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Source of isopentenyl diphosphate for taxol and baccatin III biosynthesis in cell cultures of *Taxus baccata*

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Abstract

To achieve a better understanding of the metabolism and accumulation of taxol and baccatin III in cell cultures of *Taxus*, three cell lines (I, II and III) of *T. baccata* were treated (on day 7) with several concentrations of fosmidomycin (100, 200 and 300 μM), an inhibitor of the non-mevalonate branch of the terpenoid pathway, or mevinolin (1, 3 and 5 μM), an inhibitor of the mevalonate branch, in both cases in presence and absence of 100 μM methyl jasmonate (MeJ). They were compared with lines treated only with the elicitor MeJ as well as an untreated control with respect to growth, viability and production of taxol and baccatin III. The results show that the cell line type was an important variable, mainly for taxane accumulation. The blocking effect of fosmidomycin on taxane production was significantly greater than that of mevinolin in all the cell lines, clearly suggesting that the isopentenyl diphosphate (IPP) used for the taxane ring formation was mainly formed via the non-mevalonate pathway. However, the significant reduction in the content of taxol (on average 3.8-fold) and baccatin III (on average 4.3-fold) in line I when treated with the elicitor together with mevinolin concentrations of 5 and 1 μM, respectively, also suggests that both non-mevalonate and mevalonate pathways are involved in the biosynthesis of the two taxanes as a result of cytosolic IPP and/or other prenyl diphosphate transport to the plastids. The observation that the inhibitory effect of fosmidomycin or mevilonin on taxol and baccatinn III yield does not interfere with methyl jasmonate elicitation is discussed.

Keywords: *Taxus baccata*; Cell lines; Taxol; Baccatin III; Isopentenyl diphosphate; Fosmidomycin; Mevinolin; Methyl jasmonate

1. Introduction

Taxol is a highly substituted, polyoxygenated cyclic diterpenoid characterised by the taxane ring system, which presents a very intense antitumoral activity. As the supply of taxol from nature is very limited, methods have been developed for the semisynthesis of taxol from related taxanes, such as baccatin III [1], present in substantial quantities (from 0.2 to 1 g/kg) in the leaves of the European yew, *Taxus baccata*, and other yews (e.g. Himalayan yew, *Taxus wallichiana*), which is a renewable starting material. However, this methodology requires the continued use of precursors from natural sources and significant amounts of solvents, both of which present environmental problems.

Another alternative for obtaining taxol and its synthetically useful progenitors is plant cell culture. Cell suspension [2–5] or cell immobilised systems [6,7] can provide an environmentally friendly way to produce taxol and enhance its productivity. The complex chemical structure of taxol means that total chemical synthesis is not commercially viable at present, and an efficient and economical supply of the drug must rely on biological production systems for the foreseeable future [8]. Up-regulation of the taxol biosynthetic pathway by overexpression of selected genes in *Taxus* cells can potentially address the supply issue. In all cases, improving the biological production yields of taxol depends critically upon a detailed understanding of the biosynthetic pathway.

In plants, isopentenyl diphosphate (IPP), the universal precursor of terpenoids, is synthesized either via the classical cytosolic mevalonate pathway or via the non-mevalonate plastid 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erytritol 4-phosphate (MEP) pathway [9]. Whereas the IPP derived from

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the mevalonate pathway is generally used in the biosynthesis of sesquiterpenes, phytosterols and triterpenes, the IPP derived from the MEP pathway is employed in the biosynthesis of monoterpenes, diterpenes and tetraterpenes. According to Eisenrich et al. [10], the IPP involved in the biosynthesis of the taxane ring, which has a diterpenic structure, is formed through the non-mevalonate pathway. However, other work [11,12] shows that when *Taxus* plants are supplied with labelled mevalonate, high rates of radioactively labelled taxol are obtained. Furthermore, our recently obtained results using a *Taxus media* cell suspension supplemented with mevalonate suggest that both biosynthetic routes could be involved [4]. Therefore, although many researchers have shown that diterpenes are synthesized via the non-mevalonate pathway in different systems [13,14], it might be a mistake to accept this as the general route. According to Adam and Zapp [15], a distinct division between the mevalonate and non-mevalonate pathways may not always exist for a given end-product because the biosynthesis of certain plant terpenoids appears to involve both routes. The involvement of the two pathways may be a result of the transport of prenyl diphosphate intermediates between the different sites of terpenoid biosynthesis [16,17].

Considering that IPP is not only an essential precursor but also the first intermediate in taxane biosynthesis, it is important to elucidate its source in this process in order to know to what extent the cytosolic mevalonate and plastid MEP (non-mevalonate) pathways contribute to the formation of the taxane ring system of taxol and baccatin III in *Taxus* cell cultures. In this context, it is of interest that mevinolin and fosmidomycin are two inhibitors that block the mevalonate and non-mevalonate pathways, respectively. Mevinolin (6α-methylcompactin) is a compound belonging to the statins group, which competitively inhibits the binding of natural substrate hydroxymethylglutary-CoA (HMGCoA) to the active site of the enzyme hydroxymethylglutary-CoA reductase (HMGR), and, consequently blocks the synthesis of cytosolic IPP [18]. Fosmidomycin, an antibiotic, herbicidal and antimalarial compound, is an inhibitor of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DOXP reductoisomerase) blocking the plastid non-mevalonate pathway [19].

In this study, using three different cell lines of *T. baccata*, the dependence of methyl jasmonate-induced and non-induced taxol and baccatin III production on IPP derived from the mevalonate and non-mevalonate pathways was detected by selectively blocking the IPP biosynthesis with specific inhibitors. The effects of the inhibitors were analysed in terms of cell growth, cell viability and content of taxol and baccatin III.

