Chapter 5

Micropropagation in Altered Growth Condition-Biochemical Studies

5.1 Studies on different biochemical parameters

In a liquid culture system, plants may encounter stress signals that induce atypical growth patterns, rendering them less conducive to transplantation. When plant tissues are immersed in such an environment, they undergo osmotic and oxidative stresses, resulting in elevated levels of antioxidant enzymes and other chemical compounds. These alterations in the plant's stress response mechanisms can impact their general well-being and readiness for transplantation (Fujii *et al*., 2011). To combat environmental stresses, plants activate diverse biochemical processes. For instance, they accumulate compatible solutes and trigger detoxification enzymes to alleviate the adverse impacts of these stressors (Hasanuzzaman *et al*., 2012). Therefore, a comprehensive understanding of these factors across various growth stages and in different culture systems is crucial. Such knowledge is fundamental for devising strategies to enhance a plant's adaptive capacity during the transition from *in vitro* to *ex vitro* environments (Pandey *et al*., 2019). In recent years, there has been a surge in interest in exploring these transformations and adaptations. Researchers are increasingly dedicated to unravelling how plants respond to the challenges posed by different culture systems, particularly concerning the shift from controlled *in vitro* conditions to the unpredictable *ex vitro* environment (Carvalho and Amâncio, 2019). To gain a deeper understanding of the regeneration process, researchers have shifted their focus to variations in antioxidant enzymes. Specifically, these variations have been extensively explored in the context of shoot organogenesis (Saha and Dutta Gupta, 2018; Chen *et al*., 2020; Zayova *et al*., 2020), somatic embryogenesis of oil palm (Cui *et al*., 1999), and *ex vitro* acclimation of regenerated plants (Kshirsagar *et al*., 2021). Numerous studies have documented changes in the concentration of metabolites and enzyme activities during *in vitro* growth. These investigations have yielded valuable insights into the dynamic biochemical processes underlying plant

development in controlled laboratory conditions (Mendoza *et al*., 2018; Khan *et al*., 2019; Nazir *et al*., 2019).

Carbohydrate biosynthesis and metabolism are critical for plant growth during *in vitro* propagation, as they directly influence the energy and building blocks necessary for cell division, elongation, and overall development. A thorough examination of carbohydrate-related processes is crucial for understanding and enhancing plant growth in controlled *in vitro* environments, where nutrient availability can vary significantly from natural conditions (De Moraes *et al*., 2016; Wu *et al*., 2019). Various carbohydrates, including soluble sugars, starch, glucomannans, and fructans, have been identified in bulbs of lachenalia (Bach *et al*., 2015) and lily bulbs (Wu *et al*., 2021). Carbohydrate accumulation is markedly influenced by both the inorganic and organic components of the culture medium, playing a substantial role in regulating plant cell growth due to its osmotic properties. Changes in carbohydrate levels can induce osmotic stress, profoundly impacting various aspects of plant tissue culture, including callus growth, colony formation, shoot regeneration, somatic embryogenesis, and adjustments in ion transport (such as ion extrusion or uptake). These metabolic alterations also influence carbon metabolism, eliciting diverse responses in plants to osmotic stress at molecular, cellular, and whole-plant levels (Llanes *et al*., 2018). Previous studies have explored carbohydrate content and concentration in various bulb tissues under different environmental conditions and growth stages, offering valuable insights into the dynamic regulation of carbohydrates in bulbs during development and in response to environmental changes (Chaves *et al*., 2020; Cioć *et al*., 2021). It is important to note the scarcity of information regarding carbohydrate metabolism in geophytes, particularly during *in vitro* cultivation (Wu *et al*., 2016).

Oxygen is vital for the survival of aerobic organisms, including plants. However, under stressful conditions, plants often experience an increase in toxic reactive oxygen species (ROS), such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide. The cumulative damage caused by these ROS is termed oxidative stress, which is among the most harmful factors affecting plants (Hasanuzzaman *et al*., 2020). To counteract the detrimental effects of ROS, plants have evolved various antioxidant defense mechanisms. One of the key components of

these defense is peroxidase (POD) and superoxide dismutase, an essential enzyme that protects plants from oxidative damage. Peroxidases may can contain a heme cofactor in their active sites, or redox-active cysteine or selenocysteine residues. Changes in peroxidase activity have been associated with wide array of physiological processes involved with auxin function and cell wall synthesis. The association with auxin and lignification made peroxidase analysis informative in response to external stimuli such as light, temperature, irritation and wounding, parasites and pathogens and variation in ion status. Peroxidases are heme containing proteins that utilize H_2O_2 in the oxidation of various organic and inorganic substrates. Peroxidases utilizing guaiacol as electron donor *in vitro* are guaiacol peroxidases and participate in developmental processes, lignification, ethylene biosynthesis, defense, wound healing, etc. Peroxidase which participates in lignin biosynthesis might built up a physical barrier against biotic and abiotic stress. (Bhaduri and Fulekar, 2012). Superoxide dismutases (SODs) are essential enzymes that protect plants from oxidative stress by converting harmful superoxide radicals into less toxic molecules (García-Caparrós *et al*., 2021). Measuring SOD activity is a useful indicator for assessing a plant's micronutrient status.

Total phenol production is integral to plant biology and physiology, exerting significant influence on various facets of plant growth, development, and environmental interactions. Phenols, encompassing flavonoids and phenolic acids, serve as essential constituents of a plant's antioxidant defense system. They effectively scavenge reactive oxygen species (ROS), safeguarding plant cells from oxidative stress (Tuladhar *et al*. 2021). Additionally, phenols play a vital role in the plant's defense against microbial pathogens. Upon pathogen invasion, plants frequently elevate the production of phenolic compounds, which hinder pathogen growth and serve as signalling molecules to activate defense responses. Phenolic compounds serve as secondary messengers in diverse signal transduction pathways, modulating gene expression and regulating numerous physiological processes, including the activation of stress-responsive genes and the coordination of plant defense mechanisms. When plants are subjected to abiotic stresses such as drought, salinity, or extreme temperatures, alterations in phenol production are often observed. Phenolic compounds aid plants in adapting to adverse environmental conditions by

regulating osmotic balance, reducing water loss, and preserving cellular integrity. In the context of our experiment on plant tissue culture, monitoring total phenol production can serve as a valuable indicator of the physiological status of cultured plant tissues. It provides insights into the stress response, growth potential, and overall health of the cultured plants under different conditions (Mushtaq and Fauconnier, 2024).

Chlorophyll plays a pivotal role in photosynthesis, the fundamental process through which plants, algae, and certain bacteria convert light energy into chemical energy, specifically glucose (sugar). The production of chlorophyll *in vitro*, or within plants cultivated in controlled laboratory environments, can be influenced by several factors. Maintaining optimal chlorophyll levels is crucial for the photosynthetic capacity and overall health of the plants. The decrease in chlorophyll content observed in *in vitro* conditions can be attributed to various factors, including chloroplast malformation resulting from limited gaseous exchange and heightened relative humidity within the culture vessels (Martins *et al*., 2015). Ventilation of the culture vessels would facilitate improved gaseous exchange, promoting the healthy growth of plantlets.

Proteins, as essential biomolecules, are central to the complex process of plant growth during *in vitro* propagation. These macromolecules are crucial as they form the structural basis of cells and participate in numerous biochemical pathways. A thorough investigation into protein-related processes is indispensable for comprehensively understanding and improving plant growth in controlled *in vitro* conditions, where nutrient availability may significantly vary from natural environments (Zhao *et al*., 2019). In plant biology, a diverse array of proteins plays critical roles in regulating growth, development, and responses to environmental stimuli. Proteins engaged in signal transduction, transcriptional regulation, and enzymatic activities constitute vital components of the molecular apparatus governing plant growth. For example, transcription factors like MYB and bHLH proteins have been demonstrated to regulate gene expression associated with growth and differentiation in plant tissue cultures (Liu *et al*., 2013). Moreover, enzymes such as kinases and phosphatases perform crucial functions in signal transduction pathways, shaping cellular responses to external stimuli (Wang *et al*., 2019). Moreover, the

proteomic makeup of plant tissues across various environmental conditions and growth stages continues to be a subject of ongoing research. Prior studies have scrutinized the protein profiles of diverse plant tissues to glean insights into the dynamic regulation of proteins during growth and in reaction to shifting environmental factors (Manivannan *et al*., 2015; Chauhan *et al*., 2018). However, there exists a significant gap in our comprehension of how protein metabolism is regulated in geophytes, particularly when cultivated under differing growth conditions in *in vitro* systems.

5.1.1 Materials and methods

The process of micropropagation undergoes significant transformations across diverse growth environments and in mature plants in their natural habitats. These transformations involve substantial modifications in the biochemical composition, including carbohydrates, proteins, phenol, chlorophyll, and the antioxidant enzyme peroxidase (POD) and superoxide dismutase (SOD). The study of these changes is crucial for understanding plant growth and development.

From healthy leaves obtained from propagating shoots, rooted, and mature plantlets, as well as fresh twigs from field-grown plants carefully transported to the laboratory under cool conditions, a meticulous process was followed. The leaves were meticulously cleaned to eliminate any debris or residual medium, and excess moisture was eliminated by gentle pressing with tissue paper. Subsequently, the leaves were finely fragmented using an appropriate buffer or solvent, followed by centrifugation. The resulting supernatant, the liquid fraction post-centrifugation, was utilized as the plant extract for further analysis. Utilizing a spectrophotometer, specifically the Shimadzu UV1800, absorption readings of the mixtures were taken to quantify various reactions. Qualitative assessments were conducted at specific wavelengths, with reference to established standard curves aiding in the interpretation of the results. These standard curves provided a benchmark for identifying the properties of the substances under investigation.

