Increased puerarin biosynthesis during *in vitro* shoot formation in *Pueraria tuberosa* grown in growtek bioreactor with aeration

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Abstract Puerarin accumulation during shoot cultures in static and liquid medium with or without aeration is described in *Pueraria tuberosa* (Roxb. ex Willd.) DC. Maximum shoot induction from nodal explants was achieved on Murashige and Skoog's medium supplemented with 1.13 μM TDZ and 0.25 μM IBA after 4 weeks of growth. Puerarin content was higher in shoot cultures grown in liquid medium as compared to static medium. When shoots were grown in growtek bioreactor with different aeration volume, maximum puerarin content (1484 $\mu\text{g/g}$ DW) was recorded with 20 % v/v aeration which was ~ 2.3 fold higher than puerarin content recorded in control cultures (cultures grown in growtek without aeration). Aeration requirement for organized (shoot) cultures was different for growth and puerarin accumulation.

Keywords Puerarin · *Pueraria tuberosa* · Secondary metabolites · Growtek

Introduction

Pueraria tuberosa (Indian Kudzu, family Leguminosae) is a perennial woody climber. It is geographically distributed in India, Nepal and Pakistan. Some of the isoflavonoids present in the *P. tuberosa* are puerarin, daidzein, genistein and genistin (Goyal and Ramawat 2007). Among these

isoflavonoids, puerarin (7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β -D-glucopyranoside) is the major isoflavonoids present in root tubers of Indian Kudzu. *Pueraria* species are popular Chinese herbal medicine with antioxidative and antithrombotic effects. It has been reported that puerarin exhibits effective role in the treatment of hypertension, arteriosclerosis, diabetes mellitus and metabolic syndrome (Luo et al. 2010; Teng et al. 2009; Zhu et al. 2010). Some reports suggested that puerarin can reduce the cellular melanin content by significantly inhibiting cellular tyrosinase activity *in vitro* (Choi et al. 2010). Establishment of these wide-ranging biological properties of puerarin leads to increased market demand.

In vitro cultures provide a potential alternative to the mass harvesting of plants for the purpose of obtaining crude drug extracts. Wide-spread harvesting of *P. tuberosa* for its tubers restricts its reproduction and regeneration and thereby made it a threatened species (Rathore and Shekhawat 2009). *In vitro* culture protocols can be adapted to produce the compounds of interest which are simpler to isolate than from *in vivo* plants. Greater product yield in a short period of time is possible with *in vitro* cultures, relative to cultivated plants, as they can be oriented to secondary product accumulation rather than growth (Reppert et al. 2008). Organized cultures can produce secondary metabolite either in higher amounts or compounds specific to differentiation as demonstrated in shoot cultures of *Catharanthus roseus* and *Ruta graveolens*, which produced vincristine and platydesminium in organized cultures only (Ramawat 2007). The organ cultures are relatively more stable in production of secondary metabolites than undifferentiated callus and cell cultures (Cui et al. 2010; Shilpa et al. 2010).

Bioreactor design is an integral part of biotechnology and each design tries to make conditions more favorable for

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Fig. 1 *In vitro* shoot multiplication in *P. tuberosa*. **a** Germinated seedling on MS+BAP 4.43 μ M and IAA 1.43 μ M, **b–c** Shoot regeneration from nodal explant, **d** Elongated shoots on MS+TDZ 1.13 μ M and IBA 0.25 μ M, **e** Shoot regeneration in growtek bioreactor supplied with 20 % aeration

production of different metabolites (Bhanja et al. 2007). In bioreactor, aeration volume is the most important factor because it is correlated with agitation and dissolved oxygen in the media, which regulates the biomass and secondary metabolite production (Ahmed et al. 2008; Senoussi et al. 2009). Our previous investigations of *P. tuberosa* involve callus and cell suspension cultures for the production of isoflavonoids (Goyal and Ramawat 2008a, b; Sharma et al. 2009) and *in vivo* analysis (Vaishnav et al. 2006). A recent report (Rathore and Shekhawat 2009) described method of micropropagation and puerarin content in *P. tuberosa*. The

present work demonstrates increased puerarin biosynthesis during shoot formation in *P. tuberosa* grown in static medium and in liquid medium using growtek bioreactor with aeration.

