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Isoflavonoids production in callus culture of *Pueraria tuberosa*, the Indian kudzu

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Isoflavonoid contents of different plant parts and callus tissues of the Indian Kudzu, *Pueraria tuberosa* (Roxb.ex.Willd.) DC are presented. The initial cultures were slow growing, associated with browning of the tissues. The production of four isoflavonoids (puerarin, genistin, genistein and daidzein) in the callus cultures of *P. tuberosa* was studied by manipulating the plant growth regulators and sucrose concentration in the medium. Organogenesis was not recorded in callus on any of these treatments. Tuber and stem accumulated puerarin, a glycoside of daidzein, at high amounts, 0.65% and 0.054% respectively. However, the daidzein content of the callus tissues grown on Murashige and Skoog medium containing BA (20.9 μ*M*) and sucrose (60 gl^{-1}) was significantly higher (0.056%) than *in vivo* plant material (0.02%) and other comparable culture systems like *Genista* and *Pueraria lobata*.

Keywords: Callus culture, Isoflavonoids production, *Pueraria tuberosa*

Plant cell cultures have been considered to be an attractive source of biologically active compounds and attempts have been made in order to increase their accumulation[1]. Isoflavonoid production has been reported in cell cultures and hairy root cultures derived from a number of species, such as *Pueraria lobata*[2,3], *P. phaseolides*[4]. *Genista tinctoria*[5], *Glycine max*[6,7], *Psoralea* sp.[8] and *Maackia* sp.[9].

During the past decade, interest in polyphenols, including isoflavonoids has increased considerably because of its beneficial effects in cardiovascular diseases, postmenopausal symptoms and cancer[10-13]. Some of these isoflavonoids present in the *Pueraria tuberosa* (Roxb.ex.Willd). DC[14] are puerarin, diadzein, genistein and genistin. *P. tuberosa* is a perennial climber, belonging to family fabaceae. The tubers of this plant are widely used in various formulations in the Indian system of medicine (Ayurveda). Besides this, kudzu root (*Pueraria lobata*) is a well-known Chinese herbal medicine, which is being extensively investigated. "Puerariae radix" or dried roots of *P. lobata* are widely used as a drug under the name of Gegen or Kakkon in traditional Chinese and Japanese medicine[15]. The pharmacological studies of puerarin show hypothermic, spasmolytic, hypotensive, anti-arrhythmatic activities and protective effect against cerebral ischemia[16] and Parkinson disease[17]. It also significantly decreases myocardial oxygen consumption, and improves microcirculation in patients suffering from cardiovascular disease. Recent investigations on daidzein have demonstrated antithrombotic, antiallergic, potential antidiabetic and antidipsotropic properties[18].

The other compound of interest is the genistein, which is a promising anticancer agent that inhibits platelet aggregation and induces apoptosis[19]. Genistein and daidzein both show phytoestrogenic activities[20] and exhibit effective antioxidant properties[21].

In the present communication, we have reported the culture initiation and effect of auxin, cytokinin and sucrose variation on the isoflavonoids production in callus culture of *P. tuberosa.*

Materials and Methods

Plant material—The root tubers of the plant, *Pueraria tuberosa*, were collected from the Sitamata Reserve Sanctuary in this region and maintained in the botanical garden of the institute. Plant material (leaf and petiole) of *P. tuberosa* was first washed with liquid detergent then it was surface sterilized in ethanol for 30 sec. After it, they were immersed in 0.1% of aqueous solution of $HgCl_2$ for 10 min, rinsed four times with sterilized distilled water. The plant material was then cut down to make suitable size

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explants (~1 cm) and inoculated onto the surface of static medium.

Callus cultures—Callus cultures of *P. tuberosa* were initiated by placing the leaf and petiole explants on to the Murashige and Skoog[22] medium with different combination and concentration of auxins [2,4-dichlorophenoxy acetic acid (2,4-D); 1-naphthalene acetic acid (NAA); indole 3-butyric acid (IBA)] and cytokinin (benzyl adenosine, BA). The *pH* of the medium was adjusted to 5.8 and autoclaved at 121°C for 15 min. The cultures were maintained at 25°±0.2°C and under white fluorescent light (Philips cool TL 36 W, 220 V) with a total irradiance of 36 μ mol $m^{-2}s^{-1}$ for 16 h photo-period and 55-60% relative humidity. For each treatment, 25 explants or three culture flasks were used as replicates. The callus produced was subcultured after every fourth week onto the fresh medium. For determining the growth index (GI), calli were carefully removed from the flasks, fresh weight was determined, and then were dried in an oven at 60°C to a constant weight and dry weight was determined. GI was calculated as – [fresh weight-initial weight/initial weight].

