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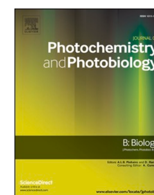

Gene expression pattern and taxane biosynthesis in a cell suspension culture of *Taxus baccata* L. subjected to light and a phenylalanine ammonia lyase (PAL) inhibitor

Mahsa Bamneshin [a], Mohammad Hossein Mirjalili [b,*], Mohammad Reza Naghavi [c], Rosa M. Cusido [d], Javier Palazón [d,*]

[a] *Department of Agricultural Biotechnology, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran*
[b] *Department of Agriculture, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, 1983969411 Tehran, Iran*
[c] *Division of Biotechnology, Department of Agronomy and Plant Breeding, College of Agricultural and Natural Resources, University of Tehran, Karaj, Iran*
[d] *Laboratorio de Fisiologia Vegetal, Facultat de Farmacia, Universitat de Barcelona, Barcelona, Spain*

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ABSTRACT

Taxus baccata L. cell culture is a promising commercial method for the production of taxanes with anti-cancer activities. In the present study, a *T. baccata* cell suspension culture was exposed to white light and 2-aminoindan-2-phosphonic acid (AIP), a phenylalanine ammonia lyase (PAL) inhibitor, and the effects of this treatment on cell growth, PAL activity, total phenol content (TPC), total flavonoid content (TFC), taxane production and the expression of some key taxane biosynthetic genes (*DXS*, *GGPPS*, *T13OH*, *BAPT, DBTNBT*) as well as the *PAL* were studied. Light reduced cell growth, whereas AIP slightly improved it. Light increased PAL activity up to 2.7-fold relative to darkness. The highest TPC (24.89 mg GAE/g DW) and TFC (66.94 mg RUE/g DW) were observed in cultures treated with light and AIP. Light treatment also resulted in the maximum content of total taxanes (154.78 μg/g DW), increasing extracellular paclitaxel and cephalomannin (3.3-fold) and intracellular 10-deacetyl paclitaxel (2.5-fold). Light significantly increased the expression level of *PAL*, *DBTNBT*, *BAPT*, and *T13αOH* genes, whereas it had no effect on the expression of *DXS*, a gene active at the beginning of the taxane biosynthetic pathway. AIP had no significant effect on the expression of the target genes. In conclusion, the light-induced activation of *PAL* transcription and altered expression of relevant biosynthetic genes reduced cell growth and increased the content of total phenolic compounds and taxanes. These findings can be applied to improve taxane production in controlled cultures and bioreactors.

1. Introduction

Plant specialized metabolites (SMs), mainly produced during defense reactions against a variety of biotic and abiotic stresses, are the source of many pharmaceutical products [1]. Biotechnological platforms such as plant cell cultures constitute an efficient and commercial system to produce valuable SMs using metabolic engineering techniques [2]. However, insufficient knowledge of their frequently complex biosynthetic pathways is a major challenge to improving production [3].

Taxanes, diterpenoids produced by the genus *Taxus* L., are used to treat a range of cancers, including lung, ovarian, cervical, melanoma, and AIDS-related Kaposi's sarcoma, especially paclitaxel (Taxol®), whose therapeutic use has been approved by the US Food and Drug Administration (FDA) [4]. The anti-cancer effects of taxanes are unique for two reasons. Initially, they bind to tubulin α and β dimers, which stabilizes them and prevents microtubule dynamics and cell division. In addition, taxanes bind to and block the apoptosis-inhibiting Bcl-2 protein, thereby increasing apoptosis and anti-cancer effects [5]. The extraction and production of paclitaxel from natural sources is not cost-effective and cannot meet the growing global demand for anticancer drugs because of i) the low concentration in plant organs (concentration 0.01–0.03% of the dry weight of the inner bark of mature trees); ii) high cost of extraction, and iii) the slow growth of yew trees and their limited population in natural habitats. Over the past two decades, plant cell culture has been introduced and developed as the most effective method for producing paclitaxel and intermediate taxanes such as baccatin III

* Corresponding authors.
E-mail addresses: m-mirjalili@sbu.ac.ir (M.H. Mirjalili), javierpalazon@ub.edu (J. Palazón).

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and 10-deacetylbaccatin III (10-DBA), which are used in the semi-synthesis of paclitaxel and taxotere [2].

Many studies have investigated the influence of various factors on the expression of taxane biosynthetic genes in cell cultures of *Taxus* species [6,7]. It has been clearly established that the use of elicitors is one of the most effective techniques to increase taxane production [8]. Despite the biological and economic importance of taxanes, five of the nineteen putative stages of their biosynthetic pathway remain to be elucidated [9]. Although taxane production levels vary between *Taxus* species, their biosynthetic pathway seems to be the same throughout the genus [6]. Paclitaxel biosynthesis can be divided into early, middle, and late stages. Initially, geranyl geranyl diphosphate (GGPP) is formed from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by geranyl geranyl diphosphate synthetase (GGPPS). Taxadiene synthase catalyzes the cyclization of GGPP to taxa 4(5),11(12)–diene, which is then converted by the taxane 5α- hydroxylase (T5αOH) to taxa-4(20),11(12)-dien-5α-ol. At this stage, the pathway branches and the latter product is transformed by T13αOH to taxa-4(20),11(12)-dien-5α-13α-diol or alternatively by the taxadiene-5α-ol-acetyltransferase (TAT) to taxa 4(5),11(12)-diene-5a-yl acetate, which is subsequently converted to taxa 4(5),11(12)-diene-5a-yl acetoxy-10b-ol by taxane 10β-hydroxylase (T10BH) [10]. The intermediate stage, which involves most of the unknown genes in the taxane pathway, includes four hydroxylations at positions C1, C2, C7, and C9, as well as oxidation and epoxidation, leading to the formation of a polyhydroxylated product. In addition, two hydroxylases, taxane 7β-hydroxylase (T7OH) and taxane 2α-hydroxylase (T2OH), and the enzymes taxane-2α-O-benzoyltransferase (TBT) and 10-deacetylbaccatin III-10-*O*-acetyltransferase (DBAT) are involved in the polyhydroxylation-mediated benzoylation and acetylation resulting in the production of baccatin III (the end product of the intermediate stage). In the final stage, the side-chain precursor is formed by the phenylalanine aminomutase (PAM) and activated after binding to coenzyme A (COA) by β-phenylalanine-CoA ligase [11]. The side chain is attached to baccatin III by BAPT to form 3′N-dehydroxydebenzoylpaclitaxel. Finally, paclitaxel is biosynthesized after hydroxylation of the taxane ring at the C2' position by a recently characterized hydroxylase [5], followed by benzoylation controlled by debenzoyltaxol N-benzoyltransferase (DBTNBT) (Fig. 1).

One of the most important environmental stimuli for plants, light not only has a great impact on growth and development, but also triggers a wide range of metabolic responses, including the synthesis of SMs [12]. The effect of light on plant metabolism depends on its intensity and quality and also the plant species [13]. The positive impact of light on SMs production has been described in in vitro cultures of various plants including *Thevetia peruviana* (Pers.) K.Schum. [13], *Zingiber officinale* Rosc. [14], *Fagonia indica* Burm.f. [15], and *Linum album* Kotschy ex Boiss. [16]. Although the effects of light stimulation on the formation of phenolic compounds have been reported [15], its impact on the expression of genes involved in their biosynthesis has not been fully elucidated.

Several studies have explored the effect of various factors on taxane production in cell cultures of *Taxus* species [6–8,17,18], but the impact of light has been little studied to date [19]. Therefore, the aim of the present work was to investigate the effect of light on the metabolic profile and expression level of key genes involved in taxane biosynthesis in *T. baccata* L. cell cultures. Also, the effect of phenylalanine ammonia-lyase (PAL) on the production of phenolic compounds and taxanes in the *T. baccata* cells under conditions of light stress was investigated by applying 2-aminoindan-2-phosphonic acid (AIP), an inhibitor of PAL activity. PAL is the first enzyme in the phenylpropanoid pathway, which is associated with the biosynthesis of many SMs [20,21]. The resulting information could be useful for the development of semi-industrial production systems of these valuable anti-cancer compounds.

