Photosynthetic efficiency and in vitro growth of *Celastrus paniculatus* Willd. under varied concentrations of CO$_2$

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**Abstract**

*Introduction:* *Celastrus paniculatus* (family: Celastraceae) is a woody climbing shrub valued for its immense medicinal properties contained in its various plant parts. Over-exploitation and poor natural regeneration either by seed or other method/s have resulted into depleting population of *C. paniculatus* in natural habitats in India. Novel approaches such as liquid culture media and photoautotrophic multiplication of shoots on sugar-free media has proved useful to obtain photosynthetically active micropropagated plantlets.

**Methods:** The shoot cultures of *C. paniculatus* were multiplied on sucrose containing and sucrose-free semi-solid and liquid media. The cultures were further incubated under various concentrations of carbon dioxide (0.0 to 40.0 gm$^{-3}$). The assessment of chlorophyll fluorescence parameters ($F'_{v}/F_{m}$, $F'_{m}$, $F_{v}/F_{m}$, $F_{v}/F_{o}$ and $\Phi$PS2) under similar conditions was undertaken.

**Results:** It was observed that CO$_2$ enrichment favored shoot multiplication and elongation and biomass production in sucrose supplemented medium. CO$_2$ (10.0 gm$^{-3}$) along with sucrose (3.0%), recorded maximum growth on semi-solid and liquid media. The valuation of $F'_{v}/F_{m}$, $F'_{m}$, $F_{v}/F_{o}$ and $\Phi$PS2 revealed that an increase in the concentration of CO$_2$ resulted in a decline in all the parameters especially the $F_{v}/F_{m}$ and $\Phi$PS2. On the contrary, withdrawal of sucrose from the medium under CO$_2$ enriched conditions resulted in a moderate growth rate and biomass production. However, $F_{v}/F_{o}$ and $\Phi$PS2 were considerably improved in shoot cultures incubated under elevated concentrations of CO$_2$ (10.0 gm$^{-3}$) without sucrose in the medium indicating their photoautotrophic growth.

**Conclusion:** Liquid medium proved to be superior for overall growth and biomass production over its semi-solid counterparts. The observations of photochemical efficiency in shoot cultures grown on liquid medium were at par with their semi-solid counterparts indicating no adverse effects such as hyperhydricity.

**Keywords:** Chlorophyll fluorescence, CO$_2$ enrichment, Water content, Liquid medium

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**Introduction**

*Celastrus paniculatus* (family: Celastraceae) is a woody climbing shrub valued for its immense medicinal properties contained in its various plant parts. Oil obtained from seeds is used for the treatment of beri-beri, gout and paralysis. It is also reputed to have promotory role for intelligence and sharpening of memory. The chief phytoconstituents of medicinal value testified in *C. paniculatus* include malkangunin, celapanin, celapanigin, celapagin, celastrol, pristimerin, and zeylasterone. The methanolic extracts of *C. paniculatus* have been described to exhibit free-radical-scavenging properties and antioxidant effects in human non-immortalized fibroblasts. The powdered root is considered useful for the treatment of cancerous tumors. Besides its phytotherapeutic importance, *C. paniculatus* has been explored for its potential in herbal cosmetics. The seed oil has been reported to be effective against dandruff and premature graying of the hair by the rural and tribal communities. The seed oil has also been recommended for its use as carrier oil in several skin care formulations.

Over-exploitation and poor natural regeneration either by seed or other method/s have resulted into depleting population of *C. paniculatus* in natural habitats in India. The plant has been listed in the threatened category and therefore *ex situ* conservation efforts have been devoted through micropropagation using different pathways. All these studies involved conventional methods of micropropagation where the desired rate of shoot multiplication could not be achieved. The goal...
of micropropagation is to obtain a large number of genetically and physiologically uniform plantlets with high photosynthetic potential which are able to survive their transfer to ex vitro conditions. It is therefore, necessary that some new approaches for improvement of growth conditions and shoot multiplication are attempted in order to produce morpho-physiologically normal plantlets of *C. paniculatus* that would survive the ex vitro transplantation.

