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ORIGINAL PAPER

Impact of nutrient components on production of the phytoestrogens daidzein and genistein by hairy roots of *Psoralea corylifolia*

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Abstract Transformed hairy roots of *Psoralea corylifolia* were established by infection with *Agrobacterium rhizogenes* LBA 9402. The aim of this work was to elucidate the effects of media constituents on production of the phytoestrogenic isoflavones daidzein and genistein. *A. rhizogenes* strain LBA 9402 harboring Ri plasmid was used to transform stem segments of in vitro seedlings. The resultant hairy roots were confirmed by polymerase chain reaction (PCR) and exhibited Ri T-DNA. Transformed hairy root clones were cultured in Murashige and Skoog's (MS) medium altered with different concentrations of NH_4^+ and NO_3^- and their growth and production of isoflavones were assessed. Biomass and productivity increased when MS medium was supplemented with NH_4^+ and NO_3^- at a ratio of 20:10. Increased yield of daidzein was obtained when sucrose level in the culture medium increased, whereas decreased level of sucrose favored genistein production. The hairy roots produced the highest levels of daidzein (2.06% dry wt.) and genistein (0.37% dry wt.) in the presence of low concentrations of PO_4^{3-}. Hairy roots secreted trace amounts of daidzein and genistein into the culture medium. The present results demonstrated that the productivity of daidzein was 2.2-fold more than that of untransformed roots.

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Introduction

Psoralea corylifolia Linn. (Fabaceae) is an herbaceous medicinal plant and source of the phytoestrogens daidzein and genistein [1]. Daidzein (4′,7-dihydroxyisoflavone) and genistein (4′,5,7-trihydroxyisoflavone) (Fig. 1) elicit estrogenic and anti-estrogenic properties useful in the prevention of menopausal ailments, and suppression of breast and ovary tumors caused by hormonal imbalance [2–4]. These molecules are also reported to be associated with lipid-lowering effects and ability to inhibit low-density lipoprotein [5]. The pharmaceutical industry has shown great interest in these important isoflavones derived from medicinal plants owing to their wide range of therapeutic properties [6, 7].

The inherent genetic stability of hairy roots is reflected in their stable productivity and thereby represents an attractive alternative for in vitro production of bioactive compounds and novel compounds that are not synthesized by plants. *Agrobacterium rhizogenes*-induced hairy root cultures have been investigated as a biological tool for production of valuable bioactive compounds because of genetic stability and thereby hold immense potential and exciting prospects for the pharmaceutical industry [8–10]. Hence, hairy roots have been projected as an alternative and attractive method for the production of several plant metabolites [11]. Attempts have also been made to promote hairy root growth and their production of important compounds such as alkaloids, essential oils, and biopesticides [12–14]. More importantly, hairy roots have potential to synthesize similar or even higher levels of secondary

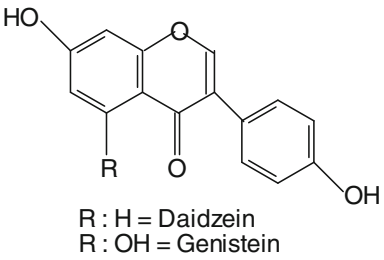

Fig. 1 Chemical structure of the phytoestrogenic isoflavones daidzein and genistein

metabolites compared with whole plants [15]. Further variations occurred in product accumulation among different hairy root clones which provides an opportunity to select elite clones with higher than average yield [13, 16].

