Bag Culture: A Method for Root-Root Co-Culture

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A method named "bag culture" was developed for co-culturing of *Linum persicum* (section *Syllinum*) and *L. austriacum* (section *Linum*) hairy roots. For this propose *L. austriacum* and *L. persicum* hairy root cultures were established using *Agrobacterium rhizogenes* in McCown medium. *L. persicum* hairy roots in bags (1 mm^2 mesh) were successfully grown together with *L. austriacum* hairy roots. The amounts of podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin (MPTOX) produced by *L. persicum* hairy root cultures were detected using HPLC. The results indicated that the amounts of both lignans and growth indexes of the two hairy roots decreased, that may be partly due to a competition between the two types of culture in using precursors of biosynthetic metabolites and the amount of culture medium which is available for each hairy root. However, MPTOX (0.17 g/100 g DW) and PTOX (0.02 g/100 g DW) levels of the *L. persicum* single culture in bag were significantly higher than of the other cultures which may be due to the immobilization effect of the bag.

Key words: Co-Culture, *Linum persicum*, *Linum austriacum*, Hairy Root Cultures

Introduction

A relatively new plant cell and tissue culture method involves the interaction of two participants leading to change the production of plant secondary metabolite levels or profiles in the resulting interaction cultures. The aim of this method, known as co-culture, could be the production of new environments that can help us to develop new characteristics in an organism that did not exist before; it also helps to improve cooperative qualities that can be useful in drug production (Guillon *et al.*, 2006). This method has been used in pharmaceutical researches for the production of diverse natural products. Some of the examples are as follows: Co-culture of *Genista tinctora* hairy root with shoot cultures of the same plant for the biotransformation of isoflavons with phytoestrogenic activity (Luczkiewicz and Kokotkiewicz, 2005), co-culture of *Linum flavum* hairy roots and *Podophyllum hexandrum* cell suspension for the production of podophyllotoxin (PTOX) (Han-wei *et al.*, 2003), co-culture of transformed shoot cultures of *Duboisia* and hairy root cultures of *Atropa belladonna* in the same medium, for the production of scopolamine (Mahagamasekera and Doran, 1998), and production of furanocumarines by the co-culture of *Ammi majus* and *Ruta graveolens* (Sidwa-Gorycka *et al.*, 2003).

In this study a co-culture method named "bag culture" is introduced for establishing root-root co-cultures of two different species of the *Linum* genus producing cytotoxic lignans.

Results and Discussion

Root-root co-cultures of *L. persicum* and *L. austriacum* were successfully established in McCown (MC) medium, a method named "bag culture". The growth indexes of the single cultures and the co-cultures were compared after 60 days of inoculation (t_{60}/t_1). The results are summarized in Table I. As it is shown in Fig. 1, two *Linum* species grew either in a single or co-culture system in liquid medium, but according to Table I the growth index of the *L. persicum* hairy root culture in MC_1 was significantly ($p \leq 0.001$) lower than that in MC_4 (Fig. 1D). The growth index of *L. austriacum* hairy roots in the co-culture system (Fig. 1A) was also decreased in comparison to the corresponding single culture (Fig. 1B), which may be due to the decrease of the medium taken in by each hairy root (Table I), *i.e.* in the co-culture system the amount of culture medium taken in by each hairy root is about half of the amount taken in by hairy roots in the corresponding single culture. However, the growth index of the *L. persicum* hairy root culture in MC_3 (Fig. 1C) was significantly higher than that in MC_4, which should be due to the root immobilizing properties of bags and so increase the biomass production.

The HPLC analysis of *L. persicum* hairy root cultures showed that the highest amount of lignans, PTOX and 6-methoxypodophyllotoxin

Table I. Growth pattern, PTOX and MPTOX levels of *Linum persicum* and *L. austriacum* hairy roots co- or single culture.

