Pure Appl. Chem., Vol. 82, No. 1, pp. 227–241, 2010.
doi:10.1351/PAC-CON-09-02-09

Enhanced production of podophyllotoxins by co-culture of transformed *Linum album* cells with plant growth-promoting fungi*

Ashish Baldi, Sunita Farkya, Abhishek Jain, Nishant Gupta, Rahul Mehra, Vatsala Datta, Ashok Kumar Srivastava, and Virendra Swarup Bisaria‡

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology-Delhi, New Delhi 110 016, India

Abstract: To investigate the plant growth-promoting effect and stimulation of lignan biosynthesis, the effect of culture filtrates/live co-culture of two arbuscular mycorrhizae-like fungi, *Piriformospora indica* and *Sebacina vermifera*, on growth of *Linum album* cells and on production of podophyllotoxin (PT) and 6-methoxypodophyllotoxin (6-MPT) was studied. For elicitation studies, different volumes of culture filtrates (autoclaved and membrane-filtered) of *P. indica/S. vermifera* were added to suspension cultures of *L. album*. The culture filtrates of both the fungi exhibited a positive effect on product formation. For co-culture experiments, both fungi were individually co-cultivated at different concentrations with *L. album* in suspension cultures for different time periods. This resulted in significant enhancement of PT and 6-MPT content in the plant cells. The activity of phenylalanine ammonia lyase (PAL) was observed to be related to the lignan accumulation, indicating its role as the key enzyme of the phenylpropanoid pathway. The study resulted in total lignan (PT and 6-MPT) production of 745.6 mg/l with a very high PT productivity of 52.4 mg/(l.d).

Keywords: arbuscular mycorrhizal-like fungi; co-culture; *Linum album*; *Piriformospora indica*; podophyllotoxins; *Sebacina vermifera*.

INTRODUCTION

Plant cell technology remains an alternative methodology for production of a wide spectrum of phytochemicals. But commercial success of this technology is still limited due to low content of desired metabolite, recalcitrant nature and slow growth rate, genotypic variations, chemical instability, and uneconomical downstream processing. Techniques like optimization of culture medium composition and environmental conditions, medium renewal, addition of biogenetic precursors and elicitation are generally used, alone or in combination, as yield enhancement strategies for biotechnological production of plant-based chemicals in plant cell cultures [1].

"Elicitor" for a plant refers to a chemical from any source that can trigger physiological and morphological responses and phytoalexin accumulation. It includes abiotic elicitors such as metal ions and inorganic compounds, and biotic elicitors from fungi, bacteria, or herbivores, plant cell wall fragments

*Paper based on a presentation at the 13th International Biotechnology Symposium (IBS 2008): "Biotechnology for the Sustainability of Human Society", 12–17 October 2008, Dalian, China. Other presentations are published in this issue, pp. 1–347.
‡Corresponding author: E-mail: vbisaria@dbeb.iitd.ac.in

as well as chemicals that are released at attack site by plants upon pathogen or herbivore attack. It is well known that the treatment of plants with elicitors or attack by incompatible pathogen causes an array of defense reactions, including the accumulation of a range of plant defensive secondary metabolites in intact plants or in plant cell culture [1,2]. Even after the intensive research on the effect of biotic and abiotic elicitors on the production of secondary metabolites in plants, the exact mechanism of elicitation is poorly understood. Various mechanisms in this regard have been hypothesized such as messenger Ca^{2+}, factors affecting cell membrane integrity, inhibition/activation of intracellular pathways, and changes in osmotic stress [2].

The majority of phenylpropanoid pathway derivatives are released by plants in excess amounts under abiotic and biotic stresses or in the presence of signaling compounds by triggering defense/hypersensitive responses [1,2]. But decrease in cell viability and hence suppressed growth of in vitro grown plant cells due to pathogenicity or hypersensitive responses upon exposure to these inducers remains a challenge in the development of commercially successful elicitation-assisted cell culture-based bioprocesses for production of desired metabolites. Therefore, selection of a microorganism for induction of secondary metabolites always remains a challenging task in plant cell technology. Furthermore, it is estimated that there are about 250 000 species of higher plants, but six times as many species of fungi [3]. Most of the plant-fungal associations are of a pathogenic nature. The interaction between plants and their pathogens is complex and may be very specific to a given combination of plants. In most of the earlier studies, either live microbial cells were applied directly to plant tissues or microbe-derived compounds or preparations were used as biotic elicitors for enhancement of secondary metabolite production in cell cultures [2,4–6]. A few researchers have also co-cultured the bacterial cells, for example, of *Phytophthora nicotianae* [7,8] and *Pseudomonas syringae* [9–11], in suspension cultures of tobacco. Enhancement of tanshinone production in *Salvia miltiorrhiza* hairy roots has been reported on co-cultivation with *Bacillus cereus* [12]. But, to the best of our knowledge, there is still no reported work on the application of live plant–fungal interaction to increase concentration of a desired compound in plant cell cultures. So in the present study, our aim was to find out the combination of plant–fungal interaction which would result in significant enhancement of secondary metabolites in cell cultures.

The most common symbiotic association of arbuscular mycorrhizal (AM) fungi with plants is known to be beneficial for both plant cells as well as fungal cells. About 80 % of the known plant species form AM associations [13]. But the cultivation of AM fungi under axenic conditions continues to be one of the most challenging goals. The advent of *Piriformospora indica* and *Sebacina vermifera*, the axenically cultivable AM-like fungi, has opened a new vista to study interaction between plant and fungal cells and to exploit their potential in elicitation of plant-based secondary metabolites of commercial interest. *P. indica* has been shown to confer growth promotion to a broad spectrum of host plants, which might be due to one or more of the following phenomena depending on the plant: elevated nitrate assimilation, starch degradation and production of the auxin, indole-3-acetic acid [14–17].

One such commercially important plant-based lead molecule is podophyllotoxin (PT), an aryltetralin lignan, presently obtained from solvent extraction of rhizomes of *Podophyllum hexandrum* and *P. peltatum*. This compound is used for production of its semi-synthetic derivatives, etoposide, teniposide, and etopophos, which are currently used worldwide for the treatment of a variety of cancers [18]. The search for alternate means to produce PT is still on due to overexploitation and endangered status of its natural source, low content, and uneconomical chemical synthesis due to the presence of chiral centers and stereochemical ring closure. Although plant cell cultures have been exploited by various researchers for commercial production of PT and related lignans, the low productivity remains the main hurdle in its commercialization [19]. The cell cultures of *Linum album* are known to produce the anticancer lignans, PT, and 6-methoxypodophyllotoxin (6-MPT) with the highest productivity [19–22]. Therefore, *L. album* cell cultures were used to establish co-cultures with living cells of *P. indica* and *S. vermifera* in suspension culture and to study their effect on lignan production in present study.

MATERIALS AND METHODS

Development of callus and suspension cultures of *L. album*

Agrobacterium rhizogenes NCIM 5140—mediated genetically—transformed high-yielding cell line of *L. album* was developed from stems of aseptically germinated seeds [20]. Cell suspension cultures were established from this cell line and cultivated in liquid B5 medium [23] supplemented with 45 g/l sucrose, 0.28 g/l ammonium sulfate, and 0.5 g/l calcium chloride [24]. The cultures were incubated on a gyratory shaker at 125 rpm and 25 ± 1 °C under 16 h/8 h light/dark photoperiod [21]. Cells (12 days old) were used as inoculum to develop the suspension cultures for elicitation and co-culture experiments.

