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In vitro propagation, micromorphological studies and ex vitro rooting of *Alternanthera philoxeroides* (Mart.) Griseb.: an important aquatic plant

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Abstract In vitro propagation methods using ex vitro rooting were developed for *Alternanthera philoxeroides* using nodal segments procured from mature plants. The explants were surface sterilized with 0.1 % $HgCl_2$ solution and inoculated on semisolid Murashige and Skoog (MS) medium containing various concentrations and combinations of plant growth regulators. All the explants responded, and maximum 8.0 ± 0.4 shoots per node with 4.7 ± 0.7 cm average length were induced from nodal meristems on MS medium supplemented with 2.0 mg/L 6-benzylaminopurine (BAP). The shoots were further multiplied (23.8 ± 1.9 shoots per explant) by repeated subculture of freshly induced shoots on MS medium augmented with 1.0 mg/L each of BAP and Kinetin, 0.1 mg/L indole-3-acetic acid (IAA) and additives. About 85 % shoots were rooted (18.0 ± 0.8 roots per shoot) in vitro on half-strength MS medium fortified with 1.5 mg/L indole-3-butyric acid (IBA). Maximum 96 % shoots were rooted using ex vitro methods, and 7.3 ± 1.0 roots per shoot induced with IBA (300 mg/L). The in vitro as well as ex vitro rooted plantlets were acclimatized successfully in the greenhouse. Biologically significant developmental changes were observed during acclimatization of plantlets, particularly in leaf micromorphology in terms of changes in stomatal frequency and stomatal index. This knowledge helps in understanding the response of the plants toward changed environmental conditions during hardening process. This is the first report on micropropagation of *A. philoxeroides* using ex vitro rooting method which can be used for the large-scale multiplication of this medicinally important aquatic plant.

Keywords *Alternanthera philoxeroides* · Ex vitro rooting · In vitro regeneration · Micromorphology

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Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog's (1962) medium
NAA	α-Naphthalene acetic acid
SFPD	Spectral flux photon density

Introduction

Alternanthera philoxeroides (Mart.) Griseb. is a perennial aquatic to semiaquatic, polymorphic herb that belongs to the family Amaranthaceae. This herb is native to the Tropical America and distributed throughout the India. *A. philoxeroides* is commonly known as alligator weed or Malancha shak in India. It has branched stem which grows up to 4 m in length. The leaves are simple and the roots occur at nodes when exposed to moist conditions. The plant blooms during the months March–June, and the inflorescence is white in color with solitary-papery head (Fedorov 1969).

Alternanthera philoxeroides is well documented as a medicinal plant, leafy vegetable and a famine food (Jain et al. 2006). The important bioactive compounds with therapeutic interest isolated from this plant are saponins, glycoside, tannin, essential amino acids, kaempferol, ferulic acid, salicylic acid and chlorogenic acid (Bhattacherjee et al. 2014). The aerial parts are rich in iron, vitamin A, proteins and dietary fibers (Dutta 2015).

The whole plant is used to treat anemia, malaria, asthma, postnatal complaints, prolapsus ani, fistulas ani, diarrhea, dysentery and puerperal fever (Rahman and Gulshana 2014). It contains carotene in abundance and used to cure night blindness and hazy vision. The plant enhances secretion of milk in new mothers and used as a remedy against intestinal cramps. It has antioxidants, antimicrobial and α-glucosidase inhibition activities (Bhattacherjee et al. 2014; Panda and Misra 2011). *A. philoxeroides* is reported as a potent antiviral herb against dengue virus (Jiang et al. 2005), epidemic hemorrhage fever virus (EHFV) (Qu et al. 1993), influenza, human herpes virus 6 (Hui-wu et al. 1995) and human immunodeficiency virus (HIV) (Lousirirojanakul et al. 2003).

