

Accumulation of Unusual Phenylpropanoids in Transformed and Non-Transformed Root Cultures of *Coreopsis tinctoria*

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Using *Coreopsis tinctoria* liquid cultures of *Agrobacterium rhizogenes* transformed roots (so-called “hairy roots”) and non-transformed roots have been established. The production of different phenylpropanoid compounds by these cultures grown in light or darkness, respectively, was compared. Whereas the composition of phenylpropanoids in the root cultures was not influenced by the culture conditions, the total production of phenylpropanoids as well as the formation of single compounds each analyzed were higher in darkness than in light. The biomass production of the transformed root cultures exceeded that of non-transformed root cultures by a factor of 2.6. At the same time compound accumulation paralleled the biomass production, so that the dark grown transformed root cultures were producing maximum yields of several phenylpropanoids, especially 1'-isobutyryloxy-eugenol-isobutyrate (7 mg per culture flask during a single culture passage).

Introduction

For more than 30 years many efforts have been made to produce secondary compounds by means of plant cell cultures. With few exceptions the cultured cells accumulate such substances in disappointingly low concentrations compared to those present in the specialized tissues of the corresponding plants [1, 2]. Many experiments have, therefore, been performed to increase production of secondary compounds *in vitro*. One possible method to achieve this aim is the *in vitro* culture of plant organs such as roots [2].

In a preceding paper we reported the presence of unusual phenylpropanoids in *Coreopsis tinctoria* [3]: derivatives of 1'-hydroxy-eugenol-isobutyrate and 1',2'-epoxy-Z-coniferylalcohol-isobutyrate. These compounds were accumulated mainly in the roots and to a much lesser extent in the leaves. Shoot axes, flower heads and fruits did not contain these phenylpropanoids. The presence of diesters of 1'-hydroxy-eugenol and 1',2'-epoxy-Z-coniferylalcohol in *Coreopsis* species [3–5] raise interesting questions about their biosynthesis (for example, the hydroxylation of the eugenol side chain or the formation of the Z-configuration of the epoxidized coniferylalcohol

compounds) and their possible biological activities. In this latter case, 1'-acetoxy-eugenol-acetat from *Alpinia galanga* (Zingiberaceae) showed a significant anti-ulcer activity in rats and a well documented anti-tumour activity against Sarcoma 180 ascites in mice [6, 7].

To investigate the biosynthesis of these phenylpropanoid types *in vitro* it is necessary to establish fast-growing root cultures with high amounts of desired phenylpropanoids. In this paper growth behaviour and the accumulation capacity for phenylpropanoids of transformed and non-transformed root cultures of *Coreopsis tinctoria* are compared.

Materials and Methods

Plants

Fruits of *Coreopsis tinctoria* NUTTAL were purchased from Samen Wagner (Heidelberg). Seedlings were cultured under aseptic conditions on a solid M&S medium without phytohormones [8]. Adult plants were grown in the greenhouse.

Bacteria

Agrobacterium rhizogenes, strain 15834 (American Type Culture Collection) was cultured in NYS medium [9] on a gyratory shaker (100 r.p.m.) at 25 °C. 48 h cultures were used for inoculation of plants.

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Inoculation of plants

Using a syringe (\varnothing 0.75 mm) the bacteria were inoculated into different sites of shoot axes of sterile seedlings (5 cm height). After 12 days the development of Transformed Roots (TR) could be observed. Transformed roots often show a considerable lateral branching. With regard to this typical phenotype the *Agrobacterium rhizogenes* transformed roots are also called "hairy roots" [2].

Liquid cultures of Transformed Roots (TR)

40 days after inoculation the transformed roots were detached from the seedlings, placed on a hormone-free solidified M&S medium [8] and inoculated with *A. rhizogenes* for a second time. After another 4 weeks side roots developing from these inoculation sites were isolated, transferred to a hormone-free solidified M&S medium containing 500 mg/l carbenicillin. By repeated transfers (7 \times) every 10 days to fresh carbenicillin-containing medium, roots devoid of any visible bacterial contamination were obtained. These roots were transferred into a liquid carbenicillin- and hormone-free M&S medium (50 ml per 200-ml-Erlenmeyer flask) and cultured on a gyratory shaker (110 r.p.m.) at 25 °C and either 1500 lux of white light or complete darkness. Every 2 weeks 0.5 g of tissue was transferred to the same fresh medium. After 15 culture passages of TR the phenylpropanoid analyses were performed.

