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Geranylgeranyl diphosphate synthase activity and taxane production in *Taxus baccata* cells

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Abstract

A *Taxus baccata* cell line has been characterized for growth, nutrient uptake, taxane production and for GGPP synthase activity. The enzyme is extracted by inclusion of non-ionic detergent and its activity parallels taxane production for the first three days. The enzyme activity reaches its peak at day 3 of culture growth, while total taxane formation rises until day 9, when the maximum taxane accumulation in the cells is observed (~0.004% dry wt). After this day the taxane excretion in the medium increases, reaching its maximum at day 19.

Keywords: Taxus baccata; Taxaceae; European yew; Biosynthesis; Taxanes; Geranylgeranyl diphosphate synthase

1. Introduction

Paclitaxel is a diterpene found in various tissues of *Taxus* species. It is widely used as an anticancer drug for breast and ovarian cancer, alone or in combination with other anticancer agents and probably it will gain approval for more types of malignancies (Slichenmyer & Von Hoff, 1991; Suffness, 1995). It is mainly extracted from the bark of the North-American species *Taxus brevifolia* and because of the low yield (~0.01% of dry wt of bark), the complexity of the paclitaxel molecule, the destructive method of extraction and the sparsity of the tree, doubts have been risen about the availability of the drug. So, the interest has been focused on advanced naturally occurring precursors of the drug that can be used for its semi-synthesis. In this sense 10-deacetyl-baccatin-III or baccatin-III is used as the core component for semi-synthesis since it is isolated from the renewable foliage of *Taxus baccata* in yields higher than that of paclitaxel (0.1% dry wt) (Denis et al., 1988) and its conversion to paclitaxel is straightforward and commercially viable. Another alternative solution to the 'supply crisis' of paclitaxel is the use of cell cultures which are a valuable tool for the production of taxanes and material of choice for biosynthetic studies.

The efficiency of paclitaxel and the urgency of the supply problem has prompted scientists to gain insight in the pathway flux towards taxane formation and to use this knowledge in order to increase the yield of useful taxanes by means of metabolic engineering. The three first steps of paclitaxel biosynthesis are now known: cyclization of GGPP (geranylgeranyl diphosphate) to taxa-4(5),11(12)-diene (Koepp et al., 1995), hydroxylation to taxa-4(20),11(12)-dien-5α-ol (Hefner et al., 1996) and the conversion of this alcohol to the corresponding acetate ester (Hezari & Croteau, 1997). Taxadiene synthase, the enzyme catalyzing the cyclization of GGPP, has been purified (Hezari, Lewis, & Croteau, 1995), its mechanism of action elucidated (Lin, Hezari, Koepp, Floss, & Croteau, 1996) and the corresponding cDNA cloned (Wildung & Croteau, 1996). Although the low level of taxadiene synthase activity in *Taxus brevifolia* stem disks suggests a crucial role for the regulation of the pathway, this could not be confirmed by using *T. canadensis* cell cultures (Hezari, Ketchum, Gibson, & Croteau, 1997).

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[3] Abbreviations used: NAA, naphthalene acetic acid; IPP, isopentenyl n *p*O*p* (isopentenyl diphosphate); DMAPP, dimethylallyl n *p*O*p* (dimethylallyl diphosphate); GPP, geranyl n *p*O*p* (geranyl diphosphate) FPP, farnesyl n *p*O*p* (farnesyl diphosphate); GGPP, geranylgeranyl n *p*O*p* (geranylgeranyl diphosphate).

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In our laboratory we have undertaken an effort to elucidate the early stages of the biosynthesis of taxanes by using cell suspension cultures of *Taxus* species. In this communication we report on the role of GGPP synthase (EC 2.5.1.29) in the formation of the taxane ring in *T. baccata* cell cultures grown in B5 medium.

