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Research Article

COMPARISON OF MAJOR SECONDARY METABOLITES QUANTIFIED IN ELICITED CELL CULTURES, NON-ELICITED CELL CULTURES, CALLUS CULTURES AND FIELD GROWN PLANTS OF *Ocimum*

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ABSTRACT

Objective: Plant cell culture technology is an attractive alternative source to whole plants for the production of valuable secondary metabolites. Elicitors are used to stimulate secondary metabolite product formation in plant cell cultures by reducing the process time for increased culture volumes and high product concentrations.

Methods: Secondary metabolites *viz.,* phenols, alkaloids and terpenoids were quantified in cell cultures of *Ocimum basilicum* L., *Ocimum sanctum* L. and *Ocimum gratissimum* L. elicited with methyl jasmonate (MeJA) and chitosan (treatments alone and in combination) and compared with non elicited cell cultures, callus cultures and field grown plants of the same.

Results: Quantification studies showed total phenols to be the highest in leaves (90 days old) of all three *Ocimum* species. Total alkaloids and terpenoids were observed as the highest at the combined elictor treatment MeJA 25 µM + chitosan 200 mg/L (8 h) for *O. basilicum,* individual elicitor treatment MeJA 25 µM (48 h) for *O. sanctum* and chitosan 50 mg/L (24 h) for *O. gratissimum*.

Conclusion: This study will serve as a platform for attempts aimed at production, screening and characterization of many economically important individual secondary metabolite compounds on an industrial scale from the three *Ocimum* species.

Keywords: *Ocimum basilicum* L., *Ocimum gratissimum* L., *Ocimum sanctum* L., Phenols, Alkaloids, Terpenoids.

INTRODUCTION

Plants are a valuable source of a wide range of secondary metabolites. Despite advancements in synthetic chemistry, biological sources are usually preferred due to lesser side effects and better biodegradability[1,2] Cell cultures show a higher rate of metabolism than intact differentiated plants due to fast proliferation of cell mass and condensed biosynthetic cycle and secondary metabolite formation can take place within a short cultivation time [3]. Plant cells are biosynthetically totipotent and be able to produce the range of chemicals found in the parent plant.

This and with other reasons, cell culture technology is found to be advantageous over conventional agricultural production [4]. A number of plant cell cultures have been established successfully such as the production of vanillin from *Vanilla planifolia* [5], mint oil from *Mentha piperata* [6] and some even on an industrial scale such as the production of rosmarinic acid (yield 36% DW) from *Salvia officinalis* [7] and shikonin (yield 12.4 % DW) from *Lithospermum erythrorhizon* [8].

Elicitation has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites [9]. Elicitors have been used to stimulate secondary metabolite product formation in plant cell cultures by reducing the process time for increased culture volumes and high product concentrations [10]. Various elicitors such as chitosan, *ß*-glucan, and yeast extracts, as well as plant hormonal chemicals such as jasmonic acid (JA) and MeJA have been used to induce biotic and abiotic stresses upon plants [11].

Ocimum is an extremely versatile group consisting of about 160 species [12] with a geographic distribution in tropical, sub tropical regions of Asia, Africa, South and Central America with the main centre of diversity in Africa [13]. Plants belonging to this genus are predominantly shrubs, undershrubs and herbs and are perennial, triennial or biennial in habit and rich sources of major secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids and essential oils [14, 15]. *O. basilicum* is an annual plant with extraordinary medicinal, aromatic and culinary properties [16], *O. sanctum* is an aromatic herb worshipped by Hindus and widely used in traditional systems of medicine [17] and *O. gratissimum* is a valuable multi-purpose medicinal plant [18].

The present investigation was aimed at quantification of major secondary metabolites *viz.,* phenols, alkaloids and terpenoids from the elicited cell cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* and have them compared with those quantified in non-elicited cell cultures, callus cultures and leaves from field grown plants.