Opine detection in transformed root cultures

Transformed roots induced by *A. rhizogenes* can be distinguished from their normal counterparts by the synthesis of opines, unique amino acid derivatives of tumour cells [10–14]. To prove the genetic transformation of roots used in our experiments, the presence of opines was demonstrated in the following way. Fresh tissues were extracted with Aq.dest. (1 ml per g tissue weight) and the solution obtained centrifuged at 12000 r.p.m. for 2 min. The supernatant was evaporated under reduced pressure at 40 °C, the residue redissolved in Aq.dest. (0.2 ml per g fresh weight of tissue) and centrifuged again. 10 μ l of the supernatant were spotted onto 3MM chromatographic paper (Whatman) and the amino acid derivatives separated by electrophoresis (400 V for 1 h) using a mixture of formic acid/acetic acid/distilled water (1:3:16) as an electrophoresis medium. The migra-

tion of opines was determined by co-migration with mannopine standard [10, 11, 13]. After drying the paper, the opines were made visible by means of alkaline silver nitrate reagent [11, 13].

Solution A: 0.5 g AgNO₃ in 250 ml acetone;

Solution B: Ethanol/20% NaOH (9:1);

Solution C: 10% Na₂S₂O₃ and 1.5% Na-metabisulphite in water.

The paper was briefly dipped into solution A and developed in solution B until brown spots appeared (about 3 min). Finally the chromatogram was fixed in solution C and washed with tap water for 20 min.

Liquid culture of non-transformed roots (CTW)

Non-transformed roots (CTW = *Coreopsis tinctoria* Wurzel) were obtained from seedlings germinated under aseptic conditions without further treatment [15] and cultured in liquid hormone-free M&S medium containing 30 g/l sucrose under conditions identical to those of transformed root cultures. After about 50 culture passages of CTW phenylpropanoid analyses were performed.

Identification of phenylpropanoids

For the identification of phenylpropanoids in plants and root cultures the compounds were isolated and their chemical structures verified by means of GC/MS coupling and by co-chromatography with phenylpropanoid standards as described before [3].

GC: Varian 3700; column: quartz capillary OV1 OB, 30 m; carrier gas: Helium; injector temperature: 250 °C; detector temperature: 260 °C; oven temperature: 140 °C; temperature program: 140 °C, after 1 min increase by 4 °C/min. MS: Finnigan Mat 311A.

Quantification of phenylpropanoids in plants and root cultures

As an internal standard amounts ranging from 0.075 to 2 mg (depending on the fresh weight of biomass and accumulation range) of epoxy-isoeugenol-isobutyrate [3, 15] were added to the biomass of a flask. The biomass was homogenized in the presence of 10–30 ml chloroform and filtered. An aliquot of each extract was subjected to HPLC separation. All data given in the Figures and Table represent averages from 2–3 independent experiments.

HPLC equipment

LDC/Milton Roy; 2 pumps Constametric I and III; injector: Rheodyne 7125; column: Lichrospher 100 CH 18/2; solvent: MeOH/water 75:25; flow 1.2 ml/min; detector: Spectro-Monitor D, 278 nm.

Results and Discussion

Inoculation of various plant organs with *A. rhizogenes*

Virulent strains of *A. rhizogenes* harbour a root-inducing plasmid (pRi). In the process of plant infection a part of this plasmid (T-DNA) is integrated into the plant nuclear DNA. Inside the plant cell plasmid-borne genes for opine and phytohormone synthesis are expressed, the phytohormone balance at the infection site is disturbed and the development of transformed roots (TR) is started [10–14]. Different plant species and different tissues of a plant show various degrees of susceptibility to an infection with *A. rhizogenes*. Therefore in preliminary experiments the susceptibility of different tissues of *C. tinctoria* was tested. Whereas transformed root formation at the shoot axis became visible beginning 12 days after inoculation, the petiole and the leaf laminae never developed roots.

Growth of transformed and non-transformed root cultures in liquid medium

From each of the 2 root types, transformed roots (TR) and non-transformed roots (CTW), two types of liquid cultures were established: one in continuous light (TR-L and CTW-L) and one in continuous darkness (TR-D and CTW-D). A comparison of the growth characteristics (root form, fresh weight) showed that the light regime did not exert an influence on root growth (Fig. 1). On the other hand the growth of the cultures was strongly influenced by the genetic status (transformed or non-transformed) of the root cells. The transformed roots responded to a much higher growth rate than the non-transformed roots. Within 12–15 days the maximum fresh weight of 12–13 g per flask was obtained and the biomass increased three fold between the 6th and the 12th day of culture passage. On the contrary, non-transformed roots achieved their maximum fresh weight of about 5 g per flask only after 26 days. So the increase of biomass of non-transformed roots was lower than that of transformed roots by a factor of 2.6.

