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# Cell line selection combined with jasmonic acid elicitation enhance camptothecin production in cell suspension cultures of *Ophiorrhiza mungos* L

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**Abstract** *Ophiorrhiza mungos* is a herbaceous medicinal plant which contains a quinoline alkaloid, camptothecin (CPT), an anticancer compound. A high-yielding cell line, *O. mungos* cell line-3 (OMC3) was selected from cell suspension cultures of *O. mungos* using cell aggregate cloning method and established cell suspension culture. OMC3 cell suspension produced significantly high biomass (9.25 ± 1.3 g/flask fresh weight (FW)) and CPT yield (0.095 ± 0.002 mg $g^{-1}$ dry weight (DW)) compared with the original cell suspension. Inoculum size of OMC3 cell suspension culture was optimised as 14 g $L^{-1}$. Media optimisation has shown that 5 % (*w*/*v*) sucrose and an increased ammonium/nitrate concentration of 40/20 mM favoured CPT production, whereas 3 % (*w*/*v*) sucrose, an ammonium/nitrate concentration of 20/40 mM and 1.25 mM of phosphate favoured biomass accumulation. Jasmonic acid, chitin and salicylic acid was used to elicit CPT production in the original cell suspension culture and achieved significantly high CPT production with jasmonic acid (JA) elicitation. Further, OMC3 cell suspension culture was elicited with JA (50 μM) and obtained 1.12 ± 0.08 mg $g^{-1}$ DW CPT and 9.52 ± 1.4 g/flask FW (190.4 g $L^{-1}$ FW). The combination of cell line selection and elicitation has produced 18.66-fold increases in CPT production together with significantly high biomass yield. The study is helpful in the scale-up studies of *O. mungos* cell suspension culture in suitable bioreactor systems for the production of CPT.

**Keywords** Camptothecin · Cell aggregate cloning · Cell line · Inoculum size · Jasmonic acid · Elicitation

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-016-7808-x) contains supplementary material, which is available to authorized users.

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## Introduction

*Ophiorrhiza mungos* (Rubiaceae) is a herbaceous medicinal plant distributed throughout the Western Ghats of India. Traditionally, *O. mungos* have been used against snakebite as the plant contains potent antisnake venom-neutralising compounds (Krishnan et al. 2014). *O. mungos* is reported to contain a quinoline alkaloid, camptothecin (CPT), with anticancer activity (Tafur et al. 1976). Due to the toxicity and low solubility of CPT extracted from natural sources, several semisynthetic analogues of CPT have been synthesised for the treatment of different types of cancers. Three of the semisynthetic analogues of CPT being used as anticancer drugs are topotecan, irinotecan and belotecan (Liu et al. 2015). CPT and its derivatives exhibit several other biological properties also (Clements et al. 1999; Uday Bhanu and Kondapi 2010; Liu et al. 2015; Huang et al. 2015).

The worldwide market value for CPT derivatives was estimated at about US$750 million in 2002, which rose to US$1 billion by 2003 and has reached to US$2.2 billion in 2008 (Lorence and Nessler 2004; Sankar-Thomas et al. 2008). This represents approximately 1 tonne of CPT in terms of natural material (Watase et al. 2004). CPT is commercially extracted from natural resources such as the seeds of *Camptotheca acuminata* and the bark of *Nothapodytes foetida* (Namdeo 2007) which have been causing a decline in their

natural population (Hombe Gowda et al. 2002). For the stable supply of CPT and for the conservation of current sources, the identification of other natural sources of CPT together with the development of various viable alternative in vitro culture methods are essential.

Cell suspension culture is one of the efficient and alternative in vitro culture method used for the production of high-value bioactive molecules (Mulabagal and Tsay 2004). Several studies have been focused on the production of secondary metabolites using cell suspension cultures but low productivity is the major drawback for large-scale production and commercialisation (Yue et al. 2016). Cell suspension culture of *O. mungos* was established from field-grown leaf explants which contained 0.06 ± 0.004 mg $g^{-1}$ dry weight (DW) of CPT as reported earlier by Deepthi and Satheeshkumar (2016). Wink et al. (2005) have reported that CPT content in cell suspension culture of *O. mungos* was low (0.004 ± 0.002 mg $g^{-1}$ DW) when compared with root and hairy root cultures. Cell suspension cultures of *C. acuminata* contained only 0.00254 mg $g^{-1}$ DW of CPT (Sakato et al. 1974). Many strategies like optimisation of various culture parameters, elicitation, precursor feeding, and cell line selection have been used to enhance the biomass and secondary metabolite yield in cell suspension cultures (Hussain et al. 2012).

Selection of high-yielding cell line is a successful method for the enhanced production of biomass and secondary metabolite. Strains producing high levels of ubiquinone 10 were isolated from tobacco cell suspension cultures by a cell cloning technique. Clonal selection from *Cytisus scoparius* callus cultures produced a high amount of vitamin B6-producing strains (Yamada and Watanabe 1980). A high nicotine-producing cell line was selected from tobacco callus by single cell cloning method (Ogino et al. 1978). *Coptis japonica* cells with high production of berberine were produced by cloning small cell aggregates (Sato and Yamada 1984).

In the present study, cell line selection by cell aggregate cloning method was attempted to select high-yielding cell line from cell suspension cultures of *O. mungos*. From the cell colonies formed, ten cell colonies (OMC1–OMC10) were selected, and among them, three cell colonies (OMC3, OMC7 and OMC9) were found highly proliferative and accordingly selected for establishing cell suspension cultures. Cell biomass and CPT production in the three cell lines were studied and found that OMC3 was superior in terms of biomass and CPT production and thus selected for further optimisation studies. The effect of inoculum size, carbon source and its concentration, concentration of total nitrogen and nitrogen sources (ammonium ($NH_4^+$) and nitrate ($NO_3^-$)) and concentration of phosphate on biomass and CPT production were studied in OMC3 cell line.

Elicitation studies were performed in the original cell suspension with various elicitors, and jasmonic acid (JA; 50 μM) was identified as the suitable elicitor for enhanced CPT production. Finally, OMC3 cell line was elicited with JA (50 μM) on the 25th day of culture period and produced an 18.66-fold increase in CPT yields on the 5th day of incubation. Cell line selection combined with JA elicitation has significantly enhanced CPT production (1.12 ± 0.08 mg $g^{-1}$ DW) with significantly high biomass production (9.52 ± 1.4 g/flask fresh weight (FW)).

The results obtained in the present study showed that cell line selection through cell aggregate cloning combined with elicitation using a suitable elicitor is an efficient strategy to obtain significantly high biomass and CPT yield. The results indicated that high $NH_4^+$ to $NO_3^-$ concentration (40/20 mM) and 5 % (*w*/*v*) sucrose in the medium favoured CPT production whereas low $NH_4^+$ to $NO_3^-$ concentration (20/40 mM), high phosphate concentration, an inoculum size of 14 g $L^{-1}$ and 3 % (*w*/*v*) sucrose favoured biomass accumulation. The knowledge gained through this study on cell biomass and CPT production will be helpful in the scaling up studies of *O. mungos* cell suspension cultures in suitable bioreactor systems for the large-scale production of CPT. This study, to the best of our knowledge, is the first report in *O. mungos* on enhanced production of CPT through the selection of high-yielding cell line combined with JA elicitation.