Recently, *in vitro* propagation using liquid medium has been attempted as an innovative and cost effective method in a large number of plants. Liquid media are ideal in micropropagation for reducing plantlet production costs, increasing rate of multiplication and for automation. Liquid culture systems can provide much more uniform culturing conditions; the media can easily be renewed without changing the containers, and sterilization is possible by microfiltration and container cleaning after a culture period is much easier. In comparison with culturing on semi-solid media, much larger containers can be used, and transfer times can be reduced.

Further, in conventional micropropagation, sucrose is the main source of carbon and energy in the nutrient medium during micropropagation of plants but its presence increases the risk of contamination and depresses photosynthetic activity leading to mixo- or heterotrophy. This causes morphological and physiological disorders in *in vitro* grown plants resulting in high rate of mortality during hardening, acclimatization and field transfer. One of the ways to circumvent this problem is the photoautotrophic multiplication of shoots on a sucrose-free medium under CO₂-enriched conditions. Photoautotrophic multiplication of shoots on sugar-free medium has proved useful particularly to avoid or reduce contamination and improve the acclimation ability in micropropagated plantlets subjected to photosynthetically active conditions. Enhanced plantlet growth and multiplication under controlled carbon dioxide enriched environment have been achieved in a number of herbaceous and woody plants.

The present study was undertaken hypothesizing that the liquid medium and CO₂ enrichment could synergistically affect the *in vitro* growth of *C. paniculatus*, both in terms of rate of multiplication as well as their photosynthetic efficiency. It was also assumed that the plantlets obtained would be of better quality having strong potential of their survival during ex vitro transplantation. Hence, the present investigation involved culture of shoots on both liquid and semi-solid media under different concentrations of CO₂. The role of presence and absence of sucrose in different media types was also assessed.

**Materials and Methods**

**In Vitro Shoot Multiplication**

Shoot cultures of *C. paniculatus* were established using the standard protocol described by Rao and Purohit. Standard MS medium (MS salts + 3.0% sucrose + 0.8% agar + 0.5 mg l⁻¹ BAP) was used for *in vitro* shoot multiplication. For liquid medium agar was completely omitted from the medium. Borosilicate glass beakers were used as mechanical supports in case of liquid medium. Clusters of pre-determined size (3-5 shoots) were used as explants for further experimentation.

**CO₂ Enrichment With and Without Sucrose During In Vitro Shoot Multiplication**

Experiments were conducted to examine the impact of controlled and enriched CO₂ environment on *in vitro* shoot growth and multiplication. For this purpose, shoot clusters were inoculated on standard shoot multiplication medium (0.0 and 0.8% agar) with 30 g L⁻¹ sucrose or without it in 100 mL culture flasks (Borosil) stoppered with non-absorbent cotton plugs. Shoot cultures were exposed to different CO₂ concentrations [0.0, 0.6, 10.0 and 40.0 g (CO₂) m⁻³] applied in transparent acrylic chambers each with a volume of 7500 cm³ (25×50×15 cm; L×B×H) and closed with lid at top and sealed with packing tape (Miracle, 5.0 cm width). Various concentrations of CO₂ in acrylic chambers were controlled as per the method described by Solárová et al. Carbon nutrition was provided as 0.6 g (CO₂) m⁻³ [0.1 M solutions of sodium bicarbonate (NaHCO₃) and sodium carbonate (Na₂CO₃) mixed in the ratio 77/23 (v/v)], 10.0 and 40.0 g (CO₂) m⁻³ [3 M solutions of potassium bicarbonate (KHCO₃) and potassium carbonate (K₂CO₃) mixed in the ratio of 50/50 and 73/27 (v/v), respectively. Carbon dioxide-free atmosphere was created by keeping a 10.0% potassium hydroxide (KOH) solution in the acrylic box. The solutions were kept inside the boxes in open Petri plates providing maximum surface area for diffusion of CO₂ and were changed every 5th day.

For each treatment, six culture flasks with sucrose in the medium (3 SCSM = sucrose containing semi-solid medium + 3 SCLM = sucrose containing liquid medium) and six without sucrose (3 SFSM = sucrose free semi-solid medium + 3 SFLM sucrose free liquid medium), each inoculated with a shoot cluster, were placed in separate acrylic boxes providing different concentrations of CO₂. The boxes containing culture vessels were kept under controlled conditions of culture room (temperature, 28 ± 2°C; light, 45 μmol m⁻² s⁻² for 16 h per day provided by white fluorescent tubes, Philips; and 50-60% RH). Similarly, twelve culture flasks (3 SCSM Control +3 SCLM and 3 SFSM + 3 SFLM) were also placed in growth room under ambient air environment for comparisons. Observations were recorded after 40 days of growth.

