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Induced High Frequency Shoot Regeneration and Enhanced Isoflavones Production in *Psoralea corylifolia*

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Abstract: High frequency shoot regeneration and evaluation of product synthesis at various stages in *Psoralea corylifolia* were investigated. *In vitro* regenerated shoots were induced from germinated seedling on MS medium supplemented with 2, 4, 8, 20 and 40 µM of thidiazuron and N^6–benzylaminopurine. The results revealed that optimum concentrations of thidiazuron (8 µM) into the medium increased shoot regeneration frequency. Root differentiation was achieved from regenerated shoots on growth regulator free MS medium with frequency of 91.2% and mean number of 4.5 roots per shoot. High concentrations of Indole-3-aceticacid (IAA) into the rooting medium resulted in slow growth. Regenerated shoots and roots enhanced isoflavones production compared to field grown plants. A reverse phase high performance liquid chromatography analysis revealed that *in vitro* regenerated shoots accumulated 0.85% dry wt of daidzein and 0.06% dry wt of genistein. Maximum daidzein (1.23% dry wt) and genistein (0.38% dry wt) were accumulated by roots which obtained from regenerated shoots, which is 6.3-fold more daidzein and 77-fold more genistein respectively than field grown plants. The regeneration protocol developed successfully in this study showed the possibility for rapid propagation of *P. corylifolia* and enhanced isoflavones production.

Keywords: *Psoralea corylifolia;* shoot regeneration; root induction; isoflavones; HPLC.

1. Introduction

Psoralea corylifolia Linn (Indian bread root) belonging to Fabaceae family is a herbaceous plant that is distributed throughout tropical and subtropical regions of the world. Powders obtained from its seeds have been used for treatment of inflammatory diseases of skin including leucoderma, leprosy and psoriasis [1]. Daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7 trihydroxyisoflavone) (Figure 1) are the major bioactive isoflavones reported from *P. corylifolia* [2, 3]. These isoflavones act as estrogen agonist or antagonist depending on estrogen concentration resulted

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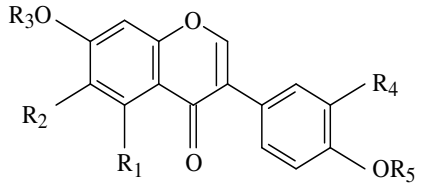
in countering postmenopausal ailments and lowering incidence of breast and ovary cancers [4]. They posses various biological properties including anti-inflammatory, anti-allergic, anti-oxidant, anti-angiogenic and DNA polymerase and topoisomerase activity inhibition [5-7]. Pre-clinical and clinical studies have shown that isoflavonoid posses lipid lowering effect and ability to inhibit low-density lipoprotein oxidation [8].

Successful development of regeneration of shoots is a prerequisite for clonal propagation and for genetic transformation. A growing demand of isoflavones from *P. corylifolia* in pharmaceutical industry has resulted in serious reduction as a consequence of unforeseen climatic conditions, which affects the production capacity and deforestation. It is observed that phenolic compounds from medicinal plants are sensitive to various environmental factors [9,10]. The major constraint in conventional propagation through seeds is the high mortality of seedlings in early stage and always the possibility of loosing the mother plant during this process. *In vitro* propagation methods offer highly efficient tools for medicinal plants useful for pharmaceutical industry [11,12].

Attempts have been made to achieve shoot proliferation from nodal explant of *P. corylifolia* [13]. However, no literature is available on quantification of product synthesis at various stages in regenerated shoots. This study reports production of isoflavones daidzein and genistein in regenerated shoots using TDZ and benzylaminopurine. The present study, also examines the efficacy of TDZ and benzylaminopurine on shoot organogenesis and isoflavones production at various stages of growth.



Isoflavones	R_1	R_2	R_3	R_4	R_5
Daidzein	H	H	H	H	H
Genistein	OH	H	H	H	H

Figure 1. Chemical structure of isoflavones daidzein and genistein.

2. Materials and Methods

2.1. Plant material and tissue culture

Seeds of *P. corylifolia* were obtained from the Botanical Garden, Department of Botany, University of Pune, India. Seeds were scarified by immersion in concentrated sulphuric acid for 1 hr and washed thoroughly with water. Thereafter they were sterilized with mercury chloride (0.05% w/v) solution for 2 min and subsequently rinsed 4 times with sterile distilled water. Surface sterilized seeds were germinated aseptically on Murashige and Skoog's medium [14].