2. Experimental

2.1. Callus Induction and Cell Suspension Culture Establishment

Callus induction was performed from young stem segments of *T. baccata* according to the method of Onrubia et al. [6]. Calli were grown and maintained on B5 solid medium [22] with the recommended additives [6]. The cultures were placed in the dark at room temperature (25 °C) and sub-cultured every 12 days to obtain sufficient calli to establish a cell suspension culture. A similar culture medium without phytagel and with antioxidants glutamine (200 mg/L), ascorbic acid (50 mg/L), and citric acid (50 mg/L) was used as the growth medium (GM) for the cell suspension culture. Finally, friable calli (16 g) were cultured in a 250 ml Erlenmeyer flask containing 80 ml GM (inoculation at a rate of 20%) and then placed in a shaker incubator at 100 rpm in the dark (25 °C). The cell suspensions were sub-cultured every 12 days [6].

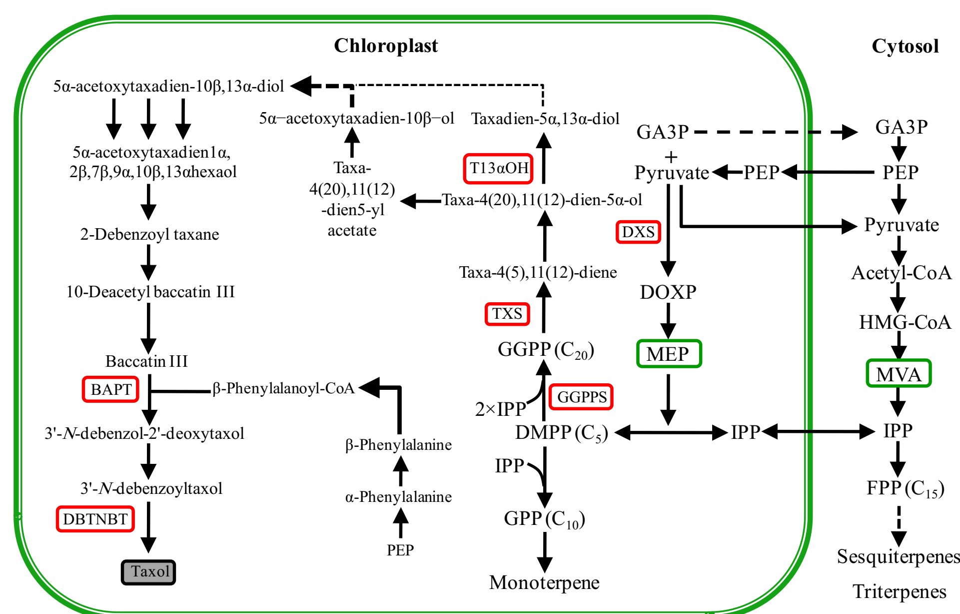

Fig. 1. Paclitaxel biosynthetic pathway. GA3P, glyceraldehydes-3-phosphate; PEP, phosphoenolpyruvate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DOXP, 1-deoxy-D-xylulose 5-phosphate; MEP, methylerythritol phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranyl geranyl pyrophosphate; TXS, taxadiene synthase; taxadiene-13α-hydroxylase (Tα13OH); BAPT, baccatin III-3-amino,C-13-phenylpropanoyl-CoA transferase; DBTNBT, debenzoyltaxol N-benzoyltransferase. The enzymes encoded by the studied genes are encircled in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Experimental Design and Treatments

In this study, a two-stage culture system was used as described previously [23]. Thus, the *T. baccata* cells were first cultured in a GM, and at day 12, when they reached the end of the exponential growth phase, the plant cells (3 g) were transferred to 175 ml flasks containing 20 ml of optimized production medium (PM). The PM consisted of B5 medium with twice the concentration of B5, sucrose, and hormones, following the method Onrubia et al. [6]. In cultures treated with AIP (10 μM), this was added to the culture media before autoclaving. AIP concentration was selected based on previous studies [46].

Additionally, at the beginning of the second stage of culture, methyl jasmonate (MeJ, 100 μM) was added to the PM by filtration, in all the studied conditions. The flasks were placed on a shaker at 100 rpm under a 12 h/12 h regime of light/darkness or continuous darkness (the control), alone or with AIP (10 μM). The light was supplied by white fluorescent lamps (Phillips, Eindhoven, Netherland) at an intensity of 80 $\mu mol/m^2.s$.

The cells were harvested at 0, 2, 7, 14, and 21 days after treatment. The culture medium was removed by filtration (Miracloth, Calbiochem, San Diego, USA) and cell fresh weight (FW) was then recorded. The cells were dried using a freeze dryer and their dry weight (DW) was recorded. To evaluate gene expression, the cells were collected from the PM at 0, 4, 12, 24, and 48 h after treatment, immediately frozen in liquid nitrogen and stored in a deep freezer (− 80 °C) until RNA isolation.

2.3. Extraction and Determination of TPC and TFC

Extraction was performed by adding 10 ml methanol 80% in water (*v*/v) to powdered dried cell samples (0.2 g) in an ultrasonic bath for 30 min at 40 °C. The extracts were then centrifuged at 7000 *g* for 20 min and the supernatant was separated [24]. A concentration of 1 mg/ml extract was used to measure phenolic compounds. TPC was measured using Folin-Ciocalteu reagent and a calibration curve was plotted using the standard gallic acid solution. The measurement was carried out at λ_{max} = 765 nm and TPC was expressed as milligrams of gallic acid equivalents per grams of DW [25]. TFC was measured according to the method of Miliauskas and Vens-kutonis [26]. The absorption was measured at λmax = 415 nm, and TFC was expressed as milligrams of rutin equivalents per grams of DW.

2.4. Protein Extraction and Determination of Phenylalanine Ammonia Lyase Activity

Crude protein was first extracted from the cells by vacuum: the cells (2 g) were homogenized in 1 g polyvinylpyrrolidone (PVP), 50 μl dithiothreitol (DTT) (1 mM), and 5 ml potassium phosphate buffer (0.1 M, pH = 8) in an ultrasonic homogenizer at 4 °C. Centrifugation was then performed at 2000 rpm for 20 min and the supernatant was used to determine protein content and also PAL activity [27]. PAL activity was calculated according to the method of Schonell [28]. In summary, 150 μl of crude protein extract was incubated with 650 μl potassium phosphate buffer (0.1 M, pH = 8) and 200 μl phenylalanine (0.1 M) at 36 °C for 1 h. Finally, the accumulation of cinnamic acid was measured at 290 nm. The protein content was measured according to Bradford [29], and the albumin content was determined based on the absorption at 750 nm. PAL activity was expressed as nanograms of cinnamic acid per milligrams of protein extract per hour.

2.5. Taxane Extraction and Chromatography Analysis

Taxanes were extracted from both freeze dried cells and culture media according to the method of Cusido et al. [30]. All extracts were then dissolved in 500 μl methanol and filtered with a 0.22 μm filter (Millipore, Billerica, MA, USA) before injection. Taxanes were measured out by ultra-performance liquid chromatography (UPLC) as described previously [31]. Chromatographic analysis was performed using a Waters Acquity Ultra Performance LC system (Waters, Milford, MA, USA) and separation was done in a SUPELCOSIL LC-F column 25 cm × 4.6 mm (Supelco, Bellefonte, USA) using a mobile phase consisting of a mixture of water (A) and acetonitrile (B) in a flow rate of 1 ml/min. Taxane standards purchased from Chromadex (Irvine, CA, USA) were separately dissolved in acetonitrile (1–100 μg/ml) and then used to plot calibration curves. The injection volume was 10 μl and the temperature was maintained at 25 °C. The alkaloids were detected at a wavelength of 225 nm. Criteria for identification included retention time, UV spectra and co-chromatography with standard compounds, and peak homogeneity determined by a photo-diode array detector after spiking with authentic standards. All injections were repeated three times ($n = 3$).

