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Lipid peroxidation, H_2O_2 content, and antioxidants during acclimatization of *Abrus precatorius* to *ex vitro* conditions

S. PERVEEN[1], M. ANIS[1,2]*, and I.M. AREF[2]

Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh 202 002, India[1]
Department of Plant Production, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia[2]

Abstract

An efficient, rapid, and reproducible plant regeneration protocol was successfully developed for *Abrus precatorius* L. using mature nodal explants excised from a 5-year-old field grown plant. The highest shoot regeneration frequency (87 %) with maximum number of multiple shoots (15.0) and shoot length (4.8 cm) were recorded on Murashige and Skoog (MS) medium amended with 2.5 μM thidiazuron, 120 mg dm^{-3} polyvinylpyrrolidone, and 0.5 μM α-naphthalene acetic acid. The best treatment for maximum root (4.0) induction was half strength MS medium supplemented with 1.5 μM indole-3-butyric acid. The *in vitro* plantlets with well-developed shoots and roots were successfully transferred into plastic cups with *Soilrite* and acclimatized in a culture room under photon flux density (PFD) of 150 μmol m^{-2} s^{-1}, thereafter transferred to a greenhouse with PFD of 300 μmol m^{-2} s^{-1}, and finally to a field with 70 % survival rate. During the acclimatization period (0 - 49 d), leaf chlorophyll and carotenoid content increased whereas malondialdehyde and H_2O_2 content decreased probably due to increasing activities of antioxidant enzymes (catalase, superoxide dismutase, glutathione reductase, and ascorbate peroxidase). Our work suggests that micropropagated plants developed an antioxidant enzymatic protective system to avoid oxidative stress during establishment under *ex vitro* environment.

Additional key words: ascorbate peroxidase, catalase, carotenoids, chlorophylls, glutathione reductase, malondialdehyde, micropropagation, reactive oxygen species, superoxide dismutase.

Introduction

Abrus precatorius (*Fabaceae*), commonly known as Crab's eye (Ratti-Hindi), is an indigenous woody climber found throughout India, even at altitude up to 1 200 m on the outer Himalayas and naturalized in all tropical countries (Dwivedi 2004). The plant is of great medicinal importance (Khare 2004, Hassan 2009) and so its populations are depleting at an alarming pace. Unfortunately, conventional propagation through seeds is rather slow and not very efficient. Hence, there is an obvious need to develop an efficient regeneration system for its effective conservation and rapid multiplication. Micropropagation methods can often be the most efficient way to clonally propagate a species without depleting wild resources and can be an important part of an integrated program for the conservation of medicinal and aromatic plants worldwide (Reed *et al*. 2011).

The environment inside the culture vessels normally used in micropropagation is characterized by high relative humidity, low photon flux density (PFD), and poor exchange of gases between the internal atmosphere and its surrounding environment which may cause physiological disorders. During the transfer to *ex vitro* conditions, *in vitro* plants are exposed to higher PFD and lower air humidity which can induce photoinhibition and water stress accompanied by production of reactive oxygen species (ROS). To combat the oxidative damage,

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Abbreviations: APX - ascorbate peroxidase; BA - 6-benzyladenine; Chl - chlorophyll; CAT - catalase; EDTA - ethylenediaminetetraacetic acid; GR - glutathione reductase; IBA - indole-3-butyric acid; KN - kinetin; KI - potassium iodide; MS - Murashige and Skoog medium; MDA - malondialdehyde; NAA - α-naphthaleneacetic acid; NBT - nitroblue tetrazolium; PFD - photon flux density; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid; TDZ - thidiazuron.
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* Corresponding author; e-mail: anism1@rediffmail.com

plants have the antioxidative enzymes comprising superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2). Therefore, it is very important to study the stresses and their control during the acclimatization of *in vitro* plants to *ex vitro* conditions.

There are several reports (Carvalho *et al*. 2001, Kadlecek *et al*. 2001, Carvalho and Amancio 2002, Faisal and Anis 2010), where the effect of various *ex vitro* conditions on growth parameters and stresses during the acclimatization of *in vitro* plants have been studied. Among environmental factors, suitable irradiance during *in vitro* as well as *ex vitro* conditions is important for the attainment of full autotrophy but avoid the risk of photoinhibition (Amancio *et al*. 1999, Pospíšilová *et al*. 1999, 2007, Van Huylenbroeck *et al*. 2000, Carvalho and Amancio 2002).

