APPLE-TREE SHOOTS AND TRANSFORMED CARROT AND APPLE ROOTS USED AS BIOCATALYSTS IN ENANTIOSELECTIVE ACETATE HYDROLYSIS, ALCOHOL OXIDATION AND KETONE REDUCTION*

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Received May 13, 1998
Accepted July 21, 1998

Dedicated to Dr Jan Fajkos on the occasion of his 75th birthday.

(±)-1-Phenylethyl (**1**), (±)-1-(1-naphthyl)ethyl (**2**), (±)-1-(2-naphthyl)ethyl (**3**) and (±)-menthyl (**4**) acetates were hydrolyzed using apple-tree shoots and hairy roots of carrot and apple-tree to afford alcohols, which, subsequntly, in the same environment, were oxygenated to ketones. Pure (*S*)-1-(2-naphthyl)ethanol ((*S*)-(–)-**7**), (*R*)-1-phenylethyl acetate ((*R*)-(+)-**1**), (*R*)-1-(1-naphthyl)ethyl acetate ((*R*)-(+)-**2**), (*S*)-1-phenylethanol ((*S*)-(–)-**5**) and (–)-menthol (1*R*,2*S*,5*R*)-(–)-**8**) have been produced.

Key words: Biotransformations; Enantioselective hydrolysis and reduction; Enzymatic resolution; Plant tissue cultures; Hairy roots; Shoots; *Malus pumila*; *Daucus carota*.

Esters of secondary alcohols are transformed both by microorganisms[2,3] and plants[4–6]. In our earlier studies some parts of plants[7–9], cell cultures of carrot[10] and whole plants[10] had been used for biotransformation of title substrates. The biotransformations resulted in separation of enantiomers.

The aim of this study was to establish the ability of apple-tree roots and shoots and carrot-roots *in vitro* culture to transform racemic esters.

The plant tissues transformed by *Agrobacterium rhizogenes* and hairy roots obtained in this way seem to be a promising tool in modern biotechnology. Hairy roots are characterized by a very efficient biomass increase and when the enzymes are released to the medium, it is possible to use them efficiently after immobilization in bioreactors. The comparison of the results of biotransformation carried out by underground and over-

* Part XLVI in the series Biotransformations; Part XLV see ref.[1].

ground parts (root, bulb, shoot and fruit) of the same plant is likely to provide more information on the potential role of its enzyme system in biocatalysis.

In the present investigation, the explants of the same (apple and carrot[8,10]) plants: genetically modified (*Agrobacterium rhizogenes*) and non-transformed carrot (*Daucus carota*) and apple-tree (*Malus pumila*) roots (underground parts of plant) as well as apple shoots (overground parts) were used. Only a few papers on the comparison of the abilities of various parts of a plant to transform exogenous substrates have been published so far. In this study, racemic acetates of secondary alcohols and methyl acetate were used as substrates. Such a selection of the substrates allowed us to examine the enantioselectivity of the enzyme systems of apple and carrot tissues.

Lactones are the compounds occurring in apple fruits. Apple (fruit) possesses the enzymatic system capable of lactonizing exogenous hydroxyacids[11]. The enzymatic system of the carrot is capable of a transformation of steroids[5,12]. Therefore, in our investigations, ricinoleic (12-hydroxyoleic) acid and testosterone were also subjected to the transformation with the use of a carrot and an apple shoot and root cultures.

EXPERIMENTAL

Chromatography

GC: Hewlett–Packard 5890, FID, carrier gas – H_2 using Chrompack WCOT Fused Silica, Chirasil-Val (0.25 mm × 25 m) capillary column for **1** (column temperature 125 °C, injection temperature 250 °C), for **2** and **3** (column temperature 100 °C/5 min; 0.5 °C/1 min; 116 °C const., injection temperature 250 °C) and Chirasil-DEX CB (0.25 mm × 25 m) for **4** (column temperature 80 °C/1 min; 1 °C/1 min; 110 °C const., injection temperature 250 °C).

TLC: Silica gel 60 F_{254} pre-coated aluminium sheets (layer thickness 0.2 mm, Merck) with n-hexane-EtOAc (8 : 1) for **1** and **2**, n-hexane–Et_2O (5 : 1) for **3** and benzene–EtOAc (10 : 3) for **4**.

CC: silica gel with n-hexane–acetone (20 : 1 for **1**, 30 : 1 for **2** and 35 : 1 for **3**).

