Chapter 5

Micropropagation In Altered Growth Condition-Biochemical Studies

5.1 Study different biochemical parameters

In a liquid culture system, plants can experience stress signals that lead to unusual growth patterns, making them less suitable for transplantation. When plant tissues are submerged in this environment, they undergo osmotic and oxidative stresses, which cause an increase in the levels of antioxidant enzymes and other chemical compounds. These changes in the plants responses to stress can affect their overall health and readiness for transplanting (Fujii *et al.,* 2011). To cope with these environmental stresses, the plant activates various biochemical processes. For example, it accumulates compatible solutes and activates detoxification enzymes to mitigate the negative effects of these stressors (Hasanuzzaman *et al.*, 2012). Hence, it is essential to have a comprehensive understanding of these factors throughout various growth stages and in different culture systems. This knowledge is a prerequisite for developing strategies that can enhance a plant's ability to adapt more effectively when transitioning from *in vitro* to *ex vitro* environments (Pandey *et al.,* 2019). In recent years, there has been a growing interest in investigating these transformations and adaptations. Researchers are increasingly focused on understanding how plants respond to the challenges posed by different culture systems, especially in the context of transitioning from controlled *in vitro* conditions to the unpredictable *ex vitro* environment (Carvalho and Amâncio, 2019). To gain a deeper insight into the process of regeneration, researchers have turned their attention to variations in antioxidant enzymes. Specifically, these variations have been extensively studied 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 occurring during *in vitro* growth. These investigations have provided valuable insights into the dynamic biochemical processes that underlie plant development in controlled laboratory conditions (Mendoza *et al.,* 2018; Khan *et al.,* 2019; Nazir *et al.,* 2019).

Carbohydrate biosynthesis and metabolism play pivotal roles in the process of plant growth during *in vitro* propagation. These aspects are of utmost significance as they directly influence the energy and building blocks required for cell division, elongation, and overall development. An in-depth exploration of carbohydrate-related processes is essential for comprehending and enhancing plant growth in controlled *in vitro* environments, where the availability of nutrients can differ significantly from natural conditions (De Moraes *et al.,* 2016; Wu *et al.,* 2019). Various carbohydrates were reported to be present in bulbs of *lachenalia* (Bach *et al.,* 2015), and lily bulb (Wu *et al.,* 2021) including soluble sugars, starch, glucomannans, and fructans. Carbohydrate accumulation is significantly influenced by both the inorganic and organic components of the culture medium, and it plays a substantial role in regulating plant cell growth due to its osmotic properties. Osmotic stress resulting from changes in carbohydrate levels can have a profound impact on various aspects of plant tissue culture, including callus growth, colony formation, shoot regeneration, somatic embryogenesis, and adjustments in ion transport (such as the extrusion or uptake of ions). These metabolic changes also affect carbon metabolism. Consequently, plants exhibit a wide range of responses to osmotic stress at the molecular, cellular, and whole plant levels (Llanes *et al.,* 2018). Previous studies have indeed explored the carbohydrate content and concentration in various bulb tissues under different environmental conditions and at different stages of growth. These investigations provide valuable insights into how carbohydrates are dynamically regulated in bulbs throughout their development and in response to changing environmental factor (Chaves *et al.,* 2020; Cioć *et al.,* 2021). It is worth noting that there is limited information available on the metabolism of carbohydrates in geophytes, particularly when they are grown in *in vitro* (Wu *et al.,* 2016).

Oxygen is crucial for the survival of aerobic life forms, including plants. However, when plants encounter stressful conditions, they often experience an increase in toxic reactive oxygen species (ROS). These ROS include the superoxide anion radical, the hydroxyl radical, and hydrogen peroxide. Collectively, injuries caused by these ROS are referred to as oxidative stresses, and they represent some of the most harmful factors that affect plants (Hasanuzzaman *et al.,* 2020). To counteract the damaging effects of ROS, plants have evolved a range of antioxidant defense

mechanisms. Among these, superoxide dismutase (SODs) plays a central role. SODs are key enzymes responsible for protecting plants from oxidative damage by converting harmful superoxide radicals into less harmful compounds (García-Caparrós *et al.,* 2021). Assessing the activity of Superoxide Dismutase (SOD) is a valuable tool for evaluating the micronutrient status of plants. Moreover, Plants grown in submerged liquid culture systems are especially vulnerable to oxidative stresses. In these controlled environments, the conditions can lead to changes in the concentrations of reactive oxygen species (ROS). This susceptibility to oxidative stresses in submerged liquid culture systems is evident through the changes observed in the activities of Superoxide Dismutase (SOD) in plants grown *in vitro* (Spinoso-Castillo *et al.,* 2017). The altered SOD activities in these *in vitro* conditions reflect the plant's efforts to manage and mitigate the oxidative stress imposed by the unique environment of liquid culture systems.

Proline is a substance that plants produce when they face extreme conditions like drought, high salt levels, or very hot/cold temperatures. It helps the plant deal with these tough situations by adjusting the water balance, getting rid of harmful oxygen compounds, and safeguarding the plant's cell walls. However, when the environment is normal and not stressful, too much proline can actually slow down the plant's growth and development. This is because it uses up resources that the plant needs to make important molecules like RNA and proteins, which are essential for its overall health and growth. proline is a helpful compound for plants in stressful situations, but it can be a bit of a double-edged sword when things are going well for the plant in its usual environment (Maitra *et al.,* 2021). As mentioned earlier, plants grown in a liquid medium tend to experience more oxidative stress. Some research on proline accumulation during *in vitro* plant culture indicates that both increases and decreases in proline levels could be used to develop strategies for improving a plant's ability to withstand these stressful conditions. This suggests that manipulating proline levels could be a valuable approach to enhance a plant's tolerance to oxidative stress when it's grown in a liquid medium (Helaly *et al.,* 2017; Ghasemlou *et al.,* 2019).

Total phenol production plays a crucial role in plant biology and physiology, significantly impacting various aspects of a plant's growth, development, and interaction with the environment. Phenols, including flavonoids and phenolic acids,

are crucial components of a plant's antioxidant defense system. They scavenge reactive oxygen species (ROS) and protect plant cells from oxidative stress. Phenols are involved in the plant's defense against microbial pathogens. Upon pathogen invasion, plants often increase the production of phenolic compounds, which can inhibit pathogen growth and act as signaling molecules for the activation of defense responses. They can act as secondary messengers in various signal transduction pathways. They modulate gene expression and regulate numerous physiological processes, such as the activation of stress-responsive genes or the coordination of plant defense mechanisms. When Plants exposed to abiotic stresses, such as drought, salinity, or extreme temperatures, often exhibit altered phenol production. Phenolic compounds help plants adapt to adverse environmental conditions by regulating osmotic balance, reducing water loss, and maintaining cellular integrity. In the context of our experiment on plant tissue culture, monitoring total phenol production can be a valuable indicator of the physiological state of cultured plant tissues. It can reflect the stress response, growth potential, and overall health of the cultured plants under different conditions.