2. Results and discussion

2.1. Cell growth

The time course of the fr. wt accumulation showed a protracted lag phase of almost 7 days and an almost linear period of growth between days 7 and 19. After the culture reached its highest fr. wt at day 19, the weight started to decline slowly and a deterioration of the quality of the culture occurred. The dry wt accumulation was essentially linear, reaching a peak at day 16 and then it decreased steadily. Although the culture hardly achieves doubling time it was preferred over other cultures because it was stable and not prone to phenolization, a common phenomenon which leads to deterioration and/or death, severely altering the characteristics of the cells. Over the 20 days the culture had an average specific growth rate of 0.20 g fr. wt/day. Even if the fr. wt hardly doubles, the specific growth rate is comparable with literature data (Shrinivasan et al., 1995; Moon, Yoo, Kim, & Byun, 1998).

The nutrient uptake analysis comprised the determination of sucrose, glucose and fructose (Fig. 1A), ammonium, nitrate and phosphate (Fig. 1B) in the medium. The limiting growth factor is most likely the carbon source. The concentration of sucrose in the medium is low compared to other *Taxus* cultures (Shrinivasan et al., 1995; Moon et al., 1998). The analysis of residual carbohydrates showed that sucrose was rapidly hydrolyzed by the cells into glucose and fructose. All the glucose was used before the cells take up fructose which is clearly demonstrated by the fact that fructose reached a maximum concentration of 56 mM just after all the sucrose and glucose had disappeared from the medium. After this point fructose was rapidly absorbed by the cells and at day 15 nearly all the fructose was used. This resulted in a slowly decreasing dry wt as the cells started to use the stored starch. Fr. wt increased until day 19. After this stage some cells died and ammonium and phosphate reappeared in the medium as a result of degradation processes. All the ammonium was taken up in three days. Phosphate was rapidly consumed until day 1. After this a plateau was reached until the cells started to grow after day 6 and subsequently phosphate concentration decreased until day 12 when all was consumed. Concerning the uptake of nitrate, at day 3 a minimum was reached after which the nitrate concentration increased until day 18 and then it gradually decreased. A possible explanation is that at day 0 the cells were transferred from a depleted medium with lower osmotic strength to fresh medium with higher ionic strength. Evidence that the cells experience a difference in osmotic conditions is the fact that the fresh weight at day 0 of 100 g/l decreases to 85 g/l at day 1. By giving away water and taking up ions the cells cope with the new environment of high osmotic strength. From day 0 to day 1 mainly phosphate and ammonium were taken up and as these ions were depleted nitrate was consumed as well (Fig. 1B). The decrease in nitrate concentration after day 18 might be due to the depletion of ammonium in the medium and in the cells, nitrate becoming the sole source of nitrogen.

2.2. GGPP synthase activity

GGPP synthase occupies an important branch-position in the diterpene biosynthetic pathway. It brings about the condensation of IPP and FPP, forming the universal precursor of diterpenes geranylgeranyl diphosphate (GGPP). This enzyme may also catalyze transferase reactions with DMAPP or GPP as the allylic donor. Additional pathways compete for its product: the formation of carotenoids, of the phytyl moiety of chlorophylls and of the side chains of quinones and tocopherols, all require the presence of GGPP as does the biosynthesis of diterpenes. Since we have embarked on an attempt to elucidate the early stages of carbon flux towards the taxane skeleton formation and to spot possible regulatory points, the key position of this enzyme prompted us to study the relation between its activity and the production of taxanes, the main diterpenes of *Taxus*.

