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Effect of methyl jasmonate and chitosan on growth characteristics of *Ocimum basilicum* L., *Ocimum sanctum* L. and *Ocimum gratissimum* L. cell suspension cultures

Rebecca Mathew* and P. Deepa Sankar

Plant Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore 32014, Tamil Nadu, India.

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The growth characteristics of *Ocimum basilicum* L., *Ocimum sanctum* L. and *Ocimum gratissimum* L. cell suspension cultures treated with elicitors, methyl jasmonate and chitosan individually and in combination were studied. Methyl jasmonate and chitosan were found to enhance accumulated cell biomass when used individually, but when administered in combination there was no significant enhancement of cell biomass. Reduction in cell growth was not observed in combined elicitor treatments. Of the two elicitors, methyl jasmonate was found to strongly influence cell growth; 25 µM of methyl jasmonate accumulated highest biomass at 12 h for *O. basilicum* and 48 h for *O. sanctum*, while 50 µM of methyl jasmonate at 8 h was found to be optimal to obtain maximal enhancement of biomass for *O. gratissimum*. Moreover, chitosan at 200 mg/L (24 h) was found to be optimal for *O. basilicum* and 50 mg/L at 24 h for both *O. sanctum* and *O. gratissimum*. Our study demonstrates that elicitors such as methyl jasmonate and chitosan could effectively enhance cell biomass in lesser time and hence can be used for effective induction of phytochemicals in *O. basilicum*, *O. sanctum* and *O. gratissimum*.

Key words: Cell suspension, elicitation, methyl jasmonate, chitosan, *Ocimum basilicum* L., *Ocimum sanctum* L. and *Ocimum gratissimum* L.

INTRODUCTION

*Ocimum* (Lamiaceae) is ranked high among the herbs for having enormous medicinal potentialities. It consists of about 160 species spread over tropical, sub-tropical and warmer parts of temperate regions of the world (Pushpagandan and Bradu, 1995). *O. basilicum* (sweet basil), *O. sanctum* (holy basil) and *O. gratissimum* (shrubby basil) are herbaceous plants with extraordinary medicinal, aromatic and culinary properties and several antioxidant compounds (Sahoo et al., 1997; Prakash and Gupta, 2005; Gopi et al., 2006). The medicinal, culinary and aromatic value of these species depends on their bioactive phytochemical constituents that produce definite physiological action in the human body (Krishnaiah et al., 2009). Some of their most important bioactive phytochemical constituents include alkaloids, flavonoids, phenolics, terpenoids and essential oils. Extraction of these secondary metabolites from naturally grown whole plants on a commercial basis involves large-scale crop cultivation (Kieran et al., 1997). However, less availability of cultivable land due to the increase in world population (Rao and Ravishankar, 2002) and the fast disappearance of natural habitats for medicinal plants along with environmental and geopolitical instabilities make it increasingly difficult to acquire plant-derived compounds (Mulabagal and Tsay, 2004).

Plant cell culture provides an alternative approach to whole plant for the production of high value secondary metabolites. Plant cells being biosynthetically totipotent, can produce the range of secondary metabolites found in the parent plant along with the following advantages...
independent of geographical and seasonal variations and environmental factors, continuous supply of products with uniform quality and yield and rapidity of production (Rao and Ravishankar, 2002). The increased production of cell biomass and secondary metabolites from plant cell cultures through elicitation has opened up a new area of research, having important economical benefits for bio industry (Angelova et al., 2006; Rao and Ravishankar, 2002). Methyl jasmonate (MeJA) derived from linolenic acid by the octadecanoid pathway has been shown to be a powerful inducer of secondary metabolites in various plants (Kim et al., 2006; Keinanen et al., 2001; Dixon and Paiva, 1995). Chitosan (β-(1,4)-glucosamine polymer) produced by the deacetylation of chitin has also been found to induce various plant defense responses (Agarwal et al., 2002). However, the effect of chitosan on induction of secondary metabolites has not been widely investigated (Kim et al., 2005).

