Plant regeneration from organogenic callus and assessment of clonal fidelity in *Elephantopus scaber* Linn., an ethnomedicinal herb

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Abstract An efficient callus induction and plant regeneration system has been standardized for an ethnomedicinal plant, *Elephantopus scaber* Linn. Two explants *i. e.* seeds and leaf segments were used for callus induction. Murashige and Skoog (MS) medium supplemented with 5.0 μM 2, 4-dichlorophenoxy acetic acid (2, 4-D) and 0.5 μM kinetin (Kn) gave the optimum frequency (89 %) of callus induction from seed explant. The results showed that the highest response in terms of percent callus regenerating (91 %) and number of shoots (56) per culture was recorded on MS medium supplemented with 6.0 μM N_6 -benzylaminopurine (BA) and 1.5 μM α naphthalene acetic acid (NAA). The best rooting of regenerated shoots was obtained on half strength MS medium supplemented with 6.0 μM indole-3-butyric acid (IBA). On this medium, 100 % of the shoots produced roots with a mean number of 3.2 roots per shoot. The positive role of vesicular arbuscular mycorrhizae (VAM) along with potting mix has been well established in the present study. Of the various potting mix employed for plant acclimatization, the highest response of 100 % plant survival was noticed when autoclaved garden soil, sand (2:1) and VAM was utilized as potting mix. Inter-simple sequence repeats (ISSR) were used to establish the clonal fidelity of regenerated plantlets and the banding profiles from callus derived plants were monomorphic and similar to those of mother plant, thus ascertaining the true-to-type nature of these plants.

Keywords Acclimatization · Callus induction · *Elephantopus scaber* · Regeneration · Rooting · VAM

Introduction

The genus *Elephantopus* (Family - Asteraceae) comprises of nearly 30 species mostly distributed in tropical countries (Cabrera and Klein 1980). This plant is commonly known as “Elephant’s foot”. It is well known as a Chinese folk medicine since the whole plant is used for the treatment of various diseases (Hiradeve and Rangari 2014). In conventional medicine this plant is used to cure diabetes, enteritis, flatulence, diuresis and is also adopted as an analgesic, astringent and antiemetic agent (Daisy et al. 2007; Lee et al. 2008). In Ayurvedic system of medicine, this herb, along with a few others is utilized to treat neoplasm of different stages (Balachandran and Govindarajan 2005). The antiproliferative activities of *E. scaber* towards carcinoma cell lines *in vitro* and human cervical cancer *in vivo* have been reported (Ichikawa et al. 2006; Xu et al. 2006). The chief constituents of *E. scaber* include flavonoids, triterpenoids, steroids and sesquiterpene lactones (Geetha et al. 2012; Lee and Shyur 2012; Mei et al. 2012).

Due to the high medicinal value of this plant, there has been an increased use and unrestricted exploitation for medicinal purposes from the natural population with no regard to the future. Moreover, no cultivation attempt to replenish this plant has been reported. Conventionally, *E. scaber* is propagated through seeds, but seed dormancy and poor seed viability restricted seed germination under natural conditions (Parashurama et al. 2013). The situation becomes further aggravated by habitat destruction and climate change. Thus, alternative propagation methods especially *in vitro* techniques

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are necessary for the rapid multiplication and conservation of this plant. Plant tissue culture technique has been recognized as an alternative method of plant propagation and production of large number of uniform, elite clones in a limited period of time (Abraham et al. 2010; Kumar and Thomas 2012). This method is also useful for the production, characterization and use of valuable secondary metabolites for pharmaceutical use (Murthy et al. 2014; Cheruvathur and Thomas 2014). There are some preliminary studies on in vitro propagation of *E. scaber* using seed and leaf derived callus organogenesis (Jagadeesh Singh et al. 2004; Rout and Sahoo 2013). However, detailed investigations are necessary for the standardization and large scale production of this medicinal plant.

One of the most important stages of micropropagation is acclimatization. A biological approach to reduce the stress in plants during transplantation is the use of vesicular-arbuscular mycorrhizae (VAM) which can provide faster growth (Elmeskaoui et al. 1995). In this investigation, we report an efficient protocol for the mass multiplication based on shoot organogenesis from callus derived from seed and leaf explants and evaluation of the role of VAM on averting the transplantation shock in *E. scaber*.