Modifications to the existing protocol (Threlfall & Whitehead, 1992) were necessary for GGPP synthase extraction and activity measurement. The first difference was encountered in the extraction procedure: following the extraction and centrifugation of the cell material IPP isomerase and GGPP synthase activities were both found mainly in the 20000*g* pellet. Inclusion of non-ionic (Brij-35, Triton X-100) or amphoteric (CHAPSO) detergent was essential for the complete extraction of the enzyme. The enzyme could not be recovered from the pellet by using Na cholate, deoxycholate, octyl glucoside, octyl thioglucoside, treatment with 1 M NaCl, 1 M $MnCl_2$ or with chaotropic reagents like NaI, NaSCN or $NaClO_4$. Interestingly, treatment of the pellet with NaCl lead to the solubilization of a part of IPP isomerase activity. By comparison, no inclusion of detergent was necessary in order to extract the enzyme from cultures of *Taxus canadensis* (results not shown). It is the second case, the first being cells of *Ginkgo billoba* L. (Carrier, Archambault, van der Heijden, & Verpoorte, 1996), that addition of detergent is essential in order to extract GGPP synthase. Here we have to mention that during subsequent puri-

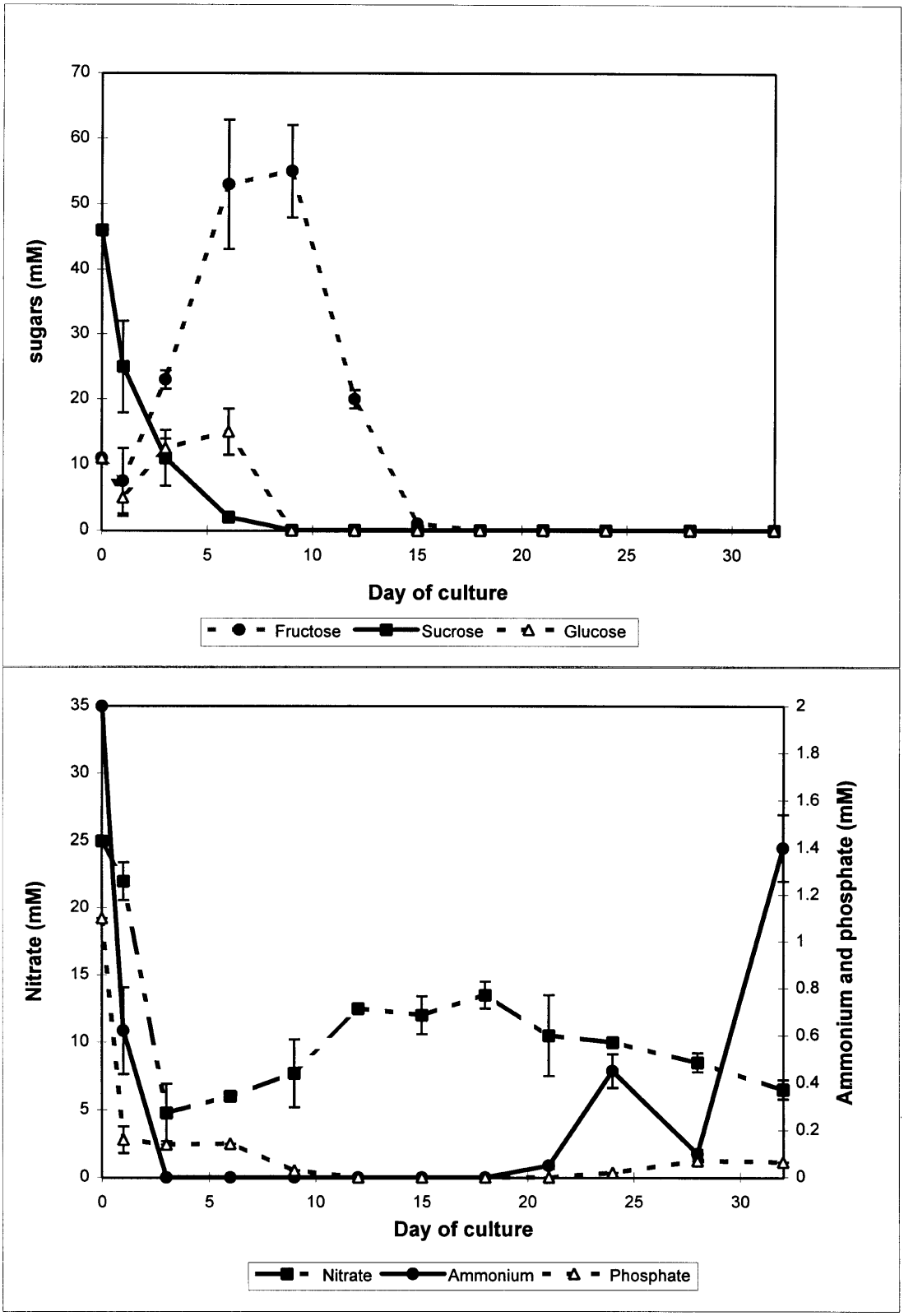

Fig. 1. Nutrient uptake of *T. baccata* cell cultures. (A) Carbohydrate concentration in the medium and (B) nitrate, ammonium and phosphate concentration in the medium.

fication steps the activity is maintained even after removal of detergent.

The second adaptation to the existing protocol is related to the assay procedure: unlike *T. canadensis* (results not shown) the ordinary assay (Threlfall & Whitehead, 1992) failed to perform, probably due to high IPP isomerase activity levels. The standard assay makes use of two incubations, one with inclusion only of [1-^{14}C]-IPP (blank) as a measure of IPP isomerase activity and the second with [1-^{14}C]-IPP and FPP and subsequent subtraction of the former from the latter. In our case, this assay performed well only when GGPP synthase activity was relatively high. In order to obtain a product profile of the incubation, we precipitated radioactive prenyl