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Full Length Research Paper

Ex Situ Conservation Method for *Clerodendrum inerme*: a medicinal plant of India

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***Clerodendrum inerme* L. (Verbenaceae), commonly known as vanajai or garden quinine is a perennial shrub. Leaves and roots of the plant are used in rheumatism and skin diseases. In Indian classical literature the plant is also reported as a substitute of quinine. Since root of the plant is used as drug, whole plant has to be destroyed; this has resulted in the depletion of the plant population. Present investigation was taken up to establish a protocol for mass production of better quality plant material, using axillary bud multiplication. The protocol will help in *ex situ* conservation of the plant. Maximum number of multiplied axillary bud was observed in 16 M 6-benzyladenine (BA) with 3% sucrose. After elongation, regenerated micro-shoots were rooted in MS medium in absence of plant growth regulators (PGR). The rooted plantlets showed 100% field survival. The regenerated plants showed similar phytochemical profile as mother plant when compared.**

Key words: *Clerodendrum inerme*, finger print profile, micropropogation.

INTRODUCTION

The resurgence of public interest in plant-based medicine coupled with the rapid expansion in plant based pharmaceutical industries has resulted in increased demand and over exploitation of medicinal plants. Indiscriminate cutting and collection of plant has threatened the survival of many important medicinal plants. To prevent this premature extinction of medicinal plants, there is an emerging need of biodiversity conservation. The most widely accepted scientific technologies for biodiversity conservation are *in situ* and *ex situ* methods of conservation. In the present investigation we report mass production protocol of *Clerodendrum inerme* using tissue culture technique, which can be used for the *ex situ* conservation of the plant.

C. inerme is a medicinal shrub belonging to the family Verbenaceae. It is an important medicinal plants reported to be used in the treatment of skin diseases, venereal infections, elephantiasis, asthma, topical burns (Anonymous I, 2001) and for rheumatism (Kirtikar and Basu, 1991). It is also used as substitute of quinine (Kirtikar and Basu, 1991). In Siddha medicine, it is used under the names of 'Chankan kuppi' and 'Pechagnan' (Sasikala et al., 1995). A glycoside ester namely Verbascoside has been isolated from the root of this plant, which has analgesic and antimicrobial properties (Fauvel et al., 1989; Rastogi and Mehrotra, 1998).

The root of *C. inerme* is used as therapeutic agent and because of this the whole plant needs to be destroyed, which has disturbed its natural population leading to unavailability of good quality plant material for therapeutic use. In this scenario there is an emerging need to systematically plan for cultivation and conservation of this plant. The present investigation describes a method which can be used for *in situ* and *ex situ* conservation of better quality plant material of *C. inerme* using tissue culture technique.

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Abbreviations: $AdSO_4$ - Adenine sulphate, BA - 6-Benzyladenine, GA - Gibberlic acid, IAA - Indole - 3 - acetic acid, IBA - Indole - 3 - butyric acid, Kn - Kinetin, MS - Murashige and Skoog, NAA - α-napthaleneacetic acid, PGR – Plant Growth Regulator.

Table 1. Shoot multiplication response.

Sucrose (%)	BA (µM)	Conc. of other PGR (µM)	No. of multiplied shoots
2	2 - 10	-	ASB
2	12	-	3
2	16	-	2
2	18	-	2
2	20	-	2
2	12	0.2 NAA	2
2	12	2 NAA	2
2	12	2 NAA + 0.5 GA	-
3	12	-	2
3	16	-	4
3	18	-	2
3	20	-	3
3	12	0.2 NAA	2
3	16	2 AdSO	3

- : None/no response.
ASB: Activation and Sprouting of bud.

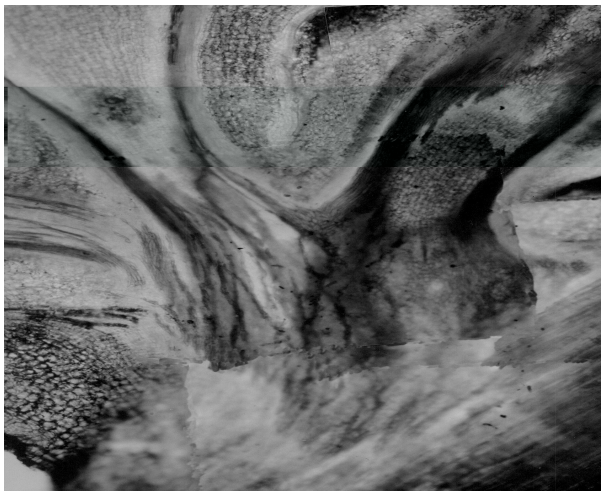

Figure 1. Photomicrograph of *C. inerme* bud multiplication.

