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Original Article

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_o, F_m, F'_m, F_t, F_v/F_m and ΦPS2) 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_o, F_m, F'_m, F_t, F_v/F_m and ΦPS2 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 ΦPS2. 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_m and ΦPS2 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

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.[1] The chief phytoconstituents of medicinal value testified in *C. paniculatus* include malkangunin, celapanin, celapanigin, celapagin, celastrol, pristimerin, and zeylasterone.[2] The methanolic extracts of *C. paniculatus* have been described to exhibit free-radical-scavenging properties and anti-oxidant effects in human non-immortalized fibroblasts.[3] The powdered root is considered useful for the treatment of cancerous tumors.[4] Besides its phytotherapeutic importance, *C. paniculatus* has been explored for its potential in herbal cosmetics.[5] The seed oil has been reported to be effective against dandruff and premature graying of the hair[6,7] by the rural and tribal communities. The seed oil has also been recommended for its use as carrier oil in several skin care formulations.[8,9]

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[10] and therefore *ex situ* conservation efforts have been devoted through micropropagation using different pathways.[11,12] All these studies involved conventional methods of micropropagation where the desired rate of shoot multiplication could not be achieved. The goal

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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.[13-19] Liquid media are ideal in micropropagation for reducing plantlet production costs, increasing rate of multiplication and for automation.[20,21] 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.[22]

Further, in conventional micropropagation, sucrose is the main source of carbon and energy in the nutrient medium during micropropagation of plants[23] but its presence increases the risk of contamination and depresses photosynthetic activity leading to mixo- or heterotrophy.[24] This causes morphological and physiological disorders in *in vitro* grown plants resulting into high rate of mortality during hardening, acclimatization and field transfer.[25,26] One of the ways to circumvent this problem is the photoautotrophic multiplication of shoots on a sucrose-free medium under CO_2-enriched conditions. Photoautotrophic multiplication of shoots on sugar-free media has proved useful particularly to avoid or reduce contamination and improve the acclimation ability in micropropagated plantlets subjected to photosynthetically active conditions.[24,27] Enhanced plantlet growth and multiplication under controlled carbon dioxide enriched environment have been achieved in a number of herbaceous,[28,29] and woody plants.[30-36]

The present study was undertaken hypothesizing that the liquid medium and CO_2 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 CO_2. 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.[37] 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 beads 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.

CO_2 Enrichment With and Without Sucrose During *In Vitro* Shoot Multiplication

Experiments were conducted to examine the impact of controlled and enriched CO_2 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 mLculture flasks (Borosil) stoppered with non-absorbent cotton plugs. Shoot cultures were exposed to different CO_2 concentrations [0.0, 0.6, 10.0 and 40.0 g (CO_2) m^{-3}] applied in transparent acrylic chambers each with a volume of 7500 cm^3 (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 CO_2 in acrylic chambers were controlled as per the method described by Solárová et al.[38] Carbon nutrition was provided as 0.6 g (CO_2)m^{-3} [0.1 M solutions of sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) mixed in the ratio 77/23 (v/v)], 10.0 and 40.0 g (CO_2) m^{-3} [3M solutions of potassium bicarbonate ($KHCO_3$) and potassium carbonate (K_2CO_3) 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 CO_2 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 CO_2. 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 [39] formula:

$$\text{Percent water content} = \frac{FW - DW}{FW} X\ 100$$

Chlorophyll Fluorescence Studies

Chlorophyll fluorescence parameters during *in vitro* growth were measured using a Pulse Modulated Chlorophyll Fluorometer (FL2 LP) of Qubit 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-noon. 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_o) from a dark-adapted leaf was measured when only the LED light (3-5 µmol $m^{-2}s^{-1}$) was turned on. Following exposure to a modulated weak light, a 0.8 s saturating pulse of more than 2800 µmol m^{-2} s^{-1} PAR was applied to the leaf and the Maximal fluorescence (F_m) of the dark adapted leaf was recorded. Quantum yield ($P = F_v/F_m$) of a dark-adapted leaf was calculated as $(F_m - F_o)/F_m$. The leaves were next irradiated with white actinic radiation (45 ± 5 µmol $m^{-2}s^{-1}$) under which the cultures were kept. Subsequently, a saturating (0.8 s of 2800 µmol m^{-2} s^{-1} 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[40]:

$$\Phi PS2 = (F_m' - F_t)/F_m'$$

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

CO_2 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 mm^2 leaf area per cluster were produced.