Optimizing the media constituents for hairy root cultures is vital to gain high production of secondary metabolites. Various factors have been studied including pH, exogenous phytohormones, and carbon source and its concentrations; temperature and light are also known to influence the root growth and production of secondary metabolites [17–20]. Nutrient culture medium constituted with different concentrations of inorganic salts plays a key role in root growth and production of bioactive compounds by hairy root cultures [21]. Nitrogen is the major limiting nutrient for plant cell cultures, and production of bioactive compounds is dependent on availability of sufficient nitrogen in the culture medium. Lourenco et al. [22] demonstrated that appropriate ratios of NH_4^+ and NO_3^- in the medium are necessary for hairy roots, and concentrations in the culture medium affect root growth and their production of secondary metabolites. From various tissue culture studies it has been reported that productivity in hairy root cultures was stimulated by altering concentrations of media constituents such as NH_4^+, NO_3^-, PO_4^{3-}, and sugar [13, 23, 24]. Muranaka et al. [25] reported that modified Heller's medium supplemented with NO_3^-, phosphate, and potassium was needed for hairy root growth of *Duboisia leichardtii*. Herein, we have focused on selection of hairy root clones and optimization of NH_4^+ to NO_3^- ratio and PO_4^{3-} concentration in the culture medium to enhanced the production of isoflavones by hairy root cultures of *P. corylifolia*.

Materials and methods

Plant materials

Seeds were obtained from *Psoralea corylifolia* plants grown in the Botanical Garden, Department of Botany, University of Pune, Pune, India. Plant specimen was authenticated by the Botanical Survey of India (BSI) and deposited in the university's herbarium, Pune, India. Seeds were scarified by immersion in concentrated sulfuric acid for 1 h followed by washing under running tap water. Seeds were disinfected with $HgCl_2$ (0.05% w/v) solution for 2 min and subsequently washed 5–6 times with sterile distilled water. Surface-sterilized seeds were cultured on half-strength Murashige and Skoog's (MS) medium [26] for germination. The pH of the medium was adjusted to 5.8 by adding 0.1 N NaOH or 0.1 N HCl prior to addition of 0.8% agar. Culture medium was autoclaved at 103.42 kPa for 20 min. Cultures were kept on photo-simulation tissue culture racks (Medipla Biotech, Navi Mumbai, India) under 16-h photoperiod (40 μmol m^{-2} s^{-1}, cool white fluorescent tubes, Philips, Holland) at 25 ± 1°C.

Agrobacterium rhizogenes cultures and hairy roots induction

Agrobacterium rhizogenes strain LBA 9402 was grown on solid YMB medium [27] supplemented with 50 mg/l rifampicin at 28°C for 48 h. Three-week-old stem portion of in vitro seedling was used for infection. Infected stem fragments were placed on the MS medium under similar culture conditions. After 2 days, shoot segments were placed on MS medium supplemented with 500 mg/l filter-sterilized cefotaxime. Hairy roots originating from the wound sites were carefully excised and cultured on fresh MS medium with reduced concentrations of cefotaxime (250–100 mg/l) till complete eradication of bacteria. Hairy root clones maintained on growth regulator free MS medium containing 30 g/l sucrose. Untransformed root cultures were initiated from in vitro seedlings and maintained on growth regulator free MS medium.

Media constituents

Addition of NH_4Cl was used to replace NH_4NO_3 along with KNO_3 to provide adequate concentrations of ammonia and nitrate as a nitrogen source. With regard to the study of nitrogen source MS medium was constituted by addition of different concentrations of ammonia (NH_4^+) and nitrate (NO_3^-). The studied ratios of NH_4^+ to NO_3^- were 0:20, 10:20, 40:20, 60:20, 20:10, 20:40, 20:60, 20:0, and 20:20 mM, the last of these as a control. Similarly, optimization of phosphate levels was studied with addition of KH_2PO_4 at different concentrations (0.625–5 mM) in MS medium. Hairy roots were also cultured on MS medium supplemented with different concentrations of sucrose (10–50 g/l).

Growth measurement

At the end of 21 days of cultivation, hairy root clones were harvested to determine the growth. A total of nine hairy

root clones were established and selected for their growth. Approximately 150 mg/50 ml of hairy roots used as inoculum of each clone into the MS medium and determined the fresh and dry weights (wt.). The growth index (GI) was calculated by using the formula $GI = W_f - W_i$, where W_f is a fresh wt. of a culture after harvest and W_i is the fresh wt. of inoculum. The doubling time (T_D) of each culture was calculated by using the following formula:

$$T_D = \ln 2(t_1 - t_0)/\ln(GI)$$

where $t_1 - t_0$ corresponds to period between inoculation and the stationary phase. Hairy roots were harvested on every fifth day to determine fresh wt. and dry wt., and details have been described elsewhere [28]. All experiments were replicated three times; four Erlenmeyer flasks were used for each treatment.

