Biotechnology and Bioprocess Engineering 2009, 14: 288-294
DOI/10.1007/s12257-008-0238-6

Enhanced Production of Phytoestrogenic Isoflavones from Hairy Root Cultures of *Psoralea corylifolia* L. Using Elicitation and Precursor Feeding

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Abstract The effect of biotic elicitors (yeast extract, chitosan), signaling molecule (salicylic acid), and polyamines (putrescine and spermidine) was studied with respect to isoflavones accumulation in hairy root cultures of *Psoralea corylifolia* L. Untreated hairy roots (control) accumulated 1.55% dry wt of daidzein and 0.19% dry wt of genistein. In precursor feeding experiment, phenylalanine at 2 mM concentration led to 1.3 fold higher production of daidzein (1.91% dry wt) and genistein (0.27% dry wt). In biotic elicitors, chitosan (2 mg/L) was found to be the most efficient elicitor to induce daidzein (2.78% dry wt) and genistein (0.279% dry wt) levels in hairy roots. Salicylic acid at 1 mM concentration stimulated the maximum accumulation of daidzein (2.2% dry wt) and genistein (0.228% dry wt) 2 days after elicitation. In case of polyamines, putrescine (50 mM) resulted in highest accumulation of daidzein (3.01% dry wt) and genistein (0.227% dry wt) after 5 days of addition. Present results indicated the effectiveness of elicitation and precursor feeding on isoflavones accumulation in hairy roots of *P. corylifolia*. This is the first report of elicitation on isoflavones production by hairy roots of *P. corylifolia*. © KSBB

Keywords: Psoralea corylifolia*, phytoestrogens, elicitation, precursor, signaling molecule*

INTRODUCTION

Psoralea corylifolia (Indian bread root) Linn. (Fabaceae) is a rich source of phytoestrogenic isoflavones daidzein and genistein [1]. Genistein is a potent growth inhibitor of human breast carcinoma cell lines MDA-468, MCF-7, and MCF-7-D-40 [2]. Recently, daidzein was shown to inhibit the proliferation of murine and human neuroblastoma cell lines *in vitro*, cell cycle progression to G_2/M phase and induce apoptosis in the neuronal tumor cells [3]. Various biological properties are associated with daidzein and genistein such as anti-inflammatory, anti-allergic, anti-oxidant, anti-angiogenic, DNA polymerase, topoisomerase-II activity inhibition, and protection against low-density lipoprotein oxidation [4-6].

The possibility of *in vitro* plant cell cultures for production of plant pharmaceuticals was studied in details as an alternate and complimentary method to whole plant extraction [7-9]. Stimulation of biosynthetic activity using elicitation and precursor feeding is most studied approach to optimize product accumulation in plant cell cultures [10-12]. Elicitors are the compounds of biological and non biological origin, which effectively induce secondary metabolite production by plant cell cultures [13,14]. Wide range of elicitors has been employed to modify plant metabolism in order to enhance productivity in plant cell/tissue cultures [15-17]. Polyamines are group of naturally accruing low molecular weight polycationic, aliphatic natural compounds associated with several important cellular processes and plant response to abiotic stress. Growth regulator or secondary hormonal messenger like activity was proposed with polyamines and related compounds [18,19]. In addition, interrelationship between polyamines and secondary metabolite production was reported in cell cultures of *Chichorium intybus*, *Hyoscyamus muticus,* and *Capsicum frutescens* [20-22].

Elicitation particularly induces plant cells to synthesize

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phytoalexins at elevated levels. In regard to this, possibility of enhanced accumulation of isoflavones by elicitation has been reported in cell suspension cultures [17,23,24]. However, there are only few reports of application of elicitors to hairy root cultures [25,26]. Isoflavone production by hairy root cultures was studied in *Psoralea* sp. and *Pueraria lobata* [27-29]. To our knowledge, so far no information is available on influence of elicitation and precursor feeding on isoflavones accumulation in *P. corylifolia* hairy root cultures. In this article, we reported the effect of chitosan, yeast extract, salicylic acid, and polyamines on phytoestrogenic daidzein and genistein production. In addition, effect of precursor feeding is also studied on hairy root cultures of *P. corylifolia*.

MATERIALS AND METHODS

Hairy Roots Initiation

Hairy root cultures were obtained by infecting stem segments of *in vitro* grown *P. corylifolia* seedlings with *Agrobacterium rhizogenes* strain LBA 9402. Hairy roots emerged at wounding sites were carefully excised and cultured on MS [30] medium supplemented with 500 mg/L filtered sterilized cefotaxime (Sigma, USA). Hairy roots were repeatedly subcultured on MS medium with reduced concentrations of cefotaxime until complete eradication of bacteria. Approximately 3~5 cm long hairy root segments were inoculated on fresh MS medium and sub-cultured after every three weeks. Transformed nature of roots was confirmed by PCR and selected for further experiments. For elicitation experiment, approximately 250 mg (fresh wt) of hairy root segments were inoculated on liquid MS medium (50 mL). Flasks were kept on gyratory shaker at 40 rpm under 16/8 photoperiod conditions (13 μmol/m^2/s, cool white fluorescent tubes, Phillips, Holland).