For total carbohydrate content estimation, leaves from various samples were ground using a mortar and pestle in a 0.2 M phosphate buffer at pH 6.2. The homogenate was then centrifuged at 3000 rpm for 15 minutes. Subsequently, 1 mL of

the supernatant was mixed with 4.0 mL of 0.2% anthrone reagent (prepared in concentrated H_2SO_4) and incubated in a water bath for 5 minutes, as described by Tandon (1976). The absorbance of the solution was then measured spectrophotometrically at 610 nm.

The total phenol content estimation was determined using the method outlined by Mahadevan (1982), utilizing Folin Ciocalteu's reagent. To do this, 500 mg of leaves, cultivated on medium, were weighed and ground with a mortar and pestle in 70% methanol. The resulting extract was centrifuged at 10,000 rpm for 15 minutes, and the clear supernatant was used for the quantitative analysis of total phenol content. For each reaction, 500 µl of the methanolic extract was placed in a test tube, to which 1.0 ml of Folin Ciocalteu's reagent (diluted 1:1 with distilled deionized water) was added, followed by 2.0 ml of a 20% (w/v) Na_2CO_3 solution. The test tubes were heated in a boiling water bath with intermittent shaking for approximately one minute. They were then cooled under running tap water. The resulting blue-coloured solution was diluted to 25 ml with distilled deionized water (DDW), and the percent transmittance was measured at 650 nm using a UV-Vis spectrophotometer (Shimadzu, Japan). The total phenol concentration in each sample was determined using a standard curve prepared from various concentrations (10-100 µg) of caffeic acid.

The total protein content was quantitatively estimated using Bradford's method (1976). 1 ml of the appropriately diluted crude tissue extract (the supernatant) was combined with 5.0 ml of Coomassie Brilliant Blue G-250 dye (Bradford reagent). The transmittance of the resulting-coloured complex was measured at 595 nm using a UV-Vis spectrophotometer (Shimadzu, Japan). The protein concentration was determined using a standard curve prepared from various concentrations of albumin.

For antioxidant enzyme, peroxidase (POD) activity was assessed by monitoring the absorbance change at 470 nm, employing guaiacol and H_2O_2 as substrates, following the method drawn by Thomas *et al*. in 1981. The substrate solution consisted of 0.5% (v/v) guaiacol in 0.1M K_2HPO_4 (pH 6.0), which was stirred for 30 minutes. Prior to use, 0.008% (v/v) 30% H_2O_2 was added. A cuvette containing 2.5 mL of substrate received 50 µL of the enzyme solution, and absorbance was recorded.

Superoxide dismutase (SOD) activity was assessed using a modified NBT method (Alam *et al*., 2021). In a 2 mL reaction mixture, 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55 µM NBT, and 0.025% Triton-x100 was prepared. Then, 40 µL of diluted sample and 20 µL of 1 mM riboflavin were added. The reaction was initiated by illuminating the samples under a 15 W fluorescent tube for 10 minutes. Duplicate tubes without light served as blanks. After the reaction, absorbance was measured at 560 nm, and enzyme activity was calculated using a standard curve derived from pure SOD.

Chlorophyll content was evaluated as a biochemical parameter during the multiplication phase in both liquid and semi-solid media across three plant species, and these results were compared to those of field-grown plants. Leaves were ground in chilled 80% acetone (Arnon, 1949), and the resulting homogenate was filtered through filter paper. The absorbance of the filtrate was measured at wavelengths of 663, 654, and 645 nm. Chlorophyll content was then calculated using the appropriate formulas:

$$\text{Total Cholophyll (mg/g)} = \frac{20.2 \times \text{A645} + 8.02 \times \text{A663}}{\text{a} \times 1000 \times \text{w}} \times \text{V}$$

$$\text{Cholophyll A (mg/g)} = \frac{12.7 \times \text{A663} - 2.69 \times \text{A645}}{\text{a} \times 1000 \times \text{w}} \times \text{V}$$

$$\text{Cholophyll B (mg/g)} = \frac{22.9 \times \text{A645} - 4.68 \times \text{A663}}{\text{a} \times 1000 \times \text{w}} \times \text{V}$$

5.1.2 Results

In examining the total sugar content across different experiments, significant variations were noted compared to control plants at various stages of plant tissue culture. During the multiplication stage, the highest total carbohydrate content was observed in plants grown in LSE medium with *Sargassum tenerrimum* with 50% concentration, reaching 271.25 mg/FW (Table 5.9).

Throughout all the experiments, the control plants were cultivated in a solid medium with 3% sucrose under normal laboratory conditions, resulting in a total carbohydrate content of 221.43 mg/FW. This value significantly differed from the total carbohydrate content observed in various experimental conditions. The study

also revealed that the addition of external CO_2 notably increased the total carbohydrate content of the plants, reaching 260.13 mg/FW compared to non-enriched plants. Additionally, the shape of the culture vessels significantly influenced the total carbohydrate content, with the highest levels observed in V7 using glass marbles as support material (258.33 mg/FW) (Table 5.2).

The highest carbohydrate content was recorded in the CO_2-enriched liquid culture treatment (260.13 mg/FW), compared to the solid medium with CO_2 enrichment (22.54 mg/FW). Additionally, the incorporation of seaweed (Table 5.9) and modifications to gelling agents (Table 5.6) significantly impacted total carbohydrate content. These findings indicate that both CO_2 enrichment and the shape of the culture vessels are crucial factors influencing plant carbohydrate content during various stages of plant tissue culture. Conversely, altering gelling agents did not significantly affect the total carbohydrate content in this experimental context. In rose explants, polyamines increased the total carbohydrate content in shoots. The highest carbohydrate levels were recorded with 30 mM concentrations of exogenous Spd and Cad (65.04 and 65.03, respectively), followed closely by Put at 30 mM (64.62). All concentrations resulted in higher carbohydrate content compared to the control (56.93) (Table 5.10).

Peroxidase (POD) represent pivotal enzymatic components is protecting plants against oxidative stress. They achieve this by catalysing the conversion of detrimental radicals into less injurious compounds. In the context of our investigation, we embarked on the quantification of POD levels across distinct growth stages and under varying *in vitro* environmental conditions. Our analysis revealed that the POD activity was notably decreased in plants cultured in a CO_2-enriched environment when subjected to a solid medium (Figure 5.3b) as opposed to a liquid medium (Figure 5.3a). Concurrently, we explored the influence of different culture vessels during the multiplication phase of plant growth. It was observed that the choice of culture vessel had a discernible impact on POD production, with solid medium vessels yielding in V7 44.88 U/mg protein (Figure 5.1b), a significant departure from the control group, which recorded 23.89 U/mg protein. When using alter gelling agent, agar (58.03 U/mg protein) (Figure 5.1a) shows significantly increase compare to other gelling agents. Notably, when comparing the effects of a liquid medium with different vessels

and support material versus semi-solid medium on plant POD production, (Figure 5.2) a significant difference was observed. Plants grown in the liquid medium displayed a production exceeding half of that observed in solid medium conditions. This observation bears implications for the enhancement of plant growth. POD activity was significantly enhanced with increasing concentrations of polyamines (PAs). Different concentrations of PAs resulted in increased POD activity compared to the control. Notably, 30 mM of PAs exhibited the highest POD activity compared to both 10 mM and 50 mM concentrations (Figure 5.10b). Interestingly, cadaverine (Cad) demonstrated higher POD activity than spermidine (Spd) and putrescine (Put). Similar results were observed with superoxide dismutase (SOD) activity, where the 30 mM concentration displayed the highest activity compared to 10 mM and 50 mM, with Cad again showing greater activity than Spd and Put (Figure 5.10c).

Phenolic compounds are the second most important group of plant products involved in defense mechanisms against biotic and abiotic stresses. Our study focused on quantitatively assessing phenolic content during various stages of micropropagation, with a particular emphasis on the multiplication stages. During the multiplication stage, we observed significant variations in phenol concentrations under different growth conditions. Plants grown in a controlled environment with a semisolid medium enriched with CO_2 exhibited a markedly higher phenol content of 198.80 mg/g FW compared to the control at 145.92 mg/g FW. Interestingly, the use of liquid medium in all treatments resulted in nearly uniform phenol production, demonstrating a consistent and significant impact on plant growth across the experimental groups, as detailed in the accompanying tables. When comparing plants grown in solid and liquid media, a substantial increase in phenol content was exclusively observed in those treated with semisolid medium. Polyamines reduced phenolic content in plantlets compared to the control level of 2.4, with the lowest phenolic levels observed at 30 mM for all three polyamines (Spd - 2.12, Put - 2.13, Cad - 2.09), and Cad showing the lowest phenolic content across all three concentrations (10 mM, 30 mM, and 50 mM) (Table 5.10).

Chlorophyll production in *in vitro* conditions, or within plants grown under controlled laboratory environments, is influenced by various factors. Attaining optimal chlorophyll levels is crucial because it directly affects the photosynthetic

capacity and, consequently, the overall health and vigor of the plants. In our study, we observed significant variations in chlorophyll content during the multiplication stage, with marked differences among the experimental treatments. These included liquid medium with different culture vessels and support materials, as well as CO_2 enrichment within both semi-solid and liquid media. In the semi-solid medium control group, the chlorophyll content was measured at 0.26 mg/g FW (fresh weight), which was higher compared to treatments with different gelling agents (Figure 5.4a). Notably, a significant increase in chlorophyll content was observed with CO_2 enrichment in the liquid culture medium (Figure 5.6), where chlorophyll content was approximately 0.300 mg/g FW. This was higher compared to the semi-solid medium control. When vessel size was altered, higher chlorophyll levels were observed in V1 with GM as the support material, recording 0.273 mg/g FW (Figure 5.7a). Additionally, liquid culture showed even higher chlorophyll content, reaching 0.304 mg/g FW in plants supported by GM in V7 (Figure 5.7a).

