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Research article

Improved biotechnological production of paclitaxel in *Taxus media* cell cultures by the combined action of coronatine and calix[8]arenes

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ABSTRACT

Paclitaxel (PTX), a widely used anticancer agent, is found in the inner bark of several *Taxus* species, although at such low levels that its extraction is ecologically unsustainable. Biotechnological platforms based on *Taxus* sp. cell cultures offer an eco-friendlier approach to PTX production, with yields that can be improved by elicitation. However, the also limited excretion of target compounds from the producer cells to the medium hampers their extraction and purification. In this context, we studied the effect of treating *T. media* cell cultures with the elicitor coronatine (COR) and calix[8]arenes (CAL), nanoparticles that can host lipophilic compounds within their macrocyclic scaffold. The highest taxane production (103.5 $mg.L^{-1}$), achieved after treatment with COR (1 μM) and CAL (10 $mg.L^{-1}$), was 15-fold greater than in the control, and PTX represented 82% of the total taxanes analyzed. Expression levels of the flux-limiting PTX biosynthetic genes, BAPT and DBTNBT, increased after the addition of COR, confirming its elicitor action, but not CAL. The CAL treatment significantly enhanced taxane excretion, especially when production levels were increased by COR; 98% of the total taxanes were found in the culture medium after COR + CAL treatment. By forming complexes with PTX, the nanoparticles facilitated its excretion to the medium, and by protecting cells from PTX toxicity, its intra-and extra-cellular degradation may have been avoided. The addition of COR and CAL to *T. media* cell cultures is therefore a bio-sustainable and economically viable system to improve the yield of this important anticancer compound.

1. Introduction

The demand for therapeutic compounds of plant origin is growing constantly, but their market availability is insufficient to cover clinical needs, resulting in the over-exploitation of many plant species and degradation of their natural habitats, which is being exacerbated by climate change. There is therefore an urgent need to increase the supply of these compounds and reduce production costs. Plant biofactories offer a bio-sustainable and highly ecological alternative to plant cultivation in the field (Choi et al., 2008). Using *in vitro* culture processes and genetic engineering techniques, these systems have the potential to enhance the production of plant bioactive compounds. However, their application has had limited success to date, mainly because of incomplete knowledge of plant secondary metabolism and its control *in vitro* (Cusido et al., 2014).

Paclitaxel (PTX), also known by the commercial name taxol, is a secondary metabolite synthesized by the yew tree, although in very low quantities (0.002% dry weight) and is one of the most effective anticancer drugs ever developed. As diterpene compound, PTX is synthesized through 19 metabolic steps from geranylgeranyl diphosphate (GGPP), which is cyclized by taxadiene synthase to taxadiene, the first intermediate bearing the taxane skeleton (Suppl. Fig. 1). Subsequent metabolic steps, involving several hydroxylases and acyl/aroyl transferases, an epoxidation and oxidation, lead to the formation of the PTX precursor, baccatin III. After the attachment of a side chain derived from β-phenylalanine at C13 OH of baccatin III and two further steps, the

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anticancer agent PTX is generated. While most of the taxane biosynthetic pathway has been elucidated, and the genes encoding the respective enzymes cloned, five steps are still without an assigned gene (McElroy and Jennewein, 2018; Sanchez-Muñoz et al., 2020) (Suppl. Fig. 1).

The antineoplastic effect of PTX and its derivatives has been demonstrated in various types of cancer and they are mainly used to treat ovarian, breast and non-small cell lung cancer, among others. In 2017, this drug had already been administered to more than one million patients, although its extreme hydrophobicity hampers its formulation (Yang and Horwitz, 2017). It has also been studied for the treatment of neurodegenerative diseases requiring microtubule stabilization, and conditions characterized by cell proliferation and angiogenesis such as psoriasis (Ehrlich et al., 2004). PTX has also been studied for its therapeutic effects in neurological disorders involving tau protein pathology such as Alzheimer's or Parkinson's disease (Zhang et al., 2005).

As secondary metabolite yields in plant cell biofactories are generally low, it is necessary to add elicitors to the culture medium (Ramirez-Estrada et al., 2016). The content of PTX and other taxanes of interest in *Taxus* spp. cell suspensions was dramatically increased by elicitation with coronatine (COR) (1 μM), a jasmonate analogue, supplied together with randomly methylated β-cyclodextrins (CDs, 50 mM), which act as a permeabilizing agent. COR clearly activated taxane biosynthesis and the expression of several relevant genes, whereas CDs enhanced the excretion of the produced taxanes to the liquid medium, which further increased production (Sabater-Jara et al., 2014; Ramirez-Estrada et al., 2015).

To maximize the yields of target compounds in plant cell cultures, a high rate of excretion from the producer cells to the culture medium is crucial. The inclusion of compounds in chemical structures such as CDs reduces the inhibitory effect of accumulation and/or toxicity on metabolite biosynthesis and offers protection from cellular and extracellular degrading enzymes (Sabater-Jara et al., 2014; Almagro and Pedreño, 2020)

Among the nanoparticles currently being explored in nanomedicine, calixarenes (CAL) are of particular interest. These artificial macrocyclic amphiphiles, which contain hydrophilic and lipophilic groups on opposite rims of the macrocyclic scaffold, have the ability to self-assemble. CAL are composed of phenolic units linked by methylene groups at meta-positions, and they form a unique basket-like shape with a hydrophobic cavity. Calix[n]arenes have variable conformations according to the number of incorporated phenolic units (generally n = 4, 5, 6 and 8).

The ease with which CAL and analogues such as resorcinarenes can be prepared, purified, and derivatized by the incorporation of various groups at both the upper and lower rim has led to successful applications. Notably, appropriate modification can significantly enhance the binding affinities between CAL and drugs of interest (Zheng et al., 2019). Several studies have shown the importance of these macrocyclic structures as a new matrix for nanomedicine. By acting as drug carriers, they increase drug stability and solubility and facilitate penetration through cell membranes.

Zhao et al. (2018) showed that fluorescent 2,7-dimethoxy-substituted calix[4]carbazole is a highly efficient nanocarrier for the poorly soluble and unstable anticancer drug curcumin. Chen et al. (2017) studied the capacity of the phosphorylated calixarene POCA4C6 for encapsulation and sustainable delivery of curcumin in mice with xenografted triple-negative breast cancer. An et al. (2019) synthesized 14 new dihomooxacalix[4]arene N heterocyclic derivatives that were used to deliver PTX in selected tumour cell lines, demonstrating the effectiveness of this system for cancer treatment. PTX delivery was successfully assayed by Mo et al. (2016) in p-phosphonated calix[4]arene vesicles conjugated with folic acid against folate receptor-positive SKOV-3 ovarian tumour cells. Renziehausen et al. (2019) showed that delivering the chemotherapeutic agent themozolomide in a p-sulphonatocalix[4]arene nanocapsule significantly boosted its anticancer action in glioblastoma cell lines and in an intracranial U87 xenograft mouse model. Zhao et al. (2015) found that calix[6]arene and calix[8]arene have a PTX entrapment efficiency of 81.6% and 90.3%, respectively, which suggests that calix[8]arene can load PTX into the inner core more effectively than calix[6]arene.

