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SYNTHETIC SEED PRODUCTION AND PHYSIO-BIOCHEMICAL STUDIES IN *CASSIA ANGUSTIFOLIA* VAHL. – A MEDICINAL PLANT

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Synthetic seed technology is an alternative to traditional micropropagation for production and delivery of cloned plantlets. Synthetic seeds were produced by encapsulating nodal segments of *C. angustifolia* in calcium alginate gel. 3% (w/v) sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ were found most suitable for encapsulation of nodal segments. Synthetic seeds cultured on half strength Murashige and Skoog medium supplemented with thidiazuron (5.0 μM) + indole-3-acetic acid (1.0 μM) produced maximum number of shoots (10.9 ± 0.78) after 8 weeks of culture exhibiting (78%) *in vitro* conversion response. Encapsulated nodal segments demonstrated successful regeneration after different period (1–6 weeks) of cold storage at 4 °C. The synthetic seeds stored at 4 °C for a period of 4 weeks resulted in maximum conversion frequency (93%) after 8 weeks when placed back to regeneration medium. The isolated shoots when cultured on half strength Murashige and Skoog medium supplemented with 1.0 μM indole-3-butyric acid (IBA), produced healthy roots and plantlets with well-developed shoot and roots were successfully hardened off in plastic pots containing sterile soilrite inside the growth chamber and gradually transferred to greenhouse where they grew well with 85% survival rate. Growth performance of 2 months old *in vitro*-raised plant was compared with *in vivo* seedlings of the same age. Changes in the content of photosynthetic pigments, net photosynthetic rate (P_N), superoxide dismutase and catalase activity in *C. angustifolia* indicated the adaptation of micropropagated plants to *ex vitro* conditions.

Keywords: Antioxidant enzymes – encapsulation – rooting – synthetic seeds – Thidiazuron

INTRODUCTION

Cassia angustifolia commonly known as Senna is a medicinally valuable drought resistant shrub of the family Fabaceae and mainly grown as a cash crop in various parts of the world. Senna is cultivated in Somalia, Arabian Peninsula and near the Nile river [19]. The leaves and pods contain sennosides A, B, C, D, kampferol, anthraquinone, essential oil, isohamnentin and emodin. These are employed in the treatment of amoebic dysentery as an anthelmintic and as a mild liver stimulant. *Cassia* is a powerful cathartic used in the treatment of constipation, working through a stimula-

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tion of intestinal peristalsis. Besides being an excellent laxative, the senna is used as a febrifuge in splenic enlargements, jaundice, tumors, bronchitis and leprosy [7].

C. angustifolia is exploited heavily from wild conditions by pharmaceutical companies and local tribes for medicinal purposes. Conventionally, it is propagated by seeds. However, low germination percentage and poor viability restricts its propagation on large scale. To cope with the heavy demand, large scale cultivation of the plant is essential, however, high seed mortality and susceptibility of the crop to frost [4] creates a hurdle which cannot be cleared with the conventional methods alone. Tissue culture technology has been successfully utilized in overcoming such problems [27]. Synthetic seed technology is a potential tool for a more efficient and cost effective rapid *in vitro* propagation system. This technology has developed considerably in recent years for *ex situ* conservation of the germplasm of elite and important medicinal plant species. The technology offers excellent scope for propagation of rare hybrids, elite genotypes and genetically engineered plants for which the seeds are either very expensive or are not available. During the last few years, considerable efforts have been made for *in vitro* regeneration of this medicinally valuable species from different explants [2–4, 33–34, 36–38]. The *in vitro* growth conditions can result in the formation of plantlets of abnormal morphology, anatomy and physiology which resulted in high percentage of plantlets death because of sudden changes after their *ex vitro* transfer [11, 17, 28]. The physiological status of *in vitro* grown plantlets during acclimatization is an important factor determining success rates [39] and is a crucial step for many species, requiring time and expensive installation that restrict the commercial application of the micropropagation processes. Sudden changes in environmental conditions during acclimatization of tissue culture raised plants generate stress through the formation of reactive oxygen species (ROS) [9, 29]. These include superoxide radical ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), which cause tissue injury [18]. To combat the danger posed by the presence of ROS, plant cells have evolved defense antioxidant mechanism. To scavenge these ROS, different mechanisms, both enzymatic and non enzymatic are present in plants [20]. Among the enzymatics, superoxide dismutase SOD and catalase CAT are efficient antioxidant enzymes [30] and their joint action prevents cellular damages caused by O_2, H_2O_2 and OH^-. Changes in CAT and SOD activity in response to adverse environmental conditions have been reported by Sgherri and Navari-Izzo [31].