Elicitor Preparation and Application

Concentrated chitosan (Sigma, USA) solution was prepared by dissolving chitosan in (1%, v/v) acetic acid. The pH was adjusted to 5.5 with 1N NaOH and sterilized by autoclaving at 120°C and 15 lbs for 20 minutes. Yeast extract was dissolved in distilled water whereas salicylic acid in ethanol and filter sterilized using 0.22 μM filter before application. For elicitation experiments, concentrated solution of elicitors were added to 18 day old cultures to give final concentrations (0.01, 0.1, 1, and 2 mM) for salicylic acid (25, 50, 100, 200, and 500 mg/L) and for yeast extract (2, 5, 10, 20, 40, and 80 mg/L) for chitosan.

For studying polyamine effect, concentrated solutions were added to 18 day old cultures to give final concentrations (25, 50, 100, and 200 μM) for spermidine and putrescine. Control cultures received equal volume of medium without any elicitor. To study the effect of treatment duration, biomass was harvested at regular interval (1, 2, 3, 5, and 7 days) after addition of elicitors. Non treated hairy roots (control) were harvested on similar day as experimental flasks. All experiments were replicated four times.

Precursor phenylalanine, was added to culture at concentration (0.05, 0.1, 0.5, 1, 2, and 5 mM) on 15 day and hairy roots were harvested after 5th day of addition.

Isoflavones Extraction

Harvested biomass was dried in an oven at 55°C for 16 h and powdered by Wiely Mill (Model No. 4276, Thomas Scientific, USA). Dried powdered material was mixed with water and 3 M H_2SO_4 (1:1, v/v) and sonicated (33 KH_z) for 10 min. Subsequently extract was kept in a water bath at 100°C for 1 h followed by addition of equal volume of ethanol. Samples were vortexed for 3 min, transferred into polypropylene micro-centrifuge (Eppendorf) tubes, and centrifuged at 12,000 × *g* for 10 min. The supernatant was transferred to clean glass vials and analyzed by High Performance Liquid Chromatography (HPLC). Similar method was adopted for analysis of isoflavones from spent medium.

Quantification of Isoflavones by High Performance Liquid Chromatography

An isocratic analytical HPLC was performed on Jasco Liquid Chromatograph (Model 980, Japan) equipped with an auto-sampler injector (Model No. Jasco AS-950, Japan) with a 25 μL loop and a variable wavelength detector (Model No. UV-975, Japan). Data collection and integration were accomplished using BORWIN software (Japan). Separations were performed on Inertsil C_{18} (250 mm × 4.6 I. D, Sigma, USA) column. The daidzein and genistein were determined by using acetonitrile:water (40:60, v/v) as a mobile phase. The flow rate was 0.6 mL/min and the elution was monitored at 250 nm. Peak identification was carried out on the basis of an authentic sample of daidzein and genistein (Sigma, USA) and chromatography co-injection.

Statistical Analysis

The influence of various treatments on isoflavones content was analyzed by one-way analysis of variance (ANOVA) using the statistical software IRRISTAT 4.0 (Biometric unit; IRRI Los Banos, Philippines). Values are mean of four replicates from two experiments. The data was processed statistically by analysis of variance (ANOVA) and difference between means of the samples analyzed by the least significant difference (LSD) at a probability level of 0.05.

RESULTS

Effect of Precursor Feeding on Isoflavone Production

The effect of precursor feeding (phenylalanine) on production of isoflavones is presented in Fig. 1. It can be seen that phenylalanine at 2 mM concentration increased the production of daidzein and genistein by 1.3 fold compared to

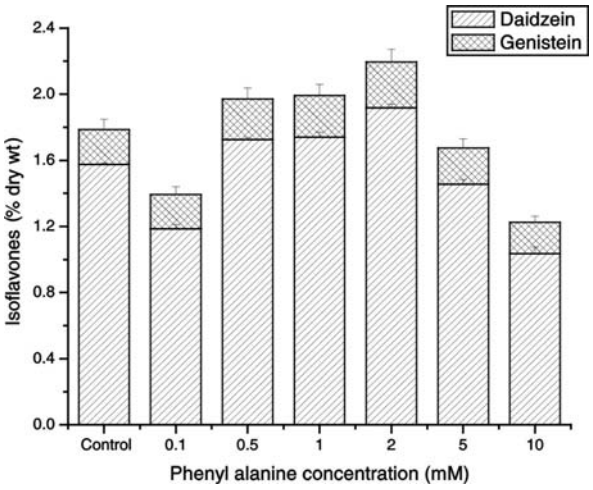

Fig. 1. Effect of precursor feeding on isoflavones accumulation in hairy root culture of *P. corylifolia* harvested on 20th day. Results are the mean of three replicates ± SD.