2. Materials and methods

2.1. Cell lines and culture conditions

The cell lines I–III of *T. baccata* used in this study were established from stable callus lines as reported earlier [20]. The lines were maintained in 175-ml flasks (Sigma, St. Louis, MO) in the dark at 25 ± 0.2 °C and 100 ± 1 rpm in a shaker-incubator (Adolf Kühner AG, Schweiz). Every 10–12 days, 1 ± 0.2 g of cells were used as inoculum in 10 ml of Gamborg's B5 medium [21] with 0.5% sucrose + 0.5% fructose, 2 mg l^{-1} of NAA and 0.1 mg l^{-1} of BAP (growth medium), which has previously been demonstrated as optimum for *T. baccata* cell growth [22]. All flasks were capped with Magenta B-Caps (Sigma).

In order to obtain high levels of taxol and baccatin III in our cell lines, which was necessary to more clearly discern the effect of the specific inhibitors fosmidomycin and mevinolin, and considering that the taxol production in *Taxus* cell cultures takes place mainly when the lineal growth phase has finished and the culture is in its stationary growth phase [23,24,4], 2 ± 0.2 g wet weight of cells grown for 12 days in the growth medium (the length of time necessary for them to enter the stationary growth phase) were transferred to 10 ml of B5 medium with 3% sucrose, 2 mg l^{-1} of Picloram and 0.1 mg l^{-1} of kinetin (production medium), which had previously been selected as optimum for both taxol and baccatin III yield of *T. baccata* cells [22]. They were then cultured for 28 days either with 100 μM methyl jasmonate (MeJ), or without the elicitor (control), or with different concentrations of fosmidomycin (100, 200 and 300 μM) or mevinolin (1, 3 and 5 μM) in presence and absence of the elicitor. Prior to application, methyl jasmonate (Sigma) was dissolved in ethanol [25], and both fosmidomycin (Invitrogen) and mevinolin (Fluka) were dissolved in cultivation medium. All compounds were sterilized by filtering through 0.22 μm sterile filters (Millipore) and added at 7 days of culture of the cell lines in the production medium to give the final concentrations considered. For analysis, six flasks from each treatment were harvested also at day 7 and then at days 14, 21 and 28.

2.2. Biomass accumulation and viability assay

Fresh weight was determined by suction filtering of suspension cultures using Miracloth filters (Calbiochem, CA). Then the cells were lyophilised to obtain dry weight and analysed to determine their content of taxol and baccatin III. Cell viability was studied by the fluorecein diacetate staining technique [26]. The cells (0.2 g wet weight) were incubated in fresh production medium (5 ml) containing fluorecein diacetate (0.1 mg ml^{-1}) for 30 min and then observed in a fluorescence microscope.

2.3. Taxol and baccatin III measurements

Taxanes were extracted from lyophilised cells and the culture medium as described by Cusidó et al. [20]. Quantification of paclitaxel and baccatin III was performed by high performance liquid chromatography (HPLC) as described in our paper [27]. Criteria for identification included retention time, UV spectra, co-chromatography with standard and peak homogeneity by photo-diode array detector when spiked with authentic standard. Taxol and baccatin III were provided by Hauser Chemicals (USA).

3. Results and discussion

3.1. Effects on cell growth

The time courses of growth of three *T. baccata* cell lines in their initial stationary growth phase cultured in a selected production medium, either without an elicitor (control) or with 100 μM MeJ, are shown in Fig. 1, together with the growth patterns resulting from the addition of different concentrations of the inhibitors fosmidomycin (100, 200 and 300 μM) or mevinolin (1, 3 and 5 μM) in presence and absence of MeJ. As can be seen, in control conditions the cell biomass of the cell lines, expressed as grams of fresh weight, increased very slightly during the 28 days of culture. Cell line I presented the lowest growth since it increased by only 36% with respect to the inoculum, achieving its highest biomass at the end of the experiment (1.36 g) with a growth rate of 0.013 day^{-1}. Cell lines II and III achieved their highest biomass after 21 days of culture, cell line III showing the highest growth capacity of 1.88 g (88% higher than the inoculum), which corresponded to a growth rate of 0.04 day^{-1}. The addition of 100 μM MeJ to the medium produced hardly any differences in growth in the three cell lines (maximum of 4%), which corresponds with a previous observation in *T. media* cell suspensions treated with the same elicitor concentration [4]. Although some researchers have indicated that 100 μM MeJ negatively affects cell growth, it has been observed that different plant cell lines respond in different ways to elicitor treatment [28] and that MeJ has contradictory effects on the growth of cell cultures [25].

These relatively small increases of biomass suggest that the three cell lines cultured with or without MeJ remained in a state of stationary growth throughout the culture period considered, which was satisfactory for our aims, since this is when the biosynthesis of taxol mainly takes place in *Taxus* cell cultures. As cells in stationary growth are characterised by limited rates of division, it has been considered that products from primary metabolism accumulate and/or become available for secondary metabolite production [29].

As shown in Fig. 1, the growth pattern of cultured cell lines was changed by the addition of different concentrations of fosmidomycin (100, 200 and 300 μM), either with or without 100 μM MeJ. Thus, whereas the highest biomass in lines treated only with MeJ or grown in control conditions was achieved after 21 days of culture, when fosmidomycin was added to the medium the highest growth was observed after 14 days. However, none of the tested fosmidomycin concentrations significantly ($p \leq 0.01$; *t*-test) affected the growth capacity of the cell lines. With respect to the addition of different concentrations of mevinolin (1, 3 and 5 μM), either with or without 100 μM MeJ, as Fig. 1 shows, the inhibitory effect on cell growth was