MATERIALS AND METHODS

Cell cultures and elicitation

Cell cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* were established and sub cultured in 100 ml of liquid Murashige and Skoog (1962) culture medium (MS) [19] with sucrose (3 %, w/v), myoinositol (100 mg/l) and supplemented with 0.5 mg/L BA and 1.0 mg/L 2,4-D. Suspension cultures were grown in gyratory rotary shakers at 120 rpm, 25 ± 1°C under dark conditions [20]. Cell suspension cultures of the three *Ocimum* species were elicited using MeJA and chitosan (individually and in combination) and their growth characteristics were studied [21].

Sample preparation for quantification studies

Callus, non-elicited, elicited cell cultures and leaves from field grown plants of *O. basilicum, O. sanctum* and *O. gratissimum* were harvested for the quantification of major secondary metabolites. Callus induced on solid MS medium with 0.5 mg/L BA and 1.0 mg/L 2,4-D was used for all three species [22]. Non-elicited cell cultures grown in liquid MS medium having 0.5 mg/L BA and 1.0 mg/L 2,4-D and harvested on the day of maximum cell weight accumulation in their growth cycle were used for all three species [20]. For elicited cell cultures, the elicitor concentration that yielded maximal cell biomass (alone and in combination) was used for each of the species and harvesting was done at their respective elicitation time. Cells were filtered from their media using Whatman's filter paper (No. 1). All samples were dried in oven (40˚C), weighed, powdered in a pestle and mortar and stored in capped vials until further use [23].

Leaves were harvested from 3 month old field grown plants of the three *Ocimum* species and dried in oven (40°C) and the dried material was powdered in a mortar and pestle and stored in capped vials until further use [24].

Quantification of secondary metabolites

Total phenolic content was determined according to Kujala *et al*. (2000) [25] on 80% methanolic extracts prepared according to Hakkim *et al*. (2007) [24]. Values were expressed in gallic acid equivalents (GAE). Total alkaloid content was determined according to Harborne (1973) [26] and total terpenoid content according to Morigiwa and Kitabatake (1986) [27].

Statistical analysis

All data are represented as mean ± SE. Data were analyzed by one way analysis of variance (ANOVA) [28] and means were compared using Duncan's multiple range test [29] at 5 % probability level using SPSS software (version 16).

RESULTS

Five passages old friable callus was used for the three *Ocimum* species. Cell content was harvested from non - elicited cell cultures on the day of maximum cell weight accumulation of their normal growth cycle which was found to be on the 32nd day of culture for *O. basilicum*, 50th day for *O. sanctum* and 46th day for *O. gratissimum* [20]. The best elicitor treatments for the three *Ocimum* species were chosen based on the highest cell weight accumulation obtained during study of growth characteristics for the elicitor treatments used individually and in combination [21]. Table 1 indicates the best elicitor treatments chosen from previous cell growth study, to be used for secondary metabolite quantification studies. Harvesting after 90 days of planting has been suggested for most of the *Ocimum* species grown under natural field conditions in order to produce the highest secondary metabolite content [15]. Leaves from field grown plants of *O. basilicum*, *O. sanctum* and *O. gratissimum* were harvested after 90 days of planting to be used for secondary metabolite quantification.

Table 1: Accumulation of highest cell biomass content at best elicitor treatments in the three *Ocimum* species

Species	Best elicitor treatment	Mean fresh weight (gm FW/5ml)	Mean dry weight (gm DW/5ml)
	MeJA (25 µM, 12 h)	0.954 ± 0.03	0.095 ± 0.002
O. basilicum	Chitosan (200 mg/L, 12 h)	0.716 ± 0.01	0.070 ± 0.002
	MeJA 25µM + chitosan 200 mg/L, 8 h	0.456 ± 0.01	0.046 ± 0.002
	MeJA (25 µM, 48 h)	0.762 ± 0.01	0.075 ± 0.001
O. sanctum	Chitosan (50 mg/L, 24 h)	0.354 ± 0.01	0.033 ± 0.001
	MeJA 100µM + chitosan 200 mg/L, 4 h	0.301± 0.01	0.030 ± 0.001
	MeJA (50 µM, 8 h)	1.464 ± 0.01	0.158 ± 0.002
O. gratissimum	Chitosan (50 mg/L, 24 h)	0.650 ± 0.01	0.065 ± 0.002
	MeJA 25µM + chitosan 100 mg/L, 24 h	0.359 ± 0.01	0.037 ± 0.0003

