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In vitro callus formation and cell line selection for production of terpenoid compounds in *Cymbopogon citratus* (DC.) Stapf

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Abstract
Callus induction was performed using young leaf base of *Cymbopogon citratus* (DC.) Stapf and was grown on MS media. Shoot and root regeneration with different hormones were tried like 2, 4-D (0.5-2 mg/L), BAP (0.5-1.0 mg/L), NAA (0.5-1.0 mg/L) and Kn (0.5-1.5 mg/L) in MS media. Methyl Jasmonate (MeJA) is used as a major elicitor for manipulating defense pathways which modifies the production of terpenoids by activating genes controlling the secondary metabolic pathways was given in varied concentrations (25, 50 and 100 µM/L). Later the analysis of secondary compound production was done using GC-MS which showed Citral as the main active component elicited in the cell culture. The percentage of Citral (50%) produced was higher in cell culture treated with 50 µM/L of MeJA.

Keywords: *cymbopogon citratus*, callus formation, cell line selection, MeJA, citral

1. Introduction

The genus *Cymbopogon* is a major aromatic plant genera belonging to the tribe Andropogoneae of the family Poaceae. The Cymbopogons are aromatic and medicinal grasses comprising about 180 species, subspecies, varieties and sub varieties (Bor, 1960; Chase & Niles, 1962; Soenarko, 1977) [1, 2, 3]. *Cymbopogon citratus* (DC.) Stapf is known to have economic importance and commonly called as western Indian lemongrass or lemongrass.

Modern biotechnological approaches like cell suspension culture serve as feasible technique for the production of secondary metabolite in economically important species (Yadav *et al*., 2000; Zheng *et al.*, 2007) [4, 5].

The *in vitro* propagation technique offer superiority over conventional method because of the production of high rate of viable callus, used for cell culture. The technique also exhibit advantage for studying elicitor-induced cell culture response, which is otherwise difficult to perform in intact plant (Honee *et al.*, 1998; Steven *et al.*, 2000; Stella and Braga, 2002) [6]. Thus, the technique of cell culture has been widely employed as a model system to investigate the production of specific secondary metabolites with scale-up potential (Buitelaar and Trapmer, 1992; Chang and Sim, 1995; Mukherjee *et al.*, 2000) [7].

Callus is an unorganized proliferative mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient medium in glass vials under controlled experimental conditions. Among the agronomic inputs, plant growth regulators have been found to favorably influence the recovery, yield and quality of essential oil, such as in Cymbopogon (Mishra and Srivastava, 1991) [8].

The biotechnological production of valuable secondary metabolites in plant cell culture is an attractive alternative to extraction from whole plant material. Although cell cultures have been established from many species, often they do not produce sufficient amount of required secondary metabolite. In such cases, the production of secondary metabolites can be enhanced by treating undifferentiated cells with abiotic or biotic elicitors such as methyl jasmonate, salicylic acid and heavy metals (Poulev, 2003) [9]. The accumulation of secondary metabolites in plants is a part of defense response against pathogenic attack, getting triggered and activated by elicitors, the signal compounds of plant defense responses (Zhao et al., 2005) [10].

Metabolic engineering of secondary metabolite require thorough knowledge of the whole biosynthetic pathway and detailed understanding of the regulatory mechanism controlling onset and flux of the pathway. Recent advances in metabolic engineering of microorganisms producing both native and heterologous secondary metabolites has allowed higher levels of production and also directed the synthesis of desired molecules (Kirsi-Marja Oksman-Caldentey and Dirk Inze, 2004) [11].

The elicitors are compounds activating the chemical defense in plants (Meenakshi and Baldev, 2013) [12]. Various biosynthetic pathways are activated in response to the elicitors used in culture. The most intensively studied elicitor for manipulating defense pathway in plants is Methyl Jasmonate (MeJA). MeJA modifies the production of terpenoids by activating genes controlling the secondary metabolic pathway.

Terpenes are chemical groups present in essential oil, which include aliphatic hydrocarbons (non-oxygenated) and terpenoids (oxygenated components or oxygenated hydrocarbons), a mixture of isomeric hydrocarbons of molecular formula $C_{10}H_{16}$, which represent the oldest group of small molecular products synthesized by plants and are probably the most widespread group of natural products. Developmentally, the rate of oil accumulation varies in Cymbopogons with leaf age and oil content. In view with the importance of *C. citratus* essential oil and their components in aromatic and pharmaceutical industries, an attempt was made to obtain the cell line for secondary metabolite production under suspension cultures.

