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EFFECT OF SUCROSE, AGAR AND PH ON *IN VITRO* PLANT REGENERATION OF *BRYONIA LACINIOSA*

UJJWALA SUPE

Department of Biotechnology,
St. Thomas College,
BHILAI (C.G.) INDIA
Corresponding author:
E-mail: ujsupe@gmail.com

ABSTRACT

Bryonia lacinosa is locally known as "Shivlingi" or Gargumaru. This herb is a boon for the childless parents. Due to its therapeutic activity and diversified uses, demand of *B. laciniosa* is increasing in Indian and international market. The aim was to study the effect of sucrose, agar and pH on *in vitro* Plant regeneration of *Bryonia laciniosa*. The selected leaves explants were sterilized with 1% Mercuric chloride for 20 min and rinsed five times with autoclaved distilled water. For shoot induction and regeneration MS medium containing different concentrations of sucrose (1.0, 2.0, 3.0, 4.0 and 5.0% w/v) were used to optimize carban source. BAP and KIN (1, 2, 3, 4 and 5 mg/ml) singly as well as in combination with different concentrations of IAA and NAA (3, 4 and 5 mg/ml) for induction and multiplication of shoots were used. MS Medium supplemented with either IBA or α-naphthyl acetic acid (NAA) at various concentrations including 0.5, 1.0 and 2.0 mg/l were used for maximum root induction. Apart from this for solidification agar (2, 4, 6, 8 and 10 gm/l) were used among different level of pH. The rooted plantlets were successfully transferred to green house for hardening process. For optimum shoot induction and multiplication in MS medium containing BAP with NAA, Sucrose 30 gm/l, agar 6 gm/l and pH 5.5 – 6.0 proved more effective. The medium having 30 gm/l sucrose showed the highest percentage of explant responded to shoot proliferation and that was 100%. The proliferation response of the explant was observed on MS medium having 6 gm/l of agar (100%). The highest percentage of explant showing proliferation was observed on the medium adjusted to pH 5.5 and 6.0. The results presented here proved to be suitable for the *in vitro* shoot multiplication of *Bryonia laciniosa*.

Figure: 01 References:27 Tables:05

KEY WORDS: Agar, *Bryonia laciniosa*, Callus, Plant regeneration, Sucrose,

Introduction

The Shivlingi plant, *Bryonia laciniosa* is a genus of family *cucurbitaceae* comprises about 12 species distributed throughout the Europe and West India. The members of *Bryonia laciniosa* are herbaceous with tuberous root and flowers in racemose[1]. *B. laciniosa* is an important medicinal plant[2]. In India, Gond and Bharia tribes of the Patalkot Valley, Madhya Pradesh worship this plant. According to them, The herbal healers (Bhumkas) prepare certain combination of herb and prescribe it to the needed person. The plant has been used in bilious attacks fevers with flatulence[3]. The plant shows many pharmacological activities like analgesic, antipyretic, anti convulsant, antimicrobial, cytotoxic, antiasthmatic, anti-inflammatory and anti fertility. Many traditional uses are also reported like adenopathy, ague, asthma, bronchitis, carbuncles, cholera, colic, consumption, convulsions, cough, delirium, fertility, headache, megalospleny, paralysis, phthisis, snake bite which are being studied till today and further research has to be done The fruits are reported to be highly medicinal and used by tribal people of Bastar for the birth of male child since many years but only little work has been done on this plant[4].

The pH affects nutrient uptake as well as enzymatic and hormonal activities in plants[5]. The optimal pH level regulates the cytoplasmic activity that affects cell division and the growth of shoots and it does not interrupt the function of cell membrane and the buffered pH of the cytoplasm[6]. The change of pH in cells or organs is due to the ions absorbed from the medium[7]. Therefore, it is necessary to optimize the sucrose concentration and pH level for maximum shoot regeneration because the sucrose concentration and pH level directly influence shoot regeneration. Agar is used in tissue culture medium as a gelling agent. But it has also some effect on growth and development of the culture depending on its concentration and brand. The lower and higher pH level hindered multiple shoot proliferation.

