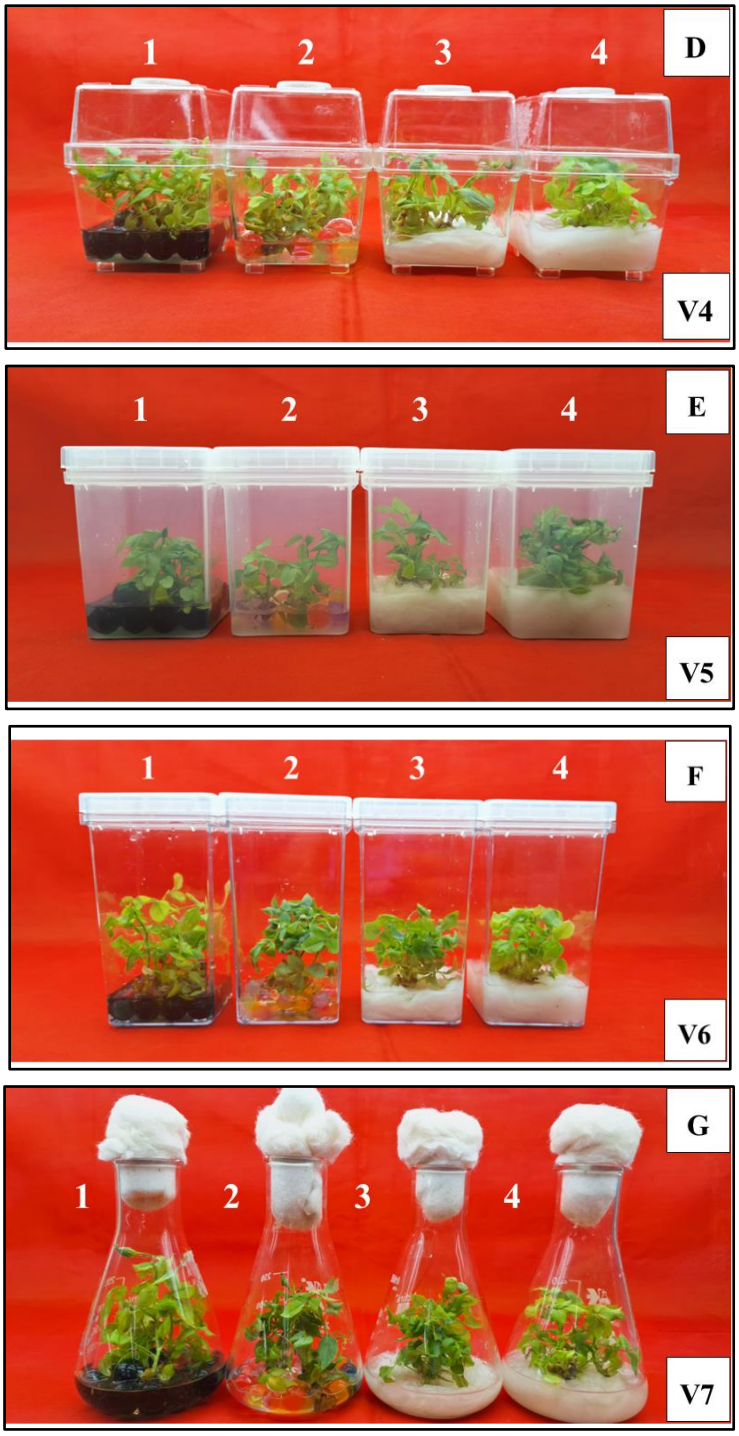

Figure 3.2(b) Effect of various supporting materials on *in vitro* growth of Rose on liquid medium was studied (1) Glass marbles, (2) Water balls, (3) Tissue paper and (4) Cotton. The culture vessels included: (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml

Figure 3.3 Effect of different culture vessels on *in vitro* growth of Rose on semi-solid medium was studied. The culture vessels included: (V1) Regular glass jar with a capacity of 200 ml, (V2) Phyta jar with a clear round container and a capacity of 150 ml, (V3) Glass jar with a capacity of 400 ml, (V4) Phyta jar with vented lids and a capacity of 150 ml, (V5) Phyta jar with a translucent square container and a capacity of 200 ml, (V6) Phyta jar with a clear square container and a capacity of 400 ml, and (V7) Conical flasks with a narrow mouth and a capacity of 250 ml