Similar results were recorded in seaweed-treated plants, with no major changes in total chlorophyll content observed (Figure 5.8). Statistical analysis further corroborated these results, indicating significant differences in total chlorophyll content among the experimental groups. These findings underscore the importance of medium composition and CO_2 enrichment in influencing chlorophyll production during the multiplication stage. Enriching culture vessels with CO_2 in the liquid medium led to a substantial increase in chlorophyll content, highlighting its potential to enhance the photosynthetic performance and overall health of the cultured plants. We found that adding polyamines (PAs) resulted in a gradual increase in chlorophyll a, chlorophyll b, and total chlorophyll content. However, further increases in PAs concentration led to a decline in chlorophyll levels, although these values remained higher than those of the corresponding control propagules (Figure 5.9).

For protein estimation, we used the Bradford method to measure total protein content across different growth conditions. Comparing plants grown in liquid and semi-solid media, we observed significant differences in protein concentration. Notably, using glass marbles (GM) as a supporting material in liquid culture medium with V6 resulted in the highest protein content at 289.56 mg/g FW. Other support materials also showed increased protein levels compared to the control: water balls in

V6 (279.16 mg/g FW), tissue paper in V7 (249.23 mg/g FW), and cotton in V6 (269.16 mg/g FW). When comparing these studies with mediums containing gelling agents, only minor changes in protein content were observed. The highest protein content was reported in agar (197.54 mg/g FW) using supporting materials. Based on these results, the same culture medium plays an important role in plant growth. In this experiment, we studied the effects of CO_2 enrichment with both liquid and solid culture growth mediums. In both studies, SFSM (81.18 mg/g FW) and SFLM (101.18 mg/g FW) showed lower protein concentrations compared to plants grown in sucrose-containing liquid and solid growth mediums. The highest protein concentration was reported in 3% sucrose with CO_2 enrichment in liquid (256.52 mg/g FW) and semi-solid (244.95 mg/g FW) growth mediums. In this study, polyamines also enhanced the total protein content in rose plantlets compared to the control, with the 30 mM concentration yielding the highest protein levels (Spd - 78.47, Put - 77.87, Cad - 73.87) relative to 10 mM and 50 mM (Table 5.10).

Table 5.1 Effect of different vessel's types on semi-solid medium and their biochemical parameters of rose during multiplication stages

Vessel's type	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
V1	265.000 c	221.433 c	150.300 c
V2	238.200 e	205.233 d	153.267 b
V3	276.033 b	237.367 b	139.800 e
V4	257.100 d	225.133 c	143.567 d
V5	225.100 f	196.100 d	171.067 a
V6	279.567 a	230.900 bc	135.067 f
V7	267.167 c	248.333 a	130.480 g
CV	0.539	2.396	0.731
SEM	0.804	3.092	0.617
CD 5%	2.439	9.378	1.872
CD 1%	3.385	13.017	2.598

(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 5.2 Effect of different vessel's types with glass marble as support material on liquid medium and their biochemical parameters of rose during multiplication stages

Vessel's type	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
V1	275.000 c	231.433 c	135.300 c
V2	248.200 e	215.233 d	138.267 b
V3	286.033 b	247.367 b	124.800 e
V4	267.100 d	235.133 c	128.567 d
V5	235.100 f	206.100 d	156.067 a
V6	289.567 a	240.900 bc	120.067 f
V7	277.167 c	258.333 a	115.480 g
CV	0.519	2.294	0.815
SEM	0.804	3.092	0.617
CD 5%	2.439	9.378	1.872
CD 1%	3.385	13.017	2.598

(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 5.3 Effect of different vessel's types with water balls as support material on liquid medium and their biochemical parameters of rose during multiplication stages

Vessel's type	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
V1	264.600 c	227.333 c	145.300 c
V2	237.800 e	211.133 d	148.267 b
V3	275.633 b	243.267 b	134.800 e
V4	256.700 d	231.033 c	138.567 d
V5	224.700 f	202.000 d	166.067 a
V6	279.167 a	236.800 bc	130.067 f
V7	266.767 c	254.233 a	125.467 g
CV	0.540	2.334	0.757
SEM	0.804	3.092	0.617
CD 5%	2.439	9.378	1.873
CD 1%	3.385	13.017	2.599

(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 5.4 Effect of different vessel's types with tissue paper as support material on liquid medium and their biochemical parameters of rose during multiplication stages

Vessel's type	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
V1	259.600 c	222.333 c	150.300 c
V2	232.800 e	206.133 d	153.267 b
V3	270.633 b	238.267 b	139.800 e
V4	251.700 d	226.033 c	143.567 d
V5	219.700 f	197.000 d	171.067 a
V6	274.167 a	231.800 bc	135.067 f
V7	261.767 c	249.233 a	130.467 g
CV	0.551	2.387	0.731
SEM	0.804	3.092	0.617
CD 5%	2.439	9.378	1.873
CD 1%	3.385	13.017	2.599

(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 5.5 Effect of different vessel's types with cotton as support material on liquid medium and their biochemical parameters of rose during multiplication stages

Vessel's type	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
V1	254.600 c	217.333 c	155.300 c
V2	227.800 e	201.133 d	158.267 b
V3	265.633 b	233.267 b	144.800 e
V4	246.700 d	221.033 c	148.567 d
V5	214.700 f	192.000 d	176.067 a
V6	269.167 a	226.800 bc	140.067 f
V7	256.767 c	244.233 a	135.467 g
CV	0.562	2.441	0.707
SEM	0.804	3.092	0.617
CD 5%	2.439	9.378	1.873
CD 1%	3.385	13.017	2.599

(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 5.6 Effect of different gelling agents and their biochemical parameters of rose during multiplication stages

Media	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
Agar	213.517 a	197.554 a	145.923 b
Phyta gel	207.454 b	184.798 b	152.805 ab
Guar gum	193.843 c	176.169 c	173.873 ab
Isobgol	184.880 d	164.881 d	196.140 a
Starch	175.148 e	145.290 e	184.473 ab
CV	1.370	1.301	13.053
SEM	1.543	1.305	12.860
CD 5%	4.861	4.113	NS
CD 1%	6.914	5.850	NS

SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation, NS- Not Significant. Means in the same columns followed by different letters are significantly different (P≤0.05) using the Duncan's Multiple Range Test

Table 5.7 Effect of CO_2 enrichment on semi-solid medium and their biochemical parameters of rose during multiplication stages

	Sugar Concentration (%)	**CO_2 Concentration (%)**	**Total protein (mg g^{-1} fwt)**	**Total carbohydrate (mg g^{-1} fwt)**	**Total phenol (mg g^{-1} fwt)**
SFSM	**0**	0.000	0.000 n	0.000 r	0.000 n
		0.030	81.187 m	65.210 q	198.807 a
		0.500	91.863 kl	78.473 o	175.900 b
		2.000	102.197 j	82.217 n	157.543 d
		GR	95.197 k	71.500 p	162.510 c
SCSM	**1**	0.000	91.187 l	80.360 no	173.807 b
		0.030	101.863 j	93.623 m	150.900 e
		0.500	105.197 j	99.478 l	132.543 h
		2.000	124.880 h	107.838 j	116.294 m
		GR	112.197 i	103.547 k	126.925 jk
	2	0.000	163.905 g	105.901 jk	153.561 e
		0.030	185.618 f	115.564 i	145.588 f
		0.500	193.161 e	126.793 h	136.599 g
		2.000	213.572 c	145.658 f	120.180 l
		GR	204.553 d	136.574 g	128.617 ij
	3	0.000	213.517 c	197.554 e	145.923 f
		0.030	226.849 b	204.962 d	136.165 g
		0.500	244.957 a	222.549 a	124.835 k
		2.000	224.905 b	214.632 b	131.216 hi
		GR	216.316 c	209.869 c	138.842 g
		CV	1.497	1.508	1.203
		SEM	1.294	1.072	0.957
		CD 5%	3.698	3.064	2.736
		CD 1%	4.948	4.100	3.662

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 5.8 Effect of CO_2 enrichment on liquid medium and their biochemical parameters of rose during multiplication stages

	Sugar Concentration (%)	**CO_2 Concentration (%)**	**Total protein (mg g^{-1} fwt)**	**Total carbohydrate (mg g^{-1} fwt)**	**Total phenol (mg g^{-1} fwt)**
SFLM	**0**	0.000	0.000 f	0.000 j	0.000 n
		0.030	101.187 e	75.210 i	168.807 a
		0.500	111.863 de	88.473 ghi	145.900 b
		2.000	122.197 cde	92.217 gh	127.543 d
		GR	115.197 cde	81.500 hi	132.510 c
SCLM	**1**	0.000	102.137 e	77.440 i	143.807 b
		0.030	115.533 cde	96.390 g	120.900 e
		0.500	125.200 cde	111.330 f	102.543 h
		2.000	136.383 cde	121.933 f	86.294 m
		GR	119.467 cde	116.140 f	96.925 jk
	2	0.000	154.847 bcde	124.833 ef	123.561 e
		0.030	177.730 bc	136.103 e	115.588 f
		0.500	200.647 ab	156.743 d	106.599 g
		2.000	213.250 ab	177.323 c	90.180 l
		GR	211.287 ab	164.867 cd	98.617 ij
	3	0.000	166.988 bcd	239.587 b	115.923 f
		0.030	256.420 a	260.133 a	106.165 g
		0.500	245.987 a	242.940 b	94.835 k
		2.000	250.227 a	239.310 b	101.216 hi
		GR	250.237 a	236.130 b	108.842 g
		CV	20.818	5.666	1.517
		SEM	19.091	4.643	0.957
		CD 5%	54.568	13.270	2.736
		CD 1%	73.018	17.757	3.662

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 5.9 Effect of different liquid seaweed extract and their biochemical parameters of rose during multiplication stages

