RAPID AND LARGE-SCALE *IN VITRO* PROPAGATION OF *ELEPHANTOPUS SCABER* L. - AN IMPORTANT MEDICINAL HERB

[1]P. Shanthi*, [2]T. Mayba Gnana Suky, [3]R. Gnana Deepa Ruby, [4]Sr. Anne Xavier and [5]P. Amudha
[1]Head and Assistant Professor, [2]Associate Professor, [3] Assistant Professor, [4]Former Head and Principal and [5]Research Scholar, PG and Research Department of Botany, Holy Cross College (Autonomous), Tiruchirappalli-02, Tamil Nadu, India.

ABSTRACT

Elephantopus scaber L. is an important herbaceous medicinal plant. It is used for analgesic, inflammation, pains and piles. The decoction of the roots and leaves is treated for diarrhea. The flowers are aphrodisiacs, tonic, and expectorant that help to cure biliousness, liver troubles and syphilis. In order to meet the increased demand, an efficient *in vitro* propagation of *E. scaber* was established. Shoot tip explants were cultured on MS basal medium fortified with different concentrations of BAP (1.0 – 5.0 mg/l) individually for multiple shoot bud regeneration. The highest frequency (93%) of multiple shoot regeneration with maximum number of shoots (95 shoots/ shoot tip) was noticed on MS medium supplemented with 3.0 mg/l BAP in second subculture. About 145 shoots/shoot tip were obtained after three subcultures on the same media composition. In the second experiment, leaf explants of *E. scaber* were inoculated into the MS medium supplemented with the combination of NAA (1.0 – 4.0 mg/l) and BAP (0.5 mg/l) for indirect organogenesis. The maximum percentage (89%) of callusing was observed at 0.5mg/l BAP and 2.0 mg/l NAA combination. The well developed leaf calli were transferred to regeneration medium containing different concentrations of BAP (1.0 – 5.0 mg/l) for shoot proliferation. A maximum of 89% of regeneration was obtained at 3.0 mg/l BAP in which 185 shoots were obtained after three subcultures. The regenerated shoots from both multiple shooting and indirect organogenesis were transferred to MS medium containing 3.0 mg/l of BAP and 1.0 mg/l GA_3 for shoot elongation. Elongated shoots (>2 cm) dissected out from the *in vitro* proliferated shoot clumps were cultured on half and full strength MS medium containing different concentrations of IAA (0.5 – 3.0 mg/l) for root induction. Highest frequency of rooting (100%) was noticed on half-strength MS medium augmented with 3.0 mg/l IAA. The rooted plantlets were successfully transferred into plastic cups containing sand and soil in the ratio of 1:2 and subsequently established in the greenhouse

Key words: *Elephantopus Scaber* L., medicinal plant, micropropagation, callus induction.

INTRODUCTION

The word medicinal plants lead to the thought of miraculous and supernatural cures. There are several thousands of drug yielding plants all over the world. The medicinal value of drug plants is due to the presence of some chemical substances (Chopra et al., 2007). *Elephantopus scaber* L., is a valuable herbaceous medicinal plant belonging to the family Asteraceae. It is useful in curing the poisoning effect due to bites of animals. It controls the disease in the blood, heart, urinary diacharges, bronchitis and small pox. The decoction of the roots and leaves is given in diarrhea. The bruised leaves with rice are given internally for swelling or pains in the stomach. The flowers are aphrodisiac, tonic, expectorant that helps to cure biliousness, liver troubles and syphilis. It is users in the preparation of laxative, analgesic, gripping, inflammation and tonic to brain (Kritikar and Basu, 1980). Bruised leaves boiled in coconut oil are applied to ulcers and eczema. The root extract is given to arrest vomiting. Root is powdered with pepper and applied for toothache (CSIR, 1949).

