# Reduction of Carbonylic and Carboxylic Groups by Plant Cell Cultures

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The transformation of aliphatic and aromatic acids to their corresponding alcohols, involving two reductive steps, is difficult to perform biologically due to its low redox potential. For this reason, the reduction of nonactivated carboxylic acids has been described for only a limited number of substrates and confined to a few microbial groups (fungi, clostridia, and archea). Nine species of cultured plant cells were able to reduce cinnamic, hexanoic, and octanoic acids to the corresponding primary alcohols with yields ranging from 2 to 80% (w/w). Aldehyde was detected only for three species during the reduction of cinnamic acid, confirming that the second reductive step from aldehyde to alcohol is faster than the first, from acid to aldehyde. Lyophilized cells from some of the cultures were used in buffer and solvent–water systems to obtain the reduction of carbonylic (ethyl acetoacetate) and carboxylic (cinnamic and hexanoic acids) groups.

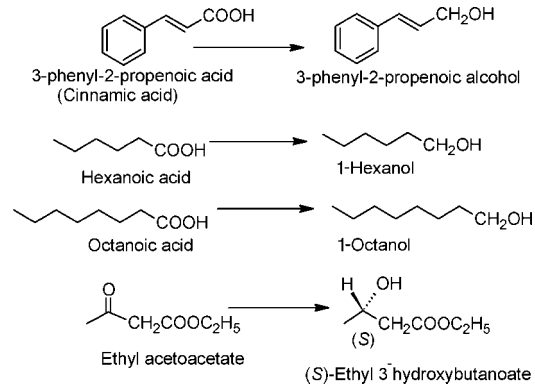
The use of plant cell cultures in biotechnology has been rising over the last 15 years. In particular, the use of different plant species for biotransformation is an increasing practice and represents an interesting route for the synthesis of useful compounds.[1,2]

Plant cell enzymes, like those from microorganisms, can catalyze regio- and stereospecific reactions such as the resolution of racemic mixtures or the transformation of prochiral compounds with advantages over chemical synthesis.[3,4] The reduction of carbonyl groups to their corresponding alcohols using plant cell cultures has been studied mainly for secondary metabolites in which the reduction occurs stereospecifically.[5,6] Most attention has been focused on the activity of a few well-known species such as *Nicotiana tabacum* and *Daucus carota*, often utilizing immobilized cells or plant enzymes and plant metabolites as substrates.[7–9] More recently, plant parts have been used directly as biocatalysts in the production of secondary alcohols.[3,10] The reductive ability of several plant species on model substrates has been previously reported showing chemical yields ranging from 20 to 100% (w/w) and enantiomeric excess (ee) ranging from 65 to 99%.[11] In this study, lyophilized cells of seven plant species were used in a buffer and in a two-phase system to stereoselectively reduce the model substrate ethyl acetoacetate.

The reduction of carboxylic groups is a biologically difficult reaction to perform due to its low redox potential. For this reason, the reduction of nonactivated carboxylic acids has been described for only a limited number of substrates and confined to a few groups (fungi, clostridia, and archea).[12–15] All plant biochemical pathways show the presence of several reductive systems, but the potential for acid reduction has never been fully investigated. The conversion of acids to their corresponding alcohols by plant cells could offer the possibility for the natural production of specialty chemicals.

The principal objective of this work was to study the ability of several plant species to perform the reduction of carboxylic and carbonylic groups of model substrates in an aqueous system with fresh and lyophilized cells and in a two-phase system with lyophilized cells.

In a preliminary study 15 plant species were screened for their ability to reduce cinnamic acid, using TLC for the detection of substrate and products. Some of them were able to consume the acid completely (*A. porro*, *H. annuus*, *P. virginalis*, *R. manii*, *S. melanogena*), accumulating alcohol in the medium or degrading it further (not investigated in this work). Most of them (eight out of 15) were able to produce cinnamic alcohol after 60 h of reaction. The main compounds detected by TLC were alcohol, acid, aldehyde, and a nonpolar compound (not identified).