Effect of varying sucrose concentration (20 to 60 g/l) with two different concentrations of BA (4.1 and 20.9 μ*M*) was studied on isoflavonoids contents and tuber formation in callus cultures or from the explants. The cultures were analyzed for isoflavonoids and the results were given as average of at least three separate analyses.

Isoflavonoids extraction and analysis—About 100-150 mg of oven dried (60°C) homogenized plant material both *in vivo* and *in vitro* were extracted in 5 ml methanol for 12 h (room temperature) 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 111V, Thermo Savant, USA). For HPLC analysis, all the extracts were redissolved in HPLC grade methanol, filtered through nylon filter (0.45 μm, 4 mm, Titan, USA) and transferred in 300 μl autosampler vials.

HPLC separation—The HPLC system used for the separation of compounds was equipped with a pump (model L2130, Merck-Hitachi), auto sampler (model L-2200, Merck-Hitachi), a UV detector (L-2400, Merck-Hitachi) controlled with "Lachrome Elite" software. Separation was accomplished on a (LichroCART)® 250 × 4 mm LiChrospher® (5 μm) RP-18 column protected by a guard column of the same material. Speed-Vac sample concentrator (model SPD 111V, Thermo Savant, USA) was used to evaporate the organic solvents after extraction. The auto sampler was programmed to inject 20 μl sample per injection.

The HPLC analysis was performed with little modifications as described by Kirakosyan[23]. The solvent system used was- Solvent A- 0.0025% trifluoroacetic acid in water; solvent B-80% acetonitrile (E. Merck, India) in solvent A. The mobile phase consisted of solvent (A) and (B). The step gradient solvent programme used was as follows: 0-2 min: 85% A and 15% B; 2-5 min: 85% A and 15% B; 5-15 min: 80% A and 20% B; 15-20 min: 50% A and 50% B; 20-30 min: 40% A and 60% B; 30-35 min: 30% A and 70% B; 35-45 min: 20% A and 80% B; 45-48 min:0% A and 100% B; 48-50 min:0% A and 100% B; 50-55: 85% A and 15% B. Separation was performed at a flow rate of 1.0 ml/min and chromatographic peaks were monitored at 254 nm**.**

Reference compounds—Standard compounds puerarin (daidzein 8-C-glucoside), genistein (5,7,4′-trihydroxyisoflavone), genistin (genistein-7-O-glucoside) and daidzein (7,4′-dihydroxyisoflavone) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results and Discussion

Induction of callus occurred from stem, petiole and leaf explants on almost all the media tested. Irrespective of initiation, callus grew very slowly (GI <1), blackened and eventually died, despite short or delayed subculturing and antioxidant use in the medium. However, polyvinyl pyrrolidone and activated charcoal prolonged the senescence and enabled the growth on high sucrose containing medium. Such browning is common in woody legumes like *Prosopis juliflora*[24,25] and in some other members of family Fabaceae like *Genista*[26], *Laburnum and Cytisus*[27,28] and *Pueraria* species[2].

Isoflavonoid contents of different plant parts of *in vivo* material are presented in Table 1. Various organs analysed like tuber, stem and leaf accumulated all the four identified isoflavonoids but the amount varied significantly with organ type. Isoflavonoid glucosyl conjugate, puerarin was maximum in tuber (0.647%) and root (0.358%). Another conjugate, genistin was also maximum in tuber (0.018%) and root (0.011%). The other isoflavonoid genistein did

not vary significantly with organ type being highest in stem (0.009%) and daidzein being highest in root (0.021%) followed by leaf (0.020%). These results showed that tubers stored most of their genistein and daidzein isoflavonoids as puerarin and genistin, the glucosyl conjugates and the other plant organs stored much lower levels of these isoflavonoids. HPLC profile of plant and callus are presented in Figs 1 and 2, respectively.

Due to the initial slow growth of callus (GI <1), the cultures were maintained on MS medium containing different plant growth regulators. The results of some selected media are shown in Table 2. Isoflavonoids contents as well as GI (2.9) of callus grown on medium containing 4.9 μ*M* of IBA and 4.1 μ*M* of BA was maximum. Daidzein content was maximum in the callus cultures maintained on all the media used, whereas puerarin content was maximum in the cultures grown on medium containing 4.9 μ*M* of IBA and 4.18 μ*M* of BA. Increase in BA from 4.1 to 20.9 μ*M* neither induced any organ nor enhanced isoflavonoids production irrespective of the auxin type.

