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High isoflavone content and estrogenic activity of 25 year-old *Glycine max* tissue cultures

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Abstract

Soy isoflavones are phytoestrogens which have been associated with several health benefits. In the present study, we report the production of isoflavones in a collection of 40 strains of soya cell cultures established in 1975. A large variability in the isoflavone composition was observed and high-producing strains, with an isoflavone content of up to 46.3 mg g^{-1} dry wt., were found. In comparison with soybeans, many callus strains had a higher isoflavone concentration (10–40 times) and a different ratio of genistin to daidzin forms. The highest producing strain was transferred to liquid medium in an Erlenmeyer flask and in a 10 l stirred-tank bioreactor where high isoflavone content (7% dry wt.), concentration (880 mg l^{-1}) and a maximum productivity estimated to 60 mg l^{-1} d^{-1} were obtained. We further studied the estrogenic activity of pure compounds compared to plant cell culture extracts in the estrogen-responsive human endometrial Ishikawa cell line. Estrogen was confirmed to be 1000–10,000 times more active than isoflavones. The estrogenic activity of the extracts correlated to their isoflavone content. The activity of the malonyl isoflavones, assessed here for the first time, was lower than the aglycones. Taken together, these results suggest that soya cell cultures can be used as an alternative source to soybeans to provide high concentrations of bioactive isoflavones.
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Keywords: *Glycine max*; Leguminosae; Soya; Callus cultures; Cell suspension culture; Isoflavones; Estrogenic activity

1. Introduction

The use of plant cell cultures for large-scale production of active compounds, such as pharmaceuticals, food additives, and cosmetics is of interest from scientific and economical points of view. Secondary metabolites such as alkaloids, terpenes, anthocyanins and anthraquinones have been reported to be produced by plant cell cultures (Alfermann and Petersen, 1995; Stöckigt et al., 1995; Fu et al., 1999). However, some well-known drawbacks are the often-low productivity and instability of the productive cell lines. Different strategies have been used to overcome these difficulties. For example, it is well known that in vitro plant cell cultivation induces a high somaclonal variability in the early derived strains, even when obtained from a single genotype that can be used for the selection of high producing strains.

Isoflavones are broadly defined as phytoestrogens, as they are plant-derived molecules that exhibit some of the metabolic functions normally associated with estrogens. Studies in humans, animals and cell culture systems suggest that dietary phytoestrogens play an important role in the prevention of breast and prostate cancer, cardiovascular disease and osteoporosis (Potter et al., 1998, Anderson and Garner, 1999; Messina et al., 2001).

Isoflavones are usually extracted from soybeans, which contain three main compounds, in four chemical forms: the aglycones daidzein, genistein and glycitein; the glucosides daidzin, genistin, and glycitin; their malonylglucoside and acetylglucoside forms (Franke et al., 1994; Wang and Murphy, 1994; Reinli and Block, 1996).

Isoflavones have been produced in plant cell cultures derived from different species, such as *Maackia* sp. (Fedoyerev et al., 2000), *Lupinus* sp. (Berlin et al., 1991), and *Psoralea* sp. (Bouque et al., 1998). Ames and Worden (1997) described a new bioreactor design, using

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immobilized soya cell cultures. Zacharius and Kalan (1990) studied the link between daidzein and glyceollin production in soya cell suspension cultures subjected to biotic stress.

In the present study, the variability in isoflavones content was studied in a collection of 40 strains of soya cell cultures established in 1975. The highest producing cell line was transferred to liquid medium to study the characteristics of growth and isoflavone production in an Erlenmeyer flask and in a bioreactor. Finally the estrogenic activity of pure compounds and plant cell cultures extracts was studied using the alkaline phosphatase assay in the human endometrial Ishikawa cell line.

2. Results and discussion

2.1. Callus cultures

Stable plant callus cultures were established more than 25 years ago from four different soybean cultivars. Of the 40 callus lines obtained, 21 were derived from root, stem and cotyledon explants of Amsoy cv., 1 from the cotyledon of Mandarin cv., 11 from the cotyledons of Altona cv. and 7 from the cotyledons of Maple Arrow cv.

The isoflavones content of the different strains was determined by HPLC and UV detection. The identity of the 6 compounds daidzin, genistin, malonyldaidzin, malonylgenistin, daidzein and genistein was verified by LC-MS and all gave intense $[M+H]^+$ ions at m/z 417, 433, 503, 533, 255 and 271, respectively. Further MS/MS experiments confirmed the identity of the isoflavones. In particular product ion mass spectra of daidzin and genistin, showed intense ions at m/z 255 and 271 corresponding to the aglycones whereas MS/MS spectra of daidzein and genistein showed expected complex fragmentation patterns.