**Water Content and Biomass Accumulation**

The fresh and dry weight were determined by weighing shoot clusters on Top Pan Electronic Balance (Contech, India) 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:

\[
\text{Percent water content} = \frac{\text{FW} - \text{DW}}{\text{FW}} \times 100
\]

**Chlorophyll Fluorescence Studies**

Chlorophyll fluorescence parameters during *in vitro* growth were measured using a Pulse Modulated Chlorophyll Fluorometer (FL2 LP) of Quibit Systems (Ontario, Canada). Fluorescence values were determined for 6 leaves (young) of almost similar leaf area growing under similar conditions. All the measurements were carried out during mid-morning. Prior to the first measurement, the leaf was dark-adapted for 30 min. Following the dark adaptation, the leaf was detached from the shoot under aseptic conditions and placed in the leaf clamp keeping adaxial surface upwards. At the beginning of each experiment, the background/minimal fluorescence \(F_m\) from a dark-adapted leaf was measured when only the LED light \((3-5 \, \mu\text{mol} \, \text{m}^{-2} \, \text{s}^{-1})\) was turned on. Following exposure to a modulated weak light, a 0.8 s saturating pulse of more than 2800 \(\mu\text{mol} \, \text{m}^{-2} \, \text{s}^{-1}\) PAR was applied to the leaf and the Maximal fluorescence \(F_v\) of the dark adapted leaf was recorded. Quantum yield \((P = F_v/F_m)\) of a dark-adapted leaf was calculated as \((F_v - F_m)/F_m\). The leaves were next irradiated with white actinic radiation \((45 \pm 5 \, \mu\text{mol} \, \text{m}^{-2} \, \text{s}^{-1})\) under which the cultures were kept. Subsequently, a saturating \((0.8 \, \text{s of 2800} \, \mu\text{mol} \, \text{m}^{-2} \, \text{s}^{-1} \, \text{PAR})\) flash was imposed to determine the maximum level in the light-adapted state \((F'_m)\). The steady-state value of fluorescence \((F_t)\) was thereafter recorded by imposing saturating pulses of high light intensity every 20 seconds for 10 minutes. The quantum efficiency of PS 2 photochemistry (ΦPS2) was calculated as follows:

\[
\Phi_{PS2} = \frac{F'_m - F_m}{F'_m}
\]

**Statistical Analysis**

Two-way analysis of variance and Tukey’s post hoc tests were performed using the SPSS 10.0 computer package (SPSS, Chicago, USA) for all sets of data, and means were compared at \(P < 0.05\).

**Results**

**CO₂ Enrichment With and Without Sucrose**

Carbon dioxide enrichment during *in vitro* multiplication of *C. paniculatus* was promotory both in case of semi-solid as well as liquid medium. Under ambient conditions of growth room, shoot clusters grown on SCSM multiplied at a rate of 3.12-folds producing ca. 3.81 cm long shoots (Table 1). During multiplication, nearly 55.44 leaves having 52.91 mm² leaf area per cluster were produced.

### Table 1. Effect of CO₂ enrichment with and without sucrose on *in vitro* shoot growth and multiplication in *C. paniculatus* shoot clusters grown on semi-solid and liquid medium (Observations were recorded after 40 days)

