ABILITIES OF SOME HIGHER PLANTS TO HYDROLYZE THE ACETATES OF PHENOLS AND AROMATIC-ALIPHATIC ALCOHOLS*

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ABSTRACT

In the biotransformations carried out under the same conditions, the whole intact plants of *Spirodela punctata*, *Nephrolepis exaltata*, *Cyrtomium falcatum*, *Nephrolepis cordifolia* and the suspension cultures of *Helianthus tuberosus*, *Daucus carota* and *Petunia hybrida* hydrolyze (partially or totally) the ester bonds of the acetates of phenols and aromatic-aliphatic alcohols and also the menthyl acetate. Nevertheless, the methyl esters of aromatic acids, structurally similar to the former substrates, do not undergo hydrolysis. At the same time, the viability of first four plants was observed for different levels of acetate concentration. The method of continuous preparative hydrolysis of the same acetates was worked out in *Cyrtomium falcatum* culture.

KEY WORDS: *Spirodela punctata*, *Nephrolepis exaltata*, *Cyrtomium falcatum*, *Nephrolepis cordifolia*, *Helianthus tuberosus*, *Daucus carota*, *Petunia hybrida*, intact plants, suspension cultures, hydrolysis of esters, shikimic acid derivatives.

INTRODUCTION

It is well known, that tissue cultures of higher plants may be applied to biotransformations similarly as microorganism cultures (Reinhard, Alferman 1980, Berlin 1988). However, for practical reason, the studies on biotransformations carried out by intact whole plants have not been reported as extensively as those on tissue cultures used as bioreagents (Pawłowicz 1989, Pawłowicz et al. 1992).

In our previous research on whole plants and plant tissue cultures used for biotransformations, some similarities in the process of hydrolysis of several selected esters were observed. Namely, the acetates of phenols and aromatic-aliphatic alcohols were hydrolyzed in a considerable degree in callus cultures of three species of orchids (*Epidendrum ochraceum*, *Cymbidium 'Saint Pierre'* and *Dendrobium phalaenopsis*) and in whole duckweed plants (*Spirodela punctata*); in the case of racemic esters the hydrolysis was enantiospecific. On the other hand, methyl esters of structurally analogous aromatic carboxylic acids were resistant to the mentioned bioreagents (Mironowicz et al. 1987, 1993).

A similar chemical activity of some species of plants in the form of tissue cultures (orchids) and the whole plants (duckweed) as bioreagents seemed to be interesting, therefore fur-

ther research has been carried out on more species able to hydrolyze the ester bonds selectively. The substrates' structure was similar to those used previously (Pawłowicz 1989, Mironowicz et al. 1993).

MATERIALS AND METHODS

In the present paper, we used the whole intact plants: *Spirodela punctata* (Pawłowicz 1989, Pawłowicz et al. 1992) and ferns: *Nephrolepis exaltata*, *Cyrtomium falcatum* and *Nephrolepis cordifolia* as bioreagents. We also used suspension cultures – *Helianthus tuberosus*, *Daucus carota* and *Petunia hybrida*.

Spirodela punctata was taken from the collection of the Department of Plant Physiology, Agricultural University of Wrocław. *Nephrolepis exaltata*, *Cyrtomium falcatum*, *Nephrolepis cordifolia*, *Helianthus tuberosus*, *Daucus carota* and *Petunia hybrida* were taken from the collection of The Tissue Cultures Laboratory of Botanic Garden, University of Wrocław.

The transformations in the culture of *S. punctata* were carried out by the method worked out by the authors (Tłomak et al. 1986, Pawłowicz 1989). The culture was prepared on the Bollard medium (Bollard 1966) and kept in conical flask in which a single plant was placed in 100 ml of medium. After two-week of preliminary cultivation continuously exposed to 1000-2000 luxes, 10 or 20 mg of the substrate dissolved in 1 ml of acetone or ethanol was added. Transformations were continued for ten to fourteen days under the same conditions

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as the culture was grown. The average biomass (as dry biomass: 12h, 105°C) used for one transformation was 56,6 mg.

