

Full Length Research Paper

# Enhanced callus induction and high-efficiency plant regeneration in *Tribulus terrestris* L., an important medicinal plant

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We described culture conditions for direct and indirect regeneration of Iranian *Tribulus terrestris* L. through epicotyl, hypocotyl and leaf explants. The explants were cultured on MS medium supplemented with different concentrations and combinations of auxin and cytokinin. The results indicated that the mean of callus induction was influenced by explant type and various phytohormones levels. The highest percentage of callus production occurred on MS medium containing 0.1 mg/l naphthalene acetic acid (NAA) and 1 mg/l 6-benzylaminopurine (BAP) from epicotyl explants (91.6%), 0.4 mg/l (NAA) + 2 mg/l (BAP) from hypocotyl explants (94.3%), and 0.4 mg/l (NAA) + 0.5 mg/l (BAP) for leaf explants (100%). Efficient shoot regeneration (22%) was achieved when the epicotyl explants were incubated on MS media amended with 0.1 mg/l (NAA) + 2 mg/l (BAP) and 0.4 mg/l (NAA) + 0.5 mg/l (BAP) within 14 days of culture. Maximum indirect shoot regeneration (28.4%) was achieved from green-yellowish calli derived hypocotyl explants on MS medium with 0.4 mg/l (NAA) + 2 mg/l (BAP) within 21 days of culture. Also, an *in vitro* regeneration system from nodal segments was developed on MS medium supplemented with different levels of 6-benzyladenine. Maximum number of shoots per nodal explants was developed on a medium containing 3 mg/l (BA) at the rate of 2.5 micro-shoots per nodal explants after 4 weeks of culture. Proliferated shoots were elongated in hormone-free MS medium and also, shoots were rooted on MS medium and MS with 2 mg/l indol-3-butyric acid.

**Key words:** *Tribulus terrestris*, plant regeneration, callus induction, plant growth regulators, MS (Murashige and Skoog) medium.

## INTRODUCTION

*Tribulus terrestris* L. (Zygophyllaceae) is a plant native of Mediterranean region, but now widely distributed in warm

regions of Europe, Asia, America, Africa and Australia (Frohne, 1999). It is known by several common names such as puncturevine, caltrop, goat head, bull's head, ground burr nut, and devil's thorn. Generally, *T. terrestris* has a considerable seed dormancy lasting over fall and winter months (Washington State Noxious Weed Control Board, 2001) with some seeds staying dormant for longer periods of time. One of the constraints of this conventional propagation is its very low germination. No reliable data are available about seed germination *in vitro* condition. Seed dormancy and low germination rate in *T.*

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**Abbreviations:** BAP, 6-Benzylaminopurine; IBA, indol-3-butyric acid; NAA, naphthalene acetic acid; BA, 6-benzyladenine; PGRs, plant growth regulators.

*terrestris* make this plant a suitable specimen for developing an *in vitro* regeneration method. Therefore, establishment of *in vitro* culture systems is an attractive approach to large-scale propagation, germplasm resource conservation, and possible future genetic modification of this plant. The occurrence of saponins, flavonoids, alkaloids, lignamides and cinammic acid amides has been reported in *T. terrestris* (Saleh et al., 1982; Bourke et al., 1992; Ren et al., 1994; Li et al., 1998). For this purpose, we have selected *T. terrestris* L., a herb of Zygophyllaceae, endowed with various medicinal properties whose fruits are used in curing urinary discharges, cough, asthma, pain, spermatorrhoea, ophthalmia, anemia, dysentery, skin and heart diseases; its leaves purify blood and used in aphrodisiac and roots are good for stomachic and appetizer (Sarwat et al., 2008). Its purported effects include increased luteinizing hormone release and thus testosterone production, increased sperm production, increased ejaculatory volume and increased libido. The original use of *T. terrestris* extract was as a tonic to treat sexual dysfunction. It is an important constituent of various medicinal preparations worldwide like Dashmularishta, Tribestan, etc., (Sarwat et al., 2008). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically plant tissue culture, are found to have potentials as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra and Ravishankar, 2002). The aim of the current research was therefore to determine the role of different combinations of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) on callus induction and shoot regeneration, and to find a suitable explant for *in vitro* multiplication of *T. terrestris*.

