Micropropagation of *Clitoria ternatea* L. (Papilionaceae) through callus regeneration and shoot tip multiplication

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ABSTRACT

Methods for rapid multiplication of *Clitoria ternatea* through callus regeneration and shoot tip cultures are described. Stem and leaf explants cultured on Gamborg's basal medium supplemented with 0.01-1 mg l^{-1} 2, 4-dichlorophenoxy acetic acid in combination with 0.01-2 mgl^{-1} kinetin or 1-3 mgl^{-1} benzyl adenine alone formed callus. These calli on transfer to Gamborg's medium supplemented with 1-2 mgl^{-1} benzyl adenine and 2-3 mgl^{-1} indole-3-acetic acid or 3-4 mg l^{-1} indole-3-acetic acid alone developed shoot buds and the number of regenerated buds was maximum (8-10) with 2 mgl^{-1} benzyl adenine and indole-3-acetic acid containing medium. Callus retained the same morphogenic potential even after repeated subculturing.. Multiple shoots were induced from the shoot tips cultured on Gamborg's medium containing 3-5 mgl^{-1} benzyl adenine, 1mgl^{-1} benzyl adenine with 2 mgl^{-1} gibberellic acid. Shoots, developed from shoot tips, multiplied further by subculturing in the same medium or from callus, were rooted in Gamborg's medium containing 1-3 mgl^{-1} 1-naphthalene acetic acid. The regenerated plants were transferred initially to vermiculite and later grown to maturity in the green house in garden soil and sand (1:3).

Key words: callus, *Clitoria ternatea*, micropropagation, organogenesis, shoot multiplication.

Abbreviations

BA : benzyl adenine
2, 4-D : 2, 4-dichlorophenoxy acetic acid
B_5 : Gamborg's medium
GA_3 : gibberellic acid
IAA : indole-3-acetic acid
Kin : kinetin
NAA : 1-naphthalene acetic acid

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Introduction

Clitoria ternatea L. (Papilionaceae) is an important medicinal legume widely used for treatment of dysentry, bronchitis, asthma and also as laxative and diuretic (Kirtikar & Basu 1975). Pharmacognostic and phytochemical properties of this plant have been studied in detail (Kulshreshtha & Khare 1967). In addition to its use in medicine, a dye is extracted from its flowers and seeds (Aiyer & Kolammal 1964). Except for a preliminary report on the growth of embryonal axis of *C. ternatea* cultured in Nitsch's basal medium (Padmanabhan 1966), no other tissue culture work is reported in this plant. The present work reports methods for rapid multiplication of *C. ternatea* from explants of mature-phase region.

Materials and methods

Stem, leaf and shoot segments were collected from 3-4 month old plants of *C. ternatea* grown in the green house. These explants were throughly washed in running tap water, 1% teepol solution (a neutral liquid detergent) for 5-10 min and surface sterilized with 0.1% (w/v) mercuric chloride solution for 10 min. The sterilized explants were washed 4-6 times in sterile distilled water and trimmed finally to 1 cm for stem explants, 1 cm^2 for leaf and 3-4 mm for shoot tips before inoculation.

The culture medium consisted of Gamborg's B_5 complete nutrients (Gamborg *et al*.1968) supplemented with 2% sucrose and gelled with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to being autoclaved at 121°C for 20 min. All the cultures were incubated at 25±2°C under a 16 h photoperiod maintained with white fluorescent light at an intensity of 3000 lux.

For callus induction from stem and leaf explants, B_5 basal medium containing 0.1-1 mgl^{-1} 2, 4-D alone or in combination with 0.1-2 mgl^{-1} Kin or 1-3 mgl^{-1} BA was used. The developed callus was subcultured in a medium containing 2-4 mgl^{-1} IAA, 1-4 mgl^{-1} BA or a combination of 2 mgl^{-1} IAA with 1-4 mgl^{-1} BA.

Shoot tips were cultured in B_5 medium containing 0.01-5 mgl^{-1} BA or 1-3 mgl^{-1} BA/Kin and 1-3 mgl^{-1} IAA/GA_3. After 30 days, the developed shoots were isolated and subcultured in the same medium for multiplication. Stage II was repeated for maximum multiplication for three subcultures and subsequently shoots of about 2 cm long were excised and cultured on B_5 medium supplemented with 1-3 mg l^{-1} NAA for rooting.

