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Influence of plant growth regulators on plant regeneration from epicotyl and hypocotyl explants of *Caesalpinia bonduc* (L.) Roxb – an ethnomedical plant

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Abstract

This study demonstrates the morphogenic potential of *Caesalpinia bonduc* (L.) Roxb, a traditional medicinal plant of the family Fabaceae/ Caesalpinaceae. The present study was designed to examine the effect of phytohormones on plant regeneration from epicotyl and hypocotyl explants of *C. bonduc*. The dormancy of the seeds was overcomed by acid scarification. Of the two explants tested, 92 percent frequency of shoot regeneration and maximum number of shoots (3.6 ± 0.3), were noticed from the epicotyl explants on Murashige and Skoog (MS) medium fortified with 6-benzyladenine (BA) 3.0 mg/L, Indole– 3- acetic acid (IAA) 1.0 mg/L and Poly vinyl pyrrolidone 100 mg/L at pH 5.8. Elongated shoots were individually rooted on half strength MS medium supplemented with 1.5 mg/L Indole -3-butric acid (IBA) and exhibited 93 % frequency of root development. The *in vitro* raised plantlets were potted and acclimatized under culture condition for four weeks and transferred to the green house. This efficient protocol will be helpful for propagation of woody climber plants belongs to the family Caesalpinaceae and could be used for genetic transformation study.

Key words: Hypocotyl, epicotyl, plant regeneration, H_2SO_4, polyvinyl pyrrolidone.

Introduction

Medicinal plants are an important source of life saving drugs for human kind, especially in developing countries. It is well known that, synthetic drugs associated with health hazards and their toxicity has renewed the interest in the use of plant-based drugs. *Caesalpinia bonduc* (L.) Roxb., (Family-Fabaceae/ Caesalpiniaceae), a prickly shrub or woody vine reaching a length of 10 m or more is also known as kalarchikai (Tamil). The name of the species (*Bonduc*) is derived from the Arabic word "*Bonduce*" meaning a "little ball" which indicates the globular shape of the seed (Kannur *et al.*, 2012). The plant is threatened and widely distributed throughout the world especially in tropical parts of India, Sri Lanka and Myanmar sparsely distributed in the deciduous forests of the Western Ghats of India. It is also reported to be critically endangered in Malaysia.

C. bonduc is one of such medicinal plants well-known for its health benefits in folk medicine. The secondary metabolite compound properties might be responsible for spectrum of phytochemical and pharmacological activities of *C. bonduc*. It is extensively used in Ayurveda, Siddha, Unani and homoeopathy since last two decades. Different parts of the plant are used to treat asthma, chronic fever, cough, headache and stomach upset (Chopra and Nayar, 1956; Nandakarni and Nandakarni, 1976). Recently conducted studies reported that, different parts of plant possess significant anti-inflammatory, analgesic, anti-bacterial, anti-diarrheal and cytotoxic activity. In addition to this, plant is known to offer multiple pharmacological benefits such as anti-pyretic, anti-diabetic, anti-carcinogenic, anti-fungal, anti-filarial, immunomodulatory, insecticidal, anxiolytic and antioxidant effects. *C. bonduc* is known to contain phenolic compounds, alkaloids, glycosides, saponin and isoflavones (Shukla *et al.*, 2009; Singh and Raghav, 2012; Billah *et al.*, 2013; Shelar *et al.*, 2014).

Conservation of medicinal plants through *in vitro* proliferation, *in situ* and *ex situ* methods was reported by Govindaraj *et al*. (2008); Radhakrishnan *et al*. (2009). There are number of reports available on the 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). There are as yet no successful report on epicotyl explants regeneration from germinating seeds of *C. bonduc*. Therefore, in the present investigation epicotyl and hypocotyl explants of *C. bonduc* were studied for regeneration.