Materials and methods

Plant material and callus induction

Mature, dry seeds of *E. scaber* were collected from field grown plants and used as explants. The seeds were removed from inflorescence, stored in muslin cloth and kept inside the fridge at 5 °C for about 1 week. The seeds were surface sterilized using 70 % ethanol for 1 min and subsequently with 20 % v/v sodium hypochlorite (NaOCl) solution for 5 min followed by 3 rinses in sterile distilled water. For leaf culture, immature leaves (about 15-days-old) were excised from in vitro grown shoots and cut into segments of about 0.5–1.5 cm in length. In vitro shoots were derived from nodal segments cultured on MS medium supplemented with 4.4 µM BA. Both seed and leaf segments were surface dried and inoculated horizontally on Murashige and Skoog (MS; Murashige and Skoog 1962) medium supplemented with various concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D; 1.0–6.0 µM) alone or in combination with kinetin (Kn; 0.2–1.0 µM) or α -naphthalene acetic acid (NAA; 0.2–1.0 µM). Data on percentage of seeds and leaves forming callus and callus growth were recorded 45 days of culture. Callus growth was evaluated by taking the fresh weight after 45 days. Fifty seeds and leaf segments were used per treatment with three replications. The friable calli derived from both explants weighing approximately 42–89 mg, were separated from the explants were isolated and subcultured

(approximately 40 mg per tube) at 4-week interval on MS medium supplemented with 2.0 µM 2, 4-D for multiplication.

Callus regeneration

For plant regeneration, organogenic, friable callus weighing 50 mg were inoculated on MS medium supplemented with various concentrations (2.0–10.0 µM) of BA, Kn or TDZ alone or in combination with NAA (0.5–2.0 µM). Callus with well differentiated shoots and leaves were scored as regenerating callus. Observations on the percentage of calli forming shoots and number of shoots and mean shoot length per responding cultures were recorded after 45 days of culture. The shoots were harvested 45 days after callus culture for rooting experiments. However, the shoots below 1.0 cm in length were excised and subcultured on MS medium containing 5.0 µM BA for shoot elongation.

Rooting and acclimatization of plants

Shoots measuring a length of 1.0–2.0 cm were used for root induction on half strength MS medium supplemented with various concentrations (2.0–8.0 µM) of NAA, indole-3-butyric acid (IBA) or IAA. Percent cultures responded, number of roots and mean root length were recorded after 45 days.

Well-rooted shoots were isolated from culture tubes after 45 days, washed thoroughly to remove agar and planted in thermocol cups (6 cm in diameter) containing potting mix. The cups were placed under high humidity in a glass house and irrigated with half-strength MS nutrient solution for 2 weeks followed by tap water. After 8 weeks, the established plants were transplanted to larger pots containing garden soil and manure.

VAM inoculation

The VAM fungus used in this study was *Glomus fasciculatum* and *Glomus macrocarpum*. Spore of *Glomus fasciculatum* and *Glomus macrocarpum* were separated from the soil taken from the rhizosphere of *Leucaena leucocephala* growing in the college campus. Sudan grass (*Sorghum halepense*) grown in earthen pots was inoculated with these spores for preparing mycorrhizal soil. Both species of *Glomus* were multiplied and maintained independently and rhizosphere soil of 6-month-old pot culture of Sudan grass was used as inoculum of VAM fungal species. Each gram of inoculum consisted of 30–40 spores plus hyphae, arbuscules, and vesicles of fungus, identified using the standard staining techniques, plus root segments of Sudan grass and soil particles. For establishment of mycorrhizal association with plantlets of *E. scaber*, about 1 g of soil inoculum of *G. fasciculatum* and *G. macrocarpum* isolate (containing hyphae, spores and colonized pieces of root) was individually mixed with the substrate and applied

below the roots, during transplantation (Thomas and Puthur 2004). Six different potting mixtures were used for acclimatization. They were autoclaved garden soil, autoclaved garden soil and VAM, autoclaved garden soil and sand (2:1), autoclaved garden soil, sand (2:1) and VAM, autoclaved garden soil and soil rite (1:2); autoclaved garden soil, soil rite (1:2) and VAM.