The cultivation of medicinal plants to meet the ever-increasing demand of raw drugs reduces pressure on natural population of plants. The conventional method of plant propagation through seeds is highly heterozygous with large variations in the growth and yield (Scher 2004). The plants propagated through vegetative methods preserve bacteria, fungi and viruses, which may affect the quality of medicinal plants and limit the success of mass multiplication and their commercial release (Chaturvedi et al. 2004). Biotechnological interventions can overcome these drawbacks. In recent years, micropropagation has emerged as a promising technique to propagate superior quality planting materials (Biotech Consortium India Ltd. 2007).

Stomata are important characteristic features of leaves for photosynthesis and transpiration. The in vitro heterotrophic environment such as medium substrates and culture conditions are responsible for poor development of internal tissues. The gradual changes in the structure of stomata in response to the altered environmental conditions have been summarized by many researchers (Pospisilova et al. 1999; Chirinea et al. 2012). The survival of tissue-cultured plants under field conditions is a limiting factor of the in vitro propagation technology. The foliar micromorphological study of in vitro and the field-

transferred plants could help to understand the response of plants toward changed conditions and improve the survival of plantlets in the soil conditions (Hazarika 2003; Yokota et al. 2007).

Rooting and acclimatization can be achieved simultaneously using ex vitro rooting methods of the in vitro regenerated shoots which increase the chances of survival of micropropagated plantlets in the field (Baskaran and Van Staden 2013). It reduces time, labor, energy and cost of production of micropropagated plantlets (Benmahioul et al. 2012). Though this plant species is medicinally important, it failed to attract the attentions of the researchers to develop efficient micropropagation protocol. Therefore, the present investigation is the pioneer attempt to develop efficient in vitro protocol for mass propagation, ex vitro rooting of shoots and foliar micromorphological (stomata) studies in *A. philoxeroides*. To the best of our knowledge, this is the first report on micropropagation of *A. philoxeroides* using nodal segments as explants and ex vitro rooting of in vitro regenerated shoots.

Materials and methods

Collection and sterilization of explants

The young, disease-free and healthy plants were collected from the east coast of the South India (Puducherry, Cuddalore, Nagapattinam and Karaikal districts) after conducting the field surveys. Plant specimens were identified by the French Institute, Puducherry. The fresh shoots were collected from the greenhouse grown plants and 2–3 cm long shoots with at least one node used as explants. The nodal explants were washed with running tap water. Special care was taken to sterilize the explants because the stem has hollowed pith. The cut ends of the explants were sealed with wax by giving dip (1–2 s) in molten (liquid) wax and surface sterilized with 90 % ethanol for 30–40 s. The explants were treated with 0.1 % (w/v) Bavistin (systemic fungicide, BASF India Ltd.) for 5 min and $HgCl_2$ (disinfectant, HiMedia, India) for 4–5 min. The treated explants were finally rinsed with autoclaved distilled water for 6–8 times under laminar air flow chamber. Both ends of the explants sealed with wax were removed using sterile scissors after sterilization and prior to inoculation.

Culture medium and in vitro environment

The nodal explants were cultured on MS basal medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose, 0.8 % agar and additives (50 mg/L ascorbic acid, 25 mg/L each of citric acid, L-arginin and adenine sulfate). The pH of the medium was adjusted to 5.8 ± 0.02 using 0.1 N HCl or NaOH prior to autoclaving. Ten ml of medium was poured in each culture tube (25 × 150 mm) and 40 ml in culture flask or culture bottles and capped with non-absorbent cotton plugs or polycarbonate cups. The medium was steam sterilized by autoclave at 1.06 kg/cm^2 pressure and 121 °C temperature for 15 min. The cultures were incubated in the culture room at 25 ± 2 °C and 50–70 % relative humidity (RH) under a photoperiod of 12 h at 40–45 µmol m^{-2} s^{-1} spectral flux photon density (SFPD) light intensity provided by cool white florescent tubes (Philips Ltd, India).