diphosphates by the method described by Reuter, Knoss, Hemmerlin, and Bach, 1997. In short, this method is based on the precipitation of prenyl diphosphates after addition of cold acetone in the presence of Mn^{2+} and Mg^{2+} and then TLC analysis on silica gel plates. In our case it could not be developed into a reliable system for quantitative assessment of enzyme activity but it afforded a valuable insight on the identity of the products. Spots were identified by using authentic markers and radio-TLC plates were visualized by using a betascope (Betascope 603, Betagen). With this method (results not shown) we concluded that while incubation with [1-^{14}C]-IPP (blank) affords FPP, GGPP and probably GPP, incubation with [1-^{14}C]-IPP and FPP (full assay) results in formation of GGPP exclusively. Since the blank reaction does not take place in the full assay, the usual assay used for GGPP synthase (Threlfall & Whitehead, 1992) underestimates GGPP synthase activity and is not applicable in our case. In order to overcome this discrepancy, we inhibited IPP isomerase by preincubating the protein extract with iodoacetamide (Shinka, Ogura, & Seto, 1974; Ogura, Nishino, Shinka, & Seto, 1985). The effectiveness of this treatment was assessed as before by precipitating the radioactive prenyl diphosphates (results not shown): no allylic diphosphate is produced after incubation with [1-^{14}C]-IPP, since IPP isomerase is inhibited by iodoacetamide, while when FPP is added in the incubation mixture, ^{14}C-GGPP is formed. The assay was checked for linearity over time between 0 and 90 min and over concentration of the substrates between 1–10 μM of IPP and 4–120 μM of FPP. No attempt for accurate K_m valuation was made, since we were dealing with a crude extract, but it seems that K_m for IPP is about 0.5 μM, while for FPP about 5 μM. The same results were obtained when instead of the non-selective iodoacetamide, 15 μm of 2-dimethylamino-1-ethyl diphosphate (NPP), a specific IPP isomerase inhibitor (Sagami, Morita, & Ogura, 1994), was used. The addition of inhibitor was omitted after chromatographic removal of IPP isomerase during subsequent purification steps.