Although the use of CAL as antibacterial and antiviral agents and drug delivery systems has been studied, more insight is required into their *in vivo* interactions and capacity to bind with animal or plant cell membranes, and the underlying mechanisms (Ryzhkina et al., 2007; Grare et al., 2010). Synthetic membrane channels based on CAL have been developed, together with other chemical structures that permit interaction with membrane components, either prokaryotic or eukaryotic (Chen et al., 2018), but more research is needed before an effective system to obtain selective transmembrane channels can be designed.

Considering the precedents outlined above, and taking advantage of the physical properties of CAL, which can carry lipophilic compounds such as PTX, the aim of this work was to study taxane production in *T. media* cell cultures using an optimum culture media (Cusido et al., 2002) elicited with COR (1 μM) and supplemented with the macrocyclic nanoparticles calix[8]arenes (CAL) (10 mg.L^{-1}). CAL were added to the culture media with or without elicitation to study their effect on the production and excretion of the target bioactive compounds. To shed light on the relationship between gene expression and taxane production, the expression pattern of several taxane biosynthetic genes was also studied: TXS, involved in the first committed step of the pathway, T7OH, which controls an intermediate step, and BAPT and DBTNBT, which mediate the last part of the pathway.

2. Material and methods

2.1. Plant material

A *T. media* cell line was established as previously described (Cusido et al., 2002). It was grown and maintained in solid Gamborg's B5 medium (Gamborg et al., 1968) supplemented with sucrose (0.5%) and fructose (0.5%), and growth regulators picloram (2 mg.L^{-1}), kinetin (0.1 mg.L^{-1}) and gibberellic acid (0.5 mg.L^{-1}), pH 5.8. The cells were cultured in growth medium at 25 °C in darkness, and subcultured biweekly to obtain enough friable and vigorous calli to establish cell suspension cultures.

2.2. Cell suspension cultures and elicitation

A two-stage culture was established as described by Cusido et al. (2002) and Ramirez-Estrada et al. (2015). After culturing the *T. media* cells in liquid growth medium for 15 days, they were transferred to a production medium (PM), which consisted of Gamborg's B5 liquid medium (Gamborg et al., 1968) supplemented with 3% sucrose and growth regulators 2.4-dichlorophenoxyacetic acid (2 mg.L^{-1}), benzylaminopurine (0.1 mg.L^{-1}), and gibberellic acid (0.5 mg.L^{-1}), pH 5.8. After 12 days in the liquid growth medium, 3 g of *T. media* cells were transferred to a 200 ml flask containing 15 ml of PM. 10 mg.L^{-1} of calix[8]arenes (CAL) (Sigma Aldrich, St Louis, USA) and 1 μM of coronatine (COR) (Sigma Aldrich, St Louis, USA) were added to the cell suspensions, separately or together, at the beginning of the second phase of culture. The CAL and COR concentrations used in this study were those found to be the most effective at increasing taxane production without seriously compromising growth in previous studies (data not shown).

2.3. Biomass accumulation and viability assay

Fresh weight was determined by filtering the cells with ILS filters. The cells were freeze-dried to obtain the dry weight and perform the taxane extraction. Cell viability was evaluated by fluorescein diacetate and propidium iodide staining techniques as described by Exposito et al. (2010). Samples were harvested after 0, 6, 12, 18 and 24 days of

treatments.

2.4. Taxane determination

Taxanes were extracted from the culture media and the lyophilized cells as previously described by Onrubia et al. (2013). HPLC analyses were performed with a Water Acquity Ultra Performance LC system (Waters, Milford, MA, USA). Taxanes were separated in a Discovery HS F5-5 column 25 cm × 4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) using a mixture of acetonitrile (A) and water (B) with the following gradient (min/A%): 0/25, 38/60, 40/100, 45/25, and 55/25 and the flow rate 1 mL min^{-1}. Identification criteria included retention time, UV spectra, and co-chromatography, with the standard peak homogeneity determined by a photodiode array detector when spiked with an authentic standard. Taxanes were quantified by integrating the corresponding peak in the standard calibration curve of each target compound.10-deacetylbaccatin III (DABIII), baccatin III (BACIII), 10-deacetyltaxol (DAT), cephalomaninne (CEPH) and paclitaxel (PTX) were provided by Abcam (Cambridge, UK). Samples for taxane determination were taken at 0, 6, 12, 18 and 24 days.

2.5. Quantitative real-time PCR (qRT-PCR)

For gene expression analysis, total RNA was isolated from 100 mg of frozen cells using the Real Plant RNA Kit (REAL, Valencia, España) following the manufacturer's instructions. The concentration of each sample was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE, USA). cDNA was prepared from 1 μg of RNA with SuperScript IV reverse transcriptase (Invitrogen, CA, USA) and qRT-PCR was performed with SYBR Green (Biorad, Hercules, CA, USA) using gene-specific primers designed with Primer3 software version 0.4.0 (Suppl. Table 1), in a 384-well platform system (LightCycler ® 480 Instrument, Roche, USA). Expression levels were normalized to the levels of TBC41 (Vidal-Limon et al., 2018; Yanfang et al., 2018), which was selected as a reference gene based on its GeNorm M-value and NormFinder stability value, previously obtained in a comparative study (Sabater-Jara et al., 2014) of TBC41, maturase K and 18S rRNA genes. For each gene studied, the relative expression levels were normalized with respect to the same cell line growing for 12 days in the optimum medium for growth (GM) (Cusido et al., 2002) without elicitation (reference value = 1). Samples for taxane gene expression were taken at 0, 4, 8, 12, 24, 48 and 72 h.

2.6. Statistics

Statistical analysis was performed with Excel software. All data are the average of three measurements ± SD. The multifactorial ANOVA analysis followed by the Turkey multiple comparison test was used for statistical comparisons. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

3. Results

In this study, taxane production and the expression pattern of relevant biosynthetic genes was assessed in *T. media* cell suspensions, together with the growth pattern and cell viability. Cell cultures were maintained in the optimum production medium, and the most efficient elicitor (1 μM COR) for increasing taxane production was added at the beginning of the experiment, alone or together with CAL (10 $mg.L^{-1}$). The effect of supplementing the cultures only with CAL was also studied. The results obtained were compared with those of the control, that is, the same cell line without an elicitor or CAL treatment.

3.1. Growth and viability of T. media cell suspension cultures

Two-phase suspension cell cultures of *T. media* were established (Cusidó et al., 2002). In the second phase, cell cultures were maintained for 24 days in the PM with or without 1 μM COR, 10 $mg.L^{-1}$ CAL or both compounds combined (COR + CAL). Cell growth was measured by determining the fresh and dry weight ($g.L^{-1}$) of the cells at days 6, 12, 18 and 24 days in the different conditions assayed. As shown in Fig. 1, the greatest increase in biomass was observed in the control cultures (57% higher fresh weight at the end of the experiment versus the beginning), although without significant differences compared to cultures treated with CAL or COR + CAL. The COR-elicited cultures exhibited a different growth pattern, the fresh weight increasing slightly from day 6–18, decreasing thereafter until the end of the culture, when the fresh weight was 1.3-fold lower than the control. Thus, the presence of COR in the culture medium significantly ($p < 0.001$) reduced biomass accumulation by approximately 30%. Notably, the results show that the presence of CAL in the medium was positive for biomass formation, inhibiting the negative effect of COR in the COR + CAL-treated cell cultures.

