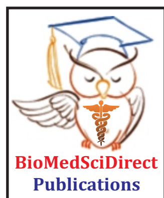

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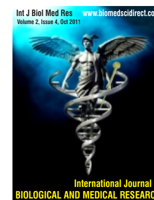

Original Article

Production of rosmarinic acid in *Ocimum sanctum (L.)* cell suspension cultures by the influence of growth regulators

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ABSTRACT

The rosmarinic acid (RA) accumulation in cell suspension cultures of Ocimum sanctum (L.) was investigated. Callus was initiated from leaf explant on Murashige and Skoog's (MS) medium supplemented with 2,4 dichlorophenoxyacetic acid (2,4-D) 1 mg/L and kinetin (KIN) 0.1 mg/L. Suspensions were established by transferring friable callus to MS liquid medium supplemented with growth regulators such as 2,4-D (1 mg/L) + KIN (0.1-0.5 mg/L), 2,4-D (0.5–2.5 mg/L), NAA (0.5-2.5 mg/L), and IAA (0.5-2.5 mg/L) individually. After stabilization of cell suspension with five subcultures, continuous culture duration of six weeks on MS liquid medium of the same growth regulator combinations resulted in progressive callus growth and RA accumulation. The highest RA (104 mg/L) content and biomass growth (17.8 g/L) was observed in the cultures supplemented with 2,4-D (1 mg/L) and KIN (0.1 mg/L). It was two-fold higher than that found in leaf callus induced on MS solid medium. RA formation was paralleled with cell growth. RA was quantified using reverse phase high performance liquid chromatography with reference standard. RA was isolated from suspension harvested biomass and characterized by spectral analysis. Key Words: Ocimum sanctum L.; Callus; Cell suspension; Rosmarinic acid; 2,4-D; Kinetin Abbreviations 2,4-D = 2,4 dichlorophenoxy acetic acid. - IAA = Indole-3-acetic acid. - NAA = Naphthalene acetic acid. - NMR = Nuclear magnetic resonance. - IR = Infra red. -UV = Ultraviolet. - HPLC = High performance liquid chromatography. - ESI-MS = Electron spray ionization – Mass spectra

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1. Introduction

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. It is a tannin-like compound and originally found in *Rosmarinus officinalis* [1]. RA has received particular attention because of its well-known biological activities including antibacterial, antiviral, and anti-inflammatory properties [2]. RA was successfully isolated from *Thymus vulgaris* [3], *Melissa officinalis* [4] 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 production of RA has been reported in cell suspension cultures of Salvia officinalis [5], *Anchusa officinalis* [6], and *Coleus blumei* [7].

The genus *Ocimum* contains 200 species of herbs and shrubs [8]. Among *Ocimum species*, *Ocimum sanctum* (L.) (Holy basil), is considered as a sacred plant in India to which several medicinal properties are attributed in ayurveda and in the Indian system of medicine [9]. Holy basil is extensively applied in the indigenous system of medicine in many Asian, African, and South American countries [10]. The leaves of the species have been reported to have anti-inflammatory, analgesic, and antistress properties [11]. A number of phenolic compounds with antioxidant activity have been identified in this plant extract [12]. Indian Materia Medica

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describes the use of *O. sanctum* in the treatment of a number of ailments like brochititis, rheumatism, and pyrexia [13]. The presence of isothymusin, carnasol, ursolic acid, eugenol, sinapic acid, and RA in *O. sanctum* has been documented previously [14]. Recently, we reported the accumulation of RA in *O. sanctum callus* cultures and its variation according to the types of explants used [15]. However, callus cultures are less suitable for studying the growth pattern and product formation than cell suspension cultures, due to limitations in homogenous mass and oxygen transfer to individual cells. Therefore, the aim of this work was to evaluate the growth and RA production pattern in cell suspension cultures of *O. sanctum* as affected by different growth regulators, as well as to develop a feasible method for isolation of RA.

2.Materials and methods

2.1.Plant material

The seeds of *O. sanctum* were purchased from the local market of Coimbatore. The plant was grown in the gardens of Bharathiar University. Green house plants were irrigated to pot capacity daily and maintained at day/night temperatures of 27–33 and 20–25^{0}C respectively. The plant was identified by the Botanical Survey of India, Southern circle, TNAU (Tamilnadu Agricultural University), Coimbatore. The specimen (Voucher specimen No. 231) was deposited in the department of Botany, Bharathiar University.

