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Evaluation of Cotyledonary Node of *Clitoria ternatea* L. for High Frequency *in vitro* Axillary Shoot Proliferation

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Abstract: The objective of this study was to establish mass multiplication protocol for *Clitoria ternatea*. An efficient protocol for *in vitro* propagation of the valuable medicinal and forage plant aparajita (*Clitoria ternatea* L.) was developed through axillary shoot proliferation. Cotyledonary node having cotyledons or devoid of cotyledons were evaluated for axillary shoot development. The effect of cytokinins, sucrose concentrations and light-dark condition on axillary shoot regeneration was investigated. Cotyledonary node with cotyledons cultured on MS medium supplemented with 1.5 mg L⁻¹ BAP yielded the maximum number (10.8) of shoots per explant. All media supplemented with 3% sucrose and incubated under 16 h light condition found most suitable for high frequency axillary shoot proliferation. Elongated shoots produced profuse rooting after transfer to half-strength MS medium supplemented with 0.2 mg L⁻¹ IBA. The rooted plantlets were successfully acclimatized and exhibited 95% survival after transfer into the field. This protocol is suitable for large scale production of plantlets of *C. ternatea*.

Key words: Germplasm conservation, medicinal plant, micropropagation

INTRODUCTION

Aparajita (*Clitoria ternatea* L.) is a valuable medicinal plant used as medhya rasayan in Indian traditional system of medicines (Sivaranjan and Balachandran, 1994). Medhya rasayana is one of the major aspects of ayurvedic pharmacology which ascribed intellect promoting activities of medicinal plants (Govindarajan *et al.*, 2005). In traditional system of medicine it is used as reputed nervine tonic. It is believed to promote memory and intelligence (Taranalli and Cheeramkuzhy, 2003). Medicinal plants used in ayurveda are important source of valuable phytochemicals used in natural product based drug development (Mukherjee and Wahile, 2006). With the advancement of ayurvedic tradition and its scientific exploration, several classes of plant species have been studied in order to evaluate their therapeutic potentials and to isolate the lead compounds. *C. ternatea* is highly palatable forage legume and generally preferred by livestock over other legume (Gomez and Kalamani, 2003). The plant contains major compound such as kaempferol, clitorin, taraxerol and a lactone - aparajitin (Anonymous, 1988). *Clitoria ternatea* seeds contain γ -sitosterol, β -sitosterol, hexacosanol and anthoxanthin (Yoganarasimhan, 2000). The plant believed to exhibits anxiolytic, antidepressant, anticonvulsant and antistress activity (Jain *et al.*, 2003). The root bark possesses diuretic, laxative, anti-inflammatory, analgesic

and antipyretic activities (Devi *et al.*, 2003). It is also used in curing stomatitis, piles, sterility in female, hematemesia, insomnia, epilepsy, psychosis, leucorrhoea and polyurea (Yoganarasimhan, 2000).

The wild population of this medicinally important plant exhibits high mortality of the seedlings and also declined very fast due to indiscriminate and illegal collection from natural habitat. Therefore, this species was included in the list of rare species by International Union for Conservation of Nature and Natural Resources (Pandey *et al.*, 1993). United States Development Agency also selected *C. ternatea* with other sixteen leguminous plants for immediate conservation (Morris, 1999). Hence, there is a need for the development of alternative plant regeneration tools for this valuable species. Plant tissue culture techniques offer a viable tool for the mass multiplication and the germplasm conservation of rare and endangered medicinal plants for meeting the pharmaceutical needs (Thorpe *et al.*, 1991; Tomar and Gupta, 1998). Cotyledonary node is suitable explant for axillary shoot proliferation. It is frequently used for propagation of several plants (Siddique *et al.*, 2006; Rajeswari and Paliwal, 2008). *In vitro* regeneration of complete plantlets from explants of seedling have been reported for large-scale propagation of several medicinal plant (Bhat *et al.*, 2001; Paramageetham *et al.*, 2004) and forage legumes (Khalafalla and Hattori, 1999).

