ORIGINAL ARTICLE

In vitro embryo culture and antimicrobial activity of *Clitoria ternatea* L.

Madhu Kumari
Department of Botany, B.R.A. Bihar University, Muzaffarpur, Bihar, India

Background: *Clitoria ternatea* L. is an important rare medicinal plant species with memory-enhancing ability used as crude drugs in many ayurvedic medicines. **Objective:** The objectives were as follows: A. To develop a protocol for rapid clonal propagation of the important medicinal climber, *C. ternatea* L., through *in vitro* tissue culture of embryo explants through callogenesis and organogenesis, and B. Antibacterial study of ethanolic extract of *in vitro* raised plant and callus mass against *Pseudomonas aeruginosa* (MTCC189), *Bacillus subtilis* (MTCC8), *Escherichia coli* (MTCC1), and *Klebsiella pneumonia* (MTCC3883), respectively. **Materials and Methods:** Explants were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations and combinations of 6-benzylamino purine (BAP), (2,4-dichlorophenoxy acetic acid (2,4-D), and α-naphthalene acetic acid (NAA) for shoot and root induction. The disc diffusion method was adopted for antimicrobial study of the plant extract. **Results:** The sub-cultured of callus on MS basal medium supplemented with BAP (2.5 mg/l) and NAA (0.5 mg/l) showed highest rate of shoot multiplication. *In vitro* shoots were rooted on to the MS basal medium supplemented with NAA (0.5 mg/l). The sub-culture of callus on MS basal medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) showed highest rate of root multiplication from callus. The antibacterial activity of the ethanolic extracts of *in vitro* grown products of *C. ternatea* L. was found to have antimicrobial activity against all tested microorganisms. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, carbohydrates, and steroids in the *in vitro* grown products of *C. ternatea* L. **Conclusion:** An efficient protocol was developed for successful micropropagation and multiple plant regeneration of an important medicinal plant *C. ternatea* L. It is a widely used in ayurvedic medicine because of its multi-potent bioactive molecules and significant antibacterial properties.

Key words: Antibacterial activity, *Clitoria ternatea* L., ethanolic extracts, growth regulator, *in vitro* multiplication, phytochemicals

INTRODUCTION

Medicinal plants have played a pivotal role in the health care of ancient and modern cultures. Screening of natural products has been the source of innumerable therapeutic agents.[1] Plant extracts have shown the presence of various chemical constituents such as flavonoids, alkaloids, steroids, tannins, saponins glycosides, and phenol compounds, which are synthesized and deposited in specific or all parts of plants.[2,3] These bioactive compounds of plants may act by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters that have an effect on humans due to compatibility in their potential target sites. There are many research groups that are now engaged in medicinal plants research.[4,5] *Clitoria ternatea* L. also known as "butterfly pea" is a multipurpose forage legume that belongs to family Fabaceae [Figure 1a]. The plant extracts has anthelmintic,[6] antipyretic, anti-inflammatory, analgesic,[7] anxiolytic, antidepressant, anticonvulsant, sedative,[8] hypoglycemic,[9] and anticancer[10] properties. This species has been listed as a rare plant species by the International Union for Conservation of Nature and Natural Resources (IUCNNR).[11] In traditional ayurvedic medicine, it has been used for centuries as a memory enhancer.[12] It has been used as an ingredient in Medhya Rasayana, a rejuvenating recipe used for treatment of neurological disorders.[13] Traditionally, the root of this plant is useful in the treatment of dysentery, severe bronchitis, asthma, and hectic fever.[14] Also, the root extract is helpful in improving learning and memory in rats.[15] The lactone-aparajitin from leaves, sitosterol from seeds, taraxerol from roots and sitosterol, and anthoxanthin are isolated from seeds.[16] The flowers and leaves of *C. ternatea* are used to make collyrium; leaves are also used in Madagascar to relieve joint pain, and hepatopathy. The seeds of *C. ternatea* have laxative effects, and are cathartic; the root juice of *C. ternatea* L. is used for treatment of chronic bronchitis and the leaves are useful in otalgia and hepatopathy, whereas seeds are cathartic.[17] It contains antifungal proteins and has been shown to be homologous to plant defensins.[18,19] The role of medicinal plants in disease prevention or

