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Micropropagation of *Cassia occidentalis* L. and the effect of irradiance on photosynthetic pigments and antioxidative enzymes

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Abstract

The objective of the current study was to develop an efficient and reproducible protocol for plant regeneration using nodal (1.0 - 1.5 cm) explants excised from a field grown mature plant of *Cassia occidentalis* L. The highest shoot regeneration frequency (80 %) with a maximum number of shoots (11.66) and shoot length (3.83 cm) after eight weeks of culture were observed on a Murashige and Skoog (MS) medium amended with 5.0 μM 6-benzyladenine, 100 μM citric acid, and 1.0 μM α-naphthalene acetic acid. A half-strength MS medium supplemented with 1.5 μM indole-3-butyric acid proved best for the induction of maximum roots (8.33) per shoot. Plantlets with well-developed shoots and roots were successfully acclimatized in plastic pots containing sterile *Soilrite* under two irradiances of 50 and 300 μmol m^{-2} s^{-1} (LI and HI, respectively) in a culture room, and after transfer to the field, the survival rate was 70 %. A significant increase in chlorophyll, carotenoid, and malondialdehyde content was found during acclimatization under both the irradiances but higher under HI. Similarly, the activities of superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase increased more under HI. Plantlets acclimatized under HI exhibited a better growth than those under LI.

Additional key words: acclimatization, ascorbate peroxidase, carotenoids, catalase, chlorophyll, glutathione reductase, superoxide dismutase.

Introduction

Cassia occidentalis L. (*Caesalpiniaceae*), commonly known as coffee senna is a valuable small legume tree that could be used for production of several medicines (Krithikar and Basu 1999). Besides, the plant has also been recommended for reclamation of land and as green manure to restore fertility in exhausted fields. The propagation of *C. occidentalis* through seeds is unreliable due to poor seed quality, erratic germination, and seedling mortality, which explain its sparse distribution. Also, seeds are not always true to type (Ran and Simpson 2005) due to a high degree of heterozygosity and the law of segregation. Hence, there is need to develop alternative propagation methods which could be beneficial in their large scale multiplication, improvement, and conservation of elite clones.

Tissue culture technology is desirable as mean for germplasm conservation to ensure the survival of medicinal plant species and rapid mass propagation for large-scale re-vegetation (Anis *et al*. 2012). However, in many cases, poor survival rates of *in vitro* plantlets after transplantation from tissue culture vials to a greenhouse or open field often limit the commercial application

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Abbreviations: APX - ascorbate peroxidase; BA - 6-benzyladenine; CA - citric acid; Car - carotenoids; CAT - catalase; Chl - chlorophyll; GR - glutathione reductase; HI - high irradiance; IBA - indole-3-butyric acid; 2-iP - 2-isopentenyl adenine; Kn - kinetin; LI - low irradiance; MDA - malondialdehyde; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloracetic acid.
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(Pospíšilová *et al*. 1999). During *in vitro* conditions, plantlets grow under controlled environment: a fixed photoperiod and weak irradiance, high air humidity, gradually decreasing medium nutrient supply, as well as low CO_2 concentration. After transfer to *ex vitro* conditions, *in vitro* plants are exposed to irradiances higher than those used under *in vitro* conditions, resulting usually in photoinhibition and/or the generation of reactive oxygen species (ROS) which include superoxide radical, singlet oxygen, hydrogen peroxide, and hydroxyl radical (Ort and Baker 2002). The uncontrolled production of ROS can cause cellular damages directly or through the formation of secondary toxic substances (Benson 2000). Plants are well equipped with numerous protective mechanisms involved in preventing oxidative damage, including superoxide dismutase (SOD), catalase (CAT), peroxidase, ascorbate peroxidase (APX), glutathione reductase (GR), *etc.* (Scandalios 1990).

In the present investigation, attempts were made to: *1*) develop and standardize a reproducible protocol for rapid *in vitro* propagation using nodal segments excised from field grown mature plants and *2*) evaluate the effect of different irradiances during *ex vitro* acclimatization of *C. occidentalis* plantlets on the antioxidant enzyme activities (SOD, CAT, GR, and APX), lipid peroxidation, and pigment content.

Materials and methods

Healthy shoots were collected from a six-month-old plant growing in the Botanical garden of the University, Aligarh, India. The twigs were washed under running tap water for 30 min, followed by the treatment with a 5 % (v/v) *Teepol* solution (a liquid detergent; *Qualigens*, Mumbai, India) for 15 min, and finally washed in tap water for 20 min. Explants were then surface disinfected with a 0.1 % (m/v) mercuric chloride (*Qualigens*) solution for 5 min under continuous shaking and then washed with at least five changes of sterile distilled water. Shoot segments containing a single node (1 to 1.5 cm) were excised aseptically and cultured on a Murashige and Skoog (MS) medium supplemented with 3 % (m/v) sucrose and 0.8 % agar (pH 5.8). Culture vials containing 20 cm^3 of the medium were autoclaved at 121 °C and 1.06 kg cm^{-2} for 15 min. All cultures were incubated at a temperature of 24 ± 2 °C, a 16-h photoperiod, an irradiance of 50 μmol m^{-2} s^{-1} (provided by cool white fluorescent tubes; 40 W, *Philips Electronics*, Kolkata, India), and a relative humidity of 60 - 70 %. The nodal segments were cultured on an MS medium supplemented with different cytokinins: 6-benzyladenine (BA), kinetin (Kn), or N-isopentenyl aminopurine (2-iP) at various concentrations (0.5, 2.5, 5.0, 7.5, or 10.0 μM) either individually or in a combination with citric acid (CA; 25, 50, 75, 100, and 125 μM) used as chelating agent. Further, optimum concentrations of each cytokinin and CA (100 μM) were used in combination with auxins, indole-3-butyric acid (IBA), or α-naphtha-leneacetic acid (NAA) at various concentrations (0.1, 0.5, 1.0, 1.5, or 2.0 μM) for a better shoot proliferation. Subculturing was performed on the same fresh medium after every two weeks. Data on the frequency of explants producing shoots per explant and on the shoot length were recorded after eight weeks of incubation in each of the experiments conducted.