The potential to micropropagate *C. ternatea* has also been emphasized by some workers (Kalamani and Gomez, 2002; Lakshmanan and Dhanalakshmi, 1990; Rout, 2005). However, none of these reports described an efficient protocol for *in vitro* regeneration in *C. ternatea*. An efficient regeneration protocol is essential for various *in vitro* manipulations such as embryo rescue and development of transgenic plants. In present investigation, explant type (cotyledonary nodes with and without cotyledon), sucrose concentration and photoperiod have been evaluated for *in vitro* axillary shoot regeneration potential.

MATERIALS AND METHODS

This study has been completed in research laboratory of Dr. K.N. Tiwari, Department of Botany, MMV, Banaras Hindu University, Varanasi during 2006 to 2010 as a research problem.

Plant material and explant source: Seeds of *C. ternatea* were collected from the campus of the Banaras Hindu University, Varanasi. Seeds were washed under running tap water for 10 min. Further seeds were agitated in freshly prepared 1% cetrimide solution for 10 min. The seeds were surface sterilized with 0.1% (w/v) HgCl_2 (Hi media, India) solution for 10 min followed by five times washing with sterilized double distilled water. The seeds were soaked in sterilized double distilled water for 8 h. Seed coats were removed with the help of sterilized fine-tipped forceps avoiding injury to the cotyledons. Seeds without seed coat were cultured on MS (Murashige and Skoog, 1962) basal medium for germination. After ten days of seed germination, the shoot tip and radicle were decapitated and remaining portion is used as cotyledonary node. Cotyledonary node explant (8-10 mm) along with either none (Fig. 1a), or two full cotyledon attached (Fig. 1b) to the embryonic axis were excised and inoculated for multiple shoot formation.



Fig. 1: (a) Cotyledonary node (explant A) derived from young seedling without any cotyledons, (b) cotyledonary node (explant B) excised from young seedling having both cotyledons, (c) limited shoot induction from the explant A cultured on MS medium supplemented with 1.5 mg L^{-1} BAP, (d) high frequency multiple shoot induction from explant B cultured on MS medium supplemented with 1.5 mg L^{-1} BAP, (e) elongated shoots of *C. ternatea* developed from cotyledonary node, (f) rooting of regenerated shoot of *C. ternatea* cultured on MS medium supplemented with 0.2 mg L^{-1} IBA, (g) well rooted plant of *C. ternatea* transferred into plastic cup containing soilrite and kept for acclimatization and (h) acclimatized plants transferred into field without any morphological variation