Material and method

Plant material

The mature pods of *P. tuberosa* were procured from Sajjangarh sanctuary near Udaipur, Rajasthan (India). The

Table 1 Effect of different concentrations of TDZ and IBA on shoot induction of *P. tuberosa* after 4 weeks of growth

TDZ (μ M)	IBA (μ M)	No. of shoots/explant (Mean $\pm$ SD)	Length of shoots (cm) (Mean $\pm$ SD)
0	0	0	0
0.45	0.25	2.3 $\pm$ 0.08 ^{de}	1.2 $\pm$ 0.20 ^{cd}
	0.50	1.6 $\pm$ 0.33 ^f	1.0 $\pm$ 0.31 ^d
1.13	0.25	4.0 $\pm$ 0 ^a	3.8 $\pm$ 0.28 ^a
	0.50	3.6 $\pm$ 0.30 ^{bc}	3.6 $\pm$ 0.43 ^a
2.27	0.25	3.9 $\pm$ 0.17 ^{ab}	2.7 $\pm$ 0.30 ^b
	0.50	3.5 $\pm$ 0.50 ^c	2.4 $\pm$ 0.29 ^b
4.54	0.25	2.4 $\pm$ 0.20 ^d	1.6 $\pm$ 0.50 ^c
	0.50	2.0 $\pm$ 0.37 ^e	1.4 $\pm$ 0.37 ^c

Means with common letter are not significantly different at $P \leq 0.05$, according to least significant (LSD) test

Fig. 2 Effect of static and liquid medium on puerarin (isoflavonoids) accumulation in shoot culture of *P. tuberosa* after 4 weeks of growth

healthy seeds were isolated mechanically from the pods with the help of cutter and washed under running tap water for 20 min to remove any adherent particles. Thoroughly washed seeds were then immersed in 5 % (v/v) teepol, a liquid detergent for 5 min, surface sterilized with 5 % (v/v) sodium hypochlorite for 10 min and finally rinsed five times with sterilized double distilled water to remove all traces of sterilant. The surface disinfected seeds were inoculated aseptically on to MS (Murashige and Skoog 1962) medium supplemented with benzyladenine (BA) 4.43 μM with indole acetic acid (IAA) 1.43 μM , 3 % (w/v) sucrose and 0.8 % (w/v) agar, pH 5.8 and autoclaved at 121 °C for 20 min. These cultures were incubated at 25 ± 0.2 °C under a 16 h d^{-1} photoperiod with 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India) with 60–65 % relative humidity. The seeds germinated within 12–15 days after inoculation.

Shoot induction

To study the shoot induction in *P. tuberosa*, nodal segments (~1.5 cm) from seedlings were cultured on MS medium with different concentrations of Thidiazuron (TDZ, 0.45, 1.13, 2.27, 4.54 μM) and indole butyric acid (IBA, 0.25 to 0.50 μM) and hormone free medium (control). Each 250 ml

flask contained 100 ml gelled medium and five explants, and six replicate cultures were maintained for each treatment. After 4 weeks of culture, number of shoots per nodal explants and length of shoots were recorded. The optimal combination was used for aeration experiment using liquid medium. Before culturing shoots in liquid medium, various combination of BAP and IAA, 2iP and IBA, Kinetin and NAA were also tried.

Production of isoflavonoids was determined in all the cultures grown in static or agitated medium. When liquid medium was used in growtek bioreactor, cultures were grown with or without aeration. Each 250 ml flask contained 100 ml medium while growtek bioreactor (1 L) contained 400 ml medium.

Aeration treatment

Growtek bioreactor (Tarson, India) is designed to support cultures on a raft in liquid medium (400 ml) and aeration was provided by inserting a needle through septum in the side tube of bioreactor. The details of design is described elsewhere (Bhanja et al. 2007). The air outlet was constructed on the lid of bioreactor through PTFE filter (Sartorius® midstar® 2000, 0.2 μm). Growtek bioreactor contained MS medium with TDZ, 1.13 μM and IBA 0.25 μM and was inoculated with 12 explants. Different aeration volume (10 %, 20 %, and 30 % v/v) was given during the growth of cultures. The shoots developed, were harvested at 4 weeks growth.

