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DEVELOPMENTAL BIOLOGY

Thidiazuron-induced high-frequency direct shoot organogenesis of *Cannabis sativa* L.

Hemant Lata · Suman Chandra · Ikhlas Khan · Mahmoud A. ElSohly

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Abstract Induction of high-frequency shoot regeneration using nodal segments containing axillary buds from a 1-yr-old mother plants of *Cannabis sativa* was achieved on Murashige and Skoog (MS) medium containing 0.05–5.0 μM thidiazuron. The quality and quantity of regenerants were better with thidiazuron (0.5 μM thidiazuron) than with benzyladenine or kinetin. Adding 7.0 μM of gibberellic acid into a medium containing 0.5 μM thidiazuron slightly increased shoot growth. Elongated shoots when transferred to half-strength MS medium supplemented with 500 mg l^{-1} activated charcoal and 2.5 μM indole-3-butyric acid resulted in 95% rooting. The rooted plants were successfully acclimatized in soil. Following acclimatization, growth performance of 4-mo-old *in vitro* propagated plants was compared with *ex vitro* vegetatively grown plants of the same age. The photosynthesis and transpiration characteristics were studied under different light levels (0, 500, 1,000, 1,500, or 2,000 μmol m^{-2} s^{-1}). An increase in photosynthesis was observed with increase in the light intensity up to 1,500 μmol m^{-2} s^{-1} and then decreased subsequently at higher light levels in both types of plants. However, the increase was more pronounced at lower light intensities below 500 μmol m^{-2} s^{-1}. Stomatal conductance and transpiration increased with light intensity up to highest level (2000 μmol m^{-2} s^{-1}) tested. Intercellular CO_2 concentration (C_i) and the ratio of intercellular CO_2 concentration to ambient CO_2 (C_i/C_a) decreased with the increase in light intensity in both *in vitro* as well as *ex vitro* raised plants. The results show that *in vitro* propagated and hardened plants were functionally comparable to *ex vitro* plants of same age in terms of gas and water vapor exchange characteristics, within the limits of this study.

Keywords Acclimatization · Gas and water vapor exchange · Nodal explant · Plant growth regulators · Plant regeneration · Water use efficiency

Introduction

Cannabis sativa L. (marijuana) is an annual herb which originated in Central Asia and spread throughout Europe and later introduced into America (Quimby 1974). It contains cannabinoids, a unique class of terpenophenolic compounds which accumulates mainly in glandular trichomes of the plant (Hammond and Mahlberg 1977). Over 70 cannabinoids have been isolated from marijuana, the major biologically active compound being Δ^9-tetrahydrocannabinol, commonly referred as THC (Mechoulam and Ben-Shabat 1999). Besides its psychoactivity, THC possesses analgesic, anti-inflammatory, appetite stimulant, and anti-emetic properties, making this cannabinol a very promising drug for therapeutic purposes, especially for cancer and AIDS patients (Sirikantaramas et al. 2005).

H. Lata (✉) · S. Chandra · I. Khan · M. A. ElSohly
National Center for Natural Products Research,
School of Pharmacy, The University of Mississippi,
University, MS 38677, USA
e-mail: hlata@olemiss.edu

I. Khan
Department of Pharmacognosy, School of Pharmacy,
The University of Mississippi,
University, MS 38677, USA

M. A. ElSohly
Department of Pharmaceutics, School of Pharmacy,
The University of Mississippi,
University, MS 38677, USA

The genetic progress of any *Cannabis* breeding programs is limited due to the difficulty in maintaining selected high THC yielding elite genotypes under field or greenhouse conditions because of the allogamous (cross-fertilization) nature of species. It is therefore impossible to maintain elite cultivar/clones by seed. Clonal collections for medicinal species are therefore advantageous because they can facilitate the conservation of targeted lines for the pharmacological properties of the compound of interest in large quantities from representative samples. This is preferable to the storage of seed, which can be highly variable and produce less efficacious genotypes (Meijer et al. 1992). Thus, advanced biotechnological approaches, including tissue culture could be extended to *Cannabis* breeding as a response (in part) to prohibition enforcement efforts that make outdoor cultivation risky. Although plant regeneration protocols have been developed for different *Cannabis* genotypes and explant sources (Loh et al. 1983; Richez-Dumanois et al. 1986; Mandolino and Ranalli 1999; Slusarkiewicz-Jarzina et al. 2005; Bing et al. 2007), considerable variation has been reported in the response of cultures and in the morphogenic pathway. A report by Fisse et al. (1981) assessed organogenesis but did not observe any direct organ formation on explants and reported that *Cannabis* calluses readily produced roots but were unreceptive to shoot formation. Mandolino and Ranalli (1999) reported occasional shoot regeneration from calluses. Feeney and Punja (2003) failed to regenerate hemp plantlets, either directly or indirectly from callus or suspension cultures.