After the evaluation of the best combination of plant growth regulators and CA, experiments were conducted to study the effect of auxins treatment on root formation. For root initiation, eight-week-old, 4 cm long shoots with fully expanded leaves developed on BA (5.0 μM) + CA (100 μM) + NAA (1.0 μM) were cultured on an MS or half MS medium alone or in combination with IBA or NAA at various concentrations (0.5, 1.0, 1.5, 2.0, or 2.5 μM). The rooting percentage, root numbers, and root lengths were recorded after four weeks.

Eight-week-old plantlets with well-developed roots were removed from the culture tubes, washed gently under running tap water, transferred to 10 cm diameter plastic pots containing sterile *Soilrite*™ (*Keltech Energies*, Bangalore, India), and covered with a polythene bag. Plantlets were watered with a half MS solution every alternate day for two weeks, followed by tap water. A total of 50 pots were divided into 2 groups (25 pots for each group) and grown under irradiances of 50 and 300 μmol m^{-2} s^{-1} (LI and HI, respectively, provided by *Philips SON-T* lamps, Turnhout, Belgium) in a culture room maintained at 24 ± 2 °C, a 60 - 70 % relative humidity, and a 16-h photoperiod. Leaflets were taken at the transplanting day (0 d) and after 7, 14, 21, and 28 d for estimations of chlorophyll (Chl) *a*, Chl *b*, carotenoids (Car), malondialdehyde (MDA), and activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX). *In vitro* developed leaves (0 to 7 d) and new leaves (14 - 28 d) were used.

Chl *a*, Chl *b*, and Car content was determined using the methods of Mackinney (1941). Fifty samples (0.5 g) from different plants (25 for each LI and HI), were collected and ground in 5 cm^3 of 80 % (v/v) acetone in a mortar and pestle. The suspension was filtered (*Whatman No. 1*) and absorbances of the solution were then measured at 645 and 663 nm for Chl, and 480 and 510 nm for Car on a UV-VIS spectrophotometer (*1700 Pharma-Spec, Shimadzu,* Tokyo, Japan).

MDA content was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Leaflets (0.5 g of about 5 - 7 small leaves) were homogenized with 1 cm^3 of 0.5 % (m/v) trichloroacetic acid (TCA) in a mortar and pestle. The homogenate was

centrifuged at 14 000 *g* for 15 min. After centrifugation, 0.5 cm^3 of the supernatant was mixed with 2.5 cm^3 of 0.5 % (m/v) TBA in 20 % TCA and incubated in a boiling water bath for 30 min. Thereafter, it was cooled immediately on ice and centrifuged at 10 000 *g* for 10 min. Absorbances at 532 and 600 nm were determined and the MDA concentration was estimated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm using a coefficient of absorbance of 156 mM^{-1} cm^{-1}.

For determination of antioxidant enzyme activities, 0.5 g of fresh leaflets was homogenized in 2.0 cm^3 of an extraction buffer [a potassium phosphate buffer containing 1 % (m/v) polyvinylpyrrolidone (PVP), 1 % (v/v) *Triton X-100*, and 0.11 g of ethylenediamine-tetraacetic acid (EDTA)] using a pre-chilled mortar and pestle. The homogenization was carried out in the dark at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged in a high-speed centrifuge (*Remi Instruments*, Mumbai, India) at 15 000 *g* for 20 min. The supernatant was used for enzyme assays using the above mentioned UV-visible spectrophotometer. Protein content in the enzymatic extracts was determined following the Bradford (1976) method using bovine serum albumin as standard.

SOD (EC1.15.1.1) activity was measured by the method of Dhindsa *et al*. (1981). The inhibition of photochemical reduction of nitroblue tetrazolium (NBT) in a reaction mixture consisting of a 0.5 M phosphate buffer (pH 7.5), 0.1 mM EDTA, 13 mM methionine, 63 mM nitroblue tetrazolium (NBT), 1.3 mM riboflavin, and 0.1 cm^3 of the enzyme extract was monitored in a test tube at 25 °C. One unit (U) of enzyme activity was defined as the quantity of enzyme that inhibited the nitroblue tetrazolium (NBT) reduction by 50 %.

CAT (EC 1.11.1.6) activity was determined from the rate of H_2O_2 decomposition as measured by the decrease of absorbance at 240 nm, following the method of Aebi (1984). An assay mixture contained a 50 mM phosphate buffer (pH 7.0) and 0.1 cm^3 of the enzyme extract in a total volume of 3 cm^3, and the reaction began by adding 10 mM H_2O_2.

