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Effect of chemical factors on production of isoflavonoids in *Pueraria tuberosa* (Roxb.ex.Willd.) DC suspension culture

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Suspension cultures of *Pueraria tuberosa*, a woody legume, have been established and using different concentrations of growth regulators, sucrose, ammonium and nitrate nitrogen, attempts have been made to improve their isoflavonoid content. The cell cultures grew well on all the treatments. Up to ~8 folds increased isoflavonoids content was recorded in the cultures grown in MS medium modified with nitrogen and supplemented with 1mg l^{-1} of kinetin.

Keywords: Cell culture, Isoflavonoid production, Genistin, Puerarin, *Pueraria tuberosa*

During the past decade, interest in polyphenols, including isoflavonoids has increased considerably because of their beneficial effects in cardiovascular diseases, osteoporosis, postmenopausal symptoms, cerebral ischemia and cancer[1-5]. The tubers of *Pueraria tuberosa* (Roxb.ex.Willd.) DC are widely used in various formulations in the Indian system of medicine (Ayurveda). Some of the isoflavonoids present in the *P. tuberosa*[6] are puerarin, daidzein, genistein and genistin. Plant cell cultures have potential to produce the molecules of interest without sacrifice of natural populations and attempts have been made in order to increase their accumulation[7,8]. Isoflavonoids production has been reported in cell cultures and hairy root cultures derived from a number of species, such as *Pueraria lobata*[9], *P. phaseolides*[10], *Genista tinctoria*[11], *Glycine max*[12], *Psoralea* sp.[13] and *Maackia* sp.[14].

A marked effect of plant growth regulators, altered nitrogen supply and sucrose concentration[15] has been studied for the optimization of secondary metabolite production in cell cultures. Accumulation of isoflavonoids in the callus cultures of *P. tuberosa* has been reported[16]. The objective of the present study is to optimize the effect of growth regulators, sucrose and nitrogen on the isoflavonoids production in the cell cultures of *P. tuberosa.*

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Materials and Methods

Cultures and Experimental setup—Callus culture was maintained on the modified MS[17] medium (KNO_3 475 mg l^{-1}, thiamine 1 mg l^{-1}) containing biotin (1 mg l^{-1}), calcium pantothenate (1 mg l^{-1}), 2,4,5-Tricholorophenoxy acetic acid (2,4,5-T, 0.1 mg l^{-1}) and kinetin (0.1 mg l^{-1}) with 3% sucrose under the same culture conditions as described earlier[16] and referred to as maintenance medium.

Cell suspension cultures were initiated by transferring 4 g of fresh soft homogenous callus into 250 ml Erlenmeyer flasks containing 100 ml of the same medium without agar. The *p*H of the medium was adjusted to 5.8 and autoclaved at 121ºC for 15 min. These cultures were incubated on a rotary shaker at 100 rpm 25º±0.2º C in dark. The cell cultures were subcultured every 4th week with inoculum density being 125 mg dry weight/ 100 ml medium (10% v/v). The cell suspensions were harvested when required, washed with distilled water and filtered under mild vacuum. The cells were weighed to obtain the fresh weight per 100 ml medium (FW) and dry weight (DW) was then determined by drying the cells at 60ºC in an oven to a constant weight. Time course of growth and production was determined up to 45 days. Three different experiments were carried out: (i) interaction effect between kinetin (0.1, 1.0 and 5 mgl^{-1}) and 2,4,5-T (0.0, 0.01 and 0.1 mgl^{-1}), (ii) effect of sucrose (3-6%), and (iii) varying ammonium and nitrate nitrogen. All the plant growth regulators and salts were added to the medium before autoclaving.

Sample preparation—Dried cells (0.1–0.2 g) were extracted in 5 ml methanol for 12 hr at 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 syringe filter (0.2μm, 4mm, National Sci. Co., USA) and transferred in 300 μl autosampler vials.

HPLC analysis—The HPLC system used for the separation of compounds was equipped with a pump (model L2130, Merck – Hitachi), autosampler (model L-2200, Merck – Hitachi) and a UV detector (L-2400, Merck-Hitachi) controlled with "Lachrome Elite" software. Separation was accomplished on a (LichroCART)® 250 × 4 mm LiChrospher® (5μm) RP-18 column protected by a guard column of the same material, and samples were concentrated *in vacuo*. The auto sampler was programmed to inject 20 μl sample injection.

The HPLC analysis was performed as described by Kirakosyan *et. al*[18] with little modifications. The solvent system used was: Solvent A- 0.0025% trifluoroacetic acid in water; solvent B- 80% acetonitrile in solvent A. The mobile phase consisted of solvent (A) and (B). The step gradient solvent programme was used as described earlier[16].

Statistical analysis—All the results are averaged over two separate analyses for isoflavonoids estimation and two consecutive experiments with three replicate flasks in each treatment for growth value determination. The results are expressed as μg g^{-1} cell dry biomass.

Results and Discussion

A cell suspension culture of a woody legume has been established including the improvement of isoflavonoid contents by suitable medium manipulation.

