International Journal on Emerging Technologies **10**(1): 35-41(2019)

ISSN No. (Print) : 0975-8364
ISSN No. (Online) : 2249-3255

In Vitro Regeneration of *Clitoria ternatea* (L.) from Nodal Explant

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(Received 22 November 2018, Revised 12 February 2019 Accepted 27 February 2019)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: *In vitro* clonal propagation of *Clitoria ternatea* has been achieved by employing decapitated embryonic axes (DEAs) explants. The explants induced multiple shoots on cytokinin-containing medium. Several cytokinins [6 -benzylaminopurine (BAP), 6-furfuryl aminopurine (KIN) and thidiazuron (TDZ)] were assayed. The best response was achieved with 8.90µM BAP in which 100% of cultures produced 6.0±0.14 shoots per explant. MS+2.88 µM gibberellic acid (GA_3) was the most suitable for shoot elongation. Regenerated shoots were rooted in half-strength Murashige and Skoog (MS) medium with 0.98 µM indole-3-butyric acid (IBA). Plantlets were successfully acclimatized and established in soil, and they were morphologically indistinguishable from the source plant. The plantlets attained maturity and flowered normally. The efficient regeneration protocol reported here provides an important method of micropropagation of this plant. Furthermore, this protocol may be used for genetic transformation of this valuable medicinal plant for its further improvement.

Keywords: Clonal propagation, *Clitoria ternatea,* Decapitated embryonic axes, Plant regeneration

Abbreviation: BAP- 6 –benzylaminopurine, GA_3- Gibberellic acid, IBA- Indole-3-butyric acid, KIN- Kinetin, NAA- α-naphthalene acetic acid, TDZ- Thidiazuron, MS- Murashige and Skoog's medium

I. INTRODUCTION

C. ternatea (Fabaceae) commonly known as aparajita or butterfly in India [1]. It is an ornamental perennial climber having medicinal properties with conscious blue or white flowers. It has diploid chromosome number 2n=16 [2]. It is found throughout the tropical regions. Propagation of *C. ternatea* through seed is unreliable due to poor germination and death of young seedling under natural conditions. It is a highly palatable forage legume generally preferred by livestock over other legumes. Overexploitation of the plant species by pharmaceutical companies has resulted in a rapid depletion of the natural stock to the extent that it is now enlisted by the International Union for Conservation of Nature and Natural Resources (IUCNNR) as a rare species [3]. The United States Development Agency (USDA) selected *C. ternatea* along among other sixteen leguminous species with value-added industrial and pharmaceutical potential for immediate conservation [4]. *Clitoria ternatea* is a high quality, protein rich legume, often refered as a protein bank and grown at low cost [5]. Live stocks tend to prefer it over other legumes and grasses and it is therefore much valued as a pasture legume. A N-fixing legume, *clitoria ternatea* is used as a green manure. It is valuable cover crop in coconut and rubber plantation. The pods are edible and used as vegetable in the Philippines [6]. Butterfly pea is used in fences and in trillises as an ornamental for its showy flower, valuable for dyeing and in ethno-medicine [5]. The extract of *C. ternatea* was found to have anxiolytic, antidepressant, anticonvulsant and anti-stress properties [7]. Oral administration with 100 mg/kg of aqueous root extract of *C. ternatea* for 80 days have proved to improve learning and memory in rats [8]. Oral treatment with alcoholic extract of aerial and root parts has been reported to increase acetylcholine content and acetylcholinesterase activitity [9]. It also has laxative and diuretic properties and treats dysentery, bronchitis and asthma [10].

Plant tissue culture is an alternative method of commercial propagation of a large number of plant species, including many medicinal plants. Strategies to regenerate plants from tissue culture of legumes have been evolved steadily during the past few years. Plant regeneration *in vitro* in *C.ternatea* has been reported from leaf [11], immature embryo and seedlings [12] and nodal segments [13] but with a limited success.

The objective of present communication was to develop an efficient and reproducible method for in vitro micropropgation and *ex vitro* establishment of this medicinally important plant species using nodal explants derived from seedlings germinated *in vitro*.

