

# Callus induction and shoot regeneration from epicotyl explants of ethnomedicinally important *Caesalpinia bonduc* (L.) Roxb

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## Abstract

An efficient plant regeneration method was established for *Caesalpinia bonduc* (L.) Roxb. by culturing immature epicotyl explants. Morphogenic calli were initiated from 96% of epicotyl explants on MS medium supplemented with BA (6-benzylaminopurine) 4.0 mg/l and NAA (Naphthalene acetic acid) 1.0 mg/l. The calli formed were excised and subcultured on MS medium fortified with 1 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) for further proliferation. Maximum percent organogenesis (84%) and average shoots per culture (5.6) was observed on MS medium fortified with 3.0 mg/l BA and 1.0 mg/l IAA (indole-3- acetic acid). Addition of indole-3-butyric acid (IBA) in ½ MS medium favored rooting of recovered shoots. Out of 30 rooted shoots transferred to soil 27 survived after acclimatization.

**Keywords:** *Caesalpinia bonduc*; Callus; Epicotyl; Ethnomedicinal plant; Regeneration

## INTRODUCTION

*Caesalpinia bonduc* is an ethnomedicinal woody climber belonging to Caesalpinaceae family. It is commonly known as fever nut or bonduc nut. *C. bonduc* is distributed in the tropical and subtropical coastal regions. All the parts of the plant including leaf, bark,

root and seed are medicinally useful. Seed is the major component of famous Ayurvedic drug- "Ayush- 64" used against malaria. Roasted seeds powdered to make a type of coffee for diabetics. A decoction of the plant is used as blood purifier and liver tonic. Plant paste is used for treatment of skeletal fractures in Sri Lanka. (Vaidyaratnam, 1994; Anonymous, 1992).

*C. bonduc* is reported to possess multiple therapeutic properties like antipyretic, antianaphylactic and antiarrhythmic (Iyengar and Pendse, 1965), antiasthmatic (Gayaraja *et al.*, 1978), antidiuretic and anthelmintic (Neogi and Nayak, 1958), antiviral (Dhar *et al.*, 1968), antiamebic and anti-estrogenic (Raghunathan and Mitra, 1982) properties. Hypoglycemic effects of *C. bonduc* have been reported with major results in rabbit (Rao *et al.*, 1994) and rat models (Chakrabarti *et al.*, 2003; Biswas *et al.*, 1997; Sharma *et al.*, 1997). Traditionally the plant is used to treat snake bite (Schaffner, 1997; Bellomaria and Kacou, 1995). A number of chemical components including flavonoids, triterpenoids, diterpenoids, and steroids have been reported from this plant (Lyder *et al.*, 1998; Peter *et al.*, 1997; Purushothaman *et al.*, 1982). The antibacterial and antifungal effects of the plant were also reported (Arif *et al.*, 2009; Simin *et al.*, 2000). Recent studies on the ethanolic extracts of *C. bonduc* introduced two new homoisoflavonoids, caesalpinianone, 6-O-methylcaesalpinianone, hematoxylool, stereochoenol A, 6'-O-acetylloganic acid, 4'-O-acetylloganic acid, and 2-O-β-D-glucosyloxy-4-methoxybenzenepropanoic acid. These compounds show different levels of glutathione S-transferase (GST) inhibitory and antifungal

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activities (Ata and Gale, 2009).

Due to its high medicinal value this plant is overexploited from the nature. According to New South Wales (NSW) Scientific Committee report (2001) this plant is on the verge of extinction and will become extinct if proper steps are not taken for its conservation and listed under endangered medicinal plant category (Hutton, 2001). To meet the increasing need for seedlings, tissue culture procedures could provide an alternative method to propagate this plant rapidly. Furthermore, the ability to regenerate plantlets *in vitro* could provide opportunities for genetic transformation and development of transgenic plants expressing novel traits. Successful *in vitro* plant regeneration and acclimatisation of regenerated plantlets in the field is an important strategy for genetic improvement of this plant species (Kannan *et al.*, 2006).