| Media       | CO₂ Conc. (g m⁻³) | No. of Shoots Per Cluster | Rate of Multiplication (in folds) | Shoot Length | No. of Leaves | Leaf Area (mm²) |
|-------------|------------------|--------------------------|-----------------------------------|--------------|---------------|----------------|
| SCSM 0.0    | 15.61¹           | 2.93²                    | 3.76¹                             | 45.84⁴      | 50.32⁴        |
| 0.6         | 18.03¹           | 3.12²                    | 3.81¹                             | 55.44⁴      | 52.91⁴        |
| 10.0        | 25.33³           | 5.98³                    | 5.76⁶                             | 90.99⁵      | 125.03⁵       |
| 40.0        | 24.12¹           | 5.61¹                    | 5.13¹                             | 86.23⁴      | 110.13⁴       |
| SCLM 0.0    | 30.12¹           | 3.31²                    | 4.12¹                             | 58.09⁶      | 65.12⁶        |
| 0.6         | 40.62¹           | 5.51¹                    | 6.11¹                             | 85.98⁵      | 81.00⁵        |
| 10.0        | 45.43³           | 7.30³                    | 8.61¹                             | 100.13³     | 210.32³       |
| 40.0        | 43.36³           | 6.85³                    | 8.42¹                             | 97.32⁴      | 170.22⁴       |
| SFSM 0.0    | 5.03¹            | 1.71¹                    | 2.00¹                             | 10.12⁴      | 45.34³        |
| 0.6         | 7.77¹            | 2.11¹                    | 2.01¹                             | 15.14⁴      | 40.03³        |
| 10.0        | 12.56³           | 3.33³                    | 3.15¹                             | 30.90⁴      | 60.67³        |
| 40.0        | 18.91¹           | 4.38³                    | 3.99¹                             | 45.21⁵      | 90.98³        |
| SFLM 0.0    | 5.51³            | 2.03³                    | 2.00¹                             | 12.22⁵      | 40.73³        |
| 0.6         | 10.01³           | 2.65³                    | 2.51¹                             | 33.20⁴      | 51.28³        |
| 10.0        | 15.56³           | 3.29³                    | 3.63¹                             | 67.23³      | 55.79³        |
| 40.0        | 27.66³           | 4.96³                    | 4.75⁵                             | 80.88bd⁶    | 130.99⁹       |
| SEM         | 1.167            | 0.2359                   | 0.296                             | 2.543       | 5.249         |
| CD 5%       | 3.335            | 0.6744                   | 0.846                             | 7.268       | 15            |
| CD 1%       | 4.464            | 0.9029                   | 1.133                             | 9.73        | 20.08         |
| CV          | 9.00             | 9.6666                   | 11.411                            | 7.3208      | 10.3253       |

SCSM, Sucrose containing semi-solid medium; SCLM, Sucrose containing liquid medium; SFSM, Sucrose free semi-solid medium; SFLM, Sucrose free liquid medium; Means followed by different letters differ significantly at 5%.
In liquid medium, all the growth parameters enhanced significantly (almost doubled) on medium containing 3.0% sucrose under ambient conditions of growth room (Figure 1 a).

The additive effect of the combination of sucrose (3.0%) and CO₂ enrichment on in vitro shoot growth and multiplication was clearly observed in SCSM as well as SCLM. Under CO₂-free conditions the growth parameters were almost similar to those cultures growing under ambient air atmosphere (Figure 1 b i, ii). Upon increasing the CO₂ concentration from 0.0 to 0.6 gm⁻³ the growth parameters also increased considerably in both SCSM and SCLM. Shoot multiplication rate, average shoot length, average number of leaves per cluster and leaf area reached to maximum at 10.0 g m⁻³ CO₂. Shoot cultures grown on SCLM under different concentrations of CO₂ evoked responses better than that was obtained on SCSM (Figure 1 di, ii). Maximum growth was observed on SCLM supplemented with 10.0 g m⁻³ CO₂. Under these conditions the shoots multiplied at a rate of 7.3-folds producing 100 leaves per cluster with average leaf area and shoot length of 210.32 mm² and 8.61 cm, respectively. A further increase in CO₂ concentration did not affect growth significantly and parameters slightly lower or of almost equal level were recorded (Figure 1 e i, ii).

In sucrose-free environment the shoot cultures grew efficiently. However, a significant decline in different growth parameters was observed when the SFSM and SFLM shoot cultures were grown under CO₂-free environment (Figure 1b iii, iv). The shoots remained stunted and turned brown within 15 days and died subsequently. The cultures incubated in the ambient atmosphere on sucrose–free semi–solid and liquid medium also could not sustain growth beyond 25 days. Improvement in in vitro growth and multiplication of shoots was observed when SFSM and SFLM cultures were grown under enriched CO₂ environment. As the concentration of CO₂ was increased from ambient to 0.6 gm⁻³ an increment in all the growth parameters was also observed. The best response was obtained when SFLM cultures were grown under 10.0 g m⁻³ CO₂ enriched environment (Figure 1d iv). At this concentration (10.0 g m⁻³), ca. 4.96-fold rate of shoot multiplication was achieved with an average of 80 leaves (130 mm² leaf area) per cluster. The shoots measured an average of 4.75 cm in length. Increase in concentration of CO₂ beyond this level did not increase growth significantly. Moreover, no visible symptoms of decline in growth were observed (Figure 1e i, iii, iv).

