Chapter 4

Micropropagation in Altered Growth Condition-Physiological Studies

Introduction

Conditions in tissue culture that encourage fast shoot growth and reproduction often lead to the development of plants that have structural and functional abnormalities. These abnormalities can have various impacts on the plant's physiology. For example, it's common to see issues with chloroplasts, which are essential for photosynthesis. In these abnormal plants, chloroplasts may not work correctly, causing poor efficiency in photosynthesis. This means the plant struggles to produce food from sunlight. Additionally, these abnormal plants can have problems with water regulation. They might accumulate too much water inside their cells, leading to issues like increased transpiration rates, which means they lose water quickly. This excessive water loss can make them inactive or even cause the absence of important photosynthetic enzymes, further hampering their ability to make energy from sunlight (Sharma *et al.,* 2020)**.** Even though these plantlets might seem healthy on the outside, they often aren't actively performing photosynthesis. In other words, they struggle to make their own food from sunlight, even if they keep growing and reproducing at a reasonable pace. However, when it comes to rooting, these plantlets usually face challenges. They either can't be rooted at all, or if they can, they don't establish themselves successfully in soil. These plants have difficulties surviving and thriving when they are planted in real soil (Monja-Mio *et al.,* 2021; Pirata *et al.,* 2022).

Several factors have been identified as contributing to the abnormal physiology of plants raised *in vitro,* and one of these factors is the composition of the growth medium. The specific mix of nutrients and substances in the medium can greatly influence how the plants develop and function (Pirata *et al.,* 2022), vessels types close or open (Manokari *et al.,* 2022). Another factor that plays a significant role in the abnormal physiology of *in vitro*-raised plants is the concentration of carbon dioxide (CO_2) in the headspace of the container where they are grown. The amount of CO_2 available to the plants can affect their photosynthesis and overall metabolic processes, which in turn can impact their growth and development (Kozai *et al.,* 2005). Indeed, there are several additional factors that can limit the physiological development of *in vitro* cultured plants. These include the use of growth

media with high sucrose and salt concentrations, as well as maintaining low light levels within the culture vessel. High levels of sucrose and salt in the growth medium can disrupt the plant's normal osmotic balance and ion regulation, affecting its overall health. Similarly, low light levels can hinder photosynthesis, which is crucial for the plant's energy production and growth (Chen *et al.*, 2019; Cioć *et al.*, 2019). It's often recommended to measure photosynthetic parameters during the *in vitro* development of plants. These measurements serve as indirect indicators of any underlying physiological disorders. By assessing photosynthetic parameters like the rate of photosynthesis, stomatal conductance, and chlorophyll fluorescence.

4.1 Studies on chlorophyll fluorescence

Chlorophyll fluorescence represents a key characteristic observed in photosynthetic organisms, offering an invaluable non-invasive technique for the assessment of photosynthesis within its natural habitat (Runcie and Riddle, 2004). The analysis of chlorophyll fluorescence stands as a straightforward yet highly effective method for evaluating photosynthetic efficiency. Within the realm of photosynthetic reactions, the light energy absorbed by chlorophyll molecules in a leaf exhibits three potential outcomes: (i) it can be utilized to propel photosynthesis (photochemistry), (ii) surplus energy may be dissipated as heat, or (iii) it can be re-emitted as light-chlorophyll fluorescence. These processes engage in a competitive interplay, where an increase in the efficiency of one process consequently leads to a decrease in yield in the other two. Therefore, by quantifying the yield of chlorophyll fluorescence, valuable insights into changes in the efficiency of photochemistry and heat dissipation can be gleaned (Maxwell and Johnson, 2000).

Kautsky (Kautsky *et al.*, 1960) observed that the transition of photosynthetic material from darkness to light induced a rapid surge in chlorophyll fluorescence within approximately 1 second. This phenomenon, elucidated subsequently, stems from the diminished electron acceptors in the photosynthetic pathway downstream of Photosystem 2 (PS 2), primarily affecting plastoquinone and particularly QA. Once PS 2 absorbs light and QA captures an electron, it cannot immediately accept another until it transfers the first electron to a subsequent electron carrier (QB). This sequence leads to a closed state of the reaction center. The presence of closed reaction centers at any given time diminishes the overall efficiency of photochemistry, resulting in a proportional increase in fluorescence

yield (Maxwell and Johnson, 2000). This reduction in photochemical efficiency is directly linked to the heightened fluorescence emission.

The quantification of chlorophyll fluorescence is a pivotal technique offering insights into a plant's capacity to endure environmental pressures and the resulting impact on the photosynthetic machinery (Maxwell and Johnson, 2000). Over time, numerous coefficients have emerged to quantify photosynthetic processes by gauging chlorophyll fluorescence, thereby elucidating photosynthetic efficiency. Among these, the quantum yield of chlorophyll fluorescence (ΔF/Fm; Genty *et al.,* 1989) stands as one of the most significant. A swift (< 1 s) application of intense light induces full reduction of the PS II electron acceptor QA. Measuring the intensity of fluorescence from light-adapted samples before and during the flash (variable fluorescence) allows for the determination of ΔF/Fm'. This value is then obtained by multiplying each ΔF/Fm' by the respective ambient irradiance (measured in units of µmol quanta $m^{-2}s^{-1}$ of photosynthetically active radiation - PAR), thus estimating the relative electron transport rate (rel ETR) (Genty *et al.,* 1989). Chlorophyll fluorescence as an indicator of photosynthetic functioning of *in vitro* grapevine and chestnut plantlets under *ex vitro* acclimatization has been reported by (Carvalho *et al.,* 2001; Schreiber and Klughammer, 2021) and chlorophyll fluorescence in micro propagated *Rhododendron* in response to different irradiances has been reported by Osorio *et al.* (2010). Several other studies involving measurements of chlorophyll fluorescence include those carried out by (Chaari-Rkhis *et al.,* 2015; Corrêa *et al.,* 2015; Pan *et al.,* 2019; Sharma *et al.,* 2020) for different purposes and during plant micropropagation.

4.1.1 Materials and methods

During the *in vitro* growth phase, chlorophyll fluorescence parameters were assessed using a Li-Cor 6400; Li-Cor, Inc., Lincoln, NE, USA facility with the help of Dr. k. k. Pal (Groundnut Research Center Junagadh). The adaxial leaf surface was maintained at a distance of 11 cm from the actinic light source using the leaf clamp of the instrument. To prevent shadowing on the leaf surface, the LED light source was positioned at a 60° angle to the leaf blade. Before the initial measurement, a 30-minute dark adaptation of the leaf was conducted. The measurement process started with the determination of the background/minimal fluorescence (F0) from a dark-adapted leaf when solely the LED light was activated, emitting a very weak light intensity (3-5 mol m^{-2} s^{-1}), inadequate for driving photosynthesis. Following exposure to a modulated weak light, a 0.8-second saturating

pulse exceeding 2800 µmol m^{-2} s^{-1} PAR was applied to the leaf, and the maximal fluorescence (Fm) of the dark-adapted leaf was recorded. The quantum yield (P = Fv/Fm) of the dark-adapted leaf was then calculated as (Fm - F0)/Fm. The leaves were subsequently illuminated with white actinic radiation (45 ± 5 µmol m^{-2} s^{-1}) in the presence of the cultures. Subsequently, a 0.8-second saturating flash of 2800 µmol m^{-2} s^{-1} PAR was administered to determine the maximum level in the light-adapted state (Fm'). The steady-state value of fluorescence (Fs) was recorded by applying saturating pulses of high light intensity every 20 seconds for 10 minutes. The quantum efficiency of PS 2 photochemistry (ΦPS2) was computed as follows (Genty *et al.,* 1989), ΦPS2 = (Fm' - Fs)/Fm'. Upon deactivation of the "actinic light," far-red irradiation (3-5 µmol m^{-2} s^{-1}) was employed to determine F0'. Subsequently, the photochemical quenching (qP) and thermal energy dissipation at the PS 2 reaction centers (qN) were determined using the equations qP = (Fm' - Fs)/(Fm' - F0') and qN = 1-(Fm' - F0')/Fm-F0 (Genty *et al.,* 1989). The apparent photosynthetic electron transport rate (ETR) was derived as ETR = 0.5 × 0.84 × (ΔF/Fm') × PPFD, where the factors 0.5 and 0.84 accommodate the presumed equal excitation of both PS 2 and PS 1 reaction centers and a light reflection of 16.0%, respectively (Schreiber *et al.,* 1994). Fluorescence values were determined for 6 leaves of almost similar leaf area growing under similar conditions. The leaves were detached prior to evaluation and placed, with adaxial surface facing upwards, on the leaf clamp.