## Materials and methods

### Cell line selection using cell aggregate cloning method

Cell suspension cultures were established from green-friable callus tissues induced from field-grown leaf explants cultured in 250 mL Erlenmeyer flask containing 50 mL 1/2 strength MS liquid medium supplemented with 3 % (*w*/*v*) sucrose, 3.0 mg $L^{-1}$ 1-naphtaleneacetic acid (NAA), 1.0 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg $L^{-1}$ kinetin (KIN) as described earlier by Deepthi and Satheeshkumar (2016). All the cultures were kept under 12 h illumination provided using cool white fluorescent tube lights of 40 W (Philips India Ltd.) at an intensity of 40 μE $m^{-2}$ $s^{-1}$ at 25 ± 2 °C. The cell cultures were agitated on a gyratory shaker (New Brunswick- USA) at 90 rpm.

Cells were plated according to a modified method described by Matsumoto et al. (1981). Cell suspension cultures on the 20th day of incubation were filtered aseptically using a nylon mesh (350 μm) to remove cell clumps of larger size. The filtrate consisted of individual cells and small cell aggregates. One millilitre ($2.5 \times 10^5$ cells) of the filtrate which contained approximately 15–16 cell aggregates was plated into sterile disposable Petri plates (10 × 1.5 cm diameter) containing 1/2 strength MS solid medium supplemented with 3 % (*w*/*v*) sucrose, 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN and the cultures were incubated for 25 days

under the laboratory conditions described earlier. The experiment was replicated in 20 Petri plates.

The number of visible colonies formed per Petri plate was counted, and the mean of five counting was taken as the average number of colonies per Petri plate. The sealed Petri plates were observed under an inverted stereo microscope and marked the positions of a total of 80 small cell aggregates to facilitate counting to ensure isolation of cell colonies. The growth of the cell colonies in the Petri plates was observed periodically under the stereo microscope and its diameter measured for a period of 25 days. Ten cell colonies (OMR1-OMR10) were selected based on their growth (diameter of the cell colony). The selected cell colonies were transferred into sterile Petri plates containing solid 1/2 strength MS medium supplemented with the same hormonal regime and cultured for 25 days. Out of the ten cell colonies, only three of them (OMC3, OMC7 and OMC9) were found to proliferate profusely to form a thick layer of cells over a period of 25 days.

### Establishment of cell suspension cultures using selected cell lines and growth curve study

FW at 0.5 g from each of the well-established cell colonies (OMC3, OMC7 and OMC9) were transferred into 250 mL Erlenmeyer flasks containing 50 mL of the same media, without agar and kept on a gyratory shaker at 90 rpm under the laboratory conditions described earlier to establish cell suspension cultures. The growth of cell suspension cultures (OMC3, OMC7 and OMC9) against different periods of incubation up to 35 days was studied in detail. To study the growth curve of the cultures, on the 25th day of culture period, 0.5 g FW cells of OMC3, OMC7 and OMC9 cell suspension was transferred to 250 mL Erlenmeyer flask containing 50 mL 1/2 strength MS liquid medium supplemented with the same hormonal regime and the cultures were kept on a gyratory shaker at 90 rpm. At every 5 days, cells were harvested, washed with sterile distilled water and blotted on a filter paper for FW determination. DW of the cells was determined after drying the cells in a hot air oven at 60 °C for 16 h. CPT was also estimated periodically. OMC3 cell line which produced the maximum biomass and CPT yield was selected for further experiments.

### Influence of different culture conditions on cell growth and CPT synthesis in OMC3 cell line

Media parameters including inoculum size, carbon source and its concentration, concentration of total nitrogen and nitrogen sources ($NH_4^+$ and $NO_3^-$) and different concentrations of phosphate were tested to study its influence on cell growth and CPT yield in the OMC3 cell line. To identify the optimum conditions, in 1/2 strength MS medium the desired component was changed while other constituents were kept constant.

OMC3 cells cultured in 50 mL 1/2 strength MS medium containing 3 % (*w*/*v*) sucrose, 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN were used for the experiments. The growth of the cells was detected periodically up to 25 days at a 5-day interval and measured the FW, DW and growth index (GI). CPT content was also estimated.

### Inoculum size

To determine the optimum inoculum size (*w*/*v*) of cells required for obtaining maximum biomass, different inoculum sizes such as 2, 6, 10, 14, 20 and 30 g $L^{-1}$ were transferred each into 50 mL 1/2 strength MS liquid medium containing 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN. Each inoculation size was repeated twice and growth was determined by measuring the FW, DW and CPT yield on the 25th day. GI was also calculated.

### Carbon source

The carbon sources used for the study were sucrose, dextrose, lactose, maltose and fructose. FW at 0.7 g (14 g $L^{-1}$ cells) was transferred to 1/2 strength MS liquid media containing 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN and different carbon sources each at 3 % (*w*/*v*), and cell growth was studied up to 25 days. Based on the results obtained with different carbon sources, further experiments were conducted to identify the optimum concentration of sucrose for high biomass and CPT production. The cells (0.7 g FW) were transferred to 50 mL MS liquid media containing the same hormonal regime and varying levels of sucrose, i.e. 1, 2, 3, 4, 5 and 6 % (*w*/*v*). The growth of cells (FW and DW) and production of CPT were recorded up to 25 days.

### Total nitrogen and ammonium/nitrate concentration

Effect of the total nitrogen content (20, 40, 60, 80 and 100 mM) in the medium on cell growth and CPT production was studied. The effect of $NH_4^+$ and $NO_3^-$ on cell growth and CPT accumulation was studied at a total inorganic nitrogen concentration of 60 mM (optimum total nitrogen content) by changing the molar ratio of $NH_4^+/NO_3^-$ (mM/mM) to 60:0, 50:10, 10:50, 40:20, 20:40, 0:60 and 30:30. $NH_4Cl$ was used as the source of $NH_4^+$ in place of $NH_4NO_3$, and $KNO_3$ was used as the source of $NO_3^-$ in 1/2 strength MS liquid media supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D, 0.5 mg $L^{-1}$ KIN and 3 % (*w*/*v*) sucrose. The growth of the cells was detected up to 25 days at a 5-day interval, and the FW, DW and CPT yields were measured.

### Phosphate concentration

To study the growth and CPT production of cells, 1/2 strength MS liquid medium supplemented with the same hormonal combination and modified with varied concentration of $KH_2PO_4$ (0, 0.1, 0.2, 0.3125, 0.625, 1.25, 2.5 mM) was used. The growth of the cells was determined up to 25 days at a 5-day interval, and the FW, DW and CPT yields were measured.

### Elicitation studies

Elicitation studies using JA, chitin (CH) and salicylic acid (SA) were carried out using the original cell suspension culture in 1/2 strength MS media supplemented with 3 % (w/v) sucrose, 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN for 15 days, and JA, which produced the highest CPT accumulation, was selected for eliciting OMC3 cell suspension cultures.

Jasmonic acid (Sigma) stock solution was prepared in DMSO (100 mg $mL^{-1}$), diluted to the desired quantity using sterile distilled water and pH adjusted to 5.8 before adding to the media using a sterile filter unit of 0.22 μm (Millipore). JA was used at different concentrations of 25, 50, 100 and 200 μM. Ten grammes of chitin (Sigma) was hydrolysed using concentrated hydrochloric acid, and chitin oligosaccharides were recovered (Rupley 1964; Nahálka et al. 1998). Hydrolysed CH powder (2.0 g) was dissolved in 50 mL sterile distilled water before adjusting the pH to 5.8 and autoclaved. CH was used at various concentrations of 25, 50, 100 and 200 mg $L^{-1}$. Salicylic acid (Sigma) was dissolved in distilled water to a final concentration of 10 mg $mL^{-1}$, pH adjusted to 5.8 and autoclaved (Kang et al. 2006). SA was used at different concentrations of 25, 50, 100, and 200 mM.