2.2 Callus induction

Leaves explants from 1-year-old *O. sanctum* plants were surface sterilized for 3–5 min in Tween 80 and for 3 min in 0.1% (w/v) $HgCl_2$ and then rinsed three times with sterilized distilled water. Sterilized explants were cut into 1 cm long pieces and cultured on Murashige and Skoog's (MS) [16] medium containing 3% sucrose (analytical grade) as a carbon source and 0.8% agar (Himedia Laboratories, Mumbai, India) for gelling. A growth regulator combination of 1 mg/L 2,4 dichlorophenoxyacetic acid (2,4-D) with 0.1 mg/L kinetin (KIN) was used. The cultures were maintained at 25 ± 2°C under 2000 lx illumination (12 h photoperiod).

2.2.HPLC analysis

A high performance liquid chromatography (HPLC) system comprising a vacuum degasser, quaternary pump, autosampler, thermostated column compartment, and photodiode array detector (PDA) was used for the quantification of RA. The column, phenomenex C_{18} 5 μm (250×4.6 mm) (Shimadzu), was maintained at 26°C. Different proportions of combined solvents such as acetonitrile/water/acetic acid (15:84:0.85) as eluant B and methanol as eluant A were used for the separation. The multiple gradient used for chromatographic separation consisted of different proportions of eluant A/B (50:50 for 1–5 min, 40:60 for 5–10 min, 30:70 for 10–15 min, 15:85 for 15–20 min). The flow rate was 1 mL/min, the sample injection volume was 50 μL, and the chromatogram was monitored at 330 nm. The peak purity of the tested sample was determined by comparing its ultraviolet (UV) spectra to that of the reference standard. Quantification was made based on the corresponding peak area recorded by chromatopac c-R6A (Shimadzu). Standard RA (Sigma Aldrich, USA) was used for the preparation of calibration curves.

2.3.Isolation and structural determination of RA

The callus biomass (30.2 g dry weight) was extracted with 80% methanol. Filtration and removal of solvent under reduced pressure at a temperature below 40°C afforded crude methanol extract (7.4 g). Further, the crude extract was fractionated using ethyl acetate and diethyl ether. The residue from the diethyl ether was discarded. The removal of solvent from an ethyl acetate phase under reduced pressure at a temperature below 40°C yielded ethyl acetate extract (3.5 g). Then, the ethyl acetate extract was adsorbed on Si gel (30 g 60-120 mesh) and RA was eluted with methanol:chlorofom in the order of increasing proportions. RA was collected in 25% methanol:chlorofom fractions. Further solvent partitioning between water and petroleum ether yielded white amorphous RA powder. The purity of isolated RA was checked by thin layer chromatography (TLC) eluting with ethyl acetate:methanol:water (100:16.5:13.5); n-butanol:acetic acid:water (10:2:1) [17] with reference standard. After obtaining a single spot in the TLC, HPLC analysis was performed to ensure the purity of isolated RA once again by using mobile phase system as described above. UV spectra were measured in methanol using an Analytic Jena UV/VIS Spectrophotometer (Germany). IR spectra were recorded on a Perkin-Elmer 650 IR spectrometer. 1H NMR spectra were recorded on Bruker DRX–300 spectrometer in dimethylsulfoxide; δ values in ppm relative to internal TMS, J values in Hz. The electron spray mass spectrum measurement was determined on a Micromass Quattro II triple quadrupole mass spectrometer (Altrincham, UK) by ESI mode at cone voltage of 40eV.

2.4.Time course of growth and RA accumulation in cell suspension cultures of O. sanctum

O. sanctum suspension cultures were established by transferring calli, at stationary growth phase (after five subcultures with one week interval), grown and stabilized on MS solid medium supplemented with 2,4-D (1 mg/L) and KIN (0.1 mg/L), to liquid MS medium containing the same composition and same hormonal supplementation and other hormonal variants such as 2,4-D (1 mg/L) + KIN (0.2-0.5 mg/L), 2,4-D (0.5-2.5 mg/L) individually, Indole-3-acetic acid (IAA) (0.5-2.5 mg/L) individually and Naphthalene acetic acid (NAA) (0.5-2.5 mg/L) individually. The cultures under different growth regulator treatments, including 2,4-D (1 mg/L) + KIN (0.1 mg/L), started with six Erlenmeyer flasks of 250 mL capacity, each one, containing 50 mL of liquid MS medium and one friable callus tissue. After 15 days, a volume of 50 mL fresh medium was added to each flask and 15 days later the content of the six flasks from each growth regulator treatment was combined and used for subculturing. After 30 days, in order to stabilize the cell suspension, subcultures were performed from each hormonal variant by transferring a volume of 10 mL suspension, to each Erlenmeyer flask of 250 mL capacity containing 100 mL of fresh medium for five times at weekly intervals. The cultures were maintained on a rotary shaker (120 rpm) at 25 2C under 2000 lx illumination (12 h photoperiod).