There are only few reports available on the micropropagation of *A. precatorius* using different explants (Biswas *et al*. 2007, Hassan 2009) but none used mature nodal explants. The aim of our experiments was to acclimatize *in vitro* raised plantlets firstly in the culture room under irradiance three times higher than during the *in vitro* growth and afterwards in the greenhouse under six times higher irradiance. Besides, during acclimatization, lipid peroxidation, content of H_2O_2 and photosynthetic pigments, and antioxidative enzyme activities were studied. The results could be further applied to large scale *Abrus* production.

Materials and methods

Healthy shoots of *Abrus precatorius* L. were collected from a 5-year-old plant growing in Botanical Garden of the University, Aligarh, India. The nodal segments (1.0 - 1.5 cm) were washed thoroughly in running tap water for 30 min, immersed in 1 % (m/v) *Bavistin* solution, and later treated with 5 % (v/v) *Teepol* solution for 20 min. After thorough washing in running tap water, the explants were surface sterilized with 0.1 % (m/v) $HgCl_2$ for 3 min followed by thorough washing with sterile distilled water in a laminar air flow before implantation. Murashige and Skoog (1962; MS) basal medium supplemented with 3 % (m/v) sucrose and 0.8 % (m/v) agar was used in all the experiments. The medium was adjusted to pH 5.8 and sterilized by autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 20 min. Cultures were incubated at 25 ± 2 °C, a 16-h photoperiod, photon flux density (PFD) of 50 μmol m^{-2} s^{-1} (cool fluorescent tubes), and relative humidity of 55 - 60 %. Nodal explants were cultured on the MS medium fortified with various concentrations of 6-benzyladenine (BA), kinetin (KN), or thidiazuron (TDZ) either individually or in combination with an adsorbent polyvinylpyrrolidone (PVP) for bud break. Afterwards, the optimum concentration of each cytokinin and 120 mg dm^{-3} PVP were used in a combination with α-naphthaleneacetic acid (NAA) at different concentrations for multiplication and proliferation of shoots. Subculturing was performed on the same fresh media after every 4 weeks. Data on the frequency of explants producing shoots, shoot number per explant, and shoot length were recorded after 8 weeks of incubation.

Shoots with fully expanded leaves were excised from the shoot clusters and transferred to different strength of MS medium (MS, 1/2 MS, 1/3 MS, and 1/4 MS) singly for root induction. Afterwards, the optimized half strength MS medium was tried in combination with either indole-3-butyric acid (IBA) or NAA at various concentrations for better rhizogenesis. Observations on rooting percentage, number of roots per shoot, and root length were recorded after 4 weeks.

Well rooted plantlets were allowed to grow on rooting medium for further 2 weeks and then they were removed from the culture vessels, washed gently with running tap water, transferred to plastic cups containing *Soilrite*, and grown in the culture room under a 16-h photoperiod, PFD of 150 μmol m^{-2} s^{-1}, temperature of 25 ± 2 °C, and high air humidity (plantlets were covered with a transparent polythene membrane) for 28 d. They were irrigated with 1/4 inorganic salts of MS medium on every alternate day for 21 d, followed by tap water. Poly bags were opened gradually and removed after 21 d to acclimatize plantlets under the culture room conditions. After 28 d, acclimatized plants were transferred to pots containing normal garden soil and grown in the greenhouse at temperature of 28 ± 2 °C, relative humidity of 60 - 80 %, a 16-h photoperiod, PFD of 300 μmol m^{-2} s^{-1}, and irrigated with tap water on every alternate day for 49 d. Afterwards, the plants were transferred to field. Leaves from *in vitro* regenerated plantlets, from those subjected to different acclimatization periods in the culture room, and from those grown in the greenhouse were used for assessment of photosynthetic pigments, MDA and H_2O_2 content, and antioxidant enzymes.

The chlorophylls (Chl) *a* and *b* and carotenoids (Car) were extracted in 80 % (v/v) acetone using the method of Mackinney (1941). Absorbances (at 663 and 645 nm for Chl and at 480 and 510 nm for Car) were estimated on UV-VIS spectrophotometer (*UV-1700 Pharma Spec*, *Shimadzu*, Kyoto, Japan). Lipid peroxidation was estimated according to malondialdhyde (MDA) production using the thiobarbituric acid (TBA) method (Heath and Packer 1968) by using a coefficient of absorbance of 155 mM^{-1} cm^{-1}. H_2O_2 content was measured after reaction with potassium iodide. Leaf tissue (0.5 g) was homogenized in 0.1 % (m/v) trichloroacetic acid (TCA) and centrifuged at 14 000 g and the homogenate was used for the determination of H_2O_2 content by the method of Alexieva *et al*. (2001). The supernatant (0.5 cm^3) was mixed with 0.5 cm^3 of 100 mM K-phosphate buffer (pH 7.8), and 2 cm^3 of reagent (1 M KI in fresh

double-distilled water. After 1 h in darkness, the absorbance was measured at 390 nm. The blank probe consisted of 0.1 % TCA in the absence of the leaf extract.

