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Short Communication

In vitro REGENERATION OF *Cinnamomum tamala* Nees FROM COTYLEDONARY SEGMENTS

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ABSTRACT

***Cinnamomum tamala* Nees., a valuable spice-yielding plant of Himalaya, is vulnerable in its natural habitat due to its over-collection for spice and medicinal purpose. In the present study, a successful attempt was made for *in vitro* shoot bud induction from cotyledons and plant regeneration. The cultured cotyledons of 10-11 wk after flowering induced meristematic response within 4-5 wk of culture where ~43% explants responded positively on MS medium fortified with 3% sucrose, polyvinyl pyrollidone (100 mg L^{-1}) and 6 µM benzyl adenine wherein ~5-6 shoot buds developed cotyledon^{-1}. The micro-shoots/shoot buds, developed on initiation medium, were maintained on MS medium fortified with 3% sucrose and 3 µM kinetin where shoot buds converted into plantlets and supported culture proliferation. Shoots (4 cm long) with fully open leaves were rooted by maintaining on MS medium enriched with 3 µM α-naphthalene acetic acid where 5-6 roots developed per micro-shoot. The rooted plants were transferred to potting mix and maintained in agro-shade ca75% shading where ~65% transplants survived after two months of transfer.**

Key words: *Cinnamomum tamala*, cotyledonary segments, *in vitro* morphogenetic response, spice yielding plant

INTRODUCTION

In vitro propagation of high value trees through organogenesis has potential for rapid propagation of *elite* germplasm and to improve quality and impart uniformity in stock (Arumugam *et al.*, 2009; Deb and Arenmongla, 2011, 2012). Plant tissue culture techniques play important role in the conservation of threatened plant taxa. *In vitro* culture of plant cells and tissue has attracted considerable interest in recent days. Various *in vitro* techniques like somatic embryogenesis, organogenesis, etc. have been employed for the propagation of forest and other economically important tree species (Deb and Tandon, 2002; Souza *et al*., 2010). *In vitro* establishment of cultures are greatly affected by various factors like explants source and type, quality and quantity of nutrient regimes, plant growth regulators, etc. (Sharma and Nautiyal, 2009; Bell *et al*., 2009; Deb and Arenmongla, 2011, 2013).

Cinnamomum tamala is an evergreen spice yielding plant and also as a source of various '*Ayurvedic*' formulation (Deb *et al*., 2012). Owing to its high medicinal value and being an important ingredient of Indian spices, *C. tamala* demand is increasing day by day and the species is being exploited from its natural pockets and entered in 'vulnerable' category throughout in Himalayan range (Deb *et al*., 2012). In this communication authors describe the *in vitro* morphogenetic potential of cotyledonary segments of *C. tamala* for *in vitro* propagation.

MATERIALS AND METHODS

Immature seeds of various developmental stages (4 to 16 wk after flowering, WAF) were harvested randomly during the years 2010-2012 from the field grown *Cinnamomum tamala* growing in the Botanical garden, Department of Botany, Nagaland University. The freshly harvested fruits were soaked in citric acid solution (100 mg L^{-1}) till use. The seeds were surface sterilized with 0.2% (w/v) $HgCl_2$ for 5 min., followed by 4-5 washings with sterile water. The cotyledons without embryonic axis from these seeds were cultured on nutrient medium with different conjunct. For initiation of culture, MS medium (Murashige and Skoog, 1962) was used as nutrient medium and fortified with sucrose (0-4%, w/v), polyvinyl pyrollidone (PVP) [0-200 mg L^{-1} with an increment of 50 mg L^{-1} as antioxidant] and plant growth regulators [PGRs] like benzyl adenine (BA), kinetin (Kn) and thidiazuron (TDZ) [0-12 µM, used either singly or in combination]. The medium was gelled using agar (0.8%, w/v) and pH of the medium adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl. About 12 mL medium was dispensed in each borosilicate test tube (25 x 150 mm) and plugged with plastic caps. The medium was autoclaved at 121°C for 20 min. at 1.05 kg cm^{-2} pressure. The cotyledons were dissected from sterilized seeds without embryonic axis. A part of cotyledons were cut into two equal halves and the other half of cotyledons cultured intact on above medium. The experiment was laid in a completely randomized design with each treatment having 20 explants and replicated three times unless otherwise mentioned; the cultures were maintained at 25±1°C in cool with 12/12 hr (light/dark) photoperiod using fluorescent light at 40 µmol m^{-2} s^{-1} intensity. The cultures were sub-cultured at 4-5 wk interval. *In vitro* response was assessed on the basis of per cent explants responded and the number of propagules formed in culture after a specific time period. The data was expressed as the mean of replicate ± standard error.