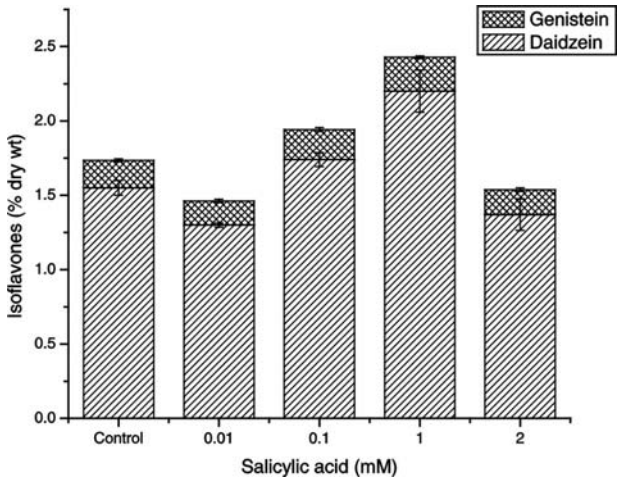

Fig. 3. Effect of salicylic acid (2nd day after addition) on isoflavones production in hairy root culture of *P. corylifolia*. Results are the mean of three replicates ± SD.

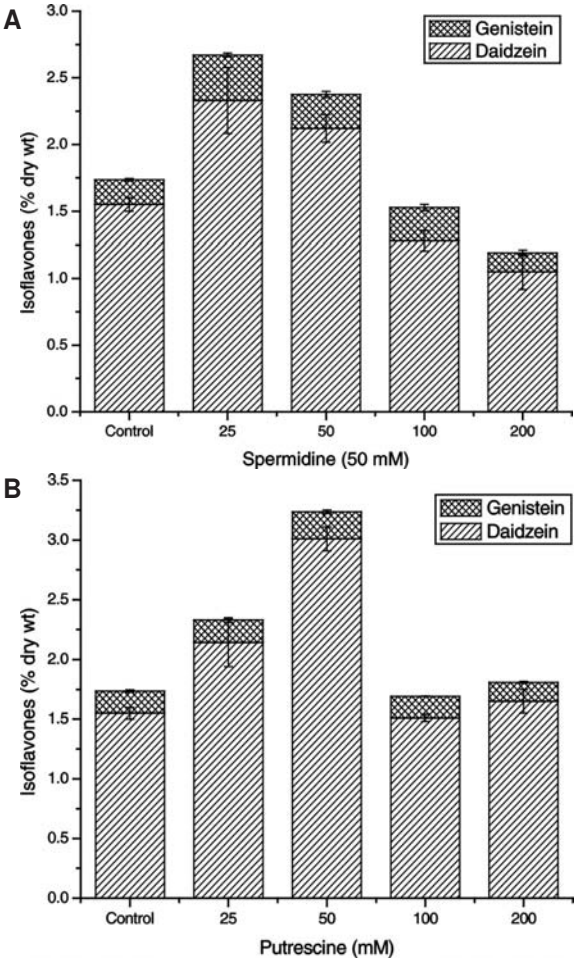

Fig. 2. Effect of polyamines (5th day after administration) on isoflavones production in hairy root culture of *P. corylifolia*. Results are the mean of three replicates ± SD.

control. Maximum concentration of daidzein (1.91% dry wt) and genistein (0.27% dry wt) was obtained in phenylalanine fed cultures. Daidzein (1.035% dry wt) and genistein (0.17% dry wt) levels greatly inhibited when concentration of phenylalanine was increased to 10 mM.

Effect of Polyamines on Isoflavones Production

Of the polyamines studied, addition of 50 mM putrescine resulted in increased accumulation of daidzein and genistein production as compared to control. Highest accumulation of daidzein (3.01% dry wt) and genistein (0.227% dry wt) was obtained after 5 days of addition. Spermidine at 25 mM concentration accumulated maximum levels of daidzein (2.33% dry wt) and genistein (0.341% dry wt). Further increase in putrescine and spermidine concentration resulted in reduced isoflavones accumulation Figs. 2A and 2B.