Further ruthless exploitation has resulted in drastic of this medicinal plant. Hence, it becomes imperative to establish a suitable protocol for rapid *in vitro* regeneration through tissue culture technique. Therefore, a suitable alternative method for large scale plant production within a short period is the use of in vitro culture technology. The micropropa-gation of plants through shoot tip culture allows recovery of genetically stable and true to type progeny. Regeneration via indirect organogenesis approach has also been attempted using leaf explants (Alagumanian et al., 2004). Higher callusing response from leaf let explants was obtained on medium supplemented with NAA (Chandra et al., 1993; 1996) and also high frequency callusing from leaf explant (Baburaj et al., 2000). In view of the above, present investigation attempts to obtain shoot regeneration from callus induced from leaf explants.

Although few tissue culture protocols have been reported in the recent past, there is no efficient regeneration protocol available for commercial scale production of this important medicinal plant. The major goal of this project was to develop an efficient protocol for large-scale production of *E. scaber* plants from an elite germplasm.

MATERIALS AND METHODS

Preparation of explants

E. scaber plants were collected from Herbal garden, Holy Cross College, Tiruchirappalli. For shoot bud induction, segments of shoot tip explants (1 – 2 cm) and leaf explants were excised from 3 months old plants and were washed in running tap water. Explants were surface sterilized with 0.1% bavistin for 15 minutes. Then, they were surface sterilized with 0.1% (w/v) mercuric chloride for 3 min followed by rinsing them for five times with sterile distilled water. Sterilized shoot tip and leaf explants were used for micropropagation and indirect organogenesis as described below.

Culture media and growth conditions

The culture medium consisted of MS (Murashige and Skoog's, 1962) salts, vitamins, 3% (w/v) sucrose and the pH of the media was adjusted to 5.7 with 0.1 N NaOH or HCl before adding of 0.8% (w/v) agar. Media (15 ml) were poured into 25 mm ×150 mm culture tubes (Borosil, Mumbai) and autoclaved at 121°C for 15 min. The cultures were incubated at 24 ± 2°C under 16/8 h (light/dark cycle) photoperiod and irradiance provided by cool-white fluorescent tubes (Philips, India).

Microropagation

Surface sterilized shoot tip explants were cultured on MS medium supplemented with different concentrations of BAP (1.0 – 5.0 mg/l) for multiple shooting.

In direct organogenesis

Callus induction

Leaf explants were inoculated into the MS medium supplemented with the combination of BAP (0.5mg/l) and NAA (1.0 - 4.0 mg/l).

Shoot organogenesis

The well developed organogenic leaf calli were transferred to regeneration medium containing different concentrations (1.0 - 5.0 mg/l) of BAP for shoot proliferation.

Shoot elongation

The regenerated shoot clumps were transferred to MS medium containing 3.0 mg/l of BAP and 1.0 mg/l GA_3 for shoot elongation.

Large scale production of shoot *buds*

For large scale plant production both in micropropagation and indirect organogenesis, the cultures were subcultured onto the fresh same media composition once in 3 weeks interval. This process was repeated for three subcultures (each 21 days) to examine the effect of subculture on

production of large scale shoot buds. *In vitro* regenerated shoot buds were harvested in three subsequent subcultures.

Rooting of elongated shoots from micropropagation and indirect organogenesis

The elongated shoots (>2.0 cm height) were transferred onto half and full strength MS medium fortified with different concentrations of IAA (0.5 – 4.0 mg/l) for root induction.

Hardening and acclimatization

Plantlets with well-developed roots were removed from the culture tubes and gently washed under running tap water to remove adhering medium. Subsequently, they were transferred to plastic cups containing vermiculite for hardening in culture condition. Then, the hardened plantlets were transferred to pot containing sterile sand and soil mixture in 1:2 ratio. The potted plantlets were initially maintained in the green house condition. After twenty days, the plantlets were successfully established in the field.

Statistical analysis

Each experiment was repeated there times and each treatment had 25 replicates. The number of explants exhibiting regeneration was identified and the number of shoots per explants was determined. The cultures were observed periodically and percent of response for shoot bud regeneration, multiple shoots development, percent of response for callus induction, shoot organogenesis and rooting. The data on number of shoots per explants were analyzed using one-way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at 5% level of significance (p= 0.05).

