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Inhibition of paclitaxel and baccatin III accumulation by mevinolin and fosmidomycin in suspension cultures of *Taxus baccata*

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Abstract

To achieve a better understanding of the metabolism and accumulation of paclitaxel and baccatin III in cell cultures of *Taxus*, inhibitors of the early steps in the terpenoid pathway were applied to a cell suspension culture of *Taxus baccata*: fosmidomycin as an inhibitor of the non-mevalonate branch of the pathway, and mevinolin as an inhibitor of the mevalonate branch. Synthesis of both taxanes in the cell suspension was first increased when cultured in the product formation medium supplemented with methyljasmonate (100 μM). The product formation medium was selected after assaying 24 different culture media. When fosmidomycin (200 μM) was added to the product formation medium together with the elicitor, the accumulation of paclitaxel and baccatin III was reduced by up to 3.0 and 1.5 times, respectively, whereas the inhibitory effect of mevinolin (1 μM) was only clearly exerted in the case of paclitaxel. Under the conditions of our experiment, we conclude that in the synthesis of both taxanes, the non-mevalonate pathway is the main source of the universal terpenoid precursor isopentenyl diphosphate (IPP).

Keywords: *Taxus baccata*; Cell culture; Taxane yield; Inhibition; Mevinolin; Fosmidomycin

1. Introduction

Paclitaxel (NSC-125973), a complex isoprenoid derivative of the *Taxus* species containing an unusual diterpene skeleton (taxane), is a compound with intense antitumoral activity. However, due to the difficulties in obtaining this compound from yew trees the clinical use of paclitaxel has been limited. Among these difficulties are its low concentration and the high cost of the extraction process (Kingston, 1994).

The limited availability of paclitaxel from its natural source has motivated the development of alternative production sources. Total synthesis is not commercially viable because of the high cost of the process. An alternative approach for obtaining paclitaxel is the semisynthesis from more abundant

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taxanes, for example, via the conversion of baccatin III isolated from the needles of yew trees (Hezari et al., 1997). However, the most promising alternative for the production of paclitaxel and related taxanes is the use of cell cultures derived from different *Taxus* species (Fett-Neto and DiCosmo, 1997).

In many plant organisms there exists a dichotomy in the biosynthesis of terpenoid precursor isopentenyl diphosphate (IPP; for a recent review see Lichtenthaler, 1999). In the cytosol, the classical mevalonate pathway produces IPP from acetyl coenzyme A for sesquiterpene and triterpene biosynthesis. The plastids are the site of the alternative non-mevalonate pathway producing IPP from pyruvate and glyceraldehyde-3-phosphate leading to plastidic isoprenoids: monoterpenes, diterpenes and tetraterpenes. The mevalonate pathway can be inhibited by the fungal metabolite mevinolin (Bach and Lichtenthaler, 1982), and the non-mevalonate pathway by the antibiotic and herbicidal compound fosmidomycin (Zeidler et al., 1998). To be exact, fosmidomycin acts by inhibiting the 1-deoxy-D-xylulose-5-phosphate reducto-isomerase (DOXP reducto-isomerase) and mevinolin by blocking the 3-hydroxy-3-methylglutaryl-Coenzym A reductase (HMG-CoA reductase) in the indicated pathways.

According to Eisenreich et al. (1996), their studies on taxol biosynthesis show conclusively that the taxane ring system is not synthesized via mevalonate, which is consistent with its diterpenic nature. However, previous work carried out by Lansing et al. (1991) and Zamir et al. (1992) shows that when *Taxus* plants are supplied with labeled mevalonate, high rates of radioactively labeled paclitaxel are obtained. At the same time, our recently obtained results using a cell suspension of *T. media* supplemented with mevalonate suggest that under the conditions of our experiment both pathways could be involved (Cusidó et al., 2002). In this context, it is of interest that Soler et al. (1993) have shown that plastids are capable of cytoplasmic IPP uptake.