The objective of this study was to develop an efficient and improved method for rapid *in vitro* propagation of *Bryonia laciniosa* using leaves via tissue culture techniques which provide viable alternative methods for the mass production of healthy plants with uniform characteristics. The attempt of this present study was to determine the effect of sucrose agar and pH on *in vitro* shoot formation and multiplication of *Bryonia laciniosa* over the cultural period. For commercial micropropagation of *Bryonia laciniosa* requires to develop protocol which will be able to produce and shoot multiplication may be affected by sucrose, agar and pH of the shoot induction medium.

Material and Methods

Establishment of aseptic cultures

Leaves (0.5 to 1 cm long) of *Bryonia laciniosa* were used as the source of explants for establishing the *in vitro* cultures. These leaves were washed thoroughly with running tap water. Again washed with detergent and then rinsed five times with autoclaved distilled water. For surface disinfections, the explants were immersed in 1% Mercuric chloride for 20 min and rinsed five times with autoclaved distilled water and inoculated in shoot proliferation media.

Culture media and culture condition

MS basal medium[8] fortified with various concentrations of plant growth hormones was used in all the experiments. Different concentrations of sucrose (1.0, 2.0, 3.0, 4.0 and 5.0% w/v) were used to optimize most appropriate one. The medium was adjusted to different pH levels viz 5.0, 5.4, 5.8, 6.2 and 6.6 using 1.0N NaOH or HCl and sterilized by autoclaving at 121°C and 15 lb/inch2 pressure for 20 min. Cultures were incubated at 25±2°C under 16/8 h (light/dark) photoperiod provided by cool white fluorescent tubes.

Adventitious shoot formation and multiplication

Explants were cultured on MS medium augmented with various concentrations of BAP and KIN, (1, 2, 3, 4 and 5 mg/ml) singly as well as in combination with different concentrations of IAA and NAA (3, 4 and 5 mg/ml) for induction and multiplication of shoots. Sub culturing was required periodically on the same fresh media after (21 days) week to avoid basal callusing. Data on the frequency of shoots formation, shoot number were recorded.

***In vitro* rooting of shoots**

Shoots with fully expanded leaves were transferred to MS Medium supplemented with either IBA or α-naphthyl acetic acid (NAA) at various concentrations including 0.5, 1.0 and 2.0 mg/l. Observations on percent of root formation, number of roots per shoot and root length were recorded.

Transfer of plant to soil

The rooted plants were transferred to small pots containing autoclaved sand for one month under greenhouse conditions. They were nourished with Hoagland solution daily. Then these plants were shifted to soil containing 50% of compost for one to two months. The hardened plants were transplanted for field trials.

Statistical analysis

A completely randomized design was employed in all experiments. Regeneration experiments comprised 10 explants, the transformation experiment consisted of 25 explants in replicates, and each experiment was performed

at least three times. All data were statistically analyzed using analysis of variance (ANOVA) and the means differing significantly were compared using Duncan's multiple range test at 5% probability level. Variability around the mean was represented as ±standard deviation.

Results and Discussion

Effect of plant growth regulators on shoot formation and multiplication

The potential of various plant growth regulators on *in vitro* shoot formation from leaves were explored and is summarized in Tables 1, 2, 3 and 4. Data presented in Table 1 depicts that MS medium containing 4 mg/ml BAP showed higher rate of shoot formation (83.0%) as compared to other cytokinins used in this study (Table-1). In addition, significantly greater number of shoot buds was observed on this medium (Figure 1A). At the same concentration, KIN showed 75.0% response having an average of 5.96 ± 0.41 shoot buds per culture vial. MS medium supplemented with BAP gave a better response than KIN.

Effect of different conc. of sucrose on shoot formation

Sucrose is an important factor for *in vitro* shoot proliferation. In this experiement different concentration of sucrose in MS medium were used for multiple shoot regeneration and development. Shoots were taken from *in vitro* cultures that grew on a particular medium composition for the present investigation. Shoots were cultured on MS medium having 4 mg/l BAP and 4 mg/l NAA at five different concentration of sucrose viz. 10, 20, 30, 40 and 50 gm/l and with a control treatment of without sucrose. After 6 weeks of culture percentage of explant showing proliferation, number of total shoots/culture and average length of shoots were as shown Table-1.

Among the different sucrose concentration in MS medium, the medium having 30 gm/l sucrose showed the highest percentage of explant responded to shoot proliferation and that was 100 %. The medium having 30 gm/l sucrose also produced the optimum result for number of usable shoot/culture and average length of shoots (Figure- 1A). The medium containing 50 gm/l sucrose showed the lowest percentage of explant number showing proliferation, number of usable shoots/culture and average shoots length.

