Journal of Medicinal Plants Research Vol. 2(9), pp. 242-245, September, 2008
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875© 2008 Academic Journals

Full Length Research Paper

In vitro propagation of *Crotalaria verrucosa* L. an important ethnobotanical plant

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Accepted 16 September 2008

An efficient and reproducible procedure for the large scale propagation of *Crotalaria verrucosa*, an important ethno botanical herb is described. Multiple shoots were produced from cotyledonary nodes of 15 days old aseptic seedlings on Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ) or benzyladenine (BA). Highest number (6.0) of shoots per cotyledonary node was recorded on MS medium with 0.1 µM thidiazuron after 4 weeks of culture. Proliferating shoot cultures were established by repeatedly sub culturing the original cotyledonary node on fresh medium (MS + 0.1 µM TDZ) after harvesting newly formed shoots. The shoot forming capacity of explants was influenced by the type and concentration of the growth regulator in the medium. Roots formed on excised shoots when these were cultured on quarter strength MS medium containing 1.0 µM indole-3-acetic acid (IAA). Plantlets were acclimatized in vermiculite and established in soil.

Key words: Cotyledonary node, *crotalaria*, micro propagation, thidiazuron.

INTRODUCTION

Crotalaria verrucosa, a member of the Leguminosae, is a much branched herbaceous plant found in tropical regions of Burma, the Malaya and China. Popularly known as `ghelegherinta' by the local yanadi tribes, villagers and herbalists, this ethno botanical herb is known to have medicinal properties. The juice of leaves is supposed to be efficacious in diminishing salivations and its leaves, tender stalks were prescribed by the doctors both internally and externally in case of scabies and impetigo (Watt, 1972). However, the natural regeneration potential of this herb is very poor due to low seed viability, because the seeds and developing capsules are often found on the ground, loss due to rodents and flooding is considerable. In recent years, there has been an increased interest in *in vitro* culture techniques which offers a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora and Bhojwani, 1989; Lewu et al., 2006; Tefera and Wannakrairoj, 2006). In order to increase regeneration efficiency, cotyledonary nodes and shoot tip explants were derived from *in vitro* raised aseptic seedlings. The use of *in vitro* raised plants provides juvenile explants that often have a better regenerability than explants derived from mature tissue (Thorpe, 1993). In view of its ethno botanical importance, there is a need for organized cultivation and conservation of the wild stock of *C. verrucosa*. The present paper describes a simple protocol for the rapid and large scale propagation of *C. verrucosa in vitro* through shoot proliferation from cotyledonary nodes derived from seedlings. To the best of our knowledge this is the first report on the micropropagation of *C. verrucosa* using the tissue culture technique.

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Abbreviations: BA-N^6, Benzyladenine; IAA, Indole-3-acetic acid; IBA, Indole3-butyric acid; MS, Murashige and Skoog medium; NAA-1, Napthalene acetic acid; TDZ, Thidiazuron.

MATERIALS AND METHODS

Mature pods were collected from plants of *C. verrucosa* growing at the Botanical Garden of the Department of Botany, Sri Venkateswara University, Tirupati, India. Seeds were removed from

Figure 1A. Differentiation of shoot buds on cotyledonary node on MS + 0.1 µM TDZ.

Figure 1B. Multiple shoots developed from a cotyledonary node on MS + 0.1 µM TDZ.

the capsules and washed with 5% teepol detergent solution for 10 min. The seeds were then disinfected by immersing in 70% (v/v) ethanol for 1 min followed by immersing in 0.1% aqueous mercuric chloride ($HgCl_2$) for 5 min. After five rinses in sterile distilled water, the seeds were transferred to 25 x 150 mm test tubes (Borosil, India) each containing 15 ml of water-agar medium (0.8% agar and 3% sucrose). The pH of the medium was adjusted to 5.8 before autoclaving. The culture tubes were incubated at 25 ± 2°C under light source at 35 µmol m^{-2} s^{-1} irradiance level provided by white fluorescent tubes (Phillips, India) and 60% relative humidity (RH). Fifteen days old seedlings served as the source of explants. Cotyledonary nodes and shoot tips were cultured in 25 x 150 mm test tubes containing Murashige and Skoog medium (1962). Different concentrations of TDZ, BA (0.1 - 10.0 µM) used alone and in combination with auxins (IAA, IBA and NAA).

