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IN VITRO PROPAGATION OF AN ENDANGERED MEDICINAL PLANT *BRYONIA LACINIOSA* L.THROUGH LEAF EXPLANT

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Abstract:

An endangered medicinal plant *Bryonia laciniosa* L. belongs to the family Cucurbitaceae was selected to regenerate the young plants through tissue culture techniques using leaf explants. The leaf explants gave differential response to different concentrations of 2, 4- D and produced green to dark green compact calli. The maximum amount of callus tissue per explants was produced on MS medium with 2.0 mg/l, 2, 4-D with 60 % response by showing dark green, hard, compact calli. In order to induce differentiation, leaf derived tissues cultured on BAP produced shoot buds on MS medium supplemented with 2 + 2.0 mg/l, 2, 4- D + BAP combination. Efficiency of the media was assessed in terms of enhancement of shoot bud production. Spontaneous rooting was observed in medium containing NAA and IBA combination. The highest mean number of roots per culture was noted in half strength MS medium supplemented with 1 + 0.8 mg/l NAA + IBA combination. The in vitro grown complete plantlets were then successfully transferred from the culture room to thermocol cups through a process of successive phases of acclimatization.

Key Words: *Bryonia, In Vitro* Propagation, Growth Regulators, Callogenesis, Shoot & Root Induction

Introduction:

Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. Greater demand for these plants especially for the purpose of medicine is one of the causes of their rapid depletion from primary habitats. Micro propagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation (Boro *et al*., 1998). There has been progress in tissue culture studies in Cucurbitaceae members such as *Zehneria scabra* (Anand and Jeyachandran, 2004) *Diplocyclos palmatus* (DaSilva *et al*., 2005; Alpana *et al*., 2011; Caroline *et al*., 2012 and), and *Cucurbito pepo* (Pal *et al*., 2006). It is commonly known as lollipop climber and it is also known as "Shivlingi" in India *Bryonia* plants are used to treat adenopathy, asthma, headache, phthisis, tuberculosis etc.The bioactive molecule goniothalamin isolated from this plant showed potent cytotoxicity, weak antibacterial and significant antifungal activity against a wide range of gram positive and gram negative bacteria and fungi (Mosaddik and EkramulHaque, 2003). Traditional healers use the leaves and the seeds of this plant for treatment of fevers. It is also taken in impotency and used as a tonic. Whole plant is used to treat ague, bronchitis, carbuncles, cholera, colic, consumption, convulsions, cough, delirium, fertility, megalopleny, paralysis and snake bite. The chloroform extract of *Bryonia laciniosa* has exhibited a significant anti-inflammatory activity (Gupta *et al*., 2003).Analgesic and antipyretic activity of methanol extract of *Bryonia laciniosa* also has been shown in standard animal models (Sivakumar*et al*.,2004).

Plant Collection:

In the present study, herbal plants were surveyed in the Kundri hill of Thukkanayakkan Palayam range of Sathyamangalam forest. From the survey *Bryonia* (*Bryonia* meaning to sprout in Greek) *laciniosa* was selected and the leaves were used for conservation techniques. *Bryonia laciniosa* is an annual scaberulous climbing herb, tendril bifid, stem angular, leaves deeply palmately five lobed, flowers monoecious male and female fascicled, often in the same axils, calyx campanulate, lobes ovate, stamens three, free, inserted, ovary inferior, style slender, stigma three, papillose, deeply two lobed, pistillodes in male flowers, fruit ovoid, brick-red when ripe with white vertical lined, many seeded berry (Gamble and Fischer, 1915- 1936). It is used commonly as an aperient medicine and tonic and is globally distributed in the paleotropics. The over exploitation even results in ecological imbalance. Therefore, intensive studies on medicinal plant species pertaining to their conservation are desperatively needed. Hence, an *in vitro* regeneration technique was studied.

Materials and Methods:

Explants Preparation:

The young leaf explants of field grown plants of *Bryonia laciniosa* were collected, thoroughly washed under tap water for 10 minutes and treated with liquid detergent for 10 minutes, followed by dipping in 5% (v/v) savlon solution (an aqueous solution containing 0.3% (w/v) chlorhexidine gluconate and 3% (w/v) cetimide as active ingredient) for 10 minutes. They were then washed several times with distilled water. After rinsing with 70% ethanol for less than 60 seconds, they were surface sterilized with 0.1 % (w/v) mercuric chloride solution for 6 minutes and washed with sterile distilled water 4-5 times to remove all the traces of mercuric chloride.

