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Elicitor-mediated induction of anthraquinone biosynthesis and regulation of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase activities in cell suspension cultures of *Cinchona robusta* How.

Ana C. Ramos-Valdivia[1,2], Robert van der Heijden[1], Robert Verpoorte[1]

[1]Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, P.O. Box 9502, NL-2300 RA Leiden, The Netherlands
[2]Departamento de Biotecnología, Centro de Investigación y Estudios Avanzados (CINVESTAV), Apto Postal 14-740, CP 07000, México D.F., México

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Abstract. Treatment of a *Cinchona robusta* How. cell suspension culture with a homogenate of *Phytophthora cinnamomi* resulted in cessation of growth and a rapid induction of the biosynthesis of anthraquinone-type phytoalexins. The strongest induction of anthraquinone biosynthesis was obtained when the elicitor was added in the early growth phase of the growth cycle. The accumulation of anthraquinones was accompanied by a tri-phasic response in the activity of isopentenyl diphosphate (IPP) isomerase (EC 5.3.3.2): phase I was characterised by a rapid induction of activity, reaching a maximum at 12 h after elicitation. During phase II, IPP isomerase rapidly decreased to levels below those found in untreated cells. At phase III, IPP isomerase activity increased again, reaching a second maximum at about 72 h after elicitation. During phase I, the activity of farnesyl diphosphate synthase (EC 2.5.1.10) was found to be suppressed. Extraction and assay conditions were optimised for IPP isomerase. The presence of Mn^{2+} in the incubation buffer resulted in a marked increase in the activity of the enzymes obtained from cells in phase I. The induction of IPP isomerase in combination with a concomitant inhibition of farnesyl diphosphate synthase might result in an efficient channeling of C_5-precursors into phytoalexin biosynthesis.

Key words: Anthraquinone – *Cinchona* – Farnesyl diphosphate synthase – Isopentenyl diphosphate isomerase – Phytoalexin – *Phytophthora*

Introduction

Cinchona (Rubiaceae) plants are the source of the therapeutically important alkaloids quinine and quinidine. Besides alkaloids, triterpenes, flavonoids, proanthocyanidins and anthraquinones have been isolated from these plants (Verpoorte et al. 1988). The anthraquinones in *Cinchona* play a role as phytoalexins and their accumulation in infected parts of *Cinchona* trees is connected with a reduction of the alkaloid content (Wijnsma et al. 1986). This might be due to the fact that alkaloid and anthraquinone biosynthesis share common intermediates, namely the isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and the precursor of aromatic compounds, chorismic acid (Wijnsma 1986).

Dimethylallyl diphosphate is a central intermediate in the biosynthesis of terpenoid and meroterpenoid compounds. In the terpenoid pathway, DMAPP and IPP are interconverted by the enzyme IPP isomerase (EC 5.3.3.2). The DMAPP is condensed with one IPP molecule by geranyl diphosphate (GPP) synthase (EC 2.5.1.1) to give geranyl diphosphate, the precursor for monoterpenoids and the terpenoid moiety of the *Cinchona* alkaloids (Leete and Wemple 1969). Condensation of DMAPP with two molecules of IPP in a reaction catalysed by farnesyl diphosphate (FPP) synthase (EC 2.5.1.10) yields FPP, the precursor of phytosterols and triterpenes. In rubiaceous plants, DMAPP forms the C-ring of the anthraquinone skeleton (Leistner 1985).

An approach to gain insight into the regulatory sites of secondary metabolism in *Cinchona* is to study the mechanisms involved in the regulation of competitive branches in the relevant biosynthetic pathways. Since DMAPP finds itself on a branch point of metabolic pathways, IPP isomerase may thus play an important regulatory role in controlling the availability of C_5 units. Here we report on the changes of the activities of IPP isomerase and FPP synthase in *C. robusta* cells treated with an elicitor preparation from *Phytophthora cinnamomi* (*Pcin*). The *Cinchona-Pcin* system was characterised with respect to biomass and anthraquinone accumulation. In addition, studies were performed to elucidate the possible requirement of de-novo protein synthesis for the elicitor-mediated induction of anthraquinone biosynthesis.