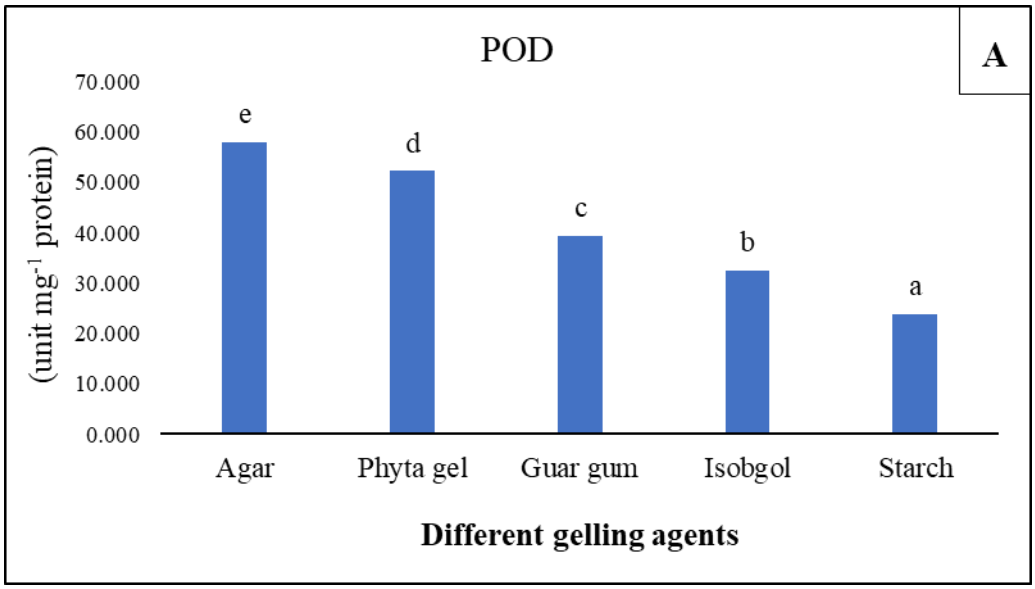
Liquid Seaweed Extract (LSE)	Concentration of LSE (% v/v)	Total protein (mg g^{-1} fwt)	Total carbohydrate (mg g^{-1} fwt)	Total phenol (mg g^{-1} fwt)
Caulerpa	10	119.000 h	221.230 m	1.020 def
racemosa	30	127.000 g	227.620 k	0.887 f
	50	129.000 f	224.320 l	1.320 abc
Ulva lactuca	10	131.000 def	234.650 g	1.060 cdef
	30	132.000 de	235.650 f	1.150 bcdef
	50	135.333 c	239.520 d	1.280 abcd
Sargassum	10	136.000 c	237.650 e	1.150 bcdef
tenerrimum	30	136.000 c	245.620 b	1.270 abcd
	50	188.000 a	271.250 a	1.450 a
Sargassum	10	130.000 ef	241.350 c	1.120 bcdef
wightii	30	136.000 c	229.950 h	1.220 abcde
	50	129.000 f	235.690 f	1.350 ab
Gracilaria edulis	10	132.000 de	228.650 j	1.120 bcdef
	30	139.000 b	234.250 g	1.240 abcde
	50	132.000 de	229.360 i	1.320 abc
MS medium with standard PGR		132.000 d	239.770 d	1.290 abcd
MS medium without standard PGR		89.000 i	196.623 n	0.990 ef
CV		0.846	0.147	11.793
SEM		0.647	0.199	0.081
CD 5%		1.859	0.571	0.233

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 5.10 Effect of different polyamines concentration on biochemical parameters in rose micropropagules grown under *in vitro* conditions

Polyamines	**Polyamines concentration (mM)**	**Total carbohydrate (mg g^{-1} fwt)**	**Total phenol (mg g^{-1} fwt)**	**Total protein (mg g^{-1} fwt)**
Control	0	56.933 a	2.400 a	69.370 a
Spermidine	10	61.973 bc	2.210 a	75.870 f
	30	65.043 d	2.120 a	78.470 g
	50	63.973 cd	2.163 a	71.070 b
Putrescine	10	61.733 bc	2.207 a	74.870 ef
	30	64.623 d	2.130 a	77.870 g
	50	63.523 cd	2.173 a	72.080 bc
Cadaverine	10	60.833 b	2.180 a	72.870 cd
	30	65.033 d	2.093 a	73.870 de
	50	63.733 cd	2.117 a	71.970 bc

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



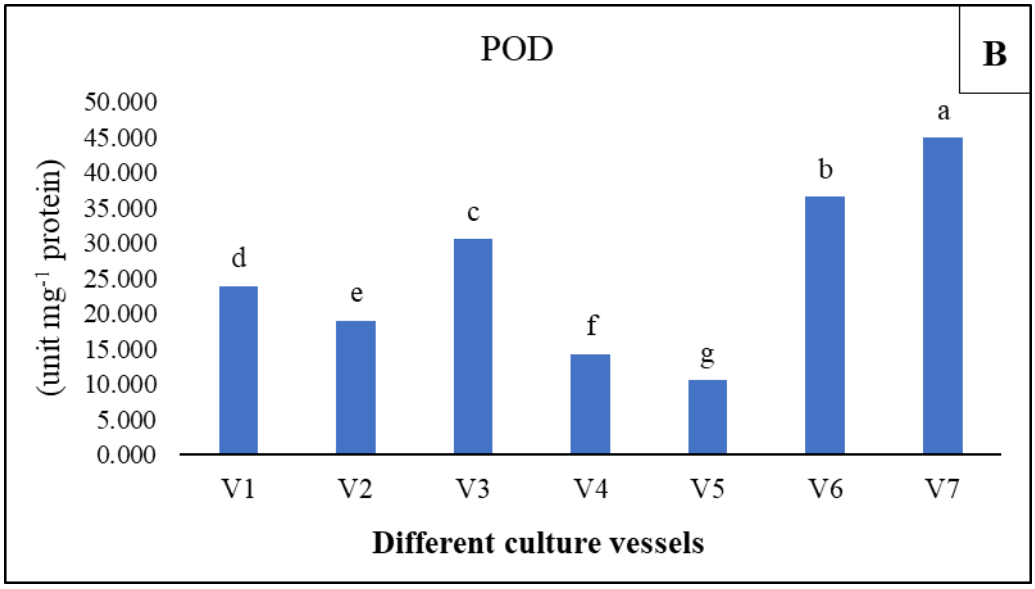


Figure 5.1 Peroxidase (POD) activity of micropropagated Rose during the multiplication stages of plantlets under various growth conditions. The figure show (A) Effects of different gelling agents and (B) Effects of different culture vessels

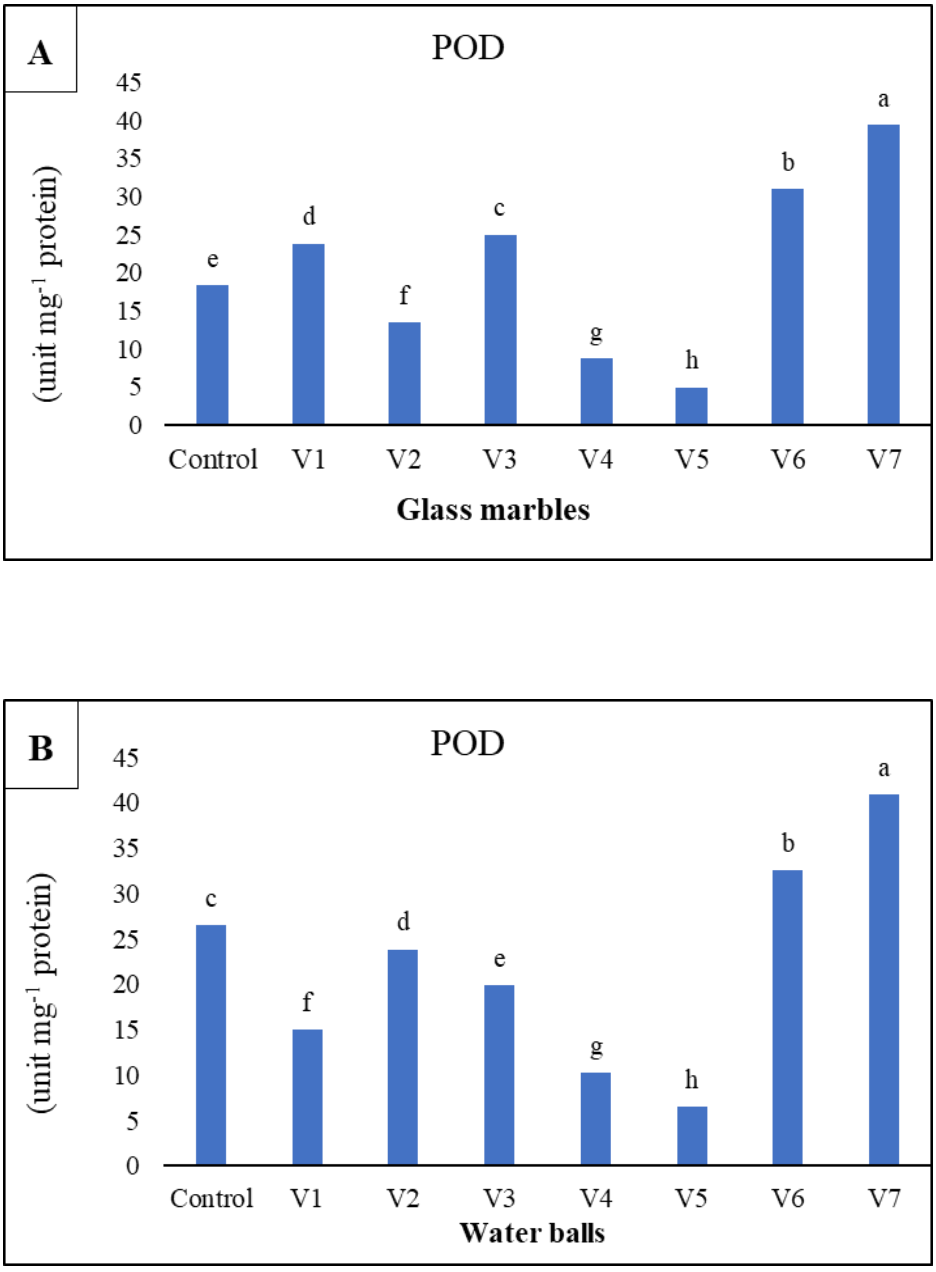
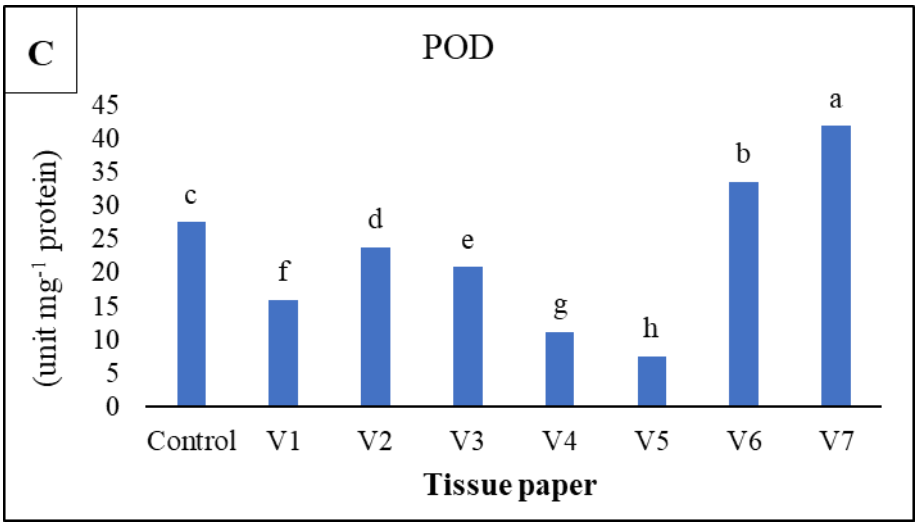