The aim of this work was to provide more information concerning the origin of the taxane ring system of paclitaxel and baccatin III in a cell suspension culture of *Taxus baccata*. For this purpose, mevinolin and fosmidomycin have been used as tools to block the HMG-CoA reductase and the DOXP reducto-isomerase in the mevalonate and non-mevalonate branch of the terpenoid biosynthetic pathway, respectively. It is important to understand the origin of the taxane ring system before addressing yield improvement strategies such as metabolic engineering of paclitaxel and baccatin III biosynthesis.

2. Materials and methods

2.1. Development of an effective product formation medium and establishment of cell culture

In order to obtain high levels of paclitaxel and baccatin III in our *T. baccata* cell suspension, necessary to clearly discern the effect of the specific inhibitors mevinolin and fosmidomycin, as a first step we developed an effective product formation medium. For this, we tested the effects of different basal media [Gamborg's B5 (Gamborg et al., 1968) and McCown's Woody Plant Medium (WPM; Lloyd and McCown, 1980)] and sources of sugar (3% sucrose and 0.5% sucrose + 0.5% fructose), auxin [2 mg l^{-1} of 2,4-D, 1-naphthaleneacetic acid (NAA) and Picloram, respectively] and cytokinin [0.1 mg l^{-1} of kinetin, zeatin, 6-benzylaminopurine (BAP) and *m*-Topolin, respectively] on the yield of both taxanes during the culture of the callus line used to establish the suspension. Of the 48 possible resultant culture media, only 24 were in fact tested (Table 1). The selection was performed following a fractional factorial design based on an orthogonal array (Dean and Voss, 1999). For assays of all these culture media, callus pieces (300 ± 30 mg) from the callus line were inoculated onto petri dishes containing 20 ml of medium. All cultures were maintained in a growth chamber at 25 °C in darkness during 28 days. After this, 24 callus pieces grown in each different culture media were harvested for analysis. Our results (Table 1) revealed that Gamborg's B5 medium (B5) with 3% sucrose, 2 mg l^{-1} of Picloram and 0.1 mg l^{-1} of kinetin was optimum for both paclitaxel and baccatin III yields (product formation medium), while the same basal medium

Table 1
Growth and accumulation of paclitaxel and baccatin III in callus pieces of *T. baccata* cultured for 28 days in petri dishes containing 24-ml of one of 24 different culture media selected as indicated in Section 2 from the 48 possible media resulting from the combination of two basal media (B5 and WPM) and two sources of sugar (S and S/F), four of cytokinin (Kin, Zea, BAP and MT) and three of auxin (2,4-D, NAA and P)

Culture media	Basal media	Source of sugar	Plant growth regulators		Growth	Taxane production	
			Cytokinin	Auxin	Fresh weight (g)	Paclitaxel (mg g DW^{-1})	Baccatin III (mg g DW^{-1})
I	B5	S		2,4-D	0.80±0.12	0.30±0.03	0.05±0.01
II			Kin	NAA	0.84±0.16	0.13±0.01	0.02±0.00
III				P	0.72±0.25	1.68±0.28	0.35±0.02
IV				2,4-D	1.05±0.27	0.14±0.01	0.04±0.01
V			BAP	NAA	1.01±0.25	0.84±0.07	0.11±0.02
VI				P	0.63±0.19	0.45±0.05	0.09±0.00
VII		S/F		2,4-D	0.72±0.12	0.03±0.00	0.07±0.01
VIII			Zea	NAA	0.65±0.13	0.25±0.02	0.09±0.01
IX				P	0.83±0.16	0.60±0.07	0.11±0.02
X				2,4-D	1.01±0.27	0.16±0.02	0.04±0.00
XI			MT	NAA	1.29±0.26	0.47±0.05	0.08±0.01
XII				P	0.87±0.13	0.23±0.02	0.32±0.02
XIII	WPM	S		2,4-D	0.87±0.12	0.05±0.01	0.05±0.01
XIV			Kin	NAA	0.86±0.21	0.06±0.01	0.06±0.01
XV				P	0.47±0.06	0.28±0.03	0.07±0.01
XVI				2,4-D	0.98±0.15	0.04±0.01	0.04±0.01
XVII			BAP	NAA	0.86±0.21	0.05±0.01	0.05±0.01
XVIII				P	0.96±0.28	0.05±0.01	0.08±0.01
XIX		S/F		2,4-D	0.68±0.13	0.07±0.01	0.06±0.01
XX			Zea	NAA	0.44±0.04	0.13±0.02	0.06±0.01
XXI				P	0.45±0.07	0.16±0.01	0.03±0.00
XXII				2,4-D	0.43±0.10	0.02±0.01	0.02±0.00
XXIII			MT	NAA	0.70±0.18	0.09±0.01	0.08±0.01
XXIV				P	0.49±0.09	0.02±0.00	0.03±0.00