Table 1. Effect of CO_2 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	CO_2 Conc. (g m^{-3})	No. of Shoots Per Cluster	Rate of Multiplication (in folds)	Shoot Length	No. of Leaves	Leaf Area (mm^2)
SCSM	0.0	15.61^{i}	2.93^{gh}	3.76^{gh}	45.84^{j}	50.32^{k}
	$_{Am}$Con	**18.03^{h}**	**3.12^{g}**	**3.81^{g}**	**55.44^{h}**	**52.91^{k}**
	0.6	21.74^{gh}	4.18^{f}	4.31^{e}	77.32^{e}	80.12^{h}
	10.0	25.33^{f}	5.98b^{c}	5.76^{c}	90.99^{bc}	125.03^{d}
	40.0	24.12^{f}	5.61^{c}	5.13^{c}	86.23^{c}	110.13^{ef}
SCLM	0.0	30.12^{e}	3.31^{gh}	4.12^{f}	58.09^{gh}	65.12^{ij}
	Am	40.62^{c}	5.51^{cd}	6.11^{b}	85.98^{c}	81.00^{h}
	0.6	35.20^{d}	6.23^{bc}	6.34^{b}	85.24^{c}	81.29^{h}
	10.0	**45.43^{a}**	**7.30^{a}**	**8.61^{a}**	**100.13^{a}**	**210.32^{a}**
	40.0	43.36^{b}	6.85^{ab}	8.42^{a}	97.32^{a}	170.22^{b}
SFSM	0.0	5.03^{l}	1.71^{j}	2.00^{k}	10.12^{k}	45.34^{l}
	Am	7.77^{k}	2.11^{i}	2.01^{k}	15.14^{k}	40.03^{l}
	0.6	12.56^{j}	3.33^{gh}	3.15^{i}	30.90^{j}	$60.67j^{k}$
	10.0	**20.11^{gh}**	**4.87^{de}**	**4.68^{d}**	**45.80^{ij}**	**95.12^{fg}**
	40.0	18.91^{h}	4.38^{ef}	3.99^{fg}	45.21^{i}	90.98^{g}
SFLM	0.0	5.51^{l}	2.03^{ij}	2.00^{k}	12.22^{k}	40.73^{l}
	Am	10.01^{j}	2.65^{hi}	2.51^{j}	33.20^{j}	51.28^{k}
	0.6	15.56^{i}	3.29^{gh}	3.63^{h}	67.23^{f}	55.79^{k}
	10.0	**27.66^{f}**	**4.96^{de}**	**4.75^{d}**	**$80.88d^{e}$**	**130.99^{cd}**
	40.0	26.98^{f}	4.01^{fg}	4.81^{d}	78.97^{e}	123.45^{d}
	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	9.6866	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 CO_2 enrichment on *in vitro* shoot growth and multiplication was clearly observed in SCSM as well as SCLM. Under CO_2-free conditions the growth parameters were almost similar to those cultures growing under ambient air atmosphere (Figure 1 b i, ii). Upon increasing the CO_2 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} CO_2. Shoot cultures grown on SCLM under different concentrations of CO_2 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} CO_2. 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 mm^2 and 8.61 cm, respectively. A further increase in CO_2 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 CO_2-freeenvironment (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 CO_2 environment. As the concentration of CO_2 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} CO_2 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 mm^2 leaf area) per cluster. The shoots measured an average of 4.75 cm in length. Increase in concentration of CO_2 beyond this level did not increase growth significantly. Moreover, no visible symptoms of decline in growth were observed (Figure 1e iii, iv).

Figure 1. Effect of CO_2 enrichment on *in vitro* shoot multiplication in *c. paniculatus* grown on semi-solid and liquid medium (a) Ambient air of growth room; (b) 0.0 gm^{-3} CO_2; (c) 0.6 gm^{-3} CO_2 (d) 10.0 gm^{-3} CO_2; (e) 40.0 gm^{-3} CO_2.