A final comment is reserved for the optimum conditions of the assay: unlike the high pH values recommended by some authors, probably reflecting the proton concentration of the plastidial stroma pH, values higher than 8 dramatically decrease GGPP synthase activity of *T. baccata*. Furthermore, divalent cations are typically required as cofactors. A combination of 4 mM of Mn^{2+} with 2 mM of Mg^{2+} appears to perform best. A reducing environment (2 mM DTT) and a high concentration of glycerol (20%), are important factors for optimum activity. Potassium fluoride is included in the assay mixture in order to inhibit the action of phosphatases.

2.3. GGPP synthase activity and taxane accumulation

Fig. 2(A) shows the GGPP synthase activity and the taxane accumulation during the growth of the culture. Enzyme activity increased from day 1, reached a maximum at day 3 (27.2 μkat/kg of protein) and thereafter declined steadily. Little data was found in order to correlate the enzyme activity with growth of cell cultures of other plants, so we can not put this value into perspective. It is almost 300 to 1000 times higher than the activity of cell cultures of *Ginkgo biloba* (Carrier et al., 1996) and little higher than germinating seedlings from *Cucurbita pepo*, where the activity reached 7.4 μkat/kg at day 3 (day of maximum activity) (Ogura et al., 1985).

With regard to the determination of taxanes we selected monoclonal antibodies specific for the taxane ring in order to have a full measure of the amount of the compounds bearing a complete taxane skeleton. The initial formation of total taxanes (cells + medium) is reflected in the activity of the enzyme and until day 3 taxane production parallels the enzyme activity. The enzyme activity increased until day 3 and thereafter it started to decline, but since GGPP molecules kept on accumulating the taxanes continued to form until day 9. After day 9 no net accumulation of taxanes takes place and from this point onward, they started to excrete in the medium (Fig. 2B). Although the concentration in the medium rose steadily till day 19, there was a net loss of total taxanes. In this sense, if results are expressed as summed taxanes in cells and medium, there is an inverse relationship between growth and taxane production (Fett-Neto, Zhang, & DiCosmo, 1994). The opposite stands if results are expressed in taxanes in the medium. The taxane production profile in cells closely resembles the baccatin-III profile reported for *T. baccata* cultures by Moon et al., while the taxane production in medium looks like the paclitaxel profile reported by the same authors (Moon et al., 1998).

We should mention that our results seemingly are not in accordance with those recently reported by Hezari et al., who traced taxadiene synthase activity in cultures of *T. canadensis* (Hezari et al., 1997): if taxanes start to accumulate from the onset of the growth cycle (as our study indicates), then the same holds for the activity