II. MATERIALS AND METHODS

A. 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 [14] basal medium for its further germination. The 10 days old seedlings were served as the source of nodal explants.

B. Culture media and culture conditions

MS medium was used in all experiments. The medium was 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. Media were autoclaved at 1.06 kg cm^{-2} at 121℃ for 15 min. The

cultures were incubated at 24±2℃ under 16 hr. photoperiod of 50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India).

C. Induction of multiple shoots

The nodal segment were cultured on MS basal medium supplemented with BAP (0.0-22.1µM), KIN (0.460-23.20 µM) and TDZ (0.04-4.54 µM) for multiple axillary shoot proliferation (Table 1), combinations of BAP (0.443-22.1 µM) and NAA (0.537-5.37 µM) (Table 2) or KIN (0.460-23.20 µM) and NAA (0.537-5.370 µM) (Table 3) were also evaluated for axillary shoots regeneration potential. The frequency of responding explants, mean number of shoots/explant and mean shoot length (cm) were recorded after four weeks of culture.

Table 1: Effect of various concentrations of cytokinins on shoot regeneration from nodal explants of *Clitoria ternatea* L. after 4 weeks of culture initiation.

Cytokinins	Concentration (µM)	Regeneration frequency (%)	Number of shoots/explant (Mean±S.E.)	Length of shoot (cm) (Mean±S.E.)
BAP	0.0	68	2.30±0.23^{bc}	3.00±0.39^{bc}
	0.44	70	2.60±0.32^{bc}	2.80±0.31^{bc}
	2.22	85	3.80±0.50^{a}	5.00±0.14^{a}
	4.40	82	3.00±0.39^{ab}	4.00±0.48^{ab}
	8.90	78	2.10±0.47^{bcd}	3.20±0.37^{bc}
	17.75	75	2.00±0.26^{bcd}	2.90±0.38^{bc}
	22.10	68	2.30±0.23^{bc}	3.00±0.39^{bc}
KIN	0.46	48	0.80±0.16^{e}	2.00±0.26^{c}
	2.23	50	1.20±0.20^{de}	3.50±0.50^{b}
	4.60	52	1.80±0.47^{cd}	3.80±0.50^{b}
	9.30	58	2.50±0.22^{bc}	3.00±0.39^{bc}
	18.50	55	0.80±0.16^{e}	2.90±0.32^{bc}
	23.20	50	0.50±0.13^{e}	0.70±0.19^{d}
TDZ	0.04	10	0.20±0.10^{ef}	0.24±0.28^{de}
	0.09	35	0.32±0.12^{ef}	0.30±0.11^{de}
	0.22	40	0.45±0.11^{e}	0.50±0.13^{d}
	0.53	30	0.30±0.14^{ef}	0.35±0.25^{de}
	2.68	0	0	0
	5.37	0	0	0

Each mean is based on three replicates, each of consisting 20 culture tubes. The values marked with the same letter do not differ significantly (Duncan's multiple range test, P < 0.05).

Table 2: Effect of BAP and NAA combinations on shoot regeneration from nodal explants of *Clitoria ternatea* L. after 4 weeks of culture initiation.

