Chapter 23
Fungal Elicitors for Enhanced Production of Secondary Metabolites in Plant Cell Suspension Cultures

A. Baldi, A.K. Srivastava, and V.S. Bisaria

23.1 Introduction

Plant cell culture systems are viable alternatives for the production of secondary metabolites that are of commercial importance in food and pharmaceutical industries. However, relatively very few cultures synthesize these compounds over extended periods in amounts comparable to those found in whole plants. Various strategies have been employed to increase the production of secondary metabolites in cell cultures as well as in hairy root cultures for commercial exploitation. These include manipulation of culture media (hormonal and nutrient stress) and environmental conditions (temperature, pH and osmotic stress), precursor addition, elicitation and combination of these strategies. Nowadays, genetic manipulation of biosynthetic pathways by metabolic engineering has also become a powerful technique for enhanced production of desired metabolites.

The recent developments in elicitation of cell cultures have opened a new avenue for the production of these compounds. Secondary metabolite synthesis and accumulation in cell cultures can be triggered by the application of elicitors to the culture medium. Elicitors can be defined as signalling molecules triggering the formation of secondary metabolites in cell cultures by inducing plant defence, hypersensitive response and/or pathogenesis related proteins. Depending on the origin, elicitors can be classified in two classes: biotic and abiotic. Elicitors of biological origin are called biotic elicitors. These include polysaccharides, proteins, glycoproteins or cell-wall fragments derived from fungi, bacteria and even plants.

A. Baldi, A.K. Srivastava, and V.S. Bisaria (✉)
Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, New Delhi, India
e-mail: vbisaria@dbeb.iitd.ac.in

A. Varma and A.C. Kharkwal (eds.), *Symbiotic Fungi*, Soil Biology 18,
DOI: 10.1007/978-3-540-95894-9_23,

Among these, fungal elicitors have been most widely studied for enhancement of synthesis of commercially important compounds from plant cell cultures. Elicitors of non-biological origin are called abiotic elicitors, which include metal ions, UV light and chemically defined compounds. Recently, the term 'abiotic stress' is also being used for abiotic elicitors.

Many studies have shown that both biotic elicitors and abiotic stresses enhance secondary metabolite synthesis in plant cell cultures. However, no elicitor has been found to have a general effect on many culture systems, and no system has been found to respond to all elicitors. So it is necessary to screen various elicitors for a particular system for production of a desired compound. Moreover, the concentration of elicitors, and the incubation time required for maximum elicitation, differ with the kind of elicitor and the culture system. Therefore, screening is a must to arrive at a suitable elicitor, and to determine its concentration and contact time for maximal response in terms of secondary metabolite accumulation. Another important aspect is the time of addition of a elicitor to the culture. Optimal induction occurs when the elicitors are added to cultures at late exponential or early stationary phase of plant cell growth. So the effect of any elicitor for maximum response depends on the age of culture, concentration of elicitor and incubation time with the elicitor. Keeping this in view, there has been a strong need for the discovery of useful elicitors and for novel screening methods that would allow substances having elicitation capability to be rapidly and easily screened.

In most of the studies done to date to enhance the synthesis of commercially important plant-derived compounds by the addition of fungal elicitors to cell cultures, pathogenic fungi have been used. Fungi, which mainly induce hypersensitive response in plant cells, result in activation of plant defence pathways and thereby increase phytoalexin production. Another significant plant–fungi interaction reported is the symbiotic relationship between arbuscular myccorhizal fungi and plant cells/organs. But due to the inability of these symbiotic fungi to grow in a synthetic media, it has not been possible to study their effect for elicitation, if any. With the discovery of *Piriformospora indica* (Varma and Franken 1997), a novel endophytic axenically cultivable fungus, which mimics the capability of arbuscular myccorhizal fungi, a new era has opened for enhanced production of plant-based secondary metabolites in cell cultures by elicitation with this fungus.

Podophyllotoxin is one of the most promising secondary metabolites from medicinal plant research, due to its pronounced cytotoxic activity. It is currently being used for the synthesis of anticancer drugs such as etoposide, teniposide, and etopophos, which are used for the treatment of testicular and lung cancers and certain leukemias (Stahelin and Wartburg 1991; Imbert 1998). The isolation of podophyllotoxin from the rhizomes of *Podophyllum peltatum* and *P. hexandrum* (Berberidaceae) plants is not a very ideal production system. The *P. hexandrum* rhizomes may contain ca. 4% of podophyllotoxin on a dry weight basis, while *P. peltatum* contains still lower amounts of it. The supply of this compound has become increasingly limited due to intensive collection, lack of cultivation, long juvenile phase and poor reproduction capabilities (van Uden 1992). The species *P. hexandrum* is also listed in Appendix II of CITES (Convention for International Trade in Endangered Species). This appendix lists species that are not necessarily

threatened now with extinction, but which may become so unless trade is closely controlled (World Conservation Monitoring Centre 2001).

