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Biosynthesis of Sterols and Triterpenes in Cell Suspension Cultures of *Uncaria tomentosa*

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Pectin administered to *Uncaria tomentosa* cell suspension cultures, was found to increase the production of triterpene acids (ursolic and oleanolic acid), however, neither growth nor sterol accumulation were affected. Cell cultures showed that pectin treatment caused a rapid threefold increase in the activities of enzymes involved in the biosynthesis of C_5 and C_{30} isoprenoid, such as isopentenyl diphosphate isomerase and squalene synthase. The activity of a farnesyl diphosphatase, which could divert the flux of farnesyl diphosphate to farnesol, was two times lower in elicited than in control cells. Elicited cells also transformed more rapidly a higher percentage of [5-^{3}H]mevalonic acid into triterpene acids. Interestingly, addition of terbinafine, an inhibitor of squalene epoxidase, to elicited cell cultures inhibited sterol accumulation while triterpene production was not inhibited. These results suggest that in *U. tomentosa* cells, both the previously mentioned enzymes and those involved in squalene 2,3-oxide formation play an important regulatory role in the biosynthesis of sterols and triterpenes.

Keywords: Farnesol — Isopentenyl diphosphate isomerase — Squalene synthase — Sterols — Triterpenes — *Uncaria tomentosa*.

Abbreviations: DMAPP, dimethylally diphosphate; FPP, farnesyl diphosphate; HMGCoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; IPP-isomerase, isopentenyl diphosphate isomerase; KF, potassium fluoride; NNm, Nitsch–Nitsch modified medium; SQS, squalene synthase.

Introduction

Uncaria tomentosa (Willd.) DC, commonly known as cat's claw, is widely used in traditional Peruvian medicine (Obregón-Vilches 1995). Oxindole alkaloids (Laus et al. 1997), sterols and triterpenes, i.e. ursolic acid, its isomer oleanolic acid and quinovic acid have been isolated from the bark and plants of *U. tomentosa* (Aquino et al. 1991). These pentacyclic triterpenes possess pharmacological (Liu 1995, Kashiwada et al. 1998) and agrochemical properties (Varanda et al. 1992).

Triterpenoids are a large class of natural isoprenoids present in higher plants, which exhibit a wide range of biological activities. Some plants contain large quantities of triterpenes in their latex and resins, and among their physiological functions has been proposed a chemical defense against pathogens and herbivores (Brown 1998). Plant cell cultures can respond to elicitor treatments with the induction of triterpenoid phytoalexins (van der Heijden et al. 1989), while this pathway could be inhibited using specific enzyme inhibitors such as terbinafine, which inhibits squalene 2,3-oxide formation (Yates et al. 1991). Both strategies are useful tools for further biochemical analysis.

Triterpenoid and sterol biosynthesis exhibits a common route from C_5 isoprenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate to the formation of the C_{30} units squalene and squalene 2,3-oxide (Fig. 1). However, three putative precursors have been postulated for IPP formation i.e. mevalonate (Brown 1998), deoxyxylulose (Rohmer et al. 1993) and in some plants, amino acids (Suga et al. 1980, Koops et al. 1991). Experiments using labeled compounds revealed that mevalonate is the preferential precursor for sterol (Baisted 1971, Trojanowska et al. 2000) and pentacyclic triterpene (van der Heijden et al. 1989, Akashi et al. 1994) biosynthesis. The enzyme hydroxymethyl glutaryl CoA (HMGCoA) reductase, which catalyzes mevalonate formation, is a limiting step in sterol formation (Bach and Lichtenthaler 1982).

It has been postulated that active biosynthesis of triterpenes occurs when sterol formation has been satisfied, for example, during exponential cell growth by relaxation of the control mechanism of precursor production to sterols (Kamisako et al. 1984).

Various studies of triterpene biosynthesis in plants propose a metabolic regulatory role at the level of post-squalene 2,3-oxide enzymes (Henry et al. 1992, Fulton et al. 1994) (Fig. 1). Moreover, it has been suggested that the presence of multifunctional triterpene synthases (Morita et al. 2000, Kushiro et al. 2000) would account for the co-occurrence of different types of triterpenes. However, other enzymes, such as IPP isomerase EC 5.3.3.2, squalene synthase (SQS) EC 2.5.1.21 (Fulton et al. 1994) and branch point enzymes such as allyl prenyldiphosphatases (Croteau et al. 1987) could play an important role during growth and maximum triterpene accumulation.