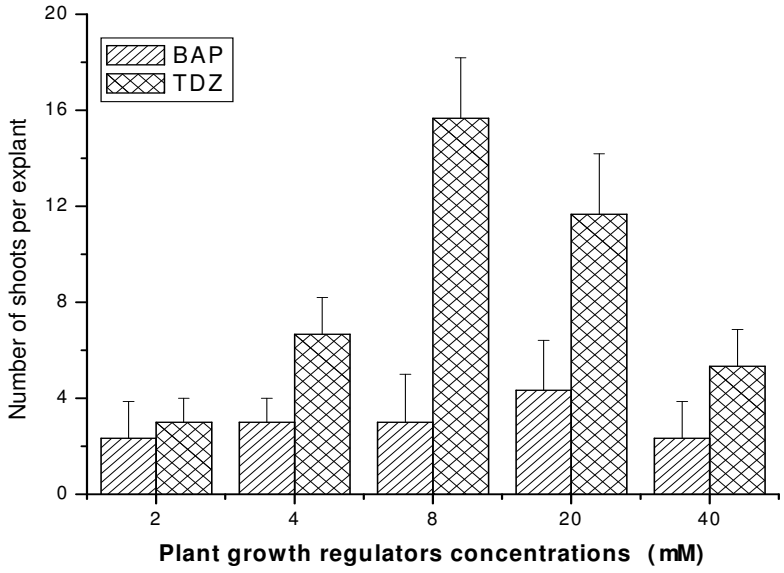


Figure 2. Effect of TDZ and BAP on shoot regeneration in *P. corylifolia.* (Results are mean of three experiments with 20 replicates each).

2.2. Culture media and conditions

MS media was fortified with different concentrations of plant growth regulators and 3.0% sucrose. The pH of the medium was adjusted to 5.8 by 0.1 N NaOH or HCl before adding 0.8% agar (Hi-Media, India). Culture medium was autoclaved at 121 ^{0}C at 103.42 kPa for 20 min. Cultures were maintained at 25±1 ^{0}C for 16 hr light provided by cool white fluorescent tubes (Phillips, Holland) to provide 40 µmol $m^{-2}s^{-1}$ light intensity.

2.3. Multiple shoots proliferation media

Three-week-old *in vitro* cotyledonary nodes of germinated seedlings were used as explants. MS medium supplemented with 2, 4, 8, 20 and 40 µM TDZ and BAP each was used for shoot regeneration.

2.4. Root differentiation

For root induction, regenerated shoots derived from TDZ supplemented media, were excised and cultured on ½ concentration of MS macro element, micro element and vitamin or MS medium with or without 0.025, 0.05, 0.5 µM indole-3-acetic acid (IAA). Percentage of rooting, number of roots per explants and root length were calculated at the end of 4th week of culture. All experiments were repeated for three times consisting of twenty replicates of two explants.

2.5. Extraction of isoflavones

For determination of isoflavones regenerated shoots were harvested at different developmental stages including shoot primordial stage, elongated shoots and regenerated roots. Field grown plants were harvested and analysed separately for isoflavone contents. Harvested plant materials was dried in an oven at 55^{0}C for 16 hr and powdered by Wiely Mill (Model No. 4276, Thomas Scientific, USA). Dried powdered material was mixed with 3M H_2SO_4 and sonicated at 33 KHz for 10 min. Samples were incubated in a water bath at 100 ^{0}C for 60 min followed by addition of equal volume of distilled

ethanol. Samples were centrifuged at 12000x*g* for 10 min and supernatant was directly checked in High Performance Liquid Chromatography (HPLC) for the isoflavone contents.

2.6. Quantification of isoflavones on HPLC

The HPLC analysis was performed on Jasco Liquid Chromatograph (Model 980, Japan) equipped with 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. Separations were performed on Inertsil C_{18} (250 mm x 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. Validation of quantitative method was performed with samples for five times. The results of the five injections from the same samples at five concentrations (0.01µg–0.5µg) showed similar retention time. The analytical operation was completed in 20 min.