Figure 3.4 Temporary immersion system designed in plant biotechnology laboratory to study *in vitro* growth of Rose

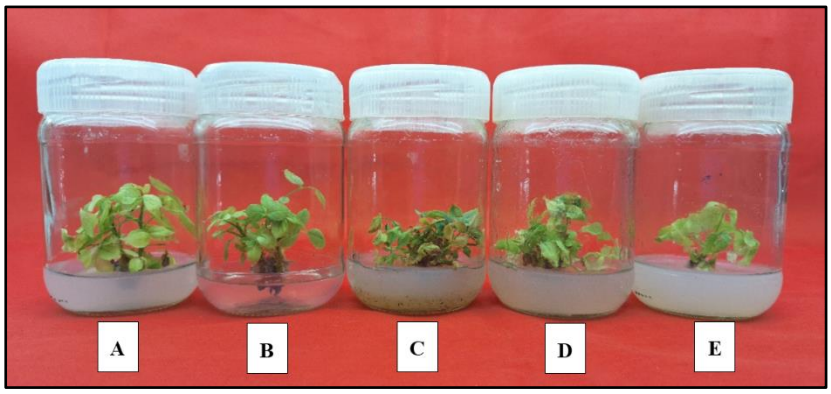

Figure 3.5 Effect of different gelling agents on *in vitro* plant growth during the multiplication stage of Rose was studied. The gelling agents included: (A) Agar, (B) Phyta gel, (C) Isabgol + agar, (D) Guar gum + agar, and (E) Starch + agar