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Address for correspondence: Dr. Madhu Kumari, Department of Botany, B.R.A. Bihar University, Muzaffarpur, Bihar, India.
E-mail: madhu_kumari58@yahoo.com
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control has been attributed to the antioxidant properties of their constituents.[20] The main objective of our study was to establish an efficient, quick, and reliable protocol for *in vitro* regeneration of *C. ternatea*. To fulfill the increasing demand of this potent medicinal plant, *in vitro* culture is an alternative method for conservation of this diminishing plant population. Some studies have reported *in vitro* plant regeneration and micropropagation of *C. ternatea*.[21-23] Knowledge of the chemical constituents of *in vitro* grown plants is desirable because such information will be of value for synthesis of complex chemical substances. In the present work, a preliminary qualitative phytochemical analysis was carried out using a crude extract of *in vitro* grown plants of *C. ternatea* L. The available literature on the medicinal properties of this plant mainly focuses on field grown plants. The plant, roots, and callus were successfully raised under *in vitro* tissue culture conditions to study the antimicrobial activity of *in vitro* raised *C. ternatea* L. plant. In this report, we describe the procedure for proliferation, and maintenance of *in vitro* grown plants and callus, on MS medium supplemented with different growth regulator. The antimicrobial activity of the ethanolic extract of *C. ternatea* L., as well as its preliminary phytochemicals screening, has been studied.

MATERIALS AND METHODS

Source of Explants

In order to obtain plants *in vitro*, a protocol was developed for rapid clonal propagation of *C. ternatea* L. through embryo culture. Mature seed pods were collected from the campus of Department of Botany, B.R.A. Bihar University, Muzaffarpur. The seeds were washed with a 5% (v/v) detergent solution Teepol) for 10 min followed by rinsing under running tap water for several times. The seeds were further treated with 70% alcohol for 1 min followed by 0.1% (w/v) mercuric chloride treatment for 5 min, aseptically. The seeds were soaked in sterile distilled water and allowed to sprout on wet blotting paper. After a period of 24 h, the embryo was excised from the seeds and washed with sterile distilled water, which was later dried using sterile blotting paper. Dried embryo obtained in this manner was used as explant for raising *in vitro* cultures.

Growth Medium and Culture Condition

Murashige and Skoog's (MS) medium[24] was used as the nutrient source with or without growth hormones such as 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzylamino purine (BAP), and α-naphthalene acetic acid (NAA) either individually or in combination for culture explants. Sucrose 3% and agar 0.8% (Hi media) were used as carbon source and gelling agent, respectively. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 20 min. The cultures were maintained in the culture room at 25°±2°C under white fluorescent light.

Shoot Organogenesis and Elongation

For multiple shoot induction the embryo explants were culture on MS medium supplemented with BAP (0.5-2.5 mg/l) alone or combination with kinetin (0.5-1 mg/l) [Table 1].

