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Micropropagation of *Canavalia cathartica* of coastal sand dunes

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Among the tender pods, cotyledons of ripened beans, germinated dry seeds and hypocotyls used for *in vitro* culturing, cotyledons and hypocotyls of germinated dry seeds responded positively. A maximum of 78% callus induction in large quantity was seen in cut ends of cotyledons fortified with 1 mg/l BAP within four weeks followed by formation of embryos in six weeks, but failed to develop roots in spite of supplementing IAA. At 0.5 mg/l each of BAP and 2iP induction of shoot buds in 30% cotyledon explants was seen. In 40% of hypocotyls, 1 mg/l each of BAP and 2iP induced large amount of friable calli within four weeks. In hypocotyls placed upright position in medium with 0.5 mg/l and 1 mg/l each of BAP and 2iP, leafy shoots were induced up to 50% and 42.5% respectively. A highest of 62.5% of hypocotyl explants exhibited rooting on culturing with 1 mg/l each of NAA and IAA, but higher concentrations failed to induce roots. Treatment of the stem cuttings with IBA at 1 mg/l for 30 min increased rooting response, while 0.25 mg/l NAA resulted in high rooting response. *In vitro* and *ex vitro* micropropagation techniques for *C. cathartica* could be employed to supplement the traditional propagation methods of plant breeding. Attempts are successful to initiate callus (cotyledon and hypocotyl), shoot bud induction (cotyledon), root formation (hypocotyl) and somatic embryogenesis (cotyledon). Induction of friable calli from cotyledon and hypocotyls will be useful to extract valuable phytochemicals such as con A, canavanine and canaline.

Key words: *Canavalia cathartica,* wild legume, coastal sand dunes, *in vitro* propagation, *ex vitro* culturing

Introduction

Among the members of Fabaceae, the perennial creeper *Canavalia cathartica* Thouars [Synonym: *C. microcarpa* (DC.) Piper; *C. turgida* Graham ex A. Gray; *C. virosa* (Roxb.) Wight et Arn.; *Dolichos virosus* Roxb.; *Lablab microcarpus* DC.] is the second dominant member after *C. maritima* on the coastal sand dunes (CSD) of southwest coast of India (Arun *et al*., 1999; Sridhar and Bhagya, 2007). Another landrace of *C. cathartica* grows in the mangroves of southwest coast of India, which differs from CSD variety in

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agrobotanical, nutritional and antinutritional features (Bhagya *et al*., 2007; Seena *et al*., 2007; D'Cunha, 2009). *Canavalia cathartica* is a wild ancestral form of *C. gladiata*, which has wide pantropical distribution (Purseglove, 1974; Arun *et al*., 1999; Seena *et al*., 2007). Sastrapradja *et al*. (1981) reported a natural hybrid of *C. cathartica* × *C. gladiata*, while artificial hybridization of *C. cathartica* with *C. ensiformis* decreased the pollen fertility in F1 and F2 progenies. *Canavalia ensiformis* has been considered one among 17 leguminous species possessing potentially useful phytochemicals by the Plant Genetic Resources Conservation Unit (PGRCU), USA (Morris, 1999). Besides nutritional and antinutritional values, *Canavalia* spp. are known for several valuable phytochemicals and toxins (e.g. anticancer agent, trigonelline; cytotoxic aminoacid, canavanine; antiviral lectin, concanavalin A) (Ghosh *et al*., 1985; Ramírez *et al*., 1992; Sato *et al*., 1993; Backstrom-Sternberg and Duke, 1994; Swaffar *et al*., 1995, Jayavardhanan *et al*., 2006; Sridhar and Seena, 2006; Seena and Sridhar, 2006).