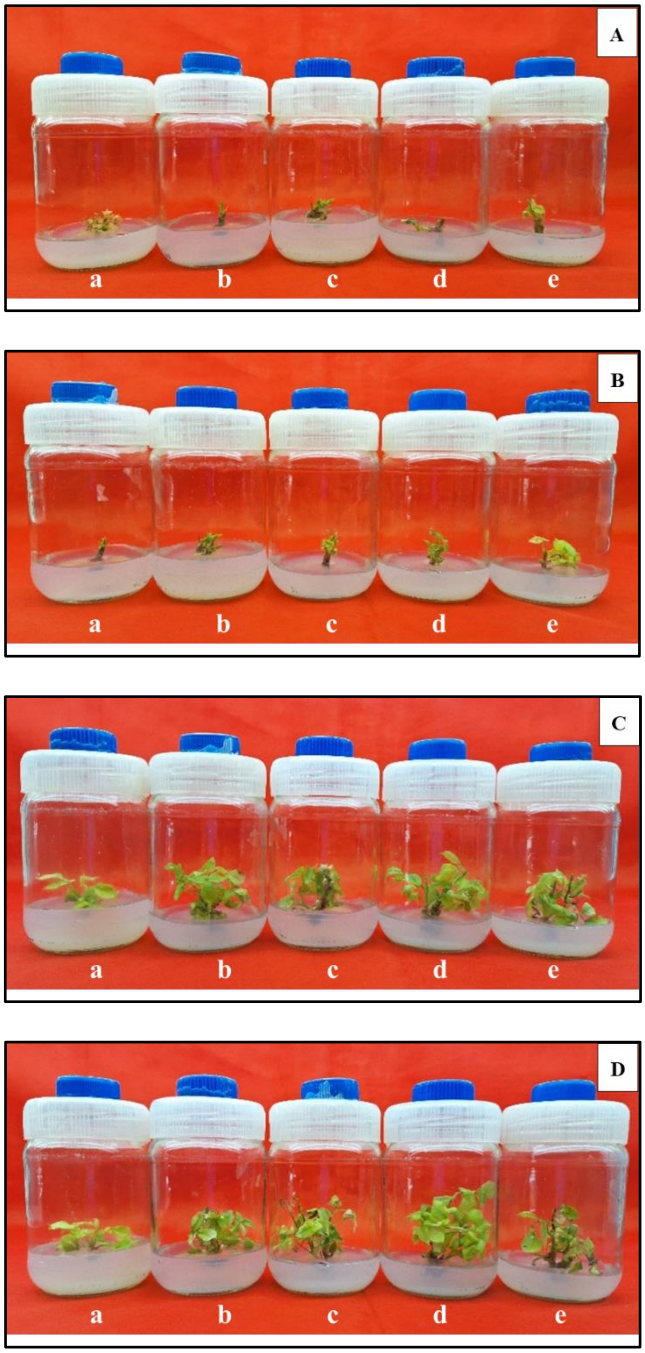

Figure 3.6 Effect of CO_2 enrichment on shoot multiplication of Rose plant grown on sucrose free semi-solid medium (SFSM) and sucrose containing semi-solid medium (SCSM). (A) SFSM with 0% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR; (B) SCSM with 1% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR; (C) SCSM with 2% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR; (D) SCSM with 3% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR

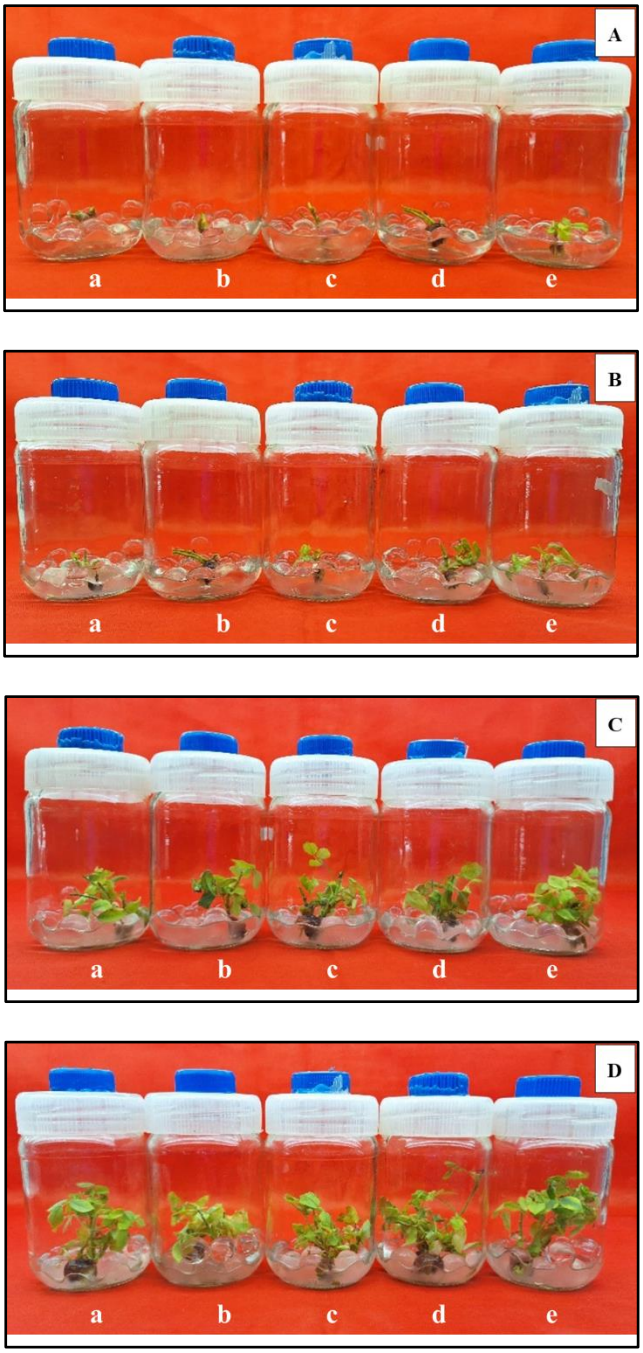

Figure 3.7 Effect of CO_2 enrichment on shoot multiplication of Rose plant grown on sucrose free liquid medium (SFLM) and sucrose containing liquid medium (SCLM). (A) SFLM with 0% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR; (B) SCLM with 1% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR; (C) SCLM with 2% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR; (D) SCLM with 3% sucrose- (a) 0% CO_2, (b) 0.03% CO_2, (c) 0.5% CO_2, (d) 2% CO_2 and (e) GR