The isoflavone content at the time of subculture of the 40 different strains and of their four soybean cultivars of origin, is reported in Fig. 1. Large variability, up to 500-fold, was observed within the strains, ranging from 0.10 mg g^{-1} dry wt. (no. 12807) to 46.3 mg g^{-1} dry wt (no. 13406) of isoflavones. In contrast, the diversity among different soybean cultivars, grown both in fields and in greenhouse, was much smaller (1.38–2.27 mg g^{-1} and 4.85–6.27 mg g^{-1} dry wt. respectively, data not shown) in agreement with the data by Wang and Murphy (1994) regarding the isoflavone content of different varieties of American and Japanese soybeans. It was not possible to show an effect of the cultivar of origin due to the small size of the callus population. Interestingly, many of the strains analyzed had an isoflavone concentration much higher than the whole soybeans. This allowed the recovery of high producing strains, such as no. 13406 originating from Maple Arrow cv. that contained 46.3 mg g^{-1} dry wt. of isoflavones, approximately 25 and 10 times greater than the soybean of origin grown in field and in greenhouse, respectively. This amount represented 7% of the dry matter, which is very high for a plant secondary metabolite. This result is in agreement with the report of Bouque et al. (1998) who found that in *Psoralea* species (callus culture or genetically transformed "hairy-root" cultures) the daid-

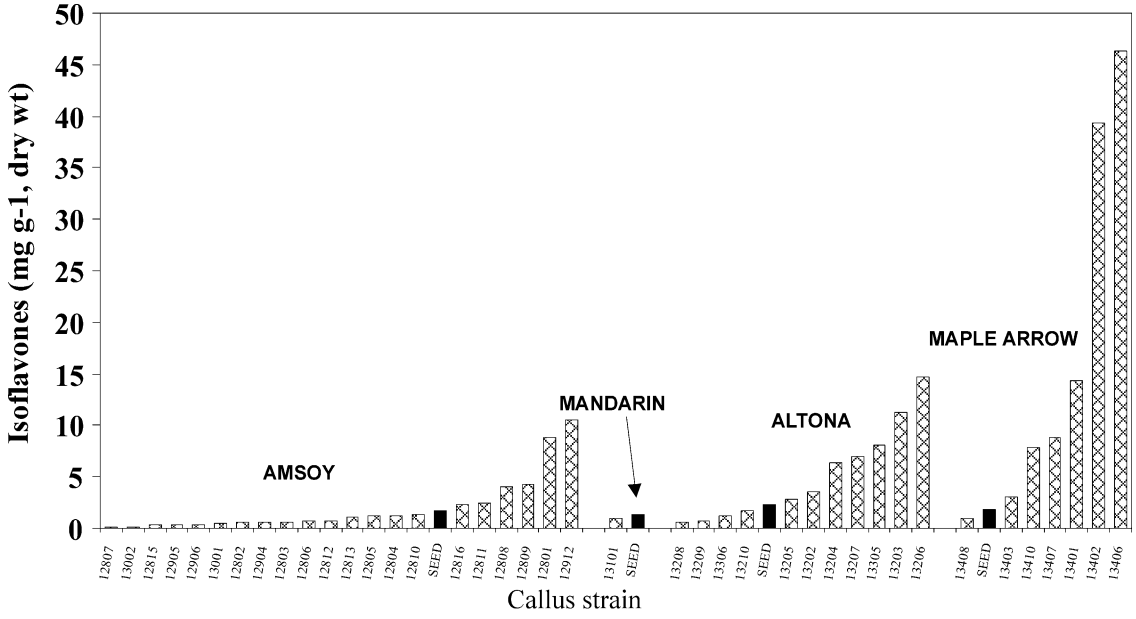

Fig. 1. Isoflavone content of the 40 different callus strains and of their four soybean cultivars of origin. The soya cell cultures presented a large variability in their isoflavone content, ranging from 0.10 mg g^{-1} dry wt (no. 12807) to 46.3 mg g^{-1} dry wt. (no. 13406). The diversity among the different soybean cultivars, Amsoy, Mandarin, Altona and Maple Arrow, was much smaller (1.38–2.27 mg g^{-1} dry wt). Total isoflavones content was quantified by HPLC at the time of subculture.

zein concentration *in vitro* was always much higher than *in planta*.

In the case of the Amsoy cv. the callus strains originated from explants of different parts of the plant (roots, stem and cotyledons) but the intraspecific variability could not be related to the origin of the callus. Moreover, even calli derived from the same explant (strains no. 12801 and no. 13002, for example) showed different colours (white and green, respectively) and different isoflavone content (8.789 mg g^{-1} dry wt. and 0.161 mg g^{-1} dry wt., respectively).

As already observed in seeds (Graham et al., 1990; Liggins et al., 1998; Murphy et al., 1999), the major isoflavone forms in the callus strains were glycosides and malonyl conjugates (>95% of total). Nevertheless, the ratio between the genistin and daidzin forms was extremely variable from one strain to another, within and outside a given cultivar. In contrast, in all the soybeans analyzed the concentrations of the genistin forms were always higher than the daidzin forms. As an example, Fig. 2 represents the composition of two callus strains from Maple arrow cv. and seeds from plants cultivated in field and in greenhouse. Strain no. 13406 was richer in daidzin forms (ca. 70% of total) and strain no. 13407 contained more genistin forms (ca. 70% of total) while both the seeds had the same composition (ca. 40% of daidzin forms and ca. 60% of genistin forms). The selection of the strain is thus important to obtain a specific and exclusive balance in isoflavone forms.