2.6. RNA Isolation and cDNA Synthesis

RNA extraction was carried out using the CTAB-based method described by Abbasi Kejani et al. [32]. The quantity and quality of isolated RNA were determined by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA) and agarose gel electrophoresis (1%). To manufacture cDNA, the first 2 μg of each sample was treated with DNaseI (Fermentas GmbH, St. Leon-Rot, Germany) to remove genomic DNA contamination from the extracted RNA. The reverse transcription reaction was performed using 1 μg of the total RNA of each sample and the M-MuLV RT Kit (Lithonia Fermentas) according to the manufacturer's instructions. For cDNA analysis, a polymerase chain reaction (PCR) of all cDNAs was performed using 18S RNA as a reference gene and the product was observed by agarose gel electrophoresis. All cDNA samples were diluted to a ratio of 0.2 and stored in a freezer at −80 °C.

2.7. qPCR Analysis

The primers of studied genes were designed by Primer3 software version 0.4.0 (Table 1). The amplification efficiency of each pair of primers was determined according to the Qiagen method and by 10-fold serial dilution of cDNA. Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green Mastermix (Solis BioDyne, Estonia) and the 384-well platform system (LightCycler®480 Instrument, Roche, USA). Reaction conditions were as previously described [6]. The TBC41 gene, which has a stable expression in *T. baccata*, was used as a reference gene and the data were normalized. Relative expression of each gene was obtained using the expression of the same sample that was grown in GM for 12 days without treatment. The reported values are the average of three technical replications of the three biological experiments.

Table 1
Sequence of specific primers utilized for quantitative Real-Time PCR analysis.

Designation	Primer sequence (5′ → 3′)	Amplicon size (bp)
TBC41	F: CAAGAAGAAAGAGTCAGCAAATGG R: GGAACGACATGACATTATGAATAGC	91
DXS	F: TGGCCCTGCACCCCCTGT R:GCCCACATCAAAGCAGCGTTCT	568
GGPPS	F:CCGGTGTGTGGGGCTTCTGTTT R:TTTGCTTTCTCCAGGCCCATCA	144
T13OH	F:GCCCTTAAGCAATTGGAAGT R:CAGAGGAATGGCGTTTAGAG	100
BAPT	F: TAAGCACTCTACAACAACAATGG R: GCATGAACATTAGTATCTTGATTCC	111
DBTNBT	F: CGGGGGGTTTGTTGTGGGATTA R: TTAGCCTCTCCCCTCGCCATCT	105
PAL	F: ACGGTTTGCCTTCTAAT R: CATCCTGGTTGTGTTGC	132

3. Results and Discussion

3.1. Effect of Light and AIP on Cell Growth

The highest amount of biomass was observed at the end of the culture at day 21: the cell DW increased in conditions of darkness (31.2%), darkness plus AIP (33.3%), light (27.7%), and light plus AIP (28.3%) (Fig. 2a). Biomass production was higher in darkness than in light ($P \leq 0.05$), the cell DW being 21% higher at day 14 (Fig. 2b). The use of AIP slightly improved growth, although the difference was not significant. The AIP treatment increased cell DW in light and darkness by up to 14 and 9%, respectively, with a maximum impact at day 7, decreasing thereafter. Thus, at day 21, there was no difference in biomass in cultures with or without AIP.

The DW and FW values followed a similar pattern throughout the experiment, suggesting that the lower cell weight in conditions of light was due to a reduction in viable cells and not osmotic changes. These results were also confirmed by cell survival rates (data not shown). Our results agree with those of Fett-Netto et al. [19], who reported that cell growth in a *T. cuspidata* Siebold & Zucc. suspension culture was higher in darkness than in white light.

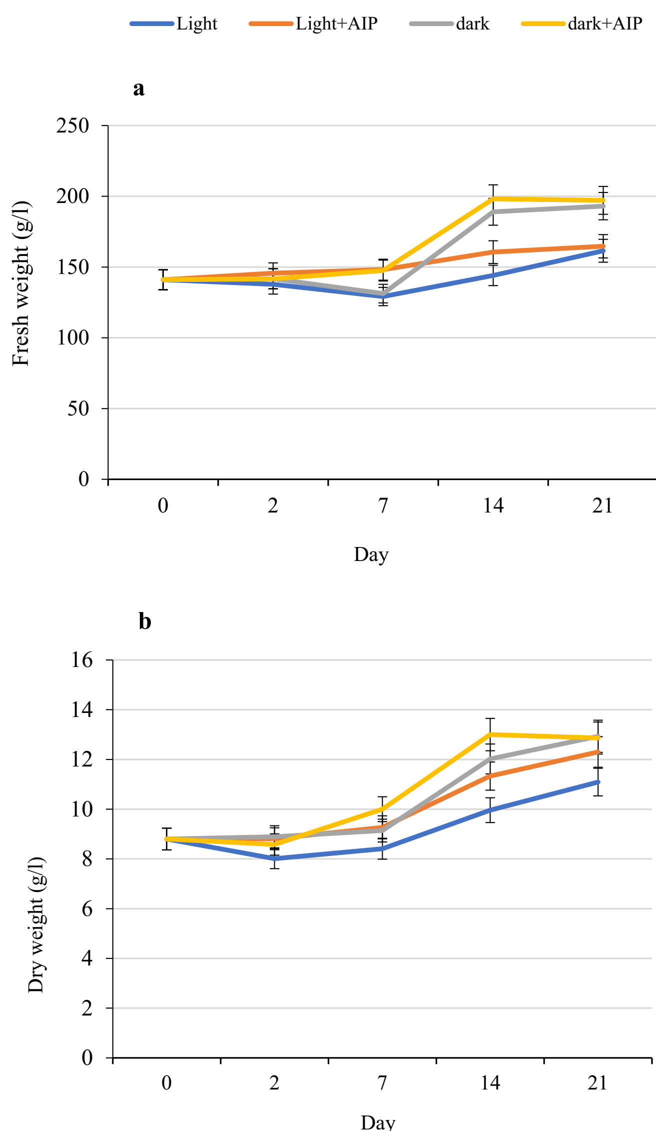

Fig. 2. Growth of methyl jasmonate-elicited *Taxus baccata* cells exposed to white light or darkness for 21 days with and without AIP measured as (a) fresh weight (g/l) and (b) dry weight (g/l). Each value is the average of three biological replications ± SD.

The improvement in growth observed after the inhibition of PAL activity by AIP is in line with the results of Andi et al. [20], who reported that the presence of phenylalanine reduced *Vitis vinifera* L. cell growth in light or dark conditions. There are many reports of biomass depletion with increasing phenylalanine levels in darkness, including in *Vitis amurensis* Rupr. cell cultures [33], but no studies suggest that this effect can be reversed by exposure to light. This could be due to increased photosynthesis in response to light and a higher production of salicylic acid mediated by phenylalanine, which affects various biochemical and physiological processes [34].