Treatments replicated thrice. Values are mean ± SE found significant by ANOVA

***O. basilicum* L.**

Highest total phenolic content (Figure 1a) was observed in the leaves (69 ± 0.57 mg gallic acid equivalents (GAE) /gm DW). This was much significantly higher than those observed for *in vitro* grown cell cultures. Among the *in vitro* grown cell cultures, the highest phenolic content 33 ± 0.57 mg GAE/gm DW was observed in the cell culture elicited with MeJA (25 µM, 12 h). Cell culture elicited with chitosan (200 mg/L, 12 h) showed its total phenolic content to be at 32 ± 0.57 mg GAE/gm DW which was almost the same as observed in MeJA elicited cell culture.

However, combination of MeJA and chitosan showed a decrease in total phenolic content (25.6 ± 0.33 mg GAE/gm DW). The lowest total phenolic content was observed in the callus culture which was found to be 20 ± 0.57 mg GAE/gm DW.

When quantified for total alkaloid content (Figure 1b), the highest (0.22 ± 0.01 gm/gm DW) was observed again in the cell culture elicited with both MeJA and chitosan (MeJA 25µM + chitosan 200 mg/L, 8 h). The second highest total alkaloid content was observed in the MeJA elicited cell culture (19 ± 0.01 gm/gm DW). Alkaloid content was observed to be almost the same for callus culture, non-elicited cell culture, chitosan elicited cell culture and the plant material of *O. basilicum.*

In the case of total terpenoid content (Figure 1c), cell culture elicited with both MeJA and chitosan (MeJA 25µM + chitosan 200 mg/L, 8 h) was again observed to have the highest which was found to be 0.45 ± 0.02 gm/gm DW. Low alkaloid content was observed for callus culture (19 ± 0.01 gm/gm DW), chitosan elicited cell culture (19 ± 0.01 gm/gm DW) and the leaves (19 ± 0.02 gm/gm DW) of *O. basilicum*.

The combined elicitor treatment of MeJA and chitosan (MeJA 25µM + chitosan 200 mg/L at 8 h) was found to exhibit higher secondary metabolite content when compared to individual elicitor treated cell cultures in cell cultures of *O. basilicum.* Total phenolic content alone was observed as the highest in the leaves of *O. basilicum.*

***O. sanctum* L.**

Leaves of *O. sanctum* were observed to show the highest total phenolic content, which was 61.3 ± 0.66 mg GAE/gm DW (Figure 1a). The lowest was observed in the callus culture, which was found to be 28 ± 0.57 mg GAE/gm DW. Among the *in vitro* grown cell cultures, the highest was obtained in the cell culture elicited with chitosan (33.3 ± 0.33 mg GAE/gm DW).

Cell culture elicited with MeJA was again found to show the highest (0.33 ± 0.01 gm/gm DW) when quantified for total alkaloid content (Figure 1b). The lowest was observed in the cell culture elicited with chitosan (14 ± 0.01 gm/gm DW). In the case of total terpenoid content (Figure 1c), cell culture elicited with MeJA was again found to show the highest (0.43 ± 0.03 gm/gm DW). The lowest was observed for the cell culture elicited with both the elicitors (0.12 ± 0.03 gm/gm DW).

Thus, *O. sanctum* cell cultures elicited with MeJA (25 µM at 48 h) were observed to show the highest in alkaloid and terpenoid content whereas, the highest total phenolic content was found in the leaves.

***O. gratissimum* L.**

As observed in *O. basilicum* and *O. sanctum*, total phenolic content was observed to be the highest (57.7 ± 0.33 mg GAE/gm DW) in the plant material of *O. gratissimum* (Figure 1a). Among the *in-vitro* grown cultures, cell culture elicited with chitosan was observed to show the highest (44 ± 0.57 mg GAE/gm DW). Lowest phenolic content was observed in the callus culture, which was 35.3 ± 0.33 mg GAE/gm DW. In general, all the *in-vitro* grown cell cultures of *O. gratissimum* were observed to show a higher phenolic content than the *in-vitro* grown cell cultures of *O. basilicum* and *O. sanctum*.