GR (EC 1.6.4.2) activity was assayed by the method of Foyer and Halliwell (1976) modified by Rao (1992) based on glutathione dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. An assay mixture contained a 50 mM potassium phosphate buffer (pH 7.5), 1.0 mm Na_2EDTA, 0.2 mM (NADH), and 0.5 mM glutathione disulfide (GSSG). The reaction was started by the addition of the enzyme extract and allowed to run 5 min at 25 °C. The activity was calculated using the coefficient of NADPH absorbance of 6.22 mM^{-1} cm^{-1}.

APX (EC1.11.1.11) activity was determined at 25 °C by recording the decrease in absorbance at 290 nm due to ascorbic acid oxidation to dehydroascorbate by H_2O_2 according to the method of Nakano and Asada (1981). A reaction mixture contained a 50 mM phosphate buffer (pH-7.5), 0.5 mM ascorbate, and 0.1 mM EDTA, and 0.1 mM H_2O_2 was added to start the reaction.

The experiments were laid out in a completely randomized design. Ten replicates were taken in each experiment and each experiment was performed thrice. Data were subjected to the one-way analysis of variance (*ANOVA*) using *SPSS v. 16* (*SPSS Inc.,* Chicago, USA) and pair wise means were compared using the Tukey's multiple range test at $\alpha = 0.05$.

Results

The nodal explants (Fig. 1*A*) cultured on the MS basal medium served as control and did not show axillary bud proliferation even after four weeks of culture, whereas the addition of growth regulators favored axillary bud induction. The effect of various cytokinins (BA, Kn, and 2-iP) at different concentrations (0.5 - 10.0 µM) showed a marked effect on multiple shoots induction from the nodal explants. Among the different cytokinins tested, 5.0 µM BA was found to be most effective and produced 6.0 ± 0.57 shoots with the length of 1.5 ± 0.11 cm in 60 % of the cultures (Table 1), whereas Kn or 2-iP showed 50 or 30 % regeneration frequency with only 5.0 ± 1.15 or 3.6 ± 0.66 shoots per explant, respectively, after 8 weeks of culture (Table 1).

Browning has been a major drawback in leguminous plants. In *C. occidentalis*, browning was observed during 28 d of culture and was even extended up to 8 weeks. Therefore, to circumvent the problem, the medium was supplemented with CA at different concentrations. Among the various concentrations of CA used, 100 µM CA along with the optimum concentration of each cytokinin was found effective in inhibiting browning by controlling phenolic exudation. The maximum 70 % shoot formation frequency with 8.0 ± 0.57 shoots per explant and 2.9 ± 0.11 cm shoot length was observed on a medium containing BA (5.0 µM) along with 100 µM CA (Table 2).

The synergistic influence of auxins with cytokinin was evident when the optimal concentration of each cytokinin and 100 µM CA was combined with different concentrations (0.1 - 2.0 µM) of NAA or IBA. Among all cytokinin-auxin combinations, the maximum shoot formation frequency (80 %) with 11.6 ± 0.88 shoots per explant and the longest shoots (3.83 ± 0.08 cm) was observed on a medium containing 5.0 µM BA, 100 µM CA, and 1.0 µM NAA after 8 weeks of culture (Fig. 1*C*). This was considered to be the optimal growth regulator combination for maximum shoots regeneration in

Table 1. Effects of different concentrations of cytokinins on multiple shoot induction from nodal segments after eight weeks of culture. Means ± SE, n = 10, means within columns with different letters are significantly different ($P \leq 0.05$) according to the Tukey's multiple range test.

BA [µM]	Kn [µM]	2-iP [µM]	Response [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
0.5	-	-	26.60 ± 0.67^{f}	2.00 ± 0.57^{def}	0.63 ± 0.24^{d}
2.5	-	-	33.40 ± 0.67^{d}	4.33 ± 0.88^{abcd}	1.00 ± 0.15^{abcd}
5.0	-	-	53.20 ± 0.86^{a}	6.00 ± 0.57^{a}	1.50 ± 0.11^{a}
7.5	-	-	35.60 ± 0.50^{c}	4.66 ± 0.88abc	1.13 ± 0.12^{abcd}
10.0	-	-	23.20 ± 0.58^{g}	3.33 ± 0.88^{bcdef}	0.90 ± 0.17^{bcd}
-	0.5	-	20.00 ± 0.54^{h}	2.00 ± 0.57^{def}	0.90 ± 0.15^{bcd}
-	2.5	-	33.20 ± 0.66^{d}	3.00 ± 0.57^{bcdef}	1.06 ± 0.26^{abcd}
-	5.0	-	50.00 ± 0.44^{b}	5.00 ± 1.15^{ab}	1.30 ± 0.17^{ab}
-	7.5	-	30.00 ± 0.70^{e}	4.00 ± 0.57^{abcde}	0.90 ± 0.05^{bcd}
-	10.0	-	23.20 ± 0.66^{g}	1.66 ± 0.33^{ef}	0.70 ± 0.10^{cd}
-	-	0.5	16.00 ± 0.70^{i}	2.00 ± 0.57^{def}	0.76 ± 0.17^{bcd}
-	-	2.5	20.80 ± 0.73^{h}	3.33 ± 0.88^{bcdef}	1.10 ± 0.15^{abcd}
-	-	5.0	30.00 ± 0.44^{e}	3.66 ± 0.66^{abcdef}	1.20 ± 0.11^{abc}
-	-	7.5	13.40 ± 1.81^{j}	2.33 ± 0.88^{cdef}	0.96 ± 0.18^{bcd}
-	-	10.0	11.60 ± 0.40^{j}	1.33 ± 0.33^{f}	0.90 ± 0.05^{bcd}