Abbreviations: DMAPP = 3,3-dimethylallyl diphosphate; FPP = farnesyl diphosphate; GPP = geranyl diphosphate; IPP = isopentenyl diphosphate; NPP = N,N-(dimethylamino)ethyl diphosphate; *Pcin* = *Phytophthora cinnamomi,* PVPP = polyvinylpolypyrrolidone

Correspondence to: R. van der Heijden; E-mail: heijden@lacdr.leidenuniv.nl; Fax: 31 (71) 527 45 11

Materials and methods

Chemicals. [1-^{14}C]Isopentenyl diphosphate (IPP) with a specific activity of 1.96 GBq · mmol^{-1} was purchased from Amersham Nederland BV (s-Hertogenbosch, The Netherlands). Unlabelled IPP and DMAPP were synthesised according to Davisson et al. (1985). E,E Farnesol (96%), 3-methyl-3-buten-1-ol (isopentenol, 97%), 3-methyl-2-buten-1-ol (dimethylallyl alcohol, 99%) and squalene (97%) were obtained from Aldrich Chemie (Zwijndrecht, The Netherlands), and geraniol (99.5%) and iodoacetamide were from Fluka Chemie (Bornem, Belgium). Alkaline phosphatase from calf intestine grade II (140 U · mg^{-1} at 37°) was purchased from Boehringer Mannheim BV (Almere, The Netherlands). Dithiothreitol (DTT) was from Merck Nederland BV (Amsterdam, The Netherlands). Leupeptin, cycloheximide, bovine serum albumin (fraction V) and polyvinylpolypyrrolidone (PVPP) were from Sigma (Zwijndrecht, The Netherlands). N,N-(dimethylamino)-ethyl diphosphate (NPP) was a generous gift from Prof. C.D. Poulter (Utah University, Salt Lake City, USA). Sephadex G-25 M (PD-10) columns were from Pharmacia Biotech (Roosendaal, The Netherlands). All the other chemicals were of highest purity commercially available.

Cell cultures. Cell suspension cultures of *Cinchona robusta* How. were initiated in 1985 from seedling stem explants and were grown in B5 medium (Gamborg et al. 1968) containing 2% sucrose, 2 mg · l^{-1} 2,4-dichlorophenoxyacetic acid, 0.2 mg · l^{-1} kinetin and 50 mg · l^{-1} cysteine and were subcultured weekly by adding 15 ml of the suspension into 50 ml of fresh medium. The cultures were grown in the light at 25 °C on gyratory shakers at 120 rpm. For all the experiments, 250-ml Erlenmeyer flasks containing 50 ml of medium were used. The flasks were inoculated with weighed amounts of cells (between 5 and 6 g FW). The cells were taken from a culture which was in the exponential phase of the growth cycle.

Elicitation experiments. *Phytophthora cinnamomi* (ex *Cinchona robusta*; *Pcin*, CBS No. 403.48) was purchased from Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands). The biomass of *Pcin* was produced in a 5-l Wilson-type bioreactor (Wilson 1976), containing 4 l sucrose-asparagine medium (Keen 1975). The bioreactor was inoculated with 100 ml *Pcin* suspension. After 9 d the culture was autoclaved and, after filtration, the biomass was lyophilised, homogenised and stored at −20°. An autoclaved *P. cinnamomi* preparation (40 mg mycelium DW in 2 ml of water) was added per 50 ml of *C. robusta* cell cultures.

Time course studies. Flasks containing cell cultures of *C. robusta* were prepared and inoculated as described above. They were allowed to grow for 0, 2, 4, or 8 d before addition of the elicitor. Control cultures were left untreated. At different intervals after addition of the elicitor, one control flask and duplicate flasks of the elicitor-treated cultures were harvested. Biomass and medium were separated by filtration over Miracloth (Omnilabo Int. BV, Breda, The Netherlands). Five grams (FW) of the biomass of each flask was used for the preparation of enzyme extracts (see below), the remainder was lyophilised. Aliquots of the dry biomass were used for the determination of the anthraquinone content and biomass accumulation (g DW · l^{-1}). The medium of each flask was collected, and stored at −20 °C before determination of the anthraquinone content.

Preparation of protein extracts. After filtration, the cells (5 g FW) were washed with water and frozen in liquid nitrogen. The frozen cells were homogenised in a Waring blender for 3 × 20 s at maximum speed. Immediately, per gram (FW) was added: 0.15 g of PVPP and 0.6 ml of extraction buffer (200 mM Tris-HCl, pH 7.5; 2 mM EDTA; 2 mM DTT; 5% glycerol; 10 μM leupeptin). The homogenate was squeezed through Miracloth and centrifuged at 10 000 *g* for 30 min. The supernatants were desalted using Sephadex G-25 M (PD-10) columns, which were equilibrated and eluted with 100 mM Tris-HCl buffer (pH 7.5) containing 2 mM DTT, 5% glycerol and 10 μM leupeptin.

Protein determination. Protein concentrations were determined according to Peterson (1977) with bovine serum albumin as standard.