2. Materials and methods

2.1 *In vitro* culture

2.1.1 Callus induction

Young leaf base (2-1.5 cm) of *C. citratus* was used as explant source. Explants were surface sterilized, disinfected with Mercuric chloride (0.01%) for 10 min and immersed in potassium permanganate soln (50mg/L) for 20-30min. The meristematic region of sterile explants were removed aseptically and excised into small discs (3-5mm) and cultured onto Murashige and Skoog (1962) (MS) solid basal medium amended with growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D), Kinetin (Kn) and Benzylaminopurine (BAP), incubated at 25°C using a 16h photoperiod for four weeks (Fig 1).

2.2 Elicitation of secondary metabolites

2.2.1 Preparation of MeJA stock solution

MeJA stock solution (10mM) was prepared in 50% (v/v) ethanol, filter-sterilized and added to 10days old cell suspension culture at the concentration of 25, 50 and 100µM/L. Culture with equal volumes of ethanol was maintained as control.

2.3 Establishment of cell suspension culture

2 g of the friable callus (3 weeks old) was transferred aseptically to 250mL Erlenmeyer flask containing 50mL liquid MS basal medium supplemented with 1.5mg/L 2,4-D, same composition as of callus induction media. The cultures were agitated at 120 rpm and incubated using 16h photoperiod at 24±2°C. The cell culture obtained after 10 days (log phase of growth) of incubation were used to study the effect of the elicitor MeJA on growth characteristics of cell suspension.

2.31 Growth assessment of suspension culture: Growth of cells in suspension culture was measured in terms of fresh and dry weight. Harvesting of suspension cells was performed aseptically in triplicates at 2, 4, 6, 8, 10 and 12th day of incubation after the addition of MeJA. The cells in the suspension cultures were filtered through a filter paper (Whatman® No.1, diameter 90mm) under vacuum. The dry weight (DW) was obtained by drying the fresh cell mass at 50°C in an oven until constant weight, and both the dry weight and the fresh weight (FW) were recorded.

2.4 Analysis of the secondary compound production

For analysis of the secondary compound production, the oven-dried calli were extracted with 5mL of 80% aqueous methanol and homogenized with mortar and pestle. The extract was subjected to GC-MS analysis.

2.41 Gas Chromatographic-Mass Spectral analysis: GC-MS analysis of the extract was performed on an Agilent Technologies gas chromatograph Model 5973N equipped with mass selective detector. A CPSil8CB column (30m × 0.25mm × 0.25 Mm film thickness) coated with dimethylpolysiloxane with 5% diphenyl as the stationary phase. Helium was used as the carrier gas at a flow rate of 1mL per min (constant flow). Temperature programming was done from 50°C (2 min) to 280°C at 10°C per min. Injector and detector temperature were maintained at 250°C and 280°C respectively. Samples of 1µL dissolved in hexane were injected using a split ratio of 10:1. Mass spectra were recorded in the EI mode at 70eV in the m/z range of 30-450.

2.5 Identification of compounds

The essential oil components were identified by comparison of the mass spectra of the peaks with those of compounds from literature (Adams, 2007) [13] and those stored in NIST library. Peak area percentages were computed from GC peak areas without using correction factors.

2.6 Statistical analysis

Experiment was repeated in triplicates with ten replicates. All data are represented as mean±SD. Data were analyzed by one way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at 5 % probability level using SPSS software (version 16).

3. Results & Discussion

During this study, the problems associated to obtain axenic culture of *C. citratus* were observed to be due to the intact association of endophytic microorganisms with the plant. Thus, to overcome the endophytic growth, a modified protocol was developed whereby the surface sterilizing agent sodium hypochlorite was replaced by potassium permanganate and mercuric chloride which yielded 95% of aseptic cultures devoid of endophytic microorganisms.

The leaf base of *C. citratus* was used as explant source and amended with various growth regulators such as 2, 4-D, Kn and BAP which are tabulated (Table 1). Among the different concentrations and combination of hormone tested, 2, 4-D (1.5 mg/L) was found to be the most effective hormone in eliciting hard and friable callus, which was further used for cell culture.

The secondary metabolites from essential oil was elicited using different concentrations of MeJA under cell suspension culture. The culture showed an enhancement of accumulated cell biomass from the time of addition (12^{th} day to 22^{nd} day). The growth curve of elicited cell suspension culture of *C. citratus* in terms of accumulated biomass content (fresh and dry wt.) is shown (Table 2, Fig 3). The growth of cell suspension culture at different intervals of time was compared with the growth curve of non-elicited cultures (Control). During the study it was noted that for the addition of MeJA in culture, there was significant accumulation (fresh weight) of secondary metabolites when compared to the culture under Control. A maximum increase in fresh weight of cells was observed at 50µM concentration of MeJA and attained stationary phase on the 20th day. Similarly, the dry weight of cell culture also increased in correspondence to the fresh weight. The suspended cell aggregates showed creamish white to creamish yellow color initially and later turned light brown during MeJA treatment at various concentrations (Fig 2).