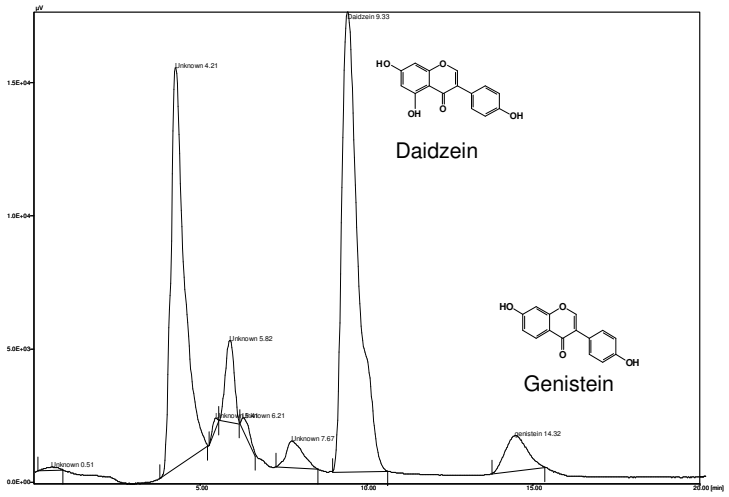


Figure 3. HPLC chromatogram revealed presence of daidzein and genistein in shoots and roots extracts.

3. Results

3.1. Shoot regeneration

Multiple shoot proliferation started after 18-20 days of cultures with distinguished shoots after four weeks of culture. Shoot proliferation occurred in form of small shoot clusters on MS medium containing any concentration of TDZ without callus formation. From Fig. 2 we can see the efficacy of various concentrations of TDZ and BAP on shoot proliferation in cotyledonary nodal sectors of germinated seedlings of *P. corylifolia.* Maximum numbers of 15 shoots were recorded at concentrations of 8 µM TDZ in MS medium which mean shoot length of 2.5 cm in 4 weeks. Maximum concentrations of BAP (20 µM) in to the culture medium proliferated low frequency of shoot induction and developed into a normal plantlets. It was also observed that scanty callus at the base after 4 weeks of cultivation. Comparison of the efficiency of shoot induction rate showed that TDZ was remarkably effective than BAP.

Efficiency of shoot regeneration showed variation in presence of different concentrations of TDZ and BAP into culture medium. From Figure 2, indicated that the TDZ was efficient for shoot induction. Therefore, regenerated shoots induced by TDZ were selected for further study and used for estimation of daidzein and genistein contents.

3.2. Root initiation from regenerated shoots

Root differentiation was observed after about three weeks of culture period. Maximum of 91.2% shoots were rooted with an average of 4.5 roots per shoot and average length of 10.1 cm on growth regulators free MS medium (Table 1). However, medium supplemented with low concentration of IAA (0.025 µM) showed 83.4% roots with 4.2 roots per shoot and an average length 6.8 cm. Addition of IAA at high concentrations reduced the percentage of rooting and elongation. On the other hand, medium devoid of growth regulators enhanced the elongation and developed maximum roots. The rooting percentage and elongation decreased with increased concentrations of IAA in the culture medium. Low concentration of IAA showed elevated branched roots, whereas with increased concentration of IAA into the culture medium led to slow growth and rapid necrosis.

Table 1. Rooting response of *P. corylifolia* on MS and MS medium fortified with various concentrations of IAA. (Results are mean of three experiments with 20 replicates each).

Medium	Percentage of rooting (%)	Number of roots per shoot	Root length (cm)
½MS	41.6	3	2.4
MS	91.2	5.4	10.1
MS + IAA (0.5 µM)	35.7	2.1	2.3
MS + IAA (0.05 µM)	58.1	2.8	2.7
MS + IAA (0.025 µM)	83.4	4.2	6.8

3.3. Production of daidzein and genistein

In the present study, we also quantified daidzein and genistein yield in roots and at various stages of regenerated shoots. The regenerated shoots at specific stages were isolated, processed and evaluated for isoflavones. The concentrations of daidzein and genistein reached 0.33 and 0.02% dry wt, respectively during shoot primordial stage. *In vitro* regenerated shoots of about >5 cm length accumulated 0.85% dry wt daidzein and 0.068% dry wt genistein. It was found that the rate of accumulation of isoflavones was associated with progressive stages of regenerated shoots. The highest content of yield was accumulated in roots obtained from regenerated shoots. Chromatographic analysis revealed that highest levels of 1.23% dry wt of daidzein and 0.38% dry wt of genistein, are several fold more compared to *ex-vitro* grown plants (Figure 3).