In all cases the concentration of cytokinins was 0.1 mg l^{-1} and that of auxins was 2 mg l^{-1}. S, 3% sucrose; S/F, 0.5% sucrose +0.5% fructose; Kin, Kinetin; Zea, zeatin; MT, *m*-Topolin; P, Picloram. Values are the mean of 24 determinations±S.E. Inoculi were callus pieces of 0.30±0.03 g fresh weight.

(B5) with 0.5% sucrose+0.5% fructose, 2 mg l^{-1} of NAA and 0.1 mg l^{-1} of BAP (growth medium) was optimum for callus growth, although due to the high standard error there may be other candidates.

The cell suspension was established from the considered callus line, as reported earlier (Cusidó et al., 1999). The cell suspension was maintained in the above mentioned growth medium, which was optimum for the growth of the callus line. Routine maintenance of the culture was performed in 175-ml flasks (Sigma) by transferring every 10–12 days 1±0.2 g of cells to 10 ml of fresh medium. All flasks were capped with Magenta B-Caps (Sigma) and incubated in the dark at 25 °C and 100 rpm in a shaker-incubator (Adolf Kühner AG, Schweiz).

2.2. Treatments with methyljasmonate and specific inhibitors

The second step in improving the yield of paclitaxel and baccatin III in our *T. baccata* cell suspension was to test the effect of adding methyljasmonate (100 μM) to the selected product formation medium. This concentration was previously established as optimum for paclitaxel biosynthesis by Ketchum et al. (1999) and Cusidó et al. (2002). Methyljasmonate was added to the

culture in 2.5 μl of ethanol per ml of culture (Yukimune et al., 1996). Equal volumes of ethanol were added to all cultures. Cells were cultivated for 22 days in a 175-ml flask containing 10-ml of selected product formation medium with methyljasmonate, or without as a control, and maintained in a shaker-incubator at 100 rpm in the dark at 25 °C. Inoculi were 1 ± 0.2 g of cells harvested from a donor suspension grown for 13 days in the growth medium, which was the length of time necessary for it to enter the stationary growth phase (see Fig. 1A). Secondary metabolite production in plant cell cultures is a process not usually dependent on growth. This is the case of paclitaxel production in *Taxus* cell cultures, where the production of this secondary product takes place mainly when the lineal growth phase has finished and the culture is in its stationary growth phase (Fett-Neto and DiCosmo, 1997).

To evaluate and compare the effect of the inhibitors mevinolin and fosmidomycin on the cell suspension capacity to synthesize paclitaxel and baccatin III in the above indicated culture conditions with methyljasmonate, which clearly increased the production of both taxanes (see Fig. 2), cultivation with mevinolin (1 μM) or fosmidomycin (200 μM) was performed. These inhibitor concentrations were among those previously tested by Hagen and Grünewald (2000) in *Haematococcus pluvialis* (which accumulates secondary carotenoids) and selected by us after various assays. During these, we could observe that less than 200 μM fosmidomycin exerted no effect, but more than this amount progressively reduced the cell growth, and that although concentrations of mevinolin higher than 1 μM (up to 5 μM) did not increase its inhibitory effect, they decreased cell growth considerably. This latter effect had been observed by Bach and Lichtenthaler (1983). Fosmidomycin was a gift from A. Boronat (Barcelona University), and was dissolved in cultivation medium prior to application to the cell suspension. Mevinolin was purchased from Sigma. Before application, it was converted to the water-soluble sodium salt as described in Bach and Lichtenthaler (1983).