Herein, investigation was carried out to study the influence of two elicitors, MeJA and chitosan, used individually and in combination on the growth characteristics of cell suspension cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* under *in vitro* conditions for the rapid production of major secondary metabolites.

**MATERIALS AND METHODS**

**Plant cell culture**

Seeds of *O. basilicum*, *O. sanctum* and *O. gratissimum* were received from University of Agricultural Sciences (UAS), Bangalore, India. Callus was initiated and sub cultured from cotyledonal leaves of *O. basilicum*, *O. sanctum* and *O. gratissimum* on solid Murashige and Skoog (MS) culture medium (1962) with sucrose (3%, w/v), myoinositol (100 mg/L) supplemented with 0.5 mg/L benzyladenine (BA) and 1.0 mg/L 2,4-dichlophenoxacylic acid (2,4-D) (Mathew and Sankar, 2011a). Cell suspension cultures of *Ocimum* species were established and maintained in 250 ml Erlenmeyer flasks containing 100 ml of liquid MS culture medium 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. Sub culture was performed on day 32 for *O. basilicum*, day 50 for *O. sanctum* and day 46 for *O. gratissimum*, where maximum accumulation of cell biomass was observed (Mathew and Sankar, 2011b).

**Elicitor treatment**

**Methyl jasmonate**

Methyl jasmonate (Sigma-Aldrich, Germany) was dissolved in ethanol, filter-sterilized (Acrodisc® Syringe Filter 0.22 μM, PALL Life Sciences, USA) and added to the sub cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* at final concentrations of 25, 50, 100 and 150 μM. Culture with equal volumes of ethanol and cultures not elicited with MeJA were maintained as control variants for each of the *Ocimum* species. Samples were taken at time 0 (when the elicitor was added) and after 4, 8, 12, 24, 36, 48, 72, 96 and 120 h of culture (Georgiev et al., 2007).

**Chitosan**

Chitosan (Sigma-Aldrich, Germany) was prepared as suggested by Popp et al. (1997). Chitosan was dissolved in 5% (v/v) 1 N hydrochloric acid (HCl) through gentle heating and continuous stirring. pH was adjusted to 5 with 1 N sodium hydroxide (NaOH) and the final concentration was adjusted to 10 mg/ml. The solution was stirred to dissolve chitosan further and then autoclaved for 15 min at 121°C. The solution was kept at 4°C prior to use. Chitosan was added to the sub cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* at final concentrations of 50, 100, 200 and 300 mg/L. Cultures with equal volumes of 5% (v/v) 1 N HCl and cultures not elicited with chitosan were maintained as the control variants for each of the *Ocimum* species (Chang et al., 1998). Samples were taken at time 0 (when the elicitor was added) and after 4, 8, 12, 24, 36, 48, 72, 96 and 120 h of culture.

**Combination of elicitors**

Combinations of MeJA and chitosan (Zhang et al., 2000) were added to the sub cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum*. Samples were taken at time 0 and after 4, 8, 12, 24, 36, 48, 72, 96 and 120 h of culture.

**Growth analysis**

Growth characteristics of the elicited and control cultures of *Ocimum* species were analyzed in terms of fresh weight. Harvesting of suspension cells was performed aseptically in triplicates at 0, 4, 8, 12, 24, 36, 48, 72, 96 and 120 h of culture. Fresh weight was determined by transferring known volume of cell culture to pre-weighted centrifuge tube. Tube was spun at 200 g until the supernatant was free of cells. Supernatant was discarded without disturbing the pellet and then the centrifuge tube was weighed with the cells (Dixon and Gonsalves, 1994).

**Statistical analysis**

Experiment was repeated three times and triplicates were maintained for individual species. All data are represented as mean ± SE. Values were recorded up to 120 h of culture. Data were analyzed by one way analysis of variance (ANOVA) at 5% probability level using SPSS software (version 16).

