

Physiological and biochemical parameters influencing *ex vitro* establishment of the *in vitro* regenerants of *Albizia lebbek* (L.) Benth.: an important soil reclaiming plantation tree

S. Perveen · M. Anis

Received: 19 July 2014 / Accepted: 31 March 2015 / Published online: 30 April 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Present approach demonstrates a speedy and competent *in vitro* regeneration method for *A. lebbek*. The hypocotyl explants (HP) excised from a 15-day-old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations (0.5, 2.5, 5.0, 7.5, 10.0 and 12.5 μM) of 6-Benzyladenine (BA), Kinetin (Kn) and 2-Isopentenyl adenine (2-iP) singly as well as in combination with α -naphthalene acetic acid (NAA) or Indole-3-butyric acid (IBA) or Indole-3-acetic acid (IAA; 0.1, 0.5, 1.0, 1.5 and 2.0 μM). The best shoot regeneration rate (88 %) with number of shoots (34.00 ± 1.15) and shoot length (6.30 ± 0.05 cm) per explant was obtained on MS + BA (7.5 μM) + NAA (0.5 μM) after 8 weeks of culture. While, the explants cultured onto the MS + TDZ (Thidiazuron; 0.5, 1.0, 2.5, 5.0 and 7.5 μM) singly showed maximum shoot proliferation response of 77 % when, TDZ exposed cultures were transferred on the BA (7.5 μM) + NAA (0.5 μM) supplied MS medium producing maximum no. of shoots (20.30 ± 0.32) and shoot length (5.15 ± 0.08 cm) after 8 weeks of culture. The regenerated

shoots were rooted by using *ex vitro* rooting method and successfully acclimatized by using a simple acclimatization procedure of 70 days, primarily in soilrite for 28 days and finally in soilrite + garden soil (1:1) for 70 days under culture room conditions (150 PPF). This acclimatization period under the optimized conditions of culture room ensures a high survival rate (80 %) of the plantlets in the field conditions. Photosynthetic pigments (Chlorophyll a, b and carotenoid content) evaluated during acclimatization period showed a decreasing trend in the initial 14 days afterwards a significant increase in the subsequent period of 21–70 days. While all the stress parameters MDA and H_2O_2 content showed decreasing trend all through the acclimatization, concurrently all the tested antioxidative enzymes (SOD, CAT, APX and GR) exhibited an increasing trend.

Keywords Hypocotyl explants · Adventitious shoots · Direct regeneration · *Albizia lebbek* · Antioxidative enzyme

Abbreviations

BA	6-Benzyladenine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
2iP	2-Isopentenyl adenine
Kn	6-Furfurylaminopurine
PGRs	Plant growth regulators
MS	Murashige and Skoog (1962) medium

S. Perveen · M. Anis (✉)
Plant Biotechnology Laboratory, Department of Botany,
Aligarh Muslim University, Aligarh 202 002, India
e-mail: anism1@rediffmail.com

M. Anis
Department of Plant Production, College of Food and
Agricultural Sciences, King Saud University,
Riyadh 11451, Saudi Arabia

NAA	α -Naphthalene acetic acid
TDZ	Thidiazuron
HP	Hypocotyl explant
Chl	Chlorophyll
Car	Carotenoid
P _N	Photosynthetic rate
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate peroxidase
GR	Glutathione reductase

Introduction

Albizia lebbek (Fabaceae) is an imperative multi-use perennial tree of the genus *Albizia* indigenously found in India, Bangladesh, Nepal and Pakistan etc. (Orwa et al. 2009). It owns several medicinal evidences such as anti-inflammatory, analgesic, antifertility and antimicrobial properties. In Indian traditional medicinal systems, the leaves, flowers, seeds, bark and roots are used to treat cough, boils, eye flu, gingivitis, piles, diarrhea, gonorrhoea, hypoglycemia, abdominal tumors, lung problems, bronchial asthma and other allergic disorders (Rätsch and Christian 1998). Economically, it is used for fuel, timber, furniture, erosion control (Singh et al. 2004) and as a shade tree in tea, coffee and cardamom plantations (Saha and Ahmed 2009).

Unsystematic and illicit erosion, low natural rejuvenation, constricted habitat specificity and insufficient conventional methods resulted in the rigorous depletion of *A. lebbek* population (Perveen et al. 2011). Therefore, keeping in views the outputs of the tissue culture techniques in woody trees, a number of efforts have been made for the in vitro propagation of *A. lebbek* by using hypocotyl, root, cotyledon, leaflet, stem, petiole and seed explants (Gharyal and Maheshwari 1983; Mamun et al. 2004; Gharyal and Maheshwari 1990; Perveen et al. 2011, 2012, 2013b). For clonal plantlets production in vitro propagation through axillary shoots proliferation is a useful technique, while adventitious bud induction is a promising tool in order to obtained high frequency of plantlets at commercial level in a shorter period of time.

Moreover, the particular in vitro culture conditions i.e., high relative humidity, low CO₂ concentration, low light intensity and massive of sugar etc., results in the

formation of plantlets with anomalous physiology, morphology and anatomy (Ali et al. 2005). Transfer of these plantlets to ex vitro conditions having high light intensity, low relative humidity, high CO₂ and temperature etc., might easily impaired them by hasty changes. Thus, the success of in vitro propagation depends on efficient transplantation protocols ensuring the establishment of in vitro plants under ex vitro environment (Hazarica 2006). Furthermore, study of changes in the physiological and biochemical parameters during acclimatization of the in vitro plants provide information on the effect of changed environmental conditions during acclimatization and helps in the standardization of acclimatization protocol (Van Huylbroek et al. 2000; Kadlecck et al. 2001; Perveen et al. 2013a).

Hence, in vitro propagation of *Albizia* via adventitious bud induction followed by concerning the difficult stages of ex vitro acclimatization may prove highly efficient for the commercial protocol establishment. Therefore, the objectives of this study were (1) to develop a highly efficient in vitro protocol for routine plant regeneration in the *A. lebbek* by using HP explants and (2) to assess the information on the changes in physiological and biochemical parameters during the acclimatization of in vitro raised plantlets under ex vitro conditions and its neutralization by biochemical characteristics.

Materials and methods

Seed germination and establishment of aseptic seedling

The mature fruits of *A. lebbek* were collected from a 20 years old tree growing in the botanical garden of the Aligarh Muslim University, Aligarh, India. The healthy seeds, excised from the fruits were washed thoroughly in running tap water for 30 min. These were immersed in 1 % (w/v) solution of Bavistin, a fungicide, for 15 min to remove adherent particles and later treated with 5 % (v/v) Teepol solution for 20 min. Thereafter, the seeds were rinsed thrice with sterile distilled water followed by surface sterilization in 0.1 % (w/v) HgCl₂ solution under laminar flow hood for 15 min. Finally, the seeds were washed thrice with sterile distilled water to remove the traces of HgCl₂ solution. The disinfected seeds were inoculated on MS (Murashige and Skoog 1962) basal medium for seed germination. Hypocotyl segments

(1.0–1.5 cm) (Fig. 1a) excised from 15-day-old seedlings were used as explants.

