Genista tinctoria Hairy Root Cultures for Selective Production of Isoliquiritigenin

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Hairy root cultures were established after inoculation of *Genista tinctoria in vitro* shoots with *Agrobacterium rhizogenes*, strain ATCC 15834. In transformed roots of *G. tinctoria* grown in Schenk-Hildebrandt medium without growth regulators the biosynthesis of isoflavones, derivatives of genistein and daidzein, and flavones, derivatives of luteolin and apigenin, characteristic for the intact plant, was completely inhibited. The only compound synthesized in *G. tinctoria* hairy roots was isoliquiritigenin (2.3 g/100 g DW), a daidzein precursor absent in the intact plant. This compound was stored entirely within cells and it was not until abscisic acid was added (37.8 μM supplement on day 42) that approx. 80% of it was released into the experimental medium. The paper discusses the effect of abscisic acid on the growth of *G. tinctoria* hairy root cultures, the biosynthesis of isoliquiritigenin and the way it is stored. A prototype basket-bubble bioreactor was designed and built to upgrade the scale of the *G. tinctoria* hairy root cultures. With immobilized roots and a new aeration system, large amounts of biomass were obtained (FW_{max} 914.5 g l^{-1}) which produced high contents of isoliquiritigenin (2.9 g/100 g DW). The abscisic acid-induced release of the metabolite from the tissue into the growth medium greatly facilitated subsequent extraction and purification of isoliquiritigenin.

Key words: Abscisic Acid, Basket-bubble Bioreactor, Isoliquiritigenin

Introduction

For many years, *in vitro* plant cultures have been seen as an alternative source of biologically active compounds (Verpoorte *et al.*, 1998; Bourgaud *et al.*, 2001). However, despite numerous experiments, only a few led to the successful development of *in vitro* plant systems capable of producing significant amounts of secondary metabolites (*e.g.* shikonin, berberin). The reason for the frequent failure rate is that plant cell cultures are genetically unstable and very often produce low yields of secondary metabolites (Ramachandra Rao and Ravishankar, 2002). A lot of potential, on the other hand, is seen in transformed root cultures. Hairy roots obtained by infecting plants with *Agrobacterium rhizogenes* are unique in their genetic and biosynthetic stability. They are not only able to anergise growth regulators but are also characterized by fast growth, which seems to make them appropriate for the production of valuable secondary metabolites (Shen *et al.*, 1988; Wysokińska and Chmiel, 1997; Giri and Narasu, 2000).

One of the many groups of natural compounds of plant origin which have valuable pharmacological properties are isoflavones (Dixon and Steele, 1999; Qiang *et al.*, 2001). Thanks to their affinity to estrogen receptors β they exhibit both estrogenic and anti-estrogenic effects. Therefore, plant extracts of isoflavones are successfully used to treat menopause-related conditions (Dixon and Steele, 1999; Qiang *et al.*, 2001; Radzikowski *et al.*, 2004). Moreover, pharmacological investigations of genistein, daidzein and their derivatives proved that these compounds can inhibit formation and growth of cancers which are directly related to the estrogenic balance within the body (Radzikowski *et al.*, 2004).

Biotechnological research previously carried out by the authors of this paper showed that *in vitro* cultures of *Genista tinctoria* L. accumulate some of the highest concentrations of phytoestrogens ever produced *in vitro* (Łuczkiewicz and Głód, 2003). They exceed in this respect not only the intact plant, but also soy bean products, which have traditionally been considered to be the main source of isoflavones (Federici *et al.*, 2003). The phyto-

Abbreviations: ABA, abscisic acid; DW, dry weight; FW, fresh weight; SH, Schenk-Hildebrandt medium (Schenk and Hildebrandt, 1972); SH_0, Schenk-Hildebrandt medium without growth regulators.

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chemical analysis of the obtained suspension, embryo and shoot cultures of *G. tinctoria* showed that the ratio of isoflavonoid accumulation in *in vitro* cultures was influenced greatly by tissue differentiation (Łuczkiewicz and Głód, 2005).

The main purpose of this work was to obtain hairy root cultures of *G. tinctoria* and to determine whether the process of transformation with a wild strain of *A. rhizogenes* affects the metabolism of the tissue in terms of flavonoid production. The obtained root cultures were then compared with the intact plant in terms of phytoestrogen production to determine whether hairy root cultures of *G. tinctoria* are a promising system for secondary metabolite production.