Chlorophyll Fluorescence Studies
Changes in chlorophyll a fluorescence parameters of C. paniculatus during in vitro multiplication on semi-solid and liquid medium under CO₂ enriched conditions are presented in Table 2.

The dark adapted leaves of SCSM under varied concentrations of CO₂ exhibited a Fₘ ranging between 0.23-0.39. Upon increasing the concentration of CO₂ from 0.6 to 40.0 gm⁻³ in SCSM the Fₘ of the leaves also increased from 0.33 to a maximum of 0.39 at 40.0 gm⁻³ of CO₂. In SCLM leaves nearly equal values of Fₘ were recorded at 0.0 and 0.6 gm⁻³ of CO₂ in comparison with SCSM leaves whereas significant differences were noticed at Am, 10.0 and 40.0 gm⁻³ of CO₂. Complete absence of both sucrose and CO₂ significantly reduced the Fₘ to a minimal level of 0.10 and 0.25 in leaves harvested from SFSM and SFLM, respectively. Elevating the carbon dioxide concentration thereafter increased the Fₘ significantly in both the media types with maximum values of 0.59 (at 10.0 gm⁻³ of CO₂ in SFSM) and 0.68 (at 40.0 gm⁻³ of CO₂ in SFLM). However, all the values of Fₘ more or less fell into the range (0.2-0.4) of a non-stressed plant in case of sucrose containing medium as well as sucrose-free medium.

The maximal fluorescence (Fₘ') under dark conditions also followed a similar trend as recorded for the basal fluorescence (Fₘ). In the leaves excised from shoots cultured on SCSM medium the Fₘ' increased from 0.71 to 0.89 as the CO₂ concentration increased from 0.0 to 40.0
The presence and absence of sucrose in the medium significantly influenced the Fm/Fm (maximal quantum yield) of the leaves. An Fm/Fm of 0.60 was obtained in cultures growing under ambient air of atmosphere on SC medium. The Fm/Fm declined with increasing the concentration of CO₂ in semi-solid medium reaching to almost minimum of 0.56 in SC cultures grown at 40.0 gm⁻³. In leaves growing in complete absence of CO₂ in SC medium the Fm/Fm severely reduced to 0.54. On the contrary, in the leaves of shoots grown on SCM a significant improvement in the Fm/Fm was recorded at all the concentrations of CO₂ as compared to its semi-solid counterparts. A maximum Fm/Fm of 0.70 was observed at 10.0 gm⁻³ of CO₂ which declined to 0.64 upon further increasing the CO₂ concentration to 40.0 gm⁻³. Both sucrose-free and CO₂-free conditions led to a severe reduction in the Fm/Fm in semi-solid as well as liquid medium. Increase in the CO₂ (0.6-40.0 gm⁻³) concentration in sucrose-free conditions improved the Fm/Fm reaching to 0.65 in SFM and SFLM at 10.0 gm⁻³ of CO₂.

The ΦPS2 was also variably affected by the presence and absence of CO₂ and sucrose in the medium. A maximum ΦPS2 of 0.56 was recorded in SC medium incubated under the ambient air of atmosphere which declined significantly with increasing concentrations of CO₂ from 0.6 to 10.0 gm⁻³. In sucrose containing liquid medium also maximum ΦPS2 was recorded at a concentration of CO₂ reaching to ca. 0.66 in SFM and SFLM at 10.0 gm⁻³ of CO₂.

