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ORIGINAL ARTICLE

# *Piriformospora indica* enhances the production of pentacyclic triterpenoids in *Lantana camara* L. suspension cultures

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**Abstract** The stimulation of secondary metabolite synthesis by the endophytic fungus, *Piriformospora indica*, was investigated for the production of triterpenoids—ursolic acid (UA), oleanolic acid (OA) and betulinic acid (BA)—in the suspension cultures of *Lantana camara*. Growth and product formation profiles of the cell suspension cultures of *L. camara* were established for optimum elicitation strategy. The elicitation potential of *P. indica* was compared with typical elicitors like methyl jasmonate (MJ), chitin and chitosan. MJ at 62.5 µM and 2.5 % (v/v) filter-sterilized culture filtrate of *P. indica* enhanced the triterpenoids production to similar level. Filter-sterilized culture filtrate of *P. indica* at 2.5 % (v/v) increased the concentration of UA by 3.5-fold (3869.3 µg/g DW), OA by 5.6-fold (1425.7 µg/g DW) and BA by 7.8-fold (117.02 µg/g DW) in the cell cultures of *L. camara*.

**Keywords** Betulinic acid · Biotic elicitor · *Lanatana camara* · *Piriformospora indica* · Oleanolic acid · Triterpenoids · Ursolic acid

## Introduction

*Piriformospora indica*, a root endophytic fungus, is similar to arbuscular mycorrhizal fungi; however, it can be axenically cultivated on a variety of synthetic media (Singh et al. 2013). The fungus has been reported to increase the growth of a diverse varieties of plants such as *Bacopa monniera*, *Nicotiana tabaccum*, *Artemisia annua* and *Zea mays* (Varma et al. 1999), *Withania somnifera* and *Spilanthes calva* (Rai et al. 2001), *Azadirachta indica* (Singh et al. 2003) and *Coleus forskohlii* (Das et al. 2012) as well as the production of bioactive compounds in a wide variety of whole plants such as spilanthol in *S. calva* (Rai et al. 2004), asiaticoside in *Centella asiatica* (Satheesan et al. 2012), aristolochic acid in *Aristolochia elegans* Mart (Bagde et al. 2013), and artemisinin in *A. annua* (Sharma and Agrawal 2013). In addition, it has also been shown to impart beneficial effects on secondary metabolite synthesis in plant cell cultures, e.g. on production of podophyllotoxin in cell suspension cultures of *Linum album* (Baldi et al. 2010) and in hairy root cultures of *L. album* (Kumar et al. 2013).

*Lantana camara* L. (Family Verbenaceae), commonly known as wild or red sage, has been reported to be a reservoir of diverse classes of bioactive compounds like monoterpenes, sesquiterpenes, triterpenes and flavonoids (Ghisalberti 2000). Srivastava et al. (2010) studied the production of pentacyclic triterpenoids—betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA) in the cell cultures of *L. camara* and their significant cytotoxic effect on HeLa cell lines. UA and OA have been reported to be antioxidant, anti-inflammatory, antitumor, antimicrobial, hepatoprotective and antifungal (Liu 2005). BA has antimalarial, anti-inflammatory, anti-HIV and cytotoxicity activities on HeLa cell lines (Alakurtti et al. 2006).

Therefore, it is of interest to investigate the effect of biotic elicitors derived from *P. indica* on the production of pentacyclic triterpenoids in the cell cultures of *L. camara*. For this, the growth and triterpenoids production profiles of *L. camara* in the suspension cultures were studied. The elicitation strategy included the addition of appropriate

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levels of elicitors present in *P. indica* to the suspension cultures of *L. camara* with a view to get the maximum concentration of the bioactive compounds.

## Materials and methods

### Growth and triterpenoids production profiles of *L. camara*

Callus culture of *Lantara camara* (Accession Number GUBH 79865, Guwahati University, Guwahati, India) was maintained on MS medium as described by Srivastava et al. (2011). The suspension cultures of *L. camara* were established with 2.0 g (fresh weight) of green friable callus in 50 ml liquid MS medium with 5 µM BA, 1 µM 2,4-D and 1 µM NAA contained in 250 ml Erlenmeyer flask. The pH was adjusted to 5.8 before autoclaving at 121 °C at 15 psi for 20 min. The cultures were incubated on gyratory shaker at 120 rpm and 25 ± 2 °C under dark conditions (Srivastava et al. 2011). The cultures were subcultured every 8th day in the same medium with 3.0 g/l (DW basis) inoculum. For kinetic study, the flasks were harvested every alternate day till 16th day to analyze dry cell weight and triterpenoids (UA, OA and BA) contents.