All compounds were sterilized by filtering through 0.22-μm sterile filters (Millipore) and added to the production medium prior to inoculation to give the final concentrations considered. For analysis, six flasks from each treatment were harvested on day 4 and then at 3-day intervals in the case of the elicitor alone, and on days 4, 10, 16 and 22 in the case of the elicitor together with mevinolin or fosmidomycin.

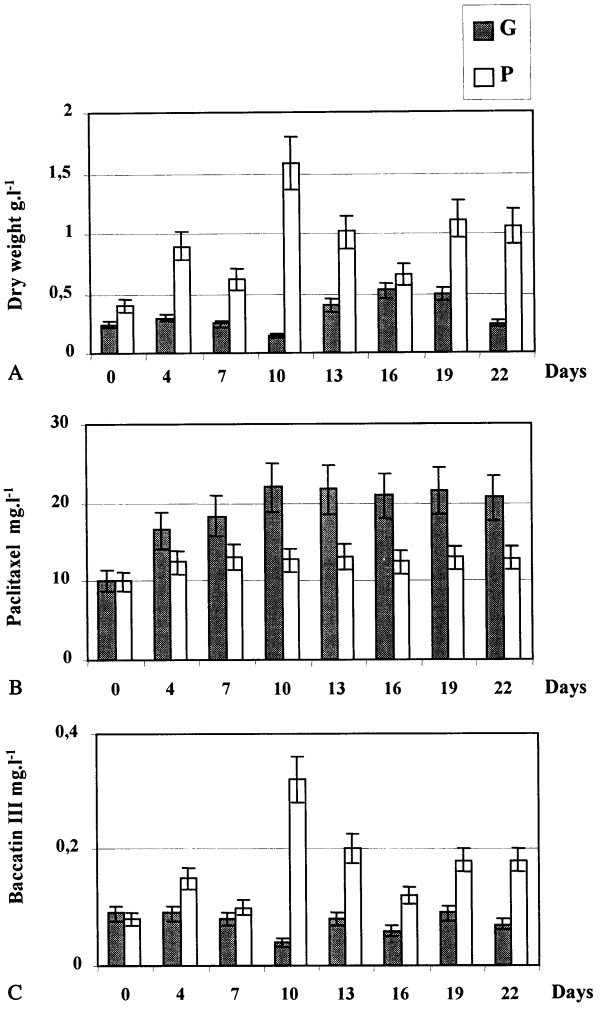

Fig. 1. Time courses of biomass accumulation (A) and total yield (cell-associated + extracellular) of paclitaxel (B) and baccatin III (C) by a cell suspension of *T. baccata* grown for 22 days in 175-ml shake flasks containing 10-ml of culture medium. In all cases the inoculum consisted of 100 g cell fresh weight per l. G, growth medium; P, product formation medium. Data represent average values from six replicates ±S.E.