The major practical problem with woody plant tissue culture is the contamination of explants due to microorganisms present on the surface as well as in the internal tissues of the explant source. Hence special precautions are to be taken when explants are collected from field grown elite plants. Exogenous microorganisms can be effectively eliminated by surface sterilization. Although endogenous damage and insect attack cannot be easily eliminated and pose serious problems (Blake, 1988; Leifert, 1990). The contaminants appear at later stages of growth during *in vitro* culture (Bastiens, 1983; James and Thurbon, 1978). In contrast, seedling explants are considered as an excellent material for culture initiation and the degree of contamination is comparatively low (McComb and Bennett, 1986). To date not much research work has been carried out in *C. bonduc* despite a preliminary work on the adventitious bud formation from mature stem explants by Kannan *et al.*, 2006. This is probably due to its recalcitrant nature coupled with media browning during the initial stage of culture. One among the principal factors determining tissue culture responses in strongly recalcitrant species is the developmental stage of the explants at the time of culture establishment (Gentile *et al.*, 2002). There are a number of recent reports on successful plant regeneration from juvenile epicotyl explants of various woody legumes including *Tetrapleura tetraptera* (Ayisire and Amoo, 2004); *Parkia biglobosa* (Amoo and Ayisire, 2005); *Albizia odoratissima* (Rajeswari and Paliwal, 2008) and *Piliostigma thonningii* (Ayisire *et al.*, 2009). Therefore the present study aims at the application of tissue culture techniques to develop an effective protocol for regeneration of plants from epicotyl explants collected from germinating seeds of *C. bonduc*.

## MATERIALS AND METHODS

The mature dried seeds of *C. bonduc* were collected from the natural populations near Kallanai Dam (Grand Anaicut), bank of Cauvery river, Tiruchirappali, Tamilnadu in South India. The selected healthy seeds were immersed in water for five minutes and washed thoroughly. The seeds were washed with Teepol (a detergent and antiseptic) solution for five minutes and then soaked in 1% Bavistin for ten minutes followed by rinsing the seeds in distilled water and allowed to dry.

The seeds were surface sterilized with 20% V/V Sodium Hypochlorite (NaOCl) for eight minutes followed by three rinses in distilled water. Just before culture the lower side of the seeds was cut open without injuring the embryo to support rapid growth of the embryo. The seeds were cultured in autoclaved flasks containing cotton soaked in distilled water. All the cultures were kept under normal photoperiod with a light intensity of 25  $\mu$  mol m<sup>2</sup>/s in culture room at temperature 25±2°C.

The epicotyl region measuring a length of about 1.5 cm was isolated from the seven days old seedlings and cultured on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/l naphthalene acetic acid (NAA) or 1 mg/l 2, 4-Dichlorophenoxyacetic acid (2, 4-D) alone or in combination with various concentrations (2.0-6.0 mg/l) of 6-benzylaminopurine (BA) for callus induction. The calli were isolated after 45 days and subcultured on MS medium supplemented with 1 mg/l 2, 4-D for multiplication. For callus regeneration, the calli were subcultured on MS medium supplemented with either BA (1.0-4.0 mg/l) or 1.0-4.0 mg/l kinetin (KN) in combination with 1 mg/l indole-3-acetic acid (IAA). Shoot elongation was carried out on MS medium supplemented with BA (3 mg/l) and GA<sub>3</sub> (0.5 mg/l). Elongated shoots above 2.5 cm were transferred to MS medium supplemented with either 2.0-8.0 mg/l indole-3-butyric acid (IBA) or NAA (2.0-8.0 mg/l) for rooting.

MS medium supplemented with 30 g/l sucrose and 8 g/l agar was used as basal medium. The pH of the media was adjusted to 5.8 prior to autoclaving at 1.06 kg/cm<sup>2</sup> and 121°C for 20 min. The culture tubes were incubated at 25 ± 2°C under a 16 h photoperiod with a photosynthetic photon flux density of 60  $\mu$  mol m<sup>2</sup>/s supplied by two Philips TL 40 W fluorescent tubes. At least twenty-four cultures were raised for each treatment and all experiments were repeated three times. Analysis of variance and Duncan's multiple range test was used for comparison among treatment means.

Well rooted shoots were taken from the medium and washed in sterile distilled water to remove all traces of agar. The plants were then transferred to small plastic cups (6 cm diameter) containing garden soil mixed with sand (1:1). The cups were covered with polythene bags to retain 70-80% relative humidity and maintained at  $25\pm 2^{\circ}\text{C}$  with 16-h/d photoperiod for 4 weeks in the culture room. The cups were then transferred to a shade for another three weeks and eventually to field.