Biocatalysts

Cultures of M7 Apple Rootstock Shoots as Biocatalysts

Proliferating cultures of M7 apple shoots were obtained from isolated meristems. Meristems were collected from field growing plants in the period of their intensive growth in June and July. After being disinfected, isolated meristems were inoculated on Murashige Skoog (MS) medium[13] containing 0.2 mg/dm^3 of kinetin and 0.2 mg/dm^3 of indole-3-acetic acid (IAA). For multiplication purposes, developing shoots were transferred on the Quoirin and Lepoivre (QL) medium[14] with half a macronutrient concentration, enriched with benzyladenine (BA, 0.5 mg/dm^3), indole-3-butyric acid (IBA, 0.1 mg/dm^3) and gibberellic acid (GA_3, 2.0 mg/dm^3). Saccharose (20 g/dm^3) and agar (10 g/dm^3) were added to the medium. Before sterilization, pH was adjusted to 5.8–6.0 with 0.1 M NaOH. Cultures were maintained in a growth chamber at 25–27 °C and illuminated (2 500 lx) by using fluorescent Day Light type tubes in a 16 h photoperiod.

Apple shoots 2.0–2.5 cm long were taken from 10 week old culture. They were inoculated in 200 cm^3 flasks containing 25 cm^3 of a liquid medium. Ten shoots were transferred to each flask and then

substrates were added. The medium composition was the same as that stimulating multiplication of shoots, the only difference being the agar omission.

Transformation of Plant Tissues with A4 *Agrobacterium rhizogenes* Strain

Carrot – Daucus carota. Edible roots of carrot after being disinfected (20% Monochloramine, 20 min) were cut into 1.5 cm slices. Explants of dimensions 1.0 × 1.5 cm, consisting of phloem, xylem and cambium, were isolated. The apical ends of the explants were immersed into a 7-day culture of A4 *Agrobacterium rhizogenes* strain and inoculated on MS agar medium with inverted orientation. For the growth of bacteria population Rhizogelose medium (in 1 dm^3: yeast extract 5 g, casamino acids 0.5 g, mannitol 8 g, $(NH_4)_2SO_4$ 2 g, NaCl 5 g, Bactoagar 15 g, biotin 0.8 cm^3/dm^3, pH 6.6) was used. After 2–3 weeks formed hairy roots were cut out and transferred to a liquid QL medium with half a macronutrient concentration, containing IAA in the amount of 1.0 mg/dm^3. In order to eliminate the *Agrobacterium*, two antibiotics were added to the medium: Carbeniciline and Cefotaxim (Polfa, Tarchomin, Poland), 500 mg/dm^3 of each. After 8 days (two changes of medium), the transformed roots were subcultured to a medium without antibiotics and the sterility of cultures was subsequently visually checked.

Non-transformed roots were obtained from the same explants as the transformed ones. They were cultured on the agar MS medium with a half concentration of macronutrients and the addition of IBA (0.5 mg/dm^3). The roots regenerated from explants on that medium and those obtained *via* the somatic embryogenesis were used for the comparative studies.

According to Pierik[15] adventitious roots are mainly formed on the basal side of explants, which results in more efficient formation of the roots when the apolar inoculation is used (upside down), due to better oxygen availability.

M 7 Apple rootstocks – Malus pumila. Transformed apple roots were obtained by the infection of the shoot sections followed by their inoculation in inverted orientation on a medium lacking phytohormons, in which the concentration of salts was half of that of the QL medium. After 6 weeks, the arising hairy roots were transferred to the medium containing antibiotics. Non-transformed comparative roots were induced on the QL medium with a half concentration of macronutrients and the addition of IBA (1.0 mg/dm^3).

The cultures of hairy roots and those genetically unchanged of both species under investigation serving for biotransformation, were grown on a liquid QL medium with the reduced amount of macronutrients (1/2) and some addition of IAA (1 mg/dm^3).

Transformations of root tissues was confirmed by opine test[16]. For biotransformation experiments to each flask 5–7 fragments of the hairy roots 3–6 cm long were used.

Biotransformation

The 200 cm^3 flasks containing plant material (approximately 0.01–0.28 g DW) were placed on a shaker (WU 4 type, approximately 80 rpm, 8 h a day) at 25–27 °C and illuminated by the light of intensity of 1 500 lx. Ten days after the placement of the plant material in a fresh medium (25 cm^3) 5 mg of the substrate (0.003 mol of **1**, 0.0023 mol of **2** and **3**, 0.0025 mol of **4**) dissolved in 0.5 cm^3 of acetone was added. Dissolving of the substrates one day before application prevented the culture from possible contamination that could be introduced with the chemicals used. The ten days' period of the delay was necessary to adapt the cultures to a fresh medium and, as well, to eliminate probable contamination. After 14 days of incubation with substrates, the medium and plant material were extracted separately with $CHCl_3$. As it was established that the transformation takes place both in plant tissues and in the medium due to the presence of exzoenzymes, in the next biotransformations they

were both extracted together. The composition of the reaction mixture was determined by TLC and GC.