During the 15th day of incubation (exponential phase of cell growth), the cell suspension culture was treated with the desired elicitor at the above-mentioned concentrations. Sterile distilled water was added as a control in the same quantities *in lieu* of the elicitor. For each treatment, three replicates were done and the experiment was repeated twice. Cell growth (FW and DW) and CPT content were estimated in the cultures treated with elicitors and in the control cultures on 1st, 5th, 10th and 15th days of elicitation. Cell viability was also estimated using Evans blue stain (0.05 %) according to the method described by Baker and Mock (1994).

### Jasmonic acid elicitation in OMC3 cell line

OMC3 cells were cultured in 1/2 strength MS media supplemented with 3 % (*w*/*v*) sucrose, 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN, and JA (50 μM) was fed into the OMC3 cell suspension culture on the 25th day of culture period (period of maximum growth) and biomass and CPT production were estimated after 5 days of incubation.

### Extraction and camptothecin quantification

The cells were harvested periodically, dried at 60 °C for 16 h in a hot air oven, ground and extracted with 150 mL methanol in a soxhlet apparatus for 6 h at 60 °C and was concentrated using a rotary evaporator (Heidolph). The concentrate was dissolved in 3 mL methanol, filtered using a 0.22-μm filter (Millipore) and used for high-performance liquid chromatography (HPLC) analysis. HPLC analysis was done as per the method described by Fulzele et al. (2001). Isocratic analytical HPLC was performed on a Gilson 321 HPLC system using a Kromasil 100C (250 × 4.6 mm) C-18 column, maintained at room temperature. The mobile phase was acetonitrile/water (45:55 *v*/v) at a flow rate of 1 mL $min^{-1}$. CPT was identified at 254 nm in a detector (Gilson UV/VIS 156) attached to the HPLC system based on a co-chromatography with authentic CPT (Sigma-Aldrich, USA). CPT quantification was done by comparison with the standard curve plotted using different concentrations of authentic CPT in methanol (HPLC grade, Merck), each taken in triplicates.

### Statistical analysis

All the experiments were repeated thrice; the data analysis was done twice and is presented as mean ± standard error. The data were analysed using analysis of variance (ANOVA) using 95 % confidence interval. The statistical significance between contrasting treatments was assessed by Duncan's multiple range test ($P = 0.05$). All analysis was done using the software IBM SPSS statistics 21.

## Results

### Cell line selection using cell aggregate cloning method and establishment of cell suspension culture

Per Petri plate, 8.2 ± 6.4 cell colonies were formed, and thus the plating efficiency was calculated as 51.23 ± 5.2 %. The division of plated single cells was negligible and did not form any colonies. Small cell aggregates divided very fast and developed cell colonies in 25 days (Fig. S1 in Electronic supplementary material). A total of 80 cell colonies were marked in the entire Petri plates altogether. The size of the cell colonies varied from ≈1.0 to 5.0 mm. Ten cell colonies (OMC1-OMC10) which attained the highest diameter in a period of 25 days were selected and transferred to fresh 1/2 strength MS solid medium. Out of them, only three cell colonies (OMC3, OMC7 and OMC9) exhibited luxurious growth. Whereas, other cell colonies did not proliferate further and turned brown in colour within a period of 2 weeks. Cell colonies of OMC3 were pale green in colour, OMC7 cell colonies were pale yellow coloured and OMC9 cell colonies were pale brown

coloured (Fig. S2 in Electronic supplementary material). Among the three cell lines, it was observed that in OMC7 and OMC9, the cell aggregates were compact but in OMC3 cell colonies, the single cells or group of cells were friable. Cell suspension cultures established using the selected cell lines showed profuse growth in a culture period of 25 days (Fig. 1).

## Growth pattern of the cell suspension cultures established from the selected cell lines

Growth pattern of the cell suspension cultures (OMC3, OMC7 and OMC9) and CPT production were recorded for a period of 35 days at a 5-day interval (Fig. 2). Growth started after 10 days and continued up to 30 days. Growth pattern of OMC7 and OMC9 cell lines were similar with maximum biomass and CPT production on the 30th day of incubation. But in OMC3 cell line, maximum biomass and CPT production was obtained on the 25th day of incubation.

High cell biomass was obtained in OMC3 (9.25 ± 1.3 g/flask FW) compared with other cell lines (OMC7, 7.5 ± 0.9 g/flask FW; OMC9, 6.8 ± 1.0 g/flask FW). CPT accumulation was also estimated during different periods of growth in all the cell lines. Maximum CPT (0.095 ± 0.004 mg $g^{-1}$ DW) was detected in OMC3 cell line which was higher than that in OMC7 (0.064 ± 0.002 mg $g^{-1}$ DW) and OMC9 (0.062 ± 0.005 mg $g^{-1}$ DW) cell lines.

## Optimisation of different culture conditions for the enhanced cell growth and CPT production in OMC3 cell line

### *Inoculum size*

Inoculum size of the culture system against fixed volume of nutrient media (50 mL) was studied. Varied inoculum sizes of cells in 50 mL nutrient media were tested, and the results obtained are given in Table 1. It was found that 14 g $L^{-1}$ (0.7 g/flask) was required for obtaining maximum biomass (10.5 ± 0.8 g/flask FW) and CPT production (0.143 ± 0.005 mg $g^{-1}$ DW) over 25 days of incubation. When inoculum size was small, cell growth was very low. It was also noted that the biomass yield had significantly decreased when the inoculum size was above 14 g $L^{-1}$. CPT production was remarkably affected by both low and high inoculum size.

### Carbon source

Results on the growth of OMC3 cell line in media supplemented with different carbon sources (3 %, *w*/*v*) in 1/2 strength MS liquid medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN are presented in Fig. 3a. Among the different carbon sources used, maximum cell growth was recorded in media supplemented with 3 % (*v*/*v*) sucrose. Cell growth in media containing 3 % (*v*/*v*) dextrose was similar to that of sucrose during the early stages of growth but later cell growth was retarded. Cells grown in media containing sucrose and dextrose were greenish yellow and highly proliferative compared with medium containing other carbon sources. Cells cultured in media supplemented with fructose, maltose and lactose turned brown in colour and became necrotic after 20 days of incubation. According to the results, sucrose was chosen as the best carbon source and cells were grown in medium containing various concentrations of sucrose to study its effect on cell growth and CPT yield.

Fourteen grammes per litre of cells were cultured in MS liquid media supplemented with sucrose at varied concentration (1–6 %), and the results are presented in Fig. 3b. Among the different concentrations of sucrose tested, maximum cell growth (10.32 ± 1.2 g/flask FW) was obtained in media containing 3 % (*w*/*v*) sucrose. The concentration of sucrose affected cell growth and CPT production as evident from the results. Cell growth was significantly reduced at sucrose concentration by >5 % (*w*/*v*) and <3 % (*w*/*v*) of sucrose. However, maximum CPT production (0.23 ± 0.004 mg $g^{-1}$ DW) was recorded in cells cultivated in media containing 5 % (*w*/*v*) sucrose.

