Transformation of *Catalpa ovata* by *Agrobacterium rhizogenes* and Phenylethanoid Glycosides Production in Transformed Root Cultures

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Transformed root cultures of *Catalpa ovata* were established following shoots infection with four agropine strains of *Agrobacterium rhizogenes*. Frequency of root formation was dependent on the bacterial strain and the presence of acetosyringone in the incubation medium. It is the first report concerning the possibility of transforming *Catalpa ovata* by *A. rhizogenes*. Both transformed and untransformed root cultures of *C. ovata* were studied for their growth and phenylethanoid glycoside production. As with the roots of intact plants, *cis*- and *trans*-verbascoside as well as martynoside were produced in transformed and untransformed root cultures of *C. ovata*. In hairy roots, total (*cis* + *trans*) verbascoside production could be stimulated up to three-fold of that of roots of 6-month-old plants grown in a greenhouse, by using an appropriate root line cultured in liquid ½ B5 Gamborg medium containing indole-3-butyric acid (0.1 mg/l) in the dark but not light conditions. Transformed and untransformed root cultures of *C. ovata* were also found to have 10 times higher martynoside production than roots of intact plants.

Introduction

Catalpa ovata G. Don. (Bignoniaceae) is a medicinal plant widely used in the Far East for the treatment of asthma and cough and also for its diuretic and wound healing properties (Koriyone and Kimura, 1962). The trees are known for production of naphthoquinones, iridoid glycosides and flavonoids (Inoue *et al.*, 1979; Suzuki *et al.*, 1991; Okuda *et al.*, 1975). In our recent communication micropropagation of *C. ovata* and the formation of iridoid glycosides (catalpol and catalposide) in regenerated plants have been reported (Lisowska and Wysokińska, 2000).

This work reports for the first time the transformation of *C. ovata* through *Agrobacterium rhizogenes*. Infection of dicotyledonous plants by *A. rhizogenes* usually results in the formation of hairy roots at the site of wounding due to transfer of a DNA fragment of the Ri plasmid from the bacteria to plant cells. Transformed root cultures are of interest for study of secondary metabolite formation or for high production of specific secondary metabolites (Gori and Narasu, 2000). We describe the isolation and accumulation of phenylethanoid glycosides in *C. ovata* hairy root cultures transformed with different strains of *A. rhizogenes*. Culture conditions, such as the presence of auxin in the culture medium as well as the effects of light on root growth and phenylethanoid production were investigated. Phenylethanoid glycoside concentrations were also determined in cultures of untransformed roots and roots of 6-month old, intact plants of *C. ovata*.

Phenylethanoids are known to have some interesting properties, such as antibacterial (Shoyama *et al.*, 1986), antiviral (Kernan *et al.*, 1998), antiproliferative (Herbert *et al.*, 1991) and immunosuppressive activity (Kimura *et al.*, 1987), together with important antioxidant and radical scavenging effects (Zheng *et al.,* 1993; Wang *et al.*, 1996). Although the compounds are distributed among plant species belonging to Bignoniaceae family, they have not been reported in *Catalpa* plants so far.

Materials and Methods

Establishment of transformed root cultures

Axenic *C. ovata* shoots were used for direct infection by the *Agrobacterium rhizogenes*. The

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shoots were obtained from 4-week old shoot cultures growing on Schenk and Hildebrandt (1972) (SH) agar medium supplemented with indole-3-acetic acid (IAA) (0.1 mg/l) and benzylaminopurine (BAP) (1 mg/l). Four agropine-type *Agrobacterium* strains (A4, LBA 9402, ATCC 15834, TR 105) were used for the generation of hairy roots. Bacteria were grown on YMB agar medium (Vervliet *et al.*, 1975) at 26 °C for two days and then were applied to *C. ovata* shoots by wounding with sterile needle dipped into bacterial culture. The infected shoots were incubated on hormone-free agar solidified SH medium supplemented with 200 μmol/l acetosyringone or without this compound. Shoots wounded with a sterile needle without bacteria were placed on the same media served as a control. 15–20 shoots were used for infection with each of four *Agrobacterium* strains. The experiments were repeated three times. After two weeks the inoculated and control shoots were transferred into the SH basal medium supplemented with 500 mg/l ampicillin. Three weeks later, the frequency and number of roots emerging from the inoculation site were recorded. All cultures were kept at 26 °C under continuous light ($40\ \mu m \cdot m^{-2} \cdot s^{-1}$).