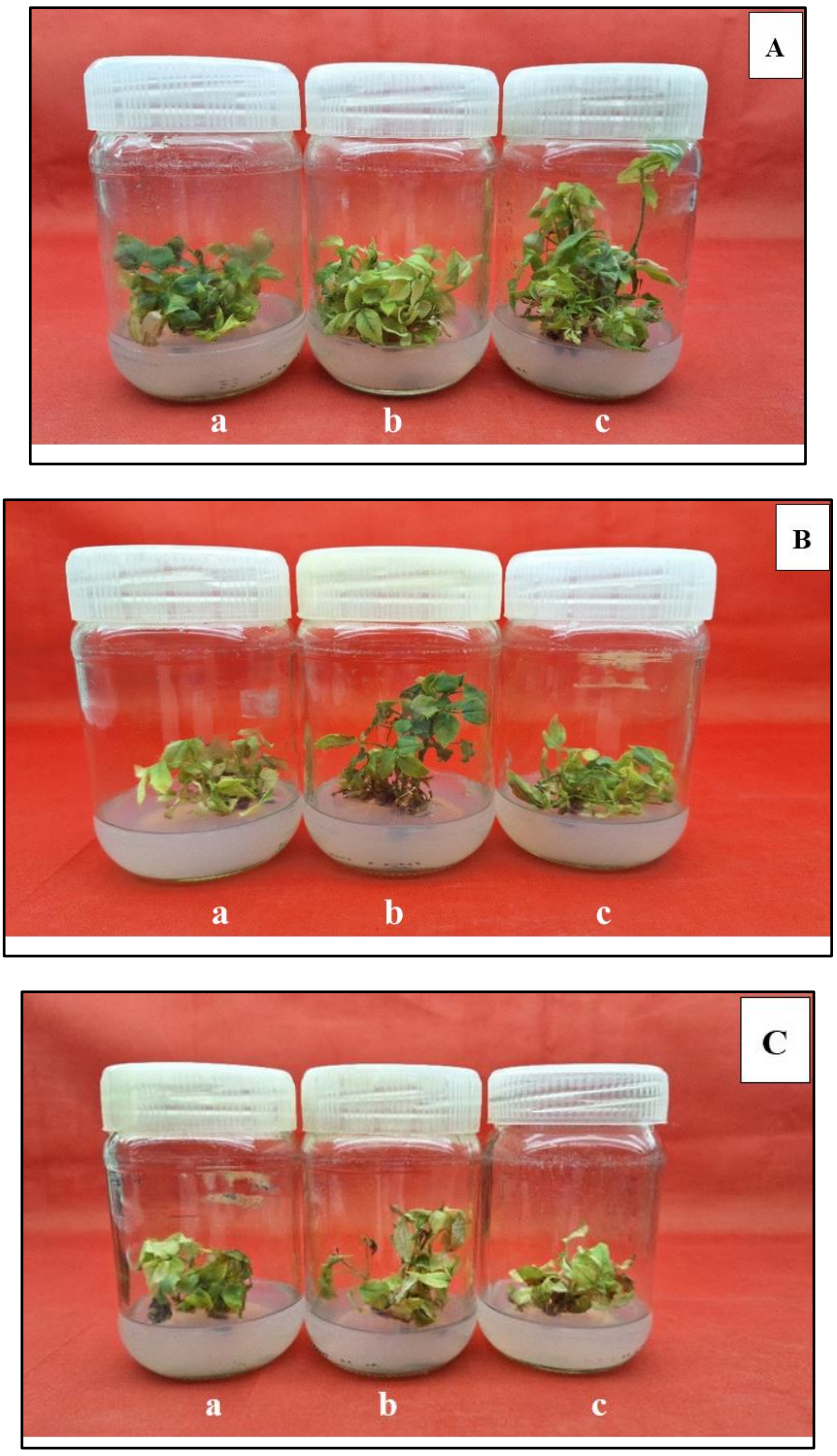

Figure 3.8(a) Effect of different Liquid Seaweed Extract (LSE) with different concentration were used on *in vitro* growth at multiplication stage of Rose. LSE used- (A) *Sargassum tenerrimum* (a) 10%, (b) 30% and (c) 50%; (B) *Sargassum wightii* (a) 10%, (b) 30% and (c) 50%; (C) *Gracilaria edulis* (a) 10%, (b) 30% and (c) 50%