Growth regulator (µM)		Explant developing axillary shoots (%)	Number of shoots/explant (Mean±S.E.)	Length of shoot(cm) (Mean±S.E.)
BAP	NAA			
0.44	0.53	45	2.50±0.22^{fg}	2.60±0.32^{cdef}
0.44	1.07	50	2.55±0.31^{efg}	2.60±0.31^{cdef}
0.44	2.68	65	2.85±0.32^{defg}	2.80±0.32^{bcdef}
0.44	5.37	60	2.10±0.23^{fgh}	2.00±0.26^{f}
2.22	0.53	62	3.00±0.39^{defg}	2.80±0.32^{bcdef}
2.22	1.07	65	3.20±0.37^{cdef}	3.35±0.40^{bcde}
2.22	2.68	70	3.80±0.46^{cd}	3.75±0.46^{bcd}
2.22	5.37	80	4.20±0.50^{bc}	4.00±0.48^{b}
4.40	0.53	90	5.90±0.28^{a}	5.70±0.32^{a}
4.40	1.07	82	5.20±0.14^{ab}	5.60±0.32^{a}
4.40	2.68	78	5.00±0.14^{ab}	5.40±0.15^{a}
4.40	5.37	75	4.85±0.19^{b}	5.20±0.14^{a}
8.90	0.53	70	3.75±0.46^{cd}	3.88±0.47^{b}
8.90	1.07	65	3.60±0.46^{cde}	3.50±0.45^{bcde}
8.90	2.68	60	2.50±0.22^{fg}	3.00±0.39^{bcdef}
8.90	5.37	58	2.20±0.24^{fgh}	2.60±0.30^{cdef}
17.75	0.53	70	3.60±0.46^{cde}	3.80±0.47^{bc}
17.75	1.07	64	3.00±0.39^{defg}	3.30±0.39^{bcde}
17.75	2.68	50	2.00±0.26^{gh}	2.40±0.31^{ef}
17.75	5.37	C	0	0
22.10	0.53	75	2.85±0.32^{defg}	3.00±0.34^{bcdef}
22.10	1.07	68	2.30±0.23^{fg}	2.55±0.23^{def}
22.10	2.68	65	1.20±0.20^{h}	1.80±0.47^{f}
22.10	5.37	0	0	0

Each mean is based on three replicates, each of consisting 20 culture tubes. The values marked with the same letter do not differ significantly (Duncan's multiple range test, P < 0.05).

D. Effect of silver nitrate ($AgNO_3$) on shoot multiplication
To evaluate the efficacy of silver nitrate on shoot multiplication, its different concentrations (0.58-5.88 µM) (Table 4) were supplemented in MS medium containing 4.40 µM BAP and 0.537 µM NAA. The frequency of responding explants, mean number of shoots explant and mean shoot length (cm) were recorded after four weeks of culture.

E. Rooting and acclimatization
Elongated shoots (4-5 cm) with two to three pair of leaves were harvested and transferred to half MS medium supplemented with different concentrations (0.49-7.38 µM) IBA (Table 5). Data were recorded on percentage of rooting, mean number of roots and root length 4 weeks after transferring onto rooting medium. Plantlets with well developed shoots and each were removed from the culture medium, washed gently under running tap water and 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 illumination of 20 µmol $m^{-2}s^{-1}$ 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. The acclimatized plantlets further transferred to pots containing autoclaved gardens oil and sand (1:1) and kept into greenhouse conditions before transfer into the field.

E. Statistical analysis
Experiments were set up in a completely randomized block design and each experiment usually had three replicates. The number of culture tubes containing single explant per replicate was twenty culture tubes. 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 new multiple range test (DMRT) at a $P < 0.05$ level [15].

Table 3: Effect of Kinetin and NAA combinations on shoot regeneration from nodal explant of *Clitoria ternatea* L. after 4 weeks of culture initiation.

