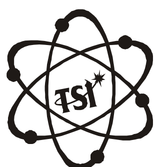
ISSN : 0974 - 7435 Volume 6 Issue 10



Trade Science Inc.

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 6(10), 2012 [295-298]

Induction of callus and cell suspension cultures of three *Callistemon* species

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ABSTRACT

Several *Callistemon* species (bottle brush plants; Myrtaceae) are well known for use in folk medicine. Explants of *C. viridiflorous* (*Cv*), *C. comboynensis* (*Cc*), and *C. lanceolatus* (*Cl*) were placed for callus induction on MS medium with different combinations of phytohormones. A mixture of NAA and kinetin was the optimum combination for establishing calli of *Cv*, *Cc*, and *Cl*. In case of *Cl* callus induction, BAP in combination with NAA and kinetin in combination with 2,4D may be used. For induction of cell cultures, aliquots of *Cv*, *Cc*, and *Cl* callus material were collected in the active growth phase and transferred to liquid MS medium, supplemented with 0.9 mg·L^{-1} kinetin and 1.1 mg·L^{-1} NAA.

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KEYWORDS

Callistemon;
Indole acetic acid (IAA);
Benzyl amino purine (BAP);
Kinetin (K);
2,4-dichlorophenoxy acetic acid (2,4-D).

INTRODUCTION

The genus *Callistemon* is a shrub belonging to the family Myrtaceae. It contains 34 species of beautiful evergreen shrubs and small trees. The majority of the *Callistemon* species is endemic to the more temperate regions[1]. A synonym of this genus is *Melaluca*[2,3]. The species are commonly known as bottle brushes because of their cylindrical brush-like flowers resembling the traditional bottle brush.

Some species of this genus are used as a tea substitute and have a refreshing flavor[4]. In folk medicine, the genus *Callistemon* is known for its anticough, antibronchitis, insecticidal, antifungal, antibacterial, antiinflammatory, analgesic, antinociceptive, anticonvulsant and antidiabetic effects[5-7]. Phytochemical investigations of members of the genus resulted in the identification of *C*-methyl flavonoids, flavonol glycosides, phenolic acids, hydrolysable ellagitannins, triterpenoids, and phloroglucinol derivatives[8-11]. Due to over-exploitation for their volatile oil and secondary metabolites, there is a great need to develop alternative strategies of conservation and industrial production of the bioactive compounds from these plants. *In vitro* cultures have the potential to form secondary metabolites and to exhibit bioactivity comparable to the original plant. Cultured cells may serve industrial purposes, e.g. by immobilization in a matrix for use in bioreactors. Besides the ge-

netic potential of the donor plant for callus induction and growth in *in vitro* cultures, a medium containing sufficient nutrients, such as the preferred MS medium, is required[12].

MATERIALS AND METHODS

Medium and phytohormones

Murashige Skoog (MS) medium[13] was used as artificial medium for *in vitro* tissue cultivation. MS supplemented with sucrose (30 g.L^{-1}) and agar (8 g.L^{-1}) at pH 5.8 was used for *in vitro* propagation of *Cv, Cc* and *Cl*. Phytohormones like 1-naphthyl acetic acid (NAA), 6-benzyl aminopurine (BAP), kinetin (K) and dichlorophenoxy acetic acid (2.4-D) were used for callus induction. The various hormone combinations used for the study of callogenic response are given in TABLE 1.

Preparation of sterile *Cc* seedlings

Seeds were sterilized for 5 minutes in 70% ethanol followed by centrifugation. They then were incubated for 5 minutes in 6% sodium hypochlorite/0.1% Tween 20, washed twice with autoclaved distilled water, and spread as a suspension with sterile solution of 8% sucrose in water with 0.8% agar in Petri dishes. Seeds may exhibit dormancy which can be overcome by exposure to low temperature for a certain time[14]. After exposure to the cold for two days, the seeds were incubated in the dark at 25°C for 48 hours and afterwards under light-dark regime of 16 h light/8 h dark at 25°C. Ten-day-old seedlings were transferred to MS medium (without phytohormones).

