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ORIGINAL PAPER

Synergistic effect of morphactin on cytokinin-induced production of isoflavonoids in cell cultures of *Pueraria tuberosa* (Roxb. ex. Willd.) DC

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Abstract Isoflavonoids, the functional molecules of Fabaceae, are under clinical trials against cancer, osteoporosis and cardiovascular diseases. In this study, the efficacy of different plant growth regulators was evaluated for optimizing the production of isoflavonoids in *Pueraria tuberosa.* The cultures were maintained in Murashige and Skoog's medium containing 0.1 mg l^{-1} 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 0.1 mg l^{-1} kinetin. The addition of 5.0 mg l^{-1} N^6-(2-Isopentenyl) adenine (2iP) resulted in about ~32-folds increase in production of isoflavonoids, while about ~23-folds increase was recorded in the absence of kinetin in the maintenance medium. A maximum yield of isoflavonoids (~80 mg l^{-1}; 82-folds increase) was obtained in cultures grown at 0.1 mg l^{-1} morphactin and 5.0 mg l^{-1} of 2iP. However, 2,4,5-T in combination with 2iP was ineffective for their production. Among different plant growth regulators tested, maximum yields of puerarin, genistin, daidzein and genistein were 17.4, 15.9, 69.0 and 0.04 mg l^{-1}, respectively. The study suggested that the presence of two cytokinins or 2iP with morphactin in the culture medium markedly enhanced the production of isoflavonoids in *P. tuberosa*.

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Abbreviations

2iP	6-(γ,γ-Dimethylallylamino)purine
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
DM	Dry mass
Morphactin	Chloroflurenol-butylester
MS	Murashige and Skoog's medium

Introduction

Besides showing antihypercholesterolemic activity, isoflavonoids have been implicated in cancer and osteoporosis prevention (Yan et al. 2006). Current clinical trials of isoflavonoids in medicine (Choi et al. 2007) encourage research into developing efficient controlled production methods where phytoestrogens are either synthesized chemically, or produced in vitro (Ren et al. 2001). Isoflavonoids enjoy a restricted distribution in the plant kingdom, and are mostly limited to the family Fabaceae (Dixon and Sumner 2003). They have been reported in cell and hairy root cultures derived from a number of species, such as *Pueraria lobata* (Fang et al. 2006; Li and Zhang 2006), *P. phaseoloides* (Kintzios et al. 2004), *P. thomsonii* (Maojun et al. 2006), *Genista tinctoria* (Luczkiewicz and Glod 2005), *Glycine max*

(Tuominen and Musgrave 2006), *Psoralea corylifolia* (Abhyankar et al. 2005) and *Maackia* spp. (Fedoreyew et al. 2000).

Phytohormones play a crucial role in the regulation and coordination of plant growth, morphogenesis and metabolism (Suri and Ramawat 1995; Ramawat and Mathur 2007 and references therein). Growth modulation may also affect the accumulation of secondary metabolites. Cytokinin causes cellular differentiation (Suri and Ramawat 1995), while morphactin acts slowly but systematically modifying further developmental pathways (Sankhla et al. 1975). Morphactin has also been shown to induce profuse flowering in mango (Blaikie et al. 2004) and production of guggulsterones in callus cultures of *Commiphora wightii* (Tanwar et al. 2007).

Tubers of *Pueraria tuberosa* (Roxb. ex. Willd.) DC are widely used in various ayurvedic (ancient Indian system of medicine) formulations, and contain isoflavonoids, viz., puerarin, daidzein, genistein and genistin (Dev 2006). Earlier we reported not only the production of isoflavonoids (Vaishnav et al. 2006), but also the effects of nutrients on their accumulation (Goyal and Ramawat 2007) in callus/cell cultures of *P. tuberosa*.

Plant cell and callus cultures have always been an attractive means for the production of bioactive molecules (Ramawat and Mathur 2007). Altering various physiochemical factors that influence a specific secondary metabolite pathway in the system can optimize the production of desired molecules. Indeed, we have earlier shown a marked effect of morphactin on guggulsterones production in vitro (Tanwar et al. 2007). In the present study, we investigated the effects of plant growth regulators on isoflavonoids production in cell cultures of *P. tuberosa*.