Effect of pH value on shoot regeneration

Concentration of agar in medium can effect the culture growth and development of shoots. Seeds were cultured on MS medium having 4 mg/l BAP and 4 mg/l NAA at five different strength of agar viz. 2, 4, 6, 8 and 10 gm/l to standardize the optimum agar strength for maximum growth and development of shoots. Data on shoot proliferation and shoot elongation were recorded after 6-7 weeks of culture as presented in Table-2. The percentage of explant showing proliferation was lowest in medium having the highest concentration of agar (10 gm/l) and that was only 30 %. Whereas, the highest proliferation response of the explant was observed on medium MS medium having 6 gm/l of agar and frequency was 100% (Figure-1B). Number of shoots/culture and average shoot length was highest on the medium that contained 6 gm/l agar.

In vitro multiple shoot development depends upon some other factors rather than cytokines, auxins and gibberellins. The pH of the culture medium is an important factor for the *in vitro* proliferation and healthy culture growth. I*n vitro* grown shoots that grew on medium containing 4 mg/l BAP and 4 mg/l NAA were used in the present study. Shoots were cultured on MS medium adjusted to five different level of pH viz. 4.5, 5.0, 5.7, 6.0 and 6.5 (Table-3). Among these pH levels, the highest percentage of explants showing proliferation was observed on medium adjusted to 5.5 to 6.0 and that was 100%. The second highest percentage of explants showing proliferation was observed on medium having 5.0 pH.

Rooting of micro shoots

Individual shoots were excised and transferred to MS basal medium supplemented with IBA and NAA (0.5, 1.0 and 2.0 mg/l) for rooting. After 4 weeks, data were recorded on percentage of rooting ad mean no of roots/shoot. MS medium fortified with IBA was found superior to NAA with respect to induction of roots

(Figure-1C).The best rooting was achieved in MS medium fortified with 1.0 mg/ml IBA where fairly good root number (6.03 ± 0.71)

Hardening and acclimatization

Well rooted plants with 4-6 leaves were carefully removed from culture vessels, washed under running tap water to remove the agar and transferred to pots containing sand and soil. Sterilized double distilled water was given for 1 week and the hardened plants were kept in green house. After 1 month these plants were transferred in earthen pots and kept in open field (Figure.-1D).

TABLE-1: Effect of various plant growth regulator (BAP, KIN, IAA and NAA) on shoot induction of *Bryonia laciniosa* after four weeks of culture

Growth regulator (mg/ml)				% regeneration	Mean number of shoots
BAP	KIN	IAA	NAA		
1				40	2.75 ± 0.35
2				50	5.25± 0.48
3				70	7.25± 0.23
4				75	8.26 ± 0.41
5				64	7.65 ± 0.34
	1			55	1.97±0.62
	2			67	2.67±0.71
	3			73	3.69±0.71
	4			83	5.95±0.41
	5			72	3.85±0.34
4		3		60	4.00±0.70
4		4		70	7.75±0.70
4		5		68	6.85±0.19
4			3	55	12.0±0.36
4			4	89	24.4±0.42
4			5	67	7.25±0.51

TABLE-2: Effect of sucrose on proliferation of shoots from seeds of *in vitro* proliferated shoot.

Sucrose Conc.(gm/l)	% Explant responded	No. of shoot/culture	Average length of shoot (cm)
0	-	-	-
10	64	3.6±0.2	4.2±0.3
20	75	4.5±0.3	4.4±0.4
30	100	5.8±0.3	5.4±0.5
40	84	5.2±0.4	5.2±0.6
50	50	4.1±0.3	4.3±0.3

TABLE-3: Effect of agar on induction and development of shoot from seed of *in vitro* proliferated shoot.