Another way light can reduce growth is by inducing phenolic biosynthesis [20,35]. When cell cultures are treated with elicitors, the enhanced SMs production requires carbon consumption at the expense of growth [35]. Phenolic compounds can also bind to various growth-related enzymes and block their activity. Phenol-induced growth inhibition has been previously reported in the genus *Taxus* [36]. The inhibitory effect of light on plant cell growth can also be mediated by the negative regulation of HMG-CoA, which is involved in mevalonate synthesis. Mevalonate is active in regions of cell division and is involved in the biosynthesis of growth regulators such as gibberellin and cytokinin. A reduction in the expression and activity of microsomal HMG-CoA in response to light has been reported in *Taxus* sp. [19]. Additionally, it has been shown that the reduced growth of MeJ-treated *Taxus* cells is due to taxane production and is not a direct effect of MeJ; variable taxane contents can therefore also explain differences in growth [37]. Light-induced reduction of plant cell growth is not due to a longer cell cycle but a lower number of dividing cells [19].

3.2. PAL Activity

As can be seen in Fig. 3, the maximum PAL activity was at the early growth stage. At day 1, PAL activity in MeJ-elicited cells exposed to light or darkness was 7.2- and 2.6-fold higher than in the control (day zero and GM without MeJ), respectively. The increased activity in dark conditions seems to be due to the presence of MeJ. The lowest PAL activity in all the treatments was observed at day 14. PAL activity was significantly higher in light than in darkness, peaking at day 1 and decreasing over time: thus, at days 1, 2, and 7, PAL activity was 2.71-, 1.73-, and 1.14-fold higher, respectively, than in darkness. PAL activity was effectively inhibited by AIP in both light regimes, above all at day one, when the reduction was 3.6-fold in light and 2.3-fold in darkness.

It has recently been reported that enzymes of the phenylpropanoid pathway have a longer half-life when expressed in darkness than in continuous light [38]. In agreement with this observation, between days 2 and 14, PAL activity remained almost constant in dark-grown cells, otherwise decreasing.

PAL, the first enzyme in the phenylpropanoid pathway, catalyzes the deamination of phenylalanine, which is converted to a free ammonium ion and cinnamic acid. PAL is activated in response to a range of biological and non-biological stresses and conditions [39], including light. For example, PAL activity increased in strawberries exposed to white light [40], and in *Rehmannia glutinosa* (Gaertn.) DC. after red and blue light exposure [41]. Consistent with our study, in which the highest PAL activity occurred in the first 24 h of culture, several enzymes of the phenylpropanoid pathway have a short half-life and decrease rapidly after reaching their peak activity [42]. In *Petroselinum hortense* Hoffm. cells cultured in light, PAL activity peaked at 17 h and then decreased [43]. The enhancing effect of phenylalanine on taxane production in *Taxus* sp. has been reported [36].

Biochemical evidence shows that light increases transcript levels of phenylpropanoid biosynthetic genes such as PAL and chalcone synthase [44] and subsequent protein synthesis. The higher taxane production in the light-grown cells may also be associated with increased gene transcription and PAL activity. Interestingly, Sreelakshmi [45] described

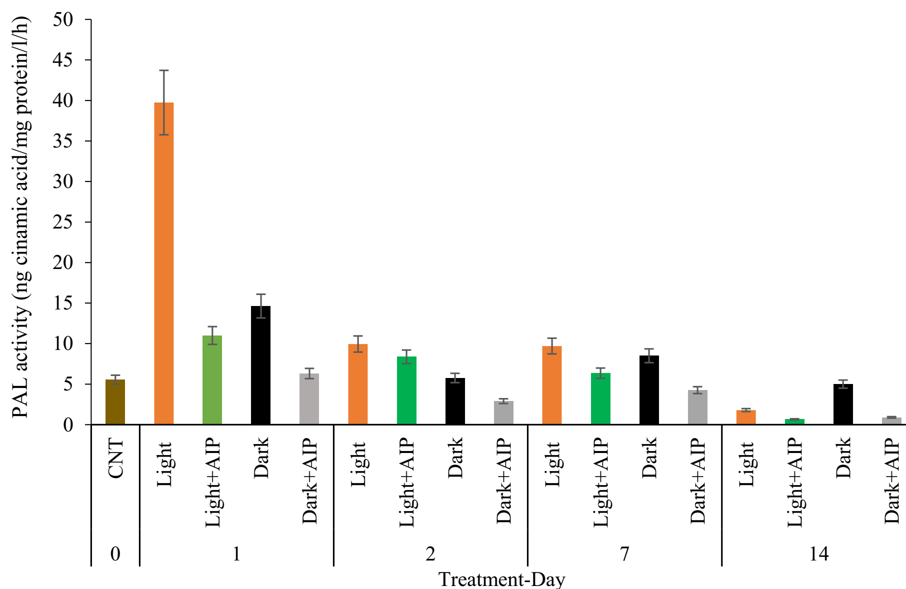

Fig. 3. Time course of change in PAL activity in methyl jasmonate-elicited *Taxus baccata* cells exposed to white light or darkness for 21 days with or without AIP. CNT, control indicates growing medium without MeJ on day zero. The data are the average of three technical replications of three separate biological experiments ± SD. Means with different letters were significant at the 5% level by Duncan's test.

that light induced a parallel increase in PAL activity and protein synthesis, and that the subsequent decrease in protein levels after the peak of PAL activity was due to protein phosphorylation and not degradation. It may therefore be argued that phosphorylation and dephosphorylation of the PAL protein could explain its increase at day 21.

AIP is a potent inhibitor of PAL [46], although the underlying mechanism remains to be fully elucidated. It has been suggested that the inhibition, which is time-dependent, may arise from the molecular reorganization required for the specific binding of the AIP conformer by PAL [46]. The effects of AIP on plant cultures have been widely studied. For example, the addition of AIP to the culture medium of *Artemisia annua* L. resulted in a significant reduction in tissue browning, a condition associated with phenolic compound production, and correspondingly a lower phenolic content and higher growth [47]. The addition of AIP to *Cistanche deserticola* Y.C.Ma cultures also reduced PAL activity and phenolic content [48].

3.3. Effect of Light and AIP on TPC and TFC

The TPC was high in the light-grown cells, reaching maximum levels at the end of culture (day 21): 24.89 GAE/g DW (with AIP) and 18.60 mg GAE/g DW (without AIP). These values were 3- and 2.2-fold higher than in the control (day zero, GM) (Fig. 4a). In contrast, in dark-grown cells (with or without AIP), there was little change in TPC.

A similar pattern was observed for TFC, which showed a strong positive correlation with TPC. Thus, the maximum TFC was found in light-grown cells at day 21: 66.94 DUE/g DW (with AIP) and 51.39 mg DUE/g DW (without AIP), 5.5- and 4.2-fold higher than the control