### Use of methyl jasmonate, chitin and chitosan as elicitors

Methyl jasmonate (MJ) with a purity of 95 % from Sigma was dissolved in 96 % ethanol and filter-sterilized (Kuźma et al. 2009). It was added to the culture medium to give final concentrations of 25, 62.5, 125, 250, 625 and 1250 µM. Stock solution of chitin (≥95 % acetylation) from shrimp shells (Sigma) was prepared by dissolving in 1 M acetic acid and then diluting it with distilled water, pH adjusted to 5.8 with 1 M NaOH and sterilized by autoclaving at 15 psi g and 121 °C for 20 min. Chitosan (≥75 % deacetylated) from crab shells (Sigma) was dissolved in 5 % (v/v) HCl and left overnight on stirrer to dissolve completely (Wiktorowska et al. 2010). Chitin and chitosan were added separately to the culture medium to give final concentrations of 25, 50, 100, 250 and 500 mg/l.

### Preparation of culture filtrate (autoclaved and filter-sterilized) and cell extract of *P. indica* as a source of biotic elicitors

*Piriformospora indica* (DSM 11827, Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany) was obtained from Prof. Ajit Varma, Amity University, Noida, India. The fungus was cultivated on Kaefer medium (Kaefer 1977) at pH 6.5, temperature 30 ± 1 °C on a rotatory shaker at 200 rpm. The fungal cultures were harvested in the late log phase (6th day) to prepare biotic elicitors as described by Baldi et al. (2010). Elicitors were added to the growing suspension cultures of *L. camara* on 10th day and harvested on 12th day to analyze for dry cell weight and triterpenoids contents.

### Co-culture of live *P. indica* cells with *L. camara*

The live 5-day old fungal cells of *P. indica*, grown in Kaefer medium, were inoculated at different concentrations (0.5, 1.0, 2.5, 5.0 and 7.5 g/l on DW basis) to different growth stages of *L. camara* (10th and 11th day) and harvested on 12th day to analyze for plant biomass and triterpenoids contents.

### Dry cell weight (DW) estimation

The biomass, harvested by vacuum filtration using a Buchner funnel and washed with distilled water, was dried to a constant weight at 60 ± 2 °C and reported as g/l (DW basis) (Godoy-Hernández and Vázquez-Flota 2012).

### Extraction and quantification of triterpenoids from cell cultures of *L. camara*

The triterpenoids were extracted and quantified as reported by Srivastava and Chaturvedi (2010). Quantification was done on Agilent 1200 HPLC system (Agilent Technologies, USA) with Syncronis$^{TM}$ C18 column (250 × 4.6 mm; 5 µm; Thermo Scientific, USA), mobile phase of acetonitrile/water (80/20) at a flow rate of 1.0 ml/min and analyzed by diode array detector at 209 nm. The samples were filtered through 0.2 µm PTFE syringe filters before injecting 20 µl of it for HPLC analysis. The triterpenoid peaks in cell extract of suspension cultures of *L. camara* were identified with the retention times of authentic standards purchased from Sigma-Aldrich (St. Louis, MO) with purity of >90 % for UA, >97 % for OA and >98 % for BA.

### Determination of fungal and plant biomass in the co-culture experiment

The fungal and plant biomass were determined as described in detail by the authors' laboratory (Baldi et al. 2008).

### Statistical analysis

Each experiment was repeated at least three times. Values were expressed as mean ± SD. Data were analyzed using IBM SPSS Statistic software. All mean comparisons were subjected to a one-way analysis of variance (ANOVA). In all cases the confidence coefficient was set at $P < 0.05$ level.

## Results and discussion

### Growth and triterpenoids production in suspension cultures of *L. camara*

The growth cycle of 16 days can be divided into three growth phases namely a lag phase (0–4th day), an exponential phase (4–12th day) and a stationary phase (12–16th day) as evident from the growth profile (Fig. 1). The maximum biomass (19.71 g/l DW) was achieved on 12th day and thereafter it remained constant. Triterpenoids accumulation was intracellular and was not identified in the culture medium. The triterpenoids content increased from 4th to 12th day, after which it decreased. The production was thus growth-associated. Similar production profile has been reported in the production of podophyllotoxin in the suspension cultures of *L. album* (Baldi et al. 2010). Maximum UA content was 1034.0 µg/g DW and OA content 248.50 µg/g DW on 12th day of cultivation. BA was present in trace amount (15.73 µg/g DW). The total triterpenoids production was 25.28 mg/l, which was slightly higher than reported under the same culture conditions (21.6 mg/l) in the literature (Srivastava et al. 2011). The growth and triterpenoids production profiles indicated that 12th day is the optimum time for the harvest of the suspension cultures to get maximum biomass and triterpenoids production.

