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Full Length Research Paper

In vitro propagation of *Clitoria ternatea* L.: A rare medicinal plant

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A rapid micro-propagation protocol was established for rare medicinal plant *Clitoria ternatea* L. Multiple shoot formation was induced from shoot tip, node and cotyledonary node explants on Murashige and Skoog (MS) medium containing 2.0 mg/l BAP. Nodal explants gave the largest number (11.1) of shoots followed by cotyledonary node and shoot tip. MS medium containing 0.5 mg/l GA_3 was found most suitable for shoot elongation. *Ex vitro* rooting was achieved when the basal cut ends of elongated shoots were dipped in 250 mg/l IBA solution for half an hour followed by transplantation in plastic cups containing sterilized soilrite. The acclimatized plantlets were successfully established in the field with 90% survival rate. The plantlets are growing well without any phenotypic aberrations.

Key words: *Clitoria ternatea,* aparajita, axillary shoot proliferation.

INTRODUCTION

Clitoria ternatea L. is a member of the family Papilionaceae, commonly known as 'Aparajita' or Girikarnika'. It is a perennial climber widely used in the traditional Ayurvedic system of Indian medicine for treating a wide variety of ailments (Anonymous, 1988). It has slender downy stems with leaves having 5 - 7 leaflets elliptical to oblong in shape and flowers are usually solitary, bright blue or sometimes white with an orange center and is a very good source of anthocyanins (Terahara et al., 1996).

In the traditional system of medicine 'Aparajita' is considered as a 'Medhya' drug to improve intelligence and enhance memory function (Kulkarni et al., 1988; Jain et al., 1993). It is also used in the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors (Jain and De Filipps, 1991).. The plant contains several glycosides e.g., malvidin-3-β-glycoside, deiphinidin-3-β-glycoside (Srivastava and Pandey, 1977). The conventional method of propagation of *C. ternatea* is through seed and is not attractive approach for producing a large number of elite plants within short period. Further its requirement for 'Ayurvedic' health industry is met solely from the wild natural population which leads to their gradual depletion, as a result of which *C. ternatea* is now listed as a rare species by International Union for Conservation of Nature and Natural Resources (Pandey et al., 1993).

Therefore, the measures to develop micropropa-gation protocols for elite stocks of *C. ternatea* are urgently needed. Some studies have reported *in vitro* plant regeneration and micropropagation of *C. ternatea* (Lakshmanan and Dhanalakshmi, 1990; Malabadi and Nataraja, 2001; Rout, 2005).

Micropropagation through culture of explants having pre-existing meristem is powerful options which allow multiplying genetically stable and true-to-type progeny of the species that are rare, endangered and difficult to propagate (Cassel et al., 1999; Nepovim and Vanek,

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Abbreviations: MS, Murashige and Skoog; ***BAP,*** N^6-Benzylaminopurine; ***GA_3,*** Gibberellic acid; ***GR,*** Growth regulator; ***IBA,*** Indole-3- butyric acid; ***NAA,*** α-Naphthalene acetic acid; **DMRT,** Duncan's multiple range test.

Table 1. Morphogenetic response of different explants of *Clitoria ternatea* cultured on MS medium supplemented with BAP and NAA.

Growth regulators (mg/l)		Shoot - tip		Node		Cotyledonary node	
NAA	BAP	Responding Frequency (%)	Number of shoots per explant	Responding Frequency (%)	Number of shoots per explant	Responding Frequency (%)	Number of shoots per explant
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0	1.0	82.0	4.8^{f}	92.0	10.1^{g}	100.0	6.4^{e}
0.1	1.0	75.0	4.4^{f}	87.0	6.1^{de}	95.0	7.5^{f}
0.5	1.0	72.0	2.7^{bc}	100.0	6.2^{d}	80.0	6.0^{e}
0.0	2.0	92.0	7.1^{h}	100.0	11.1^{h}	100.0	9.8^{h}
0.1	2.0	80.0	5.4^{g}	100.0	10.3^{g}	95.0	8.2^{g}
0.5	2.0	75.0	3.0^{cd}	100.0	9.4^{f}	85.0	7.0^{f}
0.0	3.0	62.0	3.7^{de}	80.0	6.1^{de}	90.0	5.4^{e}
0.1	3.0	60.0	4.7^{f}	72.0	5.9^{d}	85.0	4.8^{d}
0.5	3.0	57.0	3.1^{cd}	68.0	4.7^{c}	85.0	3.2^{cd}
0.0	4.0	60.0	3.7^{de}	75.0	9.0^{f}	80.0	5.0^{d}
0.1	4.0	57.0	3.1^{cd}	68.0	8.3^{ef}	75.0	4.2^{d}
0.5	4.0	50.0	2.7^{bc}	60.0	8.3^{ef}	70.0	3.2^{cd}
0.0	5.0	50.0	3.2^{d}	65.0	5.8^{d}	75.0	3.5^{c}
0.1	5.0	48.0	3.0^{cd}	59.0	2.9^{b}	65.0	2.3^{b}
0.5	5.0	40.0	2.9^{c}	50.0	2.7^{b}	60.0	1.3^{a}
0.0	10.0	30.0	1.1^{a}	59.0	3.5^{c}	65.0	2.0^{b}
0.1	10.0	28.0	1.0^{a}	48.0	2.2^{a}	50.0	1.1^{a}
0.5	10.0	20.0	0.9^{a}	42.0	2.0^{a}	40.0	1.0^{a}