To determine activities of antioxidant enzyme, 0.5 g of fresh leaf tissue was homogenized in 2.0 cm^3 of extraction buffer (pH 7.5) containing 1 % (m/v) PVP, 1 % (v/v) *Triton X-100*, and 0.11 g of ethylenediamine tetraacetic acid (EDTA) using a pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15 000 *g* for 20 min. The supernatant was used for enzyme assays. Extraction was carried out in dark at 4 °C. SOD activity was assayed (Dhindsa *et al*. 1981) by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture consisting of 0.5 M phosphate buffer (pH 7.5), 0.1 mM EDTA, 13 mM methionine, 63 mM NBT, 1.3 mM riboflavin, and 0.1 cm^3 of the enzyme extract in test tubes was incubated under 15 W fluorescent lamp (*Philips*, Kolkata, India) at 25 ºC for 15 min. Absorbance was measured at 560 nm. CAT 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 (1984). The assay mixture contained 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 activity was measured using the protocol of Rao (1992) based on glutathione-dependent oxidation of 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 (GSSG). The enzyme extract (0.1 cm^3) was added to begin the reaction and the reaction was allowed to run at 25 °C for 5 min. APX activity was measured by monitoring the decrease in absorbance at 290 nm within 1 min according to Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA, and 0.1 cm^3 of the enzyme extract. The protein content in the enzymatic extracts was determined following the Bradford (1976) assay using bovine serum albumin as a standard.

The experiments were laid out according to a completely randomized design. Each treatment consisted of 10 replicates and all the experiments were repeated thrice. The data were analyzed statistically using one way *ANOVA* and pair wise means were compared using Tukey's multiple range test at $P = 0.05$.

Results

The nodal explants did not show any sign of bud break when cultured on a plant growth regulator-free MS medium and failed to regenerate shoots even after 4 weeks of culture. The explants cultured on 2.5 µM TDZ containing medium showed their first response by initial enlargement of the existing axillary bud followed by bud

Table 1. Effect of different concentrations of cytokinins on multiple shoot induction from nodal segments after 8 weeks of culture. Means ± SE, $n = 3$. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

TDZ [µM]	BA [µM]	KN [µM]	Response [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
0.5			60	4.10 ± 0.51^{ab}	1.83 ± 0.03^{ab}
2.5			70	6.33 ± 0.88^{a}	2.03 ± 0.08^{a}
5.0			40	4.30 ± 1.42^{ab}	1.44 ± 0.07^{cd}
7.5			30	1.66 ± 0.66^{b}	1.13 ± 0.06^{de}
	2.5		25	0.66 ± 0.33^{b}	0.32 ± 0.05^{ghi}
	5.0		35	1.33 ± 0.88^{b}	0.53 ± 0.09^{fgh}
	7.5		40	2.33 ± 0.78^{ab}	0.86 ± 0.08^{efg}
	10.0		60	4.00 ± 1.15^{ab}	1.56 ± 0.11^{bc}
	12.5		32	2.80 ± 1.35^{ab}	1.00 ± 0.07^{de}
		2.5	15	0.33 ± 0.10^{b}	0.13 ± 0.12^{i}
		5.0	22	0.68 ± 0.33^{b}	0.26 ± 0.11^{hi}
		7.5	36	1.33 ± 0.38^{b}	0.43 ± 0.13^{ghi}
		10.0	51	2.66 ± 0.21^{ab}	0.93 ± 0.03^{def}
		12.5	30	1.80 ± 0.57^{b}	0.60 ± 0.14^{fgh}