The variability observed within the strain collection, cultivated under the same culture conditions, may be due to the somaclonal variation induced during the initiation of the cultures 25 years ago. In the absence of regular analysis during these 25 years, it is difficult to claim the stability of the strains for such a long period, but the high-producing strain (no. 13406), did not show any change in the accumulation of isoflavones over the past two years. This data is very important when considering the difficulty of obtaining a reproducible source of isoflavone extracts from soybean. Strong physiological and genetic variability affects the isoflavones content of the seeds that have been shown to vary 3–8 fold among years and 2–3 fold among varieties (Eldridge and Kwolek, 1983; Wang and Murphy, 1994).

Taken together, these results confirm the hypothesis that plant tissues cultivated in vitro represent an unorganized, dedifferentiated stage in which the cells present a high variability in gene expression (Stöckigt et al., 1995). Besides cultivated plants, plant cell cultures can thus represent an interesting source of phytochemicals and even of novel compounds that are not produced or have not yet been detected in differentiated plants.

2.2. Suspension cultures

The high isoflavone content observed in the Maple arrow strain no. 13406 led us to choose this strain to run kinetic experiments in liquid medium for the characterization of isoflavone production. Calli were transferred in liquid medium and cultivated under the same culture conditions as those on solid medium but with the addition of agitation.

Cell suspensions grew with a $\mu max = 0.158$ d^{-1} leading to a biomass production of up to 500 g l^{-1} fr. wt. and 14.5 g l^{-1} dry wt. (Fig. 3). The lag phase was relatively long (3–4 days) maybe due to the small size of the inoculum (25 g l^{-1}). Indeed, this phase could be reduced to 1–2 days if the inoculation was 50 g l^{-1} (data not shown). The cell growth kinetics were similar for the fr. wt. and the dry wt., with the exception that the dry wt stopped increasing at day 15 while the fr. wt. increased

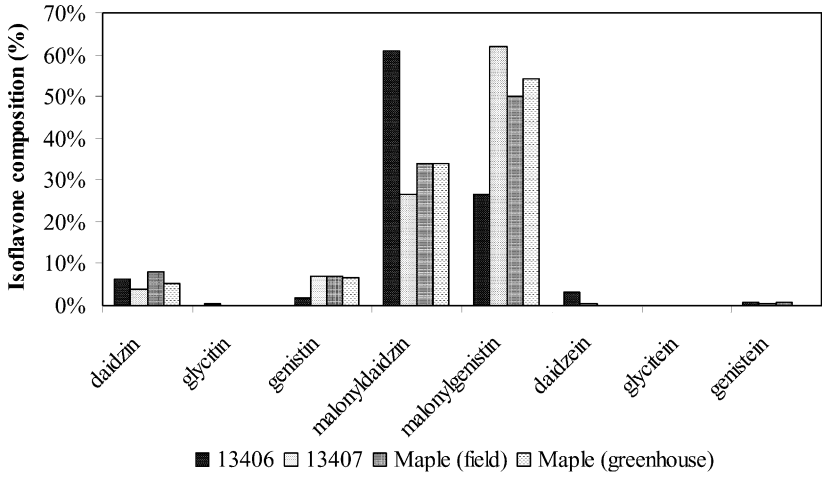

Fig. 2. Isoflavone composition of two callus strains (no. 13406 and no. 13407) and seeds from plants cultivated in field and in greenhouse of Maple arrow cv. Strain no. 13406 was richer in daidzin forms (ca. 70% of total) while strain no. 13407 contained more genistin forms (ca. 70% of total). Both seeds had the same composition (ca. 40% of daidzin forms and ca. 60% of genistin forms). Isoflavone composition was analyzed by HPLC and expressed as% of the total main isoflavone forms.

up to day 20. This means that the cells stopped growing but became larger in volume, accumulating liquid, a phenomenon very often observed at the end of the growth phase in plant cell culture.

The initial sucrose was hydrolysed regularly to glucose and fructose, with the glucose being consumed preferentially (Fig. 3). The absence of sugars after day 15 correlated well with the beginning of the stationary phase of growth. Indeed, the addition of sucrose at the end of the growth phase led to a higher biomass (data not shown), showing that sugars are one of the limiting factors for growth. The culture conditions can be optimized considering that the μmax obtained in this study was lower than for other cell cultures such as *Peganum harmala* (0.235 d^{-1}, Courtois et al., 1988) or tobacco (0.696 d^{-1}, Verdelhan des Molles et al., 1999).

The transfer to liquid medium was not detrimental to isoflavone production. In fact, an isoflavone concentration of up to 72 mg g^{-1} dry wt. was obtained, which was higher than in callus culture (Fig. 4). No isoflavones were detected in the culture media. The isoflavone concentrations were much higher than those already published. Ames and Worden (1997) obtained 10–200 μg g^{-1} dry wt. trying to overcome the limitation of production due to the intracellular storage of isoflavones by using a magneto-fluidized bioreactor. Zacharius et al. (1990) found amounts from 600 μg g^{-1} dry wt up to 15–20 mg g^{-1} dry wt., mainly genistein.