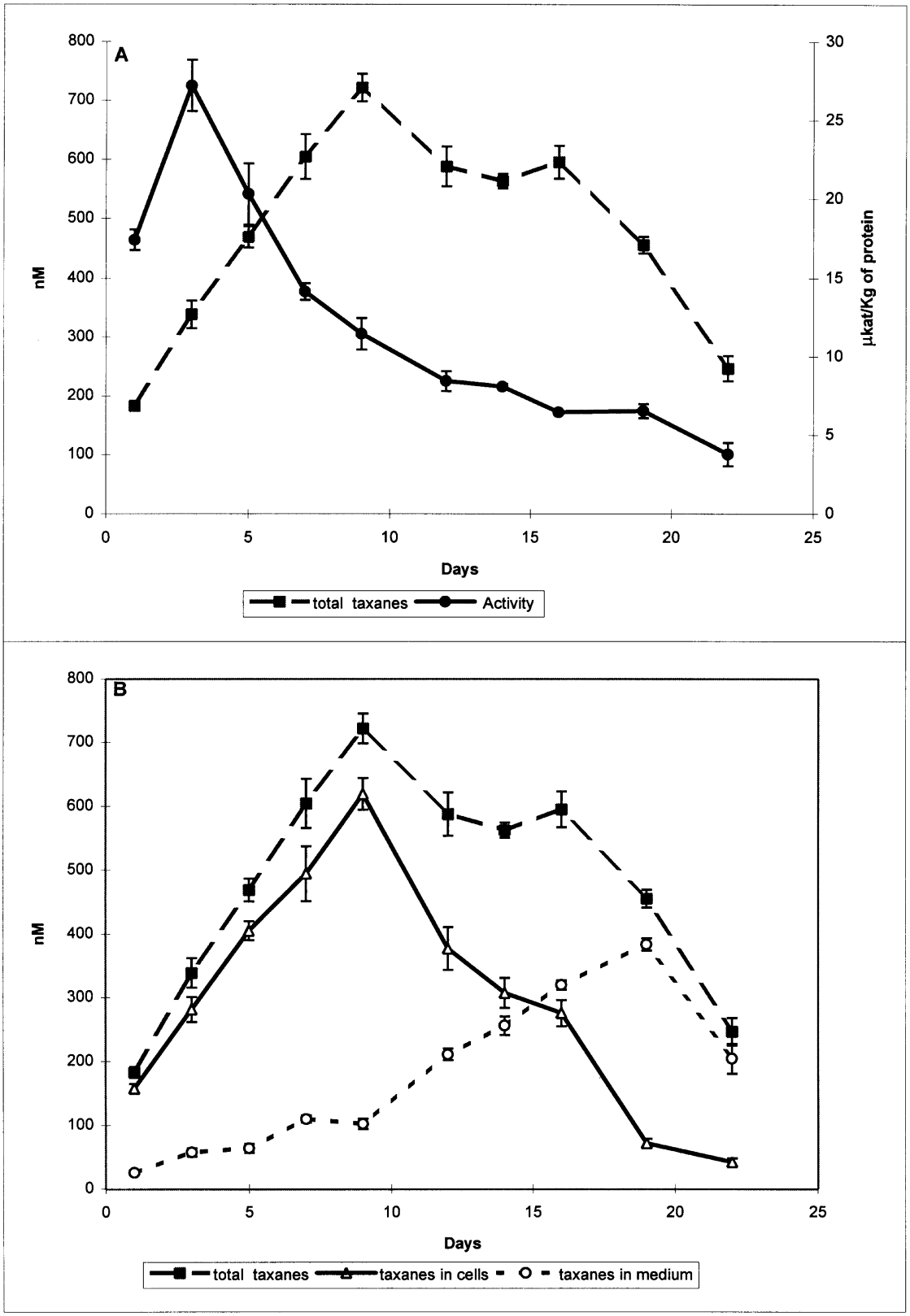

Fig. 2. (a) GGPP synthase activity and total taxane accumulation during growth cycle of *T. baccata* cultures. (B) Taxane accumulation in the cells, in the medium and in cells + medium (total taxanes).

of taxadiene synthase, otherwise taxanes simply cannot form. Oddly, the activity of this enzyme picks up after day 7 (Hezari et al., 1997). Several reasons can account for this disparity, the difference of the species being just one of them. This is probably due to the differences in the subculturing methods and in the culturing conditions: our cultures are maintained under constant light, while the *T. canadensis* cells are kept in the dark. Additionally in our case *T. baccata* cells have different growth characteristics than the *T. canadensis* ones, the latter maintaining steady growth for 35 days while the former just reaching doubling time in less than three weeks before starting to deteriorate, implying a different metabolic profile. Furthermore the way of subculturing differs: while we subculture after the cells have reached their stationary phase and GGPP synthase and taxane production are hardly noticeable, Hezari et al. subculture when taxadiene synthase and paclitaxel in the medium are at their peak. The analytical methods and targets are different since we concentrate on occurrence of total taxanes determined by use of immunoassay. Interestingly the time period when most of the taxanes accumulate in the medium of *T. baccata* (days 9–16) coincides with the period when taxadiene synthase activity picks up in *T. canadensis*.