In this paper we report for the first time the induction of *G. tinctoria* hairy root cultures able to accumulate large amounts of a single isoflavonoid precursor-isoliquiritigenin. The growth characteristics were described for the obtained cultures on the particular days of the experiment. Moreover the metabolism of roots was analyzed in terms of isoflavonoids and the results were compared with the intact plant. The feeding experiment showed the effect of the addition of abscisic acid to the experimental medium on the production and storage of isoliquiritigenin. The *G. tinctoria* hairy root system was then upgraded from Erlenmeyer flask to bioreactor. In order to obtain a large-scale culture system for isoliquiritigenin production a prototype basket-bubble bioreactor was built (construction details are presented in the paper). The calculations of productivity parameters make it possible to evaluate the suitability of the basket-bubble bioreactor for large-scale production of isoliquiritigenin.

Materials and Methods

Plant material

Seeds of *G. tinctoria* (Botanic Gardens, Glasgow) obtained in the year 2000 were used in the development of seedlings, shoot cultures and *in vitro* plantlets. Samples of these seeds are deposited at the herbarium of the Medicinal Plant Garden of the Medical University of Gdańsk. The seeds were surface sterilized in 0.1% aqueous solution of $HgCl_2$ for 45 min, rinsed three times with sterile, double distilled water and placed in petri dishes, on sterile paper soaked in GA_3 (28.9 μM) and kinetin (46.0 μM). The dishes were sealed with parafilm and kept in the dark at (25 ± 2) °C and after a week seedlings for transformation were obtained. The plantlets of *G. tinctoria* were grown on Schenk-Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) without any growth regulators, supplemented with 3% w/v sucrose and solidified with 0.7% w/v of agar. *G. tinctoria* shoot cultures were grown on SH medium supplemented with 9.84 μM 2iP, 0.99 μM TDZ, 3% w/v sucrose and solidified with 0.7% w/v of agar, for 5 weeks. All *in vitro* cultures were maintained at (25 ± 2) °C under continuous light (intensity 88 ± 8 μmol m^{-2} s^{-1}).

Bacteria strains

Agrobacterium rhizogenes, agropine strains LBA 9402 and ATCC 15834 were grown on solid yeast tryptone broth (TY) medium (Łuczkiewicz *et al.*, 2002) supplemented with 200 μM acetosyringone in the dark, at 26 °C. The 24-h grown bacteria cultures were used for tissue infection.

Transformation and induction of hairy root cultures

Using a syringe (Ø 0.75 mm) the bacteria were inoculated into the hypocotyl region of 7-day-old seedlings, the petioli and leaf laminae of *G. tinctoria* plantlets as well as into the shoot primordia of *in vitro* cultures. After inoculation, the explants were transferred into SH agar medium without growth regulators and cultured in darkness (25 ± 2) °C. Simultaneously, non-infected plant material of *G. tinctoria* was grown under the same conditions as the infected plant tissues. After 14 d, when hairy roots had developed, they were excised from the original explants and transferred to an antibiotic-containing medium; the same medium as described above, but supplemented with filter sterilized 50 mg l^{-1} of tetracycline. Excised hairy roots were kept and subcultured three times on this medium (20 d each) to fully eradicate the bacteria. Finally, bacteria-free, actively growing hairy roots were cut into small segments and subcultured in SH medium (Schenk and Hildebrandt, 1972) with 3% w/v sucrose.

G. tinctoria hairy root cultures for flavonoid production were grown using SH medium without growth regulators and supplemented with 3% w/v sucrose. A total of 5 g fresh weight (FW) of root biomass was inoculated in each 250 ml Erlenmeyer flask containing 100 ml of the medium on an orbital shaker and grown for 60 d. In order to

trigger the release of isoliquiritigenin from the root tissue into the growth medium on day 0 or 42 of the experiment the media were supplemented with 37.8 μM of abscisic acid (ABA). Hairy roots in all media (SH_0 and SH with ABA) were grown on an orbital shaker at 150 rpm [(25 ± 2) °C, with continuous light, intensity of 88 ± 8 μmol m^{-2} s^{-1}]. Three sample flasks from each culture type (for biomass and media analysis) were collected every two days during the whole experiment. Fresh weight (FW) and dry weight (DW) of all biomasses were recorded and flavonoid extraction, from plant matrix and media, performed as described before (Łuczkiewicz *et al.*, 2004). The results were analyzed statistically with *t*-Student's test for comparison of the mean and to assess differences. Flavonoid content (HPLC analysis) and DW values of all three replicates were determined after biomass and media lyophilization (Łuczkiewicz *et al.*, 2004).

