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Full Length Research Paper

In vitro multiple shoot induction through axillary bud *of Cocculus hirsutus* (L.) Diels: A threatened medicinal plant

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In this study, we describe a method for rapid *in vitro* multiplication for conservation of a threatened medicinal plant, *Cocculus hirsutus* (Menispermaceae) via enhanced axillary bud proliferation from nodal explants collected from young shoots of a mature elite plant. The physiological effect of different plant growth regulators such as 6-Benzylaminopurine (BAP), kinetin (Kn), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) on *in vitro* multiplication was studied. Multiple shoots were induced *in vitro* from the nodal segments of stem on Murashige and Skoog's (MS) medium containing BAP or Kn alone or in combination. Maximum number of shoots (45 ± 0.69 shoots per explant) was observed on the medium containing BAP (0.5 mg/L) along with additives like adenine sulphate (50.0 mg/L) and glutamine (150 mg/L). The shoots were best elongated on medium containing 0.25 mg/L BAP along with additives. Elongated shoots were rooted on ¼ MS medium supplemented with 0.5 mg/L IBA. The rooted *in vitro* raised plantlets were acclimatized in growth chamber. The method described here can be successfully employed for large-scale multiplication, long term *in vitro* conservation and for further investigation of medicinally active constituents of *C. hirsutus*. This is the first report on *in vitro* multiplication of *C. hirsutus.*

Key words: *Cocculus hirsutus*, *in vitro* multiplication, threatened, nodal explants.

INTRODUCTION

Medicinal plants are source of various alkaloids and other chemical substances essential for mankind. The exploitation of tissue culture technique in medicinal plants is indeed desirable for their *in vitro* propagation and extraction of important chemical compounds. (Tabata, 1977) *Cocculus hirsutus* (L.) Diels is an important climber mainly found in tropical and subtropical climates. Traditionally, the plant was patronized for its unique property of healing all types of cuts, wounds and boils. The roots and leaves of *C. hirsutus* have great medicinal value and are used both internally, as well as externally.

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Abbreviations: BAP, 6-Benzylaminopurine; **Kn,** kinetin; **IBA,** indole-3-butyric acid; **IAA,** indole-3-acetic acid.

The root is bitter and used as alterative, emollient, demulcent, tonic, antiperiodic in fever, in malaria, joint pains, in treatment of skin diseases, constipation and kidney problems (Chopra et al., 1996). Decoction of the root mixed with long pepper is used in chronic rheumatism and syphilitic cachexia (Chadha, 1950; Nandkarni, 1976). It is also used in the treatment of gonorrhea, spermatorrhoea, urinary troubles, diarrhoea and hyperglycemia (Kirtikar and Basu, 2002). The leaves of the plant have been evaluated for antihyperglycemic (Badole et al., 2006), antibacterial (Panda et al., 2007), diuretic, laxative and toxicity (Ganapaty et al., 2002) effects.

In recent times, the demand of *C. hirsutus* has increased owing to its medicinal property. This led to indiscriminate harvesting that threatened its status in the biodiversity. Hence, it has become imperative to establish a suitable protocol for its micropropagation. To our

knowledge, there is no report regarding tissue culture studies for conservation of this species. This investigation, therefore, aims to develop a rapid and high frequency shoot regeneration system from nodal segments containing axillary buds of *C. hirsutus* for providing continuous supply of a better source of elite plants to be used as standard material in the field of drug research, as well as in the manufacturing of drugs.

MATERIALS AND METHODS

Plant material

Nodal segments containing axillary buds were collected from the plants growing in Kulish Smriti Van, Jaipur and its identity was confirmed by comparing with voucher specimen available in the University of Rajasthan, Jaipur.

Sterilization of explant

C. hirsutus twigs with three to four nodes used as explants were washed thoroughly with two to three drops of Tween -20 (a commercial liquid detergent) and were rinsed in running tap water for 30 minutes. To overcome the problem of *in vitro* oxidative browning, the explants were given a pretreatment with an antioxidant solution comprising ascorbic acid (50 mg/L), polyvinylpyrrolidone (100 mg/L) and citric acid (100 mg/L) for One hour. The explants were then rinsed with distilled water and brought under laminar air flow hood for surface sterilization. The explants were then treated with 0.1% mercuric chloride for One minute and washed with sterile distilled water (three to four times) to remove traces of mercuric chloride.

Culture medium and conditions for plant regeneration

Under a laminar flow cabinet, explants were inoculated aseptically on MS (Murashige and Skoog's 1962) medium supplemented with various concentrations of BAP and Kn alone or in combination. The pH of media were adjusted to 5.85, supplemented with 0.8% agar and 30 g/L sucrose before autoclaving at 121°C for 20 min under pressure of 15 Psi. The media were left to cool in the culture room until use. All cultures were maintained at 16:8 h light photoperiod of 2000 lux using fluorescent lamps at 25 ± 2°C. Results were observed at regular intervals and data were collected from three independent experiments with six replicates each and presented as average ± standard deviation (SD).