Water Content and Biomass Accumulation
CO₂ enrichment in addition to sucrose in the medium
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Photoautotrophic growth of *Celastrus paniculatus*

significantly promoted the fresh and dry weight of shoot cultures with the water content values ranging from 80.92% to 89.13% (Table 3). Liquid medium further added to the effect produced on the biomass and moisture accumulation by CO$_2$ enrichment and sucrose in the medium. Sucrose–free and CO$_2$-enriched conditions were also stimulatory in terms of biomass production. The water content of such cultures registered an increment over sucrose–containing cultures. Approximately, 80% moisture accumulation was recorded in SCSM cultures incubated under ambient air and CO$_2$-free conditions. In SCLM cultures a total water content of 84.61 and 86.53% was recorded in CO$_2$-free and ambient air conditions, respectively. An improvement in total fresh and dry weight was also observed under similar conditions. Maximum fresh and dry weight accumulation was recorded on SCSM at 10.0 gm$^{-3}$ of CO$_2$. However, the moisture content of these cultures was also high. A further increase in CO$_2$ concentration from 40.0 gm$^{-3}$ did not play any noticeable effect on biomass production, but the water content was considerably increased. Improved in *in vitro* growth and multiplication was obtained when sucrose-free cultures were grown under a controlled and CO$_2$-enriched environment. Low sucrose contents promoted the photosynthetic rate of Quince shoots.

**Discussion**

CO$_2$-enrichment in semi-solid as well as liquid medium greatly stimulated *in vitro* shoot growth and multiplication in *C. paniculatus*. Lack of both the carbon sources (CO$_2$ and sucrose) caused gradual deterioration of cultures and subsequent death in SFSM and SFLM due to starvation. Improvement in *in vitro* growth and multiplication was obtained when sucrose-free cultures were grown under a controlled and CO$_2$-enriched environment. Low sucrose contents promoted the photosynthetic rate of Quince shoots.

Water accumulation was more in SFLM cultures falling within the range of 91–95% as compared to SFSM (89.0–90.0%).

### Table 3. Percent water content and other growth parameters in *C. paniculatus* grown under CO$_2$-enriched conditions (Observations were recorded after 40 days)

| Media Type | CO$_2$ conc. (g m$^{-3}$) | Fresh Weight (g/Cluster) | Dry Weight (g/Cluster) | Percent Water Content |
|------------|--------------------------|--------------------------|------------------------|-----------------------|
| SCSM       | 0.0                      | 1.248$^f$ 0.238$^e$     | 80.92$^a$              |                       |
|            | Am                       | 1.386$^b$ 0.276$^a$     | 80.29$^a$              |                       |
|            | 0.6                      | 1.756$^c$ 0.299$^d$     | 82.97$^{abc}$          |                       |
|            | 10.0                     | 2.341$^d$ 0.412$^{bc}$  | 82.40$^{ab}$           |                       |
|            | 40.0                     | 2.073$^e$ 0.405$^c$     | 80.46$^a$              |                       |
| SCLM       | 0.0                      | 2.561$^d$ 0.394$^c$     | 84.61$^{bcd}$          |                       |
|            | Am                       | 3.030$^c$ 0.408$^b$     | 86.53$^{bce}$          |                       |
|            | 0.6                      | 3.231$^e$ 0.404$^c$     | 87.49$^{abcde}$        |                       |
|            | 10.0                     | 4.350$^d$ 0.540$^c$     | 87.58$^{bde}$          |                       |
|            | 40.0                     | 3.975$^a$ 0.432$^b$     | 89.13$^{b cde}$        |                       |
| SFSM       | 0.0                      | 0.110$^k$ 0.010$^j$     | 93.90$^{hi}$           |                       |
|            | Am                       | 0.430$^b$ 0.044$^a$     | 90.69$^{bhi}$          |                       |
|            | 0.6                      | 0.745$^g$ 0.069$^h$     | 90.73$^{bhi}$          |                       |
|            | 10.0                     | 1.470$^d$ 0.150$^c$     | 89.79$^{bhi}$          |                       |
|            | 40.0                     | 1.350$^a$ 0.140$^b$     | 89.62$^{bhi}$          |                       |
| SFLM       | 0.0                      | 0.215$^a$ 0.010$^j$     | 95.34$^{a}$            |                       |
|            | Am                       | 0.555$^a$ 0.043$^a$     | 92.25$^{a}$            |                       |
|            | 0.6                      | 0.991$^c$ 0.080$^b$     | 91.92$^{b}$            |                       |
|            | 10.0                     | 1.982$^a$ 0.110$^a$     | 94.45$^{a}$            |                       |
|            | 40.0                     | 1.566$^d$ 0.090$^b$     | 94.25$^{a}$            |                       |
| SEM        |                          | 0.05477 0.9539          | 3.205                  |                       |
| CD 5%      |                          | 0.1565 2.726            | 9.192                  |                       |
| CD 1%      |                          | 0.2096 3.65             | 12.33                  |                       |
| CV         |                          | 41.6745 1.8749         | 16.3203                |                       |

