



Short communication

Relative examination of antioxidative enzymatic activities in plantlets of *Cardiospermum halicacabum* L. differentiated from hypocotyls in in vivo and ex vitro environment



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ABSTRACT

A plant regeneration protocol was devised for *Cardiospermum halicacabum* by means of aseptically extracted 7 days old hypocotyls forming adventitious shoots on Murashige and Skoog (MS) medium harmonized with 0.7 μ M thidiazuron (TDZ) producing a maximum of 18.20 ± 0.98 number of shoots in 94% cultures following 4 weeks. Subsequent subculturing for five passages, on a medium without plant growth regulators, tempted the highest shoot number (40.00 ± 1.15) with an average shoot length of 6.53 ± 0.49 cm after the fourth subculture. Histological sections confirmed the formation of multiple buds from hypocotyl explants. The expression of antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase was found to be higher in acclimatized plants than in the in vitro cultured ones suggesting the involvement of these enzymes in shoot differentiation and in growth under external environment partly due to their ability to cope up with oxidative stress.

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1. Introduction

Cardiospermum halicacabum L. is one of the most important medicinal plants used in traditional ayurvedic system of medicine in several parts of India for the treatment of rheumatoid arthritis. It belongs to family-Sapindaceae and widely distributed in tropical and sub-tropical America, Africa, and Asia. Alternatively, it has been used for the treatment of nervous diseases, reduce hardened tumors, asthma, as a demulcent in orchitis and in dropsy. Leaves are emetic, stimulant and their decoctions are given for the treatment of piles, diarrhoea, as an infusion for general sores and to reduce obesity [1].

Techniques of micropropagation are employed generally with a particular view to increase the number of individuals in species rapidly countered with reproductive problems or those facing extreme reduced populations. *C. halicacabum* is one such plant facing threat to their natural population. Regardless of its outstanding pharmacological utility for treating many ailments for centuries, yet it is best known to modern society as a weed.

Consequently, governments have developed vegetation management programs and bi-laws aimed at eradicating specific weeds. This presents a paradox for the eradication of novel medicines for ailments that plaque our society. Balloon vine is an example of such controversy because it is considered to be a pan tropical weed and a traditional medicinal herb [2].

Adding together, the plant is conventionally propagated all the way through seeds but finds restrictions due to low germination rate, low viability, and delayed rooting of seedlings. Furthermore, payable to its large scale unobstructed exploitation of whole plant to meet its ever-increasing demand by the pharmaceutical industries, coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this valuable medicinal plant has been strikingly depleted.

The in vitro culture protocol devised for micropropagation of *C. halicacabum* has been presented in literature with successful plant regeneration using either callus [3,4] or using meristematic explants such as nodal segments [5]. However, there was no report published based on direct plant regeneration from hypocotyls explants. This paper reports, for the first time a protocol to regenerate plants through hypocotyl culture of *C. halicacabum* focusing on the origin and mode of development of the regenerated shoot buds by means of histological analysis.

In recent years, there has been a growing interest in the functional significance of ROS and the concomitant antioxidant

Abbreviations: TDZ, thidiazuron; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase.

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response in growth, development and differentiation of plant cells. Manifestation of ROS in the plant cells is in general allied with the free radical processes involved in the development of plant, as well as its interface with the external surroundings. Furthermore, these free radicals have an important role in the metabolism and development of aerobic organisms; however, their uncontrolled production leads to oxidative stress. Under in vitro conditions, plants are exposed to low photosynthetic photon flux density (PPFD) and high humidity conditions. Once transferred to greenhouse, plants experienced water stress because of higher PPFD and low humidity environment. A synergic action of high irradiance and water stress reduces the capacity of a photosynthetic system to utilize incident radiation and causes oxidative stress through the formation of reactive oxygen species (ROS) or active oxygen species (AOS). These include superoxide radicals ($O_2^{\bullet-}$), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\bullet) which causes tissue injury. These are highly reactive species and can seriously disrupt normal metabolism through oxidative damage to membrane lipids, protein pigments and nucleic acid and ultimately results in cell death. To counter the hazardous effect of reactive oxygen species under stress, plants have developed or have evolved a complex antioxidative defense mechanism system which involves both enzymatic and non-enzymatic metabolites antioxidant such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) which are efficient antioxidant enzymes. The antioxidant metabolism is enhanced during differentiation in vitro, and antioxidant profiles also vary throughout different phases of culture [6]. The production of ROS has been associated with plant recalcitrance during in vitro culture [7]. In this work, we also match up the altered levels of antioxidant enzymes produced during the culture conditions with those of ex vitro regenerated plants and their part in thriving plant to external environmental conditions.