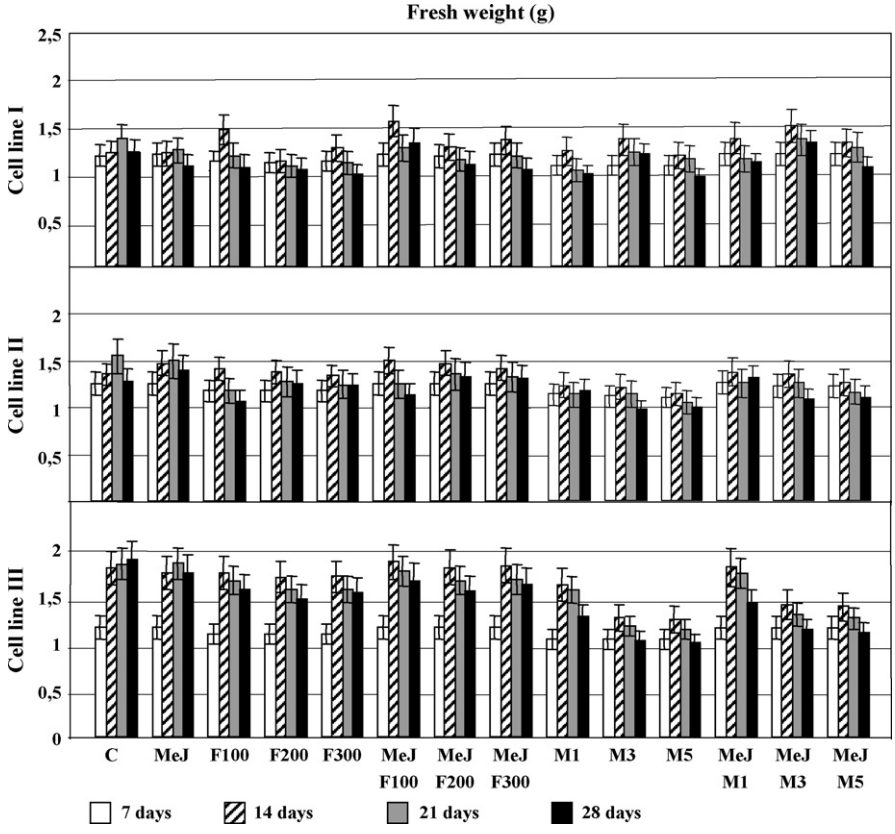

Fig. 1. Time courses of biomass accumulation in *T. baccata* cell lines. Cells in their initial stationary growth phase were cultured for 28 days in a selected production medium for the yield of taxol and baccatin III, either with 100 μM methyl jasmonate, or without the elicitor (control), or with different concentrations of fosmidomycin (100, 200 and 300 μM) or mevinolin (1, 3 and 5 μM) in presence and absence of the elicitor. The different treatments were initiated on day 7, and in all cases the inoculum consisted of 100 g of cells l^{-1}. F, fosmidomycin; M, mevinolin; MeJ, methyl jasmonate. Data represent average values from six separate experiments ± S.D.

only clearly noticeable in cell lines II and III. In presence of the elicitor the average inhibition was 15.2% and 21.7%, respectively, and in its absence 20% and 31.3%, respectively. On the contrary, none of the mevinolin concentrations had much affect on the growth capacity of cell line I, suggesting that this line was less sensitive or more resistant to the effect of the inhibitor. From these results it may be inferred that under the conditions of this work, the biomass was affected to a greater degree by the cell line type and composition of the culture medium than by the presence of the inhibitors. In contrast, Hemmerlin et al. [30] have reported that the treatment of tobacco Bright Yellow-2 cells by both fosmidomycin and mevinolin leads to growth reduction, although the former has much less effect. Taking into account that we have studied a different species, our contrasting results may also be partly due to the initial stationary growth phase of our *T. baccata* cell lines and that they were grown in a selected production medium promoting taxol and baccatin III production but not cell growth.

3.2. Effects on cell viability

Given that the different concentrations of fosmidomycin (100, 200 and 300 μM) or mevinolin (1, 3 and 5 μM) did not produce significant differences (<10%) among the viability percentages of our cell lines, grown either with or without 100 μM MeJ in the production medium, the corresponding values in Table 1 represent the average of viability percentages obtained for the three concentrations used of each inhibitor, both with and without the elicitor. Additionally, since the different treatments were initiated at day 7 of the culture, the viability percentage values obtained on this day were taken as a reference.

As can be seen in Table 1, the highest viability, after 7 days of culture without MeJ (control), corresponded to cell line II (75%), and the lowest to cell line I (55%). These viability levels were maintained or decreased very slightly during the culture period considered (28 days). The addition of 100 μM methyl jasmonate to the production medium did not significantly ($p \leq 0.01$) change the viability in lines II and III, but line I presented a decrease of 36%, 30% and 22% at days 14, 21 and 28, respectively. As cell line I presented the highest taxol accumulation (see Fig. 2), in agreement with previous observations [5], this decrease in cell viability might be due to an increase of taxane accumulation rather than being a direct effect of the elicitor treatment.

When fosmidomycin and mevinolin were added to the cultured cell lines in presence or absence of 100 μM MeJ, the cell viability decreased only in lines II and III. This reduction of viability, reflected in the decrease in cell growth (see Fig. 1), was greater with the addition of mevilonin. Since the latter blocks the mevalonate pathway leading to cytosolic IPP, the reduction of growth and viability caused by its addition to the culture medium could be a consequence of a decrease in some growth-related terpenoids, such as β-sitosterol and stigmasterol (the two major phytoesterols with primary roles in membrane architecture [31]), formed directly from the cytosolic IPP.

3.3. Effects on taxol and baccatin III production

The time course of total content (cell-associated + extracellular) of taxol and baccatin III of the three different cell lines cultured in their initial stationary growth phase in a specific production medium was followed for 28 days in the various treatments, adding the inhibitors fosmidomycin and mevinolin either with or without 100 μM MeJ, or adding only the elicitor, or nothing at all (control). Since the different treatments were initiated on day 7 of the culture, these contents were determined on this day and then weekly until the end of culture period.