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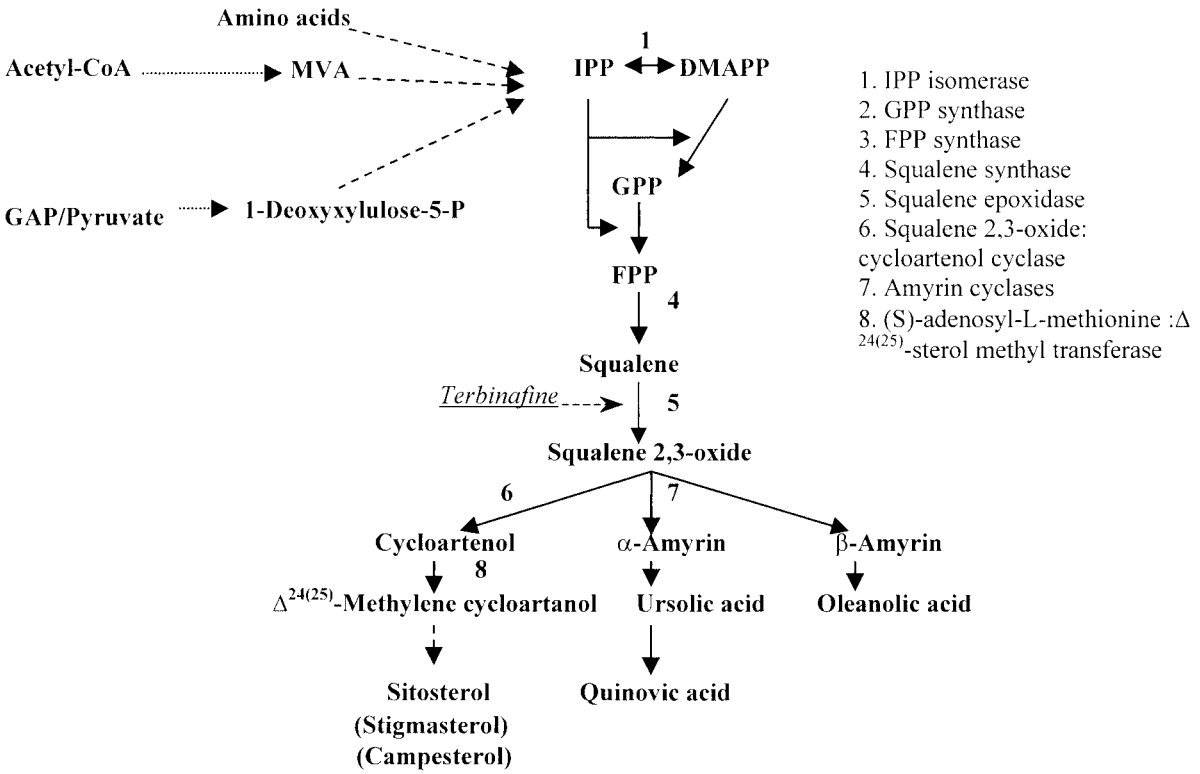

Fig. 1 General overview of sterol and pentacyclic triterpene pathways in *U. tomentosa*.

The present study investigates the biosynthesis of sterols and triterpenes in elicited *U. tomentosa* cell cultures, including the modulation of enzyme activities involved in C_5 and C_{30} isoprenoid (squalene and squalene 2,3-oxide) formation. We also report incorporation experiments using [5-^{3}H]mevalonate a putative triterpenoid precursor in these cell cultures.

Results and Discussion

Pentacyclic triterpenoid production using elicitors

U. tomentosa cell cultures were elicited using different biotic elicitors (Table 1). One hundred-twenty h after elicitation with pectin, the content of ursolic–oleanolic and quinovic acids increased 9.5 and 16 times respectively, in comparison to control cultures. When fungal elicitors were used, such as *Trichoderma* sp., the content of ursolic–oleanolic and quinovic acids increased 2–3 times, whereas *Pestalotia* sp., *Alternaria tenuis* and *Epicoccum nigrum* elicitors did not favor ursolic–oleanolic acid production. *A. tenuis* increased the production of the more oxidated triterpene, quinovic acid by four times.

Several reports have shown that the use of yeast and fungi as elicitors provoked an accumulation of triterpenoid phytoalexins (van der Heijden et al. 1989, Yoon et al. 2000).

In our system of *U. tomentosa* the largest effect even higher than that obtained with fungi in the production of pentacyclic triterpenoids, was by a sterilized suspension of *Citrus*

Table 1 Comparative triterpene production in elicited *U. tomentosa* cell cultures [a]

Elicitor treatment	Ursolic–oleanolic acid production [b] (μg g^{-1} dry weight)	Quinovic acid (μg g^{-1} dry weight)
Control	80.0	22.4
Pestalotia sp.	49.7	–
Trichoderma sp.	160.1	80.9
Alternaria tenuis	57.9	82.0
Epicoccum nigrum	37.3	–
Pectin	760.0	356.0

[a] Cultures after 120 h of elicitation.
[b] Oleanolic acid was 30% maximum of the total.

fruit pectin (Aldington and Fry 1993) with 9.3% degree of esterification. The *Citrus* pectin is a polymer formed by homogalacturonan, however, after sterilization at 121°C and pH 4.63, a variety of oligogalacturonic fragments must have formed (Ranganna et al. 1983) and acted as a signal (Dumville and Fry 2000, Ridley et al. 2001) when interacting with *U. tomentosa* cell cultures.