Large-scale G. tinctoria hairy root culture system

A basket-bubble bioreactor was made of two identical glass domes (Rasotherm, Germany), 100 mm high, largest internal diameter 180 mm, connected with 8 clamps (Fig. 1). The joint between the glass domes was additionally sealed with a silicon gasket (dimensions DN 150, Schott-Duran, Mainz, Germany). In order to immobilize root cultures inside the growth vessel, a stainless steel (18/8) basket was placed, mesh diameter 8 mm (Bochem Laborbedarf, Weilburg, Germany). The basket, 100 mm in diameter and 80 mm in height, was placed concentrically inside the vessel, so that the clearance between the bottom of the basket and the bottom of the lower dome was 20 mm (Fig. 1). Hairy roots of *G. tinctoria* (ca 25.0 g) were evenly placed in the basket. After filling the growth vessel with 500 ml of the SH medium the roots were fully immersed in the growth medium. The culture was aerated with forced air flow from the bottom of the growth vessel via a silicon hose, 4 mm in diameter and 1 mm wall thickness (Deutsch-Neumann, Berlin, Germany) using a membrane pump with flow regulation (Maxima R Air Pump, 6 W, 6 PSI, Rolf C. Hagen Inc., Montreal, Quebec, Canada). The air flow through the culture was 800 ml/min. In order to ensure sterile conditions for the cultures, sterilizing filters were used (Millex Vent Filter, 50 mm diameter, PTFE membrane, 0.2 mm pore size; Millipore Corporation, Bedford, USA) both on the inflow ducts and at the outflow from the growth vessel (Fig. 1). In order to prevent loss of media during aeration, prior to passing through sterilizing filters, the air was forced through a water washer (Fig. 1).

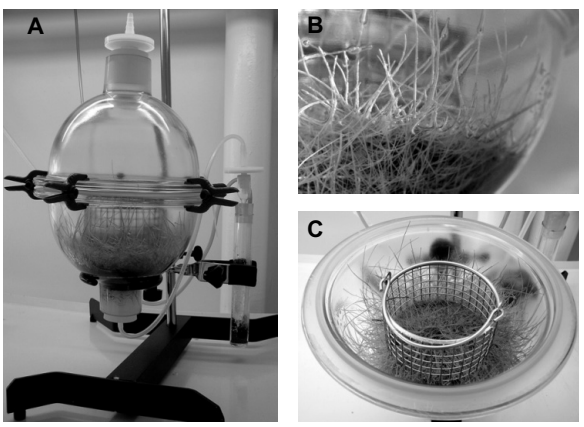

Fig. 1. *Genista tinctoria* hairy root cultures grown in the basket-bubble bioreactor in liquid SH medium with 37.8 μM ABA supplemented on day 42 [(25 ± 2) °C; light intensity 88 μmol m^{-2} s^{-1}]. (A) The basket-bubble bioreactor, general view; (B) the basket-bubble bioreactor, side view; (C) the basket-bubble bioreactor, upper view.

G. tinctoria hairy roots were cultivated in the bioreactor under the same conditions as in Erlenmayer flask. In order to cause the release of isoliquiritigenin from the root biomass into the growth medium, on day 42 of the experiment the medium was supplemented with 37.8 μM ABA. The biomass and the media were collected on day 6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 by fully unloading the bioreactor in order to determine the FW, DW and to carry out phytochemical analysis of the biomass and the media. The experiment was repeated 3 times and the results were analyzed statistically with *t*-Student's test.

Opine detection in transformed root cultures

To prove the genetic transformation of *G. tinctoria* hairy roots the fresh tissues were extracted with double distilled water (1 ml g^{-1} tissue weight) and the solution obtained was centrifuged at 1100 × *g* for 5 min. The supernatant was evaporated under reduced pressure at 40 °C, the residue re-dissolved in water (0.2 ml g^{-1} FW of tissue) and centrifuged again. 10 μl of the supernatant was spotted onto 3MM chromatographic paper (Whatman) and the

amino acid derivatives separated by electrophoresis (400 V for 1 h) using a mixture of formic acid/acetic acid/water (1:3:16) as an electrophoresis medium. The migration of opines was determined by co-migration with opine standard. After drying, the opines were visualized by means of alkaline silver nitrate reagent (Thron *et al.*, 1989; Łuczkiewicz *et al.*, 2002).