After 5 weeks roots developing at the wound site were cut off and transferred individually into liquid B5 medium (Gamborg *et al.*, 1968) diluted to half of normal concentration of salts (½ B5), containing 30 g/l sucrose and 500 mg/l ampicillin to eliminate the bacteria. Bacteria-free root cultures were established after five subcultures at 10-day intervals. Among an axenic root cultures, four lines (one from each *A. rhizogenes* strain) designated HR-1 (induced by LBA 9402), HR-2 (by ATCC 15834), HR-3 (by TR 105) and HR-4 (by A4) were chosen for the experiments. They were grown in ½ B5 or ½ SH liquid media supplemented with 0.1 mg/l indole-3-butyric acid (IBA) and routinely subcultured (1–2 cm long pieces of hairy root – *ca.* 0.01 g dry weight – put into 300 ml Erlenmeyer flask containing 80 ml of the medium) every 5 weeks by at least 18 passages. All cultures were kept on a rotary shaker (100 rpm) at 26 °C in the dark, except for the light experiment carried out with line HR-1 in which continuous cool white fluorescent lamps ($40\ \mu m \cdot m^{-2} \cdot s^{-1}$) were used.

Establishment of untransformed root culture

Roots (about 2–3 cm long) were excised from 3-week-old aseptic seedlings of *C. ovata*. The roots were grown in ½ B5 liquid medium supplemented with sucrose (30 g/l) and IBA (0.1 mg/l). They were subcultured every 5 weeks and maintained under the same conditions as these used for hairy root cultures.

Growth index

For biomass increase of transformed and untransformed roots, growth index (GI) was calculated as a ratio of dry weight after 35 days of culture to dry weight of inoculum.

Isolation and identification of phenylethanoid glycosides

Dried and powdered hairy roots (500 g) were extracted twice with boiling methanol. Extracts were combined, dried under reduced pressure and dissolved in hot (80°) water. After filtration the aqueous solution was purified with petrol ether. The aqueous phase was extracted successively with (6×50 ml) benzene, ethyl acetate and *n*-butanol. Ethyl acetate and *n*-butanol extracts were concentrated and chromatographed on silica gel column (70–230 mesh, Merck) as follows.

Ethyl acetate dry extract (1.1 g) was chromatographed using chloroform – methanol (3 : 1, v/v). The fractions were analysed by TLC (silica gel 60 F_{254} plates, with solvent system chloroform – methanol 3 : 1, v/v). For glycosides detection TLC plates were sprayed with 1% vanillin /10% H_2SO_4 reagent or a saturated solution of ceric sulfate in 65% H_2SO_4 and then heated to 120 °C for 5–15 min. The relevant fractions were combined and further separated by preparative TLC to afford 27 mg of martynoside. *n*-Butanol dry extract (0.95 g) was chromatographed on silica gel column using methanol-chloroform-water (75:25:2, v/v) solvent system. The combined fractions after TLC control were repeatedly chromatographed on silica gel column with ethyl acetate: methanol (4:1, v/v) to give verbascoside (16 mg). Identification of martynoside and verbascoside was carried out using ^{1}H-NMR spectroscopy (Bruker 200 MHz CD_3OD) and MS-FAB spectrometry (Finnigan-MAT 95). Their spectral data were compared with

those of authentic samples. Original samples of martynoside and verbascoside were obtained earlier in our laboratory (Skrzypek *et al.*, 1999).

Quantification of phenylethanoid glycosides

Dried and powdered hairy roots (0.5 g) were extracted with methanol (1h, 2×30 ml). The solvent was evaporated *in vacuo* and the residue was dissolved in methanol (5 ml). After filtration on Millipore filter (0.22 μm), the filtrate was analysed by HPLC (Beckmann, computer system – System Gold). The analysis was performed on C-18 ODS column (4.6 × 250 mm). An isocratic elution with 0.04 M *o*-phosphoric acid:acetonitrile (4:1, v/v) was used at a flow rate 1 ml·min^{-1}. The detection was performed at 332 nm.

The identification of phenylethanoid glycosides was done by comparison of the chromatographic peak retention times with those of authentic compounds. Three compounds observed in the HPLC chromatogram were identified as *cis*-verbascoside (Rt 5.02 min) *trans*-verbascoside (Rt 5.38 min) and martynoside (Rt 16.28 min). For quantitative analysis the external calibration method was used. Three calibration curves were established with *cis*- and *trans*-verbascoside and martynoside over the range 10–300 μg/ml. The results presented as a percentage of dry weight are means of three experiments. Untransformed roots cultured *in vitro* and roots of 6-month-old *in vitro* regenerated plants grown in pots were treated in an analogous way.

