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Multiple Shoot Regeneration through Axillary Meristem Culture in *Cassia Tora* L.

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Abstract

The present investigation was carried out to develop an efficient protocol for regeneration of *Cassia tora* L. through axillary meristem culture. In this experiment different concentration and combinations of auxin and cytokinin were used in full strength of MS medium to observe the shoot regeneration and root proliferation. All nodal explants cultured on MS medium supplemented with various concentrations of cytokinin (BA, Kn and TDZ) individually as well as the combination developed shoots. Nodal explants cultured on MS media fortified with BAP alone produced multiple shoots at a higher frequency as compared to the other media supplemented with combination of BAP and TDZ/ Kn. Among the various concentrations of BAP tested, 2.0 mg/l BAP showed the highest shoot regeneration frequency with maximum number of shoots (8.66 ± 0.88) and shoot length. Further for complete plantlet development these micro shoots were transferred to the medium supplemented with ½ strength of MS salts with 3.0 mg/l IBA. In this medium 65-70% root development was observed. Well rooted plants were acclimated and successfully established onto natural conditions with 35% survival rate.

Keywords: *Cassia Tora* L., Regeneration, Polyvinylpyrrolidon, Anthraquinone.

Introduction

Cassia tora L. commonly known as foetid cassia is a medicinally valuable drought-resistant herb of the family fabaceae and is mainly grown as a cash crop in various parts of the world (Anonymous 1992). It is a native of America and Asia and has been naturalized in India. *C. tora* is a very important plant in Indian and Chinese system of medicine. The leaves and pods of cassia are chief source of anthraquinone glycosides known as sennosides, which are extensively used as a laxative in pharmaceutical industry (Izhaki, 2002; Xie *et al.*, 2007). Besides being an excellent laxative, it is also used as a febrifuge in splenic enlargement, anaemia, typhoid, cholera, biliousness, Jaundice, gout, rheumatism, tumours, foul breath, bronchitis and in leprosy (Chan *et al.*, 1976; Maity *et al.*, 1998; Upadhyay *et al.* 2000; Kim *et al.* 2004). The plant is eco-friendly and has been recommended for developing wastelands, as it does not require frequent irrigation. Its drought resistance, quick establishment and perennial nature provide permanent green cover on sand in vegetation deficient arid zones (Sharma *et al* ., 1999).

In nature, this species is propagated only through seeds. However, poor seed viability and low germination frequency restricts its propagation on a large scale. Furthermore, the lack of proper cultivation practices, destruction of their habitat and ruthless exploitation of this plant has led to a rapid decline in wild population of *C. tora L.* Despite the commercial interest in sennoside produced by this species of Cassia, there are only a few reports available on plant regeneration, which too have shown only low number of shoots and could not be useful for large scale production and cultivation of elite clones (Hasan *et al.* 2008). Therefore, there is a need to devise a method for the development of a large-scale multiplication protocol for commercial production of this medicinal plant *via* optimization of medium, pH, cytokinins and auxins ratio and subculture passages, followed by successful outdoor establishment of regenerated plants.

In vitro propagation has proven as a potential technology for mass scale production of various medicinal plant species (Lui and Li 2001; Wawrosch *et al.* 2001; Faisal *et al.* 2003; Martin 2003; Azad *et al.* 2005;

Hassan and Roy 2005). So far the literature, single report has been published on *in vitro* propagation of *C. tora* through axillary meristem culture (Quraishi et al., 2011). However, in the present investigation through axillary meristem culture we have obtained 7-9 shoots per explants, which could be used as a potential tool for large scale production of this medicinal plant.

Aim of the Study

Present study is done to develop an *in vitro* multiplication protocol for commercial production of this medicinal plant *via* optimization of medium, pH, cytokinins/auxins ratio and subculture passages, followed by successful outdoor establishment of regenerated plants.

Methodology

Plant Material

Plants were collected from the campus of University of Rajasthan and they were maintained in pots of a greenhouse. Nodal segments were excised and washed with running tap water for 15 min and again washed by using 2 drops of teepol and rinsed for 25 min under running tap water. After this nodal segments were again rinsed 3 times with sterile distilled water. Before inoculation the nodal segments were dipped in 0.1% solution of $HgCl_2$ for 2 min and repeatedly washed with sterile distilled water. Leaves of each explant were removed and nodal segments were inoculated in flasks containing around 40 ml of MS (Murashige and Skoog 1962) medium supplemented with hormones, respectively.