MATERIALS AND METHODS

Nodal segments with healthy axillary buds were collected in the morning hours and were cleaned with polysorbate 80 and distilled water. They were surface sterilized with mercuric chloride 0.1% (w/v), cut into desired size and inoculated onto the MS medium (1962) supplemented with different concentrations and combinations of PGRs (BA, Kn, $AdSO_4$, NAA, IAA, IBA) along with sucrose as energy source. The pH of the medium was adjusted to 5.7 before autoclaving. Medium was solidified with 0.8% agar. The cultures were maintained at 24 ± 2°C temperature, 16/8 h photoperiod. For histological observations, saffranin stained thin hand cut section (~10 µm) of multiplied axillary bud was used.

Regenerated microshoots, after attaining 5 - 7 cm height, were excised and transferred for rooting in MS medium of full and half strength along with different PGRs at various concentrations. For hardening, the rooted plantlets were transferred into pots containing mixture of sterilized sand and soil at various ratios. This was maintained at 28°C temperature with 85-90% humidity.

Phytochemical finger print profile of methanolic extracts of field grown and culture grown plants was developed using precoated TLC silicagel 60 F_{254} plate (Merck, India). Various polar and non-polar mobile phases were tried to develop a suitable mobile phase for maximum band separation. The TLC plates developed in standardized mobile phase were observed under UV 254 and 366 nm for any quench and fluorescence bands. The plates were then visualized with Anisaldehyde sulfuric acid reagent (Wagner, 1996).

RESULTS AND DISCUSSION

The activation and multiplication of axillary bud was observed in BA amongst all the cytokinine used. Lower concentrations of BA (up to 10 µM) supported activation and sprouting of dormant axillary bud while higher concentrations supported multiplication, which was best at 16 µM BA concentration. Similar results were obtained by Mansor et al. (2003), who obtained significant increase in shoot multiplication of *Balanites aegyptiaca* with higher level of BA 2.5 mg/l, in contrast to this Afaque et al. (1996) and Philomina and Rao (2000) got good shoot multiplication at lower concentration of BA (0.4 - 1.1 µM). Favorable synergistic effects of cytokinin and auxin for shoot multiplication has been demonstrated in many plants like *Santolina canescens* (Casado et al., 2002), *Bupleurum fruticosum* (Fratenale et al., 2002) and *Randia dumetorum* (Ferdousi et al., 2003). In our study, addition of auxin with BA and change of cytokinin from BA to Kn could not improve the result (Table 1). Concentration of sucrose plays an important role in axillary bud multiplication in axinic culture. Keeping this in view, the other set of experiment with increase concentration of sucrose (3%) was planned. Results of these experiments show (Table 1) that increase level of sucrose could increase the multiplication rate. The highest (1 to 3) being in 16 µM BA (Figure 1). Further high concentration of sucrose (4%) was inhibitory for multiplication. The multiplied shoots were allowed to grow up to a height of 5–7 cm then they were excised and subjected to root induction. The best response towards root formation amongst various treatments was obtained in MS basal medium with 2% sucrose (Figure 2) in terms of number and quality of root induced as well as the time taken for root induction. Rooted plantlets were transferred to pots for hardening containing sterilized sand and soil at the ratio of 1:1, 1:2, 1:3 (w/w). All the rooted plantlets established well in 1:1 sand: soil ratio showing 100% field survival. The field acclimatized plants exhibited normal growth and development; no morphological variation was noticed (Figure 3).

Table 2. TLC details of methanolic extract of *C. inerme* at 254 and 520 nm.