Inter simple sequence repeats (ISSR) analysis

ISSR analysis was performed to ascertain the clonal fidelity of in vitro regenerated plants with mother plant. Of the 25 ISSR primers used for the present study to test genetic stability of 17 randomly selected callus regenerated plants with mother plants (control), only 12 primers produced clear and reproducible bands (Table 1). Total genomic DNA of the callus derived plants and mother plant was extracted from young leaf tissue by using the cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson (1980). Quality and quantity of DNA was evaluated by gel electrophoresis and spectrometric inspection. The polymerase chain reaction (PCR) was performed in a volume of 25 μ l containing 100 ng genomic DNA, 500 nM ISSR primer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs and 1U Taq polymerase (Bangalore Genei, Bangalore, India). The amplification was carried out in a gradient thermal cycler (Eppendorf, Gradient thermal cycler, Germany). The sequential steps involved, 1 cycle of 2 min at 95 °C, 2 min at 53 °C and 2 min at 72 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 53 °C and 2 min at 72 °C. The last cycle was followed by 7 min extension at 72 °C. Amplification was repeated twice to confirm reproducibility of the results and the products were separated in a 2 % agarose gel using 1 $\times$ TAE buffer and stained with 2 μ l

ethidium bromide (EB; from 10 mg/ml stock). The fragments were scored as 1=present and 0=absent using a gel documentation system (Bio-Rad, Hercules, USA) that formed the binary matrix. The most intense and reproducible DNA bands were considered for analysis.

Culture conditions

Explants were cultured in test tubes (25 $\times$ 150 mm) for all steps of callus induction, regeneration, and maintenance. The pH of all media was adjusted to 5.8 with 1.0 N HCl or 1.0 N NaOH before autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 15 min. The cultures were maintained in a culture room with a 16 h/8 h light/dark photoperiod at 23 $\pm$ 2 °C. Light was supplied at intensity of 80 μ mol $m^{-2}s^{-1}$ supplied by two Philips TL 40W cool-white 154 fluorescent lamps. For shoot regeneration and rooting experiments, each treatment consisted of 20 tubes and all experiments repeated 3 times. The data were presented as mean and its standard deviation (mean $\pm$ SD) and comparisons of means were carried out with Duncan's multiple range test (DMRT; Duncan 1995) at 0.05 % significance.

Results

Callus induction

Although callus induction was observed in both explant types, seeds produced better callus induction than leaf segments. There was no callus induction on MS basal medium (control). The average time required for callus initiation varied from 12 to 17 days. The intensity of callus induction and percent response was significantly influenced by the type of explants and plant growth regulators (Table 2). On various concentrations of 2, 4-D (1.0–6.0 μ M), the frequency of explants producing callus varied from 38 to 56 % and 33–49 % in seed and leaf segment explants respectively. The optimum response (56 and 49 % in seed and leaf segment respectively) was observed on 5.0 μ M 2, 4-D. Similarly, the fresh weight of the callus showed variations depending on the type of explants and concentration of 2, 4-D (Table 2). Inclusion of NAA (0.2–1.0 μ M) to 2, 4-D medium did not found effective as the percent response and fresh weight did not show significant variation except at 0.2 μ M. However, a combination of Kn (0.2–1.0 μ M) and 2, 4-D (5.0 μ M) significantly improved the callus induction response. The highest callus induction frequency and fresh weight was obtained on MS medium supplemented with 5.0 μ M 2, 4-D and 0.5 μ M Kn. On this medium 89 and 62 % explants responded with a mean fresh weight of 89 and 74 mg in seed and leaf segment respectively (Table 2; Fig. 1a–c). The leaf segments enlarged in size and callus induction was mainly confined to cut regions only. The

Table 1 ISSR banding pattern of micropropagated and field-grown mother plant of *E. scaber*