Culture media and culture condition

MS (Murashige and Skoog 1962) basal medium fortified with 3 % (w/v) sucrose, 0.8 % (w/v) agar (Qualigens, Mumbai, India) was used in all the experiments. The medium was adjusted to pH, 5.8 using 1N NaOH or HCl and sterilized by autoclaving at 121 °C and 1.06 kg cm⁻² pressure for 20 min. Cultures were incubated at 25 ± 2 °C under 16/8 h (light/dark) photoperiod provided by cool white fluorescent tubes (Phillips, India) with a photon flux density of 50 μmol m⁻² s⁻¹ at 55–60 % relative humidity.

Adventitious shoot induction and multiplication

HP explants excised from 15-day-old seedlings were cultured onto MS medium augmented with various

concentrations of BA, Kn, 2-iP (0.5, 2.5, 5.0, 7.5, 10.0 and 12.5 μM) or TDZ (0.5, 1.0, 2.5, 5.0 and 7.5 μM) singly as well as in combination with different concentrations of NAA or IBA or IAA (0.1, 0.5, 1.0, 1.5 and 2.0 μM) for induction and multiplication of shoots.

Histological analysis

To confirm the regeneration of multiple shoot buds from the nodal 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 saffranin and fast green



Fig. 1 In vitro regeneration and plantlet establishment of *A. lebbbeck*. **a** Hypocotyl explants (1.0–1.5 cm) obtained from 15 days old aseptic seedling. **b** Emergence of direct adventitious shoot buds from hypocotyl (HP) explants on MS + BA (7.5 μM) after 4 weeks of culture. **c, d** Histological section showing the direct induction of adventitious shoot buds (SB) in

HP. **e** Cultures showing elongation and proliferation of adventitious shoots from HP on MS + BA (7.5 μM) + NAA (0.5 μM) after 8 weeks of incubation. **f** Acclimatized plantlets in sterile soilrite after 4 weeks. **g** Acclimatized plantlets in sterile soilrite + normal garden soil (1:1) after 10 weeks. **h** 4 month old plants acclimatized in garden soil under field conditions

solutions. The sections were examined under an optical microscope (CH20i, Olympus, Tokyo, Japan).

Rooting and acclimatization

For rooting 4–5 cm shootlets were rooted and acclimatized in soilrite by utilizing Perveen et al. (2013b) method. After 4 weeks of soilrite acclimatization, plants were transferred to pots containing a mixture of garden soil [loam to clayey (Khan et al. 2012)] + soilrite (1:1) and maintained in the culture room conditions (25 ± 2 °C under 16/8 h light–dark conditions with a PPFD of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$) up to the next 6 weeks, followed by green house transfer (garden soil) under normal day length conditions.

Fully developed leaves obtained at transplantation day (day 0, control) and after 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of acclimatization were used for the evaluation of different physiological and biochemical parameters (chl a/b, car content, P_N , MDA, H_2O_2 , SOD, CAT, APX and GR) as mentioned below.

Photosynthetic pigments estimation

The chl (a and b) and car contents were extracted using the method of MacKinney (1941). A total of 0.5 g of fresh leaf tissues from inter-venal areas was ground in 5 ml of 80 % acetone. The suspension was filtered with Whatman filter paper number 1. Absorbance of chlorophyll at 663, 645 nm and carotenoid at 480 and 510 nm was estimated on UV–Vis Spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan).

Net photosynthetic rate (P_N)

The net photosynthetic rate (P_N) was measured on fully expanded leaves using a portable Infra-red Gas Analyzer (IRGA, LI-COR 6400, Lincoln, USA) on the basis of net exchange of CO_2 between leaves and atmosphere by enclosing the leaves in a leaf chamber and monitoring the rate of CO_2 concentration changed over short time intervals. The net photosynthetic rate was expressed as $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$.

Estimation of MDA content

Lipid peroxidation was estimated according to malondialdehyde (MDA) production using the thiobarbituric

acid (TBA) method (Heath and Packer 1968) by using a coefficient of absorbance of $155 \text{mM}^{-1} \text{cm}^{-1}$.

Estimation of H_2O_2 content

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 $14,000 \times g$ and the homogenate was used for the determination of H_2O_2 content by the method of Alexieva et al. (2001). The supernatant (0.5cm^3) was mixed with 0.5cm^3 of 100 mM K-phosphate buffer (pH 7.8), and 2cm^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.

Antioxidant enzyme extraction and assay

To determine the activities of antioxidant enzymes, 0.5 g of fresh leaf tissue was homogenized in 2.0cm^3 of extraction buffer (pH 7.5) containing 1 % (m/v) PVP, 1 % (v/v) Triton X-100, and 0.11 g of ethylenediaminetetraacetic acid (EDTA) using a pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at $15,000 \times g$ 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 0.1cm^3 of enzyme extract in test tubes was incubated under 15 W fluorescent lamp (Philips, Kolkata, India) at 25 °C 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 0.1cm^3 of enzyme extract in a total volume of 3cm^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 $25 \text{ }^\circ\text{C}$ 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 included 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 enzyme extract.

The protein content in enzymatic extracts was determined following the Bradford (1976) assay using bovine serum albumin as a standard.

Statistical analysis

The experiments were based on a completely randomized design with 20 explants per treatment and all the experiments were repeated thrice (=three replicates, 60 explants per treatment). The data on various parameters were subjected to one-way analysis of variance (ANOVA) using SPSS version 16 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at $P = 0.05$. The results were expressed as the mean \pm SE.

Results

Effect of cytokinins on adventitious shoot proliferation

The ability of HP segments to produce shoots varied depending upon the PGRs supplied in the MS medium. The first visible change in the explants was a slight enlargement in size within the first week followed by multiple shoot bud induction (without an intervening callus formation) at the cut ends of the explants after 2 weeks of culture on MS + BA, Kn and 2-iP singly. After a period of 2 weeks, shoot buds started to stretch over the whole explant surface.

All the tested levels of BA supplied in MS medium were found more effective than Kn and 2-iP, for the regeneration of adventitious shoot buds from HP explants. Maximum shoot regeneration response 81 % with number of shoots (22.00 ± 1.10) was observed on MS + BA (7.5 μM) containing medium after

4 weeks of culture (Table 1; Fig. 1b). While, at the same level (7.5 μM), Kn and 2-iP produces maximum 68 and 59 % regeneration response respectively (Table 1).