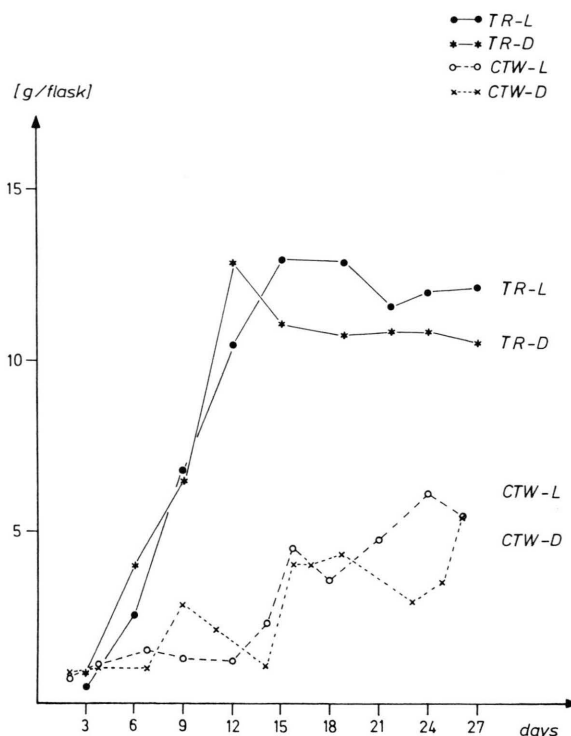


Fig. 1. Growth kinetics of transformed (TR) and non-transformed (CTW) root cultures during a single culture passage in light (L) and darkness (D), respectively.

Opine production by transformed root cultures

To prove the transgenic character of TR of *Coreopsis tinctoria* used in our experiments opine tests have been performed. In freshly established transformed root cultures considerable amounts of agropine and mannopine could be detected. After repeated subcultures the opine production decreased; after 6 passages opines were no longer detected. This apparent loss of opine production during long-term *in vitro* culture of transformed roots has also been found with other species [12, 13].

Histology of transformed and non-transformed root cultures in liquid medium

Normal, non-transformed roots from intact plants and from liquid culture (CTW) and cultured transformed roots (TR) were examined for morphological and anatomical traits. Macroscopically the 3 types of roots did not show great differences, with the exception of a much more pronounced ramification of the

transformed roots. The 3 types of roots showed a comparable anatomical structure: an intact root tip is covered by a calyptra which is coloured deep red, probably by the accumulation of anthocyanins. 2–3 cm proximal to the root tip a root hair zone is present. The central bundle is of the typical radial type. Near the endodermis there are 3 to 4 intercellular spaces per cross section which are filled with essential oil.

Accumulation of phenylpropanoids in transformed and non-transformed root cultures

The infection of plants with *A. rhizogenes* leads to a new genetic status of the tissues concerned. As a consequence the growth pattern of these tissues is changed. At the same time changes in the accumulation of secondary compounds may be initiated. To consider this possibility, both transformed and non-transformed roots cultured *in vitro* have been compared with respect to their production and accumulation of phenylpropanoids in light and darkness. The phenylpropanoids formed under these conditions have been identified and characterized by HPLC, GC and GS-MC coupling.

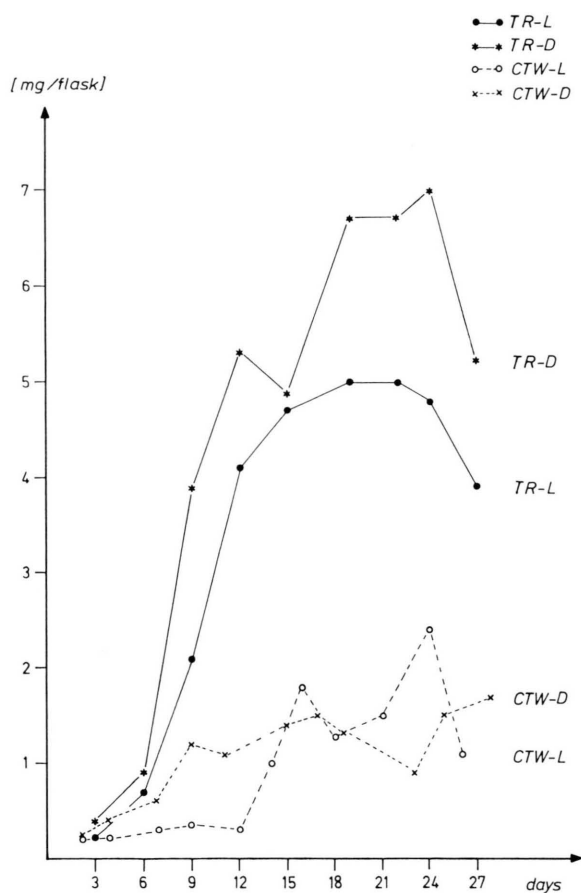
In Table I the phenylpropanoids detected and the maximum amounts of phenylpropanoids accumulated during a single culture passage are listed. Neither light conditions nor the genetic status of the cultured roots influenced the number of phenylpropanoids accumulated. Compounds **1** to **4** have