Results and Discussion

Root formation mediated by A. rhizogenes

C. ovata shoots were susceptible to the infection with different strains of *A. rhizogenes*. The infection usually resulted in callus formation on the wounded surface followed by appearance of roots (Fig. 1). The first putatively transformed roots were visible 10 days after inoculation. They were formed within 4 weeks. No callus or roots formation was observed in uninoculated control shoots.

The percentage of *C. ovata* shoots forming roots ranged from 0 to 83% depending on the strain of *A. rhizogenes* and on the presence of acetosyringone in the inoculation medium. The A4 and ATCC 1584 strains were the best ones for inducing

Fig. 1. Hairy roots induced by the infection of aseptically grown shoots of *Catalpa ovata* with *A. rhizogenes* (ATCC 15834), 5 weeks after infection (bar = 1 cm).

hairy roots of *C. ovata*. The infection of shoots with these strains induced root initiation in approximately 80% of the explants treated (Table I). It has been shown that the plasmids pRi 15834 and pRi A4, present in ATCC and A4 strains, respectively, are almost identical (Huffmann *et al.*, 1984). Transformation of *C. ovata* (expressed as a number of explants forming roots at the inoculation site after 5 weeks) by LBA 9402 strain occurred at approximately two-fold lower frequency compared to results obtained using ATCC 15834 and A4 strains. Among the four *A. rhizogenes* strains tested, the TR 105 was generally unable to initiate roots on *C. ovata* shoots. Only with the addition of acetosyringone, 7% of explants developed callus and single root within 4 weeks after infection with the strain. The addition of acetosyringone to SH inoculation medium enhanced also the virulence of LBA 9402 strain. In the presence of this compound the percentage of *C. ovata* shoots forming roots increased to 58% as compared to 41% without acetosyringone (Table I).

Table I. Effect of various strains of *Agrobacterium rhizogenes* and acetosyringone on the frequency of root induction on *Catalpa ovata* shoots.

Bacterial strain	Inoculation medium	% of explants producing roots	Mean number of roots per explant *
LBA9402	SH	41	1.9
	SH + AcS	58	1.6
ATCC15834	SH	78	2.8
	SH + AcS	73	2.0
A4	SH	83	1.8
	SH + AcS	78	2.3
TR105	SH	n.r.	
	SH + AcS	7	1.0

Root induction was determined 5 weeks after inoculation.
AcS – acetosyringone.
SH – Schenk and Hildebrandt (1972) agar medium without acetosyringone.
SH + AcS – Schenk and Hildebrandt agar medium with acetosyringone (200 μmol/l).
* Mean number of roots per explants was calculated by dividing the total number of roots by the number of explants forming roots.
n.r. – no response.

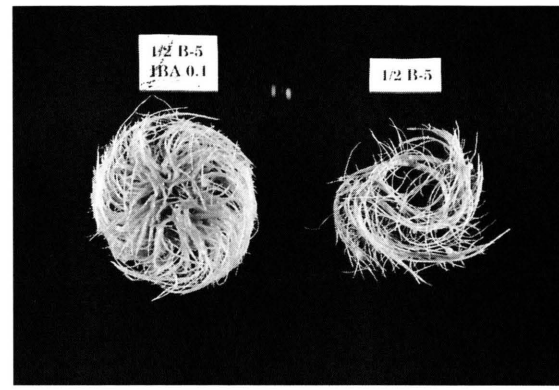

Fig. 2. Hairy roots of *Catalpa ovata* (HR-1 line) cultured in ½ B5 liquid medium with indole 3-butyric acid (0.1 mg/l IBA) (left) or without IBA (right) for 5 weeks, in the dark (bar = 1 cm).

Studies of growth of transformed and untransformed roots

Four axenic hairy root lines (HR-1 ~ HR-4), each one obtained through inoculation with a different bacterial strain, were selected and examined on their growth and phenylethanoid production (Table II). Transformation of these roots was proved by the detection of opines (mannopine and agropine). The roots were grown in ½ B5 and ½ SH liquid media in the dark. However, isolated roots grew poorly in hormone-free media (Fig. 2). For example, GI of HR-1 line reached 6 after 5 weeks. Therefore, auxin (IBA) at low concentration (0.1 mg/l) was added to the culture media. Under these conditions the roots grew well; their GI ranged from 10 to 37 depending on root line and basal medium. Slow growth of transformed roots in hormone-free media is not rare among recalcitrant plants. For example, it has been demonstrated that *Panax ginseng* hairy roots required phytohormones in the medium for satisfactory growth (Ko *et al.*, 1994). Tanaka (1999) has found that a low concentration of auxin (0.1 mg/l α-naphthaleneacetic acid) is required for growth as well as initiation of hairy roots on *Vinca minor*. According to Gaudin and Jouanin (1995) auxin is necessary for *aux* gene expression (bacterial genes encoding auxin biosynthesis located on TR-region of T-DNA) and the level of expression is correlated with its concentration. *Aux*-gene silencing might be one of the reasons why exogenous auxin is needed for growth of *C. ovata* hairy roots in *in vitro* culture.