Assay of IPP isomerase activity. The assay was based on the acid lability of the allylic diphosphates. The method described by Satterwhite (1985) was slightly modified. The incubation mixture, with a total volume of 200 μl, contained 100 mM Tris-HCl buffer (pH 7.5), 18 μM (370 kBq · μmol^{-1}) [1-^{14}C]IPP, 1.5 mM $MnCl_2$, 1.5 mM $MgCl_2$, 2 mM DTT and 25 mM KF. The reaction was initiated by addition of 10 μl (10–20 μg protein) of crude desalted enzyme preparation. After incubation for 10 min at 30 °C, 0.20 ml methanol-HCl (4:1 v/v) and 0.5 ml water were added and incubation was continued for 15 min at 37 °C. The incubation mixture was saturated with NaCl. The allylic prenols were extracted with 2 × 1 ml of toluene. The combined extracts were dried with dry sodium sulphate. A 1-ml aliquot of the toluene layer was removed and mixed with 10 ml Opti-Fluor (Packard Instrument BV, Groningen, The Netherlands) and radioactivity was determined using a Tri-Carb 4530 liquid scintillation counter (Packard Instruments BV, Groningen, The Netherlands). Substrate blanks were performed by adding the methanol-HCl before addition of the enzyme.

The assays in which IPP isomerase inhibitors were added, were performed in the same way, except that before addition of substrate the crude desalted enzyme was preincubated for 10 min in the presence of 10 mM iodoacetamide or 25 μM NPP.

Assay of FPP synthase activity. The incubation conditions for FPP synthase were identical to those of IPP isomerase, except for the addition of 100 μM DMAPP as second substrate. After incubation, diphosphates were hydrolysed by the action of alkaline phosphatase, followed by separation (see below) and subsequent quantification of the incorporation of radioactivity in farnesol.

Determination of biosynthetic capacity to form allylic compounds. The incubation conditions for determining the capacity of the enzyme preparations to incorporate [1-^{14}C]IPP into allylic compounds (DMAPP, GPP and FPP) were similar as those for the IPP isomerase assay, except the total volume was 2 ml. The reaction was initiated by addition of 100 μl of desalted protein extract. The incubation, performed in duplicate, continued for 30 min at 30 °C. Aliquots of 0.2 ml were taken at times indicated in Fig. 3. Similar incubations were performed in the presence of 100 μM DMAPP, added as second substrate. Products were analyzed by HPLC.

Analysis of enzyme product formation by HPLC. After incubation, the diphosphates were hydrolysed by addition of 200 μl of 200 mM Tris (pH 9.5) and 10 μl (14 U) of alkaline phosphatase (1 U releases 1 μmol of 4–nitrophenol in 1 min at 37 °C and pH 9.8, in the presence of 0.97 M diethanolamine, 0.5 mM Mg^{2+} and 11 mM 4–nitrophenyl phosphate) suspension to the mixtures. Samples were incubated for 3 h at 37 °C. Alcohols were extracted by shaking the mixture with 200 μl of hexane. The samples were centrifuged at 10 000 · *g* and stored for 20 min at −20°. The organic layer was removed and 8 μg of dimethylallylalcohol, geraniol, E,E-farnesol and isopentenol, (dissolved in hexane) were added as carriers. Extracts were then analysed by HPLC. The HPLC system consisted of a Hypersil column (5 μm; 250 mm long, 4.6 mm i.d.; Shandon, Life Sci. Int. Benelux, Veldhoven, The Netherlands) using 1.5% *n*-butanol in hexane as eluent at a flow rate of 0.5 ml · min^{-1} (Suga and Endo 1991). The alcohols were monitored at 210 nm. They were collected and the radioactivity was determined by liquid scintillation counting (LSC).

Estimation of the anthraquinone content. For each culture, 100 mg of lyophilised biomass and 15 ml of culture medium were extracted and the anthraquinone content was determined as described by Schulte et al. (1984). The UV/VIS-spectra were recorded from 350 to 700 nm and the absorbance was measured at 410 nm. Subsequently, the extracts were concentrated and acidified with phosphoric acid to pH 3. Then anthraquinone aglycones were extracted

as described by Harkes et al. (1985) and the spectra and absorbance were recorded as described above. The anthraquinone content was determined relative to rubiadin, $\lambda_{max}^{ethanol}$ nm (log ε): 410(3.90).

Addition of protein synthesis inhibitors. Inhibitors were added to the cell cultures 30 min prior to the addition of *Pcin*. Cycloheximide was added from a stock solution of 2.5 mg · ml^{-1} in 70% ethanol to give a final concentration of 35 μM, actinomycin D from a stock solution of 2.5 mg · ml^{-1} in 95% ethanol to give a final concentration of 8 μM. Control cell cultures were left untreated. Cells of each treatment were harvested at 12, 24, 48 and 72 h after elicitation and IPP isomerase and FPP synthase activities were assayed. Anthraquinone contents were determined in samples harvested 24 and 72 h after elicitation. The experiments were performed in triplicate.