Type of culture	Plant species	DW [g]	FW [g]	Growth index*	PTOX [g/100 g DW]	MPTOX [g/100 g DW]
Co-culture (bag culture)						
MC_1	*L. austriacum*	1.14 ± 0.01	16.71 ± 0.26[a]	3.29 ± 1.44[a]	–	–
	L. persicum	0.62 ± 0.06[b]	26.60 ± 4.59[b]	4.43 ± 0.25[b]	–	0.09 ± 0.01[c, d]
Single culture						
MC_2	*L. austriacum*	1.80 ± 0.06	35.67 ± 0.82	5.95 ± 0.13	–	–
MC_3	*L. persicum*	1.23 ± 0.12[b]	22.95 ± 6.58[b]	3.83 ± 0.82[b]	0.01 ± 0.00	0.14 ± 0.04
MC_4 (in bag)	*L. persicum*	6.99 ± 0.44	43.05 ± 3.58	7.18 ± 0.45	0.02 ± 0.01	0.17 ± 0.01

[a] Significant differences with $p \leq 0.01$ *vs.* MC_2;
[b] significant differences with $p \leq 0.001$ *vs.* MC_4;
[c] significant differences with $p \leq 0.01$ *vs.* MC_3;
[d] significant differences with $p \leq 0.01$ *vs.* MC_4.
* Growth index is the ratio of FW at the 60th day to FW at the first day (t_{60}/t_1); FW, fresh weight (day 60); DW, dry weight (day 60).
–, No peak at the retention time of this compound was detected.
MC_1, *L. persicum* and *L. austriacum* hairy roots co-culture; MC_2, *L. austriacum* hairy roots single culture; MC_3, *L. persicum* hairy roots single culture; MC_4, *L. persicum* single culture placed in the bag.

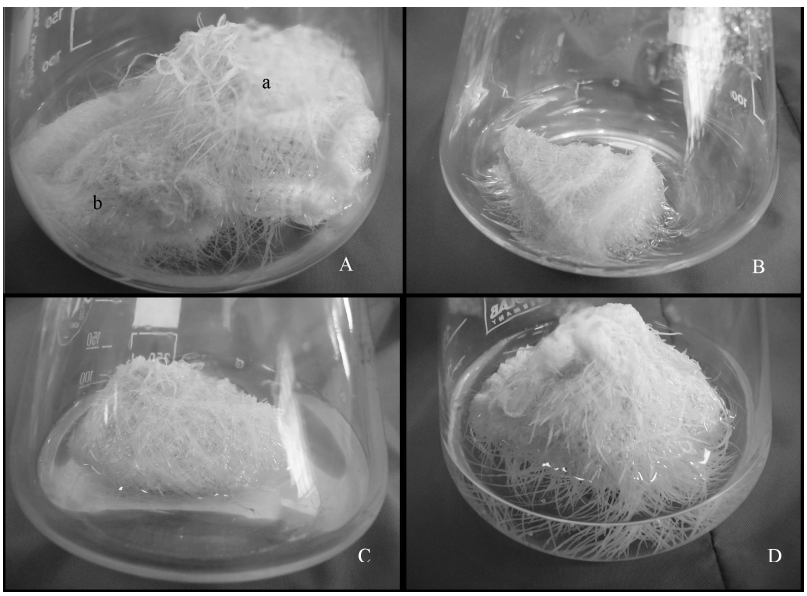

Fig. 1. (A) Co-cultures of *Linum persicum* (a) and *L. austriacum* (b) hairy roots. (B) Single culture of *L. austriacum* hairy roots. (C) Single culture of *L. persicum* hairy roots. (D) Single culture of *L. persicum* hairy roots in bag.

(MPTOX), was found in roots in MC_4 medium (MPTOX: 0.17 g/ 100 g DW and PTOX: 0.02 g/ 100 g DW) which may be due to immobilization effects of the bag. Lignans content of *L. persicum* hairy root co-cultures were significantly ($p \leq 0.01$) lower than those in hairy roots of the corresponding single culture (Table I). *L. persicum* belongs to the *Syllinum* section. This section is promising for the production of aryltetralin lignans like PTOX and MPTOX (Mohagheghzadeh *et al.*, 2003), whereas *L. austriacum* belongs to the *Linum* section which produces arylnaphthalene lignans like justicidin and isojusticidin (Mohagheghzadeh *et al.*, 2002). These two types of lignans are produced in a similar phenylpropanoid biosynthetic pathway (Umezawa, 2003). So a competi-