Maintenance and growth of fungi

P. indica and *S. vermifera* cultures (kindly provided by Prof. Ajit Varma, Amity Institute of Herbal and Microbial Studies, Noida, India) were maintained on modified Kaefer-agar medium [25] at pH 6.5 and 30 ± 1 °C. Liquid medium of the same composition without agar was used to grow the fungi. The fungal cultures were grown in 100 ml of the medium contained in 500-ml Erlenmeyer flasks at 30 ± 1 °C on a gyratory shaker at 200 rpm. The 5-day-old cultures were used as inoculums to grow the fungi.

Elicitor preparation and its addition to *L. album* cultures

The cultures of the two fungi were grown as described above. The cultures were harvested after they reached their late log phase, i.e., 6 days. The fungal biomass was collected by passing through a filter paper, and the spent medium was centrifuged at 5000 g for 15 min to remove suspended particles and filtered through Whatman No. 1 filter paper. The clear solution so obtained was designated as culture filtrate and stored at 4 ± 1 °C for further use. The culture filtrates of the two fungi were divided into two parts: one part was autoclaved at 15 psig for 20 min and designated as autoclaved culture filtrate (AF) and another part was filtered through 0.22 μm membrane filter and designated as filtered culture filtrate (FF).

The two fungal elicitor preparations viz. AF and FF were added at different concentrations (1, 2.5, 5, 7.5, and 10 % v/v) on the 10^{th} day to growing cultures of *L. album*. The control received an equal volume of the medium. Culture conditions for growing *L. album* in shake flasks remained the same as described above. The cultures were harvested on the 12^{th} day and analyzed for growth, cell viability, lignan production, and phenolics content.

Co-culture of *L. album* with the two fungi

L. album cell suspensions were developed in 50-ml modified B5 medium as described above. The live 5-day-old fungal cells were aseptically added at different concentrations (0.5, 1.0, 2.5, 5.0, and 7.5 g/l on a dry cell weight (DCW) basis) into the suspension culture of *L. album* on 9^{th}, 10^{th}, and 11^{th}, days of cultivation. The flasks, in triplicate, were harvested on the 12^{th} day and analyzed for growth, lignan, phenylalanine ammonia lyase (PAL) enzyme activity, cell viability, and phenolics content.

Extraction and estimation of lignans

The lignans, PT and 6-MPT, were extracted in methanol. The extract was analyzed by high-performance liquid chromatography (HPLC) (Agilent Technologies HP 100) on C_{18} column (Waters, USA, 250 × 4.6 mm). The simultaneous separation and analysis of lignans were carried out at a flow rate of 0.8 ml/min with acetonitrile:phosphoric acid (0.01 %) in water (72:28) as mobile phase at 290 nm wavelength [21].

Determination of cell viability

Triphenyl tetrazolium chloride (TTC) (8 g/l) was dissolved in sodium phosphate buffer (0.05 M, pH 7.5). The TTC reagent was added to the plant cells in 2:1 ratio and the plant cells were incubated for 18–22 h in dark at 25 ± 2 °C. After incubation, the cells were pelleted and washed with distilled water. The red formazan product was extracted from the pelleted cells by incubating with 3 ml ethanol (95 % v/v) for 30 min including brief heating (up to 60 °C) for 15 min. The absorbance was measured at 485 nm by a spectrophotometer using ethanol as blank [26]. The cell viability was expressed in percentage on the basis of the absorbance of the sample in comparison to that of the control culture (considered to be 100 %). *L. album* cultivated in axenic cultures was the control culture.

PAL enzyme assay

Fresh weight of plant (1 g) was frozen and homogenized using liquid nitrogen in a precooled mortar and pestle at 4 °C. Three milliliters of borate buffer (0.1 M, pH 8.0) containing 5 % v/v glycerol and 50 mM β-mercaptoethanol was added to the powdered cells. Samples were subjected to ultrasonication for 1 min. The homogenate was centrifuged at 10 000 g for 30 min at 4 °C and the supernatant was collected. For activity assay, 1 ml of 50 mM phenylalanine was added to 0.5 ml of supernatant and allowed to incubate at 40 ± 2 °C for 60 min. The reaction was stopped by addition of 0.2 ml 6 N HCl. The reaction mixture was extracted by addition of 4 ml toluene by vortexing for 15 s. This mixture was then centrifuged at 1000 g for 10 min to separate the phases. The toluene phase was analyzed for the *trans*-cinnamic acid recovered against a toluene blank at 290 nm. PAL enzyme activity was expressed as μkat (μmoles of cinnamic acid formed per s) per kg protein. Total proteins were extracted in Tris buffer [27], and its estimation was based on the principle of protein-dye binding using bovine serum albumin as standard [28].

Phenolics assay

Phenolics content in culture filtrate was determined by slight modification in the method described by Yuan and co-workers [29]. For this, 2 ml culture filtrate was extracted with 10 ml ethyl acetate. Then 5 ml of ethyl acetate extract was taken and evaporated to dryness. The residue left was redissolved in 3 ml 75 % v/v ethanol. The absorbance was measured at 280 nm against 75 % v/v ethanol as blank. One unit of phenolics accumulated was defined as the amount equivalent to 1 μg salicylic acid absorbance at 280 nm.

Determination of fungal and plant growth

Chitin was selected as a parameter to determine the growth of fungal cells in co-culture experiments as this molecule is present in fungal cells only and not in plant cells. Air-dried biomass from each of the fungal cultures/total biomass samples was acid-hydrolyzed using hydrochloric acid treatment [22]. Estimation of chitin as chitosamine using 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) was performed [22].

Total biomass (plant + fungal) as g/l on a DCW basis was determined for each set of co-culture experiment. The plant biomass produced in co-culture experiments was then determined by subtracting the fungal biomass from the total biomass.

Estimation of residual sucrose

Residual sucrose concentration was measured by the method of Dubois et al. [30].

RESULTS AND DISCUSSION

Growth and production kinetics

The growth, lignan production, and substrate consumption profiles of *L. album* cells in suspension culture were established and are given in Fig. 1.

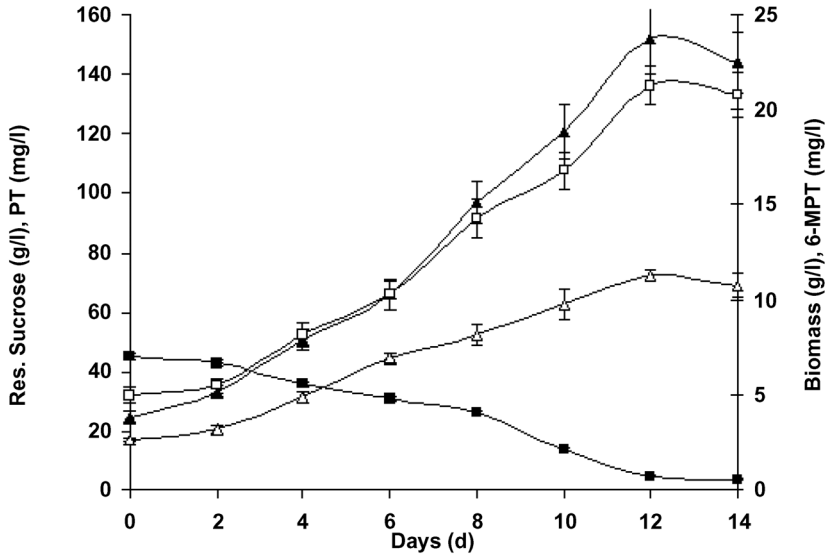

Fig. 1 Kinetics of growth, lignan production, and substrate consumption in *L. album* suspension cultures. Average values are given, error bars are represented by vertical lines. (□: biomass, ■: residual sucrose, ▲: PT, and △: 6-MPT).

