Acta Physiol Plant (2008) 30:891–896
DOI 10.1007/s11738-008-0181-3

SHORT COMMUNICATION

Establishment of adventitious root co-culture of Ginseng and Echinacea for the production of secondary metabolites

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Received: 31 December 2007 / Revised: 3 April 2008 / Accepted: 28 April 2008 / Published online: 9 July 2008

Abstract We have successfully established the co-culture of ginseng (*Panax ginseng* C.A. Meyer) and echinacea [*Echiancea purpurea* (L.) Moench.] adventitious roots for the production secondary metabolites. Adventitious roots of ginseng and echinacea were cultured in different proportions (5 g L^{-1}; 4:1, 3:2 and 2:1 ginseng and echinacea, respectively) in 5-L capacity airlift bioreactors containing 4 L Murashige and Skoog medium supplemented with 25 μM indole-3-butyric acid and 50 g sucrose L^{-1} and maintained at 25°C in the dark for 40 days. Results showed the negative effect of echinacea adventitious roots on the growth of ginseng roots, however, by limiting the inoculum density of echinacea, it was possible to establish the co-cultures. To enhance the accumulation of secondary metabolites, co-cultures were treated with 200 μM methyl jasmonate after 30 days of culture initiation. Methyl jasmonate elicitation promoted the accumulation of ginsenosides in the co-cultures. It was possible to produce ginsenosides and caffeic acid derivatives in higher amounts by establishing co-cultures with higher inoculum proportion of ginseng to echinacea (4:1 and 3:2) followed by elicitation treatment. This work demonstrates the effectiveness of interspecies adventitious root co-cultures for the production of plant secondary metabolites.

Keywords Adventitious roots · Co-cultures · Echinacea · Ginseng · Secondary metabolites

Communicated by S. Lewak.

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Introduction

Higher plants produce a great variety of secondary metabolites, which have been used as pharmaceuticals, nutraceuticals and food additives. Recent advances in the techniques and applications of plant cell and organ cultures have made it possible to produce these valuable secondary metabolites in large scale. Moreover, plant cell culture systems provide various ways to boost yields of desired metabolites, conveniently and cost effectively (Rao and Ravishanakar 2002).

Ginseng (*Panax ginseng* C.A. Meyer) is a valuable medicinal plant and has been shown to have various bioactive effects on human health such as antitumor, antineoplastic, antimitogenic, anti-stress, antiageing, and immunomodulation activities (Attele et al. 1999). The major secondary metabolites of ginseng are an array of triterpene saponins, collectively called ginsenosides (Dewick 2001), which are divided into three groups based on their structure, that is the Rb group (protopanaxadiol including Rb1, Rb2, Rc, Rd and others), Rg group (protopanaxatriols including Rg1, Rg2, Re, Rf and others) and the Ro group (oleanolic acid).

Echinacea (*Echinacea purpurea* (L.) Moench) is another medicinal plant, which has shown antioxidative, antibacterial, antiviral and antifungal properties, and is used in the treatment for common cold, as well as respiratory and urinary diseases (Barrett 2003). The main bioactive

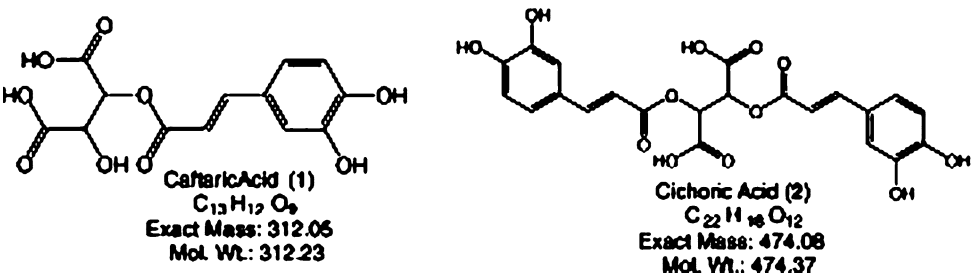

Fig. 1 Structure of caftaric acid and cichoric acid

constituents are caffeic acid derivatives, alkylamides, polyacetylenes and polysaccharides (Bauer 1999). With regard to caffeic acid derivatives, several compounds have been identified from the hydrophilic fractions of *E. purpurea*, which revealed that caftaric acid, chichoric acid (Fig. 1) and chlorogenic acid are the important ones.