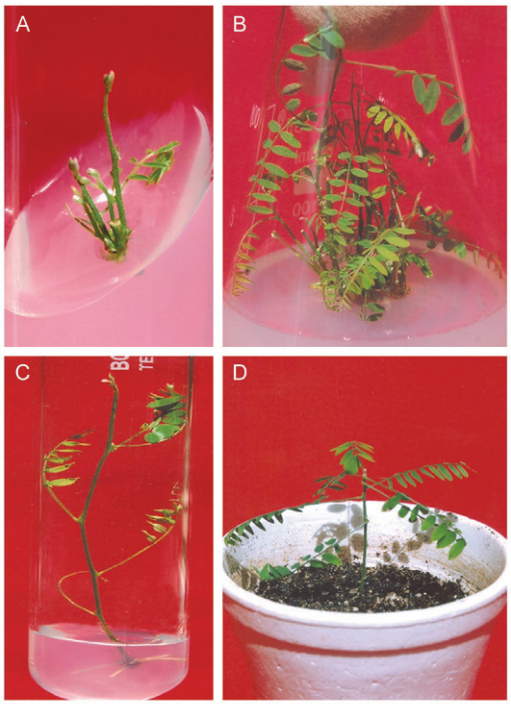

Fig. 1. *In vitro* regeneration and plantlet establishment of *A. precatorius*. *A* - Proliferation of multiple shoots on MS + 2.5 µM TDZ. *B* - Multiplication of shoots achieved from nodal explants cultured on MS + 2.5 µM TDZ + 0.5 µM NAA + 120 mg dm^{-3} PVP. *C* - Rooted plantlet after 30 d on rooting medium. *D* - An acclimatized plant in *Soilrite* after 28 d in the culture room.

Table 2. Effect of various concentrations of PVP with optimal concentration of TDZ (2.5 μM), BA (10 μM), and KN (10 μM) on shoot induction and multiplication after 8 weeks of culture. Means ± SE, n = 3. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

PGR	PVP [mg dm-3]	Response [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
TDZ	20	70	5.40 ± 0.23^{de}	2.20 ± 0.57^{ef}
TDZ	50	75	6.00 ± 0.17^{bcd}	2.66 ± 0.88^{bcd}
TDZ	100	78	6.80 ± 0.20^{ab}	2.96 ± 0.82^{b}
TDZ	120	81	7.40 ± 0.15^{a}	3.76 ± 0.14^{a}
TDZ	150	65	6.50 ± 0.17^{bc}	2.50 ± 0.15^{de}
BA	20	68	3.80 ± 0.12^{hi}	1.45 ± 0.05^{h}
BA	50	71	4.50 ± 0.11^{fgh}	1.83 ± 0.09^{g}
BA	100	74	5.00 ± 0.18^{efg}	2.61 ± 0.11^{cd}
BA	120	78	6.50 ± 0.13^{bc}	2.93 ± 0.12^{bc}
BA	150	66	5.20 ± 0.14^{def}	2.67 ± 0.10^{bcd}
KN	20	55	2.20 ± 0.11^{bc}	1.16 ± 0.16^{h}
KN	50	59	3.00 ± 0.21^{ij}	1.30 ± 0.04^{h}
KN	100	64	4.20 ± 0.13^{gh}	2.03 ± 0.14^{fg}
KN	120	67	5.70 ± 0.22^{cde}	2.60 ± 0.05^{cd}
KN	150	56	3.80 ± 0.18^{hi}	1.83 ± 0.08^{g}

break within 20 - 25 d and multiple shoots were induced within 30 d. After 8 weeks, the same medium proved to be the most effective among all cytokinins tested as it induced maximum regeneration percentage (70 %) with six shoots reaching length 2 cm (Fig. 1*A*). MS medium containing 10 μM BA or 10 μM KN exhibited 60 and 51 % regeneration frequency with the formation of only 4 and 2.6 shoots, respectively (Table 1).

During initial days of culture, exudation of phenols in the medium occurred and reappeared even after several subculturing and caused necrosis of shoots after 8 weeks of incubation. Therefore, after standardizing optimal hormonal concentration, the medium was supplemented with an adsorbent polyvinylpyrrolidone (PVP). Of the various concentrations of PVP tested, 120 mg dm^{-3} was found to be the most effective in the MS medium amended with 2.5 μM TDZ, 10 μM BA, and 10 μM KN (Table 2). This treatment considerably enhanced the production of healthy shoots.

To induce shoot multiplication and proliferation, NAA at different concentrations was added along with the optimal concentrations of each cytokinin and PVP. Maximum shoot formation frequency (84 %) with 15.3 shoots per explant and longest shoot (4.8 cm) was observed on a medium containing 2.5 μM TDZ, 0.5 μM NAA, and 120 mg dm^{-3} PVP after 8 weeks of culture (Fig. 1*B*). The presence of NAA (0.5 μM) with 10 μM BA proved to be the optimum in 79 % of cultures with maximum 10.0 shoots. KN in combination with NAA (0.5 μM) was least effective as it exhibited only 69 % regeneration (Table 3).