Culture Media and Conditions

MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar was used during the investigation. The pH of the medium was adjusted to 5.8 ± 0.2 using 1 N NaOH/HCl. The culture flasks containing the media were autoclaved at 121°C at a pressure of 15psi for 20 min. After inoculation all the cultures were maintained at 26±2°C under 16/8 h photoperiod with 55±5% relative humidity. Illumination was provided by cool white fluorescent tubes in culture shelves to render constant light intensity of 2000-3000 lux.

Shoot Induction and Multiplication

For multiple shoot induction, nodal segment were placed on MS medium supplemented with Kinetin (Kn), 6-Benzyladenine (BAP) and thidiazuron (TDZ) alone or in combination at different concentrations. Concentration of Kn/BAP was between 0.5- 6.0 mg/ l and for TDZ it was between 0.1-2.0 mg/l. The percent regeneration, number of shoots produced and shoot length was recorded after 3-4 weeks of culture.

Root Formation

In vitro regenerated shoots measuring 4–5 cm in length with two pairs of fully expanded leaves were cultured in ½ MS medium supplemented with indole-3-butyric acid (IBA) at different concentrations (0.5- 5.0 mg/l). Data were recorded on percentage of rooting, number and length of roots after 4 weeks of culture.

Hardening and Acclimatization

Plantlets with well developed shoot and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing vermicompost and autoclaved soil (1:3). Potted plantlets were covered with transparent beakers to ensure high humidity and watered regularly with half strength of MS salt solution for 2 weeks. Beakers were removed after 2 weeks in order to acclimatize plants to field conditions. Plantlets were gradually exposed from partial light to sun light. After 4 weeks, acclimated plants were transferred to pots containing normal garden soil and maintained in the greenhouse under natural light.

Statistical Analysis

The data were collected after 3-4 weeks for shoot regeneration and rooting experiments. There were 8 replicates per treatment and one explant was cultured per flask. The experiments were repeated thrice. The results were analyzed statistically using SPSS Ver 10 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at P = 0.05. The results are expressed as a mean ± SE of three experiments.

Results

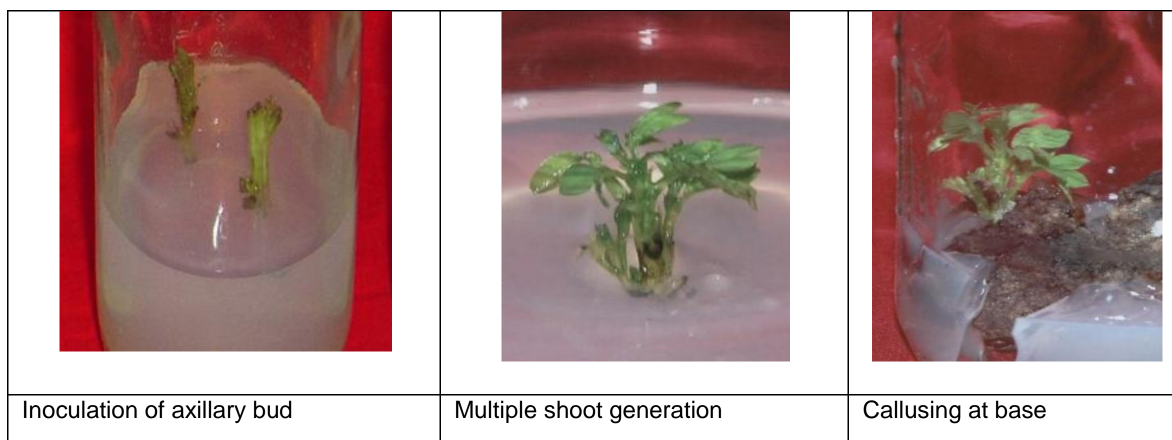
In the present research studies regeneration potential of nodal segments was explored on MS medium supplemented with various plant growth regulators and results are summarized in table given below.

Medium	**:**	MS + Sucrose (3.0%) + BAP/Kn (0.5-6.0 mg/l)/TDZ (0.1-2.0 mg/l)	
Inoculum	**:**	Nodal segments	
Incubation	**:**	**At 26±2°C under 16/8 hours period for 3-4 weeks**	
Cytokinin level (mg/l)			**Number of shoot buds per explants* Mean±$t_{0.05}$ S.E.(X)**
Kn	TDZ	BAP	
Control (0)	0	0	0
0.5			1.66±0.33
1.0			1.33±0.33
2.0			3.00±0.57
3.0			2.00±0.57
4.0			Nil
	0.1		Nil
	0.25		2.00±0.57
	0.5		4.66±0.12
	1.0		3.66±1.20
	2.0		2.66±0.66
		0.5	Nil
		1.0	2.66±0.66
		2.0	**8.66±0.88**
		3.0	4.66±0.88
		4.0	4.00±1.15