## MATERIALS AND METHODS

### Plant materials

Mature fruits of *T. terrestris* were harvested during July, 2010 from Kermanshah province of Iran.

### Seed germination

In the present study, a mixed method was used for seed germination and sterilization. Seeds were treated with captan (1%) as an antifungal agent for 30 min and then sterilized with 5% sodium hypochlorite for 5 min and 0.1% mercuric chloride for 3 min, followed by rinsing with sterile distilled water three times. The sterilized seeds were maintained in culture bottles each containing 25 ml of Murashige and Skoog (MS) and half MS basal medium with 0.7% (v/v) agar and 3% (g/l) sucrose and maintained at 32°C under 16 h light and at 27°C under 8 h dark condition. In another part of these experiments, seeds were cultured in a mixture of soil peat, clay, and sand in the same condition.

Explants such as epicotyl, hypocotyl and leaf were used from 2 weeks old plants grown in a greenhouse. Then, these tissues were surface-disinfected with 0.1% mercuric chloride for 5 min and

thoroughly washed with sterilized distilled water three times. Also, stems (approximately 1 cm in length) were harvested from greenhouse grown plants. The nodal segments (approximately 1 cm) were dissected from the stems and were kept under running tap water for about 20 min and treated with captan for 30 min, then rinsed with sterile distilled water.

### Media and culture conditions for callus and shoot development

The surface sterilized explants were inoculated on sterile medium. The culture media used throughout the experiments for tissue culture consisted of MS (Murashige and Skoog, 1962) basal salts supplemented with various concentrations and combinations of phytohormones like NAA, BAP, and indol-3-butyric acid (IBA) with 100 mg/l casein hydrolysate. The pH of media in all case was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were kept at 25°C under cool-white light with a 16-h photoperiod (40 to 60 mmol/m/s) and 50% relative humidity and sub-cultured on fresh media at 14 days interval. Callus and direct organogenesis were achieved by placing the mentioned explants on MS medium supplement with 0.1, 0.2, and 0.4 mg/l NAA in combination with 0.5, 1, and 2 mg/l BAP. The percentage of the explants producing calli and regenerated shoots were recorded on the basis of visual observation. Regenerated shoots were excised and transferred to hormone-free MS medium for rooting. Also for promoting development of the roots, the plantlets were transferred to MS Medium and MS medium supplemented with 2 mg/l IBA. Finally, plantlets were transferred to soil pots.

### Statistical analysis

The experiment was laid out as a factorial experiment (3×3×3) based on completely randomized design with three replications and each replication was made by using 3 Petri dishes per medium which contains 9 explants. In the first experiment, the rate of callus induction and shoot regeneration from epicotyl, hypocotyl and leaf explants were measured in front of different concentrations of NAA (0.1, 0.2 and 0.4 mg/l) and BAP (0.5, 1 and 2 mg/l) phytohormones. In another experiment, the rate of shoot initiation from nodal segments was investigated onto MS media supplemented with 3, 4 and 5 mg/l 6-benzyladenine (BA). An analysis of variance (ANOVA) was carried out for all traits and then mean comparisons were performed using Duncan's multiple range test (Duncan, 1955) at  $P=0.05$ . Traits such as percentage of callus, rate of shoot regeneration and root percentage were recorded in the experiments.

## RESULTS

Approximately 28% of collected seeds were germinated after 10 to 14 days *in vivo* condition. Also in another part of investigation, seeds were not germinated in MS basal medium and 1/2 MS with 0.7% (g/l) agar and 3% sucrose was maintained at 32°C under 16 h light and at 27°C under 8 h dark condition (*in vitro* condition).