Figure 5.2(a) Peroxidase (POD) activity of micropropagated Rose during the multiplication stages of plantlets under various growth conditions. The figure shows (A) Effects of different culture vessels with glass marbles as support material, (B) Effects of different culture vessels with water balls as support material



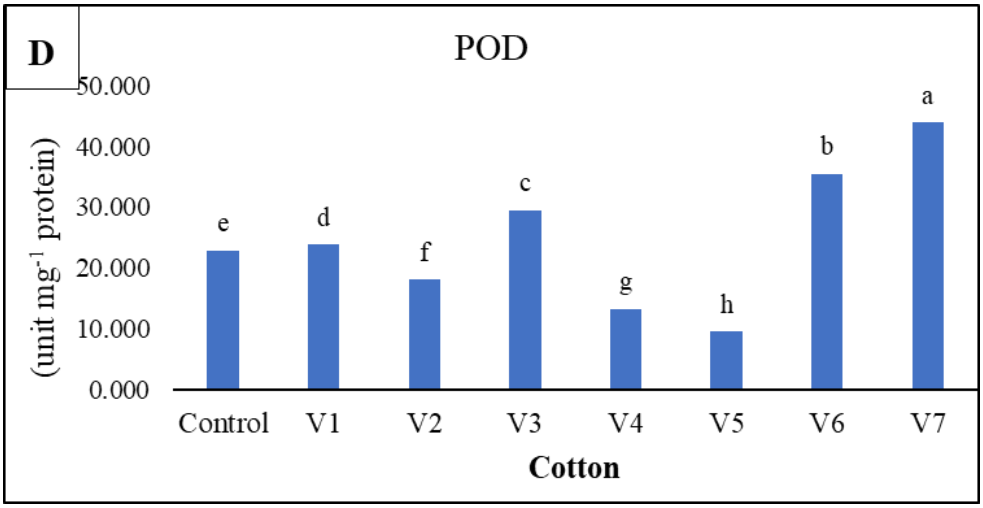
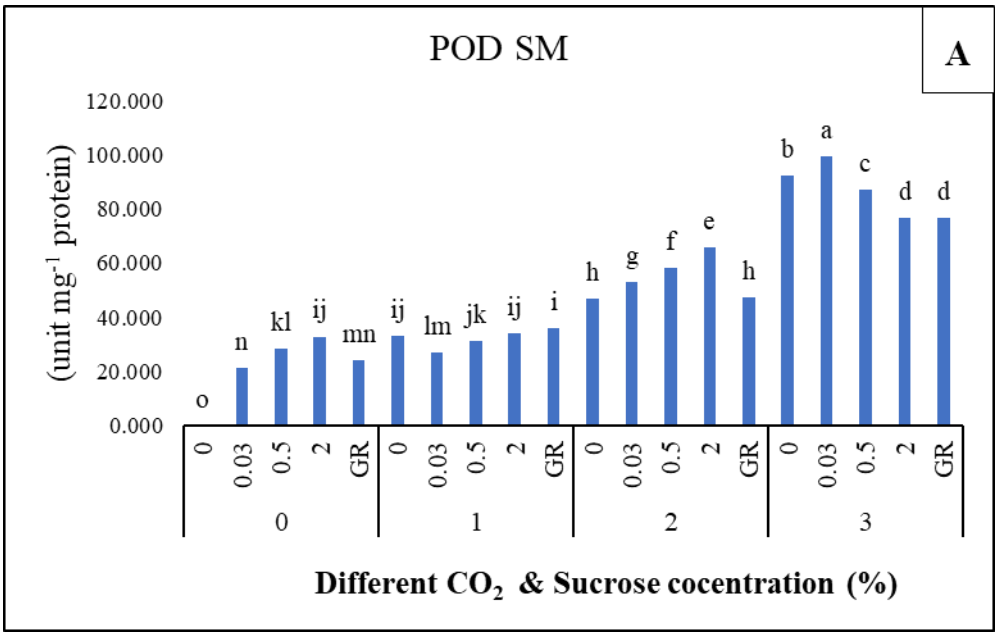


Figure 5.2(b) Peroxidase (POD) activity of micropropagated Rose during the multiplication stages of plantlets under various growth conditions. The figure shows (C) Effects of different culture vessels with tissue papers as support material and (D) Effects of different culture vessels with cotton as support material



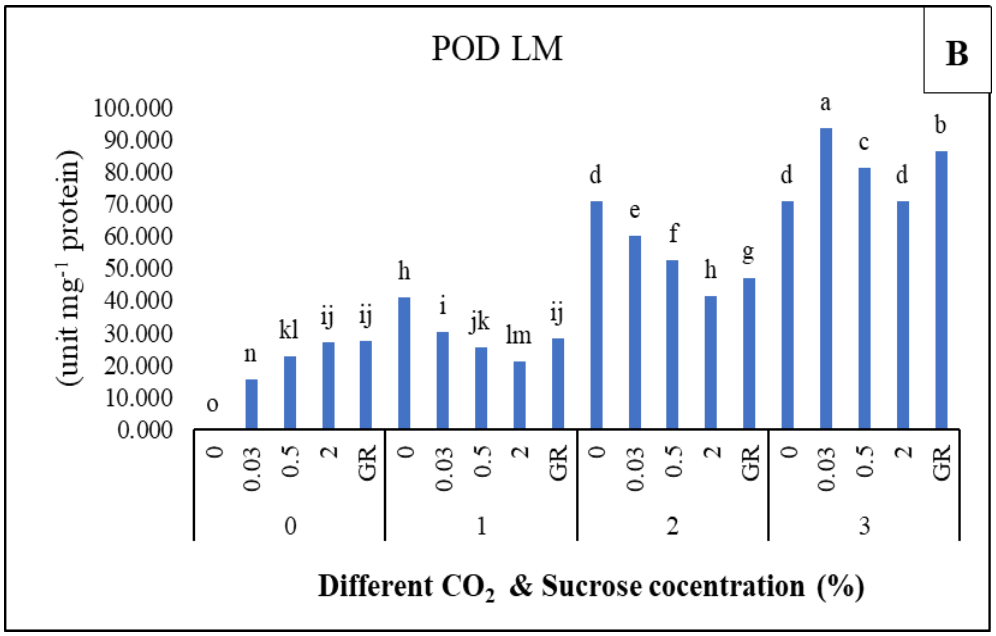


Figure 5.3 Peroxidase (POD) activity of micropropagated Rose during the multiplication stages of plantlets under CO_2 enrichment condition. In figure shows the (A) Effects of CO_2 enrichment with SS medium and (B) Effects of CO_2 enrichment with liquid medium