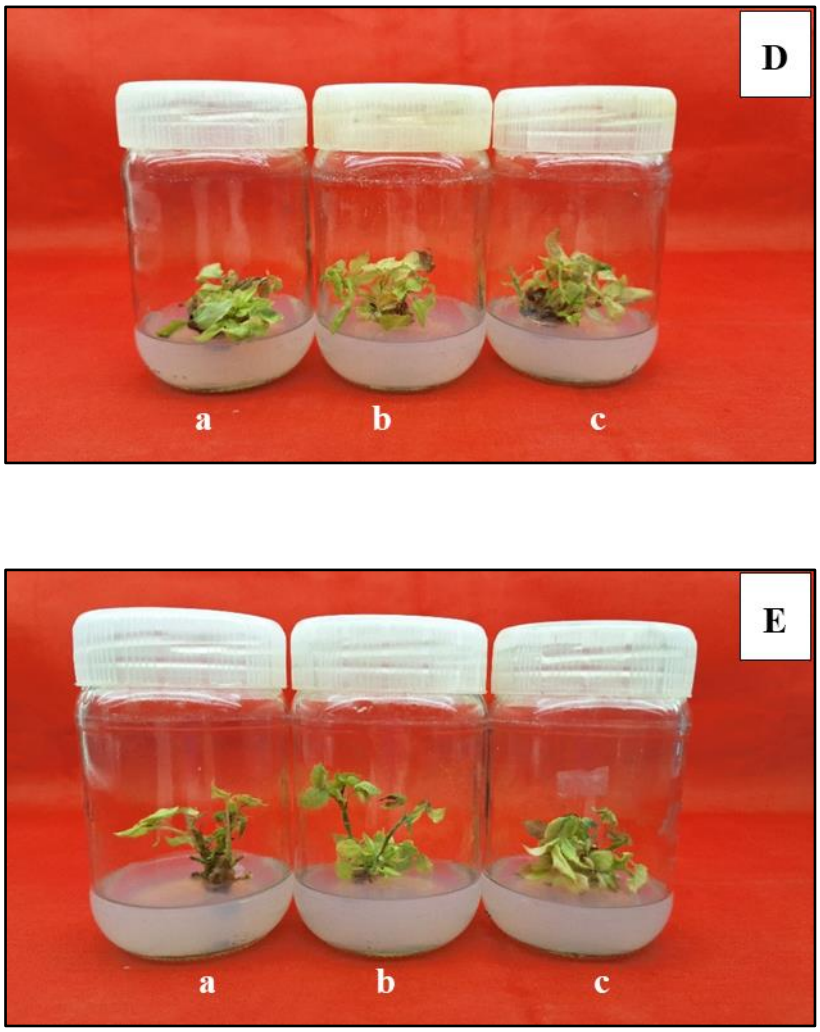

Figure 3.8(b) Effect of different Liquid Seaweed Extract (LSE) with different concentration were used on *in vitro* growth at multiplication stage of Rose. LSE used- (D) *Caulerpa racemose* (a) 10%, (b) 30% and (c) 50%; (E) *Ulva lactuca* (a) 10%, (b) 30% and (c) 50%

Figure 3.9 Effect of semi-solid and liquid medium on *in vitro* rooting of Rose. (A) Rooting in semi-solid medium and (B) Rooting in liquid medium