RESULTS AND DISCUSSION

Micropropagation

After 5 days of inoculation, shoot buds initiated from each shoot tip explants when they were cultured on MS medium (Fig. 1a) A maximum of 93% of response was observed at 3.0 mg l^{-1}BA. The regenerated shoots showed elongation on MS medium containing 3.0 mg l^{-1}BA and 1.0 mg l^{-1} GA^3. A maximum of 96 shoot explants in second harvest (Fig. 1b). In three subsequent harvests, 145 plantlets were produced per explants (Table 1). The shoots were dark green and length in 0.5 - 1.2cm. Ads were not effective in inducing multiple shooting. From this study it is inferred that, BA plays a key role in inducing multiple shoot production. Various studies had also showed shoot tip culture in *Arnica montana* on MS and B5 media (Conchou et al., 1991) *Gremlin arborea* on MC Cown's medium (Thirunavoukkarasu and Debate 1998). In our study BA was a promising cytokine for multiple shoot induction. Similar observations were reported in *Chlorophytum borivillianum* (Purohit et al., 1994) and in *Ocimum species* (Sitakanta Pattnaik et al., 1996).

In various studies, the shoots were produced in the presence of 2 cytokinins on *Macrotyloma uniflogum* (Varisai Mohamed et al., 1998). Study a maximum of 145 shoots was obtained in the presence of single cytokines (BA).

In direct organogenesis

Callus formation from leaf explants

Leaf explants showed swelling after 5 days of inoculation (Fig. 2a). Later on, the entire surface of the explants was covered by proliferated callus. The maximum percentage (89%) of callusing was observed at 0.5mgl^{-1} BA and 2.0 mgl^{-1} NAA Combination (Table 2). Similar findings were reported in *Solanum surattense* (Baburaj and Thamizhchelvan, 1991), *Gomphrena officinalis* (Mercier et al., 1992), *Cicer arietinum* (Barna and Wakhlu, 1994) and *Aeshynomence sps* (Roy and Mroginski, 1996). But in some reports explained MS with BA response for callus in *Datura metal* (Muthukumar and Arockiasamy, 1998**).**

Regeneration and elongation of shoots from leaf callus

The green compact calli were transferred to MS medium containing different concentrations 1.0 - 5.0 mgl^{-1} of BA for regeneration of shoots. After 15 days of inoculation the regeneration was observed (Fig. 2b). A maximum of 89% of regeneration was obtained at 3.0 mgl^{-1} BA. The regenerated shoot clumps were transferred to medium containing 3.0 mgl^{-1} and 1.0 mgl^{-1} GA_3 for elongation. The elongated shoots were harvested at 20 days interval. A maximum of 92 shoots callus was observed during second subculture (Fig. 2c) and the number of shoots decreased in following harvest. Thus from a single explants, a maximum of 185 shoots were obtained within 120 days (Table 3). Regeneration of plantlets from leaf callus has been reported in several medicinal plants like *Solanum trilobatum* (Shanthi and Annexavier, 2003), *Datura metal* (Muthukumar and Arockiasamy, 1998).

Rooting of regenerated shoots from micropropagation and indirect organogenesis

The regenerated shoots were excised and transferred to half and full strength MS medium fortifed with various concentrations 5.0 - 4.0 mgl^{-1} of IAA. Roots were initiated after 10 days of inoculation. Of these two types of media (half and full) used, a maximum of 100% rooting was observed at 3.0 $mg1^{-1}$ of IAA on half strength MS medium (Table 4). After 20 days of inoculation, 10 roots formed per shoot and it were long, linear light green in color with numerous lateral roots (Fig. 2d). The average length of the root was measured to be 3.5 - 4.5 cm long (Fig. 1d). The full strength medium did not show any response on rooting. The half strength medium was proved to be the best for rooting. Our observations are in accordance with the report on *Trachyspermum ammi* (Jyothi Sardana *et al.*, 1998), *Eclinta prostrate* (Kamala and Jegadeesan, 1999), and *Actinides delicious* (Kumar *et al.*, 1998). However, the combination of NAA, IAA and IBA were essential for rooting in *Holarrahena antidysenterian* (Golam Ahmed *et al.*, 2001) and *Enicosterma littorale* (Shanti and Anne Xavier, 2003).