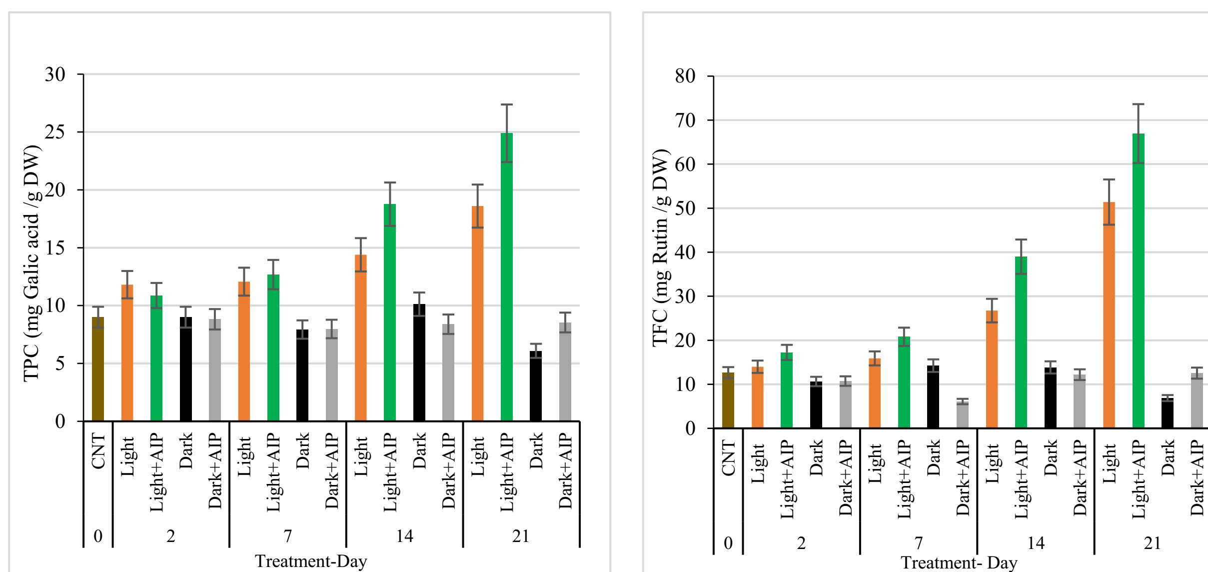

Fig. 4. Total phenolic content (TPC) and total flavonoid content (TFC) of methyl jasmonate-elicited *Taxus baccata* cells exposed to white light or darkness for 21 days with or without AIP. Each value is the average of three replicates of three independent biological experiments. CNT, control indicates growing medium without MeJ on day zero. Columns with different letters have a significant difference at the level of 5%.

value (12.64 mg RUE/g DW), respectively (Fig. 4b). Overall, light significantly increased the production and accumulation of TPC and TFC compared to darkness ($P \leq 0.05$). Light may cause stress conditions in suspension cultures and stimulate the production of phenolic metabolites, as observed in cell suspension cultures of *Artemisia absinthium* L. [12] and *T. peruviana* [13].

As the first enzyme in the phenylpropanoid pathway, PAL plays a key role in phenolic biosynthesis, and an increase in phenolic production in the presence of higher amounts of PAL has been reported [16,20]. It was therefore expected that the use of AIP would reduce TPC and TFC levels, but our results showed no significant positive relationship between PAL suppression and phenolic production in *T. baccata* cells exposed to white light.

Our results are consistent with those of Su et al. [21], who reported that in *Pueraria thomsonii* Benth. cell culture under low light stress, there was no significant positive relationship between PAL suppression by AOPP and phenolic compound production; the phenolic content in the presence of AOPP was even higher than in the control. Their results showed that when the PAL pathway was inhibited, the isochorismate synthase (ICS) pathway took over, and that both metabolic routes are alternatively involved in the biosynthesis of salicylic acid. Finally, it was suggested that there may be an unknown pathway associated with the ICS pathway also involved in phenolic biosynthesis. Further research is required to examine this possibility in *Taxus* cell cultures.

3.4. Effect of Light and AIP on Taxane Production

As shown in Fig. 5, the maximum content of total taxanes (intracellular + extracellular) was achieved in *T. baccata* cell cultures at day 7. Overall, the most effective treatment to increase taxane production was exposure to white light. The difference with dark-grown cells was highest at day 14, when total taxanes were 1.9-fold (light without AIP) and 2-fold higher (light plus AIP), decreasing thereafter. The application of AIP reduced total taxanes up to 42 and 15% in light and dark treatments at days 2 and 7, respectively, subsequently not making a significant difference.

The capacity of *Taxus* species and cell lines to secrete taxanes into the culture medium can vary considerably (between 10 and 90%) [49]. In the present study, the highest taxane secretion occurred at day 7 (peak point of taxane production), when in light-grown cells the extracellular taxanes was 1.7 times more than the intracellular taxanes. The addition of AIP reduced extracellular taxanes by up to 33% in light (up to day 14) and 21% in darkness (up to day 7). In addition to affecting SM production, light also plays a key role in regulating the secretion mechanism [50], and increases the excretion capacity of cells [51]. Although the mechanism of taxane excretion into the culture medium is not fully understood, it clearly requires the activity of ABC transporters, which may be induced by light. Metabolite excretion is critical for the mass production of taxanes in bioreactors, because it promotes sustained yields without reducing cell viability and facilitates extraction and purification.

Variations in intracellular and extracellular taxanes (paclitaxel, cephalomannine (CFM), 10-diethyltaxol (10-DAT), baccatin III, and 10-diacetylbaccatin III (10-DAB), are shown in Fig. 6a and b. Most of the taxanes that accumulated in the *T. baccata* cell culture medium were side chain-bearing (paclitaxel, 10-DAT, and CFM), and those without were only observed in the two first days (Fig. 6a). In *Taxus* cell cultures, baccatin III and 10-DAB, intermediates in the pathway of paclitaxel and other taxanes with side chains, are formed in the early stages, and the side chain is attached after a few days, mainly in the presence of an elicitor [6].

The highest extracellular content among all the taxanes was observed for 10-DAT, regardless of the treatment used, and it reached a peak (48.47 μg/g DW) in light-exposed cultures at day 7. Maximum extracellular levels of paclitaxel (35.51 μg/g DW) and CFM (12.48 μg/g DW) were also observed under light at day 7. Compared to darkness, white light increased extracellular levels of paclitaxel and CFM 3.3-fold and DT 2.2-fold at day 14. The use of white light plus AIP reduced the level of extracellular CFM, paclitaxel, and 10-DAT up to 46% (day 2), 35% (day 14), and 32% (at second day, 7th day and 14th day), respectively. In darkness at day 2, the negative effect of AIP on these taxanes was less intense, or not observed, and extracellular taxane levels were 17% lower in conditions of light plus AIP. However, over time, this tendency was reversed, with taxane levels being 14 and 42% higher under light plus AIP at days 7 and 14, respectively. Regarding paclitaxel and 10-DAT, levels were up to 2.1- and 1.4-fold higher (day 14), respectively, and CF up to 2.6-fold higher (day 21) in light compared to darkness after AIP addition.

The highest rate of taxane excretion and secretion occurred at day 7, when the highest production rate of total CFM, 10-DAT and paclitaxel was also observed. As taxanes with a side chain are generally toxic, their secretion to the culture medium allows plant cells to continue taxane production [7]. The level of intracellular taxanes in light was 1.5-fold higher than in darkness at days 2 and 7. The effect of AIP on intracellular taxane levels was highest at day 2, when the reduction was 50% in light and up to 10% in darkness; little effect was observed at other times.

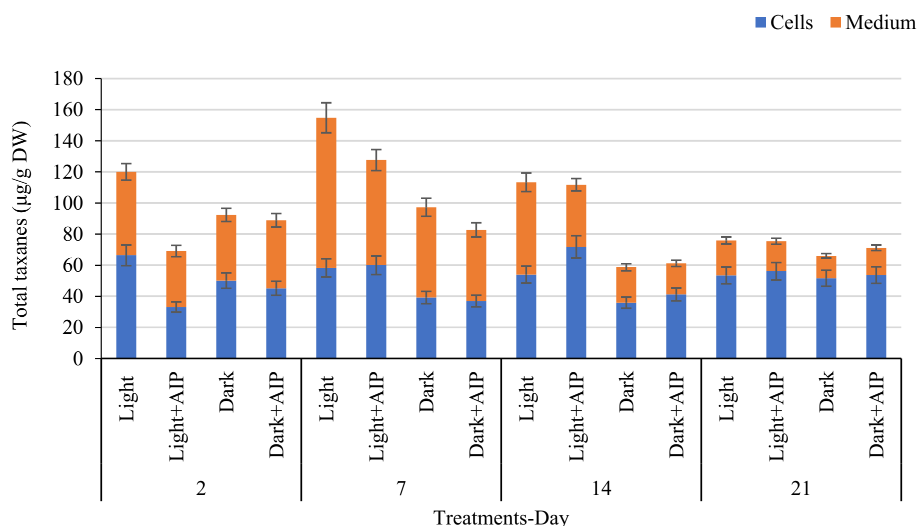

Fig. 5. Total intracellular and extracellular taxanes in methyl jasmonate-elicited cell culture of *Taxus baccata* exposed to white light or darkness for 21 days with or without AIP. Each value is the average of three technical replications of three separate biological tests ± SD.