Effect of Different Elicitors on Isoflavones Accumulation

Isoflavones content in hairy roots were analyzed under the influence of salicylic acid, yeast extract, and chitosan. In case of signaling molecule, salicylic acid at 1 mM concentration stimulated the maximum accumulation of daidzein (2.2% dry wt) and genistein (0.228% dry wt) 2 days after elicitation. Further increase in salicylic acid concentration significantly reduced levels of daidzein and genistein (Fig. 3). Highest level of daidzein (1.93% dry wt) and genistein (0.23% dry wt) was obtained in yeast extract (200 mg/L) treated hairy roots 2 days after elicitation compared to control. Higher concentration of yeast extract (500 mg/L) in culture medium significantly reduced daidzein (0.77% dry wt) and genistein (0.1% dry wt) accumulation in hairy roots (Fig. 4).

Chitosan was found to be the most efficient elicitor to in-

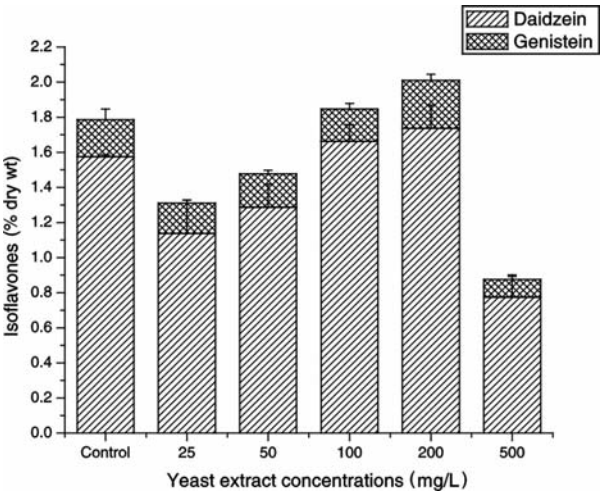

Fig. 4. Effect of yeast extract (2nd day after addition) on isoflavones accumulation in hairy root culture of *P. corylifolia*. Results are the mean of three replicates ± SD.

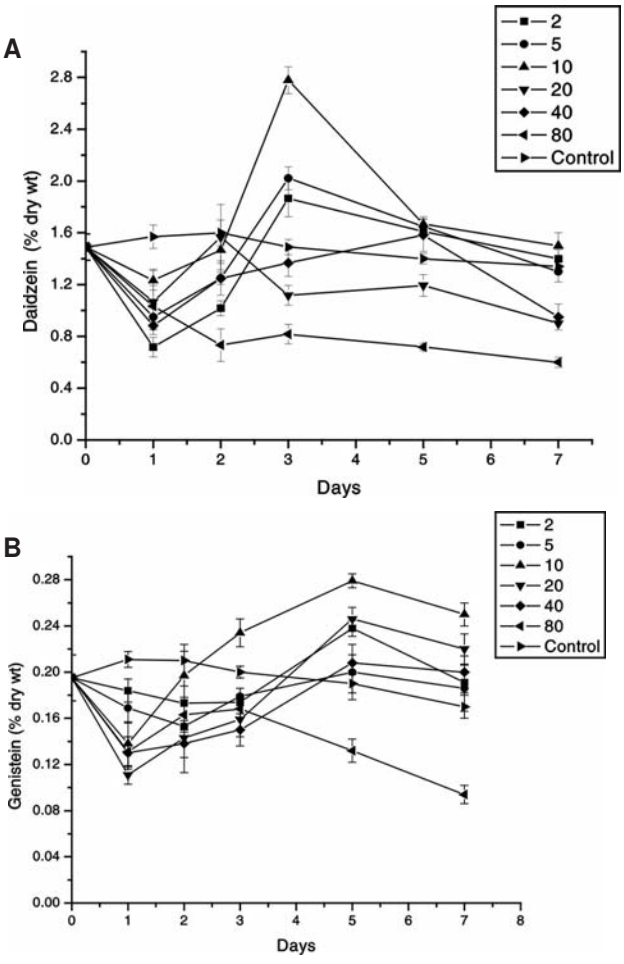

Fig. 5. Effect of chitosan (mg/L) on daidzein (A) and genistein (B) accumulation in hairy root culture of *P. corylifolia*. Results are the mean of three replicates ± SD.

Table 1. Effect of elicitation treatment on biomass accumulation in hairy root culture of *P. corylifolia*

Treatment	Effective concentration and day	Fresh wt (g/L)	Dry wt (g/L)
Control	–	171.83 ± 10.49^{bc}	18.40 ± 0.46^{c}
Phenyl alanine	2 mM (5)	181.35 ± 13.28^{c}	18.94 ± 0.32^{c}
Salicylic acid	1 mM (2)	104.11 ± 14.72^{a}	11.49 ± 0.19^{a}
Yeast extract	200 mg/L (2)	118.87 ± 9.57^{a}	12.30 ± 1.46^{a}
Chitosan	10 mg/L (4)	169.46 ± 11.88^{b}	15.61 ± 0.32^{b}
Spermidine	25 mM (5)	160.24 ± 13.43^{b}	14.09 ± 0.72^{b}
Putrescine	50 mM (5)	159.74 ± 8.45^{b}	14.86 ± 0.41^{b}