Hardening of rooted plantlets

Rooted planets were transferred to plastic cups containing vermiculite for hardening (Fig. 1e & 2e). The cups were covered with perforated poly there bags to maintain humidity. One week of the plantlets transferred to green house condition with 80% healthy plants survivability.

CONCLUSION

In conclusion, the protocol describes rapid shoot regeneration from shoot tip and leaf explants, which can ensure stable supply of this medicinally valuable plant of any seasonal variation.

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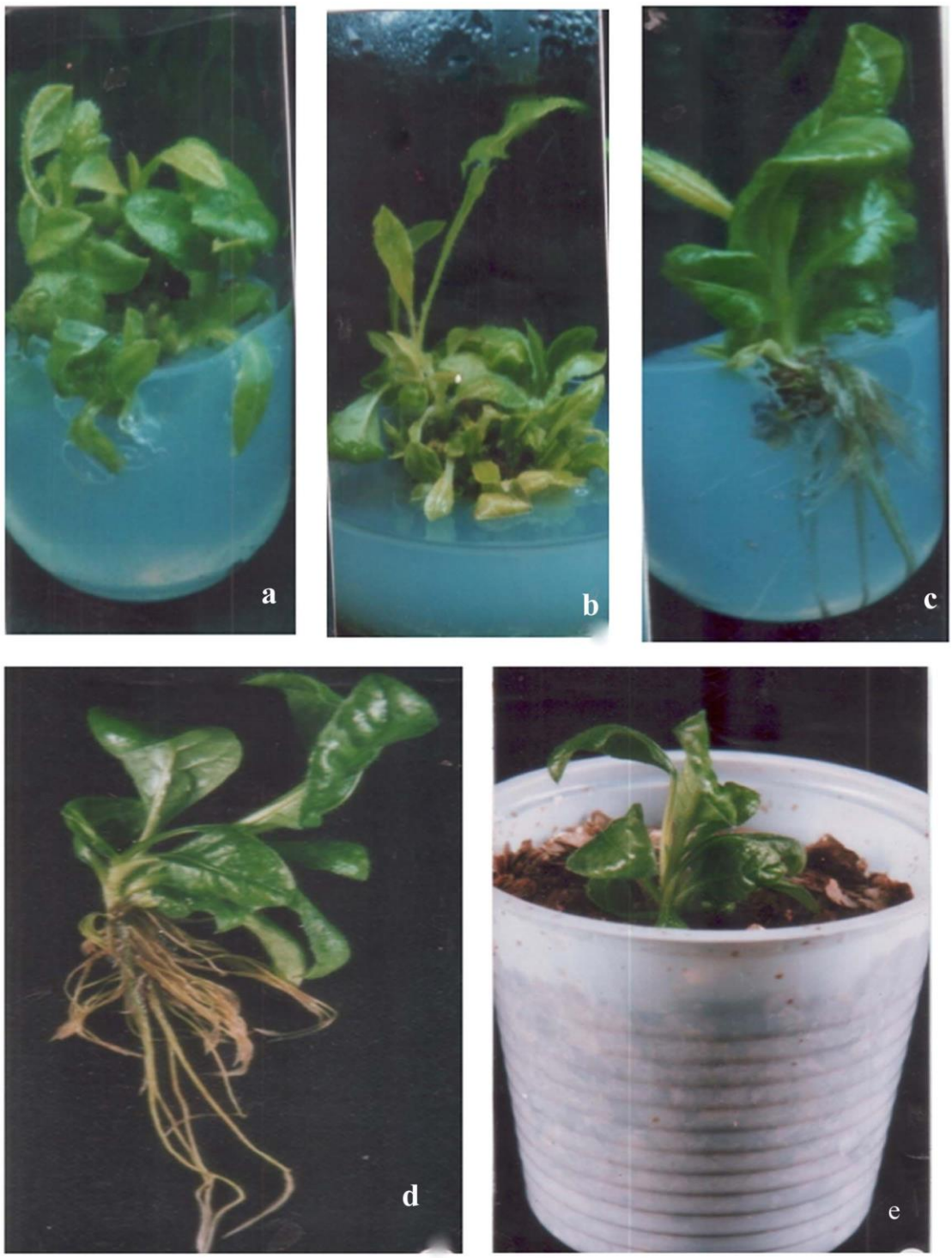