Materials and methods

Cultures and experimental setup

Callus cultures of *Pueraria tuberosa* (Roxb. ex. Willd.) DC were maintained on modified MS (Murashige and Skoog 1962) medium (475 mg l^{-1} KNO_3 and 1.0 mg l^{-1} thiamine) containing biotin (1.0 mg l^{-1}), D-calcium pantothenate (1.0 mg l^{-1}), 2,4,5-T (0.1 mg l^{-1}), kinetin (0.1 mg l^{-1}), sucrose (30 g l^{-1}) and agar (8 g l^{-1}; BDH, Poole, United Kingdom) under the same culture conditions, as described earlier (Vaishnav et al. 2006). Cell suspension cultures were initiated by transferring 4 g of fresh soft homogenous calli into a 250 ml Erlenmeyer flask containing 100 ml medium of the same composition without agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. These cultures were incubated on a rotary shaker (100 rpm) at 25 ± 0.2°C in the dark, and were subcultured at an interval of 4 weeks at an inoculation density of 125 mg dry mass 100 ml^{-1} medium (10% v/v).

To investigate the effects of plant growth regulators on the production of isoflavonoids, five independent sets of experiments were laid out: (1) effect of 2iP (0.1, 0.5, 1.0 and 5.0 mg l^{-1}) in the presence of 2,4,5-T (0.1 mg l^{-1}) and kinetin (0.1 mg l^{-1}); (2) interaction effect between 2,4,5-T (0.0, 0.01 and 0.1 mg l^{-1}) and 2iP (0.1, 0.5, 1.0 and 5.0 mg l^{-1}); (3) effect of morphactin (0.1, 0.5, 1.0 and 5.0 mg l^{-1}) in the presence of 2,4,5-T (0.1 mg l^{-1}) and kinetin (0.1 mg l^{-1}); (4) interaction effect between morphactin (0.1, 0.5, 1.0 and 5.0 mg l^{-1}) and kinetin (0.0, 0.1 and 1.0 mg l^{-1}); and (5) interaction effect between morphactin (0.1, 0.5, 1.0 and 5.0 mg l^{-1}) and 2iP (0.1, 0.5, 1.0 and 5.0 mg l^{-1}). All the plant growth regulators were co-autoclaved with the medium.

Sample preparation

The cultures were harvested on the 30th day after inoculation, washed with distilled water and filtered under mild vacuum. The cells were weighed to obtain the fresh mass per 100 ml medium, and dry mass (DM) was then determined by drying the cells at 60°C in an oven to a constant weight. Dried homogenized cells (100–150 mg) were extracted in 5 ml methanol for 12 h and analyzed by HPLC, as described earlier (Vaishnav et al. 2006).

HPLC analysis

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 × 4 mm C_{18} (5 m) 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 by Kirakosyan et al. (2003). 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 solvents 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 two separate analyses from two flasks for the estimation of isoflavonoids and two consecutive experiments with six replicate flasks in each treatment for growth value determination. The results were expressed as μg g^{-1} cell dry mass. For individual isoflavonoid content, the data were analyzed by two-way ANOVAs followed by mean separation using post hoc least significant difference (LSD) test at $P \leq 0.01$.

Results and discussion

The medium containing morphactin and 2iP caused about 80-folds increase in the accumulation of isoflavonoids in *P. tuberosa* cells grown in vitro. The increase in isoflavonoids accumulation up to 0.6%, as obtained in this study, has never been reported for cell suspension cultures of any of the species of *Pueraria*. This relatively high level isoflavonoids content was equivalent to that present in 2-year-old in vivo-tubers of *P. tuberosa* (Vaishnav et al. 2006). No marked changes in cell morphology were observed in any of the treatments tested.

Effect of cytokinin

The results showed that a significant increase in isoflavonoids production occurred with increasing concentrations of 2iP (Table 1). A 20% increase in culture growth (15.6 g l^{-1}) over the control (12.7 g l^{-1}) was observed with increasing concentrations of 2iP. The isoflavonoids content increased up to 2,282 μg g^{-1} DM, resulting in a yield of 32 mg l^{-1} (29-folds increase over the control) when the medium was supplemented with 1.0 mg l^{-1} 2iP. However, a significant decline in isoflavonoids content occurred at 5.0 mg l^{-1} 2iP.

To evaluate the individual effect of 2iP as the sole source of cytokinin, kinetin was withdrawn from the medium and instead 2,4,5-T was incorporated (Table 2). Maximum growth (15.1 g l^{-1}) and isoflavonoids production (1,486 μg g^{-1} DM) were recorded in cultures grown at 5.0 mg l^{-1} 2iP alone (yield = 22.4 mg l^{-1}). This suggested that 2,4,5-T even at a low concentration (0.01 mg l^{-1}) reduced the isoflavonoids content. An enhanced production of isoflavonoids was recorded in cultures grown in the presence of two cytokinins along with an auxin (2,4,5-T), and this might be due to synergistic effects of cytokinins present in the medium.