Sl. no.	Primer	Primer sequence (5'–3')	Total no of scorable bands
1	ISSR-3	GAGAGAGAGAGAGAGAYC	14
2	ISSR-4	TGTGTGTGTGTGR	9
3	ISSR-6	ATGATGATGATGATGATG	8
4	ISSR-9	CTCCTCCTCCTCCTCCTC	7
5	ISSR-12	TCTCTCTCTCTCTCTCC	10
6	ISSR-18	GAGAGAGAGAGAGAGAYT	15
7	ISSR-21	CTCTCTCTCTCTCTCTRA	17
8	ISSR-22	GACAGACAGACAGACA	10
9	ISSR-23	AGAGAGAGAGAGAGAGV	13
10	ISSR-26	AGAGAGAGAGAGAGAGYA	12
11	ISSR-28	GAGAGAGAGAGAGAGA	9
12	ISSR-30	GACGACGACGACGAC	11
Total			135

Table 2 Effect of various plant growth regulators on callus induction from seed and leaf explants of *E. scaber* 45 days after culture

Plant growth regulators (μM)			% response		Fresh weight (mg)*	
2, 4-D	Kn	NAA	Seed	leaf	Seed	leaf
0.0	0.0	0.0	0.0g	0.0e	0.0f	0.0e
1.0			38f	33d	55 $\pm$ 5.6e	43 $\pm$ 5.8d
2.0			41e	38d	56 $\pm$ 4.9e	46 $\pm$ 6.5d
3.0			48e	41c	68 $\pm$ 4.7d	43 $\pm$ 5.5d
4.0			51d	47c	65 $\pm$ 5.9d	42 $\pm$ 4.8d
5.0			56d	49c	71 $\pm$ 4.9c	56 $\pm$ 6.5c
6.0			50d	38d	73 $\pm$ 7.4c	53 $\pm$ 8.3c
5.0	0.2		75b	56b	81 $\pm$ 8.8b	65 $\pm$ 8.9b
5.0	0.5		89a	62a	89 $\pm$ 7.3a	74 $\pm$ 7.2a
5.0	1.0		68c	51b	80 $\pm$ 6.8b	67 $\pm$ 5.4b
5.0		0.2	65c	50b	81 $\pm$ 9.7b	65 $\pm$ 6.9b
5.0		0.5	54d	52b	83 $\pm$ 7.6b	62 $\pm$ 4.7b
5.0		1.0	51d	55b	79 $\pm$ 6.6bc	61 $\pm$ 7.5b

* The values represent the means ($\pm$ SE) of three independent experiments. At least 50 explants were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

Medium: MS

seed produced callus all over the surface. The color of the callus remained white and no morphological differences were observed between calli derived from both the explants (Fig. 1d).

Shoot regeneration from callus

The callus formed from seed and leaf explants was subcultured on MS medium supplemented with various concentrations (2.0–10.0 μM) of BA, Kn or TDZ alone or in combination with NAA (0.5–2.0 μM) for shoot regeneration (Table 3). The calli enlarged in size and developed small green spots on its surface after one week of culture on regeneration medium. These spots later transformed in to green shoots and several shoots were appeared on the surface of the callus 3 weeks after culture on regeneration medium (Fig. 2a, b). Among the three cytokinins used individually, BA was comparatively superior in terms of percent explants producing shoots and mean shoot number. The optimum medium for shoot regeneration contained 6.0 μM BA, on which 56 and 50 % callus produced shoots with a mean number of 33 and 28 shoots per callus from seed and leaf segments respectively (Table 3). Enhanced regeneration was observed when NAA provided in the medium combined with cytokinin. The maximum callus producing shoots (91 and 71 % from seed and leaf derived callus), average

Fig. 1 Callus induction from seed and leaf segment explants of *E. scaber*. **a.** Seeds on MS medium supplemented with 5.0 μM 2, 4-D and 0.5 μM Kn at the time of culture. Bar=3.0 mm. **b.** Callus induction from seed explants on MS medium supplemented with 5.0 μM 2, 4-D and 0.5 μM Kn 45 days after culture. White, friable callus has been induced from the surface of the explant. Bar=4.0 mm. **c.** Callus developing from leaf explants on MS medium augmented with 5.0 μM 2, 4-D and 0.5 μM Kn 45 days after culture. Bar=0.7 cm. **d.** Callus multiplication on MS medium supplemented with 2.0 μM 2, 4-D. Bar=0.4 cm

number of shoot buds (56 and 43 from seed and leaf derived callus) per callus and average shoot length (1.4 and 1.2 cm) was obtained on MS medium containing 6.0 μM BA and 1.5 μM NAA 45 d after culture (Table 3; Fig. 2c, d).