Extraction and analysis of isoflavonoids

Shoots were dried at 50 °C for 16 h and ground until powder. Dried powder samples (200 mg) were extracted with 5 ml methanol for 12 h on a test tube rotator, centrifuged at 2000 rpm for 10 min and then the supernatant was collected and evaporated by speed-vac sample concentrator (model SPD 111 V, Thermo Savant, USA). For HPLC analysis, all the extracts were redissolved in HPLC grade methanol, filtered through nylon filter

Table 2 Effect of different aeration volume on the growth and puerarin accumulation in *P. tuberosa* shoots grown in growtek bioreactor (400 ml medium) containing MS medium with 1.13 μM TDZ and 0.25 μM IBA

Aeration volume	No. of shoots/explant	Length of shoots (cm)	Isoflavonoid content $\mu\text{g g}^{-1} \text{DW}$				Total Isoflavonoid
			Puerarin	Genistin	Daidzein	Genistein	
Control	4.6 $\pm$ 0.54 ^a	3.8 $\pm$ 0.43 ^a	643 $\pm$ 18 ^d	104 $\pm$ 13 ^d	36.7 $\pm$ 1.8 ^d	32.6 $\pm$ 3.0 ^a	817 $\pm$ 35 ^d
10 % v/v	4.8 $\pm$ 0.44 ^a	4.0 $\pm$ 0.30 ^a	1106 $\pm$ 38 ^b	238 $\pm$ 15 ^b	77.3 $\pm$ 3.5 ^b	24.1 $\pm$ 0.5 ^{bc}	1445 $\pm$ 57 ^b
20 % v/v	5.0 $\pm$ 0 ^a	4.2 $\pm$ 0.44 ^a	1484 $\pm$ 47 ^a	251 $\pm$ 12 ^a	126 $\pm$ 7.2 ^a	22.0 $\pm$ 0.9 ^c	1883 $\pm$ 67 ^a
30 % v/v	Callus	–	936 $\pm$ 13 ^c	155 $\pm$ 8.0 ^c	63.3 $\pm$ 4.2 ^c	27.2 $\pm$ 1.2 ^b	1181 $\pm$ 26 ^c

Means with common letter are not significantly different at $P \leq 0.05$, according to least significant (LSD) test

Fig. 3 Shoot culture in a growtek bioreactor

(0.45 μM , National, USA). The HPLC system programmed to inject 20 μl samples per injection. The HPLC system used for the separation of compounds was equipped with a pump (model L2130; Hitachi, Tokyo, Japan), auto sampler (model L-2200; Hitachi) and a UV detector (L-2400; Hitachi) controlled with “Lachrome Elite” software. The separation was performed on a 250 $\times$ 4 mm C_{18} , 5 μm , reverse-phase column (LichroCART; Merck KGaA, Darmstadt, Germany) protected by a guard column of the same material. The HPLC analysis was performed with little modifications, as described earlier (Goyal and Ramawat 2008a, b). The solvent system used was: solvent A, 0.0025 % trifluoroacetic acid in water and solvent B, 80 % acetonitrile (E. Merck, Mumbai, India) in solvent A. The mobile phase consisted of solvent A and B. The step-gradient programme of solvent A was as follows: 0–2 min, 85 %; 2–5 min, 85 %–80 %; 5–15 min, 80 %–50 %; 15–20 min, 50 %–40 %; 20–30 min, 40 %–30 %; 30–35 min, 30 %–20 %; 35–45 min, 20 %–0 %; 45–48 min, 0 %; 48–50 min, 0 %–85 %; 50–55 min, 85 %. The separation was performed at a flow rate of 1.0 ml min^{-1} , and chromatographic peaks were monitored at 254 nm.

Statistical analysis

All results were averaged over three separate analyses from three flasks and each experiment was done in duplicate. Results were reported as the mean $\pm$ standard deviation (SD) and analyzed by the least significant difference (LSD) test at $P \leq 0.05$ using prism statistical software.

Results and discussion

Fresh seed of *P. tuberosa* germinated readily on to MS medium supplemented with BAP 4.43 μM and IAA 1.43 μM (Fig. 1a). On other combinations of PGRs, poor seed germination was observed (Data not shown). Time period of <4 weeks resulted in poor growth and low accumulation of puerarin while time period of >5 weeks resulted in necrosis of shoots without further increase in puerarin content. Hence, time period of 4 weeks was selected for subculture.