For total alkaloid content (Figure 1b), cell culture elicited with chitosan was again found to show the highest (0.45 ± 0.01 gm/gm DW). The lowest was observed in the callus culture, which was found to be 0.15 ± 0.01 gm/gm DW. For total terpenoid content (Figure 1c), the highest was again observed in the chitosan elicited cell culture, which was found to be 0.42 ± 0.04 gm/gm DW. The lowest (14 ± 0.02 gm/gm DW) was again observed in the callus culture.

On the whole, *O. gratissiumum* cell cultures elicited with chitosan (50 mg/L at 24 h) were found to show the highest for all the secondary metabolites measured except for total phenolic content, where it was found to be the second highest with plant material being the highest.

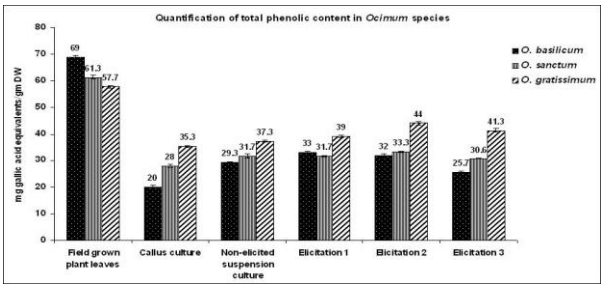

Fig. 1a: Quantification of total phenolic content in *Ocimum* species

Treatments replicated thrice. Values are mean ± SE. Means followed by the same number are not significantly different by Duncan's multiple range test at 5% probability level.

O. basilicum L. - Elicitation 1 - MeJA 25µM, 12 h, Elicitation 2 - Chitosan 200 mg/L, 12 h, Elicitation 3 - MeJA 25µM + chitosan 200 mg/L, 8 h

O. sanctum L. - Elicitation 1 - MeJA 25µM, 48 h, Elicitation 2 - Chitosan 50 mg/L, 24 h, Elicitation 3 - MeJA 100 µM + chitosan 200 mg/L, 4 h

O. gratissimum L. - Elicitation 1 - MeJA 50 µM, 8 h, Elicitation 2 - Chitosan 50 mg/L, 24 h, Elicitation 3 - MeJA 25 µM + chitosan 100 mg/L, 24 h.

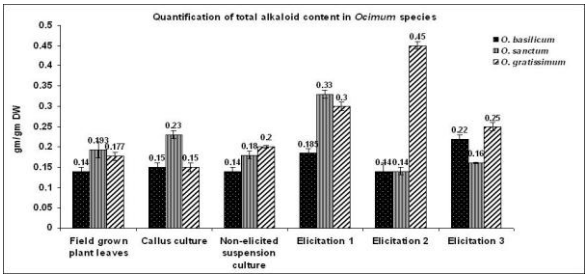

Fig. 1b: Quantification of total alkaloid content in *Ocimum* species

Treatments replicated thrice. Values are mean ± SE. Means followed by the same number are not significantly different by Duncan's multiple range test at 5% probability level.

O. basilicum L. - Elicitation 1 - MeJA 25µM, 12 h, Elicitation 2 - Chitosan 200 mg/L, 12 h, Elicitation 3 - MeJA 25µM + chitosan 200 mg/L, 8 h

O. sanctum L. - Elicitation 1 - MeJA 25µM, 48 h, Elicitation 2 - Chitosan 50 mg/L, 24 h, Elicitation 3 - MeJA 100 µM + chitosan 200 mg/L, 4 h

O. gratissimum L. - Elicitation 1 - MeJA 50 µM, 8 h, Elicitation 2 - Chitosan 50 mg/L, 24 h, Elicitation 3 - MeJA 25 µM + chitosan 100 mg/L, 24 h