Induction and proliferation of shoots in vitro

The nodal explants were cultured on MS medium enriched by additives with different concentrations of BAP and Kin ranging from 1.0 to 5.0 mg/L to evaluate the appropriate cytokinin for bud breaking of *A. philoxeroides*. The cultures were further multiplied by repeated transfer of mother explants, and subculturing of in vitro produced shoots on fresh medium. The explants along with regenerated shoots were cultured on both semisolid and liquid medium with comparatively lower concentrations of cytokinins and auxins for multiplication of shoots. The MS medium supplemented with various concentrations and combinations of cytokinins (BAP and Kin ranging from 0.5 to 3.0 mg/L), and auxins was used for further proliferation of shoots. The cultures were incubated in culture room at 25 ± 2 °C temperature and 40–45 μmol m^{-2} s^{-1} SFPD light intensity for 12 h/day.

In vitro rooting of shoots

Around 5–6 cm long in vitro raised shoots were excised and transferred to full- and half-strength MS medium containing 3 and 1.5 % (w/v) sucrose and solidified with 0.8 % (w/v) agar. The medium was further supplemented with different concentrations of rooting hormones such as IBA and NAA ranging from 0.5 to 5.0 mg/L. Initially the cultures were maintained under diffused light (30 μmol m^{-2} s^{-1} SFPD) for a week and then shifted to normal culture conditions for 3 weeks. The well-established rooted plantlets were cautiously removed from the culture vessels, washed with water to remove the remains of agar and transferred to eco-friendly paper cups containing sterile soilrite® (a combination of perlite with peat moss and exfoliated vermiculite procured from KelPerlite, Bangalore, India) and irrigated with 1/4th MS macro-salts aqueous solution.

Ex vitro roots induction

To examine the induction of roots from the in vitro regenerated shoots under ex vitro conditions, the healthy shoots of 5–6 cm length were excised and the base of the shoots (3–4 mm) was externally treated with root inducing solutions of IBA and NAA at different concentrations (100–500 mg/L) for 5 min. The auxins-treated shoots were transferred to sterile soilrite® containing paper cups and moistened with 1/4th MS macro-salts aqueous solution. The setup was initially placed in the greenhouse to maintain high relative humidity (70–80 %), low temperature (26–28 ± 2 °C) at 55–57 μmol m^{-2} s^{-1} SFPD.

Hardening of in vitro and ex vitro rooted plantlets and field transfer

In vitro and ex vitro rooted plantlets were removed from the vessels after 4 weeks and shifted to nursery polybags (12 cm length × 6 cm diameter) containing soilrite®, organic manure and garden soil (1:1:1) and maintained under the greenhouse environment for 2 weeks. These were regularly irrigated with 1/8th MS macro-salts solution, where low temperature (26–28 ± 2 °C) and high humidity (70–80 %) were maintained near the pad section. The plantlets were shifted to high-temperature (30–32 ± 2 °C) and low humidity (55–65 %) zone of the greenhouse (fan section) after 2 weeks. The acclimatized and hardened plants were then shifted to nursery in another 4 weeks.

Foliar micromorphological study (stomata) of in vitro and field-transferred plants

Experiments were conducted to study the stomatal index (SI) of leaves of the plants under in vitro condition after 4th subculture of multiplication phase and after 6th week of field-transferred plants. Plants were randomly selected (10 from each stage of plantlets) between third and seventh leaves for the micromorphological experiments from both environments for all the experiments. To observe the types of stomata, epidermal peels were separated (Johansen 1940) and preserved in 50 % ethanol and stained in 1 % safranine (Loba Chemie, India) aqueous solution, and stomatal index was calculated as per standard methods (Wallis 1985). Photomicrographs were taken with the aid of microscope (Labomed iVu 3100, USA) and analyzed by the software Pixel Pro.

Statistical analysis of data

All the experiments in the present investigation were conducted with a minimum of ten replicates per treatment and repeated thrice. Observations were recorded after the time period of 4 weeks of interval. Data were subjected to analysis of variance , and the significance of differences was calculated by Duncan's multiple range test using SPSS software (version 16.0).