Among the various strengths of MS media tested,

Table 3. Effect of various concentrations of NAA with TDZ (2.5 μM), BA (10.0 μM), KN (10.0 μM), and PVP (120 mg dm^{-3}) on shoot multiplication and elongation after 8 weeks of culture. Means ± SE, n = 3. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

PGR	NAA [μM]	Response [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
TDZ	0.1	78	9.00 ± 2.08^{abc}	4.23 ± 0.14^{b}
TDZ	0.5	84	15.30 ± 1.85^{a}	4.80 ± 0.32^{a}
TDZ	1.0	71	11.00 ± 2.30^{ab}	4.00 ± 0.28^{bc}
TDZ	1.5	61	8.66 ± 1.76^{abc}	3.80 ± 0.17^{bc}
TDZ	2.0	50	7.00 ± 1.15^{abc}	2.96 ± 0.21^{de}
BA	0.1	73	6.33 ± 1.82^{abc}	3.00 ± 0.23^{de}
BA	0.5	79	10.00 ± 1.15^{abc}	3.73 ± 0.39^{bc}
BA	1.0	66	5.00 ± 1.00^{c}	$3.40 \pm 0.23c^{d}$
BA	1.5	56	3.33 ± 0.88^{bc}	2.90 ± 0.28^{de}
BA	2.0	48	2.31 ± 0.88^{c}	2.86 ± 0.26^{def}
KN	0.1	65	5.33 ± 0.88^{bc}	2.50 ± 0.18^{efg}
KN	0.5	69	6.00 ± 2.51^{abc}	2.83 ± 0.12^{def}
KN	1.0	60	4.33 ± 1.45^{bc}	2.50 ± 0.23^{efg}
KN	1.5	52	3.66 ± 1.76^{bc}	2.30 ± 0.17^{fg}
KN	2.0	48	2.33 ± 1.45^{c}	1.96 ± 0.26^{g}

Table 4. Effect of MS strength and IBA concentrations on rooting in *in vitro* microshoots after four weeks of culture. Means ± SE, n = 3. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

MS + IBA [μM]	Response [%]	Number of roots [shoot^{-1}]	Root length [cm]
MS	40	0.66 ± 0.23^{b}	0.23 ± 0.88^{cde}
1/2 MS	55	1.33 ± 0.32^{b}	0.35 ± 0.33^{cde}
1/3 MS	32	0.32 ± 0.12^{b}	0.20 ± 0.11^{de}
1/4 MS	10	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}
1/2 MS + IBA (0.5)	38	1.35 ± 0.33^{b}	0.55 ± 0.05^{c}
1/2 MS + IBA (1.0)	49	2.33 ± 0.88^{ab}	1.35 ± 0.20^{b}
1/2 MS + IBA (1.5)	60	4.00 ± 1.15^{a}	2.00 ± 0.17^{a}
1/2 MS + IBA (2.0)	53	1.00 ± 0.00^{b}	1.03 ± 0.14^{b}
1/2 MS + IBA (2.5)	41	0.38 ± 0.31^{b}	0.50 ± 0.11^{cd}
1/2 MS + IBA (0.5)	32	0.69 ± 0.23^{b}	0.35 ± 0.04^{cde}
1/2 MS + IBA (1.0)	40	1.00 ± 0.01^{b}	0.40 ± 0.02^{cde}
1/2 MS + IBA (1.5)	51	1.66 ± 0.34^{ab}	0.56 ± 0.10^{c}
1/2 MS + IBA (2.0)	38	0.82 ± 0.57^{b}	0.35 ± 0.02^{cde}
1/2 MS + IBA (2.5)	30	0.33 ± 0.31^{b}	0.15 ± 0.04^{e}

in vitro rooting was found to be superior on 1/2 MS as compared to full strength with maximum 55 % root formation. To enhance the rooting efficiency, effect of auxins (IBA and NAA) at different concentrations were also tested. The best rooting response with 60 % of root regeneration frequency and maximum of 4 roots with 2.0 cm length was obtained in 1/2 strength MS medium augmented with 1.5 μM IBA after 4 weeks of culture

(Fig. 1*C*). Fast growing thick roots were produced on IBA containing medium whereas the NAA supplemented medium induced thin delicate roots with slow growth and resulted in callus formation which was considered to be undesirable for *ex vitro* establishment.