Results

Standardisation of the elicitation procedure. The *C. robusta* cell suspension culture was selected as it does not accumulate anthraquinones under standard culture conditions. A homogenate of *Pcin* proved to be a potent elicitor (van der Heijden et al. 1988) and was selected for further experimentation. After large-scale culture of *Pcin*, a stock preparation was prepared. Based on cell viability studies, as observed after fluorescein diacetate staining (Widholm 1972) and on the capacity to induce anthraquinone biosynthesis, the effective concentration range of the *Pcin* preparation was determined. Addition of 2 mg *Pcin* DW · ml^{-1} culture was lethal for the cells. A concentration of 0.8 mg *Pcin* · ml^{-1} suspension culture yielded high levels of anthraquinones with maintained high cell viability. The elicitor preparation proved to be very stable, after three years of storage at −20 °C it still showed full activity. Furthermore, the elicitor remained active after autoclaving. The *C. robusta-Pcin* system as described below, comprising the effects on growth, anthraquinone accumulation, IPP isomerase and FPP synthase activities, showed itself to be very stable and to give reproducible results. During the last five years, these elicitation experiments have been repeatedly performed, resulting in identical responses.

Extraction and cofactor requirement of IPP isomerase and FPP synthase. Anthraquinones may cause severe interference in the extraction of active enzymes and in the methods for determination of protein concentrations (Heide and Leistner 1983). Addition of 150 mg PVPP · (g FW)$^{-1}$ of anthraquinone-containing *C. robusta* cells increased the recovery of IPP-isomerase activity by 30%. No further increase in activity was obtained when using 200 and 250 mg PVPP · (g FW)$^{-1}$. Addition of 2 mM EDTA, 10 μM leupeptin and 5% glycerol to the extraction buffer increased the recovery of the activity by a further 15%. After PVPP treatment and gel filtration over Sephadex G25 (PD-10 columns), similar total protein concentrations were found in extracts obtained from elicited and untreated cells. Using this procedure, FPP synthase was also efficiently extracted.

A divalent metal ion such as Mn^{2+} or Mg^{2+} as cofactor is required for IPP isomerase activity (Poulter and Rilling 1981). The highest activity was observed when an extract of elicited *C. robusta* cells was incubated in the presence of 1.5 mM of both Mg^{2+} and Mn^{2+} (data not shown). Addition of DTT (2 mM) and KF (25 mM, an inhibitor of acidic phosphatases) had a stimulatory effect on the formation of allylic diphosphates; only 48% and 70% of the activity was recovered when DTT or KF, respectively, were omitted from the incubation buffer. For KF, addition of 25 mM was the optimum, lower and higher (tested up to 100 mM) KF concentrations resulted in lower product yields (data not shown).

For IPP isomerase assays, incubation times of 10 min were used to obtain a constant rate of product formation during incubations in the presence of 5–30 μg protein.

No appreciable difference in activity was observed when the incubation buffer for the FPP synthase assay contained 1.5 mM of Mn^{2+} (100%) or 1.5 mM of both Mg^{2+} and Mn^{2+} (97%). However, with only 1.5 mM of Mg^{2+} in the buffer, 70% of the activity was obtained.

Effects of elicitation on growth, anthraquinone accumulation, IPP isomerase and FPP synthase activities. The capacity of the elicitor to induce anthraquinone biosynthesis was studied in relation to the growth cycle of the culture. *Pcin* was added to cultures immediately after inoculation (t = 0 d) and to cultures which had been growing for 2, 4, 6 and 8 d. When the elicitor was added at t = 0 d, the final biomass yield was strongly reduced, as compared to untreated cells, and the accumulation of anthraquinones only started after more than 2 d after elicitation (data not shown). Elicitation of a 4-d-old culture resulted in a rapid and strong induction of anthraquinone biosynthesis; growth was fully suppressed (Fig. 1A, 1B). The maximum level of anthraquinone accumulation in the cells [about 12 μmol · (g DW)$^{-1}$] was reached within 72 h. In this culture less than 2% of the total anthraquinone content was found in the medium. Of the total anthraquinone content 45–50% was recovered as aglycones. Addition of *Pcin* to 6- or 8-d-old cultures did not affect biomass accumulation, but the accumulation of anthraquinones was slower and less when compared with elicitation of 4-d-old cultures (data not shown). Phytochemical analysis of cells, harvested 72 h after elicitation, revealed that the major anthraquinone-aglucon present was 1,4,7-trihydroxy-6,8-dimethoxy-2-methylanthraquinone (Ramos-Valdivia 1996).