Canavalia cathartica is an endangered plant species on the CSD as well as mangroves of the southwest coast of India due to human interference (Seena *et al*., 2007; Sridhar and Bhagya, 2007). Being potential nutritional, antinutritional and pharmaceutical value, landraces of *C. cathartica* deserves different strategies of conservation. Besides its protection in their natural habitats, micropropagation and tissue culture techniques may provide opportunities to profitably exploit these genetic resources as food and pharmaceuticals. Therefore, the current study aimed at preliminary evaluation of tissue culture potential of *C. cathartica* of CSD using *in vitro* and *ex vitro* methods.

Materials and methods

Explants for in vitro study

Dry seeds, tender pods and ripened beans of *C. cathartica* were gathered during post-monsoon and summer (December 2007 – May 2008) from the CSD of Someshwara (12º47′N, 74º52′E) of the southwest coast of India. The hilum of dry seeds was mechanically scarified and incubated for germination on moist sand beds up to 72-120 hr. Explants of tender pods, cotyledons of ripened beans, cotyledons of germinated dry seeds and hypocotyls were used for *in vitro* culturing. They were washed in running water to eliminate extraneous matter and surface sterilized [carbendazim (Northern Minerals Ltd., Mumbai), 0.2% w/v, 10 min; mercuric chloride (Qualigens, India), 0.1% w/v, 7 min; sodium lauryl sulphate (Qualigens, India), 0.1% w/v, 7 min] followed by rinses in sterile distilled water and excised aseptically into desired size. Anthers from flower buds were also used as explants.

Nutrient medium

Analytical grade ingredients (Hi-Media Laboratories, India; Merck, India) and sterile double distilled water were used to prepare the MS medium (Murashige and Skoog, 1962). The plant growth regulators (PGR) [6-benzyl amino purine (BAP), 6-γ,γ-dimethylallylaminopurine (2iP), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4–D), indole acetic acid (IAA), indole butyric acid (IBA) and kinetin (KIN)], vitamins (pyridoxine HCl, thiamine HCl, nicotinic acid) and glycine were incorporated into the medium. Stock solutions of macronutrients, micronutrients, vitamins, glycine and PGR were prepared using sterile double distilled water and preserved at 4°C. The PGR were weighed and dissolved in a few drops of 0.1 N NaOH or ethanol before making up to desired volume and preserved at 4°C. The basal medium was prepared using required amount of stock solutions, sucrose and myo-inositol in a beaker containing 250 ml double distilled water, mixed, diluted up to 950 ml, pH was set to 5.7 ± 0.1, and the volume was made up to 1000 ml and mixed. To this basal medium, required quantity of PGR and agar were transferred, melted using microwave oven, dispensed into culture vials (Borosil, India) and autoclaved. Anthers were aseptically cultured in Nitsch medium (Nitsch, 1969) along with BAP and 2,4-D.

Explant transfer and incubation

The surface sterilized explants were aseptically transferred to culture vials in a laminar flow workbench (Klenzaids, India). Single explant was transferred to each culture tubes, while 4-5 explants per conical flasks. All culture vials were incubated at uniform humidity (75 ± 5%), temperature (25 ± 2°C) and under cool daylight fluorescent tubes (10-15 $\mu E/m^{-2}/s^{-1}$, 16 hr photoperiod monitored by luxmeter) (Voltkraft MS 1500, Germany).

Ex vitro study

For *ex vitro* study, juvenile shoots with 2-3 nodes (0.2-0.4 × 20-30 cm) were collected from the CSD of Someshwara were used. Forty shoots were excised into 15 cm segments, the immersing end (inter nodal region) was cut obliquely and rinsed in running tap water to remove the extraneous matter. The obliquely cut ends were immersed in vials containing different concentrations of IBA and NAA (0-7.5 mg/l) for 30 min. The hormone-treated shoots were blotted and transferred to polythene bags containing mixture of sand, soil and vermiculite (1:2:1 v/v/v). The segments without hormone treatment served as control. The planted shoots were maintained under shade (temperature, 28 ±

2°C; humidity, 70-80%) by spraying water once a day. The rooting response (number of roots per plant and root length) of each cutting was assessed after three weeks. Another set of shoot segments was treated similarly with different doses of auxins (IBA and NAA) as pulse treatment (duration: 15, 30, 60 and 120 min) and the response was assessed.