Considering the limitations, a protocol for *in vitro* tissue culture of nodal segments containing axillary buds is needed to propagate elite *C. sativa* clones. Our goal is to develop a secure and stable *in vitro* clonal repository of elite medicinal plant germplasm that will ensure future availability of desirable pharmacological active chemotypes.

Thus, the first objective of our study was to develop a tissue culture system that will allow large scale clonal production of screened and selected high yielding elite clones. We have refined a *Cannabis in vitro* propagation protocol to be a tool to micropropagate high-yielding elite clones for field plantings. This procedure will be used to increase seed production of genetically superior lines. Furthermore, the micropropagation protocol of *C. sativa* may be a useful tool for multiplication and maintenance of high-yielding elite plants, saving space and time in the selection program.

Plant survival, growth, and productivity are intimately coupled with the aerial environment through processes such as energy exchange, loss of water vapor in transpiration, and uptake of carbon dioxide in photosynthesis (Stoutjesdijk and Barkman 1992). The water vapor exchange rate affects the energy budget and transpiration of leaves and, consequently, the physiology of the whole plant (Chandra and Dhyani 1997). Therefore, data on physiological parameters such as gas and water vapor exchange are likely to provide valuable information regarding the suitability of tissue culture raised plants for field plantations.

The second objective of our study was to evaluate the performance of *in vitro* propagated and hardened plants, on the basis of selected physiological parameters, in comparison to those of *ex vitro* vegetatively grown plants from same mother plant of the same age. The effects of different light intensities on photosynthesis and water vapor exchange characteristics have also been examined.

Materials and Methods

Plant material. Nodal segments containing axillary buds (~1 cm in length) were used as an explant for initiation of shoot cultures. Explants were obtained from healthy branches of a screened and selected (on the basis of its chemical profile using gas chromatography–mass spectrometry) high-yielding *C. sativa* variety (MX-1) grown in a indoor cultivation facility housed at Coy-Waller laboratory, University of Mississippi. Vegetative cuttings were also taken from the same 1-yr-old mother plant for the comparison between vegetatively propagated (VP) and *in vitro* propagated plants (IVP) of same age (age of the IVP plants were taken from the day they were transferred to soil).

Sterilization. Explants were surface-disinfected using 0.5% NaOCl (15% *v*/*v* bleach) and 0.1% Tween 20 for 20 min. The explants were washed in sterile distilled water three times for 5 min prior to inoculation on the culture medium.

Inoculation. Disinfected explants were inoculated on Murashige and Skoog's medium (Murashige and Skoog 1962) containing 3% (*w*/*v*) sucrose, 0.8% (*w*/*v*) type E agar (Sigma Chemical Co., St. Louis, MO) supplemented with various concentrations of cytokinins—benzyladenine (BA), kinetin (Kn), and thidiazuron (TDZ) ranging from 0.05 to 9.0 μM (Table 1) or in combination with gibberellic acid (GA_3; Table 2) adjusted to pH 5.7. Sterile medium was dispensed (25 ml) in glass culture vessels (4-cm diameter × 9.5-cm height, baby food jars with magenta B caps). Shoots that originated from the explants were subcultured after 30 d. Shoots taller than 2.5 cm were transferred to 1/2-MS medium supplemented without and with 500 mg l^{-1} of activated charcoal and different auxins—Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA) at 2.5 and 5.0 μM concentrations (Table 3) for root induction. All cultures were incubated at 25±2°C with 16-h photoperiod under fluorescent light with a photon flux of ≈52 μmol m^{-2} s^{-1}. All experiments were repeated at least three times with six explants, with one explant per jar. The cultures were observed constantly