3.3.1. Utilization of fosmidomycin as an inhibitor

Regarding the taxol production at day 7 in control conditions (Fig. 2), cell line I was the most productive (7.0 mg l^{-1}), followed by lines III (4.0 mg l^{-1}) and II (3.1 mg l^{-1}). The taxol production of cell line I clearly decreased during the experiment,

Table 1
Comparison of cell viability in *T. baccata* cell lines

Days	Cell line	Control	Methyl jasmonate	Fosmidomycin	MeJ + F	Mevinolin	MeJ + M
7	I	55	–	–	–	–	–
	II	75	–	–	–	–	–
	III	65	–	–	–	–	–
14	I	60	39	62	58	57	50
	II	70	69	58	54	50	47
	III	63	60	54	51	45	42
21	I	50	35	64	56	58	53
	II	70	67	48	45	42	39
	III	60	50	46	42	40	40
28	I	48	38	65	59	56	55
	II	69	66	60	59	40	35
	III	57	55	48	38	41	40

Cells in their initial stationary growth phase were cultured for 28 days in a selected production medium for the yield of taxol and baccatin III, either with 100 μM methyl jasmonate, or without the elicitor (control), or with different concentrations of fosmidomycin (100, 200 and 300 μM) or mevinolin (1, 3 and 5 μM) in presence and absence of the elicitor. The different treatments were initiated on day 7, and in all cases the inoculum consisted of 100 g of cells l^{-1}. Data represent the average of viability percentages obtained in each case for the three inhibitor concentrations in presence and absence of elicitor from six separate experiments. F, fosmidomycin; M, mevinolin; MeJ, methyl jasmonate.

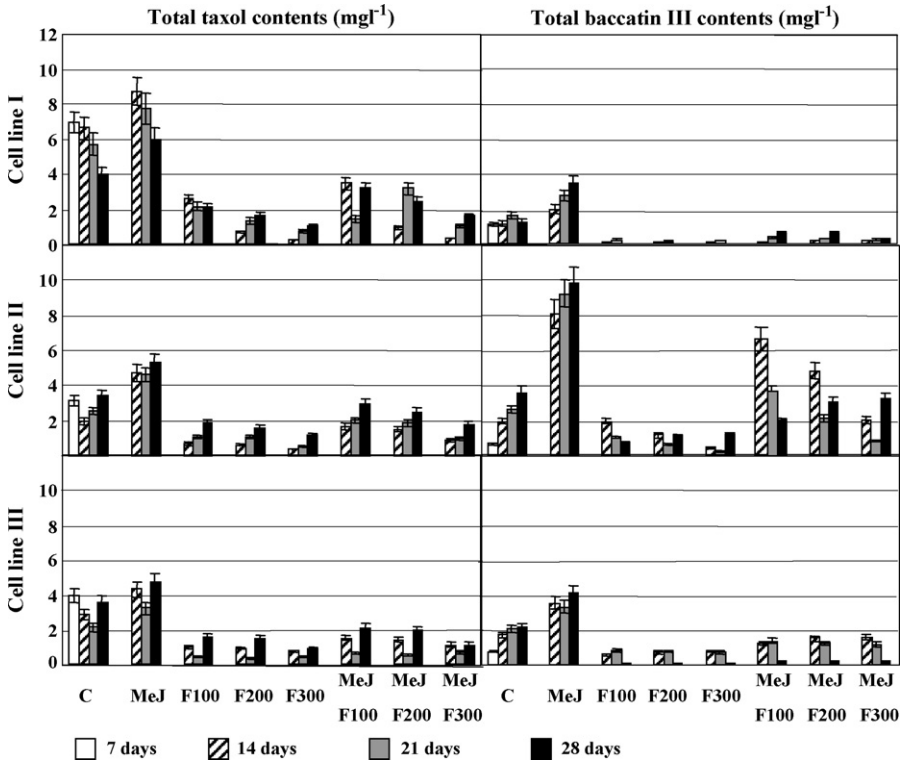

Fig. 2. Comparison of total content (cell-associated + extracellular) of taxol and baccatin III in *T. baccata* cell lines using fosmidomycin as the inhibitor. Cells in their initial stationary growth phase were cultured for 28 days in a selected production medium for the yield of taxol and baccatin III, either with 100 μM methyl jasmonate, or without the elicitor (control), or with different concentrations of fosmidomycin (100, 200 and 300 μM) in presence and absence of the elicitor. The different treatments were initiated on day 7, and in all cases the inoculum consisted of 100 g of cells l^{-1}. F, fosmidomycin; M, mevinolin; MeJ, methyl jasmonate. Data represent average values from six separate experiments ± S.D.

being 42% lower at the end of the culture than on day 7. The production pattern of lines II and III differed from that of line I in that the taxol yield was at its highest (line II) or had decreased only slightly (line III) at the end of the experiment.

From Fig. 2, it can also be seen that the addition of 100 μM MeJ to the production medium increased the taxol accumulation in the three cell lines studied, although this increase depended on culture age and to a higher degree on line type. In line I, the highest taxol accumulation (8.8 mg l^{-1}) was obtained 7 days after the elicitor addition (that is, day 14), being 24.5% higher than in the control. The highest taxol yields of lines II (5.7 mg l^{-1}) and III (4.7 mg l^{-1}) were achieved at the end of the culture period (day 28), the levels being 35% and 24% higher, respectively, than in the control. Our results agree with those obtained by Eilert et al. [32], who postulate that the response of cell cultures to elicitation with MeJ differs according to the cell line considered.

