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BIOTRANSFORMATION OF GERANIOL BY PHOTOAUTOTROPHIC, PHOTOMIXOTROPHIC AND HETEROTROPHIC PLANT CELL SUSPENSIONS

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Key Word Index—*Euphorbia characias*, Euphorbiaceae, *Nicotiana tabacum*, Solanaceae, *Catharanthus roseus*, Apocynaceae, *Glycine max*, Leguminosae, plant tissue culture, biotransformation, geraniol, photoautotrophy, photomixotrophy, heterotrophy

Abstract—Cell suspension cultures of four plant species have been maintained under different carbon and energy supply regimes, i.e photoautotrophy, photomixotrophy and heterotrophy, and have been assayed for their capacity to biotransform geraniol The reactions of interconversion of geraniol into nerol, or oxidation of alcohols into their respective aldehydes, were mainly determined by the plant species, whatever the modes of culture were. A rapid metabolism of monoterpenic alcohols to unidentified compounds was observed. In one cell suspension (*Glycine max*), a high biotransformation activity (40–60%) into neral and geranial was detected

INTRODUCTION

Several studies have reported on the capacity of plant cell suspensions to biotransform an exogenous monoterpenic alcohol [1–4], but the influence of photosynthetic activity on this capacity is yet unknown As the mevalonic pathway of isoprenoid biosynthesis seems to be mainly located in the chloroplast [5, 6], the utilization of chlorophyllous cell suspensions, now obtained from various plant species [7], and capable of high levels of photosynthetic activity [7, 8], is a suitable, but still undeveloped, approach in the study of biotransformation processes From four plant species, we initiated cell suspensions cultivated in different regimes of carbon and energy supply, i.e photoautotrophy, photomixotrophy and heterotrophy, and we investigated their capacity to biotransform a monoterpenic alcohol such as geraniol.

RESULTS AND DISCUSSION

Biotransformation of exogenous geraniol has been performed with eight different cell suspensions. Their growth characteristics are indicated in Table 1 All cell suspensions were tested in early stationary phase of growth Geraniol was added to methanol (2% v/v), rather than ethanol as used in ref [1] because of its lower toxicity on several plant cell suspensions [9] The final amount of methanol alone in the medium (0 5% v/v) was not toxic for the cells For each cell suspension, 100 mg/l geraniol maintained cell viability higher than 80% of the initial one, 24 hr after inoculation (not shown) Beyond this concentration, the viability decreased rapidly, and at 500 mg/l complete death of cells was observed after 8 hr of incubation A similar 100 mg/l maximal amount of geraniol is also reported in *Vitis vinifera* [1] and *Pelargonium fragrans* [10] cell suspensions, both in stationary phase of growth

Time-course variations for the biotransformation of geraniol in photoautotrophic, photomixotrophic and heterotrophic cultures are presented in Table 2 For photoautotrophic cultures, we observe in *E characias* suspension a decrease of the total amount of products, mainly because of geraniol disappearance, while the aldehyde content remains roughly stable after 8 hr Interconversion of geraniol into nerol shows a constant ratio (about 50–60%) In *N. tabacum* suspension, we can observe only the disappearance of geraniol without any significant biotransformation

For photomixotrophic cultures, the same results as in photoautotrophic cultures are obtained in *E characias* suspension an interconversion of geraniol into nerol (at a constant rate of $55 \pm 10\%$) and a late increase in the relative amount of citral (geranial + neral). In *N tabacum* suspension, the total amount of products decreases, mainly because of geraniol disappearance, the amount of nerol remains stable, *ca* 10 ± 4 mg/l In *C roseus* suspension, we observe only the disappearance of geraniol, another compound (data not shown) different from citrol (geraniol + nerol) and citral was detected after 24 hr, and its retention time was similar, by GC analysis, to that of citronellol This would be in agreement with other studies [11] using *Vitis vinifera* cell suspensions For heterotrophic cultures, in *E characias* and *C roseus* cell suspensions, we observe the same variations as in photomixotrophic cultures In *G max* suspension, a slight decrease of total products after 24 hr is observed, compared with the high ones of other plant species The main phenomenon is the very fast formation of aldehydes and their very high and stable (80–85%) relative proportion in total products The citral composition remains also constant with a neral/geranial ratio around 3/4