The time course study on growth and lignan accumulation revealed that the PT and 6-MPT synthesis in suspension culture increased up to the 12th day, which was associated with an increase in biomass indicating predominantly growth-associated nature of product formation. This phenomenon has also been observed for lignan production by cell suspension cultures of *P. hexandrum* [31] and *L. album* [32]. The present studies showed that lignan accumulation was intracellular and no traces were detected in the culture medium. The maximum PT and 6-MPT concentrations of 152.1 mg/l and 11.3 mg/l, respectively, were obtained on the 12th day of cultivation, with maximum cell growth of 21.3 g/l, which declined thereafter. The decrease in lignan content after the 12th day could be due to either by the initiation of its degradation process, or by a change in its chemical structure. These growth and production levels were significantly higher than earlier reports on *L. album* cell suspension cultures [20,22,31–36] due to application of a high-yielding genetically transformed cell line and optimized culture medium [24].

Effect of biotic elicitor(s) on growth and PT production in *L. album* suspension cultures

The biotic elicitors derived from the fungi, *P. indica* and *S. vermifera*, were tested by adding various concentrations to cell suspension culture of *L. album* on the 10th day of cultivation (late exponential phase) for 48 h. Many workers have studied elicitation by biotic means in various plant cell culture-based biosystems and have reported suppression of cell growth in most of the elicitor-treated cultures. Therefore, biotic elicitor preparations of the two AM-like fungi were added at late log phase in an attempt to first allow the substrate to be converted into cell biomass without any inhibition and then adding the elicitor preparations to see their effects on lignan synthesis. This was expected to make use of these biotic agents effectively against greater cell density, which might lead to higher lignan biosynthesis. Furthermore, the exposure time of the elicitor can be an important factor for product formation also. Comparatively, a shorter exposure time is generally required to induce the process of synthesis of secondary metabolites in plant cell cultures. A longer exposure time could have harmful effects on the production of desired metabolites either due to their degradation or due to stimulation of other biosyn-

thetic pathways. Such phenomenon was observed when the cells of *Catharanthus roseus* were exposed to elicitor extracts of *Trichoderma viride*, *Aspergillus niger*, and *Fusarium moniliforme* for various time intervals. About three-fold increase in ajmalicine production by *C. roseus* cells elicited with extracts of *T. viride* and about two-fold increase in cells elicited with *A. niger* and *F. moniliforme* was observed with an exposure time of 48 h; however, further increase of exposure time resulted in decrease of ajmalicine content [37]. This pattern has also been reported by Rijhwani and Shanks [38], Moreno et al. [39], and Negeral and Javelle [40].

The overall effect of biotic elicitors on cell growth and PT and 6-MPT production by *L. album* is summarized in Table 1. The results indicate that even at late exponential growth phase, the biotic elicitors used in present studies had a suppressing effect on *L. album* cell growth. Addition of different biotic elicitor preparations to the suspension cultures, except FF of *S. vermifera,* significantly suppressed the cell growth at the concentrations tested. The reduction in biomass was responsible for the decreased intracellular lignan concentration (mg/l) despite slight increase in lignan content on cell mass basis (mg/g DCW). For example, the addition of AF of *P. indica* at 2.5 % (v/v) level resulted in slight enhancement of PT content (8.81 mg/g DCW) as opposed to control cultures (7.14 mg/g), but the concentration of PT decreased from 152.1 mg/l in control cultures to 141.0 mg/l in elicited cultures. Elicitor concentration is an important factor for the induction process for enhanced production of secondary metabolites. Various elicitor preparations affected differently the production of lignans.

Table 1 Effect of various biotic elicitors on growth of and lignan production by *L. album* cultures.

Concentration	DCW (g/l)	Phenolics content (mg/ml)	PT (mg/g DCW)	PT (mg/l)	6-MPT (mg/g DCW)	6-MPT (mg/l)
Control (without elicitor)						
	21.3 ± 0.4	75.5 ± 4.4	7.14 ± 0.04	152.1 ± 1.1	0.53 ± 0.04	11.3 ± 0.9
Piriformospora indica						
AF-1.0 %	16.0 ± 1.6	124.9 ± 8.2	7.05 ± 0.02	112.8 ± 11.6	0.19 ± 0.02	3.0 ± 0.6
AF-2.5 %	16.0 ± 1.4	127.8 ± 4.6	**8.81 ± 0.04**	141.0 ± 13.0	0.30 ± 0.01	4.8 ± 0.6
AF-5.0 %	16.0 ± 1.2	143.4 ± 7.9	8.05 ± 0.02	128.8 ± 10.0	0.32 ± 0.02	5.1 ± 0.7
AF-7.5 %	15.5 ± 1.0	148.6 ± 6.9	5.08 ± 0.04	78.7 ± 5.7	0.18 ± 0.01	2.8 ± 0.3
AF-10.0 %	15.5 ± 1.2	158.4 ± 13.4	4.15 ± 0.04	64.3 ± 3.5	0.15 ± 0.01	2.3 ± 0.2
FF-1.0 %	16.0 ± 1.2	129.9 ± 8.9	**8.02 ± 0.06**	128.3 ± 10.6	0.09 ± 0.02	1.6 ± 0.3
FF-2.5 %	16.0 ± 1.6	137.5 ± 11.4	6.44 ± 0.04	103.0 ± 11.1	0.16 ± 0.04	2.5 ± 1.0
FF-5.0 %	15.5 ± 1.4	146.8 ± 8.2	6.07 ± 0.06	94.1 ± 9.5	0.18 ± 0.04	2.8 ± 0.9
FF-7.5 %	16.0 ± 1.2	154.5 ± 5.7	5.99 ± 0.02	95.8 ± 7.5	0.21 ± 0.02	3.3 ± 0.6
FF-10.0 %	15.5 ± 1.0	168.6 ± 4.8	4.76 ± 0.02	73.8 ± 5.1	0.12 ± 0.02	1.8 ± 0.4
Sebacina vermifera						
AF-1.0 %	16.5 ± 1.2	284.9 ± 14.2	5.80 ± 0.06	95.7 ± 8.0	0.31 ± 0.03	5.1 ± 0.9
AF-2.5 %	16.0 ± 1.4	175.5 ± 8.1	5.10 ± 0.05	82.7 ± 6.9	0.19 ± 0.01	3.0 ± 0.4
AF-5.0 %	16.0 ± 1.0	161.1 ± 6.4	4.85 ± 0.04	77.6 ± 5.5	0.17 ± 0.02	2.7 ± 0.5
AF-7.5 %	15.5 ± 1.2	156.5 ± 9.3	4.05 ± 0.02	62.8 ± 5.2	0.17 ± 0.02	2.6 ± 0.5
AF-10.0 %	15.5 ± 1.0	133.1 ± 12.2	3.70 ± 0.01	55.5 ± 5.7	0.16 ± 0.02	2.4 ± 0.5
FF-1.0 %	21.0 ± 1.8	321.7 ± 20.2	6.54 ± 0.02	137.3 ± 12.2	0.08 ± 0.00	1.7 ± 0.1
FF-2.5 %	21.0 ± 1.0	340.2 ± 16.8	7.05 ± 0.04	148.0 ± 7.9	0.20 ± 0.04	4.2 ± 1.1
FF-5.0 %	20.5 ± 1.0	354.6 ± 14.2	8.02 ± 0.06	164.4 ± 9.3	0.98 ± 0.06	20.1 ± 2.3
FF-7.5 %	20.5 ± 0.8	358.7 ± 12.9	**9.78 ± 0.04**	**200.5 ± 8.7**	0.49 ± 0.06	10.0 ± 1.7
FF-10.0 %	20.0 ± 0.6	340.4 ± 8.4	6.67 ± 0.05	133.4 ± 5.0	0.20 ± 0.04	4.0 ± 0.9