Rooted plantlets with fully expanded leaves and well-developed roots were transferred to *Soilrite* (Fig. 1*D*) and grown in the culture room conditions for 28 d followed by their establishment in pots containing natural soil and growth in the greenhouse up to 49 d. Due to these two acclimatization periods, about 70 % of the micropropagated plants survived after transfer to the field and did not show any visible defects in morphological and growth characteristics when compared with their respective donor plants.

In vitro raised plantlets of *Abrus precatorius* exhibited a considerable increase in content of Chl (*a* and *b*) and Car throughout the acclimatization period (0 - 49 d). However, there was a reduction in photosynthetic pigments in plantlets at day 7 but on subsequent days, the new leaves appeared and resulted in a significant increase in both Chl (*a* and *b*) and Car content at day 21 compared to day 0. No significant increase was observed at day 28 and 35 as compared to day 21 (Table 5). After 28 d, when plantlets were transferred to the greenhouse, a further increase in Chl *a*, Chl *b*, and Car up to 42 d was observed and subsequently stabilized at 49 d.

Table 5. Changes in content of chlorophylls *a* and *b* and carotenoids in plantlets acclimatized at PFD of 150 µmol m^{-2} s^{-1} (for 28 d) and then 300 µmol m^{-2} s^{-1} (up to 49 d). Means ± SE, n = 3. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

Time [d]	Chl *a* [mg g^{-1}(f.m.)]	Chl *b* [mg g^{-1}(f.m.)]	Car [mg g^{-1}(f.m.)]
0	0.200 ± 0.01^{cd}	0.113 ± 0.01^{de}	0.123 ± 0.08^{b}
7	0.183 ± 0.14^{d}	0.098 ± 0.02^{e}	0.110 ± 0.05^{b}
14	0.250 ± 0.17^{c}	0.160 ± 0.04^{cd}	0.133 ± 0.07^{b}
21	0.345 ± 0.01^{b}	0.200 ± 0.11^{c}	0.160 ± 0.15^{ab}
28	0.360 ± 0.03^{b}	0.210 ± 0.12^{bc}	0.180 ± 0.11^{ab}
35	0.370 ± 0.11^{b}	0.218 ± 0.01^{bc}	0.205 ± 0.12^{ab}
42	0.434 ± 0.08^{a}	0.271 ± 0.13^{ab}	0.223 ± 0.21^{ab}
49	0.452 ± 0.02^{a}	0.290 ± 0.08^{a}	0.250 ± 0.13^{a}

A time dependent variation in the lipid peroxidation and H_2O_2 content were found in the plantlets during the acclimatization period (0 - 28 d in the culture room and 28 - 49 d in the greenhouse). An increased MDA and H_2O_2 content was observed in the plantlets in early days of acclimatization. However, after 7 d, a decreasing trend was observed in MDA and H_2O_2 content up to 49 d (Table 6). Furthermore, acclimatized plantlets exhibited a significant increase in SOD and CAT activities up to 21 d in the culture room and from 28 to 42 d after transfer to the greenhouse. However, there was a non-significant increase in SOD activity between day 21 and 28 and it was reduced at day 49. Besides, the activities of both APX and GR also increased in the plantlets from 0 to 49 d of acclimatization (Table 7).

Table 6. Changes in MDA and H_2O_2 content and SOD activity in plantlets acclimatized at PFD of 150 µmol m^{-2} s^{-1} (for 28 d) and then 300 µmol m^{-2} s^{-1} (up to 49 d). Means ± SE, n = 3. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

Time [d]	MDA [µmol g^{-1}(f.m.)]	H_2O_2 [µmol g^{-1}(f.m.)]	SOD [U mg^{-1}(prot.)]
0	8.80 ± 0.11^{ab}	7.10 ± 0.05^{ab}	0.93 ± 0.09^{e}
7	9.10 ± 0.05^{a}	7.20 ± 0.13^{a}	1.03 ± 0.05^{e}
14	8.60 ± 0.07^{abc}	6.80 ± 0.07^{b}	1.50 ± 0.08^{d}
21	8.30 ± 0.11^{bcd}	6.40 ± 0.21^{c}	1.90 ± 0.14^{c}
28	8.13 ± 0.08^{bcde}	6.30 ± 0.16^{c}	2.00 ± 0.07^{c}
35	8.00 ± 0.15^{cde}	6.27 ± 0.11^{c}	2.40 ± 0.11^{b}
42	7.70 ± 0.17^{de}	5.83 ± 0.03^{d}	3.03 ± 0.03^{a}
49	7.52 ± 0.28^{e}	5.66 ± 0.12^{d}	2.93 ± 0.12^{a}

Table 7. Changes in CAT, APX, and GR activities [mmol mg^{-1}(prot.) min^{-1}] in plantlets acclimatized at PFD of 150 µmol m^{-2} s^{-1} (for 28 d) and then 300 µmol m^{-2} s^{-1} (up to 49 d). Means ± SE, n = 3. Means sharing the same letter within columns are not significantly different ($P \leq 0.05$) according to Tukey's multiple range test.