The chemical synthesis of podophyllotoxin is possible (Hadimani et al. 1996), but largely hampered by the complicated stereochemical ring closure necessary to attain this compound. Synthetic production therefore only yields restricted quantities at high cost.

This supply problem forms the drive for a large number of scientists to search for alternative sources of podophyllotoxin. An ideal resource would be a fast-growing and easy to cultivate plant cell or organ culture with a high lignan content. Tissue cultures of *Podophyllum* species turned out to be quite recalcitrant or low-yielding (Chattopadhyay et al. 2001, 2002a, b, 2003a–c; Farakya et al. 2004); therefore, in recent years tissue culture of Iranian flax, *Linum album*, has become an attractive alternative. These cell cultures have been reported to produce lignans with highest productivity (Baldi et al. 2007). This chapter provides details for enhanced production of podophyllotoxin in cell suspension cultures of *Linum album* by elicitation with culture filtrate and cell extract of *P. indica*.

23.2 Development of Plant Cell Cultures

23.2.1 Germination of Seeds

1. Treat seeds of *L. album* with 1% Savlon (Johnson and Johnson, USA) for 5 min.
2. Rinse seeds with sterile double distilled water four to five times.
3. Transfer seeds into a 100 ml Erlenmeyer flask containing 50 ml of 70% ethanol, and treat for 1 min.
4. Remove ethanol after treatment, and rinse the seeds with sterile double distilled water thrice.
5. Transfer seeds in a 100 ml Erlenmeyer flask containing 25 ml of 0.01% mercuric chloride, and treat for 2 min.
6. Remove mercuric chloride solution, and rinse the seeds with sterile double distilled water thrice.
7. Transfer one seed/culture tube containing 20 ml of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 1% agar for germination using a sterile forceps over flame. Gently press the explants into the media for good contact.
8. Plug the culture tubes with a sterile cotton plug over flame.
9. Transfer the tubes into a culture room at 25 ± 2°C under complete darkness, and allow the seeds to germinate.

23.2.2 Initiation of Callus Cultures

1. Collect stem portions from 30-day-old *in vitro* germinated plants of *L. album*, and place the explants (stem) in a sterile Petri dish.
2. Cut stem portions to 1 cm × 1 cm size with the help of sterile blade.

3. Place one explant in one Petri dish containing 15 ml of MS medium (Murashige and Skoog 1962) solidified with 1% agar and supplemented with 0.4 mg l^{-1} NAA (naphthalene acetic acid) for callus initiation using a sterile forceps.
4. Cover the petri dish with lid and seal it with parafilm.
5. Transfer Petri dishes in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux.
6. Observe the callus initiation.

23.2.3 Initiation of Suspension Cultures

23.2.3.1 Inoculum Preparation

1. Transfer fresh and friable cells (20 days old) from callus culture (5 g l^{-1} on dry cell weight basis) into a 250 ml Erlenmeyer flask containing 50 ml MS media containing 0.4 mg l^{-1} NAA with the help of sterile spatula over flame.
2. Plug the culture tubes with sterile cotton plug over flame.
3. Place inoculated flasks on a gyratory shaker rotating at 125 rpm in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux.
4. Allow the cells to grow in suspension culture for 12 days.
5. Centrifuge the grown cells in sterile centrifuge tubes at 5,000 rpm for 10 min.
6. Decant the spent media and use cells to inoculate suspension cultures for elicitation setup.

23.2.3.2 Development of Suspension Culture

1. Transfer these cells (5 g l^{-1} on dry cell weight basis) into a 250 ml Erlenmeyer flask containing 50 ml MS media containing 0.4 mg l^{-1} NAA with the help of sterile spatula over flame.
2. Place inoculated flasks on a gyratory shaker rotating at 125 rpm in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux and allow the cells to grow for 12 days.

23.3 Development of Elicitors

23.3.1 Preparation of Elicitors

1. Transfer cells of arbuscular myccorhiza like fungus, *Piriformospora indica*, to a 250 ml Erlenemeyer flask containing 50 ml of MYPG media of following composition:

Malt extract: 3 g l^{-1}
Yeast extract: 3 g l^{-1}
Peptone: 5 g l^{-1}
Glucose: 10 g l^{-1}
(pH: 6.2)

2. Incubate the inoculated flasks on an incubator shaker, rotating at 200 rpm, at 25 ± 2°C in 16 h/8 h light/dark cycle, and allow the cells to grow till the end of the log phase (6 days).
3. Collect the culture broth by passing the fungal culture through a Whatman No. 1 filter paper.
4. Centrifuge the culture broth at 5,000 rpm for 15 min and remove fungal cells, if any.
5. Farakya et al. (2005) suggested the following technique for preparation of fungal elicitors.