Growth regulator (µM)		Explant developing axillary shoots (%)	Number of shoots/explant (Mean±S.E.)	Length of shoot (cm) (Mean±S.E.)
KIN	NAA			
0.46	0.53	10	0.60 ± 0.60^{hi}	0.80 ± 0.20^{gh}
0.46	1.07	15	0.80 ± 0.20^{ghi}	1.10 ± 0.28^{fgh}
0.46	2.68	28	1.75 ± 0.44^{cdefg}	1.48 ± 0.39^{efgh}
0.46	5.37	18	0.90 ± 0.17^{ghi}	1.00 ± 0.20^{gh}
2.32	0.53	25	0.80 ± 0.20^{ghi}	0.85 ± 0.22^{gh}
2.32	1.07	32	1.00 ± 0.17^{fghi}	0.95 ± 0.17^{gh}
2.32	2.68	40	1.55 ± 0.40^{cdefgh}	2.50 ± 0.22^{abcd}
2.32	5.37	25	1.35 ± 0.27^{defghi}	2.00 ± 0.35^{bcdef}
4.60	0.53	30	1.85 ± 0.44^{cdef}	1.75 ± 0.44^{cdefg}
4.60	1.07	38	2.00 ± 0.37^{bcde}	1.75 ± 0.36^{cdefg}
4.60	2.68	45	2.20 ± 0.24^{bcd}	2.30 ± 0.23^{abcde}
4.60	5.37	40	2.12 ± 0.36^{bcd}	2.65 ± 0.21^{abc}
9.30	0.53	60	2.50 ± 0.22^{abc}	2.90 ± 0.26^{ab}
9.30	1.07	67	3.20 ± 0.36^{a}	3.00 ± 0.36^{a}
9.30	2.68	64	2.85 ± 0.31^{ab}	3.20 ± 0.32^{a}
9.30	5.37	54	2.10 ± 0.36^{bcd}	2.40 ± 0.39^{abcde}
18.50	0.53	50	2.00 ± 0.37^{bcde}	2.50 ± 0.22^{abcd}
18.50	1.07	30	1.45 ± 0.38^{defghi}	1.68 ± 0.43^{defg}
18.50	2.68	20	0.50 ± 0.11^{i}	0.60 ± 0.15^{h}
18.50	5.37	8	0.50 ± 0.11^{i}	0.70 ± 0.15^{h}
23.20	0.53	20	0.80 ± 0.16^{ghi}	1.00 ± 0.20^{gh}
23.20	1.07	30	1.10 ± 0.20^{efghi}	1.30 ± 0.23^{fgh}
23.20	2.68	10	0.60 ± 0.14^{hi}	0.80 ± 0.16^{gh}
23.20	5.37	0	0	0

Each mean is based on three replicates, each of consisting 20 culture tubes. The values marked with the same letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

Table 4: Effect of $AgNO_3$ on multiple shoot formation from nodal explants of *Clitoria ternatea* L. cultured on MS medium supplemented with 4.40 µM BAP and 0.53 µM NAA after 4 weeks of culture initiation.

$AgNO_3$ concentration (µM)	Explant developing axillary shoots (%)	Number of shoots/explant (Mean±S.E.)	Length of shoot (cm) (Mean+S.E.)
0.58	74	4.00 ± 0.43^{c}	5.10 ± 0.11^{c}
1.52	85	5.10 ± 0.11^{bc}	5.75 ± 0.15^{bc}
2.94	92	6.70 ± 0.38^{a}	7.85 ± 7.90^{a}
4.70	88	6.00 ± 0.46^{ab}	6.80 ± 0.40^{b}
5.88	80	6.00 ± 0.46^{ab}	5.80 ± 0.46^{bc}

Each mean is based on three replicates, each of consisting 20 culture tubes. The values marked with the same letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

Table 5: Effect of different concentrations of IBA on root Induction from regenerated shoots of *C. ternatea* cultured on half-MS medium.

Medium	IBA (µM)	Rooting frequency (%)	Number of root/explants (Mean± S.E.)	Length of roots (Mean± S.E.)
½ MS	0.0	20	2.09±0.20^{de}	2.40±0.22^{d}
½ MS	0.49	68	3.16±0.30^{c}	3.60±0.33^{c}
½ MS	0.98	90	6.00±0.42^{a}	6.12±0.47^{a}
½ MS	2.46	80	3.50±0.22^{b}	4.38±0.25^{b}
½ MS	4.90	70	3.15±0.12^{c}	4.10±0.21^{bc}
½ MS	7.38	40	2.10±0.11^{d}	2.10±0.17^{e}

Each mean is based on three replicates, each of consisting 20 culture tubes. The superscript indicate significant difference between mean (P<0.05) comparison by DMRT.