Chlorophyll plays a crucial role in photosynthesis, the fundamental process by which plants, algae, and some bacteria convert light energy into chemical energy in the form of glucose (sugar). Chlorophyll production *in vitro,* or within plants grown in a controlled laboratory environment, can be influenced by various factors. Achieving optimal chlorophyll levels is important for the photosynthetic capacity and overall health of the plants. The decrease in chlorophyll content observed in *in vitro* conditions can indeed be attributed to various factors, including chloroplast malformation resulting from limited gaseous exchange and elevated relative humidity within the culture vessels (Martins *et al.,* 2015; Sáez *et al.,* 2016). Culture vessel ventilation would allow better gaseous exchange and lead towards healthy growth of plantlets.

Proteins, as fundamental biomolecules, play a pivotal role in the intricate process of plant growth during *in vitro* propagation. These macromolecules are of paramount significance as they serve as the building blocks for cellular structures and are intricately involved in various biochemical pathways. An exhaustive exploration of protein-related processes is essential for a comprehensive understanding and

enhancement of plant growth in controlled *in vitro* conditions, where nutrient availability can markedly differ from natural settings (Zhao *et al.,* 2019). In plant biology, various proteins are crucial for regulating growth, development, and responses to environmental cues. Proteins involved in signal transduction, transcriptional regulation, and enzymatic activities are essential components of the molecular machinery orchestrating growth in plants. For instance, transcription factors such as MYB and bHLH proteins have been shown to modulate gene expression related to growth and differentiation in plant tissue cultures (Liu *et al.*, 2013). Additionally, enzymes like kinases and phosphatases play critical roles in signal transduction pathways, influencing cellular responses to external stimuli (Wang *et al.,* 2019).

Furthermore, the proteomic composition of plant tissues under different environmental conditions and growth stages remains an area of active research. Previous studies have examined the protein profiles of various plant tissues to gain insights into the dynamic regulation of proteins during growth and in response to changing environmental factors (Manivannan *et al.,* 2015; Chauhan *et al.,* 2018). However, there is a notable gap in our understanding of how protein metabolism is modulated in geophytes, especially when cultivated in different growth condition in *in vitro* systems.

5.1.1 Materials and methods

Throughout the various phases of micropropagation in different growth condition, as well as in fully grown plants in natural conditions, significant alterations occur in the composition of biochemical substances such as carbohydrates, protein, proline, chlorophyll and the enzyme superoxide dismutase (SOD). These changes are of particular interest for understanding the growth and development of plants. were studied.

Following four stages were chosen for the biochemical studies:

Stage M – *In vitro* shoot multiplication

Stage R – *In vitro* rooting

Stage A – *In vitro* hardening

Stage F – Field grown mature plants

Healthy leaves were gathered from multiplying shoots, rooted, and hardened plantlets. We also collected fresh twigs from plants grown in the field and kept them cool during transport to the lab. The leaves underwent a cleaning process to get rid of any dirt or medium stuck to them, and excess water was removed by pressing them with tissue paper. Subsequently, the leaves were broken down into tiny pieces using the right buffer or solvent, followed by centrifugation. The liquid that settled at the top after centrifugation, known as the supernatant, was used as the plant extract and subjected to analysis. To measure various reactions, we used a spectrophotometer to read the absorption of the mixtures (Shimadzu UV1800). Qualitative estimations were performed at the appropriate wavelength, and we made these estimations by referring to the corresponding standard curves. These standard curves served as a reference to determine the characteristics of the substances we were studying.

Leaves collected from various samples were homogenized with the help of pestle mortar in 0.2 M phosphate buffer (pH 6.2) and centrifuged at 3000 rpm for 15 min. One ml of the supernatant was mixed with 4.0 ml of 0.2 percent anthrone reagent (in concentrated H_2SO_4) and placed on water bath for 5 min (as described by Tandon, 1976). Absorbance was read spectrophotometrically at 610 nm.

Proline in the plant sample was determined following Bates *et al.* (1973). Proline was selectively extracted from 0.5 g fresh tissue, using 10 ml aqueous sulphosalicylic acid (3%). The homogenate was filtered using Whatman No. 1 filter paper. To 2.0 ml of fresh extract, was added 2.0 ml of glacial acetic acid and 2.0 ml of acid ninhydrin (1.25 g ninhydrin dissolved in a mixture of 30 ml warm glacial acetic acid and 20 ml of 6 M phosphoric acid with agitation and used within 24 h when stored at 4°C). The mixture was heated in boiling water bath for 1 h. The reaction was terminated by transferring the reaction tubes to ice and to it was added 4.0 ml toluene followed by stirring on a cyclomixer (Remi) for 20–30 s. After thawing it to room temperature, the toluene layer (upper one, red in colour) was separated and the absorbance was measured at 520 nm. The proline concentration was determined from standard curve.

Measurement of SOD activity was essentially based on methods of Beauchamp and Fridovich (1971). For extraction of SOD enzyme, leaves were thoroughly ground in ice–cold mortar in chilled extracting medium (5 ml/g fresh leaf

tissue) containing 0.1 M K_2HPO_4 and 0.1 M ethylenediamine tetra acetic acid, sodium salt (EDTA); pH 8.0. The resulting homogenate was centrifuged at 13,000 X g for 20 min at 4 °C. Aliquots of the supernatant (hereafter referred to as crude SOD extract) were taken for determination of SOD activity. The reaction mixture (2.5 ml) in the SOD assay comprised of 1.3 □M riboflavin, 13 mM L–methionine, 0.05 M Na_2CO_3, pH 10.2, 63 □M *p*–nitroblue tetrazolium chloride (NBT, Hi–Media) and 0.1 ml crude SOD extract. The reaction mixture was incubated in a light chamber for 15 min. Under these conditions, riboflavin is excited by a photon and is able to oxidize an electron donor molecule (here L–methionine). This donation of an electron results in the production of a superoxide ion (O^{2-}) which reduces NBT, giving an insoluble purple blue formazan. The change in color was measured spectrophotometrically at A_{560}. The enzyme SOD competes for the superoxide ion resulting in to a reduction in the level of formazan being produced. One unit of SOD is defined as the amount of enzyme necessary to produce a 50% inhibition of the maximum inhibition. It should be noted that even extremely concentrated forms of the enzyme cannot lead to a 100 percent inhibition, and so the 50 percent mark is defined as being the midpoint of no inhibition and maximal (but not complete) inhibition.