### Effect of addition of methyl jasmonate during different stages of growth cycle of *L. camara*

MJ is one of the signaling molecules from octadecanoic pathway that acts as signal transducer for the perception of elicitation leading to activation of biosynthetic machinery of the secondary metabolites in plant cells. Therefore, MJ was chosen to study the impact of its addition in mid log phase (8th day) and late log phase (10th day) of the growing suspension cultures of *L. camara* for biomass and triterpenoids production. A dose-dependent and duration-dependent decrease in biomass was observed. A greater decrease in biomass was observed as the concentration of elicitor or the exposure time increased (Table 1). Higher concentrations of the elicitor incited hypersensitive responses, which was inferred through change in color of the biomass and the media. Elicitors cause physiological and morphological responses in cells and suppression of biomass has generally been observed in elicited cell cultures (Savitha et al. 2006; Bahabadi et al. 2011). Although the maximum production of UA and OA were not significantly different ($P > 0.05$) by the addition of 62.5 µM MJ on 10th day and 250 µM MJ on 8th day, the respective biomass accumulations were significantly different ($P < 0.05$) (Table 1). The suspension cultures in the late log phase (10th day) were most susceptible to elicitation as they required very low amount of elicitor (62.5 µM) as compared to the mid log phase cultures that required four times greater concentration of MJ (250 µM) to produce similar effects. When MJ was added at 250 µM on 8th day, the maximum total triterpenoids production of only 69.14 mg/l was achieved, due to decrease in biomass. However, when MJ was added at 62.5 µM on 10th day, it resulted in maximum total triterpenoids production of 85.12 mg/l.

Thus the strategy for addition of elicitors in late exponential growth phase (10th day) was found to be better in the present study. Elicitation at late log phase allowed the maximum conversion of substrate into biomass and substantial improvement in production of triterpenoids. The addition of elicitors in late exponential phase has been reported to increase the production of secondary metabolites by several investigators (Rijhwani and Shanks 1998; Savitha et al. 2006; Kuźma et al. 2009). We believe that the biosynthetic machinery of *L. camara* for the triterpenoids production was fully activated by late log phase. Hence, 10th day was chosen for the addition of fungal elicitors to the growing suspension cultures of *L. camara*.

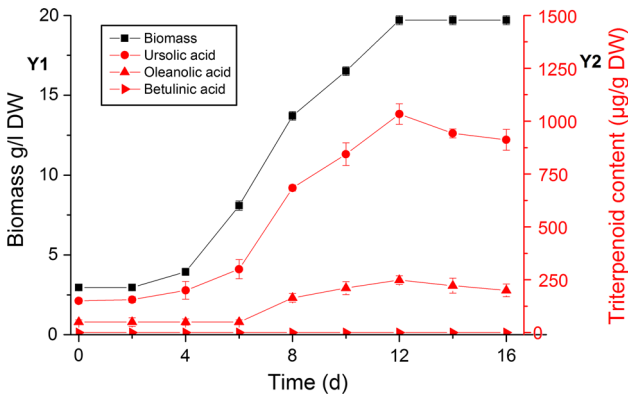


**Fig. 1** Growth and triterpenoids production profile in cell suspension culture of *L. camara*. Data represents mean values ± SD of three replicates. Biomass (g/l DW) on the *Y1* axis and triterpenoids content (µg/g DW) on *Y2* axis

### Effect of different biotic elicitor preparations from *Piriformospora indica* on growth and triterpenoids production in the suspension cultures of *L. camara*

The suspension cultures of *L. camara* were elicited with autoclaved culture filtrate, filter-sterilized culture filtrate and fungal extract of *P. indica* on 10th day and the response was measured in terms of biomass and production of UA, OA and BA. A dose-dependent decrease in biomass was observed as the concentration of the biotic elicitors increased (Fig. 2). Baldi et al. (2010) also reported a