Isopentenyl diphosphate isomerase was assayed in *C. robusta* cultures, treated on day 4 of the growth cycle with *Pcin* (Fig. 1C). Activity of IPP isomerase showed a tri-phasic response to elicitation. During phase I there was an induction of enzyme activity. At 12 h after elicitation, the specific IPP isomerase activity in elicitor-treated cells was between 2 and 8 times higher than in untreated cells. In phase II, IPP isomerase activity decreased to lower levels than observed in the untreated cells. During phase III, IPP isomerase activity in elicited cells started to rise again, reaching the level in untreated cells at about 50 h after elicitation. The levels in the elicited cells continued to increase, while in untreated cells the levels decreased. The IPP isomerase was also

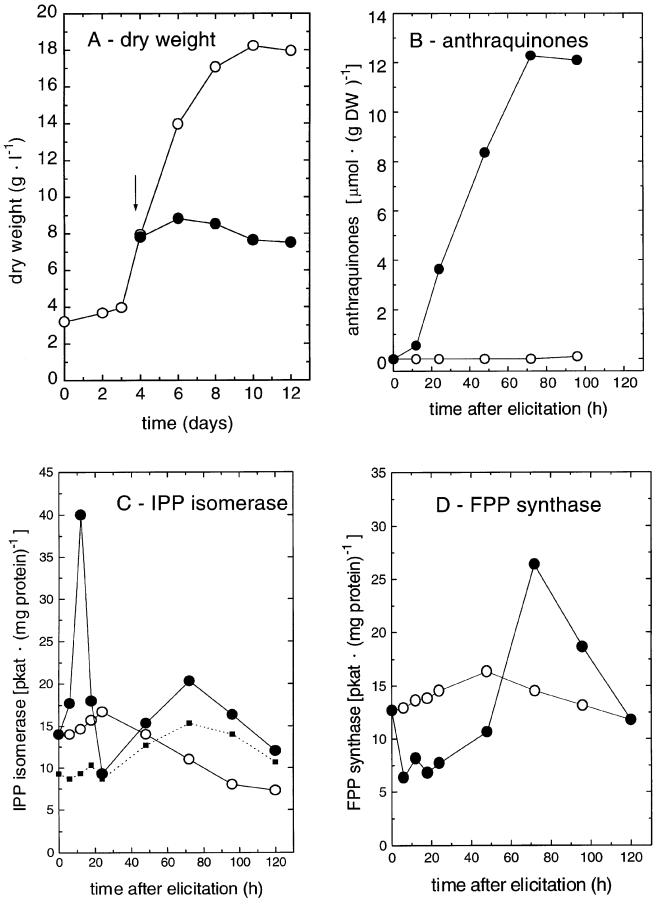

Fig. 1A–D. Accumulation of biomass (**A**), totals of anthraquinones in biomass and medium (**B**), and time courses of IPP isomerase (**C**) and FPP synthase (**D**) activities in a *Cinchona robusta* suspension culture after addition of an elicitor preparation of *Phytophthora cinnamomi*. The elicitor was added to 4-d-old-cultures (indicated by an *arrow* in **A**). *Open symbols*, untreated cells; *closed symbols*, elicited cells. *Dotted line* (**C**): IPP isomerase activity assayed in the presence of Mg^{2+} (1.5 mM, Mn^{2+} was omitted)

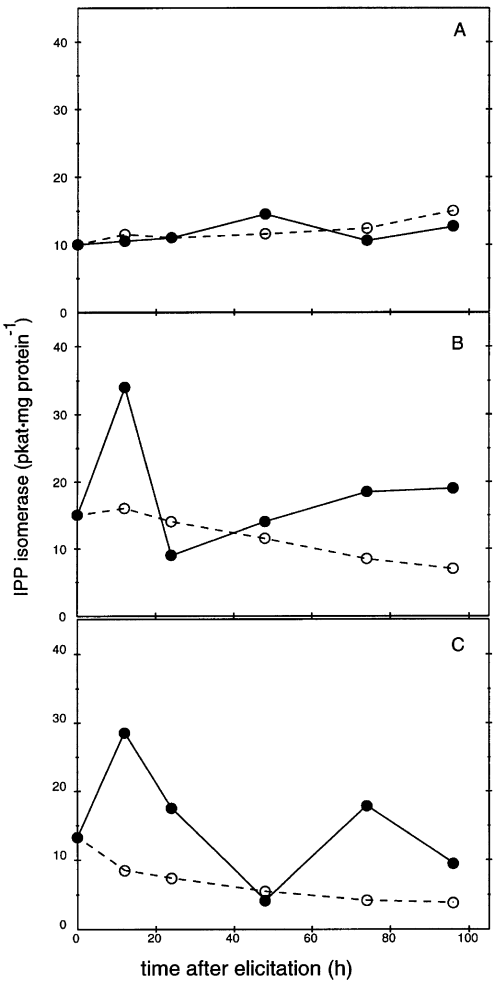

Fig. 2A–C. Time course of IPP isomerase activity in a *Cinchona robusta* suspension culture after addition of an elicitor preparation of *Phytophthora cinnamomi*. The elicitor was added to 0-(**A**), 6-(**B**) and 8-d-old cultures (**C**; *open symbols*, untreated cells, *closed symbols*, elicited cells). For IPP isomerase activity after addition of the elicitor at day 4, see Fig. 1C

