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RESEARCH ARTICLE

INDIRECT ORGANOGENESIS FROM STEM DERIVED CALLUS OF *CAESALPINIA BONDUC* (L.) ROXB– A MEDICINAL PLANT OF WESTERN GHATS

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ABSTRACT

An *in vitro* protocol was developed for multiple shoot induction via indirect organogenesis from the mature stem explants of *Caesalpinia bonduc* (L.) Roxb (*Caesalpinaceae*), a scrambling woody liana and medicinal plant. Callus was initiated from stem explants 15 days after inoculation, when 2, 4-dichlorophenoxyacetic acid (2, 4-D) was used alone at 0.25 to 3 mgL^{-1} on Murashige and Skoog (MS) medium. After 20-25 days on the same medium callus formation and shoot bud differentiation was noticed from the entire surface of explants .Callus induction was optimized at 2 mgL^{-1} 2,4-D and 0. 2 mgL^{-1} 6-benzyladenine (BA) in 96.66% of all explants. After culturing callus for 15 days on MS medium with 1-6 mgL^{-1} BA and 0.1–1 mgL^{-1} indole-3-butyric acid (IBA), small green protuberances formed over the entire surface of the callus which later developed into small shoots. 4 mgL^{-1} BA with 0.5 mgL^{-1} IBA was the optimal hormonal combination for multiple shoot organogenesis with a mean of 36.6 ± 1.17 shoots/explant and a mean shoot length of 7.09 ± 0.23 per callus-forming explant. Rooting of the small shoots was achieved on MS medium fortified with 0.2-2.0 mgL^{-1} IBA and optimized with 0.6 IBA mgL^{-1}. Roots formed from the base of shoots after 3 weeks of culture and 80% of the regenerants could be acclimatized.

INTRODUCTION

Caesalpinia bonduc (L.) Roxb. is a scrambling woody liana belongs to the family Caesalpinaceae and grows up to 1.5 m in height (unsupported) and 6 m or more in extension. Conventionally, the plant is propagated only through seeds which are thick, glossy and hard. Longer duration of dormancy (up to 5 years) and thick seed coat make it to geminates with very poor viability. In Ayurveda system of medicine seeds are the potential drug for treating tuberculosis, cancer, eye sores, haemorrhages, leprosy, inflammations, asthma, toothache and fever (Said, 1970; Gopalan 1976), Pharmacologically, therapeutic effect of seeds are screened for anti-microbial activity (Simi *et al.,* 2000; Asif Saeed *et al.,* 2001; Arif *et al.,* 2009), anti-hypoglycaemic, anti-hyperglycaemic, anti-hypolipidemic (Sharma *et al.,* 1997), anti-tumour, antioxidant (Gupta *et al*., 2004), anti-pyretic and analgesic activities (Archana *et al.,* 2005), anti- diabetic activity (Chakrabarti *et al.,* 2003; Kannur *et al.,* 2006), anti-filarial activity Gaur *et al.,* 2008), anxiolytic activity (Venkat Rao *et al.,* 2008), anti-inflammatory, anti-pyretic and analgesic (Shruti Shukla *et al.,* 2009), immunomodulatory activities (Shruti Shukla *et al.,* 2009). There is an alarming reduction of the population in the wild due to longer duration of seed dormancy, unscientific exploitation of the plant parts like seeds and bark for medicinal purposes and destruction of habitat by anthropogenic activities (Hutton, 2001). In vitro technique is a promising tool for ex situ conservation of germplasm of threatened medicinal plants that can be used for the extraction of active compounds from the in vitro grown tissue with out depleting the natural population. The major problem with woody plant tissue culture is the exudation of phenolic compounds leading to inhibition of explants growth and contamination of explants due to microorganisms present on the surface as well as in the internal tissues of the explants. Despite the earlier investigations (Kannan *et al.,* 2006) reported the direct organogenesis from mature stem explants, the present paper revealed that indirect organogenesis of *C. bonduc* for conservation of endangered species in Western Ghats through multiple shoot bud differentiation and plantlet regeneration from the stem calli.

MATERIALS AND METHODS

The mature stem explants of C. *bonduc* were collected from an healthy plant grown in the forest range of Bhadra Wild Life Sanctuary (1km from Kuvempu University), Karnataka, India. The explants were thoroughly washed with running tap water for 5 min and surface sterilized thoroughly with diluted Tween 20 for 2 - 3 min then with 70% ethanol for 5 min followed by 3 to 4 times rinses with tap water and finally with double distilled water. The explants were disinfested with 0.1% mercuric chloride for 10 – 15 min followed by 3 - 4 time wash with double distilled water. The stem was aseptically cut in to 1 – 1.5 cm long segments and was carefully inoculated onto the culture media.