Figure 3.10 Acclimatization of *in vitro* grown Rose plant

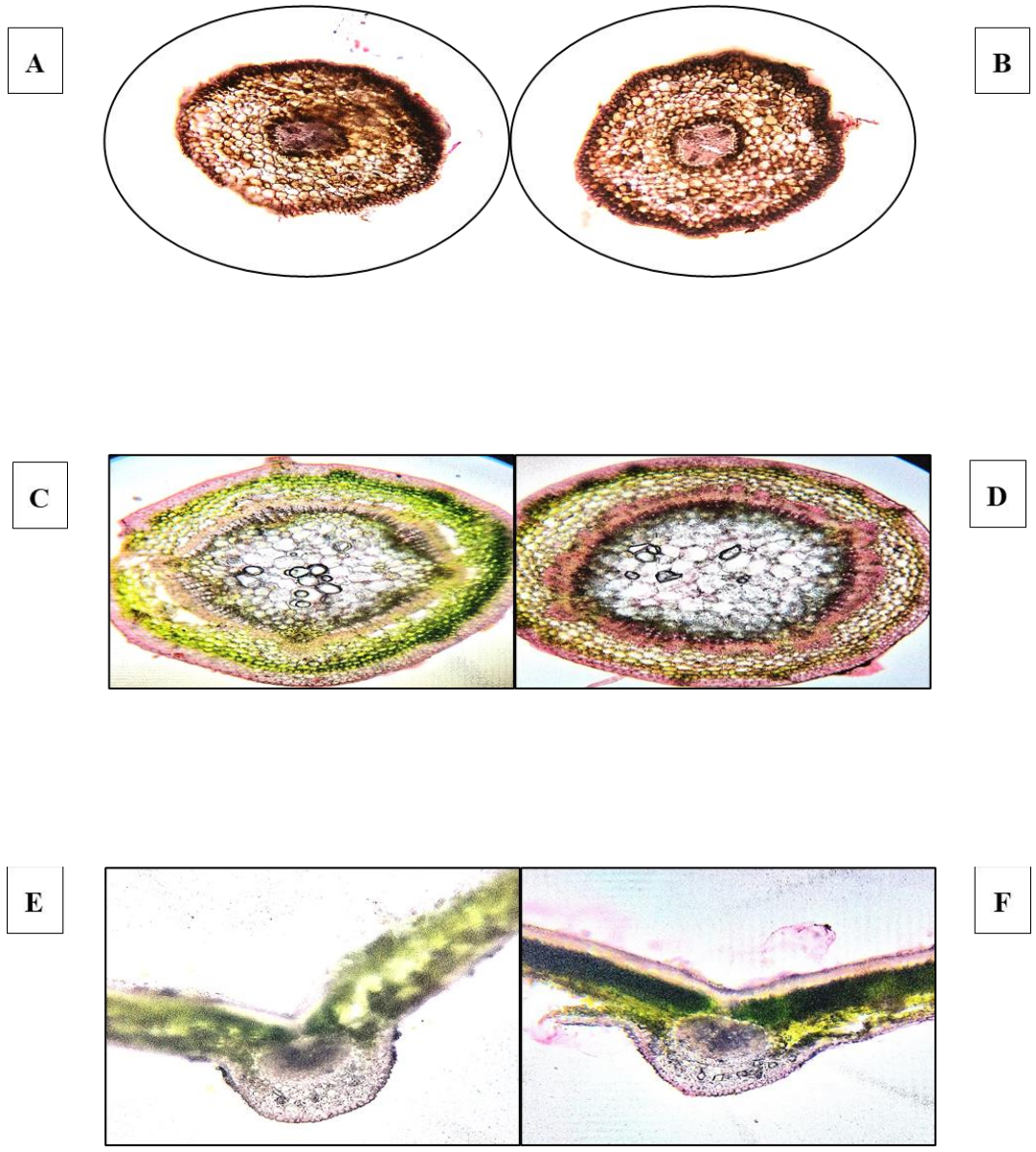

Figure 3.11 Transverse section through shoot, root and leaf of Rose during *in vitro* growth (multiplication stages) on semi-solid and liquid medium: (A) root TS grown on semi-solid medium; (B) root TS grown on liquid medium; (C) shoot TS grown on semi-solid medium; (D) shoot TS grown on liquid medium; (E) leaf TS grown on semi-solid medium; and (F) leaf TS grown on liquid medium