It is important to note that the isoflavone accumulation began at the end of the growth phase (day 10) and continued even after the end of this phase (day 15) during the stationary phase of growth (day 22), with a rate of 5.3 mg g^{-1} dry wt. day^{-1}. This demonstrated that even when the cells stopped growing, the secondary metabolite synthesis continued together with the increase of the cell volume. This is not surprising since isoflavones are known to accumulate within the vacuole. The total isoflavones production was 880 mg l^{-1} (Fig. 4), but it may

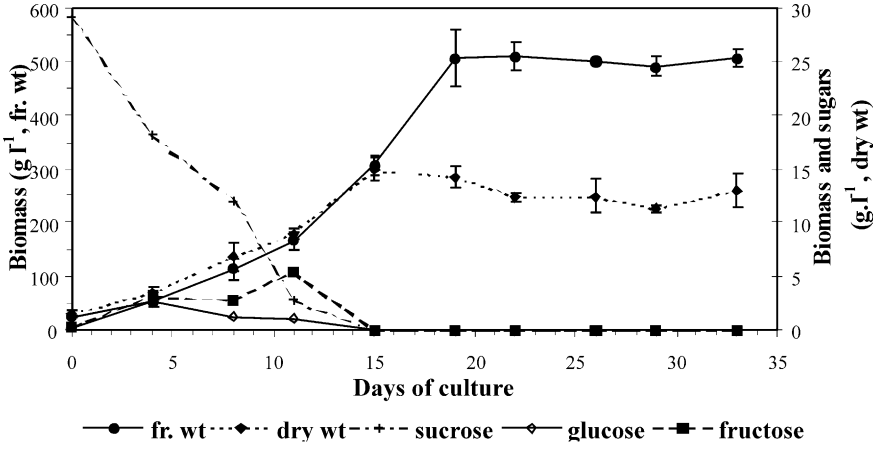

Fig. 3. Biomass production (fr. wt. and dry wt. basis) and sugar consumption in suspension culture of strain no. 13406. The biomass production reached 500 g l^{-1} fr. wt and 14.5 g l^{-1} dry wt. The dry wt increased up to day 15 while the fr. wt increased up to day 20. Initial sucrose was hydrolysed to glucose and fructose and no sugars were detected after day 15. The strain no. 13406, originated from Maple arrow cv. was transferred in liquid medium and cultivated in a 250 ml Erlenmeyer flask on an orbital shaker. Biomass growth and sugars were measured periodically taking an aliquot of cell suspension.

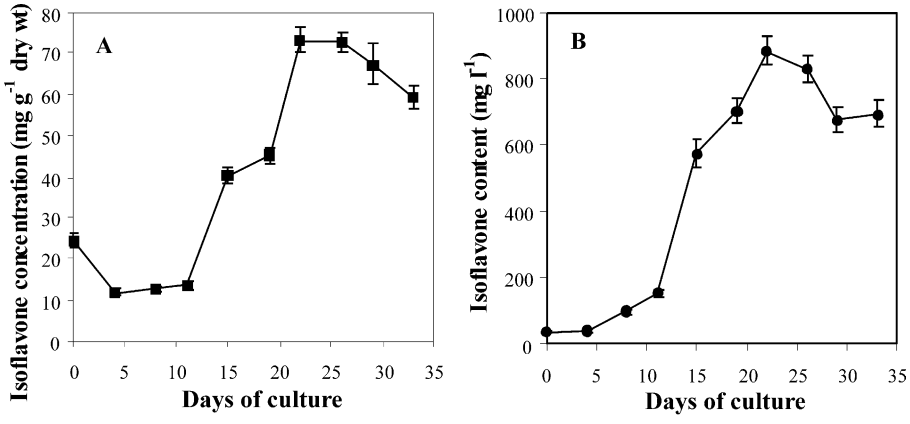

Fig. 4. Isoflavone production in suspension culture of strain no. 13406. An isoflavone concentration of up to 72 mg g^{-1} dry wt (A) and a total isoflavones content of up to 880 mg l^{-1} (B) was obtained. Isoflavone production was assessed by HPLC on aliquots of cell suspension cultivated in a 250 ml Erlenmeyer flask on an orbital shaker.

be postulated that with a larger tank more isoflavones could be produced.

The isoflavone composition during the liquid culture is reported in Fig. 5. With the exception of slight differences observed during the first days of cultures, corresponding to the lag phase and beginning of the growth phase, the ratios remain stable, with 70% of malonyl–daidzin, 20% of malonyl–genistin and 10% of other forms (mainly daidzin and genistin and traces of daidzein and genistein). These values were very similar to those observed in the callus culture.

Similar results were obtained in preliminary experiments in a classical 10 l stirred-tank bioreactor (data not shown).

For the optimization of the isoflavone production, key factors such as the medium composition, the influence of light and the bioreactor design must be studied in more depth. Nevertheless, the high production costs for plant cell suspension cultures in traditional tank bioreactors limit the economic development of such a technique. The use of new generation of culture processes such as non-autoclaved systems (Hsiao et al., 1999) or specific plastic bags (Singh, 1999) could improve the productivity and therefore reduce the cost of production.