The dry weight values (Suppl. Fig. 2) corroborated the fresh weight results, the highest corresponding to control cultures without significant differences compared to the CAL- or CAL + COR-treated cultures. The negative effect of COR on growth was also observed.

The viability of the control cell cultures, measured as the percentage of living cells in relation to the total, remained high until day 6 (more than 96%) (Suppl. Fig 3)), after which it decreased slightly until the end of the experiment (87%). The addition of COR to the culture medium induced a progressive reduction in cell viability (75% at day 24), which would account for the parallel decrease in biomass accumulation. On the other hand, in cultures treated with CAL or COR + CAL, the viability decreased at day 6, but without a significant difference compared to the control, due to the initial cell interaction with the treatments. The similar viability of control and CAL-treated cultures suggests that CAL did not interfere with the growth processes. Moreover, as cell viability was considerably lower when COR was applied alone than with CAL, the capacity of CAL to form inclusion complexes with taxanes seems to have mitigated the toxic effect of the secondary metabolites on cell metabolism.

3.2. Total taxane production

The total taxane contents in the *T. media* cell cultures (control, supplemented with 1 μM COR, 10 $mg.L^{-1}$ CAL or 1 μM COR + 10 $mg.L^{-1}$ CAL) were determined by UPLC throughout the experiment as the sum of DABIII, BACIII, DAT, CEPH and PTX.

As shown in Fig. 2A, the highest taxane accumulation in all the culture conditions was obtained at the end of the experiment at day 24. This is the usual pattern of taxane biosynthesis/accumulation, which mainly takes places after more than 2 weeks of culture (Vidal-Limon et al., 2018).

As expected from previous studies (Onrubia et al., 2013; Ramirez-Estrada et al., 2015; Vidal-Limon et al., 2018), COR elicitation effectively increased taxane production, and the total taxane contents at

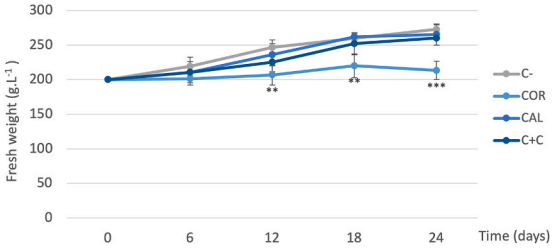

Fig. 1. Growth of *T. media* cell cultures measured as fresh weight ($g.L^{-1}$). In all cases the inoculum was 30% (w/v). Data represent average values from three separate experiments ± SD. COR: 1 μM coronatine: CAL: 10 $mg.L^{-1}$ calix[8] arenes; C + C: 1 μM coronatine and 10 $mg.L^{-1}$ calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

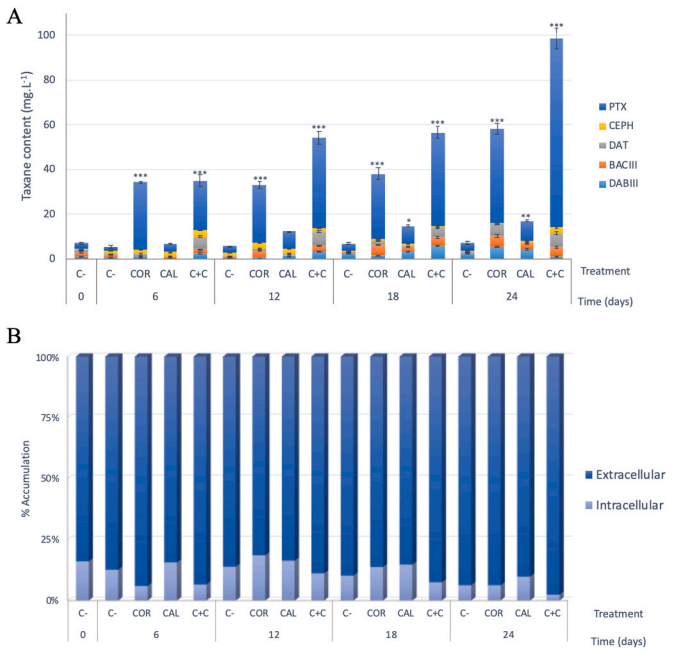

Fig. 2. A) Total and individual taxane content (cell-associated + extracellular) of a *T. media* cell line cultured for 24 days in a production medium. Data represent average values from three separate experiments ± SD. COR: 1 μM coronatine: CAL: 10 mg L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg L^{-1} calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.
B) Taxane production (expressed as the percentage of extracellular and cell-associated taxanes) in *T. media* cell suspension cultures elicited for 24 days in a production medium. Each value is the mean of 3 determinations ± SD. COR: 1 μM coronatine: CAL: 10 mg.L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg.L^{-1} calix[8]arenes.

the end of the experiment were more than 8-fold higher compared to the control. The effect of CAL was also positive, although to a lesser extent, its addition to the culture medium resulting in a 2.5-fold increase.

However, the addition of COR + CAL induced a highly significant ($p < 0.001$) (more than 15-fold) enhancement of taxane contents (Fig. 2A). It is worth noting that already at day 12, total taxane levels in COR + CAL-treated cultures were significantly higher than in cultures where the compounds were applied separately, indicating a synergistic action.

In this cell line, the producer cells readily excreted the taxanes to the liquid medium (Fig. 2B). In control conditions, the excretion percentage throughout the experiment ranged from 84% to 93%. Cell capacity for taxane excretion is known to fluctuate along the culture period and depends largely on the cell line and plant species (Navia-Osorio et al., 2002).

The addition of CAL to the culture medium did not increase the total taxane excretion, which was on average 3.8% lower compared to the control (Fig. 2B). The effect of COR was unclear, with variable excretion among samples during the experiment, although at the end the results were similar to the control. However, the addition of COR + CAL enhanced the excretion percentage throughout the experiment (ranging from 89% to almost 98%) with an average increase of 4% over the control (Fig. 2B). These results indicate that CAL have a positive effect on taxane excretion when production is more active. By hosting taxanes in their structure, the macrocyclic oligomers may facilitate their stability and excretion.

3.3. Individual taxane production

The contents of 10-deacetylbaccatin III (DABIII), baccatin III (BACIII), 10-deacetyltaxol (DAT), cephalomaninne (CEPH) and paclitaxel (PTX) in the *T. media* cell cultures were also determined individually (Figs. 2A, 3, 5 and 6).

In control conditions, BIII was more abundant than DABIII until day 12 (although never more than 2.2 mg.L^{-1}), after which DABIII predominated, probably because BIII was metabolized into other taxanes (Fig. 3). When production levels are very low, as in the control conditions, BIII and DABIII are usually the main taxanes to accumulate (Nims et al., 2006; Onrubia et al., 2013). When CAL was added to the culture medium, DABIII and BIII levels at the end of the experiment increased 2- and 4.5-fold, respectively, compared to the control, with a higher accumulation of DABIII than BIII. The strong elicitation effect of COR was already observable at day 12, and at day 24, DABIII and BIII levels were 2.5- and 8-fold higher, respectively, compared to the control. The COR + CAL treatment also significantly enhanced ($p < 0.001$) the production of both taxanes, especially DABIII (Fig. 3).