Furthermore, the histological analysis confirms the direct organogenesis pathway showing shoot differentiation from HP explants and maintaining vascular connections from parent explants (Fig. 1c, d).

Synergistic effect of cytokinins and auxins on adventitious shoot proliferation

After 4 weeks, HP explants with regenerated shoot-buds were transferred onto the shoot proliferation and elongation media containing MS + 7.5 μM BA or Kn or 2-iP with different concentrations of various auxins viz., NAA, IBA and IAA (0.1, 0.5, 1.0, 1.5, 2.0 μM) singly (Tables 2, 3, 4). Among the different combinations tested, BA (7.5 μM)+NAA (0.5 μM) was found to be the most effective, producing maximum regeneration response (88 %), number of shoots (34.00 ± 1.15) per explant and shoot length ($6.30 \pm 0.05 \text{ cm}$) after 8 weeks of culture (Table 2; Fig. 1f). While, BA combined with (0.5 μM) of IBA or IAA produced relatively low (78 and 65 %) shoot regeneration frequency (Table 2).

Similarly, optimal level (7.5 μM) of Kn or 2-iP combined with NAA (0.5 μM) produced maximum regeneration frequency of 75 and 67 % respectively after 8 weeks of culture. While the combination of Kn (7.5 μM) + (0.5 μM) IBA or IAA and 2-iP (7.5 μM) + (0.5 μM) IBA or IAA (0.5 μM) proved comparatively less efficient (Tables 3, 4).

Effect of TDZ

The effect of various concentrations of TDZ was also evaluated on the direct adventitious shoot regeneration from HP explants. Among the various levels tested TDZ at (1.0 μM) showed maximum 12.60 ± 0.33 number of shoots in 70 % cultures after 4 weeks of culture (Table 5). While an increase in TDZ level beyond 1.0 μM resulted in callus formation. The TDZ exposed hypocotyl explants sub-cultured onto the same fresh medium showed vitrification and shoots did not elongate further. To overcome this problem, explants were subcultured onto the MS + BA (7.5 μM) + NAA (0.1, 0.5, 1.0, 1.5 and 2.0 μM) singly. The highest shoot regeneration response

Table 1 Effect of different cytokinins on adventitious shoot buds induction from hypocotyl explants after 4 weeks of culture

Cytokinins (μM)			% Response	Mean no. of shoot buds/explant
BA	Kn	2-iP		
0.5			60	$9.33 \pm 0.33^{\text{d}}$
2.5			67	$12.00 \pm 0.57^{\text{c}}$
5.0			72	$17.00 \pm 0.55^{\text{b}}$
7.5			81	$22.00 \pm 1.10^{\text{a}}$
10.0			70	$16.00 \pm 0.81^{\text{b}}$
12.5			62	$10.00 \pm 0.50^{\text{d}}$
	0.5		50	$3.50 \pm 0.05^{\text{hi}}$
	2.5		55	$6.00 \pm 0.11^{\text{f}}$
	5.0		61	$9.00 \pm 0.15^{\text{de}}$
	7.5		68	$11.80 \pm 0.06^{\text{c}}$
	10.0		63	$8.00 \pm 0.28^{\text{e}}$
	12.5		58	$5.80 \pm 0.10^{\text{f}}$
		0.5	40	$2.80 \pm 0.21^{\text{ij}}$
		2.5	49	$4.43 \pm 0.03^{\text{gh}}$
		5.0	54	$5.93 \pm 0.13^{\text{f}}$
		7.5	59	$8.00 \pm 0.14^{\text{e}}$
		10.0	52	$5.00 \pm 0.05^{\text{fg}}$
		12.5	45	$2.20 \pm 0.13^{\text{j}}$

Values represent mean \pm SE. Means sharing the same letter within columns are not significantly different ($P = 0.05$) using DMRT

Table 2 Effect of different auxins amended with an optimal concentration of BA ($7.5 \mu\text{M}$) in MS medium on shoot proliferation and multiplication from hypocotyl explants after 8 weeks of culture

Auxins (μM)			% Response	Mean no. of shoots/explants	Mean shoot length (cm)
NAA	IBA	IAA			
0.1			82	$28.00 \pm 0.28^{\text{b}}$	$6.00 \pm 0.17^{\text{b}}$
0.5			88	$34.00 \pm 1.15^{\text{a}}$	$6.30 \pm 0.05^{\text{a}}$
1.0			79	$26.00 \pm 0.57^{\text{c}}$	$5.90 \pm 0.14^{\text{b}}$
1.5			70	$19.00 \pm 0.53^{\text{de}}$	$5.50 \pm 0.11^{\text{c}}$
2.0			64	$15.00 \pm 1.10^{\text{e}}$	$5.00 \pm 0.08^{\text{d}}$
	0.1		72	$17.30 \pm 0.33^{\text{ef}}$	$4.70 \pm 0.22^{\text{c}}$
	0.5		78	$20.00 \pm 0.15^{\text{d}}$	$5.00 \pm 0.15^{\text{d}}$
	1.0		67	$16.00 \pm 0.24^{\text{fg}}$	$4.56 \pm 0.03^{\text{c}}$
	1.5		60	$12.00 \pm 0.22^{\text{h}}$	$4.00 \pm 0.11^{\text{fg}}$
	2.0		57	$8.66 \pm 0.32^{\text{ij}}$	$3.70 \pm 0.09^{\text{gh}}$
		0.1	60	$10.00 \pm 0.34^{\text{i}}$	$3.90 \pm 0.13^{\text{fgh}}$
		0.5	65	$12.60 \pm 0.16^{\text{h}}$	$4.10 \pm 0.18^{\text{f}}$
		1.0	57	$7.33 \pm 0.23^{\text{j}}$	$3.73 \pm 0.03^{\text{h}}$
		1.5	50	$4.56 \pm 0.20^{\text{k}}$	$3.00 \pm 0.20^{\text{i}}$
		2.0	45	$3.33 \pm 0.19^{\text{k}}$	$2.73 \pm 0.03^{\text{j}}$

Values represent mean \pm SE. Means sharing the same letter within columns are not significantly different ($P = 0.05$) using DMRT

(77 %) with number of shoots (20.30 ± 0.32) and shoot length (5.15 ± 0.08 cm) was obtained on MS + BA ($7.5 \mu\text{M}$) + NAA ($0.5 \mu\text{M}$) after 8 weeks of culture (Table 6).