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The culture media consisted of MS salts (Murashige *et al.,* 1962) with sucrose 3% (w/v) and various auxin e. g. 2, 4-D, IBA and cytokinins e.g. BA at appropriate concentrations, either single or in combinations. For callogenic and caulogenic media consisted with a range of 0.25 to 3 mgL^{-1} 2, 4-D, and 0.1 to 0.5 mgL^{-1} BA. For multiple shoot induction, the organogenic callus media supplemented with the range of 1 - 6 mgL^{-1} BA and 0.1 - 1mgL^{-1} IBA. Elongated small shoots of 6 –7 cm were aseptically excised and transferred to rhizogenic media fortified with 0.2- 2.0 mgL^{-1} IBA. All plant growth regulators were added to the medium before autoclaving. The pH of the medium was adjusted to 5.6 to 5.8, autoclaved at 121° C at 15 psi (1.06 kg/cm^2) pressure for 20 min. The Cultures were maintained in 12 h photoperiod with a light intensity of approximately 2000 Lux at 25 ± 2^0 C with 65 to 70% relative humidity. Analysis of variance of caulogenic frequency and mean separations were carried out using Duncan's Multiple Range Test (Duncan, 1955). The nature and percentage of response were recorded at an interval of one week and sub-culturing was periodically carried out at 45 days intervals. The regenerated plantlets were maintained in polythene bags filled with garden soil, saw dust, and cattle dung manure in the ratio 1:1:2 for a period of 4 - 5 weeks at 70 – 80% relative humidity in green house and subsequently transferred to potted soil.

RESULTS AND DISCUSSION

The stem segments of C. *bonduc* inoculated on MS medium augmented with 2, 4-D, BA showed the sign of organogenic response and became enlarged to thrice of their original size. Callus initiation was noticed from excised part of the explants after 15 days of inoculation, at the concentration of 0.25 to 3 mgL^{-1} of 2, 4-D alone. On further incubation on the same medium entire explants converted into callus and differentiation of shoot buds from the primary callus mass was noticed in the form of greenish protuberances (Fig. 1A).

Table 1: Effect of 2,4-D and BA on the frequency of callus formation from the stem explants of *Caesalpinia bonduc*

Growth regulators		Frequency of callus formation %
2,4-D mgL^{-1}	BA mgL^{-1}	
0.75	0.0	10.00
0.75	0.1	46.66
0.75	0.2	23.33
0.75	0.3	26.66
0.75	0.4	00.00
0.75	0.5	00.00
1.0	0.0	26.66
1.0	0.1	36.66
1.0	0.2	33.33
1.0	0.3	73.33
1.0	0.4	26.66
1.0	0.5	16.66
2.0	0.0	56.66
2.0	0.1	76.66
2.0	0.2	96.66
2.0	0.3	66.66
2.0	0.4	23.33
2.0	0.5	16.66
3.0	0.0	43.33
3.0	0.1	66.66
3.0	0.2	36.66
3.0	0.3	00.00
3.0	0.4	00.00
3.0	0.5	00.00

The value of each combination consisted of percentage of callus induction from the mature stem of 3 x10 replicates.

In most of the dicotyledonous species this synthetic auxin promoted only callus proliferation and hindered the caulogenic potentialities whereas, in culture of stem explants of C. *bonduc* 2, 4-D stimulated both callogenesis and caulogenesis (Paek *et al.,* 2002; Santos *et al.,* 2002; Aftab *et al.,* 2008). The frequency of caulogenesis increases with the augmentation of BA at the range of 0.1 to 0.5 mgL^{-1}. The caulogenic frequency was highest (96.66 %) at the concentration of 2 mgL^{-1} 2, 4-D and 0.2 mgL^{-1} BA as shown in Fig. 1B and Table 1. However the interaction of 2, 4-D and BA favored only multiplication and dedifferentiation of shoot buds. Further elongation of shoots was not observed. The interaction of BA at the concentration of 4 mgL^{-1} with 0.5 mgL^{-1} IBA, favored further elongation of shootbuds. After 15 days of culture on regeneration medium, the buds with simple leaves grew up in to pinnately compound leaves. In four week old culture, shoot buds sprouted from the surface of the explants grew up well with green leaves. In addition, small green protuberances arose all over the surface of the callus which later developed into shootlets with compound leaves (Fig. 1C). In six week old culture, a multiple shoot differentiation was noticed with a mean of 36.6 ± 1.17 shoots counted per explants. The aseptic isolation and transformation of shoot clump on to the same media resulted better proliferation of shoots with pinnate leaves (Fig. 1D). One of the possible roles of higher concentration of cytokinin in the organogenic stage is to nullify the effects of auxin on shoot organogenesis and elongation (Vidya *et al.,* 2005). The effect of interaction of higher levels of BA (1 to 6 mgL^{-1}) with lower levels of IBA (0.1 to1. 0 mgL^{-1}) on multiple shoot induction and elongation was assessed in Table 2. At increased concentration of BA (above 6 mgL^{-1}) dedifferentiation of shoots and phenolic oxidation of callus in to black mass was noticed.