The following amounts of plant material per one flask (dry mass, 105 °C, 12 h) after the experiment were determined: apple shoots – 0.28 g, transformed and non-transformed carrot and apple roots – 0.01–0.014 g. Each reaction for every substrate was carried out repeatedly in 3–7 flasks to confirm the results and/or to obtain sufficient amounts of products for further study. Stability of the substrates in the media was checked.

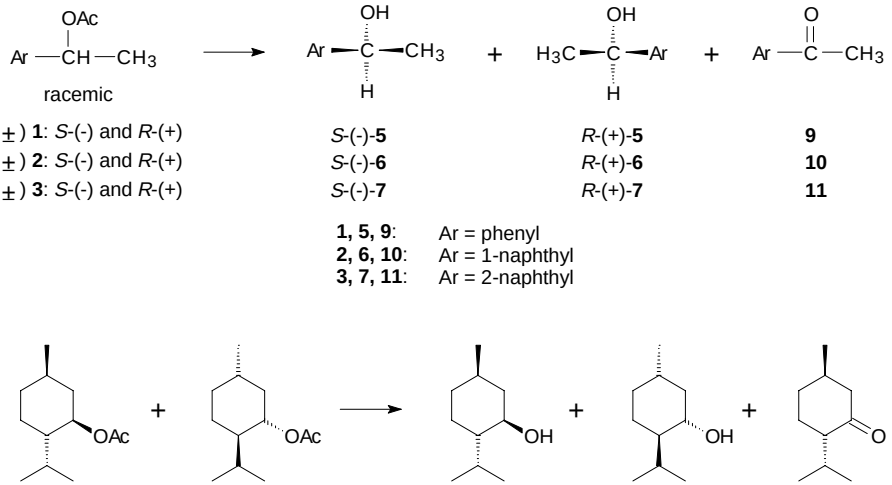
The amount of the substrate used for biotransformation (5 mg/25 cm^3 of medium) was established on the basis of the previous experiments[10] with the use of the whole, intact ferns as biocatalysts transforming substrates **1**–**4**. At the substrate concentration of 0.12% (30 mg/25 cm^3 of medium) the survival of plants during the biotransformation was 5 to 54 days, depending on the substrate used and the fern species (*Nephrolepis exaltata*, *N. cordifolia* and *Cyrtomium falcatum*). For a 14-day transformation, with the use of the material under examination, the concentration of the substrate (5 mg/25 cm^3) was chosen, at which 2/3 of the plant biomass survived (few signs of necrosis of leaves were observed).

Under the same conditions, using the same biocatalysts, an attempt to transform a ricinoleic acid and testosterone was made (5 mg/0.5 cm^3 of acetone, 25 cm^3 of a medium in 200 cm^3 flasks).

Control biotransformation with the use of enzymes secreted to the medium. Before adding the substrate to the plant culture, the biocatalysts were removed and, after 14 days, the culture was extracted with $CHCl_3$.

RESULTS AND DISCUSSION

The following racemic acetates were used for biotransformations: 1-phenylethyl (**1**), 1-(1-naphthyl)ethyl (**2**), 1-(2-naphthyl)ethyl (**3**) and menthyl (**4**). Each acetate was hydrolyzed to an appropriate alcohol which was subsequently oxygenated to a ketone (Scheme 1).



SCHEME 1

Biotransformations by Apple-Tree Shoots

Biotransformations of acetates (±)-**1**, (±)-**2**, (±)-**3** and (±)-**4** by apple-tree shoots result in hydrolysis yielding 98% of **5**, 15% of **6**, 9% of **7** and 11% of **8**, respectively. The alcohol obtained in this way was oxidized to ketone only in the case of acetate (±)-**1**: the transformation products are enantiomeric alcohols **5** and acetophenone (**9**) yielding 2%; other alcohols **6**, **7**, **8** are not oxidize to ketones.