Table 1. *In vitro* responses to different concentrations of cytokinins on multiplication and proliferation of nodal explants in *C. sativa*

Growth Regulator	Conc. (μM)	Average no. of shoots	Average shoot length (cm)	% of explant producing shoots
BA	0.05	2.2 ef	2.6 de	50.0
	0.25	2.6 def	3.1 cde	66.6
	0.50	3.0 de	3.2 cde	72.2
	2.5	5.2 bc	2.8 de	72.2
	5.0	6.3 b	3.4 cde	61.1
	7.0	2.8 def	3.2 cde	55.5
	9.0	2.6 def	2.6 de	33.3
Kn	0.05	1.3 f	2.5 e	61.1
	0.25	1.6 ef	2.6 de	72.2
	0.5	2.6 def	2.5 e	77.7
	2.5	5.6 bc	3.5 cd	83.3
	5.0	4.2 cd	2.8 de	50.0
	7.0	2.2 ef	2.7 de	55.5
	9.0	1.6 ef	3.1 cde	44.4
TDZ	0.05	4.2 cd	3.5 cd	94.4
	0.25	6.6 b	3.7 c	94.4
	0.5	12.6 a	7.1 a	100.0
	2.5	11.1 a	6.1 b	94.4
	5.0	5.8 b	3.2 cde	83.3
	7.0	2.5 def	2.8 cde	77.7
	9.0	1.5 ef	2.5 e	72.2

Data represent the mean of three replicates with six explants for each treatment. Means followed by same *letter* do not differ statistically at $p=0.05$ according the Tukey test

for any response. The parameters evaluated were the average number of shoots per explant, average shoot length, percentage of explants producing shoots, average number of roots per explant, average root length, and percentage of rooted plantlets. The data were submitted to statistical analyses by analysis of variance followed by the Tukey test with the level of significance set at 5% using SAS version 9.1 (SAS Institute, Cary, NC).

Acclimatization. Rooted shoots were carefully taken out of the medium and washed thoroughly in running tap water to remove all traces of medium attached. Plantlets were pre-incubated in coco natural growth medium (Canna Continental, Los Angeles, CA) in thermocol cups (Walmart Stores, Inc.) for 10 d. The cups were covered with polythene bags to maintain humidity and kept in a grow room and later acclimatized in sterile potting mix-fertilome (Canna Continental) in large pots. All these plantlets were kept under similar environmental conditions grown in an indoor cultivation facility housed at Coy-Waller laboratory, University of Mississippi. Light was provided with full spectrum 1,000-W high density discharge (HID) lamps (Sun Systems, CA) hung on the top of plants. A hot air suction fan was attached, and about 1-m distance between plants and bulb was maintained to avoid heating due to HID bulbs. Using an automatic electric timer, artificial day/night cycle was regulated with a 16-h photoperiod. Grow room temperature and relative humidity was kept nearly 25–30°C and 60%, respectively. Plants were watered regularly and individually to maintain sufficient moisture content in the pots.

Gas and water vapor exchange. Four months after transfer to soil, four sets of each type of plants (*in vitro* propagated plants, IVP as well as *ex vitro* vegetatively propagated, VP plants of the same age taken from the same mother plant) were used for carbon assimilation and water vapor exchange studies. Measurements were carried out on five upper undamaged, fully expanded, and healthy leaves of each plant with the help of a closed portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, NE). Following preliminary experiments on these plants, ~30°C was found to be the optimal temperature for growth, and therefore, all subsequent gas and water vapor exchange measurements were carried out at 30±0.5°C. To determine the effect of light on gas and water vapor exchange, leaves were exposed to different photosynthetic photon flux densities (PPFD) viz., 0, 500, 1,000, 1,500, and 2,000 μmol m^{-2} s^{-1} with the help of an artificial light source (Model LI-6400-02; light emitting silicon diode; LI-COR), fixed on the top of the leaf chamber. Photosynthetically active radiation was

Table 2. *In vitro* responses to different concentrations of cytokinins and GA3 on shoot regeneration of *C. sativa*