Rooting and acclimatization

The in vitro regenerated shootlets were rooted successfully by using previously standardized ex vitro rooting

Table 3 Effect of different auxins amended with an optimal concentration of Kn (7.5 μ M) in MS medium on shoot proliferation and multiplication from hypocotyl explants after 8 weeks of culture

Auxins (μ M)			% Response	Mean no. of shoots/explant	Mean shoot length (cm)
NAA	IBA	IAA			
0.1			70	15.00 \pm 1.11 ^b	4.10 \pm 0.05 ^b
0.5			75	18.00 \pm 0.57 ^a	4.50 \pm 0.11 ^a
1.0			67	13.60 \pm 0.33 ^{bc}	4.00 \pm 0.12 ^{bc}
1.5			60	11.30 \pm 0.21 ^{de}	3.70 \pm 0.05 ^{de}
2.0			57	9.33 \pm 0.23 ^{fg}	3.00 \pm 0.10 ^g
	0.1		62	12.60 \pm 0.16 ^{cd}	3.50 \pm 0.05 ^e
	0.5		68	15.00 \pm 0.51 ^b	3.83 \pm 0.03 ^{cd}
	1.0		59	10.00 \pm 0.28 ^{ef}	3.20 \pm 0.13 ^f
	1.5		52	8.00 \pm 0.13 ^g	2.83 \pm 0.03 ^{gh}
	2.0		47	5.00 \pm 0.50 ^h	2.10 \pm 0.09 ^j
		0.1	50	8.00 \pm 0.22 ^g	2.70 \pm 0.19 ^{hi}
		0.5	54	10.00 \pm 0.45 ^{ef}	3.00 \pm 0.08 ^g
		1.0	48	6.00 \pm 0.15 ^h	2.50 \pm 0.08 ⁱ
		1.5	00	0.00 \pm 0.00 ⁱ	0.00 \pm 0.00 ^k
		2.0	00	0.00 \pm 0.00 ⁱ	0.00 \pm 0.00 ^k

Values represent mean \pm SE. Means sharing the same letter within columns are not significantly different ($P = 0.05$) using DMRT

Table 4 Effect of different auxins amended with an optimal concentration of 2-iP (7.5 μ M) in MS medium on shoot proliferation and multiplication from hypocotyl explants after 8 weeks of culture

Auxins (μ M)			% Response	Mean no. of shoots/explants	Mean shoot length (cm)
NAA	IBA	IAA			
0.1			62	11.00 \pm 0.57 ^b	3.23 \pm 0.17 ^b
0.5			67	13.00 \pm 0.52 ^a	3.80 \pm 0.05 ^a
1.0			60	10.00 \pm 0.21 ^{bc}	3.00 \pm 0.11 ^c
1.5			57	7.00 \pm 0.12 ^d	2.73 \pm 0.03 ^d
2.0			52	4.83 \pm 0.16 ^f	2.30 \pm 0.05 ^{ef}
	0.1		55	6.83 \pm 0.16 ^d	2.50 \pm 0.10 ^e
	0.5		58	9.00 \pm 0.50 ^c	2.93 \pm 0.03 ^{cd}
	1.0		52	5.76 \pm 0.14 ^{ef}	2.13 \pm 0.13 ^f
	1.5		47	4.83 \pm 0.15 ^f	1.73 \pm 0.08 ^g
	2.0		42	3.20 \pm 0.10 ^g	1.10 \pm 0.12 ⁱ
		0.1	38	5.70 \pm 1.10 ^f	1.80 \pm 0.13 ^g
		0.5	41	6.76 \pm 0.12 ^{de}	2.10 \pm 0.06 ^f
		1.0	36	3.20 \pm 0.05 ^g	1.33 \pm 0.17 ^h
		1.5	00	0.00 \pm 0.00 ^h	0.00 \pm 0.00 ^j
		2.0	00	0.00 \pm 0.00 ^h	0.00 \pm 0.00 ^j

Values represent mean \pm SE. Means sharing the same letter within columns are not significantly different ($P = 0.05$) using DMRT

protocol and hardened off inside the culture room (in substrate soilrite) for 4 weeks (Perveen et al. 2013b). The primary hardened plants on soilrite for 4 weeks (Fig. 1f), were transferred to garden soil + soilrite mixture (1:1) showed survival rate of about 80 % after 6 weeks of transplantation (Fig. 1g, h). The plants grew well and did not show any variation in morphology and growth characteristics when compared with mother plant.

Estimation of physiological parameters during acclimatization

Photosynthetic pigments

Throughout the ex vitro acclimatization period of 10 weeks, in vitro raised plantlets showed a considerable increase in chl (a and b) and car content.

Table 5 Effect of different concentrations of TDZ on multiple adventitious shoot buds induction from hypocotyl explants after 4 weeks of culture

TDZ (μM)	% Response	Mean no. of shoot buds/explants
0.5	65	9.67 ± 0.65^b
1.0	70	12.60 ± 0.33^a
2.5	67	11.30 ± 0.21^a
5.0	59	7.33 ± 0.23^c
7.5	60	5.50 ± 0.57^d

Values represent mean \pm SE. Means sharing the same letter within columns are not significantly different ($P = 0.05$) using DMRT

However, there was a reduction of 14.2 and 36.3 % in Chl a and b pigments respectively after 14 days of transfer in soilrite as compared to day 0 plants but on subsequent days, the new leaves appeared and resulted in a significant increase of 67 and 118 % in both Chl a and b contents respectively at day 28. Again a significant decrease in photosynthetic pigments was observed after 28 days with the change in potting substrate [garden soil + soilrite mixture], but thereafter a significant increase in Chl a and b pigments was observed (Fig. 2). Similarly, with the increase in number of days of acclimatization (0–70 days), the carotenoid content was found to increase (62.8 %) up to the day 63 and get stabilized beyond 63 days (Fig. 2).

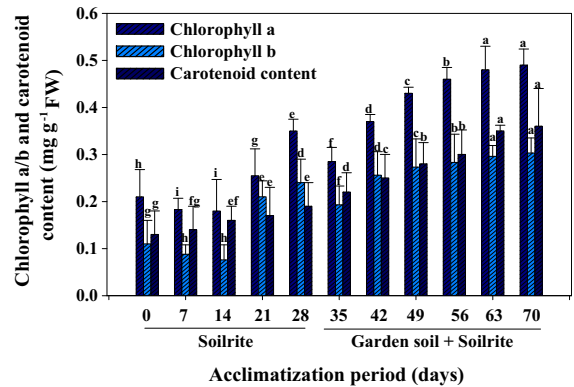
Net photosynthetic rate (P_N)

The P_N rate was found to decrease (26.6 %) during the first 2 weeks of acclimatization in the soilrite. While, after 2 weeks, it started to increase (30.2 %) up to 28 days. Transfer of the plants in changed substrate, again resulted in a significant decrease (09 %) in P_N

Table 6 Effect of different concentrations of NAA supplied with 7.5 μM BA, on shoot multiplication from TDZ-exposed hypocotyl explants after 8 weeks of culture