Each mean is based on two replicates, each of which consisted of 20 culture tubes(culture age; 6 weeks).The alphabets indicate significant difference between means($P < 0.05$); comparison by DMRT.

1998). During past few years, there has been an increase interest for *in vitro* multiplications and germplasm conservation of rare, endangered, aromatic and medicinal plants (Tiwari et al., 1998; Villalobos and Engelmann, 1995). The main objective of our study is to establish an efficient, quick and reliable protocol for *in vitro* regeneration of C*. ternatea*. In this communication we have described reproducible and rapid method for *in vitro* multiplication of *C. ternatea* through high frequency axillary shoot proliferation from different explants followed by successful establishment of regenerated plants in the field.

MATERIALS AND METHODS

Preparation of explants

Healthy shoots of *C. ternatea* were collected from Ayurvedic garden of Banaras Hindu University, Varanasi (India) and washed thoroughly in 1.0% (v/v) cetrimide solution (ICI India Ltd., Chennai, India) for 10 min. Surface sterilization was done as follows: A quick dip in 70% ethanol for 30 s and thereafter 5 min treatment with 0.1% (w/v) mercuric chloride (E-Merck India Ltd., Mumbai) followed by a quick dip in 70% ethanol for 30 s and finally six washes with sterilized double distilled water. The shoot tips and nodes (5 mm) were excised from this surface sterilized shoots. Seeds of *C. ternatea* have hard seed coat so for isolation of cotyledonary node surface sterilized seeds were soaked over night in sterilized double distilled water.

Culture media and conditions

MS (1962) media supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose was used during the investigation. The pH of the medium was adjusted to 5.8 with 1 N NaOH or 1 N HCl. The media were dispensed into culture tubes and autoclaved at 15 lbs (1.04 kg/cm^2) for 15 min at 121°C. The cultures were incubated at 24 ± 2°C under 16 h photoperiod of 50 µE $m^{-2}s^{-1}$ irradiance provided by cool-white fluorescent tubes (Philips, India).

Shoot organogenesis and elongation

For multiple shoot induction shoot tips, node and cotyledonary nodes were cultured on MS medium supplemented with 0 - 10.0 mg/l BAP alone or in combination with NAA (0.1 and 0.5 mg/l) (Table 1). Data on percentage of responding explants and number of shoots development per explant were recorded after six weeks of culture initiation.

Regenerated shoots were transferred to MS medium supplemented with 0 - 1.0 mg/lGA_3 for shoot elongation and growth. The data were recorded after four weeks of culture initiation (Table 2).

Ex vitro root formation and acclimatization

For *ex vitro* root induction, elongated shoots of 5 – 6 cm were

Table 2. Influence of GA_3 on elongation of shoots *C. ternatea*.

GA_3 (mg/l)	Frequency of elongation (%)	Mean shoot length (cm)	Number of nodes/ shoot
0.0	40	3.5^a	2.7^a
0.1	100	4.4^b	4.1^b
0.2	100	5.4^c	4.7^c
0.5	100	6.8^d	5.7^d
1.0	100	3.7^a	4.2^b

Each mean is based on two replicates, each of which consisted of 20 culture tubes (culture age: 4 weeks). The alphabets indicate significant difference between means (P<0.05); comparison by DMRT.

Table 3. The efficiency of root induction from regenerated shoots of *C. ternatea* dipped in IBA solution for half an hour.