To our best knowledge, no report is available on the development of synthetic seed system for clonal propagation of *C. angustifolia* using nodal segment. However, to date, there have not been any reports either published on physiological and biochemical studies of this species. The present investigation reports on the optimized parameters for the production and conservation of synthetic seeds to study their conversion storage under *in vitro* conditions and to optimize the various physiological and antioxidative enzymes activity during acclimatization of micropropagated plants.

MATERIALS AND METHODS

Explant source

Nodal segments (1–2 cm) collected from 2-year-old mature plant of *C. angustifolia* growing in the botanical garden of the King Saud University, Riyadh were used to initiate *in vitro* cultures.

Surface sterilization

Nodal explants were washed thoroughly under running tap water for at least 30 min, to remove adherent particles, treated with 5% (v/v) labolene™ (Qualigens, Mumbai, India) for 20 min and finally rinsed with sterilized distilled water. The explants were then surface sterilized with freshly prepared 0.1% (w/v) $HgCl_2$ (Qualigens, Mumbai, India) solution for 5 min followed by five rinses with sterile distilled water to remove any traces of sterilant.

Encapsulation

Sodium alginate (Qualigens, Mumbai, India) at different concentrations (2, 3, 4 and 5%) (w/v) was added to Murashige and Skoog [26] liquid medium. For complexation 25, 50, 75, 100 and 125 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) solution was prepared using MS liquid medium. The pH of the gel matrix and the complexing agent was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. Encapsulation was accomplished by mixing the nodal segments with sodium alginate solution and with a pipette by droping them into $CaCl_2 \cdot 2H_2O$ solution. The droplets containing the explants were held for at least 20 min, to achieve polymerization of the sodium alginate. The calcium alginate beads containing the nodal segments were retrieved from the solution and rinsed twice with autoclaved distilled water to remove the traces of $CaCl_2 \cdot 2H_2O$ and thereafter cultured on different medium.

Growth media and culture conditions

The encapsulated nodal segments were planted onto Petri dishes containing Murashige and Skoog (MS), half-strength MS and half-strength MS medium supplemented with different concentrations of thidiazuron (TDZ) (0.5, 2.5, 5.0, 7.5 and 10.0 μM) and an auxin indole-3- acetic acid (IAA) (1.0 μM). After sprouting of shoots, the encapsulated nodal segments were transferred to 100 ml culture flask containing the same medium as the one on which they developed shoots. The medium was supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar and pH was adjusted

to 5.8 prior to autoclaving at 121 °C for 20 min. Cultures were maintained at 24 ± 2 °C under 16/8 h light : dark conditions with a photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

Low temperature storage

A set of encapsulated nodal segments were transferred to Petri dishes containing water and agar medium and stored in a refrigerator at 4 °C. Seven different low temperature exposure times (0, 1, 2, 3, 4, 5, and 6 weeks) were evaluated for regeneration. After each storage period, encapsulated nodal segment were cultured on half-strength MS medium supplemented with 5.0 μM TDZ and 1.0 μM IAA for conversion into plantlets. The percentage of encapsulated nodal segments forming shoots and roots were recorded after 8 weeks of culture to regeneration medium.

Rooting and acclimatization

For rooting, the *in vitro*-regenerated shoots (3–4 cm) were harvested from proliferating cultures and transferred to 1/2 MS medium amended with IBA (1.0 μM). After rooting, rooted shoots were washed gently under running tap water to remove the nutrient medium and subsequently transferred to plastic cups containing sterile soilrite (Keltech Energies Ltd., Bangalore, India). Potted plantlets were covered with transparent polythene bags to ensure high humidity and watered every 3 days with half-strength MS solution for 2 weeks. After 4 weeks, acclimatized plants were transferred to pots containing normal garden soil and maintained in a greenhouse under normal day-length conditions.