Co-culture of different organs together has been attempted in the recent years so that the co-culture provides the opportunity for metabolites produced by one organ to be excreted into medium and taken up by another organ for further biochemical conversion. Genetically transformed shooty tetromas and hairy roots of *Atropa bellodona* and *Duboisia* hybrid were co-cultured for biotransformation of tropane alkoloids in flasks and bioreactors (Subroto et al. 1996), Whereas Sidwa-Gorycka et al. (2003) have established co-culture of hairy roots of *Ammi majas* and cell/shoot suspension culture of *Ruta graveolens* to verify possible interaction of metabolic pathways of coumarins. In the present study, we have established co-culture of adventitious roots of ginseng (*Panax ginseng* C.A. Meyer) and echinacea (*Echinacea purpurea* (L.) Moench) with two major objectives: (1) Production of two different secondary metabolites simultaneously by adventitious root co-cultures. (2) To verify how such co-cultures affect biomass yield and productivity of ginsenosides and caffeic acid derivatives.

Materials and methods

Plant material

The ginseng (*Panax ginseng* C. A. Meyer) adventitious roots were maintained by sub-culturing once in 40 days in 4 L working capacity balloon type bubble bioreactors containing Murashige and Skoog (Murashige and Skoog 1962) liquid medium (without NH_4NO_3) supplemented with 25 μM indole-3-butyric acid (IBA) and 50 g L^{-1} sucrose (Kim et al. 2004). Echinacea (*Echinacea purpurea* (L.) Monech) adventitious roots were grown in half strength MS liquid medium (5:25 mM ammonium and nitrate ratio), supplemented with 9.8 μM IBA and 50 g L^{-1} sucrose (Wu et al. 2007a). Cultures were maintained in the dark at 25°C.

Co-cultures

Co-cultures were initiated using adventitious roots of ginseng and echinacea in different proportions (5 g L^{-1}; 4:1, 3:2 and 2:3 ginseng and echinacea, respectively) in bioreactors (balloon type bubble bioreactors of 5 L capacity) using MS liquid medium (without NH_4NO_3) supplemented with 25 μM indole-3-butyric acid and 50 g L^{-1} sucrose. Cultures of ginseng and echinacea adventitious roots were also established individually as control cultures. The cultures were aerated with filter sterilized air at 0.1 vvm. All cultures were maintained for 40 days at 25°C, under dark. There were five replicates in each experiment and experiments were repeated twice.

Elicitation

In another set of experiment, ginseng and echinacea adventitious roots were established individually and as co-cultures as above. An amount of 200 μM methyl jasmonate was added to the cultures after 30 days of incubation. All other culture treatments are as above.

Determination of root weight and growth ratio

Root fresh weight (FW) and dry weight (DW) were determined as follows. Roots were separated from the medium by passing through a 1 mm stainless steel sieve. Root FW was measured after rinsing once with sterile water and blotting away surface water, and root DW was recorded after roots were dried to a constant weight at 60°C for several days. The growth index was calculated using following equation.

$$\text{Growth Index} = \frac{\text{Harvested dry weight (g)} - \text{Inoculated dry weight (g)}}{\text{Inoculated dry weight (g)}}$$

Extraction and analysis of secondary metabolites

Extraction of ginsenosides was done according to the method of Kim et al. (2007) and analysis was carried by following the method of Yu et al. (2002). The ginsenoside fraction was analyzed using HPLC systems with an Altec

Table 1 Comparison of growth and accumulation of secondary metabolites in the adventitious roots of ginseng and echinacea grown as single culture and in the co-cutlure

Inoculum ratio (5 g L^{-1})	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth index	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)
Single culture					
Ginseng (G)	106.25 a	10.09 a	19.1	2.09 d	0.15 d
Echinacea (E)	83.00 b	9.79 a	10.7	55.99 a	33.06 a
Co-culture					
4G:1E	57.88 d	5.85 c	14.5	20.93 c	11.27 c
3G:2E	71.76 c	7.75 c	15.9	40.69 b	24.38 b
2G:3E	76.71 c	8.47 b	18.5	49.51 a	30.17a

Mean separation within columns by Duncan's multiple range test at 5% level. Adventitious roots were cultured in 5-L airlift bioreactors containing 4 L of MS medium supplemented with 25 μM IBA and 50 g L^{-1} sucrose and cultures were maintained in dark for 40 days

Platinum C18 column (particle size 1.5 μm, 33 × 7 mm), eluting with water/acetonitrile at 3:1 (v/v) for 10 min then at 63:37 (v/v) for 25 min with a flow rate of 1.2 mL min^{-1}. Ginsenoside was detected at 203 nm. Authentic samples of ginsenosides were from Chromadex Inc., USA. Total ginsenoside content was calculated as sum of ginsenoside fractions.