Study of photosynthetic and gas exchange parameters

For studying different photosynthetic and gas exchange parameters *viz.* Maximum photosynthetic rate (Amax); Maximum carboxylation rate (Vcmax); Intercellular CO_2 concentration (Ci) (where Rubisco and RuBP regeneration are equally limiting *i.e.* Ac=Aj); Chloroplast CO_2 concentration (Cc) (where Rubisco and RuBP regeneration are equally limiting *i.e.* Ac=Aj); ratio between the electron transport rate (J) and the maximum rate of Rubisco carboxylation (Vcmax) (*i.e.* J/Vcmax); total conductance between intercellular spaces and chloroplast (gm), we used portable gas exchange system (Li-Cor 6400; Li-Cor, Inc., Lincoln, NE, USA). Throughout our measurements, we maintained a consistent temperature of 25°C. To ensure accurate readings and data, we closely monitored and regulated leaf vapor pressure deficits, maintaining values within the targeted range of 0.5 to approximately 1.6 kPa. Banana shoots were taken out just before the reading to avoided photoinhibition, water stress or triose-phosphate utilization limitation. For calculation of

all the parameters we followed the model equations and parameters as defined in Ethier and Livingston (2004).

Experimentations

1. Photosynthetic efficiency during different stages of micropropagation on semi-solid and liquid medium compared with field grown leaves.
2. Photosynthetic efficiency during *in vitro* multiplication under CO_2 enriched environment.

Experiment 1.

4.1.2 Results

A study of the fluorescence peaks in the Kautsky's plots, as acquired through the Qubit's fluorometer, elucidated a substantial array of distinctions between leaves cultivated in the field and those cultured *in vitro* (Figure 4.1 a). The dark-adapted control leaves exhibited F0 and Fm values of 0.50 and 3.02, respectively. Upon exposure to light, a maximal fluorescence (Fm') of 3.0 was achieved, diminishing to a steady-state fluorescence (Ft) of 0.56 within a duration of five to eight minutes. The calculated value of ΦPS2 was 0.81, and an ETR rate of 55.27, under approximately 200 µmol m^{-2} s^{-1} of incident light, was determined for field-grown leaves. The fraction of open reaction centers was established at 0.13 (with F0' at 0.4), and non-photochemical quenching exhibited negligible significance (Table 4.1).

The F0 values observed in both SM and LM leaves were elevated compared to the control, whereas a decline in their Fm values was noted following a brief exposure to high light intensity. The minimum values of Fv/Fm (0.76 and 0.77 at SM and LM, respectively) occurred during the multiplication phase of the *in vitro* period. These values were 0.90 times lower than the control (0.84 in field-grown leaves) but remained within the standard normal range (0.85-0.60). These observations indicated an approximate 15-23% reduction in intrinsic quantum efficiency during this stage. The Fm' values were lower than the control for both SM (0.19) and LM (0.29) during *in vitro* multiplication. The values of φPS2 recorded in SM, LM, and control leaves were 0.79, 0.77, and 0.81, respectively. This suggests that SM leaves could absorb 73.17% of incident light, while LM leaves redirected 90.71% of light energy towards underlying photochemical processes, compared to 93.75% in the control. In *in vitro* multiplying plants on both media types, ETR was poor, approximately 6 times less than in field-grown plants.

Comparisons of outcomes obtained from cultures in semi-solid (SM) and liquid medium (LM) revealed that the medium consistency did not exert a statistically significant influence on the photochemical quenching of fluorescence, as depicted in Figure 4.1 b and c. This is evidenced by the F'0 values of 0.12 and 0.21 for SM and LM, respectively. The proportion of open reaction centers (qP) during this phase was akin to the control in both SM (0.91) and LM (0.92) leaves. However, Minimal loss of fluorescence (qN) due to thermal dissipation was observed. Upon subjecting the shoots to *in vitro* rooting (Figure 4.1 d, e), a reduction in F0 and Fm was observed in both SR and LR leaves. Nevertheless, at this stage, both SR and LR leaves demonstrated a Maximum Quantum Efficiency (Fv/Fm) of SR (0.80) and LR (0.82) representing 1.05 and 1.03 times increase compared to SM and LM leaves, respectively. The intrinsic efficiency loss was mitigated to 13.0%. An elevation in the ΦPS2 values was noted in SR leaves (0.83; 93% light harvesting capacity), while LR leaves exhibited a increase (0.85) in the same. The rooting phase did not impact the ETR rates of growing shoots significantly. The qP values of SR leaves were nearly comparable to the control and SM leaves. Non-photochemical quenching during the *in vitro* rooting phase remained insignificant. Subtle variations in one or two fluorescence parameters were discernible when comparing SR and LR leaves.

Experiment 2

Alterations in chlorophyll a fluorescence parameters of *Musa acuminata* during *in vitro* propagation under elevated CO_2 concentrations are delineated in Table 4.2. The F0 and Fm values were assessed in dark-adapted leaves collected from semi-solid medium enriched with sucrose (SCSM) under varying CO_2 atmospheres, including ambient air, CO_2-free, and CO_2-enriched conditions. Optimal readings were observed in the CO_2-free environment with sucrose-containing medium. At the specified concentration, F0-0.35 and Fm-1.5 values resulted in a Fv/Fm ratio of 0.77. The ratio of ΦPS2 (0.82) was only marginally lower, at 1.07 times less than Fv/Fm. Leaves under these conditions exhibited maximum ETR values (13.55 µmol m^{-2} s^{-1}), representing a 1.05-fold increase compared to the control, and qP values of 0.90. It is noteworthy that both Fv/Fm and ΦPS2 remained unaffected by subsequent increments in CO_2 concentration. Across all SCSM leaves, these parameters exhibited relatively constant values within the range of 0.77-0.82 (Fv/Fm) (figure 4.1). Elevating the CO_2 concentration beyond 10.0 gm^{-3}, specifically at 40.0 gm^{-3}, exerted a noteworthy impact on the photochemical parameters. Under this concentration,

the maximal fluorescence (Fm) exhibited a significant reduction, registering a decrease by a factor of 1.45. Consequently, there was a substantial decline in both the Fv/Fm and ΦPS2 ratios. Concurrently, parameters like Electron Transport Rate (ETR) and Photochemical Quenching (qP) reached their minima in these leaves, accompanied by a conspicuous dissipation of absorbed light as heat (qN=0.15).

The liquid medium exhibited variable effects on the photochemical processes within *in vitro* proliferating cultures cultivated under diverse concentrations of carbon dioxide (CO_2). The Fv/Fm ratio observed in leaves cultured in CO_2-free and ambient air environments demonstrated remarkable similarity in SCLM. Additionally, the values for F0, Fm, Fm', and Fs was analogous under both aforementioned CO_2 concentrations. Nonetheless, a moderate decline in Fm was observed in SCLM leaves cultivated under normal atmospheric conditions. The highest quantum efficiency (Fv/Fm=0.15) (Figure 4.3) was documented at a CO_2 concentration of 0.6 gm^{-3}. This result was nearly equivalent to the value of 0.2 recorded at 0.0 gm^{-3} of CO_2. The photosynthetic parameters exhibited a decreasing trend beyond a CO_2 concentration of 10.0 gm^{-3}, reaching a minimum level at 40.0 gm^{-3}. At an incident luminous flux intensity of approximately 50 µmol photons m^{-2} s^{-1}, the rates of Electron Transport Chain (ETR) ranged between 10-13 µmol electrons m^{-2} s^{-1}. The peak percentages of accessible reaction centers were documented at CO_2 concentrations of 0.0 and 0.6 gm^{-3}. No thermodynamic dissipation was discerned during the observations.

The photochemical parameters assessed in the absence of sucrose and under CO_2-enriched conditions revealed a state of photoautotrophic growth in shoot cultures. Conversely, a noteworthy decline in all fluorescence values was evident when exposed to CO_2-free and ambient air conditions within the SFLM medium. Elevating the CO_2 concentration, ranging from 0.6 to 40.0 gm^{-3}, exhibited a proportional enhancement in the Fv/Fm ratio. Optimal outcomes were achieved at the highest concentration of 40.0 gm^{-3} CO_2. At this specific concentration, the recorded values for F0 and Fm were 0.30 and 2.8 (Figure 4.4), respectively. In this study, leaves subjected to a concentration of 10.0 gm^{-3} of CO_2 exhibited optimal efficiency, with a maximum Fv/Fm of 0.89 and a ΦPS2 of 0.93. Furthermore, a noteworthy reduction in sensible heat losses was evidenced by the low qN values (0.14) associated with this concentration. Interestingly, the highest values of Electron Transport Rate (ETR) were recorded at 11.01 µmol m^{-2} s^{-1}, along with a substantial

quantum yield of photosystem II (qP) at 0.92. Remarkably, negligible non–photochemical quenching was observed under these conditions, emphasizing the intricate interplay between CO_2 concentration and photosynthetic parameters in influencing plant physiological responses.