   (a) Divide the culture broth into two portions. Use one portion after passing it through 0.22 μm filter and designate it as 'filtered culture broth'.
   (b) Autoclave another portion of culture broth at 15 psig and 121°C for 20 min and designate it as 'autoclaved culture broth'.
   (c) Wash the fungal mat several times with sterile double distilled water and allow it to dry at 40 ± 2°C in a hot air oven to constant weight.
   (d) Crush the dry cells in a mortar pestle.
   (e) Suspend 10 g of dry cell powder in 100 ml of double distilled water (pH 5.7) and autoclave it at 15 psig and 121°C for 20 min for hydrolysis.
   (f) Centrifuge the hydrolysate at 5,000 rpm for 10 min
   (g) Collect the supernatant and designate it as 'cell extract'.
   (h) Store the fungal elicitor preparations at 4°C till further use.

   Methodology for preparation of fungal elicitors is represented as Fig. 23.1.

23.3.2 Addition of Elicitors

1. Add all three fungal elicitors separately on v/v basis (generally in a range of 1–10%, v/v) to suspension cultures of *L. album* at different phases of growth with the help of sterile pipette.

(Note that the time of addition can be at the start of cultivation, end of lag phase, middle and/or late log phase or early stationary phase. Depending on the time of addition of elicitor to the medium and harvest time of culture, one can test the effect of various times of incubation also, e.g. if the elicitor is added on the 8th and 10th days of a cultivation that ends on the 12th day, the times of incubation will be 96 and 48 h respectively.)

2. Harvest the flasks, in duplicate, at the end of cultivation, and analyse for the effect of fungal elicitors on growth and product accumulation.
3. Measure the responses in terms of biomass (g l^{-1}, on dry cell weight basis), podophyllotoxin accumulation (mg l^{-1}) and overall volumetric productivity of podophyllotoxin (mg $l^{-1}d^{-1}$).

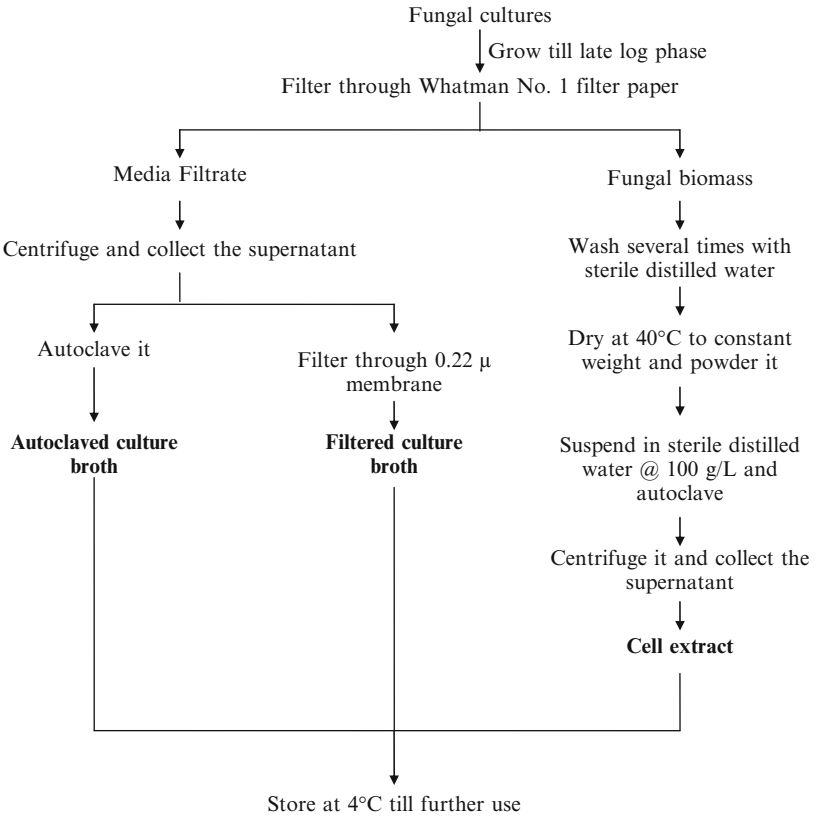

Fig. 23.1 Preparation of fungal elicitors

23.4 Analysis

23.4.1 Growth in Terms of Dry Cell Weight

1. Harvest individual flasks, in duplicate, at the end of cultivation.
2. Filter the cell suspension through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum.
3. Wash the cells with double distilled water.
4. Drain fully under vacuum.
5. Transfer the cells to a pre-weighed Petri dish. Dry cells at $25 \pm 2°C$ to a constant weight (approx. 48 h).
6. Reweigh the cells with a Petri plate.
7. Calculate biomass produced/50 ml media by subtracting weight of the empty Petri dish from total weight of Petri dish and dried cells. Express the weight of cell biomass as g l^{-1} dry cell weight.

