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6. Biotechnological production of taxanes: A molecular approach

Rosa M. Cusidó[1], Heriberto Vidal[1], Ana Gallego[2], Maryam Abdoli[3] and Javier Palazón[1]

[1]Secció de Fisiologia Vegetal, Facultat de Farmàcia, Universitat de Barcelona E-08028 Barcelona, Spain; [2]Departament de Ciències Experimentals i de Salut, Universitat Pompeu Fabra, E-08003 Barcelona, Spain; [3]Department of Plant Breeding & Biotechnology Faculty of Agriculture Tarbiat Modares University, Tehran, Iran

Abstract. Plant cell cultures constitute a promise for the production of a high number of phytochemicals, although the majority of bioprocesses that have been developed so far have not resulted commercially successful. An overview indicates that most of the research carried out until now is of the empirical type. For this reason, there is a need for a rational approach to the molecular and cellular basis of metabolic pathways and their regulation in order to stimulate future advances.

The empirical investigations are based on the optimization of the culture system, exclusively considering input factors such as the selection of cellular lines, type and parameters of culture, bioreactor design and elicitor addition, and output factors such as cellular growth, the uptake system of nutrients, production and yield. In a rational approach towards the elucidation of taxol and related taxane production, our group has studied the relationship between the taxane profile and production and the expression of genes codifying for enzymes that participate in early, intermediate and late steps of their biosynthesis in elicited *Taxus* spp cell cultures. Our results show that

Correspondence/Reprint request: Dr. Rosa M. Cusidó, Secció de Fisiologia Vegetal, Facultat de Farmàcia Universitat de Barcelona, E-08028 Barcelona, Spain. E-mail: rcusido@ub.edu

elicitors induce a dramatic reprogramming of gene expression in *Taxus* cell cultures, which likely accounts for the enhanced production of taxol and related taxanes and we have also determined some genes that control the main flux limiting steps. The application of metabolic engineering techniques for the production of taxol and taxanes of interest is also discussed.

Introduction

One of the most important characteristics of plants is their ability to form compounds that, though not essential for completing the plant's life cycle, are extremely useful for their adaptation to the environment. Many of these secondary compounds are considered of high value by the chemical and pharmaceutical industry due to their therapeutic and adaptogenic properties. It is estimated that 50% of drugs used in the preparation of medicines are natural products of plant secondary metabolism. Therefore, having been used as drugs since ancient times, by all the cultures ever to exist on our planet, plants remain an inexhaustible source of new medicines [1].

The natural exploitation of medicinal plants by means of their culture and extraction of their main active elements is fraught with geographical, environmental, economical and political risks and difficulties. So it is hardly surprising that one of the main objectives of plant biotechnology is the large-scale production of secondary compounds with industrial applications. The biotechnological production of secondary metabolites using different *in vitro* techniques has been the focus of considerable research in recent years. Besides their use as pharmaceuticals, fragrances, colorants, aromatics and pesticides, secondary metabolites have recently been recognized as important disease-preventative ingredients in food, resulting in the term nutraceuticals [2].

The advantages of this technological approach over conventional agriculture include: independence from geographical and seasonal variations and environmental factors as well as from political interference; a defined production system that ensures a continuous supply of products of uniform quality and quantity; the possibility of obtaining new compounds not originally formed in the cultivated plant; an efficient recovery of the product of interest; an extremely fast production and also stereo- and regiospecific biotransformations from inexpensive precursors.

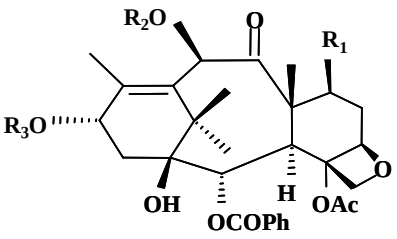
Although *in vitro* culture of plant cells and tissues is routinely used in agriculture for plant breeding and micropropagation, its application in the commercial production of pharmaceuticals and other secondary compounds of interest is limited to only a few procedures [3-5].

An essential requirement for the application of *in vitro* plant culture techniques in industrial-scale production of therapeutic compounds is that its cost is lower than culturing the intact plant or other procedures. Given the

complexity of these techniques, together with the costs involved in sterilization and use of bioreactors, the new technologies can only be assigned to the production of compounds of high added value.

Taxol (Fig. 1) is a secondary compound of great interest due to its wide spectrum of antineoplastic action. The main limitation to its therapeutic use is a limited supply from its natural source, the inner bark of *Taxus* species [6]. One of the most successful anticancer drugs to be developed in the past 50 years, taxol produced by Bristol-Myers Squibb (BMS) reached worldwide sales of $1.5 billion in 1999. Although this company subsequently reported a 24% decrease in taxol sales, from $422 millions in 2006 to $385 millions in 2007 [BMS 2008 Annual Report], this reduction is primarily due to patent expiry and increased generic competition in Europe, as well as generic entry in Japan during the third quarter of 2006 [1]. The total market for taxol remains well above $1 billion per year [www.strategyr.com/Bulk Paclitaxel Market Report.asp] and continues to expand, with new clinical uses anticipated [7].

To combat the patent expiries, supergeneric versions of taxol, such as Cell Therapeutics' Xyotax (polyglutamate paclitaxel) and Abraxis Oncology's Abraxane (nanoparticle albumin-bound paclitaxel) have been developed, offering significant advantages over taxol in terms of adverse effects and drug delivery.



