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Micropropagation of *Tribulus terrestris* Linn.

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A simple and efficient method for high frequency direct shoot regeneration from nodal explants of *Tribulus terrestris* Linn. (Family — Zygophyllaceae), a medicinally important plant is described in this paper. Woody Plant Medium (WPM) supplemented with 4.0 mg/l BA was found to be most effective in inducing bud break, growth and also in initiating multiple shoot proliferation at the rate of six to seven micro-shoots per nodal explant after four weeks of culture. A high frequency multiplication rate was established by repeated sub-culturing. Excised shoots were rooted on MS basal medium without any growth regulators and got established after a hardening process with 80% survival. Micropropagated plants grew well under field conditions, attained maturity and flowered. No phenotypical differences were observed among regenerated plants.

Keywords: Direct shoot regeneration, Medicinal plant, Micropropagation, Nodal explants, Tissue culture, *Tribulus terrestris*, Woody Plant Medium.

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Introduction

Tribulus terrestris Linn. (Family — Zygophyllaceae) is a procumbent herb native to Mediterranean region and distributed throughout the tropical regions from sea level to 3500 m altitude. This plant is being used in Ayurveda and Chinese medicine for a variety of ailments[1, 2]. It is reported to be toxic against the causal organism of typhoid fever and hepatoprotective[3,4]. Due to the diuretic action of the fruits, it is found to be highly beneficial for urolithiasis[5]. It is also cooling, demulcent, tonic and aphrodisiac, promotes strength and digestive power and is useful in cough, difficult breathing, diabetes, piles, rheumatism, dropsy, burning sensation, impotence and heart diseases[6].

This plant is conventionally propagated through seeds. Based on the studies conducted on seed propagation, the germination percentage was very low under natural and laboratory conditions[7,8]. Due to its high medicinal value and increasing demand, *in vitro* studies have great importance in the propagation and genetic improvement programmes in this species. A little information is available on somatic embryogenesis and plant regeneration via callus in this plant[9]. Direct regeneration of shoots without any intermediate callus phase can yield clonal plants for large-scale propagation and for genetic transformation studies. This paper reports an efficient protocol for clonal propagation through axillary bud proliferation from mature nodal explants, followed by successful *ex vitro* establishment of micropropagated plants.

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Materials and Methods

Plant material and surface sterilization

Shoots were collected from 1 to 2 year old *T. terrestris* plants maintained in the Herbal Garden of Arya Vaidyasala, Kottakkal, Kerala, India. The excised double node shoots were washed thoroughly in water and Tween 20-a wetting agent (5 drops in 100 ml) for 10 min by constant agitation, leaves were removed and the shoots were cut into one-node segments and washed thoroughly with tap water. The explants were surface sterilized with 0.1% (w/v) mercuric chloride for 10 min followed by repeated washings with sterile distilled water. Under aseptic conditions the nodes were placed vertically on the culture medium.

Media and culture conditions

The nutrient medium consisted of full strength WPM[(Ref. 10)] with 3% (w/v) sucrose, 0.7% agar and supplemented with various concentrations of BA (0.5, 1.0, 2.0. 3.0, 4.0, 5.0, 6.0 mg/l) and Kinetin (0.5, 1.0, 2.0, 3.0 mg/l) individually for shoot proliferation. For rooting MS basal medium was used[11]. The *p*H of the

medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. The cultures were grown at 25±2°C under 12 hours photoperiod with a light intensity of 35-40 µmol/m^2/s supplied by cool white fluorescent lamps (Philips, India).

Shoot proliferation and rooting

Single node explants were cultured on to various combinations of shoot induction medium. All the cultures were transferred to fresh medium every 4 weeks for production of new shoots. The percentage of explants producing shoots and the number of differentiated shoots per explant was recorded periodically. Well differentiated micro-shoots were excised individually (3-4 cm long) from shoot clusters and inoculated in root induction medium.

Hardening and acclimatization

Rooted shoots were taken out without damaging the roots, washed to remove all traces of media and planted in thermocol cups filled with moist coarse sand. These were kept in a humid chamber maintained in a shaded nursery. The plants were taken out after 4 weeks and kept in the nursery for acclimatization. After one month, survived plants were transferred to field.

Data analysis

All the experiments were repeated thrice with ten explants for each treatment. Standard errors of means were calculated and statistically significant mean differences were determined by the least significant difference (LSD) test.

Results and Discussion

Woody Plant Medium (WPM) was effective than MS basal medium for giving favourable responses and hence it was used for multiplication trials. The nodal explants responded with 70% bud break within two weeks in woody plant WPM salts supplemented with 1.0 mg/l BA (Plate 1a). Only single shoot was initiated from each explant in this medium that also prohibits the elongation of shoots. After four weeks the nodes were excised from these cultures and subcultured for multiplication on woody plant medium supplemented with various concentrations of BA and kinetin (Table 1). Along with this, the nodes from mature plants were directly used for the multiplication experiments. Both of them responded similar in all the media, but the nodes from the *in vitro* cultures responded early. WPM with 0.5mg/l by BA did not show good response compared with higher concentrations. The higher concentrations of