Under sucrose-free conditions in semi-solid medium, outcomes akin to those observed in Sucrose-Containing Liquid Medium (SCLM) were noted. Nevertheless, SFSM exhibited moderately diminished fluorescence parameters and photochemical yield compared to SFLM. Notably, neither CO_2-free conditions nor exposure to ambient air elicited any discernible photosynthetic response. It was observed that at a concentration of 0.6 gm^{-3} of CO_2, a substantial portion of absorbed light energy was dissipated as heat, with a quantification of qN=1.39. Enhancements in photosynthetic parameters were discerned exclusively beyond this concentration threshold. At a CO_2 concentration of 10.0 gm^{-3}, the basal and maximal fluorescence values were recorded as 0.35 and 1.5 (Figure 4.5), respectively, resulting in an Fv/Fm ratio of 0.77. Notably, a substantial elevation in the Electron Transport Rate (ETR) was observed, reaching 12.55 µmol m^{-2} s^{-1} at this specific concentration. An examination of quenching parameters elucidated that the photochemical quenching registered a value of 0.15, accompanied by a negligible presence of heat dissipation (non-photochemical quenching). Notably, leaves cultivated in an environment containing 40.0 gm^{-3} of CO_2 exhibited an elevated Fv/Fm ratio, albeit with a significant reduction in both F0 and Fm values.

4.2 Studies on water relations

Many authors have reported that liquid growth medium can have a stimulating effect on the growth of shoots in *in vitro* plant cultures. This means that when plants are grown in a liquid medium rather than a solid one, they tend to show increased shoot growth (Gatambia *et al.,* 2016; Gatti *et al.,* 2017; Nápoles Borrero *et al.,* 2017; Arigundam *et al.,* 2020; Hwang *et al.,* 2022; Muhammet Dogan, 2022; Shekhawat *et al.,* 2022). The degree of solidification or the texture of the growth medium significantly impacts not only the ability of shoots to regenerate but also their water management. When shoots are cultured in a liquid medium, they tend to have a higher water content compared to those grown on a more solidified medium (Malik *et al.,* 2018). Sreelakshmi *et al.,* (2021) quantified the water content, specifically focusing on hyperhydricity, in cultures of *Dianthus chinensis L.* One of the primary reasons tissue culture-derived plantlets often don't survive after being

transferred out of the controlled lab environment (*ex vitro*) is excessive drying out, known as desiccation (Zein El Din *et al.,* 2020). This happens because the plantlets struggle to maintain the right balance of water, leading to a malfunction in their water management system. This disruption in their "water housekeeping" balance can result in mortality, making it a critical factor to address when transitioning these plants from the lab to more natural or greenhouse conditions (Gaspi *et al.,* 2013). Plantlets from various species often experience excessive water loss and rapid wilting if they are not kept in a high-humidity environment during their transition from *in vitro* culture to *ex vitro* conditions (outside the controlled lab setting). Numerous studies have been carried out to investigate and understand the dynamics of water loss during the acclimatization of micro propagated plants to natural environments was reported by *P. algarbiensis and P. almogravensis* (Gonçalves *et al.,* 2017), *Trichosanthes kirilowii* (Duan *et al.,* 2020), and *Genipa americana* L. (de Souza *et al.,* 2021).

4.2.1 Materials and methods

Water loss

We conducted an experiment using detached leaves of approximately the same size from five different plants at various stages of micropropagation. These leaves were obtained from plants grown on two different types of growth media. Initially, we weighed these leaves while they were still fresh, and then we monitored their rate of water loss. To allow for transpiration, we positioned the leaves with their abaxial side facing upward on a clean bench at a room temperature ranging from 25 to 28°C and a relative humidity (RH) of 35 to 40%. We took measurements of each leaf's weight at 15-minute intervals for a total of 3 hours. After completing this transpiration experiment, we oven-dried all the leaves at 60°C to determine their dry mass. The calculation of water loss was performed according to the method described by (Salomon *et al.,* 2014).

$$WL\ (\%) = (FWt_0 - DW_t) - (FW_t - DW_t) \times 100 / (FWt_0 - DW_t)$$

where WL is the percentage of water loss, FWt_0 is fresh weight at time zero, FW_t is fresh weight after time, and DW_t is dry weight.

Biomass accumulation and water content

The fresh and dry weight were determined by weighing shoot clusters on Top Pan Electronic Balance (Shimazu) wet and after drying overnight at 60°C temperature in a hot

air oven, respectively. The percent water content and dry weight were determined using the following formula:

We determined both the fresh and dry weights of the shoot clusters using a Top Pan Electronic Balance (Shimazu). First, we weighed the clusters when they were still wet. Then, we placed them in a hot air oven and dried them overnight at a temperature of 60°C. After this drying process, we weighed the clusters again to obtain their dry weight. To calculate the percent water content and dry weight, we used the following formula:

Percent Water Content = [(Fresh Weight - Dry Weight) / Fresh Weight] * 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 analyzed during the following experiments:

Experimentations

1. Role of liquid medium
2. Role of different gelling agent
3. Role of vessel and closure type
4. Role of seaweed
5. Role of CO_2 enrichment semi solid medium
6. Role of CO_2 enrichment liquid medium

4.2.2 Results

Water loss

In the initial 30-minute period, a swift desiccation, accounting for 38.74% water loss, was discerned in the leaves of the SM grown. Conversely, the LM leaves exhibited notable resilience, displaying minimal wilting. As time progressed, the pace of water loss escalated, culminating in a cumulative loss of 49.52% for the SM leaves and a comparably lower 31.08% (1.59 times less) for the LM leaves over the course of 3 hours (refer to Table 4.3). During the initial 30 minutes to 180 minutes of retention in the LR stage, leaves at the *in vitro* rooting stage demonstrated minimal water content reduction, registering only a modest decrease from 3.5% to 17.86%. In contrast, the SR stage manifested a notably elevated water loss compared to the LR phase. The *in vitro* hardening phase witnessed the highest water loss, reaching a peak of 69.00% and 67.07% in SH and LH leaves, respectively, following a three-hour period of air drying.

Biomass accumulation and water content

Study 1, 2, 3 and 4

In the assessment of the growth performance of *in vitro* cultivated plants, the quantification of fresh weight (FW) and dry weight (DW) provides a comprehensive understanding. The collective mass of micropropagules directly correlates with the plant's functionality, responding to factors such as photosynthetic capacity, nutritional elements, and environmental influences. Notably, a significant enhancement in both fresh and dry weight, as well as water content, was evident in shoot clusters cultivated in a liquid medium compared to the control condition of agar-based solid medium. On the solid medium, the recorded total fresh weight was 2.02 grams with a dry weight of 0.104 grams (see Table 4.4). Shoots on this substrate exhibited a 94% water content. Contrastingly, when shoots were nurtured in a liquid medium utilizing gel rite (GM) for structural support, an increase in fresh weight (3.33 grams) and dry weight (0.146 grams) was observed. Notably, these cultures also displayed a higher water content of 96.23%. The utilization of tissue paper as a support matrix showcased a considerable surge in water content (96.19%) in conjunction with elevated total fresh weight (3.4 grams) and dry weight (0.129 grams).

However, when employing pebbles as a supporting material in the liquid medium, while there was a promotion in wet and dry weight accumulation, the water content remained relatively similar to that of the control condition. An intriguing observation was the appearance of delicate and excessively moist shoot formations, known as hyperhydric shoots, despite the increased weight accumulation in this context. In studies 2 and 3, the implementation of different gelling agents and variations in culture vessels exhibited a noteworthy increase in both total fresh weight and dry weight. Specifically, the use of Phytagel as a gelling agent resulted in a substantial rise in total fresh weight, registering at 2.75 grams, and dry weight, measuring 0.229 grams, in comparison to other gelling agents (refer to Table 4.5). This indicated a higher quantitative value alongside a water content of 91.59%. Conversely, a reduced fresh weight was observed in the medium containing starch. Examining the influence of culture vessels on the overall mass of the plants, it was observed that V1 displayed the maximum fresh weight at 3.04 grams and dry weight at 0.105 grams, accompanied by a water content of 96.52% (refer to Table 4.6). In contrast, lower fresh and dry weights were observed in V6 (control), amounting to 2.0 grams and 0.114 grams, respectively.