It has been reported that the addition of oligogalacturonides to different cell cultures increases the accumulation of monoterpenes and diterpenes (Bruce and West 1982, Croteau et al. 1987), shikonin (Tani et al. 1993) and anthraquinones (Dörnenburg and Knorr 1994). There are no reports that pectin or oligogalacturonides can stimulate accumulation of pentacyclic triterpenoids.

The percentage of cell viability (87.8±3.3) and biomass concentration (13.8±0.4 g liter^{-1}) of the cultures was not affected with the addition of 0.1% pectin w/v. Cellular morphology did not change with elicitation. The pH of the medium during the growth cycle of both control and elicited cell cultures was similar, between 4 and 6 (data not shown), and coincide with pH values reported for other triterpene acids produced by the *Psychotria carthagenensis* cell cultures (Lopes et al. 2000).

Effect of pectin elicitation on sterols and pentacyclic triterpenoid production

As in the plant, sitosterol (Senatore et al. 1989) was the main sterol present in *U. tomentosa* cell cultures. The production of sitosterol was not affected by the addition of pectin (Fig. 2A). During exponential growth the content of this phytosterol rose from 44 to 109.8±11.6 and 115.0±8.6 μg g^{-1} dry biomass in control and elicited cultures, respectively. Active sterol synthesis occurs following the exponential phase of cellular growth to provide sterols for new membranes of the cells in division and cellular stability (Hartmann 1998). The sitosterol content of *U. tomentosa* cell cultures was 2.5 and 10 times lower than in tobacco and *Morinda citrifolia* cells (Dyas et al. 1994), so that the content of free sterols is different among cell cultures of plant species of the same family.

In control cultures, the cell content of ursolic–oleanolic acids increased during the stationary phase of plant cell growth (Fig. 2B). The highest content of these triterpenoids was 2183 μg g^{-1} dry biomass, 16 d after inoculation. The biosynthesis profile of these triterpenoids is similar to that found in suspension cultures of *Datura innoxia*, *Luffa cylindrica* and *Lycopersicon esculentum* (Kamisako et al. 1984). In all these cell systems, maximal triterpene accumulation coincided with the ceased of the sitosterol biosynthesis.

In elicited cultures, the cell content of ursolic–oleanolic acids started 48 h before initiating the stationary phase, and the highest concentration was 1.5 times higher than in controls. These elicited cell cultures produced ursolic acid and oleanolic acid in a 71 : 29 ratio, determined from the ^{1}H NMR integrals of the methyl signals at C-27 for both substances (to be published).