Chlorophyll Fluorescence Studies

Changes in chlorophyll *a* fluorescence parameters of *C. paniculatus* during *in vitro* multiplication on semi-solid and liquid medium under CO_2 enriched conditions are presented in Table 2.

The dark adapted leaves of SCSM under varied concentrations of CO_2 exhibited a F_o ranging between 0.23-0.39. Upon increasing the concentration of CO_2 from 0.6 to 40.0 gm^{-3} in SCSM the F_o of the leaves also increased from 0.33 to a maximum of 0.39 at 40.0 gm^{-3} of CO_2. In SCLM leaves nearly equal values of F_o were recorded at 0.0 and 0.6 gm^{-3} of CO_2 in comparison with SCSM leaves whereas significant differences were noticed at Am, 10.0 and 40.0 gm^{-3} of CO_2. Complete absence of both sucrose and CO_2 significantly reduced the F_o 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 F_o significantly in both the media types with maximum values of 0.59 (at 10.0 gm^{-3} of CO_2 in SFSM) and 0.68 (at 40.0 gm^{-3} of CO_2 in SFLM). However, all the values of F_o 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 (F_m) under dark conditions also followed a similar trend as recorded for the basal fluorescence (F_o). In the leaves excised from shoots cultured on SCSM medium the F_m increased from 0.71 to 0.89 as the CO_2 concentration increased from 0.0 to 40.0

Table 2. Effect of CO2 Enrichment on chlorophyll fluorescence parameters of *C. paniculatus* (Observations were recorded after 40 days of growth)

Media	CO_2 Conc. (gm^{-3})	Fo	Fm	F'm	Ft	Fo'	Fv/Fm	ΦPS2
SCSM	0.0	0.31^{cd}	0.71^{f}	0.68^{e}	0.47^{d}	0.36^{c}	0.54^{e}	0.27^{j}
	Am	**0.23^{e}**	**0.58^{g}**	**0.55^{f}**	**0.24^{f}**	**0.22^{d}**	**0.60^{c}**	**0.56^{d}**
	0.6	0.33^{cd}	0.81^{e}	0.74^{d}	0.48^{d}	0.38^{c}	0.59^{c}	0.35^{h}
	10.0	0.35^{c}	0.84^{e}	0.73^{d}	0.41^{d}	0.37^{c}	0.58^{cd}	0.43^{f}
	40.0	0.39^{b}	0.89^{d}	0.84^{c}	0.43^{d}	0.38^{c}	0.56^{de}	0.49^{ab}
SCLM	0.0	0.29^{d}	0.85^{d}	0.86^{c}	0.37^{e}	0.36^{c}	0.66^{b}	0.56^{d}
	Am	0.38^{b}	1.10^{c}	1.05^{b}	0.56^{c}	0.44^{b}	0.65^{b}	0.47^{a}
	0.6	0.32^{cd}	0.93^{c}	1.15^{b}	0.35^{e}	0.35^{c}	0.67^{b}	0.65^{e}
	10.0	**0.42^{ab}**	**1.38^{a}**	**1.26^{a}**	**0.67^{a}**	**0.55^{a}**	**0.70^{a}**	**0.47^{a}**
	40.0	0.32^{cd}	0.89^{d}	0.81^{d}	0.52^{c}	0.43^{b}	0.64^{b}	0.34^{h}
SFSM	0.0	-	-	-	-	-	-	-
	Am	-	-	-	-	-	-	-
	0.6	0.43^{ab}	1.22^{b}	1.19^{a}	0.63^{b}	0.40^{b}	0.65^{b}	0.47^{a}
	10.0	0.59^{f}	1.30^{b}	1.21^{a}	0.74^{g}	0.62^{e}	0.55^{e}	0.39^{g}
	40.0	**0.38^{b}**	**1.19^{c}**	**0.88^{c}**	**0.38^{e}**	**0.33^{f}**	**0.66^{b}**	**0.51^{bc}**
SFLM	0.0	-	-	-	-	-	-	-
	Am	-	-	-	-	-	-	-
	0.6	0.48^{a}	0.92^{d}	0.89^{c}	0.66^{a}	0.51^{a}	0.48^{f}	0.26^{j}
	10.0	**0.51^{a}**	**1.44^{a}**	**1.33^{a}**	**0.64^{ab}**	**0.55^{a}**	**0.65^{b}**	**0.52^{c}**
	40.0	0.68^{g}	1.12^{c}	1.11^{b}	0.81^{g}	0.69^{f}	0.43^{g}	0.30^{i}
	CV	35.48	28.87	28.32	31.02	34.41	0.00	0.00