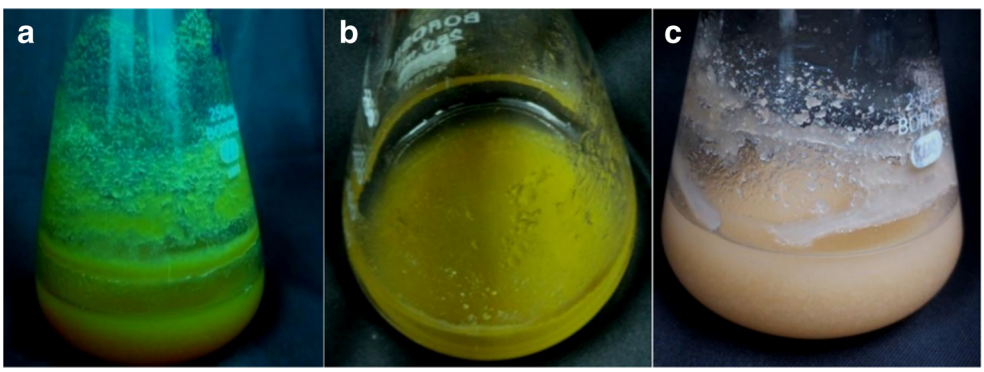


**Fig. 1** Suspension culture of selected cell lines established in 1/2 strength MS liquid medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KN on the 25th day of incubation. **a** OMC3. **b** OMC7. **c** OMC9

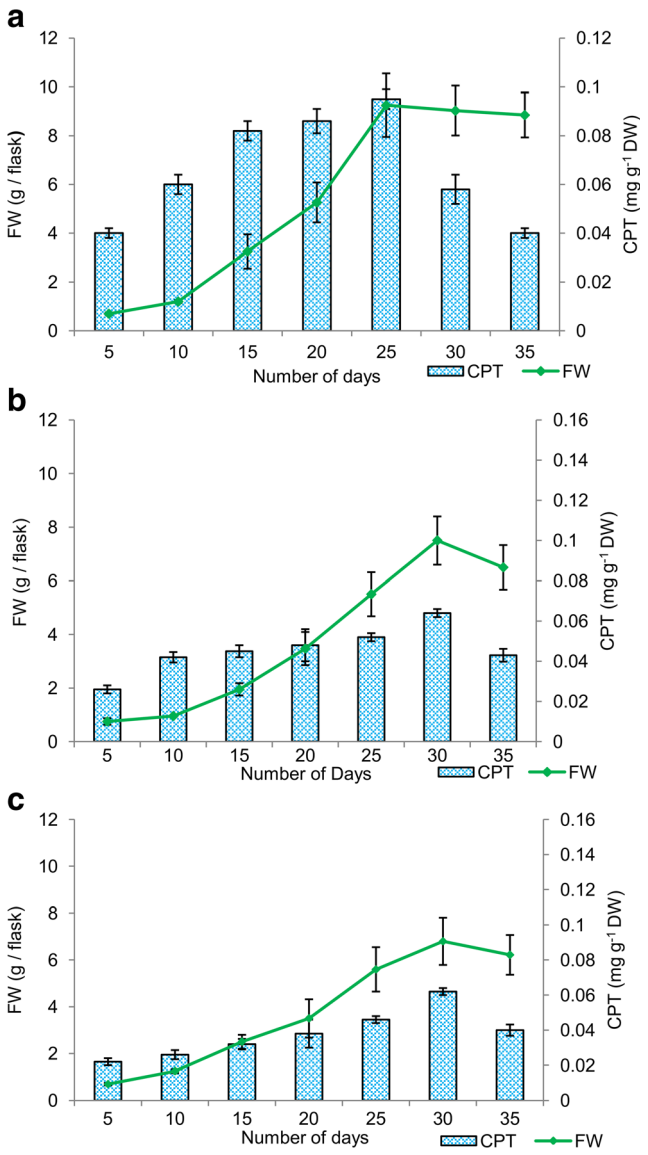


**Fig. 2** Growth curve of selected cell lines cultured in 1/2 strength MS liquid medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN for 35 days at a 5-day interval. Data represents mean ± standard error **a** OMC3. **b** OMC7. **c** OMC9

**Table 1** Influence of various inoculum sizes (2, 6, 10, 14, 20, 30 g $L^{-1}$) on biomass and CPT production in OMC3 cells cultured in 1/2 strength MS media supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN for a period of 25 days

| Inoculum size (g $L^{-1}$) | FW (g/flask) | DW (g/flask) | CPT (mg $g^{-1}$ DW) |
|---|---|---|---|
| 2 | 0.8 ± 0.2f | 0.08 ± 0.03f | 0.024 ± 0.002e |
| 6 | 2.2 ± 0.2e | 0.25 ± 0.04e | 0.042 ± 0.002d |
| 10 | 7.75 ± 0.9b | 0.8 ± 0.08b | 0.091 ± 0.003b |
| 14 | 10.5 ± 0.8a | 1.6 ± 0.15a | 0.143 ± 0.005a |
| 20 | 6.5 ± 0.9c | 0.62 ± 0.06c | 0.062 ± 0.005c |
| 30 | 5.5 ± 0.9d | 0.58 ± .048cd | 0.023 ± 0.03e |

Data represents mean ± standard error. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA

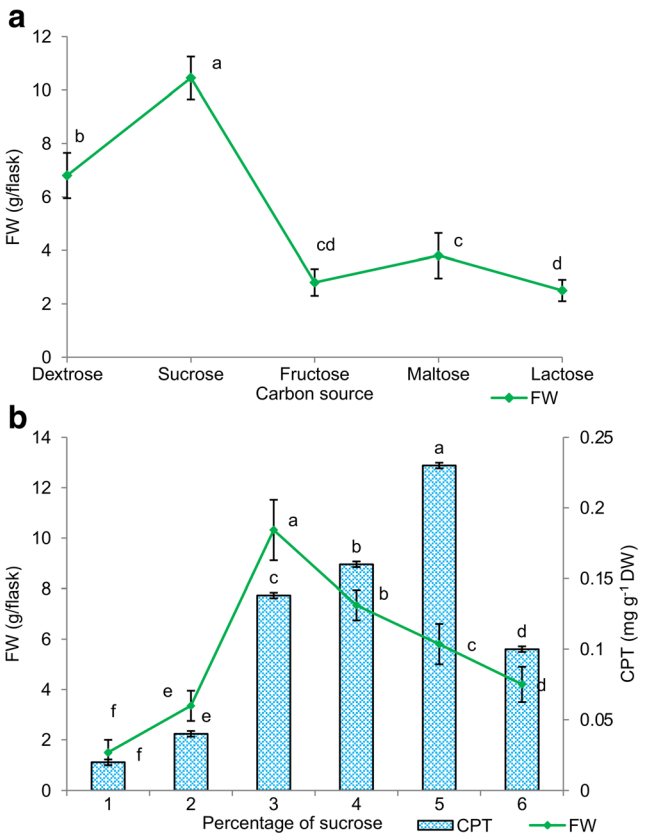


**Fig. 3** **a** Effect of different carbon sources on growth in OMC3 cell suspension culture grown in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN as on the 25th day of incubation. **b** Effect of different concentration of sucrose on growth and CPT yield in OMC3 cell suspension culture grown in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN and incubated for 25 days

## Total nitrogen and ammonium/nitrate concentration

Total nitrogen content at 60 mM in the 1/2 strength MS medium produced high cell biomass (10.42 ± 0.92 g/flask FW) and CPT (0.137 ± 0.0054 mg $g^{-1}$ DW) yield (Fig. 4a). Among the varied proportion of $NH_4^+/NO_3^-$ combinations (60/0, 50/10, 10/50, 40/20, 20/40, 0/60, 30/30 mM) tested, the highest cell growth (12.56 ± 1.2 g FW/flask) was obtained in medium containing 20/40 mM concentration of $NH_4^+/NO_3^-$ (Fig. 4b). When the proportion of $NH_4^+/NO_3^-$ is more than 40/20 mM, there was a drastic decline in cell growth. It was noted that high concentration of CPT (0.35 ± 0.005 mg $g^{-1}$ DW) was recorded in media containing $NH_4^+/NO_3^-$ at 40/20 combination.