No callus initiated from the different explants on hormone-free MS medium. The surface of the epicotyls, hypocotyls and leaves showed swelling and friability; pale green callus developed from the cut ends within 10 to 14 days of inoculation on MS medium supplemented with plant growth regulators (PGRs); eventually growing

**Table 1.** Analysis of variance (ANOVA) for callus formation and shoot regeneration of *Tribulus terrestris*.

SOV	df	MS (mean square)	
		Callus induction	Shoot regeneration
NAA	2	2087.57**	362.74**
BAP	2	38.62 <sup>ns</sup>	508.09**
NAA×BAP	4	566.13*	157.52*
Explant	2	5342.37**	303.23**
NAA×Explant	4	2072.5**	100.41**
BAP×Explant	4	1103.89**	169.72**
NAA ×BAP ×Explant	8	276.09 <sup>ns</sup>	154.48**
Error	54	206.12	2.41

ns, \* and\*\*: non-significant, significant at the 0.05 and 0.01 probability levels, respectively. S.O.V: stands for source of variation, and df: stands for degree of freedom.

**Table 2.** Effect of different concentrations of NAA and BAP on callus induction from different explants of *T. terrestris*.

Plant regulator concentration		Explant producing callus		
NAA	BAP	Epicotyl ( )	Hypocotyls ( )	Leaf ( )
0.1	0.5	65 <sup>cdefg</sup>	44 <sup>ghi</sup>	47.6 <sup>fghi</sup>
	1	91.6 <sup>abc</sup>	71 <sup>bcdefg</sup>	33 <sup>hi</sup>
	2	64.3 <sup>cdefg</sup>	65.3 <sup>cdefg</sup>	55 <sup>efgh</sup>
0.2	0.5	88.65 <sup>abcd</sup>	80.3 <sup>abcde</sup>	44 <sup>ghi</sup>
	1	90.65 <sup>abc</sup>	77.6 <sup>abcde</sup>	33.2 <sup>hi</sup>
	2	82.3 <sup>abcde</sup>	77.6 <sup>abcde</sup>	22 <sup>i</sup>
0.4	0.5	82 <sup>abcde</sup>	70 <sup>bcdefg</sup>	100 <sup>a</sup>
	1	84.8 <sup>abcd</sup>	60.4 <sup>defg</sup>	61.6 <sup>defg</sup>
	2	67.3 <sup>bcdefg</sup>	94.3 <sup>ab</sup>	73.3 <sup>abcdef</sup>

Value within a column followed by different letters are significantly different at the 0.05 probability level, analyzed by Duncan's multiple range test.

to cover the whole explants after 4 weeks of culture.