**Table 1** Effect of addition of methyl jasmonate during different stages of growth cycle of *L. camara* on growth and triterpenoids production

| Elicitor conc (μM) | Biomass (g/l DW) | UA (μg/g DW) | OA (μg/g DW) | BA (μg/g DW) |
|---|---|---|---|---|
| Control | 19.71 ± 0.20$^{a}$ | 1034.1 ± 48.4$^{a}$ | 248.5 ± 20.12$^{a}$ | 15.73 ± 10.10$^{a}$ |
| MJ added on 10th day; exposure time 48 h | | | | |
| 25 | 18.81 ± 0.24$^{a}$ | 2001.1 ± 44.3$^{d}$ | 539.0 ± 10.3$^{b}$ | 51.23 ± 24.20$^{b}$ |
| 62.5 | 18.71 ± 0.24$^{a}$ | 3193.9 ± 49.2$^{e}$ | 1188.1 ± 130.5$^{d}$ | 169.58 ± 14.41$^{d}$ |
| 125 | 18.67 ± 0.34$^{a}$ | 2006.0 ± 129.4$^{d}$ | 729.4 ± 53.2$^{c}$ | 47.64 ± 10.67$^{b}$ |
| 250 | 17.87 ± 0.43$^{bc}$ | 1616.1 ± 64.8$^{c}$ | 240.5 ± 0.9$^{a}$ | 58.63 ± 0.21$^{b}$ |
| 625 | 16.78 ± 0.33$^{c}$ | 1025.4 ± 37.6$^{a}$ | 310.0 ± 17.3$^{a}$ | 53.14 ± 8.23$^{b}$ |
| 1250 | 16.52 ± 0.28$^{c}$ | 719.3 ± 7.0$^{b}$ | 549.9 ± 36.6$^{b}$ | 113.02 ± 6.44$^{c}$ |
| MJ added on 8th day; exposure time 96 h | | | | |
| 62.5 | 18.07 ± 0.08$^{a}$ | 1366.6 ± 26.2$^{ac}$ | 539.0 ± 10.35$^{b}$ | 151.96 ± 25.19$^{d}$ |
| 125 | 17.58 ± 0.56$^{bc}$ | 1156.2 ± 7.3$^{a}$ | 475.4 ± 1.43$^{b}$ | 52.86 ± 11.59$^{b}$ |
| 250 | 16.19 ± 0.24$^{c}$ | 3088.1 ± 76.4$^{e}$ | 1044.9 ± 20.90$^{d}$ | 170.03 ± 2.07$^{d}$ |
| 625 | 15.96 ± 0.41$^{cd}$ | 2001.1 ± 44.3$^{d}$ | 776.5 ± 6.60$^{c}$ | 247.34 ± 38.87$^{f}$ |
| 1250 | 15.01 ± 0.14$^{d}$ | 1228.7 ± 37.1$^{a}$ | 823.1 ± 24.90$^{c}$ | 130.62 ± 3.95$^{cd}$ |

Data represents mean values ± SD of three replicates. Within each column, means with same letters are not significantly different ($P > 0.05$)

suppressing effect of culture filtrate of *P. indica* on the growth of suspension cultures of *L. album*. But Kumar et al. (2013) reported a 1.4-fold increase in the biomass of hairy roots of *L. album* on the addition of 2 % v/v filter-sterilized culture filtrate of *P. indica* for an exposure time of 96 h. Obviously, the elicitation effect depends on the type of plant cells, their physiological status, and the dose and duration of the elicitor, amongst other factors. A maximum of 3.2-fold increase in UA (3489.0 μg/g DW), 4.6-fold increase in OA (1164.4 μg/g DW) and 8.7-fold increase in BA (131.10 μg/g DW) was achieved on the addition of autoclaved culture filtrate at a concentration of 2.5 % v/v. The filter-sterilized culture filtrate, at a concentration of 2.5 % v/v, elicited the culture with a maximum of 3.5-fold increase in UA (3869.3 μg/g DW), 5.6-fold increase in OA (1425.7 μg/g DW) and 7.8-fold increase in BA (117.02 μg/g DW) (Fig. 2). The concentration of BA, which was negligible in the control cultures, was significantly increased in all elicited cultures ($P < 0.05$). Greater enhancement was achieved in OA production by addition of filter-sterilized culture filtrate than autoclaved culture filtrate at a concentration of 2.5 % v/v ($P < 0.05$). This indicates the elicitor moieties in the culture filtrate of *P. indica* were heat-labile and may have lost their elicitation capability on autoclaving. Similar observation was made by Kumar et al. (2013) where a maximum of 3.8-fold increase in podophyllotoxin (PT) and 4.4-fold increase in 6-methoxypodophyllotoxin (6-MPT) concentration was achieved upon the addition of 3.0 % v/v filter-sterilized culture filtrate of *P. indica* to the hairy roots of *L. album* but a maximum of 2.4-fold increase in PT and 3.3-fold increase in 6-MPT concentrations were observed upon the addition of 3.0 % v/v autoclaved culture filtrate of