Growth and isoflavonoids accumulation—The growth and isoflavonoids accumulation was studied in suspension cultures of *P. tuberosa* grown for 45 days in the maintenance medium (Fig.1) where the medium supported consistent growth of the cultures. The maximum biomass was obtained on 30th day (~ 9 fold increase). The growth profile of the cultures showed initial slow growth, may be due to low inoculum density. On the third day of the experiment, all of the identified isoflavonoids were present in lower concentration than in the inoculum i.e. on the 0 day of growth. Isoflavonoids accumulation began after six days and continued up to 27th day (Fig 1a) with maximum isoflavonoids content recorded on the 30th day of culture (80.78 μg g^{-1}). Prolongation of stationary phase up to 39 days resulted in decrease in isoflavonoids content but growth remained stable with creamish-yellow cultures. Individually both glycone and aglycone form of the isoflavonoids showed same accumulation pattern, with maximum concentration at different culture period (Figs 1b, 1c). It was observed that genistin (46.35 μg g^{-1}) and its aglycone genistein (8.51 μg g^{-1}) accumulated in between 30th to 33 th day. However, puerarin and its aglycone daidzein were accumulated at 36th to 39th day of culture when the growth was in the stationary phase. This demonstrated that when cells stopped growing, the secondary metabolite synthesis continued together with the increase of the cell volume. This is not surprising since isoflavonoids are known to accumulate within the vacuole[19].

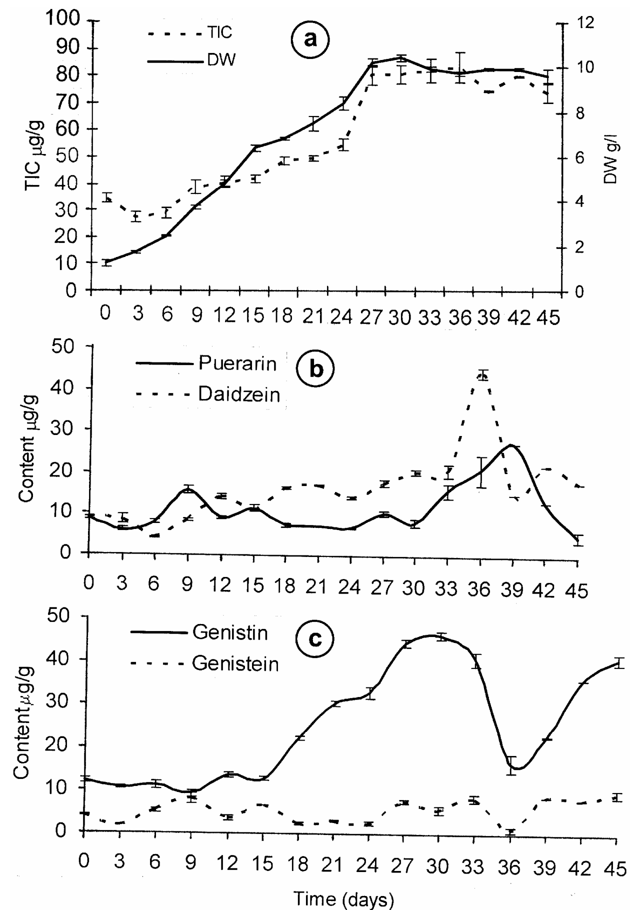

Fig. 1a-c—Growth and isoflavonoid production in cell suspension cultures of *P. tuberosa*

Interaction of 2,4,5-T and kinetin—The cell growth was maximum (14.2 g l^{-1}) in the cultures supplemented with 0.01 mg l^{-1} of 2,4,5-T and 0.1 mg l^{-1} of kinetin (Table 1). Maximal growth was observed in the medium supplemented with kinetin (0.1 mg l^{-1}) and 2,4,5-T. The isoflavonoids content was maximum (621 μg g^{-1}) in the cultures supplemented with (1 mg l^{-1}) kinetin. It was observed that with all the concentrations of 2,4,5-T (0, 0.01, 0.1 mg l^{-1}), 1 mg l^{-1} kinetin was optimal for isoflavonoids production. Increased kinetin concentration up to 5 mg l^{-1} resulted in decreased isoflavonoids content than that recorded at 1 mg l^{-1} but was higher than that obtained at the 0.1 mg l^{-1}.

Effect of NH_4^+/NO_3^- ratio—There was no profound effect on the cell growth by the altered ratio of NH_4^+/NO_3^- (Table 2). The cell growth was maximum (12.7 g l^{-1}) in the maintenance medium. The total nitrogen level (50 mM) was opted from the maintenance medium having 20.6 mM NH_4^+ and 25.3 mM NO_3^- and recorded optimal for the growth. The use of NH_4^+ (NH_4^+/NO_3^- : 50/0) as the sole nitrogen source was unfavorable for the growth and synthesis of isoflavonoids. The biomass and isoflavonoids content were improved with the increase in the amount of nitrate nitrogen and was maximum (112.24 μg g^{-1}) when ammonium was withdrawn completely from the medium (NH_4^+/NO_3^- : 0/50).