The utilization of *Sargassum tenerrimum*'s Liquid Seaweed Extract (LSE) at a 50% concentration (v/v) within the growth medium significantly contributed to a substantial increase in biomass. This specific concentration notably amplified the fresh weight, measuring at 5.61 grams, and the dry weight, recorded at 0.71 grams, as indicated in Table 4.7. This concentration exhibited a particularly advantageous impact on biomass augmentation. Furthermore, across all Liquid Seaweed Extracts (LSE) investigated, an overall increase in biomass was observable at a 30% concentration. This broader observation suggests a general trend of positive effects on the biomass of the plant specimens when exposed to various concentrations of Liquid Seaweed Extracts. Conversely, it's pivotal to highlight that the application of *G. corticata's* Liquid Seaweed Extract exhibited a contrasting effect, displaying a detrimental impact on biomass accumulation. The recorded values stood at 2.48 grams for fresh weight and 0.564 grams for dry weight, signifying a noteworthy reduction in biomass compared to the control or alternative concentrations of seaweed extracts.

Study 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 in the medium intensified the impact of CO_2 enrichment, resulting in higher fresh and dry weights compared to cultures grown in sucrose-free mediums (refer to Table 4.8 and 4.9). When comparing shoot cultures in solid and liquid mediums, it became evident that the liquid medium significantly stimulated both biomass and moisture accumulation during the *in vitro* multiplication of banana plants. Under CO_2-free conditions in SCSM cultures, the minimum fresh weight and dry weight were recorded at 2.12 grams and 0.105 grams, respectively, with a water content of 95.05%. Cultures exposed to ambient air in the growth room exhibited similar behaviour. With an increase in CO_2 concentration from 0.6 to 40.0 gm^{-3}, there was a notable favouring of wet and dry weight accumulation, reaching its peak at 40.0 gm^{-3}, along with a rise in total moisture content. Physiological parameters in SCLM cultures surpassed those of SCSM cultures across all CO_2 concentrations. The concentration of 10.0 gm^{-3} in the liquid medium was most favourable for fresh weight (5.63 grams) and dry weight (0.094 grams) accumulation, leading to an overall increase in water content (96.42%). In solid medium, a fresh weight of 4.4 grams and a dry weight of 0.185

grams were observed. In sucrose-free conditions (SFSM and SFLM), despite not favouring biomass production as much as sucrose-containing cultures, the percent water content ranged between 84-98%. A notable observation was that although sucrose-free conditions did not enhance biomass production compared to sucrose-containing cultures, the water content of all sucrose-free cultures was lower than their respective sucrose-containing counterparts. Cultures grown under CO_2-free and growth room conditions exhibited a high-water content of approximately 95% in both semi-solid and liquid mediums, accompanied by the appearance of severely hyperhydric shoots. With an increase in CO_2 concentration, an increase in fresh and dry weight along with water content was consistently recorded. Maximum wet weight (0.647 grams) was produced by SFLM cultures under 40.0 gm^{-3} of CO_2, with a water content of 91.42%. SFSM cultures under 10.0 gm^{-3} of CO_2 exhibited a fresh weight of 0.680 grams and a dry weight of 0.059 grams, with the water content (91.02%) being lower than that of the control (SCSM under ambient air).

4.3 Carbonic anhydrase enzyme activity

Carbonic anhydrase (CA), which is also known as carbonate hydrolyase (EC 4.2.1.1), is an enzyme that contains zinc and plays a crucial role in facilitating the reversible conversion of bicarbonate into carbon dioxide (CO_2). This enzyme is found in a wide range of organisms, including animals, plants, archaea, and eubacteria. Its presence is associated with various physiological processes, including ion exchange, maintaining acid-base balance, catalyzing carboxylation and decarboxylation reactions, and facilitating the movement of inorganic carbon between the cell and its surrounding environment, as well as within the cell itself. In essence, carbonic anhydrase is a versatile enzyme that contributes to several essential cellular and metabolic processes in living organisms across different domains of life (DiMario *et al.,* 2018; Supuran, 2018). In several plant species, it has been observed that there is a noteworthy linear relationship between the relative growth rate (RGR) of plantlets and their carbonic anhydrase (CA) activities. This suggests that there is a direct correlation between the CA activity of plantlets and their growth rates in vitro. In simpler terms, the more active the carbonic anhydrase in a plantlet, the higher its net photosynthetic rate, and consequently, the faster it grows (Ahmad *et al.,* 2018).

4.3.1 Materials and methods

For the analysis of enzyme activity, we followed a procedure originally outlined by (Wilbur and Anderson, 1948) with slight modifications to suit our experiment's specific needs. Leaves weighing approximately 1.0 gram were collected from *s* grown cultures that

were being grown on both liquid and semi solid growth medium. These leaves were then ground up in 10 ml of a buffer solution containing 20 mM Tris-Cl (pH 8.0). Similarly, leaves from field-grown plants leaves extracts were also prepared using the same method. To separate the extracts, they were subjected to centrifugation at 8000 RPM for 10 minutes at a temperature of 4ºC. From the supernatant, we took a 500 µL sample and added it into 30 mL of Tris-Cl Buffer. To this mixture, we promptly added 15 mL of CO_2-saturated water, which was obtained from a local supplier. We closely monitored the pH of this solution and recorded the time it took for the pH level to decrease from its initial value of 8.0 to 6.3.

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

4.3.2 Results

Table 4.10 delineates the Carbonic Anhydrase (CA) activities observed in the leaves of banana plants. The field-grown plant, considered as the control, exhibited the lowest CA activity at 31.75 WAU (Weighted Activity Units) per gram of fresh tissue among the leaves examined. In the semi solid medium (SM) leaves, there was a notable increase in enzyme activity, displaying a 1.45-fold rise compared to the control, with a total activity registered at 45.23 WAU per gram of fresh tissue.

Conversely, the liquid culture (LM) leaves displayed a significantly higher unit enzyme activity. This medium exhibited an enzyme activity of 70.12 WAU, marking a 1.74-fold increase compared to the SM leaves and a remarkable 2.2-fold increase compared to the LM leaves. Similar outcomes were observed in the context of CO_2 enrichment. When the plants were enriched with increased CO_2 concentrations, the semi-solid medium exhibited an activity of 52.63 WAU per gram of fresh (table 4.11) tissue, representing an approximately 1.63-fold increase compared to the control. Conversely, with plant growth in a solid medium under CO_2 enrichment, the recorded CA activity was notably higher at 88.42 WAU per gram of fresh weight, marking a remarkable 2.85-fold increase compared to other leaves.

Table 4.1 Various parameters of chlorophyll fluorescence in banana during different stages of micropropagation under semi–solid and liquid culture medium.

Sample	Fo	Fm	F'm	Ft	Fo'	Fv/Fm	ΦPS2	ETR	qP	NPQ
SM	0.3	1.23	0.9	0.19	0.12	0.76	0.79	9.72	0.91	0.065
LM	0.32	1.4	1.27	0.29	0.21	0.77	0.77	9.65	0.92	0.077
SR	0.15	0.75	0.7	0.12	0.1	0.80	0.83	9.53	0.97	0.05
LR	0.12	0.68	0.65	0.1	0.11	0.82	0.85	9.60	1.02	0.085
F	0.5	3.2	3	0.56	0.4	0.84	0.81	55.27	0.94	0.31

Mean followed by different letters differ significantly at 5 %. SM – in vitro multiplication stage on semi–solid medium; LM – in vitro multiplication stage on liquid medium; SR– in vitro rooting stage on semi–solid medium LR– in vitro rooting stage on liquid medium; F– Mature plant growing in the fiel

Table 4.2 Effect of CO_2 Enrichment on chlorophyll fluorescence parameters of banana (Observations were recorded after 30 days of growth).