To improve upon the isoflavonoids contents and possibility of finding a medium for tuber formation, sucrose concentration was varied in MS medium in conjunction with BA (Table 3). No tuber formation was observed in the cultures up to 10 weeks of growth on any of the treatment used. Maximum GI (3.9 and 4.5) was recorded in the tissues grown on the medium containing 30 g l^{-1} of sucrose with BA 4.1 and 20.9 μ*M*, respectively. GI value of the cultures decreased with low or high sucrose concentration in the medium. Daidzein content increased drastically with increased sucrose concentration in the medium from 46 to 561 μg/g dry weight basis. Increase in BA from 4.1 to 20.9 μ*M* significantly influenced the isoflavonoids content of the tissues increasing 150%. The fluctuations in the content of some isoflavonoids, while increasing the sucrose level can be correlated

Table 1—Comparison of puerarin, genistein, genistin and daidzein content (μg g-1 dry weight) between the different organs
[Values are mean ± SE of 3 replications]

Plant material	Puerarin	Genistin	Genistein	Daidzein
Leaf (August)	68.74±3.03	60.23±8.99	61.89±4.19	44.81±8.10
Leaf (November)	89.25±7.96	104.47±9.87	73.68±6.66	204.73±7.92
Stem	546.64±3.65	44.72±3.82	86.82±2.99	60.42±1.58
Root	3575.09±22.46	111.52±1.39	35.60±1.43	215.92±1.18
Tuber	6465.0±19.80	175.27±12.37	23.27±11.06	66.47±3.32

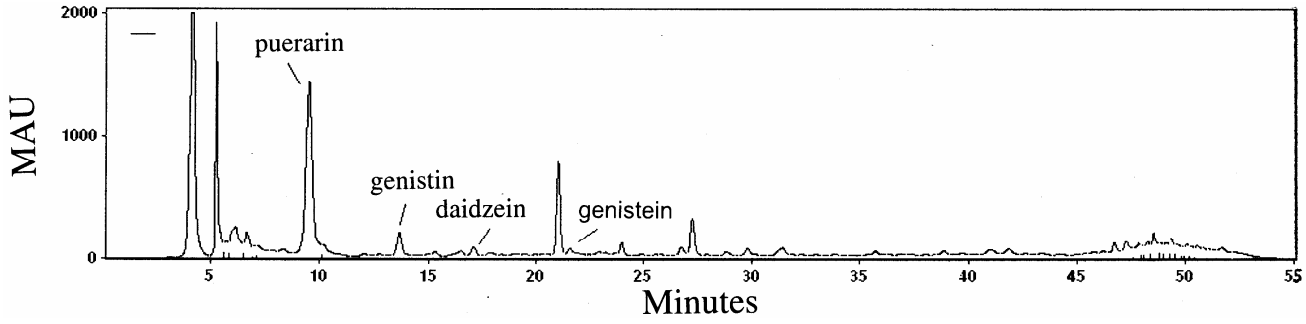

Fig. 1—HPLC profile of *Pueraria* tuber showing puerarin, genistin, daidzein and genistein

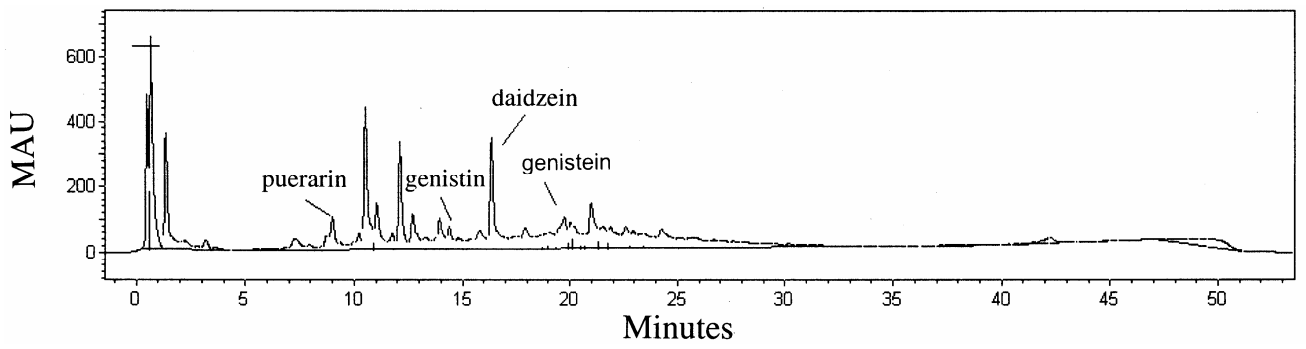

Fig. 2—HPLC profile of *Pueraria* callus showing puerarin, genistin, daidzein and genistein

with the fact that the pathways of primary and secondary metabolism often compete for the nutrients and precursors and often are mutually exclusive[29]. So, the increase in concentration of one isoflavonoids can influence the concentrations of other isoflavonoids.

