Chapter 7
Effect of *Piriformospora indica* on Enhanced Biosynthesis of Anticancer Drug, Podophyllotoxin, in Plant Cell Cultures of *Linum album*

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7.1 Introduction

7.1.1 Piriformospora indica*: A Root Endophytic Fungus*

Arbuscular mycorrhizal fungi (AM fungi) represent the most ancient symbiosis with the plants. However, the AM fungi are non-cultivable outside the host plant. The absence of an authentic pure culture creates a problem in its production and, therefore, impedes the biotechnological applications of AM fungi. A root endophyte designated as *Piriformospora indica* was discovered from the desert soil of northwest India, which is similar to AM fungi. Like AM fungi, it has a broad and diverse spectrum and exerts plant growth-promoting effects on the host plants. But the most important advantage of *P. indica* over AM fungi is that it is a facultative symbiont and can be easily cultivated axenically on a variety of synthetic media (Varma et al. 2001). *P. indica* promotes the growth of plants and improves their productivity, enhances the uptake of phosphorus from the soil, increases the drought tolerance of host plants, delays the wilting of leaves, prolongs the aging of callus tissue, protects the plants from the attack of pathogens, and relieves the stress conditions caused by acidity, desiccation, and heavy metal toxicity (Podila and Varma 2004; Zuccaro et al. 2009; Yadav et al. 2010). A review of literature

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substantiates that the fungus *P. indica* has extraordinary potential for growth promotion of plants by colonization of roots (Oelmüller et al. 2009; Varma et al. 2001). The fungus has exerted growth-promoting effects on a variety of mono- and dicotyledonous plants including medicinal and several economically important plants like *Bacopa monnieri* (Sahay and Varma 1999, 2000), *Azadirachta indica*, *Coffea arabica* (Singh et al. 2002, 2003), *Withania somnifera*, *Spilanthes calva* (Rai et al. 2001), *Tridex procumbens*, *Abrus precatorius* (Kumari et al. 2004), *Adhatoda vasica* (Rai and Varma 2005), and *Chlorophytum borivilianum* (Chauhan et al. 2006). In addition to fungal biomass, the growth-promoting effects have also been shown by the culture filtrate of *P. indica* (Varma et al. 1999, 2001).

7.1.2 Hairy Root Culture and Elicitation

Plants are inexhaustible source for a variety of chemicals such as flavors, fragrances, natural pigments, pesticides, and pharmaceuticals (Chattopadhyaya et al. 2004). Many of these valuable secondary metabolites are synthesized by plant kingdom only. Therefore we have to depend on plants for the production of these valuable secondary metabolites (Rao and Ravishankar 2002). The most important problem being faced today regarding these plant-derived chemicals is their affordability and availability. The conventional process of production of these secondary metabolites by the cultivation of the whole plant followed by the extraction of the desired product has a number of disadvantages such as low productivity and non-availability of these secondary metabolites from the plants throughout the year. Therefore there is a need to enhance their production so that their cost may come within the reach of common man. Plant cell culture provides an alternative to the whole plant cultivation for the production of these valuable secondary metabolites, which not only improves the productivity but is also independent of geographical and climatic factors and ensures the continuous supply of products, uniform quality, and yield (Georgiev et al. 2007). Furthermore, in a number of plant cell cultures, the amount of secondary metabolite produced is more than in the whole plant (Ravishankar and Rao 2000).

A route for improving secondary metabolite production is by transformation using the natural vector system of *Agrobacterium rhizogenes* (a gram-negative bacterium) which infects a variety of plants and causes the nepotistic plant disease known as "hairy root" disease. These transformed roots are genetically stable (Aird et al. 1988) that lead to stable and high-level production of secondary metabolites (Banerjee et al. 1995). They grow as fast as or even faster than the normal roots (Flores et al. 1999) and can be used as continuous source for the production of valuable secondary metabolites. Hairy roots do not require conditioning of the medium and can be grown in hormone- and vitamin-free culture medium. Moreover, the biosynthetic capacity of hairy roots is equivalent or sometimes more than the corresponding plant roots, and the hairy roots can be grown in bioreactors (Farkya and Bisaria 2008). Hairy roots also have been found to accumulate those

secondary metabolites which are found only in the aerial part of intact plant. For example, artemisinin which accumulates in the aerial part of *Artemisia annua* plant is also produced by hairy roots (Giri et al. 2000). There are a number of cases where secondary metabolites are synthesized at higher levels in hairy roots than in the corresponding untransformed roots (Bonhomme et al. 2000; Jacob and Malpathak 2004; Zhang et al. 2004).

One of the most effective strategies for enhancing secondary metabolite production in plant tissue and cell cultures is the treatment of plant cells with various elicitors (Qian et al. 2006). An elicitor may be defined as a substance which, when added in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds, and this improved biosynthesis of metabolites due to addition of trace amounts of elicitors is known as elicitation (Radman et al. 2003). Elicitors can be divided into two categories: biotic and abiotic. Biotic elicitors have a biological origin which include polysaccharides derived from plant cell walls (pectin or cellulose) and microorganisms (especially fungi and their extracts), while abiotic elicitors have a non-biological origin which include chemicals such as methyl jasmonate, arachidonic acid, copper sulfate, and silver nitrate.