**RESULTS AND DISCUSSION**

**Plant cell culture**

Callus was initiated from cotyledonal leaf explant material of *O. basilicum*, *O. sanctum* and *O. gratissimum* cultured on solid MS media supplemented with 1.0 mg/L 2,4-D + 0.5 mg/L BA. Friable calli was obtained by sub culturing callus masses every 4 weeks onto fresh solid MS media supplemented with 1.0 mg/L 2,4-D + 0.5 mg/L BA (Mathew and Sankar, 2011a). Friable callus was obtained at the 5th sub culture.

Cell suspension culture was initiated by transferring actively growing friable calli (after 15th day of 5th sub culture) to 250 ml Erlenmeyer flasks containing 100 ml of liquid MS media with 1.0 mg/L 2,4-D + 0.5 mg/L BA. The cell biomass accumulation expressed in gram fresh
weight and dry weight per 5 ml suspension was studied. A typical growth curve was obtained with lag phase for 16 days, log phase from day 18 and stationary phase on the 34th day for *O. basilicum*, 52nd day for *O. sanctum* and 48th day for *O. gratissimum*. Significant fresh weight accumulation was observed for 14 days for *O. basilicum*, 32 days for *O. sanctum* and 28 days for *O. gratissimum*. Maximum increase in fresh weight was observed on 32nd day for *O. basilicum*, 50th day for *O. sanctum* and 46th day for *O. gratissimum*. Cell cultures were sub cultured on the day of maximum cell biomass accumulation (found between mid log phase and early stationary phase) of each of the species to enhance aggregation and secondary metabolite production (Mathew and Sankar, 2011b).

**Elicitation**

Elicitation studies conducted with different concentrations of MeJA and chitosan on cell suspension cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* showed an enhancement of accumulated cell biomass from their time of addition (0th hour). The growth curves of elicited cell suspension cultures of the three *Ocimum* species are shown in Figures 1, 2 and 3 and were compared with the growth curve of non-elicited cell cultures in terms of accumulated biomass content and passage of time. MeJA and chitosan have been reported to increase secondary metabolites majorly phenols and terpenoids in *O. basilicum* plant (Kim et al., 2005, 2006). These metabolites are produced through shikimate, mevalonate and non-mevalonate pathways. Further reports also suggest the effect of these octadecanoid elicitors on isoprene metabolite biosynthesis and accumulation in a number of plants namely, the interaction of jasmonates with wounding and/or fungal elicitation in *Hyoscyamus muticus* root cultures (Singh et al., 1998), *Solanum tuberosum* (Choi et al.,1994) and *Tessaria absinthiodes* (Kurina and Donadel, 2003).

**Effect of MeJA**

Effect of MeJA on growth characteristics of *O. basilicum* cell suspension culture was studied (Figure 1a). MeJA at different concentrations triggered rapid cell biomass accumulation in significantly less amount of time when compared to the log phase of non elicited cell suspension culture. Maximum enhancement of cell biomass was observed at 12 h for different concentrations of MeJA used. Low concentration of MeJA (25 µM) showed the highest accumulation of biomass (0.954 g ± 0.03 FW/5 ml) and 150 µM showed the lowest (0.394 g ± 0.011 FW/5 ml). In *O. sanctum* (Figure 1b), highest accumulation of cell biomass was observed with 25 µM (0.762 g ± 0.01 FW/5 ml) at 48 h and the lowest with 150 µM (0.274 g ± 0.01 FW/5 ml) at 36 h. While in *O. gratissimum* (Figure 1c), MeJA at 50 µM produced maximal biomass at 8 h (1.464 g ± 0.1 FW/5 ml) and 150 µM at 12 h produced the lowest (0.601 g ± 0.02 FW/5 ml). Among the three *Ocimum* species treated with methyl jasmonate, *O. gratissimum* accumulated the highest biomass content. MeJA at 25 µM produced the highest cell content at 12 h for *O. basilicum* and 48 h for *O. sanctum*. Meanwhile, 50 µM of MeJA at 8h was found to be optimal to obtain maximal enhancement of biomass for *O. gratissimum*. Similar study conducted on *Lavandula vera* MM cell cultures showed the highest biomass accumulation for 50 µM MeJA at 4 h of elicitation (Georgiev et al., 2007).