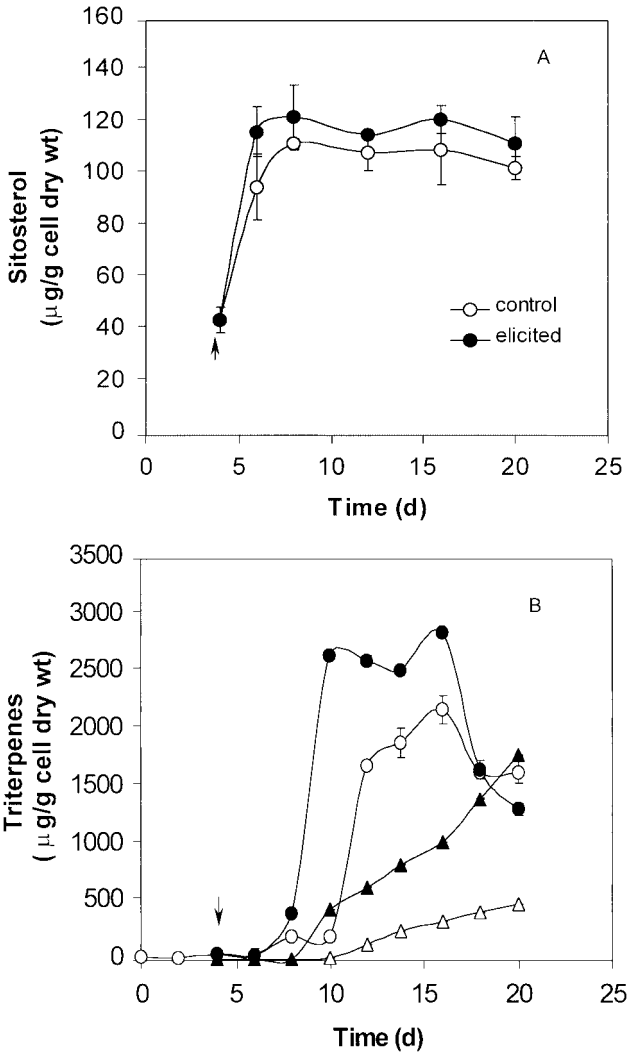

Fig. 2 Time course of (A) sitosterol and (B) triterpene acid accumulation in control (open symbols) and elicited (closed symbols) cultures of *U. tomentosa*. Ursolic–oleanolic acid (circles) and quinovic acid (triangles). Values are expressed as means of three replicates with standard deviations.

In both control and elicited cell cultures triterpenoid production declined after 16 d of initiation to 1,741.3±462 and 1,700.4±443 μg g^{-1} dry biomass, respectively. This decline in the biosynthesis of ursolic–oleanolic acids (Brown 1998) might be due to their biotransformation to quinovic acid, which production in elicited cells increased twice between the 16th and 20th days (Fig. 2B). This response of triterpene production in the presence of pectin might be generated by a sensation of damage around the plant cell wall involved in the plant defense mechanisms and induced by jasmonates (Doares et al. 1995). In control and elicited cell cultures, the accumulation of α- and β-amyrin were similar and 15–20 times lower than the ursolic and oleanolic acids concentration. Pectin-elicitation of *U. tomentosa* cell cultures did not increase the amyrin production.

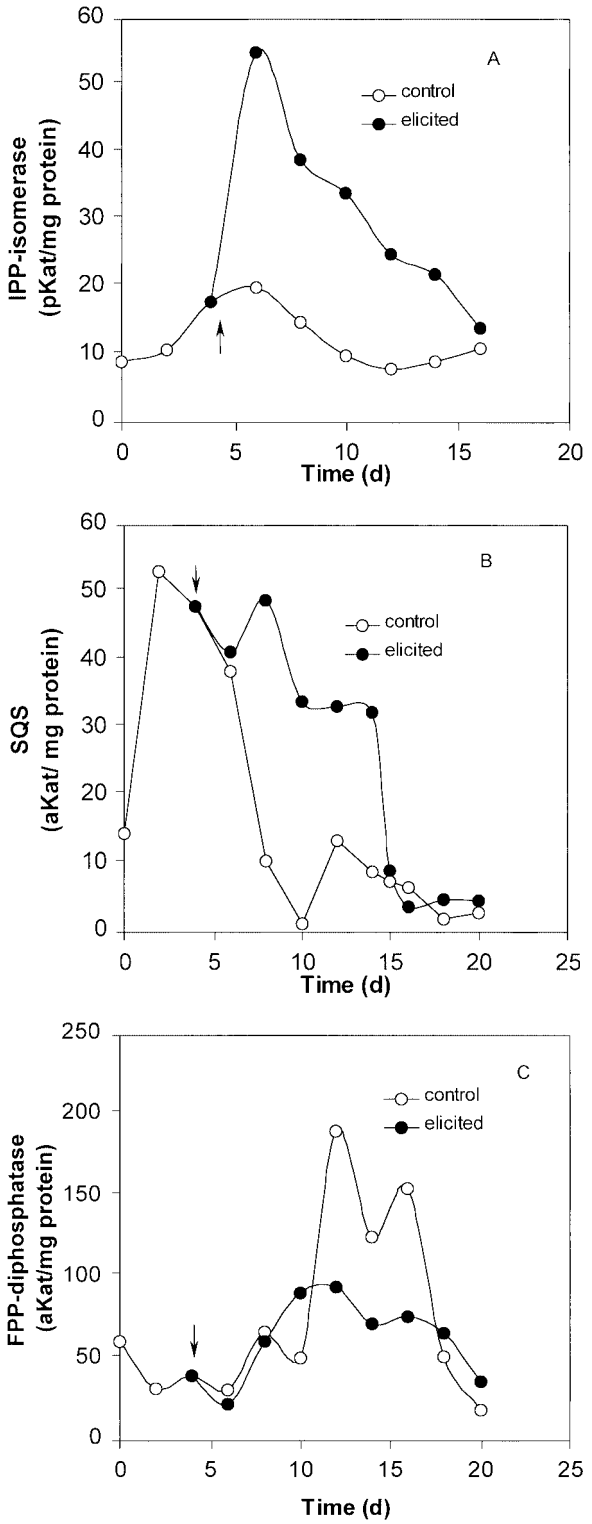

Fig. 3 Effect of pectin addition on the activities of (A) IPP isomerase; (B) squalene synthase and (C) farnesyl diphosphatase in control (open symbols) and elicited (closed symbols) cultures of *U. tomentosa*. The time of pectin administration is indicated by an arrow.