Results and discussion

Initiation of cultures

All the nodal explants cultured on the medium were responded within week with cent percent bud breaking. Nodal shoots are the favorable explants in the establishment of cultures and regeneration of shoots in *A. sessilis* (Gnanaraj et al. 2011; Das and Borua 2013). Singh et al. (2009) and Preetha et al. (2013) established the cultures of *A. sessilis* and *A. versicolor* using leaf and intermodal explants, respectively. Since the stem has hollow pith (Dutta 2015) except nodal region, extra care (sealed with wax) has been taken while sterilizing the explants to save the explants from the adverse effect of the sterilant.

Bud break from the nodal meristems was observed irrespective of the hormonal concentrations tested with the MS medium. But maximum response in terms of number of shoots (8.0 ± 0.4) per node and length (4.7 ± 0.7 cm) of the shoots was recorded on MS medium supplemented with 2.0 mg/L BAP (Fig. 1a, b; Table 1). 6-Benzylaminopurine and additives played crucial role in initial response of the explants. Comparatively, less number of shoots were recorded on MS medium containing higher concentrations of BAP (3.0–5.0 mg/L) and with all the concentrations of Kin. These observations are contrary to the findings of Preetha et al. (2013) in *A. versicolor*, Singh et al. (2009) and Das and Borua (2013) in *A. sessilis*. The superiority of BAP over other cytokinins for induction of shoots was also reported in *A. sessilis* by Subbarayan et al. (2010) and Gnanaraj et al. (2011).

Multiplication of shoots in vitro

The amplification of shoots was achieved through continuous subculture of in vitro regenerated shoots with mother explants on fresh medium after 4-week intervals. MS

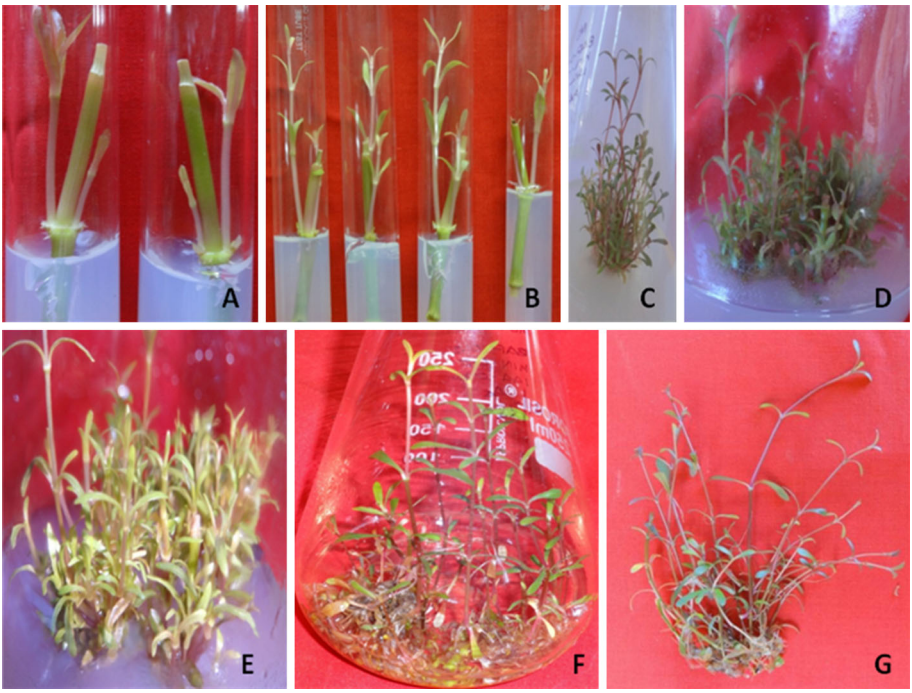

Fig. 1 **a**, **b** Induction of shoots from the nodal meristems of the explants. **c–e** Multiplication of shoots on semisolid MS medium. **f**, **g** Elongated shoots in liquid MS medium

Table 1 Effect of cytokinins (BAP and Kin) on induction of shoot buds from nodal stem explants of *A. philoxeroides*