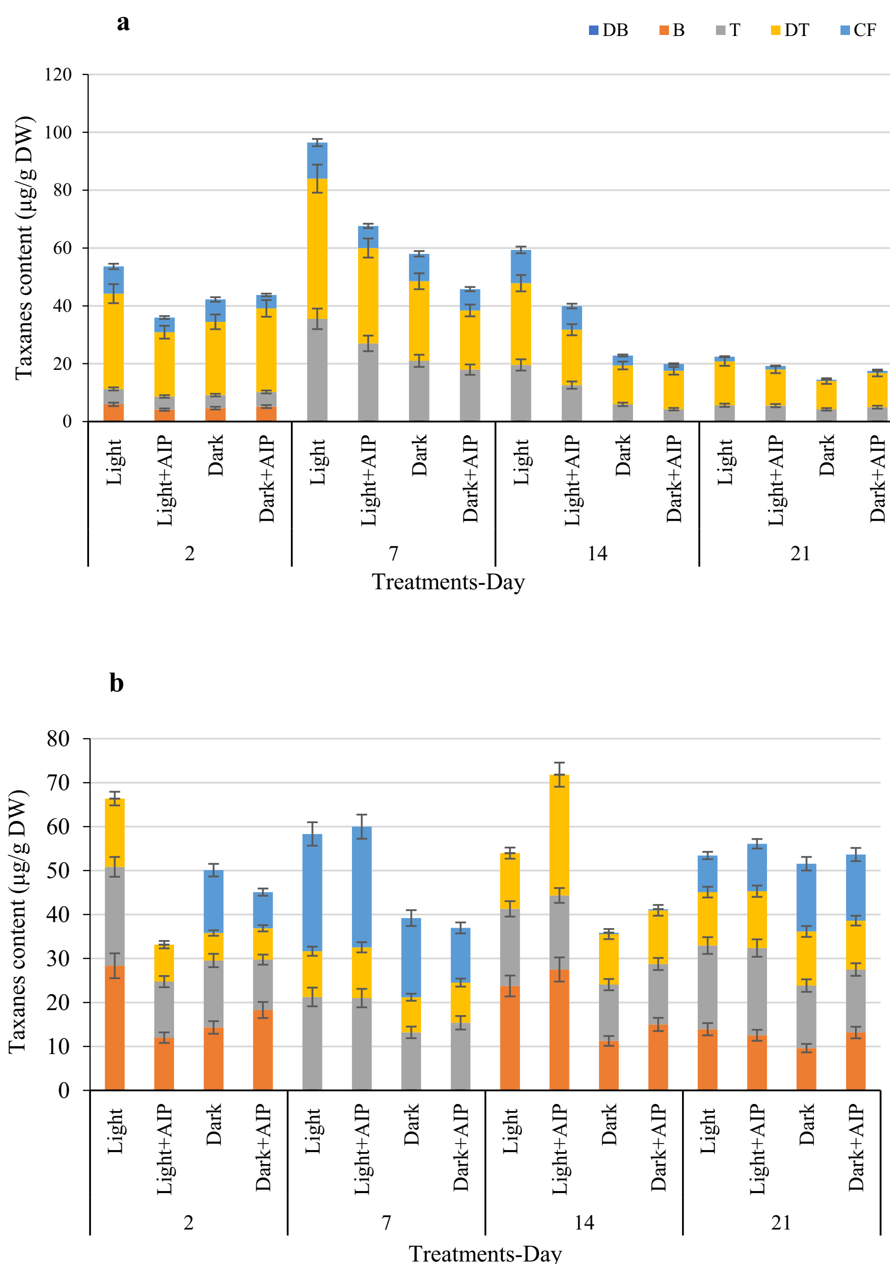

Fig. 6. Extracellular (a) and intracellular (b) content of 10-deacetyl baccatin III (DB), baccatin III (B), paclitaxel (T), 10-deacetyl taxol (DT), and cephalomanine (CF) in a *Taxus baccata* cell culture exposed to white light or darkness for 21 days with or without AIP. Each value is the average of three technical replications of three separate biological tests ± SD.

Maximum levels of intracellular CFM, 10-DAT, paclitaxel (all side chain-bearing) and also baccatin III were 2-, 1.6-, 2.5-, and 1.5-fold higher in light than in darkness, respectively. The use of AIP plus light reduced the intracellular amount of baccatin III by up to 58%, and paclitaxel and 10-DAT by up to 45% at day 2. In darkness, AIP reduced CFM (throughout the experiment) and paclitaxel (only on day 2) but had no adverse effect on other taxanes (Fig. 6b).

In the light plus AIP treatment, the intracellular level of total taxanes at day 2 was 50% lower than in dark-grown cells, but this was reversed over time, until at days 7 and 14, the respective levels were 35 and 43% higher. Regarding individual intracellular taxanes, light plus AIP was also more favorable than darkness: baccatin III and 10-DAT levels were 2.4-fold higher at day 14, and paclitaxel and CFM were 1.6- and 1.5-fold higher at day 7.

In contrast with our results, Fett-Netto et al. [19] reported that white light reduced paclitaxel production in a *T. cuspidata* cell suspension culture, perhaps because it was applied continuously. They concluded that light can reduce paclitaxel levels because of a negative effect on the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) and mevalonate.

As mentioned before, the greatest inhibitory effect of AIP on PAL activity was observed in the first 24 h in all treatments. Moreover, the presence of AIP reduced taxane levels to a greater extent in darkness than in light except at day 2. This suggests that the PAL pathway influences taxane biosynthesis in some way, because with the cessation of PAL, the superiority of light over darkness was disappeared. It should be mentioned that α-phenylalanine is the precursor of the taxane side chain. The positive effect of phenylalanine on SM production has been widely reported. For example, high concentrations of phenylalanine (3 mM) increased the biosynthesis of nordihydroguaiaretic and p-coumaric

acids in a *Larrea divaricate* Cav. cell suspension culture [52]. Also, the use of 3 and 6 mM phenylalanine increased the production of paclitaxel in *Corylus avellana* L. [53], and the application of 1 mM phenylalanine improved stilbene production in *Vitis vinifera* cell suspension cultures up to 4-fold in darkness and 3-fold in light [20].

3.5. Gene Expression

The expression levels of three genes involved in the early stage of paclitaxel biosynthesis (*DXS*, *GGPP* and *T13OH*) and two genes from the late stage (*BAPT* and *DBTNBT*), as well as those of *PAL* from the phenylpropanoid pathway, were determined by qRT-PCR. Transcripts were measured at different times during 48 h after treatment application (Fig. 7). The enzymes DXS and GGPP are located within the plastid,