Results and Discussion

Establishment of hairy root cultures

According to literature data different plant species and different tissues of a plant show various degrees of susceptibility to infection with *A. rhizogenes* (Thron *et al.*, 1989; Giri and Narasu, 2000). Moreover, despite several attempts to obtain hairy roots of plants from the Fabaceae family, it is still believed that *Agrobacterium*-mediated transformation of legumes is very difficult (Zhang *et al.*, 1999; Bednarek *et al.*, 2001). The main reason is the specific regulatory mechanisms related to the interactions of legumes with nitrogen fixing symbiotic rhizobia bacteria (Bednarek *et al.*, 2001). That is why the research into obtaining *G. tinctoria* hairy roots involved using two wild *A. rhizogenes* agropine strains: LBA 9402 and ATCC 15834. In addition, various tissues of *G. tinctoria* were used as initial material (hypocotyl regions of seedlings; 2-year-old shoot cultures; leaves and stems of 2-month-old plantlets grown *in vitro*). Hairy roots of *G. tinctoria* were only obtained when the ATCC 15834 strain was used and only when prior to inoculation the bacteria were grown on TY tryptone medium supplemented with 200 μM of acetosyringone. As previously observed by Stachel *et al.* (1985) and Królicka *et al.* (2001) this phenomenon can be explained by the activation of *A. rhizogenes vir* genes expression by acetosyringone.

Hairy roots occurred only on 32% of nodal segments of *G. tinctoria* shoot cultures. At the same time the hypocotyl region of seedlings as well as the petioli and the leaf laminae of *G. tictoria* never developed hairy roots. In case of *G. tinctoria* the obtained result, *i.e.* the hairy root cultures, was clearly the effect of using the right bacteria strain with the particular plant tissue. Only rootless 2-year-old shoot cultures of *G. tinctoria*, which have different hormonal status than seedlings and *in vitro* plantlets, proved to be susceptible to bacterial infection. Unlike the roots and shoots of the intact plant (Łuczkiewicz and Głód, 2003) *in vitro* shoots of *G. tinctoria* contained no simple flavones, derivatives of luteolin and apigenin (Łuczkiewicz and Głód, 2005). Perhaps this fact makes *in vitro* shoots exceptionally susceptible to infection by *A. rhizogenes*. The role played by flavonoids, and isoflavonoids in particular, in the relations between the plants of the Fabaceae family and symbiotic and pathogenic bacteria involves highly complex processes, which are widely discussed in numerous reports (Paiva *et al.*, 1994; Zhang *et al.*, 1999; Bednarek *et al.*, 2001). This experiment did not focus on explaining this process, so the above thesis must remain an assumption only.

After three passages on SH_o media with antibiotic, hairy root cultures were free of bacteria cells. The hairy roots obtained in this way displayed the characteristic plagiotropic growth and high incidence of lateral branching. To prove the transgenic character of *G. tinctoria* hairy roots an opine test was performed. In freshly established transformed root cultures considerable amounts of agropines were detected. The control explants (not infected) did not exhibit any tendency to form roots. Moreover, intact roots excised from *G. tinctoria in vitro* plantlets did not exhibit any elongation or growth when cultured in the same liquid medium as the hairy roots.

Growth and isoliquiritigenin production in G. tinctoria hairy roots grown in SH_0 medium

The use of Schenk-Hildebrandt medium without growth regulators yielded fast-growing cultures of *G. tinctoria* hairy roots. The maximum weight of the biomass (801.3 g l^{-1} FW and 40.6 g l^{-1} DW) was achieved on day 34 of the cycle (Table I). Since the *G. tinctoria* roots still displayed considerable vitality, the experiment was continued until day 60 in order to establish a complete growth profile. The growth cycle of *G. tinctoria* hairy roots has all the characteristic phases, with a rapid exponential phase and a weak necrotic phase (Table I). Despite growing in the light, the roots did not turn green or develop round callus-like structures, a phenomenon reported by many authors for hairy roots grown in the light (Pitta-Alvarez and Giulietti, 1995).

Phytochemical analysis of roots and the media from various stages of the experiment showed that the production of isoflavones, derivatives of genistein and daidzein, and simple flavones, deriva-

Table I. Changes in fresh (FW) and dry weights (DW) of *Genista tinctoria* hairy roots grown in SH medium without growth regulators or supplemented with 37.8 μM ABA[a].