a – Length of path light in the cell

V – Volume of the extract in ml

W – Fresh weight of the sample in g

Chlorophyll contents were also analyzed as a biochemical parameter during the multiplication phase on liquid and semi–solid medium in all the three plant species. The results were further compared with their field grown counterparts. Leaves were ground in excess of chilled 80% acetone (Arnon, 1949). Homogenized mixture was filtered through filter paper. Absorbance of the filtrate was read at 663, 654 and 645 nm. The chlorophyll contents were calculated by using the following formulae:

$$\text{Total Chlorophyll (mg/g)} = \frac{20.2 \times A_{645} + 8.02 \times A_{663}}{a \times 1000 \times W} \times V$$

$$\text{Chlorophyll a (mg/g)} = \frac{12.7 \times A_{663} - 2.69 \times A_{645}}{a \times 1000 \times W} \times V$$

$$\text{Chlorophyll b (mg/g)} = \frac{22.9 \times A_{645} - 4.68 \times A_{663}}{a \times 1000 \times W} \times V$$

5.1.2 Results

In the context of the total sugar content in different experiments, a pronounced variation was observed when compared to control plants at different stages of plant tissue culture. During the multiplication stage, the highest total carbohydrate content was recorded, amounting to 319.3 mg/FW, (Table 5.3) in plants grown in a liquid culture medium enriched with CO_2 Enrichment. Subsequently, in the semi-solid media (Table 5.2), the plants exhibited a higher total carbohydrate content compared to those grown in either a liquid culture or different vessel conditions.

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 175 mg/FW. This value was significantly different from the plant total carbohydrate content observed in various experimental conditions. In this study, it was also observed that the provision of external CO_2 had a notable impact on the total carbohydrate content of the plants, resulting in a content of 391.3 mg/FW when compared to non-enriched plants. Simultaneously, the shape of the culture vessels exhibited a significant influence on the total carbohydrate content, with the highest levels found in vessel 5 (210.66 mg/FW) (Table 5.4).

During the rooting stage, an increase in carbohydrate content was observed in the control plants (211 mg), liquid culture medium (255 mg), and vessels (228 mg). The highest carbohydrate content was observed in the CO_2-enriched liquid culture treatment (391 mg) in comparison to the solid medium with CO2 enrichment (235 mg). On the other hand, the addition of seaweed (table 5.6) and altering gelling agents (table 5.5) show a significant effect on total carbohydrate content when compared to the control plant but when we compare that to different culture vessel and CO_2 Enrichment it is not much more significant. These findings suggest that both CO_2 enrichment and the shape of the culture vessels play pivotal roles in influencing plant carbohydrate content during different stages of plant tissue culture. Additionally, the use of seaweed and altering gelling agents did not significantly impact the total carbohydrate content in this experimental context**.**

In the realm of *in vitro* plant propagation, Proline emerges as a pivotal participant in the intricate orchestration of plant growth. These crucial macromolecules, Proline, are not only fundamental as the elemental building blocks

for cellular structures but are also intimately woven into a myriad of essential biochemical pathways. In the course of our investigation into Proline dynamics during different stages of micropropagation and under varying environmental conditions, we made several noteworthy observations. Different growth condition effect on Proline Production. In liquid culture (8.79 µg/g) (figure 5.1) with Glass marble as a supporting matrix, we noted a moderate proline accumulation, which was higher compared to the CO_2-enriched liquid culture. This indicates that the absence of CO_2 enrichment is conducive to a relatively higher Proline content. As well as Culture Vessels (10.67 µg/g) provided an environment that supported a notable proline accumulation, indicating the influence of the vessel and its surroundings on proline production. These results were consistent throughout different growth stages. We are finding proline content in multiplication stage with different culture condition. In liquid culture with CO_2 Enrichment (10 gm m^{-3} with 3% Sucrose) (5.26 µg/g), the Proline content was found to be at its minimum. This finding indicates that the presence of CO_2 enrichment in a liquid culture medium appears to hinder Proline accumulation during plant growth. In Solid Medium (9.13 µg/g), we observed a slightly higher Proline content compared to the CO_2-enriched liquid culture medium. The solid medium, with its physical support and potentially not more favorable conditions, allows for a higher Proline accumulation. In Control Plants (12.56 µg/g), the Proline content was higher than in the CO_2-enriched and without in liquid culture that suggesting that typical growth conditions promote greater Proline production during micropropagation. These results were consistent throughout different growth stages. In the rooting stage, we noted a 2-3% increase in total proline accumulation compared to the multiplication stage. These findings illuminate the intricate relationship between Proline and plant growth in the context of *in vitro* propagation. They highlight the significant impact of growth conditions, with CO_2 enrichment and growth medium type playing important role in plant growth.

Superoxide Dismutases (SOD) represent pivotal enzymatic components is protecting plants against oxidative stress. They achieve this by catalyzing the conversion of detrimental superoxide radicals into less injurious compounds. In the context of our investigation, we embarked on the quantification of SOD levels across distinct growth stages and under varying *in vitro* environmental conditions. Our

analysis revealed that the SOD activity was notably reduced in plants cultured in a CO_2-enriched environment when subjected to a solid medium (107 U/mg FW) (Figure 5.2a) as opposed to a liquid medium (119 U/mg FW) (Figure 5.2b). 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 SOD production, with solid medium vessels yielding in V1 and V5 428.750 U/mg FW (Figure 5.1b), a significant departure from the control group, which recorded 459.025 U/mg FW. When using alter gelling agent phyta gel (392.67 U/mg FW) (figure 5.1c) shows significantly decrease compare to other gelling agents. Notably, when comparing the effects of a liquid medium versus a solid medium on plant SOD production, (figure 5.1a) 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.

During the rooting stage of development, a noteworthy increase in SOD activity was documented. This increase is indicative of heightened production of reactive oxygen species (ROS) by the plant, marking a critical aspect of the plant's response to its environment.

Phenolic compounds represent the second most significant group of plant products engaged in plant defense mechanisms against both biotic and abiotic stresses. In our study, we focused on the quantitative assessment of phenolic content at various stages of micropropagation, specifically during the multiplication and rooting stages.

During the multiplication stage, we observed noteworthy variations in phenol concentrations among different growth conditions. When subjected to a controlled environment with semisolid medium enriched with CO_2, plant samples exhibited a significantly higher phenol content, registering at 103 ug/g, in comparison to the control (133.46 ug/g), which displayed a phenol concentration of 95 mg/g. Notably, the application of liquid medium across all treatments resulted in nearly uniform phenol production, indicating a uniform and significant impact on plant growth across the experimental groups, as summarized in the accompanying table. Conversely, in the rooting stage, we noted a general increase in phenol production across all experimental treatments, as detailed in the provided table. When comparing plant samples subjected to solid and liquid media, we found that a substantial elevation in

phenol content was exclusively evident in plants treated with semi solid medium. This finding underscores the role of growth medium composition in modulating phenol production during the rooting stage.