Effect of sucrose—Different concentrations of sucrose supplemented in the medium retarded cell growth with the increase in sucrose concentration, while the isoflavonoids content showed a steady increase with sucrose concentration (Table 3). Maximal isoflavonoids content was recorded in the medium supplemented with 6% of sucrose (598 μg g^{-1}) and maximal growth was recorded in the medium supplemented with 3% of sucrose (12.7 g l^{-1}).

Optimization of isoflavonoids content through alteration in medium components has also been reported in the cell culture of *P. lobata,* where manipulation of various physiochemical factors in the medium resulted in increase in isoflavonoids content[9]. Besides this, most of the studies for isoflavonoids production are on callus and organized cultures of *Pueraria* species. In the present study, the isoflavonoids production increased by ~8 folds in the cell cultures of *P. tuberosa,* which can be advantageous over other systems as this system can be utilize for developing the technology for scale up production of isoflavonoids through bioreactors.

The concentration of individual isoflavonoid was maximal on different days of culture, suggesting their inter-conversion, however, this requires more studies to draw conclusion. Phenoxy acids and ammonium are known to inhibit cellular differentiation[20] and production of secondary metabolites[7]. This was

Table 1—Effect of 2,4,5-T and kinetin interaction on the isoflavonoids production (μg g^{-1} DW) in cell cultures of *P. tuberosa*

2,4,5-T (mgl^{-1})	Kinetin (mgl^{-1})	Culture dry biomass (gl^{-1})	Puerarin	Genistin	Daidzein	Genistein	Total	Yield (μg l^{-1})
0	0.1	13.6	42.2±2	7.4±0	6.1±1	3.0±0	59	797
0	1.0	10.6	95.2±2	408.1±2	114.4±4	3.0±0	621	6578
0	5.0	12.8	54.6±0	23.6±0	43.9±1	3.7±0	126	1611
0.01	0.1	14.2	20.9±0	11.3±0	7.4±0	4.4±0	44	624
0.01	1.0	11.5	64.1±1	139.7±0	28.7±8	3.9±0	237	2720
0.01	5.0	11.4	69.0±2	107.7±3	26.8±1	15.9±1	220	2502
0.1	0.1	12.7	9.1±0	48.9±0	17.4±0	2.8±0	78	992
0.1	1.0	12.6	71.7±0	159.2±2	38.9±2	6.3±0	276	3477
0.1	5.0	10.8	25.1±1	89.7±1	32.4±1	6.0±0	153	1655

Table 2—Effect of NH_4^+/NO_3^- ratio on the isoflavonoids production (μg g^{-1} DW) in cell cultures of *P. tuberosa*

NH_4^+ (mM))	NO_3^- (mM)	Culture dry biomass (gl^{-1})	Puerarin	Genistin	Daidzein	Genistein	Total	Yield (μg l^{-1})
20.6	25.3	12.7	9.1±0	48.9±2	17.4±1	2.8±0	78	992
0	50	10.0	13.7±0	64.5±2	25.0±1	9.1±1	112	1122
10	40	11.0	30.9±0	36.5±2	24.3±1	1.9±0	94	1030
20	30	11.5	15.4±0	10.9±1	17.9±1	7.9±0	52	601
25	25	11.1	13.1±0	18.4±1	11.9±1	4.3±0	48	530
30	20	10.7	6.9±0	14.9±1	14.9±2	7.8±0	45	476
40	10	10.1	13.1±0	18.4±1	5.6±0	2.8±0	39	402
50	0	9.2	9.1±0	15.0±2	10.9±0	3.2±0	38	351

Table 3 — Effect of various concentration of sucrose on the isoflavonoids production (μg g^{-1}DW) in cell cultures on *P. tuberosa*

Sucrose	Culture dry biomass (gl^{-1})	Puerarin	Genistin	Daidzein	Genistein	Total	Yield (μg l^{-1})
3%	12.7	9.1±1	48.9±0	17.4±0	2.8±0	78	992
4%	12.0	12.9±0	63.9±1	18.8±0	17.2±1	113	1450
5%	9.1	50.2±1	114.5±5	82.6±1	30.6±1	278	2515
6%	9.0	217.9±4	151.6±7	75.0±4	153.8±2	598	5385

evident in *P. tuberosa* cell cultures. In contrast to this, guggulsterones production in *Commiphora wightii*[21] and phaseolin and kievetone synthesis in shoot cultures of *Phaseolus vulgaris*[22] was supported by ammonium nitrogen in the medium. The medium for *C. wightii* also contained 2,4-D. This auxin also induced production of daidzein in callus cultures of five species of the genus *Psoralea*[23]. The beneficial role played by 2,4-D in the process of isoflavone biosynthesis was also recorded in *Maakia amurensis*[14]. This auxin is reported to block the key enzyme of the phenylpropanpoid pathway-chalcone synthesis[24]. Isoflavonoids production increased on higher concentration of carbohydrate similar to that used in the production medium for *Vitis vinifera*[25]. The experiments are in progress using these results for shake flasks and bioreactor to produce isoflavonoids.

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