Day of culture	Medium type					
	SH_0[b]		$SH_{ABA\ 0}$[c]		$SH_{ABA\ 42}$[d]	
	FW [g l^{-1}]	DW [g l^{-1}]	FW [g l^{-1}]	DW [g l^{-1}]	FW [g l^{-1}]	DW [g l^{-1}]
0	50.3 ± 2.1	2.7 ± 0.09	50.5 ± 1.7	2.3 ± 0.09	50.7 ± 1.9	2.02 ± 0.08
4	61.7 ± 2.4	3.7 ± 0.18	59.3 ± 2.5	2.0 ± 0.1	59.7 ± 1.9	2.3 ± 0.09
16	604.3 ± 24.3	28.2 ± 1.5	87.4 ± 4.1	2.6 ± 0.22	611.5 ± 19.4	25.6 ± 1.2
34	801.3 ± 25.3	40.6 ± 2.9	76.4 ± 4.8	2.2 ± 0.19	803.4 ± 21.8	32.1 ± 2.3
60	632.4 ± 22.2	26.5 ± 1.0	58.3 ± 2.7	1.1 ± 0.16	477.6 ± 16.4	19.1 ± 0.8

All cultures were maintained for 60 d under continuous light [(25 ± 2) °C; light intensity 88 μmol m^{-2} s^{-1}].
[a] Each value represents the mean ± SD of three samples.
[b] SH_0, SH liquid medium without growth regulators.
[c] $SH_{ABA\ 0}$, SH liquid medium with 37.8 μM ABA, supplemented on day 0.
[d] $SH_{ABA\ 42}$, SH liquid medium with 37.8 μM ABA, supplemented on day 42.

tives of luteolin and apigenin, characteristic for the intact plant (Łuczkiewicz and Głód, 2003), was completely inhibited. The only flavonoid found in the biomass was isoliquiritigenin (Fig. 2), which is absent in the intact plant (Łuczkiewicz and Głód, 2003). The storage of this compound was entirely intracellular, since no isoliquiritigenin was found in the media (Fig. 2). It can be assumed that in hairy roots an enzymatic inhibition occurs at the level of isoliquiritigenin, which is a direct precur-