RESULTS AND DISCUSSION

Axillary bud break was achieved in all aseptic cultures on MS medium supplemented with (0.25 to 2.5 mg/L) BAP. The morphogenetic differentiation of explants towards axillary bud multiplication was markedly influenced by the concentration of the growth regulator (BAP) in the medium. BAP treatment along with additives such as adenine sulphate (50.0 mg/L) and glutamine (150 mg/L) significantly increased bud break and the number of shoots that proliferated. At 0.5 mg/L concentration of BAP along with additives, maximum number of shoots (45 ± 0.69) proliferated and yielded a cluster of shoots within four weeks (Table 1 and Figure 1C). As the concentration of BAP was increased, the number of shoots decreased. In MS medium without any growth regulator, no axillary bud break response was seen. However, on MS medium with different concentrations of Kn (0.5 to 2.5 mg/L), multiple shoot bud formation was also observed. The highest number of shoot buds (20 ± 0.75) (Table 1) was produced on medium with 0.5 mg/L of Kn along with additives but the number of shoot buds were fewer than in medium containing BAP and shoots were stunted in growth. The results are in line with researches indicating the efficiency of BAP for shoot culture initiation and multiplication (Uranbey et al., 2003; Khawar et al., 2005; Tiwari et al., 2007; Meena and Patni, 2007; Jain et al., 2009; Akbas et al., 2011).

Moreover, in the case of MS medium supplemented with combination of BAP and Kn, axillary bud break was observed in all aseptic cultures. The combination of BAP (0.5 mg/L) and Kn (0.5 mg/L) along with additives like adenine sulphate (50.0 mg/L) and glutamine (150 mg/L) produced 30 ± 0.56 shoots per explant (Table 1). When both the cytokinins that is BAP (0.5 to 2.5 mg/L) and Kn (0.5 to 2.5 mg/L) were supplemented in combination, this proved better than Kn alone in producing multiple shoots, although the number of shoots were less compared to BAP alone (0.5 mg/L). Similar observations were recorded by Sobhakumari and Lalithakumari (2003), Sevimay et al. (2005), Girija et al. (2006), Rao et al. (2006), Sharma et al. (2007), Akbas et al. (2009) and Negi and Saxena (2011).Furthermore, the proliferated *in vitro* axillary shoots were excised from mother explants and sub cultured on MS medium supplemented with either (0.5mg/L) BAP or Kn or combination of BAP and Kn for further *in vitro* shoot multiplication. After every four weeks of subculture, it was noticed that the best *in vitro* shoot multiplication with sizeable development was obtained on 0.5 mg/L BAP supplemented medium. The multiple shoots obtained on various shoot induction media (Table 1) did not elongate on the same media. Therefore, the multiple shoots were transferred on MS medium containing low concentration of cytokinin, BAP (0.25 mg/L) (Figure1D). This led to the conclusion that media with lower concentration of BAP (0.25 mg/L) favored the elongation of shoots. Higher cytokinin concentrations promoted shoot multiplication and at lower concentrations shoot elongation was observed. Similar effects of lower concentrations of cytokinin on shoot elongation have been reported also by Kaur et al. (1998), Dave and Purohit (2002), Parmaksiz and Khawar (2006), Arya et al. (2008), Basalma et al. (2008a), Basalma et al. (2008b), Gayathri et al. (2009) and Uranbey et al. (2010).

The regenerated shoots were excised and placed on MS medium supplemented with different concentrations (0.25 to 2.5 mg/L) of IBA and IAA for rooting. IBA was more effective in promoting root induction than IAA (Table 2). The optimum rooting efficiency for shoots as well as the highest number of roots per shoot was obtained on ¼ MS medium supplemented with 0.5 mg/L

Table 1. Effects of type and concentration of plant regulators on *in vitro* shoot proliferation from nodal explants of *Cocculus hirsutus* after four weeks of culture.

Plant growth regulators (mg/L)		Number of shoot/explants (mean ± SD)	Percentage (%) of shoot regeneration
BAP	Kn		
0.0		0.0	0.0
0.25		25 ± 0.08	45
0.5		45 ± 0.69	90
1.0		30 ± 0.42	60
1.5		21 ± 0.37	35
2.0		16 ± 0.05	27
2.5		9 ± 0.25	15
	0.0	0.0	0.0
	0.25	11 ± 0.26	20
	0.5	20 ± 0.75	65
	1.0	16 ± 0.58	48
	1.5	12 ± 0.28	25
	2.0	6 ± 0.39	16
	2.5	4 ± 0.08	10
0.0	0.0	0.0	0.0
0.25	0.25	18 ± 0.89	37
0.5	0.5	30 ± 0.56	70
1.0	1.0	25 ± 0.43	52
1.5	1.5	15 ± 0.45	25
2.0	2.0	10 ± 0.22	20
2.5	2.5	8 ± 0.32	12