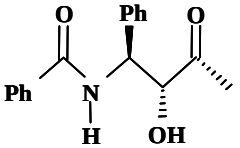
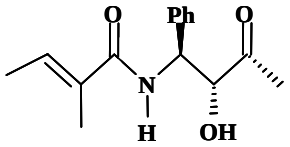
Taxol ®	**R_1 = OH; R_2 = Ac; R_3 =**	
Cephalomaninne	**R_1 = OH; R_2 = Ac; R_3 =**	
Baccatin III	**R_1 = OH; R_2 = Ac; R_3 = H**	
10-Deacetylbaccatin III	**R_1 = OH; R_2 = H; R_3 = H**	

Figure 1. General structure of taxol and related taxoids. Ac: acetyl group; Ph: phenyl group.

Sales of Abraxane rose to $275 millions in 2009 [www.BioPortafolio.com: emerging oncology treatments: a focus on targeted therapeutics, supergeneric reformulations and supportive care] reflecting the growing market for taxol and its derivatives. Consequently, taxol and its semisynthetic precursors, including baccatin III, 10-deacetylbaccatin III and cephalomaninne (Fig. 1), are considered to be high added value secondary compounds.

Plant cell cultures constitute a promise and almost a reality for the controlled production of a considerable number of secondary compounds of interest, including taxol and its derivatives, although most of the bioprocesses developed up to now have not turned out to be commercially viable. Much of the research in this field carried out during the last decades has been empirical, concentrating on optimizing the culture system via known in-put factors (cell line selection, culture media, culture conditions, elicitors, bioreactor culture, etc.) and out-put factors (growth, production, yield, etc.) However, the most successful strategies and technologies depend on a rational approach to the molecular bioprocesses of plant cells, based on the understanding of biosynthetic pathways and their regulation.

1. Empirical studies

Biotechnological production of taxol has been studied since the early 1990s. Early work carried out by different research teams [8-10] showed that calli and cell suspensions obtained from young stems of *Taxus* sp. were able to produce taxol at least to the same extent as the intact plant.

In order to increase the productivity of taxol and related taxoids in cell and tissue cultures, various strategies, including optimisation of culture conditions, selection of high-producing cell lines, use of elicitors, and addition of precursors, have been examined by many researchers [11-18]. There are excellent reviews about the biotechnological production of taxol and current knowledge of its metabolism in cell cultures [19-25].

Establishment of *Taxus* spp. cell cultures

In order to obtain cell suspensions, initially on a small scale and then at bioreactor level, it is first necessary to establish fast-growing callus cultures from which the cell suspensions are derived. We obtained *Taxus* callus cultures from young stems of 3-4 year-old yew trees cultured in optimum conditions [26]. In order to develop a biotechnological system for an efficient taxol production, we optimized the culture conditions by assaying several basic media, plant growth regulators, sugar supplements, etc. As secondary metabolite production in plant cell cultures does not usually depend on

growth, a two-stage culture system was established. Plant cells are first cultured in a medium optimized for their growth, which is then replaced by a production medium that principally stimulates the biosynthesis of secondary metabolites [27]. This system has the added advantage of permitting the addition of biosynthetic precursors and elicitors when the secondary metabolite production is at its highest, that is, during the culture's second stage. Taxol production was clearly enhanced by transferring cells from the optimum growth medium to the optimum medium for taxane production.

Elicitor treatments

A high increase in taxol and baccatin III production was observed in the MeJ-supplemented culture medium of different T. baccata cell lines (Fig. 2). We have also supplemented the culture medium with a variety of elicitors, finding methyl jasmonate (MeJ) and coronatine (Cor) to be the most effective in promoting taxane biosynthesis and accumulation [27-31]. When comparing the maximum levels of taxane production in *T. media* cell cultures,

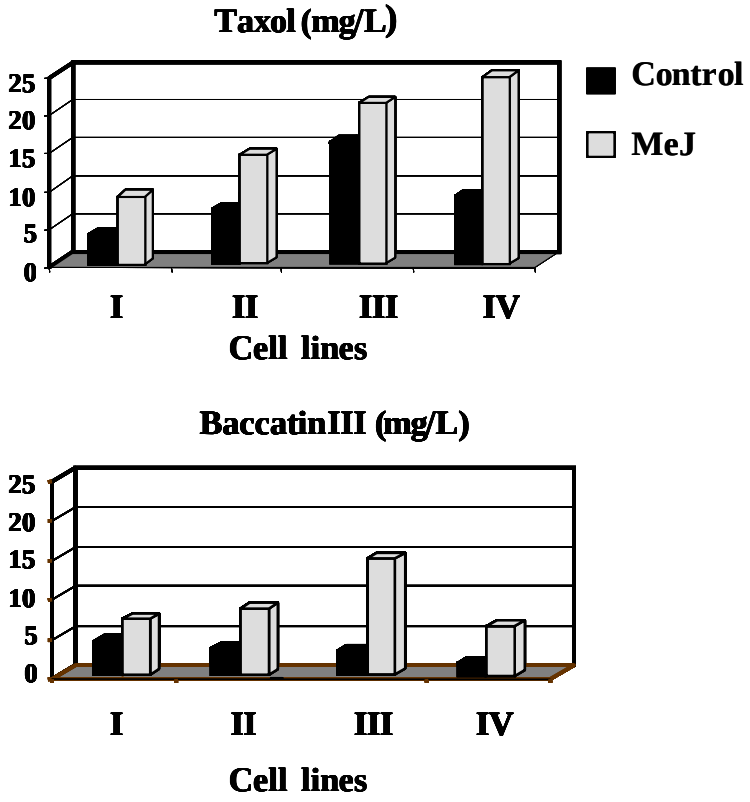


Figure 2. Taxol and baccatin III production in four different *T. baccata* cell lines treated with methyl jasmonate (MeJ, 100 μM) after 21 days of culture in the optimum medium for production.