2.3. Estrogenic activity

The estrogenic activity of pure isoflavone standards and of isoflavone crude extracts obtained from soya callus strains was determined using a bioassay in human endometrial Ishikawa cells. This cell line possesses an endogenous estrogen-inducible alkaline phosphatase (ALP). The estrogenic activity is thus assessed by determining the rate of *p*-nitrophenol generated from *p*-nitrophenylphosphate, subsequent to the treatment with test compounds (Littlefield et al., 1990; Pisha and Pezzuto, 1997).

Preliminary experiments were performed to examine the applicability of the Ishikawa/ALP bioassay to the evaluation of estrogenic activities in crude soybean callus culture extracts. An isoflavone extract obtained from a pool of soybean cell cultures, estrogen (17β-estradiol) and malonyl-genistin were tested at concentrations previously determined to be sufficiently high to exhibit maximal activity. Importantly, the crude plant cell culture extract was not cytotoxic to the Ishikawa cells and the same levels of ALP activity were induced by the isoflavone pure standard and crude extract and by estrogen (data not shown). To ensure that the alkaline phosphatase activity induced in the Ishikawa cells depends only on the isoflavone concentration of the sample tested, a plant extract with a very low isoflavone concentration and a hexane plant extract (free of isoflavones) were tested. In both cases no enzymatic activity was detected (data not shown).

Two crude soya cell culture extracts of known isoflavone content were tested: extract A, from callus n°13203 and extract B from callus n°12805, containing 11.3 mg g^{-1} and 1.2 mg g^{-1} dry wt. of isoflavones, respectively. These two extracts were compared in dose response assays with pure isoflavones and an estrogen control. The two dose-response curves obtained (Fig. 6) depended only on the isoflavone concentration of the extracts and presented a shape similar to those of the standards (data not shown). This means that the estrogenic activity was directly correlated to the isoflavone content of the extracts and that no other compounds in the crude extracts interfered with the estrogenic bioassay.

The EC_{50} values calculated from the curves, which represent the concentrations at which the compounds exert one half of their maximum effects on ALP activity in Ishikawa cells, are reported in Table 1. In agreement with published data (Markiewicz et al., 1993) estradiol was found to be 1000–10,000 times more active than isoflavones. The aglycones showed significantly higher

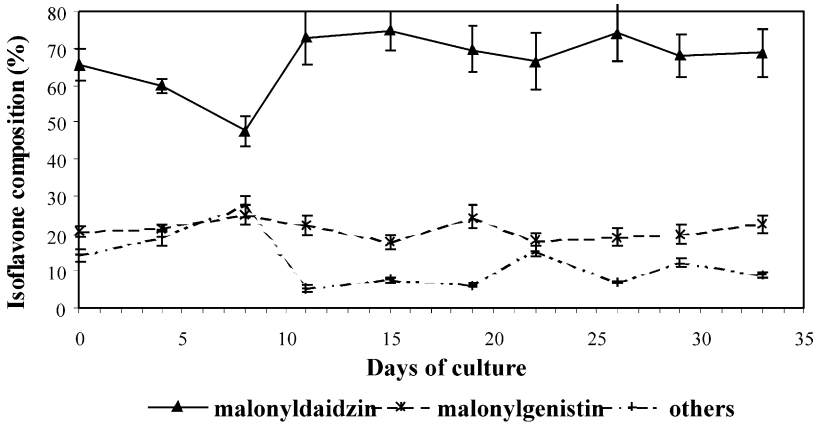

Fig. 5. Isoflavone composition during suspension culture of strain no. 13406. The isoflavone composition of the suspension culture (70% of malonyl–daidzin, 20% of malonyl–genistin and 10% of other forms) was very similar to the callus culture of the same strain. Isoflavone composition was analyzed by HPLC on aliquots of cell suspension cultivated in a 250 ml Erlenmeyer flask on an orbital shaker.

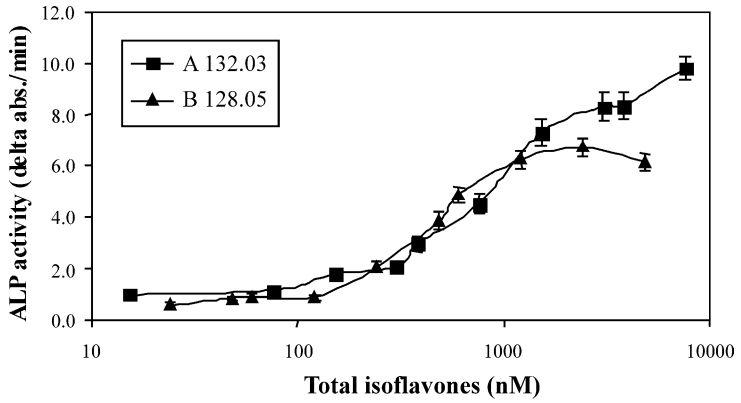

Fig. 6. Estrogenic activity of two crude soya cell culture extracts in the Ishikawa cell line. Ishikawa cells were cultured for 72 h with extract A (from callus no. 13203 containing 11.3 mg g^{-1} dry wt. of isoflavones) and extract B (from callus no. 12805 containing 1.2 mg g^{-1} dry wt. of isoflavones) and the estrogenic activity was assessed by determining the rate of *p*-nitrophenol generated from *p*-nitrophenylphosphate added as a substrate. The two dose-response curves obtained depended only on the isoflavone concentration of the extracts.

activity than the glycosides and genistein displayed an activity approximately 6 times higher than daidzein. Nevertheless, the glycoside forms did show some activity. In particular, the activity of the malonyl conjugate was determined for the first time. Similar to the aglycones, malonylgenistin showed an approximately 6 times higher activity than malonyldaidzin.