SCSM, Sucrose containing semi-solid medium; SCLM, Sucrose containing liquid medium; SFSM, Sucrose free semi-solid medium; SFLM, Sucrose free liquid medium.
growth in the present case. This can be attributed to the added effect of endogenous carbon level. The shoots grew sustainably on sucrose-free medium under CO$_2$-enriched conditions. The cultures grown under additional CO$_2$ supply appeared to have luxury consumption and fertilizing effect in the cultures grown in vitro. The photoautotrophic growth on sucrose-free medium has been observed in Cannabis, Hypericum perforatum, Cymbidium and Carica papaya. Regardless of the sugar contents in the medium, an increase in CO$_2$ concentration greater than the optimal concentration resulted in decreased growth in a few cases. This might be because of down regulation of photosynthesis found in plants growing under CO$_2$ enrichment or may be due to their incubation under PAR limited conditions. In the present investigation, the combination of 3.0% sucrose and controlled and enriched CO$_2$ environment proved to be a better option for in vitro shoot growth and multiplication in C. paniculatus. Similar synergistic effects of combinations of sucrose and CO$_2$ on in vitro shoot growth were observed in tobacco, Wrightia tomentosa, apple, C. papaya and Actinidia delicosa. Besides this, it was also observed that liquid medium was superior in overall growth over semi-solid medium in CO$_2$-enriched conditions. CO$_2$ enrichment in liquid culture system was promotory for growth in Uniola paniculata and Musa. The photosynthetic capacity of C. paniculatus was assessed while providing CO$_2$ enrichment to the cultures. Significantly varied responses in terms of all the fluorescence parameters were obtained under these conditions. Measurements of F$_{v}$/F$_{m}$ and ΦPS2 provided an indirect estimation of the photosynthetic efficiency of the cultures. Higher values of these parameters are suggestive of photosynthetically active cultures. The value of F$_{v}$/F$_{m}$ fell in the moderate range of 0.6-0.7 in the present case, which has been considered as values obtained for stressed plants. This anomaly can be explained by the fact that plants are considered to be under stable stress if the value of F$_{v}$/F$_{m}$ falls beyond 0.5. Values nearing to 0.6 revealed that the cultures were under transient stress which can be reverted back to normal when appropriate conditions arise. Our results suggested that a lower F$_{v}$/F$_{m}$ indicating transient stress on our cultures could be due to their incubation under low light intensities. As the cultures would undergo hardening and acclimatization, non-stressed F$_{v}$/F$_{m}$ values might be obtained.

On sucrose supplemented medium CO$_2$ enrichment significantly influenced the F$_{v}$/F$_{m}$, ΦPS2 and other fluorescence parameters of shoot cultures. However, maximum photochemical yield was recorded in the in vitro multiplying cultures obtained from SCSM and SCLM growing under CO$_2$-free conditions or at lower concentration of carbon dioxide. The photochemical parameters (F$_{v}$, F$_{m}$, F$_{v}'$, F$_{m}'$, F$_{v}$/F$_{m}$, and ΦPS2) continued to decline with increase in concentrations of carbon dioxide. Further, a considerable reduction in the photochemical efficiency was recorded in SCSM supplemented with maximum amounts of CO$_2$ i.e. 40.0 gm$^{-3}$. This goes well with the hypothesis that excess sugars cause the down regulation of photosynthesis. A down regulation of photosynthesis was observed when sugars were fed to suspension cultured cells or tobacco leaves, or when the balance between the production and the consumption of carbohydrates was disturbed. Sugar in the medium has been reported to reduce the Rubisco activity, and thus the photosynthetic efficiency of in vitro plantlets.

Cultures of C. paniculatus grown under CO$_2$-free and sucrose-free conditions demonstrated extremely poor photosynthetic capability as indicated by their extremely low F$_{v}$, F$_{m}$, F$_{v}'$, F$_{m}'$, F$_{v}$/F$_{m}$ and ΦPS2. The experimental results obtained by Fujimura et al and Infante et al suggested that insufficient CO$_2$ supply into the vessel limited the photosynthesis.

