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Co-cultures of shoots and hairy roots of *Genista tinctoria* L. for synthesis and biotransformation of large amounts of phytoestrogens

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Abstract

Shoot and hairy root *Genista tinctoria* co-culture was established in Schenk and Hildebrandt medium supplemented with 24.6 μmol l^{-1} indole-3-butyric acid (IBA) to produce large amounts of isoflavones of phytoestrogenic activity. The different tissue inoculation ratios were tested to achieve the best growth of *G. tinctoria* shoots and hairy roots in the co-culture system. In the co-culture, hairy roots produced large amounts of a single isoflavone—isoliquiritigenin (2473.8 mg/100 g dry weight (DW)), which is a daidzein precursor absent in the intact plant. Owing to the addition of abscisic acid (37.8 μmol l^{-1}—on day 42), isoliquiritigenin was almost completely released into the growth medium, from which it was used by the shoots to produce significant amounts of daidzin and daidzein. *G. tinctoria* shoots in a co-culture system, like in classic single cultures, maintained the ability to produce significant amounts of genistin (6941.5 mg/100 g DW) and its derivatives. At the same time, as a result of the described bioconversion of isoliquiritigenin, they synthesised 38 times more daidzin than the intact plant (1647.5 mg/100 g DW). This result suggests that *G. tinctoria* co-culture may be used to produce large amounts of isoflavone phytoestrogens. A prototype basket-bubble bioreactor was designed and built to upgrade the scale of the *G. tinctoria* co-culture. The new device significantly improved the growth parameters and the productivity of both tissues.

Keywords: Abscisic acid; Bioconversion; Basket-bubble bioreactor; Daidzin; *Genista tinctoria*; Genistin; Hairy roots; In vitro cultures; Isoliquiritigenin; Shoots

1. Introduction

One of the key problems facing modern medicine is effective treatment of menopause-related conditions and a variety of tumours dependent on estrogen balance within the body [2–4]. In order to prevent such postmenopausal symptoms as hot flushes, vulvovaginal complaints, osteoporosis and cardiovascular diseases, the hormone replacement therapy (HRT) becomes increasingly popular. Unfortunately, long-term exposure to estrogens (HRT therapy) is related to high risk of breast and endometrial cancers [2–6], encouraging the search for safe alternatives to HRT [7–9]. A group of compounds of potential interest are phytoestrogens, among which are isoflavones. Due to their affinity to estrogen receptors β, in addition to treatment of menopause-related symptoms, these compounds may prevent breast, colon, prostate and thyroid cancers [4,5,9].

Biotechnological research, carried out so far, showed that the biomasses of *Genista* plants are able to synthesise some of the largest amounts of phytoestrogens ever obtained in vitro [10,11]. They accumulate many times more of these compounds, not only compared to the respective intact plants, but also to soy products, which have so far been seen as the main source of isoflavones [11,12].

We have previously reported conditions for efficient growth and effective production of isoflavones in cell and organ cultures of *Genista tinctoria* [13].

In vitro shoots of *G. tinctoria* were particularly active in accumulating compounds (14 different isoflavones) which belong to the metabolic pathway of 5-hydroxyisoflavones

Abbreviations: ABA, abscisic acid; DW, dry weight; FW, fresh weight; HPLC, high performance liquid chromatography; IBA, indole-3-butyric acid; SH, Schenk and Hildebrandt [1]

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(genistein derivatives) with relatively low production of 5-deoxyisoflavones (daidzein and daidzin) [13]. At the same time, hairy roots of *G. tinctoria* synthesised selectively only a single isoflavone compound—isoliquiritigenin, 80% of which was released into the culture medium in response to supplementation with abscisic acid (ABA) [14]. Since isoliquiritigenin is a direct precursor of daidzein, the authors decided to test whether co-culturing shoots and hairy roots of *G. tinctoria* can result in the bioconversion of isoliquiritigenin to derivatives of daidzein in the shoots. The main purpose of the experiment was, therefore, to obtain a shoot/root co-culture of *G. tinctoria* which could produce not only large amounts of genistein and its derivatives, like in single shoot cultures, but also significant concentrations of daidzein and daidzin.

In this paper, the conditions were developed for efficient growth of shoot and hairy root co-culture of *G. tinctoria*. Also, the metabolism of shoots and roots in the co-culture was analysed in terms of isoflavones and the results were compared with respective single cultures. Then, the *G. tinctoria* co-culture system was upgraded from Erlenmeyer flask to bioreactor.

