Chapter 4

Micropropagation in Altered Growth Condition-Physiological Studies

Introduction

Conditions conducive to rapid shoot growth and reproduction in tissue culture often result in the emergence of plants exhibiting structural and functional abnormalities. These irregularities can manifest in various ways, significantly impacting the physiological functions of the plant. One common manifestation involves abnormalities in chloroplasts, pivotal for photosynthesis. In such instances, chloroplasts may malfunction, diminishing photosynthetic efficiency, thereby impeding the plant's ability to convert sunlight into nourishment. Furthermore, these anomalous plants may encounter challenges in regulating water content. Excessive water accumulation within cells can arise, leading to complications such as heightened transpiration rates, exacerbating water loss. Excessive water loss can render the plants inactive, potentially leading to the depletion of crucial photosynthetic enzymes, thereby further compromising their capacity to harness energy from sunlight (Sharma *et al*., 2020). Despite outward appearances of health, these plantlets often exhibit diminished photosynthetic activity, struggling to synthesize their own nutrients from sunlight, even amidst continued growth and reproduction. Moreover, rooting poses significant challenges for these plantlets. They may either fail to root altogether or, if successful, struggle to establish themselves firmly in soil. Consequently, these plants encounter difficulties in both survival and optimal growth when transplanted into natural soil conditions (Monja-Mio *et al*., 2021; Pirata *et al*., 2022).

Multiple factors contribute to the aberrant physiology observed in plants cultivated *in vitro*, with one significant aspect being the composition of the growth medium. The precise blend of nutrients and substances within the medium profoundly influences the developmental trajectory and functionality of the plants (Pirata *et al*., 2022), as well as the behaviour of vessel types, whether they remain open or close

(Manokari *et al*., 2022). Furthermore, the concentration of carbon dioxide (CO_2) in the container's headspace where the plants are cultured also plays a crucial role in their abnormal physiology. The availability of CO_2 directly impacts photosynthetic rates and overall metabolic processes, thereby exerting profound effects on growth and development (Kozai *et al*., 2005). Indeed, various other factors can impede the physiological progress of plants cultured *in vitro*. Factors contributing to the hindrance of physiological development encompass the utilization of growth media characterized by elevated concentrations of sucrose and salt, alongside the maintenance of subdued light levels within the culture vessel. Elevated sucrose and salt levels in the growth medium can disturb the plant's natural osmotic equilibrium and ion regulation, consequently impacting its overall well-being.

4.1 Studies on water relations

Numerous studies have highlighted the stimulating influence of liquid growth medium on shoot growth in *in vitro* plant cultures. When plants are cultivated in a liquid medium rather than a solid one, they typically exhibit enhanced shoot growth (Gatambia *et al*., 2016; Gatti *et al*., 2017; Nápoles Borrero *et al*., 2017; Arigundam *et al*., 2020; Hwang *et al*., 2022; Muhammet, 2022; Shekhawat *et al*., 2022). The degree of solidification or the consistency of the growth medium not only affects shoot regeneration but also influences water management. Shoots cultured in a liquid medium tend to possess higher water content compared to those grown in a more solidified medium (Malik *et al*., 2018). Sreelakshmi *et al*. (2021) specifically quantified water content, focusing on hyperhydricity, in *Dianthus chinensis* L. cultures. A significant factor contributing to the frequent failure of tissue culture-derived plantlets to thrive post-transfer from the controlled laboratory environment (*ex vitro*) is excessive dehydration, termed desiccation (Zein El Din *et al*., 2020). This occurs due to the plantlets' challenges in sustaining the proper water balance, resulting in dysfunction within their water management system. The disturbance in this "water housekeeping" equilibrium can lead to mortality, underscoring its importance as a critical consideration when transitioning these plants from laboratory settings to more natural or greenhouse conditions (Gaspi *et al*., 2013). Plantlets of various species commonly encounter excessive water loss and rapid wilting if they are not maintained in a high-humidity environment during their transition from *in vitro* culture to *ex vitro*

conditions (outside the controlled lab setting). Numerous studies have delved into investigating and comprehending the dynamics of water loss during the acclimatization of micropropagated plants to natural environments. For instance, Gonçalves *et al*. (2017) reported on this phenomenon in *P. algarbiensis* and *P. almogravensis*, while Duan *et al*. (2020) explored it in *Trichosanthes kirilowii*.