Among the studied genes, TXS (taxadiene synthase) and T7OH (taxane 7β hydroxylase) are the only two involved in the biosynthesis of DABIII and BIII, neither of which bear the taxane side chain (Fig. 4). Based on our previous studies, transcript accumulation was quantified only until 72 h of elicitation, after which levels decrease (Expósito et al., 2010; Onrubia et al., 2010).

A clear increase in the expression of the TXS gene was observed in the elicited *T. media* cultures, reaching a maximum at 24 h, when the mRNA accumulated in Cor- and COR + CAL-treated cultures was significantly higher ($p < 0.001$; 20- and 30-fold higher, respectively, than in the control (Fig. 4). The high transcript level of TXS suggests that the precursor taxa-4 (5), 11 (12)-diene was formed in sufficient quantity to increase the production of the downstream taxanes studied. In CAL-treated cultures, TXS expression at 24 h was the same as in the control, but at 12h it was triple the control value, an increase that could explain the moderate enhancement of DABIII and BIII levels. Nevertheless, the TXS expression level at 12 h was 3- and 4-fold lower than in COR- and COR + CAL-treated cultures, respectively.

The T7OH gene was expressed most strongly in cultures treated with COR at 8–24 h and COR + CAL at 4–24 h, declining thereafter, as expected based on previous work. Transcript levels were much lower in the cultures treated only with CAL, although slightly higher compared to the control (4 times higher than the control values) Although COR treatment induced the maximum T7OH transcript accumulation, it was not as long-lasting as the effect of COR + CAL (Fig. 4). A higher supply of precursors due to this increased expression, maintained throughout the 72 h, could explain the highest total taxane yield achieved by COR + CAL elicitation.

The taxanes bearing a side chain attached at C13 of the taxane skeleton studied here were CEPH, DAT and PTX (Figs. 5 and 6).

The production of CEPH in control cell suspensions was highest (approximately 5–6 mg.L^{-1}) at days 6 and 12 of culture. Its subsequent

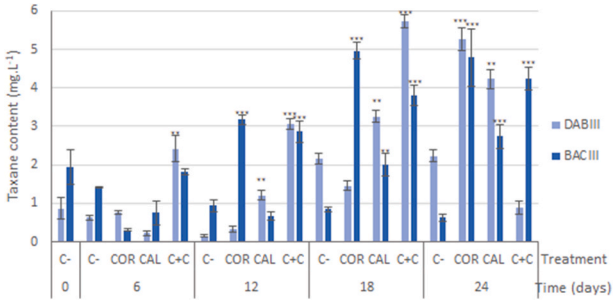

Fig. 3. Effect of coronatine and calix[8]arenes, administered separately and together, on DABIII and BACIII production in *T. media* cell cultures grown for 24 days in a production medium. Each value is the mean of 3 determinations ± SD. COR: 1 μM coronatine: CAL: 10 mg.L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg.L^{-1} calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

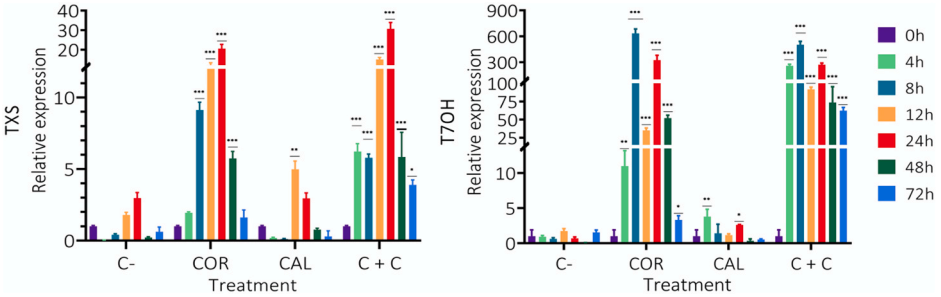

Fig. 4. Relative expression level of the TXS gene and T7OH gene in *T. media* cell cultures elicited for 0, 4, 8, 12, 24, 48 and 72 h. Each value is the mean of 3 determinations ± SD. COR: 1 μM coronatine: CAL: 10 mg.L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg.L^{-1} calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

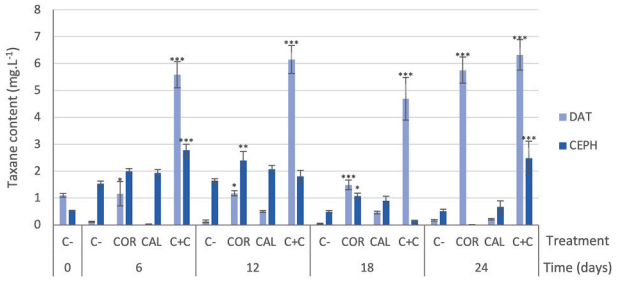

Fig. 5. Effect of coronatine and calix[8]arenes, administered separately and together, on DAT and CEPH production in *T. media* cell cultures grown for 24 days in a production medium. Each value is the mean of 3 determinations ± SD. COR: 1 μM Coronatine: CAL: 10 mg.L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg.L^{-1} calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

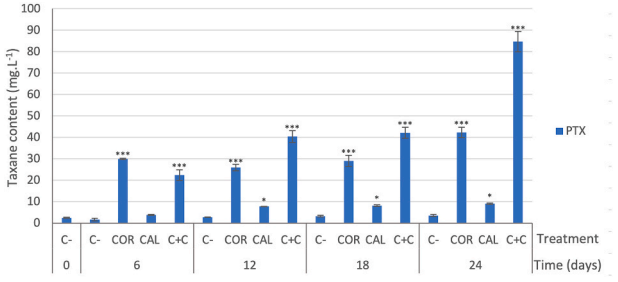

Fig. 6. Effect of coronatine and calix[8]arenes, administered separately or together, on paclitaxel production in *T. media* cell cultures grown for 24 days in a production medium. Each value is the mean of 3 determinations ± SD. COR: 1 μM coronatine: CAL: 10 mg.L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg.L^{-1} calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

decline until the end of the experiment at day 24 (0.5 mg.L^{-1}) was possibly due to rapid degradation or transformation. The addition of CAL, and to a greater extent COR, enhanced CEPH accumulation until day 12 (when values were 2.4 mg.L^{-1} (COR), 2.1 mg.L^{-1} (CAL)), after which a declining trend began. In contrast, a positive effect of COR + CAL was observed only at days 6 and 24 (2.8 mg.L^{-1} and 2.5 mg.L^{-1}, respectively) (Fig. 5).

The effectiveness of COR, as an elicitor, was apparent in its effect on DAT levels, especially when applied with CAL. The highest amount of DAT was observed at day 24 in the COR + CAL-treated cultures, when it was significantly higher ($P < 0.001$; 37-fold) than the control value (Fig. 5). DAT biosynthesis and metabolism has still not been fully elucidated, so its role in PTX biosynthesis is not clear.