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Table 1 Growth characteristics of plant cell suspensions used in stationary phase for geraniol biotransformation experiments

Growth conditions	Cell suspension	fr wt *	dry wt†	Chlorophyll‡
Photoautotrophy (CO_2 + light)	*E. characias*	78	7.1	568
	N. tabacum	71	3.9	70
Photomixotrophy (sucrose + light)	*E. characias*	117	6.5	236
	N. tabacum	184	4.0	32
	C. roseus	110	4.5	11
Heterotrophy (sucrose + darkness)	*E. characias*	71	7.1	
	C. roseus	110	8.6	
	G. max	45	10.3	

* Cellular concentration of the culture (g fr wt l)
† Dry weight content (%)
‡ Chlorophyll content (μg/g fr wt)

This case illustrates a general observation from our experiments aldehydes, when they appear, seem to be more stable than alcohols after 24 hr. Two pathways have been proposed for nerol and citral formation: firstly, a direct isomerization of geraniol into nerol, followed by an oxidation of alcohols into their respective aldehydes by an alcohol dehydrogenase or alcohol oxidase [2]; it was elsewhere proposed [11] that after geraniol oxidation, an interconversion of aldehydes followed by a reduction of neral into nerol could occur. Our results cannot discriminate between the two pathways, as the variations of nerol/citral ratio cannot be precise if the aldehydes are intermediate or final products.

Except in our *C. roseus* suspension, we did not detect citronellol as in *V. vinifera* [11] and *L. angustifolia* [4], nor geranyl acetate as in *V. vinifera* [11]. The variability of geraniol biotransformation ability among plant genera is convenient with the variability already reported, for example on cell cultures of various *Citrus* species for biotransformation of a sesquiterpenoid [12].

In measuring the geraniol disappearance in cell-free medium (not shown), we found that evaporated geraniol after 24 hr represented only 35% of the initial amount. So the geraniol evaporation was too low to account for the decrease of total products in the seven suspensions of *E. characias*, *N. tabacum*, and *C. roseus*. It can be concluded that metabolism of total products into unidentified compounds must be involved in their disappearance, as described for *L. angustifolia* cell suspension [4].

Oxidative degradation is a possible pathway for metabolism. Its mechanism is based upon double-bond epoxidation, and enzymatic systems implicated in this metabolic pathway have recently been described [13]. Glucosylation is an important pathway in metabolism of monoterpenoid alcohols, and their glucosides are commonly found in essential oil of whole plants. This possibility of disappearance was investigated by using β-glucosidase to recover the corresponding alcohols (not shown), but no monoterpenoid alcohol was found.

In conclusion, carbon and energy supply conditions during culture do not seem to greatly affect the geraniol biotransformation capacity of the cells. Interconversion of geraniol into nerol, and levels of aldehyde formation are determined by the plant species. While metabolism of total products seems to be the main pathway in our cell suspensions, one cell suspension (*G. max*) presents a high level of biotransformation (40–60%) of geraniol into geranial and neral.

EXPERIMENTAL

Cell suspension culture stocks. All the cell suspensions tested were originally developed in the laboratory. Photomixotrophic cell suspensions of *Euphorbia characias* were cultivated in a modified MS [14] liquid medium containing NAA (1 mg/l), BAP (1 mg/l) and sucrose (15 g/l) as described [15]; those of *Nicotiana tabacum* in a modified MS medium with NAA (2 mg/l), BAP (0.2 mg/l) and sucrose (15 g/l), and those of *Catharanthus roseus* in a modified Gamborg medium [16] with NAA (1 mg/l), BAP (1 mg/l) and sucrose (15 g/l).

Photoautotrophic cell suspensions of *E. characias* and *N. tabacum* derived from photomixotrophic cultures by transition on modified MS medium, without growth regulators and sucrose, were grown in a 1 l bubble-column aerated (5 l/hr) with 2% CO_2 enriched air; they were routinely subcultured during several months every 2 weeks by adding 250 ml of suspension into 750 ml of fresh medium.

Heterotrophic cell suspensions derived from heterotrophic callus cultures. *E. characias* and *C. roseus* suspensions were cultivated respectively in the same medium as photomixotrophic cultures, except sucrose at 30 g/l. Heterotrophic cell suspension of *Glycine max* was cultivated as *E. characias* suspension.