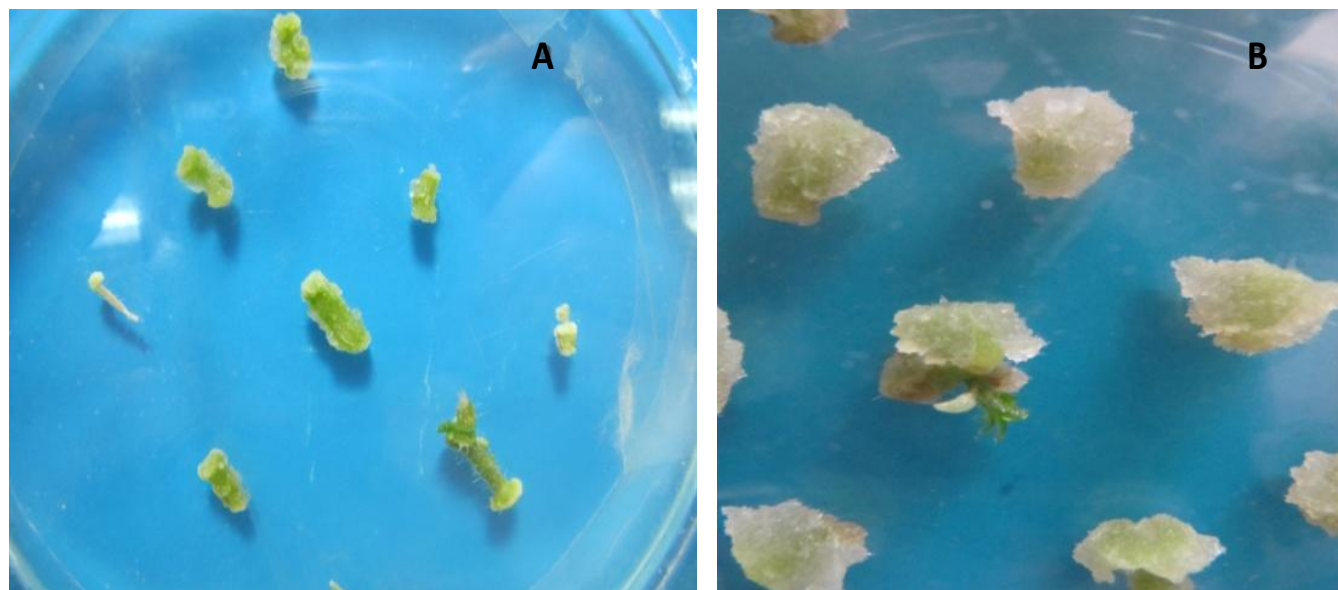
The rate of callus formation and regeneration were determined after 4 weeks. The callus induction and shoot regeneration were variable and depended on the combination of growth regulators applied and explant types. Significant and non-significant differences among main levels of NAA, BAP concentrations and their interactions have been represented in Table 1. The results showed that all interactions between the BAP and NAA concentrations were significant at the 0.05 probability level, except the interaction between explants×NAA×BAP for callus induction (Table 1).

The percentage of explants producing callus ranged from 22 to 100%. The highest percentage of callus induction occurred with 0.4 mg/l NAA and 0.5 mg/l for leaf explants (100%), with 0.4 mg/l NAA + 2 mg/l BAP for hypocotyls segments (94.3%) and for epicotyl explants

(91.6%) with 0.1 mg/l NAA + 1 mg/l BAP (Table 2).

Maximum shoot regeneration (22%) for epicotyl was obtained on MS media supplemented with 0.1 mg/l NAA + 2 mg/l BAP and 0.4 mg/l NAA + 0.5 mg/l BAP within 14 days of culture (Figure 1a; Table 3). Shoot regeneration for hypocotyl segments was observed within 21 days of culture, and maximum regeneration for this explant (28.4%) was measured on medium with 0.4 mg/l NAA + 2 mg/l BAP (Figure 1b; Table 3). As a result, according to Table 3, the leaf explant could not produce any shoots.

Also, the results of shoot development and shoot per explant from nodal explants showed that the maximum shoots initiation from nodal (54%) was obtained on MS medium containing 3 mg/l BA, and the highest number of shoots per nodal was developed on the same medium at the rate of 2.53 micro-shoots per nodal after 4 weeks of culture (Figure 2; Table 4).



**Figure 1.** Callus formation and *in vitro* shoot regeneration of *Tribulus terrestris* L., a) direct shoot regeneration for epicotyl on MS media supplemented with 0.1 mg/l NAA + 2 mg/l BAP and b) indirect regeneration of shoots from the hypocotyls on medium with 0.4 mg/l NAA + 2 mg/l BAP.

**Table 3.** Effect of different concentration of NAA and BAP on shoot regeneration from hypocotyl and epicotyl of *Tribulus terrestris* after 14 to 21 days of culture.