*Nicotiana tabacum* showed good growth and good reductive activity, and it was chosen for further TLC assays aimed at selecting the best reaction medium. Reduction yields in the phosphate buffer at pH 5.8 were higher than in the transformations performed in the other media (growing media, buffer at pH 6.4, 7.0, and 5.8 with glucose). This indicates that the presence of glucose is not mandatory for the regeneration of the cofactors involved in the reduction of acid. Glucose is, however, necessary in the reduction of ketones,[11] suggesting the presence of two different reductive systems for the production of primary and secondary alcohols. Phosphate buffer at pH 5.8 without glucose was, therefore, chosen for subsequent work.

The yields of the alcohols obtained by reduction of cinnamic acid, hexanoic acid (caproic acid), and octanoic acid (caprylic acid) with different plant species are reported in Table 1. These results show that all species studied were able to reduce at least one of the three acids considered, albeit with low yields. In all cases, part of the substrate was metabolized in a different way or the product was further degraded, and the products were not identified by our GC analysis. Aldehydes were detected by GC as intermediates of the reduction from acid to alcohol in the first 24 h, and in low yields, during the reduction of cinnamic acid, for only three species: *A. chinensis*, *H. annuus*, and *S. melanogena*. This confirms that, in this first intermediate step of acid reduction to alcohol, aldehydes are not accumulated in the medium and that probably the second reductive step from aldehyde to alcohol is faster than the first, from acid to aldehyde, due to the higher cell toxicity of the aldehyde.

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**Table 1.** Reduction of Cinnamic, Hexanoic (Caproic), and Octanoic (Caprylic) Acids in Buffer with Different Plant Species Obtained after 120 h of Reaction (by GC analysis)

| | % yield[a] | | |
|---|---|---|---|
| plant species | cinnamic acid | hexanoic acid | octanoic acid |
| *Actinidia chinensis* | 10 | <5 | <5 |
| *Daucus carota* | 6 | <5 | 5 |
| *Helianthus annuus* | 12 | 80 | 6 |
| *Nicotiana tabacum* | 10 | 35 | 0 |
| *Phytolacca decandra* | 0 | <5 | <5 |
| *Polygonum persicaria* | 8 | <5 | 6 |
| *Rauwolfia manii* | 10 | 60 | 5 |
| *Solanum melanogena* | 0 | <5 | <5 |
| *Tagetes patula* | 7 | 0 | 5 |

[a] Reduction yields are expressed as a ratio (w/w) of alcohol to alcohol plus acid determined by gas chromatography on the crude extracts using an internal standard. No other compounds were detected at 120 h by GC analysis.

**Table 2.** Reduction of Cinnamic and Hexanoic (Caproic) Acids Obtained after 120 h of Reaction Using Lyophilized Cells of Different Plant Species in a Two-Phase System by GC Analysis

| | % yield[a] | | | |
|---|---|---|---|---|
| | cinnamic acid | | hexanoic acid | |
| plant species | buffer | biphasic system | buffer | biphasic System |
| *Actinidia chinensis* | 10 | 6 | 0 | 0 |
| *Daucus carota* | 0 | 5 | <5 | <5 |
| *Helianthus annuus* | 8 | 6 | <5 | <5 |
| *Nicotiana tabacum* | 10 | 10 | 10 | <5 |
| *Polygonum persicaria* | 5 | 5 | 0 | 0 |
| *Solanum melanogena* | 5 | <5 | <5 | <5 |
| *Tagetes patula* | 0 | 6 | 0 | <5 |

[a] Reduction yields are expressed as a ratio (w/w) of alcohol to alcohol plus acid determined by gas chromatography on the crude extracts using an internal standard. No other compounds were detected at 120 h by GC analysis.