The effect of fosmidomycin on the total taxol content of cell lines simultaneously induced by 100 μM MeJ is also depicted in Fig. 2, which shows that the higher the concentration of fosmidomycin, the more severe was the inhibition of taxol. The most marked reduction in taxol yield was observed in cell line I, which was the most productive. Its total taxol content after 14, 21 and 28 days of culture in presence of 300 μM fosmidomycin was 22.8, 7.0 and 3.7 times lower, respectively, than the values achieved when only induced by 100 μM MeJ. In presence of the same fosmidomycin concentration (300 μM), cell lines II and III lowered their taxol yield after 14, 21 and 28 days, 5.3-, 4.8- and 2.9-fold, and 3.8-, 4.5- and 4.2-fold, respectively, when compared with the values achieved in presence of 100 μM MeJ alone.

Considering that fosmidomycin is a specific inhibitor of DOXP reductoisomerase and that it consequently blocks the synthesis of plastidic IPP involved in the formation of the taxol–taxane ring, from the above results it can be presumed that the translocation of IPP from cytosol to the plastids was involved in the taxol biosynthesis in our cell lines, as in no case was this completely inhibited. In comparison, it has recently been shown in *Capsicum* chromoplasts that 100 μM fosmidomycin completely inhibits the conversion of labelled 1-deoxy-D-xylulose 5-phosphate (DOXP) into the tetraterpene β-carotene [33]. Our presumption is supported by the fact that in the non-elicited lines, although the addition of the inhibitor (Fig. 2) resulted in a significantly ($p \leq 0.01$) reduced taxol yield, it was never completely inhibited. According to Fellermeier et al. [33], fosmidomycin inhibits the formation of terpenoids from DOXP, whereas it does not affect the conversion of IPP into terpenoids.

Moreover, when comparing the taxol concentration of the elicited and non-elicited cell lines, in both cases in the presence of fosmidomycin (Fig. 2), it can be seen that the total taxol content was clearly higher in the former, which indicates effective elicitation by MeJ. In this context, it is worth noting that the favorable effect of this elicitor on taxol biosynthesis is due to the activation of enzymes located at the cyclization step of geranyl-geranyl diphosphate to taxa-4(5), 11(12)-diene or fur-

ther down the pathway [25–34]. Due to the dependence of plastid IPP uptake on IPP concentration [16], it can be assumed that the IPP translocation and the taxol production of our cell lines, when cultured in the presence of fosmidomycin, depended on the cytoplasmic IPP concentration, and simultaneous induction by 100 μM MeJ.

Regarding the total baccatin III content achieved by the three studied cell lines in control conditions (Fig. 2), it was generally lower than the total taxol content. The most taxol-productive cell line, line I, presented baccatin III levels four- to six-fold lower than those of taxol throughout the culture period. The addition of 100 μM MeJ to the production medium also activated the baccatin III production. At its highest (after 28 days of culture), the baccatin III yield achieved by the elicited cell lines I, II and III was, respectively, 2.7-, 2.7- and 1.9-fold greater than in the same lines without elicitation. The cell line most induced for baccatin III production by MeJ was line II, which after 14 and 21 days of elicitation (21 and 28 days of culture) achieved levels of up to 9 and 10 mg l^{-1}. It is of interest that, in relation to the control (without MeJ), the increase of both taxanes was considerable, but what is more significant is that taxol increased more than baccatin III in the case of line I and *vice versa* in line II. On the basis of previously reported studies by Yukimune et al. [25], this fact suggests that in line I MeJ contributed to taxol production not only by activating the biosynthetic steps from geranyl-geranyl diphosphate to baccatin III, but also those from baccatin III to taxol.

The yield of baccatin III, like that of taxol, was markedly reduced by the addition of fosmidomycin together with 100 μM MeJ to the production medium, especially at the concentrations of 200 and 300 μM. However, the response to this inhibitor also clearly depended on the cell line considered. Thus, cell line I presented the greatest sensitivity to the fosmidomycin action, its baccatin III levels being reduced by up to 9.8-, 11.5- and 13-fold at days 7, 14 and 21 after the addition of 300 μM fosmidomycin. The lowest decrease of baccatin III content was observed in cell line II, its production falling during the same culture period by only 3.9-, 10.8- and 3.0-fold, respectively, even when the inhibitor was added at its highest concentration. Cell line III presented an intermediate sensitivity.

When comparing the concentration of baccatin III of the elicited and non-elicited lines in presence of fosmidomycin (Fig. 2), it can be seen that, as for taxol, the total content was always higher in the elicited lines. Aside from indicating effective elicitation by MeJ, this clearly suggests that fosmidomycin acts as a specific inhibitor of the non-mevalonate pathway producing IPP but without affecting the conversion of IPP into the aforementioned taxanes. At the same time, the fact that the addition of the inhibitor in non-elicited lines significantly ($p \leq 0.01$) reduced but did not completely inhibit the baccatin III yield, supports the observation that in our cell lines the translocation of IPP from cytosol to plastids was also involved in baccatin III biosynthesis.

3.3.2. Utilization of mevinolin as an inhibitor

As previously mentioned, mevinolin competitively inhibits the binding of the natural substrate hydroxymethylglutary-CoA to the active site of the enzyme hydroxymethylglutary-CoA reductase, and consequently, blocks the synthesis of cytosolic IPP. Thus, if cytosolic IPP transport to the plastids is involved in the biosynthesis of the taxane ring of taxol and baccatin III, the presence of mevinolin should have inhibited the synthesis of both taxanes in our *T. baccata* cell lines.