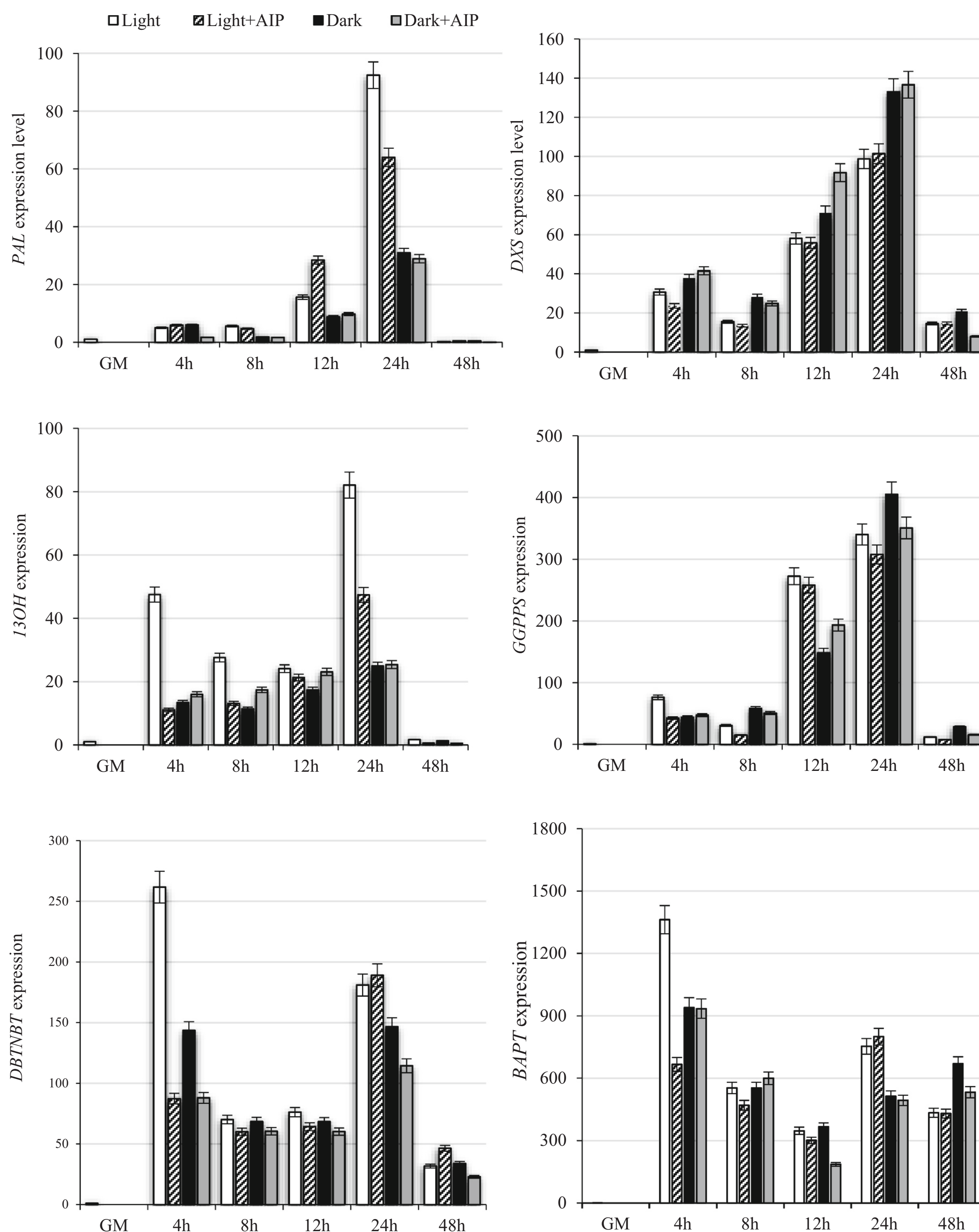

Fig. 7. The expression level of genes involved in the biosynthesis of taxanes in methyl jasmonate-elicited *Taxus baccata* cell cultures growing in production medium exposed to light or darkness, with or without with AIP for 48 h. Each value is the average of three technical replicates of three separate biological tests ± SD. Means with different letters are significant at the 5% level by Duncan's test.

while T13αOH, BAPT, DBTNBT, and PAL are located within the cytosol. The genes encoding those enzymes have previously been studied in different *Taxus* species under different treatments.

PAL: In agreement with enzymological studies that show the maximum activity of PAL at the beginning of plant cell growth, we observed the highest expression of PAL in the first 24 h of culture (Fig. 7). Among the different treatments, light significantly increased *PAL* gene transcription, which was 3-fold higher than in darkness at 24 h. Adding AIP to the culture medium of light-grown cells reduced *PAL* transcript levels, which were up to 31% lower at the peak of expression (24 h). Light is known to increase transcripts of enzymes in the phenylpropanoid pathway [44], and has been reported to enhance *PAL* gene expression in cell cultures of *Linum album* [16] and *Camellia sinensis* (L.) Kuntze [54].

DXS: Although taxanes are derived from the plastidic methylerythritol 4-phosphate (MEP) pathway, there are conflicting reports on the metabolic route of taxane precursors IPP and DMAPP, for which both the plastidic MEP pathway [55] and cytosolic mevalonate pathway [56] have been proposed. Both mevalonate and non-mevalonate pathways have also been described for the biosynthesis of taxanes [57], but it is currently assumed that the taxane chemical structure is formed mainly through the MEP pathway. The first step in this route is the formation of IPP from pyruvate and D-glyceraldehyde-3-phosphate catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS). As shown in Fig. 7, light did not increase *DXS* gene expression, which at 24 h was 34% higher in dark-grown cells. AIP had no apparent effect on *DXS* expression in either light or darkness.

Although the DXS enzyme seems not to play an important role in taxane biosynthesis in *Taxus* species cultured in vitro [17], it is known to be involved in the biosynthesis of plastidic isoprenoids (monoterpenes, diterpenes, and tetraterpenes) [58]. Nevertheless, Exposito et al. [17] observed an increase in *DXS* expression by feeding *T. baccata* cell cultures with paclitaxel. Li et al. [59] stated that a high expression of *DXS* can increase the isoprenoid flow channel to downstream pathways and high expression of *GGPPS* can regulate the balance of precursors in favor of diterpenoid metabolism.

GGPPS: GGPPS, which is made up of three IPP and one DMAPP molecule [60], is another important enzyme in the taxane pathway, as it forms the intermediate that is cyclized by taxadiene synthase to form the taxane skeleton (taxa-4(5),11(12)-diene). Although the *GGPPS* gene does not limit the rate of taxane biosynthesis, it may play a regulatory role [61]. At the peak of expression (24 h), *GGPPS* transcription was practically the same in all four treatments, but at 12 h, it was 84% higher in light than in darkness. Like the *DXS* gene, AIP had no apparent effect on *GGPPS* gene expression in any treatment.

In a study with *Taxus media* cells elicited with coronatine or MeJ, although the *GGPPS* gene expression rate was the same in both treatments, the peak of transcript production occurred earlier with coronatine. It was therefore argued that earlier access to biosynthetic intermediates could explain the higher rate of taxane production in coronatine-treated cells [18]. In contrast, Onrubia et al. [62] stated that although the expression of *GGPPS* in a MeJ-treated *T. baccata* cell suspension culture was significantly enhanced, it could not be assumed that this would lead to a higher taxane production, because GGPP is a precursor of all diterpenoids in the plants. In a study on the enhancing effect of light on volatile terpene production in *Camellia sinensis*, the expression level of the first gene involved in the biosynthetic pathway, geranyl diphosphate (GDP), did not increase, unlike that of subsequent genes, such as those encoding terpene synthases [54].

T13αOH: *T13αOH* gene transcription was 3.5-fold higher in cultures exposed to light than darkness at 4 h, decreasing at 8 h and reaching a new peak at 24 h, when transcript levels were 3.3-fold higher than in dark-grown cells. The application of AIP reduced the transcription rate by 76% in light-grown cells at 4 h after treatment; this inhibitory effect then decreased, only to increase again, with a reduction of 42% at 24 h (relative to light alone). In contrast, AIP had no significant effect on dark-grown cells.

The high level of *T13αOH* gene expression in light-treated cells may indicate that in these conditions, taxane biosynthesis occurs mainly through the step catalyzed by the enzyme T13αOH rather than the alternative pathway controlled by TAT. In a *T. cuspidata* cell suspension culture, Nimes et al. [10] suggested that the 5α-13α-diol pathway catalyzed by T13αOH was more favorable than the one via the 5a-yl acetate substituent controlled by TAT. Similarly, Onrubia et al. [18] reported that in MeJ-elicited *T. baccata* cell cultures, paclitaxel biosynthesis occurs mainly through the pathway catalyzed by T13αOH.