Shoot elongation and rooting

The emerging shoots were excised from shoot clumps (Fig. 3a) and smaller shoots were subcultured on elongation medium (5.0 μM BA) and attained a mean length of 2.6 cm in 30 days. No rooting was observed on half strength MS basal medium. IBA was comparatively better than other auxins for root induction. Root induction was slow and root primordia appeared in 3 weeks after transfer to IBA. Among the different concentrations tested, maximum number of roots (3.2) per shoot and highest percentage of rooting (100 %) was achieved at 6.0 μM IBA after 45 days of culture (Table 4; Fig. 3b). However, the highest root length (3.2 cm) was obtained on half strength MS supplemented with 2.0 μM IBA.

Table 3 Effect of various concentrations of auxins and cytokinins on shoot induction from callus of *E. scaber*

Plant growth regulators (μM)				% response		Mean no of shoots*		Mean shoot length*	
BA	KN	TDZ	NAA	Seed	Leaf	Seed	Leaf	Seed	Leaf
0.0	0.0	0.0	0.0	0.0	0.0	0.0e	0.0e	0.0	0.0
2.0				32g	35f	24 $\pm$ 3.9c	19 $\pm$ 4.3d	0.7 $\pm$ 0.3	0.8 $\pm$ 0.3b
4.0				43f	37f	29 $\pm$ 4.3c	23 $\pm$ 4.1c	0.8 $\pm$ 0.4	0.7 $\pm$ 0.4b
6.0				56e	50d	33 $\pm$ 4.8c	28 $\pm$ 4.0c	0.9 $\pm$ 0.3	0.9 $\pm$ 0.4b
8.0				44f	41e	29 $\pm$ 5.3c	26 $\pm$ 4.6c	0.7 $\pm$ 0.4	0.8 $\pm$ 0.2b
10.0				40f	38f	22 $\pm$ 5.5c	22 $\pm$ 3.8c	0.8 $\pm$ 0.3	0.7 $\pm$ 0.3b
	2.0			33g	30f	29 $\pm$ 5.1c	21 $\pm$ 3.9c	0.9 $\pm$ 0.3	0.9 $\pm$ 0.4b
	4.0			36g	33f	32 $\pm$ 3.8c	29 $\pm$ 4.2c	1.1 $\pm$ 0.2a	0.8 $\pm$ 0.2b
	6.0			41f	36f	36 $\pm$ 4.5c	31 $\pm$ 4.1b	1.2 $\pm$ 0.3a	0.7 $\pm$ 0.2b
	8.0			48f	44e	41 $\pm$ 4.8b	37 $\pm$ 3.9b	0.9 $\pm$ 0.1b	1.0 $\pm$ 0.3a
	10.0			40f	37f	34 $\pm$ 5.0c	33 $\pm$ 3.8b	1.1 $\pm$ 0.3a	1.1 $\pm$ 0.4a
		2.0		38f	31f	21 $\pm$ 5.2d	35 $\pm$ 5.3b	0.8 $\pm$ 0.2b	0.9 $\pm$ 0.3b
		4.0		48f	38f	38 $\pm$ 3.8c	37 $\pm$ 4.9b	0.9 $\pm$ 0.4b	0.8 $\pm$ 0.4b
		6.0		43f	34f	31 $\pm$ 4.9c	35 $\pm$ 5.7b	1.3 $\pm$ 0.5a	0.6 $\pm$ 0.3b
		8.0		41f	30f	26 $\pm$ 5.1d	38 $\pm$ 3.8b	1.1 $\pm$ 0.4a	0.8 $\pm$ 0.2b
		10.0		40f	31f	22 $\pm$ 4.4d	33 $\pm$ 4.4b	1.4 $\pm$ 0.5a	0.9 $\pm$ 0.1b
6.0			0.5	73c	62c	21 $\pm$ 2.8d	30 $\pm$ 4.3b	1.2 $\pm$ 0.6a	1.0 $\pm$ 0.2a
6.0			1.5	91a	71a	56 $\pm$ 4.3a	43 $\pm$ 4.9a	1.4 $\pm$ 0.4a	1.2 $\pm$ 0.3a
6.0			2.0	82b	68b	42 $\pm$ 4.5b	35 $\pm$ 4.0b	1.0 $\pm$ 0.6a	1.1 $\pm$ 0.4a
	8.0		0.5	65d	55d	32 $\pm$ 4.9c	21 $\pm$ 5.3c	1.2 $\pm$ 0.4a	0.9 $\pm$ 0.3b
	8.0		1.5	69c	57c	36 $\pm$ 5.3c	29 $\pm$ 5.1c	1.1 $\pm$ 0.5a	0.8 $\pm$ 0.2b
	8.0		2.0	76c	63c	34 $\pm$ 5.5c	26 $\pm$ 4.4c	1.2 $\pm$ 0.6a	0.7 $\pm$ 0.3b
		4.0	0.5	58e	61c	38 $\pm$ 5.4c	24 $\pm$ 4.8c	1.3 $\pm$ 0.7a	0.9 $\pm$ 0.4b
		4.0	1.5	63d	63c	31 $\pm$ 4.3c	28 $\pm$ 4.6c	1.3 $\pm$ 0.5a	0.8 $\pm$ 0.3b
		4.0	2.0	66d	64b	39 $\pm$ 4.4c	22 $\pm$ 3.9c	1.2 $\pm$ 0.4a	0.7 $\pm$ 0.2b