IPP isomerase, SQS and farnesyl diphosphatase enzymatic activities in U. tomentosa elicited cultures

In control cell cultures a high activity of IPP isomerase (Fig. 3A) and SQS (Fig. 3B) occurred during the exponential growth phase. However, after 4 d of elicitation, enzyme activity was 3 times higher than in control cell cultures. It has been reported that IPP isomerase and SQS activities were increased during maximal growth rate due to intensive sterol production (Hartmann et al. 2000, Ramos-Valdivia et al. 1998). The high activity of these enzymes in elicited cells correlates with the high triterpene production during the stationary phase of cell growth. Further studies of the enzyme involved in triterpene formation in elicited *Tabernamontana divaricata* cell cultures also showed an induction of IPP and SQS activities (Fulton et al. 1994). The SQS activity observed after elicitation in *U. tomentosa* differed from that obtained in tobacco cell cultures, when these were elicited with fragments of fungi. In this system the interruption of cellular growth was correlated with a suppression of SQS activity and previous to sesquiterpene production (Vögeli and Chappell 1988).

In preliminary SQS assays farnesol was formed, even if the acid phosphatase inhibitor KF (Qureshi et al. 1973) was added to the protein extracts. In plants and mammals the presence of an acid farnesyl diphosphatase which hydrolyzes farnesyl diphosphate (FPP) to farnesol (Meigs and Simoni 1997, Pérez et al. 1983) has been reported. In *U. tomentosa* cells we detected the activity of a Mg^{2+} dependent farnesyl diphosphatase, which was not inhibited with KF. In control cultures the activity of this diphosphatase increased during the stationary growth phase (Fig. 3C) while, in elicited cultures, the diphosphatase activity was two times lower. A low farnesyl diphosphatase activity in elicited cells was obtained by Croteau et al. (1987) in elicited pine saplings. Farnesol content within the cell has been associated with the recycling of FPP, regulation of isoprenoid production and apoptosis (Edwards and Ericsson 1999, Hemmerlin and Bach 2000). Therefore, in *U. tomentosa* the greater activity during the stationary cell growth phase could be related with cellular apoptosis.

Incorporation of [5-^{3}H]mevalonic acid in elicited U. tomentosa cell cultures

In control cultures, the distribution of radioactivity from [5-^{3}H]mevalonic acid was higher in phytosterols (20%) than pentacyclic triterpenoids (14%) (Fig. 4). In contrast, elicited cell cultures incorporate more radioactivity into pentacyclic triterpenoids (23%) than into phytosterols (15%). The high incorporated radioactivity shows that, in *U. tomentosa* cell cultures, mevalonate is a precursor of phytosterols and triterpene acids. Moreover, elicited cells rapidly transform a higher percentage of mevalonate to triterpenoids than control cells. In cell and organ cultures of *Rabdosia japonica* Hara (Seo et al. 1988), *Taraxacum officinale* (Akashi et al. 1994) and *Avena sativa* cv. *image* (Trojanowska et al. 2000) mevalonate was the precursor of pentacyclic triterpenoids and sterols. When lovas-

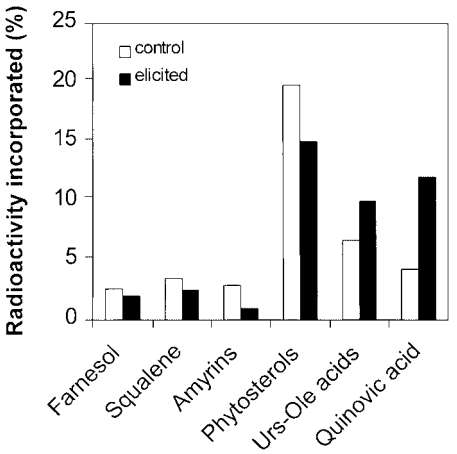

Fig. 4 Distribution of radioactivity incorporated from (*R*, *S*)-[5-^{3}H]mevalonic acid after 144 h into farnesol, squalene, amyrins, phytosterols, ursolic–oleanolic acids and quinovic acid by control and elicited *U. tomentosa* cell cultures.

tatin, a specific HMGCoA reductase inhibitor, was added to *U. tomentosa* cell cultures in the initial exponential growth phase, the biosynthesis of phytosterols and ursolic–oleanolic acid was inhibited by about 80% (data not shown). It has been reported that lovastatin inhibits the synthesis of sterols and mevalonate-derived secondary metabolites in cell and plant cultures (Bach and Lichtenthaler 1982). Biosynthesis inhibition of phytosterols and pentacyclic triterpenoids in *U. tomentosa* by lovastatin confirms mevalonate as the initial precursor of these compounds.