The micro-shoots were developed from the cultured cotyledons/segments. Segments were maintained on optimum initiation medium for another two passages. The tiny shoots were then transferred on different strengths MS medium fortified with sucrose (0-4%), different PGRs such as BA and Kn (0-9 µM), either singly or in combination. The micro-shoots were separated at every sub-culture and transferred on fresh regeneration medium. Micro-shoots (3-4 cm long) with well expanded leaves from regeneration medium were rooted by transfer on MS medium enriched with 3% sucrose and NAA (0-8 µM) and maintained under normal laboratory conditions for 7-8 wk.

The rooted plantlets were hardened by maintaining them on half strength MS inorganic salts and full organic additives with 2% sucrose and under normal laboratory condition for 7-8 wk. The hardened plants were transplanted onto plastic pots containing a mixture of soil, sand, decayed wood powder in 1:1:1 ratio and topping with a moss layer. The potted plants were maintained in agro-shade with ca 75% shade for 7-8 wk before their transfer to the field.

RESULTS AND DISCUSSION

Culture initiation

In the present study *Cinnamomum tamala* cotyledons were collected from seeds of 4-16 WAF. *In vitro* morphogenetic response from cotyledons largely depended on the developmental age at which they were harvested and cultured on nutrient medium. Cotyledons up to 6 WAF did not show any morphogenetic response. The cotyledons of 7-9 WAF exhibited swelling but failed to form shoot buds. The meristematic loci formation followed by shoot buds formation was achieved from the cotyledons of 10-11 WAF. Morphogenetic response declined significantly beyond 12 WAF of age.

A key factor for successful *in vitro* culture initiation greatly depends on choosing the right developmental stage of fruits/seeds. The earliest stage at which the embryos/cotyledons could be

cultured successfully varies with genotype and local conditions. The *C. tamala* seeds reached maturity after 16 WAF, and cotyledons of 10-11 WAF supported optimum morphogenetic response (~44% explants responded positively and formed shoot buds). The cotyledons were cultured on MS medium fortified with various concentrations of PVP as antioxidant besides other adjuncts. Optimum response was achieved in medium supplemented with 100 mg L^{-1} PVP and 3% sucrose.

Table 1: Effect of PGRs on *in vitro* morphogenetic response of cotyledons of *C. tamala*

