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ORIGINAL RESEARCH PAPER

Catechin production in cultured cells of *Taxus cuspidata* and *Taxus baccata*

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Abstract The main polyphenols in callus and cell suspension cultures of *Taxus cuspidata* and *T. baccata* were (+)-catechin and (−)-epicatechin, while lignans, such as (+)-taxiresinol, (+)-isotaxiresinol, (+)-isolariciresinol and (−)-secoisolariciresinol, were present in trace amounts. *T. cuspidata* cells contained 1.7% (+)-catechin and 2.4% (−)-epicatechin on dry wt basis but when stimulated with methyl jasmonate produced 3.4% catechin and 5.2% epicatechin. These are the highest levels of these metabolites obtained in plant cell cultures.

Keywords (+)-Catechin · Cell culture · (−)-Epicatechin · Methyl jasmonate · *Taxus*

Introduction

Catechins are potent agents for the treatment and prevention of inflammation-related cardiovascular diseases and autoimmune diseases, because they are critically involved in the suppression of proinflammatory signaling pathways (Suzuki et al. 2009; Al-Hanbali et al. 2009). In cultured plant cells, the maximal content of (+)-catechin (2.9% dry wt) was found in *Polygonum hydropiper* cell suspension cultures (Ono et al. 1998; Nakao et al. 1999). Likewise, the maximal content of (−)-epicatechin (3.9% dry wt) was found in *Camellia sinensis* cultured cells (Shibasaki-Kitakawa et al. 2003).

Although cell cultures from the *Taxus* species have been extensively studied as producers of taxane diterpenoids, little is known about their ability to produce other secondary metabolites. In addition to taxane diterpenoids, many non-taxane compounds have been isolated from the seeds, roots, bark and leaves of *T. cuspidata*, including fifteen lignans, seven flavonoids and five phenolic compounds (Wang et al. 2010).

This study aimed to investigate the composition and content of polyphenols in cell cultures of two *Taxus* species.

Materials and methods

Plant material and cell cultures

Samples of *Taxus cuspidata Sieb.* and *Zucc.* were collected from the southern regions of the Primorsky Territory (Russian Far East). *T. cuspidata* callus

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cultures were established from roots, needles and young shoots as described previously (Fedoreyev et al. 1998). Calli were cultivated on $W_{B/A}$ medium (Bulgakov et al. 2002) supplemented with the following components (mg/l): thiamine-HCl (0.2), nicotinic acid (0.5), pyridoxine-HCl (0.5), meso-inositol (100), peptone (100), sucrose (25000), agar (6000), 6-benzylaminopurine (0.5) and α-naphthaleneacetic acid (2.0) at 25°C in the dark at 30-day subculture intervals. The actively growing callus culture of root origin was selected and designated as the Tc-R1 line. The suspension culture of the Tc-R1 line was grown in 250 ml Erlenmeyer flasks containing 60 ml of the $W_{B/A}$ liquid medium at 21-day intervals in the dark at 25°C in an orbital shaker (100 rpm). A cell suspension culture of *Taxus baccata* was established and maintained using conditions identical to those described here for *T. cuspidata*. The growth index of *Taxus* callus and suspension cultures was calculated as $W_f - W_i/W_i$ where W_f is the final biomass and W_i represents the inoculum biomass. Growth indexes were calculated for 30-days callus cultures and 21-days suspension cultures.

Elicitation with methyl jasmonate (MeJA)

Sterile solutions of MeJA were added to the autoclaved media aseptically in desired concentrations. Stock solution of MeJA in DMSO was prepared at a concentration of 0.1 g/ml w/v. In the control flasks equal volumes of DMSO were added.

Co-cultivation of *T. cuspidata* cells with *Agrobacterium*

The *A. tumefaciens* strains and *T. cuspidata* suspension cultures were co-cultivated as described previously (Bulgakov et al. 2010). The suspension cultures of *T. cuspidata* at the late exponential stage of growth (7 days) were co-cultivated with *A. tumefaciens* GV3101 strains containing pPCV002 (empty vector) or pPCV002-*rolC*.

Analysis of polyphenols

The polyphenol standards, such as (+)-catechin (Porter et al. 1982), (−)-epicatechin (Markham and Ternai 1976), (+)-taxiresinol, (+)-isotaxiresinol, (+)-isolariciresinol and (−)-secoisolariciresinol (Das et al. 1994; Chattopadhyay et al. 2003; Erdemoglu et al. 2004) were previously isolated from mature organs of *T. cuspidata* (Veselova et al. 2007) and *T. cuspidata* cell cultures during the course of the present series of investigations and identified by ^{1}H and ^{13}C NMR and by mass-spectroscopy. Optical rotations and CD spectra of the reference compounds were recorded. The purity of the reference compounds was >98% by the peak area normalization method using HPLC UV.