Chlorophyll production in *in vitro*, or within plants cultivated under controlled laboratory conditions, is subject to modulation by a range of factors. Achieving optimal chlorophyll levels is of paramount importance as it directly impacts the photosynthetic capacity and, consequently, the overall vigor of the plants. In our study, we observed substantial variations in chlorophyll content during the multiplication stage, with notable disparities among the experimental treatments, specifically liquid medium, culture vessels, and CO_2 enrichment within semi-solid medium (figure 5.3). In the semi-solid medium control group, the chlorophyll content was determined to be 0.45 mg/g FW (fresh weight) this is compare to other gelling agent lower (Figure 5.5c), when phytagel use in growth medium total chlorophyll (0.565 mg/g FW) show significantly increase. Notably, a marked increase in chlorophyll content was observed in the presence of CO_2 enrichment in the liquid culture medium (figure 5.4), where the chlorophyll content was recorded at approximately 0.95 mg/g FW. This observation signified a nearly twofold augmentation in chlorophyll content as compared to the semi-solid medium control. When we change vessels size for plan growth its show higher chlorophyll in V1 (0.678 mg/g FW) (figure 5.5b) shows the significantly increase simultenasly liquid culture show higher chlorophyll (0.723 mg/g FW) content in GM supported plant (figure 5.5a). Same result record in seaweed treated plant there is no major change in total chlorophyll contain observe in (figure 5.6) The results were further corroborated by statistical analysis, indicating a significant difference in total chlorophyll content among the experimental groups (refer to the table 5.7). These findings underscore the importance of the medium composition and CO_2 enrichment in influencing chlorophyll production during the multiplication stage. The enrichment of culture vessels with CO_2 in the liquid medium led to a substantial increase in chlorophyll content, highlighting its potential for enhancing the photosynthetic performance and health of the cultured plants.

In the context of protein, we follow Bradford methos for the estimation of total protein from the different growth condition. In this investigation when we compare

protein content in Liquid and solid growth medium grown plant we found not major change in concentration , in study 1 when we use different supporting material in liquid culture medium GM (279.083 mg/g FW) supporting plant shows higher value and other support matrix pebbles (275.75 mg/g FW), tissue paper (252.41 mg/g FW) also show increase of protein compare or control plant (240.75 mg/g FW) but when we use only liquid medium without supporting matrix its show lower concentration compare to control. When we compare study 1 with gelling agent contain medium shows minor change in protein contain, higher protein contains reported in phytagel (255.58 mg/g FW) using supporting material. Same we reported in Study 3 and 4 but in study 3 when we increase vessels size it will impact on protein contain. Vessels 1 (272.41 mg/g FW) were reported in study and lower vessels 2 (255.25 mg/g FW) but all the vessels show significantly increase in protein contain compare to control vessels. Base on this result same culture medium play important role in plant growth we observed in study 5 and study 6 in this experiment we study CO_2 Enrichment with liquid and solid culture growth medium effect on plant growth. In both studies SFSM (160.25 mg/g FW) and SFLM (166.33 mg/g FW) show lower concentration of protein compare to plant grown in sucrose contain in liquid and solid growth medium. The higher protein concentration were reported in 3% sucrose with CO_2 enrichment in liquid (354.66 mg/g FW) and solid (266.00 mg/g FW) growth medium.

Table 5.1 Effect of semi–solid and liquid culture medium on some biochemical parameters of banana during multiplication stages of growth.

Medium	Total Carbohydrate (mg/g Fresh tissue)	Total Phenol (mg/g Fresh tissue)	Total protein (mg/g Fresh tissue)
GM	215.550 a	80.080 e	279.083 a
Tissue paper	185.550 c	93.427 d	252.417 b
PA	197.550 b	113.447 c	275.750 a
Liquid	175.550 e	133.467 b	240.750 c
Control	180.550 d	153.487 a	229.083 d
SEM	1.591615	2.709204	1.828536
CD 5%	1.519594	1.795363	2.696448
CD 1%	*4.580551*	*5.657305*	*8.496682*

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

Table 5.2 Effect of co_2 enrichment on solid medium on some biochemical parameters of banana during multiplication stages of growth.

	Sucrose	CO_2 conc. ($g\ m^{-3}$)	Total Carbohydrate mg/g Fresh tissue	Total Phenol mg/g Fresh tissue	Total protein mg/g Fresh tissue
SFSM	0.0 %	0	0.000 j	0.000 l	0.000 n
		0.6	100.149 h	50.867 j	173.250 k
		10	121.251 g	80.267 h	161.250 m
		40	129.250 f	80.267 h	190.500 i
		GR	63.350 i	31.733 k	160.750 m
SFSM	1.0 %	0	120.750 g	156.333 b	165.500 l
		0.6	160.200 d	112.000 f	180.750 j
		10	173.350 c	77.467 h	208.500 g
		40	148.750 e	67.200 i	221.750 f
	2.0 %	0	144.750 e	189.933 a	204.250 h
		0.6	174.850 c	137.667 c	234.750 e
		10	193.250 b	119.467 de	236.250 e
		40	194.500 b	103.600 g	246.750 c
	3.0 %	0	157.000 d	105.467 g	211.000 g
		0.6	190.200 b	123.200 d	247.500 c
		10	235.550 a	111.533 f	266.000 a
		40	193.250 b	117.600 e	255.500 b
		GR	175.550 c	133.467 c	240.750 d
		SEM	2.657933	2.954633	2.954633
		CD 5%	1.975694	1.704026	1.704026
		CD 1%	5.601738	4.887415	4.887415
		CV	7.460078	6.553567	6.553567

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 5.3 Effect of CO_2 Enrichment on liquid medium on some biochemical parameters of banana during multiplication stages of growth.

	Sucrose	CO_2 conc. ($g\ m^{-3}$)	Total Carbohydrate mg/g Fresh tissue	Total Phenol mg/g Fresh tissue	Total protein mg/g Fresh tissue
SFLM	0.0%	0	0.000 k	0.000 k	0.000 n
		0.6	166.333 i	36.333 i	231.000 k
		10	196.667 h	57.333 g	215.000 m
		40	215.000 g	57.333 g	254.000 i
		Gr	97.333 j	22.667 j	214.333 m
SCLM	1.0%	0	195.000 h	111.667 b	220.667 l
		0.6	264.667 e	80.000 ef	241.000 j
		10	287.000 d	55.333 g	278.000 g
		40	245.333 f	48.000 h	295.667 f
	2.0%	0	240.667 f	135.667 a	272.333 h
		0.6	292.667 d	98.333 c	313.000 e
		10	325.667 b	85.333 de	315.000 e
		40	315.000 c	74.000 f	329.000 c
	3.0%	0	265.333 e	75.333 f	321.000 d
		0.6	319.333 bc	88.000 d	281.333 g
		10	391.333 a	79.667 ef	330.000 c
		40	325.000 b	84.000 de	354.667 a
		GR	286.667 d	95.333 c	340.667 b
		SEM	3.77933	3. 4633	3.9633
		CD 5%	2.97594	2.7040	2.4026
		CD 1%	5. 1738	5.88741	5. 7415
		CV	8. 0078	7.55367	8.553567

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 5.4 Effect of culture vessels on some biochemical parameters of banana during multiplication stages of growth.

