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In vitro propagation and cell suspension culture of *Callistemon citrinus* L.

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Abstract

The bottle brush tree, *Callistemon citrinus* is a shrub that is nearing extinction. An attempt to revive this plant and propagate it *in vitro* has been performed for large scale production. Calli cultured on MS medium with different combinations of cytokinins and auxins *viz*. 1.0 mg/l Kinetin along with 1.0 mg/l IAA; 1.5 mg/l Kinetin along with 1.0 mg/l IAA; 1.0 mg/l Kinetin along with 1.0 mg/l IBA, and 2.0 mg/l Kinetin along with 1.5 mg/l IBA. All combinations were very effective in inducing callus. 0.5 mg/l kinetin along with 0.5 mg/l IAA was very effective for shoot regeneration and shoot elongation. The developed shoots when transferred to MS medium, supplemented with 1.5mg/l IBA, shows root development within a week. Axillary bud proliferation is also observed in the explants inoculated in MS medium supplemented with combinations of kinetin and IAA.

Keywords: *Callistemon citrinus*, Indole Acetic Acid (IAA), Indole Butyric Acid ((IBA), Benzyl amino purine(BAP).

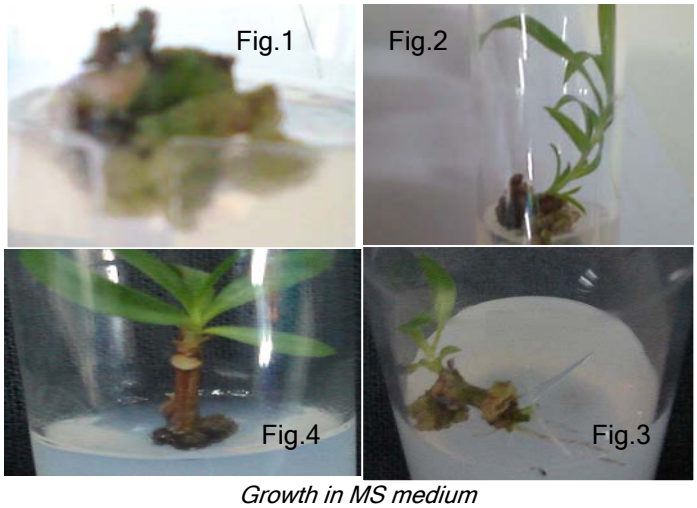
Introduction

The bottle brush tree, *Callistemon citrinus* is a shrub belonging to the family, Myrtaceae. The synonym of the plant is *C. lanceolatus.* Due to the over exploitation for its volatile oils and secondary metabolites, there is a need to develop alternate strategies for conservation and industrial production of bioactive compounds from this plant (Vogler *et al.,* 1998). The leaf of the plant is used as a tea substitute and it has a refreshing flavor too. The standardized oil from the leaves of *C. citrinus* has been proved to have anti-nociceptive and anti-inflammatory effects in experimental animals (Sudhakar *et al*., 2004). The oil of *C. citrinus* stimulates the analgesic activity with aspirin and pentazocine. The oil reduces the paw volume in case of paw edema. Many phenolic compounds and cross reactive allergenic components have been identified in this plant (Stanaland *et al*., 1986; Mahmoud *et al*., 2002). The essential oils from *C. citrinus* exhibit higher activity than the synthetic antibiotics like miconazole and clotrimazole (Brophy *et al*., 1998; Sharma *et al*., 2006). The plant has been proved to exhibit anti-candidal activity (Dutta *et al*., 2007). Studies have also proved that the methanol extract of the plant has anti-thrombin activity (Chistokhodova *et al*., 2002).

C. citrinus has a phytotoxin (leptospermone) belongs to the family of β-triketones and acts as a natural herbicide (Vogler *et al.,* 1998). This plant is usually propagated through seeds and cuttings; meanwhile, tissue culture technique has also been attempted. The most suitable medium for callus induction of *C. citrinus* is Murashige and Skoog medium (MS), supplemented with 5.0 mg/l of 6- BAP, 4.0 mg/l of Kinetin, 2.0 mg/l of IBA and 30g/l of sucrose (Lin Che *et al*., 2005).