Plant regulator concentration		Explant producing shoot		
NAA	BAP	Epicotyl (%)	Hypocotyls (%)	Leaf (%)
0.1	0.5	0	0	0
	1	0	0	0
	2	22 <sup>b</sup>	10 <sup>d</sup>	0
0.2	0.5	0	0	0
	1	0	0	0
	2	0	0	0
0.4	0.5	22 <sup>b</sup>	0	0
	1	0	0	0
	2	15.3 <sup>c</sup>	28.4 <sup>a</sup>	0

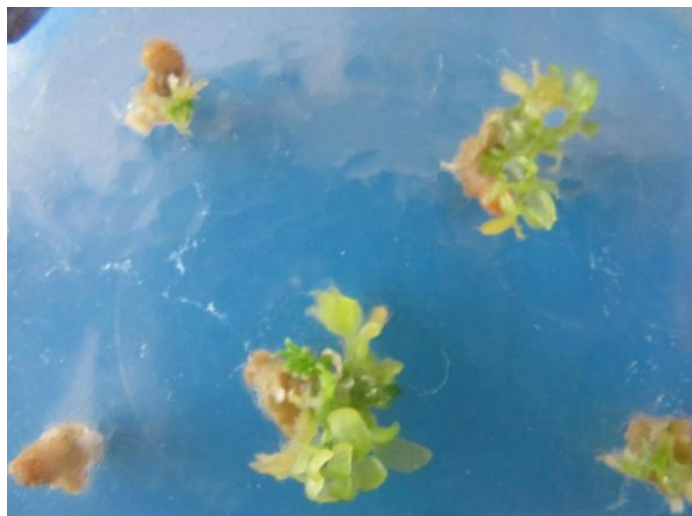
Value within a column followed by different letters are significantly different at the 0.05 probability level, analyzed by Duncan's multiple range test.

Shoots were excised from the explants and subculture on MS medium for the development of plantlets. To promote the development of roots, the plantlets were transferred to MS medium alone or supplemented with 2 mg/l IBA (Figure 3a). The rooting of regenerated and elongated shoots was observed on MS with IBA and without IBA after 14 to 30 days of culture and the percentage of rooted shoots fluctuated between 70 and 100%. Rooted plantlets were then transferred to a bed of sterile, moist sand and perlite (Figure 3b).

## DISCUSSION

Due to its high medicinal value and increasing demands, *in vitro* studies have great importance in the propagation and genetic improvement programmer in this species.

In nature, there are many mechanisms producing the crack of the tegumentary barrier in legumes, as temperature oscillation and the alternance of dry and wet periods (Quinlivan, 1971; Roston, 1978), bacteria and other soil microorganism action, and the chemical