Vessel type	Total Carbohydrate (mg/g Fresh tissue)	Total Phenol (mg/g Fresh tissue)	Total protein (mg/g Fresh tissue)
C	175.550 d	133.467 a	244.083 c
V_1	228.215 a	73.407 e	272.417 a
V_2	193.105 c	100.100 c	255.250 bc
V_3	191.350 c	86.753 d	261.917 ab
V_4	196.616 c	106.773 b	269.250 a
V_5	210.660 b	89.423 d	268.583 a
SEM	1.73718	2.688139	2.593991
CD 5%	1.730659	1.525931	3.922572
CD 1%	5.142053	4.701861	12.08665
CV	7.045046	6.591673	16.94462

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

Table 5.5 Effect of different gelling agents on some biochemical parameters of banana during multiplication stages of growth.

Medium	Total Carbohydrate (mg/g Fresh tissue)	Total Phenol (mg/g Fresh tissue)	Total protein (mg/g Fresh tissue)
Agar	175.550 b	133.467 b	12.560 b
Phyta Gel	195.550 a	134.801 b	10.792 c
Guar gum	177.550 b	149.483 a	12.932 ab
Isabgol	165.550 c	146.813 a	11.054 c
starch	160.550 d	153.487 a	14.188 a
SEM	1.737175	2.64393	1.948273
CD 5%	1.519594	2.192171	2.802529
CD 1%	4.580551	6.907673	NS
CV	6.332577	9.825374	NS

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 seaweed extract on some biochemical parameters of banana during multiplication stages of growth.

Liquid Seaweed Extract (LSE)	Concentration of LSE (% v/v)	Total carbohydrate mg/g Fresh tissue	Total phenol mg/g Fresh tissue	Total protein mg/g Fresh tissue
Caulerpa racemosa	10	176.950 b	1.030 h	183 h
	30	191.950 d	1.230 e	189 e
	50	213.655 a	1.300 d	210 d
Gracilaria corticata	10	184.705 f	1.230 e	225 e
	30	185.905 e	1.450 b	231 b
	50	190.555 d	1.530 a	234 a
Caulerpa paspaloides	10	153.355 i	1.300 d	240 d
	30	156.580 hi	1.470 b	246 b
	50	174.550 h	1.520 a	267 a
Ulva lactuca	10	151.705 i	1.030 h	216 h
	30	158.935 hi	1.120 g	225 g
	50	199.900 c	1.250 e	231 e
Sargassum tenerrimum	10	153.250 i	1.240 e	249 e
	30	183.355 f	1.360 c	267 c
	50	214.945 a	1.500 a	275 a
Caulerpa sertularioides	10	148.450 i	1.050 h	231 h
	30	177.205 g	1.110 g	237 g
	50	183.670 f	1.360 c	240 c
Sargassum wightii	10	177.250 g	1.140 g	234 g
	30	188.845 d	1.190 f	246 f
	50	197.005 c	1.250 e	249 e
Gracilaria edulis	10	164.200 h	1.060 h	237 h
	30	181.345 g	1.160 f	255 f
	50	176.950 b	1.030 h	183 h
SEM		1.73718	2.688139	2.593991
CD 5%		1.730659	1.525931	3.922572

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 5.7 Effect of semi–solid and liquid culture medium on some biochemical parameters of banana during different stages of growth.

Stage	Medium	Total Carbohydrate (mg/g FW) ± SD	SOD Activity (U mg/g FW) ± SD	Proline Content (µ moles/g FW ± SD	Chlorophyll Content (mg/g FW) ± SD		
					Chl a	Chl b	Total Chl
M	**SM**	175.00 ±8.85	392.51 ±14.11	10.64 ±0.32	0.188±0.02	0.383±0.023	0.565±0.03
	LM	215.00 ±10.99	511.80±14.60	13.11±2.29	0.24±0.01	0.49±0.02	0.723±0.04
R	**SM**	390.09±5.10	180.91±4.72	17.70±1.47	–	–	–
	LM	420.82±12.90	203.86±6.88	28.40±5.60	–	–	–
A	**SM**	240.07±10.92	245.61±43.02	08.55±2.16	0.322±0.02	0.269±0.02	0.742±0.13
	LM	270.77 ±12.61	350.10±19.98	15.66±1.51	0.439±0.01	0.526±0.03	1.268±0.03
F		320.34 ±0.18	244.44±4.16	5.45 ±0.02	0.655±0.04	0.764±0.04	1.723±0.08
	SEM	0.7753	11.4436	1.4793	0.0186	0.0203	0.0548
	CD 5%	23.517	34.7105	4.487	0.0645	0.0703	0.1895
	CD 1%	32. 662	48.2078	6.2318	0.0976	0.1064	0.2871
	CV	7.48	5.01	4.04	6.83	6.76	7.62
M	**SM**	175.00 ±8.85	392.51 ±14.11	10.64 ±0.32	0.188±0.02	0.383±0.023	0.565±0.03

M– Multiplication Stage; R– Rooting Stage; A–in vitro hardening Stage; F– Field grown Mature Plant; SM – Solid Medium and LM – Liquid Mediu