7.1.3 *Podophyllotoxin (A Secondary Metabolite with Cytotoxic Activity) and* Linum album

Lignans are the dimerization products of phenylpropanoid pathway intermediates which are connected by the central carbons of their side chain. Podophyllotoxin (PT) is a naturally occurring lignan, which is a pharmacologically important compound for its cytotoxic properties. Though PT itself is too toxic for therapeutical use, but its three semisynthetic derivatives, etoposide, etopophos, and teniposide, are widely used as anticancer drugs and have shown good clinical effects against several types of neoplasms including testicular and small-cell lung cancers, lymphoma, leukemia, and Kaposi's sarcoma (Schacter 1996). Therefore PT is widely used for the commercial semisynthetic production of these derivatives.

Linum album is a perennial herbaceous plant which produces PT and its derivatives. The main lignan in *L. album* is PT (Smollny et al. 1998), and the suspension cultures of this plant are known to produce these lignans with highest productivity (Baldi et al. 2007). For the enhanced secondary metabolite production, transformed cell and organ cultures have been reported to be an alternative to suspension cultures (Giri et al. 2000; Baldi et al. 2008a). For example, hairy root cultures of *Linum flavum* have been reported to produce 6-methoxypodophyllotoxin (6-MPT) in 2–5 and 5–12 times higher concentration compared to non-transformed root and undifferentiated cell suspension cultures, respectively (Oostdam et al. 1993). Hairy root cultures of *L. album* have also been reported to produce lignans (PT and 6-MPT) (Farkya and Bisaria 2008). Baldi et al. (2010) studied the effect of

culture filtrates (autoclaved and membrane filtered) and live cells of *P. indica* on growth and lignan production by the cell suspension cultures of *L. album*. The maximum improvement in the growth (28.4 g/L DCW) and lignan content (PT: 20.1 mg/g DCW; 6-MPT: 2.3 mg/g DCW) was achieved on addition of live cells of *P. indica* at a concentration of 1.0 g/L for an exposure time of 24 h. A similar kind of study was carried out by Kumar et al. (2011a) who investigated the effect of addition of autoclaved and filter-sterilized culture filtrate of *P. indica* to the growing *L. album* hairy root cultures on growth and lignan production. They also found that the addition of culture filtrate resulted in a significant enhancement in growth and lignan content of *L. album* hairy root cells. The present article deals with the effect of culture filtrate, cell extract, and live cells of *P. indica* on growth and lignan production by *L. album* hairy root cultures.

7.2 Materials and Methods

7.2.1 Culture Maintenance and Growth of P. indica

The culture of *P. indica* was provided by Prof. Ajit Varma (Amity Institute of Herbal and Microbial Studies, Noida, India), and the stock culture was maintained on slants containing Kaefer-agar medium at pH 6.5 and 30 ± 1 °C. The seed culture was grown in a 500 mL Erlenmeyer flask containing 100 mL potato dextrose broth with an initial pH of 6.5 at 30 ± 1 °C on a rotary shaker at 200 rpm for 4 days. For elicitation and co-culture experiments, the fungus was grown on Kaefer medium for 8 days using the cultivation conditions mentioned above (Kumar et al. 2011b).

7.2.2 Development, Culture Maintenance, and Growth of L. album *Hairy Roots*

Agrobacterium rhizogenes LBA 9402-mediated genetically transformed high yielding hairy root line LYR2i of *L. album* was developed from cotyledon segments of aseptically germinated seeds (Farkya and Bisaria 2008). The root line was maintained by sub-culturing fresh root mass of 2.5 g/L, every 12th day in 250 mL Erlenmeyer flask containing 50 mL of Gamborg liquid medium (Gamborg et al. 1968) supplemented with 30 g/L sucrose on a gyratory shaker at 60 rpm and 25 ± 1 °C under 16/8 light/dark photoperiod. The pH of the media was adjusted to 5.8 before autoclaving. Hairy roots (12 days old) were used as inoculum to develop the suspension cultures. For elicitation and co-culture experiments, hairy roots were grown in Gamborg medium, containing 32.5 g/L sucrose and 0.75 g/L calcium chloride, with an inoculum level of 5.0 g/L on dry weight basis, incubated

on a gyratory shaker at 125 rpm and 25 ± 1 °C under 16/8 h light–dark regime. The intensity of the light used in all the experiments was 1,200 Lux. The fresh weight used for inoculation was calculated from the ratio of fresh weight to dry weight, which was equal to 12 (Farkya and Bisaria 2008; Kumar et al. 2011a).

7.2.3 Measurement of Hairy Root Growth

Hairy root growth was estimated by measuring dry cell weight (DCW) which was determined by drying hairy root cells on Whatman no. 1 filter paper at 25 ± 1 °C until a constant weight was achieved (Kumar et al. 2011a).

7.2.4 Estimation of Lignans and Phenylalanine Ammonia Lyase Activity

Lignans and phenylalanine ammonia lyase (PAL) activity in hairy root cells of *L. album* were measured by using the method followed by Kumar et al. (2011a). PAL enzyme activity was expressed as μkat (μmoles of cinnamic acid formed per sec) per kg protein.