With respect to taxol, it is evident that in line I the addition of 1, 3 or 5 μM mevinolin to the production medium, with or without 100 μM MeJ, resulted in a notable inhibition of its total content throughout the culture period (Fig. 3). As can be seen, compared with the values achieved when line I was treated by the elicitor alone, the average decrease in taxol was 2.9-, 3.3- and 3.8-fold, respectively, and compared to the line grown in control conditions (without the elicitor), the average decrease was 2.9-, 3.3- and 3.7-fold, respectively. However, in contrast with the marked reduction of taxol accumulation observed in all three lines when treated with fosmidomycin, both with or without the elicitor (see Fig. 2), the addition of mevinolin surprisingly resulted in higher taxol values in line II at the end of the culture (28 days), while the reduction in line III was less marked. The increase in taxol in line II was particularly notable when treated with 100 μM MeJ and the highest mevinolin concentration. In agreement with previous observations [35], this might be ascribed to a mevinolin tolerance of the cell line, which, consequently, might result in a much higher cytoplasmic IPP concentration, particularly at the end of the culture. Cell cultures of *Solanum xanthocarpum* selected for their enhanced tolerance to mevinolin exhibit an HMGR enzyme activity 2.5-fold higher than normal cell lines, and the author suggests a probable amplification of HMGR in the presence of mevinolin as the underlying cause [36]. Our data agree with this suggestion, although much remains to be tested to validate this interpretation.

In contrast to its effect on taxol, mevinolin notably reduced the total baccatin III content in the three lines cultured in presence of 100 μM MeJ. In comparison with the content of lines grown only with the elicitor, it was, on average, 4.3-, 2.3- and 2.0-fold lower in the presence of 1, 3 or 5 μM mevinolin, respectively (Fig. 3). However, with respect to the values in the control conditions, the total baccatin III content of line II remained largely unchanged in presence of mevinolin alone, and was always significantly ($p \leq 0.01$) higher in the presence of the inhibitor together with 100 μM MeJ. These results support the previous observation that under our cultivation conditions, cell line II was tolerant to mevinolin.

On the other hand, as observed with fosmidomycin (Fig. 2), when comparing both taxol and baccatin III concentration in the elicited and non-elicited lines treated with mevinolin (Fig. 3), total content was clearly higher in the elicited lines, which consequently also indicates effective elicitation by 100 μM MeJ. This was particularly evident for line II, which presented levels of total taxol and baccatin III that were, on average, 1.8- and 3.6-fold lower when growing with mevinolin alone. Considering the aforementioned mevinolin tolerance of this cell line, from these results it could also be deduced that its higher taxol and baccatin III contents were a consequence of higher IPP cytoplasmic

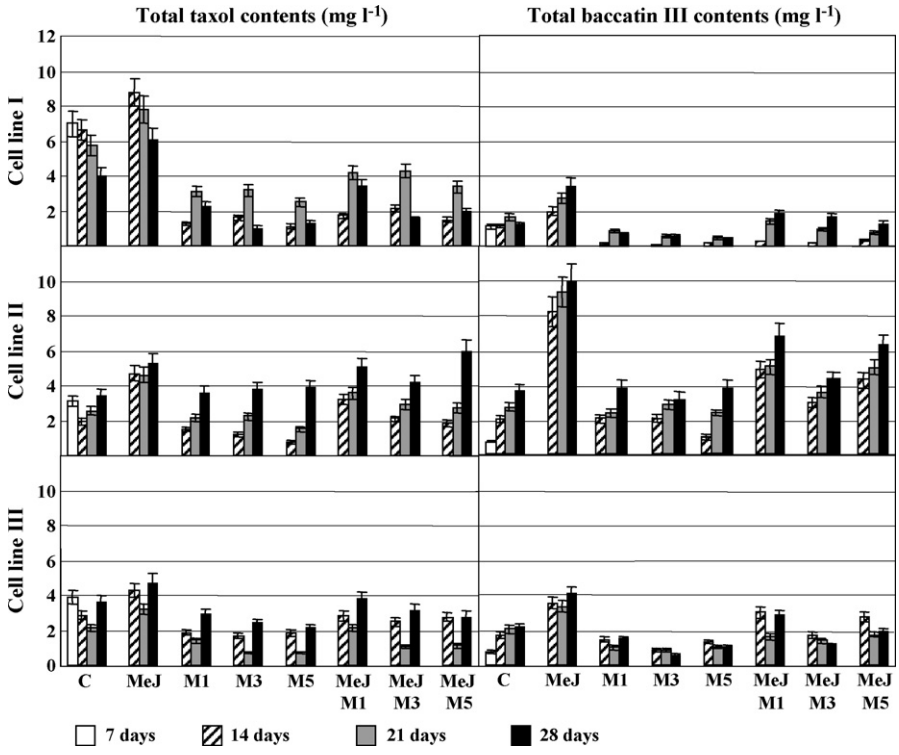

Fig. 3. Comparison of total content (cell-associated + extracellular) of taxol and baccatin III in *T. baccata* cell lines using mevinolin as the inhibitor. Cells in their initial stationary growth phase were cultured for 28 days in a selected production medium for the yield of taxol and baccatin III, either with 100 μM methyl jasmonate, or without the elicitor (control), or with different concentrations of mevinolin (1, 3 and 5 μM) in presence and absence of the elicitor. The different treatments were initiated on day 7, and in all cases the inoculum consisted of 100 g of cells l^{-1}. F, fosmidomycin; M, mevinolin; MeJ, methyl jasmonate. Data represent average values from six separate experiments ± S.D.

concentration. It should be noted that according to Soler et al. [16], the IPP uptake into the plastids depends on the IPP concentration.