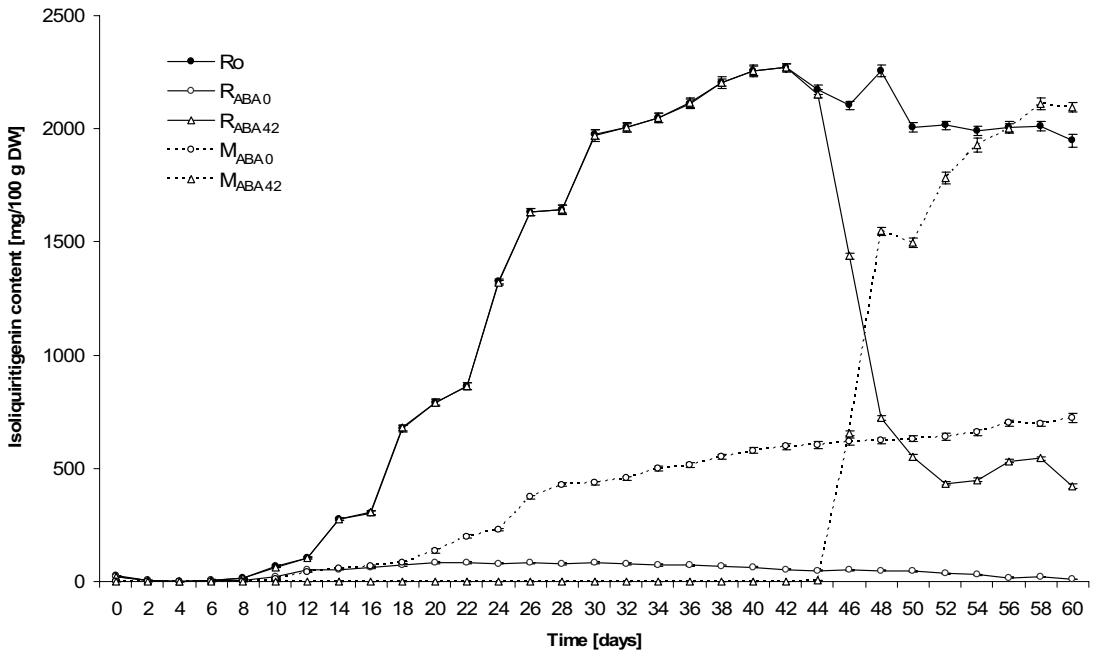

Fig. 2. Isoliquiritigenin content in *Genista tinctoria* hairy root cultures (measured in the biomass and the media) grown in SH medium without growth regulators (SH_0) or supplemented with 37.8 μM ABA (isoliquiritigenin content in mg/100 g DW plant material). Ro, isoliquiritigenin content in hairy roots grown in SH liquid medium without growth regulators (SH_0); $R_{ABA\ 0}$, isoliquiritigenin content in hairy roots grown in SH liquid medium with 37.8 μM ABA, supplemented on day 0; $M_{ABA\ 0}$, isoliquiritigenin content in SH media with 37.8 μM ABA, supplemented on day 0; $R_{ABA\ 42}$, isoliquiritigenin content in hairy roots grown in SH liquid medium with 37.8 μM ABA, supplemented on day 42; $M_{ABA\ 42}$, isoliquiritigenin content in SH media with 37.8 μM ABA, supplemented on day 42.

sor of daidzein (Dixon *et al.*, 1995; Qiang *et al.*, 2001). Existing reports and previous research carried out by the authors of this paper showed that metabolites from genistein and daidzein metabolic pathway are synthesized simultaneously and independently without competing for biosynthesis precursors (Shimada *et al.*, 2000; Łuczkiewicz and Głód, 2005). Apart from isoliquiritigenin, the root biomass investigated contained no derivatives of genistein or its direct precursor naringenin (Fig. 2). This indicates an enzymatic inhibition of the metabolic pathways of genistein, dominant in the intact plant (Łuczkiewicz and Głód, 2003). As a result the *G. tinctoria* hairy roots became a selective, rich source of isoliquiritigenin (2271.2 mg/100 g DW, day 42) (Fig. 2). The productivity calculated for this culture (719.97 mg l^{-1}) and the specific productivity (17.14 mg l^{-1}) indicate *G. tinctoria* hairy roots as a possible profitable source of this compound (Table II). Since the biosynthesis of isoliquiritigenin was most intensive in the stationary phase a rather long growth cycle is needed (Table I, Fig. 2).

Growth and isoliquiritigenin production in G. tinctoria hairy roots grown in SH medium supplemented with ABA

The inhibition of isoflavone production may indicate that *G. tinctoria* hairy roots might have a different hormonal status than the intact plant possibly caused by growing the biomass *in vitro* or the infection with *A. rhizogenes* bacteria. The research of cotyledons of *Phaseolus vulgaris* proved that in the investigated tissue material it was possible to significantly increase the production of isoflavonoid phytoalexins (phaseolin and kievitone) by simple incubation of the plant material with abscisic acid. It was suggested that ABA did not induce a stress situation but rather interfered with the regulation of phytoalexin biosynthesis. As a result the cotyledons of *P. vulgaris* produced 10 times more isoflavones than the control (Goossens and Vendrig, 1982). Based on the above report, a decision was made to check whether *G. tinctoria* hairy roots grown on SH medium supplemented with ABA would have a changed isoflavone production profile.

Table II. Comparison of maximum productivity of isoliquiritigenin (biomass and media) in *Genista tinctoria* hairy root cultures grown in Erlenmeyer flasks and in the basket-bubble bioreactor[a].

Type of culture	Max. content [mg/100 g DW]	Day of culture	Culture biomass [g l^{-1}]	Productivity[b] [mg l^{-1}]	Specific Productivity[c] [mg l^{-1}]
Culture in Erlenmeyer flask (SH medium without ABA supplement)	2271.2	42	31.7	719.97	17.14
Culture in Erlenmeyer flask (SH medium with ABA supplement on day 0)	732.7	60	1.1	8.06	0.13
Culture in Erlenmeyer flask (SH medium with ABA supplement on day 42)	2652.9	58	18.9	501.39	8.64
Culture in the basket-bubble bioreactor (SH medium with ABA supplement on day 42)	2887.9	54	25.4	733.52	13.58

[a] All measurements throughout the table are quoted for the day on which maximum concentration of isoliquiritigenin was recorded.

[b] $\text{Productivity} = \dfrac{\text{max. content for isoliquiritigenin in mg/100 g DW} \times \text{culture biomass in g } l^{-1}}{\text{100 g DW}}$ [mg l^{-1}].