The EC_{50} values of the crude soya cell culture extracts (880 nM and 413 nM for extract A and extract B, respectively) were found to be between that of the pure malonyl-genistin (350 nM) and malonyl-daidzin (1880 nM). In fact, malonyls are the main form of isoflavones present in the extracts. Hydrolysis of the extracts with β-glycosidase to generate the aglycone forms would be expected to increase the estrogenic activity.

Several in vitro test systems have been developed for the assessment of steroidal and non-steroidal (phytoestrogens) estrogenic activities, recently reviewed by Diel et al. (1999). They are mainly based on receptor binding assays (Kuiper et al., 1998; Garrett et al., 1999), cell proliferation assays (Sathyamoorthy and Wang, 1997) and gene reporter assays (Collins et al., 1997; Shiizaki et al., 1999). All these systems present some drawbacks, for example, the binding to the receptor cannot distinguish between agonistic and antagonistic actions, the cell proliferation may be influenced by non-estrogenic substances and gene reporter assays represent a gene construct in an artificial cell environment. The model we used here is based on the modulation of the endogenous alkaline phosphatase activity in Ishikawa cells which represents a functional response to physiological doses of estrogenic compounds.

The results obtained in the present study demonstrated that this bioassay was compatible with crude extracts from soya cell cultures and so it can be successfully used in the future as a screening method to assess the presence of bioactive phytoestrogens in plant extracts. Indeed, Liu et al. (2001) have recently included this method in a panel of in vitro assays for the evaluation of the estrogenic activities of eight botanical preparations commonly used for the treatment of menopausal symptoms.

Table 1
EC_{50} values of pure isoflavones, estrogen and soya callus culture extracts tested in the Ishikawa bioassay[a]

		EC_{50} (nM)
Isoflavones	Genistein	134
	Daidzein	676
	Genistin	110
	Daidzin	630
	Malonyl-Genistin	350
	Malonyl-Daidzin	1880
Soya callus culture extracts	Ext. A	880
	Ext. B	413
Estrogen	17β-Estradiol	0.089

[a] EC_{50} values were calculated from the dose-response curves and represent the concentrations at which the compounds exerted one half of their maximum effects on ALP activity in Ishikawa cells.

3. Conclusions

A large variability in the isoflavone content of a collection of soya cell strains was observed and high-producing strains, with an isoflavone content up to 46.3 mg g^{-1} dry wt., were identified. High isoflavone content (7% dry wt.), concentration (880 mg l^{-1}) and a maximum productivity estimated to 60 mg l^{-1} day^{-1} were obtained in an Erlenmeyer flask as well as in a 10 l stirred-tank bioreactor. The Ishikawa/ALP bioassay was successfully used to measure the estrogenic activities of pure isoflavones and soya cell extracts. The activity of the extracts correlated to their isoflavone content. Thus, the present study reports a high production of bioactive phytoestrogens in soya plant cell cultures.

4. Experimental

4.1. Plant tissue cultures

Tissue culture strains of *Glycine max* (L.) Merr. were initiated from four different cultivars on the medium described by Gamborg et al. (1968) supplemented with 20 g l^{-1} sucrose, 7 g l^{-1} agar (bacto-agar Difco) and 1 mg l^{-1} 2,4-D. The pH was adjusted to 5.8 prior to autoclaving (30 min at 115 °C). 40 strains were subcultured on the same medium in Petri dishes (10 cm diameter) monthly at 26 °C under light (16 h photoperiod, 36 μmol.m^{-2} s^{-1}, cool white fluorescent lamps, Sylvania Groslux, Germany). 21 strains originated from Amsoy cv., 11 from Altona cv., 7 from Maple Arrow cv. and 1 from Mandarin cv.

The strain no. 13406, originated from Maple arrow cv. (deposited according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany under the ref. 134 06-DSM 14883) was transferred in a liquid medium with the same composition as for the tissue cultures but without agar and and with the addition of 30 g l^{-1} of sucrose. The culture was carried out with an inoculum of 5 g l^{-1} fr. wt. in 100 ml medium in a 250 ml Erlenmeyer flask placed on an orbital shaker (New Brunswick Scientific, Edison, USA) with a shaking diameter of 20 min at 100 rpm and subcultured every two weeks.

Soya seeds of the same cultivars were analyzed directly or after one cycle in greenhouse.

4.2. Analysis

To assess the biomass growth the dry weight was measured periodically after freeze-drying of an aliquot of fresh cells.

Sugars (Sucrose, glucose, fructose) were determined enzymatically with the Assay Reagent Kit from Roche Molecular Biochemicals, USA (no. 716260).