Establishment of tissue cultures of *Cv, Cc* and *Cl*

Successful callus regeneration protocols for explants from *C. lanceolatus*, *C. rigidus,* and *C. viminalis* were reported by Paul *et al*. (2010), Lin *et al*. (2005), Cheng *et al*. (2007), and Shipton (1982)[15-18]. We tried here to simulate the same circumferences and applied similar conditions with some modifications to optimize the regeneration of *Cv* and *Cc* in addition of *Cl* as control for callus induction. Different concentrations of various phytohormones with different combinations were tested (TABLE 1). Entire leaves of *Cv* and *Cl* were washed for 2 minutes in 70% ethanol followed by 3 minutes in 6 % sodium hypochlorite/0.1% Tween 20[14]. The explants were washed thoroughly twice with distilled sterile water. Sterile leaves of *Cv* was injured or cut into 0.5 cm pieces. Leaves of *Cl* were cut into 1 cm pieces. Ten-day-old seedlings of *Cc* were injured. Explants were then aseptically inoculated in a 15x100 mm Petri dish containing about 25 ml of solid callus induction medium (MS medium) supplemented with different concentrations of phytohormones (TABLE 1). The dishes were sealed with Parafilm and kept in the dark for the first week at 25°C, then under 16 h light/8 h dark at 25°C for 2-3 weeks to induce callus formation.

RESULTS AND DISCUSSION

The composition of the culture medium is considered to be the most important factor governing growth and development of plant material. Plant tissue and cell culture media are generally made up of some or all of the following components: macroelements, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar (s) as carbon source, other organic supplements, growth regulators (phytohormones), and solidifying agents or supporting systems. The most critical factor for successful callus or cell growth and differentiation is the level of phytohormones. Four classes of growth regulators have been reported to be crucial in plant tissue culture: auxins, cytokinins, gibberellins, and abscisic acid[19-24].

An effective and reproducible procedure for regeneration of plants from cell and tissue culture is essential for studies involving determination of active constituents, analysis of biosynthetic pathways, and transfer of genes. Thus, the first step is establishment of a suitable protocol for generating tissue cultures of *Cv, Cc*, and *Cl*. Here, establishment of callus cultures of these three species is demonstrated. The response of explants was found to depend on the type of culture medium employed (TABLE 1).

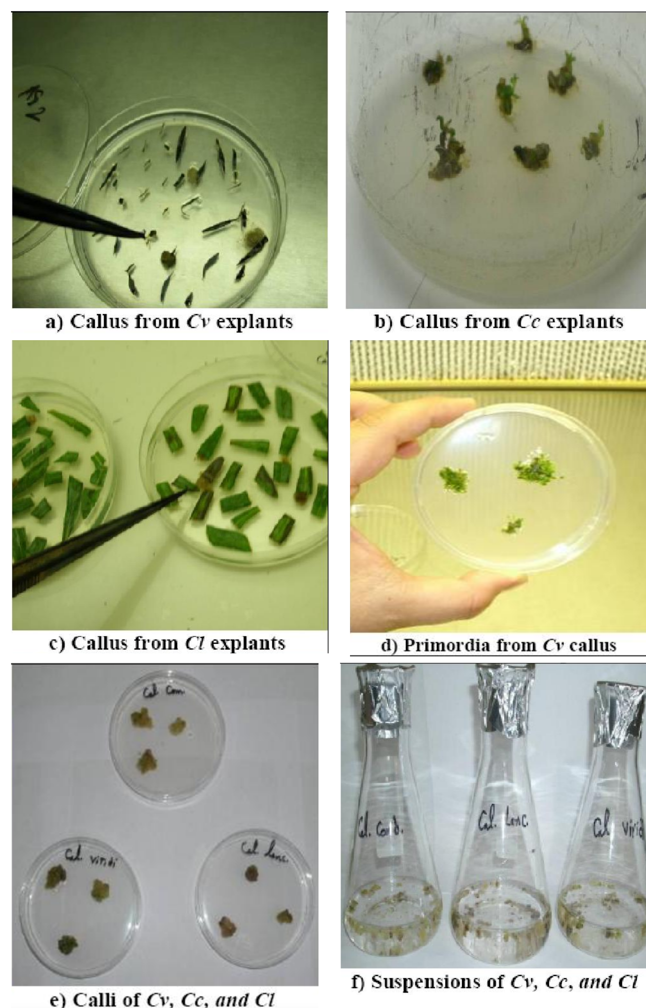
Calli from leaf explants of *Cv, Cc*, and *Cl* were obtained 3-4 weeks after explants were brought into contact with sterile solid nutrient medium for induction. The growth of callus tissue was visible at excision and injured sites (Figure 1 a-c). Numerous shoot primordia emerged from the callus of *Cv* within the next three weeks in M2 medium (Figure 1d). The media M2, M12,