## Phosphate concentration

Influence of phosphate on cell growth and CPT production was studied in detail, and the results are presented in Fig. 5. 1/2 strength MS medium containing 1.25 mM phosphate produced good cell growth (12.84 ± 1.1 g/flask FW) compared with other concentrations. When the concentration of

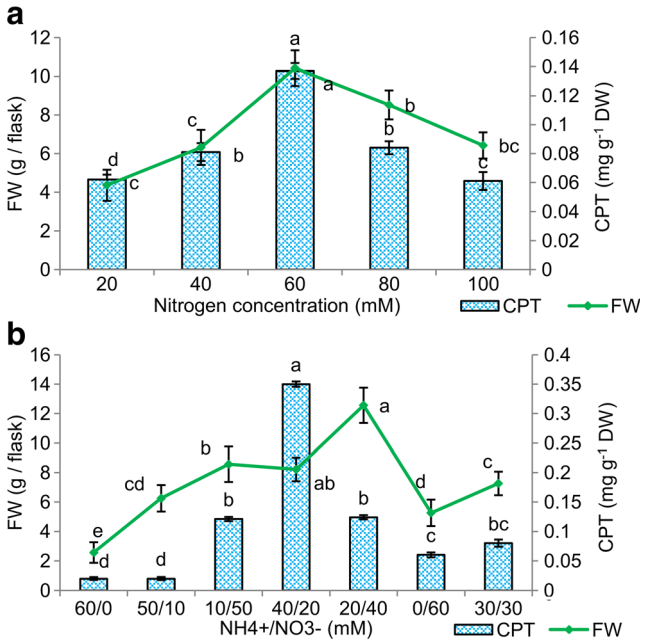


**Fig. 4** **a** Effect of total nitrogen concentration on cell growth and CPT production in OMC3 cell suspension culture grown in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN over a period of 25 days. **b** Effect of different concentrations of $NH_4^+$/ $NO_3^-$ on cell growth and CPT yield in OMC3 cell suspension culture grown in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN incubated for 25 days

phosphate was increased to 2.5 mM, cell growth reduced. Similar CPT yield was recorded in 0.3125, 0.625 and 1.25 mM of phosphate. An increase in the phosphate concentration above 0.3125 mM did not have an impact on enhancing CPT production. The increase in phosphate concentration above the optimum level had a negative effect on both cell growth and CPT production.

### Elicitation studies

CPT and biomass yield varied according to the concentration of elicitors in the medium and incubation period with the

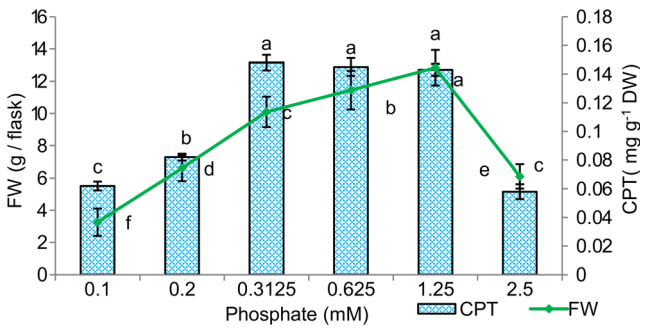


**Fig. 5** Cell growth and CPT production in OMC3 cell suspension culture grown in 1/2 strength MS liquid medium containing different concentrations of phosphate, 3 % (*w*/*v*) sucrose and 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN incubated for a period of 25 days

elicitors (Table 2). The maximum amount of CPT (0.92 ± 0.084 mg $g^{-1}$ DW) was produced when elicited with 50 μM JA, on the 5th day of incubation; 0.5 ± 0.025 mg $g^{-1}$ DW CPT was obtained in cells treated with 100 mg $L^{-1}$ CH on the 10th day of incubation. 0.55 ± 0.032 mg $g^{-1}$ DW CPT was produced in cells treated with 50 μM SA on the 5th day of incubation. There was a progressive decrease in cell growth and cell viability with increasing concentration of JA, CH and SA compared with the control cultures (Fig. 6).

### Elicitation of OMC3 cell suspension culture with jasmonic acid

A significantly high CPT (1.12 ± 0.08 mg $g^{-1}$ DW) and biomass yield (9.52 ± 1.4 g/flask FW) was obtained in OMC3 cell suspension on the 5th day of elicitation when elicited with JA (50) on the 25th day of the culture period. Biomass yield from OMC3 cell line was maximum on the 25th day of culture which when combined with JA elicitation produced an 18.66-fold increase in CPT production together with high biomass yield. HPLC chromatograms of methanol extracts of OMC3 and original cell suspension culture with and without JA elicitation peaked with CPT are shown in Fig. 7. A comparative account on CPT and biomass produced in original cell suspension and OMC3 cell culture when elicited with JA is given in Table 3.

## Discussion

### Cell line selection and establishing cell suspension cultures

Very low CPT content (0.06 ± 0.004 mg $g^{-1}$DW) was reported in *O. mungos* cell suspension culture induced from callus tissues derived from field-grown leaf explants (Deepthi and Satheeshkumar 2016). To obtain high CPT and biomass yielding cell suspension culture, cell line selection was performed. Selection of high-yielding cell line and further establishment of cell suspension culture have been successfully employed in many studies for the production of useful secondary metabolites (Matsumoto et al. 1981; Fujita et al. 1985; Liang et al. 2006). In the present study, cell line selection was performed on *O. mungos* cell suspension culture according to cell aggregate cloning method. Eighty cell colonies were selected, and out of them, only ten survived after subculturing and only three of them (OMC3, OMC7 and OMC9) proliferated well for further studies. Among the three cell lines, OMC3 cell line was highly proliferative with high CPT content which might be due to the selection of the cell lines with unique genotypic features. Selection of highly productive cell lines in various cell suspension cultures is possible due to the heterogeneity in the biochemical activity of cells in the suspension culture (Larkin and Scowcroft 1981). There are many

**Table 2** Effect of various concentrations of elicitors (JA, SA and CH) on biomass and CPT production in original cell suspension culture of *O. mungos* cultured in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN during 1, 5, 10 and 15 days after elicitor feeding