Values were recorded at maximum isoflavones accumulating stage [Incubation period (days)].
Means with common letters (a~c) within column are not significantly different at $p \leq 0.05$ according to LSD test.

duce isoflavones production (Fig. 5). The lowest applied dose of chitosan (2 mg/L) slightly enhanced the production while 10 mg/L treatment of chitosan significantly increased the daidzein (2.78% dry wt) and genistein (0.279% dry wt) level in hairy roots. Maximum levels of daidzein and genistein was obtained after 3rd and 5th day of elicitation, respectively. Further increase in incubation period reduced isoflavones accumulation Figs. 5A and 5B. Chitosan at 80 mg/L concentration was found to be detrimental for daidzein (0.81% dry wt) and genistein (0.13% dry wt) accumulation.

Table 1 represents changes in biomass accumulation after various treatments. Of the studied treatments, salicylic acid (11.49 g dry wt/L) and yeast extract (12.30 g dry wt/L) significantly reduced biomass accumulation compared to control (18.40 g dry wt/L). Moderate amount of daidzein and genistein leaching was observed after various elicitation treatments. Effective treatment of elicitor concentration and incubation period on isoflavones leaching in medium is presented in Table 2. Maximum concentrations of daidzein (33 mg/L) and genistein (8.2 mg/L) were leached out 3 days after elicitation by salicylic acid treated cells compared to control.

DISCUSSION

In hairy root cultures of *P. corylifolia*, phenylalanine feeding showed positive effect on isoflavones production. Precursor feeding strategy was used to enhance the biosynthesis of secondary metabolites in hairy root cultures of *Catharanthus roseus* and *Beta vulgaris* [31,32]. Isoflavones daidzein and genistein are originated from phenylalanine, a upstream metabolic precursor through phenylpropanoid pathway. Considering this phenylalanine supplementation expected to increase metabolic flux through phenyl-propanoid biosynthetic pathway and elevated level of target compound. Phenylalanine supplementation has been reported to enhance secondary metabolite production in cell cultures of Strawberry and *Taxus baccata* [11,12]. Results obtained are in

Table 2. Effective treatment and incubation period (day) for isoflavones leaching in medium by hairy root culture of *P. corylifolia* in response to elicitation and precursor feeding

Treatment	Effective concentration and day	Daidzein (mg/L)	Genistein (mg/L)
Control	-	4 ± 0.2[a]	3.3 ± 0.3[a]
Phenyl alanine	0.5 mM (5)	10.8 ± 0.5[d]	3.2 ± 0.4[a]
Salicylic acid	1 mM (2)	33 ± 1.5[f]	8.2 ± 0.6[e]
Yeast extract	500 mg/L (5)	5.6 ± 0.6[b]	5.6 ± 0.2[d]
Chitosan	10 mg/L (5)	7 ± 0.4[c]	4.2 ± 0.3[b]
Spermidine	50 mM (5)	18.4 ± 0.7[e]	3.2 ± 0.3[a]
Putrescine	50 mM (5)	6 ± 0.5[b]	5.2 ± 0.1[c]

Means with common letters (a~f) within column are not significantly different at $p \leq 0.05$ according to LSD test.

accordance with these findings. However appropriate concentration of precursor and time of addition of precursor are crucial factors. In addition feedback inhibition to the metabolic pathway due to excess precursor is necessary for consideration during product optimization. [33].

Polyamines administration influenced daidzein and genistein accumulation in hairy root cultures of *P. corylifolia*. Polyamines were reported to be involved in the interaction with ethylene and abiotic stress tolerance [34]. Furthermore it was believed that phenylpropanoid pathway provide conjugation partners for polyamines which also contribute to regulatory machinery of plant growth [35]. Interaction between polyamines and secondary metabolism was investigated in plant cell cultures. Stimulatory effect of polyamines on growth and secondary metabolite production was observed to be mediated through increased endogenous polyamines level in hairy root cultures of *Cichorim intybus* and *Beta vulgaris* [20,36,37]. Among polyamines studied, putrescine administration significantly stimulated isoflavones accumulation in hairy root cultures. Putrescine addition has been shown to have stimulatory effect on secondary metabolites [20,36,37]. Similar, stimulatory effect of putrescine was also observed on enhancement of growth and capsaicin production in the cell suspension cultures of *Capsicum frutescens* [22].

Yeast extract, chitosan, and salicylic acid elicit production of daidzein and genistein at various concentrations tested. Salicylic acid is an important plant signaling compound associated with stimulation of pathogen induced plant defense response and accumulation of pathogenesis related compounds [15,38-40]. Stimulatory effect of salicylic acid was commonly observed on overproduction of secondary metabolites in plant cell cultures [41,42].