Physiological and biochemical studies

Plant growth was recorded by total shoot and root length, shoot, root fresh and dry mass and number of leaves per plant determined after 2 months of transplanting of *in vitro*-propagated plants and compared with control plants of same age.

Relative water content (RWC) of micropropagated plants were determined after 2 months of transplanting and compared with control plants of same age by using the formula

$$\text{RWC}\ (\%) = (\text{FM} - \text{DM}) \times 100/(\text{SM}-\text{DM})$$

where FM = fresh mass, DM = dry mass and SM = saturated mass.

A set of tissue culture raised healthy plantlets were transplanted in sterile soilrite and placed in culture room at 24 ± 2 °C and 16/8 h photoperiod at 55–60% relative humidity under controlled conditions. Leaf samples were taken at transplantation day

(0), and after 7, 14, 21 and 28 d and stored in liquid nitrogen for physiological and biochemical studies.

P_N was measured on fully expanded leaves using portable Infra Red Gas Analyzer (IRGA, LI-COR 6400, Lincoln, USA) on the basis of net exchange of CO_2 between leaf and atmosphere by enclosing the leaf in the leaf chamber, and monitoring the rate at which the CO_2 concentration changed over a short time intervals. The net photosynthetic rate was expressed as μmol CO_2 m^{-2} s^{-1}.

The chlorophylls (chl) a and b and carotenoid contents were estimated by the method of Mckinney [23] and McLachlan and Zalik [24], respectively.

Super oxide dismutase (SOD)

SOD (superoxide: superoxide oxidoreductase, EC 1.15.1.1) activity was measured by the method of Dhinsa et al. [12] with slight modifications in concentrations. SOD activity in the supernatant was assayed by its ability to inhibit the photochemical reduction. The assay mixture consisting of 1.5 ml reaction buffer, 0.2 ml of methionine, 0.1 ml enzyme extract with equal amount of 1 M Na_2CO_3, 2.25 mM NBT solution, 3 mM EDTA, 60 μM riboflavin and 1.0 ml of DDW was taken in test tubes which were incubated under the light of 15 W fluorescent lamp for 10 min at 25/28 °C. Blank A containing all the above substances of reaction mixture along with the enzyme extract, was placed in the dark. Blank B containing all the above substances of reaction mixture except enzyme was placed in light along with the sample. The reaction was terminated by switching off the light, and the tubes were covered with a black cloth. The non-irradiated reaction mixture containing enzyme extract did not develop light blue colour. Absorbance of samples along with blank B was read at 560 nm against the blank A. The difference of %reduction in the colour between blank B and sample was then calculated. 50% reduction in colour was considered as one unit of enzyme activity and the activity was expressed in Enzyme Units (EU) mg^{-1} protein.

Catalase (CAT)

Catalase (H_2O_2:H_2O_2 oxidoreductase: EC 1.11.1.6) activity in the leaves of regenerated plantlets was determined by the method of Aebi [1] with slight modifications in concentrations. Reaction mixture containing 0.5 M Potassium phosphate buffer, 3 mM EDTA, 0.1 ml enzyme extract, and 3 mM H_2O_2. The reaction was allowed to run for 5 min. CAT activity was determined by monitoring the disappearance of H_2O_2 by measuring a decrease in absorbance at 240 nm. CAT activity was calculated by using extinction coefficient (Σ) 0.036 mM^{-1} cm^{-1} and expressed in enzyme units (EU) mg^{-1} protein min^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol of H_2O_2 per min at 25 °C.

Protein content

The total soluble protein content of the leaves of regenerated plants was estimated following the method of Bradford [8] using Bovine Serum Albumin (BSA, Sigma, USA) as standard.

Statistical analysis

All the experiments were conducted with a minimum of 20 replicates per treatment. The experiments were repeated three times. The data was analyzed statistically using SPSS Ver. 14 (SPSS Inc., Chicago, USA). The significance of differences among means were carried out using Duncan's multiple range test at $P = 0.05$. The results are expressed as a means ± SE of three experiments.

