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Enhanced plumbagin production in elicited *Plumbago indica* hairy root cultures

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Elicitation of *Plumbago indica* hairy roots with yeast carbohydrate fraction, chitosan, manganese chloride, copper chloride and methyl jasmonate exhibited significant elevation (~1.2 to 2 fold) of plumbagin production in shake flask culture as compared with control. Chitosan and methyl jasmonate elicitation also caused simultaneous plumbagin leaching into culture media. Three days' exposure of chitosan (200 mg l^{-1}) and methyl jasmonate (80 μM) together synergized total plumbagin yield to its maximum 11.96 ± 0.76 mg g^{-1} DW in shake flask culture. In bioreactor cultivation, a significant raise in fresh root biomass was recorded on day 20 as compared with control shake flask culture. Three days' exposure of chitosan (200 mg l^{-1}) and methyl jasmonate (80 μM) with 20 days old bioreactor-culture significantly improved total plumbagin production to 13.16 ± 1.72 mg g^{-1} DW with simultaneous plumbagin leaching into bioreactor media.

[**Key words:** *Plumbago indica*; Hairy roots; Elicitors; Bioreactor; Chitosan; Methyl jasmonate]

Plants are considered as chemical factories for biosynthesis of a huge array of structurally diverse bioactive secondary metabolites. The isolation of bioactive compounds from whole plants creates pressure on natural germplasm. Therefore, the biotechnological production of valuable secondary metabolites in plant cell culture is a striking choice. It offers attractive alternatives to classical technologies for the production of plant-based metabolites without hampering natural flora. Moreover, the production of plant metabolites in plant cell culture is independent of seasonal and geographical variations. *In vitro* culture systems provide various ways to boost the yields of desired metabolites conveniently and cost-effectively. Elicitation is one of the most effective approaches for increasing the production of secondary metabolites in plant cell culture (1,2). Elicitors are signals compounds triggering the formation of secondary metabolites by activating the pathways in response to exogenous stresses (3). Though plant cell cultures could be a potential source of wide varieties of valuable metabolites, but the recovery of metabolites from culture medium and down-stream processing is very much challenging. Low hydrophilicity of most of secondary metabolites restricts their release into the medium from *in vitro* maintained cell culture. Thus, it is necessary to remove the water insoluble products from the culture medium without disturbing the cell metabolic activities. In this connection, the extracellular leaching is also important.

Plumbago indica (family Plumbaginaceae), a dicotyledonous plant, is well known for its ethnomedicinal values. *P. indica* is a rich source of therapeutically active, root specific, natural napthoquinone plumbagin. Plumbagin has been reported to possess filaricidal (4) anticancer (5), cardiotonic (6), antimalarial (7), antimicrobial (8) and antifertility (9) activities. Among six different species of *Plumbago*, *P. indica* is the richest source of plumbagin (10). The annual requirement for plumbagin in Indian subcontinent is about 7 metric tonnes (11). Increasing demand of plumbagin in both domestic and international markets has led to the overexploitation of *P. indica* from natural habitat. Presently, this plant has become a rare categorized (12). Traditional agricultural methods take several years to achieve desired level of plumbagin in the roots of this seedless plant (11). On the other hand, synthetic approach of plumbagin production is not commercially promising (13,14). In this situation, intervention of modern biotechnological approaches to enhance plumbagin production through plant cell culture is the only way to fulfill market demands, as well to save this plant from becoming extinct. During past few decades, several attempts were made to improve plumbagin production through suspension (15) and adventitious root culture (16), but the product yield was small in industrial aspect. Since the synthesis of plumbagin is linked to root differentiation, undifferentiated cell cultures did not produce this metabolite efficiently. In this respect, hairy or transformed root cultures have several advantages over normal cell and organ cultures (17). Hairy root culture of *P. indica* has been successfully established in the Medicinal Plant Laboratory, Bose Institute, Kolkata, India (18). *P. indica* hairy roots were able to grow faster than non-transformed roots in hormone-free media and produced plumbagin at comparatively higher levels (18) than non-transformed roots. Since the increasing market demand necessitates further improvement of plumbagin production for viable commercial exploitation, the present study was undertaken to enhance plumbagin production in *P. indica* hairy roots under the influences of different biotic and abiotic elicitors. Since the plumbagin production is intracellular and growth associated, it was further aimed to culture hairy roots in a bioreactor (19) to obtain significant

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higher root biomass. Conventional bioreactors for cell suspension cultures cannot be used for hairy roots because the later form root clumps with intertwined and self-immobilized morphologies, which resist the percolation of oxygen into the hairy roots, leading to poor growth and metabolite production (20). In this study, *P. indica* hairy roots were cultured in a special bioreactor with continuous air supply. After a significant raise in fresh root biomass, hairy roots were exposed to selected and optimized elicitors for enhanced plumbagin production.