PGR conc. (µM)			Morphogenetic response*	No. of shoot bud primordia/ explants**	Morphogenetic response (%) (±SE)***
BA	Kn	TDZ			
0	0	0	Sw	-	09.2 (±0.6)^{g}
3	-	-	Sw-Sb	3.3 (±0.3)^{d}	22.5 (±1.5)^{e}
6	-	-	Sb	6.6 (±0.6)^{a}	43.2 (±1.4)^{a}
9	-	-	Sb	3.0 (±0.3)^{d}	16.6 (±1.2)^{f}
12	-	-	Sw-Ca-Sb	2.2 (±0.2)^{e}	14.2 (±0.7)^{f}
-	3	-	Sw-Sb	2.0 (±0.5)^{e}	38.0 (±1.7)^{c}
-	6	-	Sw-Sb	4.3 (±0.6)^{c}	40.6 (±1.3)^{b}
-	9	-	Sw	-	15.6 (±1.3)^{f}
-	12	-	-	-	-
-	-	3	Sw	-	16.2 (±0.7)^{f}
-	-	6	Sw-Sb	4.4 (±0.2)^{c}	33.3 (±1.6)^{d}
-	-	9	Sw-Ca-Sb	5.6 (±0.4)^{b}	44.5 (±1.7)^{a}
-	-	12	Sw-Sb	3.3 (±0.2)^{d}	22.2 (±1.2)^{e}
3	-	3	Sb	3.3 (±0.3)^{d}	34.2 (±1.0)^{d}
6	-	3	Sb	4.6 (±0.6)^{c}	41.0 (±1.5)^{b}
9	-	3	Sb	4.0 (±0.3)^{c}	36.6 (±1.2)^{c}
12	-	3	Sw-Sb	3.2 (±0.2)^{d}	26.3 (±1.6)^{e}
-	3	3	Sw-Sb	2.0 (±0.3)^{e}	23.2 (±1.8)^{e}
-	6	3	Sw	2.1 (±0.6)^{e}	22.0 (±1.5)^{e}
-	9	3	Sw	-	18.0 (±1.5)^{f}
-	12	3	Sw	-	20.2 (±1.2)^{e}

*Ca: callus, Sb: shoot buds, Sw: swelling; BA: benzyl adenine, Kn: kinetin, TDZ: thidiazuron; **on MS medium enriched with 3% sucrose, PVP (100 mg L^{-1}) & cotyledons of 10-11 WAF;

*** Standard error from mean [Data given is mean of 3 replicates; The figures followed by the same letter in the same column are at par at the threshold of 5% (Newman-Keuls± SE).

The intact cotyledons were found better over cotyledon segments. Within 2 wk of culture, the responding cotyledons/ segments started swelling and invoked meristematic loci (Fig. 1a,b), followed by shoot buds formation (Fig. 1b). The presence of cytokinin was found obligatory for morphogenetic response. Amongst the 3 PGRs tested, BA across the concentrations proved superior followed by TDZ and Kn (Table 1). Under a given set of conditions, optimum response was achieved on medium fortified with BA (6 µM) alone. Under this condition, ~43% cotyledons responded positively with 6.6 shoot buds invoked/ cotyledon, followed by TDZ (9 µM) where ~44% morphogenetic response was achieved with ~5.6 shoot buds formed/cotyledons. Incorporation of TDZ along with BA and Kn did not improve the morphogenetic response.

Many stimuli are communicated across the plant body by PGRs which consequently play an important role in diverse aspects of plant growth and development (Mereier *et al*., 2003; Deb and Arenmongla, 2012). At cellular level, cytokinins affect division, expansion and differentiation. Cytokinins are necessary in concerts with auxin in many cases for cell division at G1-S and G2-M transition in a variety of cultured plant cells as well as in plants. Progression through the cell cycle is central to cell proliferation and fundamental to growth and development of higher plants (Abhyankar and Reddy, 2007; Dhavala and Rathore, 2010).

Plant regeneration, rooting and potting of regenerates

The shoot buds developed from cultured cotyledons were maintained on MS medium fortified with sucrose (0-4%) and two different cytokinins (BA & Kn), either singly or in combination, for plant regeneration. The incorporation of sucrose was obligatory for plantlet regeneration and culture proliferation. The lower concentrations of sucrose delayed culture differentiation. At 1% sucrose differentiation started after 35 days of transfer on regeneration medium where ~1.4 shoot buds explants^{-1} developed. With increase in sucrose concentration, the culture differentiation and proliferation improved remarkably with optimum regeneration and shoot bud formation on MS medium containing 3% sucrose (Fig. 2). The carbon source serves as energy and osmotic agent to