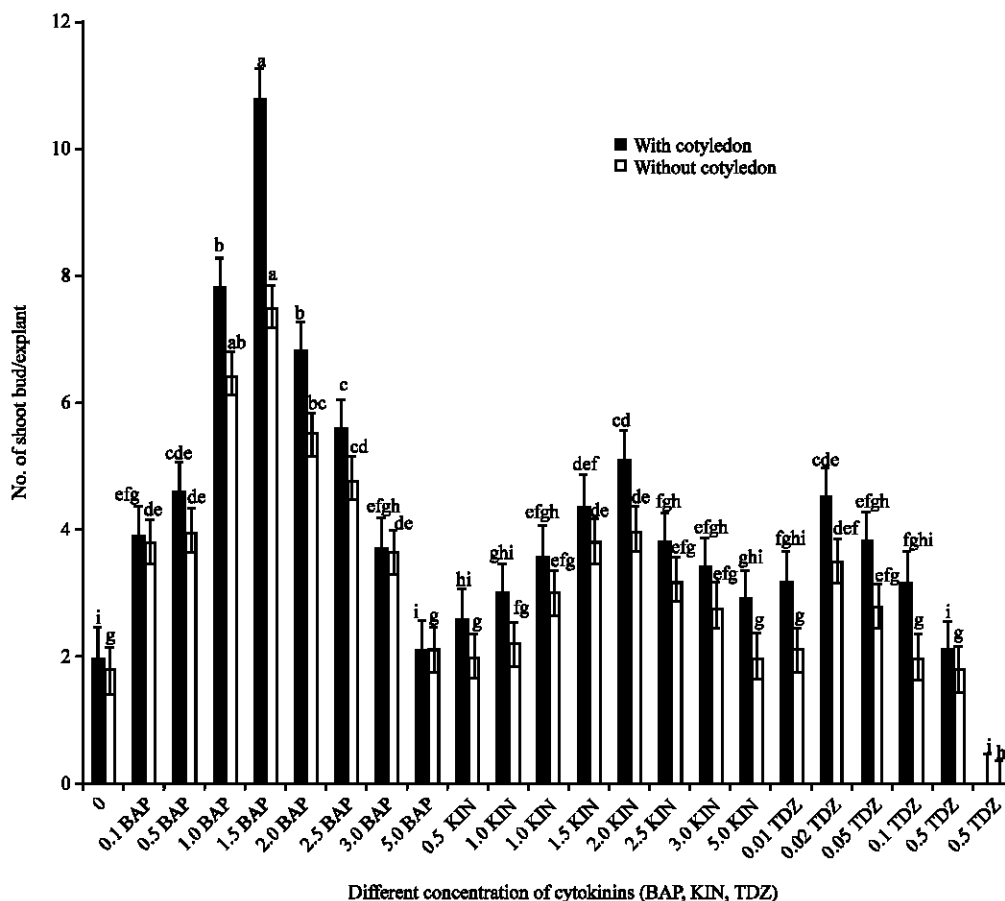


Fig. 2: Effect of different concentration of cytokinins on shoot regeneration from cotyledonary node with or without cotyledon of *C. ternatea* after 4 weeks of culture initiation. (Each mean is based on three replicates, each of consisting 20 culture tubes. The alphabets indicate significant difference between mean ($p < 0.05$); Comparison by DMRT)

Table 1: Effects of sucrose concentration on shoot regeneration from cotyledonary node of *C. ternatea* after 4 weeks of culture initiation

Sucrose ($g L^{-1}$)	Regeneration frequency (%)	No. of shoots (Mean \pm SE)
10	70	4.35 \pm 0.18 ^d
20	90	5.75 \pm 0.22 ^c
30	100	10.80 \pm 0.25 ^a
40	100	7.10 \pm 0.28 ^b

Each mean is based on three replicates, each of consisting 20 culture tubes. The superscripts indicate significant difference between mean ($p < 0.05$); Comparison by DMRT

Media and culture conditions: MS media were used in all experiments. The media were supplemented with 3% (w/v) sucrose, 0.8% agar (w/v) and the pH of the media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving. Both types of explants were cultured on MS media without any Plant Growth Regulators (PGR) or it was supplemented with BAP (0.10-5.0 $mg L^{-1}$), KIN (0.10-5.0 $mg L^{-1}$) and TDZ (0.01-1.0 $mg L^{-1}$) for multiple axillary shoot proliferation (Fig. 2). Cotyledonary nodes with two cotyledons (explant B) were cultured on MS media supplemented with 1.5 $mg L^{-1}$ BAP and

Table 2: Morphogenetic responses of cotyledonary node explants of *C. ternatea* cultured on MS medium (containing 3% sucrose) supplemented with 1.5 $mg L^{-1}$ BAP and incubated at different light conditions

Light condition	Regeneration frequency (%)	No. of shoots/ explant (Mean \pm SE)	Length of shoot (cm)
Continuous dark	42	4.50 \pm 0.22	3.15 \pm 0.20 ^f
Light-dark (16-8 h)*	100	10.0 \pm 0.30	8.20 \pm 0.18 ^a
Continuous light*	38	5.50 \pm 0.38	3.88 \pm 0.33 ^b

Each mean is based on three replicates, each of consisting 20 culture tubes. The superscripts indicate significant difference between mean ($p < 0.05$); Comparison by DMRT. *Incubated under a cool-white-fluorescent light providing a quantum flux density of 50 $\mu mol/m^2/sec$

various concentration of sucrose (1, 2, 3 and 4%) (Table 1). This explant cultured on MS medium supplemented with 1.5 $mg L^{-1}$ BAP and 3% sucrose were incubated under different light condition (continuous dark, continuous light and 16 h light and 8 h dark) (Table 2). Media were autoclaved at 1.06 $kg cm^{-2}$ at 121 $^{\circ}C$ for 15 min. The cultures were incubated at 24 \pm 2 $^{\circ}C$ under 16 h photoperiod of 50 $\mu mol/m^2/sec$ irradiance provided

Table 3: Effect of different concentration of IBA on root induction from regenerated shoots of *C. ternatea* after 4 weeks of culture initiation