In order to determine the effect of growth regulator supplementation on the biomass production and its RA accumulation at the end of the fifth subculture, suspensions were maintained continuously for six weeks without subculturing. The calli growth and RA accumulation in cell suspension were determined by harvesting the biomass periodically (once a week, throughout the six-week period) from the suspension by filtration. Data were expressed as mean SD of each three replicates.

2.5.Quantitative analysis of RA in cell suspension cultures of O. sanctum

Biomass was harvested from the suspension by filtration and the dry weight was determined [18]. RA content of dried biomass was determined as described earlier [15]. RA content and the dry weight were recorded on weekly basis. Data were expressed as mean ± SD of each three replicates.

2.6.Statistical analysis

All data were expressed as mean ± SD of the number of experiments (n=3). The statistical significance was evaluated by one-way ANOVA, and significance of the individual comparison was obtained by Duncan's multiple range test (DMRT).

3.Results and discussion

3.1.Characterization of RA

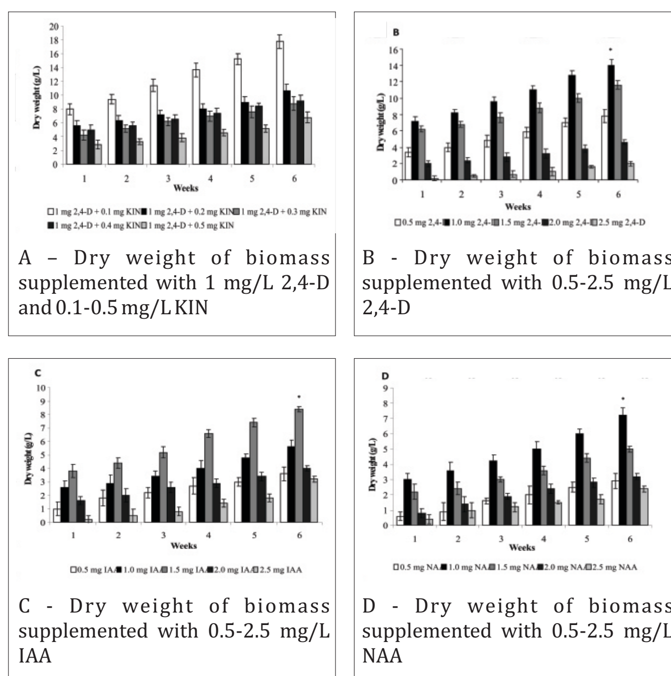
The isolated RA showed UV absorbance at 330 nm, which could be due to phenolic acid with aromatic rings [19]. The purity of isolated RA was checked by TLC and HPLC analysis. The IR spectrum showed that this compound has hydroxyl (OH), esteric (C=O) and aromatic rings. The elemental analysis profile of RA at 40eV showed a molecular radical ion peak at 361 [M] +. The mass spectra of the isolated RA agreed with the earlier reports [20, 21]. There is sparse information about NMR spectroscopy of RA in dimethylsulfoxide but the application of other spectroscopic methods (UV, IR, and ESI-MS) was very useful for the structural elucidation of this compound. NMR spectroscopy of RA in dimethylsulfoxide resulted in the appearance of protons of phenolic and acidic groups (analytical data not shown).

3.2.Callus growth and RA accumulation in cell suspension cultures

Owing to the pharmacological value of RA, extensive research has been carried out for its production in suspension cultures of Coleus blumei [22], Anchusa officinalis [6], *Orthosiphon arsitatus* [23], *Lithosperum erythrorhizon* [17], and *Lavendula* vera [24]. In genus *Ocimum*, *O. basillicum* suspension cultures have been exclusively documented for RA production [25]. In this study, we have reported for the first time, RA synthesis in *O. sanctum* cell suspension cultures. In our previous study, we showed that O. sanctum callus cultures are able to accumulate RA in larger amount in comparison with other compounds, depending on the types of explant (leaves, stems, and inflorescence) and growth regulator combination (2,4-D and KIN) used [15]. However, in order to reach a conclusion on RA production and calli growth pattern associations in *O. sanctum,* it is necessary to fine-tune the type and different concentrations and combinations of growth regulators used for callus growth and RA synthesis in cell suspension culture.

In the present study, callus growth was satisfactory in suspension compared to solid medium (data not shown). All callus lines exhibited progressively increased growth and RA accumulation during the exponential growth phase, which was observed throughout the experimental culture period (Figure 1 & 2). This result was in agreement with previous study of Kintzios et al (1999) [26]. This is not surprising, since the secondary metabolism pathways are often restricted to an individual species or genus, which might be activated only during particular stages of growth and development [27].