2. Materials and methods

2.1. Establishment of aseptic seedlings

Seeds of *C. halicacabum* were collected from the plants growing in the botanical garden of the university. The seeds were washed thoroughly under running tap water for 30 min followed by treatment with 5% (v/v) Labolene, a liquid detergent for 15 min. The seeds were then rinsed thoroughly and treated with 0.1% (w/v) $HgCl_2$ for 5 min. After rinsing 5–6 times with sterilized distilled water, the seeds were inoculated in Murashige and Skoog's medium [8] for germination. Hypocotyl segments excised from 7 days old aseptic seedling were used as an explant.

2.2. Culture media and conditions

MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar or 0.25% (w/v) gelrite was used during the investigation. The pH of the medium was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving. The media were dispensed in 25 mm × 150 mm test tubes (Borosil, India) each containing 20 ml of medium and cotton plugs (single layered cheese cloth stuffed with non-absorbent cotton) were used as closures. Glasswares, culture media, and instruments were sterilized by autoclaving at 121 °C at ~105 kPa for 20 min. All the cultures were maintained at 24 ± 2 °C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by 40 W cool white fluorescent lamps (Philips, India) and with 60–65% relative humidity.

2.3. Shoot induction and multiplication

For multiple shoot induction, excised hypocotyl explants were inoculated on MS medium augmented with various cytokinins, BA (0.5, 2.5, 5.0, 7.5, and 10.0 μM) and TDZ at lower concentrations (0.1, 0.3, 0.5, 0.7, and 0.9 μM) individually. Initially, cultures were subcultured onto the same fresh medium after every 2 weeks resulted in fasciated, distorted, stunted, and clumped shoots which did not elongate further. So, in order to avoid this problem, these clusters were transferred and subcultured onto the hormone free MS medium just to nullify the negative effects of TDZ. Subculturing was done on hormone free MS medium after every 2 weeks and the data of each subculture passages was recorded. The percentage of explant producing shoots, number of shoots per explant and shoot length were recorded after 4 and 8 weeks of culture.

2.4. Root formation

In vitro rooting method was employed using protocol established by Jahan and Anis [5].

2.5. Acclimatization

Plantlets with well developed roots and shoots were removed from the culture medium and washed gently under running tap water to remove any adherent gel from the roots and transferred to thermo cups containing sterile soilrite. These were kept under diffuse light conditions (16:8 h photoperiod) covered with transparent polythene bags to ensure high humidity, irrigated after every 3 days with half-strength MS salt solution (without vitamins) for 2 weeks. Polythene membranes were removed after 2 weeks in order to acclimatize the plantlets and after 4 weeks they were transferred to earthen pots containing garden soil and vermicompost (1:1) and maintained in a greenhouse under normal day length conditions.

2.6. Antioxidant enzymes assay

To determine antioxidant enzyme activity, 0.5 g fresh leaf tissue, collected from 2 and 4 weeks regenerated adventitious shoots and from 2 and 4 weeks micropropagated plantlets, respectively, was homogenized in 2.0 ml 0.5 M phosphate extraction buffer (pH7.5) containing 1% polyvinylpyrrolidone, 1% Triton X-100, and 0.1 g ethylenediaminetetraacetic acid (EDTA) using a prechilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 rpm for 20 min. The supernatant was used for protein determination and enzyme assays. Extraction was carried out in the dark at 4 °C. A high-speed centrifuge (Remi Instruments Ltd., Goregaon East, MH, India) and UV–visible spectrophotometer (Shimadzu, Kyoto, Japan) were used.

