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Plant tissue culture: An alternative for production of useful secondary metabolites

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Abstract
Current study involves investigation of rosmarinic acid (RA) accumulation in callus cultures of *Ocimum gratissimum* (L.). *Ocimum*. is belongs to family: *Lamiaceae*, popularly known in India as Rama tulasi, tulasi is a traditional medicinal plant. Single node explants were inoculated on basal MS (Murashige and Skoog) medium containing 3% (w/v) sucrose, supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA) for direct plant regeneration. Maximum numbers of shoot were observed on the medium containing 0.5 mg/l BAP and 0.25 mg/l IAA after four weeks of culture. Callus was initiated from *in vitro* cultures of leaf explant on Murashige and Skoog's (MS) medium supplemented with 2,4 dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin (KIN). In the present study profuse callus was obtained on MS medium supplemented with 2, 4-D (1 mg/l) + BAP (0.5 mg/l). RA was isolated from callus harvested biomass and characterized by chromatographic techniques like TLC and HPTLC.

Keywords: *Ocimum gratissimum L*. Callus; Rosmarinic acid; 2, 4-D; Kinetin; BAP

1. Introduction
Plants are valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides and food additives. Over 80% of available drugs approximately 30,000 known natural products are of plant origin [1]. With the rapid increase in world population, extreme pressure on the available cultivable land, fast disappearance of natural habitats for medicinal plants together with environmental and geopolitical instabilities; it is increasingly difficult to acquire plant-derived compounds [2]. Plant cell cultures are an attractive alternative source to whole plant for the production of secondary metabolites [3]. Plant cell cultures have the following advantages over conventional agricultural production *viz.,* independent of geographical and environmental variations, defined production system with continuous supply of products, uniform quality, yield, rapidity of production and production of novel compounds. In recent years, various plant cell culture systems have been exploited for the enhancement of high value metabolites [4].
Rosmarinic acid (RA), an ester of caffeic acid and 3, 4 dihydroxy phenyllactic acid, is a natural antioxidant most commonly occurring in the species of *Lamiaceae* and *Boraginaceae*. RA has received particular attention because of its well-known biological activities including antibacterial, antiviral and anti-inflammatory properties [5]. RA was successfully isolated from *Thymus vulgaris* [6], *Melissa officinalis* [7] and other species. Because of its high medicinal value and demand, considerable effort is needed to develop an economically feasible method for production of RA.
The genus *Ocimum* is ranked high among the herbs for having enormous medicinal potentialities. It consists of about 160 species spread over tropical, subtropical and warmer parts of temperate regions of the world [8]. *Ocimum gratissimum L.* (Rama tulasi or Nimma tulasi) is a valuable multipurpose medicinal plant which belongs to the family *Lamiaceae*. Traditionally, this plant is being widely used for the treatment of various ailments including rheumatism, paralysis, epilepsy, high fever, diarrhea, sunstroke, influenza, gonorrhea and mental illness [9]. The plant exhibits antimicrobial [10], Antifungal [11], antibacterial [12], antimalarial [13] and antiprotozoal [14] activities. An investigation was carried out to initiate and study the growth characteristics of callus cultures of *O. gratissimum* under *in-vitro* conditions for the production of the major secondary metabolite (Rosmarinic acid).

2. Materials and Methods

2.1. Plant Material

Young disease free single nodal explants (2-2.5 cm) were collected from plant growing in the Central Institute of Medicinal and Aromatic Plants (CIMAP) resource centre, Boduppal, Hyderabad, Telangana, India. Explants were washed thoroughly under running tap water and then treated with surfactant Tween 80 (10 drops for 100 ml sterilized distilled water) followed by rinsing for three to five times in sterile distilled water. The nodal segments were then surface disinfested with 70% alcohol for 1 min followed by immersion in 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 5-6 min and finally rinsed with autoclaved sterile distilled water (five to six times) in a flow chamber. The surface sterilized explants were trimmed at cut ends and about 1-1.2 cm prior to inoculation on culture media.