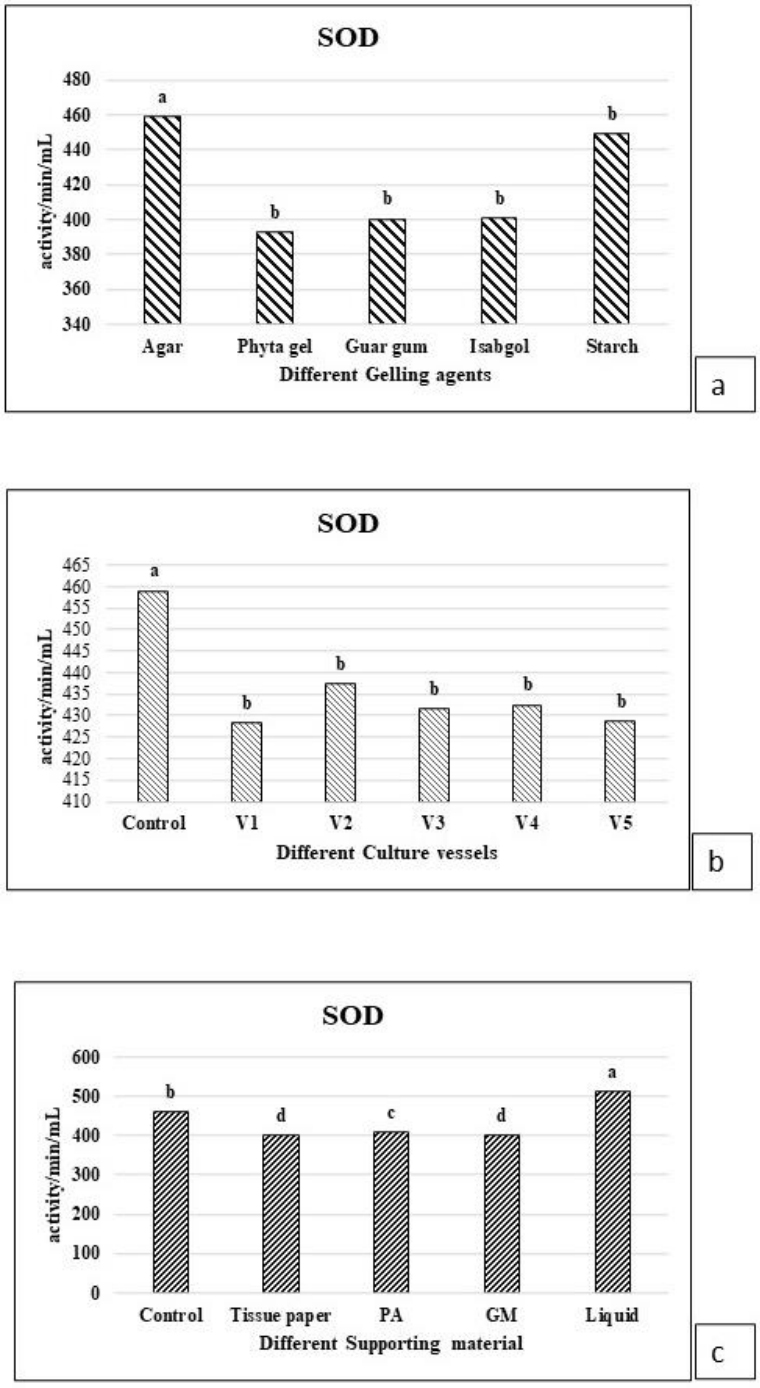


Figure: 5.1 Superoxide Dismutase (SOD) activity of micropropagated *Musa acuminata* during the multiplication of plantlets under various growth conditions. The figure shows (A) the effects of different gelling agents; (B) the effects of different culture vessels; and (C) the effects of different supporting materials.

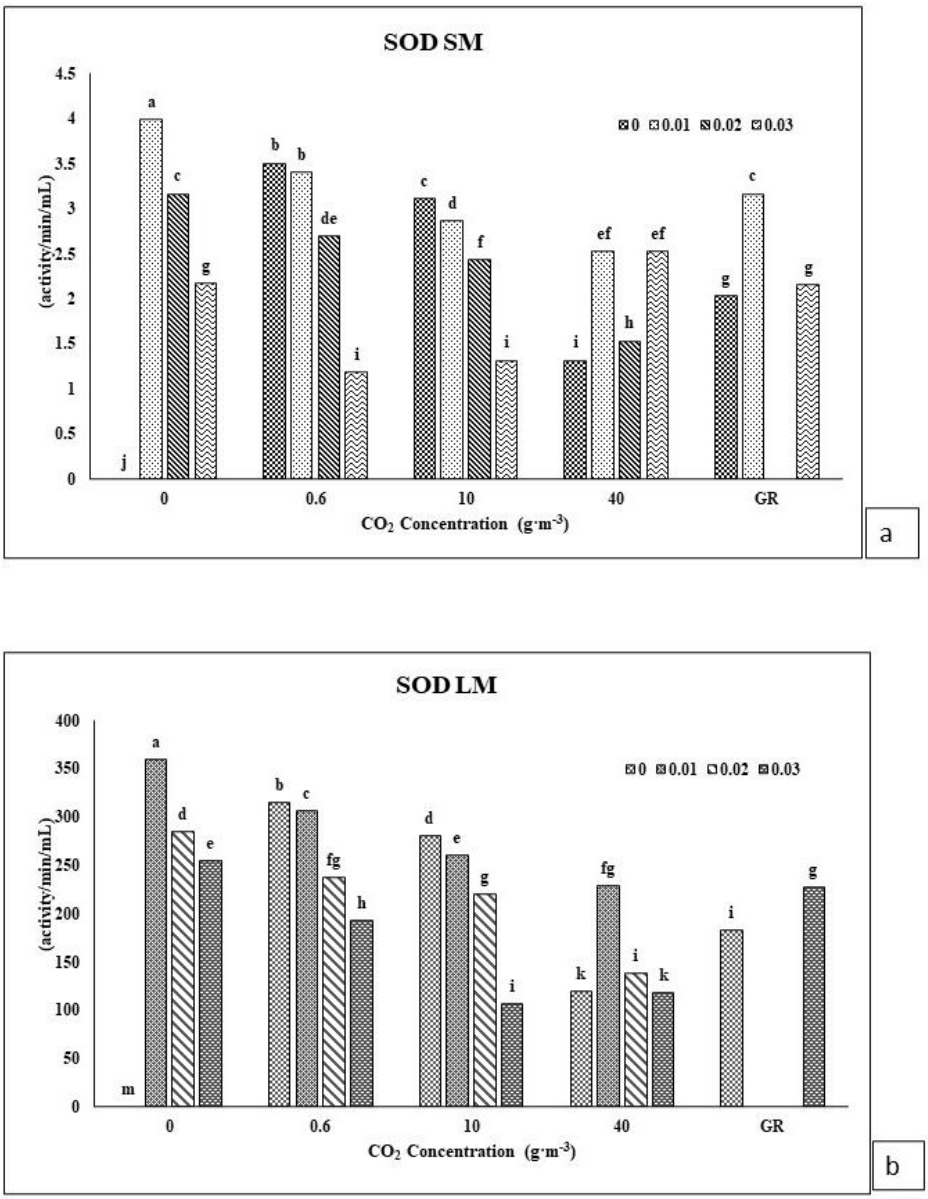
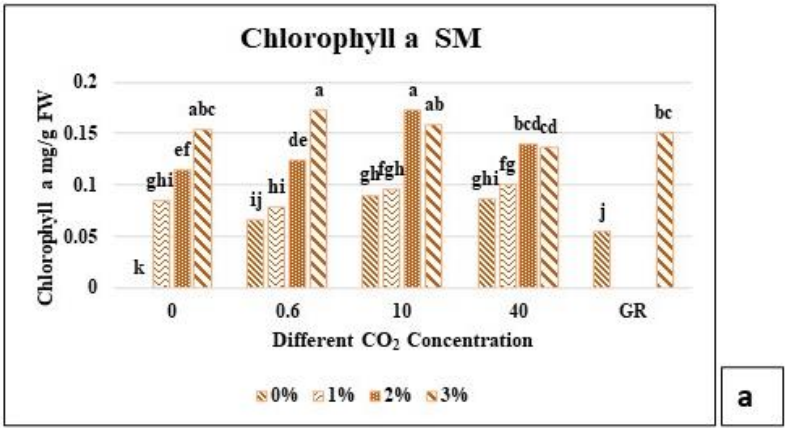
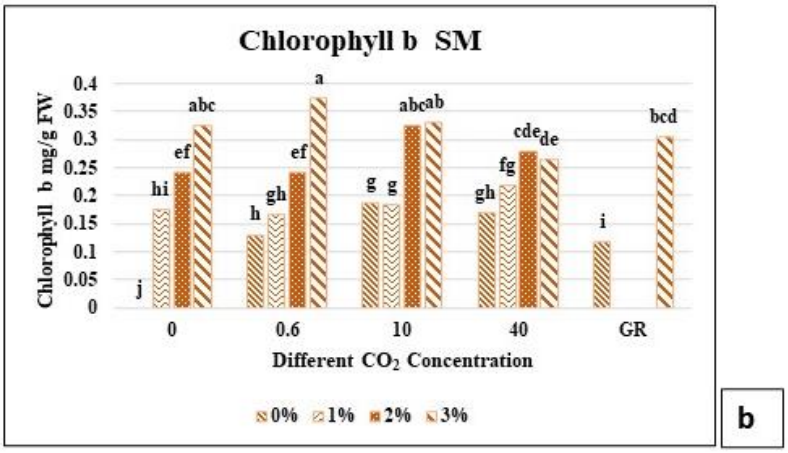