Growth regulator	Conc. (μM)	Average no. of shoots	Average shoot length (cm)	% of explant producing shoots
BA + GA3	0.5+7.0	3.2 f	2.5 e	50.0
	2.5+7.0	3.6 ef	3.5 d	66.6
	5.0+7.0	4.5 def	3.6 d	33.3
Kn + GA3	0.5+7.0	3.2 f	2.6 e	44.4
	2.5+7.0	5.5 d	2.1e	66.6
	5.0+7.0	4.8 de	2.5 e	55.5
TDZ + GA3	0.5+7.0	13.8 a	7.9 a	83.3
	2.5+7.0	11.2 b	7.0 b	94.4
	5.0+7.0	7.3 c	5.0 c	77.7

Data represent the mean of three replicates with six explants for each treatment. Means followed by same *letter* do not differ statistically at $p=0.05$ according the Tukey test

Table 3. *In vitro* responses to different concentrations of auxin on root induction of *C. sativa*

Growth regulator	Conc. (μM)	Average no. of roots	Average root length (cm)	% Rooted plantlets
IAA[a]	2.5	1.25 b	1.4 b	33.3
	5.0	1.6 b	1.3 b	44.4
IBA[a]	2.5	4.8 a	4.8 a	94.4
	5.0	3.8 a	4.7 a	83.3
NAA[a]	2.5	2.2 b	2.3 b	55.5
	5.0	2.3 b	2.4 b	44.4

Data represent the mean of three replicates with six explants for each treatment. Means followed by same *letter* do not differ statistically at $p=0.05$ according the Tukey test

[a] Medium used is half-strength Murashige and Skoog salts (1/2-MS) supplemented with 500 mg/l activated charcoal

recorded with the help of a quantum sensor kept in the range of 660- to 675-nm wave radiation fixed at the leaf level. The rate of dark respiration was measured by maintaining the leaf in the cuvette at zero irradiance. The leaf chamber was covered with a black cloth throughout the course of respiration measurements to avoid any external radiation. Air flow rate (500 mmol s^{-1}), CO_2 concentration inside the leaf chamber (350±5 ppm), temperature (30± 0.5°C), and relative humidity (55±5%) were kept nearly constant throughout the experiment. Since steady-state photosynthesis is reached within 30–45 min, the leaves were kept for about 45–60 min under each set of light conditions before the observations were recorded. Water use efficiency (WUE) was calculated as a ratio of the rate of photosynthesis and transpiration.

Results and Discussion

Shoot proliferation and multiplication. Of the three cytokinins used, TDZ was most effective for shoot proliferation (Table 1). TDZ, a synthetic phenylurea, is considered to be one of the most active cytokinins for shoot induction in plant tissue culture (Huetteman and Preece 1993). Reports suggest that TDZ induces shoot regeneration better than other cytokinins (Thomas 2003; Thomas and Puthur 2004; Husain et al. 2007). The nodal segments proliferated shoots within 14 d of culture on MS medium containing 0.05–9.0 μM TDZ without intervening calluses. The optimal response in terms of percentage of explants producing shoots with maximum shoot length and the highest number of shoot per explant was recorded on MS medium supplemented with TDZ (0.5 μM; Fig. 1*a*). On this medium, 100% of the cultures responded with an average of 13 shoots per culture (Table 1). The regenerated shoots elongated within 2 wk of culture. However, TDZ at higher concentrations than 5.0 μM resulted in suppressed shoot formation. Magioli et al. (1998) have reported the superiority of lower concentration of TDZ in *Solanum melongena* using a similar explant. Such a response may perhaps be

Figure 1. (*a*) Shoot multiplication of *C. sativa* on MS. (*b*) Rooting on 1/2MS medium supplemented with 500 mg l-1 activated charcoal and 2.5 μM IBA. (*c*) Well rooted plantlet prior to soil. (*d*) *Ex vitro* vegetatively propagated (*VP*) and *in vitro* vegetatively propagated (*IVP*) plants. *Bars* represent 1.22 cm (*a*), 1.31 cm (*b*), 2.3 cm (*c*) and 32 cm (*d*).

due to the increase in the levels of endogenous cytokinins by the effect of the growth regulator used. Hare and Staden (1994) reported that TDZ has a capacity to inhibit (at least partly) the action of cytokinin oxidase, which in turn may increase the levels of endogenous cytokinins. However, higher concentrations caused reduction in shoot length. Cytokinins commonly stimulate shoot proliferation and inhibit their elongation. Therefore, inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity (Huetteman and Preece 1993).