NAA (μM)	% Response	Mean no. of shoots/explant	Mean shoot length (cm)
0.1	62	17.60 ± 0.34^b	4.75 ± 0.09^b
0.5	77	20.30 ± 0.32^a	5.15 ± 0.08^a
1.0	66	16.00 ± 0.57^c	4.62 ± 0.18^b
1.5	61	11.00 ± 0.48^d	3.80 ± 0.10^c
2.0	52	8.66 ± 0.57^e	3.15 ± 0.04^d

Values represent mean \pm SE. Means sharing the same letter within columns are not significantly different ($P = 0.05$) using DMRT

**Fig. 2** Changes in the levels of photosynthetic pigments (Chl a and b) and carotenoid content in the micropropagated plantlets acclimatized in soilrite (from 0 to 28 days) followed by garden soil + soilrite (1:1) transfer (from 28 to 70 days). Bars represent the mean \pm SE. Bars denoted by the alphabets are significantly different ($P = 0.05$) using DMRT

rate at day 35 compared to day 28, followed by steady increase (45 %) in the subsequent period (Fig. 3).

Estimation of biochemical parameters during acclimatization

MDA and H_2O_2 content

An increase in the MDA and H_2O_2 content (3 and 2 %) respectively was observed in the plantlets in early 14 days of acclimatization, subsequently, a decreasing trend (23.1 and 30.3 %) respectively was observed up to the day 70, as compared to the day 0 plants (Fig. 4).

Antioxidant enzymes

Plantlets exhibited a significant increase in SOD, CAT, GR and APX activity reaching a maximum on day 63 and thereafter got stabilized (Fig. 5). While,

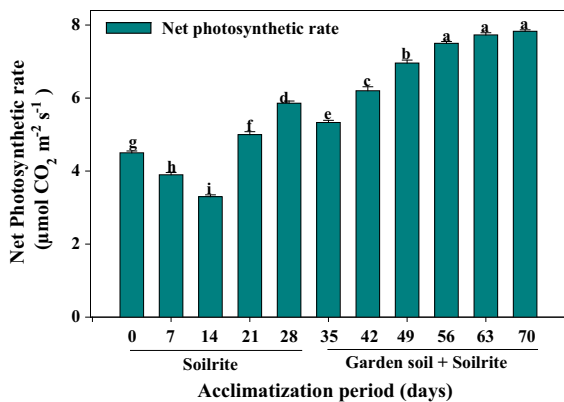


Fig. 3 Change in net photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-2}$) of micropropagated plantlets acclimatized in soilrite (from 0 to 28 days) followed by garden soil + soilrite (1:1) transfer (from 28 to 70 days). Bars represent the mean \pm SE. Bars denoted by the alphabets are significantly different ($P = 0.05$) using DMRT

GR was found increasing up to the day 70 of acclimatization (Fig. 5).

Discussion

Successful genetic improvement of the plants for the useful trait expression (quality improvement in multipurpose trees) requires a suitable plant regeneration protocol. Plant production in vitro via adventitious shoot organogenesis on various juvenile explants (bearing high morphogenetic potential and low contamination rates) is the best system to obtain transgenic with ease (Yang et al. 1996). HP explants being juvenile and lacking pre-existing meristems can be suitably subjected to growth regulator manipulations to result in de novo plant regeneration as reported in number of plant species viz., *Annona squamosa* (Nagori and Purohit 2004), *Euonymus japonicus* (Shang et al. 2006) and *Vigna subterranea* (Mongomake et al. 2009).

In vitro organogenesis requires the moderation of cell differentiation by the application of appropriate plant growth regulators (Benson 2000). In the present study, BA followed by TDZ was found to be the most efficient cytokinin than Kn and 2-iP for maximum adventitious shoot buds production (without callus formation) from aseptic HP explants of *A. lebbek*. The effectiveness of BA for adventitious organogenesis from HP explants has been demonstrated in

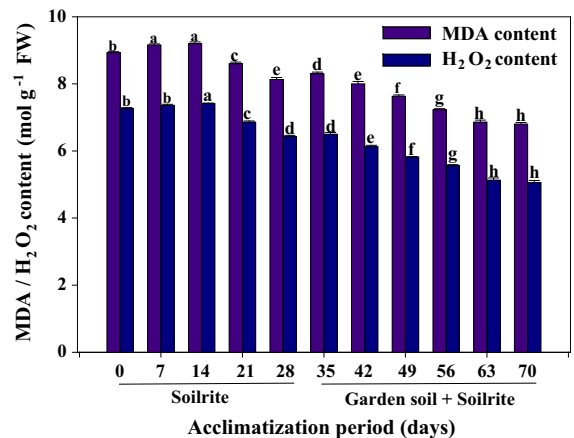


Fig. 4 Changes in the levels of MDA and H₂O₂ content in micropropagated plantlets acclimatized in soilrite (from 0 to 28 days) followed by garden soil + soilrite (1:1) transfer (from 28 to 70 days). Bars represent the mean \pm SE. Bars denoted by the alphabets are significantly different ($P = 0.05$) using DMRT

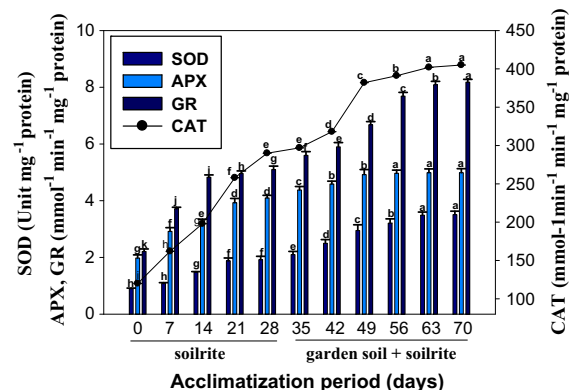


Fig. 5 Changes in the levels of SOD (Unit mg⁻¹ protein), CAT, APX and GR (mmol min⁻¹ mg⁻¹ protein) content in micropropagated plantlets acclimatized in soilrite (from 0 to 28 days) followed by garden soil + soilrite (1:1) transfer (from 28 to 70 days). Bars represent the mean \pm SE. Bars denoted by the alphabets are significantly different ($P = 0.05$) using DMRT

number of woody tree species including *Sesbania rostrata* (Jha et al. 2002) and *Fraxinus nigra* (Beasley and Pijut 2013). While, in consistence with our finding, Parimalan et al. (2007) in *Bixa orellana* and Annapurna and Rathore (2010) in *Embelia ribes* found TDZ as effective cytokinin for in vitro adventitious shoot regeneration.