[c] $\text{Specific productivity} = \dfrac{\text{productivity}}{\text{number of days after which the max. concentration of isoliquiritigenin was reached}}$ [mg l^{-1}].

Abscisic acid added to the SH medium in the amount of 37.8 μM had a very significant effect on both the growth of *G. tinctoria* roots and the production of isoliquiritigenin. The FW and DW measured on the particular days of the experiment indicate that ABA clearly inhibited the growth of the investigated root culture. Although the culture gradually grew until day 18 of the cycle, the maximum amount of tissue (FW 92.3 g l^{-1}; DW 2.9 g l^{-1}) was approx. 9 times smaller than in the experiment when ABA was not added. Due to the progressive necrotic changes on day 60 the amount of FW (58.3 g l^{-1}) was nearly the same as the inoculated biomass (50.5 g l^{-1}) (Table I).

In the presence of ABA, hairy roots did not synthesize any derivatives of daidzein or genistein. This clearly indicates that in the examined tissue of *G. tinctoria* ABA did not play the expected regulatory function in the flavonoid pathways. The only compound found in the roots was isoliquiritigenin. Its concentration, compared to the culture grown in SH_o medium (2271.2 mg/100 g DW, day 42), was 27 times smaller (84.7 mg/100 g DW, day 20) (Fig. 2). This fact is undoubtedly related to the generally negative effect of ABA on the growth of *G. tinctoria* hairy roots. Phytochemical analysis of media sampled from the various days of the cycle showed, however, that in this case, unlike in the culture grown on SH_o medium, isoliquiritigenin was gradually released into the growth media. In effect, the level of the measured metabolite in the media was even 9 times higher than in the root biomass (723.5 g/100 g DW, day 60) (Fig. 2). It can be, therefore, inferred that while ABA did not affect the metabolic pathways of isoflavones in transformed hairy roots of *G. tinctoria*, it essentially changed the storage status of the synthesized isoliquiritigenin. The medium used in this growth system functioned as an active "sink", *i.e.* storage compartment similar to extracellular vacuoles. The authors do not know any reports on the role of ABA in the secondary metabolism of higher plants which would describe any change in the storage of bioactive compounds after external application of this growth regulator, so it is difficult to conclude whether this phenomenon is related to the change of cell permeability or whether it is a more complex mechanism. From the technological point of view, the release of a secondary metabolite from the tissue into the growth medium is highly desirable, as it eliminates the need to extract the compound from the very complex plant matrix.

The maximum amount of isoliquiritigenin in media supplemented with ABA (732.7 g/100 g DW, day 60, Fig. 2, Table II) was still approx. 3 times lower than in the roots of *G. tinctoria* grown without the addition of this growth regulator (Fig. 2). Since the culture was gradually dying, resulting in the small amount of tissue mass obtained and a long growth cycle, the productivity (8.06 mg l^{-1}) and specific productivity (0.13 mg l^{-1}) was very low in this growth system (Table II).

In order to obtain isoliquiritigenin production at the same level as in the experiment where ABA was not added and then cause the release of the metabolite into the growth media, abscisic acid was added to the medium on day 42 of the cycle. By this time the *G. tinctoria* root culture was already in the stationary phase and the concentration of isoliquiritigenin in the tissue was at its peak (Table I, Fig. 2). In this way the negative effect of ABA on the growth of *G. tinctoria* roots was eliminated (FW_{max} 803.4 g l^{-1}; DW_{max} 32.1 g l^{-1}, day 34), and beginning with day 44, gradual release of isoliquiritigenin into the media was achieved. As a result of this experiment, on day 58 ca 2109.2 mg/100 g DW of isoliquiritigenin was obtained in the medium, which is about 80% of the total amount of this compound synthesized at this point by the culture (Fig. 2). For this reason hairy root cultures of *G. tinctoria* may be considered to be a highly valuable production system from which isoliquiritigenin is easy to isolate, given its extracellular storage mode.