Transferring these cultures to MS media supplemented with TDZ and 7.0 μM GA_3, a slight increase in shoot growth and plantlet formation was observed (Table 2). However, the increase was found statistically insignificant. Some reports indicated that GA_3 is conducive for *in vitro* shoot regeneration (Chakraborty et al. 2000) or for promotion of growth, biomass production, and xylem fiber length (Ericksson et al. 2000). Furthermore, GA_3 can act as a replacement for auxin in shoot induction, and thus, a ratio of cytokinin–GA_3 may be decisive for differentiation in certain plant tissues (Sekioka and Tanaka 1981). The presence of GA_3 in combination with kinetin or BA did not restore growth to suppressed cultures (Table 2). *In vitro* raised shoots could be used to induce further multiple shooting by culturing individual nodes with axillary buds. A good number (8–12) of shoots were obtained through recurrent subculture without any loss of vigor of the regenerated shoots.

Induction of rooting. Root initiation of *C. sativa* was difficult when elongated shoots (2–3 cm long) excised from parent culture were grown on half-strength MS medium (1/2-MS) without activated charcoal. The inclusion of activated charcoal was effective in root induction. Roots developed within 10 d in shoots placed on 1/2-MS with activated charcoal supplemented with IBA with no intervening calluses. However, on IAA and NAA, profuse callus formation took place at the base of shoots. Rooting response was least on medium supplemented with IAA. Similar findings on the beneficial effect of activated charcoal have also been reported (Encina et al. 1994; Figueiredo et al. 2001; Mohamed-Yasseen 2001; Lata et al. 2002, 2003). Although *C. sativa* shoots rooted on all media augmented with different concentrations of auxin (IAA, IBA and NAA), the presence of IBA resulted in significantly higher rooting percentage with almost a twofold increase obtained in average number of roots and root length than IAA or NAA (Table 3). The percentage of rooting was 80–95% on media 1/2-MS containing 2.5–5.0 μM IBA and 500 mg l^{-1} activated charcoal (Fig. 1*b*, Table 3). The promotory effect of IBA on *in vitro* rooting of shoots has also been obtained in different plants (Abrie and Staden 2001; Thomas 2003; Thomas and Puthur 2004).

Acclimatization. Rooted plantlets were successfully transferred to thermocol cups containing potting mix and new growths were observed after 2 wk. The plants attained 14- to 16-cm height within 6 wk of transfer (Fig. 1*c*,*d*). These plants exhibited 95% survival rate 8 wk after transfer. The acclimatized plants exhibited normal development and no gross morphological variation was observed. The three-step