Analytical HPLC

Extraction and analysis were performed as described previously (Veselova et al. 2007). The dried and powdered cell culture sample (100 mg) was extracted with 96% EtOH (3 ml) for 2 h at 40–45°C. The analytical HPLC was carried out using an Agilent Technologies 1100 Series HPLC system equipped with a VWD detector ($\lambda = 280$ nm). Extracts were analyzed using a ZORBAX Eclipce® XDB-C-8 column (5 µm, 150 × 4.6 mm) thermostated to 30°C. The mobile phase consisted of 1% aqueous acetic acid (A) and acetonitrile containing 1% acetic acid (B). For the analyses, the following gradient steps were programmed: 0–6 min, 10–27% B; 6–20 min, 27–70% B; and 20–25 min, 70–27% B. The flow rate was 1 ml/min. The data were analyzed using the ChemStation® program var. 09 (Agilent Technologies, Germany). Dihydroquercetin dissolved in ethanol was used as an internal standard.

Results and discussion

Callus cultures were obtained from the roots, needles and young shoots of *T. cuspidata*. The behavior of initial callus cultures was similar to that described in the literature (Wickremesinhe and Arteca 1993). The calli possessed heterogeneous morphology and slow growth with brown areas originating on the periphery of the cells, whereas the cells in close contact with the nutrient medium were pale brown. A 2-year selection of the actively growing aggregates yielded homogenous calli of pale brown color. An actively growing callus line of root origin, designated Tc-R1, was chosen for further analysis. The growth index of the Tc-R1 callus line was calculated to be 8.5–9.5.

Table 1 Polyphenol content (lignans and catechins) in *T. cuspidata* trees and the Tc-R1 callus line (% dry wt)

Samples	(−)-Secoiso-lariciresinol	(+)-Isotaxiresinol	(+)-Isolariciresinol	(+)-Taxiresinol	(+)-Catechin	(−)-Epicatechin	Total polyphenols
Needles	tr	0.07 ± 0.02	–	tr	0.16 ± 0.04	0.45 ± 0.08	0.68 ± 0.1
Young shoots without needles	0.05 ± 0.01	0.06 ± 0.01	–	0.03 ± 0.01	0.23 ± 0.05	0.53 ± 0.10	0.90 ± 0.12
Bark	tr	0.06 ± 0.02	tr	0.05 ± 0.01	0.79 ± 0.13	0.34 ± 0.07	1.24 ± 0.14
Heartwood	1.15 ± 0.11	1.96 ± 0.18	0.29 ± 0.06	2.04 ± 0.22	tr	tr	5.44 ± 0.48
Roots	0.21 ± 0.05	0.17 ± 0.08	tr	0.36 ± 0.07	0.23 ± 0.04	0.15 ± 0.03	1.12 ± 0.09
Tc-R1 callus[a]	tr	tr	tr	tr	1.70 ± 0.43	2.41 ± 0.36	4.11 ± 0.72

Samples from wild-growing mature trees were collected in the Khasan Territory of the Primorye Region in Russia

Results presented as mean values ± standard error based on four independent measurements

tr trace amounts

[a] Mean values based on five determinations obtained during 1-year cultivation of the Tc-R1 line. Calli were cultivated in 100 ml Erlenmeyer flasks containing 30 ml of the $W_{B/A}$ agarized medium for 30 days

Polyphenol content was measured in different parts of the *T. cuspidata* plants (Table 1). *T. cuspidata* plants contained lignans, such as (+)-taxiresinol, (+)-isotaxiresinol, (+)-isolariciresinol and (−)-secoisolariciresinol. The Tc-R1 callus culture contained only trace amounts of these compounds. However, this culture produced a large amount of (+)-catechin and (−)-epicatechin (Table 1).

To investigate the biosynthetic capacity of the Tc-R1 line, suspension-cultured cells were grown in the presence of MeJA. MeJA significantly increased the accumulation of catechins, but decreased cell growth (Table 2). (+)-Catechin and (−)-epicatechin content was 3.4 and 5.2% dry wt, respectively. To our knowledge, these are highest levels of catechins for plant cell cultures. Additional amounts of catechins were released into the cultural medium ranging from 5.3 to 10% of the total cell catechins. Other commonly used methods for improving polyphenol production, such as treating with salicylic acid, increasing the sucrose content and changing the phytohormone concentrations, were ineffective or only slightly increased the catechin content (data not shown).

To study polyphenol content in *T. baccata* cells, we used a 3-year-old cell suspension culture (growth index 9.5) from our collection. In this case, stimulating with 30 μM MeJA was the most effective and caused an increase in total (+)-catechin and (−)-epicatechin content from 4 to 8.1% dry wt, which was accompanied by suppressed cell growth (Table 3). Again, (+)-catechin and (−)-epicatechin were the principal polyphenols in these cells. Interestingly, the relative content of (+)-catechin and (−)-epicatechin in the *T. baccata* cells was reversed compared to the *T. cuspidata* cells. While the *T. cuspidata* cultured cells always contained more (−)-epicatechin than (+)-catechin, the *T. baccata* cells contained more (+)-catechin.