Planting Medium and Culture Conditions:

Basal nutrient medium consisted of MS salts and vitamins (Murashige and Skoog, 1962) along with 3% (w/v) sucrose and 0.8% agar. pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min at 121℃ and 15 lbs/Psi to decontaminate the medium. After inoculation all cultures were incubated in controlled conditions with 25± 2℃, 16h light/8h dark photoperiod and light intensity provided by cool white fluorescent tubes.

Induction of Callus:

The surface sterilized leaf explants were cut in to small 1cm pieces with a sterilized surgical blade and then inoculated on MS basal medium which was supplemented with different concentrations(0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) of 2,4-D individually for induction of calli. The hard compact calli were selected for shoot bud organogenesis. Sub culturing of leaf derived calli on fresh medium was done after 23 days of its development for the induction of shoot.

Proliferation of Shoot Buds:

In order to induce differentiation the callus tissues were sub cultured on medium supplemented 2.0 mg/l of 2,4-D with different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) of Benzyl amino purine(BAP).

Rooting in Micro Shoots:

For rooting, the micro shoots up to 2 to 3 cm long were excised aseptically and individually transferred to root induction medium containing half strength MS basal medium supplemented with various concentrations (0.6, 0.8 and 1.0 mg/l) of NAA individually and in combinations with different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/l) of IBA. The data were recorded after 4 weeks of transfer.

Acclimatization:

For acclimatization, the mouth of the culture tubes were kept open for one day in the culture room and they were then kept outside the culture room for 6 hours of the following day. Later on, they were kept outside the culture room for 12 hours. Finally the seedlings were taken out of the culture tubes and rinsed with running tap water for complete removal of medium attached to roots. The *in vitro* raised well rooted plantlets were transferred to thermocol cups containing soil rite and then to pots containing a mixture of soil and compost (2:1) following successive phases of acclimatization. For each set of experiment 10 replicates/concentration were studied. All the experiments were repeated 3 times. Data were recorded periodically for any noticeable change. The data were subjected to statistical analysis for computation of the Standard Error (SE) of the Mean.

Results and Discussion:

In the present study, the leaf explants gave differential response to different concentrations of 2, 4- D and produced green to dark green compact calli. The maximum amount of callus tissue per explant was produced on MS medium with 2.0 mg/l, 2, 4-D with 60 % response by showing dark green, hard, compact calli. Similar kinds of proliferation of callus in 2, 4- D supplemented media were also noted in *Arabidopsis* (Kumar *et al*., 1983); *Canavalia brasiliensis* (DaSilva e*t al*., 2005) and *Diplocyclos* (Caroline *et al*., 2012). In order to induce differentiation, leaf derived tissues cultured on BAP produced shoot buds on MS medium supplemented with 2 + 2.0 mg/l, 2, 4- D + BAP combination. Efficiency of the media was assessed in terms of enhancement of shoot bud production. Such enhancement of shoot bud has been noted in *Zehneria scabra* (Anand and Jeyachandran, 2004) cultured on BAP and IAA. Spontaneous rooting was observed in medium containing NAA and IBA combination. The highest mean number of roots per culture was noted in half strength MS medium supplemented with 1 + 0.8 mg/l NAA + IBA combination. This is in line with the findings of Henselova (2002) who reported that IAA, IBA and NAA are the most suitable auxin which can promote high percentage of rhizogenesis and thus it is widely accepted that auxin play a vital role in the production of adventitious roots in different plants. The *in vitro* grown complete plantlets were then successfully transferred from the culture room to thermocol cups through a process of successive phases of acclimatization. Most of the regenerated plants survived. Phenotypic variations were not observed and the plants behaved normally. The overall result of the preliminary study revealed that the propagation of *Bryonia laciniosa* is possible through induction of organogenesis in leaf originated tissue which was dependent on growth regulator supplements in the medium.

Effect of 2, 4-D on Callus Induction:

Preliminary experiments revealed that the growth regulators are essential for induction of callus. Callus was successfully initiated on MS medium in combination of 2, 4-D at 1.5, 2.0 and 2.5 mg/ l concentrations after 20 days of inoculation. The percent response was higher at 2.0 mg/ l than 2.5 mg/ l concentration and this concentration produced dark green friable calli (TABLE- I).