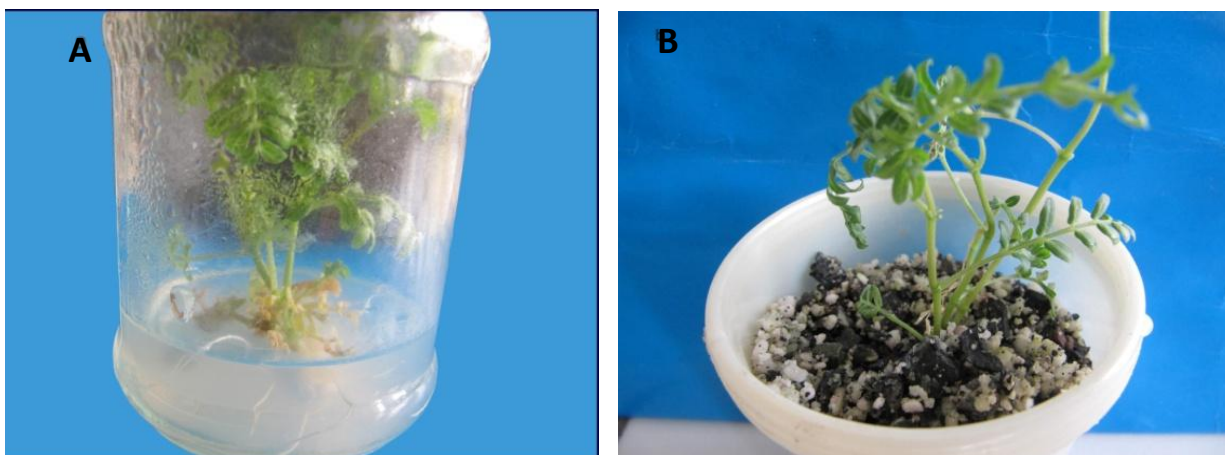


**Figure 2.** Shoot multiplication from node explants on MS medium with 3 mg/l BA after 4 weeks.

**Table 4.** Effect of BA (cytokinin) on shoot induction from nodal explants of *Tribulus terrestris*.

BA (mg/l)	Explant initiating shoots (%)	Number of shoots/explant
3.0	54 <sup>a</sup>	2.53 <sup>a</sup>
4.0	46 <sup>ab</sup>	2.06 <sup>a</sup>
5.0	30 <sup>b</sup>	2.53 <sup>a</sup>

Value within a column followed by different letters are significantly different at the 0.05 probability level, analyzed by Duncan's multiple range test.



**Figure 3.** a) Transferring of regenerated shoots to hormone-free MS medium for developing and rooting after 4 weeks and b) transferring rooted plantlets to soil.

scarification suffered through the herbivore digestive system (Pereiras et al., 1985). In the current study, it seems that soil bacteria and other microorganisms stimulate germination of *T. terrestris* but physical scarification of seed coat and chemical treatment (with

H<sub>2</sub>SO<sub>4</sub>) did not have any effect on germination.

Petkov (2010) reported that cyanobacteria and microalgae, being photosynthesizing organisms, enrich the soil with oxygen, thus increased seed germination of *T. terrestris*.

A review of the *T. terrestris* literature revealed that *in vitro* plant regeneration from cotyledonary leaves of young seedling (Ali et al., 1997) and somatic embryogenesis from stem-derived callus of *T. terrestris* (Mohan et al., 2000) required a few changes of media fortified with tested exogenous plant growth regulators.

Another study showed maximum embryo formation from leaf explants of *T. terrestris* on MS medium containing 5  $\mu\text{M}$  BA and 2.5  $\mu\text{M}$  NAA with 75 mg/l casein hydrolysate. The embryogenic callus culture of this species might offer a potential source for production of important pharmaceuticals (Nikam et al., 2009).

In general, the explants type, its orientation in the culture medium, and PGRs play a key role in regulating the differentiation process (Chawla, 2000). Selection of suitable explants at correct development stage plays a key role in the successful establishment of culture under *in vitro* conditions. Morphological integrity of explants along with the proper choice of plant growth regulators strongly influence induction of optimal callus and shoot regeneration (Khawar et al., 2005).

We also suggested that the balance of auxins and cytokinins is a decisive morphogenic factor. The present results showed that high concentration of BAP and low concentration of NAA was efficient for the induction of callus and subsequently shoots regeneration.

Reports of auxin and cytokinin combinations supporting organogenesis differentiation in other species have been well documented (Lisowska and Wysonkiska, 2000; Pereira et al., 2000; Pretto and Santarém, 2000; Tokuhara and Mii, 1993; Tisserat and Jones, 1999; Roy and Banerjee, 2003; Janarthanam and Seshadri, 2008).

In this study, leaf segments cultured on growth regulator free and MS medium supplemented with BAP and NAA failed to show any regeneration (organogenesis) response but remained green up to 2 weeks.

It seems the epicotyl explant of *T. terrestris* has a great organogenic potential for direct shoot regeneration. An important advantage of direct organogenesis is the potential for maintaining genomic stability of regenerated plants, whereas regeneration via an intermediated callus phase increases the possibility of somaclonal variations (Reddy et al., 1998; Tang and Guo, 2001).