As habitual in unelicited *Taxus* cell cultures (Cusido et al., 2002; Kim et al., 2005), PTX levels in the control cultures remained very low throughout the experiment, reaching a peak at day 18 (9.1 mg.L^{-1}). The addition of CAL to the medium had a slight positive effect on the PTX yield, which was never more than 2.8-fold higher than in the control. In contrast, COR had a rapid and strong impact on PTX production, resulting in levels of 42.3 mg.L^{-1} at the end of the study, 12-fold higher than the control (Fig. 6).

However, the highest PTX production was obtained when COR was administered together with CAL. After 12 days of treatment, PTX levels never dropped below 40 mg.L^{-1}, reaching 85 mg.L^{-1} at the end of the experiment, a yield 23-fold higher than in the control. Notably, at day 12, PTX contents in the COR + CAL-treated cultures were higher than the sum of yields achieved by the separate elicitation treatments, indicating a synergic effect (Fig. 6).

While the TXS and T7OH genes are involved in the biosynthesis of all the studied taxanes, the BAPT and DBTNBT genes directly influence the production of side chain-bearing DAT, CEPH and PTX, so their expression level was also studied (Fig. 7).

CAL had little impact on BAPT and DBTNBT gene expression, BAPT showing a low peak at 12h (0.7-fold higher than in the control). Consequently, these genes, which are active in the last elucidated steps of the PTX pathway, were not responsible for the CAL-induced increase in PTX yield. In contrast, their transcript levels were clearly enhanced by COR + CAL: at 4 and 24 h after elicitation, the increase was 5- and 4.8-fold for BAPT and 4.2- and 12.5-fold for DBTNBT, respectively (Fig. 7). This increased expression probably accounts for the very high PTX accumulation in the elicited *T. media* cell cultures.

An ABC gene was also studied (Suppl. Fig. 4). As described above, CALs are macrocycles able to host hydrophobic compounds in their cavities, thereby increasing their stability, solubility and cell membrane transfer. ABC transporters play an important role in the removal of secondary metabolites from the active cytoplasm, allowing them to accumulate in locations where their toxicity does not interfere with cell viability (Fornale et al., 2002; Sun et al., 2013).

As shown in Suppl.Fig. 4, the expression level of the ABC gene was enhanced by the presence of CAL in the medium, especially after 4 and 72 h, when it was 2.2- and 2.6-fold higher than in the control. However, elicitation with COR and COR + CAL had a far stronger effect, mainly at 4 h, 8 h and 24 h: the highest values were achieved at 4h when the mRNA accumulation for the ABC gene had increased 5.2- and 3.7-fold, respectively. In this case, no synergic effect between COR and CAL was observed.

4. Discussion

The *T. media* cell line used in this experiment was maintained in a two-stage culture (Cusido et al., 2002; Palazon et al., 2003) in which the biomass obtained using a medium optimized for growth (30% fresh weight/V) was transferred to an optimum medium for taxane production, where different elicitation conditions were studied. Growth

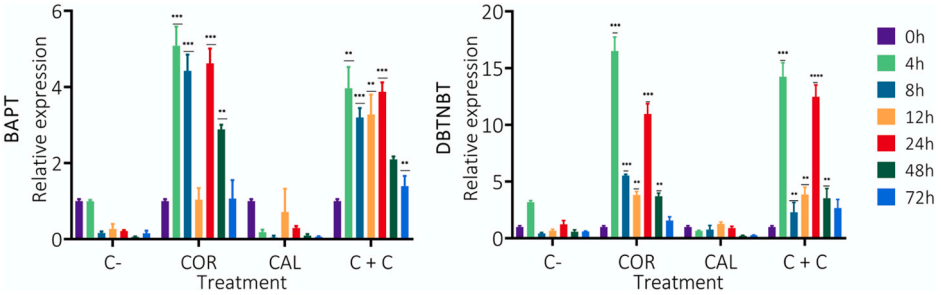

Fig. 7. Relative expression level of the BAPT gene and DBTNBT gene in *T. media* cell cultures elicited for 0, 4, 8, 12, 24, 48 and 72 h. Each value is the mean of 3 determinations ± SD. COR: 1 μM coronatine: CAL: 10 mg.L^{-1} calix[8]arenes; C + C: 1 μM coronatine and 10 mg.L^{-1} calix[8]arenes. A p-value of <0.05 (*), <0.005 (**), <0.001 (***) was assumed for significant differences.

continued in the PM, but at a much lower rate than in the first stage (data not shown).

Growth of CAL-treated cells was very similar to the control. Studies in animal cells have shown that C[n]As are able to pass through membranes and accumulate in the cytosol (Renziehausen et al., 2019). Also, simple clusters of C[n]As in an aqueous medium do not damage the membrane integrity. Although they may have a depolarizing effect and change the membrane permeability, the cell structure in the presence of CAL was maintained (Ryzhkina et al., 2007).

The COR-induced enhancement of taxane production (above all PTX) was accompanied by a dramatic decrease in growth, but not when co-administered with CAL. Growth inhibition by elicitors, including COR, has been observed previously (Onrubia et al., 2013; Sabater-Jara et al., 2014; Ramirez-Estrada et al., 2015; Vidal-Limon et al., 2018). However, the formation of a CAL-PTX complex may have allowed the cell metabolism to remain active, thereby counteracting the negative effect of COR on growth, which stayed similar to the control. Similar results were found when *Taxus* spp cell cultures were treated with COR and CDs, since COR reduced the growth capacity of both *T. globosa* and *T. media* cell lines, but CDs apparently inhibited this adverse effect (Ramirez-Estrada et al., 2015).

Likewise, CAL protected the cells from the negative impact of COR on cell viability, which remained high throughout the COR + CAL treatment. In previous studies, (Onrubia et al., 2013; Vidal-Limon et al., 2018), we reported a clearly negative effect of COR on *Taxus* spp. cell culture viability.

Total taxane production was increased by both COR and CAL when applied separately; in the case of CAL, the increase was only slight, the yield never exceeding 17 mg.L^{-1}. However, the production achieved by the dual treatment (98 mg.L^{-1}) was far higher than the sum of yields induced by its separate components, indicating a synergic action. Similar results were obtained when we studied the co-administration of CDs with COR (Ramirez-Estrada et al., 2015) or CDs with methyl jasmonate (Sabater-Jara et al., 2014), both in *T. media* cell cultures. In the former study, the total taxane production when the two compounds were supplied together was 4.4-fold higher (nearly 80 mg.L^{-1}) than the combined yield when they were added separately; in the second study it was more than 2-fold higher (around 140 mg.L^{-1}). Based on these results, it was inferred that the elicitors COR and methyl jasmonate exerted a synergic effect with CDs. By sequestering the taxanes, the CDs protected the cells from metabolite toxicity, and prevented PTX degradation by cellular or extracellular enzymes (Sabater-Jara et al., 2014; Ramirez-Estrada et al., 2015). The instability of PTX and related taxanes has not been extensively studied, but their enzymatic degradation and/or transformation is known to occur due to the presence of several hydrolytically sensitive ester groups (Shimoda et al., 2008; Tian and Stella, 2008; Exposito et al., 2010). It may be surmised that CAL could act in a similar way to CDs, forming inclusion complexes with PXT and inhibiting its degradation in the extracellular medium. Notably, whereas CDs had to be supplied to the culture medium at a concentration of 50 mM (65 g.L^{-1}) to be effective, similar outcomes were obtained with only 10 mg.L^{-1} of CAL, an important factor for reducing culture costs.