tion may be occur between these two species for consuming precursors of biosynthetic compounds, and therefore PTOX and MPTOX levels decreased, however, more investigations are needed. Sometimes the biosynthesis of a valuable metabolite by hairy roots is limited by the availability of its precursor. One solution of this problem is to add the desired product to the culture medium, but this can be costly if the precursor is difficult to synthesize or to obtain from other natural sources. In this context, the co-culture system has proven to be a judicious alternative for the production of the anti-tumour drug podophyllotoxin by *Podophyllum hexandrum* cell suspension cultures (Han-wei *et al.*, 2003). In addition, some co-culture systems were used for increasing the amount or changing plant secondary metabolites; shoot-root and suspension-root co-culture systems were established in previous works (Luczkiewicz and Kokotkiewicz, 2005; Mahagamasekera and Doran, 1998; Subroto *et al.*, 1997), while here, a root-root co-culture system named "bag culture" was established for the first time. This method can be used to overcome bottlenecks and flux imbalances in biosynthetic pathways, by supplying exogenous intermediates that are relatively close to the desired product.

Experimental

Plant materials

The collection of *L. austriacum* L. and *L. persicum* Ky. ex Boiss. seeds and seed germination were described previously (Mohagheghzadeh *et al.*, 2002, 2003).

Hairy root cultures

Shoots of *L. persicum* and *L. austriacum* were transferred to MS medium and infected by *Agrobacterium rhizogenes* (ATCC 15834). Infected tissues were placed into sterile petri dishes containing McCown medium (Lloyd and McCown, 1980) supplemented with 5% sucrose and 0.8% agar. After 2 d the infected shoots were washed in cefotaxime sodium (Hanmi-Korea, Seoul, Korea) solution (500 mg l^{-1}) and transferred into McCown medium containing 5% sugar, 0.8% agar and 500 mg l^{-1} cefotaxime to inhibit the growth of *Agrobacterium*. Every 2–3 weeks hairy roots were subcultured into fresh culture medium containing cefotaxime. After culture establishment, well-grown hairy roots were transferred to 250-ml flasks containing liquid McCown medium supplemented with cefotaxime (500 mg l^{-1}). Cultures were maintained under permanent darkness on a rotary shaker (80 rpm) at (25 ± 2) °C and refreshed with liquid medium containing cefotaxime antibiotics every 2 weeks.

Co-culture

L. persicum and *L. austriacum* hairy roots were co-cultured in 250-ml flasks containing liquid McCown medium supplemented with 5% sugar at pH 5.6 before autoclaving (MC_1). To facilitate the isolation of hairy roots at the end of the experiment, cotton bags with 1 mm^2 mesh size were used. Bags were washed with boiling water and sterilized before using in the co-culture experiment. *L. persicum* hairy roots were put into the bag and *L. austriacum* hairy roots were placed out of the bag, the inoculum ratio was 1:1 (6 g biomass of *L. persicum* hairy roots and 6 g of *L. austriacum* hairy roots). As control systems, 6 g of *L. austriacum* hairy roots (MC_2) and 6 g biomass of *L. persicum* hairy roots (MC_3) single cultures were used. To investigate the effect of the bag on the growth pattern of *L. persicum* hairy roots, 6 g hairy root cultures of *L. persicum* were placed into the bag and established as single culture (MC_4). There were 3 flasks which contained 50 ml liquid McCown medium for each culture. All cultures were incubated under permanent darkness on a rotary shaker (80 rpm) at (25 ± 2) °C. Cultures were refreshed every week and harvested after 1 month.

Growth parameters

After passing the culture period, resulting roots were removed carefully and their fresh weight (FW), dry weight (DW) and growth indexes were measured. Growth index was measured as the ratio of FW at the 60th day to FW at the first day (t_{60}/t_1).

Lignan analysis

For the lignan assay, root samples were removed and outer portions of were selected for measuring the lignan contents by HPLC.

Lignan extraction was carried out as described by Empt *et al*. (2000). The HPLC analysis was performed using a Waters instrument equipped with a UV detector at 290 nm, a column of 250 mm length and 4.6 mm inner diameter coupled to a guard column of 40 mm length and 4.6 mm inner diameter, both filled with Nucleosil 100 C_{18}, 5 μm (particle size) as described earlier (Mohagheghzadeh *et al.*, 2003). PTOX was quantified and MPTOX was estimated according to commercial PTOX (Roth, Karlsruhe, Germany, 3946.1).

For all parameters one-way analysis of variance (ANOVA) with tukey post hoc test was applied with a critical value of $p \leq 0.05$.

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