Direct shoot organogenesis without callus formation obtained from HP explants is beneficial as compared to indirect shoot organogenesis, as it

generally results in a lower frequency of somaclonal variation (Annappurna and Rathore 2010). Hence, the structural analysis is an important step to corroborate adventitious shoot bud regeneration as it is an extremely useful approach in plant morphogenesis studies (Saha et al. 2012). The HP explants showed high mitotic division on cytokinin supplemented medium resulting in the proliferation of meristematic zones nearby epidermis. As expected, vascular connections were observed to occur between meristematic regions and mother explants as established by Neto et al. (2003) in *Bixa orellana*. Therefore, histological studies indicated that in vitro plant regeneration in *A. lebbbeck* has developed through direct adventitious organogenesis.

The combination of various auxins with cytokinins enhances the shoot induction and multiplication rate from HP explants. Among all the combinations tested BA + NAA was found to be the most favorable producing maximum shoot proliferation and elongation, which is in accordance with the findings in other plant species, *Artirrhinum majus* (Cui et al. 2004) and *Psoralea corylifolia* (Baskaran and Jayabalan 2010; Tiwari and Pathak 2012). Gharyal and Maheshwari (1983) found NAA and IAA as best auxins with cytokinins for organogenesis from HP explants of *A. lebbbeck* but the results (number of shoots) were comparatively less productive than our observations, it may be because of the supplementation of B₅ basal medium, which was found less effective than MS medium for in vitro *A. lebbbeck* production (Perveen et al. 2011).

The shoot buds induced in HP explants on TDZ-containing medium showed enhanced shoot induction and multiplication rate when cultured onto the secondary medium containing BA + NAA. The efficiency of BA + NAA on TDZ-exposed cultures for enhanced shoot induction and elongation was also confirmed by Khan and Anis (2012) in *Salix tetrasperma* and Ahmed and Anis (2012) in *Vitex trifolia*.

In vitro regenerated shootlets of *A. lebbbeck* rooted by ex vitro rooting method gave the highest number of root regeneration response as compared to in vitro rooting (Perveen et al. 2011; Perveen et al. 2013b) and is less time consuming because of rooting and acclimatization took place simultaneously. The importance of ex vitro rhizogenesis over in vitro rooting have been reported by many researchers (Rogers and Smith 1992; Nas and Read 2004).

To safeguard in vitro grown plants against water stress and to encourage autotrophy, acclimatization under ex vitro conditions is one of the main processes to promote their field survival and physiological competence (Pospisilova et al. 1999; Hazarika 2003). For successful photoautotrophic acclimatization, a transitional environment is usually supplied for a duration ranging from one to several weeks using environmental controls, which has successfully improved the survival percentage of number of in vitro raised plant species under ex vitro environment (Xiao and Kozai 2004; Perveen et al. 2013a). In the same way, present study standardized an acclimatization period of 10 weeks for *A. lebbbeck*. The primary hardened plantlets on soilrite for 4 weeks when transferred onto the mixture of soilrite + garden soil (1:1) showed good survival rate of 80 % after 10 weeks of acclimatization and did not show any visible defects in morphological and growth characteristics when compared with their respective donor plants. Similar findings have been reported by Balaram et al. (2009) in *Swertia chirata* and Chabukswar and Deodhar (2005) in *Garcinia indica*, where sequential hardening was found highly effective.

Furthermore, evaluation of physiological and biochemical characteristics during the hardening process may be helpful for the development of competent transplantation protocols for the in vitro raised plants and will help to make assessments on adjusting under ex vitro environmental conditions (Hazarika 2006). In the present study, during the course of acclimatization of regenerants initial decline in chl (a and b), car contents and P_N rate (during 14 days) is in accordance with the findings of Kadlecik et al. (1998) in *Nicotiana tabacum*, wherein photomixotrophically grown plantlets exhibited decrease in chl a and b content during first week of ex vitro transplantation. Correspondingly, P_N rate was found decreasing in *Solanum tuberosum* and *Spathiphyllum floribundum* regenerants in the initial days of transplantation and started increasing thereafter (Baroja et al. 1995; Van Huylenbroeck and Debergh 1996).

According to Borkowska (2001), during the ex vitro acclimatization of strawberry plants, newly formed leaves (during first week of acclimatization) exhibited low photochemical activity and with the progression of acclimation they become more active. Similarly in this study, newly formed leaves showed significant decline in chl (a and b) content at day 7 and 14 as

compared to day 0 plants followed by an increase showing functional activeness of the newly originated leaves with the passage of acclimatization. While, a decline in photosynthetic parameters during initial 14 days of acclimatization, may be because of the low photochemical activity of the persistent leaves or newly formed leaves (Perveen et al. 2013a). These results are in accordance with the reports on *Calathea louisae* (Van Huylbroek et al. 2000) and *Tylophora indica* (Faisal and Anis 2010). Likewise, in *Calathea louisae* and *Spathiphyllum floribundum* substantial increase in P_N was measured with the fully developed new leaflets (Van Huylbroek et al. 1998, 2000).

Light intensities has a crucial role for autotrophic plant development, as transfer of in vitro (50 PPF) plants to ex vitro at increased light intensities (150 PPF) favoured leaf expansion with autotrophic plant development mainly with regard to chlorophyll content as reported by Carvalho et al. (2002) in *Vitis vinifera*. Likewise, transfer of *A. lebbeck* plantlets under threefold (150 PPF) light intensity than in vitro condition (50 PPF) favours proper acclimatization of the plantlets as proved by the enhanced levels of photosynthetic parameters. Similar results have been reported in *Gardenia jasmiinoides* by Serret et al. (2001) and *Ulmus minor* by Dias et al. (2013). While, an increase in carotenoids level particularly signifies that plants sustained the light stress as play a key role in protecting of chlorophyll pigments under stress conditions (Ali et al. 2005).

Generally, water stress and photoinhibition promotes the production of reactive oxygen species (ROS) and in consequence of oxidative stress during the acclimatization of plants to ex vitro conditions (Pinto et al. 2011). ROS being inevitable byproducts of aerobic metabolism cause lipid peroxidation, protein degradation, damage of DNA; therefore, their production and removal must be controlled (Batkova et al. 2008). The extent of the damaging effects of ROS depends on the effectiveness of the antioxidative systems which include antioxidative enzymes viz., Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX) and Glutathione reductase (GR) (Perveen et al. 2013a). Therefore, an increase in SOD and CAT activities in the process of acclimatization suggests an up-regulation of the plant protective mechanism against oxidative stress. These results are in consistent with the earlier findings of Faisal and Anis (2010) in *Tylophora indica*, Varshney and Anis

(2012) in *Tecomella undulata* and Perveen et al. (2013a) in *Abrus precatorius*. Similarly, elevation in activities of both APX and GR suggests chloroplast-based detoxification of ROS (Perveen et al. 2013a). Hence, plants with higher content of antioxidants are usually better adapted to stress (Mitrovic et al. 2012).