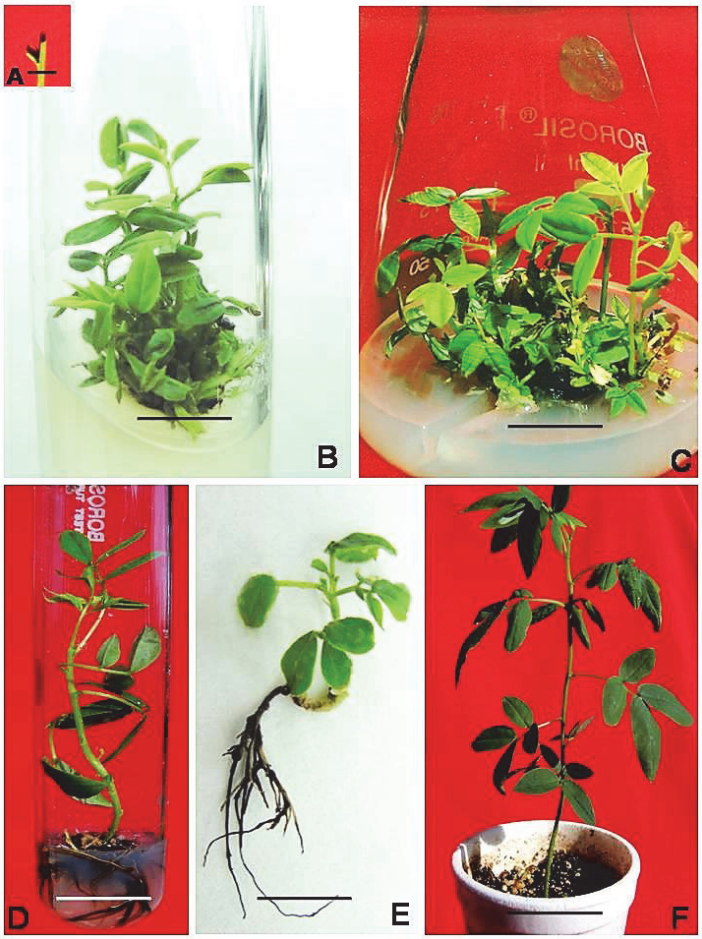

Fig. 1. Direct multiple shoot regeneration from nodal explants, and complete plantlet establishment of *Cassia occidentalis*. *A* - nodal explants, *B* - multiple shoots on an MS medium supplemented with 5.0 µM BA, *C* - multiplication and elongation of shoots on an MS medium augmented with 5.0 µM BA, 100 µM CA, and 1.0 µM NAA, *D* - an *in vitro* rooted plantlet, *E* - an *in vitro* thick root plantlet, *F* - an acclimatized plant in *Soilrite*. The *scale bars* represent 0.31 cm for *A* and 1 cm for *B, C, D, E,* and *F*.

Table 2. Effects of various concentrations of citric acid (CA) with the optimal concentration of different cytokinins on shoot induction and multiplication after eight weeks of culture. Means ± SE, n = 10, means within columns with different letters are significantly different ($P \leq 0.05$) according to the Tukey's multiple range test.

BA [μM]	Kn [μM]	2-iP [μM]	CA [μM]	Response [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
0.5	-	-	25	27.00 ± 0.57^{g}	3.66 ± 0.88^{bc}	1.50 ± 0.23^{def}
2.5	-	-	50	30.00 ± 0.40^{f}	4.33 ± 0.33^{bc}	1.80 ± 0.11^{bcd}
5.0	-	-	75	40.00 ± 0.91^{c}	5.33 ± 0.88^{ab}	2.00 ± 0.05^{bc}
7.5	-	-	100	70.00 ± 0.70^{a}	8.00 ± 0.57^{a}	2.90 ± 0.11^{a}
10.0	-	-	125	33.50 ± 0.86^{e}	4.00 ± 1.15^{bc}	1.70 ± 0.10^{bcde}
-	0.5	-	25	23.50 ± 0.64^{h}	2.66 ± 0.88^{bc}	1.20 ± 0.17^{f}
-	2.5	-	50	33.00 ± 0.91^{e}	3.33 ± 1.33^{bc}	1.76 ± 0.08^{bcd}
-	5.0	-	75	36.25 ± 0.62^{d}	3.66 ± 1.20^{bc}	1.90 ± 0.05^{bcd}
-	7.5	-	100	60.00 ± 0.70^{b}	5.66 ± 0.66^{ab}	2.03 ± 0.03^{b}
-	10.0	-	125	20.00 ± 0.40^{i}	2.00 ± 0.57^{c}	1.66 ± 0.08^{bcde}
-	-	0.5	25	13.50 ± 0.50^{j}	1.66 ± 0.88^{c}	1.13 ± 0.23^{f}
-	-	2.5	50	20.75 ± 0.75^{i}	2.73 ± 0.81^{bc}	1.20 ± 0.15^{f}
-	-	5.0	75	23.00 ± 0.91^{h}	3.33 ± 1.20^{bc}	1.53 ± 0.16^{cdef}
-	-	7.5	100	30.00 ± 0.91^{f}	4.00 ± 0.57^{bc}	1.66 ± 0.08^{bcde}
-	-	10.0	125	26.50 ± 0.64^{g}	2.00 ± 1.00^{c}	1.30 ± 0.20^{ef}