Of the three model acids, cinnamic acid is the most commonly found in plant systems and was expected to be the preferred substrate. Although it was reduced with good molar conversion by the majority of the plant species investigated, with a maximum of 12% with *H. annuus*, the highest reduction yields were obtained with linear hexanoic acid using *H. annuus* and *R. manii*. Conversion yields of 80 and 60%, respectively, were obtained, followed by 35% with *N. tabacum*. Octanoic acid, on the contrary, was reduced by seven species, but the conversions were all low (<10%), suggesting a higher toxicity of the acid or low specificity of the enzyme. The results reported in Table 1 refer to conversion yields after 120 h, but the results after 24 h and subsequent intervals were only slightly lower than those reported. The concentration of cells utilized was quite high (350 mg/mL), but the extent of the acid conversion suggests that a high concentration of free plant cells is a good system to obtain primary alcohols.

Under the hypothesis that plant cells can maintain their enzymatic ability after lyophilization (and, thus, be more "user-friendly"), the reductions of carbonylic and carboxylic groups were carried out using lyophilized cells in a buffer and in a two-phase system (buffer−iso-octane). Different aliphatic hydrocarbon solvents (pentane, hexane, heptane, and iso-octane) have previously been evaluated on microbial systems, all showing good transformation results.[16] As all the tested solvents showed similar conversions after 5 days, iso-octane was chosen for the plant cell reductive systems because of its low volatility compared with the others.

In the two-phase system, substrates and products could be present in both phases, which were analyzed separately. The results of the reductions of carboxylic and carbonylic groups are reported in Tables 2 and 3, respectively.

Freeze-dried cells, used at the same concentration as the fresh cells, were able to reduce both acids, although lower yields were obtained, suggesting a deactivation/denaturation effect of the lyophilization on the enzyme. This effect is particularly marked with hexanoic acid, which showed very good molar conversions with fresh cells (80% with *Helianthus annuus* and 35% with *Nicotiana tabacum*) but less than 5% with *Helianthus annuus* and 10% with *Nicotiana tabacum* in the buffer system. In the biphasic system these low conversion yields are usually attributed to an inhibition of the solvent. However, in this case, the conversion yields with lyophilized cells in buffer systems are similar to those obtained in the two-phase systems (buffer−solvent), suggesting a less remarkable effect of the solvent on the enzymes involved in the reactions. Furthermore, the transformation yields of cinnamic acid with *A. chinensis* and *N. tabacum* are not much lower than those obtained with fresh cells, suggesting the presence of different reductive systems for aromatic and linear acids, which are affected differently by the lyophilization. No other compounds were detected in these transformantions at 120 h apart from acids and alcohols.

**Table 3.** Reduction of Ethyl Acetoacetate and Enantiomeric Excesses of the Alcohols Obtained after 120 h of Reaction Using Lyophilized Cells of Different Plant Species in a Buffer and a Two-Phase System by GC Analysis

| | ethyl acetoacetate | | | |
|---|---|---|---|---|
| | buffer | | biphasic system | |
| plant species | % yield[a] | enantiomeric excesses (% ee) | % yield[a] | enantiomeric excesses (% ee) |
| *Actinidia chinensis* | 45 | ≥98 *S* | 85 | ≥98 *S* |
| *Convolvulus sepium* | 40 | 65 *S* | 70 | ≥98 *S* |
| *Daucus carota* | 85 | ≥98 *S* | 80 | ≥98 *S* |
| *Helianthus annuus* | 30 | ≥98 *S* | 40 | ≥98 *S* |
| *Nicotiana tabacum* | 25 | ≥98 *S* | 20 | ≥98 *S* |
| *Polygonum persicaria* | 35 | ≥98 *S* | 45 | ≥98 *S* |
| *Solanum melanogena* | 30 | ≥98 *S* | 70 | ≥98 *S* |

[a] Reduction yields are expressed as a ratio (w/w) of alcohol to alcohol plus acid determined by gas chromatography on the crude extracts using an internal standard. No other compounds were detected at 120 h by GC analysis.