BAPT: The enzyme BAPT binds the beta-phenylalanine side chain to baccatin III, leading to the formation of the 3'-N-debenzoyl-2′-deoxytaxol side chain [63]. The highest increase in *BAPT* expression was measured in the cells treated with light, where transcript levels were 31% higher than in dark-grown cells at 4 and 24 h. The higher expression level at 4 h can be attributed to the effect of transcription factors, as an increase in substrate concentration requires more time.

The half-life of *BAPT* transcripts was short at 4 h, and even shorter at the second peak at 48 h, which may be due to substrate accumulation. The accumulation of baccatin III (the BAPT substrate) was highly correlated with the *BAPT* transcript level, especially at day 2 of treatment. The addition of AIP to light-grown cells caused a 50% reduction in *BAPT* transcripts at 4 h but had no significant effect in the other treatments. The increase in *BAPT* transcription in light-grown cells may explain the pronounced increase in their production of 10-DAT, paclitaxel and CFM.

The biosynthesis of 10-DAT probably requires baccatin III and beta-phenylalanoyl-CoA in the presence of BAPT. However, it is not clear whether 10-DAT is a degradation product of paclitaxel or is formed through a side reaction in the paclitaxel biosynthetic pathway [7]. CFM, which differs from paclitaxel only in the N-tigloyl or N-benzoyl group at the C3 ‘position, is another by-product whose formation requires the enzyme BAPT, but not DBTNBT [6]. As the amount of paclitaxel was higher than CFM, it can be concluded that the metabolic steps leading to the formation of CFM occurs less efficiently than paclitaxel biosynthesis.

DBTNBT: Paclitaxel is obtained from the action of DBTNBT on N-debenzoylpaclitaxel [64]. CFM and 10-DAT are formed by the modification of N-debenzoylpaclitaxel or paclitxel by transferases or other enzymes. The highest increase in the expression level of *DBTNBT* was observed in light, when at 4 h it was 45% higher than in darkness, likely due to the effect of transcription factors. The presence of AIP led to a 66 and 38% decrease in the level of *DBTNBT* transcripts in the light and dark treatments at 4 h, respectively, but no other effect was observed. Therefore, the effect of AIP at the beginning of the experiment may be due to the stress induced by its addition.

BAPT and DBTNBT are directly responsible for the binding of baccatin III to β-phenylalanoyl CoA, and the benzoylation of 3′N-debenzoylpaclitaxel, respectively. Although DBTNBT is involved only in the formation of paclitaxel, the BAPT also catalyzes the formation of other taxanes with a side chain. The quantity and activity of both genes was apparently enough for the production of high levels of cyclic taxanes (CFM, 10-DAT, paclitaxel) in the *T. baccata* cell culture. Also, the high rate of taxanes with a side chain (paclitaxel, CFM, and 10-DAT) indicates that the two terminal transferases are not limiting factors.

The expression patterns of *BAPT* and *DBTNBT* genes were almost identical, both showing peaks at 4 and 24 h, as previously observed in *T. cuspidate* and *T. baccata* cell cultures treated with MeJ [6,10]. Both *BAPT* and *DBTNBT* are reported to play a significant role in the improvement of paclitaxel biosynthesis [10,65]. However, Katkovcinova et al. [66] found that paclitaxel accumulation is not associated with *DBTNBT* gene transcription, and that higher expression levels led to a delay in paclitaxel intracellular accumulation.

In previous studies, the expression of key taxane biosynthetic genes in *Taxus* species cell cultures increased within 6 h of elicitation, remained high for about 24 to 30 h, and returned to baseline values after 48 h. However, the peak of metabolite production was observed 2 to 15

days later, indicating a delay of several days between mRNA accumulation and taxane production. Therefore, it has been suggested that the activity of enzymes related to these genes may continue for a long time after gene expression has declined [4,10]. In the present study, the peak expression for *PAL*, *T13αOH*, *DXS*, and *GGPPS* genes was at 24 h and for *BAPT* and *DBTNBT* genes at both 4 and 24 h. The expression level of all studied genes declined after increase. Thus, light may have a positive adjustment first and then a negative one, in agreement with the results obtained in the MeJ-elicited *T. media* f. hicksii Rehder cells [7,10].

In the present study, contrary to expectations, the regulatory effect of light on gene expression was stronger on genes at the end of the taxane biosynthetic pathway than on those involved in early steps. There are several possible explanations. a) Light needs more time to affect plastid genes such as *GGPPS* and *DXS*. b) MeJ has an inhibitory effect on plastid transcriptional activities [67]. c) The non-mevalonate cytosolic pathway may have been more effective than the MEP plastid pathway in the formation of taxane precursors, although this should be confirmed by further research. Regarding AIP, little effect was observed on the expression of taxane biosynthetic genes except *T13αOH*.

A positive correlation between the expression level of genes involved in the biosynthesis of paclitaxel and its production has been already reported [7,62,65]. However, post-transcription and post-translational factors, as well as post-biosynthesis events such as storage and degradation can also be influential. Paclitaxel biosynthesis begins in the plastids and involves hydroxylations in the endoplasmic reticulum and acylation within the cytosol [65]. Therefore, more knowledge about the intracellular transport of paclitaxel and its precursors and the mechanism of extracellular secretion will help to identify the factors influencing taxane production. In addition, molecular and metabolic effects are critically dependant on the cell line biosynthetic capacity, cell growth stage, culture medium composition, and use of elicitors [8]. Also, the effect of transcription factors such as TcWRKY1 and TcAP2 on taxane biosynthetic genes in cells treated with MeJ has been demonstrated [65].

The simultaneous effect of light on gene expression and increased production of SM has been described previously. The enhancing effect of red and blue lights on the expression level of PAL, cinnamoyl-CoA reductase, pinoresinol lariciresinol reductase, PAL activity, and accumulation of podophyllotoxin in cell suspension cultures of *Linum album* has been reported [16]. Blue and red light also increased the expression level of genes involved in the biosynthesis of volatile fatty acid derivatives, volatile phenylpropanoids/benzenoids and volatile terpenes in tea leaves [54]. In contrast with these positive effects, light was found to inhibit the biosynthesis of stilbenes in *Vitis vinifera* [20].

4. Conclusion

Based on the results obtained, it seems that light affects *T. baccata* cell cultures in two ways. First, light increases the expression level of genes involved in phenylpropanoid metabolism and stimulates PAL activity. Enhancement of PAL activity has been associated with higher phenolic production, followed by a lower cell growth and a higher yield of taxanes, especially those bearing a side chain. Suppression of PAL activity by AIP led to improved growth, but unexpectedly TPC and TFC levels increased. Thus, there was no positive significant relationship between PAL inhibition and phenolic biosynthesis in *T. baccata* cells under light treatment. This suggests there may be an alternative unknown pathway responsible for phenolic production.

The greatest effect of AIP on taxane yields was also observed in the early days of culture, when PAL activity was negligible. In general, AIP seemed to be effective only for seven days, after which it was metabolized. On the other hand, it can be concluded that light has a regulatory effect on the expression of taxane biosynthetic genes in *T. baccata* cell cultures, leading to an increase in taxanes, especially those with a side chain. The enhanced yield of taxanes in light-grown cells is associated with increased transcription of key genes and PAL activity. Further insights would be provided by the study of physiological reactions that occur within chloroplasts during light exposure. The findings reported here confirm the complexity of regulating taxane metabolism.

Credit Authorship Contribution Statement

Mahsa Bamneshin: Investigation, Methodology, Data curation, Writing – original draft. Mohammad Hossein Mirjalili: Conceptualization, Supervision, Formal analysis, Data interpretation, Writing – review & editing. Mohammad Reza Naghavi: Methodology, Data curation, Formal analysis. Rosa M. Cusido: Supervision, Formal analysis, Data curation, Writing - review & editing. Javier Palazon: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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