Sr. No.	R_f value at 254 nm*	Presence of bands		R_f value at 520 nm	Presence of bands	
		In vivo	*In vitro*		*In vivo*	*In vitro*
1	0.09	+	-	0.02	+	+
2	0.17	+	+	0.05	+	+
3	0.37	+	+	0.08	+	-
4	0.42	+	+	0.11	+	-
5	0.55	+	-	0.16	+	+
6	0.59	+	-	0.20	+	+
7	0.66	+	+	0.26	+	+
8	0.72	+	+	0.29	+	-
9	0.76	+	-	0.34	+	+
10	0.86	+	-	0.43	+	+
11	0.90	-	+	0.47	+	+
12	0.94	+	+	0.53	-	+
13				0.58	+	+
14				0.66	+	+
15				0.75	+	+
16				0.82	+	+
17				0.88	+	-
18				0.95	+	-
19				0.97	+	+

- :indicates absence of band, + : indicates presence of band.
*Band No. 13 to 19 was not observed at UV 254 nm.

Figure 2. Rooting of regenerated *C. inerme* shoot.

Figure 3. Hardened plantlet of *C. inerme*.

Regenerated plantlets were subjected to phytochemical investigation for evaluation of their chemical profile. Methanolic extract of aerial parts of culture grown and field grown plant were investigated for TLC finger print profile. Among various combinations of polar and non-polar mobile phase tried, the maximum resolution was found with toluene: ethyl acetate: methanol at the ratio of 7:2.5:0.5, v/v/v. The TLC plate showed no fluorescence bands when observed under UV at 366 nm whereas twelve quench bands were seen at UV 254 nm. In both samples, after derivatization with Anisaldehyde sulfuric acid reagent, nineteen bands of different colour was seen on the plate (Figure 4) showing the presence of various compounds. R_f value of each band was calculated (Table 2). It was observed that all the major bands are present in culture grown plants, showing the similar biosynthetic po-

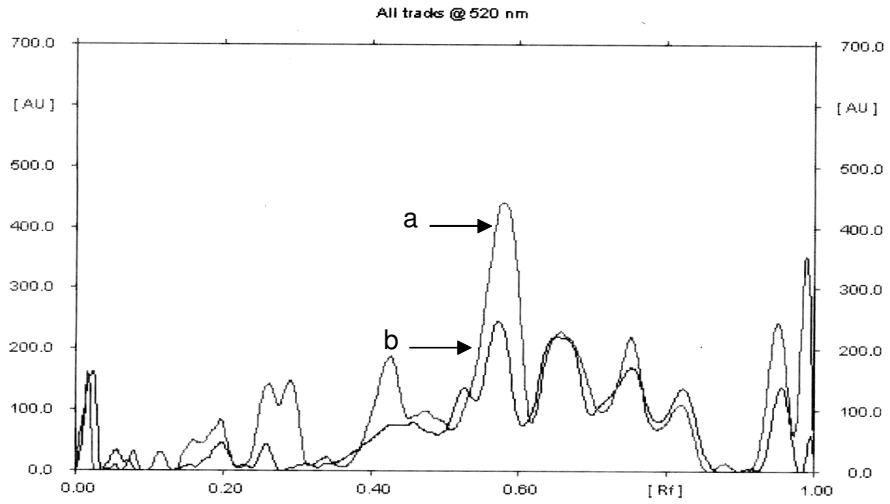

Figure 5. TLC Densitometric profile of (a) field grown and (b) tissue culture grown plant.

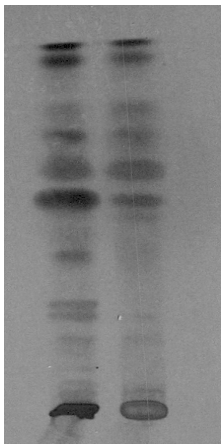

Figure 4. TLC finger print profile of methanolic extract of *C. inerme* a: *in vivo*, b: *in vitro*.

tential of culture grown and field grown plants indicating that the similar genetic makeup has been inherited by regenerated plants and confirms the true to type cloning (Figures 4 and 5). A new band was observed at R_f 5.3, while a few minor bands found to be absent in culture grown plants when observed after derivatization with Anisaldehyde sulfuric acid reagent.

This could be due to environmental factor. The present studies establish an efficient protocol for *ex situ* conservation of *C. inerme*.

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