RESULTS AND DISCUSSION

Synthetic seed production

The morphology of encapsulated beads in respect to shape, texture and transparency varied with different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$. A 3% sodium alginate produced clear and uniform beads, while higher concentrations resulted in the production of hard beads and showed considerable delay in germination. On the contrary, sodium alginate concentration below 3% was also not suitable because beads were fragile and difficult to handle (Table 1). Of the various concentrations of $CaCl_2 \cdot 2H_2O$ tested, 100 mM was found to be optimum for the production of uniform synthetic seeds with desired texture. Lower concentration of $CaCl_2 \cdot 2H_2O$ not only prolonged the ion exchange (polymerization) duration but also resulted in the formation of fragile calcium alginate beads that were difficult to handle (Table 2).

In vitro response of the encapsulated nodal segments inoculated in different concentration of TDZ and IAA is summarized in Table 3. Nodal segments encapsulated in 3% sodium-alginate and 100 mM $CaCl_2 \cdot 2H_2O$ exhibited re-growth within 2 weeks of incubation on MS, 1/2 MS, and 1/2 MS medium augmented with various concentrations and combinations of TDZ and IAA. Only few shoots emerged from encapsulated nodal segments in full and half strength MS basal medium. Half strength MS medium supplemented with TDZ (5.0 µM) and IAA (1.0 µM) gave the maximum frequency (78%) of conversion of encapsulated nodal segments into plantlets with maximum (10.9 ± 0.78) shoots after 8 weeks of culture. Though roots were also formed in this medium but these roots were thin and not sufficient to handle. Our results showed consistency with the reports [22, 35]. Well-developed roots were obtained by transferring the individual shoots to rooting media, i.e. 1/2 MS medium augmented with 1.0 µM IBA. This is in contrast to the reports in *Tylophora indica*

Table 1
Effect of sodium alginate concentration on conversion of encapsulated nodal segments of *C. angustifolia* after 8 weeks of culture on half strength MS medium

Sodium alginate (% w/v)	% conversion response into plantlets
2	Fragile beads
3	39
4	32
5	29

Different concentration of sodium alginate added to MS medium.

Table 2
Effect of different concentration of $CaCl_2 \cdot 2H_2O$ in the presence of optimal sodium alginate concentration on conversion of encapsulated nodal segments of *C. angustifolia* after 8 weeks of culture on half strength MS medium

Sodium alginate (% w/v)	Calcium chloride (mM)	% conversion response into plantlets
3	25	Fragile beads
3	50	Fragile beads
3	75	35
3	100	39
3	125	33

Different concentrations of $CaCl_2 \cdot 2H_2O$ added to MS medium.

Table 3
Effect of different MS strength and concentrations of TDZ along with IAA on conversion of encapsulated nodal segments of *C. angustifolia* after 8 weeks of culture

Treatments (µM)	% conversion response into plantlets	Mean number of shoots
MS	22	1.4 ± 0.13^{f}
½ MS	32	3.2 ± 0.20^{e}
½ MS + TDZ (0.5) + IAA (1.0)	42	5.0 ± 0.34^{cd}
½ MS + TDZ (2.5) + IAA (1.0)	57	8.5 ± 0.45^{b}
½ MS + TDZ (5.0) + IAA (1.0)	78	10.9 ± 0.78^{a}
½ MS + TDZ (7.5) + IAA (1.0)	64	8.2 ± 0.43^{ab}
½ MS + TDZ (10.0) + IAA (1.0)	50	6.0 ± 0.19^{c}

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

and in *Vitex negundo* where shoot and root formation took place in the same media [5, 15]. Higher concentration of TDZ decreased the conversion frequency of encapsulated beads into plantlets.

Storage duration

The regeneration frequency was clearly influenced by storage time. Table 4 shows the effect of different storage duration of encapsulated nodal segment at 4 °C for 0, 1, 2, 3, 4, 5 and 6 weeks. The synthetic seed stored at 4 °C for a period of 4 weeks resulted in maximum conversion frequency (93%) with an induction of 14.8 ± 0.51 shoots after 8 weeks of culture under *in vitro* conditions on half strength MS medium supplemented with TDZ (5.0 µM) and IAA (1.0 µM). With an increase in storage time to more than 4 weeks, the conversion frequency decreased considerably (Table 4). Decline in conversion response could be attributed to inhibition of tissue respiration by the alginate matrix, or a loss of moisture due to partial desiccation during storage as reported earlier [10, 14, 15].