4. Conclusions

Using three different cell lines of *T. baccata*, induced or not by MeJ, and several concentrations of the specific inhibitors fosmidomycin and mevinolin, we set out to study the dynamics of taxol and baccatin III production and their dependence on IPP derived from the classical mevalonate pathway in the cytosol and the alternative MEP pathway in plastids. Among the literature about the translocation of IPP from the cytosol to plastids for the taxane ring biosynthesis, the work of Srinivasan et al. [23] suggests that IPP formed through the mevalonate pathway could collaborate in the taxol production during the early growth phase of *Taxus* cells. On the contrary, Wang et al. [37], after supplementing two translocator inhibitors to *T. chinensis* cell suspensions, proposes that IPP translocation through plastidic membranes only takes place in the late growth phase of culture. However, to our knowledge, with the exception of our preliminary study [22], there is no information about the reduction of taxane production in *Taxus* cell cultures caused by the two metabolic inhibitors.

On the basis of our results, it can be inferred that the cell line type was an important variable for taxol and baccatin III production, but the biosynthesis of both taxanes was reduced by fosmidomycin to a much greater extent than mevinolin, irrespective of the presence of 100 μM MeJ. These results show that the biosynthesis of the IPP responsible for the taxane ring formation takes place mainly via the non-mevalonate pathway, which fosmidomycin blocks. However, since the taxane production also decreased in cultures treated with mevinolin, it would seem both pathways are involved, suggesting that cytosolic IPP is transported to the plastids to be used as a precursor of the taxane ring. In such a case, IPP and/or other prenyl diphosphates need to pass through the plastid membranes. Soler et al. [16] have shown that plastids of *Vitis vinifera* are capable of cytoplasmic IPP uptake, and De-Eknamkul and Potduang [38] have reported that the isoprene units used for the synthesis of phytosterols in *Croton sublyratus* callus cultures have a double origin, suggesting an active exchange between the plastidial and cytosolic compartments. The studies of McCaskill and Croteau [17] also provide evidence for a significant amount of exchange of IPP or other prenyl diphosphates between subcellular compartments, depending on the tissues, the development stage of involved cells and the plant culture system.

In addition, the fact that the taxol and baccatin III yield was never completely inhibited by fosmidomycin also shows that their biosynthesis could rely on the IPP or other precursors coming from the mevalonate pathway. At the same time, the reduction of growth observed in our mevinolin-treated cell lines

could have contributed to an increase in the accumulation and/or availability of terpenoids formed through the non-mevalonate pathway. Bearing in mind that we use cells in their stationary growth phase growing in a selected production medium that promotes taxol and baccatin III production but not cell growth, our *T. baccata* cell lines could have reacted by stimulating mechanisms involved in the exchange of intermediates in the biosynthesis of isoprenoids. We believe that our results might help to understand the participation of both IPP sources in taxane biosynthesis in order to develop rational strategies for the bioengineering of taxol and baccatin III production.