IBA (mg/l)	Responding frequency (%)	Number of roots/shoot	Root length (cm)
150	40	7.2^b	5.4^b
250	90	15.6^d	10.5^d
350	75	10.4^c	7.4^c
500	30	5.1^a	3.2^a

Each mean is based on two replicates, each of which consisted of 20 culture tubes (culture age: 4 weeks). The alphabets indicate significant difference between means (P < 0.05); comparison by DMRT.

harvested and their basal portion were dipped in different concentrations of IBA (150 – 500 mg/l) (Table 3) for half an hour and then planted in plastic cups containing sterilized soilrite (Kel Perlite, Vishwasnagar, Karnataka, India). The potted plantlets were covered with transparent polythene bags and kept in culture room at 24 ± 2°C under 16 h illumination with cool fluorescent light (20 µE $m^{-2}s^{-1}$). The plantlets were irrigated every day with 1 - 2 ml tap water. The polythene covers were removed after 3 weeks and pots were kept in the culture room for another 2 weeks and later transferred to normal laboratory conditions (2 weeks). The acclimatized plantlets were then transplanted to the field conditions. Data were recorded on percentage of rooting, number and length of roots after 4 weeks of *ex vitro* transplantation.

Statistical analysis

Experiments were set up in a randomized block design and each experiment usually had two replicates; each of which consisted of 20 culture tubes. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test (DMRT) at the 5% probability level according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Shoot induction

Morphogenetic responses of the explants (shoot tip, node and cotyledonary node) of *C. ternatea* cultured on MS medium containing BAP either alone or in combination with NAA are summarized in Table1. Explants cultured on MS medium without cytokinin failed to induce shoot proliferation. MS medium supplemented with different concentrations of BAP (0 – 10 mg/l) resulted in the induction of axillary shoots. The frequency of responding explants and the number of axillary shoots per explant increased with an increased in the concentration of BAP up to 2 mg/l. These results indicated that BAP played an important role in the induction of multiple shoots. However, BAP at higher concentrations not only reduced the number of shoots formed but also resulted in stunted growth of the shoots. The stimulating effect of BAP on multiple shoot formation has been reported for several medicinal plant species (Tiwari et al., 1998; Wang et al., 2004; Espinosa et al., 2006).

Analysis of variance revealed a significant effect (P < 0.05) of concentrations of BAP and explants for number of axillary shoots produced per explant. MS medium supplemented with 2 mg/l BAP supports maximum number of shoot formation from node (11.1; Figure 1A), cotyledonary node (10.7; Figure 1B) and shoot tip (7.1; Figure 1C). Addition of NAA with BAP did not improve the shoot proliferation. Similar results were reported in *Centella asiatica* (Raghu et al., 2007), *Vitex negundo* (Usha et al., 2007) and *Gynura procumbens* (Keng et al., 2009).

Shoot elongation

Axillary shoots developed from different explants they did not elongate further. Therefore, young shoots were transferred to MS medium supplemented with GA_3 (0.0 – 1.0 mg/l) to ensure their normal elongation. Analysis of variance revealed (Table 2) a significant effect of treatments on shoot length and number of nodes/ shoot. All the shoots elongated on the GA_3 treatments but optimum shoot growth in terms of shoot length (6.8 cm) and number of nodes per shoot (5.7) was recorded on MS +