C. occidentalis, whereas the combination of 5.0 μM BA + 100 μM CA + 1.0 μM IBA was less effective (Table 3).

Regenerated shoots failed to produce roots on the full strength MS basal medium. The addition of auxins in the half MS medium facilitated better rhizogenesis. Of the two auxins tested, the best rooting response (Fig. 1*D*) with 80 % of root regeneration frequency, 8.3 ± 0.88 number of roots, and 2.6 ± 0.08 cm root length was obtained in the half MS medium supplemented with 1.5 μM IBA (Table 4). The roots were thick with secondary root hairs which helped in establishing the plantlets in soil. On the other hand, the half MS medium supplemented with NAA produced thin and delicate roots causing only a 50 % regeneration frequency (Table 4).

Regenerated plantlets (Fig. 1*E*) with a well-established root system were transferred to sterile *Soilrite* and grown under 300 or 50 μmol m^{-2} s^{-1} (HI or LI) for 21 d. The plantlets from both the treatments were morphologically similar, with well-developed leaves and without any symptoms of necrosis. The HI exposure plantlets showed a higher survival and better growth than the plantlets under LI. Under both the irradiances, new leaves appeared around 14 d and reached full expansion after about 21 d of acclimatization. Further, when the HI treated plantlets were transferred to garden soil, 70 % of the plantlets could survive in the field, whereas only 50 % of the LI treated plantlets survived. A considerable increase in the content of photosynthetic pigments (Chl *a*, Chl *b*, and Car) was observed in the *in vitro* derived plantlets after the acclimatization period (Fig. 2*A,B,C*) under both HI and LI. However, a decrease in the pigments content during the first seven days of the acclimatization was found, but on the subsequent days when new leaves appeared, a significant increase ($P \leq 0.05$) was recorded (Fig. 2 *A*). At the end of the acclimatization, an increase in Chl *a* was 74 and 75 % under Li and HI, respectively. Likewise, Chl *b* also gradually increased up to 65 and 56 % at HI and LI, respectively (Fig 2*B*). The Car content also showed a positive response to HI compared to LI (Fig. 2 *C*).

Lipid peroxidation (measured as MDA content) was higher in the HI treated plantlets than in the LI grown plantlets indicating a higher degree of oxidative stress. The maximum accumulation of MDA was observed after the first week of the acclimatization, and a slight decrease afterwards (Fig. 2*D*).

Under HI, the SOD activity increased slightly during the first two weeks after transfer of the plantlets in *Soilrite*, followed by a significant increase ($P \leq 0.05$) on day 28 (Fig. 2*E*). A slight increase of SOD activity was also detected under LI (Fig. 2*E*). The CAT activity increased gradually during the whole period of the acclimatization (Fig. 2*F*). A significantly higher CAT activity under HI than under LI was observed at day 28. The GR and APX activities also increased during the acclimatization period in comparison with day 0, however, similarly under HI and LI (Fig. 2*G,H*).

Discussion

The *in vitro* regeneration of direct axillary shoots is an essential method for the production of elite plant genotypes and to avoid formation of somaclones. In nature, axillary buds remain dormant depending on the

Table 3. Effects of optimum concentrations of cytokinins (BA, Kn, and 2-iP), 100 μM citric acid (CA), and various concentrations of auxins (NAA or IBA) on shoot multiplication and elongation after eight weeks of culture. Means ± SE, n = 10, means within columns with different letters are significantly different ($P \leq 0.05$) according to the Tukey's multiple range test.