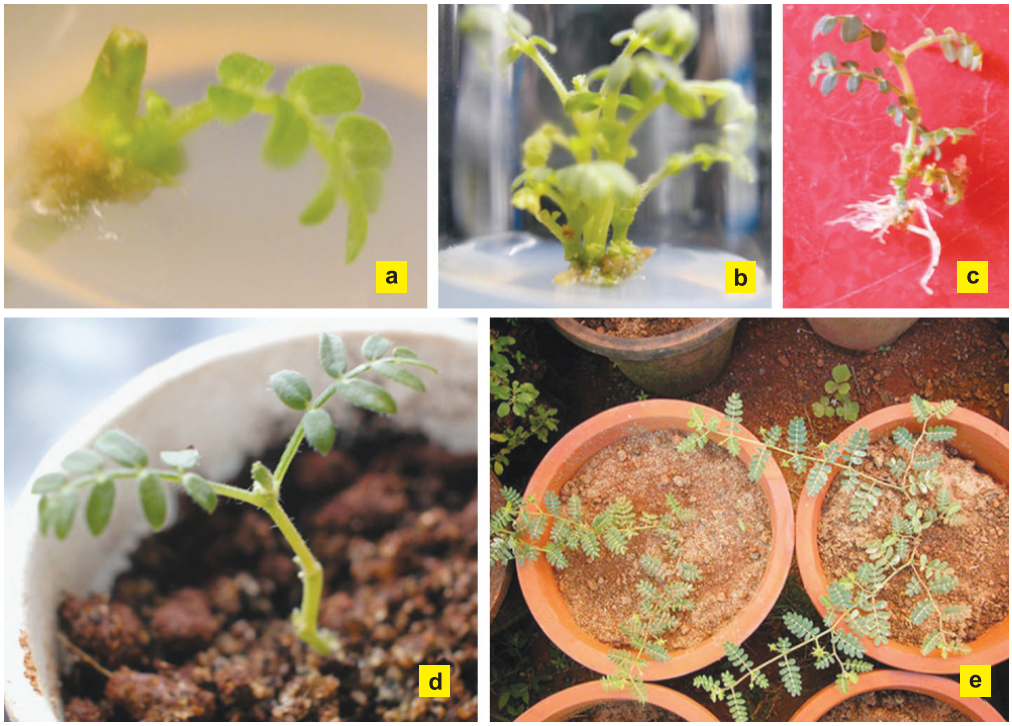

Plate 1 a-e—Micropropagation of *Tribulus terrestris*. a-Culture initiation on WPM supplemented with 1.0 mg/l BA; b-Shoot multiplication on WPM with 4.0 mg/l BA; c- Root induction on MS growth regulator free medium; d & e- Acclimatized plantlets.

Table 1— Effect of Woody Plant Medium with two different cytokinins individually on shoot induction from nodal explants of *Tribulus terrestris*

Cytokinins (mg/l)	Explant initiating shoots (%)	Number of shoots/explant (mean ± SE)	Shoot length (cm) (mean ± SE)
BA			
0.5	32.6[b]	0.3 ± 0.7[a]	0.9 ± 0.7[a]
1.0	62.8[d]	1.9 ± 0.7[b]	1.2 ± 0.3[a]
2.0	74.6[e]	2.3 ± 0.4[c]	1.5 ± 0.2[b]
3.0	82.4[f]	4.8 ± 0.9[e]	2.4 ± 0.6[c]
4.0	72.8[e]	6.3 ± 0.7[f]	2.6 ± 0.7[d]
5.0	48.6[c]	3.2 ± 0.3[d]	1.6 ± 0.8[b]
6.0	22.5[a]	1.7 ± 1.5[b]	1.1 ± 0.3[a]
Kinetin			
0.5	0.0	0.0	0.0
1.0	16.4[a]	0.9 ± 0.7[a]	1.8 ± 0.5[a]
2.0	32.6[b]	1.2 ± 1.2[a]	2.2 ± 1.3[a]
3.0	49.3[c]	1.3 ± 0.2[a]	2.3 ± 1.6[a]

Note: Values represent means of ± standard error of ten explants per treatment in three repeated experiments. Data were recorded after 28 days of culture.
Means followed by different letters within cytokinins (in the same column) differ significantly ($P< 0.05$) by LSD

BA showed increased multiplication but with lower rate of shoot elongation. From this study the maximum number (6-7 numbers in average) of shoots/node were obtained in WPM supplemented with 4.0mg/l BA (Plate 1b). Further increase in BA concentration resulted in a decline in the number of shoots with callusing. The significance of optimum concentration of BA for inducing maximum number of shoots has been reported in *Rotula aquatica* Lour., *Holarrhena pubescens* Wall. ex G. Don, *Tinospora cordifolia* (Willd.) Miers. ex Hook. f & Thomas. and *Aegle marmelos* Correa ex Roxb., etc[12-15].

For root induction, *in vitro* obtained shoots (2-3 cm long) were transferred to MS growth regulator free medium (Plate 1c). This result is in contrast with the observation of Mohan *et al*[16] in the same species that the auxins were very much needed for rooting. The induction of the root in the MS basal medium was also reported in some other species like *Withania somnifera* Dunal[17] and *Hemidesmus indicus* R. Br[18]. Plants with thin fibrous roots were established within 20 days of culture. The rooted plantlets were successfully transferred to the pot with 80% survival (Plate 1d & e).

Conclusion

The present study demonstrates a simple and efficient method for high frequency direct shoot regeneration from nodal explants of *T. terrestris*. The nodal explants responded with 70% bud break in the media containing WPM salts supplemented with 1.0 mg/l BA within two weeks of inoculation. Further, within 4 weeks, maximum number (6-7 numbers in average) of shoots/node could be obtained in WPM supplemented with 4.0 mg/l BA and these shoots could be rooted in MS growth regulator free medium. Following the procedures described here, approximately 6-7 well rooted plants could be produced developed from a single explant, within 2 months after culture initiation. By repeated subculturing of nodal explants to fresh medium large scale multiplication is possible. Use of nodal explant ensures clonal multiplication of selected species, and explant prepared from *in vitro* raised shoots can be employed as propagules for further multiplication. This efficient and easy to handle protocol can be used for propagating this plant at large scale and also for further genetic improvement programmes.

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