Confirmation of transformation

Total genomic DNA of hairy roots of *P. corylifolia* was isolated by the method described elsewhere [29]. Plasmid DNA from *A. rhizogenes* strain LBA 9402 was extracted by alkaline lysis [30]. Polymerase chain reaction (PCR) amplifications of the isolated DNA samples were carried out by using primers specific for the ORF-13 coding sequence of T_L DNA and *mas*1′ sequence of T_R DNA of pRiLBA9402. The primers used for amplification of the ORF-13 coding sequence were (+) 5′CAG CTT CTA AAT GTG GAG GCC and (−) 5′CCT TGC CGA TTG CCA GTA TGG C. These primers amplified a 498-bp fragment [31]. For amplification of *mas*1′ sequence, primers used were (+) 5′CGG TCT AAA TGA AAC CGG ACG and (−) 5′GGC AGA TGT CTA TCG CTC GCA CTC C which defined the 970-bp domain [32].

DNA amplification was performed on an Eppendorf thermal cycler. Each reaction mixture (25 µl) consists of 50 ng of plant genomic DNA (or 25 ng of plasmid DNA), 2.5 µl of 10× *Taq* DNA polymerase buffer, 50 µM each of dNTPs, 0.2 µM primers, and 0.5 U *Taq* polymerase. The amplification program involves an initial denaturation step at 94°C for 2 min followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1.5 min at 72°C with a final extension of 7 min. PCR products were examined by electrophoresis on 1.5% (w/v) agarose gel.

Extraction of phytoestrogenic isoflavones

Harvested biomass was dried in an oven at 55°C for 16 h and powdered by using a Wiely Mill, followed by extraction with 3 M H_2SO_4 and sonication (33 kHz) for 10 min. Subsequently, the extract was kept in a water bath at 100°C for 60 min; finally an equal volume of distilled ethanol was added to the sample. Samples were vortexed for 2 min and centrifuged at 12000×*g* for 15 min. The supernatant was transferred to clean glass vials and directly analyzed by HPLC.

The spent medium was filtered and extracted with chloroform three times. The chloroform fraction was dried (Na_2SO_4), filtered (Whatman no. 1), and subsequently evaporated on a BÜCHI Rotavapor (model no. 111, Switzerland) at 45°C to obtain a concentrated residue and stored at 4°C prior to quantification by HPLC.

Quantification of phytoestrogenic isoflavones daidzein and genistein by HPLC

The HPLC was performed on a 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). Separations were performed on Inertsil C_{18} (250 × 4.6 mm 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. Data collection and integration were accomplished by using BORWIN software (Japan). This method is sensitive and accurate with good reproducibility. The results of the five injections from the same samples at the five concentrations (0.01–0.5 µg) showed similar retention time. Peak identification was carried out by using authentic samples of daidzein and genistein (Sigma, USA).

Results

Establishment of hairy root clones

Hairy roots were successfully induced by infection with *A. rhizogenes* LBA9402 to stem in vitro seedlings of *P. corylifolia*. Hairy roots emerged from the infected sites within 12–15 days with 30% transformation frequency and maintained on growth regulator free MS medium. Among 25 hairy root clones only 9 clones were selected on the basis of their growth rate. The selected transformed clones showed rapid growth and tendency of profuse branching and active elongation, whilst untransformed roots did not show similar growth, elongation, or branching pattern.

Molecular analysis

Hairy roots were analyzed by PCR for confirmation of integration of T-DNA in the plant genome. PCR amplification with specific primers was used to show the presence of T_R-DNA of the Ri plasmid in the genomic DNA of hairy roots of *P. corylifolia*. DNA isolated from hairy roots

induced by LBA 9402 when amplified with primers specific for ORF 13 and *mas*1′ sequence showed the expected fragments. PCR analysis confirms the successful integration of T-DNA in the genome of transformed roots, while DNA from untransformed roots (used as control) did not show any amplification.