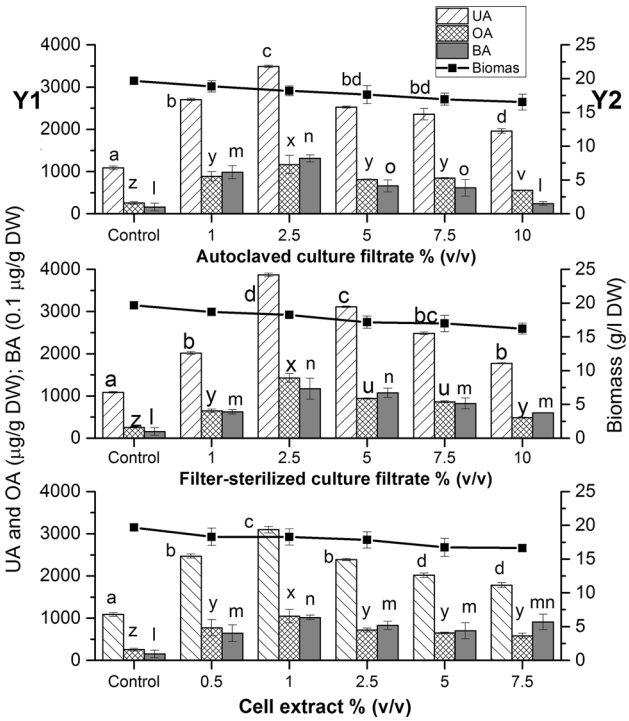


**Fig. 2** Growth and triterpenoids production in cell suspension cultures of *L. camara* treated with various biotic elicitors from *P. indica*. Data represents mean values ± SD of three replicates. UA, OA and BA contents are represented on the *Y1* axis with UA and OA contents in μg/g DW and BA content in 0.1 μg/g DW, and biomass (g/l DW) is represented on the *Y2* axis. Within each set of experiments, *bars* with *different letters* are significantly different ($P < 0.05$)

*P. indica*. A positive effect on lignan accumulation in the cell cultures of *L. album* has also been reported upon the addition of autoclaved culture filtrate of *P. indica* at 2.5 %

v/v (Baldi et al. 2010). The culture filtrate of *P. indica* also improved the overall growth of *A. elegans* Mast with increase in total biomass by 136 % and active ingredient aristolochic acid content by 7.6–28.8 % in comparison to the untreated control plants (Bagde et al. 2013). Treatment of *Helianthus annus* Sun gold and *H. annus* Japanese gold varieties with culture filtrate of *P. indica* in the greenhouse enhanced the overall growth and seed production of the plants, with an increase in oil content of the seeds by 50–70 % (Bagde et al. 2011). The culture filtrate of *P. indica* contains many known constituents such as indole acetic acid (IAA), flavonoids, cell wall degrading enzymes (such as, cellulases and xylanase) and unknown constituents such as oligosaccharides, hormones, enzymes and peptides, which could act as elicitors (Sirrenberg et al. 2007; Adya et al. 2013).

Addition of fungal extract of *P. indica* at a concentration of 1.0 % v/v led to a maximum enhancement of 2.8-fold increase in UA (3096.0 µg/g DW), 4.1-fold increase in OA (1046.5 µg/g DW) and 7.4-fold increase in BA in cell cultures of *L. camara* in comparison to the control cell cultures (Fig. 2). Similar results have been obtained in *L. album* where a maximum enhancement of 2.1-fold in PT and 3.2-fold in 6-MPT concentration was achieved on the addition of cell extract of *P. indica* to the hairy roots of *L. album* at concentration of 1 % v/v for an exposure time of 48 h (Kumar et al. 2013).