Effect of plant growth regulators

Shoot induction by various concentrations of TDZ along with IBA incorporated in MS medium is presented in Table 1. Shoot induction and multiplication was observed on almost all the media used, which proliferated further on transfer to the medium of the same composition (Fig. 1b, c). No shoots were observed in the explants grown on the control medium. Explants also produced callus which could not be differentiated on any of the medium or maintained separately. Maximum number of elongated shoots (4 in numbers, 3.8 cm long) was recorded on the medium supplemented with 1.13 μM TDZ and 0.25 μM IBA. These shoots were gregarious in habit (Fig. 1d). Increased concentration of IBA in the medium was inhibitory to shoot formation with same concentration of TDZ. Effect of TDZ was also reported for shoot regeneration and isoflavonoid production in *Psoralea corylifolia* (Shinde et al. 2009).

Fig. 4 HPLC profile of shoot culture of *P. tuberosa* grown in growtek bioreactor showing isoflavonoids with major peak of puerarin and its structure

Besides this, the other media tried for shoot induction using liquid medium resulted in callus formation.

About four shoots per explant were produced in liquid and static cultures. Puerarin and other isoflavonoids accumulation in shoot cultures grown on static and in liquid medium is presented in Fig. 2, which shows that shoots grown in liquid medium accumulated high amount of puerarin (761.08 $\mu\text{g/g}$ DW) and genistin as compared to shoots grown on static medium. Daidzein and genistein content were higher in static shoot cultures (Fig. 2).

Effect of aeration

The results obtained with aeration volume (10 %, 20 %, and 30 % v/v) for healthy shoot induction and enhanced puerarin production are presented in Table 2. Increase in aeration volume up to 20 % (Fig. 1e) was associated with increase in number of shoots per explant. However, further increase in aeration beyond 20 % v/v supported callus growth. With 20 % v/v aeration, 5 shoots per explants were produced with maximum puerarin (1484 $\mu\text{g/g}$ DW) and other Isoflavonoids (Fig. 3). This puerarin content was ~2.3 fold higher than that recorded in control shoots cultures (cultures grown in growtek without aeration).

Present work showed advantage of liquid culture over the static cultures in shoot formation and puerarin accumulation. The bioreactor grown shoots accumulated ~1.9 mg/g DM total isoflavonoids and ~1.5 mg/g DM of puerarin, which was ~2 fold higher than the previous report (Rathore and Shekhawat 2009). The content of different plant parts reported by Rathore and Shekhawat (2009) appears to be low as compared to our earlier report (roots 3.57 mg/g DM, tuber 6.46 mg/g DM, Vaishnav et al. 2006). Further, they did not present any data about peak purity and validation of analytical method which might have further lowered the value. In bioreactors, aeration volume is one of the most important components affecting the cultures growth and productivity. High cell growth and secondary metabolites accumulation was recorded in *Morinda citrifolia* cultures grown in a bioreactor supplied with 10 % and 30 % v/v aeration, respectively (Ahmed et al. 2008). Aeration volume of 30 % was found beneficial for anticancer lignans production and syringin production in *Linum album* (Baldi et al. 2008) and *Saussurea medusa* (Xu et al. 2008) cell cultures, respectively. However, in the shoot cultures of *P. tuberosa*, maximum shoots and puerarin accumulation was recorded in the cultures supplied with 20 % v/v aeration as also evident by HPLC profile (Fig. 4). This might be due to differentiated nature of the cultures as compared to cell cultures. Such work has not been reported in differentiated cultures.

In present work increased puerarin, genistin and daidzein production was recorded with aeration while the production of genistein declined with increased aeration, which was

found higher in the cultures grown on static medium. Similar to *M. citrifolia* (Ahmed et al. 2008), higher biomass was also obtained in *Scopolia parviflora* with high aeration volume, and alkaloid content increased with low aeration (Min et al. 2007). Aeration is not always beneficial for high secondary metabolites accumulation but sometimes it depends on the condition of culture stage and selected explants source (Ahmed et al. 2008; Senoussi et al. 2009). In present work shoot cultures have been used for the accumulation of puerarin and high puerarin content was associated with increased aeration up to 20 %. In cell cultures, high aeration was associated with decline of total anthraquinones (Ahmed et al. 2008). However, in some cases high aeration improves oxygen transfer, a process that improves growth and secondary metabolites accumulation (Zhong et al. 1993). It may be concluded from the present work that organized (shoot) cultures have different aeration regimen for the growth and natural products accumulation, which requires further work.

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