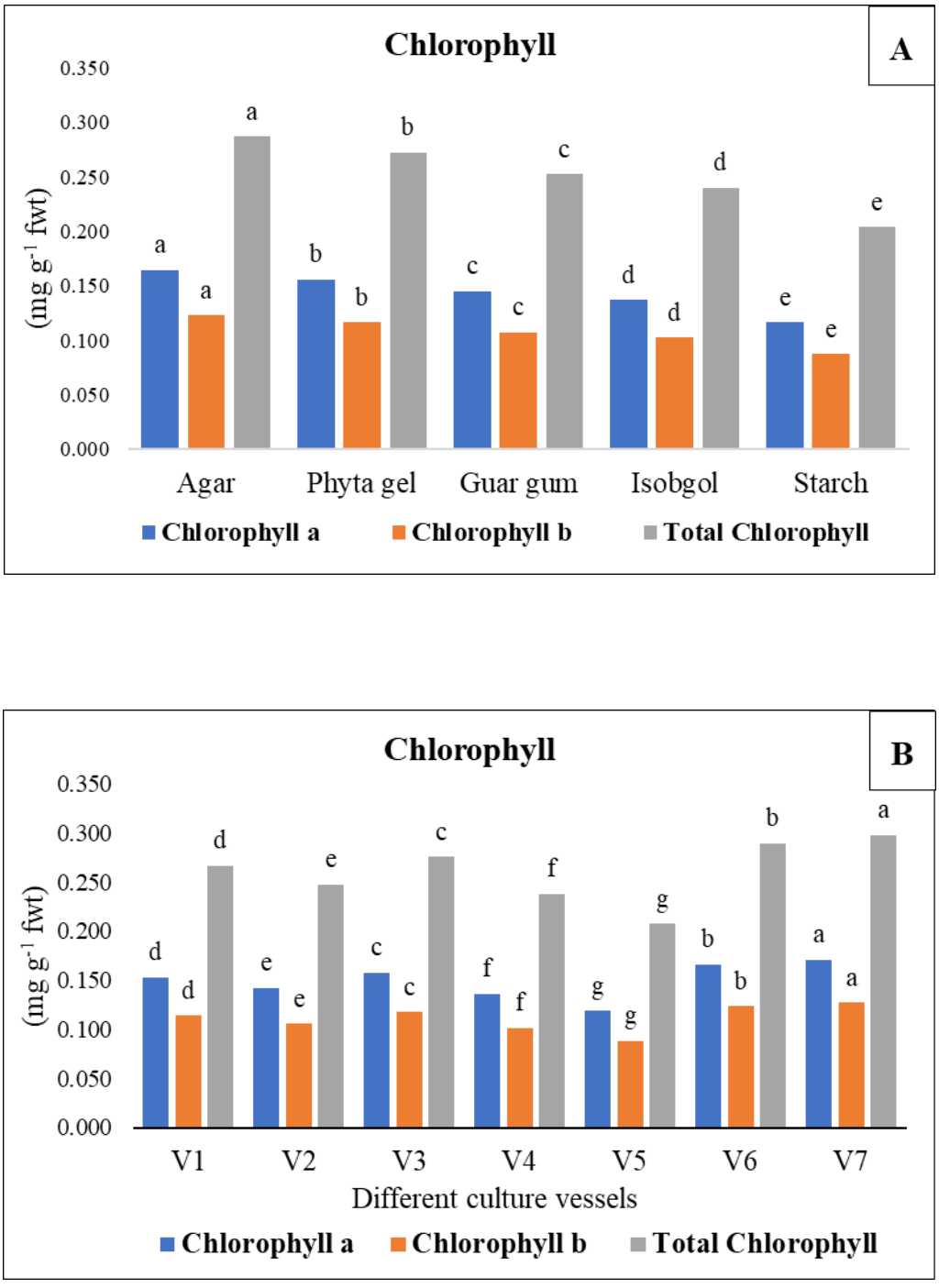


Figure 5.4 Chlorophyll activity of micropropagated Rose during multiplication stage of plantlet under different growth condition. The figure shows (A) Effects of different gelling agents and (B) Effects of different culture vessels

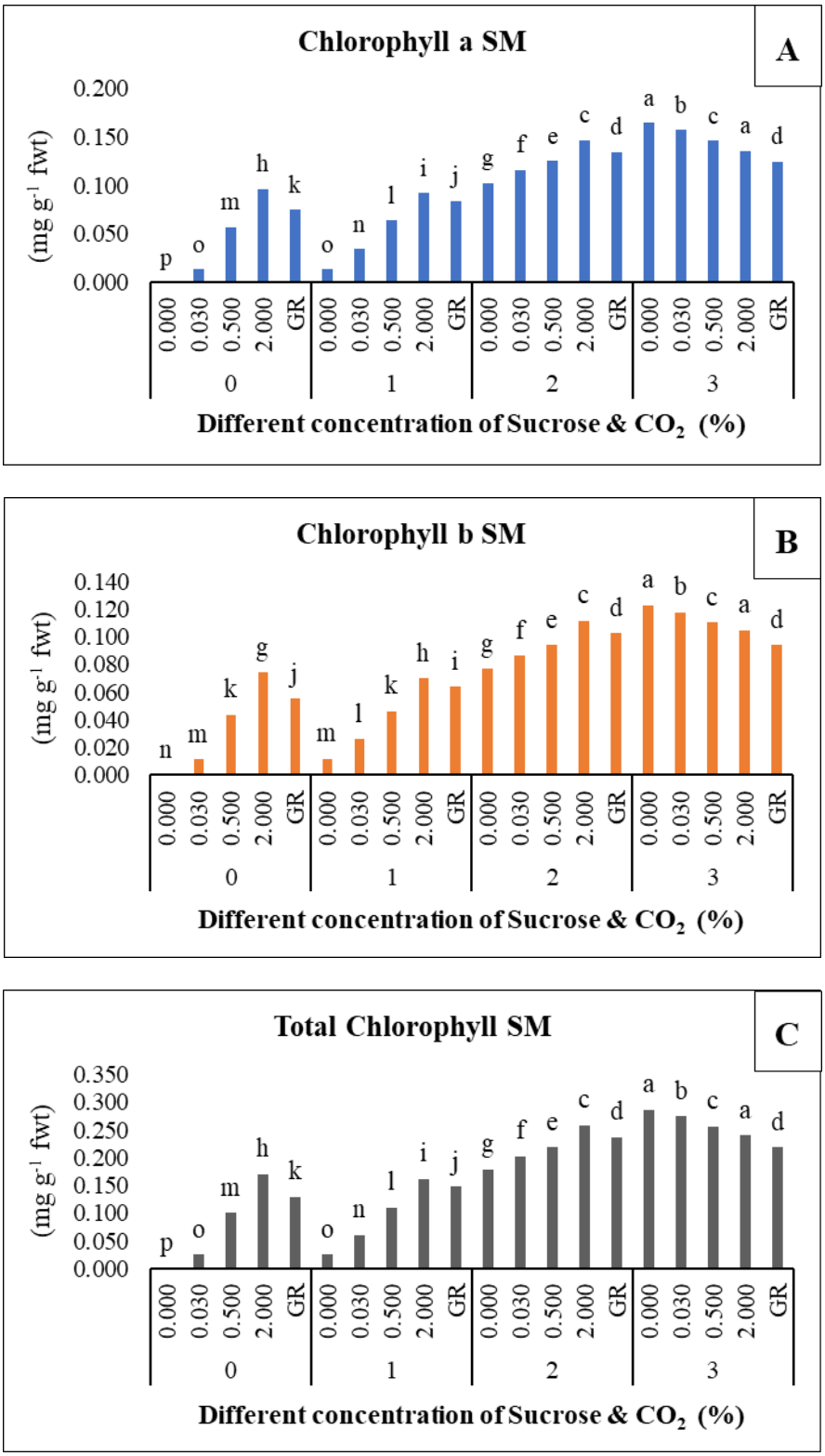


Figure 5.5 Chlorophyll activity of micropropagated Rose during the multiplication stages of plantlets under CO_2 enrichment condition on semi-solid medium. The figure shows (A) Chlorophyll a, (B) Chlorophyll b and (C) Total chlorophyll

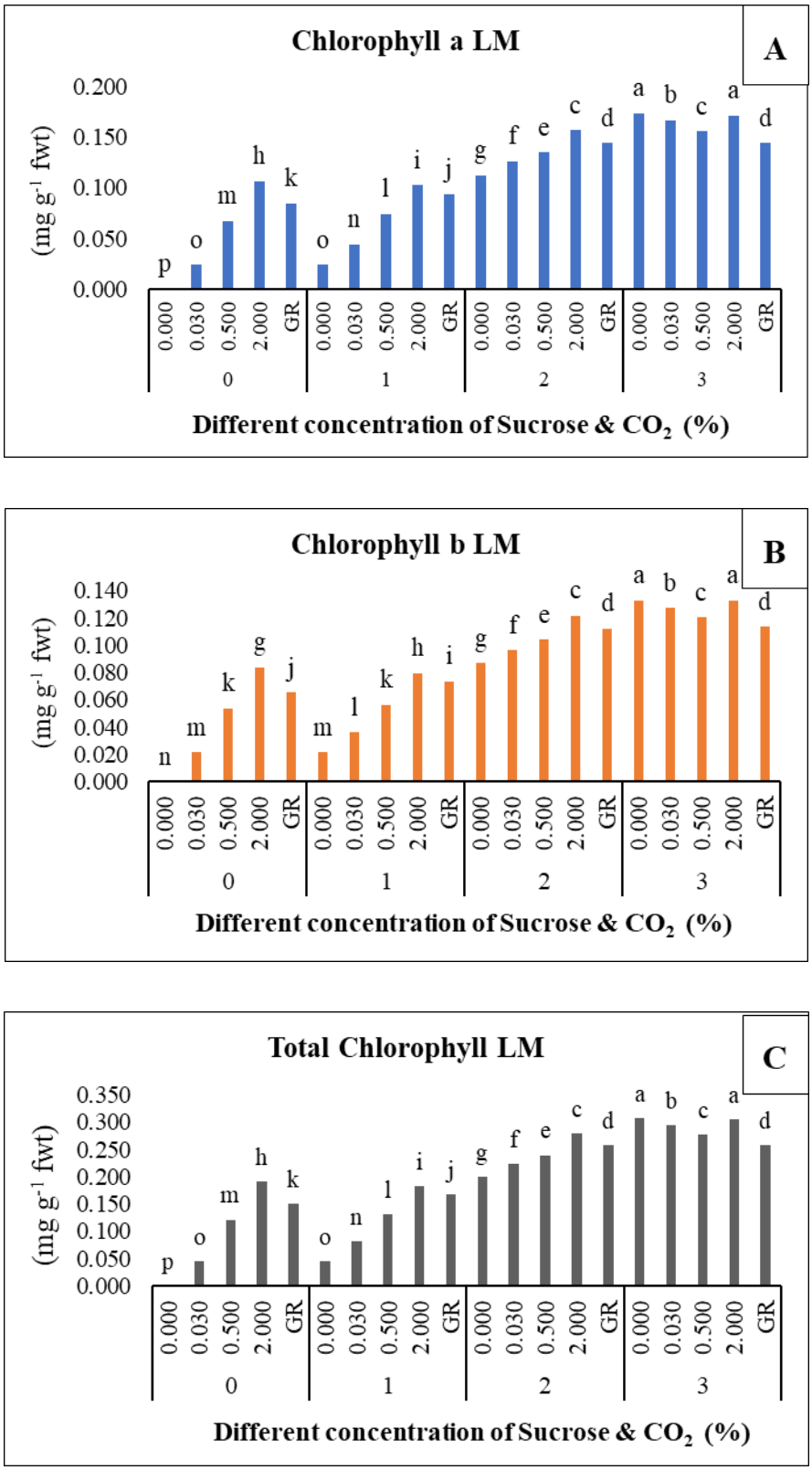


Figure 5.6 Chlorophyll activity of micropropagated Rose during the multiplication stages of plantlets under CO_2 enrichment condition on liquid medium. The figure shows (A) Chlorophyll a, (B) Chlorophyll b and (C) Total chlorophyll