Increase in MeJA concentration showed a gradual decrease in accumulated biomass for all the three species. Concentrations at and above 100 µM of MeJA showed a decrease in cell biomass enhancement, which could be due to the fact that high elicitor concentrations lead to cell death (Rijhwani and Shanks, 1998). In *L. vera* MM cell suspension cultures, all concentrations of methyl jasmonate were reported to show enhancement of accumulated biomass only at 4 h of addition and a lower enhancement of biomass accumulation at 100 to 150 µM of MeJA (Georgiev et al., 2007). Non-elicited cell culture and cultures with equal volumes of ethanol (as that of methyl jasmonate added to elicited cell cultures; culture with highest volume of ethanol represented) were used as control variants for each of the species. Both non-elicited cell culture and cultures with ethanol were observed to follow similar growth pattern, thereby ruling out any effect that may be produced by ethanol on cell growth.

**Effect of chitosan**

Figure 2a shows the effect of chitosan on *O. basilicum* cell cultures. Concentrations from 50 to 200 mg/L of chitosan influenced biomass accumulation in narrow borders and the highest accumulation of cell biomass (0.716 g ± 0.01 FW/5 ml) was observed for 200 mg/L at 12 h. A drop in biomass enhancement was observed at concentration 300 mg/L. Kim et al. (2005) reported an increase in the height and weight of *O. basilicum* plant by 17 and 12% with the treatment of chitosan up to 0.1%; concentrations above 0.1% were reported to decrease the growth of sweet basil plant. Similar observations were also made in other reports such as soya bean sprout, *Vitis vinifera* L. and laver (No et al., 2003; Kim et al., 2003; Barka et al., 2004).

Increase in cell content of *O. sanctum* (Figure 2b) on treatment with chitosan was comparatively less when compared to that of *O. basilicum* and *O. gratissimum*. Cell biomass of 0.354 g ± 0.01 FW/5 ml was observed as the highest for 50 mg/L of chitosan at 24 h. The lowest (0.089 g ± 0.01 FW/5 ml) was observed at 300 mg/L.
Figure 1. The dynamics of biomass changes in Ocimum species after treatment with different concentrations of methyl jasmonate. Experiment was repeated three times, with triplicates maintained for each treatment on individual species. Values are mean ± SE, and were found significant by ANOVA.
Figure 2. The dynamics of biomass changes in *Ocimum* species after treatment with different concentrations of chitosan. Experiment was repeated three times, with triplicates maintained for each treatment of individual species. Values are mean ± SE, and found significant by ANOVA.
Figure 3. The dynamics of biomass changes in Ocimum species on combined elicitor treatments. Experiment was repeated three times, with triplicates maintained for each treatment of individual species. Values are mean ± SE and found non-significant by ANOVA.
Gradual decrease in biomass enhancement was observed from 100 to 300 mg/L of chitosan. In *O. gratissimum* (Figure 2c), 50 mg/L of chitosan enhanced biomass content to 0.650 g ± 0.011 FW/5 ml at 24 h; lowest enhancement (0.395 g ± 0.016 FW/5 ml) was observed for 300 mg/L at 24 h.