Materials and methods

The mature dried seeds of *C. bonduc* (Fig.1a & b) were collected from Kollidam river bank, Tiruchirappalli District, Tamil Nadu, India. The plant was authenticated with the type specimen available in the Rapinat Herbarium. St. Joseph's College (Autonomous), Tiruchirappalli, Tamil Nadu. The dormancy of the seeds was overcomed by acid scarification using conc. H_2SO_4 for 45 min. The treated seeds were washing under running tap water for about 10 min. Then seeds were sterilized with detergent for 5 minutes followed by washed under running tap water for 10 minutes. Immersed in an aqueous solution of 5 % Teepol for 5 min, later the seeds were treated with 4 % of Sodium hypochloride solution for 5 minutes and washed with distilled water followed by 0.1 % of mercuric chloride and washed with sterile water 3 times.

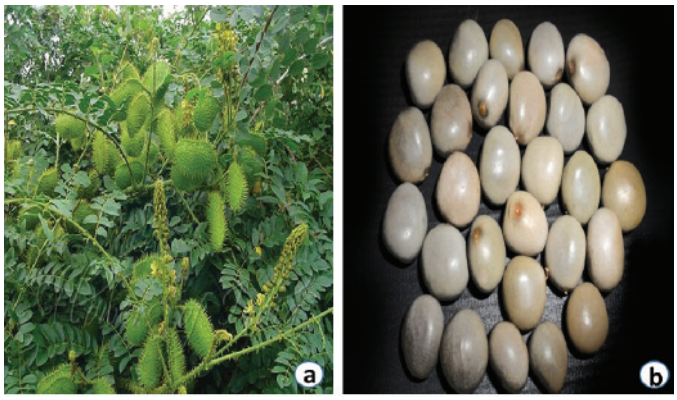

Fig. 1. a: *C. bonduc* plant. b: *C. bonduc* seeds

Shoot initiation and multiplication: The treated seeds were inoculated on the MS medium (Murashige and Skoog, 1962) supplemented with 3 % (w/v) sucrose and 0.8 % (w/v) agar. The medium was adjusted to pH 5.7 with 1 N NaOH or 1 N HCl prior to the autoclave at 121 ºC for 20 min. For direct multiple shoot induction, 10-12 days of the epicotyl, hypocotyl explants were excised and inoculated on the medium fortified with 100 mg/L polyvinyl pyrrolidone (PVP) to arrest phenolic contamination, BAP, Kin, TDZ and IAA for the shoot induction. The explants were supplemented with the different concentrations of BAP, Kin, TDZ and IAA compared with control (without any hormone). All the cultures were incubated at 25± 2 ºC under cool –white light with a 16 hrs photoperiod and 8 hrs dark period and subcultured on fresh media at 15-20 days of interval.

Rooting, hardening and acclimation: Data were collected at the end of 45 days of culture on percentage shoot regeneration and number of shoots per responding explant. For the root initiation the shoots were transferred to the half strength medium 3 % (w/v) sucrose and 0.8 % agar (w/v) supplemented with Indole -3-butyric acid (IBA) (0.5-2.0 mg/L) or NAA (0.5-2.0 mg/L) and control (without any hormone). The *in vitro* raised plantlets were potted and acclimatized under culture condition for four weeks before their transfer to green house.

Statistical analysis: Data were analyzed by using one-way analysis of variance (ANOVA) to assess treatment differences and interactions using SPSS programme (16.0). The mean values of treatments were subjected to DMRT (Duncans Multiple Range Test) ($P \leq 0.05$). Data are represented as Mean ± Standard Error of three replications.

Results and discussion

Shoot multiplication: Establishment of an efficient *in vitro* regeneration system is dependent on the type of explant used. Shoot proliferation from the epicotyl and hypocotyl explant was visible after 15-20 days of inoculation. *In vitro* generated explants of *C. bonduc* were used for multiple shoot induction and proliferation. MS medium supplemented with different concentrations of cytokinins such as TDZ, BAP, and KIN (1.0-4.0 mg/L) were used for shoot induction (Table 1 and 2). Interestingly, all cytokinins used in the present study showed similar shoot response. Similar studies are also reported by Eapen and George (1993) in groundnut, Hossain *et al.* (1994) in *Aegle marmelos*, Mendoza and Futsuhara (1990) in mungbean, Pandey and Bansal (1992) in soybean and Barna and Wakhlu (1994) in chickpea, Venkatachalam *et al.* (1998) in groundnut and in *Celastrus paniculatus* Willd. by Moola and Ranjitha Kumari (2019).