Medium	IBA (mg L ⁻¹)	Rooting frequency (%)	No. of root/explant -----Mean±SE-----	Length of root (cm)
½MSB	0.0	20	2.20±0.23 ^d	2.60±0.27 ^d
½MSB	0.1	68	3.18±0.38 ^c	3.80±0.53 ^c
½MSB	0.2	90	6.20±0.58 ^a	6.15±0.57 ^a
½MSB	0.5	80	3.55±0.24 ^b	4.48±0.35 ^b
½MSB	1.0	70	3.20±0.18 ^c	4.12±0.24 ^{bc}
½MSB	1.5	40	2.00±0.12 ^{bc}	2.20±0.17 ^c

Each mean is based on three replicates, each of consisting 20 culture tubes. The superscripts indicate significant difference between mean ($p < 0.05$); Comparison by DMRT

by cool white fluorescent tubes (Philips, India). The frequency of responding explants, mean number of shoots/explant and mean shoot length (cm) were recorded after 4 weeks of culture initiation.

Rooting and hardening: The elongated shoots (4-5 cm) having 2 or 3 expanded leaves were excised and transferred to root induction medium. Elongated shoots were cultured on half-strength MS medium supplemented with IBA (0.0-1.5 mg L⁻¹) (Table 3). The frequency of rooting, mean number of roots per explant and mean root length were recorded after 4 weeks of culture initiation. Plantlets with well developed roots were taken out from the culture vessels, washed thoroughly under running tap water. The rooted plantlets were transferred to plastic cups containing sterilized soilrite. The cups were covered with transparent polythene bags and irrigated daily with 1-2 mL of sterilized MS salt solution for six days followed by sterilized tap water; the plants were maintained in culture room at 24±2°C and 16 h day⁻¹ illumination of 50 µmol/m²/sec provided by cool-white fluorescent tubes. After two weeks the polythene bags were gradually removed, the plants were kept in the culture room for another two weeks. Further plantlets were transplanted into earthen pot containing garden soil and kept under shade in a net house for another two weeks before transferring to outside under the field conditions.

Statistical analysis: Experiments were set up in a completely randomized block design and each experiment usually had three replicates. The number of cultures per replicate was twenty. The analysis of variance (ANOVA) 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 $p < 0.05$ level according to Gomez and Gomez (1984).

RESULT AND DISCUSSION

Multiple shoot formation: Different types of cotyledonary node explants of *C. ternatea* were cultured on cytokinin-

containing MS basal media for multiple shoot formation. The number of multiple shoots induction was highest when the explant having both cotyledons (Fig. 1d). Complete removal of both cotyledons caused a delayed response of axillary shoot regeneration and the number multiple shoots were reduced (Fig. 1c). Similar results have been reported by Gulati and Jaiwal (1994) in *Vigna radiata*. In *V. radiata* it was demonstrated that cotyledon size affected regeneration ability of the explant. In *Vigna* sp. *in vitro* induction of multiple shoot and plant regeneration is influenced by attachment of cotyledon with embryonic axes (Sen and Guha-Mukherjee, 1998). It thus appears that cotyledons provide endogenous signals for bud development following decapitation of the shoot tip. Experimental data provided by other authors also support this hypothesis (Polisetty *et al.*, 1997). Explant cultured on MS media without growth regulator (GR) did not support multiple shoot formation.

Frequency of responding explants and mean number of shoots per explant is higher with the cotyledonary nodes having both cotyledons (Fig. 1d). All cytokinins (BAP, KIN and TDZ) tested support the axillary shoot formation (Fig. 2). All concentrations of BAP, KIN and TDZ (except 1.0 mg L⁻¹) support shoot differentiation. BAP was found to be superior to KIN and TDZ in terms of the number of shoots produced per explant and shoot length. In general by increasing the concentrations of GRs the responding frequency of explants and the number of shoot regeneration per explant increased. After optimum concentration, response was gradually decreased. Analysis of variance revealed that morphogenetic response of two types of explant in terms of number of shoots regeneration is significantly influenced by concentrations and types of cytokinin supplemented in media. Maximum number of shoots (10.8) (Fig. 1d) and shoot length (8.5 cm) (Fig. 1e) was recorded on MS medium supplemented with 1.5 mg L⁻¹ BAP. The stimulating effect of BAP on bud break and multiple shoot formation has been reported earlier for several medicinal and aromatic plant species (Tiwari *et al.*, 2001; Sivanesan, 2007; Keng *et al.*, 2009; Kalimuthu *et al.*, 2010).