## RESULTS

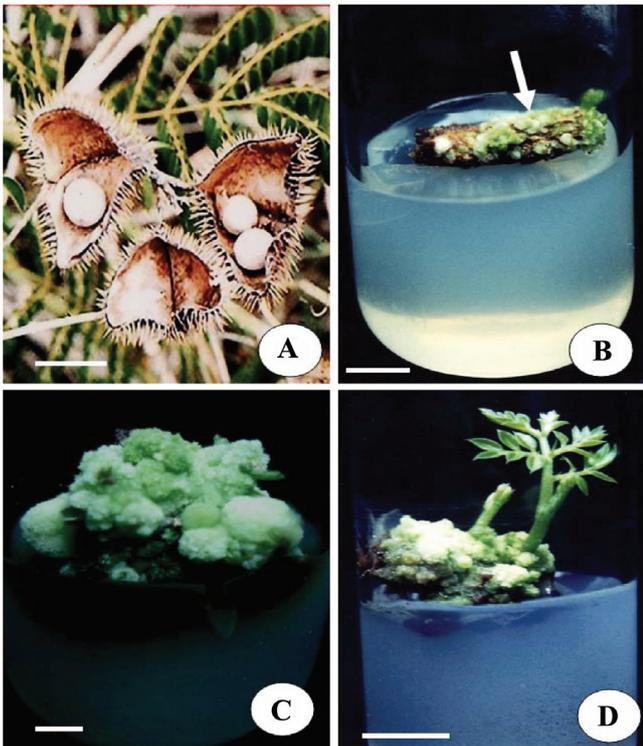
The mature dried seeds (Fig. 1 A) maintained 85-90 percent viability one month after collection. The seeds germinated after three days. Epicotyl region measuring a size of about 1.5 cm was isolated from 7 days old

seedlings and used for further culture experiments. The epicotyl explants were cultured on MS medium supplemented with NAA (1.0 mg/l) or 2, 4-D (1 mg/l) alone or in combination with various concentrations of BA (2.0-6.0 mg/l).

Explants did not callus on basal medium. Some explants (12%) showed phenolic exudation and those cultures were frequently subcultured to reduce exudation and cell death. Concentration of NAA and 2, 4-D above 1 mg/l was not favorable for callus growth. The presence of auxin alone showed poor callusing from the explant. Addition of 1 mg/l NAA or 2, 4-D (1 mg/l) in conjunction with BA was vital for callus initiation. Of the two different auxins tested here, callus induction was better on a medium containing NAA than 2, 4-D. On medium containing 2, 4-D (1 mg/l) 55% of epicotyl explants produced calli. It could be further enhanced to 88% by supplementing the medium with 4 mg/l BA along with 1 mg/l 2, 4-D. Similarly NAA (1 mg/l) alone gave rise to 65% callusing. The percentage of callus induction increased considerably to 96% when NAA at 1.0 mg/l was added in conjunction with 4 mg/l BA (Data not shown).

Calli were induced from explants within 8-10 days of placement on the medium (Fig. 1B). Epicotyl explants enlarged double its original size within 9-10 d of culture and were surrounded by light green, compact, organogenic calli throughout the outer surface of the explant including both the cut ends. The calli formed on epicotyl explants were excised and subcultured after 45 days on MS medium fortified with 1 mg/l 2, 4-D for further proliferation. On this medium morphogenic, yellowish-green friable calli grew repetitively during monthly subcultures and retained its regeneration potential.

Shoot organogenesis was observed from callus tissues obtained from epicotyle explants, when it was subcultured on MS medium supplemented with 1-4 mg/l BA or kinetin (KN; 1-4 mg/l) in combination with 1 mg/l IAA. Comparatively BA produced better response than KN when used in combination with IAA (Table 1). The small shoot clumps were started appearing on the calli 2 weeks after culture (Fig. 1C). Optimum shoot regeneration (84%) was observed on MS medium containing 3 mg/l BA and 1 mg/l IAA with an average number of 5.6 shoots per culture (Fig. 1D). Shoot regeneration was asynchronous and of the different shoots arose; only one or two shoots elongated (height ranging from 0.5 to 1.6 cm) and growth of the other shoots suppressed. BA at 3 mg/l was found to be giving optimum response for shoot regeneration.