Effect of cytokinins on shoot multiplication of *C. bonduc*: In this study, different concentrations of cytokinins with auxin were used for shoot multiplication. Among the different concentrations tested, the highest shoot multiplication was achieved at BAP+IAA 3.0+1.0 mg/L concentration with number of shoots (3.6±0.3), and high frequency of response (92±0.5 %) obtained from epicotyl explants (Table 3) whereas in hypocotyl explants (Table 4), the highest shoot multiplication was achieved at BAP+IAA 3.0+1.0 mg/L concentration with maximum number of shoots (2.6 ± 0.08) and highest frequency percentage (82±1.1). In comparison with hypocotyl explants, epicotyl explants showed highest regeneration efficiency in all aspects.

Explants tested in the present study failed to elicit response in a hormone-free medium. MS supplemented with 17.75 µMol BAP and 2.46 µMol IBA induced a mean of 3.40±1.07 frequency root

Table 1. Effect of various concentrations of BAP, KN, TDZ on survival rate and shoot multiplication from epicotyl explants of *C. bonduc*

Concentration	BAP		Kin		TDZ	
	Response (%)	No. of shoots	Response (%)	No. of shoots	Response (%)	No. of shoots
1.0	26 ± 0.6^{f}	1.4 ± 0.03^{d}	20 ± 0.5^{g}	1.0 ± 0.0^{c}	20 ± 0.5^{g}	1.0 ± 0.0^{c}
2.0	36 ± 0.3^{f}	1.5 ± 0.05^{cdā}	33 ± 0.3^{ef}	1.6 ± 0.3^{abc}	33 ± 0.3^{ef}	1.6 ± 0.3^{abc}
3.0	56 ± 0.3^{c}	2.1 ± 0.0^{a}	56 ± 0.3^{bc}	2.3 ± 0.3^{a}	56 ± 0.3^{bc}	2.3 ± 0.3^{a}
4.0	40 ± 0.5^{e}	1.6 ± 0.06^{bc}	46 ± 0.3^{cd}	1.3 ± 0.3^{bc}	46 ± 0.3^{cd}	1.3 ± 0.3^{bc}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Table 2. Effect of various concentrations of BAP, KN, TDZ on survival rate and shoot multiplication from hypocotyl explants of *C. bonduc*

Concentration	BAP		Kin		TDZ	
	Response (%)	No. of shoots	Response (%)	No. of shoots	Response (%)	No. of shoots
1.0	24 ± 0.5^{i}	0.8 ± 0.1^{g}	26 ± 0.5^{i}	1.06 ± 0.03^{f}	26 ± 0.5^{i}	1.06 ± 0.03^{f}
2.0	25.3 ± 0.3^{i}	1.0 ± 0.03^{f}	27.3 ± 0.3^{ki}	1.46 ± 0.03^{cd}	27.3 ± 0.3^{ki}	1.46 ± 0.03^{cd}
3.0	34 ± 1.1^{i}	1.6 ± 0.0^{bc}	31.3 ± 1.7^{ij}	1.8 ± 0.1^{b}	31.3 ± 1.7^{ij}	1.8 ± 0.1^{b}
4.0	30 ± 1.1^{jk}	1.2 ± 0.0^{ef}	30.3 ± 0.3^{jk}	1.3 ± 0.08^{de}	30.3 ± 0.3^{jk}	1.3 ± 0.08^{de}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Table 3. Effect of BAP, Kin, TDZ with IAA concentrations on survival rate and shoot multiplication from epicotyl explants of *C. bonduc*