Growth and daidzein and genistein production by selected hairy root clones

Hairy roots transferred into growth regulator free MS medium gave an initial biomass to liquid ratio of 0.003 g/ml fresh wt. At the end of 21 days of cultivation, hairy root clones were harvested to determine their growth and isoflavones productivity. Figure 3 shows the ratio of doubling time and productivity in selected hairy root clones (PC1–PC9). Untransformed roots transferred into MS medium devoid of growth regulator gave an initial biomass to liquid ratio of 0.003 g/ml fresh wt. and were used as control. Hairy root clones PC1–PC9 attained maximum biomass on day 21 and also showed rapid doubling time compared with untransformed roots. Comparing the doubling time of transformed hairy roots and untransformed roots, the hairy root clone PC9 showed rapid growth with minimum doubling time (T_D 3.3 days), whilst untransformed roots showed relatively slow growth and maximum doubling time (T_D 11 days).

Harvested hairy root clones showed variation in the contents of isoflavones. The results obtained from HPLC analysis showed that the hairy root clones (PC4, PC5, PC7, PC8, and PC9) produced significantly higher levels of phytoestrogenic isoflavones compared with the untransformed roots (Fig. 2). Hairy root clone PC9 produced a significantly large amount of daidzein (1.45% dry wt.) on day 21. Overall the high-yield clones (PC9) accumulated approximately 2.2-fold more daidzein than that of untransformed roots. From Fig. 2 one can clearly see that PC9 hairy root clones accumulated the highest amount of isoflavones of all the established hairy root clones. In addition, PC9 clones showed minimum doubling time and rapid growth. Therefore, PC9 clones selected as the best hairy roots, and consequently further studies of the effect of nutrient components were performed with PC9 hairy root clones for growth and isoflavones production at different growth periods.

Effect of sucrose on root growth and production of phytoestrogenic isoflavones

Addition of different sucrose concentrations (10–50 g/l) into the growth regulator free MS medium affected the root growth and production of isoflavones. Figure 3 represents the kinetic study of hairy roots growth related to various

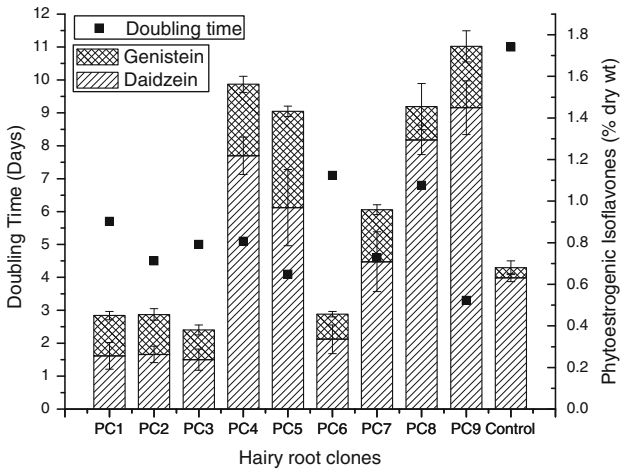

Fig. 2 Quantification of daidzein and genistein in selected hairy root clones of *P. corylifolia* versus doubling time (*PC1–9* selected hairy root clones, *Control* untransformed roots). Results are the mean of five replicates ± SD

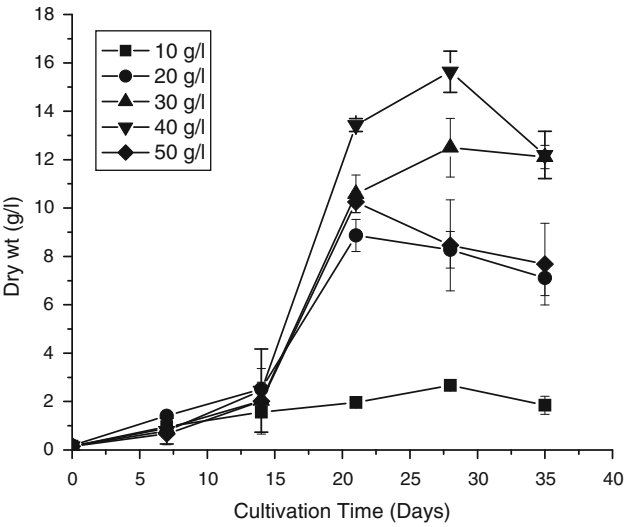

Fig. 3 Time course of influence of sucrose concentrations on growth of hairy root clone (PC9) *P. corylifolia.* Results are the mean of three replicates ± SD