we observed that the content of baccatin III in the Cor-treated cultures was 21.6- and 4.8-fold higher than in the control and MeJ-treated cultures, respectively, whereas MeJ elicitation increased baccatin III production 4.5-fold compared to the control. Taxol production under Cor treatment was 3.6- and 9.0-fold greater than in MeJ-treated and control cultures, respectively (Fig. 3) [31].

A notably high level of baccatin III accumulated in the Cor-treated cultures (52 mg/L) at day 16, when the productivity rate reached 3.3 mg L^{-1} day^{-1}. This is a very promising result, since new taxol-related compounds with improved efficacy and less toxicity are currently being sought, and most of them are being obtained semisynthetically from the natural precursor baccatin III [25].

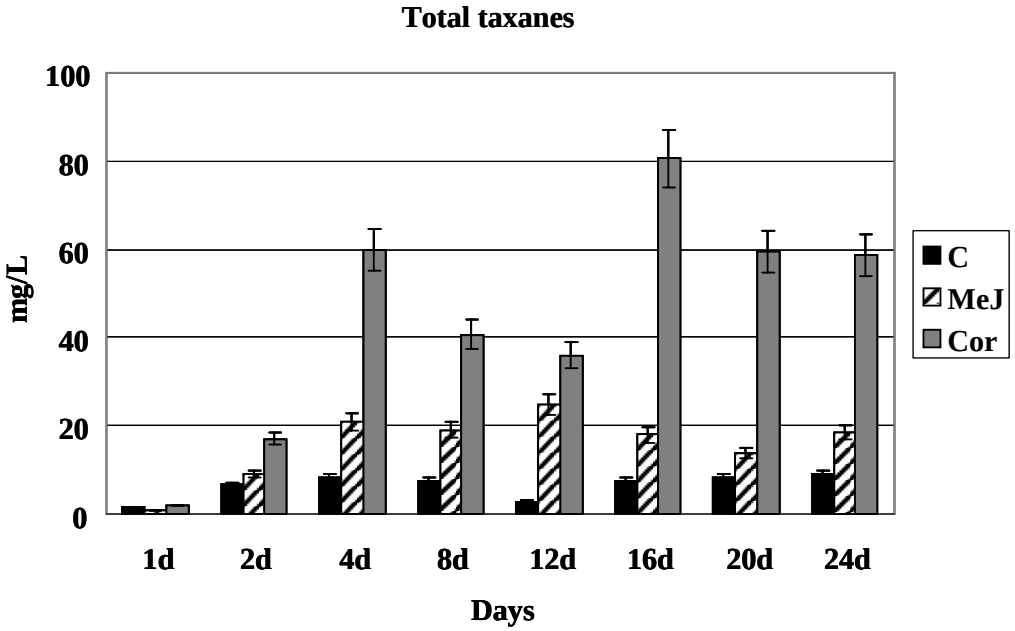


Figure 3. Total taxane content (cell associated + extracellular) in the TXS cell line growing for 24 days in the production medium without (Control, C) and with 100 μM methyl jasmonate (MeJ) or 1 μM coronatine (Cor). Data are average values from three replicates ± SD.

Scale-up to bioreactor culture

After optimizing the culture conditions, as well as environmental and physical factors, the next step for the profitable production of valuable secondary compounds is to scale up the culture. Bioreactors are used since they can be applied in large-scale production and allow a close control of culture conditions. The production of taxol and related taxanes in a variety of bioreactors has been described by several researchers [24, 32-34]. We compared the taxane levels achieved by *T. media* cell cultures grown in

Erlenmeyer flasks and a stirred bioreactor, in both cases using the same cell line and production medium supplemented with MeJ. Scaling up was found to improve the production of taxol and baccatin III 2.4- and 9 –fold, respectively (Fig. 4) [33].

Numerous studies have also reported that the immobilization of plant cell suspensions enhances the production of valuable plant metabolites [35-37]. This increase is probably due to the higher cell concentration, better cell-cell contact and more favorable conditions for cell differentiation.

In our case, taxol production clearly improved when cell suspensions were immobilized in 2% alginate beads and cultured in a bioreactor, using the optimum MeJ-supplemented medium for taxol biosynthesis. When taxol production was at its highest, at day 16, its levels were more than 5 times higher than those obtained by the same cell line growing freely in the same conditions (Fig. 5) [33].