### Co-culture of live *P. indica* cells with *L. camara*

The study was aimed to exploit the elicitation as well as growth promotional effects of *P. indica* with a view to achieve maximum enhancement of triterpenoids production in the suspension cultures of *L. camara*. Dose-dependent and duration-dependent studies for the co-cultivation of *P. indica* with *L. camara* cells were carried out. Elicitation generally decreases the growth of the plant cells due to hypersensitive responses. But *P. indica* is known for its plant growth promoting effects at field trails, green house level and in-vitro plant cell and tissue cultures of various plants (Varma et al. 1999). For example, an increase in biomass by 21.3 % was observed in the cell suspension cultures of *L. album* when it was co-cultivated with live *P. indica* cells at a concentration of 1 g/l for 24 h (Baldi et al. 2010). In the present study, however, with an increase in the initial fungal concentration beyond 2.5 g/l and also with exposure time of more than 24 h, there was significant decrease in plant biomass accumulation ($P > 0.05$) (Table 2), which might be due to competition for nutrients by the fungal cells. On the other hand, a significant enhancement of 3.5-fold (3848.9 µg/g DW) in UA, 4.0-fold (1029.6 µg/g DW) in OA and 6.5-fold (102.15 µg/g DW) in BA was achieved when the suspension cultures of *L. camara* were co-cultivated with *P. indica* cells at an initial fungal concentration of 2.5 g/l for a duration of 24 h ($P < 0.05$). Fragments of the endophytic fungi like lipopolysaccharides, polysaccharides and glycoprotein, formed by the action of plant-derived hydrolases, have been reported to act as elicitors and stimulate plant defense and secondary metabolite production (Gao et al. 2010). A maximum of 3.4-fold and 4.9-fold increase in PT and 6-MPT concentrations, respectively, was reported in cell cultures of *L. album* in comparison to control cultures, when 1.0 g/l *P. indica* was co-cultivated for 24 h (Baldi et al. 2010). Similarly a maximum increment of 2.1-fold in PT and 2.5-fold in MPT concentrations was achieved when

**Table 2** Effect of addition of different concentrations of *P. indica* in cell suspension cultures of *L. camara* on growth and triterpenoids production

| *P. indica* cells (g/l DW) | Biomass (g/l DW) | UA (µg/g DW) | OA (µg/g DW) | BA (µg/g DW) |
|---|---|---|---|---|
| Control | 19.89 ± 0.20$^{a}$ | 1085.0 ± 19.0$^{a}$ | 254.1 ± 15.12$^{a}$ | 15.73 ± 10.10$^{a}$ |
| *P. indica* added on 11th day; duration of co-cultivation-24 h | | | | |
| 0.5 | 20.40 ± 0.70$^{a}$ | 2782.9 ± 31.2$^{d}$ | 780.9 ± 20.6$^{c}$ | 47.60 ± 10.30$^{b}$ |
| 1.0 | 20.60 ± 0.10$^{a}$ | 3472.8 ± 45.9$^{e}$ | 1056.3 ± 23.5$^{e}$ | 90.56 ± 21.40$^{de}$ |
| 2.5 | 20.24 ± 0.80$^{a}$ | 3849.0 ± 21.3$^{f}$ | 1029.7 ± 21.7$^{e}$ | 102.15 ± 25.10$^{e}$ |
| 5.0 | 18.98 ± 0.70$^{b}$ | 2317.9 ± 56.3$^{c}$ | 675.6 ± 40.2$^{c}$ | 84.21 ± 15.30$^{d}$ |
| 7.5 | 17.42 ± 0.60$^{c}$ | 2214.3 ± 56.7$^{c}$ | 521.2 ± 41.3$^{b}$ | 78.25 ± 16.40$^{d}$ |
| *P. indica* added on 10th day; duration of co-cultivation-48 h | | | | |
| 0.5 | 18.32 ± 0.70$^{b}$ | 2304.0 ± 105.2$^{c}$ | 677.3 ± 20.9$^{c}$ | 42.85 ± 26.40$^{b}$ |
| 1.0 | 18.52 ± 0.60$^{b}$ | 2598.2 ± 121.1$^{cd}$ | 668.3 ± 45.7$^{c}$ | 44.71 ± 21.40$^{b}$ |
| 2.5 | 18.24 ± 10.00$^{b}$ | 2390.0 ± 21.3$^{c}$ | 854.5 ± 21.7$^{d}$ | 69.53 ± 15.60$^{c}$ |
| 5.0 | 17.46 ± 0.80$^{c}$ | 1566.0 ± 96.7$^{b}$ | 540.4 ± 45.2$^{b}$ | 36.01 ± 15.20$^{b}$ |
| 7.5 | 17.02 ± 0.70$^{c}$ | 1413.2 ± 102.9$^{b}$ | 444.2 ± 21.1$^{b}$ | 12.49 ± 13.50$^{a}$ |

Data represents mean values ± SD of three replicates. Within each column, means with same letters are not significantly different ($P > 0.05$)

fungal cells at 2.0 g/l were added to *L. album* hairy roots for an exposure time of 48 h (Kumar et al. 2013).