7.2.5 Analysis of Protein Content in Hairy Root Cells

For the estimation of protein content in hairy root cells, the method of Bonjoch and Tamayo (2001) was followed. The method was based on the principle of protein-dye binding using bovine serum albumin as standard.

7.2.6 Preparation of Elicitors and Their Addition to L. album Hairy Root Cultures

The cultures of *P. indica* were grown as described above and were harvested in log and decline phase, i.e., on the 4th day and 8th day, respectively. The filter-sterilized and autoclaved culture filtrates of *P. indica* from log and decline phase, used as elicitors, were prepared according to the method followed by Kumar et al. (2011a). The two fungal elicitor preparations were added at six different levels (0.2, 0.5, 1, 2, 3, and 5 % v/v) on the 4th, 6th, 8th, 10th, and 12th day to growing *L. album* hairy root cultures.

7.2.7 *Preparation of Cell Extract and Its Addition to* L. album *Hairy Root Cultures*

For the preparation of cell extract, the submerged culture of *P. indica* was grown as described above. The culture broth was harvested after 5 days and the fresh mass of the *P. indica* was obtained by centrifugation at 4,000 × *g* for 10 min. This fresh mass was then dried at room temperature for 5–7 days till it became completely dry. After this it was grounded to a fine powder whose 10 % suspension was prepared in distilled water. Finally the pH was adjusted to 5.8 and autoclaved. The supernatant of this autoclaved suspension was used as an elicitor (Baldi 2008). Different concentrations of solution (0.2, 0.5, 1, 2, 3, and 5 % v/v) prepared from the cell extract of *P. indica* were aseptically added to *L. album* hairy root cultures on the 12th and 13th day of growth.

7.2.8 *Co-cultivation of* P. indica *with* L. album *Hairy Root Cultures*

The live, 5-day old, fungal cells of *P. indica* were inoculated at different concentrations (1, 2, 3, 4, and 5 g/L on DCW basis) into *L. album* hairy root cultures, and the co-culture of plant and fungus was established for different periods (24, 48, 72 and 96 h). For the co-cultivation, *L. album* hairy root culture was grown in Gamborg medium supplemented with 0.2 % peptone, 0.1 % yeast extract, and 0.1 % casamino acid hydrolysate using the culture conditions described above.

7.3 Results and Discussion

7.3.1 *Effect of Culture Filtrate, Cell Extract, and Live Cells of* P. indica *on Lignan Production by* L. album *Hairy Root Cultures*

Majority of the intermediates of phenylpropanoid pathway are produced by the plants in excess in the presence of signaling compounds by triggering defense/hypersensitive responses. Phenylpropanoids like PT and 6-MPT are either induced or produced constitutively at higher levels upon exposure to microbes or microbe-derived compounds. A variety of fungal elicitors which resulted in significant enhancement of phytochemicals in plant tissue cultures have been reported (Namdeo et al. 2002; Zhao et al. 2005). But there is still no way to predict a suitable fungal biotic elicitor for enhancement of a desired metabolite. A reduction in biomass upon treatment with biotic elicitors due to hypersensitivity responses is a

major cause of concern with respect to intracellular products as lowered biomass ultimately results in comparatively lower product concentration. However, *P. indica* being a known endophytic fungus with mutual synergism with plant roots was selected for the present study to see if its interaction with *L. album* increases the growth of lignan production in *L. album* hairy root cultures. For this, various concentrations of culture filtrate (autoclaved and filter sterilized), cell extract, and live cells of *P. indica* were added to growing *L. album* hairy root cultures during different phases of growth, and their effect on plant growth, PT, and 6-MPT contents was studied to determine the optimum concentration and time of addition.

7.3.1.1 Effect of Autoclaved Culture Filtrate

Effect of addition of autoclaved culture filtrate from log and decline phase of fungal growth on lignan (PT and 6-MPT) production is summarized in Tables 7.1 and 7.2. The level of enhancement in lignan content caused by the culture filtrate from log and decline phase was almost similar. The maximum increase in lignan content was obtained when 4-day old (log phase) autoclaved culture filtrate was added at a concentration of 3 % v/v on the 12th day (when the cells were actively synthesizing secondary metabolites), i.e., for an exposure time of 48 h. PT content and concentration were maximally improved by 2.1 times (8.6 mg/g) and 2.4 times (149.3 mg/L), respectively. On the other hand, maximum enhancement of 2.9-fold in 6-MPT content (4.5 mg/g) and 3.3-fold in 6-MPT concentration (77.5 mg/L) was obtained on addition of an 8-day old (decline phase) autoclaved culture filtrate at the same concentration and for the same duration (Table 7.2) (Kumar et al. 2011a).

7.3.1.2 Effect of Filter-Sterilized Culture Filtrate

From Tables 7.3 and 7.4, it is clear that the elicitation potential shown by filter-sterilized culture filtrate was greater than that of autoclaved one. *PT and 6-MPT contents were maximally enhanced by 3.3 times (13.0 mg/g) and 3.8 times (7.2 mg/g) in comparison to control culture on addition of culture filtrate from log and decline phase, respectively, at a level of 3 % v/v for exposure time of 48 h. PT and 6-MPT concentrations were also improved by 3.8 times (233.8 mg/L) and 4.4 times (131.9 mg/L), respectively, in comparison to control culture on addition of culture filtrate from decline phase for the same duration and concentration* (Table 7.4) (Kumar et al. 2011a).