4. Discussion

Overall objective of the current study was to develop an *in vitro* system for rapid propagation of *P. corylifolia* plantlet cultures that could contain high levels of isoflavones compared to field grown plants. Shoot proliferation rates shows in the present study are obviously high and obtained 15 shoots per explants compared to earlier report by Faisal and Anis [13], who reported that TDZ at 2 µM promoted the shoot frequency and achieved maximum regenerated shoots in *P. corylifolia*. In contrast, the present results revealed that high proliferation of shoots per ex-plant achieved on media supplemented with 8 µM TDZ. It was observed that medium fortified with TDZ (2 µM) did not favor shoots proliferation. It was also found that TDZ acted as a potent growth regulator compared to BAP for shoot organogenesis in *P. corylifolia.*

TDZ did not hamper root formation once shoots are excised [15,16,17,18]. IAA and IBA are the most commonly used auxins for root induction [19,20]. High rooting and survival was achieved on growth regulator free MS medium compared to medium fortified with IAA.

Table 2. Accumulation of isoflavones in various stages of regenerated shoots and filed grown plant of *P. corylifolia*. (Results are mean six replicates ± SD)

Plant material	Daidzein (% dry wt)	Genistein (% dry wt)
***In vitro* cultures**		
Shoot primordial stage	0.332 ± 0.019	0.024 ± 0.008
Shoots (0.5 - 1.5 cm)	0.538 ± 0.048	0.041 ± 0.011
Shoots (> 5 cm)	0.850 ± 0 0.41	0.068 ± 0.019
Roots	1.238 ± 0.068	0.385 ± 0.019
***Ex-vitro* plant materials**		
Shoots	0.063 ± 0.012	0.013 ± 0.002
Roots	0.197 ± 0.017	0.005 ± 0.001

Plant cell and organ cultures were studied as an effective system for production of natural bioactive products. The global demand of plant origin bioactive compounds is very high, but not possible to fulfill by field grown plants. An attractive and very promising alternative system for commercial exploitation is plant cell cultures thereby producing high yield compared to field grown plants. Datta and Srivastava (21) demonstrated that the high degree of differentiation and maturity in the tissues of *Catharanthus roseus* was correlative to the increased vinblastine production. Similarly, active secondary products of *Hypericum perforatum* cell cultures were connected with the formation of secretory structures in regenerated vegetative buds (22). HPLC chromatogram exhibited that plant roots of *P. corylifolia* accumulated maximum amount of daidzein (0.19% dry wt). However, chromatographic analysis revealed that the roots obtained from regenerated shoots synthesized highest levels of daidzein and genistein compared to field grown plants. Our results further strengthen the fact that biosynthesis of daidzein is more restricted to roots in *Psoralea* species [1]. Fulzele et al., [23] reported that *in vitro* plantlet cultures synthesized high levels of terpenoids compared to parent plants of *A. annua*. Similarly, micro propagated *Pueraria lobata* plants produced isoflavonoid compounds compared to parent plants [9]. Liu et al., [24] reported that cell cultures of *H. perforatum* stimulated product synthesis by addition of TDZ into the culture medium compared to the controls. Similarly, our present results demonstrated that regenerated shoots and roots increased significant levels of isoflavones productivity compared to the field grown plants. The present results suggested that a possibility to establish high yielding genotypes by *in vitro* culture for production of medicinally important bioactive isoflavones.

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References

[1] F. Bourgaud, C. Nguyen, A. Guckert (1995). XXII *Psoralea* sps: *In vitro* culture and production of furanocoumarins and secondary metabolites, In: Medicinal and aromatic plants VIII, ed: Y.P.S. Bajaj, Springer-Verlag Berlin Heidelberg, pp. 388–411.

[2] P. Kaufman, P. Duke, J. Brielmann, H. Boik, J. Hoit, (1997). A comparative survey of leguminous plants as a source of the isoflavones, genistein and daidzein: Implications for human nutrition and health. *Alter. Comp. Med.* **3**, 7-12.

[3] Y. Hsu, C. Wu, J. Chem, Y. Yang, S. Wang (2001). The presence of three isoflavonoid compounds in *Psoralea corylifolia*. *J. Chromatographic Sci*. **39,** 441- 444.