The toxicity of PTX for the producer cells has been shown repeatedly (Kim et al., 2005; Expósito et al., 2010). Its separation from the cytosolic metabolism in non-toxic complexes is therefore desirable, particularly in a production system like the one established here, where PTX constituted 73% of the total studied taxanes in the COR-treated cultures, the proportion increasing to 82% after the COR + CAL treatment.

To shed light on the effects of CAL at the gene transcription level, the expression of some taxane biosynthetic genes was also determined in the *T. media* cell cultures during the first 72 h of the different treatments. In previous studies by our group (Expósito et al., 2010; Onrubia et al., 2010), the transcript levels in *Taxus* spp cell cultures were found to decrease after 72 h of elicitation. Similar results have been reported elsewhere (Nims et al., 2006).

Among the five genes targeted in the present work, the TXS and T7OH genes are involved in the production of DABIII and BIII, two taxanes without a side chain (Suppl. Fig. 1). Although the exact hydroxylation sequence has still not been determined, T7OH is known to act before the formation of DABIII and BIII, as these two taxanes present an OH group at C7 of the taxane skeleton. In the CAL-treated cultures, TXS and T7OH transcript levels were similar to the control, except that peak expression was observed at 12h instead of 4h. At 12h of culture, DABIII was more abundant than BIII, which could be due to the inefficiency of the DBAT enzyme (10-deacetyl baccatin III-10-O-acetyltransferase), which transforms DABIII to BIII (Vongpaseuth and Roberts, 2007), although the DBAT gene was not studied here. The expression of TXS and T7OH genes was strongly enhanced by the treatment with COR (20- and 30-fold, respectively) and especially COR + CAL (600- and 500-fold, respectively), but this was not reflected in DABIII and BIII levels, which increased only slightly in the cells treated with COR (2.5- and 2.7-fold, respectively) and COR + CAL (7.5- and 6.7-fold, respectively). These results indicate that neither TXS nor T7OH are flux-limiting genes in taxane biosynthesis and their expression is strongly induced by COR.

Likewise, Vongpaseuth and Roberts (2007), 6 h after adding the elicitor methyl jasmonate (100 μM) to the culture medium, observed a sustained increase in transcript levels of genes involved in the early steps of PTX biosynthesis, namely, those encoding geranylgeranyl diphosphate synthase and TXS, which controls the first committed step of the PTX pathway. Nims et al. (2006) also found that the maximum expression levels of the TXS gene and taxadiene synthase activity coincided at a very early stage of elicitation, whereas the highest taxane accumulation was reached at the end of the culture. This gap between the genetic changes induced by the elicitors and the biosynthesis of the target taxanes shows that the product of the TXS-catalyzed reaction, taxa-4 (5), 11 (12)-diene, is not a limiting substrate in the taxane pathway.

Despite the slow kinetics of TXS in the biosynthetic process (Croteau et al., 2006), the levels of taxadiene and other early intermediates rapidly decreased in the cultures, suggesting they were converted in

downstream reactions. Thus, although it is a key enzyme in taxane biosynthesis, TXS is not rate-limiting, and other slow metabolic steps may have responded to elicitation to form the target taxanes towards the end of the culture period. In previous studies, the transcript accumulation of the T7OH gene, which encodes one of the last hydroxylases in DABIII formation, was clearly enhanced by elicitation with COR and methyl jasmonate (Onrubia et al., 2013; Sabater-Jara et al., 2014) and with perfluorodecalins together with COR and CDs (Vidal-Limon et al., 2018). Additionally, the overexpression of the genes encoding T2OH, T13OH was observed when *T. media* cell cultures were elicited with COR and methyl jasmonate (Onrubia et al., 2013). The PTX biosynthetic pathway includes nine hydroxylation steps, although only six hydroxylases have been characterized to date. We recently identified a novel hydroxylase (TB506) able to hydroxylate the C 2′ position of the PTX side chain (2′C hydroxylase), one of the last metabolic steps in PTX biosynthesis (Sanchez-Muñoz et al., 2020). All the identified hydroxylases of *Taxus* spp., including TB506 are cytochrome P450-dependent enzymes with comparable mechanisms of action (Kaspera and Croteau, 2006). This would explain the similarity of their strong response to elicitation, which induces a high increase in the expression of their encoding genes.

At the end of the PTX pathway, the last identified genes are BAPT and DBTNBT. In the attachment of the side chain, taxane-C13-O-phenylpropanoyl-CoA transferase (BAPT) catalyzes the initial esterification of C13–OH with β-phenylalanoyl-CoA to give N-debenzoyl-2′-deoxytaxol (Walker et al., 2002a). Hydroxylation of the side chain at the 2′C position by a 2′C hydroxylase enzyme (Sanchez-Muñoz et al., 2020) leads to the formation of 3′-N-debezoyltaxol, a direct precursor of PTX, which undergoes benzoylation through the action of 3′-N-debezoyltaxol-N-benzoyl transferase (DBTNBT), the last enzyme in PTX biosynthesis (Walker et al., 2002b).

Although the expression of BAPT and DBTNBT (Fig. 7) was significantly enhanced by the COR and COR + CAL treatments, it was never more than 5.1- and 15.7-fold higher than the control values. The very high PTX production in the COR + CAL cultures was therefore not only a direct consequence of BAPT and DBTNBT up-regulation. The accumulation of CEPH, a taxane whose biosynthesis involves the BAPT gene, was never higher than 3 $mg.L^{-1}$ in any of the elicited cultures, suggesting a rapid conversion or low efficiency of tigloyl transferase, which catalyzes the last step in CEPH biosynthesis and the tigloylation of the side chain. More insight would be obtained by studying the expression pattern of the genes involved in CEPH formation.

In previous studies (Sabater-Jara et al., 2014; Ramirez-Estrada et al., 2015), we observed that PTX production increased dramatically when it was excreted at a high rate to the culture medium after the addition of CDs. These cyclic oligosaccharides, which have a hydrophilic external surface and a hydrophobic central cavity, are able to form inclusion complexes that can trap apolar compounds, thereby increasing their solubility and facilitating their secretion from cells. Probably calixarenes also act by forming complexes with PTX rather than as an elicitor. Unlike the elicitor COR, they scarcely increased the expression of flux-limiting genes involved in PTX formation.

To study the excretion of PTX, the predominant taxane in the cell cultures, the expression level of an ABC transporter-encoding gene was also determined. Although the ABC gene expression was higher in the cultures treated only with COR, the COR + CAL treatment induced a greater percentage of excretion. In the CAL-treated cultures, excretion was lower because there was less PTX to transport. The results show that as the production of PTX increased, so did its excretion from the cells to the medium, due to the presence of CAL in the cell suspensions.

5. Conclusion

Taken together, the results of this study support that elicitors, in this case COR (1 μM), are needed to improve taxane (mainly PTX) production in *T. media* cell cultures. The addition of the macrocyclic nanoparticles CAL (10 $mg.L^{-1}$) proved to be an effective strategy, not only for significantly increasing PTX production but also for promoting its excretion from the producer cells to the medium. Cells with a high capacity for excreting bioactive products to the culture medium permit continuous systems to be established at the bioreactor level without cell biomass destruction. Moreover, the processes required for extracting and purifying the compounds of interest are simpler, bio-sustainable and economically more viable. To the best of our knowledge, this is the first time that CAL have been used to develop a promising biotechnological system for an eco-friendly and sustainable production of the anticancer drug PTX.