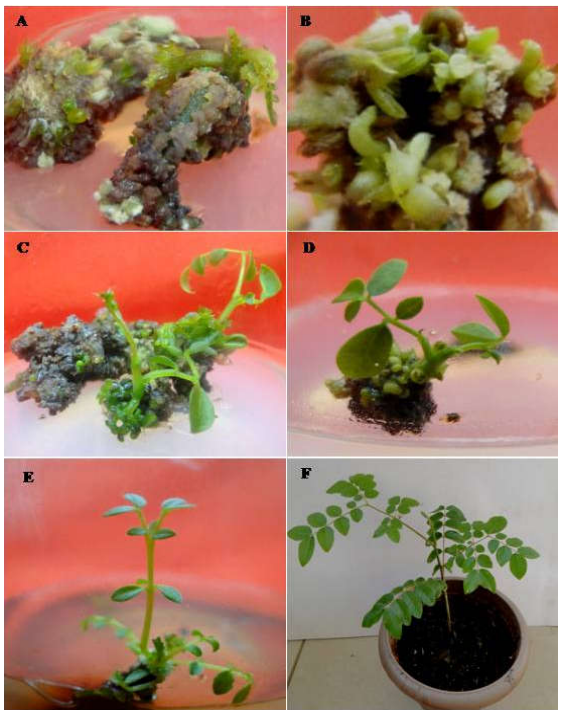

Figure 1: Indirect Organogenesis of *Caesalpinia bonduc* Roxb. from mature stem explants

A. Callus formation from the stem explants.
B. Initiation of shoot bud organization.
C. Multiple Shoot differentiation.
D. Growth of shoot with serrate margined leaves.
E. Profuse rooting from the organized shoot.
F. Hardened and soil acclimatized plantlets.

Table 2: Synergetic effect of BA and IBA on shoot bud differentiation from the stem callus of *Caesalpinia bonduc*

Growth regulators		Number of shoot buds/callus Mean ± SD	Number of shoot length/ bud Mean ± SD
BA	IBA		
2	0.0	05.40 ± 1.84	0.65 ± 0.27
2	0.1	07.00 ± 1.56	1.08 ± 0.23
2	0.2	10.50 ± 1.72	2.03 ± 0.56
2	0.3	07.50 ± 1.43	1.33 ± 0.26
3	0.0	06.80 ± 1.75	1.13 ± 0.35
3	0.1	13.60 ± 3.24	2.68 ± 0.36
3	0.2	15.20 ± 3.58	3.67 ± 0.33
3	0.3	14.30 ± 6.00	3.01 ± 0.19
4	0.0	10.20 ± 1.99	3.65 ± 0.78
4	0.1	22.90 ± 1.97	5.23 ± 0.63
4	0.2	36.60 ± 1.07	7.09 ± 0.23
4	0.3	28.30 ± 2.06	5.41 ± 0.70
5	0.0	08.10 ± 1.66	1.21 ± 0.56
5	0.1	19.50 ± 3.60	2.69 ± 0.38
5	0.2	22.30 ± 1.70	2.86 ± 0.51
5	0.3	20.10 ± 1.85	2.41 ± 0.17
6	0.0	04.20 ± 1.87	0.62 ± 0.24
6	0.1	07.70 ± 1.57	0.75 ± 0.32
6	0.2	11.00 ± 2.91	1.01 ± 0.49
6	0.3	08.90 ± 4.91	0.84 ± 0.32
F valve		84.4	207

The value of each combination consisted of mean ± S.D. of 10 replicates. The F-value is significantly different when $p < 0.05$.

Rooting of the small shoots was achieved on MS medium fortified with 0.2- 2.0 mgL^{-1} IBA respectively and optimized at 0.6 IBA mgL^{-1}. Roots were initiated from the base of small shoots after 3 weeks of culture (Fig. 1E). The individuals shoot buds with intact roots were harvested from the clump when they attained a length of 5-6 cm with 3 - 4 leaf primordia. The regenerants were successfully hardened and acclimatized on soil (Fig. 1F). One of the practical problems with woody plant tissue culture is the contamination of explants and most of researchers made sincere attempt to control the growth of endogenous and exogenous small flora of the explants (Blake *et al.,* 1988; Leifer *et al.,* 1990). In the present study also we made an attempt to establish aseptic regeneration protocol using stem explants of the mature plant. The regeneration protocol is an efficient means of ex situ conservation of this threatened woody climber and it assists sustainable maintenance of the present day rapidly dwindling germplasm on long-term basis.

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