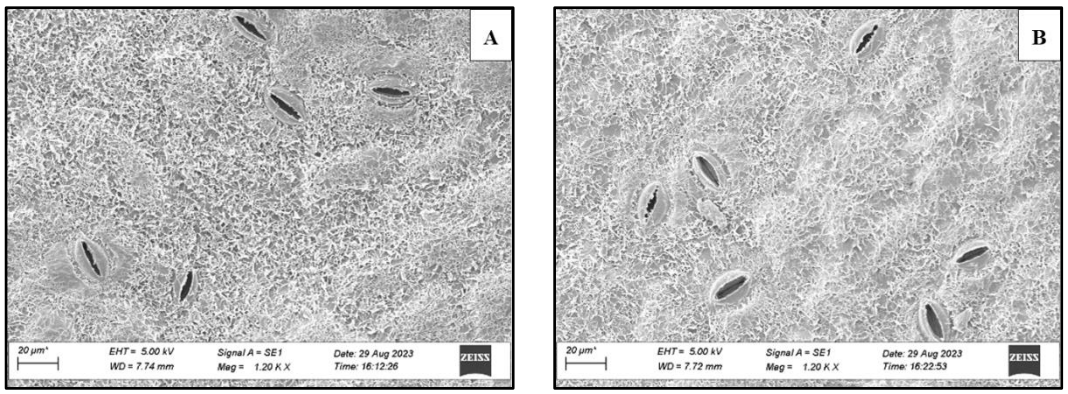

Figure 3.12 SEM analysis with magnification of 1.2 K X of leaf adaxial surface of *in vitro* gown Rose plant in (A) semi-solid medium and (B) liquid medium

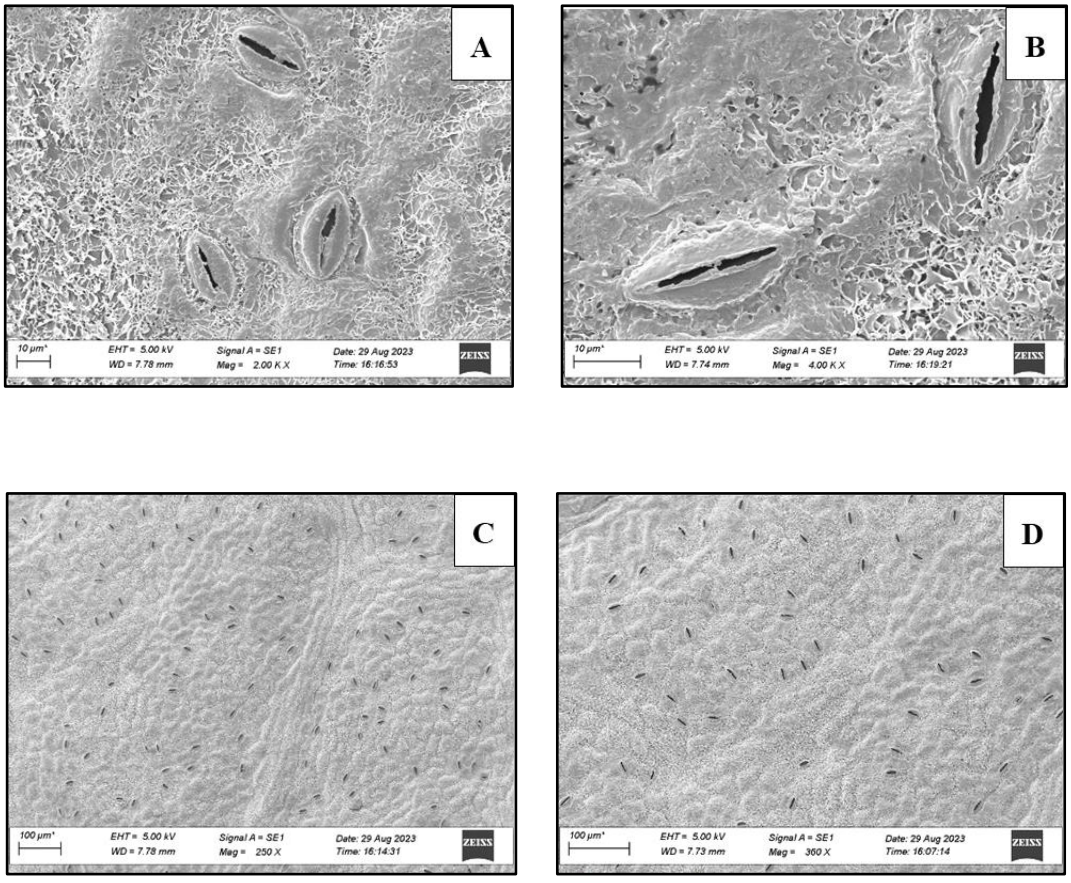

Figure 3.13 SEM analysis with magnification of leaf adaxial surface of *in vitro* gown Rose plant (A) *in vitro* grown plant in 2.0 K X, (B) *in vitro* grown CO_2 enrichment plant in 4.0 K X, (C) field grown plant in 250 X and (D) field grown plant in 360 X