| Elicitor | Concentration | Biomass (g/flask) | | | | CPT (mg $g^{-1}$ DW) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 day | 5 day | 10 day | 15 day | 1 day | 5 day | 10 day | 15 day |
| JA (μM) | 25 | 1.95 ± 0.21a | 2.45 ± 0.4b | 4 ± 0.5b | 4.0 ± 0.5b | 0.02 ± 0.0021b | 0.35 ± 0.05b | 0.25 ± 0.005b | 0.25 ± 0.002b |
| | 50 | 1.9 ± 0.23ab | 3.25 ± 0.32ab | 4.8 ± 0.8ab | 4.8 ± 0.62a | 0.06 ± 0.003a | 0.92 ± 0.084a | 0.5 ± 0.006a | 0.4 ± 0.001a |
| | 100 | 1.8 ± 0.32b | 2.4 ± 0.4b | 4.2 ± 0.7b | 4.2 ± 0.71b | 0.04 ± 0.003ab | 0.25 ± 0.03c | 0.2 ± 0.005b | 0.1 ± 0.002c |
| | 200 | 1.8 ± 0.21b | 2.3 ± 0.45b | 4.0 ± 0.6b | 4.0 ± 0.82b | 0.04 ± 0.003ab | 0.12 ± 0.025d | 0.08 ± 0.006cd | 0.1 ± 0.001c |
| | 0.0 | 1.95 ± 0.22a | 3.47 ± 0.6a | 5.5 ± 0.8a | 7.78 ± 0.92a | 0.02 ± 0.004b | 0.04 ± 0.006e | 0.06 ± 0.002d | 0.06 ± 0.002d |
| CH (mg $L^{-1}$) | 25 | 1.95 ± 0.2a | 2.45 ± 0.3bc | 4.0 ± 0.5b | 3.8 ± 0.8b | 0.02 ± 0.002b | 0.04 ± 0.003b | 0.08 ± 0.008cd | 0.06 ± 0.015c |
| | 50 | 1.9 ± 0.2a | 2.59 ± 0.23b | 4.0 ± 0.8b | 3.5 ± 0.9bc | 0.04 ± 0.003a | 0.06 ± 0.005a | 0.3 ± 0.02b | 0.25 ± 0.03b |
| | 100 | 1.75 ± 0.4b | 2.36 ± 0.3c | 3.82 ± 0.9bc | 3.0 ± 0.85c | 0.04 ± 0.003a | 0.06 ± 0.008a | 0.5 ± 0.025a | 0.4 ± 0.025a |
| | 200 | 1.25 ± 0.2c | 2.42 ± 0.23bc | 3.2 ± 0.8c | 3.0 ± 0.75c | 0.02 ± 0.004b | 0.04 ± 0.004b | 0.2 ± 0.025c | 0.2 ± 0.02b |
| | 0.0 | 1.95 ± 0.4a | 3.48 ± 0.3 | 5.5 ± 0.9a | 7.78 ± 0.65a | 0.02 ± 0.003b | 0.04 ± 0.005b | 0.06 ± 0.008d | 0.06 ± 0.006c |
| SA (mM) | 25 | 1.95 ± 0.25a | 2.58 ± 0.5b | 4.5 ± 0.8ab | 5.5 ± 0.8b | 0.02 ± 0.002c | 0.32 ± 0.025b | 0.15 ± 0.02ab | 0.068 ± 0.002ab |
| | 50 | 1.9 ± 0.3a | 3.55 ± 0.41a | 5.8 ± 0.6a | 6.5 ± 0.9ab | 0.06 ± 0.002a | 0.55 ± 0.032a | 0.3 ± 0.03a | 0.085 ± 0.002a |
| | 100 | 1.8 ± 0.25b | 3.2 ± 0.35ab | 4.2 ± 0.75ab | 5.2 ± 0.95b | 0.04 ± 0.005a | 0.25 ± 0.045c | 0.12 ± 0.04b | 0.065 ± 0.003b |
| | 200 | 1.8 ± 0.32b | 3.0 ± 0.34bc | 4.0 ± 0.45b | 4.8 ± 0.45c | 0.02 ± 0.002c | 0.1 ± 0.012d | 0.08 ± 0.008b | 0.04 ± 0.003d |
| | 0.0 | 1.95 ± 0.24a | 3.48 ± 0.24a | 5.5 ± 0.6a | 7.78 ± 0.55a | 0.02 ± 0.003c | 0.04 ± 0.01e | 0.06 ± 0.002c | 0.06 ± 0.004c |

Elicitors were fed on the 15th day of culture period. Data represents mean ± standard error. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA

methods for cell line selection which includes visual selection, clonal selection and selection of mutants (Remotti et al. 1997; Shiba and Mii 2005; Liang et al. 2006). The clonal selection includes two techniques namely, cell aggregate cloning and single cell cloning. Limitation of single cell cloning is that isolation of single cell and culturing it for obtaining cell line is very difficult compared with the cell aggregate cloning method. The colonies derived from cell aggregates can be considered true colonies because when a plant cell divide, the new cell wall get deposited within the existing cell and daughter cells remains joined together in the form of cell aggregates and separate only with the application of stress or shear forces (Dougall 1987). OMC3 cell suspension culture was selected for further studies based on superior biomass and CPT production. Significantly high biomass production (9.25 ± 1.3 g/flask FW) was achieved in OMC3 cell suspension on the 25th day of incubation compared with the original cell suspension (5.86 ± 0.42 g/flask FW). Selection using analysis of the growth of the culture system in suspension cultures and the quantification of the desired product is superior to visual selection techniques (Murthy et al. 2014).

## Effect of various culture parameters on biomass and CPT production

In this study, the effect of culture parameters like concentration of carbon source, $NH_4^+/NO_3^-$ concentration and phosphate concentration on cell biomass and CPT production was studied. The components of the culture media play an important role in controlling cell biomass and secondary metabolite production. Optimisation of culture conditions in cell suspension culture results in the accumulation of products in cells at a higher level than in native plants. There are various examples where the concentration of secondary metabolites was higher in cell suspension cultures than in the field-grown plants such as ginsenoside saponin production by cell suspension cultures of *Panax quinquefolium* (Zhong et al. 1996), rosmarinic acid in *Colleus blumei* cell suspension cultures (Zenk et al. 1977), shikonin by *Lithospermum erythrorhizon* cell suspension cultures (Fujita et al. 1981) etc. The classical approach of media manipulation for identifying the optimum condition is by changing the desired component(s) while maintaining the others at a constant level (Chattopadhyay et al. 2002).

One of the main factors affecting biomass yield of cell suspension culture is the inoculum size (Lee and Shuler 2000). A high inoculum size of 14 g $L^{-1}$ was found to be the ideal inoculum size for OMC3 cell suspension culture with a maximum biomass yield of 10.5 ± 0.8 g/flask FW. Lower inoculum size contributed to low biomass production, might be due to lack of cell-to-cell interaction. Similarly, higher inoculum size above the optimum level decreased biomass production, which could be related to the availability of sufficient

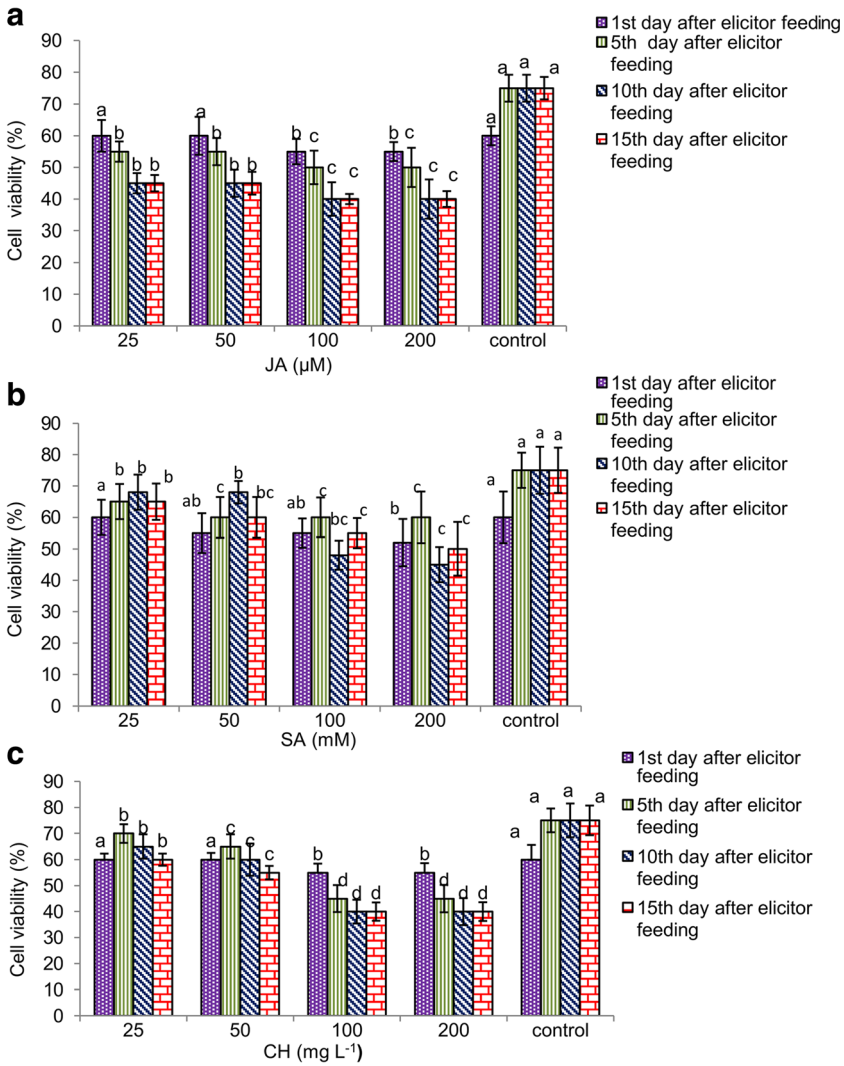


**Fig. 6** Effect of elicitor feeding on cell viability in original cell suspension cultures of *O. mungos* cultured in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN during the 1, 5, 10 and 15 day after elicitor feeding. **a** JA. **b** SA. **c** CH