Extraction and analysis of caffeic acid derivatives was done by the method of Wu et al. (2007b). The caffeic acid fractions were analyzed using an HPLC system with XTerra RP 18 column (particle size 3.0 μM, 150 ×3 mm). The mobile phase was (A) water and (B) acetonitrile. The gradient elution was modified as follows: initial 10% B for 40 min; 25% B for next 11 min; 50% B for 1 min; recycle to initial condition for 8 min with a flow rate 0.3 mL min^{-1}. Caffeic acid derivatives were detected at 330 nm. Standard caftatic acid, cichoric acid and clorogenic acid were obtained from CromaDex (Laguna Hills, CA, USA).

The content of total phenolics in root extracts was analyzed spectrophotometrically by modification of Folin–Ciocalteu colorimetric method (Wu et al. 2007c). The total flavonoid content was determined by colorimetric method (Zhishen et al. 1999).

Results and discussion

Biomass accumulation and growth index of the single cultures (ginseng and echinacea) and of the co-cultures were compared after 40 days of incubation. Growth index of ginseng adventitious roots was 19.1, whereas growth index of echinacea adventitious roots was 10.7. These growth values are similar to the earlier reported results (Kim et al. 2004; Wu et al. 2007a). Co-culture experiments were conducted using different inoculum densities of ginseng and echinacea adventitious roots, that is 4:1, 3:2 and 2:3 proportion and results are presented in the Table 1. It was not possible to measure precisely the growth index for individual adventitious roots growing in the co-cultures, as the roots were closely tangled with each other and it was impossible to separate individual root samples. Growth index was 14.5 when ginseng and echinacea co-culture ratio was 4:1. Growth index increased with an increment of inoculum ratio of echinacea, that is 3:2 and 2:3 ginseng and echinacea, however, increment of echinacea composition drastically affected the growth of ginseng roots. Careful observation of root samples revealed that higher proportions of echinacea roots could inhibit the growth of ginseng adventitious roots and this could be due to higher accumulation of phenolics and flavonoids in the echinacea roots (Table 1). Our results showed the negative effect of echinacea adventitious roots on ginseng adventitious root growth; however, by limiting the inoculum of echinacea, it was possible to establish the co-cultures in MS medium (Fig. 2a–c).

The level of ginsenosides in ginseng and caffeic acid derivatives in echinacea adventitious roots grown for 40 days in single cultures and co-cultures was estimated. Amount of individual and total ginsenosides were the highest (2.13 ± 0.36 mg g^{-1} DW) with the single cultures of ginseng. Similarly, individual and total caffeic acid derivatives were the highest (39.62 ± 1.64 mg g^{-1} DW) with the single cultures of echinacea. Accumulation of ginsenosides and caffeic acid derivatives were affected by inoculum ratios of co-cultures (Table 2). The results indicate that accumulation of ginsenosides was much reduced when compared with caffeic acid derivatives during co-cultures.

Elicitation strategy was conveniently used to enhance the ginsenoside accumulation in the adventitious root cultures of ginseng (Yu et al. 2002; Kim et al. 2004). Single cultures and co-cultures were elicitated with 200 μM methyl jasmoante after 30 days of culture with the objective of enhancing the ginsenoside levels. Methyl jasmonate