MDA is a product of lipid peroxidation and an indicator of tissue damage (Perveen et al. 2013a). Besides, H_2O_2 functions as a signaling molecule for antioxidant responses (Giampaoli et al. 2012). Hence a considerable decrease in MDA and H_2O_2 content and an augment in antioxidative enzymes (SOD, CAT, APX and GR) after 14 days of acclimatization clearly indicated the stress tolerance in *A. lebbeck* regenerants. Analogous decrease in MDA and H_2O_2 content has been observed by Chakrabarty and Datta (2008) in *Gerbera jamesonii*.

Therefore, reduction in photosynthetic pigments level and increase in antioxidative enzymes and stress indicator during the initial period (0–14 days) of acclimatization reflects the production of oxidative stress and its neutralization by the higher activities of antioxidative enzymes. While, enhancement in photosynthetic pigments and antioxidative pools from day 21–70 suggest that the micropropagated plants developed functional photosynthetic machinery to reduce oxidative stress during the acclimatization period. Hence, results prove that by using steady hardening processes plants can gradually and successfully establish in autotrophic environmental conditions from the heterotrophic environment by slowly neutralizing oxidative stresses and improving photosynthetic systems.

Our study successfully overcomes the drawback of tissue culture technique in tree species related with the establishment of in vitro plants under natural environmental conditions via gradual acclimatization process standardization. However there are several reports on *Albizia* tissue culture but none of these studies reports efficient results in comparison to our study in terms of large scale plant production as well as their autotrophic establishment. So, this study for the first time reported a highly efficient commercially usable protocol with successful autotrophic plant development as confirmed by several physiological and biochemical parameters also.

Acknowledgments Financial support from the DST-FIST (2011–2016) and UGC (DRS-I) (2009–2014) programmes, Govt of India, New Delhi, is highly appreciated.

References

- Aebi H (1984) Catalase in vitro. In: Packer L (ed) Methods in enzymology. Oxygen radicals in biological systems, vol 105. Academic Press, Orlando, pp 121–126
- Ahmed MR, Anis M (2012) Role of TDZ in the quick regeneration of multiple shoots from nodal explant of *Vitex trifolia* L.—an important medicinal plant. Appl Biochem Biotechnol 168:957–966
- Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and UV radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344
- Ali MB, Hahn EJ, Paek KY (2005) Effects of light intensities on antioxidant enzymes and malondialdehyde content during short-term acclimatization on micropropagated *Phalaenopsis* plantlet. Environ Exp Bot 54:109–120
- Annapurna D, Rathore TS (2010) Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F. Plant Cell Tissue Org Cult 101:269–277
- Balaraju K, Agastian P, Ignacimuthu S (2009) Micropropagation of *Swertia chirata* Buch. —Hams. ex Wall.: a critically endangered medicinal herb. Acta Physiol Plant 31:487–494
- Baroja ME, Aguirreolea J, Sanchez-Doaz M (1995) CO₂ exchange of in vitro and acclimatized potato plantlets. In: Carre F, Chagvardieff P (eds) Ecophysiology and photosynthetic in vitro cultures. CEA, Centre detudes de Cadarache, Saint-Paul-lez-Durance, pp 187–188
- Baskaran P, Jayabalan N (2010) Direct organogenesis from hypocotyl explants of *Psoralea corylifolia* L.—An endangered medicinal plant. IJBT 9:329–332
- Batkova P, Pospisilova J, Synkova H (2008) Production of reactive oxygen species and development of antioxidative systems during in vitro growth and ex vitro transfer. Biol Plant 52:413–422
- Beasley RR, Pijut PM (2013) Regeneration of Plants from *Fraxinus nigra* Marsh. hypocotyls. HortScience 48:887–890
- Benson EE (2000) In vitro recalcitrance: an introduction. Special symposium: in vitro plant recalcitrance. In Vitro Cell Dev Biol Plant 36:141–148
- Borkowska B (2001) Morphological and physiological characteristics of micropropagated strawberry plants rooted in vitro or ex vitro. Sci Hortic 89:195–206
- Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72:248–254
- Carvalho LC, Santos P, Amancio S (2002) Effect of light intensity and CO₂ concentration on growth and the acquisition of in vivo characteristics during acclimatization of grapevine regenerated in vitro. Vitis 41:1–6
- Chabukswar MM, Deodhar MA (2005) Rooting and hardening of in vitro plantlets of *Garcinia indica* Choisy. IJBT 4:409–413
- Chakrabarty D, Datta SK (2008) Micropropagation of gerbera: lipid peroxidation and antioxidant enzyme activities during acclimatization process. Acta Physiol Plant 30:325–331
- Cui ML, Takayanagi K, Handa T (2004) High frequency from hypocotyl and stem segments of *Antirrhinum majus* (Snapdragon). Plant Cell Tissue Organ Cult 78:51–53
- Dhindsa PS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and Lipid peroxidation and decreased levels of Superoxide dismutase and Catalase. J Exp Bot 32:93–101
- Dias MC, Pinto G, Santos C (2013) Acclimatization of micropropagated plantlets induces an antioxidative burst: a case study with *Ulmus minor* Mill. Photosynthetica 49:259–266
- Faisal M, Anis M (2010) Effect of light irradiations on photosynthetic machinery and antioxidative enzymes during ex vitro acclimatization of *Tylophora indica* plantlets. J Plant Interact 5:21–27
- Gharyal PK, Maheshwari SC (1983) In vitro differentiation of plantlets from tissue cultures of *Albizia lebbek* L. Plant Cell Tissue Organ Cult 2:49–53
- Gharyal PK, Maheshwari SC (1990) Differentiation of explants from mature leguminous trees. Plant Cell Rep 8:550–553
- Giampaoli P, Tresmondi F, Lima GPP, Kanashiro S, Alves ES, Domingos M, Tavares AR (2012) Analysis of tolerance to copper and zinc in *Aechmea blanchetiana* grown in vitro. Biol Plant 56:83–88
- Hazarica BN (2006) Morpho-physiological disorders in in vitro culture of plants. Sci Hortic 108:105–120
- Hazarika BN (2003) Acclimatization of tissue cultured plants. Curr Sci 85:1705–1712
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I kinetics and stoichiometry of fatty acid peroxidation—Arch. Biochem Biophys 125:189–198
- Jha AK, Prakash S, Jain N, Nanda K, Gupta SC (2002) Production of adventitious shoots and plantlets from the hypocotyl explants of *Sesbania rostrata* (Bremek & Obrem.). In Vitro Cell Dev Biol Plant 38:430–434
- Kadlecek P, Ticha I, Capkova V, Schafer C (1998) Acclimatization of micropropagated tobacco plantlets. In: Garab G (ed) Photosynthesis: mechanisms and effects, vol 5. Kluwer Academic Publishers, London, pp 3853–3856
- Kadlecek P, Ticha I, Haisel D, Capkova V, Schafer C (2001) Importance of in vitro pretreatment for *ex vitro* acclimatization and growth. Plant Sci 161:695–701
- Khan ML, Anis M (2012) Modulation of in vitro morphogenesis in nodal segments of *Salix tetrasperma* Roxb. through the use of TDZ, different media types and culture regimes. Agrofor Sys 86:95–103
- Khan JA, Usmani SF, Khan S (2012) Effect of fly ash on the mobility of amino acids through six typical soils of Aligarh district. JPBMS 15:1–4
- Mackinney G (1941) Absorption of light by chlorophyll solution. J Biol Chem 140:315–322
- Mamun ANK, Matin MN, Bari MA, Siddique NA, Sultana RS, Rahman MH, Musa ASM (2004) Micropropagation of woody legume (*Albizia lebbek*) through tissue culture. Pak J Biol Sci 7:1099–1103
- Mitrovic A, Janosevic D, Budimir S, Pristov JB (2012) Changes in antioxidative enzymes activities during *Tacitus bellus* direct shoot organogenesis. Biol Plant 56:357–361
- Mongomake K, Hilaire KT, Daouda K, Michel Z, Justin KY, Ochat SJ (2009) In vitro plantlets regeneration in Bambara groundnut [*Vigna subterranea* (L.) Verdc. (Fabaceae)] through direct shoot bud differentiation on hypocotyl and epicotyl cuttings. Afr J Biotechnol 8:1466–1473
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