* The values represent the means ($\pm$ SE) of three independent experiments. At least 20 cultures were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

Medium: MS; Culture period: 45 days

Transplantation and role of VAM in averting transplantation shock

The shoots having well developed roots were transferred to thermocol cups filled with six different potting mixtures i. e. autoclaved garden soil, autoclaved garden soil and VAM, autoclaved garden soil and sand (2:1), autoclaved garden soil, sand (2:1) and VAM, autoclaved garden soil and soilrite (1:2); autoclaved garden soil, soilrite (1:2) and VAM (Table 5). The plants were placed under high humidity in a growth room for 3–4 weeks (Fig. 3c) and watered every day. The survival percentage of plants in all the six potting mixtures was analyzed after 30 and 60 days of transplantation (Table 5). The plantlets transferred to garden soil showed 57 and 53 % survival rate. However, the addition of VAM to this potting mix increased the survival percentage to 70 and 63 after 30 and 60 days of transplantation. Similarly, 80 and 61 % plant survival was recorded when autoclaved garden soil and soilrite (1:2) was

used as potting mix after 30 and 60 days of transplantation. This response was further improved to 95 and 93 after 30 and 60 days by the addition of VAM to this mix. The highest plant survival rate (100 %) was obtained when autoclaved garden soil, sand (2:1) and VAM were utilized as potting mix (Fig. 3c). Microscopic assessment of roots confirmed the colonization by VAM. The acclimatized plants were transferred to field after 2 months. The VAM association significantly improved the plant growth and development as compared to other treatments.

ISSR analysis

Of the 25 ISSR primers used for the present study to test genetic stability of 17 randomly selected regenerants obtained through callus organogenesis, only 12 primers produced clear and reproducible bands (Table 1). These primers generated 135 distinct and scorable bands with an average of 11.25

Fig. 2 Various stages of callus organogenesis in *E. scaber*. **a.** Shoot induction from leaf derived callus on MS medium supplemented with Kn (8.0 μ M) and NAA (2.0 μ M) after 30 days of culture. Bar=0.2 cm. **b.** Organogenesis of seed derived callus on MS medium supplemented with 6.0 μ M BA and 1.5 μ M NAA 30 days after culture. Bar=0.5 cm. **c.** Same as in Fig. 2a 45 days after culture. Green shoots have developed from the callus. Bar=0.5 cm. **d.** same as in Fig. 2b 45 days after culture. Large number of well developed shoots were formed from the callus. Bar=0.5 cm

bands per primer, the highest (17) being against primer ISSR 21 and the lowest (8) against primer ISSR 6 (Table 1). All the bands generated by ISSR techniques were of monomorphic in nature and similar to those of the mother plant (Fig. 3d).