Results

Among the tender pods, cotyledons of ripened beans, germinated dry seeds and hypocotyls used for *in vitro* culturing, cotyledons and hypocotyls of germinated dry seeds responded positively.

Callus and shoot bud induction

The cotyledon explants in MS medium with 2 mg/l BAP produced white, green and friable calli (Fig. 1a-c). A maximum of 78% callus induction in large amount was recorded in cut ends of cotyledons fortified with 1 mg/l BAP within four weeks, while in rest of the concentrations it ranged between 18% and 33% (Table 1). In hypocotyls, 1 mg/l each of BAP and 2iP induced large amount of friable calli (40%) within four weeks (Fig. 1d). At 0.5 mg/l each of BAP and 2iP induction shoot buds was highest (30%) in cotyledon explants (Table 2; Fig. 1e). In hypocotyls placed upright position in medium with 0.5 mg/l and 1 mg/l each of BAP and 2iP leafy shoots were induced up to 50% and 42.5% respectively (Fig. 1f, 2a). Anthers cultured in Nitsch medium did not show any morphogentically differentiated structures.

Embryogenesis and rooting

Embryogenic calli and somatic embryos were seen after six weeks in cotyledon explant culture fortified with BAP 1mg/l (Fig. 2b). Although somatic embryos differentiated directly from cotyledon explants, they failed to develop roots in spite of supplementing IAA. The hypocotyl explants did not show embryogenesis, but responded positively on culturing with low concentrations of NAA and IAA. A highest of 62.5% of hypocotyl explants exhibited rooting on culturing with 1 mg/l each of NAA and IAA (Table 3; Fig. 2c, d). In basal medium, one of the hypocotyls gave rise to single root (length, 2 cm) within three weeks, while another hypocotyl treated with 0.5 mg/l each of IAA, BAP and KIN yielded five roots (length, 0.5-1 cm). However, increased concentrations of these hormones failed to induce roots in the hypocotyls.

Table 1. Callus induction in cotyledon and hypocotyl explants of *Canavalia cathartica* in MS medium supplemented with BAP and 2iP in 4 weeks.

PGR (mg/l)		Number of explants	Number of explants formed callus	Callus induction (%)	Amount of callus[a]
BAP	2iP				
Cotyledon explants					
0	0	50	NG	0	-
1.0	0	50	39	78.0	+++
2.0	0	50	9	18.0	++
0.5	0.5	30	10	33.0	++
1.0	1.0	30	6	20.0	++
1.5	1.5	50	12	24.0	++
Hypocotyl explants					
0	0	40	NG	-	-
1.0	0.0	40	NG	-	-
2.0	0.0	40	NG	-	-
0.5	0.5	40	7	17.5	++
1.0	1.0	40	16	40.0	+++
1.5	1.5	40	8	20.0	+++

NG, No growth
[a] ++, Medium; +++, Large

Table 2. Shoot bud induction in cotyledon hypocotyl explants of *Canavalia cathartica* in MS medium supplemented with BAP and 2iP in 4 weeks.

PGR (mg/l)		Number of explants	Number of explants formed buds	Shoot induction (%)
BAP	2iP			
Cotyledon explants				
0	0	50	NG	-
1.0	0	50	1	2.0
2.0	0	50	2	4.0
0.5	0.5	30	9	30.0
1.0	1.0	30	1	3.3
1.5	1.5	30	2	6.6
Hypocotyl explants				
0	0	40	NG	-
1.0	0	40	NG	-
2.0	0	40	NG	-
0.5	0.5	40	20	50.0
1.0	1.0	40	17	42.5
1.5	1.5	40	NG	-

NG, No growth

Rooting in ex vitro culture

Treatment of the stem cuttings with IBA at 1 mg/l for 30 min increased rooting response (Table 4) (Fig. 2e, f), while 0.25 mg/l of NAA resulted in high rooting response (Table 4). Rooting response was highest at these concentrations at 30 min in pulse treatment (15-120 min) (Table 5).