It was observed that, the MeJA at various concentrations (25, 50 and 100 µM/L) elicited secondary metabolite production in cell suspension culture compared to Control. Citral was the main active component elicited in the culture as identified by GC-MS analysis. The percentage of Citral (50%) produced was higher in cell culture treated with 50 µM/L of MeJA (Table 3 & Fig 4). This can be attributed to the fact that MeJA and its related derivatives have been implicated as signal transduction molecules with multifaceted effects on plant growth, development and response to stress (Staswick, 1992; Sembdner and Parthier, 1993; Reinbothe *et al.*, 1994) [14, 15]. MeJA was found to trigger secondary metabolite

formation through MEP (Methyl Erythritol Phosphate) pathway leading to the production of terpenoids (Herrmann, 1995) [16]. However, for economic production of the secondary compounds obtained further optimization of the culture is required.

Table 1: Effect of growth regulator for *in vitro* response of *C. citratus*

Source of explant	Plant growth regulator	Concentration (mg/L)	Type of response	Callus growth response*
Young leaf base	2,4-D	0.5	Friable callus	+
		1.0	Friable callus	++
		1.5	Friable callus	+++
		2.0	Friable callus	++
	2,4-D+Kn	2.0+0.5	Somatic embryos	+++
	2,4-D+BAP	2.0+0.5	Somatic embryos	+++
	2,4-D+BAP	2.0+1.0	Somatic embryos	++

*+ slow growth, ++ moderate growth, +++ excellent growth

Table 2: Effect of MeJA on *C. citratus* cell growth in suspension culture

Control		Me JA (25µM/L)		Me JA (50µM/L)		Me JA (100µM/L)	
DW	FW	DW	FW	DW	FW	DW	FW
0.69±1.02a	3.1±1.04a	0.72±1.03a	3.20±0.93a	0.88±1.03a	3.91±1.00a	0.76±1.01a	3.40±1.05a
0.77±1.03b	3.4±1.04b	0.82±1.04b	3.65±0.84b	1.01±0.73b	4.50±0.96b	0.83±0.81bc	3.70±1.00a
0.83±1.02c	3.7±0.93bc	0.88±1.01c	3.90±1.05c	1.34±0.90c	5.98±0.92c	1.03±0.93cd	4.60±1.08b
0.97±1.04d	4.3±0.81c	0.93±1.02c	4.15±1.03c	1.42±0.81cd	6.30±0.82de	1.17±1.04d	5.20±1.03bc
1.04±0.91d	4.6±1.00cd	1.06±1.02d	4.70±1.03d	1.46±1.02d	6.50±1.03e	1.26±1.10de	5.60±0.93c
1.06±1.01d	4.8±1.02d	1.07±1.02d	4.73±1.02e	1.48±1.04e	6.50±1.02e	1.28±1.12e	5.60±1.14c

DW: Dry weight, FW: Fresh weight

Values expressed as mean±SD. Data were analyzed by Duncan's MRT. Means followed by different alphabets were statistically different ($P<0.05$, n=6)

Table 3: Major chemical components of cell extract identified in GC-MS

RI	Compound	Percentage
986	6-Methyl 5-hepten-2-one	4.06
1098	Linalool	2.29
1259	Neral	23.09
1280	Geranial	26.63
1344	Epoxy-linalool oxide	13.85

RI = Retention Index on DB-5 column

Fig 1: Callus induction in *C. citratus*

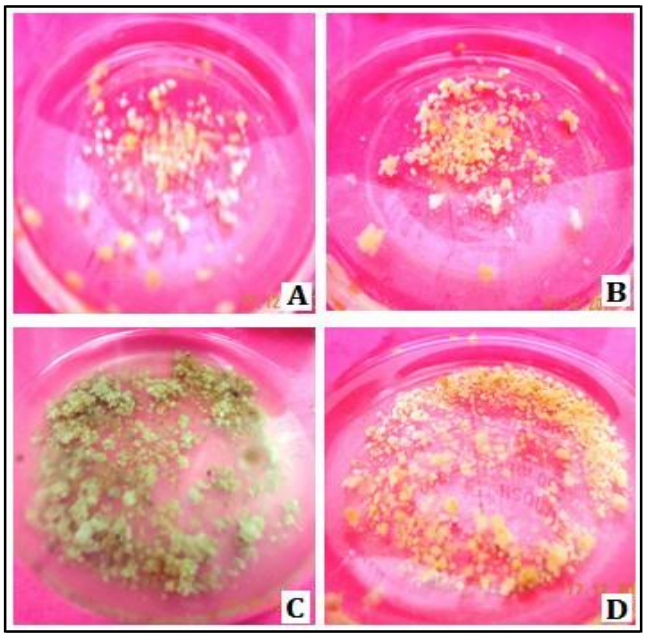

Fig 2: Cell suspension culture of *C. citratus* at different concentration of Methyl jasmonate (MeJA)
A-Control, B-MeJA 25 µM, C- MeJA 50 µM, D- MeJA 100 µM