Addition of AF of *P. indica* resulted in an increase of PT content up to a concentration of 2.5 %, after which it decreased significantly on increasing the concentration of the elicitor. Similar phenomenon of dose-dependent elicitation of PT was observed when *L. album* cells were treated with AF of *S. vermifera*. The maximum enhancement of PT concentration to 200.5 mg/l was achieved when *S. vermifera* FF was added at 7.5 % (v/v) level to *L. album* suspension cultures. However, further increase in elicitor concentration resulted in decrease of PT content significantly.

Similar phenomenon has been reported for ajmalicine accumulation in *C. roseus* suspension cultures when exposed to different concentrations of elicitor extracts of *T. viride*, *A. niger*, and *F. moniliforme*. Ajmalicine accumulation was higher in cells elicited with an optimum concentration (5.0 %) of elicitor extracts as compared to lower or higher concentrations [37]. Such dose-dependent results have also been supported by the findings of Nef-Campa et al. [41] and Rijhwani and Shanks [38]. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction [42,43]. These results also indicate that elicitor specificity is one of the most important parameters in selection of elicitation agent for optimum induction of desired secondary metabolite in plant cell cultures.

A large variation in PT content was observed with different elicitor preparations tested in this study and it is not clear as to what the mechanism behind this variation was or how was it influenced. Variation in lignan production due to addition of different fungal preparations might be attributed to the differences in elicitor moieties generated during the growth of the fungi. More work, therefore, is needed for a better understanding of the mechanism of elicitor-induced lignan production and its relationship with cell physiology. The variation in response of *L. album* cells to various biotic elicitors might also be due to the type of their interaction around the exposure site with the elicitor moiety. This would explain the observed death of plant cells and thereby decreased biomass upon exposure to the biotic elicitors tested. Similar phenomenon was observed in *Lettuu* cells infected with *Bremia lactucae* [44] and in *Taxus chinesis* cells exposed to *Fusarium oxysporum*-based elicitor preparations [29].

Activation of defense response by enhanced production of phenolic compounds is known against cell wall-derived elicitors from pathogens [45]. Increase in phenolics was, in general, also observed in this study upon addition of elicitors (Table 1). Mainly, two types of phenolics are present in plant cells, induced and constitutively present phenolics like *p*-coumaric acid, ferulic acid, sinapic acid, etc. These phenolics are biogenetic precursors of lignan and lignin biosynthesis in plant cells, and these seem to have been diverted toward lignan biosynthesis, which resulted in enhanced production of PT and 6-MPT under some situations exemplified above (Table 1).

In the present investigation, the increased production of PT provided an evidence for occurrence of positive response of the fungal elicitors in *L. album* cell culture. But the suppression of the cell growth remained to be the main hurdle for development of a plant cell culture-based bioprocess for production of PT and related lignans at significantly higher levels. Therefore, co-culture of live *L. album* cells with these AM-like fungi was used as a novel yield enhancement strategy for production of PT and 6-MPT.

Effect of co-culture on growth of plant cells

In order to exploit the plant growth promoting and elicitation potential of the fungi, different concentrations of live fungal cells (0.5–7.5 g/l) were added to the cell suspension cultures of *L. album* for different co-cultivation periods (24, 48, and 72 h). The results are summarized in Figs. 2–5 and Tables 2 and 3.

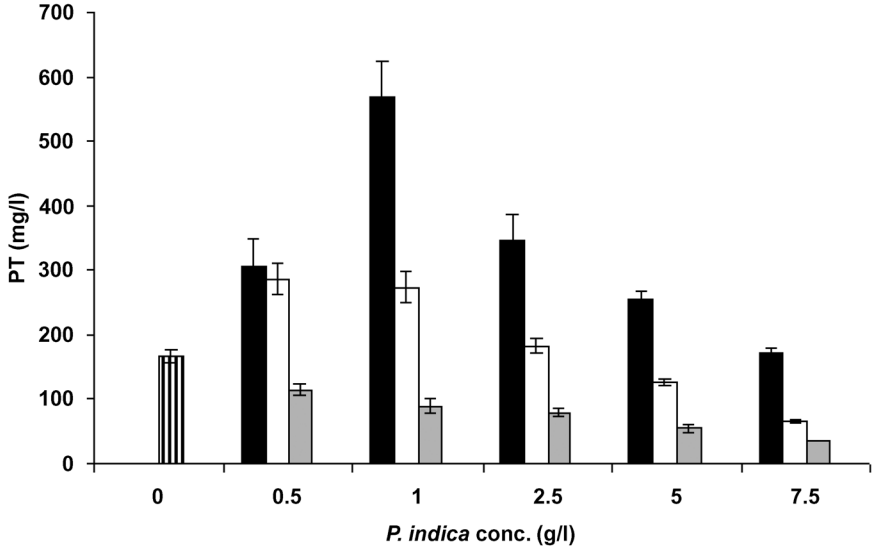

Fig. 2a Effect of *P. indica* concentration on PT accumulation in cell suspension cultures of *L. album* for different periods of co-culture. Average values are given, error bars are represented by vertical lines. (■: 24 h, □: 48 h, ▩: 72 h, and ▥: control).

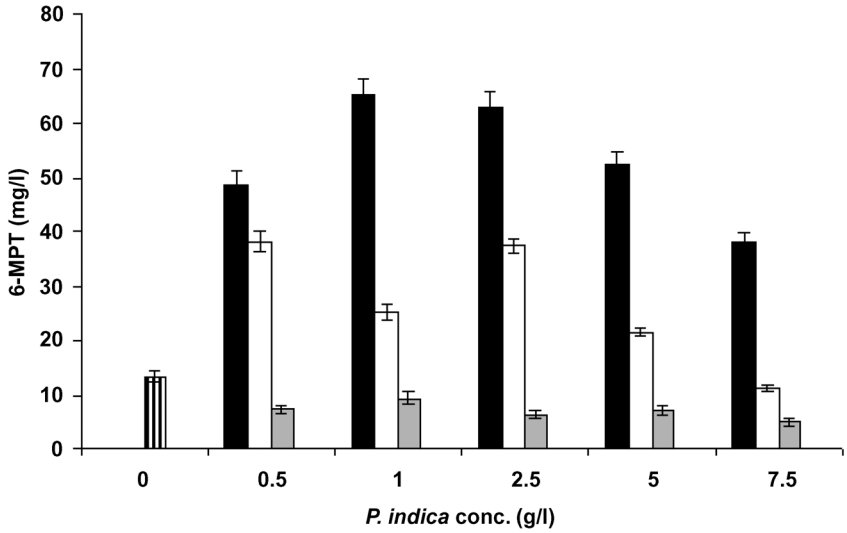

Fig. 2b Effect of *P. indica* concentration on 6-MPT accumulation in cell suspension cultures of *L. album* for different periods of co-culture. Average values are given, error bars are represented by vertical lines. (■: 24 h, □: 48 h, ▩: 72 h and ▥: control).