### Elicitation with chitin and chitosan

Chitin and chitosan may be the elicitor moieties in the fungal extract and in co-culture experiments that were responsible for the induction of defense responses in the plants cells. In the present investigation, chitin at 50 mg/l was able to induce a maximum increment of 2.3-fold in UA (2151.4 µg/g DW), 3.4-fold in OA (754.7 µg/g DW) and 7.0-fold in BA in comparison to the control cultures. Addition of chitosan at 100 mg/l gave a maximum increment of 4.3-fold in UA (4686.0 µg/g DW), 3.4-fold in OA (861.6 µg/g DW) and 3.6-fold in BA (Fig. 3). Since chitosan had a greater enhancement effect on the triterpenoid production, it indicated that acetyl groups in chitin may have an inhibitory effect in elicitation of *L. camara* cultures. Addition of chitosan at 50 mg/l to cell cultures of *Calendula officinalis* for an exposure time of 48 h reportedly enhanced OA accumulation by 5-fold (Wiktorowska et al. 2010).

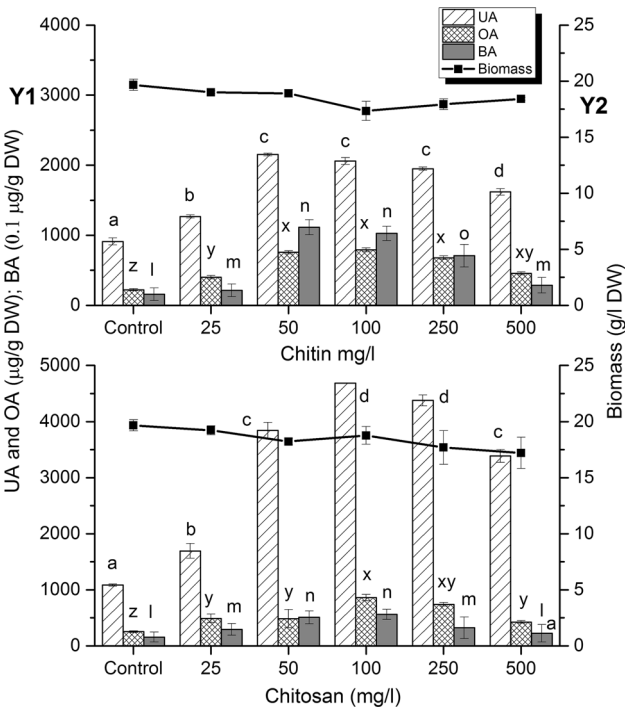


**Fig. 3** Effect of addition of chitin and chitosan as elicitors to the suspension cultures of *L. camara*. Data represents mean values ± SD of three replicates. UA, OA and BA contents are represented on the *Y1* axis with UA and OA contents in µg/g DW and BA content in 0.1 µg/g DW, and biomass (g/l DW) is represented on the *Y2* axis. Within each set of experiments, *bars* with *different letters* are significantly different ($P < 0.05$)

## Conclusions

Elicitation in the late log phase was successfully established for maximum production of triterpenoids in cell cultures of *L. camara*. Out of all the biotic elicitor preparations from *P. indica*, the filter-sterilized culture filtrate at 2.5 % (v/v) effected the maximum enhancement on the production of UA, OA and BA in the cell cultures of *L. camara*. Co-culture of live *P. indica* cells at 2.5 g/l with the suspension cultures of *L. camara* for 24 h also enhanced the triterpenoids production without any negative effect on the plant biomass. Thus, with this study on the positive effect of *P. indica* on triterpenoids synthesis in *L. camara* (Verbenaceae) and the studies on the enhanced synthesis of lignans in cell cultures (Baldi et al. 2010) and hairy roots of *L. album* (Kumar et al. 2013), it appears that the fungus has the potential of acting as a good source of elicitor in enhancing the production of useful secondary metabolites in other plant cell cultures also and this might be worth investigating.

## References

Adya AK, Gautam A, Zhang L, Varma A (2013) Characterization of *Piriformospora indica* culture filtrate. In: Varma A et al (eds) *Piriformospora indica*, soil biology, vol 33. Springer, Berlin, pp 345–375

Alakurtti S, Mäkelä T, Koskimies S, Yli-Kauhaluoma J (2006) Pharmacological properties of the ubiquitous natural product betulin. Eur J Pharm Sci 29:1–13

Bagde US, Prasad R, Varma A (2011) Influence of culture filtrate of *Piriformospora indica* on growth and yield of seed oil in *Helianthus annus*. Symbiosis 53:83–88