2.6.1. Superoxide dismutase

(SOD; EC 1.15.1.1) activity, described by Dhindsa et al. [9], was measured by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) in a reaction mixture consisting of 0.5 M phosphate buffer (pH7.5), 0.1 mM EDTA, 13 mM methionine, 63 mM NBT, 1.3 mM riboflavin, and 0.1 ml enzyme extract. The reaction mixture was irradiated for 15 min and absorbance was measured at 560 nm against the non-irradiated blank.

2.6.2. Catalase

(CAT; EC 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease of absorbance at

240 nm, following the method of Aebi [10]. The assay mixture contained 50 mM phosphate buffer (pH 7.0) and 100 µl enzyme extract in a total volume of 3 ml, and the reaction was started by addition of 10 mM H₂O₂.

2.6.3. Glutathione reductase

(GR; EC 1.6.4.2) activity was measured using the protocol described by Foyer and Halliwell [11], and as modified by Rao [12] on glutathione dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. The assay mixture contained 50 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.2 mM (NADPH), and 0.5 mM glutathione disulfide. The enzyme extract (0.1 ml) was added to start the reaction, and was allowed to run for 5 min at 25 °C.

2.6.4. Ascorbate peroxidase

(APX; EC 1.11.1.11) activity as estimated by Nakano and Asada [13] was measured via monitoring the decrease in absorbance at 290 nm within 1 min. The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.1 mM H₂O₂, 0.1 mM EDTA, and 0.1 ml enzyme extract. The activities of each enzyme were expressed in enzyme units per milligram protein per minute. The protein content in enzymatic extracts was determined following the Bradford assay [14] using bovine serum albumin as a standard.

2.7. Histological analysis

To confirm the regeneration of multiple shoot buds from the hypocotyl explants, histological examination of explants was performed after 15 days. Tissues were fixed in formalin:glacial acetic acid:ethanol 4:6:90 (v/v) solution. Fixed tissues were dehydrated through an ethanol/xylol series and embedded in paraffin wax (60 °C). Serial sections 10 µm thickness were cut using a Spencer 820 microtome (American Optical Corp., Buffalo, NY, USA) and the resulting paraffin ribbons were passed through a series of deparaffinising solutions and stained in safranin and fast green solutions. The sections were examined under an optical microscope (CH20i, Olympus, Tokyo, Japan).

2.8. Statistical analysis

All the experiments were conducted with a minimum of 10 replicates per treatment and repeated three times. The data was analyzed statistically using SPSS version 10 (SPSS Inc., Chicago, USA). The significances of differences among means was carried out using Duncan's multiple range test at $P=0.05$.

The results are expressed as a means \pm SE of three repeated experiments.

3. Results and discussion

Cardiospermum hypocotyls explant keeps a hold of an adequate amount of cellular plasticity to achieve plantlet regeneration as observed from experimentation. Adventitious shoot formation was observed for all TDZ and BA concentrations tested. A positive correlation was noted between TDZ concentration and percent shoot formation with the optimum regeneration medium supplemented with PGR. TDZ proved to be the best plant growth regulator for inducing maximum rates of shoot multiplication than BA. TDZ at a very low concentration of 0.7 µM found extremely competent in activating the maximum rate of shoot bud differentiation from hypocotyl explants upto many folds forming 18.20 ± 0.98 numbers of shoots with 2.56 ± 0.23 cm shoot length in 94% cultures after 4 weeks (Table 1; Fig. 1A and B). The histological sections revealed direct differentiations of multiple shoot buds form the hypocotyl explants (Fig 1E). Lower levels of this potent cytokinin have been recommended by Huettelman and Preece [15] for obtaining maximum shoot proliferation results which also corroborates with my results. A similar domino effect was achieved on the shoot forming capacity of *Crotalaria verrucosa* [16].