Media	CO_2 Conc. (g m^{-3})	Fo	Fm	F'm	Ft	Fo'	Fv/ Fm	ΦPS2	qP	NPQ
SCSM	**0.0**	0.35	1.5	1.4	0.25	0.2	0.77	0.82	0.96	0.35
	Am[c]	0.18	1.0	0.9	0.15	0.12	0.82	0.83	0.96	0.18
	0.6	0.10	0.6	0.58	0.12	0.08	0.83	0.79	0.92	0.1
	10.0	0.03	0.14	0.14	0.023	0.2	0.77	0.84	1.95	0.032
	40.0	0.35	1.5	1.4	0.25	0.2	0.77	0.82	0.96	0.35
SCLM	**0.0**	0.3	2.8	2.5	0.18	0.2	0.89	0.93	1.01	0.12
	0.6	0.25	1.18	1	0.2	0.15	0.79	0.80	0.94	0.029
	10.0	0.12	0.67	0.6	0.1	0.11	0.82	0.83	1.02	0.147
	40.0	0.04	0.16	0.15	0.02	0.3	0.75	0.87	-0.89	1.88
SFSM	**0.0**	0.04	0.16	0.153	0.02	0.3	0.75	0.87	-0.89	1.88
	0.6	0.12	0.67	0.6	0.1	0.11	0.82	0.83	0.92	0.14
	10.0	0.25	1.18	1	0.2	0.15	0.79	0.80	0.94	0.02
	40.0	0.3	2.8	2.5	0.18	0.2	0.89	0.93	1.01	0.12
SFLM	**0.0**	0.04	0.16	0.15	0.02	0.3	0.75	0.87	-0.89	1.88
	0.6	0.03	0.14	0.14	0.023	0.2	0.77	0.84	1.95	1.39
	10.0	0.35	1.5	1.4	0.25	0.2	0.77	0.82	0.96	0.15
	40.0	0.25	1.18	1	0.2	0.15	0.79	0.80	0.94	0.029

SCSM– Sucrose containing semi–solid medium; SCLM– Sucrose–containing liquid medium; SFSM–Sucrose–free semi–solid medium; SFLM–Sucrose–free liquid medium

Table 4.3 Percent water loss in detached leaves of banana plantlets during different phases of its micropropagation in liquid and semi–solid medium.

Time [min]	**Multiplication**		**Rooting**		***In vitro* hardened plants**	
	SM %	**LM %**	**SR %**	**LR %**	**SH %**	**LH %**
30	38.74	11.7	13.15	3.5	36.56	32.93
60	40.9	15.48	16.89	7.4	37.96	50.66
90	43.05	22.78	24.36	10.3	55.95	55.65
120	43.05	25.11	26.23	12.2	63.55	63.14
150	45.21	28.84	32.21	14.09	66.77	67.01
180	49.52	31.08	35.79	17.86	69	67.01
SEM	0.8731					
CD 5%	2.54					
CD 1%	3.123					
CV	15.804					

SM– shoots multiplied on semi–solid medium; LM– shoots multiplied on liquid medium; SR– shoots rooted on semi–solid medium; LR– shoots rooted on liquid medium; SH– shoots rooted on semi–solid medium and hardened; LH– shoots rooted on liquid medium and hardened; SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation.

Table 4.4 Percent water content and other growth parameters in banana grown under different types of liquid culture systems (Observations were recorded after 45 days)

Media type	Fresh weight (mg)	Dry weight (mg)	Percent water content
GM	3933.000 a	146.642 a	96.238 a
Tissue paper	3435.000 b	129.390 b	96.199 a
PA	2688.000 c	103.512 c	96.113 a
Liquid	1443.000 e	69.008 d	95.165 b
Control	2027.500 d	104.260 c	94.843 b
SEM	10.27468	2.990316	0.505875
CD 5%	138.9805	1.653082	0.242091
CD 1%	418.9324	4.982927	0.72974
CV	579.1709	6.888859	1.00886

SM – Solid Medium; LM – Glass bead supported Liquid Culture Medium; FP– Filter Paper Supported liquid medium; TI– Temporary Immersion; SD - Standard Deviation; SEM - Standard Error Mean; 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 gelling agents on water content and other growth parameters in banana during *in vitro* shoot multiplication (Observations were recorded after 45 days).

Media type	Fresh weight (mg)	Dry weight (mg)	Percent water content
Agar	2027.500 c	104.260 e	94.843 a
Phyta Gel	2753.100 a	229.390 b	91.599 b
Guar gum	1881.600 c	203.512 c	89.090 c
Isabgol	2404.500 b	246.642 a	89.656 c
starch	1443.000 d	169.008 d	88.170 c
SEM	9.740073	1.734951	1.189195
CD 5%	102.3652	1.653082	0.539133
CD 1%	308.5621	4.982927	1.625121
CV	426.5848	6.888859	2.246718

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 vessel and closure types on water content and other growth parameters in banana during *in vitro* shoot multiplication (Observations were recorded after 45 days)

Vessel type	Fresh weight (mg)	Dry weight (mg)	Percent water content
C	2027.500 d	114.260 b	94.349 d
V_1	3041.250 a	105.490 c	96.523 a
V_2	2230.250 cd	116.390 b	94.770 cd
V_3	2270.800 c	113.538 b	94.990 c
V_4	2635.750 b	118.964 b	95.470 b
V_5	2874.667 ab	125.163 a	95.638 b
SEM	5.886642	3.017097	0.31873
CD 5%	73.79911	1.743012	0.15185
CD 1%	219.2684	5.178755	0.451168
CV	300.4162	7.095332	0.618139

(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 4.7 Percent water content and other growth parameters in banana grown under LS (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 racemosa	10	2.810 h	0.2 h	92.883
	30	2.080 k	0.05 k	97.596
	50	2.280 e	0.3 e	86.842
Gracilaria corticata	10	2.870 h	0.5 h	82.578
	30	2.220 j	0.4 j	81.982
	50	0.980 l	0.1 l	89.000
Caulerpa paspaloides	10	2.740 d	0.5 d	81.752
	30	2.960 d	0.45 d	84.797
	50	2.870 h	0.4 h	86.063
Ulva lactuca	10	2.430 i	0.4 i	83.539
	30	3.350 e	0.4 e	88.060
	50	1.910 k	0.2 k	89.529
Sargassum tenerrimum	10	3.900 e	0.2 e	94.872
	30	3.660 f	0.4 f	89.071
	50	4.110 b	0.6 b	88.258
Caulerpa sertularioides	10	2.540 i	0.2 i	92.126
	30	3.090 g	0.3 g	90.291
	50	3.570 f	0.4 f	88.796
Sargassum wightii	10	2.330 j	0.1 j	95.708
	30	3.910 d	0.6 d	84.655
	50	2.860 h	0.2 h	93.007
Gracilaria edulis	10	4.910 d	0.6 d	87.780
	30	4.560 c	0.4 c	91.228
CV		15.35		11.90
SEM		0.40		0.17
CD 5%		1.12	0.50	0.50

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 Per cent water content and other growth parameters in banana grown under Solid growth medium CO_2 enriched conditions (Observations were recorded after 45 days).

	Sucrose	CO_2 conc. ($g\ m^{-3}$)	Fresh weight (mg)	Dry weight (mg)	Percent water content
SFSM	0	0	0.000 f	0.000 k	0.000 e
		0.6	372.500 ef	57.000 gh	84.681 d
		10	680.000 def	59.750 g	91.025 bc
		40	385.000 ef	58.750 g	84.694 d
		G**r**	297.500 ef	40.250 j	86.458 cd
SCSM	1	0	474.933 def	46.750 i	90.076 bc
		0.6	721.333 def	64.157 f	90.997 bc
		10	926.667 de	66.210 ef	92.819 ab
		40	1191.133 d	69.290 de	94.109 ab
	2	0	914.000 de	53.940 h	93.955 ab
		0.6	2085.333 c	64.730 f	96.893 a
		10	2188.000 c	70.580 d	90.645 bc
		40	2211.333 c	70.502 d	96.803 a
	3	0	2086.667 c	72.827 d	96.492 a
		0.6	3500.000 b	86.320 c	97.524 a
		10	4326.667 a	91.173 b	97.887 a
		40	4406.667 a	85.733 c	98.050 a
		GR	2153.333 c	105.413 a	95.059 ab
		SEM	25.75932	3.253093	3.397638
		CD 5%	238.9547	1.213901	1.719876
		CD 1%	685.3599	3.481659	4.932877
		CV	919.0037	4.66858	6.614527

SCSM= Sucrose containing semi–solid medium; SCLM= Sucrose containing liquid medium; SFSM= Sucrose free semi–solid 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 4.9 Percent water content and other growth parameters in banana grown under CO_2 enriched conditions in liquid growth medium (Observations were recorded after 45 days).