assayed in *C. robusta* cultures, treated on day 0, 6 and 8 with *Pcin*. The tri-phasic response was most pronounced in cultures elicited at days 4 and 6 of the growth cycle (Fig. 2). As elicitation of 4-d-old cultures also resulted in a strong induction of anthraquinone biosynthesis, this experimental set-up was selected for further studies on DMAPP metabolism.

It is noteworthy that the increase in activity measured during phase I was small when the incubations were performed using only Mg^{2+} (i.e. omitting Mn^{2+}) as cofactor (Fig. 1C), while little difference in the co-factor requirement was observed using enzymes extracted from cells in phase III.

After elicitation of a 4-day-old culture of *C. robusta*, FPP synthase activity was rapidly suppressed (Fig. 1D), in contrast to IPP isomerase induction during phase I. Within the first 24 h, the levels remained 50% lower than in untreated cells. Afterwards, FPP synthase activity increased, reaching a maximum at about 72 h. In untreated cells, FPP synthase activity remained rather constant during the growth cycle.

The specific IPP isomerase inhibitor NPP and the thiol-selective inhibitor iodoacetamide were used to confirm that the elevated recoveries of radioactivity in the IPP isomerase assays of elicited cell extracts (phase I) were directly linked to this enzyme activity. Both compounds inhibited the in-vitro IPP isomerisation completely (Table 1). In contrast, FPP synthase activity was not affected by NPP, while iodoacetamide inhibited FPP synthase by 66% and 35% in untreated and elicited cell extracts, respectively.

Biosynthetic capacity of C. robusta protein extracts to form allylic diphosphates from [1-^{14}C]IPP and both [1-^{14}C]IPP and DMAPP. Twelve hours after *Pcin* treatment, the biosynthetic capacity of the cells to form allylic C_5 and C_{15}-units are drastically changed due to the induction of IPP isomerase and the inhibition of FPP synthase. To study the consequences of these changes on the level of product formation, cell extracts were incubated with [1-^{14}C]IPP and both [1-^{14}C]IPP and

Table 1. Effect of NPP and iodoacetamide on the activity of IPP isomerase and FPP synthase [in pkat · (mg protein)$^{-1}$] from elicitor-treated *C. robusta* cells. The elicitor was added to 4-d-old cultures and cells were harvested 12 h after elicitation (mean ± SD, $n = 3$)

Inhibitor	IPP isomerase		FPP synthase	
	Untreated	Elicited	Untreated	Elicited
None	14.4 ± 0.6	38.6 ± 0.7	14.6 ± 0.5	9.9 ± 0.3
NPP (25 μM)	0.3 ± 0.1	0.3 ± 0.1	13.7 ± 0.3	10.7 ± 0.3
Iodoacetamide (10 mM)	0.2 ± 0.1	0.2 ± 0.1	4.9 ± 0.2	6.4 ± 0.2