Photomixotrophic and heterotrophic cultures were performed in 300 ml conical flasks containing 100 ml of cell suspension, closed with a polyethylene sheet, and maintained at 110 rpm on an orbital shaker; they were routinely subcultured every 2 weeks (3 ml of suspension into 97 ml of fresh medium). Photoautotrophic and photomixotrophic cell suspensions were cultivated under 100 $\mu E/m^2/sec$ photosynthetically active radiation, 18 hr light period per day and heterotrophic ones in darkness. All cultures were kept at 25°.

Growth measurements. Fresh weight was determined by harvesting cell culture samples and filtering on fiberglass filter (GF/A Whatman). Dry wt was measured after drying overnight at 90°. Cell viability was determined after colouring in an erythrosin soln [17]. Chlorophyll was extracted in an Me_2CO–H_2O mixture (4:1), and quantified according to ref. [18].

Table 2 Time-course variations for the biotransformation of geraniol by photoautotrophic, photomixotrophic and heterotrophic cell suspensions

	Time*															
	2				4				8				24			
	Go	No	Ga	Na†	Go	No	Ga	Na	Go	No	Ga	Na	Go	No	Ga	Na
Photoautotrophic cell suspension																
E characias	37 2	18.9	6.0	4 8	33.8	21 7	1 8	1 3	12 9	7 8	2 8	2.2	3 7	1.9	6.7	5 4
N tabacum	72 8	1.5	0.3	trace	50 1	1.8	1.0	0 8	42 7	2 5	0 2	trace	21 7	2 5	trace	trace
Photomixotrophic cell suspension																
E characias	25 1	11 2	5 8	3 7	12 0	7 0	2 4	1 9	11.3	7.4	8 0	6 7	2 8	1 4	2.4	1 9
N tabacum	70 3	8 2	1.0	0 5	48 9	10 6	0.3	0 1	23.0	6 0	0.2	trace	5 0	14.2	trace	trace
C roseus	86 4	1.3	1 0	0.9	74 5	2 9	1 4	1.6	45 9	3 4	0 9	0.9	9 7	1 0	0 1	trace
Heterotrophic cell suspension																
E characias	59 1	26 4	1.5	2 6	52 2	25 5	1 9	1 8	40 5	25 2	1 9	2.5	18 0	11 7	4 9	5 1
C roseus	60.8	0.8	0 8	0 5	54 2	1.9	1 0	0 1	34 1	1 9	0 8	0 6	3 2	0 1	trace	trace
G max	13 8	2 1	36 0	25.8	8 3	4.0	25 7	22.1	4 6	3.9	22 0	16 1	3 0	3 3	21 4	15 4

*Time of incubation (hr).

†Products detected in the culture (mg/l) Initial amount was 100 mg/l of geraniol, Go = geraniol, No = nerol, Ga = geranial, Na = neral

Biotransformation experiments Geraniol–MeOH mixture (2% v/v) was added to each culture flask to obtain a geraniol amount of 100 mg/l culture In order to minimize the evapn of substrate, photoautotrophic cell suspensions were removed from the bubble column into a two-tier culture flask as described in ref [19], and containing 250 ml cell suspension, in a partial pressure of 2% CO_2 Flasks were shaken at 100 rpm in light Photomixotrophic and heterotrophic cultures were kept in the same flasks as culture stocks All the flasks were closed with an additional plastic cap For each incubation duration, two flasks were gathered to perform analysis Incubation was stopped by adding liquid N_2 on fresh biomass, and samples were stored at −30

Chemical analysis After a fast thawing, cell suspensions were ground and 1 mg of *n*-hexadecane was added as an internal standard for quantitative analysis Cells and medium were extracted with distil diethyl oxide for 48 hr by using a Jalade liquid–liquid extractor

Gas chromatography of biotransformation products was performed on a Delsi IGC 121 DFL chromatograph equipped with a 25 m capillary column Carbowax 20 M and a FID Injector and detector temperatures were 210° and 240° respectively After sample injection and a delay time of 30 sec at 70° a linear temp program ranging from 70 to 200° (5°/min) was applied Carrier gas was H_2 at 30 ml/min Products were identified by their retention times and their GC–MS analysis on a Delsi DI 700 chromatograph coupled with Ribermag R 10-10 mass spectrometer and Sidar computer

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