nutrients and aeration in the medium. Low inoculum size delays the onset of log phase in plant cell suspension culture. When fresh cells are transferred to a new medium, they leak growth factors to the medium and the dispersed nature of cell suspensions makes them more prone to leakage of key growth factors/cellular contents to medium (Carvalho and Curtis 1999). This leakage is more significant when cells are transferred in low inoculums, and the cells have to resynthesise these growth factors to the level needed for the growth to resume and the cells to enter the log phase (Syono and Furuya 1968; Carvalho and Curtis 1999) So, a small inoculum size can lead to cultures with a longer lag phase. High inoculum size results in the browning of cells and cell death due to lack of aeration and nutrients. Optimum inoculum size is very important for cell growth in cell suspension so as to provide cell-to-cell interactions for growth. A previous study on the effect of inoculum size on biomass production in *Hyoscyamus muiticus* cell and root cultures indicated that cell suspension cultures require a substantially higher inoculum size. Cells inoculated at low cell concentrations displayed a typical growth reduction, whereas roots inoculated at low concentrations displayed an improvement in growth (Carvalho and Curtis 1999). The need for high inoculum size for cell suspension culture compared with root culture might be due to the high specific surface area of cells as compared with roots and also due to the undifferentiated status of cells in the suspension culture compared with the differentiated root cultures. In *Panax ginseng* cell suspension cultures, cell growth was low at a low inoculum size of 1.5 g $L^{-1}$ DW, the maximum cell growth rate was obtained at 3 g $L^{-1}$ DW of inoculum size and maximum ginseng saponin production of 275 mg $L^{-1}$ was achieved at 6 g $L^{-1}$ DW of inoculum size (Akalezi et al. 1999).

Optimum carbon source and its appropriate concentration in the culture medium are essential for the growth of cells in suspension cultures. Several reports indicated the effect of carbon source and its concentration on secondary metabolite production and altering the concentration of sucrose has shown to change the biomass yield and compound production

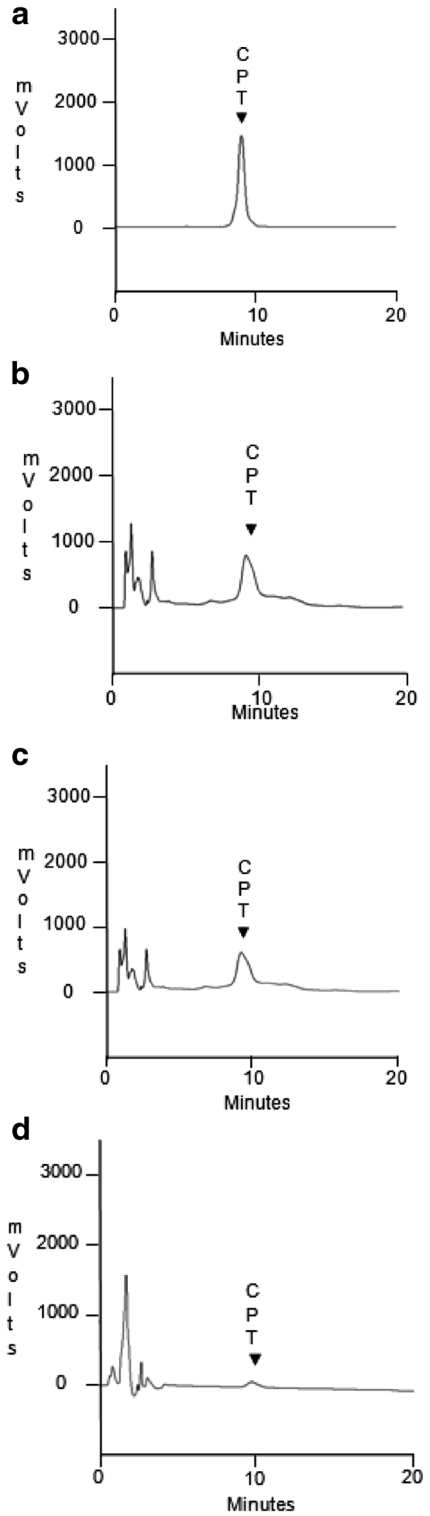


**Fig. 7** HPLC chromatogram of methanol extract of cell suspension cultures of *O. mungos* peaked with CPT. **a** Standard CPT. **b** OMC3 cell suspension elicited with JA (50 μM). **c** OMC3 cell suspension. **d** Original cell suspension culture. The detector wavelength was 254 nm

in cell suspension cultures (Bao Do and Cormier 1991a; Karwasara and Dixit 2013). The carbon source is needed in the culture media to maintain the osmotic potential and also as an energy source for all the developmental processes. Sugars, not only act as a source of cellular carbon but also as signalling molecules using a sugar sensor (hexokinase) thereby regulating hexokinase-dependent and hexokinase-independent pathways to regulate many molecular mechanisms like gene transcription, translation, protein stability etc. (Koch 1996; Rolland et al. 2006; Wang and Ruan 2013). The preference of cells to the type and concentration of carbon source in the medium has to be identified in order to get the maximum biomass and compound yield. Sucrose is the most preferred carbon source by most of the plant cell suspension cultures, followed by glucose, and this may be because of the similarity between the two sources, as sucrose is a non-reducing disaccharide of glucose and fructose. In the present study, fructose, maltose, dextrose and sucrose were used as carbon sources in tissue culture media and found that sucrose at 3 % (*w*/*v*) gave the highest biomass and sucrose at 5 % gave the highest CPT yield. The present result corroborates the study in *C. acuminata* cell suspension cultures wherein 5 % (*w*/*v*) sucrose was optimal for CPT production while cell growth together with CPT production was suppressed at higher sucrose concentration (Karwasara and Dixit 2013). *Hypericium perforatum* adventitious root cultures grown in 1/2 strength MS medium supplemented with 3 % (*w*/*v*) sucrose resulted in optimum biomass accumulation, but higher sucrose concentrations (5, 7 and 9 %, *w*/*v*) inhibited biomass accumulation due to relatively higher osmotic strength and increased the accumulation of secondary metabolites (Cui et al. 2010). Maximum CPT yield (0.23 ± 0.004 mg $g^{-1}$ DW) obtained in the present study with 5 % (*w*/*v*) sucrose in the cell suspension culture might be due to the osmotic stress induced by sucrose, when supplied at a higher concentration.