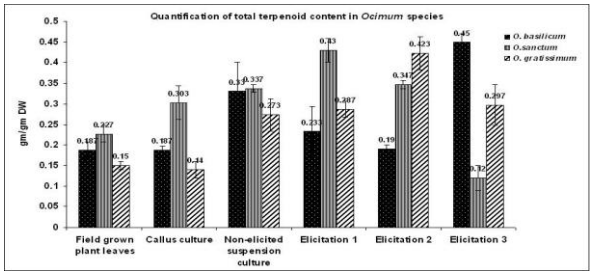

Fig. 1c: Quantification of total terpenoid content in *Ocimum* species

Treatments replicated thrice. Values are mean ± SE. Means followed by the same number are not significantly different by Duncan's multiple range test at 5% probability level.

O. basilicum L. - Elicitation 1 - MeJA 25µM, 12 h, Elicitation 2 - Chitosan 200 mg/L, 12 h, Elicitation 3 - MeJA 25µM + chitosan 200 mg/L, 8 h

O. sanctum L. - Elicitation 1 - MeJA 25µM, 48 h, Elicitation 2 - Chitosan 50 mg/L, 24 h, Elicitation 3 - MeJA 100 µM + chitosan 200 mg/L, 4 h

O. gratissimum L. - Elicitation 1 - MeJA 50 µM, 8 h, Elicitation 2 - Chitosan 50 mg/L, 24 h, Elicitation 3 - MeJA 25 µM + chitosan 100 mg/L, 24 h

DISCUSSION

With reports on MeJA and chitosan affecting the production of secondary metabolites in field grown plants as well as in cell suspension cultures of various medicinally important plant species, attempts were made at quantification of secondary metabolites in the elicited cell suspension cultures of the three *Ocimum* species and were compared with those quantified in callus, non-elicited, elicited cell cultures and leaves from field grown plants.

Earlier reports show MeJA and chitosan triggering highest total phenolic content in cell cultures [30, 31] whereas in *Ocimum*, total phenols were observed as the highest in leaves for all the three species and the next highest was observed in the elicited cell cultures. Though it was observed so, total phenolic content quantified in elicited cultures could again be considered as a success which is due to the fact that total phenols observed in all elicited cell cultures were obtained within a few hours of elicitation when compared to the leaves that were harvested from 90 day old plants. In addition to it, *Ocimum* plants can only be reharvested after 65-75 days interval to be used for extraction of any secondary metabolite [15].

In the elicited cell cultures of *O. basilicum*, combination of MeJA and chitosan (MeJA 25 µM + chitosan 200 mg/L) were found to produce the highest content of secondary metabolites rather than the individual elicitor treatments. High secondary metabolite content has also been obtained by combined elicitor treatment of MeJA and chitosan in few other suspension cultures such as the increase in podophyllotoxin content from elicitation of *Juniperus chinensis* cell cultures with MeJA and chitopentaose [32], increase in paclitaxel production from *Taxus cuspidata* var. nana cell cultures elicited with MeJA and chitoheptaose [33] and *Taxus chinensis* cell cultures elicited with MeJA and chitosan [34].

Unlike cell cultures of *O. basilicum*, MeJA (25 µM, 48 h) was found to trigger the highest secondary metabolite content in the elicited cell cultures of *O. sanctum*. MeJA has been found to trigger high concentrations of alkaloids, terpenoids and phenolics in *Nicotiana* species, *Hyoscyamus muticus* and Norway spruce stems and sweet basil [35, 36, 37, 38]. An increase in flavonoid content has been reported in cell cultures of Ohelo [39] and in blackberries [40] on elicitation with MeJA. Desired economically important products such as rosmarinic acid has been obtained from members of Lamiaceae family such as *Lavendula vera* MM cell cultures at 12 h when elicited with MeJA 50 µM [41] and *Coleus blumei* cell cultures at 16 h when elicited with MeJA 100 µM [42].