sucrose concentrations. In comparison with different concentrations of sucrose, 40 g/l sucrose enhanced the growth rate and afforded the highest dry wt. of 15.6 g/l on day 28. A decreased concentration of sucrose (10 g/l) resulted in a long lag phase and inhibited growth of hairy roots.

Figure 4 shows the time course of hairy roots that started production of isoflavones after 7 days of cultivation in the presence of different concentrations of sucrose (10–50 g/l) in MS medium. The presence of 40 g/l sucrose in the culture medium afforded maximum levels of daidzein (2.08% dry wt.) in hairy roots (Fig. 4). At the same time, the HPLC profile revealed that medium containing 20 g/l sucrose favored the maximum production of genistein

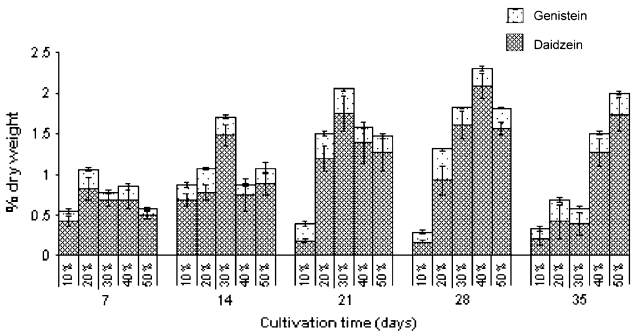

Fig. 4 Time course of influence of sucrose concentrations on daidzein and genistein production by hairy root clone (PC9) *P. corylifolia.* Results are the mean of three replicates ± SD

(0.37% dry wt.) (Fig. 4). The presence of higher concentrations of sucrose (50 g/l) in the culture medium prolongs the production of isoflavones, and the hairy roots accumulated isoflavones even after 35 days of cultivation. This shows that sucrose exists in the culture medium that supports the steady increase of productivity even on day 35. Medium containing minimum sucrose (10 g/l) inhibited the production of target compounds. HPLC profiles showed trace amounts of isoflavones secreted by hairy roots into the culture medium.

Effect of NH_4^+ to NO_3^- ratio on root growth and production of phytoestrogenic isoflavones

Nitrogen was supplied in the form of different concentrations of nitrate and ammonium ions in MS medium. Table 1 shows the different ratios of NH_4^+ to NO_3^- in MS medium used and their effects on root growth and accumulation of isoflavones. MS medium supplemented with a 20:10 mM ratio of NH_4^+ to NO_3^- produced the maximum biomass, 13.86 g/l dry wt., which is relatively higher than that achieved with the similar proportion (20:20 mM) in culture medium. Medium devoid of either NH_4^+ and NO_3^- and high concentrations (60 mM) slightly affected the growth of hairy roots.

Hairy roots were grown in the presence of various ratios of NH_4^+ to NO_3^- as a source of nitrogen that influenced the accumulation levels of target compounds. Hairy roots cultured in MS medium supplemented with a 20:10 mM ratio of NH_4^+ to NO_3^- gave the highest isoflavones productivity of 2.05% dry wt. of daidzein and 0.51% dry wt. of genistein. In contrast, the productivity declined by approximately 1.5-fold when the NH_4^+ and NO_3^- ratio was altered to 10:20 mM in MS medium (Table 1). Therefore, adequate ammonia is an important factor for enhancement of isoflavones productivity. The present results show that high concentrations of ammonia or nitrate in a ratio of 40:20 and 60:20 or 20:40 and 20:60 mM in the culture medium decreased the accumulation of phytoestrogenic isoflavones.