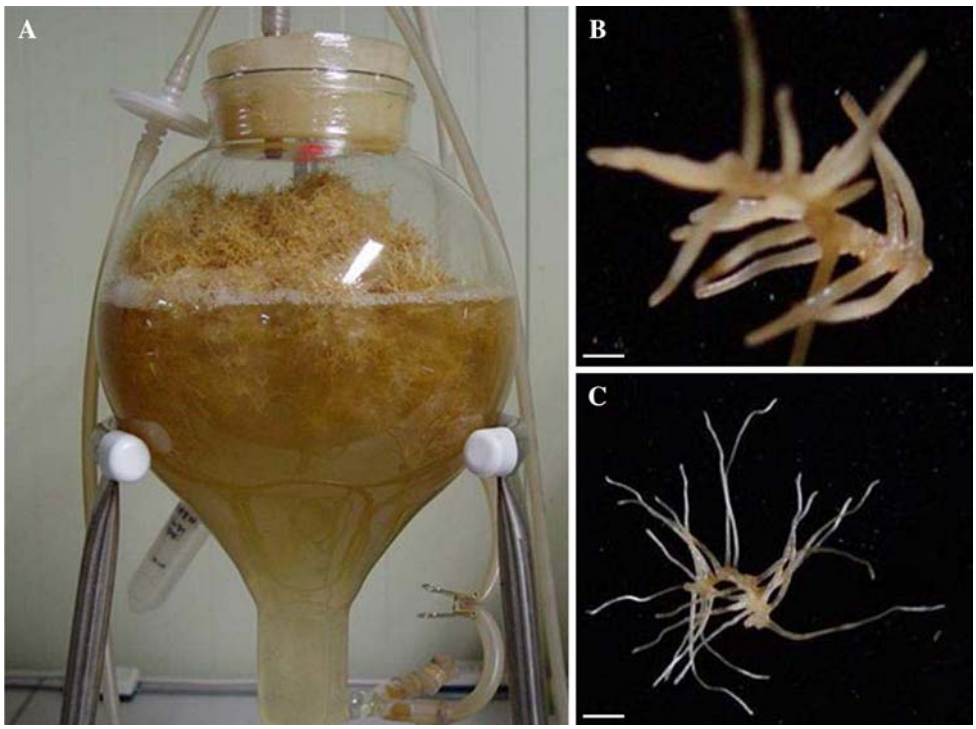

Fig. 2 Co-cultures of ginseng and echinacea adventitious roots. Co-cultures (inoculum ratio 4:1) were grown for 40 days in MS medium at 25°C in the dark. **a** Bioreactor culture after 40 days of incubation. **b** Ginseng adventitious roots (*bar* 0.4 cm). **c** Echinacea adventitious roots (*bar* 0.4 cm)

elicitation inhibited the root growth and there was reduction of both fresh and dry biomass with co-cultures when compared to single cultures (Table 3). However, it did not affect the levels of total phenolics and flavonoids.

Methyl jasmonate elicitation promoted the accumulation of individual and total ginsenosides (Table 4). Eightfold increment was evident in total ginsenoside levels in elicitated cultures when compared to cultures without elicitation. However, levels of caffeic acid derivatives were not affected by methyl jasmonate elicitation. It is interesting to report here that methyl jasmonate elicitation was responsible for differential accumulation of individual ginsenosides and Rc

Table 2 Amount of ginsenosides, caffeic acid derivatives accumulated in adventitious roots of ginseng and echinacea grown as single culture and in the co-culture

Inoculum ratio (5 g L^{-1})	Amount of ginsenosides (mg g^{-1} DW)						
	Rg1	Re	Rb1	Rd	Rg3	Rh2	Total
Single culture							
Ginseng (G)	0.58 ± 0.04	0.67 ± 0.05	0.14 ± 0.02	0.27 ± 0.03	0.13 ± 0.0	0.49 ± 0.22	2.13 ± 0.36
Co-culture							
4G:1E	0.21 ± 0.28	0.17 ± 0.03	–	–	0.18 ± 0.13	0.15 ± 0.02	0.53 ± 0.02
3G:2E	0.23 ± 0.05	0.12 ± 0.00	–	–	0.37 ± 0.0	–	0.36 ± 0.06
2G:3E	0.21 ± 0.01	0.16 ± 0.0	–	–	–	–	0.31 ± 0.01

Single culture	Amount of caffeic acid derivatives (mg g^{-1} DW)			
	Caftaric acid	Chlorogenic acid	Chichoric acid	Total
Echinacea (E)	1.12 ± 0.86	9.89 ± 0.82	28.81 ± 1.68	39.62 ± 1.64
Co-culture				
4G:1E	1.13 ± 0.13	2.98 ± 0.68	7.62 ± 1.21	11.73 ± 2.02
3G:2E	2.04 ± 0.19	5.39 ± 0.12	21.10 ± 2.32	28.54 ± 2.38
2G:3E	2.56 ± 0.05	7.22 ± 0.08	27.25 ± 1.35	37.04 ± 0.14

Adventitious roots were cultured in 5-L airlift bioreactors containing 4 L of MS medium supplemented with 25 μM IBA and 50 g L^{-1} sucrose and cultures were maintained in dark for 40 days

Table 3 Effect of methyl jasmonate elicitation on growth and accumulation of secondary metabolites in the adventitious roots of ginseng and echinacea grown as single culture and in the co-cutlure

Inoculum ratio (5 g L^{-1})	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth index	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)
Single culture					
Ginseng (G)	92.62 a	10.04 a	19.0	11.22 d	3.60 d
Echinacea (E)	80.50 b	9.10 b	17.2	60.28 a	32.93 a
Co-culture					
4G:1E	53.88 d	5.77 e	10.5	25.47 c	11.30 c
3G:2E	70.94 c	7.28 d	13.5	43.32 b	21.16 b
2G:3E	74.28 c	8.31 c	15.6	54.32 a	31.91 a