Elicitors mainly derived from cell walls of fungal pathogens are known to induce the synthesis of phytoalexins [43]. Chitosan [poly(1,4-B-D-glucopyranosamine)] is the deacetylated form of chitin, a main component of cell wall of some fungal species and of the exoskeletons of insects and crustaceans. Chitosan has been also associated with plant growth, development, and protection against microorganisms by inducing defense response in terms of secondary metabolites production [44]. The results implied that chitosan was an effective elicitor for production of daidzein and genistein in hairy root cultures of *P. corylifolia*. It was reported that chitosan induced enzymes involved in phenylpropanoid metabolism of *Vanilla planifolia* [45]. Similar stimulatory effect of chitosan on secondary metabolites was reported in *Hyoscyamus muticus* and *Panax ginseng* [16,46]. Additionally influence of yeast extract on isoflavones production has been well known. Elicitor induced enzymatic and genetic activation of isoflavonoid production was reported in *Pueraria lobata* cell cultures [17]. An increased level of isoflavones upon yeast extract elicitation was mediated through elevated levels of transcripts for enzymes flavanone 2-hydroxylase, isoflavone 2′-hydroxylase, and isoflavone synthase in cell suspension cultures of *Glycyrrhiza echinata* [47]. Similar effect of yeast extract was observed on L-phenylalanine ammonia lyase and chalcone synthase transcripts in cell suspension of *Medicago truncatula* [48].

It was observed that isoflavones were constitutively expressed in tissues and further induced in response to pathogen attack [49]. Elicitor induced accumulation of phenylpropanoids especially isoflavones were demonstrated in several legumes such as *Cicer arietinum*, *Pueraria lobata*, and soybean [17,23-24]. Results obtained here indicated for the first time that efficacy of elicitation for optimized production of phytoestrogenic isoflavones in hairy root cultures of *P. corylifolia*.

CONCLUSION

Phytoestrogens production was influenced by elicitation and precursor feeding in hairy root cultures of *P. corylifolia*. Chitosan and polyamine putrescine showed significant increase in phytoestrogen accumulation. Present results indicated effectiveness of elicitation for optimized production of isoflavones daidzein and genistein by hairy root cultures of *P. corylifolia.*

Acknowledgement The SRF provided by Department of Atomic Energy, India, to one of the author (Amit Shinde) is gratefully acknowledged.

Received October 16, 2008; accepted January 22, 2009

REFERENCES

1. Kaufman, P., J. Duke, H. Brielmann, J. Boik, and J. Hoit (1997) A comparative survey of leguminous plants as a source of the isoflavones, genistein, and daidzein: Implications for human nutrition and health. *Altern. Complement. Med.* 3: 7-12.
2. Peterson, G. and S. Barnes (1991) Genistein inhibition of the growth of human breast cancer cells: Independ-