Bagde US, Prasad R, Varma A (2013) Impact of culture fltrate of *Piriformospora indica* on biomass and biosynthesis of active ingredient aristolochic acid in *Aristolochia elegans* Mart. Int J Biol 6:29–37

Bahabadi SE, Sharifi M, Safaie N et al (2011) Increased lignan biosynthesis in the suspension cultures of *Linum album* by fungal extracts. Plant Biotechnol Rep 5:367–373

Baldi A, Jain A, Gupta N et al (2008) Co-culture of arbuscular mycorrhiza-like fungi (*Piriformospora indica* and *Sebacina vermifera*) with plant cells of *Linum album* for enhanced production of podophyllotoxins: a first report. Biotechnol Lett 30:1671–1677

Baldi A, Farkya S, Jain A et al (2010) Enhanced production of podophyllotoxins by co-culture of transformed *Linum album* cells with plant growth-promoting fungi. Pure Appl Chem 82:227–241

Das A, Kamal S, Shakil NA et al (2012) The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant, *Coleus forskohlii*. Plant Signal Behav 7:103–112

Gao FK, Dai CC, Liu XZ (2010) Mechanisms of fungal endophytes in plant protection against pathogens. African J Microbiol Res 4:1346–1351

Ghisalberti EL (2000) *Lantana camara* L. (Verbenaceae). Fitoterapia 71:467–486

Godoy-Hernández G, Vázquez-Flota FA (2012) Growth measurements: estimation of cell division and cell expansion. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant cell culture protocols, methods in molecular biology, vol 877. Humana Press, New York, pp 41–48

Kaefer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet 131:19–33

Kumar V, Sahai V, Bisaria VS (2013) Effect of *Piriformospora indica* on enhanced biosynthesis of anticancer drug, podophyllotoxin, in plant cell cultures of *Linum album*. In: Varma A et al (eds) *Piriformospora indica*, soil biology, vol 33. Springer, Berlin, pp 119–137

Kuźma Ł, Bruchajzer E, Wysokińska H (2009) Methyl jasmonate effect on diterpenoid accumulation in *Salvia sclarea* hairy root culture in shake flasks and sprinkle bioreactor. Enzyme Microb Technol 44:406–410

Liu J (2005) Oleanolic acid and ursolic acid: research perspectives. J Ethnopharmacol 100:92–94

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Rai MK, Varma A, Pandey AK (2004) Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. Mycoses 47:479–481

Rijhwani SK, Shanks JV (1998) Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14:442–449

Satheesan J, Narayanan AK, Sakunthala M (2012) Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica*. Mycorrhiza 22:195–202

Savitha BC, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2006) Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake-flask and bioreactor. Process Biochem 41:50–60

Sharma G, Agrawal V (2013) Marked enhancement in the artemisinin content and biomass productivity in *Artemisia annua* L. shoots co-cultivated with *Piriformospora indica*. World J Microbiol Biotechnol 29:1133–1138

Singh A, Singh A, Kumari M, Rai MK, Varma A (2003) Biotechnology importance of *Piriformospora indica*—a novel symbiotic mycorrhiza-like fungus: an overview. Indian J Biotechnol 2:65–75

Singh A, Rajpal K, Singh M, Kharkwal AC, Arora M, Varma A (2013) Mass cultivation of *Piriformospora indica* and *Sebacina* species. In: Varma A et al (eds) *Piriformospora indica*, soil biology, vol 33. Springer, Berlin, pp 377–392

Sirrenberg A, Göbel C, Grond S et al (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Plant 131:581–589

Srivastava P, Chaturvedi R (2010) Simultaneous determination and quantification of three pentacyclic triterpenoids—betulinic acid, oleanolic acid, and ursolic acid—in cell cultures of *Lantana camara* L. In Vitro Cell Dev Biol Plant 46:549–557

Srivastava P, Kasoju N, Bora U, Chaturvedi R (2010) Accumulation of betulinic, oleanolic, and ursolic acids in in vitro cell cultures of *Lantana camara* L. and their significant cytotoxic effects on HeLa cell lines. Biotechnol Bioprocess Eng 15:1038–1046

Srivastava P, Sisodia V, Chaturvedi R (2011) Effect of culture conditions on synthesis of triterpenoids in suspension cultures of *Lantana camara* L. Bioprocess Biosyst Eng 34:75–80

Varma A, Savita V, Sudha et al (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Wiktorowska E, Długosz M, Janiszowska W (2010) Significant enhancement of oleanolic acid accumulation by biotic elicitors in cell suspension cultures of *Calendula officinalis* L. Enzyme Microb Technol 46:14–20