Growth and isoliquiritigenin production in G. tinctoria roots grown in the bioreactor

G. tinctoria hairy root cultures grown in SH medium supplemented with ABA proved to be highly efficient in the production of isoliquiritigenin, so a decision was made to establish the culture in a special bioreactor to test the applicability of the new growth system for large-scale isoliquiritigenin production. Numerous reports indicate that the construction of the growth vessel and the aeration system have a crucial impact on both the growth parameters of root cultures and the level of secondary metabolites which they synthesize (Zobayed *et al.*, 2004). *G. tinctoria* root cultures were established in a bioreactor designed specifically for this purpose. The root inoculum was placed in

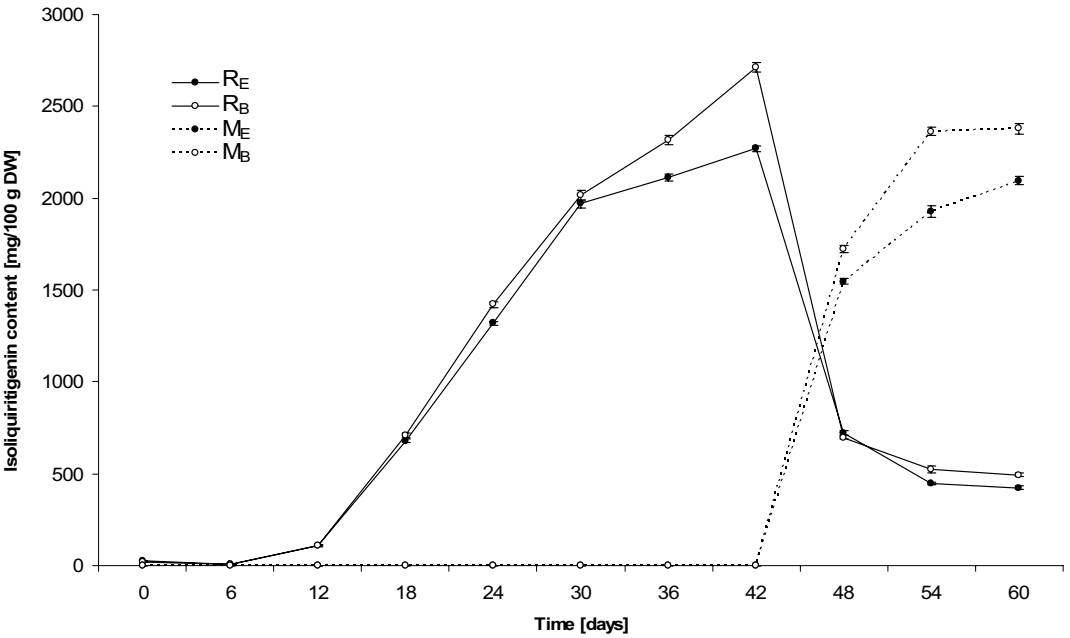

Fig. 3. Isoliquiritigenin content in *Genista tinctoria* hairy root cultures (measured in the biomass and the media) grown in an Erlenmeyer flask or in the basket-bubble bioreactor (isoliquiritigenin content in mg/100 g DW plant material). Both cultures were grown in SH medium supplemented with 37.8 μM ABA on day 42; R_E, isoliquiritigenin content in hairy roots grown in an Erlenmeyer flask; R_B, isoliquiritigenin content in hairy roots grown in the basket-bubble bioreactor; M_E, isoliquiritigenin content in SH medium of the culture grown in an Erlenmeyer flask; M_B, isoliquiritigenin content in SH medium of the culture grown in the basket-bubble bioreactor.

a stainless steel basket suspended in a glass growth vessel (Fig. 1). The culture was aerated with sterile air forced by a membrane pump, rather than by mechanic stirring, as in Erlenmeyer flasks. In effect, the immobilized roots coiled around the mesh of the basket and could freely grow upwards. In this way the entire root biomass gained good access to the nutrients in the medium while being evenly aerated. As result the growth parameters achieved for hairy roots in the basket-bubble bioreactor (FW_{max} 914.5 g l^{-1}, day 36) were better than when the tissue was grown in Erlenmeyer flasks (FW_{max} 803.4 g l^{-1}, day 36) (Table I). Also maintained were the kinetics of isoliquiritigenin production in the root tissue and the ABA-induced extracellular mode of storing this secondary metabolite in the medium (Figs. 2 and 3). Basically, moving *G. tinctoria* hairy roots from Erlenmeyer flasks into the bioreactor increased the amount of isoliquiritigenin (Fig. 3, Table II). As a result, *G. tinctoria* hairy roots grown in the basket-bubble bioreactor seem to be a valuable system for *in vitro* accumulation of isoliquiritigenin. The production parameters achieved in the bioreactor (productivity 733.52 mg l^{-1} and specific productivity 13.58 mg l^{-1}) were the highest in the whole experiment.

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