The rooted plants were hardened in B_5 liquid basal medium and subsequently transferred to vermiculite and later to a mixture of garden soil and sand (1:3).

Results and discussion

Stem and leaf explants formed callus in B_5 medium containing 0.1, 0.5 and 1 mgl^{-1} 2, 4-D, 1-3 mgl^{-1} BA alone or 0.1-1 mgl^{-1} 2, 4-D in combination with 1-2 mgl^{-1} Kin. The friable white callus developed from both the explants in 0.01-1 mgl^{-1} 2, 4-D and 0.01-2 mgl^{-1} Kin or 1-3 mgl^{-1} BA alone containing medium became nodular and green when transferred to B_5 +0.1 mgl^{-1} IAA and 0.01 mgl^{-1} BA after 40 days of culture. Shoot buds developed from the green compact callus, 50 days after subculturing in the medium containing 3-4 mg l^{-1} IAA alone or 2-4 mgl^{-1} IAA in combination with mgl^{-1} BA (Figs.1 & 2). The callus derived from 2, 4-D and Kin containing medium developed shoot buds in the medium with 1-2 mgl^{-1} BA and 2-4 mgl^{-1} IAA, while the callus induced in B_5 1-3 mgl^{-1} BA

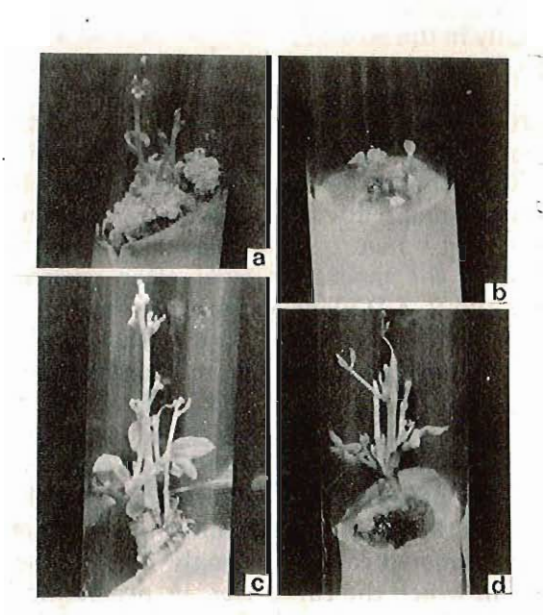

Fig.1 Micropropagation of *Clitoria ternatea*

a. Stem callus in B_5 medium containing 3 mgl^{-1} BA showing development of shoots. b. Leaf callus in B_5 medium containing 2 mgl^{-1} BA and IAA showing development of large number of shoots. c. Shoot tip cultured in B_5 with 4 mgl^{-1} BA showing development of multiple shoots. d. Multiple shoots developed from shoot-tip cultured in a medium containing 5 mgl^{-1} BA (Note the cluster of shoots at the nodes).

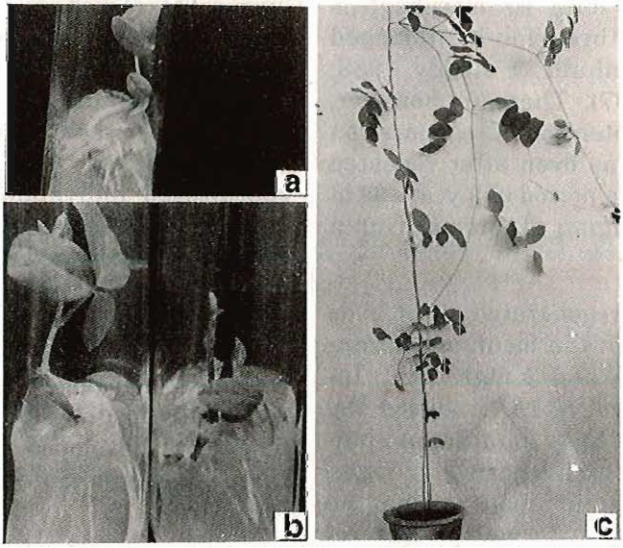

Fig. 2. Micropropagation of *Clitoria ternatea*

a. Isolated shoot rooted in B_5 medium with 1mgl^{-1} NAA (Note the poorly developed roots). b. Shoots cultured in medium containing 3 mgl^{-1} NAA showing profuse root development. c. Plant in pot after 3 months.

produced shoot buds only in the medium containing 3-4 mg l^{-1} IAA alone.