Both the alcohols (±)-**5**, (±)-**6**, (±)-**7** and (±)-**8** and ketones **9**, **10**, **11** and **12** were also subjected to transformations under the same conditions. The results obtained in the experiments confirmed that only 1-phenylethanol ((±)-**5**) and acetophenone (**9**) were transformed, whereas the other alcohols and ketones remained untransformed. During a 14-day period of transformation of alcohol (±)-**5** 2% of acetophenone (**9**) was obtained. (*S*)-(–)-Alcohol was oxidized a slightly faster than its (*R*)-enantiomer, predominating in the reaction mixture (e.e. 16%). Acetophenone (**9**), transformed under the same conditions, was reduced to alcohol **5** (2%). The formation of (*S*)-(–)-alcohol was faster (e.e. 60%).

The 14-day transformation of racemic 1-phenylethyl acetate ((±)-**1**) by apple-tree shoots resulted in total transformation of the substrate and for this reason, the products of transformations were analysed after 1, 2 and 5 days of incubation (Fig. 1).

Initially, the amount of acetophenone (**9**) produced in hydrolysis of 1-phenylethyl acetate ((±)-**1**) increases, but then it decreases due to the reduction to alcohol **5**, in which (*S*)-enantiomer predominates (e.e. 11%).

Separation of enantiomers occurs during the hydrolysis of 1-phenylethyl acetate ((±)-**1**) (Fig.1): (*S*)-(–) acetate is totally transformed after 50 h, while 20% of pure (*R*)-(+) enantiomer (e.e. 100%) remains unreacted and can easily be separated using column chromatography[8].

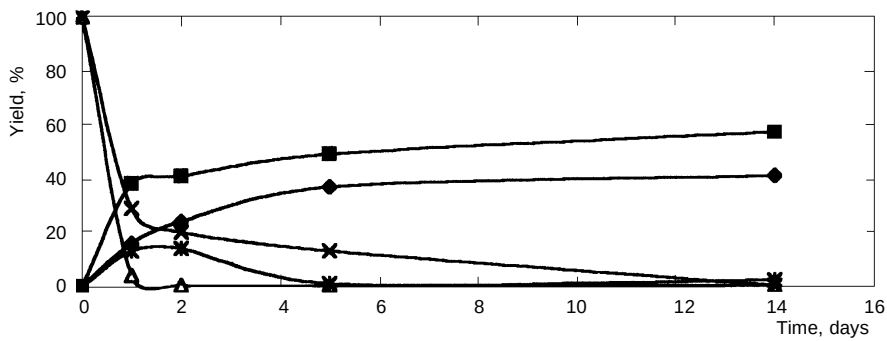


FIG. 1

Biotransformation of 1-phenylethyl acetate **1** in the presence of apple shoots; Δ *S*-(–)-**1**, * **9**, × *R*-(+)-**1**, ◆ *R*-(+)-**5**, ■ *S*-(–)-**5**

The hydrolysis of racemic menthyl acetate ((±)-**4**) also results in separation of enantiomers: after 14-day transformation, the only enantiomer produced (11%) is (1*R*,2*S*,5*R*)-(–)-**8** (e.e. 100%).

The yield of hydrolysis of (±)-**2**, (±)-**3**, (±)-**4** by apple shoots (the overground parts of plant) is 6–7 times lower than that obtained in the presence of transformed apple roots (Table I). It is also 4–8 times lower than that of the hydrolysis carried out by the enzymes present in the fruit of the same plant[8] (apples).

Biotransformation of all the acetates under investigation takes place inside the plant tissues, which means that both the substrates and products are transported through the cell membranes. It was also confirmed by separate extraction of the homogenized plant material and of the medium. Quantitative analysis of the two extracts after the transformation of 1-phenylethyl acetate ((±)-**1**) shows that concentration of the products (in particular acetophenone (**9**)) is higher in the plant material than in the medium.

Additionally, it was shown that enzymes released to the medium by a biocatalyst (apple shoots) cause biotransformation of acetates **1**–**4**. Substrates (**1**–**4**) were added to the flasks containing the medium after the removal of the apple shoots having been cultured there for 14 days and then shaken for next 14 days. The yield of acetate hydrolysis was then comparable to the yield obtained after the transformation in the presence of apple shoots, which points to the uptake of the compounds added to the plant cultures (the presence of substrates in apple shoots), and to enzyme secretion to the medium as well.

In addition to the above, it was found that apple shoots are not able to transform neither ricinoleic acid to lactone nor testosterone.

Biotransformations by Apple-Tree and Carrot Roots

Table I shows the results of biotransformations carried out by genetically modified (hairy roots) and unmodified apple (*Malus pumila*) and carrot (*Daucus carota*) roots.