Biosynthetic capacity of protein extracts

Protein cell extracts of elicited and control *U. tomentosa* cultures synthesized labeled geraniol, farnesol, squalene, α- and β-amyrin, phytosterols and ursolic–oleanolic acids after addition of [1-^{14}C]IPP. When [1-^{14}C]IPP was replaced by [1-^{3}H]FPP results were qualitatively similar (Table 2). The high percentage of radioactivity incorporated into farnesol might impede the incorporation of higher levels of radioactivity into phytosterols and ursolic–oleanolic triterpene acids. Radioactivity was not incorporated in quinovic acid. In Table 2 the presence of α- and β-amyrin and ursolic–oleanolic acids formed from [1-^{14}C]IPP or [1-^{3}H]FPP shows the participation of α-and β-amyrin synthases (Henry et al. 1992, Fulton et al. 1994) and putative oxidases (van der Heijden et al. 1989) even though the incubation mixture was not optimal for these enzymatic activities. Recently findings indicated that multifunctional triterpene synthases would be involved in the formation of different skeletal types of triterpenes as both ursene and oleanane types (Kushiro et al. 2000, Husselstein-Muller et al. 2001). The absence of quinovic acid in in-vitro conditions suggested that the activity of the putative oxidase which formed this triterpene acid in vivo (Fig. 4) was not present. The biosynthetic capacity of *U. tomentosa* extracts was comparable with other cell cultures that transform IPP or FPP into isoprenoid products, which include more than six enzymatic steps (van der Heijden et al. 1989).

Effect of terbinafine in cell suspension cultures

The growth index and accumulation of ursolic and oleanolic acids in pectin-elicited *U. tomentosa* cultures treated with 0.1 or 0.2 mg liter^{-1} terbinafine were not affected. However, cell viability (data not shown) and sitosterol content decreased (4.5 times) (Table 3). In addition, squalene content was slightly increased in the cell culture treated with 0.2 mg ml^{-1} terbinafine. In contrast to the findings in celery (Yates et al. 1991), in *U. tomentosa* cultures the growth index did not decrease, probably considering that squalene was not highly accumulated in the cells (Table 3). These results point to a differential effect of terbinafine on the biosynthesis of sterols and triterpenes, perhaps due to a different inhibitory concentration of the putative isoforms of squalene epoxidase. In the fungus *Ustilago maydis* (Orth and Sisler 1990), terbinafine inhibits sterol accumulation but stimulates pentacyclic triterpenoid formation. Treatment with other squalene epoxidase inhibitors as tolnaftate have been found to inhibit sterol synthesis, cellular plant growth and pentacyclic triterpenoids (Cho et al. 1993). On the other hand, after 24 h of terbinafine (0.2 mg ml^{-1}) addition, cellular morphology and color in elicited cell cultures were modified (from small to large cells and from green-yellow to dark brown).

Table 2 Distribution of radioactivity after incubation with [1-^{14}C]IPP and [1-^{3}H]FPP

Crude extract (hours after elicitation)	Geraniol (% of radioactivity)	Farnesol (% of radioactivity)	Squalene (% of radioactivity)	Phytosterols (% of radioactivity)	Amyrins [a] (% of radioactivity)	Ursolic–oleanolic acid (% of radioactivity)
Control (48)						
[1-^{14}C]IPP	8.0	16.1	3.6	5.8	16.7	2.2
[1-^{3}H]FPP	–	20.0	0.1	47.0	3.0	2.3
Elicited (144)						
[1-^{14}C]IPP	3.6	48.4	2.8	16.2	1.6	0.9
[1-^{3}H]FPP	–	36.0	0.1	28.4	1.4	3.1

[a] α-amyrin and β-amyrin.

Table 3 Effect of terbinafine in pectin elicited cell cultures [a]

Treatment	Growth index	Squalene (μg g^{-1} dry weight)	Sitosterol (μg g^{-1} dry weight)	Triterpenes [b] (μg g^{-1} dry weight)
Control	2.86	16.52	117.20	2151.20
Terbinafine				
0.1 mg ml^{-1}	2.62	13.03	38.76	1968.44
0.2 mg ml^{-1}	2.14	22.15	26.34	1966.47

[a] Cells harvested at 312 h after terbinafine addition.
[b] Ursolic and oleanolic acids.

In *U. tomentosa* cells, a relation between the flux of biosynthesis for sterols and pentacyclic triterpenoids may be present. IPP-isomerase and SQS activity revealed a double demand of production, first during formation of the primary metabolite sitosterol and later of the secondary metabolites ursolic–oleanolic and quinovic acids. This biosynthetic flux may form two enzymatic complexes (Srere 1987) where one participates in the formation of sterols and the other in the formation of pentacyclic triterpenoids from the initial precursor mevalonate. Under certain conditions, farnesyl diphosphatase activity could branch the FPP flux towards farnesol. Finally, our results suggest that in the triterpene acid formation of *U. tomentosa* cells, a putative regulatory role of the post squalene 2,3-oxido enzymes also occurs.