Extraction and analysis: 1 g of freeze-dried cell cultures or soybeans were ground in a mortar, mixed with 1 g of celite and packed into a 25 ml plastic column, between two frits. 50 ml of methanol/water (80/20, v/v) were sucked through the sample in the column and the extract obtained was filtered through a 0.45 μm membrane filter prior to injection in the HPLC system. First evaluation of the isoflavone content was performed at the time of subculture (1 month). Experiments were performed in triplicate. For kinetics on solid medium, 3 Petri dishes were harvested per experimental points and analyzed separately. For kinetics in liquid medium, the same procedure was followed, using three 250 ml Erlenmeyer flask per experimental point.

The HPLC analysis of isoflavones was performed using a Nucleosil 100-C_{18} column (250 cm×4.3 mm, Macherey-Nagel, Germany). Elution was carried out at a flow rate of 1.0 ml min^{-1} at room temperature, with a linear gradient composed of (A) pure acetonitrile and (B) 0.05% phosphoric acid in water. Following injection of 20 μl of sample, solvent A was increased linearly from 10 to 50% for 40 min, then 50–100% for 3 min and held at 100% for 5 min. The column was reequilibrated running for 10 min at the initial conditions before proceeding with the next injection. Analyses were monitored with a photodiode array detector at 258 nm, scanning between 220 and 400 nm for peak identification. Controls were daidzin, genistin, daidzein and genistein from Extrasynthèse, France, and malonyldaidzin and malonylgenistin were purified from soybean extracts. Retention times were 12, 15.7, 16.3, 19.7, 21.4 and 26.8 for daidzin, genistin, malonyldaidzin, malonylgenistin, daidzein and genistein, respectively.

HPLC-MS analyses were performed on a Waters Alliance 2690 system, coupled to a Micromass "Quattro-LC" with Z-spray source interface fitted with an electrospray probe. The source block temperature was set to 150 °C, the sprayer voltage to 3.1 kV and the desolvation temperature to 250 °C. Nitrogen was used as desolvation gas (690 l/h) and cone gas curtain (180 l/h). The HPLC separation of the isoflavones was performed using a Nucleosil 100-C_{18} column (250 cm×2.1 mm, Macherey-Nagel, Germany). Elution was carried out at a flow rate of 0.3 ml min^{-1} at 32 °C and one tenth of the flow was admitted to the MS using a post-column splitter. Solvents used were A) water and B) acetonitrile, both acidified with 0.5% of acetic acid. A 20 μl injection volume was used for the identification of the isoflavones using the following gradient: 10% B for 5 min, increasing to 90% B in 250 min, then staying at 90% B for 5 min. Full scan mass spectra were recorded over the mass range m/z 100–600 Da with a scan time of 1 s. Product ion mass spectra were acquired over the same mass range. Nitrogen was used as the collision gas and the collision energy was set at 20 V. Data acquisition and data evaluation were performed using the Micromass MassLynx software, v3.5 SP367.

4.3. Estrogenic activity assay

The human endometrial Ishikawa cell line was used as an in vitro bioassay to test the estrogenic activities of pure isoflavone standards and of isoflavone crude extracts obtained from soybean callus strains (Littlefield et al., 1990; Pisha and Pezzuto, 1997).

The Ishikawa cells were routinely cultured in minimum essential medium (DMEM/F12) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 1 mM sodium pyruvate. Cells were plated at 1.5×106 cells/75 cm^2 surface area at 37 °C and passaged twice weekly. Two days before starting an experiment, near-confluent cells were changed to an estrogen-free medium, DMEM/F12

without phenol red and with 10% (v/v) charcoal stripped serum. After 24 h the cells were harvested with 0.25% trypsin and plated in 96-well microtiter plates in estrogen-free medium at a density of 2.5×104 cells/well. The day of the experiment the medium was replaced with fresh estrogen-free medium containing different concentrations of the test compounds in a final volume of 150 μl. The cells were then incubated at 37 °C for 72 h.

After washing and freeze-thawing the cells (15 min at −80 °C followed by 5–10 min at room temperature) 150 μl of a *p*-nitrophenylphosphate solution were added to the cells and the alkaline phosphatase activity was monitored with an ELISA plate reader at 405 nm.

Cell growth and cytotoxicity were also determined by a Neutral Red assay.

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References

Alfermann, A.W., Petersen, M., 1995. Natural product formation by plant cell biotechnology. Plant Cell, Tissue Organ Cult. 43, 199–205.

Ames, T.T., Worden, R.M., 1997. Continuous production of daidzein and genistein from soybean in a magnetofluidized bed bioreactor. Biotechnol. Prog. 13, 336–339.

Anderson, J.B., Garner, S.C., 1999. Phytoestrogens and bone. Baillieres Clin. Endocrinol. Metab. 12, 543–557.

Berlin, J., Fecker, L., Ruegenhagen, C., Sator, C., Strack, D., Witte, L., 1991. Isoflavone glycoside formation in transformed and non-transformed suspension and hairy-root cultures of *Lupinus polyphyllus* and *Lupinus hartwegii*. Z. Naturforsch. C 46, 725–734.