Fig. 1. a). Initiation of multiple shoots from shoot tip explant;
b). Proliferation of multiple shoots from shoot tip explant;
c) & d). Rooting of plantlets; e). *In vitro* hardended planlets

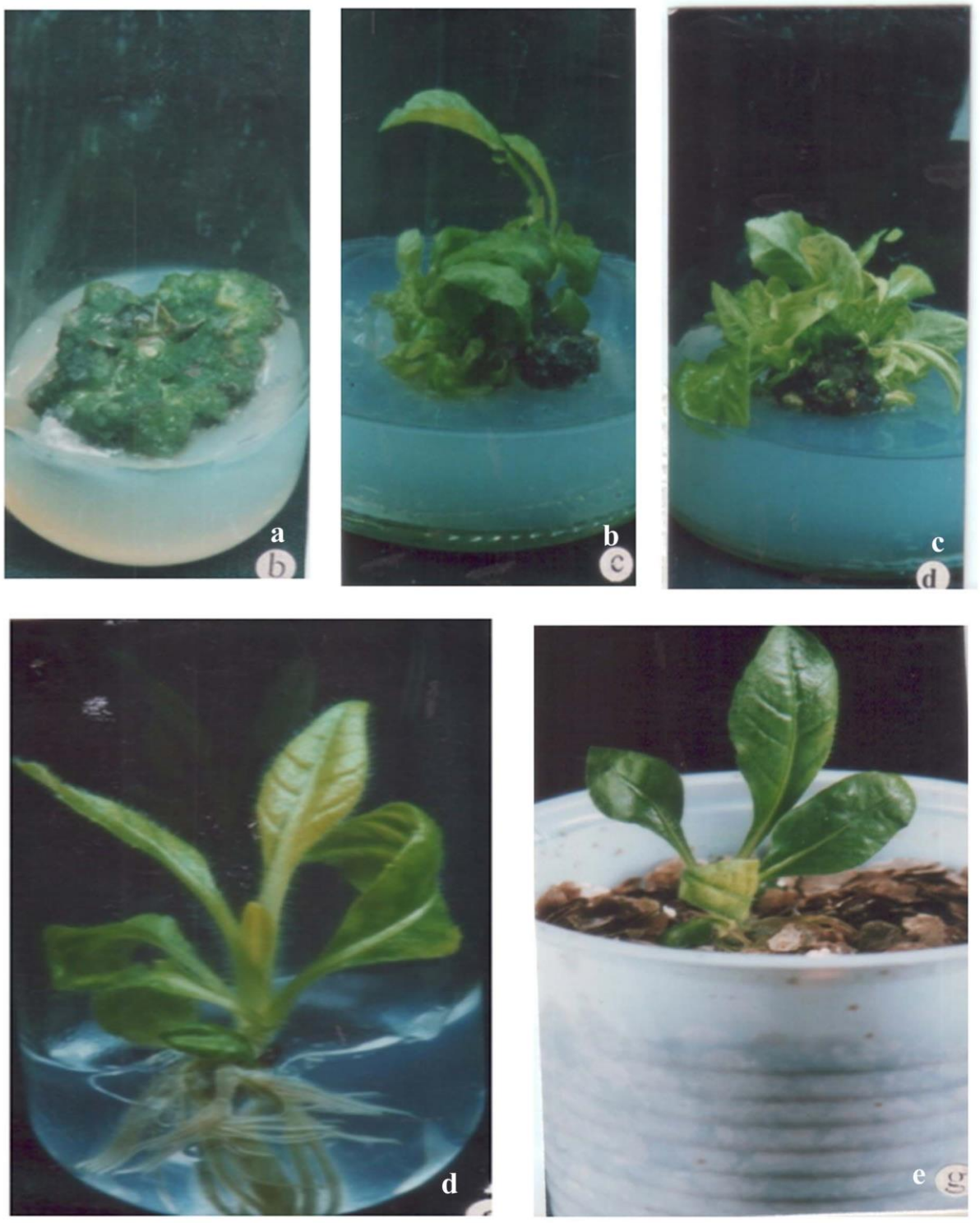

Fig. 2. a). Callus initiation from leaf explant;
b). Regeneration of shoots from callus derived from leaf explant;
c). Multiplication of regenerated shoots d). *In vitro* hardended planlets

Table 1

Effect of different concentrations (1.0- 5.0 mg/l) of BA on multiple shooting of *Elephantopus scaber* from shoot tip explants.

Hormone. Conc. (mg/l) BA	percentage of response	No of shoots / explant (Mean ± SD)			Length of shoots (cm) (Mean ± SD)
		Ist harvest	IInd harvest	IIIrd harvest	
1.0	39	4.1 ± 0.2	18.6 ± 0.4	11.3 ± 0.8	0.5 ± 0.4
2.0	61	8.0 ± 0.6	30.2 ± 0.1	12.3 ± 0.2	0.7 ± 0.1
3.0	93	15.8 ± 0.1	96.6 ± 0.4	34.0 ± 0.8	1.0 ± 0.6
4.0	72	7.4 ± 0.2	24.1 ± 0.1	15.1 ± 0.1	0.8 ± 0.2
5.0	34	5.2 ± 0.1	17.0 ± 0.2	12.3 ± 0.2	0.6 ± 0.1

Table 2

Effect of different concentrations (1.0- 5.0 mg/l) of NAA in combination with BA (0.5mg/l) on callus induction of *Elephantopus scaber* from leaf explants.

Hormone conc. (mg/l)		percentage of callusing	Morphology of callus