	Sucrose	CO_2 conc. ($g\ m^{-3}$)	Fresh weight (g)	Dry weight (mg)	Percent water content
SFLM	0	0	0.000 f	0.000 i	0.000 e
		0.6	0.630 ef	62.667 ef	84.681 d
		10	1.040 ef	66.333 de	91.025 bc
		40	0.647 ef	65.000 de	84.694 d
		G**r**	0.530 ef	40.333 h	86.458 cd
SCLM	1	0	0.747 ef	49.000 g	90.076 bc
		0.6	1.067 ef	70.333 cde	90.997 bc
		10	1.333 def	73.000 cd	92.819 ab
		40	1.807 cde	77.000 bc	94.109 ab
	2	0	1.300 def	56.333 fg	93.955 ab
		0.6	3.400 bc	70.167 cde	96.893 a
		10	2.933 bcd	77.667 bc	90.645 bc
		40	3.433 bc	77.567 bc	96.803 a
	3	0	2.733 cd	78.533 bc	96.492 a
		0.6	4.500 ab	95.400 a	97.524 a
		10	5.533 a	101.467 a	97.887 a
		40	5.633 a	94.667 a	98.050 a
		G**r**	3.233 bc	81.767 b	95.059 ab
		SEM	0.47	9.88	
		CD 5%	1.43	29.97	