Material and Methods

Chemicals

(*R*, *S*)-[5-^{3}H]mevalonic acid (33 Ci $mmol^{-1}$) and [1-^{14}C]isopentenyl diphosphate (55 mCi $mmol^{-1}$) were obtained from Sigma (St. Louis, MO, U.S.A.). [1-^{3}H]FPP (50 Ci $mmol^{-1}$) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Radioactivity in solution was measured by liquid scintillation (LS) spectrometry LS5801LS and cocktail Ready Value, Beckman (Fullerton, CA, U.S.A.). Squalene, farnesol, β-sitosterol, ursolic acid and oleanolic acid were purchased from Sigma (St. Louis, MO, U.S.A.), α- and β-amyrin and quinovic acid were a gift from Dr. R. Verpoorte, Leiden University, The Netherlands. All chemical products and mineral salts were of the highest purity.

Plant material and cell culture methods

U. tomentosa cell cultures initiated from stem explants were maintained in Nitsch–Nitsch (NN) (Nitsch and Nitsch 1969) modified medium (NNm) (Feria 1998) added with 2 mg $liter^{-1}$ 2.4-D, 2 mg $liter^{-1}$ kinetin and 20 g $liter^{-1}$ sucrose. Cells were subcultured with a 3-fold dilution every 10 d. Cultures were grown on an orbital shaker at 110 rpm and 25±2°C under a light intensity of 400 lux.

Elicitation

Four fungal strains *Trichoderma sp.*, *Pestalotia sp.*, *E. nigrum* and *A. tenuis* were isolated from seeds and plants of *U. tomentosa* and grown in NN medium (Nitsch and Nitsch 1969) containing 20 g $liter^{-1}$ sucrose. Cultures were incubated at 30°C on gyratory shaker at 110 rpm for 48 h and autoclaved. The mycelium was separated by filtration and washed with distilled water and freeze-dried.

Erlenmeyer flasks (250 ml) containing 50 ml of NNm medium were inoculated with 5 g fresh cells and incubated at 110 rpm in the light (400 lux) at 25±2°C. A 50 mg *Citrus* fruit pectin (Sigma, St. Louis, MO, U.S.A.) or dry mycelium suspension in 1.5 ml of water pH 4.63 was autoclaved (for 15 min at 121°C) and applied to the cultures of 4-day-old *U. tomentosa* cells.

Sterol and pentacyclic triterpenoid extraction

Freeze-dried cells (0.2 g) were suspended in 10 ml of 5 mM HCl, left to rest for 2 h and filtered. Cells were neutralized (pH 7) with 0.5 M NaOH, washed with neutral water and dried at 56°C. Isoprenoids were extracted with 10 ml $CHCl_3$ at 50°C for three times. $CHCl_3$ was evaporated and the extracts were stored at 4°C.

Isoprenoid analysis

For quantitative analysis of triterpene acids, squalene and sterols, samples (20 μl) were subjected to HPLC analysis in a Varian chromatograph Prostar 330 with a photodiode array detector (Varian, Walnut Creek, CA, U.S.A.) equipped with a reversed-phase C_{18} column (250×4 mm i.d. Spherisorb 5 μm ODS2) at 25°C, with the solvent system methanol–water (98 : 2) at a flow of 0.9 ml min^{-1} and detected at 205 nm. Retention times were as follows: ursolic acid and oleanolic acid 6.4 min, quinovic acid 9.4 min, β amyrin 22.4, α amyrin 24.3 min, sitosterol 31.7 min and squalene 35.2 min. Calibration curves with theirs respective standards were made. TLC was analyzed in silica gel 60 thin-layer plates (20×20 cm, Merck) developed according to Parkash and Singh (1980) in benzene–methanol–ethyl acetate (119 : 14 : 7) and revealed with anisaldehyde–sulfuric acid reagent. Identifications were done by comparing their retention factor (*Rf*) with cold standards for squalene (*Rf* 0.77), ursolic acid (*Rf* 0.38), oleanolic acid (*Rf* 0.39), geraniol (*Rf* 0.43), farnesol (*Rf* 0.46), α- and β-amyrin (*Rf* 0.61), β-sitosterol (*Rf* 0.51) and quinovic acid (*Rf* 0.32). Radioactivity in zones was scraped and measured by LS.