The cultures were maintained under conditions similar to those described earlier for seed germination. Newly formed shoots were excised after 4 weeks of culture and the original explants were transferred onto fresh medium for subculture for further proliferation of shoots. Sub culturing was performed at 4 week intervals. *In vitro* formed shoots were transferred to full-strength, half-strength and quarter-strength MS medium containing 3% sucrose and 0.8% agar. All media were supplemented individually with 0.1 - 3.0 µM of IBA, IAA or NAA. Each treatment consisted of 20 replicates and the experiment was conducted thrice. Frequency of shoot regeneration, mean number of shoots per explant, mean length of the shoot were analyzed ($P \leq 0.05$) by Tukey test (Steele and Torrie, 1980) using Soft ware Package for Social Sciences (SPSS) version 7.5. Results of the rooting experiment (frequency of root regeneration, mean number of roots per shoot and mean length of the root) were compared with standard error (SE) of the mean.

RESULTS AND DISCUSSION

Shoot tips produced only a single shoot when they were cultured on MS medium with TDZ or BA. Only cotyledonary nodes produced multiple shoots. The superiority of cotyledonary node in micropropagation has been documented in other plants such as *Dalbergia sissoo* (Pradhan et al., 1998). Single shoot was formed from each axil of the cotyledonary nodal explants when they were inoculated on MS basal medium. Similar result was reported in Leucaena *leucocephala* (Dhawan and Bhojwani, 1985). Hence, this observation showed that cytokinins were indispensable for shoot multiplication. Within 10 days following inoculation, shoot buds differentiated in cotyledonary nodal explants when grown on MS with TDZ (Figure 1A). It was also observed that type and concentration of cytokinins had a significant effect on the frequency of shoot regeneration and shoot number (Table 1). Though, explants cultured on either TDZ or BA, differentiated multiple shoots, yet the highest number of shoots (6.0) per explant was recorded on TDZ supplemented MS medium (Figure 1B). TDZ at a concentration of 0.1 µM induced a mean no. of 6.0 shoot per explant with 93.3% frequency of shoot regeneration. The stimulatory effects of TDZ on bud break and shoot regeneration has been reported earlier by Singha and Bhatia (1988). Although, high concentration of TDZ (5.0 and 10.0 µM) induced more number of shoot buds but they failed to elongate. The formation of stunted shoots on TDZ supplemented medium has been reported earlier by Preece and Imel (1991). 3.6 shoots per expant were also induced in the cotyledonary nodes on MS medium with BA at 2.0 µM. Although, the mean number (3.6) of shoots per explant was low with BA, it was able to produce shoots with highest length (4.2 cm) (Table 1). Addition of auxins (IAA or IBA or NAA) to the MS medium supplemented with 0.1 µM TDZ failed to improve the shoot regeneration and caused undesirable callus. Garland and Stoltz (1981) also supported that in a number of cases, cytokinins alone are enough for optimal shoot multiplication. A proliferating shoot culture was established by repeatedly sub culturing the original explant after harvesting the newly formed shoots at every 4 week intervals. The beneficiary effect of sub culturing in shoot multiplication was earlier reported by Dewan et al.

Table 1. Effect of different concentrations of TDZ, BA on shoot proliferation from cotyledonary nodal explants of *C.verrucosa* after 4 weeks of culture.

Growth regulator concentrations (µM) in MS	Frequency of shoot regeneration (%)	Mean number of shoots per cotyledonary node	Mean length of the shoot (cm)
TDZ			
0.1	93.3^{e}	6.0^{d}	3.0^{c}
0.5	85.0^{d}	4.4^{c}	2.6^{c}
1.0	73.3^{c}	3.6^{ab}	2.4^{b}
2.0	70.0^{bc}	2.8^{ab}	2.0^{b}
5.0	61.7^{b}	2.2^{a}	1.6^{ab}
10.0	48.3^{a}	1.8^{a}	0.6^{a}
BA			
0.1	63.3^{b}	2.2^{b}	2.3^{ab}
0.5	70.0^{c}	2.6^{bc}	2.7^{b}
1.0	76.7^{cd}	3.0^{c}	3.2^{c}
2.0	80.0^{d}	3.6^{d}	4.2^{cd}
5.0	55.0^{a}	1.8^{a}	2.2^{ab}
10.0	53.3^{a}	1.2^{a}	1.8^{a}

Values represented in the table are the mean of 3 replicates of 20 explants.
Mean values having the same letter in each column do not differ significantly at P≤0.05 (Tukey test).