Effect of phosphate on root growth and production of phytoestrogenic isoflavones

Hairy root clones (approximately 150 mg fresh wt.) were transferred to MS medium fortified with different concentrations of phosphate (0.625–5 mM) and cultivated for 21 days. Figure 5 shows the resultant root growth and production of isoflavones. Hairy roots were harvested on day 21 and we found that all concentrations of phosphate were suitable for growth, but it was impaired in the absence of phosphate in the culture medium. Increased phosphate concentrations increased the growth rate and afforded 13.59 g/l dry wt. biomass on day 21. This indicated that phosphate could be an important nutrient constituent for hairy roots growth. In contrast, the HPLC profile and growth study demonstrated that decreased levels of phosphate in the culture medium increased the accumulation of isoflavones by hairy roots. Hairy roots cultured in MS medium containing a low concentration of phosphate (0.625 mM) produced volumetric yield of daidzein (2.06% dry wt.) and genistein (0.37% dry wt.). Spent medium did not contain isoflavones.

Discussion

Hairy roots induced by *A. rhizogenes* provide an alternative system for the production of secondary metabolites because of their higher degree of genetic and biochemical stability. Recently, various valuable bioactive compounds have been produced by hairy root cultures [33, 34]. Our experimental data showed that substantial variation in isoflavones accumulation was recorded in different hairy root clones. Variation in root growth and isoflavones productivity could be attributed to the phenomenon of transformation such as copy number, size, and chromosomal location of T-DNA fragment integrated in the plant genome [35]. Shen et al. [36] demonstrated that the variation in growth and morphological traits could also be related to difference in expression of *rol* genes, which alter the endogenous auxin level among hairy root clones. Our present results demonstrated that hairy root clone (PC9) initiated from *P. corylifolia* accumulated higher levels of daidzein (1.45% dry wt.) than hairy root clones from other species of *Psoralea* [16]. This indicated that the genotypic variation could be responsible for the difference in accumulation of daidzein by different *Psoralea* species. The higher levels of isoflavones detected in hairy roots of *P. corylifolia* compared with the other species of *Psoralea*

Table 1 Effect of NH_4^+ and NO_3^- ratio on root growth and production of isoflavones by hairy root cultures of *Psoralea corylifolia*

NH_4^+ to NO_3^- ratio (mM)	Growth (g/l dry wt.)	Daidzein (% dry wt.)	Genistein (% dry wt.)
0:20	7.088 ± 0.425	1.530 ± 0.063	0.093 ± 0.010
10:20	8.120 ± 0.880	1.359 ± 0.135	0.214 ± 0.038
20:20	10.583 ± 0.779	1.567 ± 0.207	0.222 ± 0.092
40:20	12.915 ± 0.679	1.359 ± 0.135	0.214 ± 0.038
60:20	8.470 ± 1.140	0.774 ± 0.145	0.070 ± 0.092
20:0	6.482 ± 0.541	1.375 ± 0.104	0.086 ± 0.009
20:10	13.860 ± 0.880	2.055 ± 0.254	0.513 ± 0.044
20:40 (MS)	11.750 ± 0.489	1.162 ± 0.167	0.327 ± 0.014
20:60	8.670 ± 0.936	0.973 ± 0.089	0.059 ± 0.024

Results are the mean of three replicates ± SD

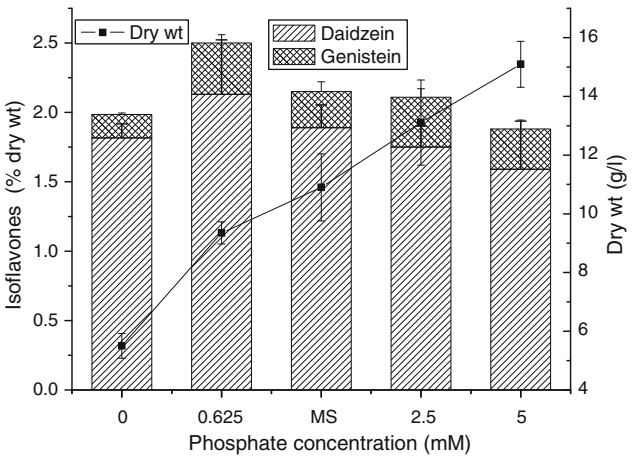

Fig. 5 Influence of phosphate concentrations on root growth and phytoestrogenic isoflavones production by hairy root clone (PC9) *P. corylifolia*. Results are the mean of three replicates ± SD

highlight the potential of our root clones for the production of isoflavones daidzein and genistein.