Means followed by different letters differ significantly at 5%

gm^{-3}, respectively. The F_m of leaves obtained from SCSM was lower than the F_m of leaves obtained from SCLM at 0.0, 0.6 and 10.0 gm^{-3} of CO_2 whereas it was higher at the ambient CO_2. In SCLM, maximum F_m (1.38) was recorded at 10.0 gm^{-3} of CO_2 which declined to 0.89 at 40.0 gm^{-3} of CO_2. Withdrawal of sucrose and carbon dioxide from the medium yielded minimum F_m in both (0.25) SFSM and (0.11) SFLM media. These minimal values reached to a maximal value of 1.30 and 1.44 in SFSM and SFLM media incubated at 10.0 gm^{-3} of CO_2, respectively. A decline in F_m at 40.0 gm^{-3} of CO_2 was recorded in both the media types.

Exposure of leaves to light yielded variable values of F'_m and F_t. The maximal fluorescence (F'_m) also followed the similar pattern as observed for F_o and F_m. Sucrose in the medium yielded a F'_m value within a range of 0.55 to 0.84 in SCSM leaves and 0.81 to 1.26 in SCLM leaves. On the contrary an F'_m ranging from 0.23 to 1.21 (SFSM leaves) and 0.11 to 1.44 (SFLM leaves) was recorded in sucrose medium. Maximum F'_m (1.33) was recorded on 10.0 gm^{-3} of CO_2 in SFLM leaves. The F_t values were at par with the corresponding F_o's indicating that the cultures were not under permanent stress.

The presence and absence of sucrose in the medium significantly influenced the F_v/F_m (maximal quantum yield) of the leaves. An F_v/F_m of 0.60 was obtained in cultures growing under ambient air of atmosphere on SCSM medium. The F_v/F_m declined with increasing the concentration of CO_2 in semi-solid medium reaching to almost minimum of 0.56 in SCSM cultures grown at 40.0 gm^{-3}. In leaves growing in complete absence of CO_2 in SCSM medium the F_v/F_m severely reduced to 0.54. On the contrary, in the leaves of shoots grown on SCLM a significant improvement in the F_v/F_m was recorded at all the concentrations of CO_2 as compared to its semi-solid counterparts. A maximum F_v/F_m of 0.70 was obtained at 10.0 gm^{-3} of CO_2 in SCLM which decreased to 0.64 upon further increasing the CO_2 concentration to 40.0 gm^{-3}. Both sucrose-free and CO_2-free conditions led to a severe reduction in the F_v/F_m in semi-solid as well as liquid media. Increase in the CO_2 (0.6-40.0 gm^{-3}) concentration in sucrose-free conditions improved the F_v/F_m reaching to ca. 0.66 in SFSM and SFLM at 10.0 gm^{-3} of CO_2.

The ΦPS2 was also variably affected by the presence and absence of CO_2 and sucrose in the medium. A maximum ΦPS2 of 0.56 was recorded in SCSM incubated under the ambient air of atmosphere which declined significantly with increasing concentrations of CO_2 from 0.6 to 10.0 gm^{-3}. In sucrose containing liquid medium also maximum ΦPS2 was recorded at a lower concentration of CO_2 (0.65 at 0.6 gm^{-3} of CO_2). Complete absence of both the carbon sources significantly reduced this parameter in shoot cultures grown on both the media types. Elevated concentrations of CO_2 resulted in an increase in ΦPS2 to *ca.* 0.5 at 10.0 and 40.0 gm^{-3} of CO_2 at SFLM and SFSM, respectively.

Water Content and Biomass Accumulation

CO_2 enrichment in addition to sucrose in the medium

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 increase 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 SCLM at 10.0 gm^{-3} of CO_2. However, the moisture content of these cultures was also high. A further increase in CO_2 (40.0 gm^{-3}) concentration in SCSM and SCLM medium did not play any noticeable effect on biomass production, but the water content was considerably increased. In SCSM also the concentration of 10.0 gm^{-3} CO_2 was best for the growth of cultures.