[4] C.S. Hwang, H.S. Kwak, H.J. Lim, S.H. Lee, Y.S. Kang, T.B. Choe, H.G. Hur, K.O. Han (2006). Isoflavone metabolites and their *in vitro* dual functions: They can play estrogenic agonist or antagonist depending on the estrogen concentration. *J. Steroid Bioch. and Mol. Biol*. **101**, 246-253.

[5] N.J. Sun, S. H. Woo, J.M. Cassady, R.M. Snapka, (1998). DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. *J. Nat. Prod.*. **61**, 362-366.

[6] M. Qiang Ren, G. Kuhn, J. Wegner, J. Chen, (2001). Isoflavones, substances with multi-biological and clinical properties. European *J. Nutrition*. **40**, 135-146.

[7] L.Coward, N.C. Barnes, K.D.R. Setchell, S. Barnes (1993). Genistein, daidzein and their β-glycosides conjugates: anti-tumor isoflavones in soybean food from America and Asian diets. *J. Agricultural and Food Chem.* **41**,1961-1967.

[8] L. W. Lissin, J. P. Cooke (2000). Phytoestorgens and cardiovascular health. J. *American College of Cardiology*. **35**, 1403-1410.

[9] B. Thiem, (2003). *In vitro* propagation of isoflavone-producing *Pueraria lobata* (willd). Ohwi. *Plant Sci.* **165**,1123-1128.

[10] P.C. Santos-Gomes, R. M. Seabra, P. B. Andrade, M. M. Fernandes-Ferreira (2002). Phenolic antioxidant compounds produced by *in vitro* shoots of sage (*Salvia officinalis* L.). *Plant Sci*. **162**, 981-987.

[11] S. Murch, S.E. Peiris, C.Z. Liu, P.K.Saxena, (2004). *In vitro* conservation and propagation of medicinal plants. *Biodiversity*. **5**, 19-24.

[12] Z.G. Pan, C.Z. Liu, P.K. Saxena (2003). Efficient plant regeneration from protoplasts of Egyptian medicinal plants *Artemisia judaica* L and *Echinops spinosissimus* Turra. *Plant Sci*. **165**, 681-687.

[13] M. Faisal, M. Anis (2006). Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia*. *Bilogia Plant.***50**, 437-440

[14] T. Murashige, F. Skoog (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**, 475-479.

[15] S. He, C. Liu, P.K. Saxena, (2007). Plant regeneration of an endangered medicinal plant *Hydrastis Canadensis* L. *Sci. Hort.* **113**, 82-86.

[16] L. V. Hiregoudar, H. N. Murthy, B. P. Hema, E. J. Hahn, K. Y. Paek (2003). Multiple shoot induction and plant regeneration of *Feronia limonia* (L.) Swingle. *Sci. Hort.* **98**, 357-364.

[17] D. I. Ledbetter, J. E. Preece (2004). Thidiazuron stimulates adventitious shoot production from *Hydrangen quercifolia* Bartr. Leaf explants. *Sci. Hort.* **101**,121-126.

[18] C. A. Huetteman, J. E. Preece (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant cell Tiss. Org. Cult.* **33**, 105-119.

[19] T. D. Thomas, J. T. Puthur (2004). Thidiazuron induced high frequency shoot organogenesis in callus from *Kigelia pinnata* L. *Bot. Bull. Acad. Sin.* **45**, 307-313.

[20] S. Uranbey (2005). Thidiazuron induced adventitious shoot regeneration in *Hyoscyamus niger*. *Biol. Plant*. **49**, 427-430.

[21] A. Datta, P.S. Srivastava (1997). Variation in vinblastine production by *Catharanthus roseus* during *in vivo* and *in vitro* differentiation. *Phytochemistry.* **46**, 135-137.

[22] G. Pasqua, A. Pinarosa, B. Monacelli, A.R. Santamaria, M.P. Argentieri (2003). Metabolites in cell suspension cultures, calli and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. *Plant Sci*. **165**, 977-982.

[23] D. P. Fulzele, M.R. Heble, P. S. Rao (1995). Production of terpenoids from *Artemisia annua* L. plantlet cultures in bioreactor. *J. Biotechnology.* **40**, 139-143
[24] X.N. Liu, X. Q. Zhang, J.S. Sun (2007). Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hyperiicum perforatum*. *Plant Grow. Regul.* **53**, 207-214.