MATERIALS AND METHODS

Plant material A fast growing hairy root clone (H13) of *P. indica* established in Medicinal Plant Laboratory, Bose Institute, India (18) was used as source material. The root clone was obtained by infecting the midribs of leaf explants of *in vitro* grown *P. indica* with *Agrobacterium rhizogenes* strain ATCC 15834 (18). Hairy roots (1.5 g l^{-1}) were sub-cultured in 50 ml of hormone-free liquid MS medium (21) with 3% sucrose at 25 ± 2°C in dark on an orbital shaker at 70 rpm for 20 d.

Preparation of elicitors The fungal cultures used for the elicitation were *Fusarium solani, Aspergillus niger* and *Rhizopus oryzae* (22). The fungi were maintained in potato-dextrose agar slant at 30 ± 2°C. The cultures were transferred into 250 ml Erlenmeyer flasks containing 50 ml of potato-dextrose broth at 30 ± 2°C on an orbital shaker at 70 rpm. After one week, cultures were harvested, filtered and dried at 60°C for 24 h. The dry cell powders were dissolved separately in double distilled water (10 g l^{-1}) and autoclaved for 15 min at 121°C.

The carbohydrate fraction isolated from yeast (*Saccharomyces cerevisiae*) extract was prepared by ethanol precipitation method (23). Briefly, 50 g of the yeast extract was dissolved in 250 ml double distilled water. Ethanol was added to 80% (v/v). After incubation at 6°C for 4 d, the precipitate was collected. The process was repeated thrice and the precipitate was dissolved in 200 ml double distilled water, yielding the crude preparation that was used without further purification.

Chitosan (Hi media, India) was purified by the method described Kim et al. (24) with little modification. Briefly, 1 g chitosan was dissolved in 90 ml, 0.1 N acetic acid and the solution was centrifuged for 20 min at 6700 × g. After centrifugation, the supernatant was precipitated by adjusting its pH to 8.0 with 5 N NaOH. The precipitate was washed repeated with double distilled water and lyophilized. One gram lyophilized chitosan was dissolved in 100 ml, 0.1 N acetic acid and the pH of the solution was adjusted to 5.0.

Methyl jasmonate (95% pure) in 96% ethanol was purchased from Sigma Aldrich, USA and filtered through a syringe filter (25 μm, Gelman Sciences, Ann Arbor, MI, USA).

Chloride salts of manganese, zinc, lead, cobalt, nickel, silver, copper and calcium were used for the elicitation. Stock solutions were prepared separately by dissolving 1 g of salt in 100 ml double distilled water. The pH of individual salt solutions was adjusted to 5.5 (25). The solutions were autoclaved for 15 min at 121°C.

Selection and optimization of elicitors Elicitation studies were carried out with selected fungal biomass (1, 2 and 3 mg l^{-1}), yeast carbohydrate fraction (1, 2 and 3 mg l^{-1}), chitosan (100, 200 and 300 mg l^{-1}), inorganic salts (100, 200 and 300 mg l^{-1}) and methyl jasmonate (20, 40, 80 and 100 μM). Twenty days' old hairy roots (0.075 g on fresh weight basis) were transferred to fresh liquid MS medium containing selected concentrations of elicitors. One set of shake flask without elicitor served as control. Intracellular plumbagin content and plumbagin leaching was estimated on days 1, 3 and 7.

To study the synergistic effect of elicitors, *P. indica* hairy roots were cultured with the exposure of selected elicitors (based on the effect of individual elicitors on plumbagin accumulation in shake flask cultures) in combination, namely chitosan (200 mg l^{-1}) + methyl jasmonate (80 μM), yeast carbohydrate fraction (1 mg l^{-1}) + chitosan (200 mg l^{-1}) + methyl jasmonate (80 μM) and yeast carbohydrate fraction (1 mg l^{-1}) + chitosan (200 mg l^{-1}) + Manganese chloride (200 mg l^{-1}) + Copper chloride (100 mg l^{-1}) + methyl jasmonate (80 μM). Plumbagin content and extracellular plumbagin leaching was estimated on days 1, 3 and 7.

Bioreactor cultivation of *P. indica* hairy roots with elicitors After selection and optimization of the elicitors in shake flasks, 3 g l^{-1} of *P. indica* hairy roots were cultivated in a 3 l bioreactor (length 22 cm and diameter 14 cm) with a working volume of 1.75 l. The reactor was provided with openings for air inlet, air outlet, inoculation port and sampling port. The air was supplied through a glass sparger, molded into a circular shape with pores of size 1 mm at the bottom of the reactor. The reactor was provided with an autoclavable perforated plastic basket, which was open on the top. The basket was placed at a height of 7 cm from the bottom of the reactor vessel on a stainless steel stand. The distance between the sparger and the basket was 4 cm. Air was sparged at a rate of 30.4 $cm^3 s^{-1}$. The bioreactor was maintained in dark at 25 ± 2°C. The reactor containing 1.75 l phytohormone-free liquid MS medium with 3% sucrose was inoculated with hairy roots (5.25 g on fresh weight basis) of *P. indica*. After 20 d, the fresh root biomass, dry root biomass, plumbagin content and total plumbagin yield were determined. Then the culture within the bioreactor was treated with elicitors i.e. chitosan (200 mg l^{-1}) + methyl jasmonate (80 μM) which were selected from shake flask studies. One set without elicitor served as control. The cultivation was continued for 3 d (based on the results of shake flask culture) and the results were recorded.