Chitosan (50 mg/L, 24 h) was observed to show the highest in secondary metabolite content quantified in elicited cell cultures of *O. gratissimum*. Low concentration of chitosan (50 mg/L) was found to trigger the highest secondary metabolite content in *O. gratissimum*. Chitosan at 200 mg/L was reported to cause an increase in menthol production at day 12 of elicitation in *Mentha piperita* cell culture [43]. Chitosan has also been found individually to increase the alkaloid content in cell cultures of *Eurycoma longifolia* [44] and in hairy root cultures of *Brugmansia candida* [45, 46]. Chitosan has been reported to have led to the rapid formation of phenylpropanoid derivatives in dark incubated *Cocos nucifera* endosperm cell suspension cultures [30]. Application of chitin and chitosan to soybean leaf tissues have been reported to cause increased activity

of PAL and TAL, the key enzymes of phenylpropanoid pathway [47]. The products of PAL and TAL are modified through phenylpropanoid metabolism to precursors of secondary metabolites including lignin, flavonoid pigments, and phytoalexins, all of which play key roles in a range of plant-pathogen interactions [48].

The octadecanoic pathway represents a series of metabolic steps through which JA and its volatile counterpart MeJA are synthesized following oxidation of linolenic acid. This pathway has long been proposed as a part of the signaling cascade that mediates plant defense responses after elicitation with oligouronide and polypeptide signals, resulting from insect and pathogen invasions [49]. Lopoxygenase (LOX) is a key regulator in plants' wound recognition and is involved in the biosynthesis of physiological active compounds. It catalyzes the oxygenation of polyunsaturated fatty acids, such as linoleic and linolenic acids, to produce fatty acid hydroperoxides, which serve as intermediates in the formation of bioactive compounds, such as jasmonic acid [50]. The LOX gene was reported to be dramatically induced in response to the MeJA treatment in sweet basil plant [31].

This MeJA has been found to trigger secondary metabolite formation through the shikimate pathway. The shikimate pathway leads to the production of the aromatic amino acids namely phenylalanine, tryptophan and tyrosine which in turn lead to the formation of a number of secondary metabolites such as phenylpropanoids from phenylalanine, lignins, flavonoids, phytoalexins and alkaloids from tyrosine involving a series of other pathways such as the mevalonate and non-mevalonate for the production of terpenoids [51]. The DAHPS gene encoding the first enzyme of the shikimate pathway has been reported to be induced by meja and physical wounding [52, 53].

Accumulation of endogenous JA and MeJA has also been observed after treatment of cell cultures with fungal elicitors [54, 55]. Application of chitosan (which is the deacetylated derivative of chitin) to plants through cut stems, led to a rapid increase in jasmonic acid content, confirming the activation of the octadecanoic pathway. Chitosan was reported to increase the endogenous levels of 2-oxo-phytodeionic and jasmonic acids of rice, leading to the activation of the octadecanoic acid pathway [56].

CONCLUSION

The evolving importance of the secondary metabolites has resulted in a high level interest in the possibility of altering their production through cell culture technology. With the establishment of cell culture technology, several strategies were put forward for the enhanced production of metabolites in less amount of time when compared to the normal cell cultures. One such strategy was the use of elicitors to trigger cell growth and secondary metabolite formation in less time.

MeJA and chitosan were found to trigger the high volumes of the major secondary metabolites such as phenols, alkaloids and terpenoids in the cell cultures of the three *Ocimum* species because of their effective action on the shikimate pathway, the pathway mainly responsible for the production of secondary metabolites from the primary metabolites. Phenols such as rosmarinic acid have been widely studied in *Ocimum* species and other members of Lamiaceae. Alkaloids constitute most of the valuable drugs such as powerful painkillers and as well as used as stimulants and narcotics.

Terpenoids are important commercially as aroma substances and pharmaceuticals. Along with phenylpropanoids, terpenoids constitute the essential oils which are also used as pharmaceutical, flavor and aroma agents and well noted among *Ocimum* species. This study mainly serves as a platform to build further investigation of screening and isolation of economically important individual compounds from the elicited cell cultures of *O. basilcium* L., *O. sanctum* L. and *O. gratissimum* L on an industrial scale.

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CONFLICT OF INTERESTS

Nil

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