Cultures grown on sucrose–free medium incubated under 0.0 gm^{-3} CO_2 and ambient air faced severe consequences of growth retardation. A significant decline in fresh and dry weight was also observed under such conditions, with enormous amount of water accumulation. Increasing the CO_2 concentration from 0.6 gm^{-3} to 10.0 gm^{-3} thereafter led to increase in biomass production reaching to almost stable values at 40.0 gm^{-3} of CO_2. The water content of shoots also increased moderately. Water accumulation was more in SFLM cultures falling within the range of 91–95% as compared to SFSM (89.0–90.0%).

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.[41,42] 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.[43] Even a slight increase in the CO_2 concentration from ambient to 0.6 gm^{-3} significantly enhanced the overall

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
	0.0	1.248^{f}	0.238^{e}	80.92^{a}
	Am	**1.386^{f}**	**0.276^{d}**	**80.29^{a}**
SCSM	0.6	1.756^{e}	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}
	0.0	2.561^{d}	0.394^{c}	84.61^{bcd}
	Am	3.030^{c}	0.408^{c}	86.53^{cde}
SCLM	0.6	3.231^{c}	0.404^{c}	87.49^{def}
	10.0	**4.350^{a}**	**0.540^{a}**	**87.58^{def}**
	40.0	3.975^{b}	0.432^{b}	89.13^{efg}
	0.0	0.110^{k}	0.010^{j}	93.90^{hi}
	Am	0.430^{ij}	0.044^{i}	90.69^{fgh}
SFSM	0.6	0.745^{gh}	0.069^{h}	90.73^{fgh}
	10.0	**1.470^{f}**	**0.150^{f}**	**89.79^{efg}**
	40.0	1.350^{f}	0.140^{f}	89.62^{efg}
	0.0	0.215^{jk}	0.010^{j}	95.34^{i}
	Am	0.555^{hi}	0.043^{i}	92.25^{ghi}
SFLM	0.6	0.991^{g}	0.080^{h}	91.92^{ghi}
	10.0	**1.982^{e}**	**0.110^{g}**	**94.45^{i}**
	40.0	1.566^{f}	0.090^{gh}	94.25^{hi}
	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.[43] 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[44] and fertilizing effect[45] in the cultures grown *in vitro*. The photoautotrophic growth on sucrose-free medium has been observed in *Cannabis*,[46] *Hypericum perforatum*,[33] *Cymbidium*[29] and *Carica papaya*.[47] 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[48,49] or may be due to their incubation under PAR limited conditions.[50-52] 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,[53] *Wrightia tomentosa*,[31] apple,[54] *C. papaya*[46] and *Actinidia deliciosa*.[55] 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*[56] and *Musa*.[35]

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.[57] 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_o, F_m, F'_m, F_s, 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.[58-60] A down regulation of photosynthesis was observed when sugars were fed to suspension cultured cells[61] or tobacco leaves,[62] or when the balance between the production and the consumption of carbohydrates was disturbed.[60] Sugar in the medium has been reported to reduce the Rubisco activity, and thus the photosynthetic efficiency of *in vitro* plantlets.[22,63]

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_o, F_m, F'_m, F_s, F_v/F_m andΦPS2. The experimental results obtained by Fujiwara et al[41] and Infante et al[64] 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.[25,50-52,65,66] 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.[67-69]

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 deliciosa*.[55] *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*,[70] *Hypericum perforatum*,[33] *Uniola paniculata*[56] and *Musa* plantain.[35] Higher plantlet dry mass and contents of photosynthetic pigments, facilitated *ex vitro* acclimation and growth in *Capsicum*.[71] Plantlet growth has been shown to increase by high PPF,[72] elevated CO_2 concentration.[25] The increase in water content in SCLM cultures was as a result of the high humidity in the culture vessels due to liquid medium.[73] 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[55] and Valero-Aracama et al in sea oats[56] 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.[74] Biomass accumulation was highest in Quince shoots cultured with 30 g dm^{-3} sucrose.[43] 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,*[75] *Eucalyptus,*[76] and *Cannabis*[46] 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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