Lyophilized cells performed the transformation of ethyl acetoacetate with very good results, suggesting the presence of more resilient reductive systems for the conversion of ketones to secondary alcohols than of acids to primary alcohols. Microbially catalyzed reductions of these substrates have been previously studied by our group,[16] providing an interesting reference point for the performance of plant cell cultures. Plant cell systems seem to behave in a similar manner to microbial ones: In the reduction system of plant cell cultures the hydrogen attack takes place preferentially from the *re* face of the carbonyl group to give hydroxy compounds with the *S*-chirality at the carbon atom bearing the hydroxy group.[17,18]

Ethyl acetoacetate has been shown, in a previous study, to be the preferred substrate for fresh cells, and it was used as a model substrate.[11]Ethyl acetoacetate was transformed by all the plant species with good yields, ranging from 25% with *Nicotiana tabacum* to 85% with *D. carota*. In one case, with *Helianthus annuus*, yields were higher (30%) than those obtained in the buffer system with fresh resuspended cells (<5%). The enantiomer formed was always the *S* alcohol, with enantiomeric excesses ≥98% in all cases. The presence of the solvent decreased the velocity of the reaction, although the final yields and the enantiomeric excesses were, in almost all cases, higher than in the simple buffer systems and in the previously reported study with fresh resuspended cells.[11]

These results are quite encouraging and suggest the possibility of using lyophilized cells in the reduction of carbonyl groups in solvent. The use of lyophilized cells is more practical and functional for chemists who are not familiar with biological systems and

widens the potential use of such systems for substrates of particular interest. In conclusion, several plant species behaved efficiently as reducing agents, converting different acids (aromatic and linear) into the corresponding primary alcohols with some good chemical yields.

In addition, lyophilized cells can be used in a two-phase system, demonstrating the presence of different plant dehydrogenases capable of reducing acids to alcohols with different affinity and resistance. The two-phase system was used in the reduction of ketones with results similar to those obtained with resting cells. Ethyl acetoacetate was the most easily reduced substrate, with enantiomeric excesses equal to or higher than those obtained in the aqueous buffer system.

Although the reactions have not been optimized, these results indicate that plant cell cultures are accessible agents for the production of primary and secondary alcohols and offer an alternative for the natural production of specialty chemicals in the pharmaceutical industry. In the case of specific targets of high commercial value (particularly those that are not easily obtained using microorganisms) the transformation processes could represent a realistic and economically convenient use of plant cell cultures. The results reported here are promising and worthy of further investigation.

## Experimental Section

**General Experimental Procedures.** An initial screening study using all 15 plant cell species was performed using cells from 7-day-old submerged cultures, collected by centrifugation and resuspended (350 mg/mL wet weight) in 5 mL of 0.1 M phosphate buffer pH 5.8 in the presence of 10% w/v glucose.[19] The substrate to be tested (cinnamic acid) was added neat (1g/L), and the Teflon-lined reaction vials were incubated at 25 °C on a reciprocal shaker. Samples (0.25 mL) were taken at intervals (every 24 h up to 7 days). Substrates and products were analyzed by TLC (results not reported). The transformation conditions were established using the best producer, *N. tabacum*, in different reaction systems: Gamborg medium, 0.1 M phosphate buffer pH 5.8, 6.4, and 7.0, and 0.1 M phosphate buffer pH 5.8 in the presence of 10% w/v glucose. Samples were obtained as reported below.

The reduction of cinnamic, caproic (hexanoic), and caprylic (octanoic) acids with nine of the 15 species was carried out using resting cells from 7-day-old submerged cultures resuspended (350 mg/mL wet weight) in 5 mL of 0.1 M phosphate buffer pH 5.8 with no added glucose. The substrate to be tested was added neat, 1 g/L, and samples (taken at 24 h intervals) were analyzed by GC. A suitable internal standard (1 g/L) was added prior to injection.