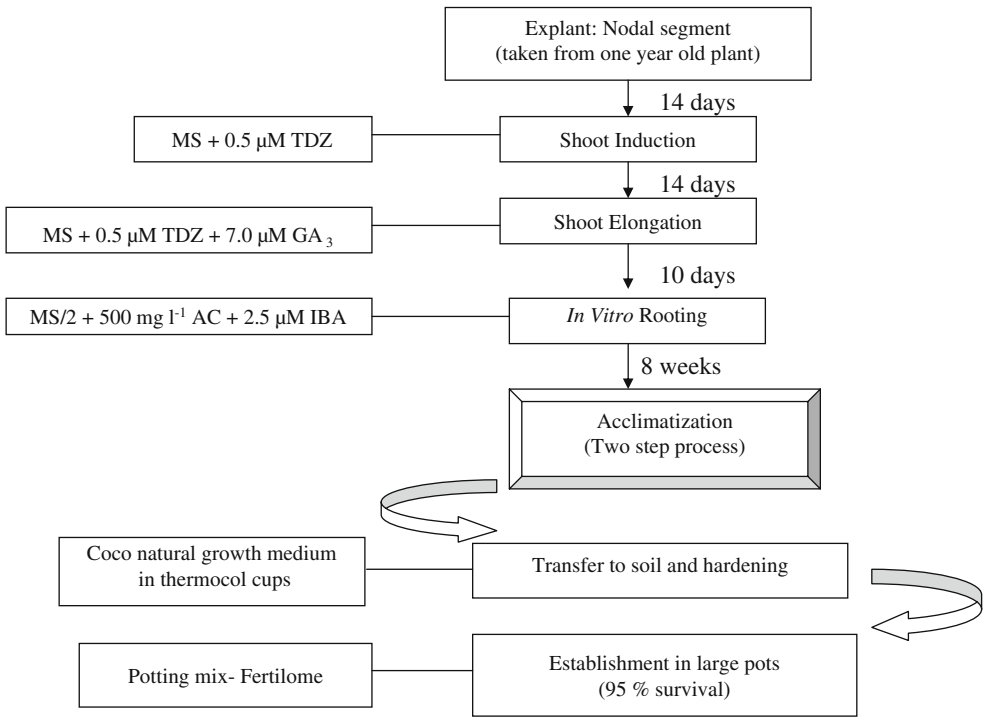

Figure 2. Schematic representation for the direct organogenesis of *C. sativa*.

plant regeneration protocol was completed within 98 d of culture (Fig. 2).

In the present investigation, a high frequency shoot organogenesis of *C. sativa* was obtained from nodal cuttings in TDZ-containing medium. Richez-Dumanois et al. (1986) induced direct multiplication of shoots from apical and axillary bud explants using BA; however, rooting was described extremely difficult and the response has been poor. On the contrary, in our experiments, the efficiency of regeneration was very high using TDZ in comparison to BA (Table 1). Also, root differentiation was observed within 10 d of culture with 95% rooting response in the present protocol developed for *C. sativa*. Plant regeneration via leaf calluses (Mandolino and Ranalli 1999) and internode, petiole, and axillary bud derived calluses (Slusarkiewicz-Jarzina et al. 2005) have been reported with other growth hormones like 2,4-D, kinetin, IBA, and BA. However, the frequency of the induced shoots with these hormones is not as high as obtained with TDZ as reported in the present work. Since the preservation of genetic stability in germplasm collections and micropropagation of elite plants is of utmost importance, the propagation of *C. sativa* through nodal explants as compared to calluses would allow recovery of genetically stable and true to type progeny plants.

Gas and water vapor exchange. The effect of different PPFD on photosynthesis (P_n), dark respiration (R_d), transpiration (E), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), ratio of intercellular to external CO_2 concentration (C_i/C_a), and WUE of *in vitro* raised and *ex vitro* vegetatively grown plants is shown in Fig. 3. Rate of photosynthesis tended to increase with increasing light intensity up to 1,500 µmol m^{-2} s^{-1} in both cases. However, the increase was more pronounced at PPFD below 500 µmol m^{-2} s^{-1}. Maximum rate of photosynthesis (A_{max}) was recorded as 26.40±4.30 µmol m^{-2} s^{-1} for *in vitro* propagated and hardened plants. However, it was slightly lower (25.6±3.90 µmol m^{-2} s^{-1}) for *ex vitro* vegetatively propagated plants at 1,500 µmol m^{-2} s^{-1}, but there was no significant difference. This difference in P_n was, however, found statistically insignificant. A comparable photosynthetic activity between seed raised plants and their tissue culture raised counterparts of same age has also been reported by Bag et al. (2000) and Purohit et al. (2002) in the case of temperate bamboo (*Thamnocalamus spathiflorus*) and Himalayan oaks (*Quercus leucotrichophora* and *Q. glauca*), respectively. Furthermore, in the present study, exchange of CO_2 was found to be adversely affected by light intensities beyond 1,500 µmol m^{-1} PPFD. Dark respiration (at 0 µmol m^{-2} s^{-1} PPFD) was ~15% higher in *ex vitro* plants as compared to *in vitro* propagated plants. The rate of transpiration (Fig. 3*a*) tended to increase