Table 1 Effect of 2iP on cell growth and isoflavonoids production in *Pueraria tuberosa* after 4 weeks' culture in MS medium containing 0.1 mg l^{-1} 2,4,5-T and 0.1 mg l^{-1} kinetin[a]

2iP (mg l^{-1})	Dry mass (g l^{-1})[b]	Isoflavonoid content (μg g^{-1} DM)[b]					
		Puerarin	Genistin	Daidzein	Genistein	Total	Yield (mg l^{-1} DM)
0.1	13.2 b	106 d	33.5 d	112 c	6.2 c	257 d	3.4 d
0.5	13.5 b	836 b	65.0 c	301 b	5.9 c	1,207 b	16.3 b
1.0	14.0 ab	871 a	264 b	1,136 a	11.4 b	2,282 a	32.0 a
5.0	15.6 a	123 c	423 a	95.2 c	18.9 a	659 c	10.3 c

[a] Control values: dry mass, 12.7 g l^{-1}; total isoflavonoids content, 78.0 μg g^{-1} DM; yield, 1.0 mg l^{-1}

[b] Means with common letters are not significantly different at $P \leq 0.01$, according to least significant difference (LSD) test

Table 2 Interaction effect between 2,4,5-T and 2iP on cell growth and isoflavonoids production in *P. tuberosa* after 4 weeks' culture in MS medium

2,4,5-T (mg l^{-1})	2iP (mg l^{-1})	Dry mass (g l^{-1})[a]	Isoflavonoid content (μg g^{-1} DM)[a]					
			Puerarin	Genistin	Daidzein	Genistein	Total	Yield (mg l^{-1} DM)
0.0	0.1	11.2 de	55.2 f–h	22.1 cd	30.1 cd	4.3 c–f	112 ef	1.3 e–g
0.0	0.5	9.9 fg	128 d	21.7 c–e	24.5 c–h	12.7 a	188 d	1.9 de
0.0	1.0	12.2 b–e	570 b	60.3 b	83.1 b	5.5 bc	719 b	8.8 b
0.0	5.0	15.1 a	1,153 a	97.4 a	233 a	2.7 f–k	1,486 a	22.4 a
0.01	0.1	8.4 h	39.2 f–i	16.9 c–i	12.7 jk	3.0 f–j	71.9 fg	0.6 f–h
0.01	0.5	13.1 bc	67.0 ef	17.6 c–h	28.3 c–f	3.3 e–i	116 c	1.5 e
0.01	1.0	9.8 fg	12.7 i	9.9 jk	15.0 h–j	4.2 c–g	41.8 g	0.4 gh
0.01	5.0	11.0 ef	196 c	18.6 c–g	32.3 c	6.9 b	253 c	2.8 c
0.1	0.1	9.4 gh	9.9 j	10.4 I–k	10.1 jk	2.5 j–k	32.8 g	0.3 h
0.1	0.5	12.0 c–e	63.8 e–g	21.6 c–f	26.2 c–g	3.7 d–h	115 e	1.4 ef
0.1	1.0	13.3 b	131.4 d	22.7 c	24.0 c–i	5.1 c–e	183 d	2.5 cd
0.1	5.0	12.4 b–d	88.8 e	15.7 d–j	30.0 c–e	5.4 b–d	140 e	1.7 de

[a] Means with common letters are not significantly different at $P \leq 0.01$, according to least significant difference (LSD) test

Effect of morphactin

The results showed that cells grew equally well with increasing concentrations of morphactin in the maintenance medium (Table 3). Maximum dry mass (15.0 g l^{-1}) and isoflavonoids production (117 μg g^{-1} DM) were recorded in cultures grown at 5.0 and 0.1 mg l^{-1} morphactin, respectively.

To study the individual effect of morphactin as the sole source of auxin, 2,4,5-T was replaced by kinetin in the maintenance medium (Table 4). The maximum growth (13.7 g l^{-1} DM) was recorded in cultures grown at 0.5 mg l^{-1} morphactin plus 0.1 mg l^{-1} kinetin. Isoflavonoids content decreased with increasing concentrations of morphactin (0.1–5.0 mg l^{-1}), irrespective of cell growth. Maximum isoflavonoids content (224 μg g^{-1} DM) was observed when the medium was supplemented with 0.1 mg l^{-1} morphactin and 1.0 mg l^{-1} kinetin, while maximum yield (2.9 mg l^{-1}) was obtained in cultures grown at 0.5 mg l^{-1} morphactin and 0.1 mg l^{-1} kinetin. It may be inferred that morphactin is effective only in combination with a cytokinin for enhancing the isoflavonoids content in cultures grown in vitro.