2.2. Culture Media and Conditions

Surface sterilized nodal segments (1-1.2 cm) were cultured on MS [15] basal medium containing 3% (w/v) sucrose for culture initiation and served as explant sources for subsequent experiments. The pH of the medium (Supplemented with respective growth regulators) was adjusted to 5.8 with 1N NaOH or 1N HCl before gelling with 0.8% (w/v) agar. In all the experiments, the chemicals used were of analytical grade. The explants initially were implanted vertically on the culture medium in test tube (150 × 25 nm) and plugged tightly with non-absorbent cotton. All the cultures were kept under cool fluorescent light (12 h photo periods, India at 25 °C ± 2 °C) and 60% - 70% relative humidity (RH).

2.3 Shoot Induction and Multiplication

For shoot induction, the nodal explants were cultured on MS medium supplemented with various concentrations of BAP (0.5 – 1.5mg/l) either individually or in combination with IAA (0.25mg/l). The regenerated shoots were excised, cut into single nodal segments and further multiplied for five to six subcultures to fresh medium of the same composition for induction of multiple shoots at an interval of 4 weeks. The frequency with which explants produced shoots, the number of shoots per explant and the shoot length were recorded after 4 weeks of culture.

2.4 Establishment of callus cultures

For callus induction, *in vitro* cultures of leaf explants were cultured on MS medium supplemented with 2, 4-D and BAP or Kinetin both alone and in combinations. Explants of *in vitro* cultures of leaf were excised as circular discs and placed on MS media supplemented with different combinations of Auxins and Cytokinins and incubated under the standard culture conditions. Friable callus was initiated in a very short period of time, 17-21 days. Callus was sub cultured every 30 days on same solid MS medium supplemented with 2, 4-D and BAP.

2.5 Isolation and characterization of rosmarinic acid by analytical techniques

The callus biomass (1.05 g dry weight) was extracted with ethanol and macerated for 24 hours. After 24 hrs, filtered. The analysis of simple phenolic acids from a plant is commonly carried out by TLC [16]. The purity of isolated RA was checked by thin layer chromatography (TLC) eluting with Formic acid: Acetone: Dichloromethane (8:2.5:85) [17]; Ethyl acetate: Acetic acid (95:5) [18] and Toluene: Ethyl acetate: Formic acid (5:4:1) [19] with reference standard.

High performance thin-layer chromatographic (HPTLC) analysis of rosmarinic acid was performed on precoated silica gel 60 F254 HPTLC plate. Ethanolic extract and rosmarinic acid standards solutions were applied on the plate and developed using a mixture of ethyl acetate: acetic acid (95:5 V/V) as mobile phase [18]. After the run the plate detection was carried out under U-V (325 nm) by use of a LAMAG TLC scanner 3 system (Camag Switzerland).

3. Results and Discussion

3.1 *In-vitro* cultures of *Ocimum gratissimum*

Nodal explants culture in MS medium individually supplemented with both BAP and KN showed remarkable response. Among cytokinins, 0.5 mg/l BAP responds well when compare to KN in medium for shoot proliferation. The maximum induction of multiple shoots was achieved from medium supplemented with 0.5 mg/l BAP and 0.25 mg/l IAA, 2 to 3 weeks after incubation. Among the concentrations tested, the best response was noticed with 0.5 mg/l BAP and 0.25 mg/l IAA.

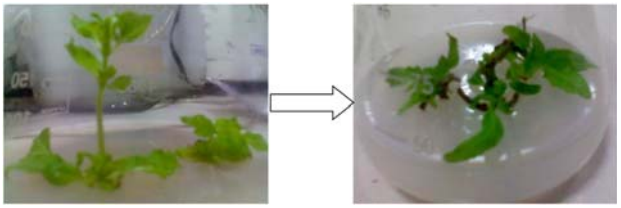

Fig 1 a **Fig 1 b**

Fig 1 a: Initial stage of *Ocimum gratissimum*
Fig 1 b: after multiplication of *Ocimum gratissimum* by in *In-vitro*

Table 1: Response of nodal explants on MS media supplemented with a combination of phytohormones.