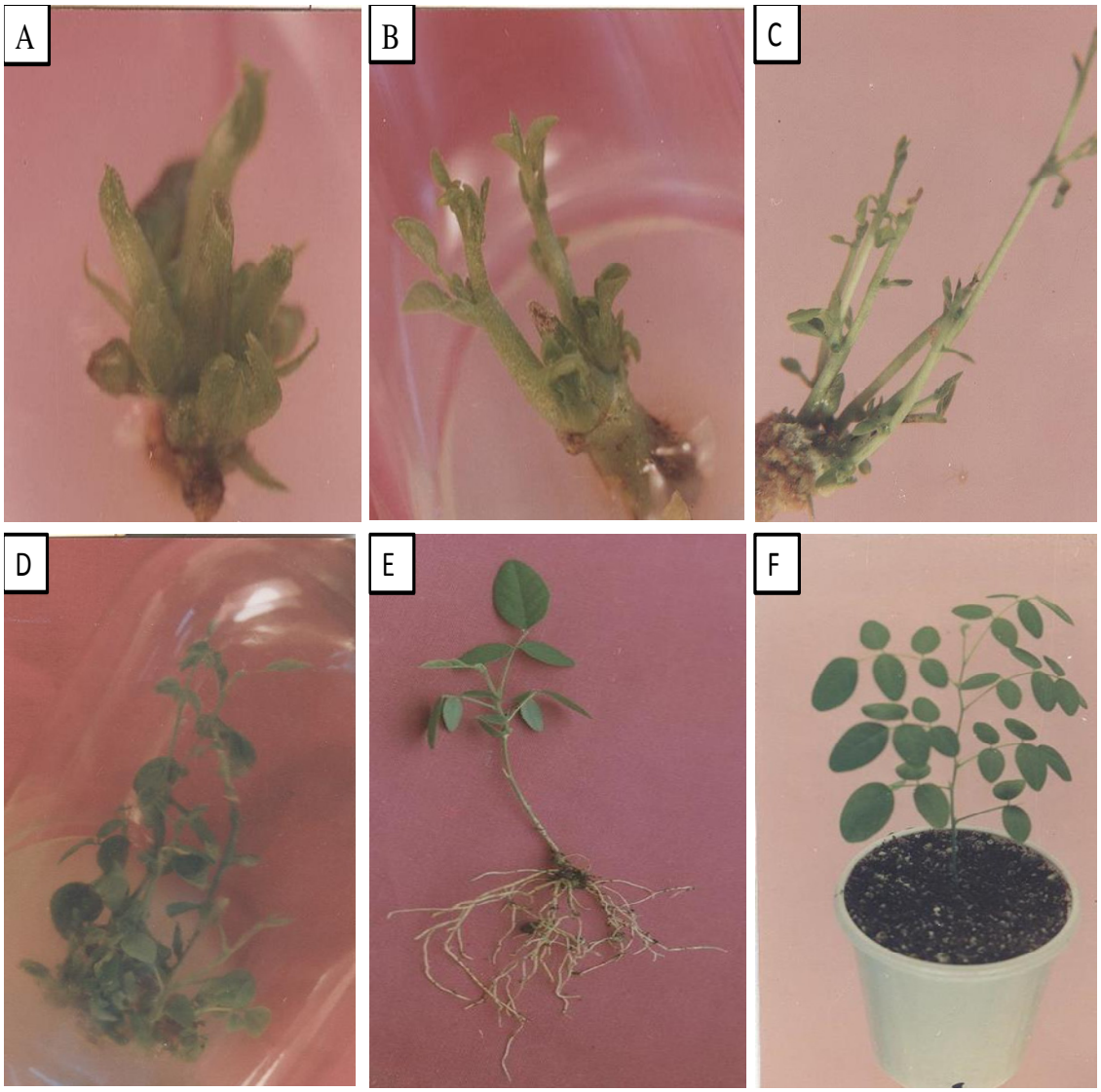

Figure 1. Axillary shoot regeneration from different explants of *Clitoria ternatea* L. A. Node cultured on MS + 2.0 mg/l BAP. B. Cotyledonary node cultured on MS + 2.0 mg/l BAP. C. Shoot tip cultured on MS +2.0 mg/l BAP. D. Elongation of shoots on MS medium containing 0.5 mg/l GA_3. E. *Ex vitro* rooted plantlet of *C. ternatea*. F. Acclimatized plantlet of *C. ternatea*.

0.5 mg/l GA_3 in four weeks old cultures (Figure 1D).

GA_3 is known to have stimulatory effect on shoot elongation in some other species also (Veltcheva and Svetleva, 2005; Rkhis et al., 2006).

Rooting and establishment in the field

Elongated shoots of 5 - 6 cm in length were separated and rooting was carried out by *ex vitro* method. Elongated shoots were dipped in different concentrations of IBA (150 – 500 mg/l) (Table 3) for half an hour and subsequently planted in plastic cups containing sterilized soilrite. Analysis of variance revealed a significant effect ($P < 0.05$) on the frequency of cultures showing root regeneration, number of roots/shoot and mean root length (Table 3). A comparison by DMRT revealed optimum frequency (90%), number of roots/shoot (15.6) and root length (10.5) (Figure 1E) in shoots who were dipped in 250 mg/l IBA solution. IBA is highly effective auxin for rooting of *in vitro* regenerated shoots in several plant species (Golegaonkar and Kantharajah, 2006; Gururaj et al., 2007). *Ex vitro* rooting was also found suitable in other species also (Arrebola et al., 1997; Siddique et al., 2006).

The acclimatized plantlets (Figure 1F) were successfully established in the field with 90% survival. There was no variation observed among field transferred plants with

respect to morphology and growth characteristics.

The significant contribution of the present report is the improvement of the rapid availability of *C. ternatea* plantlets through axillary shoot proliferation. This method could be useful for large scale multiplication as well as *in vitro* conservation of germplasm of this rare medicinal species.

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