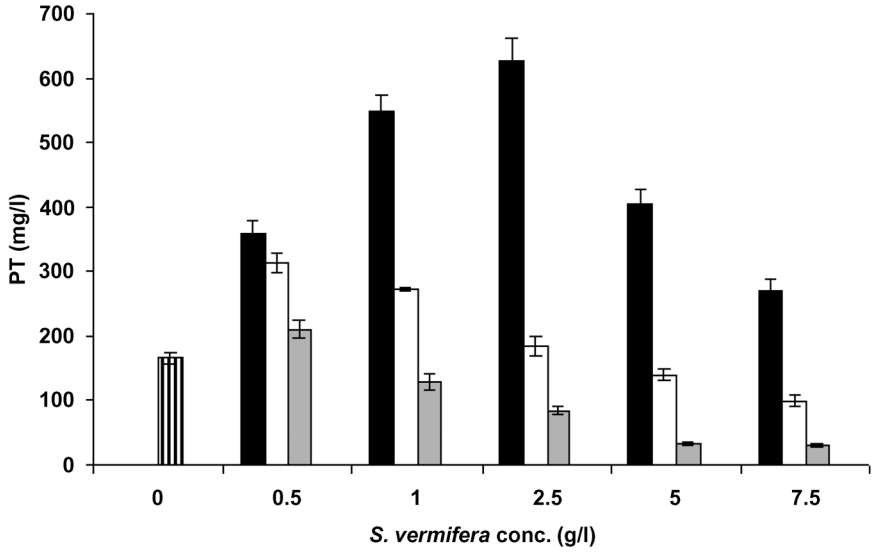

Fig. 3a Effect of *S. vermifera* concentration on PT accumulation in cell suspension cultures of *L. album* for different periods of co-culture. Average values are given, error bars are represented by vertical lines. (■: 24 h, □: 48 h, ▩: 72 h and ▥: control).

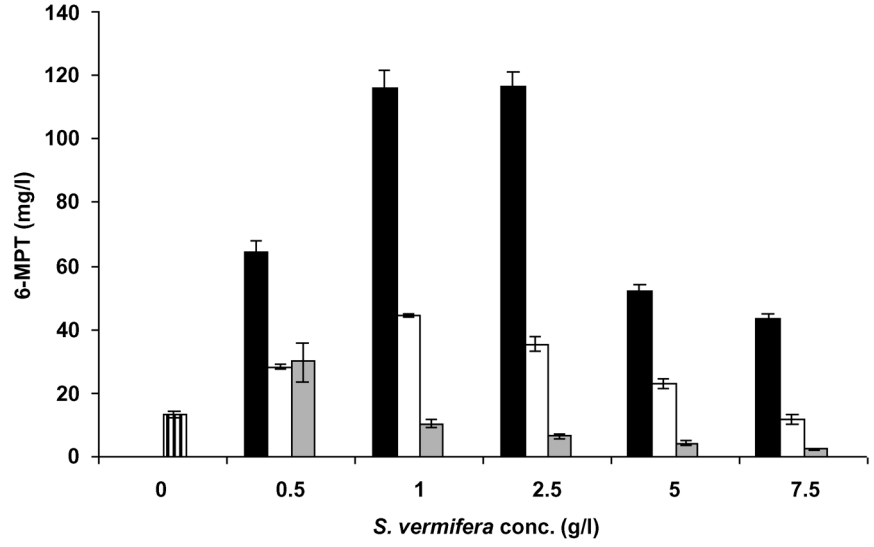

Fig. 3b Effect of *S. vermifera* concentration on 6-MPT accumulation in cell suspension cultures of *L. album* for different periods of co-culture. Average values are given, error bars are represented by vertical lines. (■: 24 h, □: 48 h, ▩: 72 h and ▥: control).

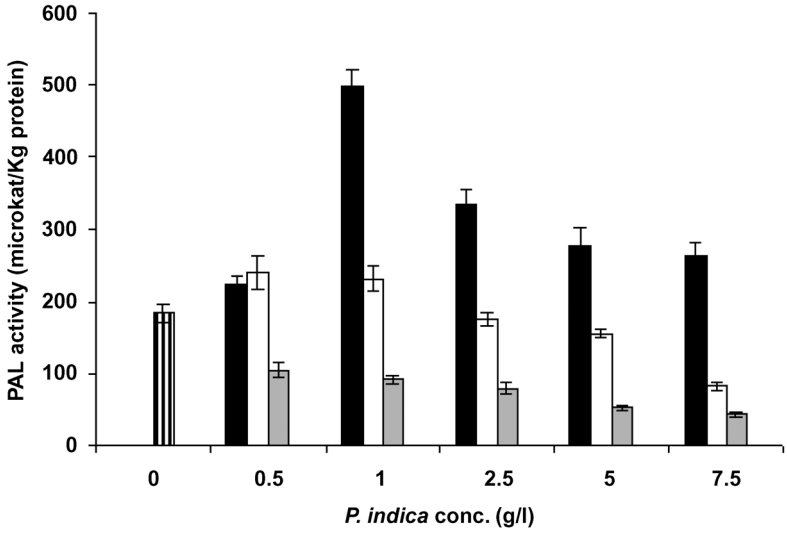

Fig. 4 Effect of *P. indica* concentration on PAL activity in cell suspension cultures of *L. album* for different periods of co-culture. Average values are given, error bars are represented by vertical lines. (■: 24 h, □: 48 h, ▩: 72 h, and ▥: control).

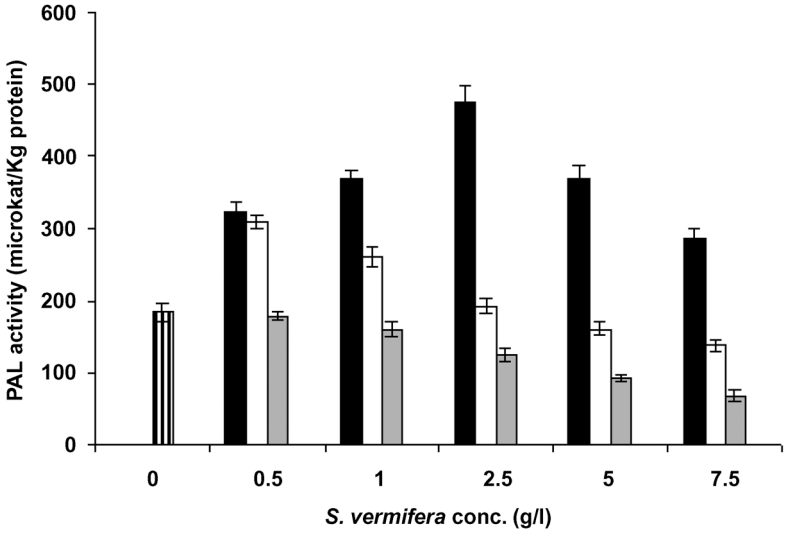

Fig. 5 Effect of *S. vermifera* concentration on PAL activity in cell suspension cultures of *L. album* for different periods of co-culture. Average values are given, error bars are represented by vertical lines. (■: 24 h, □: 48 h, ▩: 72 h, and ▥: control).