The reduction of cinnamic acid, caproic acid, and ethyl acetoacetate with seven species was carried out using lyophilized cells resuspended in buffer (phosphate buffer) or in a two-phase system (phosphate buffer/iso-octane). Lyophilized cells (90 g/L), corresponding to the fresh weight of 350 mg/mL, were resuspended in (a) 5 mL of phosphate buffer (0.1 M, pH 6.5) and (b) 5 mL of phosphate buffer (0.1 M, pH 6.5) and 5 mL of isooctane (1:1). The substrate was added to the system at the concentration of 1 g/L for the acids and 2 g/L for the ketones. Glucose was also added to the ketone reaction (10% w/v). Closed reaction vials were incubated at 25 °C on a reciprocal shaker, and samples (0.25 mL from the aequous phase and 0.25 mL from the organic phase) were taken at intervals (every 24 h up to 7 days). Substrates and products were analyzed by GC.

Lyophilized cells were obtained using the following procedure. Cells grown for 7 days in liquid cultures were harvested by filtration, washed with phosphate buffer (0.1 M, pH 6.5), homogenized, frozen at −20 °C, and finally lyophilized (Edwards Minifast mfd 01) at a plate temperature of 25 °C. Once lyophilized, the cells were stored in desiccators at room temperature.

**Analytical Procedure.** Cinnamic acid ($R_f$ 0.55), cinnamic aldehyde ($R_f$ 0.5), cinnamic alcohol ($R_f$ 0.75), and a nonpolar compound ($R_f$ 0.2) were identified in the initial screening using silica gel TLC plates. The substrates and products were developed using chloroform and methanol (85:15) in the elution system; standards of the compounds were added to the TLC. The acids and the alcohols were visualized using cerium sulfate stain (aqueous solution of 10% cerium(IV) sulfate and 15% sulfuric acid).

The reaction mixtures (buffer and media) were extracted with ethyl acetate, and the organic phase was analyzed by gas chromatography. The acids, determined as methyl esters, and the corresponding alcohols were analyzed using a Carbowax 20 M packed column (10% on Supelcoport 100/120 mesh).

The alcohols obtained by the reduction of ethyl acetoacetate were extracted with ethyl ether and dried, and the enantiomeric composition was determined transforming the alcohols extracted into the corresponding butyrate esters by reaction with butyric chloride in dichloromethane with 2% v/v pyridine.[19]

The enantiomeric composition was determined by gas chromatography on a chiral capillary column, liquid phase DMePeBeta CDX-PS086, MEGA, Legnano, Italy (internal diameter 0.25 mm, length 25 m). The absolute configuration was determined by comparison with authentic samples from Aldrich.

The aqueous phases of the two-phase systems for the reduction of the acids were extracted with ethyl acetate, and the organic phases (ethyl acetate and isooctane) were analyzed after methylation of the acids as described.

The aqueous phases of the two-phase systems for the reduction of the ketones were extracted with ethyl acetate, and the organic phases (ethyl acetate and isooctane) were analyzed directly, using a Carbowax 20 M packed column (10% on Supelcoport 100/120 mesh).

**Plant Material.** The plant cell lines used in this study were obtained from the following plant materials: *Actinidia chinensis* (stem); *Allium porro* (stem); *Convolvulus sepium* (stem); *Daucus carota* (root); *Glycine soja* (stem); *Helianthus annuus* (seed); *Nicotiana tabacum* (cell culture); *Philadelphus virginalis* (stem); *Phytolacca decandra* (cell culture); *Polygonum persicaria* (stem); *Rauvolfia manii* (cell culture); *Scorzonera hispanica* (root); *Solanum melanogena* (stem); *Tagetes patula* (cell culture) and *Vitis vinifera* (stem).

Isolation and sterilization of the material was performed following a standard protocol.[20] All suspension cultures were maintained on the following media: Gamborg medium[21] for all the species except for *N. tabacum*, *P. persicaria*, *R. manii*, and *T. patula*, which required Linsmaier and Skoog medium.[22] Both media were supplemented with 30 g sucrose/L, and 1-naphthylacetic acid (NAA, 0.5 mg/L) and 2,4-dichlorophenoxyacetic acid (2,4D, 2 mg/L) as phytohormones.

Suspension cultures were grown under standard conditions: 100 mL conical flasks containing 40 mL of medium on a rotary shaker at 100 rpm/min at 25 °C in the dark.

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