Table 4
Effect of different duration of storage (4 °C) on *in vitro* regeneration from alginate – encapsulated nodal segment of *C. angustifolia* after 8 weeks of culture to half strength MS medium supplemented with TDZ (5.0 µM) + IAA (1.0 µM)

Storage duration (weeks)	% conversion response into plantlets	Mean number of shoots
0	78	10.9 ± 0.78^{c}
1	81	11.5 ± 0.67^{bc}
2	85	12.9 ± 0.53^{b}
3	89	13.5 ± 0.42^{a}
4	93	14.8 ± 0.51^{a}
5	86	11.2 ± 0.12^{c}
6	80	9.6 ± 0.28^{d}

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

Rooted plantlets with four to five fully expanded leaves, retrieved from encapsulated nodal segments were transferred to plastic pots filled with sterile soilrite and covered with transparent polythene bags inside the culture room for 2 weeks. After one month, these were transferred in earthen pots containing garden soil and maintained in greenhouse where they grew normally.

Table 5
Comparison of some morphological features between micropropagated plants and seedlings of *C. angustifolia*

Parameters	Micropropagated plants	Seedlings
Root length (cm)	5.84 ± 0.21^{d}	6.96 ± 0.34^{d}
Shoot length (cm)	12.31 ± 0.48^{c}	15.81 ± 0.67^{c}
Root fresh mass (g)	0.12 ± 0.11^{gh}	0.16 ± 0.13^{h}
Shoot fresh mass (g)	1.36 ± 0.10^{e}	1.75 ± 0.14^{e}
Root dry mass (g)	0.05 ± 0.03^{h}	0.09 ± 0.02^{i}
Shoot dry mass (g)	0.45 ± 0.01^{f}	0.60 ± 0.04^{f}
Leaf dry mass g plant^{-1}	0.25 ± 0.02^{g}	0.32 ± 0.01^{g}
Leaf number/plant	20.12 ± 0.86^{b}	24.01 ± 0.95^{b}
Relative water content (%)	92.01 ± 1.63^{a}	67.53 ± 1.89^{a}

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

Growth performance

Comparative data on some morphological features and relative water content of *in vitro* propagated plants and seedlings is summarized in Table 5. Slight reduction in the morphology of *in vitro*-propagated plants in terms of shoot, root length and dry and fresh mass and leaf number were observed in comparison to control plants after considerable period of establishment in the greenhouse. The plantlets dehydrated quickly and experienced water stress as soon as they were transferred from the culture tubes to field conditions. RWC was decreased due to wiltness of plantlets under low humidity. However, regenerants slowly recovered from water stress with the passage of time and obtained higher relative water content than *in vivo* seedlings. Similar results have also been reported in pepper plantlets [13, 32].

Photosynthetic pigments and net photosynthetic rate

Photosynthetic parameters including chl a, chl b, carotenoid and P_N were evaluated in regenerated plantlets of *C. angustifolia* at 0 (control), 7, 14, 21 and 28 d of acclimatization (Fig. 1). Content of chl a and chl b showed an increasing trend over control plantlets throughout the study while carotenoid content first dropped from 0–14 d due to the sudden changes in environmental conditions and thereafter showed an increasing trend from 14–28 d of acclimatization. An increase in carotenoid level is reported to be involved in protecting the photosynthetic machinery form photo oxidative damage [6, 16]. P_N decreased from 0 to 7 d and thereafter showed an increasing trend with the formation of new leaves from 7–28 d of acclimatization. Photosynthetically active