Table 2 Effect of co-culture of fungal cells with *L. album* cells on phenolics content in suspension cultures.*

Conc. of fungi (g /l)	Phenolics content (µg/ml)					
	P. indica			*S. vermifera*		
	Duration of co-culture			Duration of co-culture		
	24 h	48 h	72 h	24 h	48 h	72 h
0.0	73.7 ± 10.2	73.7 ± 10.2	73.7 ± 10.2	73.7 ± 10.2	73.7 ± 10.2	73.7 ± 10.2
0.5	158.3 ± 10.2	256.0 ± 18.6	291.6 ± 22.8	138.5 ± 10.2	260.9 ± 18.4	252.0 ± 20.2
1.0	**168.5 ± 08.4**	384.3 ± 22.8	312.8 ± 26.2	150.5 ± 8.2	381.7 ± 20.4	292.8 ± 25.8
2.5	188.6 ± 10.8	348.5 ± 24.2	342.3 ± 22.8	**158.3 ± 6.4**	363.1 ± 12.8	308.9 ± 22.4
5.0	216.6 ± 06.2	338.9 ± 30.2	366.7 ± 30.2	211.0 ± 18.2	316.3 ± 11.2	348.5 ± 18.8
7.5	256.8 ± 12.4	340.5 ± 28.0	477.5 ± 28.2	235.5 ± 12.2	293.2 ± 10.8	442.0 ± 10.2

*Average values, as determined on 12^{th} d, are given; experiments were performed in triplicate.

Table 3 Summary of the best co-culture strategy for production of PT and 6-MPT by *L. album*.*

Parameters tested	Control	Co-culture with *P. indica*	Co-culture with *S. vermifera*
Exposure time (h)	–	24	24
Initial fungal conc. (g/l DCW)	–	1.0	2.5
Plant biomass (g/l DCW)	23.4 ± 1.2	28.4 ± 1.0	27.8 ± 1.0
PT content (mg/g DCW)	7.14 ± 0.04	20.08 ± 1.2	22.62 ± 0.4
PT conc. (mg/l)	167.2 ± 9.5	570.3 ± 55.9	628.9 ± 34.2
6-MPT content (mg/g DCW)	0.57 ± 0.02	2.30 ± 0.02	4.20 ± 0.01
6-MPT conc. (mg/l)	13.4 ± 1.2	65.3 ± 2.9	116.8 ± 4.5
PAL activity (µkat/kg protein)	183.5 ± 12.4	498.7 ± 22.6	476.9 ± 20.4
Cell viability (%)	96.7 ± 6.0	100.0 ± 0.0	97.8 ± 4.6

*Each value represents the mean ±S.D. of three experiments

Both fungi exhibited plant growth-promoting effect on the plant cells in suspension cultures. Plant cell growth was increased maximally by 21.3 % (28.4 g/l) in comparison to control cultures (23.4 g/l) when *P. indica* was co-cultured at a concentration of 1 g/l for 24 h. Similarly, 18.8 % enhancement in plant biomass to 27.8 g/l was obtained when *S. vermifera* was co-cultured for 24 h at a concentration of 2.5 g/l (Table 3). In contrast, the fungal biomass remained almost the same during this period. The plant growth-promoting effect of the two fungi in terms of increment in plant biomass might be either due to increased absorption of mineral nutrients by plant cells, as was observed upon mycorrhizal colonization of soybean [46] or due to reduction in incidence of disease caused by plant pathogens [47]. The exposure time of the fungal cells to the plant cells greatly affected this plant growth-promoting effect. At an exposure time of 72 h, a decrease in plant cell biomass was observed with a little increase in fungal biomass presumably due to their adaptation in the new medium (data not shown). Although a plausible explanation is not available, this could be attributed to the non-availability of nutrients to the plant cells due to their preferential uptake by the fungal cells during co-cultivation for longer duration.

P. indica, an endosymbiont, isolated from the soil of Rajasthan (India), is reported to show growth promotional activities with a wide variety of plants having commercial value like *Bacopa moniera* [48], *Withania somnifera*, *Spilanthes calva* [49], *Azadirachta indica*, *Coffea arabica* [50,51], *Tridex procumbans*, *Abrus procatorius* [52], *Adhatoda vasica* [53], and *Chlorophytum borivilianum* [54]. Survival rate and PT content of in vitro propagated plantlets of *P. peltatum* were increased when it was inoculated with AM fungi [55]. *P. indica* also enhanced flowering [49,56], increased yield and survival rates during stress conditions [15], and improved root growth in *Arabidopsis thaliana* seedling [57]. On the other hand, *Sebacina sp.* is also reported to interfere with defense signaling by ethylene inhibition and allow

Nicotiana attenuata plants to increase growth rates at the expense of herbivore resistance mediated by triose phosphate isomerases [56]. But all of these studies had been carried out on either in vitro propogated or pot/field-grown plants. Studies on organ (root) culture with AM fungi with the objective of studying plant–fungal symbiotic interaction have also been reported [58,59]. The present investigation reports the plant growth-promoting capability of these two AM-like fungi in plant cell suspension cultures of *L. album.* The induction of a nitrate reductase for enhanced nitrate assimilation in Arabidopsis on stimulation with *P. indica*, and the presence of indole acetic acid in culture filtrate of the fungus support its role as a plant growth-promoting fungus [16,60].

Effect of co-culture on production of PTs

Mutual interactions between plant and fungal cells require a sophisticated balance between the defense response of the plant and the nutrient demand of the endophyte. Hence, mutualism does not imply an absence of plant defense. Induction of defense-related gene expression has been well studied during host colonization by obligate biotrophic AM fungi. AM-like fungi can interact with plant cells by direct contact or by indirect process. Direct interactions may include physical or chemical antagonism or synergism between the fungi and plant cells. Indirect interactions may result in stimulation of production of certain chemical compounds which are antimicrobial in nature and commonly known as phytoalexin. PTs and related lignans are known to be produced as an arsenal of defense mechanism in *L. album*. Hence, the effect of co-culture of the fungi with *L. album* cells on production of the lignans was studied.

As shown in Figs. 2a,b, the maximum increments of 3.4 times (570.3 mg/l) and 4.9 times (65.3 mg/l) in PT and 6-MPT accumulation, respectively, in comparison to control cultures were obtained, when 1 g/l *P. indica* was added for 24 h. Addition of 2.5 g/l *S. vermifera* for 24 h resulted in improvement of PT and 6-MPT production by 3.76 times (628.9 mg/l) and 8.74 times (116.8 mg/l) in co-cultivation experiments (Figs. 3a,b). This could be attributed to live interaction of fungal cells with the plant cells in co-culture, which might have resulted in higher induction of the phenylpropanoids. Biomass concentrations of 1 g/l of *P. indica* and 2.5 g/l of *S. vermifera*, respectively, were found to be optimum in co-culture studies. Amongst various exposure times, an exposure time of 24 h was found to be optimum for enhanced production of PTs in co-culture system. A minimum time period is known to be necessary for AM fungi to execute their effects [61]. Induction of defense-related genes in plant cells has been reported to be the most prominent during the early stages of AM fungal penetration [62]. Therefore, exposure time of 24 h seems to have resulted in optimal overexpression of genes responsible for enhanced production of PTs.