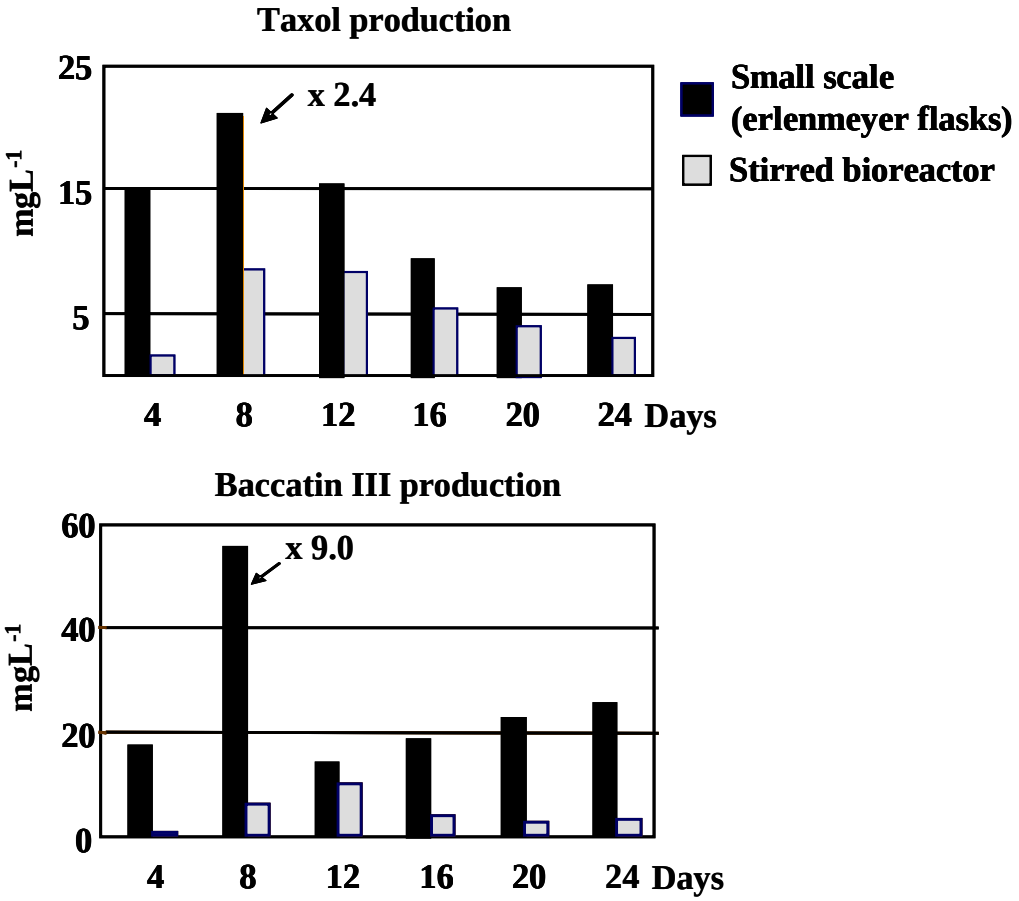


Figure 4. Total content (cell-associated + extracellular) of taxol and baccatin III in small-scale *T. baccata* cell cultures (Erlenmeyer flasks) and in a 5 L stirred bioreactor. In both cases cells were grown for 24 days in the optimum medium for production supplemented with methyl jasmonate (100 μM).

Figure 5. Configuration of a 5 L air-lift bioreactor used to culture *T. baccata* immobilized cells.

2. Rational approach

In order to convert the potential of cell cultures into a commercial reality, after empirical optimization studies, a molecular approach is required, since it is necessary to know how the factors that improve production also affect molecular processes in producer cells, such as metabolic profiles, gene expression, enzymatic activity, etc.. These studies allow a better understanding of the biosynthetic pathways and their regulation.

The taxol biosynthetic pathway consists of 19 metabolic steps, 12 of which are known and have had their corresponding genes cloned and sequenced. The functions of the remaining 7 steps are understood, but the genes involved have not yet been sequenced [38-41].

In order to understand the limiting steps of the biosynthetic production of taxol, as well as its metabolic profile, it is necessary to compare the transcriptome of the known genes involved in taxol biosynthesis in both elicited and unelicited cultures [41-44].

Transcriptomic profiling

To investigate the elicitor mode of action in taxane biosynthesis and the relationship between gene expression and the pattern of taxane production, we used qRT-PCR to profile the expression of genes encoding enzymes involved in taxol biosynthesis in *T. media* cell cultures elicited with MeJ or Cor [31]. The studied genes and the enzymes they encode were: *TXS*

(taxadiene synthase) and *T13αOH* (taxadiene 13α-hydroxylase), both involved in early synthetic steps; *T7βOH* (taxane 7β-hydroxylase), *T2αOH* (taxane 2α-hydroxylase) and *DBAT* (10-deacetylbaccatin III-10β-*O*-acetyltransferase), which control intermediate synthetic steps; and *PAM* (phenylalanine aminomutase), *BAPT* (baccatin III-3-amino-13-phenylpropanoyltransferase) and *DBTNBT* (3'*N*-benzoyl transferase), which are involved in the last synthetic steps (Fig. 6).

Gene expression was determined from 1 h to 4 days after elicitation. Subsequent transcript accumulation is not shown in the figures because we observed that the highest expression and induction thereof occurs in this early period [42, 45]. Similar results have been reported by other research groups [41, 46].