Table II. Growth and phenylethanoid glycoside contents (% of dry weight) in different lines of transformed roots of *Catalpa ovata* grown in two liquid media (½ B5 and ½ SH) for 5 weeks, in the dark.

Root line	Growth index*		Verbascoside (*cis* + *trans*)		Martynoside	
	in ½ B5	in ½ SH	in ½ B5	in ½ SH	in ½ B5	in ½ SH
HR – 1	21 ± 9	35 ± 11	1.30 ± 0.43	1.49 ± 0.03	0.48 ± 0.17	0.41 ± 0.01
HR – 2	37 ± 5	29 ± 4	1.08 ± 0.04	1.21 ± 0.02	0.44 ± 0.03	0.24 ± 0.01
HR – 3	10 ± 1	16 ± 1	2.32 ± 0.08	2.67 ± 0.09	0.49 ± 0.01	0.58 ± 0.02
HR – 4	32 ± 4	24 ± 1	3.93 ± 0.16	4.12 ± 0.07	0.49 ± 0.25	0.38 ± 0.01

Values represent means of three replications with standard error.
The media were supplemented with indole 3-butyric acid (0.1 mg/l)
* final dry weight/initial dry weight (0.01 g).

Untransformed roots of *C. ovata* obtained from germinated seedlings were cultured under the same conditions (½ B5 liquid medium supplemented with IBA 0.1 mg/l) as transformed roots. However, their growth was much lower (GI 5.2) than that obtained after infection with *A. rhizogenes*. Furthermore, after several (3–4) passages untransformed roots spontaneously developed undifferentiated callus and then the culture died.

Production of phenylethanoid glycosides in transformed and untransformed roots

Two major phenylethanoid glycosides, verbascoside and martynoside, were isolated from hairy roots of *C. ovata*. They were identified by comparing their spectroscopic data (^{1}H NMR, MS-FAB) with authentic samples. Table II shows the contents of the phenylethanoid glycosides determined by HPLC in various hairy root lines, induced by four strains of *A. rhizogenes*. The roots were cultured in ½ B5 and ½ SH liquid media containing IBA (0.1 mg/l), in the dark, for 18–20 subcultures. In hairy root lines used for analysis the verbascoside level ranged from 1 to 4% on the basis of dry weight. The line designated HR-4 induced by A4 *Agrobacterium* strain was the most productive one. Martynoside content was almost the same (0.4–0.6% of dry weight) in all hairy roots tested, with the exception of HR-2 line, in which martynoside production dropped to 0.2% of dry weight, when the roots were cultivated in ½ SH liquid medium (Table II). In other cases, the contents of martynoside as well as verbascoside in hairy roots grown in ½ SH and ½ B5 liquid media were similar. The results indicate that mineral composition of the culture medium had little effect on biosynthesis of the phenylethanoid compounds in hairy roots of *C. ovata*.

Spectral data show that verbascoside is present in extracts of hairy roots of *C. ovata* as a mixture of *trans*/*cis* isomers. The HPLC method was used for their separation. In four *C. ovata* root lines mentioned in Table II, the ratio of *cis*- to *trans*-verbascoside ranged from 1:2 to 1:5. The experiments carried out with HR-1 line showed that the level of *cis*-verbascoside production could be changed by altering culture parameters, such as the dark or the light and the presence of auxin (IBA 0.1 mg/l) in the culture medium. The roots grown under continuous light (40 $\mu m \cdot m^{-2} \cdot s^{-1}$) in ½ B5 medium containing IBA (0.1 mg/l) accumulated five times less *cis*-verbascoside than those cultivated in the same medium but in the dark (0.1% in comparison to 0.5% on the basis of dry weight, respectively) (Fig. 3). Nishimura *et al.* (1991) have reported that *cis*-verbascoside may be converted to *trans*-verbascoside in the light. However, our results demonstrate that the content of *trans*-verbascoside in hairy roots cultured in the light did not increase and its level remained the same as in roots grown in the dark (Fig. 3). This suggests that the changes in *cis*/*trans* verbascoside ratio in roots grown in the light cannot be explained only by the interconversion of geometrical isomers. Furthermore, light causes slight decrease in martynoside content (to 70% of that of the dark-grown roots) (Fig. 3). Similar results for the accumulation of phenylethanoid glycosides (*i.e.* small decrease in martynoside and large decrease in *cis*-verbascoside contents) were obtained with the dark-grown roots cultured in ½ B5 medium without auxin (Fig. 3).