As shown in Figs. 2 and 3, the accumulation of the lignans decreased after reaching a maximum at an optimum concentration of fungal cells and co-cultivation period in the present study. This might be because of conversion of PT to unknown compounds, or because of non-availability of nutrients to *L. album* cells due to their preferential uptake by fungal cells at increased concentrations or at prolonged cultivation times.

Any direct relation between lignan contents and phenolics accumulation was not observed in the present study. There was an increase in the phenolics content in the medium with an increase in exposure time and fungal concentration, however, the highest phenolics accumulation of 442.0 μg/ml being observed during co-cultivation of *S. vermifera* at 7.5 g/l level for 72 h (Table 2).

There exists increasing evidence that plant defense pathways are co-regulated by symbiotic association. They increase or decrease the level of pathogenesis-related proteins in the plant as a result of symbiosis and hence may elicit the secondary metabolite pathways [63]. The mechanism of action of symbiotic mycorrhiza like fungi as biotic elicitors may be different. It has been reported that plant defense pathways, co-regulated by symbiotic association, may be due to the secretion of some hydrolytic enzymes which are believed to have a role in defense [64], or may be due to altered levels of plant growth hormones known to act as long-distance signals [65]. It is already known that changes in the

hormonal balance of plants can modulate the expression of defense-related genes [62]. It can trigger the activation of defense genes and increase the level of pathogenesis-related proteins in the plant as a result of symbiosis [66], and hence may elicit the secondary metabolite pathways for increased phytoalexin production. This was substantiated in the present study by the observation that the activity of PAL enzyme increased significantly in *L. album*.

Effect of co-culture on PAL activity

The first step of phenylpropanoid pathway, i.e., conversion of phenylalanine to cinnamic acid by deamination, is catalyzed by PAL enzyme. This is the rate-limiting step of lignan biosynthesis and is also known as the bridge reaction between primary metabolism and natural product biosynthesis [67].

Lignan accumulation and PAL enzyme activity were found to be directly related as the maximum increase in PAL activity also gave rise to the maximum accumulation of lignans. The maximum enhancement of PAL activity by 2.71 times (498.7 μkat/kg protein) in *P. indica* treated cultures (Fig. 4) was observed in comparison to control cultures. PAL activity was found to increase maximally by 2.6 times (476.9 μkat/kg protein) in co-cultures with *S. vermifera* (Fig. 5).

Exposure of both fungi for longer duration (72 h) caused a decrease in PAL enzyme activity and consequently resulted in lesser lignan production. The overall effect of the best co-cultures strategy on production of lignans by *L. album* is summarized in Table 3.

From Table 3, it is clear that the fungi resulted in significant improvement in lignan accumulation by exhibiting plant growth-promoting activity along with increasing the intracellular lignan content. Although the highest biomass accumulation (28.4 g/l) was observed during co-cultivation of *P. indica* at 1.0 g/l concentration for 24 h, the PT content remained lower (20.08 mg/g DCW) in comparison to co-cultures with 2.5 g/l *S. vermifera* (22.62 mg/g DCW). Highest accumulations of 628.9 mg/l PT and 116.8 mg/l 6-MPT were achieved during co-cultivation of *L. album* cells with 2.5 g/l *S. vermifera* cells for 24 h. Co-culture with these plant growth-promoting fungi also avoided the hypersensitivity responses associated with biotic elicitation with their culture filtrates. This was also reflected by higher cell viability of the plant cells (96–100 %) in co-culture experiments as opposed to that with their culture filtrates (46–64 %). The present study thus resulted in very higher PT productivity of 52.4 mg/(l.d) with total lignan (PT and 6-MPT) production of 745.7 mg/l.

CONCLUSIONS

This study represents a successful approach for enhanced production of PT and 6-MPT by transformed cell suspension cultures of *L. album* on co-culturing with AM-like fungi, *P. indica* and *S. vermifera.* Significantly higher lignan accumulation was achieved in comparison to those reported earlier for any plant cell culture producing PT and related lignans. *L. album* cells were found to be susceptible to elicitation by the extracellular metabolites secreted by both *P. indica* and *S. vermifera*. The problem of suppression of cell growth upon exposure to biotic elicitors could be overcome by co-culture strategy due to plant growth-promoting action of the fungi. Simultaneous induction of symbiotic and elicitation potentials of the fungal cells to the plant cells resulted in significantly enhanced production of commercially important secondary metabolites in cell culture. The methodology is expected to provide a valuable tool to study natural symbiotic plant–fungal association at cellular level and avoid the limitations associated with establishment of axenic cultures of AM fungi.

REFERENCES

1. S. R. Rao, G. A. Ravishankar. *Biotechnol. Adv.* **20**, 101 (2002).
2. J. Zhao, L. C. Davis, R. Verpoorte. *Biotechnol. Adv.* **23**, 283 (2005).