Estimation of plumbagin by HPLC The plumbagin content was estimated by HPLC (LC-20 AT Liquid Chromatogram, Shimadzu, Japan) employing isocratic linear solvent system of water and acetonitrile (20:80, v/v) as per the method of Gangopadhyay et al. (18). Plumbagin leaching into culture media was also estimated for each set of experiment. Briefly, the 20 ml medium was collected and extracted with 20 ml ethyl acetate. The methanol soluble fraction of ethyl acetate extract was subjected to HPLC analysis to estimate plumbagin leaching. Plumbagin content was calculated as mg g^{-1} DW.

Data analysis Three replicates were made for each experimental set. Data were statistically calculated by utilizing one way ANOVA and expressed as mean ± standard deviation followed by Turkey–Kramer's *t*-test using computerized GraphPad InStat version 3.05, GraphPad Software, La Jolla, CA, USA. The values were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Effect of fungal biomass The effect of different fungi viz. *F. solani, A. niger* and *R. oryzae* on plumbagin production is shown in Table 1. The elicitation with *F. solani* and *A. niger* in hairy root culture of *P. indica* caused a slight, statistically insignificant improvement of intracellular plumbagin content in hairy roots at the dose of 1 mg l^{-1} up to 3 d. One day exposure of *F. solani* and *A. niger* at a dose of 1 mg l^{-1} showed the product yield of 5.92 ± 0.40 and 5.62 ± 0.32 mg g^{-1} DW respectively. On other hand, intracellular plumbagin content was adversely affected with *R. oryzae*. The metabolite production gradually decreased with increasing dose and exposure time with all the selected fungi. The media leaching of plumbagin was not observed in any of fungal elicitation. *F. solani, A. niger* and *R. oryzae* are commonly employed biotic elicitors for enhancing secondary metabolites in hairy root culture of different plant species (20). However, fungal elicitors produce very species-specific action of elicitation (26). In present study, insignificant alteration of intracellular plumbagin content in *P. indica* hairy roots by elicitation with the fungi suggested that the plant species is not specific toward selected fungi.

Effect of yeast carbohydrate fraction Yeast carbohydrate fraction has been employed as biotic elicitor for enhancing secondary metabolites in hairy root cultures of various species (2). Addition of yeast carbohydrate fraction exhibited positive effect on plumbagin production in *P. indica* hairy roots without altering root biomass (Table 1). Maximum increase in plumbagin accumulation (6.13 ± 0.38 mg g^{-1} DW, $p<0.05$, ~1.2 fold higher than control) was obtained at the dose of 1 mg l^{-1} on day 3. The intracellular plumbagin content

TABLE 1. Effect of different fungal elicitors and yeast carbohydrate fraction on plumbagin accumulation in *P. indica* hairy roots.

Elicitors	Concentrations (mg l^{-1})	Plumbagin (mg g^{-1} DW)		
		Day 1	Day 3	Day 7
Control	–	5.33 ± 0.21	5.31 ± 0.35	5.29 ± 0.25
F. solani	1	5.92 ± 0.40	5.66 ± 0.47	5.39 ± 0.35
	2	5.41 ± 0.17	5.39 ± 0.21	5.03 ± 0.26
	3	4.02 ± 0.22	3.88 ± 0.25	3.04 ± 0.28
A. niger	1	5.62 ± 0.32	5.42 ± 0.28	5.01 ± 0.30
	2	4.91 ± 0.18	4.22 ± 0.21	4.09 ± 0.19
	3	4.10 ± 0.24	4.01 ± 0.30	3.90 ± 0.21
R. oryzae	1	4.92 ± 0.14	4.87 ± 0.09	4.66 ± 0.12
	2	4.33 ± 0.21	4.12 ± 0.26	4.01 ± 0.19
	3	4.25 ± 0.15	4.03 ± 0.14	3.74 ± 0.18
Yeast carbohydrate fraction	1	5.46 ± 0.31	6.13 ± 0.38*	6.04 ± 0.42
	2	5.39 ± 0.23	6.01 ± 0.32	5.83 ± 0.29
	3	5.31 ± 0.24	5.88 ± 0.26	5.07 ± 0.14

Values presented as mean ± SD. Data marked with an asterisk are significantly different (elevated) with respect to the corresponding control according to Tukey's test ($p<0.05$). The final root biomass was nearly the same (0.077 ± 0.003 g on fresh weight basis) in all cases and no extracellular plumbagin leaching was observed.