For comparison, the contents of *cis*- and *trans*-verbascoside and martynoside in untransformed roots cultured *in vitro* and roots of 6-month old *C. ovata* plants grown in the greenhouse were analysed. The results shown in Table III indicate that

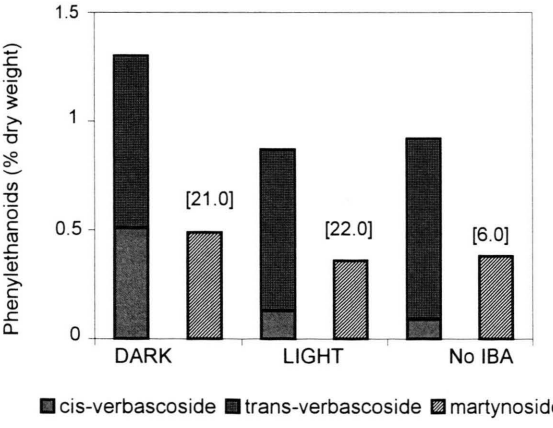

Fig. 3. The effect of light and indole-3-butyric acid on phenylethanoid glycoside content in *Catalpa ovata* hairy roots (HR-1 line).
The roots were cultured for 5 weeks in ½ B5 liquid media with indole-3-butyric acid (0.1 mg/l IBA) under light (40 $\mu m \cdot m^{-2} \cdot s^{-1}$) and dark conditions or without IBA in the dark. Values are the mean of three replications. Number in brackets indicate the growth index.

Table III. Phenylethanoid contents (% of dry weight) in untransformed root cultures and roots of 6-month old plants of *Catalpa ovata* regenerated *in vitro*.

Material	Verbascoside (*cis* + *trans*)	Martynoside
Roots grown *in vitro* *	1.69 ± 0.09	0.56 ± 0.28
Roots of regenerated plants	1.12 ± 0.15	0.05 ± 0.01

* Roots were cultured in ½ B5 liquid medium supplemented with indole 3-butyric acid (0.1 mg/l) for 5 weeks, in the dark.
Values are the mean of three replications with standard error.

untransformed root culture and roots of intact plants contained lower (2–3.6 times) amounts of verbascoside (*cis* + *trans*) compared to those observed in high-productive transformed root lines, such as HR-4. Also, *cis*- to *trans*-verbascoside ratio was quite different in untransformed (2:1) and transformed (1:2–1:4) roots, cultivated under the same conditions (*i.e.* in the dark, in ½ B5 liquid medium supplemented with IBA 0.1 mg/l). Verbascoside has been mostly described to occur in intact plants and cultured cells as *trans*- isomer (possessing *trans*- caffeoyl moiety), whereas *cis*-verbascoside (possessing *cis*-caffeoyl moiety) has been found only in small amounts so far in leaves of *Stachys sieboldii* (Nishimura *et al.*, 1991), *Lantana camara* (Mahato *et al.*, 1994) and *Lamium album* flowers (Budzianowski and Skrzypczak, 1995), as well as in cell suspension of *Penstemon serrulatus* (Skrzypek *et al.*, 1999). In contrast to the results with verbascoside, martynoside content in untransformed roots cultured *in vitro* (0.5% of dry weight) did not differ from that observed in transformed roots of *C. ovata*. In both cases this was about ten times of amount detected in roots of plants grown in the greenhouse (0.05% of dry weight) (Table III).

In conclusion, root cultures able to high and stable growth might be obtained only by genetic transformation of *C. ovata* shoots with *A. rhizogenes*. The cultures present an interesting model for studying the biosynthesis of phenylethanoid glycosides. Despite the importance of these compounds relatively little is known about their formation, storage and metabolism. Our experiments indicate, that desired biosynthetic capacity of *C. ovata* hairy roots in respect to phenylethanoid glycosides can be achieved by: 1) using verbascoside-rich line, such as HR-4; 2) addition of auxin to the culture medium; 3) culturing roots in darkness.

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