BA [μM]	Kn [μM]	2-iP [μM]	NAA [μM]	IBA [μM]	Response [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
5.0	-	-	0.1	-	30.00 ± 0.70^{l}	4.00 ± 1.00^{defghi}	2.80 ± 0.05^{b}
5.0	-	-	0.5	-	50.00 ± 0.83^{f}	6.66 ± 0.33^{bcd}	3.06 ± 0.12^{b}
5.0	-	-	1.0	-	80.00 ± 0.63^{a}	11.66 ± 0.88^{a}	3.83 ± 0.08^{a}
5.0	-	-	1.5	-	60.00 ± 0.54^{c}	8.33 ± 1.20^{b}	3.03 ± 0.12^{b}
5.0	-	-	2.0	-	36.40 ± 0.50^{j}	6.66 ± 0.88^{bcd}	2.80 ± 0.05^{b}
-	5.0	-	0.1	-	20.00 ± 0.44^{o}	4.33 ± 0.88^{defghi}	2.06 ± 0.08^{def}
-	5.0	-	0.5	-	40.00 ± 0.70^{i}	5.00 ± 0.57^{defgh}	2.40 ± 0.17^{c}
-	5.0	-	1.0	-	66.60 ± 1.50^{b}	6.00 ± 1.15^{bcdef}	2.76 ± 0.08^{b}
-	5.0	-	1.5	-	50.00 ± 0.94^{f}	4.33 ± 1.33^{defghi}	2.20 ± 0.05^{cde}
-	5.0	-	2.0	-	43.40 ± 0.81^{h}	4.00 ± 0.57^{defghi}	1.96 ± 0.08^{ef}
-	-	5.0	0.1	-	26.40 ± 0.50^{m}	2.00 ± 1.00^{i}	1.23 ± 0.12^{kl}
-	-	5.0	0.5	-	30.00 ± 0.83^{l}	2.66 ± 0.88^{hi}	1.46 ± 0.08^{hijk}
-	-	5.0	1.0	-	43.20 ± 0.37^{h}	4.00 ± 0.57^{defghi}	1.80 ± 0.05^{fgh}
-	-	5.0	1.5	-	23.40 ± 0.87^{n}	3.66 ± 0.33^{efghi}	1.63 ± 0.08^{ghi}
-	-	5.0	2.0	-	19.80 ± 0.37^{o}	2.33 ± 1.20^{hi}	1.50 ± 0.05^{hijk}
5.0	-	-	-	0.1	20.00 ± 0.54^{o}	4.00 ± 1.15^{defghi}	1.86 ± 0.14^{fg}
5.0	-	-	-	0.5	30.00 ± 0.83^{l}	4.66 ± 1.20^{defghi}	2.03 ± 0.18^{ef}
5.0	-	-	-	1.0	56.80 ± 0.86^{d}	8.00 ± 0.00bc	2.36 ± 0.08^{cd}
5.0	-	-	-	1.5	50.00 ± 0.83^{f}	6.33 ± 0.88^{bcde}	1.80 ± 0.15^{fgh}
5.0	-	-	-	2.0	46.60 ± 0.50^{g}	5.00 ± 0.57^{defgh}	1.56 ± 0.12^{ghijk}
-	5.0	-	-	0.1	20.00 ± 1.22^{o}	3.16 ± 0.44^{ghi}	1.43 ± 0.12^{ijk}
-	5.0	-	-	0.5	26.60 ± 0.40^{m}	3.66 ± 0.33^{efghi}	1.60 ± 0.05^{ghij}
-	5.0	-	-	1.0	53.40 ± 0.74^{e}	5.66 ± 0.88^{cdefg}	2.00 ± 0.05^{ef}
-	5.0	-	-	1.5	40.20 ± 1.11^{i}	3.33 ± 0.33^{fghi}	1.80 ± 0.05^{fgh}
-	5.0	-	-	20	30.00 ± 0.94^{l}	3.00 ± 0.57^{ghi}	1.56 ± 0.08^{ghijk}
-	-	5.0	-	0.1	16.60 ± 0.92^{p}	2.70 ± 0.30^{hi}	1.23 ± 0.08^{kl}
-	-	5.0	-	0.5	30.00 ± 1.09^{l}	3.66 ± 0.66^{efghi}	1.30 ± 0.05^{ijkl}
-	-	5.0	-	1.0	33.20 ± 0.73^{k}	3.66 ± 0.88^{efghi}	1.50 ± 0.05^{hijk}
-	-	5.0		1.5	20.00 ± 0.70^{o}	2.33 ± 0.33^{hi}	1.26 ± 0.08^{jkl}
-	-	5.0	-	2.0	10.00 ± 0.70^{q}	2.00 ± 0.57^{i}	1.10 ± 0.11^{l}

Table 4. Effects of auxins on *in vitro* root induction in tissue-culture-raised shoots obtained from a BA (5.0 μM) + CA (100 μM) + NAA (1.0 μM) treatment in *C. occidentalis* on a half MS medium after four weeks of culture. Means ± SE, n = 10, means within columns with different letters are significantly different ($P \leq 0.05$) according to the Tukey's multiple range test.

IBA [μM]	NAA [μM]	Rooting [%]	Number of roots [shoot^{-1}]	Root length [cm]
0.5	-	19.80 ± 0.48^{g}	2.00 ± 0.57^{c}	1.26 ± 0.12^{de}
1.0	-	43.40 ± 0.74^{c}	3.66 ± 0.66^{bc}	1.50 ± 0.15^{cd}
1.5	-	80.00 ± 0.70^{a}	8.33 ± 0.88^{a}	2.63 ± 0.08^{a}
2.0	-	40.00 ± 1.30^{d}	4.40 ± 0.83^{bc}	1.93 ± 0.08^{b}
2.5	-	30.00 ± 0.83^{f}	3.66 ± 0.33^{bc}	1.66 ± 0.08^{bc}
-	0.5	20.00 ± 0.70^{g}	2.00 ± 0.57^{c}	0.73 ± 0.18^{g}
-	1.0	30.00 ± 0.70^{g}	2.33 ± 0.88^{c}	1.10 ± 0.10^{efg}
-	1.5	50.00 ± 0.94^{b}	5.00 ± 1.00^{b}	1.23 ± 0.08^{def}
-	2.0	40.00 ± 0.89^{d}	4.00 ± 0.57^{bc}	0.86 ± 0.12^{fg}
-	2.5	36.00 ± 0.83^{e}	3.00 ± 1.00^{bc}	0.76 ± 0.12^{g}

growth pattern of the plant. However, using tissue culture, the rate of shoot multiplication is greatly enhanced in a nutrient medium containing a suitable cytokinin or cytokinin and auxin combinations (Sato and Mori 2001). Cytokinins have been shown to stimulate or accelerate the release of buds from dormancy (Sachs and