M13, and M15 induced green callus of *Cv*. While M2, M5, M12, M13, M17, and M19 induced pale green callus of *Cl*, M13 and M17 did so for *Cc*. Callus proliferation started from the cut ends of leaf lamina of *Cv* and *Cl* and from injured seedlings of *Cc* on MS medium enriched with 0.5-1.5 mg·L^{-1} kinetin and 0.5-1.1 mg·L^{-1} NAA. Callus induction was found to be best in medium supplemented with 0.5-1.1 mg·L^{-1} NAA (TABLE 1). BAP 0.5 mg·L^{-1} in combination with NAA 0.5 mg·L^{-1} and kinetin 1 mg.L^{-1} kinetin in combination with 2,4D 0.5 mg.L^{-1} could also induce callus from explants of *Cl* (TABLE 1). The data of our study well agree with previous observations made with the establishment of calli from *C. lanceolatus, C. rigidus* and *C. viminalis*[15-18].

TABLE 1 : Concentrations of phytohormones used in media for callus induction (a) and growth of calli from *Cv, Cc*, and *Cl* explants (b)

Medium (mg·L^{-1})	a				b		
	NAA	BAP	Kinetin	2,4 D	*Cv*	*Cc*	*Cl*
M0	-	-	-	-			
M1	0.5						
M2	0.5		0.5		+		+
M3	0.1	1					
M4	0.05	0.5					
M5	0.5	0.5					+
M6	0.2	1					
M7	1	2.5		0.5			
M8		2.5		0.02			
M9		1		0.02			
M10		1		0.5			
M11	0.5		2				
M12	0.5		0.6		+		+
M13	1.1		0.9		+	+	+
M14	3.5		0.9				
M15	0.5		1.5		+		
M16	0.5		2				
M17	1		0.5			+	+
M18			0.5	0.5			
M19			1	0.5			+
M20		1	0.9				

Cell suspension cultures derived from callus lead to a decrease in the generation time and an increase in the metabolic production. Innovative biotechnologies in plant cell and tissue culturing and latest achievements in metabolic engineering for improving production sustainability and efficiency of plant-derived pharmaceuticals are highly needed for industrial production of secondary metabolites in the future. Our studies provide the opportunity of carrying out research on the biosynthesis of secondary metabolites of *Cl, Cv*, and *Cc*. As documented in TABLE 1 and Figure 1e, the hormone combination of 1.1 mg·L^{-1} NAA and 0.9 mg·L^{-1} kinetin (medium M13) was the optimum medium for induction of calli of the three *Callistemon* species. Callus aliquots were therefore collected in the active growth phase (after the 15th day of subculture) and placed in liquid MS medium supplemented with 0.9 mg·L^{-1} kinetin in combination with 1.1 mg·L^{-1} NAA (medium M13). The resulting fresh cell cultures of the three *Callistemon* species (Figure 1f) were incubated on a horizontal shaker at 100 rpm and 25°C for 21



a) Callus from *Cv* explants b) Callus from *Cc* explants c) Callus from *Cl* explants d) Primordia from *Cv* callus e) Calli of *Cv, Cc, and Cl* f) Suspensions of *Cv, Cc, and Cl*

Figure 1 : *In vitro* cultures of *C. viridiflorous* (*Cv*), *C. comboynensis* (*Cc*), and *C. lanceolatus* (*Cl*)

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days, followed by studies of phenolics and their antioxidant activity, reported in a previous publication[25].

ACKNOWLEDGEMENT

Part of this research work was funded by the DFG (Deutsche Forschungs Gemeinschaft).

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