The fern culture was grown for three weeks on a complete liquid medium MS (Murashige, Skoog 1962) in the volume of 25 ml for eighteen hours photoperiod (2000lx), then 12-30 mg of the substrate was added as described above. The transformation lasted two to three weeks. The authors used 850 mg of bioreagent (presented as dry biomass).

The cultures of the cell suspensions of *H. tuberosus*, *D. carota* and *P. hybrida* were grown out in 50 ml of liquid medium MS (Murashige, Skoog 1962) containing additionally 1mg of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg of kinetin (K) in one liter of nutrient for *H. tuberosus* and *D. carota*, or 1 mg of 2,4-D and 1mg of K for *P. hybrida*. After two weeks of growth, 10-15 mg of substrate was added. The cultures were then shaken for two to three weeks under continuous exposition to illumination of 1000 luxes. The biomasses (as dry biomass) used for one transformation were as follows: 160 mg for *P. hybrida*, 80 mg for *D. carota* and 45 mg for *H. tuberosus*.

All the preparative cultures and the transformations themselves were kept under sterile conditions. Continuous shaking allowed even mixing of the bioreagent suspension and the substrate.

The products were extracted with chloroform and separated by column chromatography (SiO₂, 0,2-0,05 mm, Merck). Their structure was identified by thin-layer-, and gas chromatography. For comparison original compounds were used. The spectral method (IR) was used in special cases.

The experiments were repeated several times for confirmation of results and obtaining quantities of products sufficient for further studies.

The degrees of enantiospecificity of the hydrolysis were measured for the following racemic acetates: ($\pm$)4, ($\pm$)5 and ($\pm$)6. The reaction was interrupted before the end of the hydrolysis to determine the optical activity of alcohol and the remaining unreacted ester in post-reaction mixture. The absolute configurations were determined by the comparison of our data with data reported elsewhere (Pawłowicz 1989, Mironowicz et al. 1993).

The specific optical rotations measured in chloroform were as follows:

– after transformation in *H. tuberosus* culture:

unreacted acetate ($\pm$)4: $[\alpha]_{546}^{22}$ -3°; alcohol (+)4a: $[\alpha]_{546}^{22}$ +4°; 4a S:R=46:54; unreacted acetate 5 and alcohol 5a were optically inactive after the transformation; unreacted acetate ($\pm$)6: $[\alpha]_{546}^{22}$ -5°; alcohol 6a: [a] was not determined due to low efficiency of the hydrolysis; 6a R>S

– after transformation in *D. carota* culture:

unreacted acetate (+)4 $[\alpha]_{546}^{22}$ +4°; alcohol (-)4a: $[\alpha]_{546}^{22}$ -10°; 4a S:R=60:40; unreacted acetate ($\pm$)5: $[\alpha]_{546}^{22}$ -0,5°; alcohol (+)5a: $[\alpha]_{546}^{22}$ +20°; 5a S:R=37:63; unreacted acetate (+)6: $[\alpha]_{546}^{22}$ +5°; alcohol (-)6a: $[\alpha]_{546}^{22}$ -24°; 6a S:R=76:24.

The continuous transformation. Culture used: *C. falcatum* in 50ml of standard medium. The culture had been previously grown for three weeks. Substrate (12 mg in 25ml of nutrient) was added and the mixture was shaken for two weeks under illumination, as described above. Then the medium with the products was isolated in sterile conditions and the next portion of the substrate was added in fresh medium. The change was made four times for each substrate. The total yield was as fol-

lows: from acetate ($\pm$)4: 90-100% of alcohol 4a; from acetate ($\pm$)5: 90-95% of alcohol 5a; from acetate ($\pm$)6: 85-95% of alcohol 6a; from acetate ($\pm$)9: 70-75% of menthol (9a).

Withers's tetrazolinic test (Withers 1980) on *H. tuberosus* cells. After two-week transformation, 2ml of cell suspension were taken from each culture with substrate and from the control culture (no substrate). To the separated cell suspension 2,3,5-triphenyltetrazolinic chloride (TTC) was added. After the reduction of the reagent in the solution studied, the intensity of the reagent's colour was measured at 560nm. Absorbance of the control was 0.1.