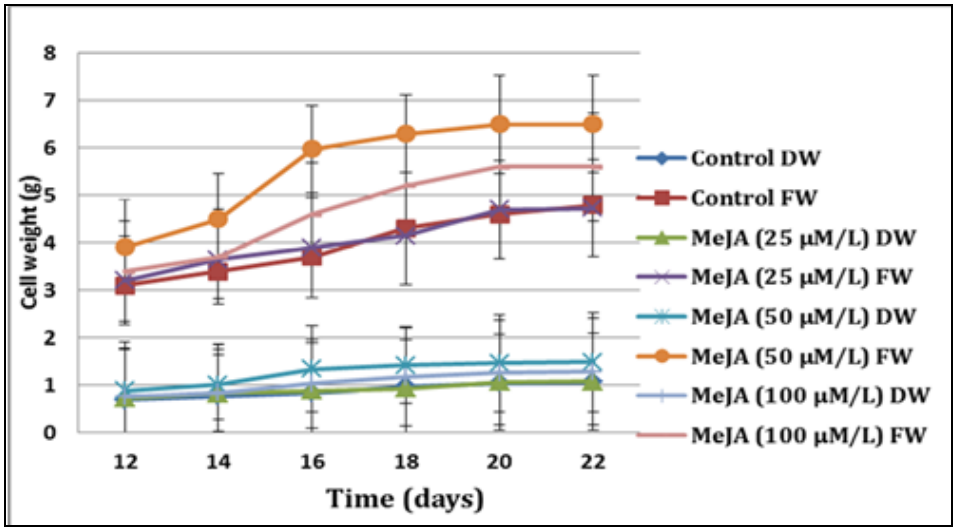

Fig 3: Fresh (FW) and dry weight (DW) of *C. citratus* cell biomass in suspension culture at different concentrations of Methyl jasmonate (MeJA) Data were analyzed by Duncan's MRT ($p<0.05$, n=6)

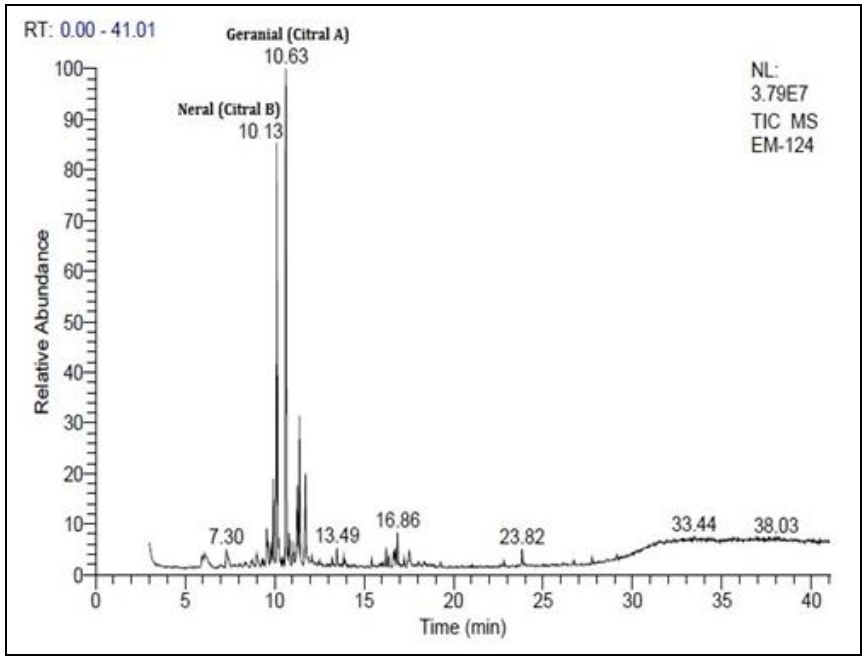

Fig 4: GC-MS profile of cell suspension extract of *C. citratus* elicited at 50µM of Methyl Jasmonate (MeJA)

4. Conclusion

Plants are rich sources of fine chemicals, largely unknown and unexplored. The recent advances in functional genomics and metabolite profiling offer unprecedented opportunities to use the biochemical capacity of plants to produce and design novel secondary compounds. The essential oil bearing species like *C.* citratus presently investigated produces secondary metabolites of medicinal and aromatic significance. The cell culture technology offers production of high-value secondary metabolites on larger scale.

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