We report the *in vitro* propagation of this plant using differential combinations of hormones. In order to revive and mass multiply, a protocol has been devised to produce callus from the explants. Moreover, an attempt has also been made to stimulate the axillary bud proliferation and initiate the shoot and root regeneration from the callus.

Fig.1. Callus development with 2 mg/l kinetin & 1.5 mg/l IAA;
Fig.2. Shoot regeneration with 1 mg/l kinetin & 1 mg/l IBA;
Fig.3. Root regeneration with 1.5 mg/l IBA;
Fig.4. Axillary bud proliferation with 2 mg/l kinetin & 1 mg/l IAA



Growth in MS medium

Materials and Methods

Medium and hormone preparation

MS supplemented with sucrose (30g/l) and agar (8g/l) at pH 5.8 was used for the *in vitro* propagation of *C. citrinus.* The various hormone combinations used for the study of callogenic response are given in the Table 1.

Surface sterilization of explants

The explants (leaf, stem, axillary bud) were washed thoroughly with tap water for 10 min, then washed with two drops of tween- 20 followed by a wash with distilled water for 10 min. The leaves were

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then cut into small pieces of 1.0 cm^2. The stems and axillary buds were cut to 1 cm in height. These explants were treated with 70% (v/v) ethanol for 20 sec. The leaves were then washed with 0.1% (w/v) mercuric chloride for 15- 20 sec, then 35- 40 sec for stems and finally, 50 sec min^{-1} for axillary buds. The explants were washed thoroughly thrice with distilled water and wiped dry with sterile Whatmann no.1 filter paper prior to inoculation.

Inoculation

The medium was autoclaved at 1.06 kg/cm^2 at a temperature of 121°C for 15 min. The required quantity of filter sterilized hormones was amended with medium kept in culture tubes. The surface sterilized explants were aseptically inoculated on the medium kept in the culture test tubes and incubated at 25±1°C with 3000 lux intensity cool white light region. After the development of shoot, the shoots were excised into single shoot and transferred to MS medium supplemented with 1.5 mg/l IBA for rooting.

Cell suspension culture

Approximately 0.1 g of callus, which still in its active growth phase (i.e., after the 15th day of subculture), was placed in 250 ml flasks containing 100 ml liquid MS medium supplemented with 1.0 mg/l kinetin and 1.0 mg/l Indole acetic acid (IAA). Cultures were incubated in 25°C in a horizontal shaker at 100 rpm for 21 days. Growth of cells were then determined by measuring absorbance at 600 nm, fresh weight, dry weight and hemocytometer reading were taken at every 3 days.

Number of generations, $n= (\log X- \log X_0) \times 3.32$

Where, X represents number of cells/ml at the end of log phase and Xo represents that at the start. Multiplication rate, r (generations/ hour), $r= n/ (t_1 - t_0)$. Generation time, g (hours) =1/r. Specific growth rate, μ ($hour^{-1}$) = 0.693/ g.

Table 1. List of hormone combinations used for callus induction, axillary bud proliferation and shoot regeneration in MS medium