Cytokinins (mg/L)		Response of explants (%)	Shoots number (mean ± SD)	Shoot length (cm) (mean ± SD)
BAP	Kin			
0.00	0.00	0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
1.00	–	79	1.8 ± 0.6^{a}	2.8 ± 0.3^{b}
2.00	–	100	8.0 ± 0.4^{c}	4.7 ± 0.7^{f}
3.00	–	84	2.4 ± 0.1^{b}	4.0 ± 0.5^{e}
4.00	–	72	2.0 ± 0.3^{b}	3.4 ± 0.0^{c}
5.00	–	66	2.0 ± 0.0^{b}	3.6 ± 0.2^{d}
–	1.00	60	0.6 ± 0.4^{a}	2.5 ± 0.7^{b}
–	2.00	76	0.8 ± 0.0^{a}	2.9 ± 0.1^{b}
–	3.00	59	1.6 ± 0.0^{a}	3.6 ± 0.5^{d}
–	4.00	54	2.0 ± 0.0^{b}	3.2 ± 0.2^{b}
–	5.00	48	2.0 ± 0.5^{b}	2.8 ± 0.4^{b}

Data were recorded after 4 weeks of inoculation. Mean separation was analyzed using SPSS software (ver. 16.0), and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

Table 2 Proliferation of multiple shoots with different concentrations of cytokinins + IAA 0.1 mg/L on MS semisolid medium after 2nd subculture of in vitro raised shoots

Cytokinins (mg/L)		Shoot number (mean ± SD)	Shoot length (cm) (mean ± SD)
BAP	Kin		
0.0	0.0	00.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.5	0.5	18.5 ± 1.4^{c}	3.2 ± 0.6^{b}
1.0	1.0	23.8 ± 1.9^{e}	5.7 ± 0.3^{d}
1.5	1.5	20.0 ± 0.6^{d}	5.0 ± 0.8^{c}
2.0	2.0	16.3 ± 1.0^{b}	5.3 ± 0.1^{c}

Data were recorded after 4 weeks of incubation. Mean separation was analyzed using SPSS software (ver. 16.0), and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

Table 3 Proliferation of multiple shoots in different concentrations of cytokinins + IAA 0.1 mg/L on MS liquid medium after 2nd subculture of in vitro raised shoots

Cytokinins (mg/L)		Shoot number (mean ± SD)	Shoot length (cm) (mean ± SD)
BAP	Kin		
0.0	0.0	00.0 ± 0.0^{a}	00.0 ± 0.0^{a}
0.5	0.5	16.3 ± 0.6^{b}	10.7 ± 0.9^{c}
1.0	1.0	18.7 ± 0.2^{c}	12.4 ± 1.4^{d}
1.5	1.5	16.0 ± 0.0^{b}	12.9 ± 0.3^{d}
2.0	2.0	15.6 ± 0.5^{b}	9.21 ± 0.8^{b}

Data were recorded after 4 weeks of incubation. Mean separation was analyzed using SPSS software (ver. 16.0), and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

medium augmented with lower concentrations of cytokinins enhanced the number of shoots. The combination of 1.0 mg/L each of BAP and Kin with 0.1 mg/L IAA proved most suitable concentration for shoots multiplication. Maximum 23.8 ± 1.9 shoots with 5.7 ± 0.3 cm length was achieved from each shoot clump within 2 weeks of incubation at 25 ± 2 °C temperature (Fig. 1c–e; Table 2), but the shoots were thin and did not exhibit further growth on this medium combination.

The healthy and lengthy shoots were produced when the shoot clumps were inoculated on liquid MS medium (without agar) with same concentrations and combination of growth hormones. The shoots were elongated enormously (12.4 ± 1.4 cm), when the shoot clumps transferred to the liquid medium (Figs. 1f, g; Table 3), which lead to the rapid depletion of the medium with respect to both organic and inorganic nutrients. The culture medium devoid of IAA was found to be less effective in further enhancement of shoots. Singh et al. (2009), Das and Borua (2013) and Preetha et al. (2013) enhanced multiple shoots formation and elongated the shoots of *A. sessilis* and *A. versicolor* on half-strength MS medium, respectively. Das and Borua (2013) multiplied maximum shoots of *A. sessilis* on MS medium with BAP 1.0 mg/L. The incorporation of IAA with BAP in the culture medium for better multiplication has also been reported by Singh et al. (2009) in *A. sessilis* and by Preetha et al. (2013) in *A. versicolor*. Additives in the culture medium played important role in prevention of shoots tip burning and defoliation.