been identified as 1'-acetoxy-eugenol-isobutyrate, 1'-isobutyryloxy-eugenol-isobutyrate, 1'-(2-methylbutyryloxy)-eugenol-isobutyrate, 1'-isovaleroyloxy-eugenol-isobutyrate; compound **6** as 1',2'-epoxy-Z-coniferyl alcohol-3'-isobutyryl-isobutyrate. On the other hand, the amounts of different phenylpropanoids accumulated were more dependent on the light conditions during the *in vitro* culture and less dependent on the genetic status of the roots. In general roots cultured in darkness (TR-D and CTW-D) surpassed roots cultured in light (TR-L and CTW-L) with respect to the total amount of phenylpropanoids accumulated as well as with respect to single compounds investigated. The cultured roots as well as the roots of intact plants accumulated mainly compound **2**. The maximum amounts detected in the root cultures (580 and 630 µg/g fresh weight) correspond to the amounts found in the roots of intact plants (400–500 µg/g fresh weight) [3, 15].

From a comparison of the accumulation kinetics of compound **2** during a single culture passage differences between the transformed and non-transformed cultures became evident. In the transformed root culture the accumulation of compound **2** parallels the increase of biomass during the complete log phase of growth. Maximum values were attained during the stationary phase of growth and remained more or less constant between day 18 and day 22 of a culture passage. By contrast, in the non-transformed root cultures compound **2** attained maximum values at

Table I. Accumulation of phenylpropanoids in transformed (TR) and non-transformed (CTW) root cultures cultivated in light (L) and dark (D). Compounds: **1** = 1'-acetoxy-eugenol-isobutyrate; **2** = 1'-isobutyryloxy-eugenol-isobutyrate; **3** = 1'-(2-methylbutyryloxy)-eugenol-isobutyrate; **4** = 1'-isovaleroyloxy-eugenol-isobutyrate; **6** = 1',2'-epoxy-Z-coniferylalcohol-3'-isobutyryl-isobutyrate. The table values mean maximum amounts (in µg/g fr.wt.) of single compounds accumulated by the root cultures at different times during one growth period.

Compounds	Transformed root cultures		Non-transformed root cultures	
	Light	Dark	Light	Dark
1	87 ± 21	171 ± 44	25 ± 7	74 ± 23
2	420 ± 36	630 ± 104	440 ± 0	580 ± 64
3/4	traces	traces	traces	traces
6	142 ± 63	152 ± 0	107 ± 35	213 ± 24
Total in % of fr.wt.	0.065%	0.095%	0.057%	0.087%



day 7 to day 13 of a culture passage and decreased rapidly to 200–300 $\mu\text{g/g}$ fresh weight. So the more rapid and more extended production of biomass in transformed roots which is paralleled by the accumulation of compound **2** leads to an increased total yield of this compound compared to non-transformed root cultures. TR-D culture accumulated a maximum value of 7 mg of compound **2** per flask during a single culture passage (Fig. 2). Among the other 4 compounds only compound **1** was produced by transformed root cultures in significantly higher amounts than by non-transformed root cultures (Table I).

The advantages of transformed root cultures of *C. tinctoria* concerning biomass production and phenylpropanoid accumulation indicate that this type of *in vitro* culture is suitable for biosynthetic studies. TR-D accumulates, in a very constant manner, 1'-isobutyryloxy-eugenol-isobutyrate (compound **2**) in high amounts (up to 7 mg/flask) for more than one year. Initial experiments using transformed root cultures for biosynthetic studies of compound **2** have already been performed successfully.

Fig. 2. Accumulation of compound **2** (1'-isobutyryloxy-eugenol-4-isobutyrate) in transformed (TR) and non-transformed (CTW) root cultures during a single culture passage in light (L) and darkness (D), respectively.

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