The expression of the *TXS* gene, which controls the first committed step of the taxol biosynthetic pathway [47], was greatly enhanced by the presence of elicitors in the culture medium (Fig. 7). Maximum *TXS* transcript levels in the Cor-elicited cultures were 4.8-fold higher than in the control, observed at 24 h in both (Fig. 7). Although values were similar under both elicitors (140 and 130 times higher than the reference value) ($p<0.05$), it is notable that *TXS* transcript levels peaked 3 days earlier in Cor-treated cultures. We had previously

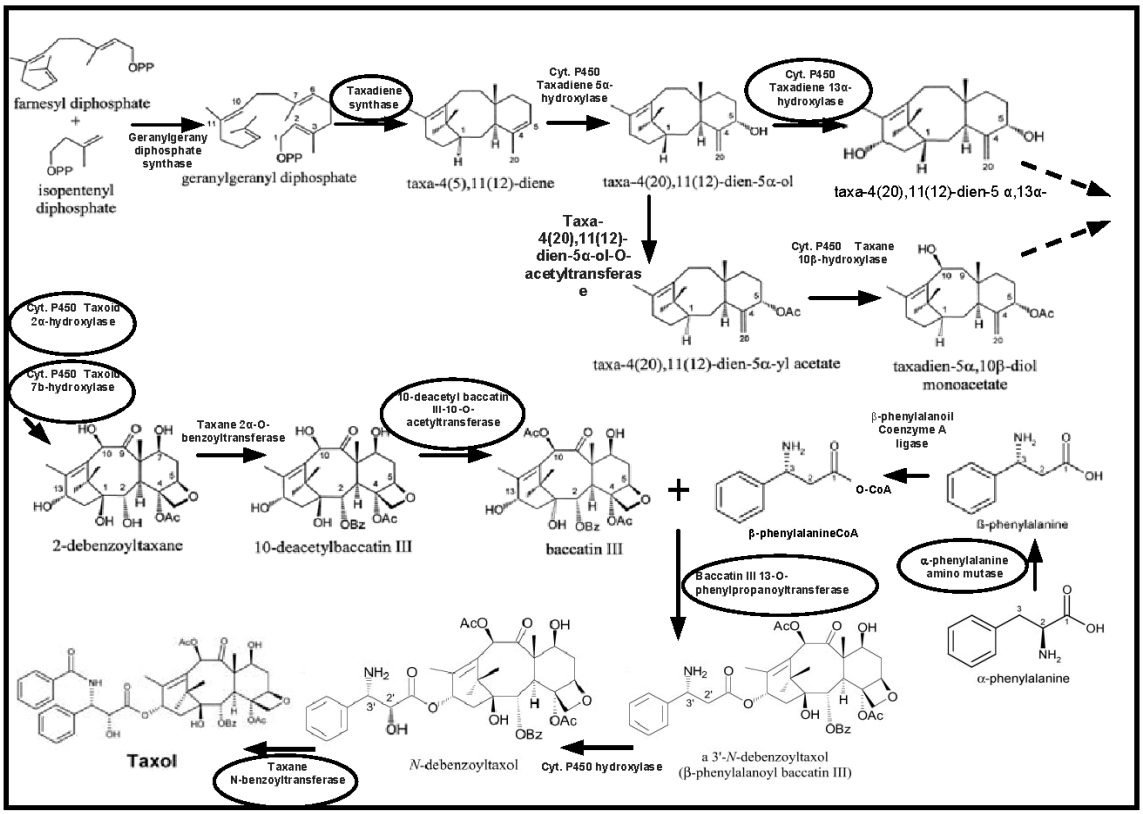


Figure 6. Taxol biosynthetic pathway. The enzymes controlling the steps discussed in the transcriptome study are highlighted with a circle.

shown that the *TXS* gene is highly induced in MeJ-elicited cell suspension cultures [42].

Taxadiene-13α-hydroxylase (T13αOH) is the enzyme that hydroxylates taxa-4(20),11(12)-dien-5α-ol to taxa-4(20),11(12)-dien-5α-13α-diol [48]. As shown in Fig. 7, significantly higher transcript accumulation of this gene was observed in Cor- compared with MeJ-treated cultures ($p>0.05$), and in both elicited cultures compared with the control ($p>0.05$). Results obtained by Nims *et al.* [41] indicate that *T13OH* expression increases during the first hours after MeJ treatment. In our case, although RNAm accumulation was evident after 1 h of elicitation, the highest level was achieved after 4 days. The difference is probably due to the use of different cell lines and culture systems.

It is known that there is a branch point after the biosynthesis of taxa-4(20),11(12)-dien-5α-ol, which can be the substrate either for an acetylation at C5OH and a subsequent hydroxylation at C10, or for an hydroxylation at C13 of the taxane skeleton, giving taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol or taxa-4(20),11(12)-dien-5α-13α-diol, respectively (Fig. 6). Although we did not study the expression of genes *(TAT* and *T10βOH*) involved in the metabolic branch leading to taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol, the high levels of the *T13OH* gene observed in our TXS cell line may suggest that, after elicitation with MeJ and Cor, taxol biosynthesis proceeds mainly through the step catalysed by the T13αOH enzyme. Nims *et al.* [41] also reported a preference for the T13αOH-side of the branch pathway in elicited *T. cuspidata* cell cultures, which confirms metabolic profiling data [49] showing that precursor flux leading to taxol is via 5α, 13α-diol through T13αOH, rather than 5α-yl-acetate derived from the alternative branch controlled by the TAT enzyme.