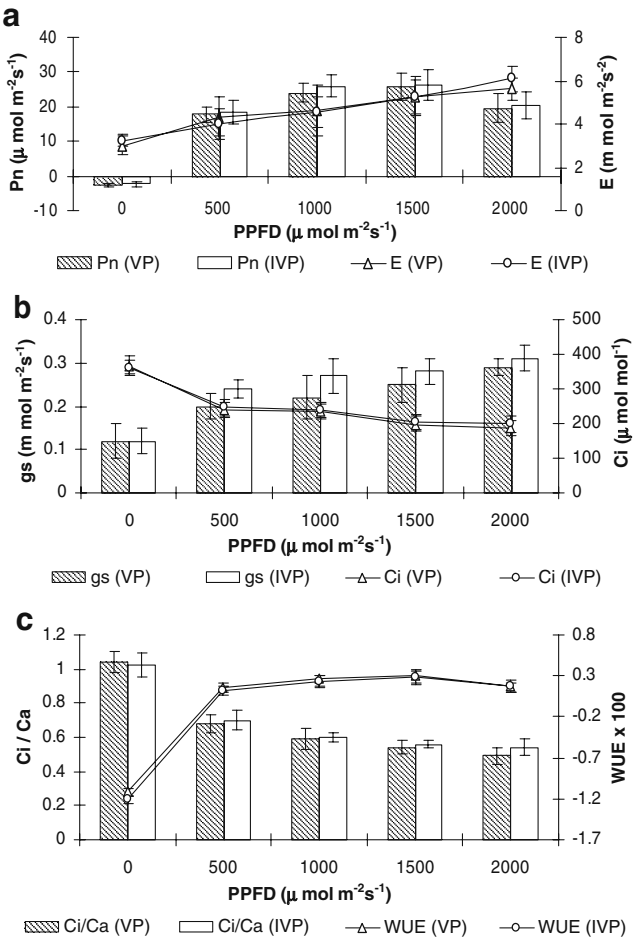

Figure 3. Comparison of gas and water vapor exchange characteristics in 4 mo old *ex vitro* vegetatively propagated (*VP*) and *in vitro* propagated (*IVP*) soil hardened plants of *C. sativa* taken from the same 1-y-old mother plant. Effect of different photosynthetic photon flux density (*PPFD*) on (*a*) photosynthesis, P_n, and transpiration, E; (*b*) stomatal conductance, g_s, and intercellular CO_2 concentration, C_i; and (*c*) ratio of intercellular to ambient CO_2 concentration, C_i/C_a and water use efficiency, WUE at 30°C and 55% relative humidity.

considerably with an increase in PPFD (up to 2,000 µmol m^{-2} s^{-1}) in both sets of plants. Similar to E, g_s also increased with PPFD in all cases up to highest level tested (2,000 µmol m^{-2} s^{-1}) and was comparable in *ex vitro* and *in vitro* raised plants. Contrary to g_s, C_i was highest, i.e., 365.00±32.82 and 360.00±21.70 µmol m^{-2} s^{-1} at zero light for *in vitro* and *ex vitro* propagated plants, respectively (Fig. 3*b*). The values gradually decreased with increase in PPFD in both cases. Variations in the C_i/C_a and WUE of both plants are shown in Fig. 3*c*. Similar to C_i, the C_i/C_a ratio at 0 µmol m^{-2} s^{-1} PPFD was maximum for both group of plants. The C_i/C_a ratio gradually decreased with PPFD up to 1,000 µmol m^{-2} s^{-1} and then became nearly constant (~0.54) for both sets of plants at higher PPFD (1,500–2,000 µmol m^{-2} s^{-1}). In both sets of plants, a gradual increase in WUE was recorded with increase in PPFD up to 1,500 µmol m^{-2} s^{-1}, and maximum values (30.00±5.80 and

28.00±9.23 for *ex vitro* and *in vitro* plants, respectively) were recorded at this light level. However, the increase was more pronounced at PPFD below 500 μmol m^{-2} s^{-1}. These results reveal that *in vitro* propagated plants of *C. sativa* appeared to be normal and was comparable to *ex vitro* vegetatively grown plants of same age with respect to the physiological functions examined in this study.

In conclusion, an efficient protocol was developed for micropropagation of *C. sativa* that can be used to regenerate a large number of plants in a short period of time with a 95% survival of rooted plantlets transplanted to soil after 8 wk according to our observations. The mass multiplication of high yielding varieties of *C. sativa* is feasible for field plantings to produce plants as the chief source of THC for the pharmaceutical industry. Furthermore, the adaptation of this micropropagation system may also be used in a breeding program that will allow improvement of this medicinal plant species through selection of plants with unusually high levels pharmaceutical compounds.

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