4.1.1 Materials and methods

Water loss

We conducted an experiment utilizing detached leaves of approximately uniform size sourced from five different plants across various stages of micropropagation. These leaves were procured from plants cultivated on two distinct types of growth media. Initially, we measured the weight of these leaves while they were still fresh and then monitored their water loss rate. To facilitate transpiration, we arranged the leaves with their abaxial side facing upward on a sanitized bench, within a room temperature range of 25 to 28°C and a relative humidity (RH) of 35 to 40%. We recorded the weight of each leaf at 15-minute intervals over a span of 3 hours. Subsequent to the transpiration experiment, we subjected all leaves to oven-drying at 60°C to ascertain their dry mass. The quantification of water loss followed the methodology outlined by Salomon *et al*. (2014).

$$WL\ (\%) = (FWt0 - DWt) - (FWt - DWt) \times 100 / (FWt0 - DWt)$$

where WL is the percentage of water loss, FWt0 is fresh weight at time zero, FWt is fresh weight after time, and DWt is dry weight.

Biomass accumulation and water content

The fresh and dry weights were determined by weighing shoot clusters on a Top Pan Electronic Balance (Shimazu) while wet and after overnight drying at a temperature of 60°C in a hot air oven, respectively. The percentage of water content and dry weight were calculated using the following formula:

$$\text{Percent Water Content} = [(\text{Fresh Weight} - \text{Dry Weight}) / \text{Fresh Weight}] \times 100$$

This formula allowed us to quantify the amount of water present in the shoot clusters and determine their dry weight.

The water content was analysed during the following experiments:

Experiments

1. Role of different culture vessel's type with semi-solid medium
2. Role of liquid medium in different vessel's type with different support materials
3. Role of different gelling agent
4. Role of seaweed
5. Role of CO_2 enrichment semi-solid medium
6. Role of CO_2 enrichment liquid medium
7. Role of polyamines

4.1.2 Results

Water loss

Studies of experiments 1 to 4

In evaluating the growth performance of *in vitro* cultivated plants, quantifying fresh weight (FW) and dry weight (DW) provides valuable insights. The total mass of micropropagules directly correlates with the plant's functionality, reflecting factors such as photosynthetic capacity, nutrient availability, and environmental conditions. Notably, shoot clusters grown in a liquid medium exhibited significant increases in both fresh and dry weight, as well as water content, compared to those cultivated in the control condition of agar-based solid medium. The use of different vessel types in semi-solid medium resulted in a significant increase in both total fresh weight and dry weight. Among the culture vessels tested, V7 displayed the highest fresh weight at 3.95 gm and dry weight at 0.39 gm, with a water content of 90.03% (Table 4.1). In contrast, V5 exhibited the lowest fresh and dry weights, measuring 1.27 gm and 0.12 gm, respectively. The use of different vessel types in a liquid medium resulted in a significant increase in both total fresh weight and dry weight. Specifically, employing glass marbles as support material in the liquid medium led to a rise in biomass. Among the culture vessels tested, V7 displayed the highest fresh weight at 4.0 gm and dry weight at 0.39 gm, with a water content of 90.03% (Table 4.2). In contrast, V5 exhibited the lowest fresh and dry weights, measuring 1.32 gm and 0.13 gm,

respectively. The use of water balls as a support material in the liquid medium led to an increase in biomass. Among the culture vessels examined, V7 showed the highest fresh weight at 3.90 gm and dry weight at 0.37 gm, with a water content of 90.30% (Table 4.3). In contrast, V5 exhibited lower fresh and dry weights, measuring 1.23 gm and 0.11 gm, respectively.

The use of tissue paper as a support material in the liquid medium led to an increase in biomass. Among the culture vessels examined, V7 showed the highest fresh weight at 3.80 gm and dry weight at 0.36 gm, with a water content of 90.31% (Table 4.4). In contrast, V5 exhibited lower fresh and dry weights, measuring 1.13 gm and 0.10 gm, respectively. Using cotton as a support material in the liquid medium led to an increase in biomass. Among the culture vessels examined, V7 exhibited the highest fresh weight at 3.60 gm and dry weight at 0.35 gm, with a water content of 90.05% (Table 4.5). In contrast, V5 showed lower fresh and dry weights, measuring 0.93 gm and 0.09 gm, respectively. Among all the vessels type glass marble use as support material give more effect results than cotton as support material in liquid medium. Using agar as a gelling agent led to a significant increase in total fresh weight, recorded at 3.18 gm, and dry weight, measured at 0.31 gm, compared to other gelling agents (Table 4.6). This indicated higher quantitative values along with a water content of 90.01%. Conversely, the medium containing starch exhibited a reduced fresh weight.