Intra-species and inter-species co-culture systems constitute a relatively new area in biotechnological research of higher plants [15,16]; therefore, there are no commercial devices available which could be used for these type of large-scale experiments. In order to obtain a large-scale *G. tinctoria* co-culture system, a prototype basket-bubble bioreactor was built, the construction details of which are described. The productivity and specific productivity of the particular derivatives of genistein and daidzein achieved with the bioreactor are discussed.

2. Materials and methods

G. tinctoria shoots used for co-cultures were grown in liquid Schenk and Hildebrandt (SH) medium [1] with 3% (w/v) sucrose and supplemented with 24.6 μmol l^{-1} indole-3-butyric acid (IBA) [13].

G. tinctoria hairy roots used for co-cultures were grown using SH medium [1] without growth regulators and supplemented with 3% (w/v) sucrose [14].

2.1. G. tinctoria shoot and hairy root co-culture—plant material and culture conditions

A *G. tinctoria* co-culture system was initiated with three different proportions of shoot fresh matter to hairy root tissue (4:1, 2:2 and 1:4). A total of 5 g fresh weight (FW) of shoot and root fragments was inoculated in each 250 ml Erlenmeyer flask containing 100 ml of SH medium with 3% (w/v) sucrose, supplemented with 24.6 μmol l^{-1} IBA. The co-cultures were maintained for 60 days on an orbital shaker set at 150 rpm (25 ± 2 °C, with continuous light intensity 88 ± 8 μmol m^{-2} s^{-1}). Three sample flasks were collected every two days during the whole experiment. After mechanical separation of the root biomass from the shoots, FW and dry weight (DW) (after lyophylization) were determined for both types of tissue. The results were analysed statistically with Student's *t*-test.

For isoflavonoid production, *G. tinctoria* shoot and hairy root co-cultures (proportion of fresh shoot matter to root tissue 4:1) were grown using the above SH medium. A total of 5 g FW of plant biomasses (4 g of shoots and 1 g of roots) was inoculated in each 250 ml Erlenmeyer flask containing 100 ml of the SH medium on an orbital shaker and cultured for 60 days. In order to induce a release of isoliquiritigenin into the growth medium, on day 42 of the experiment, the medium was supplemented with 37.8 μmol l^{-1} of ABA. The co-cultures were grown on an orbital shaker at the same conditions as above. Three sample flasks (biomass and media) were collected every two days during the whole experiment. FW and DW after lyophylization were determined separately for roots and shoots. Isoflavone extraction from plant material and media was performed after lyophylization according to the procedure described before [11].

2.2. G. tinctoria co-cultures grown in the bioreactor

The basket-bubble bioreactor was made of two identical glass domes (Rasotherm, Germany) 100 mm high, largest internal diameter 180 mm, connected with eight clamps (Photo 1). The joint between the glass domes was additionally sealed with a silicon gasket (dimensions DN 150, Schott-Duran, Germany). In order to immobilise the root and shoot cultures inside the growth vessel, two stainless steel (18/8) baskets were placed, mesh diameter 8 mm (Deutsch-Neumann, Germany). The smaller basket, 120 mm diameter and 100 mm height, was placed concentrically inside the larger basket, 150 mm diameter and 120 mm height, so that the distance between the baskets was 15 mm. Maximum clearance between the bottom of the outside basket and the bottom of the lower dome was 40 mm (Photo 1). Hairy roots of *G. tinctoria* (ca. 10.0 g) were evenly placed in the space between the baskets, and shoots (ca. 40.0 g) were placed in the inside basket. After filling the growth vessel with 1000 ml of the SH medium, the roots were fully immersed in the growth medium and the shoots were covered by the medium up to 15 mm (Photo 1). The co-culture was aerated with forced air flow from the bottom of the growth vessel via a silicon hose with 4 mm diameter and 1 mm wall thickness (Deutsch-Neumann) using a membrane pump with flow regulation (Maxima R Air Pump, 6 W, 6 PSI, Rolf C. Hagen Inc., Montreal, Que., Canada). The air flow through the culture was 800 ml/min. In order to ensure sterile conditions for the cultures, sterilising filters (Millex Vent Filter, 50 mm diameter, PTFE membrane, 0.2 mm pore size; Millipore Corporation, Bedford, USA) were used both on the inflow ducts and at the outflow from the growth vessel (Photo 1). In order to prevent loss of media during aeration,