The effectiveness of TDZ in shoot proliferation as a sole growth regulator has been well documented in a number of plant species including, *Capsicum annum* [17], *Nyctanthes arbor-tristis* [18], *C. verrucosa* [16], *C. halicacabum* [5], *Rubia cordifolia* [19], and *Tecomella undulata* [20]. Several researches have demonstrated that TDZ unlike traditional cytokinins is capable of fulfilling both cytokinin and auxin requirements of various regenerative responses in many different plant species. Such studies are supported by the fact that there may be a possibility of high natural endogenous cytokinin content within the plant species. This explanation further finds supports by the fact that adventitious root growth often appears spontaneously on plant stems of many cultivars [21]. It is likely that TDZ results in a balanced ratio of endogenous growth regulators that allows for specific mode of regeneration to take place and this is likely to be dependent on the level of TDZ provided in the medium and species. Hare and Van Staden [22] also reported that TDZ has a capacity to inhibit (atleast partially) the action of cytokinin oxidase, which in turn may increase the level of endogenous cytokinins.

When compared to purine based cytokinin i.e., BA, TDZ is found to be active at lower concentrations. Here, BA gave optimum response on 5.0 µM (Table 1). The aminopurine cyotkinins have similar effects at higher concentrations i.e., in between 1 µM and 10 µM. This range with TDZ results in excessive callus formation and cessation of shoot growth. The TDZ alone is more effective than

Table 1

Effect of plant growth regulators on the induction of multiple shoots from hypocotyls explants of *C. halicacabum* after 4 weeks of culture.

TDZ	BA	% Regeneration	Mean number of shoot/explant	Mean shoot length (cm)
0.1		40	7.0 ± 0.57^{ef}	1.26 ± 0.37^{bcd}
0.3		80	10.0 ± 1.15^{cde}	1.13 ± 0.13^{cd}
0.5		87	14.30 ± 0.68^b	2.00 ± 0.04^{ab}
0.7		94	18.20 ± 0.98^a	2.56 ± 0.23^a
0.9		60	13.00 ± 1.15^{bc}	1.60 ± 0.23^{bcd}
	0.5	33	3.66 ± 0.88^{gh}	1.00 ± 0.00^d
	2.5	60	9.66 ± 1.45^{de}	1.36 ± 0.27^{bcd}
	5.0	77	12.20 ± 1.15^{bcd}	1.86 ± 0.17^{abc}
	7.5	50	5.67 ± 1.20^{fg}	1.53 ± 0.29^{bcd}
	10.0	30	1.33 ± 0.33^h	0.90 ± 0.05^d

Values represent means \pm SE. Means followed by the same letter are not significantly different ($P=0.05$) using Duncan's multiple range test.

^{a-h}Represents significant and non significant levels according to DMRT analysis.



Fig. 1. (A and B) Induction of shoot buds from hypocotyls explants on MS + TDZ ($0.7 \mu\text{M}$) after 2 and 4 weeks of culture. (C) Proliferation and multiplication of shoots on hormone free MS medium. (D) Rooted plantlet in $1/3$ MS + IAA ($0.5 \mu\text{M}$) after 4 weeks. (E) Induction of multiple shoot buds from hypocotyls explants.

adenine-based compounds for inducing axillary shoot formation in many woody species [15]. But Palla and Pijut [23] reported adventitious regeneration from hypocotyls of *Fraxinus* only in combination with BA.

However, an over-abundance of TDZ has been shown to have negative effects in vitro, such as inhibition of shoot elongation, tight bud clusters with some leaf expansion and hyperhydricity that could be a factor limiting further

Table 2

The evaluation of morphogenetic potential of shoot culture obtained from TDZ ($0.7 \mu\text{M}$) after being tested for five subculture passages on a growth regulator free MS medium.