Bouque, V., Bourgaud, F., Nguyen, C., Guckert, A., 1998. Production of daidzein by callus cultures of *Psoralea* species and comparison with plants. Plant Cell, Tissue Organ Cult. 53, 35–41.

Collins, B.M., McLachlan, J.A., Arnold, S.F., 1997. The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. Steroids 62, 365–372.

Courtois, D., Yvernel, Y., Florin, B., Pétiard, V., 1988. Conversion of tryptamine to serotonin by cell suspension cultures of *Peganum harmala*. Phytochemistry 27, 3137–3142.

Diel, P., Smolnikar, K., Michna, H., 1999. In vitro test systems for the evaluation of the estrogenic activity of natural products. Planta Med. 65, 197–203.

Eldridge, A.C., Kwolek, W.F., 1983. Soybean isoflavones: effect of environment and variety on composition. J. Agric. Food Chem. 31, 394–396.

Fedoreyev, S.A., Pokushalova, T.V., Veselova, M.V., Glebko, L., Kulesh, N.I., Muzarok, T.I., Seletskaya, L.D., Bulgakov, V.P., Zhuravlev, Y.N., 2000. Isoflavonoid production by callus cultures of *Maackia amurensis*. Fitoterapia 71, 365–372.

Fu, T.J., Singh, G., Curtis, W.R., 1999. Plant Cell Culture for the Production of Food Ingredients. Kluwer Acad./Plenum, New York.

Garrett, S.D., Lee, H.A., Morgan, M.R., 1999. A nonisotopic estrogen receptor-based assay to detect estrogenic compounds. Nat. Biotechnol. 17, 1219–1222.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158.

Graham, T.L., Kin, J.E., Graham, M.Y., 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophtora megasperma*. Mol. Plant-Microbe Interact. 3, 157–166.

Hsiao, T.Y., Bacani, F.T., Carvalho, E.B., Curtis, W.R., 1999. Development of a low capital investment reactor system: application for plant cell suspension culture. Biotechnol. Progr. 15, 114–122.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252–4263.

Liggins, J., Bluck, L.J.C., Coward, W.A., Bingham, S.A., 1998. Extraction and quantification of daidzein and genistein in food. Anal. Biochem. 264, 1–7.

Littlefield, B.A., Gurpide, E., Markiewicz, L., McKinley, B., Hochberg, R.B., 1990. A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids. Endocrinology 127, 2757–2762.

Liu, J., Burdette, J.E., Xu, H., Gu, C., van Breemen, R.B., Bhat, K.P., Booth, N., Constantinou, A.I., Pezzuto, J.M., Fong, H.H., Farnsworth, N.R., Bolton, J.L., 2001. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. J. Agric. Food Chem. 49, 2472–2479.

Markiewicz, L., Garey, J., Adlercreutz, H., Gurpide, E., 1993. In vitro bioassays of non-steroidal phytoestrogens. J. Steroid Biochem. Mol. Biol. 45, 399–405.

Messina, M.J., Loprinzi, C.L., 2001. Soy for breast cancer survivors: a critical review of the literature. J. Nutr. 131, 3095S–3108S.

Murphy, P.A., Barua, K., Song, T., Harvey, G., 1999. Phytoestrogens in foods and plants: extraction and isolation. J. Med. Food 2, 107–109.

Pisha, E., Pezzuto, J.M., 1997. Cell based assay for the determination of estrogenic and anti-estrogenic activities. Methods in Cell Sci. 19, 37–43.

Potter, S.M., Baum, J.A., Teng, H., Stillman, R.J., Shay, N.F., Erdman jr, J.W., 1998. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. Am. J. Clin. Nutr. 68, 1375S–1379S.

Reinli, K., Block, G., 1996. Phytoestrogen content of food-A compendium of literature values. Nutr. Cancer 26, 123–148.

Sathyamoorthy, N., Wang, T.T.Y., 1997. Differential effects of dietary phyto-oestrogens daidzein and equol on human breast cancer MCF-7 cells. Eur. J. Cancer 33, 2384–2389.

Singh, V., 1999. Novel disposable bioreactor. Gen. Eng. News, March, p. 23.

Shiizaki, K., Goto, K., Ishige, A., Komatsu, Y., 1999. Bioassay of phytoestrogen in herbal medicine used for postmenopausal disorder using transformed MCF-7 cells. Phytother. Res. 13, 498–503.

Stöckigt, J., Obitz, P., Falkenhagen, H., Lutterbach, R., Endreβ, S., 1995. Natural products and enzymes from plant cell cultures. Plant Cell, Tissue Organ Cult. 43, 97–109.

Verdelhan des Molles, D., Gomord, V., Bastin, M., Faye, L., Courtois, D., 1999. Expression of a carrot invertase gene in tobacco suspension cells cultivated in batch and continuous culture conditions. J. Biosci. Bioeng. 87, 302–306.

Wang, H., Murphy, P.A., 1994. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J. Agric. Food Chem. 42, 1674–1677.

Zacharius, R.M., Kalan, E.B., 1990. Isoflavonoid changes in soybean cell suspensions when challenged with intact bacteria or fungal elicitors. J. Plant Physiol. 135, 732–736.