In our research, optimum indirect shoot organogenesis (28.4%) was achieved from hypocotyl in green-yellowish callus with 0.4 mg/l NAA and 2 mg/l BAP within 21 days of culture. Indirect organogenesis is defined as the formation of calli on explants and subsequently the development of shoots (Sharp et al., 1986). Besides, in this investigation, the highest percentage of shoot regeneration was attained on a medium containing 0.1 mg/l NAA + 2 mg/l BAP in calli derived hypocotyl and epicotyl explants. It seems that, BAP phytohormone 2 mg/l has a great potential for shoot regeneration of *T. terrestris*. Motamedi et al. (2011) reported that the highest percentage of shoot regeneration was achieved

on a range of media supplemented with 0.1 mg/l NAA + 2 mg/l BAP from cotyledon explant of *Carthamus tinctorius* Dincer cultivar.

Cytokinins are very effective in promoting direct or indirect shoot initiation. To encourage the growth of axillary buds, and reduce apical dominance in shoot cultures, one or more cytokinins are usually incorporated into the medium at proliferation stage (George et al., 2007).

Safdari and Kazemitabar (2010) results showed that the treatment containing 10  $\mu\text{M}$  BAP was found to be the best one for shoot regeneration from nodal segments of *Portulaca grandiflora* L. The treatments with NAA in combination with BAP were found to be suitable treatments for callus production from leaf explants, and shoot regeneration.

Present study showed that in nodal culture, the percentage of response and number of shoot formed per node explants was highest on medium supplemented with 3 mg/l BA and 2.53 adventitious shoots were developed per explants. The multiplication rate is lower than the earlier reports. Also, Raghu et al. (2010) reported optimum shoot regeneration from nodal explant of *T. terrestris* in woody plant medium supplemented with 4 mg/l BA with six to seven micro-shoots per nodal explant after four weeks of culture. Das and Pal (2005) used 3 mg/l BAP (equivalent to 13.3  $\mu\text{M}$ ) for shoot regeneration from lateral buds of *Bambusa balcooa*.

BAP had a significant effect on induction of multiple shoot bud in *Operculina turpethum* although callus formation was concomitant with shoot induction. Optimum shoot multiplication using BAP is reported in a number of plants (Hiregoudar et al., 2006; Sharma, 2006; Alderete et al., 2006; Alam et al., 2010b).

Also, MS medium containing 12.5 mM BA alone was effective for inducing multiple shoots of *Balanites aegyptiaca* from nodal segments in 67% of cultures (Anis et al., 2010). The stimulating effect of BA on bud break and multiple shoot formation has been reported earlier for several medicinal woody plant species viz., *Acacia tortillis* ssp. *raddiana* (Sane et al., 2001), *Acacia koa* (Skolmen and Mapes, 1976), *Leucaena leucocephala* (Dhawan and Bhojwani, 1985), *Bupleurum kanoi* (Chen et al., 2006), *Syzygium alternifolium* (Sha Villi Khan et al., 1997), *Pterocarpus marsupium* (Chand and Singh, 2004a, b; Anis et al., 2005) and *Celastrus paniculatus* Willd. (Rao and Purohit, 2006).

We observed that rooting had the better response on MS medium without any PGRs after 30 days. It seems that rooting in the absence of auxins may be attributed to endogenous auxin hormones in the plant. This results was previously reported too, by other researchers in detail for other medicinal plants (Sudharsan and Hussan, 2003; Lu, 2005; Park et al., 2011).

Finally, the system described here for continuous production of *T. terrestris* via callus induction and regenerated plants without loss of morphogenetic

capacity could be a model for micro-propagation systems, not only for the large scale of medicine plants (especially Zygophyllaceae family) but also for genetic improvement of *T. terrestris* through transformation studies. The medicinal property of this plant is mainly due to the wide spectrum of alkaloids and saponins.

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