Concentration	BAP + IAA		Kin + IAA		TDZ + IAA	
	Response (%)	No. of shoots	Response (%)	No. of shoots	Response (%)	No. of shoots
1.0 +0.5	36 ± 0.3^{f}	1.4 ± 0.03^{egf}	26 ± 0.3^{g}	1.3±0.03^{fgh}	40 ± 0.5^{gh}	1.2± 0.1^{h}
2.0 +0.5	66 ± 0.6^{d}	1.6 ±0.05^{ed}	40 ± 0.5^{de}	1.5 ± 0.0^{def}	46 ± 0.8^{fg}	1.38±0.1^{fgh}
3.0+0.5	73 ± 0.3^{c}	2.6 ± 0.08^{ae}	76 ± 0.3^{a}	2.4 ± 0.03^{b}	70 ± 0.5^{bc}	2.3± 0.0^{b}
4.0+0.5	63 ± 0.8^{c}	1.7 ± 0.03^{c}	66 ± 0.3^{ab}	1.6 ±0.03^{cd}	50 ± 0.5^{ef}	1.2± 0.03^{gh}
1.0+1.0	60 ± 0.5^{d}	2.0 ± 0.0 ^{cd}	55 ± 0.5^{e}	1.5± 0.1^{c}	63 ± 0.3^{cd}	2.0 ± 0.0^{abc}
2.0+1.0	83 ± 0.3^{a}	2.3 ± 0.3^{bc}	65.3 ± 1.4^{d}	1.2±0.3^{d}	83 ± 0.3^{ab}	2.3 ± 0.3^{ab}
3.0+1.0	92 ± 0.5^{a}	3.6 ± 0.3^{a}	83 ± 1.1^{a}	2.2±0.3^{a}	90 ± 0.3^{a}	3.0 ± 0.5^{a}
4.0+1.0	76 ± 0.3^{a}	3.0 ± 0.5^{ab}	72 ± 1.1^{c}	1.9±0.3^{b}	80 ± 0.5^{ab}	1.6 ± 0.3^{bc}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Table 4. Effect of different concentrations of BAP, Kin, TDZ with IAA on survival rate and shoot multiplication from hypocotyl explants of *C. bonduc* (L.) Robx.

Concentration	BAP + IAA		Kin + IAA		TDZ + IAA	
	Response (%)	No. of shoots	Response (%)	No. of shoots	Response (%)	No.of shoots
1.0 +0.5	37.6 ±0.8^{h}	1.3±0.3^{cd}	41± 1.1^{gh}	1.3 ± 0.3^{bc}	45±3.0^{ef}	1.0 ± 0.0^{c}
2.0 +0.5	40 ± 1.1^{gh}	1.3 ± 0.3^{cd}	43.3 ±2.8^{fg}	1.6 ± 0.3^{abc}	48.3 ±1.8^{e}	1.3 ± 0.3^{bc}
3.0+0.5	46 ± 1.1^{f}	1.6 ± 0.3^{cd}	46.6 ± 1.2^{f}	2.3 ± 0.3^{a}	54 ± 2.0^{d}	2.3 ± 0.3^{ab}
4.0+0.5	39 ± 0.5^{h}	1.0 ± 0.0^{cd}	40.6±0.6^{gh}	1.0 ± 0.0^{c}	46.3 ±1.7^{ef}	1.3 ± 0.3^{bc}
1.0+1.0	53 ± 0.5^{e}	1.9±0.05^{e}	30 ± 0.5^{fg}	1.3 ± 0.3^{bc}	64± 1.7^{c}	1.8±0.03^{e}
2.0+1.0	64 ± 1.5^{d}	2.2±0.0^{c}	43 ± 0.8^{cd}	1.0 ± 0.0^{c}	84.6 ± 1.4^{a}	1.9±0.03^{de}
3.0+1.0	82 ± 1.1^{a}	2.6± 0.08^{a}	56 ± 0.3 ^{bc}	2.0 ± 0.0^{ab}	86.6 ± 1.4^{a}	2.3±0.06^{bc}
4.0+1.0	76 ± 1.1^{b}	2.4±0.0^{b}	50 ± 0.5 ^{cd}	1.3 ± 0.3^{bc}	74 ±1.5^{b}	2.06±0.03^{d}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