In the intermediate steps of taxol biosynthesis, the enzymes taxane-2α-hydroxylase (T2αOH) and taxane-7β-hydroxylase (T7βOH) catalyse the hydroxylation at the 2C and 7C positions of the taxane skeleton, respectively [50, 51] (Fig. 6). The two corresponding genes showed similar expression patterns, which were comparable to that of the *T13αOH* gene (Fig. 7).

The expression of the *DBAT* gene, corresponding to the enzyme responsible for the transformation of 10-deacetylbaccatin III into baccatin III (Fig. 7) [52], in MeJ-and Cor-treated cell cultures was already very high at 12 h, and continued increasing until days 1-2, decreasing thereafter to values similar to those observed at 12 h. The highest values observed in both cultures were 5 and 3 times higher, respectively, than in the control (Fig. 7).

The PAM gene, which encodes the enzyme responsible for the formation of β-phenylalanine from its isomer α-phenylalanine (Fig. 6) [53], is considered to be the first gene involved in the biosynthesis of the taxol lateral

chain. The expression of this gene in control cells remained very low throughout the 4 days of the study. In the elicited cultures (Fig. 7) two expression peaks were observed. The first occurred only 1 h after elicitation, with PAM transcript levels in the MeJ- and Cor-treated cultures being 43 and 63 times higher, respectively, than the reference value. The second peak, which was the maximum PAM transcript level achieved, was observed at day 4 under MeJ and day 2 under Cor treatment, being 52 and 93 times higher, respectively, than the reference value, and 29 and 53 times higher than in the control. It is notable that Cor was almost twice as effective as MeJ for the induction of the PAM gene.

In the final steps of the metabolic pathway (Fig. 6), the enzyme baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase (BAPT) [54] is responsible for binding the lateral chain to baccatin III, leading to taxanes with β-phenylalanine as a lateral chain. The enzyme N-benzoyl transferase (DBTNBT) [55] is involved in the last metabolic step leading to taxol. Control cultures grown in the production medium presented only a slight increase in the expression of the two corresponding genes over the 4 sampled days (Fig. 7). When comparing the elicitor treatments, the highest expression level of both genes was observed under MeJ, it being 1.3 times higher than the maximum under Cor. However, Cor caused an earlier activation, as shown in Fig. 7 (days 1-2 and 4, respectively).

Our results have shown that all the studied genes were induced to a variable extent by the presence of the elicitors MeJ (100 μM) and Cor (1 μM). The elicitors enhanced the expression of genes involved in the formation of the polyhydroxylated precursor of taxol and the phenylalanoyl CoA chain, but had a more limited effect on the last two steps of the taxol biosynthetic pathway (Figs. 6 and 7). The elicited cultures clearly showed different taxane patterns and gene expression profiles, suggesting differing action mechanisms, despite the structural similarity of Cor and the active jasmonate JA-Ile.

Our metabolic studies have demonstrated that the presence of Cor in the culture medium dramatically enhanced taxane production, particularly of baccatin III and to a lower extent taxol, the highest levels of both taxanes being 4.8- and 3.6-fold greater than in the MeJ-elicited cultures, and without affecting cell growth.

Taken as a whole, our results show that both MeJ and Cor induce a dramatic reprogramming of gene expression in *Taxus* cell cultures, which likely accounts for the enhanced production of taxol and related taxanes. Compared with MeJ, Cor elicitation did not result in a significantly higher induction of the studied taxane biosynthetic genes but peak expression levels were generally observed earlier. The resulting earlier availability of precursors could be responsible for the higher amount of total taxanes achieved in cultures elicited by Cor rather than MeJ (3.3-fold higher, on average).