Nitrogen is available to the plants in the form of $NH_4^+$and $NO_3^-$. Total nitrogen in the form of $NH_4^+$ and $NO_3^-$ in the tissue culture media is identified to be playing an important role in biomass and compound production as reported in several studies (Fujita et al. 1981; Bao Do and Cormier 1991b; Chen et al. 2003; Rajesh et al. 2014). In the present study, $NH_4^+$ and $NO_3^-$ in the ratio of 20:40 was found to be suitable for maximum biomass production and at 40:20 showed high CPT levels compared with the other concentrations tested. It was noticed that cells require a higher amount of $NO_3^-$ in the medium than $NH_4^+$ for cell growth while vice versa was needed for CPT production. Similar observations are also reported in *Nothapodytes nimmoniana* cell suspension cultures (Karwasara and Dixit 2013) and *C. acuminata* cell suspension cultures (Pan et al. 2004) wherein higher concentration of $NH_4^+$ ions favoured CPT production and a higher concentration of $NO_3^-$ favoured the increase in biomass. $NH_4^+$ is easily accumulated into the plant tissue, which is toxic, may have resulted in reduced growth of cells as reported earlier in *Atropa belladonna* (Bensaddek et al. 2001). Production enhancement of CPT in cells cultured in high $NH_4^+$

**Table 3** Comparison of biomass and CPT yield in *O. mungos* original cell suspension culture and OMC3 cell line cultured in 1/2 strength MS medium supplemented with 3.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ KIN when elicited with JA (50 μM)

| Cell suspension culture of *O. mungos* | Treatment | Day of JA feeding | Day of harvesting cells for biomass and CPT yield estimation | Biomass (g/flask FW) | CPT (mg $g^{-1}$ DW) |
|---|---|---|---|---|---|
| Original cell suspension culture | nil | – | 25th day | 5.86 ± 0.42c | 0.06 ± 0.002d |
| | JA (50 μM) | 15th day | 20th day | 3.25 ± 0.32d | 0.92 ± 0.08b |
| OMC3 cell suspension culture | nil | – | 25th day | 9.25 ± 1.3b | 0.095 ± 0.004c |
| | JA (50 μM) | 25th day | 30th day | 9.52 ± 1.4a | 1.12 ± 0.08a |

Data represents mean ± standard error. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA

concentration may be due to the easy assimilation of $NH_4^+$ to glutamate which may have a role in CPT biosynthesis pathway (Yamazaki et al. 2004; Pan et al. 2004). High $NH_4^+$ in the nutrient medium can inhibit $NO_3^-$ assimilation genes thereby causing acidification of medium which resulted in poor cell growth (Crawford 1995).

Phosphorus is a macronutrient, a component of key molecules such as nucleic acids, phospholipids and ATP. It also has a role in key enzyme reactions and in the regulation of metabolic pathways in plants (Theodorou and Plaxton 1993). In the present study, biomass significantly increased with increasing concentration of phosphate while CPT yield decreased. The result is in agreement with the effect of phosphate on CPT production in cell cultures of *N. nimmoniana* (Karwasara and Dixit 2013) and ginseng saponin production in *P. quinquefolium* strain Q91625 (PQ) cell suspension cultures (Liu and Zhong 1998). In suspended cells of *Panax notoginseng*, cell growth and the saponin accumulation were greatly improved and the utilisation of sugar and nitrogen sources was also increased by increasing the phosphate concentration in the medium (Zhong and Zhu 1995).

Elicitation is an effective strategy to enhance secondary metabolite and biomass production in low-yielding cell suspension cultures and to modify cellular metabolism to enhance the secondary metabolite production by activating the defence genes (Templeton and Lamb 1988; Zhao et al. 2005). Elicitation of cell suspension cultures with a variety of elicitors was reported to be effective in enhancing secondary metabolite and biomass production (Sánchez-Sampedro et al. 2005; Veerashree et al. 2011; Kubeš et al. 2014).

Elicitation studies were performed using the original cell suspension culture, and the elicitors were added on the 15-day-old cell suspension cultures or early log phase. Among the elicitors applied to the cell suspension cultures of *O. mungos*, JA (50 μM), on the 5th day of elicitation, produced maximum CPT (0.92 ± 0.084 mg $g^{-1}$ DW). Cell growth and viability were adversely affected due to JA elicitation at higher concentration. JA is a plant response signalling compound and is a signal transducer affecting secondary metabolite production induced by various elicitors (Gundlach et al. 1992). Our result is in agreement with the JA-mediated elicitation study reported in the cell suspension cultures of *C. acuminata* where JA at 50 μM produced an 11-fold increase in CPT production on the 4th day of elicitation and thereafter decreased (Song and Byun 1998). In *Morinda eliptica* cell suspension cultures also, JA had a similar effect on cell growth and secondary metabolite production (Chong et al. 2005). JA has enhanced hypericin production in the cell suspension cultures of *Hypericum perforatum* L. (Walker et al. 2002).

CH is a polysaccharide derived from the fungal cell wall and shells of arthropod. Fungal elicitation in plant cell suspension culture was found to induce *cis*-jasmonic acid production, which acts as a message between the elicitor plant cell wall receptor and gene activation producing various signalling molecules (Mueller et al. 1993). Chitin-mediated elicitation for the enhanced production of plumbagin was reported in *Drosophyllum lusitanicum* suspension cultures (Nahálka et al. 1998). In the present study, CH (100 mg $L^{-1}$) enhanced the production of CPT to 0.5 ± 0.025 mg $g^{-1}$ DW, but cell growth decreased.

SA is a plant response signalling molecule and accumulates at the site of pathogen attack and spreads to other sites for inducing responses (Zhao et al. 2005). SA enhanced the production of CPT production to 0.55 ± 0.032 mg $g^{-1}$ DW but affected cell viability. SA-mediated enhanced production of withanolides was achieved in *Withania sominifera* (Sivanandhan et al. 2013).

## Jasmonic acid elicitation in OMC3 cell suspension culture

With the knowledge of elicitation studies performed in the original cell suspension, JA which caused maximum enhancement in CPT yield was chosen to elicit OMC3 cell suspension cultures on the 25th day of the culture period. JA was fed to OMC3 cell suspension culture on the 25th day to ensure maximum biomass yield as it was observed earlier that JA can adversely affect cell growth and viability when fed during

the early log phase (15th day). CPT (1.12 ± 0.08 mg $g^{-1}$ DW) and biomass production (9.52 ± 1.4 g/flask FW) achieved through cell line selection combined with JA elicitation were significantly higher when compared with the CPT produced in the original cell suspension. Original cell suspension produced 0.06 ± 0.004 mg $g^{-1}$ DW CPT and 5.86 ± 0.42 g/flask FW biomass on the 25th day of incubation (Deepthi and Satheeshkumar 2016). Thus, an 18.66-fold increase in CPT production was achieved when compared with the CPT produced in the original cell suspension culture. The superior CPT yield reported in the present study was higher than that reported in an earlier elicitation study using yeast extract elicitor on original cell suspension culture of *O. mungos* (Deepthi and Satheeshkumar 2016).

The strategy of combining cell line selection with elicitation resulted in an 18.66-fold increase in CPT production in OMC3 cell suspension culture. The production of CPT was higher than that reported by Namdeo et al. (2012) in field-grown plants (0.03 mg $g^{-1}$ DW) and in whole in vitro grown plants (0.768 mg $g^{-1}$ DW) of *O. mungos*. Wink et al. (2005) has also reported a low CPT content of 0.004 ± 0.002 mg $g^{-1}$ DW in *O. mungos* cell suspension culture. Significant increase in biomass production was also achieved using cell line selection.

High concentration of $NO_3^-$ and phosphate-enhanced biomass production and the increased concentration of $NH_4^+$ have enhanced CPT production in OMC3 cells. High concentration of sucrose (5 %, *w/v*) caused enhanced CPT production. Optimum inoculum size was identified as 14 g $L^{-1}$. The results obtained regarding the effects of culture parameters and major nutrients can be applied in the scale-up studies of *O. mungos* cell suspension cultures in suitable bioreactor systems. Considering the significantly high yield of CPT reported in the present study, this combination strategy can be applied in other cell culture systems also for the efficient production of secondary metabolites. Also, cell suspension cultures are of advantage that it can proliferate at a higher growth rate when compared with whole plants in the field, amenable to scale-up studies, in situ product removal and metabolic engineering studies.

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**Compliance with ethical standards** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no competing interests to declare.

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