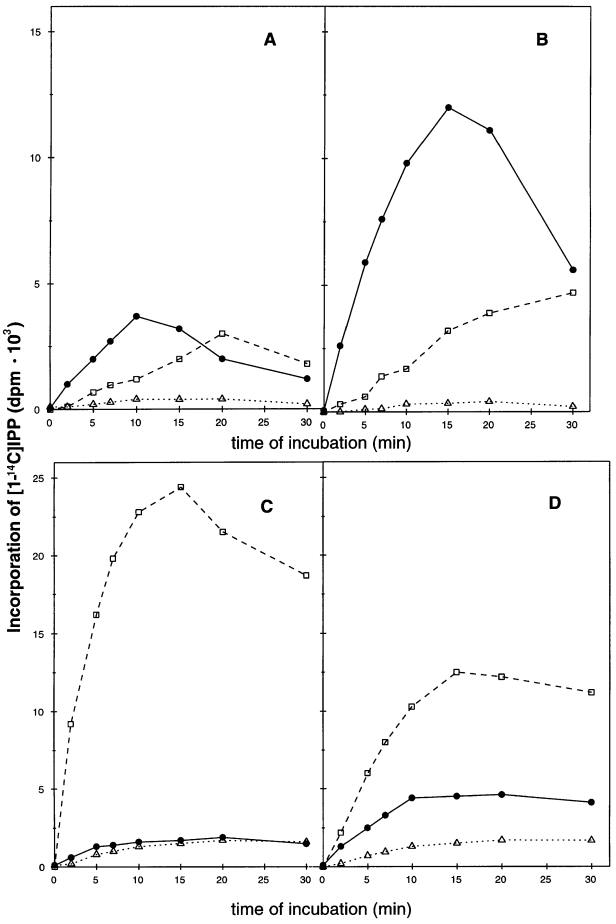

Fig. 3A–D. Product formation in incubation mixtures containing protein extracts of *Cinchona robusta* cells (165 μg protein from untreated cells, 170 μg protein from elicited cells), harvested 12 h after elicitation, and 18 μM [1-^{14}C]IPP and/or 100 μM DMAPP. After hydrolysis by alkaline phosphatases, the products were separated by HPLC. ●–● dimethylallyl alcohol; □–□ farnesol; △–△ geraniol. **A** Extract from untreated cells incubated with [1-^{14}C]IPP; **B** extract from elicited cells incubated with [1-^{14}C]IPP; **C** extract from untreated cells incubated with [1-^{14}C]IPP and DMAPP; **D** extract from elicited cells incubated with [1-^{14}C]IPP and DMAPP

DMAPP. Subsequently, product formation was determined by HPLC analysis after hydrolysis of the diphosphates.

The analysis of the in-vitro biosynthetic capacity of extracts from untreated *C. robusta* cells, incubated with IPP, revealed that allylic diphosphates of different chain lengths were formed: DMAPP (C_5), GPP (C_{10}) and FPP (C_{15}). A fourth product, with a retention time of 5.2 min in the HPLC system, was identified as presqualene.

After elicitation, the capacity of the extracts to produce DMAPP was markedly increased. Within 15 min of incubation, maximum DMAPP concentrations were reached (Fig. 3A,B). As a consequence of the increased availability of DMAPP, the formation of FPP was also increased in these incubations. During the incubation of an extract of untreated cells with both IPP and DMAPP, FPP synthase activity was predominant, reaching maximum FPP concentrations within 15 min of incubation (Fig. 3C). Incubating extracts from elicited cells with IPP and DMAPP, suppressed FPP formation by about 50% (Fig. 3D) as compared to untreated cells. The contribution of both GPP and presqualene to the total product formation was always less than 3%, highest concentrations were found using extracts of untreated cells incubated in the presence of IPP and DMAPP.

Induction of IPP isomerase activity requires de-novo protein biosynthesis. The increased activity of IPP isomerase may result from an increased catalytic efficiency or from de-novo synthesis of the enzyme. The induction of IPP isomerase activity, as observed in elicitor-treated cells, was inhibited almost completely when cells where growing in presence of the protein synthesis inhibitors cycloheximide or actinomycin D (Table 2). These inhibitors also prevented anthraquinone accumulation in elicited cells. Synthase activity of FPP was slightly inhibited in untreated cells after addition of cycloheximide and actinomycin D.

Discussion

Many plant cell cultures are able to synthesise and accumulate phytoalexins in response to microbial infection or treatment with the appropriate elicitor (Whitehead and Threlfall 1992). Cell cultures of *C. robusta* respond to *Pcin* treatment with a rapid accumulation of anthraquinone-phytoalexins. The elicitor concentration added was rather critical, the ratio between lethal and optimum dose was 2.5. Addition of 0.8 mg *Pcin* per ml of a 4-d-old *C. robusta* culture, resulted in a rapid (within 3–4 h) colour change of the culture to dark-yellow/orange, caused by the accumulation of highly oxygenated anthraquinones (Ramos-Valdivia 1996). In the early growth phase (4-d-old cells) there may be relatively large pools of precursors available as the cells are metabolically active and nutrient limitations would not be expected. This may allow a more rapid accumulation of phytoalexins than occurs when *Pcin* is added to 0-, 6- and 8-d-old cultures. In the mechanisms enabling

Table 2. Effects of cycloheximide and actinomycin D on IPP isomerase and FPP synthase activities [pkat · (mg protein)$^{-1}$, assayed 12 h after elicitation] and anthraquinone accumulation [μmol (g Dw)$^{-1}$, analysed 24 and 72 h after elicitation] in *Pcin*-treated *C. robusta* cells (mean ± SD, $n = 3$)