**Figure 1.** Shoot organogenesis from the epicotyl derived calli of *C. bonduc*. A: Seeds in the dried opened fruit just before collection. Bar=2 cm B: Epicotyl explants cultured on MS medium supplemented with IAA (1 mg/l) and BA (4 mg/l) 10 d after culture. Green friable calli started emerging all through the surface of the explants (arrow). Bar= 0.5 cm C: Calli on regeneration medium one week after culture on MS medium fortified with BA (3 mg/l) and IAA (1 mg/l). Small shoots have initiated from the sides of the calli. Bar=0.67 cm D: Emergence of shoots from the calli on MS medium fortified with BA (3 mg/l) and IAA (1 mg/l) 45 d after culture. Bar=0.67 cm.

**Table 1.** Effect of various plant growth regulators on callus regeneration from epicotyl derived calli of *C. bonduc*. Data were recorded 45 d after transferring the callus onto media. Medium MS.

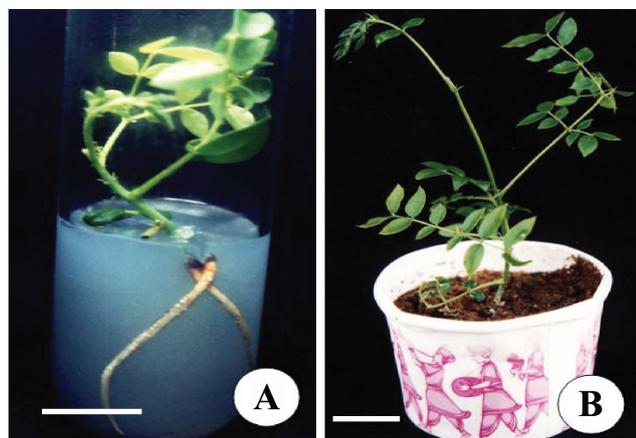
Plant growth regulators (mg/l)			Percent response <sup>a</sup>	No. of Shoots/ explants <sup>a</sup> (Mean ± S.D)	Average shoot length <sup>a</sup> (cm) (Mean± S.D)
BA	KN	IAA			
0	0	-	0	0	0
1.0	0.0	1.0	23e	1.1± 0.3c	0.5± 0.04c
2.0	0.0	1.0	57c	3.1±1.1b	0.9±0.03c
3.0	0.0	1.0	84a	5.6± 1.4a	1.6± 0.3a
4.0	0.0	1.0	81a	3.3±1.3b	1.1±0.4b
0	-	0	0	0	0
0.0	1.0	1.0	21e	1.1±0.4c	0.6±0.02c
0.0	2.0	1.0	42d	2.1±0.8c	0.8±0.04c
0.0	3.0	1.0	66b	2.8±0.6b	0.9±0.05c
0.0	4.0	1.0	62b	2.3±0.9c	1.1±0.2b

<sup>a</sup>The values represent the means (±SE) of three independent experiments. At least 24 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ( $P < 0.05$ ).

The smaller shoots were isolated and transferred to elongation medium which consists of BA (3 mg/l) and GA<sub>3</sub> (0.5 mg/l) for further growth of the shoots. Shoots attained a size of 2.5-3.0 cm on this medium after 4 wk of culture. Calli on MS basal medium without growth regulators failed to respond in all cases.

Half MS medium was more suitable for induction of roots on *in vitro* derived shoots. Lower mineral content is thought to be more suitable for *in vitro* rooting of woody species (George and Sherrington, 1984). In the present study half MS medium with various concentrations (2-8 mg/l) of two auxins (IBA and NAA)

were used for root induction. Half MS medium without auxins did not produce any roots even after 45 d. Of the two auxins tested for root induction, IBA was comparatively better than NAA. Roots were initiated in IBA containing medium within 2 weeks. NAA at lower concentrations resulted in callusing of the basal cut end and rooting was poor. The number of roots was generally low. The optimum result was observed on 4 mg/l IBA. On this medium 100% cultures responded with an average number of 3.3 roots per shoot (Fig. 2A; Table 2). Plantlets with roots were transplanted to the plastic cups (Fig. 2B) and eventually to the clay pots. 27 (90%) of the 30 plants transferred to soil survived.



**Figure 2.** Rooting and field transfer of the *in vitro* derived shoots. A: Induction of roots in *in vitro* derived shoots on ½ MS medium supplemented with IBA (4 mg/l) four wk after culture. Bar= 1 cm B: An *in vitro* derived acclimatized *C. bonduc* plant three months after transfer to soil. Bar=1.5 cm.