Since morphactin and 2iP could increase the production of isoflavonoids, their different combinations were tested (Table 5). A maximal growth of about ~14 g l^{-1} was recorded in many cultures. Isoflavonoids content decreased with increasing concentrations of morphactin (0.1–5 mg l^{-1}), but increased with increasing concentrations of 2iP (0.1–5 mg l^{-1}) in the medium. A significant increase in genistin (5,561 μg g^{-1} DM) occurred in medium containing 5.0 mg l^{-1} 2iP and 0.1 mg l^{-1} morphactin.

Table 3 Effect of morphactin on cell growth and isoflavonoids production in *Pueraria tuberosa* after 4 weeks' culture in MS medium containing 0.1 mg l^{-1} 2,4,5-T and 0.1 mg l^{-1} kinetin[a]

Morphactin (mg l^{-1})	Dry mass (g l^{-1})[b]	Isoflavonoid content (μg g^{-1} DM)[b]					
		Puerarin	Genistin	Daidzein	Genistein	Total	Yield (mg l^{-1} DM)
0.1	13.2 b	7.6 c	5.7 c	7.4 c	3.2 a	24.0 d	0.3 b
0.5	13.5 b	6.8 d	12.4 b	12.3 b	2.9 c	34.4 c	0.5 b
1.0	14.0 ab	37.2 a	60.8 a	16.5 a	2.7 d	117 a	1.6 a
5.0	15.0 a	30.4 b	3.5 d	6.4 d	3.0 b	43.3 b	0.7 a

[a] Control values: dry mass, 12.7 g l^{-1}; total isoflavonoids content, 78.0 μg g^{-1} DM; yield, 1.0 mg l^{-1}

[b] Means with common letters are not significantly different at $P \leq 0.01$, according to least significant difference (LSD) test

Table 4 Interaction effect between morphactin and kinetin on cell growth and isoflavonoids production in *P. tuberosa* after 4 weeks' culture in MS medium

Morphactin (g l^{-1})	Kinetin (mg l^{-1})	Dry mass (g l^{-1})[a]	Isoflavonoid content (μg g^{-1} DM)[a]					
			Puerarin	Genistin	Daidzein	Genistein	Total	Yield (mg l^{-1} DM)
0.1	0.0	10.3 ef	103 a	29.3 h	10.6 h	6.2 cd	149 e	1.5 d–f
0.5	0.0	12.1 bc	13.7 h	23.8 j	13.0 g	2.0 h	52.5 k	0.6 g
1.0	0.0	12.3 b	14.3 h	37.3 g	20.2 d	3.1 g	74.9 j	0.9 fg
5.0	0.0	11.5 b–e	9.9 i	26.3 i	10.2 h	2.4 gh	48.9 l	0.6 g
0.1	0.1	12.3 b	32.8 g	146 b	24.3 b	4.4 f	207 c	2.6 ab
0.5	0.1	13.7 a	61.2 d	115 c	29.5 a	5.4 de	212 b	2.9 a
1.0	0.1	10.7 d–f	57.7 e	18.8 k	13.2 g	6.8 c	96 i	1.0 e–g
5.0	0.1	9.9 f	67.5 c	61.3 e	17.7 e	42.6 a	189 d	1.9 b–d
0.1	1.0	10.6 d–f	33.3 g	166 a	22.5 c	1.7 h	224 a	2.4 a–c
0.5	1.0	11.7 b–d	78.6 b	49.4 f	9.8 h	4.8 ef	143 g	1.7 c–e
1.0	1.0	11.8 b–d	38.8 f	82.1 d	23.8 b	2.4 gh	147 f	1.7 c–f
5.0	1.0	10.9 c–f	76.8 b	23.8 j	15.2 f	8.1 b	124 h	1.4 d–g

[a] Means with common letters are not significantly different at $P \leq 0.01$, according to least significant difference (LSD) test

Table 5 Interaction effect between morphactin and 2iP on cell growth and isoflavonoids production in *P. tuberosa* after 4 weeks' culture in MS medium