CRediT authorship contribution statement

Ainoa Escrich: Formal analysis, Methodology, Software. **Lorena Almagro:** Visualization, Investigation, Writing – review & editing. **Elisabeth Moyano:** Investigation, Revision. **Rosa M. Cusido:** Visualization, Writing – review & editing. **Mercedes Bonfill:** Investigation, Revision. **Bahman Hosseini:** Methodology, Investigation. **Javier Palazon:** Visualization, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.03.047.

References

Almagro, L., Pedreño, M.A., 2020. Use of cyclodextrins to improve the production of plant bioactive compounds. Phytochemistry Rev. 19, 1061–1080. https://doi.org/10.1007/s11101-020-09704-6.

An, L., Liu, J.-D., Peng, X.-N., Zheng, Y.-G., Wang, Ch, Huang, T.-H., 2019. Preparation and in vitro bioactivity evaluation of N-heterocyclic-linked dihomooxacalix[4]arene derivatives. RSC Adv. 9, 41287–41297. https://doi.org/10.1039/C9RA06876G.

Chen, J.-Y., Haoyang, W.-W., Zhang, M., Wu, G., Li, Z.-T., Hou, J.-L., 2018. A synthetic channel that efficiently inserts into mammalian cell membranes and destroys cancer cells. Faraday Discuss 209, 149–159. https://doi.org/10.1039/C8FD00009C.

Chen, W., Li, L., Zhang, X., Liang, Y., Pu, Z., Wang, L., Mo, J., 2017. Curcumin: a calixarene derivative micelle potentiates antibreast cancer stem cells effects in xenografted, triple-negative breast cancer mouse models. Drug Deliv. 24, 1470–1481. https://doi.org/10.1080/10717544.2017.1381198.

Choi, Y.-E., Kim, Y.-S., Paek, K.-Y., 2008. Types and designs of bioreactors for hairy root culture. In: Dutta Gupta, S., Ibaraki, Y. (Eds.), Plan Tissue Culture Engineering. Springer Netherlands, Dordrecht, pp. 161–172.

Croteau, R., Ketchum, R.E.B., Long, R.M., Kaspera, R., Wildung, M.R., 2006. Taxol biosynthesis and molecular genetics. Phytochemistry Rev. 5, 75–97. https://doi.org/10.1007/s11101-005-3748-2.

Cusido, R.M., Onrubia, M., Sabater-Jara, A.B., Moyano, E., Bonfill, M., Goossens, A., Palazon, J., 2014. A rational approach to improving the biotechnological production of taxanes in plant cell cultures of *Taxus* spp. Biotechnol. Adv. 32 (6), 1157–1167. https://doi.org/10.1016/j.biotechadv.2014.03.002.

Cusido, R.M., Palazón, J., Bonfill, M., Navia-Osorio, A., Morales, C., Piñol, M.T., 2002. Improved paclitaxel and baccatin III production in suspension cultures of *Taxus x media*. Biotechnol. Prog. 18, 418–423. https://doi.org/10.1021/bp0101583.

Ehrlich, A., Booher, S., Becerra, Y., Borris, D.L., Figg, W.D., Turner, M.L., Blauvert, A., 2004. Micellar paclitaxel improves severe psoriasis in a prospective phase II pilot study. J. Am. Acad. Dermatol. 50 (4), 533–540. https://doi.org/10.1016/j.jaad.2003.09.018.

Exposito, O., Bonfill, M., Onrubia, M., Jane, A., Moyano, El, Cusido, R.M., Palazon, J., Piñol, M.T., et al., 2010. Effect of taxol feeding on taxol and related taxane production in *Taxus baccata* suspension cultures. N. Biotech. 25 (4), 252–259. https://doi.org/10.1016/j.nbt.2008.11.001.

Expósito, O., Syklowska-Baranek, K., Moyano, E., Onrubia, M., Bonfill, M., Palazon, J., Cusido, R.M., 2010. Metabolic responses of Taxus media transformed cell cultures to the addition of methyl jasmonate. Biotechnol. Prog. 26, 1145–1153. https://doi.org/10.1002/btpr.424.

Fornalé, S., Esposti, D.D., Navia-Osorio, A., Cusidó, R.M., Palazón, J., Piñol, M.T., Bagni, N., 2002. Taxol transport in *Taxus baccata* cell suspension cultures. Plant Physiol. Biochem. 40, 81–88. https://doi.org/10.1016/S0981-9428(01)01332-8.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158. https://doi.org/10.1016/0014-4827(68)90403-5.

Grare, M., Fontanay, S., Massimba Dibama, H., Mourer, M., Regnouf-de-Vains, J.-B., Finance, C., Duval, R.E., 2010. Cinétique d'action du para-guanidinoéthylcalix[4] arène, et evolution de la perméabilité membranaire. Pathol. Biol. 58, 46–51. https://doi.org/10.1016/j.patbio.2009.07.015.

Kaspera, R., Croteau, R., 2006. Cytochrome P450 oxygenases of Taxol biosynthesis. Phytochemistry Rev. 5, 433–444. https://doi.org/10.1007/s11101-006-9006-4.

Kim, B.J., Gibson, D.M., Shuler, M.L., 2005. Relationship of viability and apoptosis to taxol production in *Taxus sp.* suspensions cultures elicited with methyl jasmonate. Biotechnol. Prog. 21, 700–707. https://doi.org/10.1021/bp050016z.

McElroy, Ch, Jannewein, S., 2018. Taxol biosynthesis and production: from forest to fermenters. In: Schwab, W., Lange, B.M., Wüst, M. (Eds.), Biotechnology of Natural Products. Springer International Publisher AG, Switzerland, Cham, pp. 145–188. https://doi.org/10.1007/978-3-319-67903-7.

Mo, J., Eggers, P.K., Yuan, Z.-X., Raston, C.L., Lim, L.Y., 2016. Paclitaxel-loaded phosphonated calixarene nanovesicles as a modular drug delivery platform. Sci. Rep. 6, 23489. https://doi.org/10.1038/srep23489.

Navia-Osorio, A., Garden, H., Cusidó, R.M., Palazón, J., Alfermann, A.W., Pinol, M.T., 2002. Taxol® and baccatin III production in suspension cultures of *Taxus media* and *Taxus wallichiana* in an airlift bioreactor. J. Plant Physiol. 159, 97–102. https://doi.org/10.1078/0176-1617-00576.

Nims, E., Dubois, C.P., Roberts, S.C., Walker, E.L., 2006. Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. Metab. Eng. 8, 385–394. https://doi.org/10.1016/j.ymben.2006.04.001.

Onrubia, M., Moyano, E., Bonfill, M., Cusidó, R.M., Goossens, A., Palazón, J., 2013. Coronatine, a more powerful elicitor for inducing taxane biosynthesis in *Taxus media* cell cultures than methyl jasmonate. J. Plant Physiol. 170 (2), 211–219. https://doi.org/10.1016/j.jplph.2012.09.004.