BA	NAA		
0.5	1.0	56	Hard, Compact and dark green in color
0.5	2.0	89	Hard, Compact and dark green in color
0.5	3.0	61	Hard, Compact and dark green in color
0.5	4.0	43	Hard, Compact and dark green in color
0.5	5.0	39	Hard, Compact and dark green in color

Table 3

Effect of different concentrations (1.0- 5.0 mg/l) BA on regeneration of shoots from leaf callus of *Elephantopus scaber*.

Hormone. conc. (mg/l)	percentage of response	No. of shoots / Callus (Mean± SD)			Length of shoots (cm)
BA		Ist harvest	IInd harvest	IIIrd harvest	(Mean±SD)
1.0	18	17.2 ± 0.1	29.0 ± 0.4	14.1 ± 0.8	0.6 ± 0.6
2.0	42	21.4 ± 0.2	47.1 ± 0.8	21.0 ± 0.9	0.6 ± 0.8
3.0	89	42.0 ± 0.1	92.1 ± 0.1	51.2 ± 0.4	1.0 ± 0.1
4.0	54	27.3 ± 0.4	49.4 ± 0.8	31.0 ± 0.1	0.8 ± 0.4
5.0	22	19.4 ± 0.1	35.1 ± 0.6	20.1 ± 0.4	0.8 ± 0.0

Table 4

Effect of different concentrations (0.5 -4.0 mg/l) of IAA in half strength MS medium on rooting of shoots from micropropagation and indirect organogenesis

Hormone. conc. (mg/l) IAA	Percentage of response	Number of roots /explant (Mean±SD)	Length of roots (cm) (Mean±SD)
0.5	-	-	-
1.0	-	-	-
1.5	43	5.1 ± 0.7	3.5 ± 1.1
2.0	95	8.3 ± 1.1	4.4 ± 0.3
3.0	100	10.0 ± 0.8	4.5 ± 0.8
4.0	-	-	-