III. RESULTS AND DISCUSSION

A. Multiple shoots induction

Nodal segments are preferred explant for micropropagation due to the presence of preexisting meristems, which can be developed easily into shoots while maintain clonal fidelity [16]. Multiplication of plants through nodal segment explants has been achieved for a large number of plant species and is the basis of most of the commercial systems. Cytokinins are generally recognized as a critical factor for the initiation of shoot primordial from cultured tissues. In the present study nodal segments of *C. ternatea* cultured on MS medium supplemented with BAP (0.44-22.10 µM), KIN (0.46-23.20 µM) and TDZ (0.04-4.54 µM) (Table 1) singly to find out their influence on shoot regeneration. Data revealed that nodal explants cultured on MS medium on growth regulator free MS medium promote only two axillary shoot proliferation. Among three cytokinins (BAP, KIN and TDZ) tested only BAP and KIN support the shoot regeneration. An addition of a cytokinin was essential to induce multiple shoot formation from the explants. All concentrations of BAP and KIN support the shoot differentiation. BAP is highly efficient than KIN for shoot regeneration. The number of shoots/explant is significantly higher on BAP containing media than KIN. In general by increasing concentrations of growth regulators the responding frequency of explants and number of shoot regeneration increased. Increasing concentration of either BAP (above 2.22 µM) or KIN (above 9.30 µM) in the culture media resulted in reduction of morphogenetic response.

Analysis of variance revealed that mean shoot numbers and mean shoot length was significantly affected by the concentrations and types of cytokinin tested (Table 1). MS medium supplemented with 2.22 µM BAP showed the highest shoot regeneration frequency (85%), number of regenerated shoots (3.8 ± 0.50) and shoot length (5.0±0.14) (Fig.1a). The optimum frequency of shoot proliferation (58%) and number of shoots per explant was relatively low when the medium was supplemented with KIN (Table 1). TDZ is well established growth regulator acts as cytokinin and promotes shoot regeneration several plant species of monocots [17] and dicots [18], while it is not supportive at all for shoot regeneration from nodal segments of *C. ternatea*. Effectiveness of BAP in shoot regeneration from nodal explants has been reported in several other species [19-20]. Use of cytokinins reduce the dominance of apical meristems and induce axillary as well as adventitious shoot formation from meristematic explants [21].

B. Effect of BAP, Kinetin and NAA on shoot regeneration

Combinations of cytokinin and an auxin was found sometimes suitable than alone cytokinin for shoot organogenic response [22]. The shoot induction experiments using nodal explants were performed in the combinations of BAP (0.44 µM) and NAA (0.53-5.37µM) (Table 2) or KIN (0.46-23.20 µM) and NAA (0.53-5.37 µM) (Table 3). Data revealed that addition of NAA with BAP or KIN was more effective for direct organogenesis in terms of responding frequency of explants, number of shoots induction per explant and shoot length. BAP was found to be more effective than KIN when added in MS medium in combination with NAA in different concentrations. Highest number of shoots (5.90±0.28) was observed in explants inoculated on MS+BAP (4.40 µM) + NAA (0.53 µM) (Table 2, Fig.1b, c), whereas when KIN in combination with NAA was incorporated in MS medium the number of shoots per explant was considerably decreased. The maximum number of shoots (3.20±0.36) (Table 3) was recorded on MS medium supplemented with 9.30 µM KIN and 1.07µM NAA. Cytokinin and NAA combinations were reported in other plant species for induction of organogenesis [23]. The limitation of cytokinin (BAP/KIN) and NAA combination is development of callus at the base of nodal segment which gradually covered the entire surface of explant.

C. Effect of $AgNO_3$ on shoot multiplication

According to the experiments on *C. ternatea* micropropagation, cytokinins especially BAP, were essential to obtain maximum number of multiple shoot formation from nodal segments. In several systems micropropagation protocol used have the combination of cytokinins and auxins which supports the multiple shoot regeneration and promoted excessive amount of callus formation in the basal ends of the explants [24]. In *C. ternatea* also MS medium supplemented with 4.40 µM BAP and 0.537 µM NAA was found most suitable for axillary shoot differentiation from nodal explants with excessive callus formation. It was urgently needed to avoid the callus formation from nodal explants so that we can get callus free multiple shoot production. Auxins are known to stimulate ethylene biosynthesis [25]. An endogenous ethylene is an important regulator of diverse morphogenic processes [26] as well as in determining the regeneration response of cultured tissues [27]. Ethylene is recognized as a ubiquitous plant hormone, which influences growth and development of plants [28]. *In vitro* studies have indicated that ethylene can affect callus growth, shoot regeneration and somatic embryogenesis [29].