Hormone combinations	Conc. (mg/l)	S. No	Hormone combinations	Conc. (mg/l)
K + IAA	0.5/0.5	23	K+ IBA	2.0/1.5
K + IAA	0.6/0.5	24	K+ IBA	2.0/2.0
K+ IAA	0.7/0.6	25	K+ 2,4-D	0.5/0.5
K+ IAA	0.9/0.5	26	K+ 2,4-D	1.0/0.5
K+ IAA	0.9/1.1	27	K+ 2,4-D	1.0/1.0
K+ IAA	0.9/1.8	28	K+ 2,4-D	1.5/0.5
K+ IAA	0.9/3.5	29	K+ 2,4-D	1.5/1.0
K+ IAA	1.0/1.0	30	K+ 2,4-D	2.0/0.5
K+ IAA	1.0/0.5	31	K+ 2,4-D	2.0/1.0
K+ IAA	1.5/0.5	32	K+ 2,4-D	2.0/1.5
K+ IAA	2.0/0.5	33	K+ 2,4-D	2.0/2.0
K+ IAA	2.0/1.0	34	BAP + IAA	0.5/0.5
K+ IAA	2.0/1.5	35	BAP + IAA	0.6/0.5
K+ IAA	2.0/2.0	36	BAP + IAA	0.7/0.5
K+ IAA	1.5/1.0	37	BAP + IAA	0.9/0.5
K+ IBA	0.5/0.5	38	BAP + IAA	0.9/1.1
K+ IBA	1.0/0.5	39	BAP + IAA	0.9/1.8
K+ IBA	1.0/1.0	40	BAP + IAA	0.9/3.5
K+ IBA	1.5/0.5	41	BAP + IAA	1.0/1.0
K+ IBA	1.5/1.0	42	BAP + IAA	2.0/1.0
K+ IBA	2.0/0.5	43	BAP + IBA	3.0/1.0
K+ IBA	2.0/1.0	44	BAP + IBA	4.0/2.0

Table 2. Hormone combinations for callus induction in MS medium (Callus initiation observed after 3 weeks)

Hormone combinations	Conc. (mg/l)	S. No	Hormone combinations	Conc. (mg/l)
K+ IAA	0.5/0.5	16	K+ IBA	1.5/1.0
K+ IAA	0.6/0.5	17	K+ IBA	2.0/0.5
K+ IAA	0.7/0.5	18	K+ IBA	2.0/1.0
K+ IAA	0.9/1.1	19	K+ IBA	2.0/1.5
K+ IAA	0.9/1.8	20	K+ IBA	2.0/2.0
K+ IAA	0.9/3.5	21	K+ 2, 4-D	0.5/0.5
K+ IAA	1.0/1.0	22	K+ 2, 4-D	1.0/0.5
K+ IAA	1.5/0.5	23	K+ 2, 4-D	1.5/1.0
K+ IAA	1.5/1.0	24	K+ 2, 4-D	2.0/1.5
K+ IAA	2.0/0.5	25	BAP + IAA	0.5/0.5
K+ IAA	2.0/1.0	26	BAP + IAA	0.7/0.5
K+ IAA	2.0/1.5	27	BAP + IAA	0.9/0.5
K+ IAA	2.0/2.0	28	BAP + IAA	1.0/1.0
K+ IBA	1.0/0.5	29	BAP + IBA	4.0/2.0
K+ IBA	1.0/1.0			

Results and discussion

Callus induction

Surface sterilized explants on induction gave callus after a period of 3 weeks. The various hormone combinations (Table 1) that gave callus induction are listed in Table 2. Among the hormone combinations, 1.0 mg/l kinetin along with 1.0 mg/l IAA, 1.5 mg/l kinetin along with 1.0 mg/l IAA, 1.0 mg/l kinetin and 1.0 mg/l IBA, and 2.0 mg/l kinetin along with 1.5 mg/l IBA were highly effective in inducing callus (Fig. 1). Callus was found to be fragile and white in colour initially and as the incubation days increased the callus colour changed to pale white colour and become little hardened.

Shoot regeneration

Shoot was induced from the callus (Fig. 2). The hormone combinations that initiated shoot are presented in Table 3. The increase in the length of the shoot system as well as the number of leaves was monitored during this period. It was observed that 0.5 mg/l kinetin along with 0.5 mg/l IAA was very effective for shoot regeneration and shoot elongation. There was a geometrical increment in the length and number of leaves (from 2 to 8), within a period of 3 months in the developed shoots.

Root induction

The developed shoots were transferred to MS medium supplemented with 1.5 mg/l IBA. Root induction was initiated within a period of one week

(Fig. 3). The total number of roots obtained was five and few secondary root initiations were obtained during the period of three months.