Puerarin and genistin, the glucosyl conjugate contents were correlative with tuberization (Table 1), whereas de-differentiation (callus formation) was correlative with aglycones, daidzein and genistein content of the tissue. Increased BA and sucrose concentration though did not produce tubers in callus cultures, but enhanced total isoflavonoids production in the callus culture up to 0.093%. In the present work, the aglycones (daidzein and genistein) constituted the greater part of the isoflavonoids compounds of the tissues which was associated with dedifferentiated state without organogenesis/ tuberization. The results are significant in respect to aglycone molecules whose production exceeds that of amount present *in vivo* material as well as other systems reported for the production of isoflavonoids viz. *Genista* spp[30] (0.022% daidzein) and *P. lobata* (0.004% genistein)[31]. Total aglycone contents were 0.066% in tissue grown on the medium containing 60 g/l of sucrose, which was significantly higher to that recorded in stem and root (0.030%; sum of highest amount present in the root and stem). In *Curculigo orchioides*, increased sucrose enhanced tuber fresh weight[32], but fails to enhance tuber formation on the leaf explants[33]. Therefore, *P. tuberosa* requires further investigation to induce tuberization which can be used as a method of micropropagation and storage tissue. Such organogenesis can be used for easy transport as a storage organ propagule similar to that produced in several lilies and other medicinal plants[34]. Work on cell cultures and hairy roots is in progress to develop an efficient phytoestrogen producing system for direct

Table 2—Effect of different growth regulators incorporation in MS medium on the isoflavone content, (in µg g-1 dry weight) in callus cultures of *P. tuberosa*
[Values are mean ± SE of 3 replications]

Treatments (Conc. µ*M*)	GI	Puerarin	Genistin	Genistein	Daidzein
BA (4.1) +					
2,4-D (4.5)	1.490	154.32±5.45	75.77±0.14	70.99±0.99	366.21±4.79
NAA (5.3)	1.470	141.77±1.61	51.75±1.88	96.75±3.23	202.38±6.83
IBA (4.9)	2.865	263.63±8.07	64.21±1.24	71.85±1.46	409.73±10.30
BA (20.9) +					
2,4-D (4.5)	2.210	144.77±0.22	52.94±0.10	45.27±0.22	362.91±2.09
NAA (5.3)	1.010	128.41±2.11	45.96±0.47	81.08±1.56	196.77±3.25
IBA (4.9)	2.685	92.13±14.12	62.21±13.41	82.08±5.10	112.02±7.03

Table 3—Effects of sucrose (g l^{-1}) on isoflavonoids content, in (µg g^{-1} dry weight) callus cultures, grown on MS medium containing BA
[Values are mean ± SD of 3 replications]

Treatments Sucrose conc. (g l^{-1}) + BA	GI	Puerarin	Genistin	Genistein	Daidzein	Total isoflavonoids
BA (4.1 µ*M*) + Sucrose						
20	2.97	112.45±12.62	70.09±11.48	43.18±1.64	145.90±20.58	371.617
30	3.89	151.28±15.48	56.88±2.94	23.72±3.52	208.25±20.23	440.127
40	1.48	32.10 ±0.49	61.40±4.61	35.79±0.15	397.77±35.0	527.066
50	1.99	70.91±5.31	64.14±22.77	51.37±11.25	397.06±24.33	584.190
60	1.74	124.59±1.71	179.00±1.22	64.45±3.30	290.11±1.93	658.147
BA (20.9 µ*M*) + Sucrose						
20	1.79	448.92±42.62	119.30±11.75	44.31±10.50	46.55±6.02	659.090
30	4.46	301.56±7.20	160.05±6.39	37.16±4.65	287.43±28.75	786.208
40	1.84	162.28±30.57	143.44±19.65	81.94±8.08	322.75±43.35	710.405
50	2.51	148.79±2.96	67.05 ±8.76	53.48±9.17	456.58±56.18	725.885
60	1.75	91.81±3.99	180.27±3.01	96.22±3.86	561.19±0.89	929.477

use of *in vitro* produced biomass in the Indian system of medicine without the need to sacrifice the plant.

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