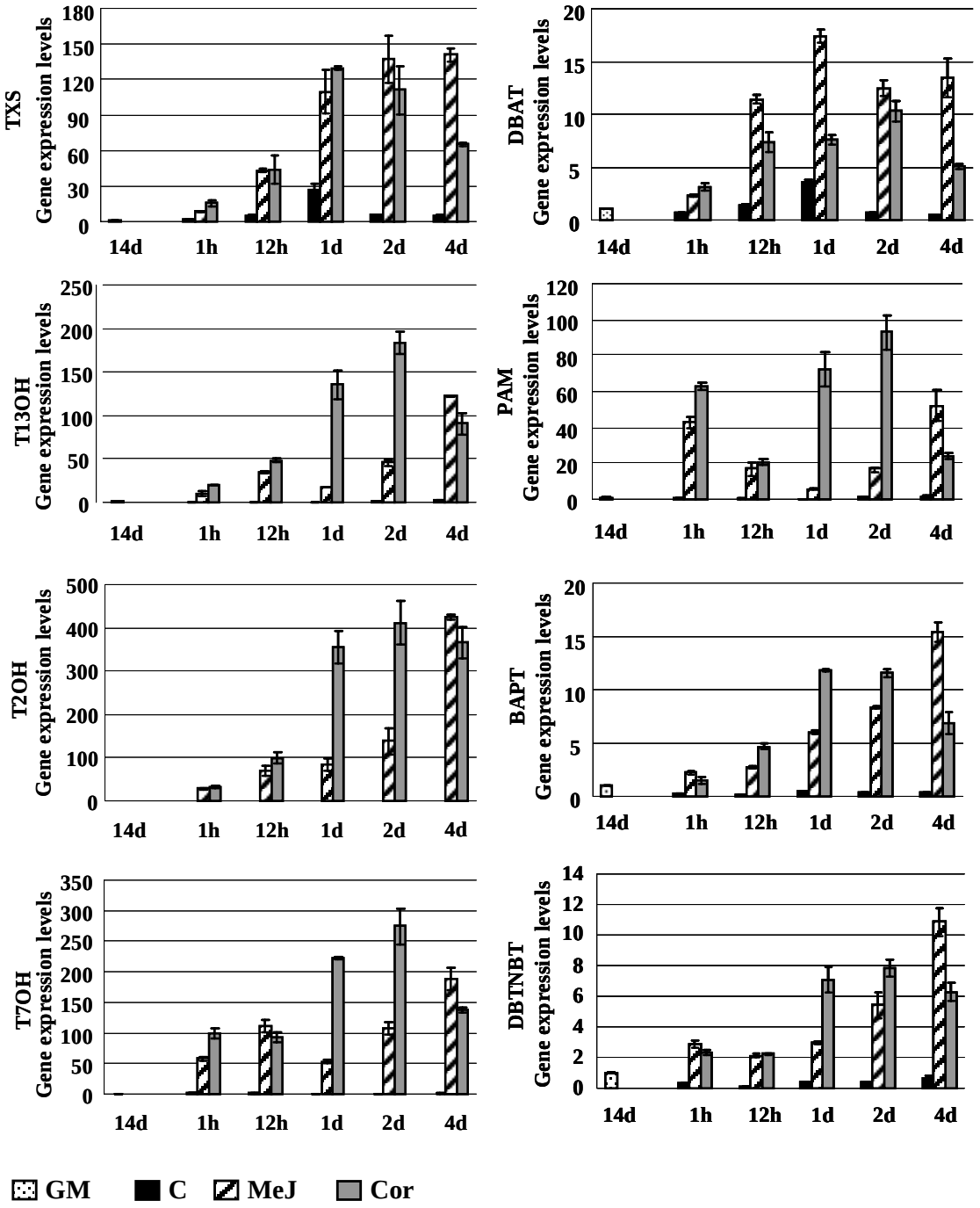


Figure 7. *TXS, T 13-OH, T 2-OH, T 7-OH, DBAT, PAM, BAPT* and *DBTNBT* gene expression in the TXS cell line cultured in the production media for 24 days under three different treatments: Control, 100 µM MeJ and 1 µM coronatine. GM: growth media; C: production media (control); Me: PM with MeJ; Co: PM with coronatine.

Determining the transcript profile of genes involved in taxol biosynthesis demonstrates that the presence of elicitors in the medium increases gene expression to varying degrees. The results also indicate that the transferases controlling the last steps of taxol biosynthesis probably also catalyze the

limiting steps, and consequently are ideal targets for metabolic engineering techniques in the enhancement of taxol production.

3. Metabolic engineering of *Taxus*

As *Taxus* species belong to the Gymnosperm class, they are very difficult to genetically transform. There have been many attempts to obtain transformed *Taxus* cell cultures using the *Agrobacterium* system or biolistic techniques, but with little success to date. In 1994, Han *et al.* [56] obtained gall cell lines after inoculating shoot segments of *T. baccata* and *T. brevifolia* with two strains of *A. tumefaciens*. In 2000, Furmanova and Syklowska-Baranek [57] established transformed root cultures of *T. x media* by directly infecting several kinds of explants with three strains of *A. rhizogenes*. In 2005, Ho *et al.* [58] described taxol production in MeJ-elicited *T. marei* cell cultures harboring the gene that encodes the enzyme 10-deacetyl baccatin III-10-O-actyl transferase (DBAT). More recently, Zang *et al.* [59], in *T. chinensis* cell cultures overexpressing the same gene, obtained a taxol production 1.7 times higher than that achieved by the untransformed cells. By using particle bombardment-mediated transformation, Vongpaseuth *et al.* [60] have successfully obtained a transient transformation of *Taxus* spp. with the aim of characterizing the genes involved in taxol biosynthesis and its regulation. Following the same transformation methodology, Li et al. [61] obtained *T. chinensis* cell cultures overexpressing a 9-*cis*-epoxycarotenoid dioxygenase gene, which encodes a key enzyme in the biosynthesis of abscisic acid (ABA), a phytohormone involved in plant responses to different types of stress. The taxol production in these transformed cell cultures was found to be 2.7-fold higher compared with untransformed cultures. However, apart from these studies, taxol production in transformed cell or root cultures has been scarcely explored.

We focused our attention on taxadiene synthase, the enzyme controlling the step that diverts GGPP from the metabolic pool to taxane biosynthesis, interested in gaining further insight into the key role it plays in taxane production overall. To this end, we established *T. media* cell cultures overexpressing the *txs* gene of *T. baccata*, but due to the difficulties of genetically transforming *Taxus* cell cultures with *A. tumefaciens,* we first obtained *T. media* transformed roots carrying this transgene (Fig. 8a), which were then dedifferentiated (Fig. 8b, c) [45]. Taxane production in the transformed cultures (Fig. 8d) was compared with an untransformed *T. media* cell line cultured in the same conditions. The highest taxane production was observed in the TXS cell line grown in the optimized production medium with MeJ (100 μM), it being 265% greater than in the untransformed control.