Our results show a correlation between the enzyme's activity and the formation of the central ring of taxanes (at least till the formation of the oxetane ring), suggesting a possible regulatory role for GGPP synthase. Furthermore there are indications suggesting that the enzyme is membrane associated. These findings call for the isolation of the enzyme, that is currently under way in our laboratory, in order to clone the corresponding gene and to proceed to localization studies. Always having in mind the limitations of extrapolating in vitro observations to in vivo reality, the potential of an increased GGPP synthase activity looks extensive. To our view, the possibility of existing regulatory steps earlier than GGPP synthase has to be taken into consideration.

Although the elucidation of the biosynthetic pathway leading to taxanes has started to develop lately, significant insights have already been gained. This approach provides the means of developing reliable strategies to increase paclitaxel yield in a sustainable and straightforward way.

3. Experimental

3.1. Materials[3]

[1-^{14}C] Isopentenyl diphosphate (IPP) with a specific activity of 1.96 Gbq mmol^{-1} was purchased from Amersham. Non-labelled IPP, dimethylallyl diphosphate (DMAPP) and farnesyl diphosphate (FPP) were synthesized according to Davisson et al. (1986) and GGPP was a generous gift from Dr. Werner Knoess (Institute of Pharmaceutical Biology, University of Bonn). Leupeptin hemisulfate was from ICN, BSA (fraction V) and PVPP from Sigma. *N*,*N*-(Dimethyl amino)-ethyl diphosphate (NPP) was a gift from Professor C.D. Poulter (Utah University, Salt Lake City, UT). PD-10 columns were purchased from Pharmacia. Precoated silica gel plates (Silica gel 60 F_{254}, Merck) were used. The TAO4 anti-taxane monoclonal antibody kit was from Hawaii Biotechnology Group. All the other chemicals were of the highest purity commercially available.

3.2. Plant material and culture conditions

Cell suspension line TBC was established in a B5M11 medium (B5 salts supplemented with 100 mg/l of *meso*-inositol, 10 mg/l thiamine dihydrochloride, 1 mg/l pyridoxine hydrochloride, 1.86 mg/l NAA and 2% sucrose) from leaves-derived callus grown in the same medium. For callus cultures 1% of agar was added to obtain solid medium. The explants were derived from the *T. baccata* 621 tree from Pinetum Blijdestein in Hilversum, in June 1995. The code refers to the herbarium specimen. Cultures were grown under continuous light of 1000 lx (Philips TL 40W/33 RS), on a gyratory shaker at 110 rpm and 25°C. All experiments were conducted with 50 ml cultures kept in 250 ml Erlenmeyer flasks, except nutrient uptake experiments that were conducted with 500 ml suspension cultures kept in 2 l Erlenmeyer flasks. The culturing cycle lasts three weeks: normally the culture medium is refreshed the second week and the cells are subcultured every third week by division of the cell mass in two parts, after decantation of the medium. In our study 30 flasks with 50 ml of culture which were at the end of the regular subculturing period, were mixed in an Erlenmeyer flask and filtered over a sintered glass filter. Afterwards 30 new flasks containing 50 ml of B5M11 medium were inoculated with 5 g of these cells and were cultured without medium refreshment for 3 weeks, after which the biomass was collected again and 30 new 50 ml cultures were initiated in the same way. The flasks were closed with cotton stoppers. Every two days, 3 flasks were harvested except for the last two sampling points and the one at day 12 which were harvested at an interval of 3 days. Collection started one day after inoculation: the content of each flask was filtered over a sintered glass filter using vacuum and the cells were weighed. Samples of approximately 2 g were freeze-dried to measure the dry weight and for taxane determination and the rest of the biomass was frozen in liquid nitrogen and stored at −80°C, until required for GGPP synthase activity measurement.