The increase in the lignan content on addition of culture filtrate could be due to the elicitation caused by the presence of some signaling molecules such as jasmonic acid, methyl jasmonate, and salicylic acid and its analogues, which can induce plant defense responses and result in secondary metabolite accumulation (Rijhwani and Shanks 1998; Ketchum et al. 1999; Ge and Wu 2005). Similar results for addition of culture filtrates from log and decline phase indicated that compounds responsible for elicitation may have been released during log phase of fungal growth itself.

Table 7.1 Effect of addition of autoclaved culture filtrate of *P. indica* on day 10 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.1 ± 0.4	62.9 ± 2.5	23.4 ± 1.2	112.9 ± 1.6
4-day old culture filtrate of *P. indica*				
0.2	16.0 ± 0.4	50.3 ± 2.7	30.8 ± 1.6	100.1 ± 2.8
0.5	16.5 ± 0.4	41.0 ± 2.9	35.0 ± 1.2	84.7 ± 3.8
1.0	17.3 ± 0.1	72.8 ± 3.7	51.7 ± 2.9	165.3 ± 6.2
2.0	18.2 ± 0.6	79.0 ± 4.2	69.6 ± 3.1	189.5 ± 5.8
3.0	17.3 ± 0.4	83.1 ± 4.5	70.9 ± 3.5	196.7 ± 3.3
5.0	16.9 ± 0.5	60.1 ± 3.1	35.1 ± 1.2	118.0 ± 4.3
8-day old culture filtrate of *P. indica*				
0.2	15.4 ± 0.7	49.3 ± 1.8	27.3 ± 1.7	88.2 ± 4.0
0.5	16.6 ± 0.6	61.7 ± 3.7	37.1 ± 2.3	113.3 ± 3.9
1.0	17.3 ± 0.1	66.5 ± 4.1	43.3 ± 2.7	162.7 ± 4.7
2.0	18.6 ± 0.5	77.5 ± 3.9	54.8 ± 3.4	152.2 ± 2.1
3.0	18.2 ± 0.3	88.0 ± 3.4	63.4 ± 2.9	182.8 ± 7.9
5.0	16.9 ± 0.2	62.1 ± 2.8	47.3 ± 1.9	143.3 ± 1.6

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly the exposure time of *L. album* with the culture filtrate of *P. indica* was 4 days

Table 7.2 Effect of addition of autoclaved culture filtrate of *P. indica* on day 12 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.1 ± 0.4	62.9 ± 2.5	23.4 ± 1.2	112.9 ± 1.6
4-day old culture filtrate of *P. indica*				
0.2	14.6 ± 0.5	81.5 ± 2.4	27.8 ± 1.2	132.6 ± 3.1
0.5	15.9 ± 0.5	99.7 ± 4.3	39.1 ± 1.7	168.4 ± 7.4
1.0	16.8 ± 0.6	115.4 ± 6.2	48.7 ± 2.1	189.0 ± 4.2
2.0	16.9 ± 0.8	124.0 ± 4.6	62.1 ± 3.3	207.9 ± 9.4
3.0	17.5 ± 0.9	**149.3 ± 6.0**	74.7 ± 3.6	227.6 ± 2.1
5.0	16.7 ± 0.4	107.6 ± 5.1	43.2 ± 1.8	164.1 ± 7.3
8-day old culture filtrate of *P. indica*				
0.2	14.9 ± 0.7	75.9 ± 3.4	34.2 ± 1.4	141.1 ± 5.6
0.5	16.5 ± 0.7	96.1 ± 4.4	47.3 ± 2.5	162.4 ± 9.9
1.0	16.8 ± 0.9	102.9 ± 3.9	53.0 ± 2.0	185.6 ± 7.8
2.0	16.9 ± 0.7	110.5 ± 4.3	66.4 ± 3.1	202.4 ± 4.0
3.0	17.4 ± 0.9	138.8 ± 6.11	**77.5 ± 2.4**	237.3 ± 8.7
5.0	16.5 ± 1.0	98.1 ± 5.2	40.5 ± 1.4	175.6 ± 6.8

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly the exposure time of *L. album* with the culture filtrate of *P. indica* was 2 days

Table 7.3 Effect of addition of filter-sterilized culture filtrate of *P. indica* on day 10 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.6 ± 0.5	60.9 ± 3.1	29.7 ± 1.0	116.7 ± 2.1
4-day old culture filtrate of *P. indica*				
0.2	17.7 ± 0.5	114.4 ± 4.4	38.1 ± 1.4	189.8 ± 7.9
0.5	18.0 ± 0.7	130.6 ± 6.0	62.1 ± 1.2	240.0 ± 5.8
1.0	19.4 ± 0.5	158.4 ± 6.1	79.8 ± 1.9	284.5 ± 7.9
2.0	20.7 ± 0.3	208.7 ± 7.1	102.3 ± 2.7	317.4 ± 7.0
3.0	19.2 ± 0.7	213.1 ± 7.2	104.8 ± 2.8	360.6 ± 4.2
5.0	17.6 ± 0.5	171.8 ± 5.6	67.7 ± 2.4	312.6 ± 3.6
8-day old culture filtrate of *P. indica*				
0.2	17.3 ± 0.2	109.5 ± 4.7	31.3 ± 1.1	178.9 ± 3.2
0.5	18.8 ± 0.6	147.6 ± 3.9	42.1 ± 1.3	212.8 ± 3.3
1.0	20.3 ± 0.2	169.9 ± 5.7	55.8 ± 1.8	238.2 ± 5.5
2.0	21.8 ± 0.2	197.1 ± 8.6	67.4 ± 2.1	268.5 ± 5.2
3.0	20.9 ± 0.3	225.7 ± 5.6	94.5 ± 4.0	317.7 ± 8.8
5.0	18.4 ± 0.5	160.3 ± 4.4	63.3 ± 1.7	257.8 ± 5.1