Subculture passage	Mean number of shoots/explant	Mean shoot length (cm)
First	18.20 ± 0.98^c	2.56 ± 0.23^c
Second	35.33 ± 0.96^b	2.66 ± 0.35^c
Third	36.86 ± 0.85^{ab}	4.53 ± 0.63^b
Fourth	40.00 ± 1.15^a	6.50 ± 0.45^a
Fifth	40.00 ± 1.15^a	6.53 ± 0.49^a

Values represent means \pm SE. Means followed by the same letter are not significantly different ($P=0.05$) using Duncan's multiple range test.

^{a-c}Represents significant and non significant levels according to DMRT analysis.

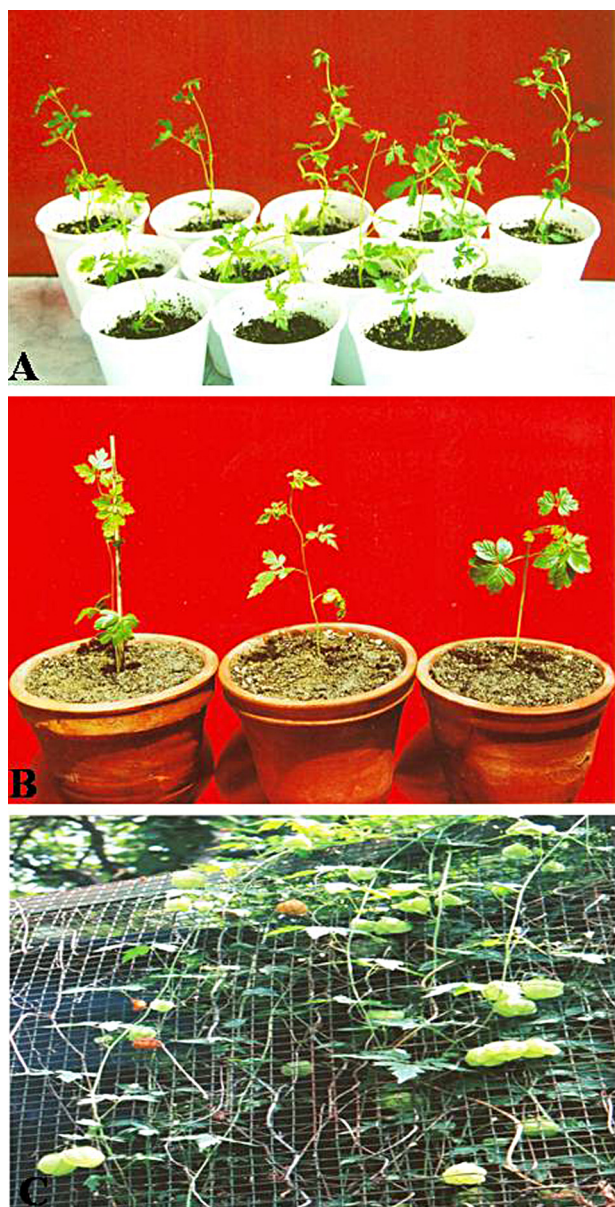


Fig. 2. (A and B) Acclimatized plant in soilrite after 4 weeks. (C) 6 Months old tissue culture raised plant in field condition.

greatest shoot length was achieved in the first four subcultures which got stabilized at fifth passage and beyond which rate of multiplication declined (Table 2; Fig 1C). This enhanced rate of shoot multiplication by subsequent subcultures substantiates with the earlier reports on *C. verrucosa* [18], *C. halicacabum* [5] and *Andrographis neesiana* [29], and *T. undulata* [20].

3.1. Rooting and acclimatization

As per the protocol devised by Jahan and Anis [5], healthy adventitious root induction was achieved on $\frac{1}{3}$ MS medium amended with IAA ($0.5 \mu\text{M}$) (Fig. 1D). Rooted plantlets with fully expanded leaves were transferred to pots containing sterile soilrite and hardened off inside the growth chamber for 4 weeks (Fig. 2A and B). Hardening of micropropagated plantlets is essential for successful establishment as regenerated plants in culture condition have been in a sheltered environment with a very high humidity, controlled light, and temperature that induces some kind of internal abnormalities. It is therefore, necessary to accustom the plants to the natural environment by a process called acclimatization. After 1 month, the micropropagated plants were planted in earthen pots containing garden soil and vermicompost (1:1) and maintained in a greenhouse. The survival rate was 80%.