Utilizing *Sargassum tenerrimum's* Liquid Seaweed Extract (LSE) at a 50% concentration (v/v) in the growth medium significantly increased biomass. This concentration notably enhanced the fresh weight, reaching 4.12 gm, and the dry weight, recorded at 0.19 gm, as shown in the table 4.7. This concentration had a particularly beneficial effect on biomass augmentation. Additionally, an overall increase in biomass was observed at a 30% concentration across all Liquid Seaweed Extracts (LSE) investigated. This broader trend suggests a general positive effect on the biomass of plant specimens when exposed to various concentrations of LSE. Conversely, it's important to note that the application of *Caulerpa racemosa's* Liquid Seaweed Extract had a detrimental effect on biomass accumulation. The recorded values were 2.89 gm for fresh weight and 0.15 gm for dry weight, indicating a

significant reduction in biomass compared to the control or other concentrations of seaweed extracts.

Studies of experiments 5 and 6

Diverse outcomes in total biomass production and water content were observed when shoot clusters were cultivated on various mediums such as SCSM, SCLM, SFSM, and SFLM, supplemented with different concentrations of CO_2. The addition of sucrose to the medium enhanced the effect of CO_2 enrichment, resulting in higher fresh and dry weights compared to cultures grown in sucrose-free mediums (Tables 4.8 and 4.9). When comparing shoot cultures in solid and liquid mediums, it was evident that the liquid medium significantly stimulated both biomass and moisture accumulation during the *in vitro* multiplication of rose plants. Under CO_2-free conditions in SCSM cultures, the lowest fresh weight and dry weight were recorded at 2.11 gm and 0.21 gm, respectively, with a water content of 90.07%. Cultures exposed to ambient air in the growth room displayed similar results. As CO_2 concentration increased from 0.03% to 2%, there was a significant enhancement in both wet and dry weight accumulation, peaking at 2% CO_2, along with a rise in total moisture content. The physiological parameters in SCLM cultures were consistently higher than those in SCSM cultures across all CO_2 concentrations. A 2% concentration in the liquid medium was optimal for fresh weight (2.17 gm) and dry weight (0.27 gm) accumulation, resulting in an overall increase in water content to 87.44%. In sucrose-free conditions (SFSM and SFLM), while biomass production was not as high as in sucrose-containing cultures, the water content ranged between 87% and 89%. Notably, although sucrose-free conditions did not improve biomass production compared to sucrose-containing cultures, the water content in all sucrose-free cultures was lower than in their sucrose-containing counterparts. Cultures grown under CO_2-free conditions and in the growth, room showed high water content, ranging from approximately 86% to 89%, in both semi-solid and liquid media, and displayed severely hyperhydric shoots. As CO_2 concentration increased, there was a consistent rise in both fresh and dry weight, along with water content. The maximum wet weight of 0.22 gm was achieved by SFLM cultures at a 2% CO_2 concentration, with a water content of 86.90%. In SFSM cultures under 2% CO_2, the fresh weight

was 0.22 gm, the dry weight was 0.023 gm, and the water content (89.94%) was lower compared to the control (SCSM under ambient air).

Studies of experiment 7

A similar trend like shoot length and numbers was observed for total biomass (fresh and dry weight), a low concentration of PAs (10 mM) led to a significant increase in biomass compared to the control. Biomass continued to increase at 30 mM and 50 mM PAs, though at 50 mM, it decreased slightly relative to the 30 mM concentration (Table 4.12). An upward trend in shoot fresh and dry weight was observed with 30 mM Spd, achieving values of 3.24 g and 0.36 g, respectively, while similar effects were noted with Put and Cad.