Thimann 1967). In the current study, the nodal explants failed to develop shoot buds in a growth regulator free medium. In contrast, when the same explants were grown on culture media containing cytokinins, axillary shoots developed and proliferated to form small clusters of secondary shoots. BA was found to be more potent compared to other cytokinins tested. BA is commonly preferred cytokinin for *in vitro* propagation, as it stimulates cell division as well as cell elongation, activates RNA synthesis, stimulates protein synthesis and enzyme activities (Al Malki and Elmeer 2010). The stimulating effect of BA on bud break and multiple shoot formation has been reported in many plants, *e.g*., *Cassia angustifolia* (Agrawal and Sardar 2003), *Tylophora indica* (Faisal and Anis 2007) and *Bauhinia tomentosa* (Naz *et al*. 2011).

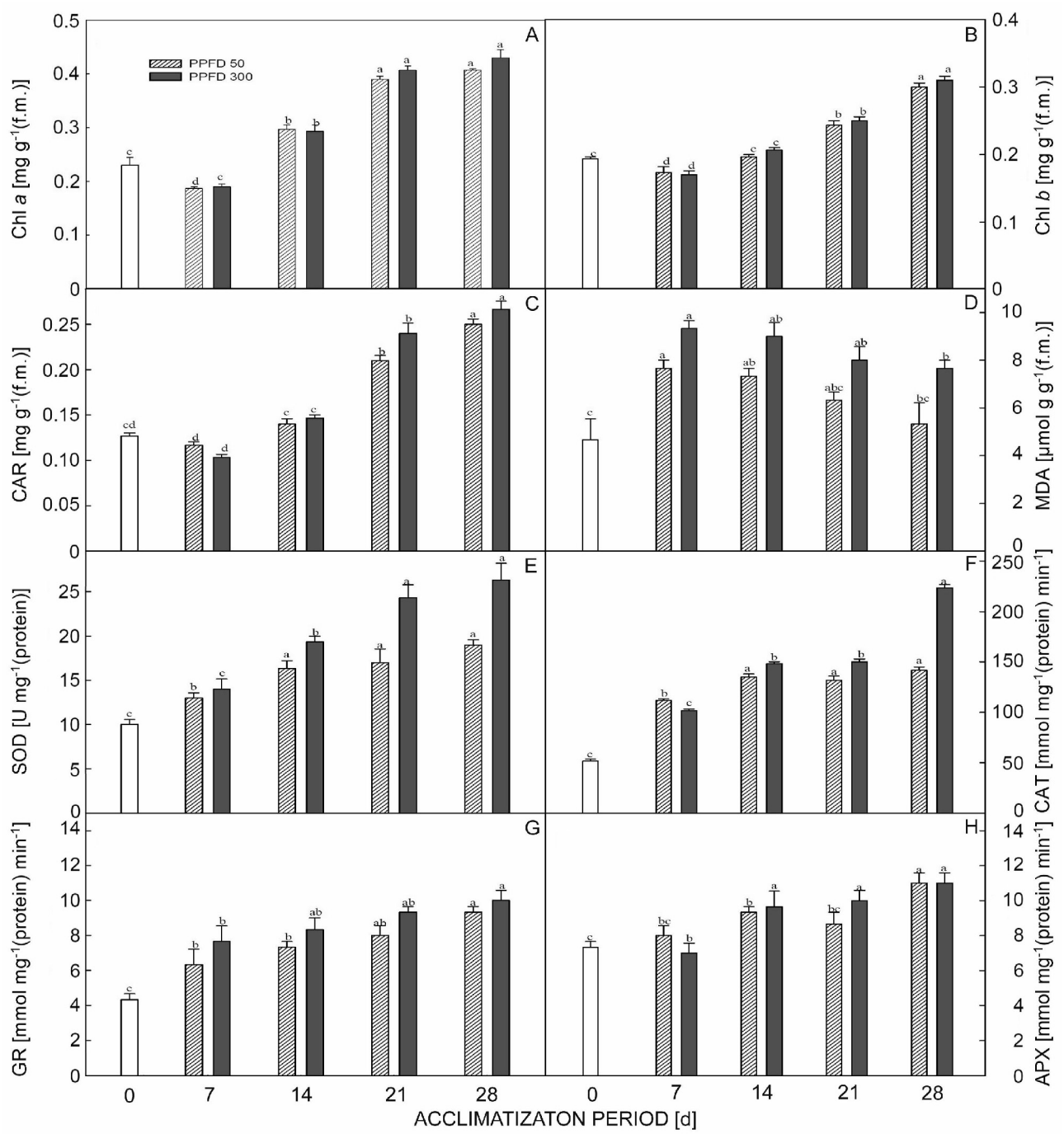

Fig. 2 Changes in the chlorophyll *a* (*A*), chlorophyll *b* (*B*), carotenoids (*C*), and MDA content (*D*), activities of SOD (*E*), CAT (*F*), GR (*G*), and APX (*H*) of micropropagated plantlets of *C. occidentalis* at PPFDs of 50 and 300 µmol $m^{-2}s^{-1}$ for 28 d. Means ± SE, $n = 10$. Means with different letters are significantly different at $P \leq 0.05$ according to the Tukey's multiple range test.