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, PAL phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly the exposure time of *L. album* with the culture filtrate of *P. indica* was 4 days

Table 7.4 Effect of addition of filter-sterilized culture filtrate of *P. indica* on day 12 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.6 ± 0.5	60.9 ± 3.1	29.7 ± 1.0	116.7 ± 2.1
4-day old culture filtrate of *P. indica*				
0.2	15.6 ± 0.7	143.4 ± 07.0	56.0 ± 2.8	216.6 ± 9.6
0.5	16.1 ± 0.9	171.1 ± 08.4	68.6 ± 2.9	261.5 ± 3.1
1.0	16.7 ± 0.6	185.9 ± 09.3	91.8 ± 3.6	298.9 ± 2.3
2.0	16.9 ± 0.5	216.4 ± 10.2	102.3 ± 3.9	321.3 ± 8.5
3.0	17.8 ± 0.8	232.1 ± 12.2	124.3 ± 5.0	353.3 ± 6.5
5.0	16.1 ± 0.4	174.7 ± 08.2	75.5 ± 3.2	277.0 ± 3.4
8-day old culture filtrate of *P. indica*				
0.2	15.8 ± 0.6	148.3 ± 06.2	42.3 ± 2.0	236.9 ± 6.9
0.5	16.4 ± 0.3	159.8 ± 07.4	64.0 ± 2.7	288.4 ± 4.7
1.0	16.9 ± 0.5	177.8 ± 08.5	81.6 ± 3.6	315.5 ± 7.2
2.0	17.2 ± 0.4	191.0 ± 10.7	111.2 ± 4.59	339.5 ± 3.7
3.0	18.2 ± 0.4	**233.8 ± 12.6**	**131.9 ± 5.05**	371.4 ± 3.1
5.0	16.5 ± 0.3	171.6 ± 06.2	63.8 ± 2.3	308.5 ± 2.2

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly, the exposure time of *L. album* with the culture filtrate of *P. indica* was 2 days

The enhancement in case of culture filtrate from the decline phase could be also due to the presence of cellular components, released because of cell lysis. The greater enhancement in lignan accumulation with filter-sterilized culture filtrate indicates that the stimulatory components responsible for this enhancement may be heat labile and may have lost their elicitation activity on autoclaving (Kumar et al. 2011a). Baldi et al. (2010) also studied the effect of autoclaved and filtered culture filtrate of *P. indica* on growth and lignan production in suspension cultures of *L. album*. They reported maximum lignan content of 9.1 mg/g DCW (PT: 8.8; 6-MPT: 0.3) on addition of autoclaved culture filtrate (at a concentration of 2.5 % v/v) for an exposure time of 48 h. A similar study was done by Bais et al. (2000) who found that the addition of culture filtrate of *Phytophthora parasitica* at a level of 0.1 % v/v to the hairy root cultures of *Cichorium intybus* resulted in a 4.1- and 3.7-fold increase in the esculin and esculetin content, respectively.

7.3.1.3 Effect of Cell Extract

Different concentrations of the extract (0.2, 0.5, 1, 2, 3, and 5 % v/v) prepared from the cells of *P. indica* were aseptically added to *L. album* hairy root cultures on the 12th and 13th day of growth. The effects on plant growth and PT and 6-MPT contents were studied to determine the optimum concentration and time of addition of the elicitor. The addition of cell extract on the 13th day of growth did not result in any significant increase in PT and 6-MPT contents while there was significant increase in PT and 6-MPT contents on addition of cell extract on the 12th day of *L. album* growth. A maximum enhancement of 2.1-fold in PT concentration (138.6 mg/L) and 3.2-fold in 6-MPT concentration (71.4 mg/L) was achieved on addition of cell extract at a level of 1 % (v/v) for an exposure time of 48 h (Table 7.5).

Elicitation by fungal culture filtrates/cell extract might be due to combined/individual effects of many compounds of fungal origin like chitin, some disaccharides, and enzymes. There are many reports where polysaccharides and oligosaccharides extracted from the cell wall and dead cell extract of pathogenic fungi have been reported as an elicitor (Wang et al. 2001; Zhao et al. 2001; Yuan et al. 2002a). For example, *Fusarium* spp. have been reported to increase the flux towards phenylpropanoid pathway which caused an enhancement in the level of cinnamic acid, coumaric acid, and ferulic acid following infection of *Musa acuminate* roots (de Ascensao and Dubery 2003). *Fusarium solani* has also been reported to enhance the accumulation of 6-methoxymellein in cell suspension culture of carrot (Marinelli et al. 1990).