Continuous subculture is also a critical event in maintaining cultures in vitro without losing vigor and growth. The cultures were subcultured regularly after 4 weeks of interval to maintain vigor and health of the shoots. Less number of shoots with stunted growth were observed when higher or lower concentrations of BAP were used than the reported concentration (Table 2). Earlier reports revealed the regeneration of shoots through callus induction in one different species of *Alternanthera* (Preetha et al. 2013; Das and Borua 2013), but direct shoots regeneration was achieved from the nodal regions of the explants in this study. The combination of cytokinins and auxins at lower concentrations suited well for the better multiplication of shoots without callus formation in present investigation.

Rooting of shoots (in vitro)

The long shoots measuring 10–12 cm in length were excised and cultured on half-strength MS medium with 1.5 % (w/v) sucrose to induce roots. Half-strength MS medium with 1.5 mg/L IBA was reported most suitable with maximum response (84.9 %) for in vitro rooting and 18.0 ± 0.8 roots with 4.82 ± 0.5 cm length induced within 20 days on this combination (Fig. 2a; Table 4). The role of reduced strength of MS salts concentration for better response during in vitro rooting was studied in number of plant species (Rathore et al. 2013a; Shekhawat and Manokari 2016b) which favor root initiation and elongation due to the scarcity of nutrients in the medium. Singh et al. (2009), Gnanaraj et al. (2011) and Das and Borua (2013) also reported rooting in *A. sessilis* on half-strength MS medium supplemented with IBA. Less number of roots (13.9 ± 0.6) were induced from the shoots when 2.0 mg/L NAA was used in the medium. Higher concentrations of auxins reported inhibitory to roots initiation from cut ends of the shoots. This might be related to the fact that higher concentrations of IBA and NAA are inhibitory to both root induction and

Fig. 2 **a** In vitro roots induction on half-strength MS medium augmented with IBA. **b** Ex vitro rooting of the shoots. **c** Plantlets in soilrite in paper cups. **d–f** Hardening of micropropagated plantlets in the greenhouse. **g** Hardened plant transferred to the field

Table 4 Effect of auxins (IBA and NAA) on in vitro root induction of shoots of *A. philoxeroides*

Auxins (mg/L)		Response	Roots number (mean ± SD)	Root length (cm) (mean ± SD)
IBA	NAA	(%)		
0.0	0.0	00.0	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}
0.5	–	42.6	9.50 ± 0.5^{d}	3.62 ± 0.3^{d}
1.0	–	69.0	13.6 ± 0.0^{g}	4.30 ± 0.0^{f}
1.5	–	84.9	18.0 ± 0.8^{h}	4.82 ± 0.5^{h}
2.0	–	75.0	11.6 ± 0.4^{f}	4.47 ± 0.3^{g}
3.0		72.7	9.41 ± 0.1^{d}	3.81 ± 0.0^{d}
–	0.5	40.5	5.25 ± 0.5^{b}	2.70 ± 1.0^{b}
–	1.0	55.3	9.27 ± 0.0^{d}	3.03 ± 0.5^{b}
–	1.5	70.0	13.9 ± 0.6^{g}	3.90 ± 0.2^{e}
–	2.0	63.8	10.6 ± 0.2^{e}	3.43 ± 0.0^{c}
–	3.0	59.6	7.20 ± 0.5^{c}	2.86 ± 0.6^{b}

Data were recorded after 4 weeks of incubation. Mean separation was analyzed using SPSS software (ver. 16.0), and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

Table 5 Effect of auxins (IBA and NAA) on ex vitro root induction from the shoots