4.2 Carbonic anhydrase enzyme activity

Carbonic anhydrase (CA), also known as carbonate hydrolyase (EC 4.2.1.1), is an enzyme crucial for facilitating the reversible conversion of bicarbonate into carbon dioxide (CO_2). This zinc-containing enzyme is ubiquitous across various organisms, including animals, plants, archaea, and eubacteria. Its role spans multiple physiological processes, including ion exchange, acid-base balance maintenance, catalysing carboxylation and decarboxylation reactions, and facilitating inorganic carbon movement within and outside the cell. Essentially, carbonic anhydrase serves as a versatile catalyst contributing to essential cellular and metabolic functions in diverse life forms (DiMario *et al*., 2018; Supuran, 2018). In several plant species, a notable linear relationship has been observed between the relative growth rate (RGR) of plantlets and their carbonic anhydrase (CA) activities. This suggests a direct correlation between plantlet CA activity and growth rates *in vitro*. Simply put, higher CA activity in plantlets corresponds to increased net photosynthetic rates and faster growth (Ahmad *et al*., 2018).

4.2.1 Materials and methods

To analyse enzyme activity, we followed a procedure originally outlined by Wilbur and Anderson (1948), making slight modifications to accommodate the specific requirements of our experiment. Approximately 1.0 gram of leaves was harvested from cultures grown on both liquid and semi-solid growth media. These leaves were then homogenized in 10 ml of a buffer solution containing 20 mM Tris-Cl

(pH 8.0). Similarly, leaf extracts from field-grown plants were prepared using the same method. To separate the extracts, centrifugation was conducted at 8000 RPM for 10 minutes at a temperature of 4°C. A 500 µL sample was extracted from the supernatant and added to 30 mL of Tris-Cl Buffer. Subsequently, 15 mL of CO2-saturated water, obtained from a local supplier, was promptly added to the mixture. The pH of the solution was closely monitored, and the time taken for the pH level to decrease from its initial value of 8.0 to 6.3 was recorded.

Enzyme activity was calculated by using the following formula:

$$WAU = 2 (To - T) / T$$

Where, To = Time without enzyme, T = Time with enzyme,
WAU = Wilburs Anderson Unit

4.2.2 Results

Table 4.10 outlines the Carbonic Anhydrase (CA) activities observed in the leaves of rose plants. The field-grown plant, serving as the control, demonstrated the lowest CA activity at 26.91 WAU (Weighted Activity Units) per gram of fresh tissue. In contrast, leaves grown in the semi-solid medium (SM) showed a significant increase in enzyme activity, with a total activity of 36.35 WAU per gram of fresh tissue. Conversely, the leaves from the liquid culture (LM) exhibited markedly higher enzyme activity. This medium recorded an enzyme activity of 62.80 WAU, which was notably higher than that observed in the SM leaves.

Similar results were noted with CO_2 enrichment. Plants exposed to higher CO_2 concentrations in the semi-solid medium showed an enzyme activity of 47.57 WAU per gram of fresh tissue (Table 4.11). In contrast, plants grown in a liquid medium under CO_2 enrichment exhibited significantly higher CA activity, reaching 75.82 WAU per gram of fresh tissue.

Table 4.1 Effect of different vessel's types on water content and other growth parameters in rose during *in vitro* shoot multiplication grown on semi-solid medium (Observations were recorded after 45 days)

Vessel's type	Fresh weight (gm)	Dry weight (gm)	Percent water content
V1	2.200 d	0.219 d	97.90
V2	1.470 e	0.149 e	98.63
V3	2.880 c	0.288 c	97.22
V4	2.187 d	0.220 d	97.91
V5	1.270 f	0.129 f	98.83
V6	3.513 b	0.353 b	96.59
V7	3.950 a	0.394 a	96.15
CV	0.471	1.039	
SEM	0.007	0.002	
CD 5%	0.021	0.005	
CD 1%	0.029	0.006	

(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 4.2 Effect of different vessel's types with use of glass marble as support material on water content and other growth parameters in rose during *in vitro* shoot multiplication grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Fresh weight (gm)	Dry weight (gm)	Percent water content
V1	2.250	0.224	97.85
V2	1.520	0.154	98.58
V3	2.930	0.293	97.17
V4	2.237	0.225	97.86
V5	1.320	0.134	98.78
V6	3.563	0.358	96.54
V7	4.000	0.399	96.10
CV	0.462	1.019	
SEM	0.007	0.002	
CD 5%	0.021	0.005	
CD 1%	0.029	0.006	