Onrubia, M., Moyano, E., Bonfill, M., Expósito, O., Palazon, J., Cusidó, R.M., 2010. An approach to the molecular mechanism of methyl jasmonate and vanadyl sulphate elicitation in *Taxus baccata* cell cultures. The role of *txs* and *bapt* genes expression. Biochem. Eng. J. 53, 104–111. https://doi.org/10.1016/j.bej.2010.10.001.

Palazón, J., Cusidó, R.M., Bonfill, M., Morales, C., Piñol, M.T., 2003. Inhibition of paclitaxel and baccatin III accumulation by mevinolin and fosmidomicyn in suspension cultures of *Taxus baccata*. J. Biotechnol. 10, 157–163. https://doi.org/10.1016/S0168-1656(02)00318-8.

Ramirez-Estrada, K., Osuna, L., Moyano, E., Bonfill, M., Tapia, N., Cusido, R.M., Palazon, J., 2015. Changes in gene transcription and taxane production in elicited cell cultures of *Taxus* × *media* and *Taxus globosa*. Phytochemistry 117, 174–184. https://doi.org/10.1016/j.phytochem.2015.06.013.

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R.M., Palazon, J., 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21 (2), 182. https://doi.org/10.3390/molecules21020182.

Renziehausen, A., Tsiailanis, A.D., Perryman, R., Stylos, E.K., Chatzigiannis, Ch, O'Neill, K., Crook, T., Tzakos, A.G., Syed, N., 2019. Encapsulation of temozolomide in a calixarene nanocapsule improves its stability and enhances its therapeutic efficacy against glioblastoma. Mol. Canc. Therapeut. 18 (9), 1497–1505. https://doi.org/10.1158/1535-7163.MCT-18-1250.

Ryzhkina, I.S., Habicher, W.D., Kiseleva, Y.V., Timosheva, A.P., Konovalov, A.I., Valitova, Y.N., Mardanova, G.N., Tsentsevitskii, A.N., Gordon, L. Kh, 2007. Aggregation and physiological effects on plant cells of amphiphilic calix[4] resorcinarenes with hydroxymethylmethylamide and carboxymethyl groups. Dokl. Biochem. Biophys. 413, 68–71. https://doi.org/10.1134/S1607672907020081.

Sabater-Jara, A.B., Onrubia, M., Moyano, E., Bonfill, M., Palazon, J., Pedreño, M.A., Cusido, R.M., 2014. Synergistic effect of cyclodextrins and methyl jasmonate on taxane production in *Taxus x media* cell cultures. Plant Biotechnol. J. 12, 1075–1084. https://doi.org/10.1111/pbi.12214.

Sanchez-Muñoz, R., Perez-Mata, E., Almagro, L., Cusido, R.M., Bonfill, M., Palazon, J., Moyano, E., 2020. Novel hydroxylation step in the taxane biosynthetic pathway: an approach to paclitaxel production by synthetic biology. Front. Bioeng. Biotechnol 8, 410. https://doi.org/10.3389/fbioe.2020.00410, 13 May.

Shimoda, K., Mikuni, M., Nakajima, K., Hamada, H., Hamada, H., 2008. Biological degradation of taxol by action of cultured cells on 7-Acetyltaxol-2″-yl glucoside. Chem. Lett. 37 (3), 362–363. https://doi.org/10.1246/cl.2008.362.

Sun, G., Yang, Y., Xie, F., Wen, J.-F., Wu, J., Wilson, I.W., Tang, Q., Liu, H., Qiu, D., 2013. Deep sequencing reveals transcriptome Re-programming of *Taxus* × *media* cells to the elicitation with methyl jasmonate. PloS One 8, e62865. https://doi.org/10.1371/journal.pone.0062865.

Tian, J., Stella, V.J., 2008. Degradation of paclitaxel and related compounds in aqueous solutions I: Epimerization. J. Pharm. Sci. 97 (3), 1224–1235. https://doi.org/10.1002/jps.21112.

Vidal-Limon, H.R., Almagro, L., Moyano, E., Palazon, J., Pedreño, M.A., Cusido, R.M., 2018. Perfluorodecalins and hexenol as inducers of secondary metabolism in *Taxus media* and *Vitis vinifera* cell cultures. Front. Plant Sci. 9, 335. https://doi.org/10.3389/fpls.2018.00335.

Vongpaseuth, K, Roberts, S.C., 2007. Advance in the understanding of paclitaxel metabolismo in tissue culture. Curr. Pharmaceut. Biotechnol. 8, 219–236. https://doi.org/10.2174/138920107781387393.

Walker, K., Fujisaki, S., Long, R., Croteau, R., 2002a. Molecular cloning and heterologous expression of the C-13 phenylpropanoid side chain-CoA acyltransferase that functions in taxol biosinthesis. Proc. Natl. Acad. Sci. U.S.A. 99 (20), 12715–12720. https://doi.org/10.1073/pnas.192463699.

Walker, K., Long, R., Croteau, R., 2002b. The final acylation step in taxol biosynthesis: cloning of the taxoid C13-side-chain N-benzoyltransferase from *Taxus*. Proc.of the Natl. Acad. Sci, USA 99 (14), 9166–9171. https://doi.org/10.1073/pnas.082115799.

Yanfang, Y., Kaikaia, Z., Liying, Y., Xing, L., Ying, W., Hongwei, L., Qiang, L., Duanfen, Ch, Devou, Q., 2018. Identification and characterization of MYC transcription factors in *Taxus* sp. Gene (675), 1–8. https://doi.org/10.1016/j.gene.2018.06.065.

Yang, C.P.H., Horwitz, S.B., 2017. Taxol®: the first microtubule stabilizing agent. Int. J. Mol. Sci. 18 (8), 1733. https://doi.org/10.3390/ijms18081733.

Zhang, B., Maiti, A., Shively, S., Lakhani, F., McDonald-Jones, G., Bruce, J., Lee, E.B., Xie, S.X., Joyce, S., Li, Ch, Toleikis, P.M., Lee, V.M.-Y., Trojanowski, J.Q., 2005. Microtubule-binding drugs offset tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model. Proc. Natl. Acad. Sci. U.S.A. 102 (1), 227–231. https://doi.org/10.1073/pnas.0406361102.

Zhao, Z.-M., Wang, Y., Han, J., Zhu, H.-D., An, L., 2015. Preparation and characterization of amphiphilic calixarene nanoparticles as delivery carriers for paclitaxel. Chem. Pharm. Bull. 63 (3), 180–186. https://doi.org/10.1248/cpb.c14-00699.

Zhao, L., Kang, L., Chen, Y., Li, G., Wang, L., Hu, Ch, Yang, P., 2018. Spectral study on conformation switchable cationic calix[4]carbazole serving as curcumin container, stabilizer and sustained-delivery carrier. Spectrochim. Acta Mol. Biomol. Spectrosc. 193, 276–282. https://doi.org/10.1016/j.saa.2017.12.037.

Zheng, Z., Geng, W-Ch, Xu, Z., Guo, D.-S., 2019. Macrocyclic amphiphiles for drug delivery. Isr. J. Chem. 59, 913–927. https://doi.org/10.1002/ijch.201900032.