Auxins (mg/L)		Response	Roots number (mean ± SD)	Root length (cm) (mean ± SD)
IBA	NAA	(%)		
0.0	0.0	00.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
100	–	76.8	2.5 ± 1.3^{b}	4.0 ± 0.5^{b}
200	–	80.3	4.8 ± 0.7^{d}	4.6 ± 1.0^{e}
300	–	96.2	7.3 ± 1.0^{f}	5.2 ± 1.3^{g}
400	–	85.7	6.0 ± 0.0^{e}	5.0 ± 0.7^{f}
500		70.9	5.4 ± 0.5^{d}	4.3 ± 0.2^{c}
–	100	50.8	3.5 ± 0.2^{c}	3.5 ± 1.0^{b}
–	200	69.4	4.0 ± 0.9^{c}	3.9 ± 0.6^{b}
–	300	72.5	5.9 ± 1.2^{e}	4.8 ± 0.2^{e}
–	400	53.8	5.4 ± 0.7^{d}	4.4 ± 0.9^{c}
–	500	50.3	5.0 ± 0.6^{d}	3.5 ± 0.4^{b}

Data were recorded after 4 weeks of incubation. Mean separation was analyzed using SPSS software (ver. 16.0), and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

elongation (Biradar et al. 2009). The increased concentrations of auxins stimulate natural ethylene production which is inhibitory to root formation.

Ex vitro rooting of in vitro regenerated shoots

The ex vitro rooting was achieved by pulse treating long (10–12 cm) and healthy shoots with highest response (96.2 %) using 300 mg/L IBA for 5 min (Fig. 2b). Maximum response in terms of rooting frequency, root number (7.3 ± 1.0) and length (5.2 ± 1.3 cm) was significantly considerable than the in vitro rooting experiments (Table 5). The

superiority of IBA on in vitro as well as ex vitro rooting over other auxins was reported by many researchers (Rathore et al. 2013b; Shekhawat and Manokari 2016a, b). The pulsed treated shoots were transferred to sterile soilrite® and moistened with one-fourth MS macro-salts aqueous solution for ex vitro roots induction in the greenhouse (Fig. 2c). The ex vitro rooting in micropropagation technique could overcome the problems during acclimatization prior to transplanting in the field conditions (Yan et al. (2010). Baskaran et al. (2009) and Shekhawat et al. (2015) have successfully achieved ex vitro rooting in *Melothria maderaspatana* and *Morinda citrifolia*, respectively. The ex vitro rooted plantlets have more vigor to tolerate stresses experienced during hardening stage.

Hardening and field transfer of plantlets

The rooted plantlets were cautiously taken out from the culture vessel, and the debris of culture medium removed with the help of a soft brush. The roots obtained by ex vitro method were reported to be having high tolerance against stress during hardening and transplanting (Rathore et al. 2013a). The hardened and acclimatized plantlets were transferred to nursery polybags (Fig. 2d–f) containing soilrite®, organic manure and garden soil and finally transferred to the natural environment. Hardened plants were successfully established in the soil under natural environmental conditions (Fig. 2g). Ex vitro rooted plantlets gained significant survival rate (92 %) than in vitro rooted plantlets.

Comparative foliar micromorphological (stomatal) studies

The stomatal peculiarities of in vitro and field-transferred leaves were compared (the study was not related to the mechanism of opening and closing of the stomata). Considerable decrease in stomatal frequency and stomatal index was reported from in vitro (24.42 ± 5.06) to field-transferred plants (16.50 ± 5.05) (Table 6). The increased stomatal index on the abaxial surface is probably an adaptation to water loss. Stomatal study explored the characteristic diacytic stomata with two subsidiary cells present right angles to the guard cells. Contiguous and anomocytic stomata were rarely observed in leaves procured from both the environments (Fig. 3). Dutta (2015) reported the presence of diacytic stomata in *A. philoxeroides*. Morphoanatomy of leaf has also been studied in *A.*