The results obtained in the study show that the enzyme system of apple roots is more active than that of carrots. The suspended cell cultures of *Daucus carota*[10], having been studied earlier, were capable of hydrolysing the investigated acetates to a larger extent (3–12 times) than the root cultures of the same plant.

Higher yields of hydrolysis of the acetates by transformed apple roots than those by non-transformed can result from genetic modifications, *e.g.* T-DNA insertion from R_i plasmid. Considering the results, one may assume that for the increase in the yields of hydrolysis genes related with biosynthesis of auxin or opine in apple hairy roots are responsible. Each hairy root, however, may represent high variability, which have not been examined in our investigations so far. In the case of both types of carrot roots such differences were not observed. It seems quite likely that this is associated with the specificity of the plant species, which can be connected, for example, with limitation in tryptophane availability.

TABLE I

Efficiency of hydrolysis of racemic acetates (±)-**1**, (±)-**2**, (±)-**3** and (±)-**4**, by means of transformed and non-transformed roots of M7 apple-tree rootstock (*M. pumila*) and carrot (*D. carota*); yields and e.e. (enantiomeric excess) are given in %

Substrate (acetate)	Contents of the reaction mixture									
	Transformed roots					Non-transformed roots				
	Alcohol		Ketone	Unreacted acetate		alcohol		ketone	unreacted acetate	
	yield	e.e.	yield	yield	e.e.	yield	e.e.	yield	yield	e.e.
	M7 apple-tree rootstock cultures									
(±)-**1**	95	4 (*S*)-**5**	5 (**9**)	0	0	57	16 (*S*)-**5**	6 (**9**)	36	100 (*R*)-**1**
(±)-**2**	87	6 (*S*)-**6**	1.5 (**10**)	11	100 (*R*)-**2**	34	52 (*S*)-**6**	1 (**10**)	65	29 (*R*)-**2**
(±)-**3**	61	100 (*S*)-**7**	39 (**11**)	0	0	9	11 (*S*)-**7**	4 (**11**)	87	48 (*R*)-**3**
(±)-**4**	72	83 (1*R*)-**8**	0 (**12**)	27	70 (1*S*)-**4**	30	94 (1*R*)-**8**	0 (**12**)	70	20 (1*S*)-**4**
	Carrot root cultures									
(±)-**1**	7	14 (*R*)-**5**	12 (**9**)	81	14 (*S*)-**1**	5 (**5**)	0	0 (**9**)	95	
(±)-**2**	1 (**6**)		0.3 (**10**)	99 (**2**)		2	65 (*R*)-**6**	0 (**10**)	98	
(±)-**3**	6 (**7**)	0	1 (**11**)	93 (**3**)	0	4	31 (*R*)-**7**	0 (**11**)	96	
(±)-**4**	0 (**8**)		0 (**12**)	100 (**4**)		4 (1*R*-**8**)	0	0 (**12**)	96	

e.e. = enantiomer excess

The transformations carried out by apple root cultures result in the separation of enantiomers due to the difference in the rate of hydrolysis. There are two possible reaction paths: either pure enantiomer of **1** or **2** remains in the unreacted substrate, or pure (or characterized by high optical purity) enantiomer of alcohol **7** or **8** is produced.

The transformations of alcohols (±)-**5**, (±)-**6**, (±)-**7**, (±)-**8** and ketones **9**, **10**, **11** and **12** were studied separately, using each of the four types of root cultures, *e.g.* transformed and non-transformed apple and carrot roots. Menthol ((±)-**8**) and menthone (**12**) were not transformed at all, while (±)-**5**, (±)-**6** and (±)-**7** were transformed in negligible amounts (1–6%; the oxidation of (*S*)-enantiomers was faster), or in trace amounts (**10** and **11**). The 1-phenylethanol ((±)-**5**) was oxidized (5%, without substrate enantioselectivity) only by transformed carrot root culture.

Reduction of acetophenone (**9**) in the culture of transformed apple-tree roots accounted for 50% of **5**, with 8% excess of (*R*)-1-phenylethanol ((*R*)-(+)-**5**). Non-transformed apple roots reduced acetophenone in 79% yield with 9% excess of the same enantiomer, ((*R*)-(+)-**5**). The effect of carrot roots on acetophenone (**9**) was different: transformed roots caused a reduction in trace amounts, while non-transformed roots produced 60% of pure enantiomer (*S*)-(–)-**5** (e.e. 100%).

Neither the hairy roots nor the non-transformed apple and carrot roots were able to affect ricinoleic acid and testosterone under the conditions described above.

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