explant (Kumar *et al.*, 2012). Owing to phenolics, most of woody species explants do not induce morphogenic response in culture condition (Furze and Cress well, 1985; Bonga, 1987). Many woody legume plants supplemented with BAP alone in different concentrations or in combination with auxin induced shoot bud organogenesis (Subotic *et al.*, 2009). BAP and IAA induced callus regeneration was reported in *Acacia sinuate* (Vengadesan *et al.*, 2000), *Citrullus lanatus* (Chaturvedi and Bhatnagar, 2001), *Argyrolobium roseum* (Khanna *et al.*, 2006) and *Vigna subterranea* (L.) Verdc. (Hilaire *et al.*, 2009).

Rooting

Root induction from *in vitro* elongated shoots of *C. bonduc*: For root induction, *in vitro* elongated shoots of *C. bonduc* were excised from regenerating cultures and transferred to half-strength MS medium supplemented with different concentrations ranging from 0.5-2.0 mg/L of auxins such as NAA and IBA. The percentage of root and number of roots per shoot were calculated (Table 5 and Fig. 2). Out of two auxins tested, IBA was found to be most effective for root induction. The highest number of roots per shoot (5.3±0.3) and high frequency (93 %) were obtained in IBA at 1.5 mg/L. Whereas, the lowest number of roots per shoot (1.3±0.3) and low frequency (50 %) were obtained in NAA (0.5 mg/L).

Table 5. Influence of IBA and NAA on rooting of *in vitro* formed shoots on half MS medium.

PGR (mg/L)		Percentage response	Number of roots explants
IBA	NAA		
0.5	0.0	63 ± 0.3^{cd}	1.6 ± 0.3^{cd}
1.0	0.0	73 ± 0.3^{bc}	2.3 ± 0.3^{cd}
1.5	0.0	93 ± 0.3^{a}	5.3 ± 0.3^{a}
2.0	0.0	56 ± 0.3^{de}	1.3 ± 0.3^{d}
0.0	0.5	50 ± 0.5^{e}	1.3 ± 0.3^{d}
0.0	1.0	53 ± 0.3^{de}	1.6 ± 0.3^{cd}
0.0	1.5	76 ± 0.3^{b}	4.3 ± 0.3^{b}
0.0	2.0	50 ± 0.5^{e}	2.6 ± 0.3^{c}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Ozean *et al.* (1992) reported that Indole-3-butyric acid is considered as the most effective growth regulator for induction of roots in legumes. Similar findings about IBA-induced rooting were observed in other leguminous species such as *Acacia sinuata* (Vengadesan *et al.*, 2000), *Swainsona salsula* (Yang *et al.*, 2001), *Cassia angustifolia* (Agarwal and Sardar, 2007) and *Vigna subterranea* (L.) Verdc. (Hilaire *et al.*, 2009). The acclimatization of plantlets resulted in a survival rate of 95 %. During the period of 6 months in the greenhouse, there was no morphological abnormalities or detectable phenotypic variation in regenerated plants when compared with mother plant.

The present study developed a time-saving protocol for rapid and high-frequency regeneration method for *C. bonduc* by using epicotyl and hypocotyl (non-meristematic) explants. Out of the two explants tested, epicotyl explants were found to be best for shoot multiplication using combination of BAP alongwith IAA (3.0+1.0 mg/L). For root induction, IBA at 1.5 mg/L concentration