Table 3. Root induction in hypocotyl explants of *Canavalia cathartica* on MS medium supplemented with NAA and IAA in 6 weeks.

PGR (mg/l)		Number of explants	Number of explants formed roots	Root induction (%)
NAA	IAA			
0	0	30	10	33.33
0.5	0.5	40	10	25.00
1.0	1.0	80	50	62.50
1.5	1.5	60	30	50.00

Table 4. *Ex vitro* rooting of stem segments of *Canavalia cathartica* treated with IBA for 30 min (mean±SE).

PGR (mg/l)	Rooting (n=40)	Mean number of roots/plant	Mean root length (cm)
IBA			
0	08	6.5 ± 0.94 (n=8)	2.38 ± 0.21 (n=52)
0.25	0	0	0
0.50	08	4.00 ± 0.00 (n=8)	1.18 ± 0.06 (n=32)
1.00	32	11.22 ± 1.62 (n=32)	4.11 ± 0.01 (n=359)
2.50	25	10.32 ± 0.33 (n=25)	4.13 ± 0.14 (n=258)
5.00	0	0	0
7.50	0	0	0
NAA			
0	08	6.50 ± 0.94 (n=8)	2.38 ± 0.21 (n=52)
0.25	24	4.33 ± 0.43 (n=24)	1.61 ±0.13 (n=104)
0.50	0	0	0
1.00	24	3.67 ± 0.79 (n=24)	1.66 ± 0.10 (n=88)
2.50	23	3.00 ± 0.30 (n=23)	1.25 ± 0.08 (n=69)
5.00	16	3.00 ± 0.23 (n=16)	2.28 ± 0.27 (n=48)
7.50	16	1.50 ± 0.12 (n=16)	4.53 ± 0.67 (n=24)

Table 5. *Ex vitro* rooting of stem segments of *Canavalia cathartica* on pulse treatment with IBA and NAA (mean±SE).

PGR (mg/l)	Pulse treatment (min)	Rooting (n=40)	Mean number of roots/plant	Mean root length (cm)
Control	-	09	6.67 ± 0.89 (n=9)	2.27 ± 0.21 (n=60)
IBA (1.00)	15	16	4.50 ± 0.13 (n=16)	7.53 ± 0.53 (n=72)
	30	24	10.33 ± 1.62 (n=24)	3.81 ± 0.01 (n=248)
	60	08	2.00 ± 0.00 (n=8)	0.25 ± 0.003 (n=16)
	120	15	4.00 ± 0.00 (n=15)	2.33 ± 0.40 (n=60)
NAA (0.25)	15	12	3.08 ± 0.06 (n=12)	3.41 ± 0.11 (n=37)
	30	29	4.14 ± 0.62 (n=29)	1.75 ± 0.06 (n=120)
	60	09	2.00 ± 0.06 (n=9)	1.30 ± 0.10 (n=18)
	120	10	2.40 ± 2.12 (n=10)	0.45 ± 0.49 (n=24)