Evans's test (Bhojwaini, Razdan 1983): one drop of Evans's blue (concentration 0.025%), the dye penetrating only into the dead cells was added to five drops of cell suspension after transformation. The comparison of colour intensity with the control culture allowed an approximate determination of the dead cell number.

RESULTS AND DISCUSSION

Table 1 shows the degree of hydrolysis of the ester bond of eight aromatic acetates and menthyl acetate (representing monoterpenes) by biological materials studied.

All the examined acetates undergo hydrolysis but to a different degree. The resistance of acetates 7 and 9 to hydrolyze in *H. tuberosus* culture is exceptional here. It is worth considering that out of ester bonds which are present in substrates 7 and 8 only the acetyl groups undergo hydrolysis. The methyl esters of the carboxylic groups in both substrates do not hydrolyze.

This observation suggested to test the ability for hydrolysis of methyl esters of aromatic acids structurally similar to the first group of substrates (Fig. 1). All of them (10, 7a, 11, 12) do not undergo the hydrolysis in the cultures of *S. punctata*, *H. tuberosus*, *D. carota* and *P. hybrida*.

Fig. 1. Examples of substrates – methyl esters of aromatic acids which do not undergo hydrolysis in cultures of *S. punctata*, *H. tuberosus*, *D. carota* and *P. hybrida*.

The presented transformations extend the previous observations (Pawłowicz et al. 1987, Pawłowicz 1989, Mironowicz et al. 1993) that in hydrolysis of esters belonging to the group of derivatives or analogues of the shikimic acid exists a regioselectivity.

Consequently, we confirm our previous results indicating that the ester bond in the acetates of phenols and aromatic-aliphatic alcohols is hydrolyzed in the following way:

Ar – phenyl group, its analogues with substituents in the aromatic ring and in Ar – CH₂.

Such hydrolysis of esters takes place with duckweed, ferns and suspension cultures of *H. tuberosus*, *D. carota*, *P. hybrida* (according to the orchids examined earlier (Mironowicz et al. 1993)).

TABLE 1.
Degree of hydrolysis of the ester bond in acetates of phenol and aromatic-aliphatic alcohols in the cultures of some selected higher plants.

Numbers, structures and names of substrates and their products	Degree of hydrolysis of acetates to free alcohols [%]						
	<i>S. punc.</i>	<i>N. exal.</i>	<i>C. falc.</i>	<i>N. cord.</i>	<i>H. tub.</i>	<i>D. car.</i>	<i>P. hybr.</i>
 phenyl acetate (1) → phenol (1a)	100	20	—	100	65	8	8
 benzyl acetate (2) → benzylic alcohol (2a)	100	100	100	100	13	50	8
 cinnamic acetate (3) → cinnamic alcohol (3a)	57	100	96	93	5	6	6
 (±) 1-phenylethyl acetate ((±) 4) → 1-phenylethanol (4a)	86 ^{x)} xx)	100	95	100	1	35	6
 (±) 1-(2-naphtyl)-ethyl acetate ((±) 5) → 1-(2-naphtyl)-ethanol (5a)	33 ^{x)}	93 - 100	95	100	4	6	6
 (±) 1-(2-naphtyl)-ethyl acetate ((±) 6) → 1-(2-naphtyl)-ethanol (6a)	53 ^{x)}	90 - 100	95	87	3	5	5
 methyl acetyl-salicylate (7) → methyl salicylate (7a)	100 ^{x)}	9	—	100	0	5	8
 methyl 2,5-diace-toxybenzoate (8) → methyl 2,5-dihydro-xybenzoate (8a)	100	—	—	—	54	64	73
 menthyl acetate ((±) 9) → menthol (9a)	100 ^{x)}	71	75	83	0	84	85

Sign "—": transformation not investigated

x) Results from Pawłowicz 1987

xx) Including 38% of acetophenone – product of oxidation of alcohol 4a

It was also observed that the ester bond of methyl esters of aromatic acid is resistant to the hydrolysis in the cultures of the plants.