Table 2 Effect of methyl jasmonate on biomass accumulation and catechin content in *T. cuspidata* cell suspension culture Tc-R1

MeJA (μM)	Dry cell weight (g/l)	(+)-Catechin (% dry wt)	(−)-Epicatechin (% dry wt)	Total (% dry wt)	Yield of catechins (g/l)
0	23.7	1.27 ± 0.13	3.07 ± 0.2	4.34 ± 0.22	1.03
1	21.0	2.33 ± 0.15	4.00 ± 0.34	6.33 ± 0.38	1.33
10	10.6	3.38 ± 0.31	5.24 ± 0.28	8.62 ± 0.45	0.92
100	4.6	2.85 ± 0.22	4.12 ± 0.16	6.97 ± 0.28	0.32

Mean values ± standard error based on three replicate samples obtained in a single experiment

The inoculum concentration was 25 g fresh wt/l (ca. 1.9 g dry wt/l). The cell suspensions were grown for 7 days. Then, MeJA was added, and the cells were cultivated in the presence of MeJA for 14 days

Table 3 Effect of methyl jasmonate on biomass accumulation and catechin content in *T. baccata* cell suspension culture

MeJA (μM)	Dry cell weight (g/l)	(+)-Catechin (% dry wt)	(−)-Epicatechin (% dry wt)	Total (% dry wt)	Yield of catechins (g/l)
0	19.8	2.61 ± 0.16	1.43 ± 0.1	4.04 ± 0.18	0.80
1	16.0	3.41 ± 0.24	2.27 ± 0.14	5.68 ± 0.28	0.91
10	7.7	3.89 ± 0.3	2.47 ± 0.22	6.36 ± 0.36	0.49
30	5.3	5.57 ± 0.33	2.55 ± 0.2	8.12 ± 0.4	0.43
100	3.9	5.32 ± 0.29	2.70 ± 0.19	8.02 ± 0.43	0.31

The experimental conditions were identical to those described in Table 2

The genetic transformation of *Taxus* species is one of the main challenges for plant biotechnology (Exposito et al. 2010). We proposed that catechins possessing antimicrobial activity could inhibit the process of transformation by suppressing agrobacterial cell growth. To test this hypothesis, we used a "white" variant of Tc-R1 line, which was selected from Tc-R1 calli. These cells contained fivefold less catechins compared to the Tc-R1 line and possessed more active growth (growth index was 13–14). For transformation, we used the *rolC* gene, which is potential activator of secondary metabolism in transformed cells from different plant families (Bulgakov 2008). During cocultivation of the Tc-R1 white cell suspension culture with *A. tumefaciens* GV3101 containing empty vector pPCV002 or pPCV002 with the *rolC* gene, a 2–3-fold increase of catechin content was observed (Table 4). At the same time, a microscopic investigation revealed almost a complete suppression of agrobacterial cell growth. This result suggests that *T. cuspidata* cells responded to *Agrobacterium* by activating catechin biosynthesis, and this event eventually led to the inhibition of bacterial growth. Although the method used (Bulgakov et al. 2010) was successful for many other plants, our attempts to transform *T. cuspidata* cells or explants with the *rol* genes failed.

Thus, *Taxus* cells in in vitro culture use a biosynthetic pathway leading to the production of only the simplest polyphenols. Biosynthesis of polyphenols in *Taxus* cells occurs in a constitutively active manner and a competition for metabolites and energy between the different groups of secondary metabolites can be the limiting factor in the production of other groups of secondary metabolites. Finally, antimicrobial catechins could hamper the genetic transformation of *Taxus* explants or cultivated cells.

Conclusions

Catechins are produced constitutively in cell suspension cultures of *Taxus* species in a high amount. Therefore, cultured *Taxus* cells may be important producers of catechins. However, strategies aimed at obtaining other *Taxus* metabolites, such as taxane diterpenoids, should try to repress the biosynthesis of polyphenols because polyphenols consume a significant amount of cell metabolites and energy.

Table 4 Stimulation of catechin biosynthesis in *T. cuspidata* cell suspension culture Tc-R1 ("white" variant) by *A. tumefaciens* strains

Treatment	(+)-Catechin (% dry wt)	(−)-Epicatechin (% dry wt)	Total
Control	0.21 ± 0.07	0.60 ± 0.04	0.81 ± 0.08
A. tumefaciens, empty vector pPCV002	0.44 ± 0.06	1.07 ± 0.06	1.51 ± 0.1
A. tumefaciens, pPCV002 + *rolC*	0.63 ± 0.04	1.69 ± 0.06	2.32 ± 0.16

Mean values ± standard error based on three replicate samples obtained in a single experiment are presented

Cell suspensions were grown for 7 days. Then, 100 μl of the bacterial suspension was added to 60 ml of *T. cuspidata* cell suspension and co-cultivated for 14 days at 25°C

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