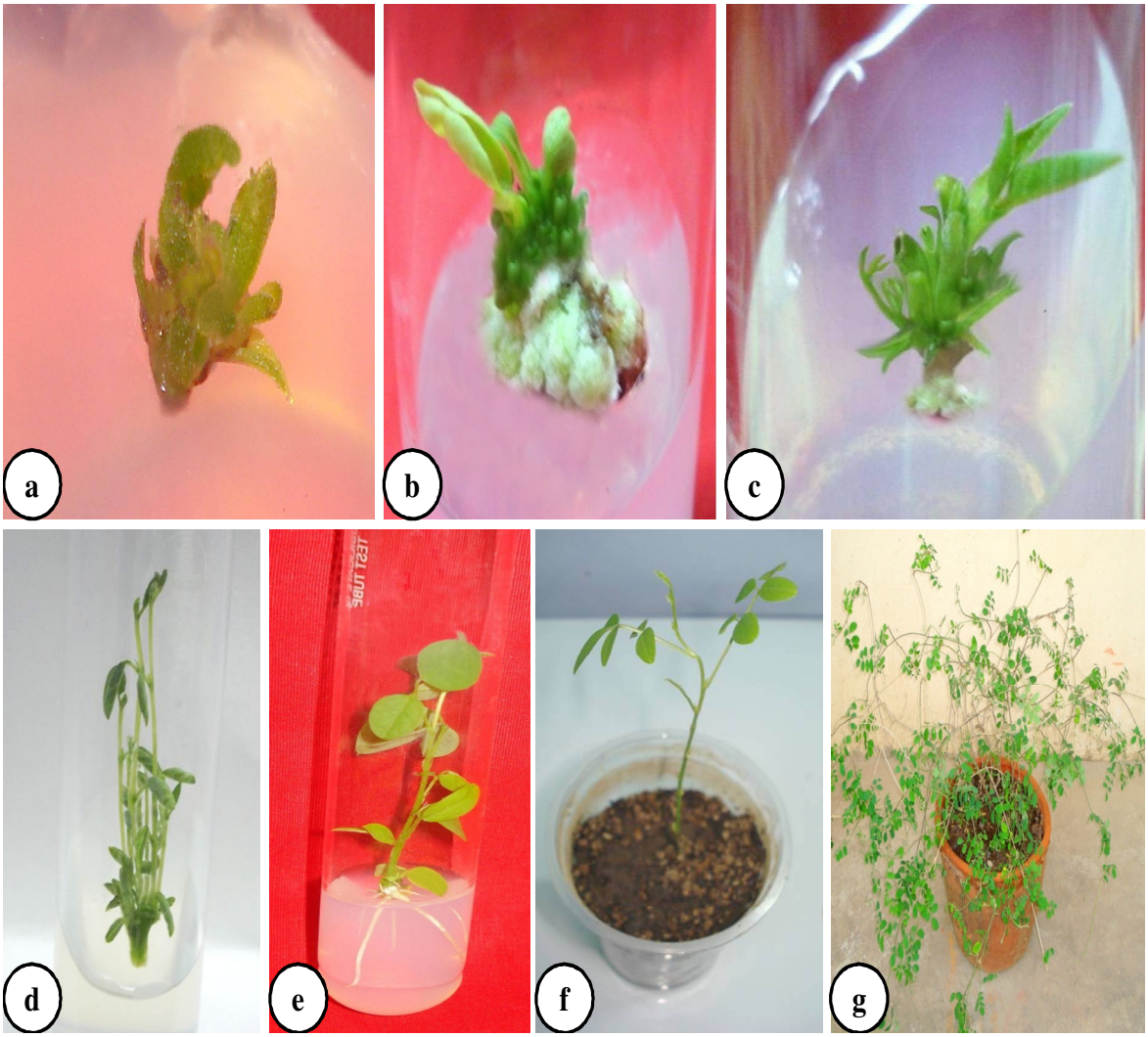

Fig. 1. *In vitro* shoot regeneration from *Clitoria ternatea* nodal explants. **(a)** multiple shoot regeneration from nodal explant on MS medium supplemented with 4.40 µM BAP **(b)** nodal explant cultured on MS medium supplemented with 4.40 µM BAP + 0.53 µM NAA showing axillary shoot induction and callus development **(c)** Enhancement of axillary shoot regeneration and inhibition of callus formation when nodal explant cultured on optimum shoot regeneration medium (MS+4.40 µM BAP + 0.53 µM NAA) supplemented with 2.94 µM $AgNO_3$ **(d)** Elongation of shoots on MS medium containing 4.40 µM BAP, 0.53 µM NAA and 2.94 µM $AgNO_3$ **(e)** Rooting of elongated shoot cultured on half- MS medium supplemented with 0.98 µM IBA **(f)** Rooted plantlet transferred into plastic cup containing sterile soilrite and kept for acclimatization **(g)** Acclimatized plantlet was transferred into pot containing garden soil.

Thus by regulating the production or action of ethylene, the growth and development of some tissue cultures can be controlled to a certain extent [30]. To avoid callus formation explants were cultured on best shoot induction media (MS+4.40 µM BAP +0.53 µM NAA) supplemented with different concentrations (0.58-5.88 µM) of $AgNO_3$ (Table 4). Results revealed that by addition of silver nitrate callus formation was suppressed and percent regeneration, number of shoots per explant and shoot length was significantly improved. Highest frequency (92%) of regeneration, maximum number of shoots per explant (6.7) and shoot length (7.85) was obtained on induction media supplemented with 2.94 µM silver nitrate (Fig.1d). Higher concentrations (>2.94 µM) of silver nitrate was inhibitor for organogenic response.

Silver nitrate has been known to inhibit ethylene action [31] so it widely used in tissue culture systems to improve regeneration of both monocot and dicot plant tissue cultures [32-33]. The usage of silver nitrate has shown to have important effects in plant tissue culture e.g., improving somatic embryogenesis [34] and organogenesis [35]. It is well established that polyamines biosynthesis accelerated after inhibition of ethylene biosynthesis due to silver ions [36]. It has been postulated that polyamines are a type of growth regulator so it promoted regeneration in several species [37].