3.3. Taxane content determination

A competitive inhibition immunoassay system (CIEIA) comprising of antitaxane skeleton monoclonal antibodies TAO4, baccatin-III-protein solid phase coating antigen and a baccatin-III standard was used. The TAO4 monoclonal antibodies can equally recognize all compounds bearing a complete taxane ring (Grothaus et al., 1995). Structures lacking the oxetane ring are practically not recognized (Heinstein & Chang, 1994): baccatin-III has an IC_{50} value of 12 ng/ml while 20-acetoxy-4-deacetyl-5-epi-20,O-secopaclitaxel (a synthetic compound with an opened oxetane ring) has an IC_{50} value >293 ng/ml (highest concentration tested). All materials and methods of the ELISA were according to the instructions of the manufacturer. Three replicates of each sample were assayed, as suggested by the manufacturer. Since the standard used was baccatin-III, results should be interpreted as baccatin-III equivalents.

The volume of the medium is considered 50 ml throughout the growth period.

3.4. Protein extraction and GGPP synthase assay

The frozen cells were harvested as described in Section 3.2 and ground in a liquid N_2-chilled Waring blender for 2×30 s at maximum speed. A fine powder was obtained. Immediately 0.15 g of polyvinylpolypyrrolidone (PVPP) and 1 ml of extraction buffer per gram of fresh weight, were added. The extraction buffer contained 50 mM Tris–HCl pH 7.6, 1% Brij-35, 2 mM DTT, 10 μM leupeptin, 4 mM $MnCl_2$, 2 mM $MgCl_2$ and 20% glycerol. The homogenate was mixed in a 30°C waterbath until temperature reached 4°C and was then passed through Miracloth and centrifuged at 20000*g* for 60 min. The supernatant was desalted by using Sephadex G-25 M (PD-10) columns, which were equilibrated before with the extraction buffer.

The assay is based on the acid lability of the allylic diphosphates (Lynen, Agranoff, Eggerer, Henning, & Moslein, 1959): allylic diphosphates, like FPP and GGPP, under acidic conditions are hydrolyzed to the corresponding apolar alcohols, while IPP is not and it remains polar. GGPP synthase activity was measured using the following incubation mixture: 50 μl of crude desalted protein extract, 50 mM Tris–HCl pH 7.6, 10 μM leupeptin, 4 mM $MnCl_2$, 2 mM $MgCl_2$, 20% glycerol and 25 mM KF in a total volume of 200 μl. Before addition of the substrates the mixture was preincubated for 10 min. in the presence of 10 mM iodoacetamide in order to inhibit IPP isomerase. The reaction was started by addition of [1-^{14}C]-IPP (4.86 μM final concentration, 55 μCi/μmol) and FPP (43.5 μM final concentration). After incubation for 30 min at 30°C, during which the formation of the product was linear with time, the enzyme reaction was stopped by addition of 500 μl EtOH:HCl (1:1). The hydrolysis of the allylic diphosphates was allowed to proceed for 20 min and then 2 ml of toluene were added in order to extract the allylic alcohols. 1 ml of the toluene layer was removed and mixed with 10 ml of Opti-Fluor (Packard) and the radioactivity incorporation was determined by liquid scintillation counting (Tri-Carb 4530, Packard). Blanks were performed with addition solely of [1-^{14}C]-IPP, protein extract and omitting acidic hydrolysis as well as incubation with [1-^{14}C]-IPP and no protein extract, showed negligible incorporation of radioactivity.

Protein concentration was determined by using the DC Protein Assay kit from Bio-Rad.

3.5. Nutrient uptake determination

Ammonium analysis was done by using a colorimetric method according to Weatherburn (1967); residual nitrate was determined with an HPLC method according to Zurhake and Wander (1985); phosphate was determined by a colorimetric method according to Gomori (1942), while sucrose, glucose and fructose levels were analyzed by means of HPLC equipped with a RI detector and a Serva DEAE column, cat. No. 242308. The mobile phase consisted of 85% ACN and 15% H_2O. The eluent was recirculating. All media were clarified by centrifugation prior to analysis.

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