- Nagori R, Purohit SD (2004) In vitro plantlet regeneration in *Annona squamosa* through direct shoot bud differentiation on hypocotyl segments. *Sci Hortic* 99:89–98
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880
- Nas MN, Read PE (2004) Improved rooting and acclimatization of micropropagated hazelnut shoots. *HortScience* 37:1688–1690
- Neto VBP, De Botelho MN, Aguiar R, Silva E, Otoni WC (2003) Somatic embryogenesis from immature zygotic embryos of annatto (*Bixa orellana* L.). *In Vitro Cell Dev Biol Plant* 39:629–634
- Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S (2009) Agroforestry Database: a tree reference and selection guide version 4.0. World Agroforestry Centre, Kenya
- Parimalan R, Giridhar P, Gururaj HB, Ravishankar (2007) Organogenesis from cotyledon and hypocotyl-derived explants of japhara (*Bixa orellana* L.). *Acta Bot Croat* 66:153–160
- Perveen S, Varshney A, Anis M (2011) Influence of cytokinins, basal media and pH on adventitious shoot regeneration from excised root cultures of *Albizia lebbbeck* L. *J Forest. Res* 22:47–52
- Perveen S, Anis M, Aref IM (2012) In vitro morphogenic response and metal accumulation in *Albizia lebbbeck* (L.) cultures grown under metal stress. *Eur J For Res* 131:669–681
- Perveen S, Anis M, Aref IM (2013a) Lipid peroxidation, H₂O₂ content and antioxidants during acclimatization of *Abrus precatorius* to ex vitro conditions. *Biol Plant* 57:417–424
- Perveen S, Anis M, Aref IM (2013b) In vitro plant regeneration of *Albizia lebbbeck* (L.) Benth. from seed explants. *For Sys* 22:241–248
- Pinto G, Silva S, Loureiro J, Costa A, Dias MC, Araujo C, Neves L, Santos C (2011) Acclimatization of secondary somatic embryos derived plants of *Eucalyptus globules* Labill.: an ultra-structural approach. *Trees* 25:292–383
- Pospisilova J, Synkova H, Haisel D, Catsky J, Wilhelmova N, Sramek F (1999) Effect of elevated CO₂ concentrations on acclimation of tobacco plantlets to ex vitro conditions. *J Exp Bot* 50:119–126
- Rätsch, Christian (1998) *Enzyklopädie der psychoaktiven Pflanzen, Botanik, Ethnopharmakologie und Anwendung*. AT Verlag, Switzerland
- Rao MV (1992) Cellular detoxifying mechanism determines age-dependent injury in tropical plants exposed to SO₂. *J Plant Physiol* 140:733–740
- Rogers RB, Smith MAL (1992) Consequences of in vitro and ex vitro root initiation for miniature rose production. *J. Hortic. Sci.* 67:535–540
- Saha A, Ahmed M (2009) The analgesic and anti-inflammatory activities of the extract of *Albizia lebbbeck* in animal model. *Pak J Pharm Sci* 22:74–77
- Saha S, Kader A, Sengupta C, Ghosh P (2012) In vitro propagation of *Ocimum gratissimum* L. (Lamiaceae) and its evaluation of genetic fidelity using RAPD Marker. *Am J Plant Sci* 3:64–74
- Serret MD, Trillas MI, Araus JL (2001) The effect of in vitro culture conditions on the pattern of photoinhibition during acclimation of gardenia plantlets to ex vitro conditions. *Photosynthetica* 39:67–73
- Shang AQ, Cai H, Yan XJ, Hu HZ, Zhao LJ (2006) Plant regeneration from in vitro cultured hypocotyl explants of *Euonymus japonicas* Cu zhi. *Agric Sci China* 5:196–201
- Singh AN, Raghubanshi AS, Singh JS (2004) Comparative performance and restoration potential of two *Albizia* species planted on mine spoil in a dry tropical region of India. *Ecol Eng* 22:123–140
- Tiwari P, Pathak M (2012) In vitro regenerative capacity of two explants, hypocotyl and leaf of *Psorelea Corylifolia* L. under various hormonal conditions. *Bionano Frontier* pp 313–316
- Van Huylenbroeck J, Debergh PC (1996) Impact of sugar concentration in vitro on photosynthesis and carbon metabolism during ex vitro acclimatization of *Spathiphyllum* plantlets. *Phys Plant* 96:298–304
- Van Huylenbroeck JM, Piqueras A, Debergh PC (2000) The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated *Calathea* plants. *Plant Sci* 155:59–66
- Varshney A, Anis M (2012) Improvement of shoot morphogenesis in vitro and assessment of changes of the activity of antioxidant enzymes during acclimation of micropropagated plants of desert teak. *Acta Physiol Plant* 34:859–867
- Xiao Y, Kozai T (2004) Commercial application of a photoautotrophic micropropagation system using large vessels with forced ventilation: system configuration, plantlet growth and production cost. *Hortscience* 39:1387–1391
- Yang J, Yu T, Cheng Y (1996) Transgenic papaya plants from Agrobacterium-mediated transformation of petioles of in vitro propagated multishoots. *Plant Cell Rep* 15:459–464