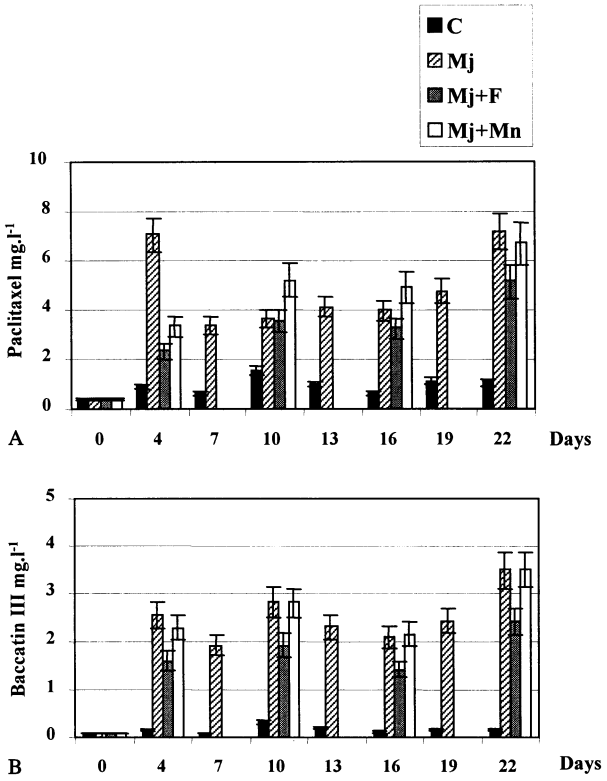

Fig. 2. Comparison of total content (cell-associated+extracellular) of paclitaxel (A) and baccatin III (B) in a cell suspension of *T. baccata* grown for 22 days in 175-ml shake flasks containing 10-ml of product formation medium without supplement (control) and supplemented with methyljasmonate (100 μM) alone and with the elicitor together fosmidomycin (200 μM) or mevinolin (1 μM). In all cases the inoculum consisted of 100 g cell fresh weight per l. Mj+F, methyljasmonate+fosmidomycin; Mj+Mn, methyljasmonate+mevinolin. Data represent average values from six replicates±S.E.

2.3. Biomass accumulation, viability assay and paclitaxel and baccatin III measurements

To determine the growth measured as fresh weight of callus cultures, callus pieces grown in each different culture media for 28 days were harvested and separately weighed. Dry weight was recorded from lyophilized callus pieces. In the case of cell suspension cultures, both fresh and dry weights were determined as reported earlier (Cusidó et al., 2002). Cell viability was followed using the method described by Duncan and Widholm (1990). Taxanes were extracted from freeze-dried callus pieces, cells and the culture medium as described by Cusidó et al. (1999). Quantification of paclitaxel and baccatin III was performed according to Grothaus et al. (1995) using an indirect competitive enzyme immunoassay (CIEIA). Paclitaxel and baccatin III–protein, coating antigen, anti-paclitaxel and anti-baccatin III monoclonal antibodies and the corresponding standards were obtained from the Hawai-Biotechnology Group.

3. Results and discussion

3.1. Cell biomass and taxane yield in selected growth and product formation media

The effects of selected growth and product formation media on growth and paclitaxel and baccatin III yield in the *T. baccata* cell suspension cultured in shake flasks for 22 days are shown in Fig. 1A–C. In the cells cultured in the growth medium, the linear growth phase appeared to begin almost immediately and lasted for 10 days. After this point, the cells entered the stationary growth phase, which lasted for the remaining 12 days. In contrast, when cultured cells in their early stationary growth phase (day 13) were transferred from the growth medium to the product formation medium for a further culture period of 22 days, in spite of a slight increase with respect to the inoculum cell biomass, the cells appeared to remain in the initial stationary growth phase throughout the culture period. The lower growth rate observed was not a consequence of cell lysis, since the viability percentage of cultured cells was more than 85% throughout the culture period considered (data not shown).

When comparing the total levels (cell-associated+extracellular) of both taxanes during the cell suspension culture in the respective growth and product formation media (Fig. 1B–C), it can be deduced that the maximum yields of paclitaxel ($1.58\ mg\ l^{-1}$) and baccatin III ($0.32\ mg\ l^{-1}$) in the product formation medium were 3.0 and 3.5-fold higher than in the growth medium, respectively. These results show the suitability of the product

formation medium for improving the yields of the taxanes considered.