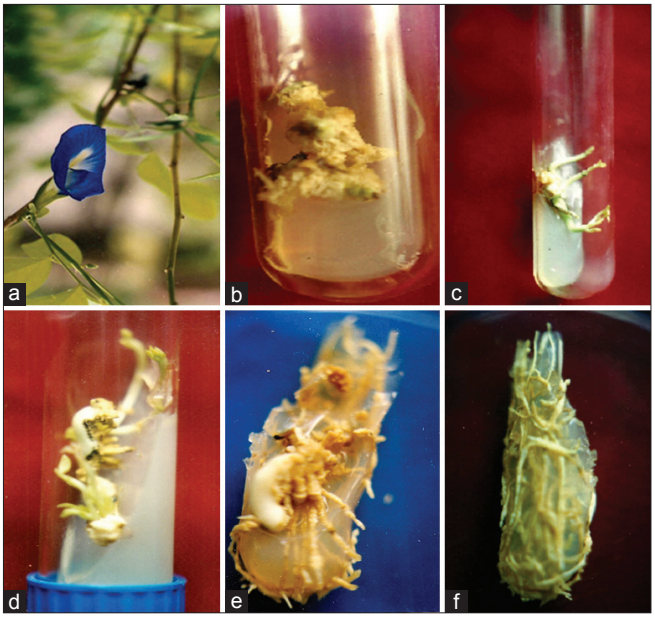

Figure 1: *In vitro* clonal propagation of *C. ternatea* L. from embryo explants. (a) A beautiful blue flower plant of *C. ternatea* L. (b) The callus emerged from embryo on MS + 2,4-D (0.5 mg/l). (c) Two to three leafy shoots emerged from embryo on MS + BAP (2.5 mg/l). (d) Multiple leafy shoots emerged from sub-culture of callus on MS + BAP (0.5 mg/l) to MS + BAP (2.5 mg/l) + NAA (0.5 mg/l). (e) Multiple roots emerged from callus on MS + BAP (1 mg/l) + NAA (0.5 mg/l). (f) The root system derived from excised embryo of *C. ternatea* after 25 days in old culture

Table 1: Effect of cytokinins and auxin and their interaction on shoot proliferation from embryo explants of *Clitoria tenatea* L.

Growth regulator	Concentration (mg/l)	% of explants showing callus	Induction of shoots per explants	Induction of roots per explants
2,4-D	0.5	100	0.0	0.0
	1.0	70	0.0	0.0
	2.5	50	0.0	0.0
BAP	0.5	100	1	0.0
	1	80	1-2	0.0
	2	70	1-2	0.0
	2.5	60	2-3	0.0
	5	70	0.0	0.0
BAP+KN	1+0.5	70	2-3	0.0
	2.5+0.5	74	3	0.0
	1+1	65	1-2	0.0
NAA	0.5	60	0.0	2-4
	1	50	0.0	1-2
BAP+NAA	2	50	0.0	0-0
Sub culture of callus derived from BAP (0.5mg/l and 0.1mg/l) into diff. con BAP+NAA	1+0.5	70	0.0	0.0
	1+0.5	-	0.0	10-12
	2+0.5	-	7-8	0.0

BAP – Benzylamino purine; KN – Kinetin; NAA – Naphthalene acetic acid

The subculture of callus was derived from BAP (0.5 mg/l) after 25 days on to MS medium supplemented with BAP (2.5 mg/l) and NAA (0.5 mg/l). Data on percentage of responding explants and number of shoots per explants were recorded after 25 days of initiation culture.

Roots Organogenesis and Elongation

For multiple roots induction embryo explants were culture on MS medium supplemented with NAA (0.5 mg/l to 1 mg/l) alone or BAP (1 mg/l) and NAA (0.5 mg/l). A bunch of roots derived from the callus [Table 1] was observed after 30-35 days of initiation of culture.

Preparation of Plant Extract

In vitro grown plants, callus, and roots were weighed and grinned with the help of a motor and pestle. About 50 g of the powdered grinded material was extracted using a soxhlet apparatus using 250 ml of ethanol as a solvent. The extracted solvent was removed from the extract under reduced pressure using a rotary vacuum evaporator. The sticky greenish-brown substance was obtained was stored in a refrigerator till use.

Antibacterial Assay

Both Gram-positive and Gram-negative bacteria were used as test organism for this study. Organisms like *Escherichia coli* (MTCC2845), *Bacillus subtilis* (MTCC441), *Klebsiella pneumonia* (MTCC3883), and *Pseudomonas aeruginosa* (MTCC189) were used for antibacterial assay. Bacteria were grown using nutrient broth medium at 37°C, for antibacterial assays. The disc diffusion method[25] was adopted for antimicrobial study of plant extract. Briefly, 20 ml of medium was transferred aseptically into each sterile petri dish and allowed to solidify. An overnight grown inoculum (100 µl) suspension was spread uniformly over the agar medium using a sterile glass rod for uniform distribution of bacteria. Sterile paper discs (~6 mm) were loaded with different concentrations of 25-400 µg of the plant extract of *C. ternatea* L. The discs were placed on the medium and incubated at 37°C for 24 h. Antibacterial activity was recorded by measuring the width of the zone of inhibition (in millimeter) formed around the disc.