(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 4.3 Effect of different vessel's types with use of water balls as support material on water content and other growth parameters in rose during *in vitro* shoot multiplication grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Fresh weight (gm)	Dry weight (gm)	Percent water content
V1	2.157 d	0.205 d	97.94
V2	1.437 e	0.135 e	98.66
V3	2.840 c	0.275 c	97.26
V4	2.150 d	0.206 d	97.95
V5	1.233 f	0.115 f	98.86
V6	3.447 b	0.336 b	96.65
V7	3.907 a	0.379 a	96.19
CV	0.881	0.907	
SEM	0.012	0.001	
CD 5%	0.038	0.004	
CD 1%	0.053	0.005	

(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 4.4 Effect of different vessel's types with use of tissue papers as support material on water content and other growth parameters in rose during *in vitro* shoot multiplication grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Fresh weight (gm)	Dry weight (gm)	Percent water content
V1	2.057 d	0.195 d	98.04
V2	1.337 e	0.125 e	98.76
V3	2.740 c	0.265 c	97.36
V4	2.050 d	0.196 d	98.05
V5	1.133 f	0.105 f	98.96
V6	3.347 b	0.326 b	96.75
V7	3.807 a	0.369 a	96.29
CV	0.918	0.947	
SEM	0.012	0.001	
CD 5%	0.038	0.004	
CD 1%	0.053	0.005	

(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 4.5 Effect of different vessel's types with use of cotton as support material on water content and other growth parameters in rose during *in vitro* shoot multiplication grown on liquid medium (Observations were recorded after 45 days)

Vessel's type	Fresh weight (gm)	Dry weight (gm)	Percent water content
V1	1.857 d	0.185 d	98.24
V2	1.137 e	0.115 e	98.96
V3	2.540 c	0.255 c	97.56
V4	1.850 d	0.186 d	98.25
V5	0.933 f	0.095 f	99.17
V6	3.147 b	0.316 b	96.95
V7	3.607 a	0.359 a	96.49
CV	1.003	0.991	
SEM	0.012	0.001	
CD 5%	0.038	0.004	
CD 1%	0.053	0.005	

(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 4.6 Effect of different gelling agents on water content and other growth parameters in rose during *in vitro* shoot multiplication (Observations were recorded after 45 days)

Media	Fresh weight (gm)	Dry weight (gm)	Percent water content
Agar	3.188 a	0.318 a	96.91
Phyta gel	3.157 b	0.315 b	96.94
Guar gum	2.177 c	0.218 c	97.92
Isobgol	2.146 d	0.214 d	97.95
Starch	2.110 e	0.209 e	97.99
CV	0.353	0.454	
SEM	0.005	0.001	
CD 5%	0.016	0.002	
CD 1%	0.023	0.003	

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 4.7 Percent water content and other growth parameters in rose grown under LSE (Observations were recorded after 45 days)

Liquid Seaweed Extract (LSE)	Concentration of LSE (% v/v)	Fresh weight (gm)	Dry weight (gm)	Percent water content
Caulerpa	10	3.130 bcde	0.148 b	96.92
racemosa	30	3.260 abcd	0.159 b	96.79
	50	2.893 cde	0.157 b	97.16
Ulva lactuca	10	3.520 abcd	0.162 b	96.53
	30	3.560 abcd	0.169 b	96.49
	50	3.480 abcd	0.162 b	96.57
Sargassum	10	3.780 abc	0.179 b	96.27
tenerrimum	30	3.890 ab	0.182 b	96.16
	50	4.120 a	0.191 b	95.93
Sargassum wightii	10	3.560 abcd	0.158 b	96.48
	30	3.650 abc	0.165 b	96.40
	50	3.460 abcd	0.148 b	96.58
Gracilaria edulis	10	3.420 abcd	0.478 a	96.72
	30	3.520 abcd	0.152 b	96.52
	50	2.331 e	0.147 b	97.73
MS medium with standard PGR		3.960 ab	0.159 b	96.08
MS medium without standard PGR		2.640 de	0.125 b	97.41
CV		13.973	78.155	
SEM		0.276	0.081	
CD 5%		0.793	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 4.8 Percent water content and other growth parameters in rose grown under semi-solid medium and CO_2 enriched conditions (Observations were recorded after 21 days)