Explant browning is usually attributed to the production of phenolic compounds released from the cut surfaces of the explants. Phenolic compounds include polyphenols and tannins which not only prevent explant development, but also lead to death of the explants. CA has a dual role as potential antioxidant and growth enhancer (acts as vitamins) in plant tissue culture systems (Nayanakantha *et al*. 2010). The enhancing effect of CA in controlling phenolic browning and accelerating organogenic potential is crucial. The application of CA in MS media have been reported as the best treatment to check browning and helps in the improvement of shoot development in *Myrica esculenta* (Bhatt and Dhar 2004) and *Santalum album* (Muthan *et al*. 2006).

The combination of cytokinin and auxins appeared to be a prerequisite for the increase of shoot development and growth in *C. occidentalis*. Both the regeneration frequency and average number of shoots per explant were enhanced significantly when compared with a single cytokinin only. All the auxins (IBA or NAA) along with optimum concentrations of cytokinins (BA, Kn, or 2-iP) were capable to produce new shoots, but the combination of 5.0 µM BA, 100 µM CA, and 0.5 µM NAA was best for maximum shoot regeneration (Table 3).

Roots initiation and development vary in their requirement of auxin type in different plant species. Our study indicates that the half MS medium supplemented with 1.5 µM NAA gave a lesser (50 %) rooting frequency, whereas quite a high percentage (80 %) of shoots rooted well when IBA was used, and it was found to be most effective at the 1.5 µM concentration. The promotive effect of IBA in rooting is well documented in *Cassia angustifolia* (Agrawal and Sardar 2006).

Successful acclimatization of plantlets is an important step in tissue culture studies (Aragón *et al*. 2014). Tissue culture raised plants are quite often susceptible to various stresses during *ex vitro* transplantation. Therefore, the plantlets were first acclimatized for 28 d in a culture room under low (50 µmol m^{-2} s^{-1}) and high (300 µmol m^{-2} s^{-1}) irradiances before transfer to the field. The plantlets of *C. occidentalis* showed a significant increase in the photosynthetic pigments under both LI and HI after 7 d of the acclimatization. Comparatively a higher Chl content was recorded under HI than LI. A continuous increase in pigment content suggests that the exposure of the plants either to LI or HI did not impair the photosystem (PS) II (Von Willert *et al*. 1995).

Enhancements in Chl *a*, Chl *b*, and Car content in new leaves of *C. occidentalis* formed during the acclimatization period are in accordance with the reports on *Calathea louisae* (Van Huylenbroeck *et al*. 2000) and *Tylophora indica* (Faisal and Anis 2010). The increase in Car content observed in both the LI and HI plants reflects a flexible functional response of the photosynthetic apparatus, since the photoprotective role of Car against photo-oxidative damage is well documented (Young 1991,Van Huylebroeck *et al*. 2000). Similar observations have been reported in different plants like *Withania somnifera* (Fatima *et al*. 2011) and *Rauvolfia tetraphylla* (Faisal and Anis 2009), where chlorophyll and carotenoid content increased during acclimatization.

MDA content is routinely used as indicator of lipid peroxidation resulting from oxidative stress (Zhang *et al*. 2007, Monteiro *et al*. 2009). The increase in the amount of MDA during the acclimatization as compared to the amount found at day 0 indicated the generation of ROS similarly as reported Faisal and Anis (2010) in *Tylophora indica*. To overcome the damage caused by ROS, plants stimulate the antioxidative mechanisms (Alscher 1989, Foyer *et al*. 1997). The current investigation demonstrates the diverse responses of the antioxidant enzymes in the plants acclimatized under LI or HI. SOD is thought to be the first line of defense against ROS. It is presented in all sub-cellular compartments and catalyzes the dismutation of superoxide radical into less harmful H_2O_2 (Zhao *et al*. 2006, Gill and Tuteja 2010). CAT decomposes this H_2O_2 and also H_2O_2 produced by photorespiration through the mitochondrial electron transport system (Scandalios 1990). Thus, SOD and CAT were more active in the HI treatment than in the LI one. These changes indicated the acclimatization of the plantlets and revealed a protection against photo-oxidation (Logan *et al*. 1998). Similar trends have been reported in *Calathea* (Van Huylenbroeck *et al*. 2000) and *Tylophora indica* (Faisal and Anis 2010). APX and GR are predominantly localized in chloroplasts which are the major site of H_2O_2 production in leaves (Foyer *et al*. 1997). GR is considered a key enzyme responsible for maintaining the reduced form of glutathione pool (Foyer *et al*. 1997). The increase in GR activity at HI exhibited protection against photooxidative stress linked to photoinhibition, as it has been previously reported by Logan *et al*. 1998 in *Cucurbita pepo* and *Vinca major* plants. Also, the increased activity of APX under both the irradiances suggests the chloroplast based detoxification of ROS produced *via* the Mehler pathway. These results are in accordance to the findings on the changes in the activities of the antioxidant enzymes in the micropropagated plantlets of *Tylophora indica* (Faisal and Anis 2010) and *Tecomella undulata* (Varshney and Anis 2012) during an acclimatization period. In fact, *C. occidentalis* was tolerant to HI during the acclimatization.

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