7.3.1.4 Effect of Live Cells of *P. indica* During Co-cultivation with *L. album*

Mutual interactions between plant and fungi do not imply absence of plant defense but require a sophisticated balance between the defense response of the plant and

Table 7.5 Effect of addition of cell extract of *P. indica* on day 12 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.4 ± 0.6	64.7 ± 2.5	22.5 ± 1.3	103.2 ± 2.1
0.2	15.8 ± 0.9	81.9 ± 4.2	29.0 ± 1.5	132.5 ± 8.0
0.5	16.3 ± 0.8	110.7 ± 5.4	38.8 ± 1.9	173.6 ± 6.9
1.0	18.1 ± 0.5	**138.6 ± 6.3**	**71.4 ± 3.7**	232.2 ± 7.5
2.0	16.8 ± 0.9	120.1 ± 4.8	58.5 ± 3.4	197.2 ± 3.4
3.0	16.3 ± 1.0	103.6 ± 3.4	46.7 ± 3.2	184.4 ± 6.6
5.0	15.5 ± 0.4	88.6 ± 2.1	32.5 ± 1.9	156.4 ± 2.6

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly, the exposure time of *L. album* with the cell extract of *P. indica* was 2 days

the nutrient demand of the endophyte. The interactions can be direct or indirect. Direct interactions may include physical or chemical antagonism or synergism between the fungi and plant cells. The indirect interactions may result in stimulation of production of antimicrobial compounds which are commonly known as phytoalexin. PT and related lignans are known to be produced as an arsenal of defense mechanism in plants. Hence the effect of co-cultivation of *P. indica* with *L. album* hairy root cultures was studied. The fungal cells were added to the late log phase of *L. album* hairy root growth, as suppression of cell growth in most of the elicitor-treated plant cell cultures has been a frequently observed phenomenon (Yuan et al. 2002b; Bennet et al. 1996). Hence, addition of the fungal cells in the late log phase will allow the plant cells to effectively utilize the available substrate to produce biomass without any inhibition, as lowered biomass will lead to the lower production of metabolites.

Co-cultivation of live *P. indica* cells with *L. album* hairy roots also resulted in enhancement of lignan concentration despite reduction in biomass. The hairy root cultures receiving 1–5 g/L fungal concentration on days 10, 11, 12, and 13 all achieved a higher PT and 6-MPT contents (mg/g) in the roots than the fungus-free control culture. The maximum increment of 2.1-fold in PT concentration (122.2 mg/L) and 2.5-fold in 6-MPT concentration (52.2 mg/L) was obtained when a fungal concentration of 2 g/L was added to a growing *L. album* hairy root cultures on the 12th day, i.e., for an exposure time of 48 h. Further increase in fungal cell concentration and exposure time resulted in significant decrease in lignan accumulation (Table 7.6). In the earlier reports based on co-cultivation, dose- and duration-dependent increase in production of secondary metabolites has been reported. For example, co-culturing of *Bacillus cereus* with hairy root cultures of *Salvia miltiorrhiza* caused a significant enhancement in tanshinone content of roots (Wu et al. 2007). Similarly, an increase in lignan content in *L. album* suspension cultures was reported upon co-cultivation with root endophytic fungi,

Table 7.6 Effect of addition of different concentrations of *P. indica* on days 10, 11, 12, and 13 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Day of addition (duration of co-culture)	Fungal conc. (g/L)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
13th day (24 h)	1.0	15.9 ± 0.9	81.5 ± 3.9	45.3 ± 2.4	158.6 ± 3.8
	2.0	15.4 ± 0.5	97.7 ± 6.0	49.7 ± 1.8	182.7 ± 1.6
	3.0	15.4 ± 0.7	77.46 ± 3.7	37.3 ± 1.9	138.8 ± 2.7
	4.0	15.1 ± 0.5	75.70 ± 4.9	35.9 ± 1.9	156.4 ± 0.6
	5.0	15.2 ± 1.2	73.83 ± 4.7	29.7 ± 1.5	126.6 ± 2.9
12th day (48 h)	1.0	14.7 ± 0.8	90.7 ± 4.5	42.3 ± 2.2	188.2 ± 5.4
	2.0	14.4 ± 0.9	**122.2 ± 7.3**	**52.2 ± 2.8**	224.3 ± 3.1
	3.0	13.3 ± 0.5	73.8 ± 4.5	36.0 ± 1.7	158.6 ± 3.9
	4.0	12.6 ± 0.4	70.2 ± 4.4	27.3 ± 1.4	154.4 ± 6.5
	5.0	12.2 ± 0.7	63.5 ± 3.5	22.0 ± 1.1	135.7 ± 2.8
11th day (72 h)	1.0	13.9 ± 0.8	65.7 ± 4.0	28.4 ± 1.7	115.8 ± 5.0
	2.0	13.4 ± 0.8	79.7 ± 4.3	39.0 ± 2.0	168.6 ± 3.3
	3.0	12.1 ± 0.8	55.6 ± 3.6	26.2 ± 1.5	142.7 ± 4.6
	4.0	11.9 ± 0.6	54.3 ± 3.3	21.3 ± 1.1	137.3 ± 5.7
	5.0	11.4 ± 0.9	50.8 ± 3.1	20.9 ± 1.1	131.4 ± 4.9
10th day (96 h)	1.0	13.1 ± 0.6	53.5 ± 3.2	22.0 ± 1.1	105.3 ± 5.4
	2.0	12.9 ± 0.6	63.7 ± 2.9	25.6 ± 1.3	117.3 ± 3.8
	3.0	11.5 ± 0.5	49.5 ± 3.1	18.6 ± 1.0	122.4 ± 6.0
	4.0	11.1 ± 0.7	43.2 ± 2.6	17.3 ± 0.9	95.5 ± 5.1
	5.0	10.9 ± 0.8	42.2 ± 2.6	15.2 ± 0.9	86.8 ± 6.1
Control	0.0	15.8 ± 1.0	59.1 ± 3.2	21.2 ± 1.1	104.8 ± 1.7