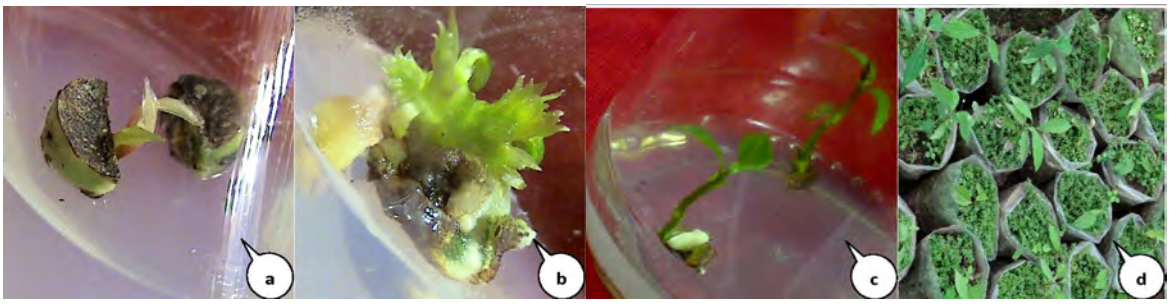

Fig. 1: Different stages of *in vitro* morphogenetic response of cotyledonary/segments of *Cinnamomum tamala*. a. Cultured cotyledon segments showing shoot buds formation; b. Multiple shoot buds formation from the intact cotyledon; c. Regenerated plantlets with fully opened leaves; d. Potted regenerates maintained in the agro-shade ready for field transfer.

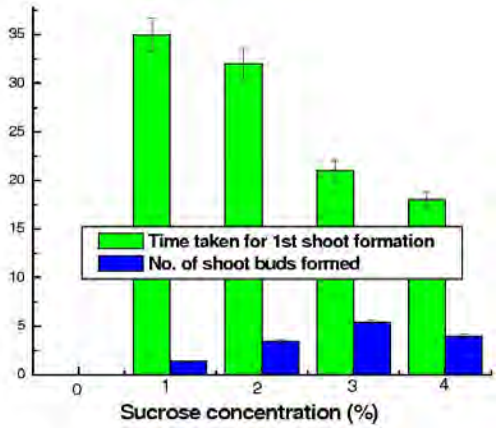

Fig. 2: Effect of sucrose on regeneration and culture proliferation of *C. tamala* (on medium fortified with optimum PGR)

support the growth of plant tissues. The process of *in vitro* culture establishment is a high energy requiring process that occurs at the expense of available metabolic substrates. In absence of organic carbonic source all the explants degenerated and optimum response was seen on medium containing 3% sucrose, while other concentrations did not support optimal culture initiation. Earlier the effect of organic organic carbon source on *in vitro* morphogenesis has been reported in *Stevia rebaudiana* (Preethi *et al.*, 2011) and in *Pogostemon cablin* (Swamy *et al.*, 2010).

For regeneration of plantlets, incorporation of any PGRs was obligatory. Of the 2 PGRs tested, BA in tested range did not support optimum culture proliferation (Table 2). BA tested singly @ 3 µM level supported 3 shoot buds formation after 8 wk of culture

Table 2: Effect of different cytokinins on plant regeneration and culture proliferation in *C. tamala*