Figure: 5.2 Superoxide Dismutase (SOD) activity of micropropagated *Musa acuminata* during the multiplication of plantlets under CO_2 Enrichment condition during multiplication stage. In figure shows the **(a)** Effects of CO_2 Enrichment with SS medium; **(b)** Effects of CO_2 Enrichment with liquid medium.





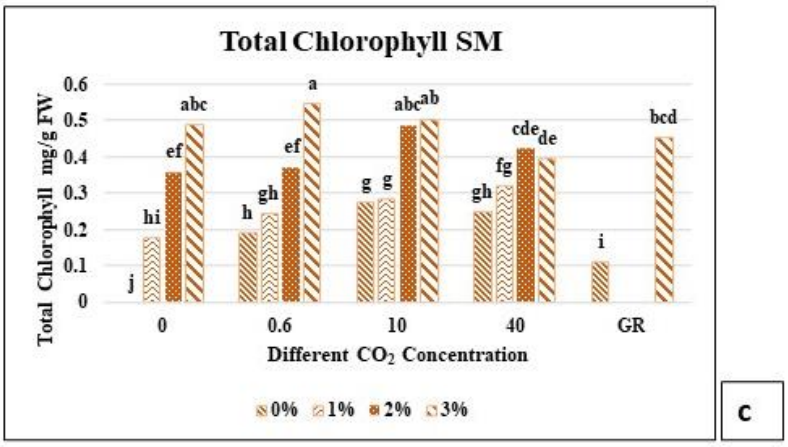


Figure:5.3 Chlorophyll activity of micropropagated *Musa acuminata* during the multiplication of plantlets under CO_2 enrichment on semi solid medium in multiplication stage. The figure shows **(A)** Chlorophyll a, **(B)** Chlorophyll b, **(C)** Total chlorophyll.

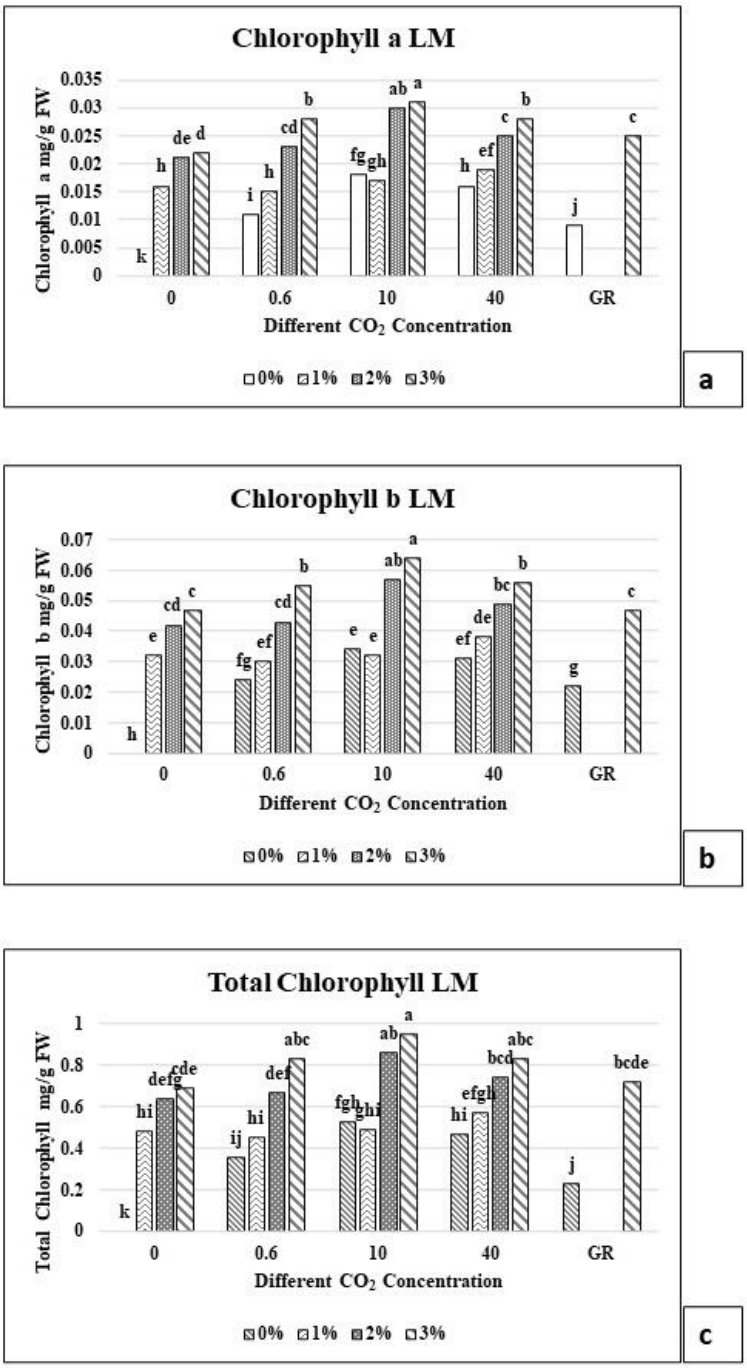


Figure:5.4 Chlorophyll activity of micropropagated *Musa acuminata* during the multiplication of plantlets under CO_2 enrichment on on liquid medium in multiplication stage. The figure shows **(A)** Chlorophyll a, **(B)** Chlorophyll b, **(C)** Total Chlorophyll