Mean separation within columns by Duncan's multiple range test at 5% level. Adventitious roots were cultured in 5-L airlift bioreactors containing 4 L of MS medium supplemented with 25 μM IBA and 50 g L^{-1} sucrose and cultures were maintained in dark for 40 days

Table 4 Effect of methyl jasmonate elicitation on amount of ginsenosides, caffeic acid derivatives accumulated in adventitious roots of ginseng and echinacea grown as single culture and in the co-culture

Inoculum ratio (5 g L^{-1})	Amount of ginsenoside (mg g^{-1} DW)								
	Rg1	Re	Rb1	Rc	Rb2	Rd	Rg3	Rh2	Total
Single culture									
Ginseng (G)	0.85 ± 0.05	1.84 ± 0.23	3.86 ± 0.23	2.64 ± 0.36	4.12 ± 0.63	3.68 ± 0.59	0.15 ± 0.03	0.84 ± 0.02	17.98 ± 2.14
Co-culture									
4G:1E	0.69 ± 0.08	0.71 ± 0.06	1.82 ± 0.04	–	–	1.16 ± 0.04	–	0.77 ± 0.0	5.16 ± 0.19
3G:2E	0.98 ± 0.10	1.06 ± 0.06	0.50 ± 0.06	–	–	0.52 ± 0.04	–	0.56 ± 0.02	3.64 ± 0.26
2G:3E	0.62 ± 0.02	0.62 ± 0.02	0.24 ± 0.2	–	–	0.36 ± 0.02	–	0.34 ± 0.01	2.18 ± 0.11

Single culture	Amount of caffeic acid derivatives (mg g^{-1} DW)			
	Caftaric acid	Chlorogenic acid	Chichoric acid	Total
Echinacea (E)	4.0 ± 0.12	6.09 ± 0.21	27.18 ± 1.54	37.27 ± 1.87
Co-culture				
4G:1E	0.84 ± 0.04	3.05 ± 0.06	8.07 ± 0.19	12.00 ± 0.21
3G:2E	2.34 ± 0.21	4.99 ± 0.01	20.90 ± 0.50	28.23 ± 0.73
2G:3E	2.18 ± 0.16	7.01 ± 0.02	26.27 ± 0.62	35.46 ± 0.79

Adventitious roots were cultured in 5-L airlift bioreactors containing 4 L of MS medium supplemented with 25 μM IBA and 50 g L^{-1} sucrose, and cultures were maintained in dark for 40 days. Cultures were treated with 200 μM methyl jasmonate after 30 days of culture

and Rb2 ginsenosides, which were not present in the cultures without elicitation were present in higher levels (2.64 ± 0.36 mg g^{-1} DW and 4.12 ± 0.63 mg g^{-1} DW respectively) in the elicitated cultures (Table 4). These observations are concurrent with results of Wang and Zhong (2002), Kim et al. (2004), who have also reported differential accumulation of ginsenosides in cell and adventitious root cultures of ginseng treated with methyl jasmonate. The results of the effect of inoculum density combined with methyl jasmonate elicitation on the levels of secondary metabolites in co-cultures are presented in Table 4. The results indicate that an increased inoculum proportion of echinacea, when compared to ginseng has led to the increased levels of caffeic acid derivatives, but it was responsible for reduced concentrations of ginsenosides. However, co-cultures of ginseng and echinacea combined with methyl jasmonate elicitation showed the possibilities of simultaneous production of ginsenosides and caffeic acid derivatives.

This work demonstrates for the first time that adventitious root co-culture is feasible from plants of different species. Our results showed the negative effect of echinacea roots on ginseng adventitious root growth and reduced accumulation of ginsenosides, when the inoculum

proportions of echinacea roots were enhanced with co-cultures. However, when limiting the inoculum of echinacea roots, it was possible to establish co-cultures successfully.

Ginsenoside accumulation was mainly affected when echiancea inoculum ratios were increased during co-cultures. However, the methyl jasmonate treatment of co-cultures elicits the accumulation of ginsenosides. It is possible to harvest ginsenosides and caffeic acid derivatives simultaneously by establishing co-cultures with higher inoculum proportion of ginseng to echinacea (4:1 and 3:2), followed by elicitation treatment.

Acknowledgments This work was financially supported by the ministry of Education and Human Resources Development; the ministry of Commerce, Industry and Energy; and the Ministry of Labor.

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