* : Values are 95% confidence limits for Mean

Nodal segments failed to develop multiple shoots in growth regulator free MS medium (control). All nodal explants cultured on MS medium supplemented with various concentrations of cytokinin (BA, Kn and TDZ) individually or in combination developed shoots. Nodal explants cultured on MS

media fortified with BAP alone produced multiple shoots at a higher frequency as compared to the other media supplemented with combination of BAP and TDZ/ Kn. Among the various concentrations of BAP tested, 2.0 mg/l BAP showed the highest shoot regeneration frequency with maximum number of shoots (8.66 ± 0.88) and shoot length. The regeneration frequencies and number of shoots declined with an increase in the cytokinin concentration beyond the optimal level. Furthermore, at all the concentrations of BAP, Kn and TDZ in the culture medium, callusing at the base of the growing shoots was observed. The effect of the optimal concentration of cytokinin (BAP at 2.0 mg/l) with various auxins (2,4-D/NAA/IAA/IBA, 0.5-6.0 mg/l) was also evaluated for multiple shoot induction. However, the combination of auxin with cytokinin showed poor responses and didn't prove to be beneficial for multiplication.



Inoculation of axillary bud	Multiple shoot generation	Callusing at base

Elongation and rooting

Elongated shoots were excised and placed in ½ strength MS medium supplemented with various concentrations of IBA for induction of roots. MS medium without growth regulators did not promote root induction. However, optimal rooting (65-70%) and growth of microshoots was observed in the medium containing 3.0 mg/l IBA, without intervening callus. Rooted plantlets were transferred to pots containing vermicompost and autoclaved soil (1:3). However, the survival rate was 35% and after one month the acclimated plants showed normal growth.

Conclusion and Discussion

In the present study it has been evaluated that in *C.tora* L. maximum shoot proliferation (8-9 shoots per explant) can be obtained by culturing axillary meristem on MS medium supplemented with 3.0 mg/l concentration of BAP. As compared to our results in previous report of bud proliferation in *C. tora*, 1-2 shoots per explant was obtained by using MS medium supplemented with BAP 2.2 µM (Quraishi *et al*., 2011). The members of the family Fabaceae have been difficult to culture *in vitro* owing to their recalcitrant nature (Trigiano *et al* 1992; Jha *et al*. 2004). Moreover, there are problems generated in micropropagation of *C. tora* due to phenol exudation. After inoculation the cutting surfaces darken and a black exudates is released into the medium, which could be growth inhibiting and results in the death of most of the explants. Addition of polyvinylpyrrolidone (PVP) or charcoal to the nutrient media failed to prevent the browning of the explants. The phenolic products released from cultured explants readily

oxidize to produce quinines and cause browning/blackening of the culture medium and/or the explant and cause necrosis and eventually death of the explants. Dipping the explants in distilled water failed to reduce explant browning. The purpose of this treatment was to dissolve the water soluble phenol and reduce browning in explants. Similarly same problem has also been encountered in some other members of the family fabaceae (Abdelwahd *el al.* 2008).

In consonance to our work the effectiveness of BAP in shoot bud differentiation has also been documented in number of plants i.e. *Azadirachta indica*, *Salvadora persica* L., *Withania somnifera* L., *Laccosperma secundiflorun*, *Vigna radiata* L., *Pongamia pinnata* L. (Shekhawat *et al* 2002; Batra *et al.* 2003; Sharma and Batra 2006; Kouakou *et al.* 2009; Yadav *et al.* 2010; Shrivastava and Kant 2010). Different culture media have also been used for *in vitro* culture of other species from the family Fabaceae (Batra and Sharma 2003; Zhao *et al.* 2003). Among all the nutrient media tried MS basal medium was the most applied one (Audichya *et al.* 1997; Cho *et al.* 2003; Makunga *et al.* 2003; Martin 2004; Pandeya *et al.* 2010).

Besides this, Fakhrai *et al.* (1989) have reported that in *Vicia faba*, BAP (2.0 mg/l) in combination with lower concentrations of NAA (0.2 mg/l) was needed for clonal propagation. However, lower concentrations of IAA (1.0 mg/l) were required with BAP (5.0 mg/l) in the effective proliferation of axillary buds in *Prosopis cineraria* (L.) Druce (Kumar and Singh 2009). Furthermore, Martin and Perez (1992) reported that in *Limonium estevei* Fdez, combination of BAP (0.1 mg/l) and IBA (1.0 mg/l) was fruitful in the regeneration of plantlets through nodal segments.

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