Time [d]	CAT	APX	GR
0	131.6 ± 6.09^{e}	1.90 ± 0.07^{g}	2.30 ± 0.19^{f}
7	181.3 ± 6.90^{d}	2.66 ± 0.08^{f}	3.50 ± 0.28^{e}
14	270.0 ± 5.77^{c}	3.36 ± 0.19^{e}	4.80 ± 0.17^{d}
21	310.0 ± 5.91^{bc}	3.80 ± 0.05^{d}	5.53 ± 0.13^{d}
28	330.0 ± 7.30^{b}	4.06 ± 0.06^{cd}	5.76 ± 0.14^{cd}
35	340.0 ± 7.70^{b}	4.33 ± 0.08^{bc}	6.70 ± 0.20^{bc}
42	390.0 ± 6.10^{a}	4.60 ± 0.17^{ab}	7.53 ± 0.29^{ab}
49	398.0 ± 5.61^{a}	4.76 ± 0.09^{a}	8.00 ± 0.30^{a}

Discussion

Different cytokinins generally express different activities in affecting axillary shoot formation *in vitro*. In the present study, TDZ was found to be the more efficient than BA and KN for attaining maximum shoot induction from mature nodal segments. TDZ has been used most frequently in recent times for the efficient shoot induction in many plants as it is less susceptible to the plant degrading enzymes and is active at lower concentrations

than the aminopurine cytokinins (Ahmad *et al*. 2006, Jahan and Anis 2009). TDZ shows high cytokinin activity in promoting growth of cytokinin-dependent cultures (Mok *et al*. 2005) and stimulates conversion of cytokinin nucleotides to more biologically active nucleotides by stimulating accumulation of endogenous purine cytokinins (Giri and Tamta 2011). Similar to our observations, the role of TDZ in shoot formation from nodal explants has been reported in many plant species such as *Arnebia euchroma* (Jiang *et al*. 2005), *Rauvolfia tetraphylla* (Faisal *et al*. 2005), *Bacopa monnieri* (Ceasar *et al*. 2010), and *Hedychium spicatum* (Giri and Tamta 2011).

The problem of exudation of phenols into medium was overcome by adding PVP. The combination of TDZ and PVP was found to be most suitable for obtaining good proliferation and quality of shoots. The combination of cytokinin and PVP have been reported as the best treatment for the inhibition of explant browning in *Isoplexis chalcantha* (Molina 2008) and *Pueraria tuberosa* (Rathore and Shekhawat 2009). Li and Qiao (2001) reported that phenolic compounds could activate polyphenoloxidase (PPO) in explants and change the metabolism of tissue cells and form brown quinone substances through their oxidation. They reported that the brown quinone substances repress the activities of other enzymes and destroy several components of the medium. However, the adsorbents like PVP could repress the oxidation of the phenolic compounds and consequently alleviate the browning.

Many authors have reported that cytokinin is required in optimal quantity for shoot proliferation in many genotypes but inclusion of a low concentration of auxin along with cytokinin increases the rate of shoot multiplication. Similarly, in the present investigation, addition of NAA at low concentration (0.5 μM) with optimum concentration of TDZ (2.5 μM) induced maximum shoot multiplication and proliferation in nodal segments. Our results corroborates with the other reports obtained in *Rhodiola rosea* (Dimitrov *et al*. 2003) and *Salvia nemorosa* (Skala and Wysokinska 2004).

Adventitious rooting in isolated microcutting of *A. precatorius* was achieved in presence of a half strength MS medium. The strength of MS medium appeared to be an important factor in influencing the rooting efficiency. Sometimes, the endogenous level of auxins present in the tissue is sufficient to induce roots in hormone free MS medium. The reduction in nutrient supply for better effect in rooting efficiency as described in the present study is in agreement with the previous experiments made in *Albizia lebbeck* (Perveen *et al*. 2011, 2012). However, sometimes a very low concentration of exogenous auxin is required for better rhizogenesis. In the present study, the half strength MS medium with optimum concentration of IBA (1.5 μM) was found to be more effective in inducing a maximum root number as compared to the MS medium with NAA. Similar to our results, the treatment with IBA has been reported to be effective in plants like *Argyrolobium roseum* (Khanna *et al*. 2006) and *Albizia lebbeck* (Perveen *et al*. 2012).