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day

P. indica and *Sebacina vermifera (*Baldi et al. 2008b, 2010). Baldi et al. (2010) reported the maximum plant biomass concentration and PT and 6-MPT contents of 28.4 g/L, 20.1, and 2.3 mg/g DCW, respectively, when live cells of *P. indica* were added at a concentration of 1 g/L for an exposure time of 24 h.

7.3.2 *Effect of Culture Filtrate, Cell Extract, and Live Cells of* P. indica *on PAL Activity*

Phenylalanine ammonia lyase (PAL) catalyzes the first step of phenylpropanoid pathway which is deamination of phenylalanine to cinnamic acid. This is the rate-limiting step of lignan biosynthesis and also acts as the bridge between primary metabolism and natural product biosynthesis. *Lignan accumulation and PAL enzyme activity were found to be directly associated as maximum lignan*

accumulation (PT + 6-MPT) coincided with maximum increase of PAL enzyme activity. PAL activity was maximally enhanced by 3.1 times (371.4 μkat/kg protein) corresponding to 3.5-fold increase in lignan content (20.1 mg/g) upon addition of filter-sterilized culture filtrate from decline phase of P. indica *at a level of 3 % (v/v) to* L. album *hairy root cultures for an exposure time of 48 h* (Table 7.4). An increase of 2.1-fold in PAL activity (237.3 μkat/kg protein) was achieved on addition of autoclaved culture filtrate under similar conditions (Table 7.2) (Kumar et al. 2011a). Similarly in case of cell extract of *P. indica*, a maximum PAL activity of 232.2 μkat/kg protein, 2.3 times higher than the control, was found on addition of cell extract at a level of 1 % (v/v) on the 12th day of *L. album* growth (Table 7.5), which also resulted in maximum increase in lignan content. In case of interaction of live fungal and plant cells during co-culture studies, the maximum increment in PAL activity (224.3 μkat/kg) and protein also coincided with highest total lignan accumulation (Table 7.6).

A correlation between changes in the levels of phenylpropanoid biosynthetic enzymes and product accumulation has been demonstrated in other plant system as well. For example, a quantitative relationship between PAL levels and phenylpropanoid accumulation was established in transgenic tobacco (Nicholas et al. 1994). A similar effect was also observed in date palm roots, carrot cell suspension cultures, *Rubus fruticosus* cell suspension cultures, and *Taxus chinensis* on treatments with elicitor preparation from *Fusarium* spp. (Marinelli et al. 1990; Modafar et al. 2001; Yu et al. 2001; Yuan et al. 2001, 2002b; Nita-Lazar et al. 2004).

7.3.3 *Effect of Culture Filtrate, Cell Extract, and Live Cells of* P. indica *on Growth of* L. album *Hairy Root Cultures*

7.3.3.1 Effect of Culture Filtrate and Cell Extract

Although the addition of culture filtrate, both autoclaved and filter sterilized, resulted in growth-promoting effect on hairy roots in suspension cultures, the increase in hairy root dry cell weight was more significant in case of filter-sterilized culture filtrate. The maximum increase of 1.4-fold (21.8 g/L) in plant cell growth was achieved upon addition of filter-sterilized culture filtrate from decline phase of *P. indica* at a concentration of 2 % (v/v) for an exposure time of 96 h (Table 7.3). In case of autoclaved culture filtrate, the maximum enhancement of 1.2 times in plant cell growth (18.6 g/L) was obtained under similar conditions (Table 7.1) (Kumar et al. 2011a). In case of cell extract of *P. indica*, the maximum increase in hairy root DCW (18.1 g/L) was obtained on addition of cell extract on the 12th day of *L. album* growth at a level of 1 % v/v (Table 7.5). Therefore, it can be hypothesized that growth-promoting effect of *P. indica* might be due to some of the extracellular metabolites released in the medium during growth of fungal cells or could be due to

the presence of growth-stimulating factors present in cell extract of *P. indica*. Exposure time and concentration of culture filtrate to plant cells greatly affected this growth-promoting effect. An exposure time of 96 h and concentration of 2 % (v/v) were found to be optimum. Addition of culture filtrate for a shorter period, i.e., 48 h, resulted in comparatively lesser increment in growth of hairy roots. This might be due to insufficient time available for growth-promoting compounds to execute their effect.