3.1.1. Antioxidants

The creation of ROS as well as their detoxification is highly synchronized in plants, and their levels are kept under firm control by a complex antioxidant system. The character played by ROS in plant growth and development is sustained by the interplay of ROS and plant growth regulators. Moreover, they have been implicated as second messenger in several plant hormone responses [30]. A comparative study has been undertaken to account the changes in the activities of antioxidant enzymes during the in vitro culture period with their ex vitro acclimatized plantlets. As observed from the data collected SOD and CAT showed a continuous increase in their activity in the in vitro regenerated shoots from 2nd to 4th weeks during the culture conditions which still sustained after 2nd–4th week of their ex vitro transfer to field conditions (Fig. 3A and B). But for SOD, an abrupt augment in the activity at 2nd week of acclimatization was observed that suggests its role in struggling oxidative stress. However, the activity of enzyme decline in the 4th week of acclimatization which advocate that the plant adjusts itself to external environmental conditions. The combined action of SOD and CAT which are the most efficient antioxidant enzymes acts on potentially dangerous superoxide radical ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) and converts it into water (H_2O) and molecular oxygen (O_2), thus averting cellular damage.

A similar line of action has been observed in the activity of APX and GR which countered the increased levels of ROS in the regenerated plantlets by growing their own level during the culture conditions and maintaining it upto 2nd–4th weeks of their transfer to ex vitro conditions (Fig. 4A and B). However, on the whole the intensity of these antioxidative enzymes in acclimatized micropropagated plants is much more than the in vitro conditions which demonstrate the determining the ability of the plant that developed functional photosynthetic machinery along with the assisting activities to survive the oxidative stress. The boost up in the activity of antioxidant enzymes activities in the micropropagated plantlets are in agreement with the outcome achieved during acclimatization of micropropagated plantlets of *Rauvolfia tetraphylla*, *Tylophora indica*, and with *T. undulata* [20,31,32].

adventitious shoot formation. Inhibition of shoot elongation may be due to high cytokinin activity of TDZ, whereas the presence of phenyl group may be a possible cause of shoot bud fasciation [15]. Similar pattern of deformities have been reported in several plants including *Daphne* sp. [24], *Ziziphus jujuba* [25], and *R. cordifolia* [19]. To overcome deleterious effect of continued presence of TDZ on growth and multiplication of shoots, these shoots were transferred to a secondary medium lacking TDZ (growth regulator free MS media). The procedure applied here substantiates and has been successfully applied in a number of plant species viz., *Morus alba* [26], *Cassia angustifolia* [27], *C. halicacabum* [5], *Cotoneaster wilsonii* [28] and *N. arbor-tristis* [18].

The effect of subculture passage was also evaluated on shoot cultures induced from TDZ. The highest number of shoots and