	Sugar Concentration (%)	CO_2 Concentration (%)	Fresh weight (gm)	Dry weight (gm)	Percent water content
SFSM	**0**	0.000	0.000 n	0.000 h	00.00
		0.030	0.205 m	0.021 g	99.90
		0.500	0.215 lm	0.022 g	99.89
		2.000	0.225 kl	0.023 g	99.88
		GR	0.220 lm	0.022 g	99.88
SCSM	**1**	0.000	0.217 lm	0.022 g	99.89
		0.030	0.226 kl	0.023 g	99.88
		0.500	0.237 jk	0.023 g	99.86
		2.000	0.257 i	0.025 fg	99.84
		GR	0.245 ij	0.025 fg	99.86
	2	0.000	0.350 h	0.036 efg	99.75
		0.030	0.425 g	0.045 ef	99.68
		0.500	0.545 f	0.054 e	99.55
		2.000	0.842 d	0.085 d	99.26
		GR	0.738 e	0.075 d	99.36
	3	0.000	1.198 c	0.122 c	98.90
		0.030	2.104 b	0.212 a	98.00
		0.500	2.127 a	0.187 b	97.96
		2.000	2.116 ab	0.210 a	97.98
		GR	2.110 b	0.207 a	97.99
		CV	1.148	15.810	
		SEM	0.005	0.007	
		CD 5%	0.014	0.019	
		CD 1%	0.019	0.025	

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 4.9 Percent water content and other growth parameters in rose grown under liquid medium and CO_2 enriched conditions (Observations were recorded after 21 days)

	Sugar Concentration (%)	CO_2 Concentration (%)	Fresh weight (gm)	Dry weight (gm)	Percent water content
SFLM	**0**	0.000	0.000 k	0.000 a	00.00
		0.030	0.201 j	0.025 b	99.92
		0.500	0.216 ij	0.028 b	99.91
		2.000	0.229 ij	0.030 b	99.90
		GR	0.210 ij	0.025 b	99.92
SCLM	**1**	0.000	0.202 j	0.025 b	99.92
		0.030	0.235 hij	0.031 b	99.89
		0.500	0.265 hi	0.032 b	99.86
		2.000	0.287 h	0.035 b	99.84
		GR	0.253 hij	0.031 b	99.87
	2	0.000	0.755 g	0.094 c	99.37
		0.030	0.958 f	0.120 d	99.17
		0.500	1.220 e	0.152 e	98.91
		2.000	1.560 d	0.195 f	98.57
		GR	1.250 e	0.155 e	98.88
	3	0.000	2.153 ab	0.271 h	97.97
		0.030	2.157 ab	0.269 h	97.97
		0.500	2.110 bc	0.265 gh	98.02
		2.000	2.177 a	0.273 h	97.95
		GR	2.060 c	0.255 g	98.07
		CV	3.402	3.689	
		SEM	0.018	0.002	
		CD 5%	0.052	0.007	
		CD 1%	0.069	0.009	

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 4.10 Carbonic anhydrase enzyme activity in the leaves of rose during *in vitro* growth on semi-solid and liquid medium, compared with leaves obtained from field grown plants (Observations recorded after 45 days)

Media	**CA Activity (WAU/g fresh tissue) ± SD**
F	26.91 ± 1.63
SM	36.35 ± 2.77
LM	62.80 ± 2.53
CV	5.618
SEM	1.363
CD 5%	4.716
CD 1%	7.146

F= Field grown leaves; SM = Semi–solid medium; LM= Liquid medium; SD - Standard Deviation; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation

Table 4.11 Carbonic anhydrase enzyme activity in the leaves of rose during *in vitro* growth CO_2 enrichment on semi-solid and liquid medium, compared with leaves obtained from field grown plants (Observations recorded after 45 days)

Media	**CA Activity (WAU/g fresh tissue) ± SD**
F	26.91 ± 1.63
SM	47.57 ± 1.94
LM	75.82 ± 1.57
CV	3.433
SEM	0.993
CD 5%	3.437
CD 1%	5.207

F= Field grown leaves; SM = Semi–solid medium; LM= Liquid medium; SD - Standard Deviation; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation

Table 4.12 Effect of different polyamines concentration on *in vitro* growth of rose in biomass accumulation during micropropagation

Polyamines	**Polyamines concentration (mM)**	**Fresh weight (gm)**	**Dry weight (gm)**
Control	0	3.188 a	0.318 a
Spermidine	10	3.218 bc	0.343 d
	30	3.248 e	0.369 g
	50	3.238 de	0.348 e
Putrescine	10	3.208 abc	0.343 d
	30	3.228 cd	0.369 g
	50	3.208 abc	0.348 e
Cadaverine	10	3.198 ab	0.328 b
	30	3.218 bc	0.358 f
	50	3.198 ab	0.335 c

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