Results show chitosan at 200 mg/L (24 h) to be optimal for *O. basilicum*, *O. sanctum* and 50 mg/L (24 h) for *O. gratissimum*. Studies conducted on chitosan elicited cell cultures of *Mentha piperita* and *Polygonum tinctorium* showed 200 mg/L of chitosan to be optimal for the accumulation of highest menthol and indirubin concentration (Chang et al., 1998; Kim et al., 1997). Non-elicited cell cultures and cultures with equal volumes of 5% (v/v) 1 N HCl (as that of chitosan added to elicited cell cultures; culture with highest volume of 5% (v/v) 1 N HCl represented) were used as the control variants. Growth pattern of cultures with diluted HCl did not differ from that of non elicited cultures.

**Combined effect of elicitors**

To observe the combined effect of elicitors on *O. basilicum*, *O. sanctum* and *O. gratissimum*, concentrations of MeJA that produced the highest cell biomass were combined with that of chitosan. Hence, 25, 50, 100 μM of MeJA were combined with 200, 300 and 50 mg/L of chitosan for *O. basilicum* (Figure 3a). Thorough enhancement of cell weight was observed from the time of addition, cell biomass changes were not significant for and among each of the combinations used. Among the three combinations, 25 μM MeJA with 200 mg/L of chitosan produced a cell mass of 0.456 g ± 0.01 FW/5 ml at 8 h, which is comparatively less than the cell weight observed for individual elicitor treatments. Moreover, for *O. sanctum*, 25, 50 and 100 μM of MeJA were combined with 50, 100 and 200 mg/L of chitosan (Figure 3b). Combination of elicitors did not show any significance. The highest cell weight obtained was 0.301 g ± 0.01 FW/5 ml at 4 h, which was for 100 μM MeJA with 200mg/L chitosan. Furthermore, 50, 25 and 100 μM of MeJA were combined with 50, 100 and 200 mg/L of chitosan for *O. gratissimum* (Figure 3c). And as observed in *O. basilicum* and *O. sanctum*, significant increase in cell weight was not observed when MeJA and chitosan were used together for *O. gratissimum*. Combination of 25 μM MeJA with 10 mg/L chitosan enhanced cell weight to 0.359 g ± 0.01 FW/5ml (as the highest), which is comparatively less to that of cell weight observed for MeJA and chitosan used individually. In general, combination of methyl jasmonate and chitosan did not enhance biomass significantly as that of individual elicitor treatments for the three * Ocimum* species, although they did not show a reduction in cell growth than that of control cultures as reported by Premjet et al. (2002) and Sudha and Ravishankar (2003). In *Taxus baccata*, though no significance was observed in cell growth under combined elicitor treatments, enhanced production of secondary metabolites was observed (Khosroushahi et al., 2006).

Elicitors reduce the process time required for increased culture volumes and attainment of high product concentrations (Barz et al., 1988). In this study, addition of MeJA and Chitosan individually and in combination were found to trigger cell biomass accumulation; the exponential phase of the cell growth cycle achieved in considerably lesser amount of time. When these elicited cultures were prolonged in their medium, gradual decrease in cell weight was observed which could be due to cell death caused by depletion of substrate in the medium. With control cultures following their regular pattern of cell growth (exponential phase observed only from day 18 for the three * Ocimum* species), gradual increase in cell weight was observed at the end of each experiment, whereas the elicited cell cultures which had already attained exponential growth within a few hours of elicitation showed a decrease in cell weight towards the end. Of the two elicitors used individually, methyl jasmonate strongly influenced cell growth and therefore could be suggested as an effective elicitor for the three * Ocimum* species. Methyl jasmonate was also suggested to be an effective elicitor for * Coleus* and * Lavandula* species of Lamiaceae family (Szabo et al., 1999; Li et al., 2005; Georgiev et al., 2007) and in * Silybum marianum* (Hasanloo et al., 2008).

With reports on MeJA and chitosan affecting cell growth and secondary metabolite production in different plant cell cultures, the present investigation on growth characteristics of MeJA and chitosan elicited cell cultures of *O. basilicum*, *O. sanctum* and *O. gratissimum* proves a greater possibility of rapid production of major secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids, and essential oil from these plants.

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