Table 6 Stomatal index of in vitro regenerated and field-transferred plants

Field no.	Stomatal index (SI) (mean ± SD)	
	In vitro plants	Field-transferred plants
1	25.35 ± 1.03^{c}	17.64 ± 0.30^{b}
2	22.97 ± 0.29^{a}	16.31 ± 0.49^{a}
3	26.47 ± 0.32^{b}	15.27 ± 0.26^{a}
4	24.35 ± 0.51^{c}	18.46 ± 0.73^{c}
5	25.92 ± 0.71^{c}	17.35 ± 1.32^{c}
6	22.66 ± 0.47^{a}	16.19 ± 0.61^{a}
7	21.73 ± 0.38^{a}	17.22 ± 0.29^{b}
8	28.57 ± 0.20^{e}	15.11 ± 0.54^{a}
9	24.65 ± 1.05^{c}	16.36 ± 0.20^{a}
10	21.62 ± 0.11^{a}	15.14 ± 0.31^{a}
Mean	24.42 ± 5.06^{b}	16.50 ± 5.05^{a}

Mean separation was analyzed using SPSS software (ver. 16.0), and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

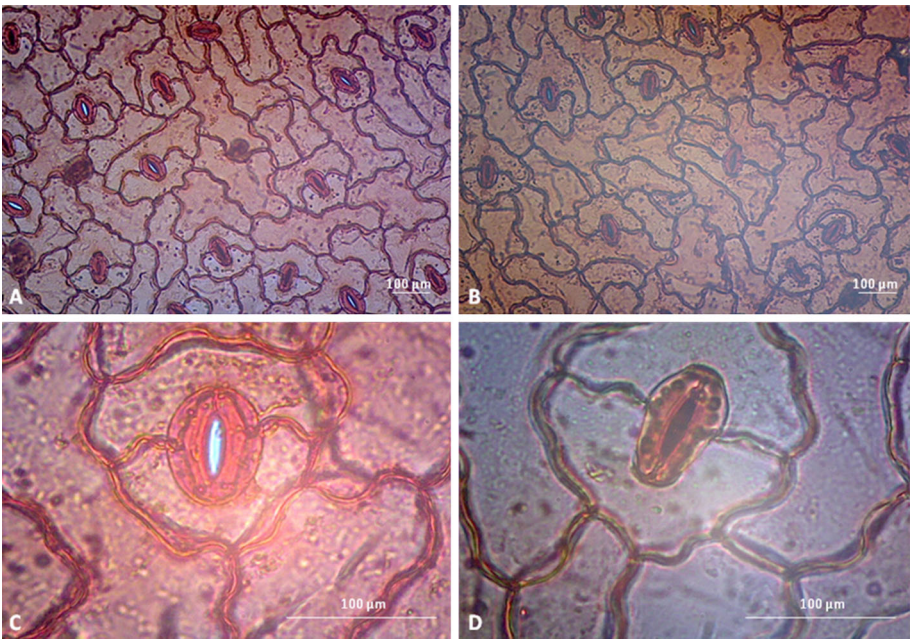

Fig. 3 Stomatal studies of *A. philoxeroides* leaves. **a**, **c** Stomatal pattern of the leaves of in vitro raised plantlets. **b**, **d** Stomatal pattern of the leaves of field-transferred plant (*scale bar* 100.0 µm)

pungens and *A. sessilis* by Gupta et al. (2012) and Debnath et al. (2014). Most of the stomata remained open under in vitro environment but not fully functional due to the heterotrophic culture conditions. Malfunctioning of stomata, poor stomatal functioning and altered morphological and anatomical characteristics of in vitro grown plants resulted in insufficient photosynthetic capacity to achieve positive carbon balance. The stomata function normal in field-transferred plants, and the opening and closing depends on the environmental factors. The gradual developments of stomata were achieved through subsequent and gradual hardening by ex vitro means and acclimatization prior to field transfer of in vitro regenerated plantlets.

Conclusion

The present study describes an efficient in vitro regeneration protocol for micropropagation of *A. philoxeroides*. It is highly reproducible system where 23.8 shoots per explants have been regenerated during multiplication stage. The shoots that were rooted ex vitro could save time, labor and energy in the in vitro production of plantlets. The results of micro-morphological studies of stomata could help in understanding the response of plantlets when these were transferred to the field environments. The protocol can be used for drug research and genetic transformation of *A. philoxeroides*.

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