Agar conc. (gm/l)	% Explant responded	No. of shoot/culture	Average length of shoot (cm)
0	-	-	-
2	30	3.5±0.2	4.4±0.3

4	45	4.5±0.4	5.1±0.4
6	100	6.8±0.7	6.7±0.4
8	76	5.8±0.4	6.1±0.3
10	27	3.1±0.1	3.7±0.2

TABLE-4: Effect of pH on development of shoot from seed of *in vitro* proliferated shoot.

pH level of medium	% Explant responded	No. of shoot/culture	Average length of shoot (cm)
4.5	45	4.7±0.3	4.4±0.4
5.0	67	5.1±0.1	5.8±0.6
5.5	100	5.9±0.2	6.5±0.4
6.0	100	5.7±0.2	6.2±0.4
6.5	42	4.2±0.1	4.0±0.3

TABLE-5: Effect of auxins on root induction of *in vitro* raised shoots of *Bryonia laciniosa*

Auxins(mg/l)		% rooting	Mean number of roots/shoot
IBA	NAA		
0.5		80	5.85±0.26
1.0		90	6.03±0.71
2.0		70	5.15±0.63
	0.5	50	2.75±0.54
	1.0	60	3.86±0.67
	2.0	40	1.87±0.19

Discussion

MS medium with 4 mg/l BAP and 4 mg/l NAA shows maximum shoot induction in present study. Ovecka et al. (2000)[9] reported that cell competence in the course of shoot bud regeneration is controlled by various internal factors such as genotype, endogenous level of auxin and cytokinin, basal medium, pH, carbohydrate uptake, etc. By increasing or decreasing the level of BAP from 1 to 5 mg/ml, the rate regeneration as well as number of shoots per culture vial was drastically reduced. Similar observations are reported by Husain and Anis (2004)[10] in *Melia azedrach*, where BAP at 10 µM showed a decrease in the rate of shoot multiplication. Such an inhibitory effect at higher concentration of BAP has also been reported by other workers irrespective of explants used[11,12]. For further enhancement in shoot multiplication response, the optimal concentration of BAP and KIN (4 mg/ml) in combination with IBA and NAA at different concentrations (3 to 5 mg/ml) was also analyzed (Table-1). The inclusion of IBA in the optimal medium enhanced the shoot multiplication response in *Bryonia laciniosa*. Kim et al. (2001)[13] suggested that the shoot forming ability of the explants is related to the *in vivo* level of endogenous auxin and cytokinin and that the differential response to different cytokinins may be because of the chemical and structural differences. Among various combinations of cytokinins- auxin combination used, the combined effect of BAP (4 mg/ml) and NAA (4 mg/ml) was best for shoot multiplication where the highest number of shoots (24.4 ± 0.42) were recorded.