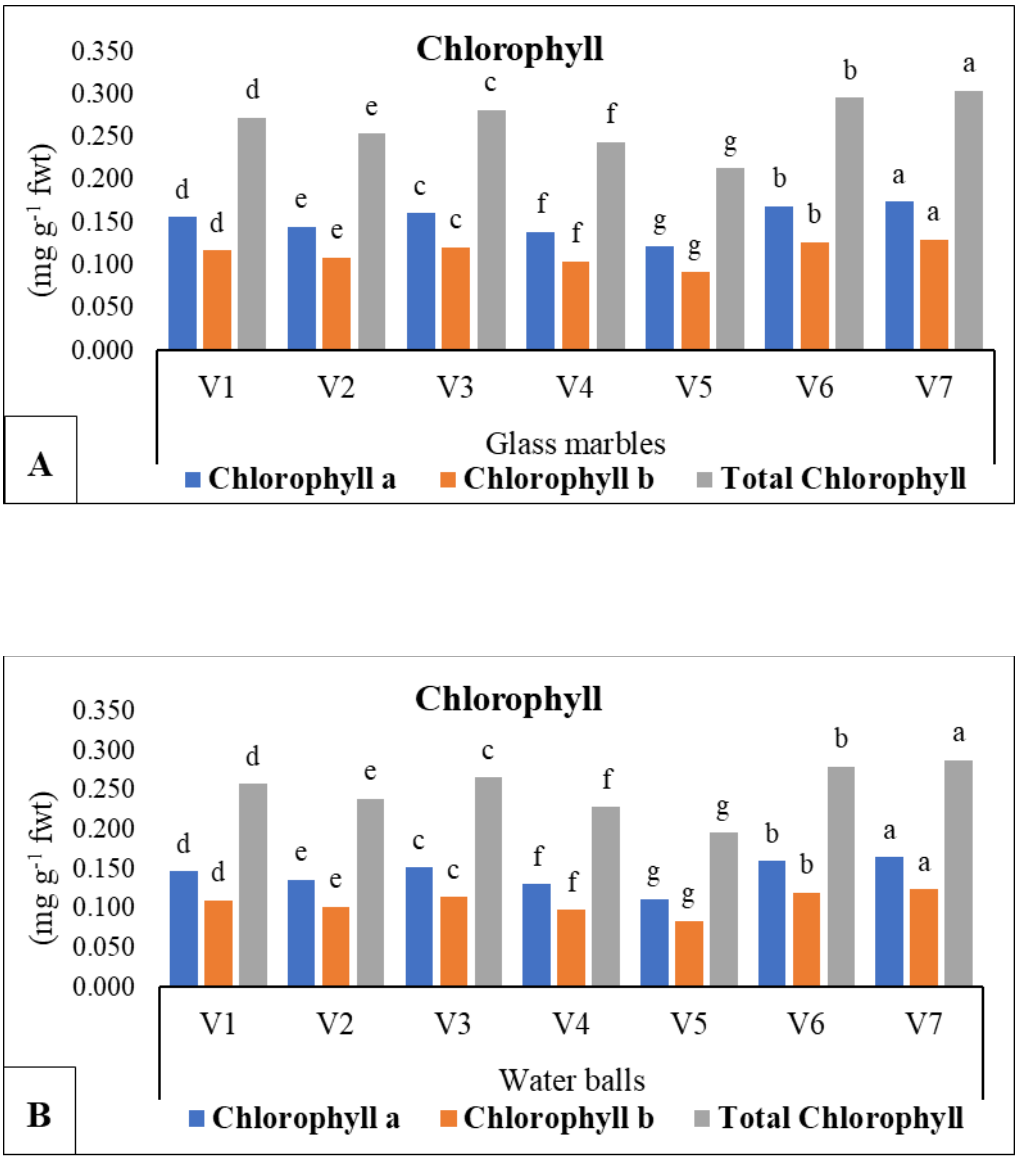


Figure 5.7(a) Chlorophyll activity of micropropagated Rose during multiplication stage of plantlet under different growth condition. The figure shows (A) Effects of different support material as glass marbles (B) Effects of different support material as water balls

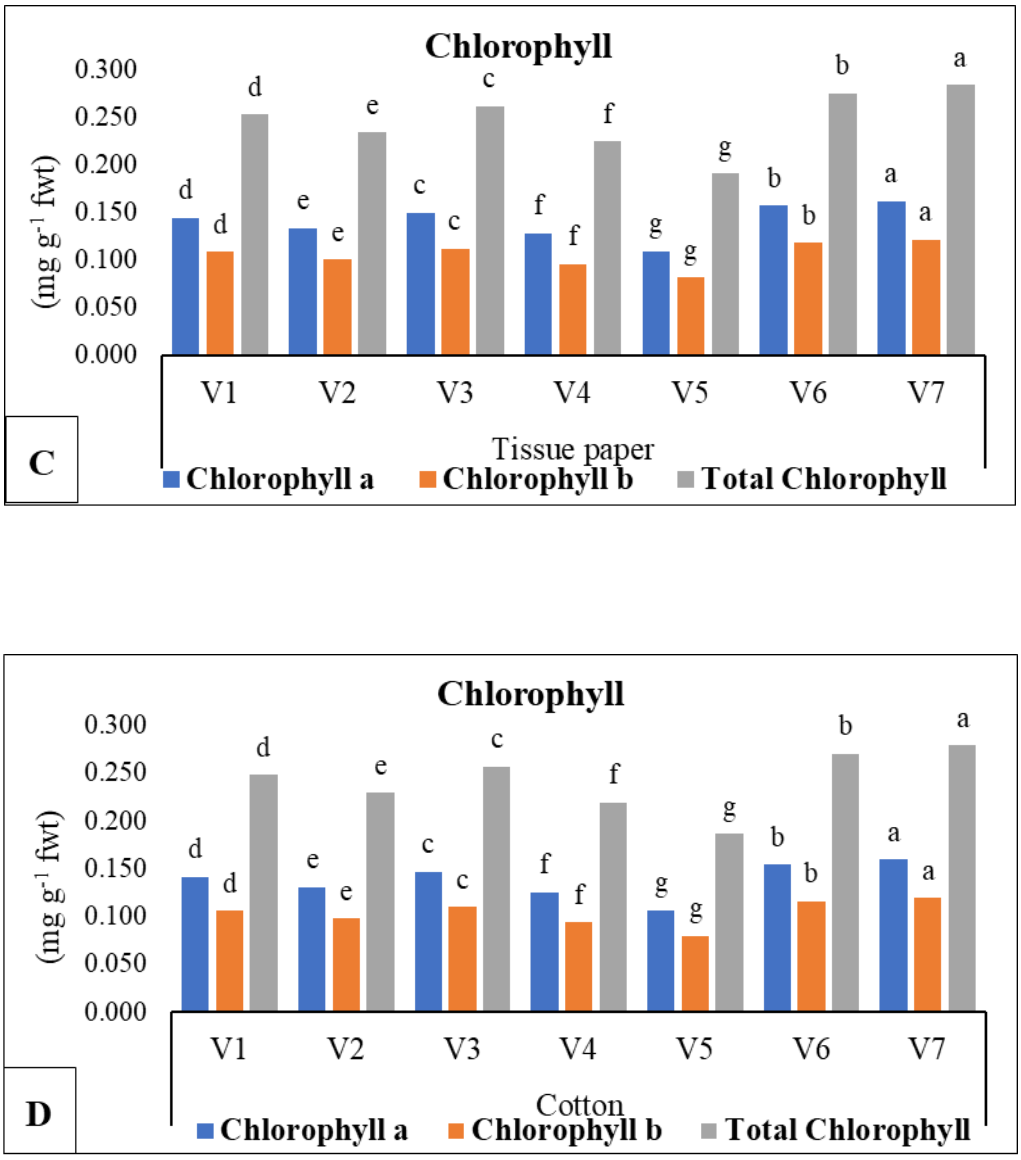


Figure 5.7(b) Chlorophyll activity of micropropagated Rose during multiplication stage of plantlet under different growth condition. The figure shows (C) Effects of different support material as tissue paper and (D) Effects of different support material as cotton