Treatment	IPP isomerase		FPP synthase		Anthraquinones		
	Untreated	Elicited	Untreated	Elicited	Untreated	Elicited	
						24 h	72 h
None	14.6 ± 0.6	33.2 ± 0.6	13.5 ± 0.5	9.5 ± 0.7	0	3.8	12.4
Cycloheximide	16.1 ± 0.9	16.6 ± 0.3	10.3 ± 0.8	8.2 ± 1.0	0	0	0
Actinomycin D	15.4 ± 0.9	16.2 ± 0.6	9.8 ± 0.5	8.7 ± 0.4	0	0	0.3

this accumulation of anthraquinones, DMAPP was expected to be an important contributor, because it is directly involved in the biosynthesis of anthraquinones and sterols. Little is known of the prenyltransferase involved in anthraquinone biosynthesis: it was suggested to prenylate 1,4-dihydroxy-naphthoic acid, a precursor derived from the shikimate pathway (Leistner 1985). Experiments in which the protein synthesis inhibitors cycloheximide and actinomycin D were added to the *C. robusta* cells, suggested that the induction of IPP isomerase activity and the induction of anthraquinone biosynthesis were a result of de-novo protein synthesis. Thus, IPP isomerase may satisfy the urgent demand for DMAPP for the biosynthesis of anthraquinone phytoalexins in response to the pathogen. The cause for the subsequent decrease in IPP isomerase activity and the inhibition of FPP synthase after elicitation is not clear. The increased activities of IPP isomerase and FPP synthase after 50 h may indicate some recovery effect, during which depleted pools of precursors are restored.

Before the enzyme activities could be determined, assay conditions were optimised. Complications due to the presence of anthraquinones (Heide and Leistner 1983) were overcome by addition of PVPP, a polymer often added to bind plant phenolics (Loomis 1974). The strongest activation of IPP isomerase was found in the presence of both Mn^{2+} and Mg^{2+}, as was also reported for plastidial IPP isomerase from tomato fruit (Spurgeon et al. 1984). During phases I and III, IPP isomerase showed differences in its requirement for Mn^{2+}, which may indicate the presence of isoforms or isoenzymes. Product formation in the IPP isomerase assay was further stimulated by inhibition of acidic phosphatases, which compete for the substrate IPP, by addition of KF. For IPP isomerase assays, short incubation times were applied, as linearity in product formation rates was rapidly lost, probably due to product inhibition and enzyme instability.

As the ratio of IPP isomerase and FPP synthase varies continuously in elicited *C. robusta* cells, product formation in cell-free incubations was analyzed. In elicited cells, IPP isomerase activity is induced resulting in an increased availability of DMAPP. This effect is enhanced by the inhibition of FPP synthase, i.e. less DMAPP is incorporated in FPP.

Since yeast IPP isomerase is inhibited by the sulphydryl alkylating agent iodoacetamide, and the presence of a thiol group in the catalytic site of IPP isomerase has been demonstrated (Reardon and Abeles 1986), it was suggested that this thiol group is the isopentene-binding domain of the enzyme (Street and Poulter 1990). N,N-(Dimethylamino)ethyl diphosphate (NPP), an ammonium analogue of the tertiary carbacationic intermediate proposed for the interconversion of IPP and DMAPP, has been shown to be a specific inhibitor of IPP isomerases from yeast (Reardon and Abeles 1986) and *Claviceps* (Muehlbacher and Poulter 1988). Also the IPP isomerase activity in the *C. robusta* cultures was inhibited by iodoacetamide and NPP, suggesting a similar reaction mechanism. The biochemistry and function of IPP isomerases have been reviewed recently (Ramos-Valdivia 1996; Ramos-Valdivia et al 1997). The activity of FPP synthase, which is suppressed in elicited cells, was less sensitive to inhibition by iodoacetamide.

To conclude, the *Pcin*-mediated accumulation of anthraquinones in *C. robusta* cells was preceded by a transient induction of IPP isomerase activity and a simultaneous decline in FPP synthase activity. These coordinated effects, resulting in an increased availability of DMAPP, enable an efficient accumulation of anthraquinone phytoalexins by channeling of C_5-units into this pathway. Similar processes have been described for cultures of other plant species. In elicited tobacco cells accumulating sesquiterpenoid phytoalexins, regulation takes places at the C_{15} level (Threlfall and Whitehead 1988; Vögeli and Chapell 1988), and in elicited *Tabernaemontana divaricata* cultures accumulating triterpenoid phytoalexins, regulation at the C_{30} level is found (van der Heijden et al. 1989; Fulton et al. 1994). The next interesting question to answer is how this channeling of DMAPP in *C. robusta* is regulated at the subcellular level, i.e. what is the role of compartmentation in the regulation of terpenoid biosynthesis. Further studies on this and the possibilities of the occurrence of isoforms in connection with anthraquinone formation in the *C. robusta* – *Pcin* system are in progress.

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