ence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun*. 179: 661-667.
3. Lo, F. H., N. K. Mak, and K. N. Leung (2007) Studies on the anti-tumor activities of the soy isoflavone daidzein on murine neuroblastoma cells. *Biomed. Pharmacother*. 61: 591-595.
4. Sun, N. J., S. H. Woo, J. M. Cassady, and R. M. Snapka (1998) DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. *J. Nat. Prod.* 61: 362-366.
5. Qiang Ren, M., G. Kuhn, J. Wegner, and J. Chen (2001) Isoflavones, substances with multi-biological and clinical properties. *Eur. J. Nutr.* 40: 135-146.
6. Coward, L., N. C. Barnes, K. D. R. Setchell, and S. Barnes (1993) Genistein, daidzein, and their β-glycosides conjugates: anti-tumor isoflavones in soybean food from America and Asian diets. *J. Agric. Food Chem.* 41: 1961 -1967.
7. Dornenburg, H. D. and D. Knorr (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. *Enzyme Microb. Technol.* 17: 674-684.
8. Bourgaud, F., A. Gravot, S. Milesi, and E. Gontier (2001) Production of plant secondary metabolites: a historical perspective. *Plant Sci.* 161: 839-851.
9. Dcosmo, F. and M. Misawa (1995) Plant cell and tissue culture: Alternative for metabolite production. *Biotech. Adv*. 13: 425-453.
10. Zhao, J., C. D. Lawrence, and R. Verpoorte (2005) Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv*. 23: 283-333.
11. Edahiro, J. I., M. Nakamura, M. Seki, and S. Furusaki (2005) Enhanced accumulation of anthocyanin in cultured strawberry cells by repetitive feeding of L-phenylalanine into the medium. *J. Biosci. Bioeng*. 99: 43-47.
12. Khosroushahi, A. Y., M. Valizadeh, A. Ghasempour, Khosrowshahli, H. Naghdibadi, M. R. Dadpour, and Y. Omidi. (2006) Improved taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biol. Int.* 30: 262-269.
13. Pitta-Alvarez, S. I., T. C. Spollansky, and A. M. Giulietti (2000) The influence of different biotic and abiotic elicitors on production of tropane alkaloids in hairy root cultures of *Brugmansia candida. Enzyme Microb. Technol.* 26: 252-258.
14. Sirvent, T. M. and D. M. Gibson (2002) Induction of hypericin and hyperfolin in *Hypericum perforatum* L. in response to biotic and chemical elicitors. *Physiol. Mol. Plant Path.* 60: 311-320.
15. Taguchi, G., T. Yazawa, N. Hayashida, and M. Okazaki (2001) Molecular cloning and heterogenous expression of novel glucosyl transferases from tobacco cultured cells that have broad substrate specificity and are induced by salicylic acid and auxin. *Eur. J. Biochem*. 268: 4086-4094.
16. Palazón, J., R. M. Cusidó, M. Bonfill, A. Mallol, E. Moyano, C. Morales, and M. Teresa Piñol (2003) Elicitation of different *Panax ginseng* transformed root phenotypes for an improved ginsenoside production. *Plant Physiol. Biochem.* 41: 1019-1025.
17. Park, H. H., T. Hakamatsuka, U. Sankawa, and Y. Ebizuka (1995) Rapid metabolism of isoflavonoids in elicitor-treated cell suspension cultures of *Pueraria lobata*. *Phytochem*. 38: 373-380.
18. Cohen, S. S. (1998) *A guide to the polyamines*. Oxford University Press, NY, USA.
19. Bais, H. P. and G. A. Ravishankar (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell Tiss. Org. Cult.* 69: 1-34.
20. Bais, H. P. and G. A. Ravishankar (2003) Synergistic effect of auxins and polyamines in hairy root of *Chicorium intybus* L. during growth, coumarin production, and morphogenesis. *Acta. Physiol. Plant* 25: 193-208.
21. Biondi, S., S. Scaramagli, K. M. Oksman-Caldentey, and F. Poli (2002) Secondary metabolism in root and callus cultures of *Hyoscyamus muticus* L.: the relationship between morphological organization and response to methyl jasmonate. *Plant Sci*. 163: 563-569.
22. Sudha, G. and G. A. Ravishankar (2002) Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites, and their molecular aspects. *Plant Cell Tiss. Org. Cult.* 71: 181-212.
23. Barz, W. and U. Mackenbrock (1994) Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer arietinum*) cell suspension cultures. *Plant Cell Tiss. Org. Cult.* 38: 199-211.
24. Dixon, R. A. (1999) Isoflavonoids: biochemistry, molecular biology, and biological functions. pp. 773-823. In: U. Sankawa (ed.). *Comprehensive natural product chemistry*. Elsevier, Oxford, UK.
25. Lozovaya, V., A. V. Lygin, O. V. Zernova, S. Li, G. L. Hartman, and J. M. Widholm (2004) Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*. *Plant Physiol. Biochem.* 42: 671-679.
26. Bourgaud, F., C. Nguyen, and A. Guckert (1995) XXII *Psoralea* species: *in vitro* culture and production of furanocoumarins and other secondary metabolites. pp. 388-411. In: Y. P. S. Bajaj (ed.). *Biotechnology in Agriculture and Forestry, Medicinal, and Aromatic Plants VIII*. Springer, Berlin, Heidelberg.
27. Bourgaud, F., V. Bouque, and A. Guckert (1999) Production of flavonoids by *Psoralea* hairy root cultures. *Plant Cell Tiss. Org. Cult.* 56: 97-104.
28. Abhayankar, G., V. D. Reddy, C. C. Giri, K. V. Rao, V. V. S. Lakshmi, S. Prabhakar, M. Vairamani, B. S. Thippeswamy, and P. S. Bhattacharya (2005) Amplified fragment length polymorphism and metabolomic profiles of hairy roots of *Psoralea corylifolia* L. *Phytochem*. 66: 2441-2457.
29. Yu, H., C. F. Liu, L. Li, and R. C. Pan (2002) *Pueraria lobata* hairy root culture *in vitro* and isoflavones production. *Acta. Phytophysiol. Sin*. 4: 281-286.

30. Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant* 15: 475-479.
31. Morgan, J. A. and J. A. Shanks (2000) Determination of metabolic rate-limitation by precursor feeding in *Catharanthus roseus* hairy root cultures. *J. Biotechnol.* 79: 137-145.
32. Bohm, H. and G. Mack (2004) Betaxanthin formation and free amino acids in hairy roots of *Beta vulgaris* var. lutea depending on nutrient medium and glutamate feeding. *Phytochem.* 65: 1361-1368.
33. Ouyang, J., X. Wang, B. Zhao, and Y. Wang (2005) Enhanced production of phenylethanoid glycosides by precursor feeding to cell cultures of *Cistanche deserticola*. *Proc. Biochem.* 40: 3480-3484.
34. Chen, W. H., C. M. Xu, J. L. Zeng, B. Zhao, X. D. Wang, and Y. C. Wang (2007) Improvement of echinacoside and acteoside production by two-stage elicitation in cell suspension culture of *Cistanche deserticola*. *World J. Microbial. Biotechnol.* 23: 1451-1458.
35. Bais, H. P., R. Madhusudan, N. Bhagyalakshmi, T. Rajasekaran, B. S. Ramesh, and G. A. Ravishankar (2000) Influence of polyamines on growth and formation of secondary metabolites in hairy root cultures of *Beta vulgaris* and *Tagetes patula*. *Acta. Physiol. Plant* 22:151-158.
36. Bais, H. P., G. Sudha, and G. A. Ravishankar (2001) Putrescine influences growth and production of coumarins in transformed and untransformed root cultures of witloof chicory (*Cichorium intybus* L. cv. Lucknow local). *Acta. Physiol. Plant* 23: 319-327.
37. Suresh, B., R. Thimmaraju, N. Bhagyalakshmi, and G. A. Ravishankar (2004) Polyamine and methyl jasmonate influnced enhancement of betalaine production in hairy root cultures of *Beta vulgaris* grown in a bubble column reactor and studies on efflux of pigments. *Proc. Biochem*. 39: 2091-2096.
38. Sandarmann, H. J., D. Ernst, W. Heller, and C. Langebartels (1998) Ozone: an abiotic elicitor of plant defense reactions. *Trends Plant Sci.* 3:47-50.
39. Jirange, D., T. L. Tootle, T. L. Reuber, L. N. Frost, B. J. Feys, J. E. Parker, F. M. Ausubel, and J. Glazebrook (1999) *Arabidopsis thaliana* PAD4 encodes lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acd. Sci., USA* 96: 13583-13588.
40. Hammerschmidt, R. and R. L. Nicholson (1999) A survey of plant defense responses to pathogens. pp. 37-53. In: A. Agrawal, S. Tuzun, and E. Bent (eds.) *Induced Defenses Against Pathogens and Herbivores*. APS Press, St. Paul, MN, USA.
41. Kittipongpatana, N., D. L. Davis, and J. R. Porter (2002) Methyl jasmonate increases the production of valepotriates by transformed root cultures of *Valerianella locusta*. *Plant Cell Tiss. Org. Cult.* 71: 65-75.
42. Wielanek, M. and H. K. Urbanek (2006) Enhanced glucotropaeolin production in hairy root cultures of *Tropaeolum majus* L. by combining elicitation and precursor feeding. *Plant Cell Tiss. Org. Cult.* 86: 177-186.
43. Szabo, E., A. Thelen, and M. Petersen (1999) Fungal elicitor preparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*. *Plant Cell Rep.* 18: 484-489.
44. Zhang, C. H., P. S. Fevereiro, G. He, and Z. Chen (2007) Enhanced paclitaxel productivity and release capacity of *Taxus chinensis* cell suspension cultures adapted to chitosan. *Plant Sci.* 172: 158-163.
45. Funk, C. and P. Brodelius (1990) Influence of growth regulators and an elicitor on phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia*. *Phytochem.* 29: 845-848.
46. Sevón, N., R. Hiltunen, K. M. Oksman-Caldentey (1992) Chitosan increases hyoscyamine content in hairy root cultures of *Hyoscyamus muticus*. *Pharmacol. Lett*. 2: 96-99.
47. Nakamura, K., T. Akashi, T. Aoki, K. Kawaguchi, and S. Ayabe (1999) Induction of isoflavonoid and retrochalcone branches of the flavonoid pathway in cultured *Glycyrrhiza echinata* cells treated with yeast extract. *Biosci. Biotechnol. Biochem.* 63: 1618-1620.
48. Suzuki, H., M. S. Srinivasa Reddy, M. Naoumkina, N. Aziz, G. D. May, D. V. Huhman, L. W. Sumner, J. W. Blount, P. Mendes, and R. A. Dixon (2005) Methyl jasmonate and yeast elicitor induce differential transcriptional and metabolic re-programming in cell suspension cultures of the model legume *Medicago truncatula*. *Planta* 220: 696-707.
49. Graham, T. L. and M. Y. Graham (1991) Glyceollin elicitors induce major shift and isoflavonoid metabolism in proximal and distal soybean cell populations. *Mol. Plant Microbe Interact.* 4: 60-68.