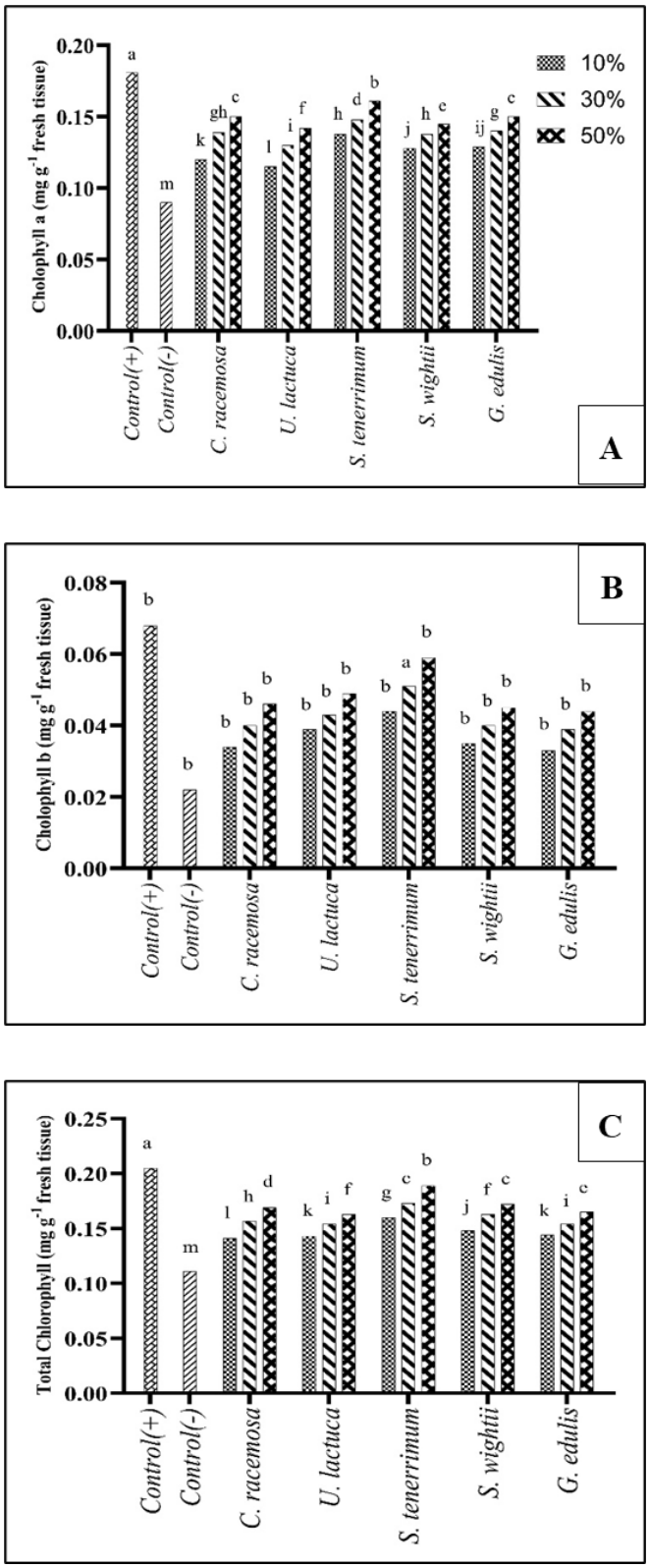


Figure 5.8 Chlorophyll activity of micropropagated Rose during multiplication stage of plantlet grown under different LSE concentrations (A) Chlorophyll a, (B) Chlorophyll b and (C) Total chlorophyll

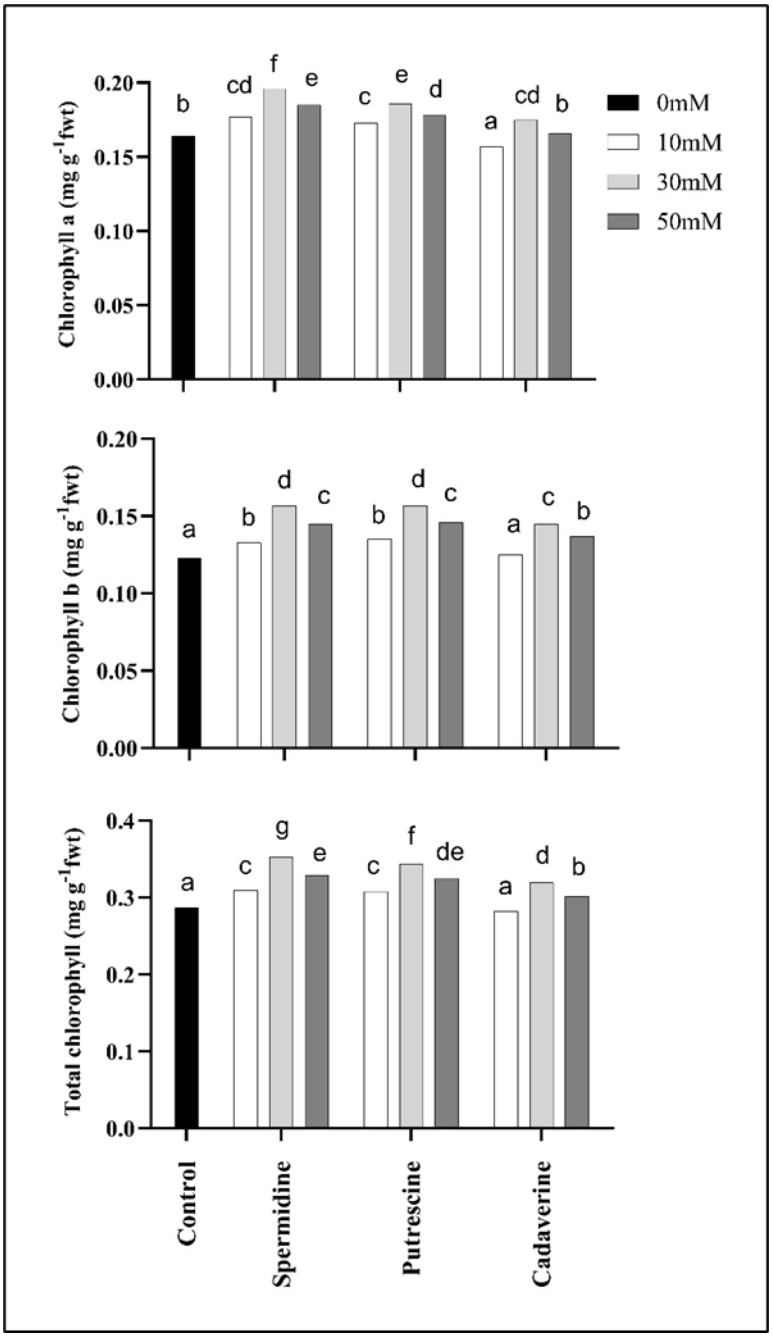


Figure 5.9 Effect of polyamines (PAs) on chlorophyll contents in rose micropropagules grown under *in vitro* conditions

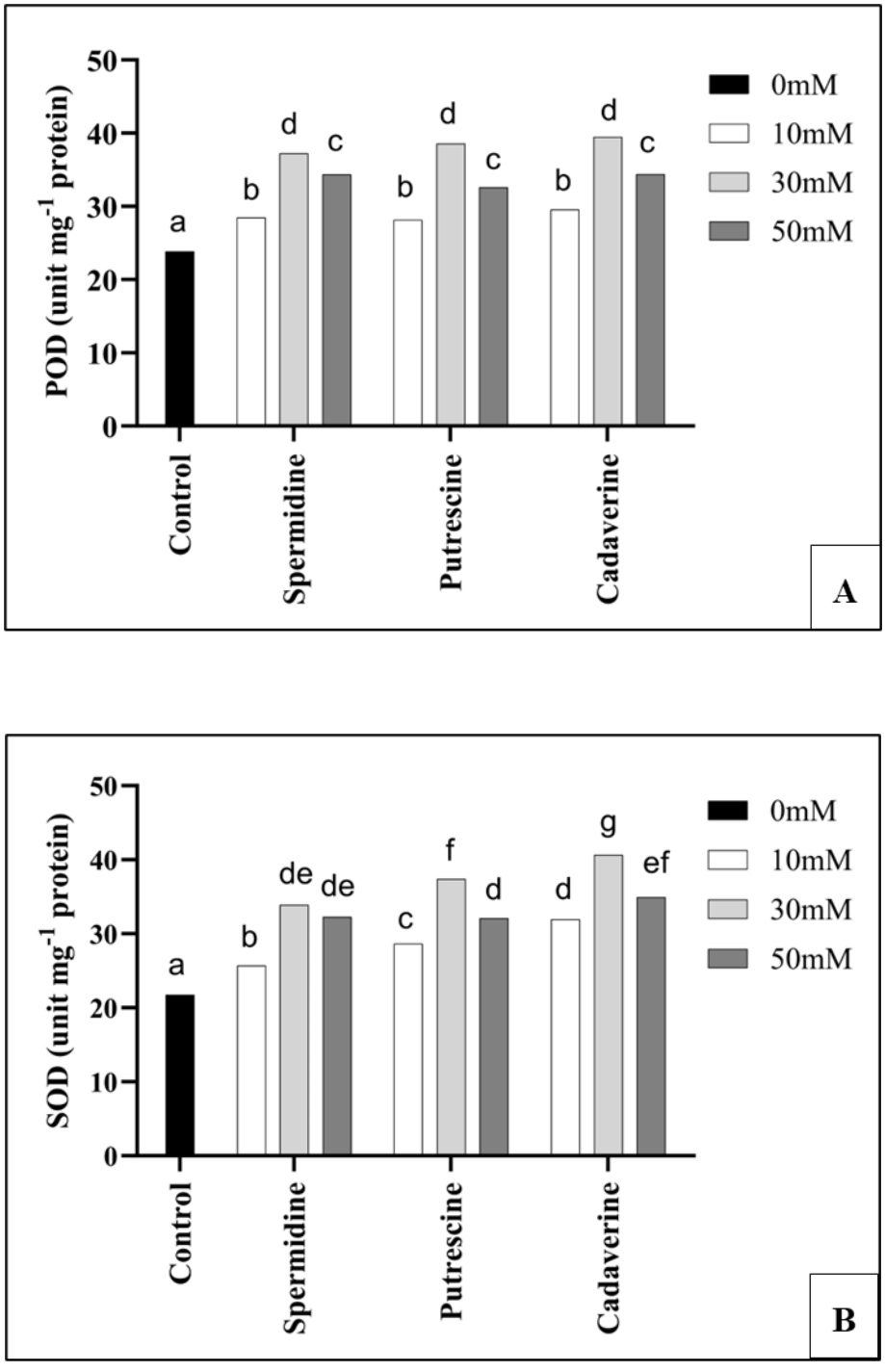


Figure 5.10 Effect of polyamines (PAs) on antioxidant enzyme like (B) peroxidase (POD) and (C) superoxide dismutase (SOD) in rose micropropagules grown under *in vitro* conditions