PGR conc. (µM)#		Shoot buds	Mean plantlet	
BA	Kn	formed explants^{-1}	height (cm.)*	Type of response**
0	0	0	0	Growth stunted and degenerated
3	0	3.2^{b}	3.2 ± 0.1^{c}	Plantlets with dark green small leaves but plantlets etiolated
6	0	2.6^{c}	4.0 ± 0.1^{b}	Plantlets slightly light green and not healthy
9	0	2.2^{c}	3.6 ± 0.2^{b}	Small leaves and slight callusing at the base
0	3	5.3^{a}	4.5 ± 0.2^{a}	Healthy plantlets with dark green and broad leaves, plantlets with few roots
0	6	3.2^{b}	3.0 ± 0.3^{c}	Poor plant growth, yellowish-green leave
0	9	2.1^{c}	2.9 ± 0.2^{c}	Stunted plant growth
3	3	3.2^{b}	4.3 ± 0.1^{a}	Dark green leaves, stunted growth with fewer roots
3	6	3.3^{b}	3.5 ± 0.3^{b}	As above
3	9	3.2^{b}	3.6 ± 0.2^{b}	As above
6	3	3.4^{b}	3.0 ± 0.2^{c}	Stunted plant growth and leaves light green
6	6	2.3^{c}	2.7 ± 0.2^{d}	As above
6	9	3.2^{b}	3.2 ± 0.5^{c}	Many small leaves crowded at top, leaves light green
9	3	2.5^{c}	2.7 ± 0.1^{d}	Broad green leaves, healthy plantlets
9	6	3.2^{b}	2.5 ± 0.2^{d}	As above
9	9	2.3^{c}	2.9 ± 0.2^{c}	Leaves light green and plantlets unhealthy

#Only significant treatments are computed; *Standard error from mean; Data scored after 8 wk of culture on regeneration medium; **On MS medium, supplemented with 2% sucrose, PVP (100 mg L^{-1}); The figures in the same column followed by same letter are statistically identical to the threshold of 5% (Newman-Keuls±SE).

Table 3: NAA stimulated *in vitro* rooting of micro shoots of *Cinnamomum tamala*

NAA conc. (µM)	No. of roots formed plantlet^{-1}	No. of secondary shoots formed/shoot	Type of response*
0	3^{d}	-	Roots were very small and degenerated.
1	4^{c}	2	Plantlets etiolated, roots short.
2	5^{b}	3	Slightly etiolated plantlets, shoots branched but short
3	6^{a}	3	Healthy plants with profuse rooting with distinct root hairs
4	4^{c}	2	Healthy roots but poor root hairs
5	4^{c}	2	Swelling at the basal part of plants as well as roots.
6	3^{d}	-	As above.
7	2^{e}	-	Roots swelled and callusing at the base of the shoot
8	2^{e}	-	As above

*On MS medium containing sucrose (3%, w/v); Data represents the mean of three replicates; Data scored after six wk of culture on the above medium;, The values followed by same letter in the same column are statistically identical to the threshold of 5% (Newman-Keuls)

culture where plant height was stunted (~3.2 cm). The incorporation of Kn alone in medium proved superior in shoot proliferation and in improving plant height with 4-5 shoot buds formed with average plant height of ~4.5 cm. Similar effectiveness of cytokinin on plant regeneration and culture proliferation has been reported by Baskaran *et al*. (2009). In *Acacia confuse*, BA, NAA and Kn in combination produced maximum shoot buds, as many as 25 shoot buds developed in culture (Arumugam *et al*., 2009).

The regenerated plants with fully opened leaves were selected for rooting (Fig. 1c). The shoots were treated differently for inducing roots. Under optimum, ~5-6 roots were formed after ~6 wk of culture on MS medium fortified with 3 µM NAA (Table 3). The role of auxins in rooting of *in vitro* raised micro-shoots have been shown in apple (Moncousin *et al*., 1992), *Quercus suber* (Manzanera and Pardos, 1990) and *Strobilanthes flaccidifolius* (Deb and Arenmongla, 2012). Auxins act as a local morphogenetic trigger in the formation of lateral roots in *Arabidopsis*, leading to the specification of founder cells of new organ from previous differentiated cells (Dubrovsky *et al*., 2008). The promotory effect of NAA on rooting is also described in *Populus euphratica* (Ferreira *et al*., 2009) where it was reported that NAA was better followed by IAA and IBA.

The rooted plants were hardened before transferring to potting mix as described in methods. The transplants were successfully transferred to an agro-shade and then to field. About 65% transplants survived after two months of transfer (Fig. 1d). The protocol developed in the present study open newer route for *in vitro* propagation of this important spice yielding species.

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