Morphactin (mg l^{-1})	2iP (mg l^{-1})	Dry mass (g l^{-1})[a]	Isoflavonoid content (μg g^{-1} DM)[a]					
			Puerarin	Genistin	Daidzein	Genistein	Total	Yield (mg l^{-1} DM)
0.1	0.1	14.6 a	8.5 k–m	21.3 k–m	8.8 l m	2.3 o	40.9 k	0.6 k
0.1	0.5	12.6 de	12.5 j–k	251 i	81.9 g	4.1 l–n	350 i	4.4 h–j
0.1	1.0	11.4 f	18.7 j	514 j	190 e	19.8 c	743 f	8.5 e–g
0.1	5.0	12.4 d–f	139 c	5,561 a	678 a	23.9 b	6,401 a	79.4 a
0.5	0.1	13.4 b–d	11.0 kl	42.5 k	13.2 l	5.3 kl	71.8 j	1.0 i–k
0.5	0.5	14.5 ab	78.4 e	324 h	39.2 ij	9.0 fg	450 g	6.5 f–h
0.5	1.0	13.3 cd	211 b	166 j	505 b	11.4 de	895 e	11.9 e
0.5	5.0	12.6 de	81.2 e	2,504 b	307 d	3.5 m–o	2,896 b	36.5 c
1.0	0.1	12.5 d–f	101.1 d	1,408 d	150 f	7.2 h–j	1,665 d	20.8 d
1.0	0.5	11.8 ef	42.0 h	303 h	45.0 hi	6.3 i–k	396 h	4.7 g–i
1.0	1.0	13.9 ab	7.2 k–m	25.5 kl	8.1 l–n	7.3 hi	48.1 jk	0.7 jk
1.0	5.0	14.7 a	49.4 g	2,350 c	422 c	37.3 a	2,859 c	42.0 b
5.0	0.1	12.1 ef	270.4 a	439 g	30.2 jk	4.8 k–m	745 f	9.0 ef
5.0	0.5	14.5 ab	32.7 i	21.8 k–m	8.1 l–n	7.9 gh	70.4 j	1.0 i–k
5.0	1.0	14.5 ab	61.0 f	220 i	33.8 I–k	10.5 d–f	325 i	4.7 g–i
5.0	5.0	11.5 ef	65.8 f	585 e	54.2 h	11.5 d	716 f	8.2 e–g

[a] Means with common letters are not significantly different at $P \leq 0.01$, according to least significant difference (LSD) test

Total isoflavonoids content of 6,401 μg g^{-1} DM amounting to a yield of 80 mg l^{-1}, although lower than that obtained from organized cultures (Kintzios et al. 2004; Maojun et al. 2006), has never been recorded in cell cultures of any of the species of *Pueraria*. Our study showed that morphactin being

effective at a low concentration was synergistic with 2iP, and its effect was independent of culture growth. Morphactins affect morphogenesis and polarity in some higher plants (Sankhla et al. 1975). Though morphological changes were not observed in the present study (data not shown), but it may be presumed that subcellular changes might be responsible for enhanced production of secondary metabolites under conditions where both morphactin and cytokinin were present without an auxin. Cytokinins are known to enhance the production of secondary metabolites and play an important role in cytodifferentiation (Suri and Ramawat 1995) and subcellular differentiation, e.g., anthocyanin production in *Camptotheca acuminata* (Pasqua et al. 2005). The effect of morphactin, though diverse, is more like an anti-auxin, and it affects vegetative as well as reproductive organs including polarity disturbance (Sankhla et al. 1975). The foliar application of morphactin has been shown to induce profuse flowering in mangoes (Blaikie et al. 2004). Morphactin-induced production of guggulsterones in callus cultures of *Commiphora wightii* has been reported earlier (Tanwar et al. 2007).

Auxin at a low concentration in conjunction with a cytokinin is always beneficial for the production of secondary metabolites, which is often enhanced further on auxin-free medium (Goyal and Ramawat 2007; Ramawat et al. 1987). A marked increase in the accumulation of isoflavonoids when kinetin was replaced by 2iP in the presence of morphactin might be because of cytokinin-like effect of morphactin (Rucker 1982). The structural differences between the two cytokinins can also be presumed to have an effect on receptor sites and stimulation. Such receptor proteins located on the membrane and in the cytoplasm are known for both auxin and cytokinins (Kulaeva and Prokoptseva 2004).

The increase in genistin, as obtained in this research, was unique to cell cultures grown in the medium containing morphactin, and this resulted in an increase in total isoflavonoids content. This factor can further be explored for the production of genistin in cell cultures. A high level of 2iP in the medium was associated with high-puerarin content. The cell cultures of *P. tuberosa* are being scaled-up in a bioreactor in our laboratory using selected combinations of medium components.

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