Growth regulators	Concentrations (mg/l)	Degree of Response
BAP	1.5	*
BAP	1.0	**
BAP	0.5	**
BAP + Citric acid	0.5	***
BAP+IAA	0.5+0.25	*****

3.2 Establishment of callus cultures

Callus was successfully initiated from cotyledonary leaf explant material cultured on MS media supplemented with 2, 4-D and BAP. When the cytokinins and Auxins were used alone, 2, 4-D at lower concentrations have shown callusing but it was very meager and BAP has shown much lesser response. Nature and proliferation of the induced calli varied accordingly with the growth regulators supplemented in the medium. BAP was suggested to be used for good development of callus with high proliferation efficiency for *O. gratissimum.* Creamy friable callus masses of *O. gratissimum* consisting of soft separable cells were obtained in culture by sub culturing callus masses on to fresh media every 4 weeks.

The media supplemented with a combination of an Auxin (2, 4-D) and Cytokinin (BAP) proved to be the best for callus induction in case of *Ocimum gratissimum*. In the present study profuse callus was obtained on MS medium supplemented with 2, 4-D (1 mg/l) + BAP (0.5 mg/l). Effect of growth regulators (Auxins and Cytokinins) for callus induction was showed in table 2.

Explants from phenolic-rich plant tissues such as these Lamiaceae plants darken when placed on the usual culture media, but the surviving tissue eventually produces a fast growing, friable, light colored callus.

Fig 2: Establishment of callus cultures on Ms media supplemented with 2, 4-D and BAP

Table 2: Effect of 2, 4-D, Kinetin and BAP for callus induction

Growth regulators	Concentrations (mg/l)	Response
2, 4-D	0.5	*
2, 4-D	1.0	*
2, 4-D	1.5	*
2, 4-D	2.0	**
BAP	0.5	**
BAP	1.0	*
2, 4-D+Kinetin	1+0.1	*
2, 4-D+Kinetin	2+0.2	**
2, 4-D+Kinetin	2+0.2	**
2, 4-D+BAP	1+0.5	*****
2, 4-D+BAP	2+0.5	***

*Poor, **Good, ***Very good, *****Excellent

3.3 Chromatographic studies

Solvent system Toluene: Ethyl acetate: Formic acid (5:4:1) in TLC, found to show proper Rf value representing the presence of RA in the callus extract similar to show as that of a standard RA Rf value. The R_f values of the standard and sample were 0.23 and 0.21 respectively. This solvent system was selected for the HPTLC analysis of RA.

3.4 HPTLC analysis of RA

HPTLC analytical procedure permits a fast and reliable determination of these phytochemicals in medicinal and aromatic plants and can be used for routine analysis. Modern densitometer afford an opportunity for fast, cheap and sensitive determinations with adequate reproducibility in the case of routine analysis involving many samples, however, these advantages can be exploited only if maximal consideration is given to the conditions applied.

The eluent system for the TLC separation of RA was selected from the literature. In the wave length range 290-330 nm, it was found that the emission peak areas exhibit a maximum at 325 nm. Selection of the emission filter was based on the conditions that it should cut off the exciting light, but allow through as much as possible of the fluorescent emission. Accordingly, filter no I was chosen, which cuts off light emission below 350 nm. In this way, the optimal noise to signal ratio can be attained.

The peaks of RA in the callus extract near to that of a standard RA peak in HPTLC chromatograms. From the above results the callus containing compound was identified as Rosmarinic acid.