(R, S)-[5-^{3}H]mevalonic acid feeding

Twenty-five-ml Erlenmeyer flasks containing 10 ml of NNm medium were inoculated with 2 g fresh cells and incubated at 110 rpm in the light (400 lux) at 25±2°C. To both elicited and control cultures, 80 μl of (*R*, *S*)-[5-^{3}H]mevalonic acid (10 μCi μmol^{-1}) were added. After 144 h of growth, cell cultures were centrifuged at 3,000×*g* for 10 min and the cell pellet was washed three times with 3 ml of water. The washed biomass was stored at –30°C. Labeled metabolites were extracted as described above.

Inhibitor addition

Lovastatin (100 μM) was added at day 3 to the cell culture. After 168 h, cell cultures were harvested and lyophilized. Lovastatin was previously dissolved in ethanol (80%), with added 0.1 M NaOH and left to rest for 2 h at 25±2°C, pH was adjusted at 7.5. Terbinafine (0.1 and 0.2 mg ml^{-1}) was applied to pectin-elicited cultures. After 312 h, cell cultures were harvested and lyophilized. A membrane-filtered solution of terbinafine (10 mg ml^{-1} in water) was used. Growth index

was calculated as follows:

Growth index = (Cell concentration after 312 h)/(Cell concentration at the time of terbinafine addition)

Protein extracts

Frozen cells were homogenized in a mortar. Immediately, per gram of fresh cell weight, 0.1 g of polyvinylpolypyrrolidone and 1 ml of extraction buffer (0.1 M potassium phosphate buffer, pH 7.2, 0.5 M sucrose, 2 mM EDTA, 10 mM dithiothreitol and 5 μM leupeptin) were added. The homogenate was squeezed through Miracloth and centrifuged at 10,000×*g* for 15 min. All steps were performed at 4°C.

Enzyme assays

IPP-isomerase activity was assayed according to Ramos-Valdivia et al. (1998) using [1-^{14}C]isopentenyl diphosphate as substrate (Sigma). SQS activity was measured as described by Threlfall and Whitehead (1992) with slight modifications. The incubation mixture, with a total volume of 250 μl, contained 50 mM potassium phosphate (pH 7.2), 0.5 M sucrose, 30 mM KF, 1 mM DTT, 25 mM $MgCl_2$, 0.5 mM NADPH and 125 μl of protein extracts (25–150 μg protein). The reaction was initiated by addition of 20 μM [1-^{3}H]FPP (50 μCi mmol^{-1}) for 35 min at 35°C, under a nitrogen gas stream and stopped with 1.25 ml $CHCl_3$–methanol (1 : 2). 1 ml water was used to wash. The evaporated organic phase was resuspended with 100 μl acetone and 60 μl was applied to thin-layer plates (Merck) and developed in cyclohexane–ethyl acetate (1 : 1), revealed with iodine vapor and identified by comparing its *Rf* with cold squalene (0.78). The areas of the silica plates corresponding to the marker were scraped and radioactivity was measured by LS.

Farnesyl diphosphatase activity assay

The incubation mixture, with a total volume of 250 μl, contained 50 mM potassium phosphate (pH 7.2), 0.5 M sucrose, 25 mM $MgCl_2$, 30 mM KF, 1 mM DTT and 125 μl protein extracts (25–150 μg protein). The reaction was initiated by addition of 20 μM [1-^{3}H]FPP (50 μCi mmol^{-1}) for 35 min at 35°C and stopped with 1.25 ml $CHCl_3$–methanol (1 : 2). Farnesol was separated as described by Threlfall and Whitehead (1992). Labeled farnesol was identified by comparing its *Rf* with cold farnesol (0.52). The areas of silica gel corresponding to the marker were scraped and radioactivity was measured by LS. Protein concentration was measured as described by Peterson (1977) with bovine serum albumin as standard. Protein extracts from elicited cultures were previously desalted using Sephadex G-25 M (PD-10) columns, eluted with 50 mM potassium phosphate buffer (pH 7.2) containing 0.5 M sucrose, 2 mM EDTA.

[1-^{14}C]IPP and [1-^{3}H]FPP incorporation in cell extracts

The incubation mixture contained 50 mM potassium phosphate buffer (pH 7.2), 0.5 M sucrose, 30 mM KF, 1 mM DTT, 0.5 mM NADPH and 250 μl protein extracts (0.37–0.98 mg protein), in a total volume of 500 μl. The reaction was initiated by addition of 0.225 mM [1-^{14}C]IPP (13 μCi mmol^{-1}) and 12.5 mM $MgCl_2$ or 20 μM [1-^{3}H]FPP (50 μCi mmol^{-1}) and 25 mM $MgCl_2$, for 1 h at 30±2.5°C and stopped with 2.5 ml $CHCl_3$–methanol (1 : 2). The separation and identification was carried out according to Threlfall and Whitehead (1992).

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