Effect of 2, 4-D and Benzyl Amino Purine (BAP) on Shoot Bud Induction:

When the hard compact calli were sub cultured on the different concentrations of BAP with 2 mg/ l 2, 4-D and growth resumed in the callus and shoot buds were best induced in 2.0 mg/ l concentration of BAP. 1.0 mg/ l, 1.5 mg/ l, 2.5 mg/ l concentrations of BAP produced less number of shoots. The days required for shoot induction ranged between 18 and 25 days. 2.0 mg/ l concentration took minimum days for inducing shoots as compared to other concentrations (Table 2).

Effect of NAA and IBA on Root Induction:

The micro shoots excised from 2, 4- D + BAP combinations were transferred to NAA concentration individually and NAA + IBA combinations. Of the various concentrations tested, 1.0 mg/ l + 0.8mg/ l concentrations of NAA + IBA combination induced higher number of roots per shoot (TABLE- II, PLATE-I).

Acclimatization:

Rooted plantlets were transferred to thermocol cups for hardening. Later the plants were exposed by gradually increasing the time of exposure. The hardened plants were transferred to earthen pots and kept in the field conditions. Initially these plants were kept in the shade for a few days before being planted out. A 70% survival rate was recorded. In conclusion, the above protocol describes a rapid *in vitro* regeneration from the leaf explants which can ensure a stable supply of this medicinally important plant species.

Table 1: Effect of 2, 4- D on Callus induction in leaf explants of *Bryonia laciniosa*

S.No	MS medium + 2, 4-D mg/l	% Response	Days Taken for Callus Induction	Nature of the Callus
1	0.5	-	-	-
2	1.0	-	-	-
3	1.5	20	23	Green friable
4	2.0	60	20	Dark Green friable
5	2.5	30	22	Green friable
6	Basal Medium	-	-	-

Table 2: Effect of 2, 4- D and BAP on shoot induction in *Bryonia laciniosa*

S.No	MS medium+ 2,4-D+ BAP mg/l	% Response	Days taken for Shoot Induction	Average No. of Shoots / Explant
1	2.0 + 0.5	-	-	-
2	2.0 + 1.0	10	25	1.1 ± 0.01
3	2.0 + 1.5	20	22	2.3 ± 0.11
4	2.0 + 2.0	30	18	3.1 ± 0.02
5	2.0 + 2.5	10	22	1.2 ± 0.01

Table 3: Effect of MS medium+ NAA+ IBA on *in vitro* rooting of regenerated shoots of *Bryonia laciniosa*

S.No	MS medium + Concentration of NAA mg/ l	% Response	Days Taken for Root Initiation	Average No. of Roots / Shoot
1	0.6	-	-	-
2	0.8	10	15	1.2 ± 0.01
3	1.0	20	14	1.3 ± 0.01
	NAA + IBA			
4	1.0 + 0.2	20	13	1.1 ± 0.11
5	1.0 + 0.4	20	12	1.3 ± 0.21
6	1.0 + 0.6	30	12	2.1 ± 0.12
7	1.0 + 0.8	50	10	4.3± 0.13
8	1.0 + 1.0	30	12	1.1 ± 0.05

Experimental Plant *Bryonia Laciniosa* L:

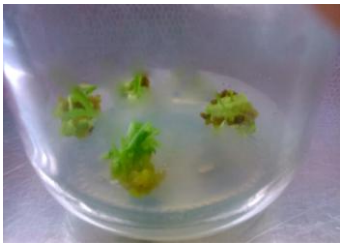
Plate 1:

In Vitro Regeneration Callogenesis, Shoot and Root Formation from Leaf of *Bryonia Laciniosa*

Inoculation of Leaf Explant

Callus Induction

Subculture of Callus

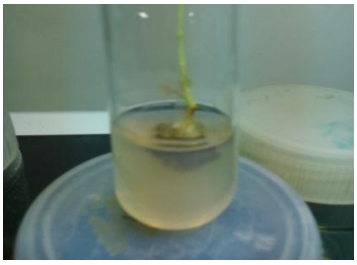


Shoot Development

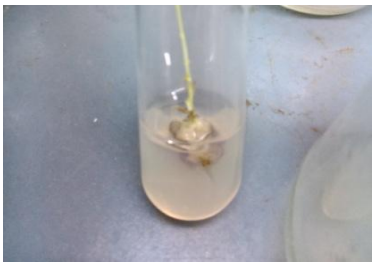
Callus Transferred to Test Tube

Shoot From Callus

Root Induction



Root Induction



Plants Transferred to the Thermocol Cup

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