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References

[1] D. Guénard, F. Guéritte-Voegelein, P. Potier, Taxol and taxotere: discovery, chemistry, and structure–activity relationships, Acc. Chem. Res. 26 (1993) 160–167.
[2] R.E.B. Ketchum, D. Gibson, R. Croteau, M.L. Shuler, The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate, Biotechnol. Bioeng. 62 (1999) 97–105.
[3] A. Navia-sorio, H. Garden, R.M. Cusidó, J. Palazón, A.W. Alfermann, M.T. Piñol, Taxol® and baccatin III production in suspension cultures of *Taxus baccata* and *Taxus wallichiana* in an airlift bioreactor, J. Plant Physiol. 159 (2002) 97–102.
[4] R.M. Cusidó, J. Palazón, M. Bonfill, A. Navia-Osorio, C. Morales, M.T. Piñol, Improved paclitaxel and baccatin III production in suspension cultures of *Taxus media*, Biotechnol. Prog. 18 (2002) 418–423.
[5] B.J. Kim, D.M. Gibson, M.L. Shuler, Effect of subculture and elicitation on instability of taxol production in *Taxus* sp. suspension cultures, Biotechnol. Prog. 20 (2004) 1666–1673.
[6] M. Seki, C. Ohzora, M. Takeda, S. Furusaki, Taxol (paclitaxel) production using free and immobilized cells of *Taxus cuspidata*, Biotechnol. Bioeng. 53 (1997) 214–219.
[7] S. Bentebibel, E. Moyano, J. Palazón, R.M. Cusidó, M. Bonfill, R. Eibl, M.T. Piñol, Effects of immobilization by entrapment in alginate and scale-up on taxol and baccatin III production in cell suspension cultures of *Taxus baccata*, Biotechnol. Bioeng. 89 (6) (2005) 647–655.
[8] G.M. Cragg, S.A. Schepartz, M. Suffmess, M.R. Grever, The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents, J. Nat. Prod. 56 (1993) 1657–1668.
[9] H.K. Lichtenthaler, The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 47–65.
[10] W. Eisenrich, B. Mewnhard, P.J. Hylands, M.H. Zenk, A. Bacher, Studies on the biosynthesis of taxol: the taxane carbon skeleton is not of mevalonoid origin, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 6431–6436.
[11] A. Lansing, M. Haertel, M. Gordon, H.G. Floss, Biosynthetic studies on taxol, Planta Med. 57 (2) (1991) A83.
[12] L.O. Zamir, M.E. Nedea, S. Belair, F. Sauriol, O. Mamer, E. Jacqmain, F.I. Jean, F.X. Garneau, Biosynthetic building blocks of *Taxus canadiensis* taxanes, Tetrahedron Lett. 33 (1992) 5235–5236.
[13] W. Knöess, B. Reuter, J. Zapp, Biosynthesis of the labdane diterpene marrubiin in *Marrubium vulgare* via a non-mevalonate pathway, Biochem. J. 326 (1997) 449–454.
[14] N. Totté, L. Charon, M. Rohmer, F. Compernolle, I. Baboeuf, J.M.C. Geuns, Biosynthesis of the diterpenoid steviol, an ent-kaurene derivative from Stevia rebaudiana Bertoni, via the methylerythritol phosphate pathway, Tetrahedron Lett. 41 (2000) 6407–6410.
[15] K.P. Adam, J. Zapp, Biosynthesis of isoprene units of chamomile sesquiterpenes, Phytochemistry 48 (1998) 953–959.
[16] E. Soler, M. Clastre, B. Bantignies, G. Maring, C. Ambid, Uptake of isopentenyl diphosphate by plastids isolated from *Vitis vinifera* L. cell suspensions, Planta 187 (1993) 324–329.
[17] D. McCaskill, R. Croteau, Some caveats for bioengineering terpenoid metabolism in plants, Trends Biotechnol. 16 (8) (1998) 349–355.
[18] T.J. Bach, H.K. Lichtenthaler, Mevinolin a highly specific inhibitor of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase of radish plants, Z. Naturforsch. 37 (1982) 46–50.
[19] T. Kuzuyama, T. Shimizu, S. Takahashi, H. Seto, Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis, Tetrahedron Lett. 39 (1998) 7913–7916.
[20] R.M. Cusidó, J. Palazón, A. Navia-Osorio, A. Mallol, M. Bonfill, C. Morales, M.T. Piñol, Production of taxol and baccatin III by a selected *Taxus baccata* callus line and its derived cell suspension culture, Plant Sci. 146 (1999) 101–107.
[21] O.L. Gamborg, R.A. Miller, Nutrient requirements of suspension cultures of soybean root cells, Exp. Cell. Res. 50 (1968) 151–158.
[22] J. Palazón, R.M. Cusidó, M. Bonfill, C. Morales, M.T. Piñol, Inhibition of paclitaxel and baccatin III accumulation by mevinolin and fosmidomycin in suspension cultures of *Taxus baccata*, J. Biotechnol. 10 (2003) 157–163.
[23] V. Srinivasan, V. Ciddi, V. Bringi, M.L. Shuler, Metabolic inhibitors, elicitors and precursors as tools for probing yield limitation in taxane production by *Taxus chinensis* cell cultures, Biotechnol. Prog. 12 (1996) 457–465.
[24] A.G. Fett-Neto, F. DiCosmo, Taxol® and taxane production by cell culture Encyclopedia of Molecular Biology and Molecular Medicine, vol. 6, VCH Verlags gesllschaft, Weinheim, 1997, pp. 10–17.
[25] Y. Yukimune, H. Tabata, Y. Higashi, Y. Hara, Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures, Nat. Biotechnol. 14 (1996) 1129–1132.
[26] J.M. Widholm, The use of fluorecein diacetate and phenosafranine for determining viability of cultured plant cells, Stain Technol. 47 (1972) 189–194.
[27] M. Bonfill, J. Palazón, R.M. Cusidó, S. Joly, C. Morales, M.T. Piñol, Influence on taxane production and 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in *Taxus media* cells, Plant Physiol. Biochem. 41 (2003) 91–96.
[28] M.A. Holden, P.R. Holden, M.M. Yeoman, Improvement by screening and selection techniques, in: R.J. Robins, M.J.C. Rhodes (Eds.), Manipulating Secondary Metabolism in Culture, Cambridge University Press, Cambridge, 1988, pp. 57–65.
[29] R.D. Hall, M.A. Holden, M.M. Yeoman, in: Y.P.S. Bajaj (Ed.), Immobilization of Higher Plant Cells, Biotechnology in Agriculture and Forestry, vol. 4, Medicinal and Aromatic Plants I, Springer-Verlag, Berlin, 1988, pp. 136–156.
[30] A. Hemmerlin, J.-F. Hoefflers, O. Meyer, D. Tritsch, I.A. Kagan, C. Grosdemange-Billiard, M. Rohmer, T.J. Bach, Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells, J. Biol. Chem. 278 (29) (2003) 26666–26676.
[31] R. Croteau, T.M. Kutchan, N.G. Lewis, Natural products (secondary metabolites), in: B. Buchanan, R. Jones (Eds.), Biochemistry and Molecular Biology of Plants, ASPB, Rockville, MD, 2000, pp. 1250–1268.
[32] U. Eilert, F. Constabel, W.G.W. Kurz, Elicitor stimulation of monoterpene indol alkaloid in suspension cultures of *Catharanthus roseus*, J. Plant Physiol. 126 (1986) 11–12.
[33] M. Fellermeier, K. Kis, S. Sagner, U. Maier, A. Bacher, M.H. Zenk, Cell-free conversion of 1-deoxy-D-xylulose 5-phosphate and 2-C-methyl-D-erythritol 4-phosphate into β-carotene in higher plants and its inhibition by fosmidomycin, Tetrahedron Lett. 40 (1999) 2743–2746.
[34] M. Hezari, R.E.B. Ketchum, D.M. Gibson, R. Croteau, Taxol production and taxadiene synthase activity in *Taxus canadensis* cell suspension cultures, Arch. Biochem. Biophys. 337 (1997) 185–190.

[35] T.J. Bach, D.H. Rogers, H. Rudney, Detergent-solubilization, purification, and characterization of membrane-bound 3-hydroxy-3-methylglutaryl-coenzyme A reductase from radish seedlings, Eur. J. Biochem. 154 (1986) 103–111.

[36] P.C. Josekutty, Selection and characterization of *Solanum xanthocarpum* cell line with augmented steroid metabolism, S. Afr. J. Bot. 64 (1998) 238–243.

[37] Y.D. Wang, Y.J. Yuan, J.Ch. Wu, Translocation of isopentenil pyrophosphate for taxol biosynthesis in suspension cultures of *Taxus chinensis* var. *mairei*, Plant Cell Tissue Organ Cult. 74 (2003) 283–288.

[38] W. De-Eknamkul, B. Potduang, Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units, Phytochemistry 62 (2003) 389–398.