Sucrose in the medium is energetically the most favorable source of carbon for cultivation of plant cell cultures and mainly for the biosynthesis of secondary metabolites. A stimulatory effect of sucrose was reported on biomass accumulation in hairy root cultures of *Centaurea calcitrapa* [22]. Similar kinds of response to the varying concentration of sucrose in the production of thiophene and steroidal alkaloids by hairy root cultures of *Tagetes laxa* and *Solanum aviculare*, respectively, have been reported [13, 37]. Sucrose concentrations too low or high affected the root growth and production of isoflavones by hairy root cultures of *P. corylifolia*.

Hairy roots are sensitive to the combinations and concentrations of medium constituents (especially NH_4^+, NO_3^-, and phosphate and carbon sources) that stimulate the root growth and production of secondary metabolites. Nitrogen, the most important element for growth of plant cell cultures, is supplied in the form of nitrate and ammonium salts. Several studies have been carried out either by varying the individual components of medium or by using medium having different ionic strength to influence the production levels [14, 38]. In the case of hairy roots of *P. corylifolia*, minimum concentrations of NO_3^- effectively enhanced the root growth and consequently increased production of isoflavones. Similarly, low concentrations of NO_3^- in the medium were reported to improve the production of secondary metabolites by hairy root cultures of *Centaurea calcitrapa* and *Atropa belldona* [22, 39]. On the contrary, reports also demonstrated that the presence of ammonia salts decreased the production of secondary metabolites by hairy root cultures *Astragalus mongholicus* [40]. In addition, hairy root cultures are more sensitive to higher concentrations of ammonia, since high levels of ammonia salts impaired the cell growth because of toxicity and repressive effects on nitrate assimilation [41]. Inorganic salt components such as NO_3^- supported both the root growth and catharanthine production by hairy roots of *Catharanthus roses* with incremental increases in concentration, whereas NH_4^+ yielded contradictory effects with respect to root growth and catharanthine production [42]. This indicated that the clonal variation in hairy roots and nutrient constituents (the ratio of NH_4^+ and NO_3^-) in the culture medium were responsible for differences in the production of isoflavones.

Pavlov et al. [19] reported that manipulation of phosphate levels in culture media could be used to stimulate the synthesis of secondary metabolites and increase culture growth. Those authors demonstrated that root growth and production of betalains by hairy root cultures of *Beta vulgaris* correlated with lower concentration of phosphate in the medium. In accordance with these earlier observations, our results showed that reduced levels of phosphates in the culture medium increased the root growth and isoflavones production by hairy root cultures of *P. corylifolia*. This indicated that the phosphate level in the culture medium correlated with production of target compounds. It was interesting to note that Taya et al. [43] reported that betalains production increased by fourfold when hairy roots of

B. vulgaris were cultured in medium devoid of phosphate. Similarly, hairy roots of *P. corylifolia* produced isoflavones in the absence of phosphate in medium but impaired the growth.

In order to assess the possibility of producing the useful compounds daidzein and genistein by using *P. corylifolia* hairy roots, several nutrient constituents were evaluated. The selected hairy root clones showed markedly improved productivity. The presence of optimum concentrations of sucrose, NH_4^+, and NO_3^- in the culture medium afforded incremental differences in the yield of daidzein and genistein. Nevertheless, adequate ammonia in the medium promotes the productivity. A low concentration of phosphate in the medium gave the highest yield of isoflavones. Selection of elite clones with higher than average product yield and coupled with optimized media constituents could help to establish a system for the production of the isoflavones daidzein and genistein.

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