3.2. Effects of product formation medium modifications

As shown in Fig. 2A–B, in relation to the control (when cultured cells in their early stationary growth phase were transferred to the product formation medium for a further culture period of 22 days), considerable differences in total paclitaxel and baccatin III content were observed in the *T. baccata* cells grown in the product formation medium supplemented with methyljasmonate (100 μM) alone or together with mevinolin (1 μM) or fosmidomycin (200 μM). When only methyljasmonate was present, the maximum total paclitaxel content was 7.09 mg l^{-1} at the end of the culture period considered (day 22), although that obtained on day 4 (6.86 mg l^{-1}) was almost as high (Fig. 2A). Compared with the maximum total paclitaxel content in the cell suspension control (1.58 mg l^{-1} on day 10), the increase was up to 4.5-fold in the presence of the elicitor. Regarding the maximum total baccatin III content (Fig. 2B), this was 0.32 mg l^{-1} on day 10 without methyljasmonate and 3.49 mg l^{-1} on day 22 with the elicitor. As can be deduced, the increase in this taxane was up to 10.8-fold in the presence of methyljasmonate. It is also of interest that in relation to the control, cell growth was not significantly affected by the presence of the elicitor alone or together with mevinolin or fosmidomycin during the period of the experiment (data not shown).

With respect to the effect of mevinolin and fosmidomycin on paclitaxel and baccatin III biosynthesis in the above indicated culture conditions, as can be deduced, the presence of fosmidomycin inhibited the biosynthesis of paclitaxel to a greater degree than that of mevinolin throughout the culture period considered (Fig. 2A). In the presence of fosmidomycin, the highest accumulations of paclitaxel occurring on days 4 and 22 of the culture were reduced by 3.0 and 1.5 times respectively, whereas in the presence of mevinolin this reduction was only 2.1-fold on day 4 and lower (1.05-fold) on day 22. Regarding the total baccatin III content (Fig. 2B), the inhibitory effect of fosmidomycin on the highest accumulations of this taxane occurring also on days 4 and 22 in the presence of the elicitor alone was 1.7 and 1.4-fold, respectively, and that of mevinolin was only very slightly apparent on day 4. During the rest of the culture period, the presence of mevinolin did not influence the stimulatory effect of methyljasmonate. In this context, it is worth noting the observation that mevinolin is incapable of entering the plastids (Bach, 1987).

On the basis of the inhibitory effect of mevinolin and fosmidomycin on the mevalonate and non-mevalonate pathways of IPP biosynthesis, respectively, the process might be expected to be sensitive to fosmidomycin if the taxane production had only the plastidic IPP as a precursor, and if the IPP source was also cytoplasmic, the process might also be expected to be sensitive to mevinolin. The fact that in our experiments both inhibitors were active in the case of paclitaxel suggests the possibility that the taxane ring system of this complex isoprenoid can also derive at least partially from cytoplasmic IPP, which concurs with the observation that the cytoplasmic IPP accumulates in plastids against a concentration gradient (Soler et al., 1993). However, as fosmidomycin was more active in its inhibitory effect, it can be deduced that the production of paclitaxel occurs mainly via the non-mevalonate pathway.

On the other hand, the fact that fosmidomycin but not mevinolin exerted a clear inhibitory effect on baccatin III yield could be the result of the lower capacity of *T. baccata* cells to synthesize this taxane. This concurs with the suggestion that paclitaxel and baccatin III are synthesized from a common precursor via two divergent pathways as previously postulated by Srinivasan et al. (1996). The recent data show that the taxane ring system of paclitaxel and related taxanes, is produced in the plastids by the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP) to taxadiene, catalyzed by the enzyme taxadiene synthase (Eisenreich et al., 1996; Lichtenthaler, 1999).

To sum up, our results indicate that although *T. baccata* cells to some extent could use the mevalonate pathway as a source of IPP for paclitaxel and baccatin III yield, the main source of the

universal terpenoid precursor for the biosynthesis of GGPP and, consequently, of both taxanes, is the plastidic non-mevalonate pathway. At present, in order to improve the yield of paclitaxel and baccatin III by overexpressing a DOXP reductoisomerase encoding gene, our first step has been to embark on the development of an efficient transformation system for *T. baccata* cells using currently available technologies for plant transformation.

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