Figure 1C. *In vitro* roots formation on MS + 1.0 µM IAA.

(1992). Repeated subculture was said to be one of the methods of maintaining juvenility. The shoot forming potential of original explant was not declined ever after 5 subcultures. Excised shoots failed to produce roots on full-strength, half-strength and quarter-strength MS basal medium. Of the three auxins supplemented to induce root formation (IAA, IBA or NAA), quarter-strength MS medium with IAA at 1.0 µM was most effective to induce rooting (Figure 1C; Table 2).

The stimulatory effect of IAA on rooting was earlier reported by Dewan et al. (1992).

For acclimatization, plantlets were removed from rooting medium 8 weeks after root initiation, and transferred to fresh tubes containing autoclaved tap water. After 10 days, plantlets were subsequently transferred to plastic pots (9 x 9 cm) containing autoclaved vermiculite, covered with perforated polythene bags to maintain humidity and were kept under culture room conditions for about 7 days. After 5 weeks, polythene bags were removed (Figure 1D) and pots were transferred to the garden and placed under shade till the new leaf appeared. Then they were planted under normal garden conditions. About 70% of the plants were successfully established in garden. The regenerated plants were morphologically uniform.

In vitro propagation can become an important alternative to conventional propagation for wide range of plant species. Conclusively, a reproducible protocol for the *in vitro* propagation of *C. verrucosa* has been developed in this study. Direct shoot multiplication is preferred for generating true-to-type plants than callus mediated regeneration. This paper supports the rapid multiplication of this important ethno botanical plant by *in vitro* culture technique. This short report provides a simple protocol for the mass propagation of *C. verrucosa* from cotyledonary nodes.

We observed that within 10 days following inoculation, shoot buds differentiated in cotyledonaryz nodal explants when grown on MS medium with 0.1 µM TDZ (Figure 1A) and maximum number of shoots per explant (6.0) were also obtained on the same medium (Figure 1B) and the shoots were subsequently rooted on quarter strength MS medium containing 1.0 µM IAA (Figure 1D). Based on the data of the present study we can conclude that the re-

Table 2. Effect of different auxins on rooting of the *in vitro* formed shoots of *C. verrucosa.*

Auxins (µM)	Frequency of shoot regeneration (%)	Mean number of roots per shoot	Mean length of the shoot (cm)
0	0	0	0
IAA			
0.1	60.0 ± 4.8	2.4 ± 0.2	2.0 ± 0.2
0.5	65.0 ± 0.9	3.8 ± 0.3	2.8 ± 0.2
1.0	85.0 ± 0.9	4.0 ± 0.2	3.4 ± 0.3
2.0	55.0 ± 1.6	2.8 ± 0.1	2.2 ± 0.1
3.0	50.0 ± 1.8	1.6 ± 0.1	1.8 ± 0.2
IBA			
0.1	55.0 ± 1.8	1.4 ± 0.1	2.0 ± 0.2
0.5	60.0 ± 1.7	1.6 ± 0.2	2.6 ± 0.3
1.0	70.0 ± 1.9	2.6 ± 0.3	3.0 ± 0.2
2.0	50.0 ± 3.3	2.0 ± 0.1	2.4 ± 0.2
3.0	45.0 ± 1.0	1.2 ± 0.2	1.4 ± 0.1
NAA			
0.1	50.0 ± 1.9	1.6 ± 0.2	1.6 ± 0.1
0.5	50.0 ± 3.3	2.0 ± 0.1	2.2 ± 0.2
1.0	65.0 ± 1.0	2.2 ± 0.2	2.7 ± 0.2
2.0	40.0 ± 1.8	1.4 ± 0.1	1.6 ± 0.1
3.0	30.0 ± 2.9	1.0 ± 0.2	1.2 ± 0.1

Values (mean ± SE) represented above are the mean of 3 replicates of 20 explant.

Figure 1D. Hardened plantlet of *C.verrucosa* after 15 weeks.

ported regeneration system is repeatable and can be easily used to propagate *C. verrucosa*.

ACKNOWLEDGEMENTS

Town Mohammad Hussain duly acknowledges the financial assistance provided by University Grants Commission (UGC), New Delhi, India.

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