Minimum Inhibitory Concentration Determination

The agar dilution method was used to determine the minimum inhibitory concentration (MIC) of all tested bacteria. A 1-ml volume of each extract containing the plant extract (25-400 µg) was added to sterile molten nutrient agar. A loop full of bacterial culture was used to inoculate the plate and incubated at 37°C for 24 h. Growth of organisms on each concentration was monitored to determine the minimum concentration that inhibited the growth of the tested organisms.

Phytochemical Screening

The ethanolic extract of the *in vitro* grown plants of *C. ternatea* L was used for qualitative phytochemical screening for identification of the various classes of active chemical constituents, using standard prescribed methods.[26,27] Positive tests were noted as present (+) and absent as (−).

RESULTS AND DISCUSSION

Medicinal plants are considered an effective and safe alternative to synthetic antibiotics. The quality and therapeutic efficacy of herbal drugs is dependent on the active constituents, which are present in the plant cell.[28] The wild stock of this important plant species has been rapidly diminishing due to over-exploitation. Natural plant propagation occurs through seed that is normally sown in the wet season. Thus, *in vitro* culture is an alternative method for conservation of this plant population. Embryo cultured on MS + 2,4-D (0.5-2.5 mg/l) only showed callus formation and it was not differentiated into root and shoot. Maximum callus induction was found in the sample treated with 2,4-D (0.5 mg/l) [Figure 1b]. However, beyond this concentration, the ratio of non-morphogenic cally increased subsequently. Increase in the size of embryo might be due to cell division and growth in the embryo in the presence of auxin. The role of auxin in enhancing the expression of a protein related to Cdc-2 type of cyclin-dependent kinases has been reported in tobacco.[29] The *in vitro* response of different auxins and cytokinins are presented in Table 1. Explants cultured on MS medium without cytokinin supplementation did not induce shoot proliferation. Varying concentrations of BAP and kinetin supplementation in the medium exhibited poor callus induction whereas shoot proliferation was enhanced. The medium supplemented with BAP alone induced only 2-3 shoots per explants [Figure 1c]. BAP at moderate concentration (1-2.5 mg/l) triggered callus formation and shoot induction [Table 1]. *In vitro* regenerated shoots were transferred onto the rooting medium containing MS medium mixed with NAA (0.5-1.0 mg/l). There was no rooting in the case of shoot planted on auxin-free basal medium. Root primordia emerged from the shoot base after 7-10 days of shoot transfer and rapid growth of root was observed. The best shoot multiplication (6-7) was observed when the explants were first cultured alone on BAP (0.5-1 mg/l) than when they were sub-cultured on medium containing BAP (2.5 mg/l) and NAA (0.5 mg/l) [Figure 1d]. The quality of shoot and all growth responses were better in the sub-culture of callus in this growth regulator combination. The excised embryo cultured on basal medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) produced significant result [Figure 1e]. The embryo swelled, gained size, with no differentiation at the plumular or radicular end for a