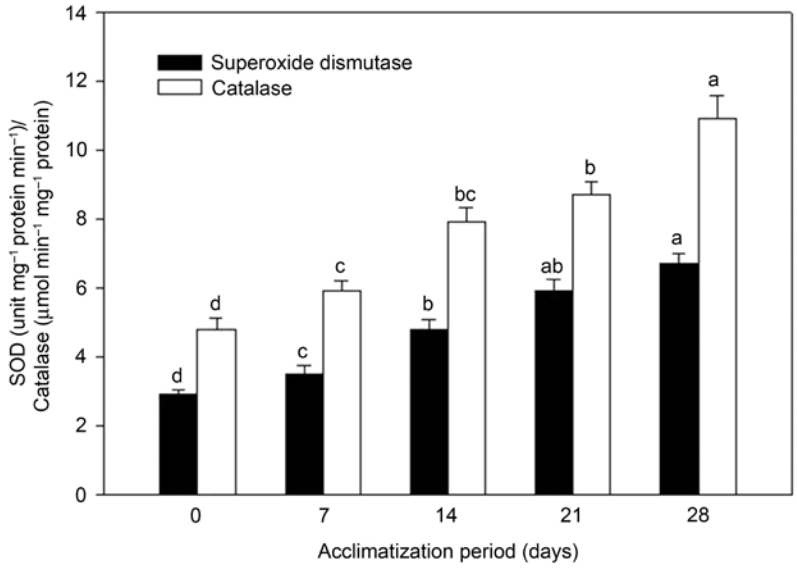

Fig. 1. Changes in superoxide dismutase and catalase activity of micropropagated plantlets of *C. angustifolia* during different days of acclimatization. Bars represents means ± SE. Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test

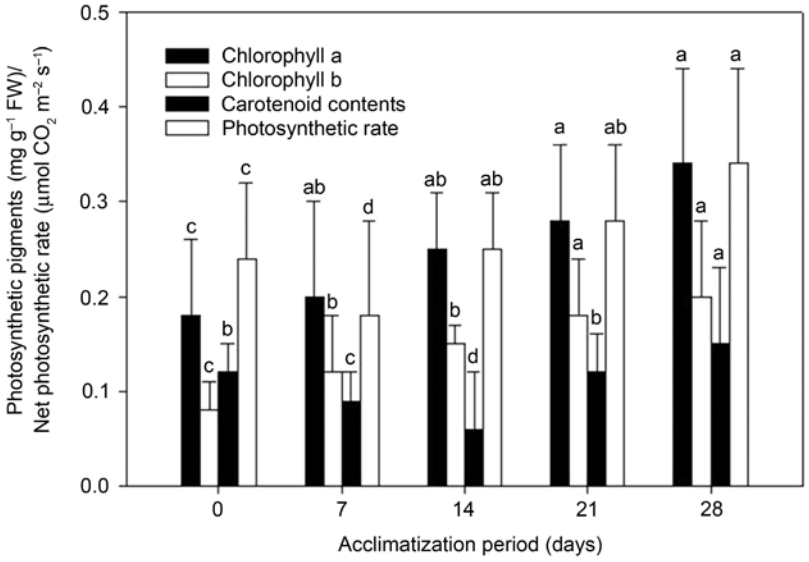

Fig. 2. Changes in photosynthetic pigments and net photosynthetic rate (P_N) of micropropagated plantlets of *C. angustifolia* during different days of acclimatization. Bars represents means ± SE. Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test

in vitro leaves have also been observed in *Spathiphyllum floribundum* plantlets [40]. After one week, P_N showed increasing trend and was found to be associated with the formation of new leaves [13].

Antioxidative enzyme activities

Acclimatized plantlets of *C. angustifolia* showed a time dependant increase in both SOD and CAT activity. SOD activity dropped first from 0–14 d and thereafter showed an increasing trend and reached maximum at 28th d of acclimatization (Fig. 2). Higher SOD activity was therefore associated with better protection against stress induced oxidative injury. Similar results were reported by Van Huylenbroeck et al. [40] and Chai et al. [9]. However, CAT activity increased significantly during 0–28 d of acclimatization (Fig. 2). Positive changes in CAT activity have often been observed in relation to mild water stress [21, 25, 35]. Thus increment in SOD and CAT activity can be considered as the protection against ROS possibly generated during acclimatization.

CONCLUSIONS

In conclusion, the present study describes a simple, reproducible and efficient protocol for synthetic seed production. This protocol may facilitate conservation, propagation and mass multiplication. Cold storage of encapsulated nodal segments offers possibility for germplasm conservation and exchange between laboratories. Changes observed in physiological and biochemical parameters during acclimatization has helped to understand better adaptation process of micropropagated plants to *ex vitro* conditions.

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