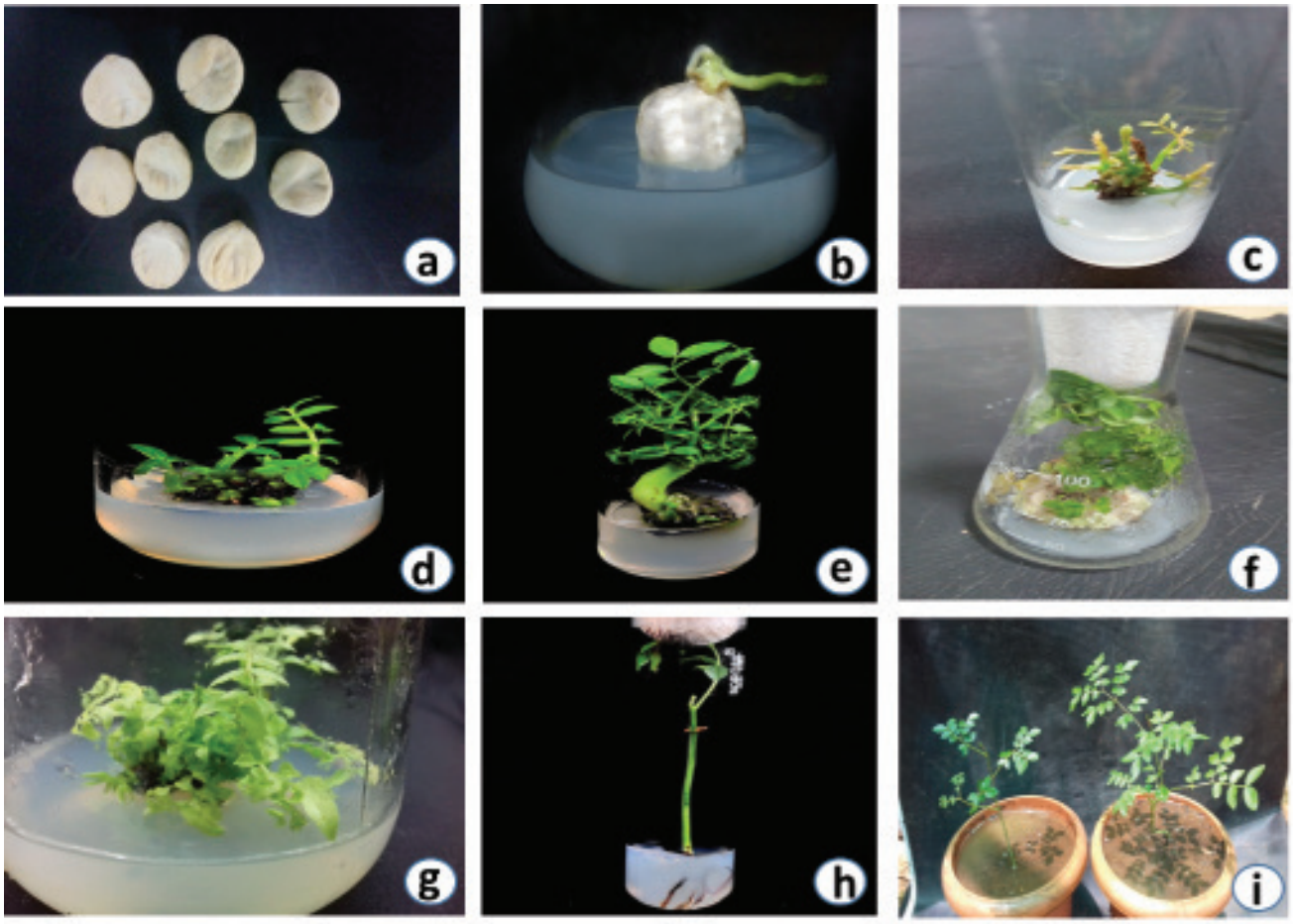

Fig. 2. Direct plant regeneration of *in vitro* cultured Epicotyl explants of *C. bonducella* (L.) Roxb. (a) Endosperm, (b) Germination of Endosperm, (c) Shoot initiation from epicotyl explants, (d) Development of shoots, (e, f and g) Shoot multiplication and elongation, (h) Rooting, (i) Hardening of plant

showed best response. Total regeneration process from the initiation of tissue culture to transplantation of regenerants into soil completed within 45 days. Furthermore, in the present study, subculture by transferring induced shoots on the same medium used for initiation would contribute to enhance the number of shoots per explant. Hence, the present study concludes that, regeneration system through epicotyl explants would be useful in *C. bonduc* propagation.

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