However, two exceptions in our observations were found:

– acetylsalicylic acid (13) in which the acetyl group did not hydrolyze in the *S. punctata* culture and salicylic acid was not formed,

– ethyl cinnamate (14) hydrolyzed to free cinnamic acid in 50% in the same culture of duckweed.

Fig. 2. Acetylsalicylic acid (13) and ethyl cinnamate (14).

Furthermore, it was noticed that two tissue cultures – *H. tuberosus* and *D. carota* were able to hydrolyze racemic acetates (±)4, (±)5, (±)6 to optically active alcohols. The hydrolysis of the racemic acetates was intentionally carried out incompletely in order to determine the optical activity of the remaining unreacted esters and alcohols formed (Table 2).

The data of the present study were compared with those obtained earlier (Mironowicz et al. 1993, Pawłowicz et al. 1987)

(Table 3). It can be seen, that the course of the enantiospecific hydrolysis process depends on the plant species.

The hydrolysis of menthyl acetate ((±)9), which does not belong to the group of shikimic acid derivatives was also investigated. Our previous experiments (Pawłowicz 1989, Pawłowicz et al. 1988) in *S. punctata* culture encouraged us to continue the studies. As presented in Table 1, the cultures of other plants transform this substrate to a high degree. The enantiospecificity was very low (Pawłowicz 1989), therefore it was not further considered.

The reactions presented, though practically quasi-preparative, did not lead to significant amounts of the products. However, continuous transformation, also in sterile conditions, with *C. falcatum* was found as a convenient bioreagent. The same plants could be used several times, but each time with a new portion of substrate and nutrient.

In transformation experiments carried out on four esters (±)4, (±)5, (±)6 and (±)9 (four times for fourteen days) the observed yield ranged from 70 to 100%, depending on the substrate used. The reaction may be repeated as long as the plant lives.

Besides, with standard two-week transformations, an attempt was made to determine the optimum time of hydrolysis in *N. exaltata* culture. Six substrates of the same concentration (0.05%) were examined. The degree of hydrolysis was measured chromatographically after 3, 6, 11, and 20 days. The results are shown in Table 4.

The observed differences suggest that the optimum time of transformation is substrate dependent and for each substrate should be determined separately.

TABLE 2. Enantiospecific hydrolysis of acetate of racemic aromatic-aliphatic alcohols (±)4, (±)5, (±)6 to alcohols in the tissue cultures of *H. tuberosus* and *D. carota*.

Substrates	Products of hydrolysis (alcohols)	Ratio of alcohols S:R	
		<i>H. tuberosus</i>	<i>D. carota</i>
acetate (±)4	(+)4a in <i>H. tuberosus</i> (-)4a in <i>D. carota</i>	46 : 54	60 : 48
acetate (±)5	5a in <i>H. tuberosus</i> (+)5a in <i>D. carota</i>	lack of enantiospecificity	37 : 63
acetate (±)6	(-)6a in <i>H. tuberosus</i> (-)6a in <i>D. carota</i>	R > S	76 : 24

TABLE 3. Comparison of enantiospecificity of the hydrolysis of racemic acetates of aromatic-aliphatic alcohols (±)4, (±)5 and (±)6 in the cultures of five higher plants species.

The observed hydrolysis	Major product: R or S alcohol				
acetate alcohol	<i>S. punctata</i> ^{x)}	<i>Cymbidium</i> 'Saint Pierre' ^{xx)}	<i>Dendrobium phalaenopsis</i> ^{xx)}	<i>H. tuberosus</i>	<i>D. carota</i>
(±)4 → 4a	R	S	S	R	S
(±)5 → 5a	R	S	R	lack of enantiosp.	R
(±)6 → 6a	R	S	R	R	S

x) data from Pawłowicz et al. 1987

xx) data from Mironowicz et al. 1993

TABLE 4. Determination of optimum time of the hydrolysis of selected acetates in *N. exaltata* culture.