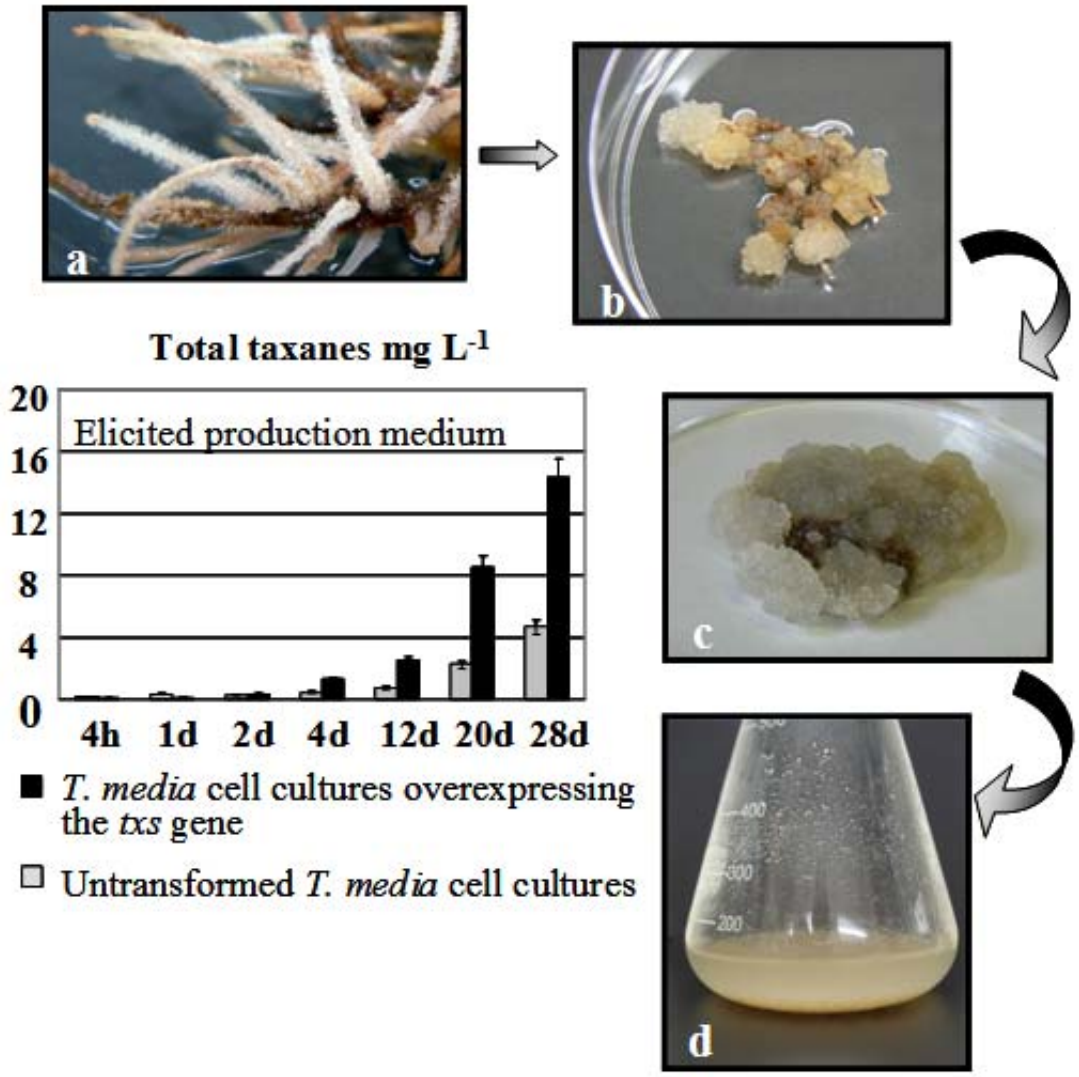


Figure 8. Steps followed to establish transformed cell cultures from transformed root cultures. Total taxane production in the *T. media* transformed cell cultures overexpressing the *txs* gene of *T. baccata*.

Notably, taxane levels as well as *txs* gene expression and TXS activity in both transformed and untransformed cell lines were clearly dependent on the elicitor action.

Knowledge of the genes involved in key steps of the taxol biosynthetic pathway and of how they are regulated, coupled with advanced metabolic engineering techniques, will allow transgenic cell cultures overexpressing target genes to be obtained, and will consequently lead to the establishment of highly productive cell cultures at an industrial level. We believe that our results could be useful for the development of rational strategies, including elicitation and metabolic engineering, to improve the yield of paclitaxel and the two related taxanes, 10-deacetylbaccatin III and baccatin III, which, as

previously mentioned, are used in the semisynthesis of the chemotherapeutic agent.

An aspect of taxane production that deserves highlighting is the mechanism of taxol excretion from cells, which could be enhanced by employing a two-phase culture system, so far not assayed in *T. baccata* cell suspensions. Future studies could combine an empirical and rational approach, assaying two-phase cultures to develop a biotechnological system for a high taxol production.

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