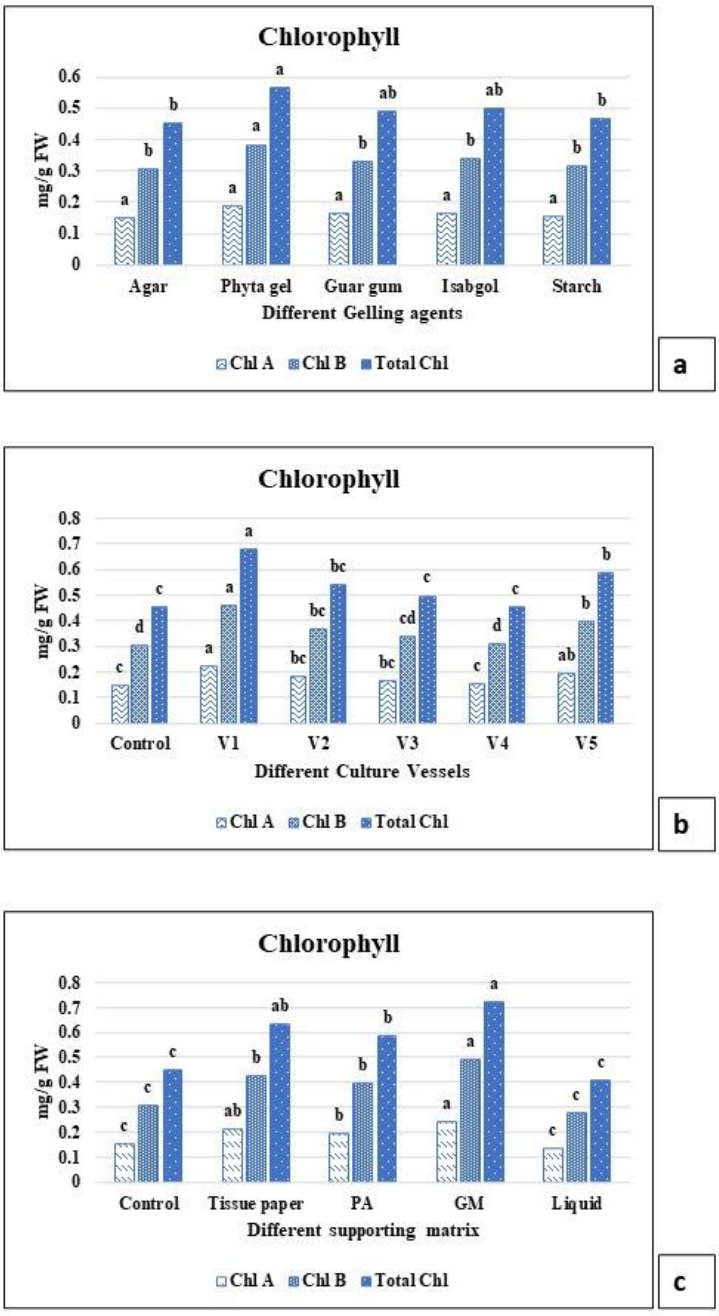


Figure:5.5 Chlorophyll activity of micro propagated *Musa acuminata* during multiplication of plantlet under different growth condition. The figure shows (A) the effects of different gelling agents; (B) the effects of different culture vessels; and (C) the effects of different supporting materials.

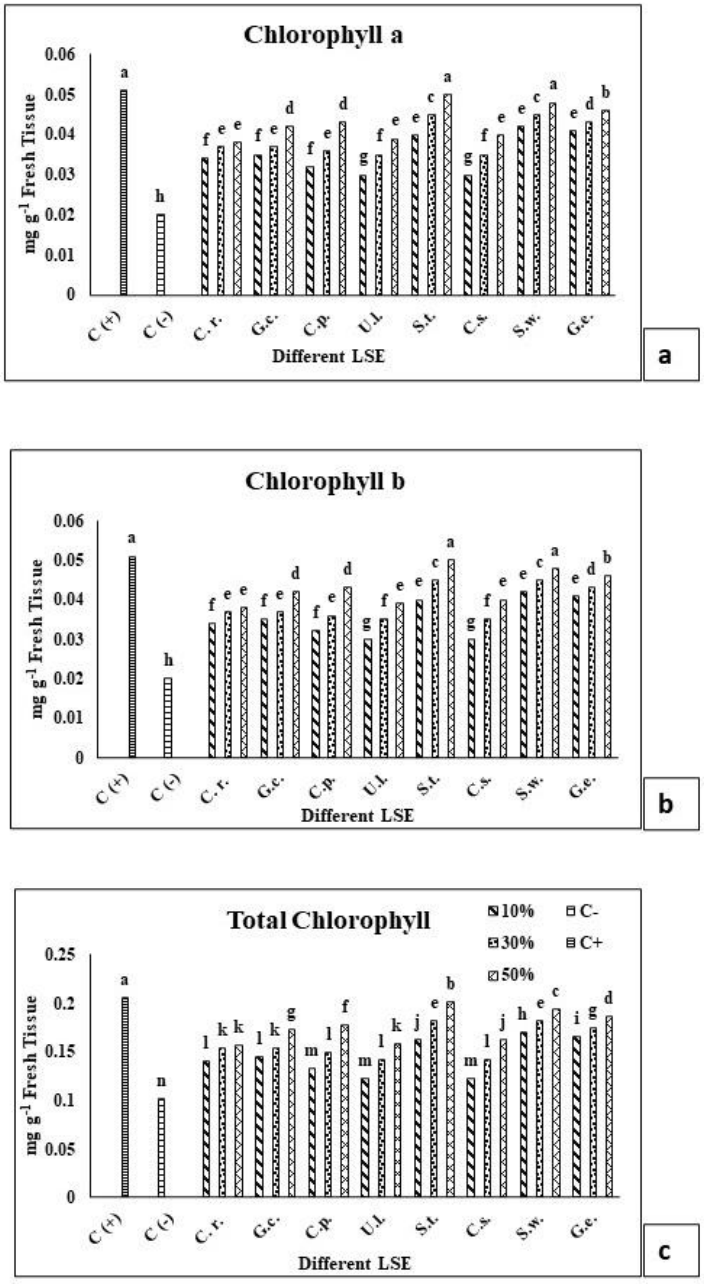


Figure: 5.6 Effect of different LSE on accumulation of Chlorophyll a (A), Chlorophyll b (B) and total chlorophyll (C) in banana micropropagules grown under in vitro conditions. C (+) represent – positive control plant grown on a standard MS medium with PGRs, C (-) represent – negative control plant grown under standard MS medium without PGRs; *C.r., G.c., C.p., U.l., S.t., C.s., S.w*. and *G.e*. represent *C. racemosa, G. corticata, C. paspaloides , U. lactuca, S. tenerrimum, C. sertularioides, S. wightii* and *G. edulis*, respectively