Effect of sucrose concentrations: Sugar has been identified as important component of tissue culture media. Sucrose is an important source of carbohydrate in the culture medium as well as acting as an osmoticum (Hartmann *et al.*, 1997). Sucrose is often assumed to be the best sugar in cell culture media. It is the main transport sugar in most plant species. Various concentrations of sucrose were tested into culture media supplemented with 1.5 mg L⁻¹ BAP for axillary shoot proliferation from the cotyledonary node having both cotyledons. At 1% concentration, responding frequency of explant and shoot

proliferation was poor. An increase in the concentration of sucrose favoured shoot development with a maximum of 10.8 shoot per explant measured at 3% sucrose. Boufleuher *et al.* (2008) also reported enhanced shoot regeneration in *Solanum sessiliflorum* when media were supplemented with 30 g L⁻¹ sucrose. Increased concentration of sucrose concomitantly decreased the number of shoot proliferation. This reduction in shoot proliferation may be due to hypoxia and ethanol accumulation caused by fast metabolism (Neto and Otoni, 2003). Inhibitory effect of higher concentration of sucrose may be due to significant decrease in the media osmotic potential (Neto and Otoni, 2003). These conditions could in turn interfere with the nutrient uptake process. This interference would most likely result in the failure of absorption or diffusion of some important elements (Jain *et al.*, 2009) ultimate it cause the reduction in shoot regeneration.

Effect of photoperiod on *in vitro* regeneration:

Photoperiod, light intensity and light quality influence plant growth and development from seed germination to flowering. Photoperiod has an effect on the development of several plant species. The influence of the photoperiod on flowering and rooting of ornamental plants under *in vivo* reported by several workers (Cameron *et al.*, 2005; Runkle and Heins, 2006). However, in several reports (Seabrook *et al.*, 1993; Kozai *et al.*, 1995; Franklin *et al.*, 2000; Kurilcik *et al.*, 2008) growth under *in vitro* conditions also influenced by light. Plant morphogenesis can be influenced by an appropriate choice of lighting, which may affect photoreceptors of the plants (Nhut *et al.*, 2003; Cameron *et al.*, 2005; Das *et al.*, 2007). The objective of this experiment was to optimize the photoperiod for the growth and development of *C. ternatea*. Cultures exposed under continuous dark or continuous light reduced the morphogenetic responses (responding frequency of explant, as well as induction of axillary shoots/explants). The shoots induced from the explants showed stunted growth and could not elongate further on the same media. The optimum photoperiod 16 h light found most suitable for *in vitro* axillary shoot proliferation as well as shoot elongation (Table 2). This data may be helpful for improving the efficiency of micropropagation of *C. ternatea*.

Rooting and acclimatization: Elongated shoots of 4-6 cm in length were separate and cultured individually on half strength MS medium with or without an auxin (IBA at 0.1 to 1.5 mg L⁻¹) (Table 3). Analysis of variance revealed a significant effect ($p < 0.05$) on the frequency of cultures showing root regeneration, number of root/shoot and

mean root length. There was poor rooting on half strength MS basal medium without an auxin, but when it was supplemented with IBA (0.1 to 1.5 mg L⁻¹) profuse rooting occurred. Devi and Srinivasan (2008) also found that MS medium containing IBA is best for root induction. A comparison by DMRT revealed optimum frequency (90%), number of roots/shoot (6.20) and mean root length (6.2 cm) in shoot cultured on half strength MS media containing 0.2 mg L⁻¹ IBA (Fig. 1f). IBA is very common auxin for inducing root formation in several species (Irvani *et al.*, 2010; Piovan *et al.*, 2010). The rooted plantlets were transferred from culture tubes into plastic cups (Fig. 1g) containing soilrite. The acclimatized plantlets were successfully established in the field (Fig. 1h) with only 5% mortality. There was no detectable variation among field transferred plants with respect to morphology and growth characteristics. The results suggest an *in vitro* protocol for regeneration of plants from cotyledonary nodes having two cotyledons will be used in *C. ternatea* improvement program that includes propagation, *in vitro* selection and/or genetic manipulation.

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