few days (6-8 days). At the end of this period, a burst of activity at the radicular end resulting in the formation of well-developed roots in large number was observed. This has proven to be a very important observation for a plant as highly valued roots are an important storage site for active phytochemicals. Embryo producing callus tissue, which differentiates into only roots, has been observed in a few cases.[30] However, the embryo of *Exocarpus cupressiformis* cultured in the presence of casein hydrolysate (400 p.p.m.) and indole acetic acid (IAA) (1 p.p.m.) showed a somewhat transition stage, producing a small root, but the plumule failed to develop into a shoot. Most significantly, hormonally induced development of embryo, producing massive roots, was achieved in the presence of BAP (1 mg/l) and NAA (0.5 mg/l) [Figure 1f]. This observation has potential for biotechnological application as two important root-derived alkaloids such as clitorin and aparajitin,[16] besides a number of pharmaceutically important active compounds, are known to exist in the roots of *C. ternatea.* These regenerated and well-rooted complete plants were transferred to a small plastic cup for hardening into an autoclaved sand and soil mixture (1:1), where the plants showed 70% survival rate. Further, the plants were transferred for acclimatization in to a field where 80% of the plants grew without any phenotypic aberration [Table 2]. Preliminary phytochemical analysis of the ethanolic extract revealed the presence of alkaloids, carbohydrates, reducing sugar, glycosides, steroids, terpenes, saponins, and flavonoids [Table 3]. Phytochemical constituents such as tannins, flavonoids, alkaloids, and several other aromatic compounds of plant that serve as defense mechanisms against predation by many microorganisms and insects were also observed.[31] The disc diffusion method is extensively used to investigate the antibacterial activity of natural substances and plant extracts. The antibacterial activity of the ethanolic extracts of *in vitro* grown products of *C. ternatea* L. is presented in Table 4. The result showed that it could inhibit the of growth of all studied bacteria with a maximum zone of inhibition of 22 mm for *P. aeruginosa*, 21 mm for *B. subtilis*, 19 mm for *E. coli*, and 13 mm for *K. pneumonia*, respectively. The point at which critical mass is reached is demonstrated by a sharply marginated circle of bacterial growth around the disk. The concentration of antimicrobial compound at this margin is called critical concentration and is approximately equal to the MIC obtained in agar dilution susceptibility tests.[32] The antibacterial activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins, and to complex with bacterial cell walls.[33] Several reports are available in support of antimicrobial activity of saponins against bacterial and fungal pathogens.[34] The plant may serve as a defence mechanism against predation by many microorganisms, insects, and herbivores. Thus it can be used as a source for developing new drugs or valuable metabolites for commercialization.

Table 2: Data on survival of transferred regenerated plants

	No. of plants	Plants survived	% response
Transferred for hardening	30	21	70
Transferred for acclimatization	20	16	80

Table 3: Qualitative analysis of the various phyto-constituents on the ethanol, extracts of *Clitoria ternatea* L.

Phytochemical test	Ethanolic extract of *Clitoria ternatea* plants
Test for alkaloids	
Dragendorff's test	++
Mayer's reagent	++
Test for saponin	++
Test for flavonoids	++
Test for carbohydrates	
Benedict's test	++
Molisch's test	++
Fehling's test	++
Test for proteins	++
Test for steroids	++
Test for terpenoids	++

Present++, Absent–

Table 4: Evaluation of antibacterial activity of the ethanolic extract of *in vitro* grown plants of *Clitoria tenatea* L.

Microorganisms	Zone of inhibition (mm) ethanol extract (µg)					Minimum inhibitory concentration (µg)
	25	50	100	200	400	
Escherichia coli	00	00	11	15	19	70
Bacillus subtilis	00	10	13	18	21	40
Klebsiella pneumonia	00	00	09	11	13	88
Pseudomonas aeruginosa	00	09	14	19	24	32

CONCLUSION

An efficient protocol was developed for successful micropropagation and multiple plant regeneration of an important medicinal plant *C. ternatea* L. It is a widely used in ayurvedic medicinal plant because of its multi-potent bioactive molecules. Phytochemical tests revealed the presence of flavonoids, saponins, alkaloids, carbohydrate, protein, steroids, and terpenoids. The antibacterial study suggests that the ethanolic extract of the *in vitro* grown plant possessed significant antibacterial activity against all tested bacteria. Extracts of this plant open up the possibility of finding new clinically effective antibacterial compounds. Further work is needed to purify the compounds from *in vitro* grown *C. ternatea* L. plant extracts. The microbicidal property of the purified phytochemical will enable the scientific community to recommend its utilization as an accessible alternative to synthetic antibiotics.

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