P. indica has shown growth-promoting and yield enhancement activities on a variety of field-grown host plants (Sahay and Varma 1999, 2000; Rai et al. 2001; Singh et al. 2002, 2003; Kumari et al. 2004; Rai and Varma 2005; Chauhan et al. 2006; Oelmüller et al. 2009). In addition, its culture filtrate has also shown growth-promoting effects in field-grown plants (Varma et al. 1999, 2001).

7.3.3.2 Effect of Co-cultivation

Co-culture of live fungal cells with hairy roots lowered the growth of hairy roots (Table 7.6). This might be attributed to induction of strong hypersensitive reactions due to interaction between live fungal and plant cells. The reduction in growth was more significant upon increasing the concentration of fungal cells and duration of the co-culture. Although co-culture of *P. indica* with suspension culture of *L. album* had resulted in growth-promoting effect (Baldi et al. 2008b, 2010), the detrimental effect with hairy roots might be due to organized structure of hairy roots which may result in lesser interaction with fungal cells. Furthermore, the decrease in growth could also be due to competition of the hairy roots with fungal cells for the nutrients. This probably led to increased unavailability of nutrients for the hairy roots due to faster uptake of nutrients by the fungal cells; the reduction in hairy root growth was more severe with longer duration of exposure and higher concentration of the fungus.

The degree of enhancement was strongly dependent on the concentration of culture filtrate, cell extract, or live cells of *P. indica* and the time of their addition. The optimal time of addition of an elicitor depends on the right combination of the phase of the growth cycle during which it is added and the exposure time to result in maximal production of the desired metabolite, as the growth stage may influence not only the response to the elicitor treatment but also the pattern of production (Eilert 1987). *Addition of culture filtrate, cell extract, and live cells of* P. indica *resulted in an increase of the lignan (PT and 6-MPT) contents up to a concentration of 3 %, 1 %, and 2 % (v/v), respectively, after which the lignans decreased significantly on increasing the concentration of the elicitors*. An elicitor dose lower than the optimum suggests that the elicitor binding sites in cells were still not fully occupied for activating the biosynthesis of the metabolites, whereas an excessive dose caused a deleterious effect on the biosynthetic capacity of the cells. The deleterious effect of high concentrations of elicitors on biosynthesis of secondary metabolites has been observed in jasmonic acid-induced indole alkaloid biosynthesis (Rijhwani and Shanks 1998) and in methyl jasmonate-induced taxol

biosynthesis (Ketchum et al. 1999). Similar phenomenon has also been reported for ajmalicine accumulation in *Catharanthus roseus* suspension cultures when exposed to different concentrations of fungal elicitor extracts of *Trichoderma viride*, *Aspergillus niger*, and *Fusarium moniliforme* (Namdeo et al. 2002). High dosage of elicitor has been reported to induce hypersensitive response leading to cell death. Therefore, an optimum level was required for induction of secondary metabolite biosynthesis in plant cell cultures (Mukundan and Hjortsø 1990; Roewer et al. 1992). These results also indicate the importance of elicitor specificity in its selection for optimum induction of desired phytochemicals in plant cell cultures.

In addition to the concentration of an elicitor, the time of addition and hence the exposure time of elicitor to plant cells are also an important factor in maximizing its elicitation potential (Ketchum et al. 1999; Wang and Zhong 2002; Tabata 2004). An elicitor requires minimum time to execute its effect, but at the same time the longer incubation of elicitor may have deleterious effects on production. *The optimum time of the 12th day (an exposure time of 48 h) for culture filtrate, cell extract, and co-culture in present studies also confirmed this.* Longer exposure time may result in conversion or degradation of induced compounds. Therefore, a shorter exposure time is generally required to elicit phytochemical production in plant cell cultures. The decrease in lignan content beyond an exposure time of 48 h may be due to the conversion or degradation of PT to some other compounds. Such phenomenon was observed when the cells of *C. roseus* were exposed to fungal elicitor extracts for various time intervals. A three-fold higher ajmalicine production by *C. roseus* cells was elicited with extracts of *T. viride* and about two-fold increase in ajmalicine by the cells elicited with *A. niger* and *F. moniliforme* was also observed for an exposure time of 48 h. However, further increase of exposure time resulted in decrease of ajmalicine content (Namdeo et al. 2002). This pattern has also been reported by Rijhwani and Shanks (1998) for hairy root cultures of *C. roseus*, by Moreno et al. (1993) for cell suspension cultures of *C. roseus*, and by Negeral and Javelle (1995) for cell suspension cultures of tobacco.

7.4 Conclusions

The results of the present study clearly show that the addition of culture filtrate and cell extract of *P. indica* can improve the growth as well as lignan production in hairy roots of *L. album*. Among the various types of elicitors used, the maximum lignan (PT and 6-MPT) accumulation in *L. album* hairy root cultures was obtained with filter-sterilized culture filtrate of *P. indica*. The enhancement in lignan biosynthesis was found to be directly related to phenylalanine ammonia lyase activity. Although co-cultivation of live *P. indica* with *L. album* hairy roots caused a decrease in growth of *L. album*, the enhancement of lignan accumulation was substantial, leading to a significant net improvement in its production. *P. indica* seems to be promising for its applications to other plant cell tissue cultures to overproduce valuable secondary metabolites.

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