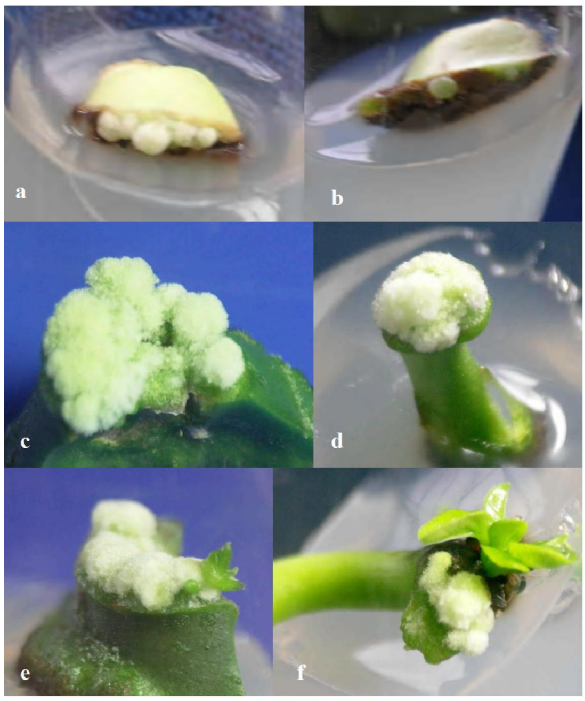

Fig. 1. Tissue induction in *Canavalia cathartica*: Cotyledon explant with white morphogenetic callus (a); Cotyledon explant with greenish morphogenetic callus (b); Cotyledon explant with profuse friable callus (c); Hypocotyl explant with white friable callus (d); Cotyledon callus with caulogenesis (e); Hypocotyle explant with callus and caulogenesis (f).

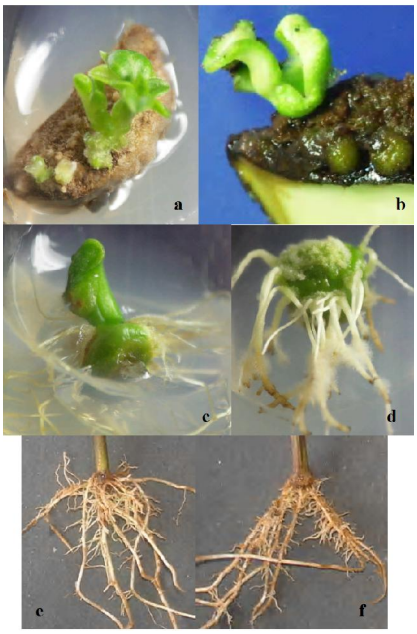

Fig. 2. Tissue induction in *Canavalia cathartica*: Cotyledon explant showing shoots in MS medium with 0.5 mg/l of BAP and 2iP (a); Somatic embryos on cotyledon callus (b); Hypocotyle with profuse branching in MS medium with 1.5 mg/l NAA and IAA (c); Hypocotyle with profuse roots and callus in MS medium with 1.5 mg/l NAA and IAA (d); *Ex vitro* rooting of juvenile shoot with 1 mg/l each of IBA and NAA treatment (e, f).

Discussion

The CSD are of great ecological and economic value as buffer zone to protect land, vegetation and agricultural properties (Maun and Baye, 1989; Martinez *et al*., 1997; Arun *et al*., 1999; Rao and Sherieff, 2002; Shareef, 2007). A wide range of economically valuable plant species (e.g. food, fodder, medicine and soil fertility) exists on the CSD of southwest coast of India (Seena and Sridhar, 2006; Sridhar and Bhagya, 2007; Sridhar, 2009). Many legumes on CSD are dependent on mutual association with microbes (e.g. rhizobia and arbuscular mycorrhizal fungi and endophytic fungi) (Seena and Sridhar, 2004, 2006; Sridhar, 2009; Arun and Sridhar, 2004). *Canavalia cathartica* has widely distributed in tropical Asia and Africa (Purseglove, 1974; Arun *et al*., 1999) including Seychelles, Japan, Papua New Guinea, Australia and Hawaiian Islands (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?310991).