Shoot development readily occurred in the callus induced in B_5 medium containing 2, 4-D and Kin when subcultured in a medium supplemented with 1-2 mgl^{-1} BA and 2-4 mgl^{-1} IAA. No regeneration occurred in the medium containing either Kin and 2, 4-D alone or in combination. Similarly, the callus induced in B_5 and BA regenerated shoot buds only when subcultured in the medium containing 3-4 mgl^{-1} IAA alone. This suggests that induction of morphogenesis in the callus depends not only on the final regeneration medium, but also in the initial hormonal combination of the medium in which the callus was induced (Mukhopadhyay & Ram 1981). Interestingly, the calli subcultured 10-12 times over a period of 10 to12 months in the same medium readily produced shoots when transferred to the regeneration medium. This is contrary to the earlier reports that in legumes, callus normally loses its morphogenic potential if cultured for a prolonged period (Narasimhulu & Reddy 1983; Kysely *et al* .1987). There are however, reports on the retention of regeneration potential of callus even after repeated subculture over a period of 2 years as in *Albizzia richardiana* (Tomar & Gupta 1988).

The number of regenerated shoot buds was maximum in the medium containing 2 mgl^{-1} IAA and 2 mgl^{-1} BA. Increasing the level of IAA reduced the number of shoot buds induced. A similar observation was reported in *Vigna aconitifolia* wherein upon raising the level of IAA, shoot formation was reduced along with a considerable decrease in the number of shoot buds (Eapen, Gill & Rao 1982). In *Arachis hypogaea,* Murashige and Skoog's (1962) medium containing IAA alone induced shoots from immature embryos (Bajaj *et al.* 1981).

Multiple shoots were induced from shoot tips cultured in B_5 medium containing 3-5 mgl^{-1} BA within 20-30 days (Fig. 1d). Lower concentrations of BA (0.01-2 mgl^{-1}) did not produce multiple shoots even after prolonged culture or subculturing in the same medium, except slight callusing at the cut ends. Maximum number of shoots was obtained in 4 mgl^{-1} BA, while the number of shoots developed was comparatively less in 3 mgl^{-1} and 5 mgl^{-1} BA containing medium after 20 days in culture (Table. 1). This is similar to the report in *Vicia faba* that 4 mgl^{-1} BA was the effective concentration in promoting high rates of shoot development (Selva, Scouffs & Briquet 1989). A combination of 1-3 mgl^{-1} BA and IAA produced friable callus which subsequently turned brown and necrotic. It is well known that most legume species require higher concentration of cytokinins than plants of other families for favourable responses (Flick, Evans & Sharp 1983). Similar, *C. ternatea* also needed a high concentration of cytokinins for maximum shoot multiplication as well as for shoot regeneration . The multiple shoots were separated and subcultured individually in the same medium for further multiplication. This process was repeated for further multiplication or the isolated shoots were transferred to B_5 medium containing 1-3 mgl^{-1} NAA for root induction. Roots were less developed in 1 mgl^{-1} NAA containing medium (Fig. 2a) while profuse roots developed from shoots cultured in medium containing 3 mgl^{-1} NAA (Fig. 2b).

The regenerated plants were hardend for 1 week in B_5 basal liquid medium on filter bridges and subsequently transferred to small plastic pots containing

Table 1. Shoot tip multiplication in micropropagation of *Clitoria ternatea*

Hormone (mgl^{-1})	No. of apices that responded *	No. of shoots developed**	No. of shoots per apex Mean ± SD	Rate of survival *** (%)
BA 0.1	22	0	0	—
BA 1	24	0	0	—
BA 3	20	140	7.0 ± 0.5	84
BA 4	19	285	15.0 ± 1.2	82
BA 5	20	180	9.0 ± 0.4	90
BA 1 + IAA 1	24	0	0	—
BA 2 + NAA 1	18	0	0	—
BA 1 + GA_3 2	25	72	2.8 ± 0.4	92
GA_3 3	24	38	1.5 ± 0.3	90
Kinetin 2	25	0	0	—
Kinetin 1+ GA_3 0.2	24	28	1.1 ± 0.1	95
Hormone free	23	0	0	—

* Out of 30 cultured apices
** After 45 days of culture
*** After 6 months in the field

vermiculite covered with plastic bags to retain humidity. After 15 days in vermiculite, the plants developed new roots and were transferred to pots containing garden soil and sand. The plants survived well in soil (Fig. 2c).

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