Axillary bud proliferation

Axillary bud proliferation was effective with different combinations of kinetin (K) and IAA (Table 4, Fig. 4).

Cell suspension culture

When callus was placed in liquid culture they disperse easily into clumps of 0.5-5.0 mm. Further

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agitation leads to fragmentation of these clumps into small cell aggregates. The parameters that are associated with cell growth are presented in Table 5. The growth curve of cell suspension culture derived from callus exhibited three different stages: (i) lag phase 0-6th day, (ii) log phase (6th -18th day) and (iii) stationary phase (18th -24th day) (Fig. 5). The doubling time is substantially reduced in cell suspension cultures.

Table 3. List of hormone combinations that induced shoot in MS medim

Hormone combinations	Conc. (mg/l)	Period for initiation	Shoot length* (cm)
K+ IAA	0.5/0.5	1 month	2.1 ± 0.1
K+ IAA	1.0/0.5	4 months	0.3 ± 0.1
K+ IAA	1.5/0.5	4 months	0.9 ± 0.1
K+ IAA	2.0/1.5	1 month	1.7 ± 0.2
K+ IBA	1.0/0.5	4 months	0.4 ± 0.1
K+ IBA	1.0/1.0	2 months	3.5 ± 0.2

**Shoot length measured after 4 months of inoculation of explants in MS medium*

Discussion

The present research work is a first attempt towards the micropropagation of *C. citrinus* when compared with other species like *C. viminalis* and *C. rigidus*. For the induction of callus, it was observed that kinetin along with IAA and Kinetin along with IBA showed significant results ie. 0.5 mg/l kinetin along with 0.5 mg/l IAA; 1.0 mg/l kinetin and 1.0 mg/l IBA when compared with hormone combinations for callus induction from *C. viminalis* (Lin che *et al*., 2005). In case of *C. rigidus* BAP (1.5 mg/l) and NAA (0.5 mg/l) was found to be the best suitable hormone combination for callus induction when compared to kinetin and IAA in *C. citrinus* (Cheng hou *et al*., 2007). Effective Auxillary bud proliferation was obtained with the hormone combinations of Kinetin and IAA similar to callus induction when compared to BAP in *Callistemon rigidus* (Lin che *et al*., 2005).

Table 4. List of hormone combinations that induced axillary bud proliferation in MS medium

Hormone combinations	Conc. (mg/l)	Period for initiation	Shoot length*(cm)
K+ IAA	0.5/0.5	1 month	0.7± 0.2
K+ IAA	1.5/1.0	1 month	0.5 ± 0.1
K+ IAA	2.0/1.0	1 month	1.5 ± 0.1
BAP+ IAA	4.0/2.0	1 month	1.2 ± 0.1

**Shoot length measured after 1 month of inoculation of explants in MS medium*

Leptospermone is considered as an unusual cyclic sesquiterpene present in *C. citrinus* (Vogler *et al.,* 1998).The present study on cells suspension culture of *C. citrinus* uoens the possibility of production of the secondary metabolite leptospermone under *in vitro* conditions for industrial production (Ying Wang, 2008). Cell suspension cultures from the callus led to a decrease in the generation time and an increase in the metabolic production. Innovative biotechnologies in plant cell and tissue cultures, and the latest achievements in metabolic engineering to improve the production sustainability and efficiency of plant derived pharmaceuticals is highly needed for industrial production of secondary metabolites in future. This is a first attempt to study on cell suspension culture of *C. citrinus* for secondary metabolite (Leptospermone) production. This has provided a unique opportunity for further research in different aspects on the biosynthesis of secondary metabolites of *C. citrinus*.

Table 5. Growth parameters of cell suspension culture

Parameters	Value
Generations, n	4
Multiplication rate, r	0.016825 ± 0.0017 generations hour-1
Generation time	60 ± 6 hours
Specific growth rate	0.0114 ± 0.0009 hour-1

0.1 g callus inoculated in 100 ml liquid MS medium

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