Elevated CO$_2$ without sucrose in the medium markedly increased the chlorophyll fluorescence parameters in C. paniculatus as compared to the CO$_2$-free conditions. Growth of plantlets on medium without saccharides enables the development of fully functional photosynthetic apparatus. These plantlets usually need elevated CO$_2$ concentration and higher irradiance than conventionally used. Our results are in conformity with the above statement. In all the plant systems maximum F$_{v}$/F$_{m}$ and ΦPS2 were obtained at either 10.0 or 40.0 gm$^{-3}$ of CO$_2$ both in SFSM and SFLM. An increase in rate of photosynthesis has been reported in various crop species by various researchers under enriched CO$_2$. Carbon dioxide enrichment with sucrose promoted fresh and dry weight accumulation in C. paniculatus. A comparison between cultures grown on semi-solid and liquid medium revealed that the latter stimulated more biomass accumulation than the former. Also, the cultures grown on liquid medium showed high percent water content. Fully photoautotrophic conditions promoted maximum plant growth in terms of both fresh and dry mass in Actinidia delicosa. In vitro culture under photoautotrophic conditions has been shown to promote the fresh and dry weight contents of several species, including, Phalaenopsis, Cymbidium kanran, and C. goeringii, Hypericum perforatum, Uniola paniculata and Musa plantain. Higher plantlet dry mass and contents of photosynthetic pigments, facilitated ex vitro acclimation and growth in Capsicum. Plantlet growth has been shown to increase by high PPF and CO$_2$ concentration. The increase in water content in SCLM cultures was as a result of the high humidity in the culture vessels due to liquid medium. Withdrawal of sucrose from the medium did not favour an increase in fresh and dry masses. This observation is consistent with the findings of Arigita et al with Kiwi explants and Valero-Aracama et al in sea oats under similar culture conditions. The reason behind this was that some cultures require an initial source of carbon from the medium until
they are capable of using CO$_2$ from the vessel headspace as their main carbon source. Sucrose markedly enhances the biomass of plantlets. Biomass accumulation was highest in Quince shoots cultured with 30 g dm$^{-3}$ sucrose. However, reduced per cent water content indicated that the cultures were not hyperhydric. Under sucrose-free conditions also, liquid medium superseded the response on semi-solid medium in terms of their fresh and dry weight. The best growth and quality of Coffea arabusta, Eucalyptus, and Cannabis plantlets were obtained from the cultures grown under CO$_2$ enrichment in sugar-free liquid medium.

Conclusions
CO$_2$ enrichment stimulated shoot growth and multiplication in C. paniculatus in semi-solid and liquid medium. The presence of both sucrose and carbon dioxide synergistically influenced the in vitro growth. However, excess of CO$_2$ beyond a certain limit retarded the growth rate. Liquid medium was superior over the semi-solid medium in terms of multiplication rate and shoot growth under CO$_2$ enriched conditions. Sucrose was responsible for the increase in biomass. In sucrose depleted medium the fresh and dry weight gain in growing cultures was not at par with those grown on sucrose containing medium in semi-solid as well as liquid media. In all the cases the per cent moisture content of shoot cultures was always higher when they were grown in liquid medium which was due to the higher relative humidity inside the culture vessel. No visual symptoms of hyperhydricity were observed in any of the cultures.

The study of chlorophyll fluorescence revealed that the presence of sucrose in the medium did not favour the photochemical yield of the plant system. This was evident from the F$_{v}$/F$_{m}$ values obtained under sucrose-free and sucrose-containing medium. When sucrose was present in the medium and the CO$_2$ concentration increased, the F$_{v}$/F$_{m}$ value either decreased or remained stable, whereas in absence of sucrose, an increase in CO$_2$ concentration always led to an increase in the F$_{v}$/F$_{m}$. This was true with other fluorescence parameters also. The photochemical yield was hypothesized as an indirect measurement for the photosynthetic efficiency of the plant system in the present investigation. Hence we can say that the presence of sucrose in the medium reduced the photosynthetic efficiency of the shoot cultures of C. paniculatus.

It was therefore, concluded that the in vitro growth of C. paniculatus was better in liquid medium under CO$_2$ enriched conditions. Withdrawal of sucrose from the medium provided photosynthetically efficient shoots. The plantlets derived from them presumably would be better equipped to survive the transplantations shocks during their ex vitro growth.

Competing Interests
None.

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