23.4.2 Extraction and Estimation of Podophyllotoxin

1. Suspend accurately weighed dried and powdered cells (100 mg) in 5 ml of methanol in 10 ml glass tube.
2. To release intracellular product, sonicate cells at 4–6°C for 15 min and allow complete extraction for 24 h.
3. Centrifuge the extract at 5,000 rpm for 10 min and collect the supernatant.
4. Allow methanol to evaporate at 25 ± 2°C (approx. 12–18 h).
5. Re-dissolve this extract in a known volume of HPLC grade methanol and filter it through 0.22 μm filter.
6. Quantify podophyllotoxin in each sample on HPLC under following conditions:

   Column: Nova Pak RP-C_{18} column (Waters, USA)
   Packing: 250 × 4.6 mm
   Mobile phase: 0.01% phosphoric acid in water: acetonitrile (72:28%, v/v)
   Flow rate: 0.8 ml min^{-1}
   Column temperature: 30°C
   Detection: Diode array detector at 290 nm
   Standard: Commercially available podophyllotoxin (Sigma, USA)

23.5 Conclusions

For elicitation studies in plant cell cultures, it is necessary to screen various elicitors and optimize their concentration and time of incubation. *P. indica* has been found to enhance podophyllotoxin in plant cell cultures by several-fold. This can provide a valuable technique in commercial production of plant-derived secondary metabolites.

References

Baldi A, Bisaria VS, Srivastava AK (2007) Biotechnological approaches for production of some plant based chemotherapeutics. In: Kayser O, Quax WJ (eds) Medicinal plant biotechnology — From basic research to industrial applications, Wiley-VCH Verlag, Weinheim, pp 117–156

Chattopadhyay S, Srivastava AK, Bhojwani SS, Bisaria VS (2001) Development of suspension culture of *Podophyllum hexandrum* for the production of podophyllotoxin. Biotechnol Lett 23:2063–2066

Chattopadhyay S, Srivastava AK, Bhojwani SS, Bisaria VS (2002a) Production of podophyllotoxin by plant cell cultures of *Podophyllum hexandrum* in bioreactor. J Biosci Bioeng 93: 215–220

Chattopadhyay S, Srivastava AK, Bisaria VS (2002b) Optimization of culture parameters for production of podophyllotoxin in suspension culture of *Podophyllum hexandrum*. Appl Biochem Biotechnol 102/103:381–393

Chattopadhyay S, Bisaria VS, Bhojwani SS, Srivastava AK (2003a) Enhanced production of podophyllotoxin by fed-batch cultivation of *Podophyllum hexandrum*. Can J Chem Eng 81:1–8

Chattopadhyay S, Bisaria VS, Srivastava AK (2003b) Enhanced production of podophyllotoxin by *Podophyllum hexandrum* using *in situ* cell retention bioreactor. Biotechnol Prog 19:1026–1028

Chattopadhyay S, Mehra RS, Srivastava AK, Bhojwani SS, Bisaria VS (2003c) Effect of major nutrients on podophyllotoxin production in *Podophyllum hexandrum* suspension cultures. Appl Microbiol Biotechnol 60:541–546

Farakya S, Bisaria VS, Srivastava AK (2004) Biotechnological aspects of the production of the anticancer drug podophyllotoxin. Appl Microbiol Biotechol 65:504–519

Farkya S, Julka A, Mehra R, DattaV, Srivastava AK, Bisaria VS (2005) Enhanced production of secondary metabolites by biotic elicitors in plant cell suspension cultures. Presented at 5th Asia Pacific Biochemical Engineering Conference, May 15–19, 2005, Jeju Island, Korea

Hadimani SB, Tanpure RP, Bhat SV (1996) Asymmetric total synthesis of (-) Podophyllotoxin. Tetrahedron Lett 37: 4791–4794

Imbert F (1998) Discovery of podophyllotoxins. Biochimie 80:207–222

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay of tobacco tissue cultures. Physiol Plant 15:473–497

Stahelin HF, Wartburg AV (1991) The chemical and biological route from podophyllotoxin glucoside to etoposide. Cancer Res 51:5–15

Van Uden W (1992) The production of podophyllotoxin and related cytotoxic lignans by plant cell cultures. Ph.D. Thesis, University of Groningen, Groningen, The Netherlands

Varma A, Franken P (1997) European Patent Office, Munich, Germany, Patent No. 97121440.8-2105, Nov 1998

World Conservation Monitoring Centre (2001) Checklist of CITES species: a reference to the appendices to the Convention on international trade in endangered species of wild fauna and flora, CITES Secretariat/World Conservation Monitoring Centre, Chatelâine-Genève