Studies on micropropagation of CSD plant species are relatively scanty. However, some studies are available on *Ammophila arenaria, Cakile maritima, Ipomoea pes-caprae* and *Kosteletzkya virginica* (Cook *et al*., 1989; Kane *et al*.,

1993; Balestri *et al*., 2001). Advanced plant breeding methods along with the *in vitro* culture techniques have helped in improvement of the quality of grain legumes (Pawan and Rana, 2003). Although specific studies on tissue culture on CSD *Canavalia* spp. are not available, some investigations have been undertaken on *Canavalia* spp. distributed in other habitats (e.g. *Canavaliia brasiliensis, C. gladiata, C. lineata, C. virosa*) (Hwang *et al*., 1991; 1996a, 1996b; Ozaki, 1993; Kathiravan and Ignacimuthu, 1999; Da Silva *et al*., 2005). Continuous exposure of leaf callus of *C. lineata* showed chlorophyll in the presence of auxins (BAP and IAA) (Hwang *et al*., 1991; Hwang *et al*., 1996a). Leaf callus of *C. gladiata* on fortifying with NAA and BAP yielded plantlets (Ozaki, 1993). In *C. brasiliensis*, cotyledon explants produced callus on supplementing 2,4-D and KIN (Da Silva *et al*., 2005). Nodal explants of *C. virosa* fortified with BAP, GA_3 and KIN yielded multiple shoots, while IBA induced roots (Kathiravan and Ignacimuthu, 1999). Kathiravan and Ignacimuthu (1999) were successful in hardening and transplanting tissue cultured plants of *C. virosa*. Although *C. virosa* is closely related to *C. cathartica*, both shoots and roots were not induced although somatic emboryos have been established from the cotyledon explants in our study. However, attempts to propagate *C. cathartica* by *in vitro* and *ex vitro* cultures are partially successful. Cotyledon explants are suitable to induce callus employing BAP and 2iP within four weeks. Cotyledon and hypocotyl explants are suitable to induce friable calli. Within six weeks somatic embryos were formed in calli obtained from cotyledon and hypocotyl explants produced profuse roots. Friable calli produced by cotyledon and hypocotyl explants and roots produced by the hypocotyl explants might be useful in secondary metabolite production. The *ex vitro* culture with pulse treatment with IBA and NAA may be helpful in successful large-scale plantlet generation for commercial revegetatation.

Reports on the plant species exist on the CSD of Indian coast and their usefulness are scanty (Beena *et al*., 2001; Rao and Sherieff, 2002; Pattanaik *et al*., 2008). Sand mining, fishing and recreation in CSD are detrimental to vegetation of the southwest coast of India. Severe human interference (e.g. deliberate fires to clear vegetation for recreation, fishing and construction of concrete or granite walls) eliminates large patches of native CSD vegetation. The CSD vegetation should be documented and protected from human interference followed by identifying potential areas for revegetation with native flora. The seeds of *C. cathartica* on the CSD go through a long period of dormancy prior to initiation of germination. A large number of propagules can be produced and used for reintroduction in their native habitat without relying on seeds. Conventional methods of propagation of *Canavalia* spp. through seeds are not reliable due to high mortality of the seedlings under hostile

conditions prevail on the sand dunes. Thus, there is an urgent need to propagate these plant species using tissue culture techniques for large scale replantation. Several legumes of CSD have a potential nutraceutical value (Sridhar and Seena, 2006; Seena and Sridhar, 2006; Sridhar and Bhagya, 2007; Pattanaik *et al*., 2008). Besides, its propagation helps in improving soil fertility as green manure, cover crop and nitrogen fixation (Seena *et al*., 2007).

Overall, there are no reports on the vegetative propagation or *in vitro* regeneration of *Canavalia cathartica* of CSD. The present study attempted micropropagation of different explants *C. cathartica* using different methods, media with growth factors and culture conditions. *In vitro* and *ex vitro* micropropagation techniques could be employed to supplement the traditional propagation methods of plant breeding. Attempts are successful to initiate callus (cotyledon and hypocotyl), shoot bud induction (cotyledon), root formation (hypocotyl) and somatic embryogenesis (cotyledon) in different explants. This study also showed the possibilities of induction of friable calli for production of phytochemicals such as con A, canavanine and canaline from *C. cathartica*.

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