Fig. 1 Callus dry weight as affected by culture duration on MS liquid medium with the aid of various concentrations of hormones (2,4-D, 2,4-D + KIN, IAA and NAA) Data were represented as mean ± SD of each three replicates. Values of individual hormone concentration of each week sharing a same letter are not significantly (P<0.05) different.



A – Dry weight of biomass supplemented with 1 mg/L 2,4-D and 0.1-0.5 mg/L KIN

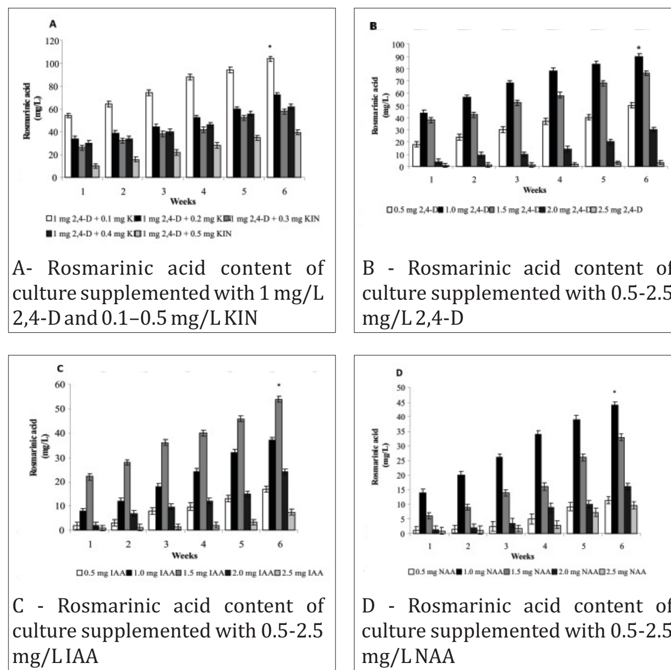
B - Dry weight of biomass supplemented with 0.5-2.5 mg/L 2,4-D

C - Dry weight of biomass supplemented with 0.5-2.5 mg/L IAA

D - Dry weight of biomass supplemented with 0.5-2.5 mg/L NAA

Fig. 2 Rosmarinic acid content as affected by culture duration on MS liquid medium with the aid of various concentrations of hormones (2,4-D, 2,4-D + KIN, IAA and NAA)

Data were represented as mean ± SD of each three replicates. Values of individual hormone concentration of each week sharing a same letter are not significantly (P<0.05) different.



A- Rosmarinic acid content of culture supplemented with 1 mg/L 2,4-D and 0.1–0.5 mg/L KIN

B - Rosmarinic acid content of culture supplemented with 0.5-2.5 mg/L 2,4-D

C - Rosmarinic acid content of culture supplemented with 0.5-2.5 mg/L IAA

D - Rosmarinic acid content of culture supplemented with 0.5-2.5 mg/L NAA

Further, in this study it was observed that RA is a growth-associated compound, its accumulation being concomitant with increases in dry weight of biomass, although the growth of callus in suspension cultures depended on the concentration and combination of added growth regulators (Fig. 1 & 2). Similarly, in Hypericum calycinum, the accumulation of adhyperforin is paralleled with cell growth and its formation increase with increased dry weight of biomass [28]. The maximal dry weight (17.8 g/L) was obtained from cultures supplemented with 2,4-D (1 mg/L) and KIN (0.1 mg/L) at the sixth week, in parallel with a maximum RA (104 mg/L) content. Similarly, the combination of 2,4-D and KIN favored callus growth and production of RA in O. basilicum [29]. The correlation coefficient between maximal dry weight of callus and RA accumulation of each concentration of growth regulators supplemented were high ($R2 = 0.9969$ – 2,4-D, $R2 = 0.9934$ – 2,4-D and KIN, $R2 = 0.9996$ – IAA, and $R2 = 0.9942$ – NAA). The highest accumulation of RA in suspension cultures of O. sanctum was two-fold higher than that found in callus induced from leaf explants on MS solid medium and 22 times higher than leaves of donor plants. The observed pattern of RA accumulation in O. sanctum suspension cultures is novel and far more complex with production peaks coinciding during intense growth phase.

4. Conclusion

In conclusion, *O. sanctum* cell suspension cultures demonstrated an outstanding potential for RA production under the conditions described in the current study, the applied plant growth regulator regime favored both cell growth and RA yield. We further plan to elucidate RA biosynthesis in *O. sanctum* cell suspension cultures by means of biotic and abiotic stress application.

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