## DISCUSSION

Phenolic exudation followed by media browning and subsequent explant death is a common problem in *C. bonduc* nodal cuttings culture (Authors unpublished work). The explant contains large quantities of phenolic compounds. The use of repeated subculture of the explants on to fresh medium or the addition of activated charcoal into medium did not yield much result. The phenolic compounds present in the explant are oxidized by polyphenol oxidases and peroxidases which cause the browning of explants. The oxidized products are highly reactive and lead to cell death (Sharma and Ramamoorthy, 2000). However, we overcome this problem by using epicotyl explants. The epi-

**Table 2.** Influence of IBA and NAA on rooting of *in vitro* formed shoots on half MS medium. Culture period 45 d.

Auxins (mg/l)		Callus formation	Percentage rooting <sup>a</sup>	Average no. of roots per shoot <sup>a</sup>	Average root length <sup>a</sup> (cm)
IBA	NAA				
0	-	-	0	0	0
2	-	-	87c	1.4±0.48c	3.3±0.2a
4	-	-	100a	3.3±0.5a	3.1±0.4a
6	-	-	100a	2.8±.71b	2.6±0.5b
8	-	-	91b	1.3±.74c	2.1±0.2b
-	0	-	0	0	0
-	2	+	69e	1.1±0.4c	1.8±0.3c
-	4	+	78d	1.8±0.3c	2.1±0.3b
-	6	-	87c	2.1±0.6b	2.2±0.2b
-	8	-	73e	1.6±0.4c	1.4±0.5c

<sup>a</sup>The values represent the means (±SE) of three independent experiments. At least 24 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ( $P < 0.05$ ).

cotyls explants produced comparatively low rate of exudation as compared to field grown explants.

For callus induction, 1 mg/l each of NAA or 2, 4-D alone or in combination with BA (2.0-6.0 mg/l) was employed. NAA and 2, 4-D are commonly used with BA for callus induction in various systems (Dhar and Joshi, 2005; Maheshwari and Kumar, 2006). In case of *Vanilla planifolia* some workers used 2, 4-D with BA for inducing optimum callusing whereas others found that NAA was suitable for callus proliferation (Funk and Brodelius, 1990; Velankar and Heble, 2004; Janarthanam and Seshadri, 2008).

Shoot organogenesis from the epicotyl derived calli was obtained on MS medium supplemented with 1-4 mg/l BA or kinetin (1-4 mg/l) in combination with 1 mg/l IAA. The superiority of BA over Kn for callus regeneration is well demonstrated in the present study. This observation is in agreement with the previous published works demonstrating BA as the most successful cytokinin for shoot organogenesis in several other systems including *Bacopa monnieri* (Tiwari *et al.*, 1998), *Holarrhena pubescens* (Sumana *et al.*, 1999), *Cynodon dactylon* (Zhang *et al.*, 2007) *Salvia officinalis* (Tawfik and Mohamed, 2007) and *Scopolia parviflora* (Kim *et al.*, 2009).

The promoting effect of auxin and cytokinin combinations on organogenic differentiation has been well documented in several systems (Dimech *et al.*, 2007; Thomas and Puthur 2004; Korocho *et al.*, 2002; Xie and Hong 2001; Pereira *et al.*, 2000; Pretto and Santarém 2000). Similarly the effects of NAA and BA for shoot development from callus observed in this study is in

agreement with earlier reports on organogenesis in *Withania somnifera* (Rani *et al.*, 2003), *Curcuma amada* (Prakash *et al.*, 2004), *Anthurium andreaeanum* (Vargas *et al.*, 2004), and *Sarcostemma brevistigma* (Thomas and Shankar, 2008).

Well developed shoots were rooted on half MS medium fortified with two auxins (IBA and NAA; 2-8 mg/l). IBA supplemented medium exhibited superior rooting than NAA. IBA is considered as the most effective growth regulator for induction of roots in legumes as stated by Ozean *et al.*, (1992). IBA stimulated rooting was observed in *Vigna radiata* (Husan and Siddiquai, 2006), *Aegle marmelos* (Nayak *et al.*, 2007), *Clitoria ternatea* (Barik *et al.*, 2007) and *Cotinus coggygria* (Metivier *et al.*, 2007).

In conclusion, the ability to regenerate whole plants from epicotyl derived calli of *C. bonduca* has been demonstrated. This is an efficient and reproducible protocol for plantlet regeneration from this important ethnomedicinal plant. It provides a promising method for the large scale *in vitro* propagation and conservation of this species.

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