The micropropagated plants are quite often susceptible to various stresses. Low photosynthesis because of low pigment content and malfunctioning of the water loss regulation are the two major constrains of tissue cultured plants during acclimatization (Fila *et al*. 2006). Therefore, the plantlets were acclimatized in the culture room for 28 d and thereafter in the greenhouse up to 49 d before transfer to field.

The regenerated plantlets exhibited an increase in content of photosynthetic pigments after one week depression during the course of acclimatization period (7 - 49 d). The findings are in consistence with the reports of Kadlecek *et al*. (1998) in *Nicotiana tabacum*, wherein photomixotrophically grown plantlets exhibited an increase in Chl *a* and *b* content after the first week of transplantation. Enhancement in Chl *a*, Chl *b*, and Car content with the formation of new leaves is in accordance with the reports on *Calathea louisae* (Van Huylenbroeck *et al*. 2000) and *Tylophora indica* (Faisal and Anis 2010). It is interesting to note that in the present investigation, an increase in Chl content was retained even after the transfer of plants to the greenhouse (day 35). This might be due to the significantly increased PFD together with increased Car content which can help to cope with possible photo-oxidative stress and protect the photosynthetic machinery (Fatima *et al*. 2011, Dias *et al*. 2013). Similar results have been found in *Tylophora indica* (Faisal and Anis 2010), where an increase in Chl and Car content was found during acclimatization. The other possible reason for steady increase in Chl content might be the hardening of plantlets under rather high PFD (150 μmol m^{-2} s^{-1}) in the culture room (from day 0 - 28) which revealed possibilities for improvement of growth in the greenhouse under PFD 300 μmol m^{-2} s^{-1}. Similar results have been reported in *Gardenia jasmiinoides* by Serret *et al*. (2001) and in *Ulmus minor* by Dias *et al*. (2013), where the initial hardening of plants under increased irradiance improved the development of photosynthetic apparatus and so increased plant growth under natural environment.

Evaluation of MDA and H_2O_2 content during acclimatization period provides further evidence of oxidative stress tolerance in *in vitro* regenerated plantlets of *A. precatorius*. Reduction in MDA and H_2O_2 content after 7 d of acclimatization and further after transfer to the greenhouse clearly indicated stress tolerance in *A. precatorius*. In addition, H_2O_2 functions as a signalling molecule for antioxidant responses (Groppa and Benavides 2008, Giampaoli *et al*. 2012). Analogous decrease in MDA and H_2O_2 content has been observed by Chakrabarty and Datta (2008) in *Gerbera jamesonii* transferred to field.

In the present investigation, an increase in SOD and CAT activities in the process of acclimatization suggests an up-regulation of the plant protective mechanisms against oxidative stress. These results are consistent with earlier findings of Faisal and Anis (2010) in *Tylophora indica*, Varshney and Anis (2012) in *Tecomella undulata*,

and Mitrovic *et al*. (2012) in *Tacitus bellus*. Similarly, an elevation in activities of both APX and GR suggests chloroplast-based detoxification of ROS (Foyer and Noctor 2005).

It is apparent that the exposure of the *in vitro* raised plants to *ex vitro* environment firstly resulted in an increase in H_2O_2 and MDA content which stimulates activities of antioxidative enzymes. Further, a decline in H_2O_2 and MDA content (after 7 d) and even after transplantation of the plants to the greenhouse might be due to the increased activities of the antioxidative enzymes which facilitate the removal of excess ROS and suppress the lipid peroxidation. This is in contrast with the findings of Langebartels *et al.* (2002) and Giampaoli *et al*. (2012). Our results suggest that the antioxidant pool is operating efficiently during growth of the *A. precatorius* plants in the culture room as well as in the greenhouse.

The present protocol of *A. precatorius* regeneration is efficient and rapid and can be adopted commercially for its mass multiplication for plantations and medicinal purposes. Also, the photosynthetic pigments and antioxidants has been shown to be important in determining the ability of the plants to survive oxidative stress and play an important role for better adaptation of plantlets transplanted from *in vitro* to *ex vitro* conditions. These factors could be a key to design adequate methods for acclimatization of other medicinally and economically important plants to *ex vitro* conditions.

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