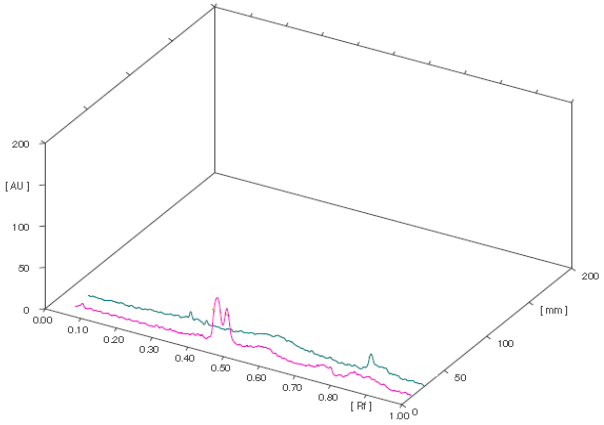

Fig 3: HPTLC chromatogram of RA

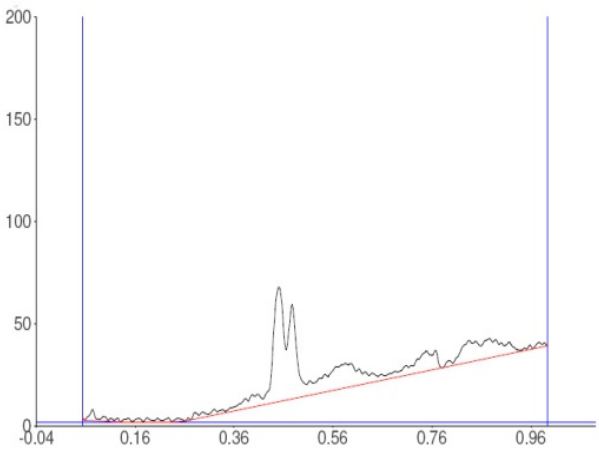

Fig 4: Densitograms of the standard Rosmarinic acid at 325 nm

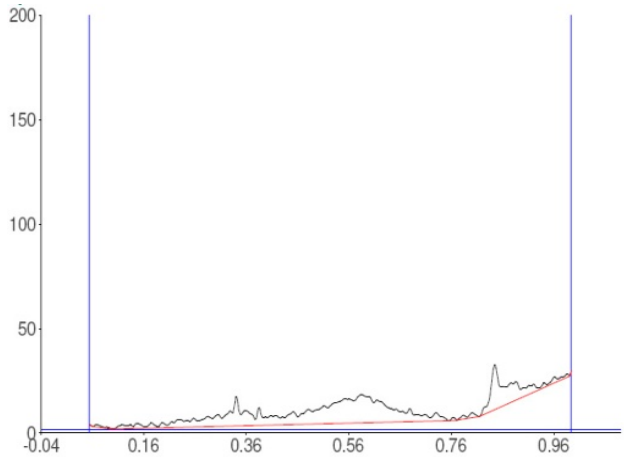

Fig 5: Densitograms of the ethanolic callus extract at 325 nm

4. Discussion and Conclusion

The present study focuses on production of Rosmarinic acid from callus cultures of *Ocimum gratissimum* (L) by using different concentrations of growth regulators. *Ocimum gratissimum* callus cultures demonstrated for RA production favored both cell growth and RA yield. Single node explants were inoculated on basal MS medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP), kinetin (KN) and indole-3-acetic acid (IAA) for direct plant regeneration. Maximum number of shoots was observed on the medium containing 0.5 mg/l BAP and 0.25 mg/l IAA after four weeks of culture. Leaf explants of *in vitro* cultures of *O. gratissimum* was inoculated on MS medium supplemented with different concentrations and combinations of 2, 4-D, BAP and Kinetin for callus induction. The media supplemented with a combination of an Auxin (2, 4-D) and Cytokinin (BAP) proved to be the best for callus induction in case of *Ocimum gratissimum*. In the present study profuse callus was obtained on MS medium supplemented with 2, 4-D (1 mg/l) + BAP (0.5 mg/l). Friable callus was initiated in a very short period of time, 17-21 days. As the phenolic compounds were more in ethanolic extract, thus ethanolic extract was chosen to check the presence of RA using TLC and HPTLC. In TLC, various solvent systems (Formic acid: Acetone: Dichloro methane; Ethyl acetate: Acetic acid; Toluene: Ethyl acetate: Formic acid) were used. Toluene: Ethyl acetate: Formic acid (5:4:1) solvent system has shown similar R_f values for standard and samples. The R_f values of sample and standard compound were 0.21 and 0.23 respectively. So the compound was identified as Rosmarinic acid. HPTLC analytical procedure permits a fast and reliable determination of these phytochemicals in medicinal and aromatic plants and can be used for routine analysis. The RA was determined qualitatively by HPTLC method.

5. References

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