D. Rooting of elongated shoots and acclimatization

Elongated shoots can be easily rooted in half MS medium containing 0.98 µM IBA and successfully acclimatized and transferred to field (Table 5; Fig.1e). IBA is higly effective in rooting of microshoots [38].

In vitro root induction and hardening were accomplished simultaneously in soilrite, whereas *in vitro* rooted shoots in agar-based medium were transferred into plastic cup containing soilrite and covered with polythene for about one week for acclimatization of the plant and irrigated with ½ MS salt solution for two weeks than followed by distilled water and new growth was observed within 3-4 weeks. After 4 weeks tissue cultured derived plantlets were successfully established and they were later transferred to pots containing soil and sand (Fig. 1f-g).

IV. CONCLUSIONS

The present study was conducted to develop a complete *in vitro* propogation protocol for valuable medicinal plant *C. ternatea* through nodal explants. During optimization, numerous cytokinins (BAP, KIN and TDZ) alone or in combination with NAA were tested to develop a high proliferation rate and to obtain healthy, multiple shoots. The addition of the ethylene inhibitor $AgNO_3$ to the best regeneration culture medium (MS+ 4.40 µM BAP + 0.537 µM NAA) helped in good auxiliary shoot proliferation and elongation, as well as low callus formation. Elongated shoots can be easily rooted in half MS medium containing 0.98 µM IBA and successfully acclimatized and transferred to field. This protocol can also be used for raising genetically uniform plants, which is important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation of elite germplasm.

ACKNOWLEDGEMENT

Author wish to thanks Head, Department of Botany, MMV, Banaras Hindu University, Varanasi for necessary support and help.

Conflict of interest

None

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How to cite this article: Mishra A.K.; Singh, Jayanti and Tiwari K.N. (2019). *In Vitro* Regeneration of *Clitoria ternatea* (L.) from Nodal Explant, *International Journal on Emerging Technologies,* **10**(1): 35-41.