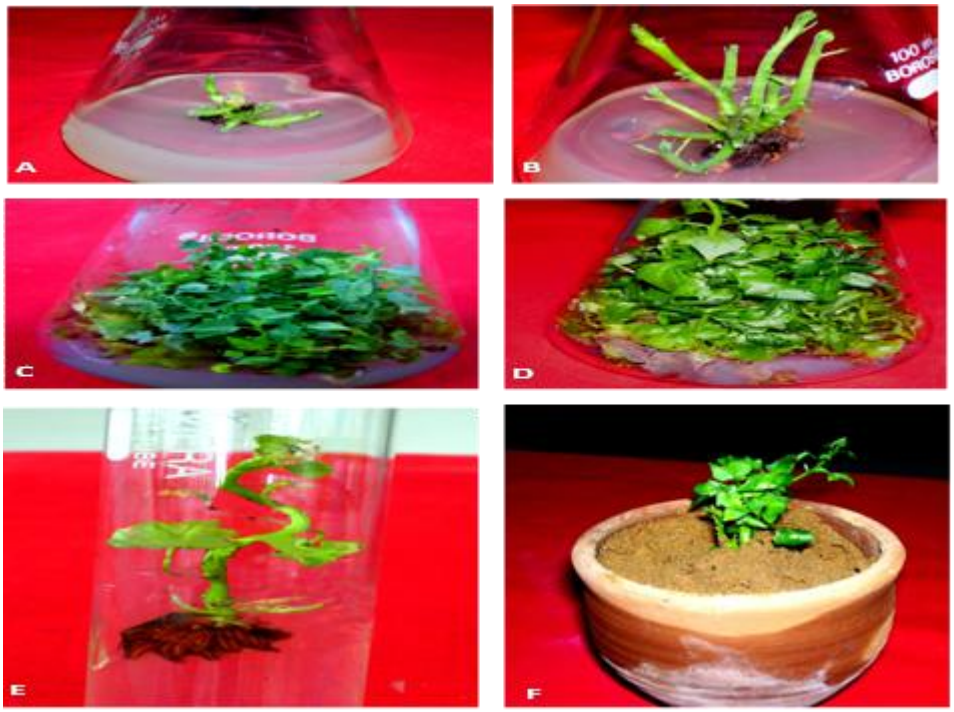

Figure 1. (A) Axillary bud proliferation on MS basal medium after four weeks of culture; (B) regeneration of shoots from nodal explants on MS + BAP (0.5 mg/L); (C) multiple shoot regeneration on MS + BAP (0.5 mg/L); (D) elongated shoots on MS + BAP (0.25 mg/L); (E) rooted shoot on ¼ MS + IBA (0.5 mg/L); (F) acclimatization of rooted plant in pots.

Table 2. Effects of auxins on rooting of *in vitro* raised shoots of *Cocculus hirsutus* after six weeks of culture.

Auxin (mg/L)	Root initiation (%)	Number of roots per shoot (mean ± SD)
IBA		
0.0	0.0	0.0
0.25	42	3.3 ± 0.12
0.5	65	8.2 ± 0.40
1.0	35	2.8 ± 0.26
2.0	20	1.5 ± 0.14
2.5	12	1.0 ± 0.31
IAA		
0.0	0.0	0.0
0.25	27	1.8 ± 0.28
0.5	45	3.5 ± 0.49
1.0	15	2.2 ± 0.37
2.0	10	1.3 ± 0.39
2.5	7	1.0 ± 0.05

IBA (Table 2 and Figure 1E). Plantlets on MS medium containing 2.5 mg/L of auxin grew slowly, turned yellowish, thick and shorter with induction of callus at the basal end. The supplementation of auxin either singly or in combination for rooting was also reported in many plant species (Gopi et al. 2006; Baksha et al., 2007; Kalidass et al., 2008; Aasim et al., 2008a; Kalidass and Mohan, 2009; Aasim et al., 2009). The addition of IBA also favored rooting in some medicinal plants (Chandra et al., 2006; Ozel et al., 2006, 2008; Sivanesan, 2007; Meena et al., 2010; Chordia et al., 2010; Nagesh and Shanthamma, 2011).The hardening process of *Cocculus hirsutus* was carried out by transferring 7 to 8 cm length rooted plantlets to plastic pots containing mixture of sterilized soil and sand 3:1 (v/v). Hardening of potted plants for two weeks in a growth chamber (Figure 1F) was found to be essential. The plantlets were nurtured with half strength MS medium (without organics) twice a week and were subsequently shifted to shade house conditions for acclimatization.

Conclusion

This study describes a procedure for *in vitro* propagation of *C. hirsutus* and results indicate that tissue culture technique can play an important role in clonal propagation of elite genotypes of *C. hirsutus* rich in secondary metabolites with high pharmaceutical properties.

To our knowledge, this is the first report on *in vitro* propagation of *C. hirsutus* via axillary shoot proliferation from nodal segments derived from field-grown material. The method can be used for rapid and mass propagation of this threatened medicinal plant.

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