3. R. J. Grayer, T. Kokubun. *Phytochem.* **56**, 253 (2001).
4. J. Ebel, E. G. Cosio. *Int. Rev. Cytol.* **148**, 1 (1994).
5. A. Namdeo. *Phcog. Rev.* **1**, 69 (2008).
6. R. Radman, T. Saez, C. Bucke, T. Keshwarz. *Biotechnol. Appl. Biochem.* **37**, 91 (2003).
7. A. J. Able, D. I. Guest, M. W. Sutherland. *Physiol. Mol. Plant Pathol.* **58**, 189 (2001).
8. A. J. Able, M. W. Sutherland, D. I. Guest. *Funct. Plant Biol.* **30**, 91 (2003).
9. L. D. Keppler, C. J. Baker, M. M. Atkinson. *Phytopathology* **79**, 974 (1989).
10. M. M. Atkinson, J. S. Huang, J. A. Knopp. *Plant Physiol.* **79**, 843 (1985).
11. M. M. Atkinson, L. D. Keppler, E. W. Orlandi, C. J. Baker, C. F. Mischke. *Plant Physiol.* **92**, 215 (1990).
12. J. Y. Wu, Ng. Janet, S. Ming, S. J. Wu. *Appl. Microbiol. Biotechnol.* **77**, 543 (2007).
13. S. E. Smith, D. J. Read. *Mycorrhizal Symbiosis*, 2nd ed., Academic Press, New York (1997).
14. A. Varma, S. Varma, N. Sudha, N. Sahay, B. Butehorn, P. Franken. *Appl. Environ. Microbiol.* **65**, 2741 (1999).
15. F. Waller, B. Achatz, H. Baltruschat, J. Fodor, K. Becker, M. Fischer, T. Heier, R. Huckelhoven, C. Neumann, D. Von Wattstein, P. Franken, K. H. Kogel. *Proc. Natl. Acad. Sci. USA* **102**, 13386 (2005).
16. I. Sherameti, B. Shehellori, Y. Venus, L. Altschemied, A. Varma, R. Oelmnller. *J. Biol. Chem.* **280**, 26241 (2005).
17. C. D. Grubb, S. Abel. *Trends Plant Sci.* **11**, 89 (2006).
18. B. Botta, G. D. Monache, D. Misiti, A. Vitali, G. Zappia. *Curr. Med. Chem.* **8**, 1363 (2001).
19. A. Baldi, V. S. Bisaria, A. K. Srivastava. In *Medicinal Plant Biotechnology: From Basic Research to Industrial Applications*, O. Kayser, W. J. Quax, (Eds.), pp. 117–156, Wiley-VCH, Weinheim, Germany (2007).
20. A. Baldi, A. K. Srivastava, V. S. Bisaria. *Biotechnol. J.* **3**, 1256 (2008).
21. A. Baldi, A. K. Srivastava, V. S. Bisaria. *Appl. Biochem. Biotechnol.* **151**, 547 (2008).
22. A. Baldi, A. Jain, N. Gupta, A. K. Srivastava, V. S. Bisaria. *Biotechnol. Lett.* **30**, 1671 (2008).
23. O. L. Gamborg, R. A. Miller, K. Ojima. *Exp. Cell Res*. **50**, 151 (1968).
24. A. Baldi. "Production of anticancer drug, Podophyllotoxin, by plant cell cultivation of *Linum album*", Ph.D. Thesis, Indian Institute of Technology Delhi, New Delhi, India (2008).
25. E. Kaefer. *Adv. Genet.* **19**, 33 (1977).
26. L. Towill, P. Mazur. *Can. J. Bot.* **53**, 1097 (1975).
27. S. Arulsekar, D. E. Parfitt. *Hort. Sci*. **21**, 928 (1986).
28. M. Bradford. *Anal. Biochem.* **72**, 248 (1976).
29. Y. J. Yuan, C. Li, Z. D. Hu, J. C. Wu, A. P. Zeng. *Process Biochem*. **38**, 193 (2002).
30. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Roberts, F. Smith. *Anal. Chem.* **28**, 350 (1956).
31. T. Smollny, H. Wichers, S. Kalenberg, A. Shahsavari, M. Petersen, A. W. Alfermann. *Phytochemistry* **48**, 975 (1998).
32. U. Empt, A. W. Alfermann, N. Pras, M. Peterson. *J. Appl. Bot.* **74**, 145 (2000).
33. V. Seidel, J. Windhovel, G. Eaton, A. W. Alfermann, R. R. J. Arroo, M. Medarde, M. Petersen, J. G. Wooley. *Planta* **215**, 1031 (2002).
34. B. van Furden, A. Hamburg, E. Fuss. *Plant Cell Rep*. **24**, 312 (2005).
35. K. Federolf, A. W. Alfermann, E. Fuss. *Phytochemistry* **68**, 1397 (2007).
36. S. Chattopadhyay, V. S. Bisaria, S. S. Bhojwani, A. K. Srivastava. *Can. J. Chem. Eng.* **81**,1011 (2003).
37. A. G. Namdeo, S. Patil, D. P. Fulzele. *Biotechnol. Prog.* **18**, 159 (2002).
38. S. K. Rijhwani, J. V. Shanks. *Biotechnol. Prog.* **14**, 442 (1998).
39. P. R. H. Moreno, R. Van der Heijden, R. Verpoorte. *Plant Cell Rep.* **12**, 702 (1993).
40. J. Negeral, F. Javelle. *Physiol. Plant.* **95**, 569 (1995).
41. C. Nef-Campa, M. F. Trousot, P. Trouslot, H. Chrestin. *Planta Med*. **60**, 149 (1994).

42. U. Mukandan, M. A. Hjorosto. *Appl. Microb. Biotechnol.* **33**, 145 (1990).
43. I. A. Roewer, N. Cloutier, R. Van der Heijden. *Plant Cell Rep.* **11**, 86 (1992).
44. M. Bennet, M. Gallagher, J. Fagg, C. Bestwick, T. Paul, M. Beale, J. Mansfield. *Plant J.* **9**, 851 (1996).
45. R. L. Nicholson, R. Hammerschmidt. *Annu. Rev. Phytopathol.* **30**, 369 (1992).
46. G. R. Safir, J. S. Boyer, J. W. Gerdemann. *Science* **172**, 581 (1971).
47. H. W. Dehne. *Phytopathol*ogy **72**, 115 (1982).
48. N. S. Sahay, A. Varma. *Curr. Sci.* **78**, 126 (2000).
49. M. K. Rai, A. Singh, D. Arya, A. Varma. *Mycorrhizae* **11**, 123 (2001).
50. An. Singh, Ar. Singh, A. Varma. *Ind. J. Biotechnol.* **1**, 372 (2002).
51. An. Singh, Ar. Singh, M. Kumari, M. K. Rai, A. Varma. *Ind. J. Biotechnol.* **2**, 65 (2003).
52. R. Kumari, G. H. Pham, M. Sachdev, A. P. Garg, A. Varma. *Nat. Prod. Rad.* **3**, 396 (2004).
53. M. Rai, A. Varma. *Elect. J. Biotechnol.* **8**, 195 (2005).
54. A. K. Chauhan, A. Das, A. C. Kharkwal, A. Varma. In *Microbiology Series—Microbes: Health and Environment*, A. K. Chauhan, A. Varma (Eds.), pp. 1–12, IK International Publishing, New Delhi (2006).
55. R. M. Moraes, Z. D. Andrade, E. Bedir, F. E. Dayan, H. Lata, I. Khan, A. M. S. Pereira. *Plant Sci.* **166**, 23 (2004).
56. O. Barazani, M. Benderoth, K. Groten, C. Kuhlemeier, I. T. Baldwin. *Oecologia* **146**, 234 (2005).
57. T. Peskan-Berghofer, B. Shahollari, P. H. Giong, S. Hehl, C. Markert, V. Blanke, G. Kost, A. Varma, R. Oelmuller. *Physiol. Plant.* **122**, 465 (2004).
58. G. Becard, J. A. Fortin. *New Phytol.* **108**, 211 (1988).
59. P. Tiwari, A. Adholeya. *Mycol. Prog.* **2**, 171 (2003).
60. A. Sirrenberg, C. Gobel, S. Grond, N. Cempinski, I. Feussner, K. Pawlowski. Abstract in Meeting Report of Research Group 546. "Signal Exchange between Root and Microorganisms." George-August-University, Gottingen (2007).
61. U. Druege, H. Baltruschat, P. Franken. *Sci. Horticult.* **112**, 422 (2007).
62. J. M. Garcia-Garrido, J. A. Ocampo. *J. Exp. Bot.* **53**, 1377 (2002).
63. L. Petruzelli, C. Kunz, R. Waldvogel, F. Meins, G. Leubner-Metzger. *Planta* **209**, 195 (1999).
64. M. J. Pozo, C. Cordier, E. Dumas-Gaudot, S. J. Gianinazzi, J. M. Barea, C. A. Aguilar. *J. Exp. Bot.* **53**, 525 (2002).
65. J. G. Murphy, S. M. Rafferty, A. C. Cassells. *Appl. Soil Ecol.* **15**, 153 (2000).
66. M. R. Lambais. In *Current Advances in Mycorrhizae Research*, G. K. Podila, D. D. Douds (Eds.). pp. 45–49, APS Press, St. Paul, MN (2000).
67. J. B. Nicholas, O. W. John, A. Meromit, T. N. Hassar. *Plant Biol.* **91**, 7608 (1994).