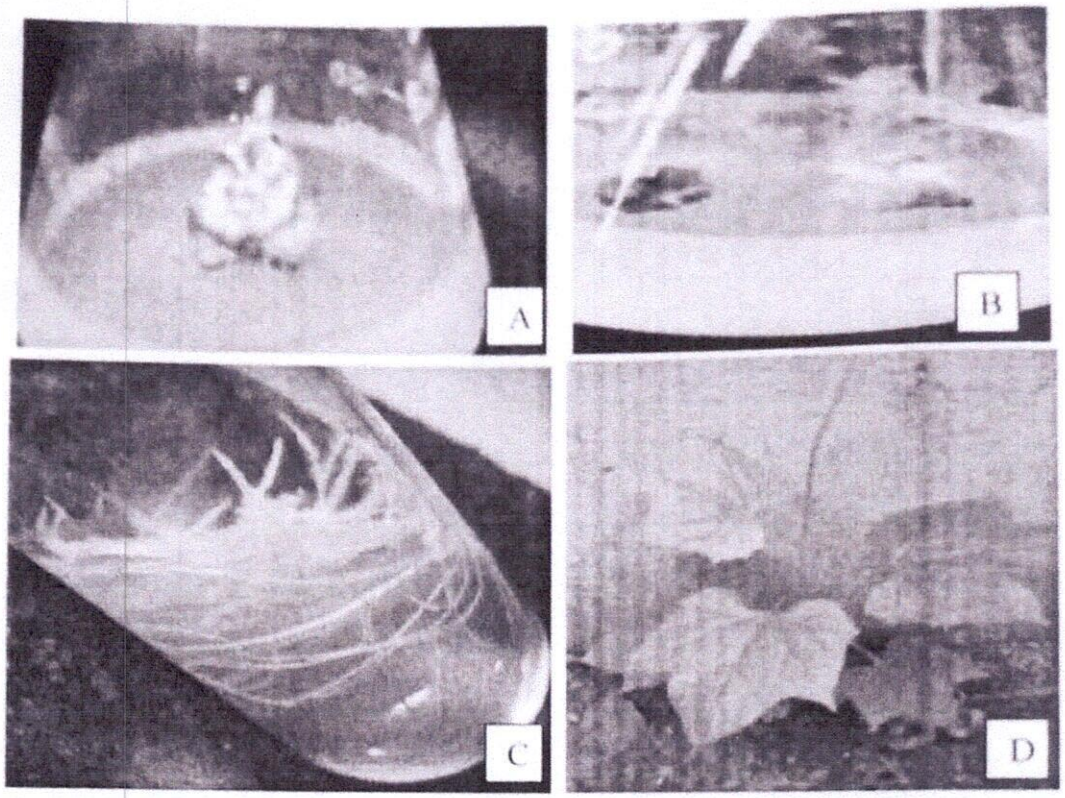

Fig.1: Effect of Sucrose, agar and pH on shoot multiplication of *Bryonia laciniosa*

A. Formation and multiplication of shoots with 4 mg/l BAP and 4 mg/l NAA with 30 gm/l sucrose after 4 weeks of inoculation.
B. Growth and shoot multiplication of shoots on MS medium supplemented with 6 gm/l agar.
C. Root formation in MS medium 4 mg/l BAP + 1 mg/l IBA pH 5.5- 6.0.
D. Matured hardened plant of *Bryonia laciniosa*

Sucrose is used as source of carbon and energy for optimum proliferation and growth of the *in vitro* grown culture. Carbon dioxide concentration and light as a source of energy are found to be low in *in vitro* conditions. To overcome this deficiency carbon source is added to the culture medium sucrose concentrations of 2% to 3% are most commonly used in tissue culture of plants [14]. Concentration of sugar is very essential for promoting plant growth. According to Debergh[15] the absence of sugar minimizes contamination problems in the culture medium so, plant can grow autotrophically *in vitro*, when sufficient carbon dioxide is supplied and light intensity is increased. However, sugar present in culture medium significantly increases production cost of micro propagated plantlets[16]. Plant cells and tissues require an optimum pH for growth and development in cultures. The optimum sucrose concentration as an efficient carbon source has been examined in tissue cultures of some plant species, such as *Asparagus racemosus* Willd[17] and *Withania somnifera*[18] in which 30 g/l sucrose enhanced shoot development. From the present investigation, it was observed that different concentration of sucrose affected in to growth of *Bryonia laciniosa* shoots variously. Complete inhibition of sprouting and development of shoots of the culture on sucrose free medium confirm the essentially of a easily accessible energy source in the proliferation medium. The *in vitro* grown shoots despite being green, do not rely on photosynthesis and grow as hetertotrophs[19]. Inhibition of chlorophyll synthesis and shoot growth on sucrose deficient medium have also been reported[20]. At 40 gm/l and 50 gm/l sucrose concentration, the shoot size was bigger but its number decreased and root growth was inhibited. The present findings also indicates that the sucrose not only acts as carbon cum energy source in the medium but also acts as an osmoticum and different

concentration of it acts as one of the controlling factor for the induction and growth of shoots.

The pH of the culture medium is an important factor for promoting shoots *in vitro*. In the absence of pH regulation, the ionization of acidic and basic groups causes considerable changes in structure that affect their function at the cellular level[7]. The pH of tissue culture media decreases by uptake of NH4+ and increases by uptake of $NO3^{-}$[21]. Any change in pH of medium may have various effects that may influence performance and development of explants [22].

From the present investigation it was revealed that both lower and higher pH level hindered multiple shoot proliferation. Comparatively less acidic gave harder gel which might have adverse effect on regeneration and proliferation of shoot. *In vitro* proliferation of *Azardiracta indica* [23], *Aloe barbadensis* [24], *Withania somnifera*[25] shoots were increased significantly when the pH of the culture medium was adjusted at 5.8 before autoclaving.

For root induction 1 mg/l IBA singly was capable to produce 6.03±0.71roots/ explants as compare to NAA (3.86±0.67).BA has been reported to have stimulatory effect on root induction in many plants *Sterculia urens*[26], *Balanites aegyptiaca*[27].

Plant regeneration through tissue culture technique would be a novable and alternative for improving the quality and faster production of *Bryonia laciniosa*. It has been demonstrated that sucrose, agar and pH of medium play important role on *in vitro* growth and development of shoots and roots

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