Discussion

In the present study, we used two explant types to induce callus *i. e.* the seed and leaf segments. The callus induction was explant-dependent and the response varied from 33 to 89 %. The seed explants exhibited better response than leaf segments. It has been observed from various previous reports that the explant source significantly influences the callus induction process. In *Brachypodium distachyon*, of the two explants (*i. e.* embryo and shoot tip) used for callus induction, the shoot explants produced better response than embryo (Thomas and Yoichiro 2011). In *Dorema ammoniacum*, various explants such as root, hypocotyls and cotyledon segments were used for callus induction and the best response was observed from root segments (Irvani et al. 2010). Similarly, in *Pseudarthria viscid* also callus formation was affected by the source of the explants and cotyledonary node was most responsive for callus induction followed by leaf explants (Cheruvathur and Thomas 2011).

In this study, 2, 4-D was found to be essential for callus induction as well as multiplication. However, the optimum callus induction was obtained on MS medium supplemented

Fig. 3 Rooting of shoots, field transfer and evaluation of clonal fidelity in *E. scaber*. **a.** An enlarged view of a shoot clump developed from the callus. Such shoots were used for root induction. Bar=0.8 cm. **b.** A rooted shoot on half strength MS medium supplemented with IBA (6.0 μ M). Note the healthy, well developed roots developing from the basal cut end of the shoot. Bar=1.0 cm. **c.** Six transplanted plants during acclimatization one month after transfer to autoclaved garden soil, sand (2:1) and VAM. Bar=2.5 cm. **d.** ISSR amplification profiles of callus derived plants and mother plant with primer ISSR 21, where L is molecular weight marker, M is mother plant (control), 1–17 plants derived from callus

with 5 μ M 2, 4-D and 0.5 μ M Kn. Our studies are in line with earlier report on callus induction in *E. scaber* where optimum callus induction was achieved on MS medium supplemented with 2, 4-D and Kn (Rout and Sahoo 2013).

Generally for callus induction and proliferation, the presence of a strong auxin like 2, 4-D is necessary. According to Bronsema et al. (2001) in maize (*Zea mays*) the minimum concentration necessary for callus induction is 1.0 μ M. 2, 4-D induced callus induction has been reported in species like *Eremochloa ophiuroides* (Yuan et al. 2009). In *E. scaber*, a combination of 2, 4-D and Kn was most effective for callus induction from seed and leaf segment explants. The high concentration of auxin in combination with low cytokinin induced callus induction has been reported in several species. In *Zoysia matrella* best callusing was obtained on MS medium

Table 4 Effect of various auxins on root induction from shoots on half strength MS medium 45 days after culture

Plant growth regulators (μM)			% response	Mean no of shoots*	Mean root length (cm)*
NAA	IBA	IAA			
0.0	0.0	0.0	0.0	0.0	0.0
2.0			30h	1.1 $\pm$ 0.4c	2.3 $\pm$ 0.4
4.0			45g	1.5 $\pm$ 0.8c	2.7 $\pm$ 0.6
6.0			36h	1.8 $\pm$ 0.7c	2.4 $\pm$ 0.6
8.0			33h	1.4 $\pm$ 0.4c	2.6 $\pm$ 0.8
	2.0		78d	2.2 $\pm$ 0.7b	3.2 $\pm$ 0.6
	4.0		92b	2.8 $\pm$ 0.6b	3.1 $\pm$ 0.7
	6.0		100a	3.2 $\pm$ 0.2a	3.0 $\pm$ 0.8
	8.0		89c	2.3 $\pm$ 0.6b	2.9 $\pm$ 0.6
		2.0	45g	1.8 $\pm$ 0.8c	2.2 $\pm$ 0.7
		4.0	56f	1.7 $\pm$ 0.7c	2.7 $\pm$ 0.8
		6.0	66e	2.1 $\pm$ 0.5b	2.0 $\pm$ 0.7
		8.0	63e	2.0 $\pm$ 0.6b	2.5 $\pm$ 0.6

* The values represent the means ($\pm$ SE) of three independent experiments. At least 20 cultures were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

augmented with 9.0 μM 2, 4-D and 0.09 μM Kn (Chai et al. 2011). However, in *Embelia ribes*, the highest callus induction was noted on MS medium supplemented with 4.5 μM 2, 4-D and 2.2 μM BA (Raghu et al. 2011).