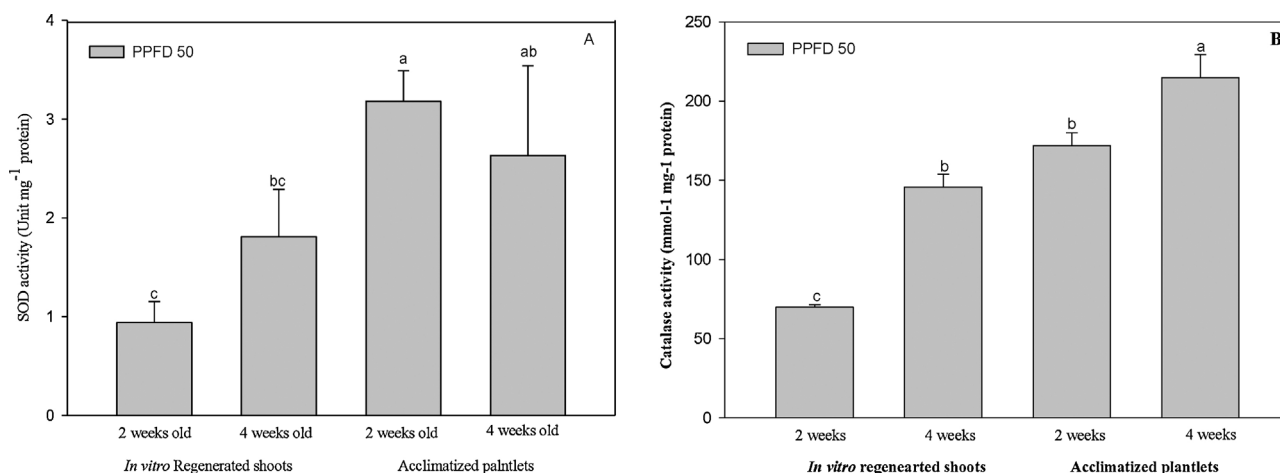


Fig. 3. Comparative account of changes in the level of SOD (A) and catalase (B) activity in in vitro and micropropagated plantlets of *C. halicacabum* acclimatized at PPFD 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Bars represent the mean \pm SE. Bars denoted by the same letter are not significantly different ($P=0.05$) using Duncan's multiple test.

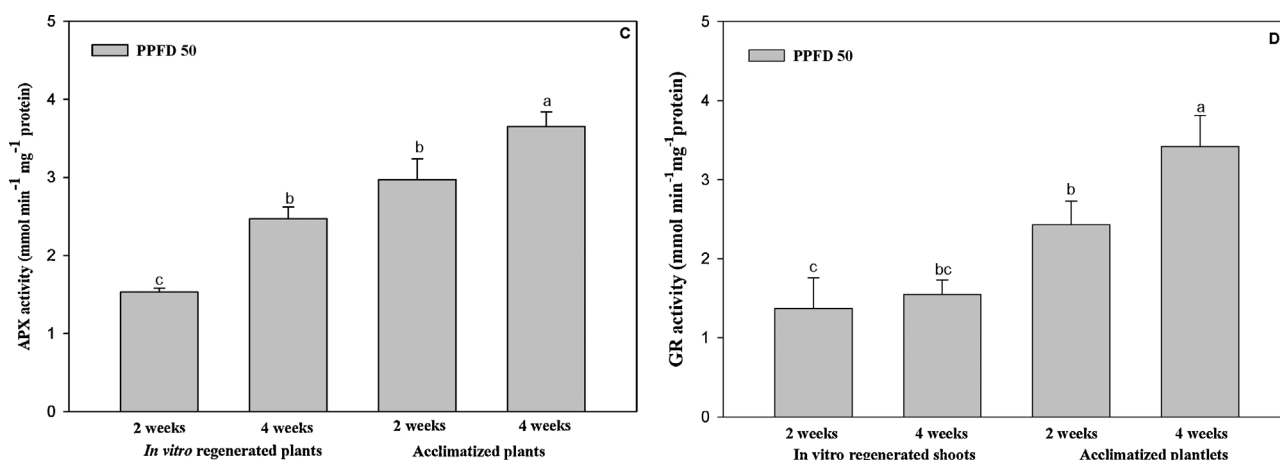


Fig. 4. Comparative account of changes in the level of APX (A) and GR (B) activity in in vitro and micropropagated plantlets of *C. halicacabum* acclimatized at PPFD 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Bars represent the mean \pm SE. Bars denoted by the same letter are not significantly different ($P=0.05$) using Duncan's multiple test.

4. Conclusion

The present paper, being the first report, the most significant outcome of the current study is the demonstration of high level aptitude of hypocotyl explants of *Cardiospermum* to regenerate adventitious shoots and successful mass micropropagation using low TDZ concentrations. Adding up together, the increased levels of antioxidant enzymes also authenticate the enhanced ability of regenerated plants to tolerate the oxidative stress. In conclusion, a reliable and commercial protocol has been developed that proved efficient mass multiplication and conservation of *C. halicacabum* L.

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