		CV	12.10	8.48	

SCSM= Sucrose containing semi–solid medium; SCLM= Sucrose containing liquid medium; SFSM= Sucrose free semi–solid 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 banana 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	31.75 ± 7.76
SM	40.23 ± 3.21
LM	70.12 ± 5.62
SEM	3.3717
CD 5%	11.6680
CD 1%	17.6761
CV	12.33

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 banana 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	31.75 ± 7.76
SM	52.63 ± 5.21
LM	88.42 ± 7.62
SEM	4.3717
CD 5%	18. 80
CD 1%	34. 61
CV	19.33

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.

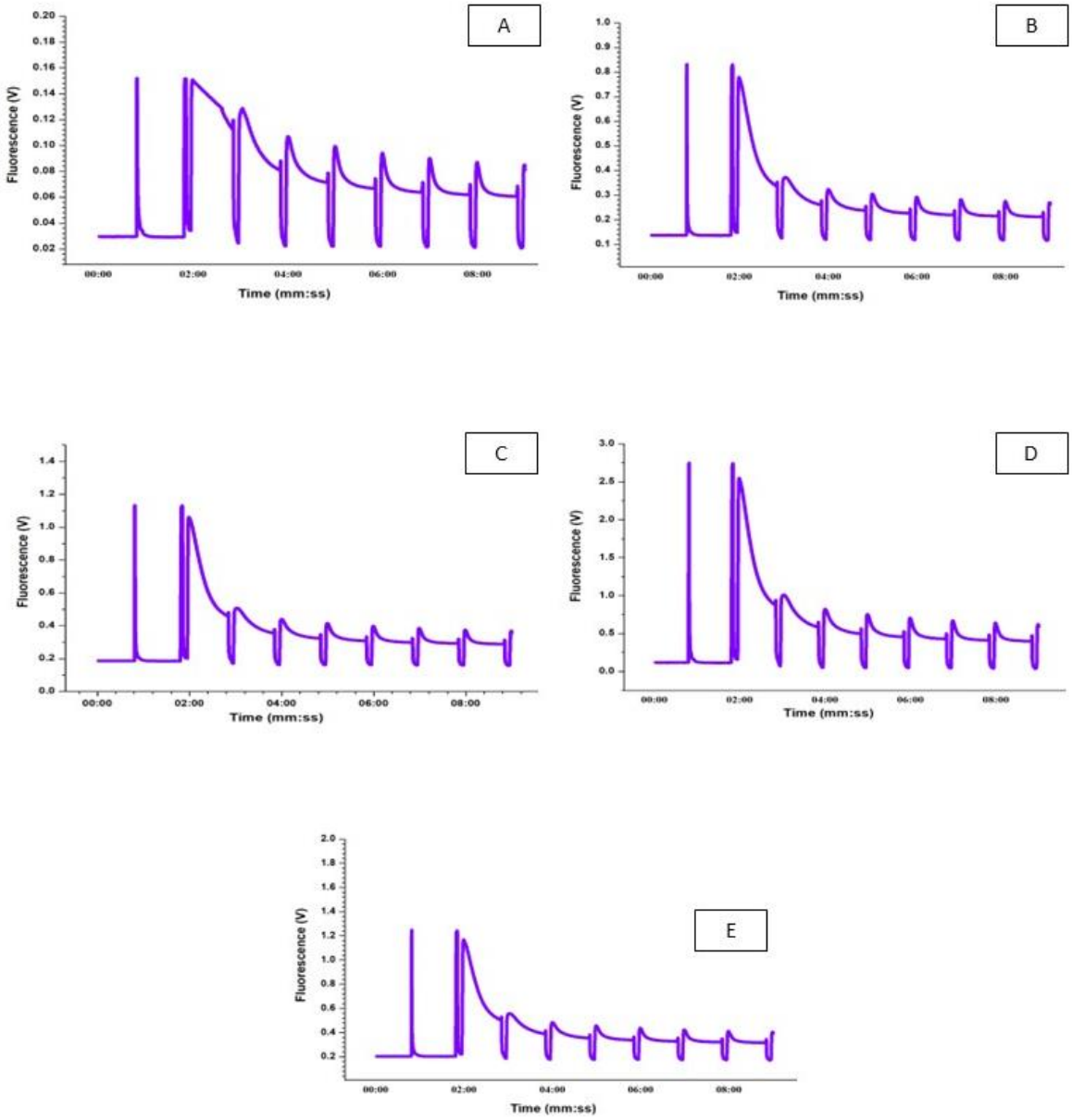
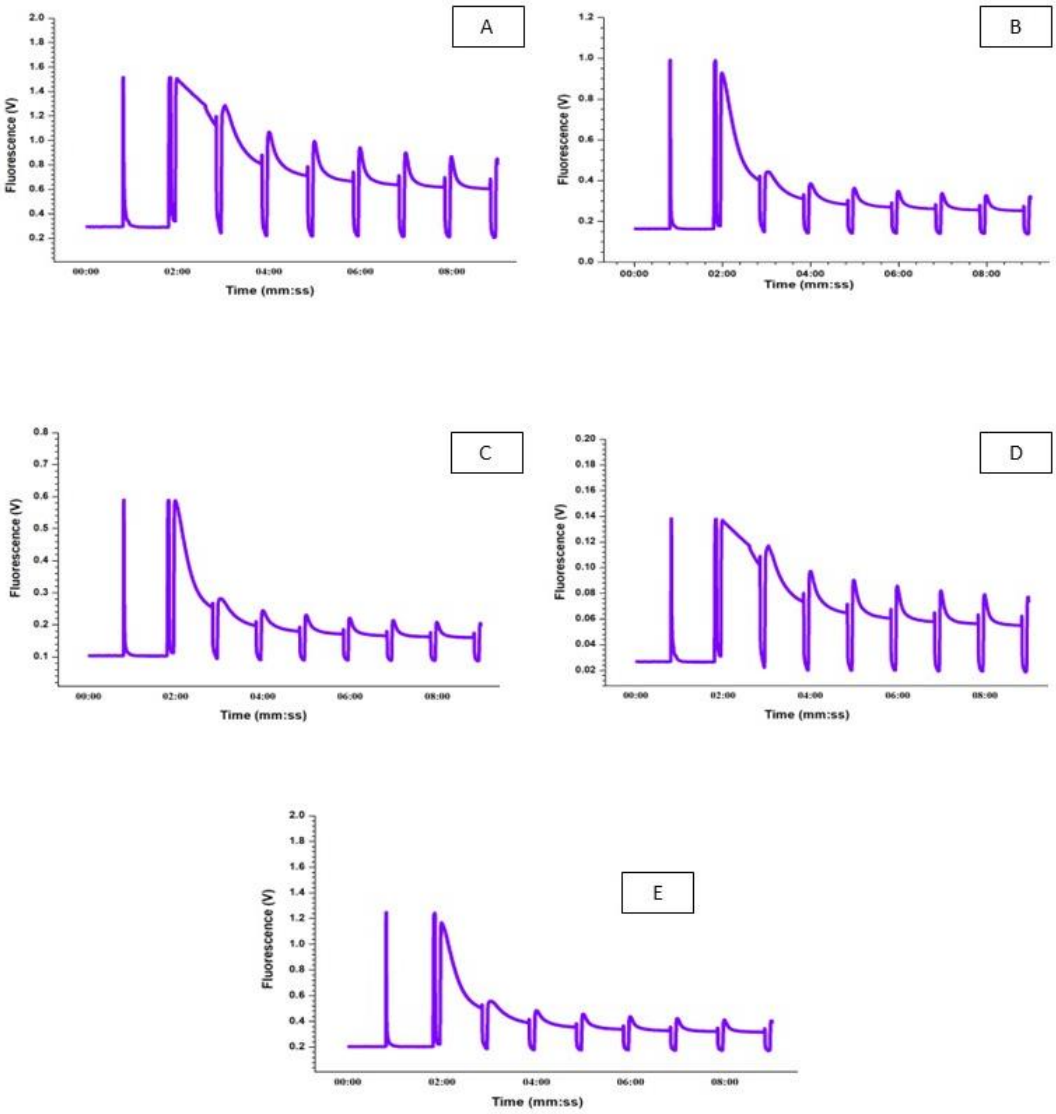
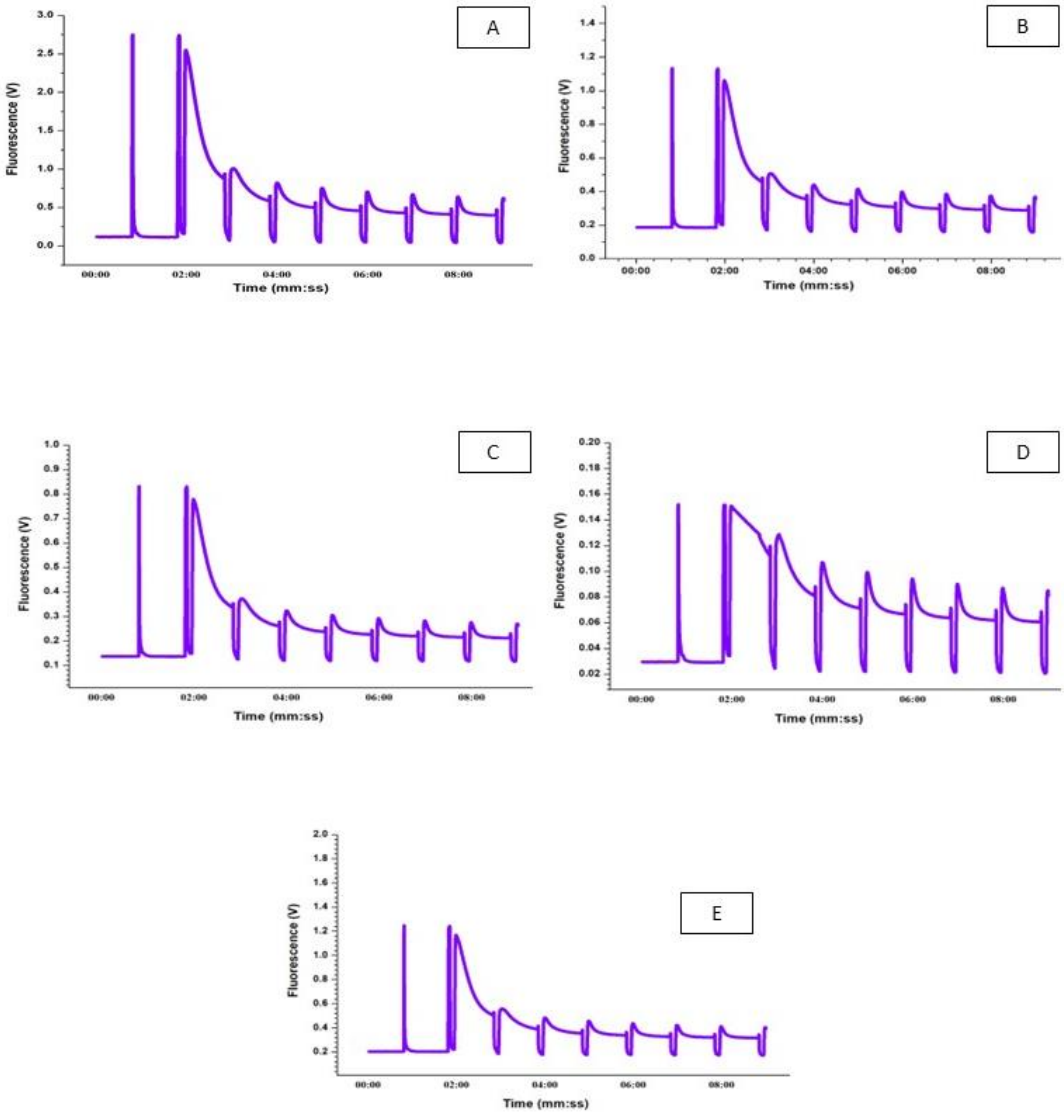