In our study, the optimum medium for shoot regeneration from callus was 6.0 μM BA and 1.5 μM NAA. This is in

Table 5 The survival percentage of micropropagated plantlets of *E. scaber* after transplantation to various potting mixtures

Potting mix	No of plants transferred	% plants survived after 30 d*	% plants survived after 60 d*
Autoclaved garden soil	40	57ef	53de
Autoclaved garden soil+VAM	43	70e	63d
Autoclaved garden soil+Soilrite (1:2)	41	80d	61d
Autoclaved garden soil+Soilrite (1:2)+VAM	40	95b	93b
Autoclaved garden soil+Sand (2:1)	42	90c	81c
Autoclaved garden soil+Sand (2:1)+VAM	40	100a	100a

Data collected 30 d and 60 d after transplantation

* The values represent the means ($\pm$ SE) of three independent experiments

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

concordance with the results of Rout and Sahoo (2013) and Jagadeesh Singh et al. (2004) where successful callus organogenesis in *E. scaber* has been reported by using a combination of BA and NAA. Similar observations of the synergistic effect of BA and NAA induced shoot regeneration from callus has been reported in several species including *Ruta graveolens* (Ahmad et al. 2010) and *Spinacia oleracea* (Nguyen et al. 2013). BA is a strong cytokinin and is routinely used either alone or in combination with low amount of auxin for shoot induction from callus (Irvani et al. 2010). According to Sharma and Wakhlu (2003), the superior role of BA over other cytokinins may be due to the capability of plant cells and tissues to metabolise natural plant growth regulators more easily than synthetic hormones.

In this study, half strength MS medium supplemented with 6.0 μM IBA was most suitable medium for root induction. Profuse callus was produced at the basal cut end of shoots which inhibits the growth and elongation of roots when full strength MS medium was used. Half strength MS medium was preferred over full strength medium in many systems for root induction (Das et al. 2013). Although we used three auxins (NAA, IBA and IAA) for root induction, the rooting response was optimum in IBA containing medium. Our result is consistent with other reports where IBA induced optimum rooting has been reported (Ghaderi and Jafari 2014).

The low transplantation success and poor initial growth are the two major obstacles during the acclimatization of in vitro derived plants. Hence, the role of VAM in averting the transplantation shock was also investigated in the present study. In our study the presence of VAM along with various potting mix significantly improved the transplantation success. Hundred percent transplanted plantlets survived when the autoclaved garden soil, sand (2:1) and VAM was used as potting mix. One of the most significant advantages of using VAM is its role in developing a strong root system (Puthur et al. 1998). Similar results of the beneficial effects of incorporating VAM fungi into the potting mix for increasing the transplantation success have been reported in several plant species such as *Kigelia pinnata* (Thomas and Jos 2004) and *Curculigo orchoides* (Sharma et al. 2008). In majority of VAM associations, the fungi form a bridge between plant and soil for the flow of energy and matter (Cardon and Whitbeck 2007). The symbiotic association between the plant and fungi has great significance to soil ecosystem functions particularly nutrient dynamics (Delian et al. 2011). Fungi colonization in cortical region of the root may be either intracellular or extracellular (Gianinazzi-Pearson et al. 1996). The association of plant and fungi has several benefits to plants such as increased water and mineral uptake (Latef 2011), tolerance to abiotic stresses and disease suppression (Evelin et al. 2009).

The ISSR investigation of callus derived plants showed no variation compared to the mother plant and thus confirming that they are genetically uniform. Our results are in agreement

with other reports where the absence of genetic variation of in vitro generated plants has been reported in other systems (Cheruvathur et al. 2012). ISSR has high ability to expose polymorphism and present high potential to establish intra and inter genomic diversity as compared to other primers (Mishra et al. 2011; Wang et al. 2012).

Conclusions

We developed an efficient system for plant regeneration of *E. scaber* using seed and leaf segment-derived callus. The best callusing was obtained from seed explants. The highest shoot regeneration (91 %) was observed on MS medium supplemented with 6.0 μM BA and 1.5 μM NAA. Transplantation success of the in vitro plantlets was significantly improved by the addition of VAM to various potting mix. The true-to-type nature of the in vitro derived plants was confirmed by ISSR analysis. The indirect regeneration system developed here could be used for the rapid multiplication and ex situ conservation of this plant. Further, this protocol may be useful in the genetic transformation of this plant.

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