Transformed substrate	Degree of hydrolysis – amount of obtained alcohol in %			
	after 3 days	after 6 days	after 11 days	after 20 days
acetate 2	100	–	–	–
acetate 3	85	98	100	–
acetate (±)4	74	93	100	–
acetate (±)5	23	30	93	–
acetate (±)6	79	80	90	100
acetate (±)9	15	18	71	–

TABLE 5. Tolerance of several species of ferns to the transformed substrate (concentration 0,12%).

Substrate in plant culture (conc. 0,12%)	Days after which the necrosis of plant takes place		
	<i>N. exaltata</i>	<i>C. falcatum</i>	<i>N. cordifolia</i>
(±)4	40	above 54	40
(±)5	22	5	22
(±)6	22	7	22
(±)9	22	42	22

The transformed substrates influenced the overall condition of the plant significantly. The first experiment revealed the toxicity of benzyl acetate (2) which consequently affected also the duckweed (*S. punctata*). It was observed that at compound concentration of 0.5%, the plant growth during the first week of transformation was identical as in the control. After the second week a minimal drop in reproduction rate was observed.

Moreover, the tolerance of three species of ferns to the transformed substrate at concentration of 0.12% was investigated. Higher doses of the substances used in the transformation process were lethal for plants. The leaves were turning yellow ultimately reaching the state of complete necrosis. The effects of four esters: (±)4, (±)5, (±)6, (±)9 are presented in Table 5.

The viability of ferns in the presence of the four mentioned acetates which were highly hydrolyzed in transformation conditions was over three weeks. *C. falcatum* seemed to be the most sensitive to substrates 5 and 6 (one week survival time).

The toxicity of substrates on cell suspensions of *H. tuberosus* after two-week transformation was examined by Withers's tetrazolinic test (Withers 1980) and Evans's test (Bhojwaini, Razdan 1983). The number of living cells was also counted under the microscope at x 160 magnification. For the experiment two types of substrates were selected: those transformed by the plant and those resistant to transformation (respectively acetates: 8 and 9). It was observed that the acetate 9 (not susceptible to hydrolysis) did not cause necrosis of *H. tuberosus* cells even at concentrations of 0.25% (absorbance identical to

the control; 90-100% of living cells observed under the microscope). At the same time, the acetate 8 (hydrolyzed in 54%) at concentration of 0.04% caused a rise of absorbance to 0.65 and reduced the number of living cells to 70-80%.

The attempts to transform selected steroids (with alcohols and esters among them) in *D. carota* culture were not successful.

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ZDOLNOŚCI WYBRANYCH ROŚLIN WYŻSZYCH DO HYDROLIZY OCTANÓW FENOLI I ALKOHOLI AROMATYCZNO-ALIFATYCZNYCH

STRESZCZENIE

W przeprowadzonych w tych samych warunkach transformacjach użyto całych (nienaruszonych) roślin wyższych *Spirodela punctata*, *Nephrolepis exaltata*, *Cyrtomium falcatum* i *Nephrolepis cordifolia* oraz kultur zawieszinowych *Helianthus tuberosus*, *Daucus carota* i *Petunia hybrida* stwierdzając, że powodują one (częściową lub całkowitą) hydrolizę estrowego wiązania w octanach fenoli i alkoholi aromatyczno-alifatycznych oraz w octanie mentylu. Estry metylowe kwasów aromatycznych, strukturalnie podobnych do wymienionych wyżej alkoholi, nie ulegają jednak hydrolizie. Jednocześnie badano wpływ stężenia octanu poddawanego hydrolizie na kondycję rośliny. Dla kultury *Cyrtomium falcatum* opracowano warunki transformacji ciągłej.

SŁOWA KLUCZOWE: *Spirodela punctata*, *Nephrolepis exaltata*, *Cyrtomium falcatum*, *Nephrolepis cordifolia*, *Helianthus tuberosus*, *Daucus carota*, *Petunia hybrida*, kultury zawieszinowe, hydroliza estrów, pochodne kwasu szikimowego.