Figure: 4.1 Kaustsky plot showing the change in chlorophyll fluorescence parameters during different stage of micropropagation of *Musa acuminata* on semi solid and liquid medium. In figure **(A)** Field grown leaves; **(B)** *in vitro* multiplication stage on semi solid medium; **(C)** *in vitro* multiplication stage on liquid medium; **(D)** *in vitro* rooting stage on semi solid medium; **(E)** *in vitro* rooting stage on liquid medium.



. Figure 4.2 Kaustsky plot showing the change in chlorophyll fluorescence parameters during micropropagation under CO_2 enrichment of *Musa acuminata* on semi solid sucrose contain medium. **(A)** 3% SCSM with 0% CO_2; **(B)** 3% SCSM with 0.03% CO_2; **(C)** 3% SCSM 0.5% CO_2; and **(D)** 3% SCSM with 2% CO_2.



. Figure: 4.3 Kaustsky plot showing the change in chlorophyll fluorescence parameters during micropropagation under CO_2 enrichment of *Musa acuminata* on sucrose contain liquid medium. **(A)** 3% SCLM with 0% CO_2; **(B)** 3% SCLM with 0.03% CO_2; **(C)** 3% SCLM 0.5% CO_2; and **(D)** 3% SCLM with 2% CO_2.

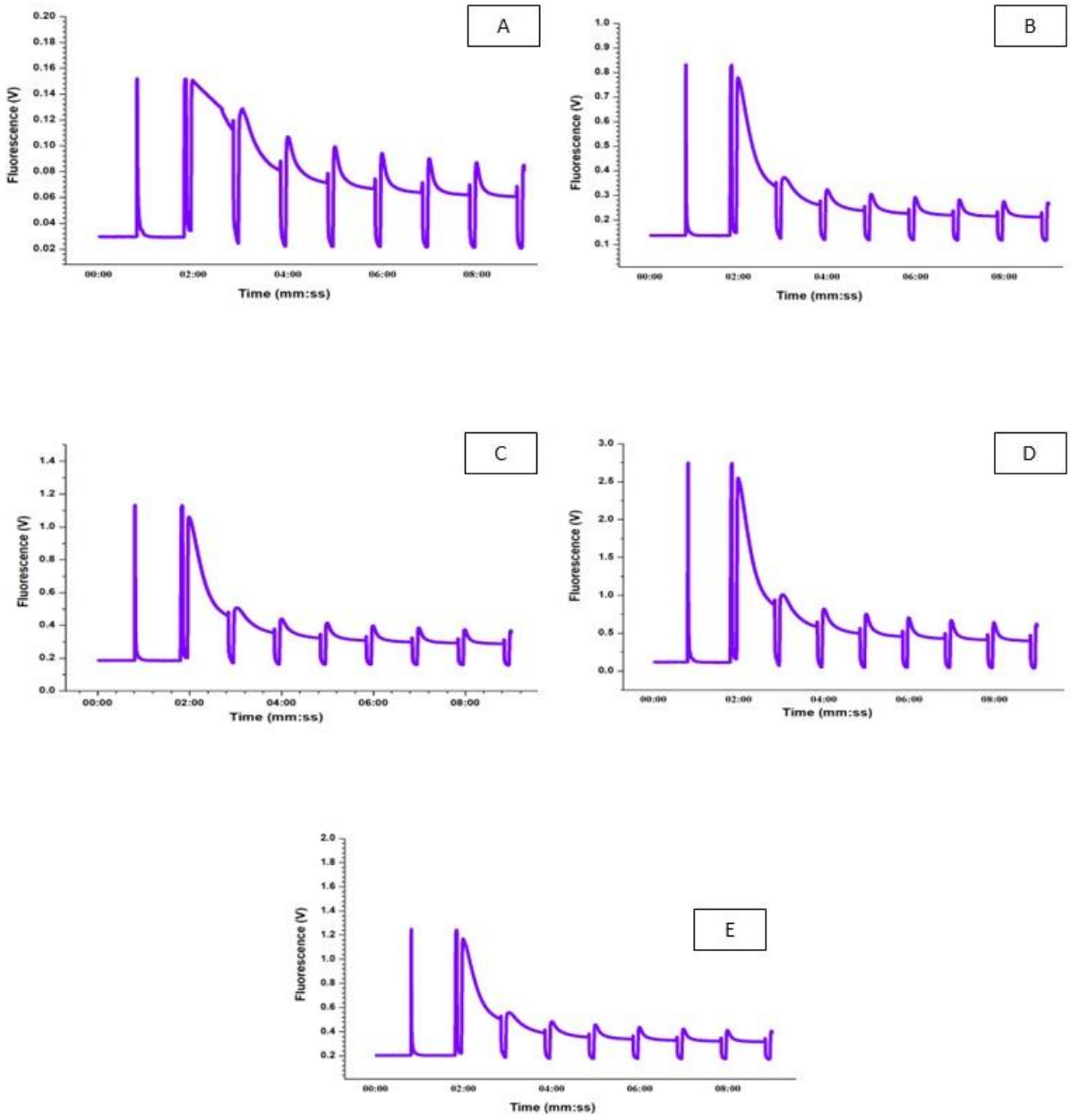


Figure: 4.4 Kaustsky plot showing the change in chlorophyll fluorescence parameters during micropropagation under CO_2 enrichment of *Musa acuminata* on sucrose free semi solid medium. **(A)** 0% SFSM with 0% CO_2; **(B)** 0% SFSM with 0.03% CO_2; **(C)** 0% SFSM 0.5% CO_2; and **(D)** 0% SFSM with 2% CO_2.

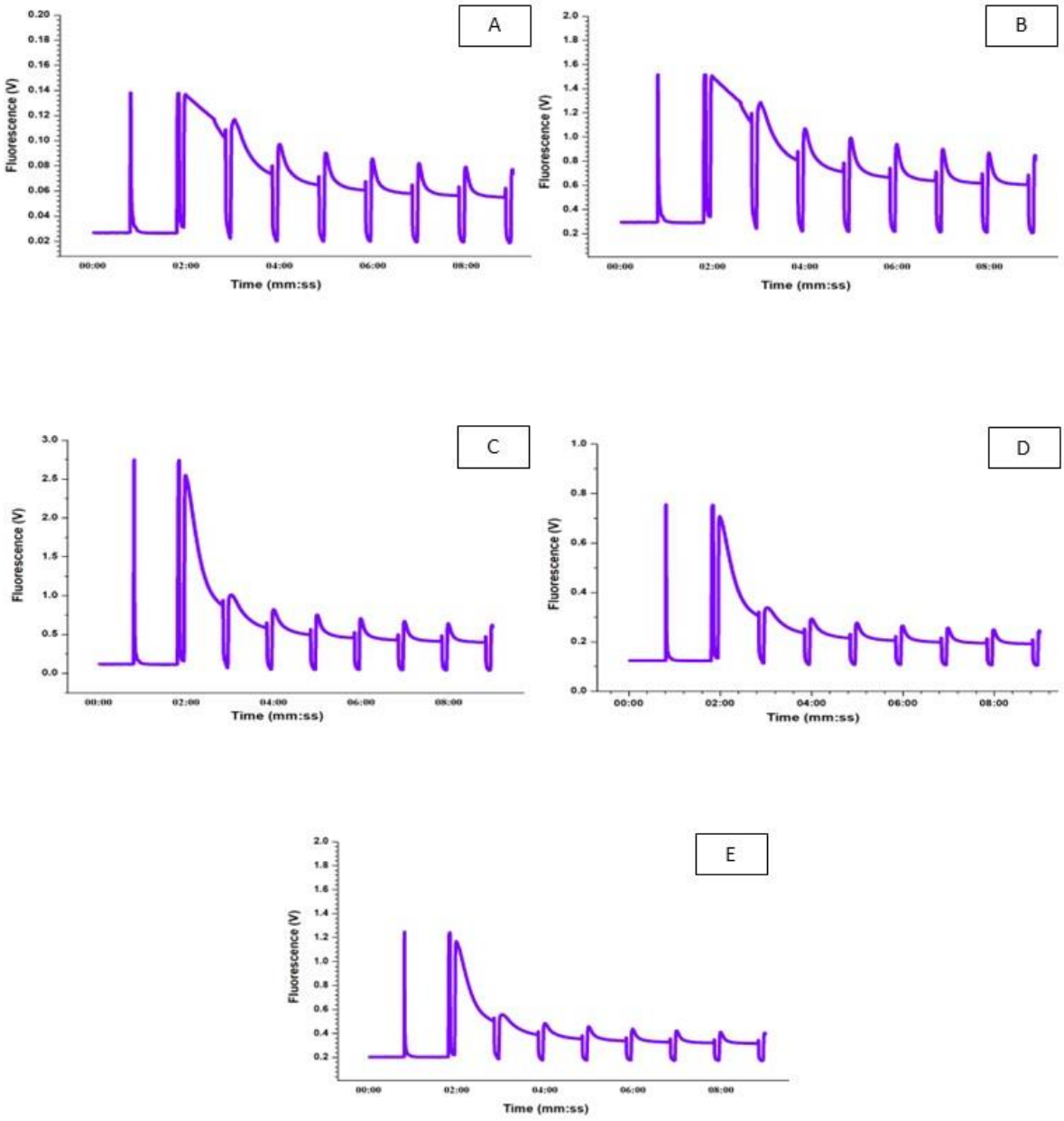


Figure: 4.5 Kaustsky plot showing the change in chlorophyll fluorescence parameters during micropropagation under CO_2 enrichment of *Musa acuminata* on sucrose free liquid medium. **(A)** 0% SFLM with 0% CO_2; **(B)** 0% SFLM with 0.03% CO_2; **(C)** 0% SFLM 0.5% CO_2; and **(D)** 0% SFLM with 2% CO_2.

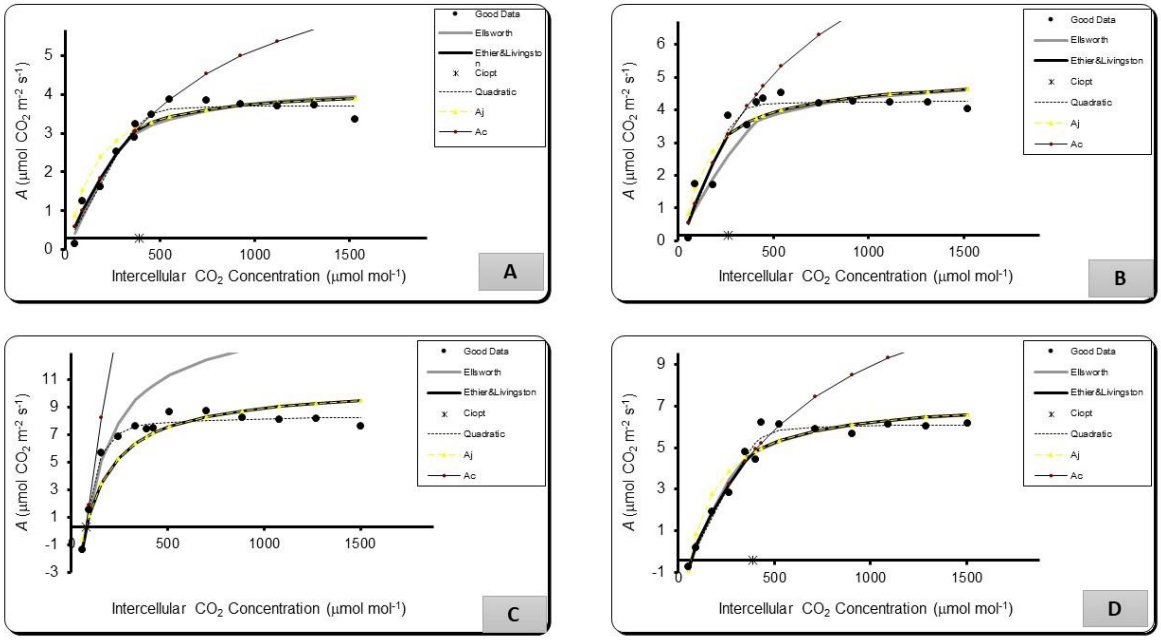


Figure: 4.6 A-Ci Curve of plant grown under CO_2 enriched conditions in 3% sucrose with different CO_2 concentration. (A) 0% CO_2; (b) 0.03% CO_2; (c) 0.5% CO_2 and (d) 2% CO_2