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ORIGINAL ARTICLE

Taxane production induced by methyl jasmonate in free and immobilized cell cultures of Mexican yew (*Taxus globosa* Schltdl)

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Abstract *Taxus globosa* Schltdl, the Mexican yew, represents a new source of taxanes, including taxol, baccatin III, 10-deacetylbaccatin III, 10-deacetyltaxol and cephalomannine. Due to the anticancer activity and other biological activities of these compounds, and their scarcity in nature, we initiated in vitro cultures of this species with the aim of developing a biotechnological process for obtaining taxol and related taxanes. In the current work, in a batch-type two-phase culture of *T. globosa*, we evaluated the effect of cell immobilization and methyl jasmonate (MeJ) elicitation in two culture media containing different plant growth regulator combinations: 2,4-dichlorophenoxyacetic + benzylaminopurine (Treatment 1: T1) and picloram + kinetin (Treatment 2: T2). The productivity and excretion rate into the culture medium of baccatin III (12.79 μg L^{-1} d^{-1}) (84 %), 10-deacetylbaccatin III (15 μg L^{-1} d^{-1}) (0 %), 10-deacetyltaxol (3.18 μg L^{-1} d^{-1}) (63 %), and cephalomannine (49.27 μg L^{-1} d^{-1}) (9 %) were increased by the effect of T1 in the free cell cultures elicited with MeJ. Cell immobilization in alginate beads did not improve the biotechnological production of these four taxanes. In contrast, the highest productivity of taxol (53 μg L^{-1} d^{-1}) was achieved in MeJ-elicited free cells under T2 and cell immobilization in these conditions increased productivity by more than twofold (130.35 μg L^{-1} d^{-1}).

Keywords Cell suspension cultures · Cell immobilization · Methyl jasmonate · Sodium alginate · Taxanes · Taxol · *Taxus globosa*

Abbreviations

WPM	Woody plant medium
PGR	Plant growth regulators
PIC	Picloram
KN	Kinetin
GA_3	Gibberellic acid
B5	Gamborg's medium
2,4-D	2,4-Dichlorophenoxyacetic
BAP	Benzylaminopurine
FW	Fresh weight
DW	Dry weight
GI	Growth index
dt	Doubling time
HPLC	High-performance liquid chromatography
MeJ	Methyl jasmonate

Introduction

Paclitaxel, the generic name of taxol, is a diterpene alkaloid obtained from the inner bark of most *Taxus* species, with a high activity against breast, lung and ovary cancer, as well as leukemia and AIDS-related Kaposi's sarcoma

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(Malik et al. 2011). The problems derived from debarking yew trees have led to the development of alternative methods for obtaining taxol, notably semi-synthesis from precursors such as baccatin III and 10-deacetyl-baccatin III, although this considerably increases the output cost (Nicolau et al. 1994; Hezari et al. 1997). An arguably more viable option to meet the current high demand for taxanes is the establishment of in vitro cultures of *Taxus* species.

Different biotechnological approaches have been evaluated to optimize taxane production, including experimenting with basal culture media, growth regulator combinations, and biotic and abiotic elicitors. Different culture systems have also been developed to perform these experiments, including two-phase cultures with free and immobilized cells, from a small scale to bioreactor level. The final goal of all these studies is to develop an eco-friendly and sustainable biotechnological platform based on highly taxane-productive *Taxus* sp. cell cultures, thereby contributing to the preservation of yew species in their natural habitats, which are currently being affected by climate change (Arias et al. 2009; Malik et al. 2011).

Taxus globosa Schltdl is the only species of the genus *Taxus* native to Mexico, hence the name "Mexican yew". Its geographical distribution is very limited, being restricted mainly to protected forests at 1000–3400 m of altitude in mountainous areas of the Nuevo León, Tamaulipas, San Luis Potosí, Querétaro, Hidalgo, Oaxaca and Chiapas states (Soto-Hernández et al. 2000; Zavala-Chávez 2001). Due to its slow growth and low population density, *T. globosa* has the status of a rare species and is currently protected by the Mexican government, which limits the collection of plant material for taxane extraction and commercialization.

Phytochemical studies on taxanes in wild *T. globosa* trees (Strobel et al. 1973; Guerrero et al. 2000) have shown that unlike other *Taxus* species, the Mexican yew has a high taxane content, particularly taxol (433 μg/g DW), which accumulates mainly in leaves and young stems (van Rozzendal et al. 2000).

In response to the growing importance of plant cell cultures for taxane production, in vitro callus and cell suspension cultures of *T. globosa* have been established from the needles and young stems of the trees (Barrios et al. 2009; Barradas-Dermitz et al. 2010; Barrales et al. 2011), but the production of both taxol and its precursors achieved to date has been very low.

In a previous study (Tapia et al. 2013), we established callus and cell suspension lines derived from *T. globosa*, which were cultured in woody plant medium (WPM) (McCown and Lloyd 1981), achieving a taxol production of up to 197.99 μg L^{-1}, up to 633.72 μg L^{-1} of baccatin III and 229.61 μg L^{-1} of 10-deacetyl taxol, the latter two compounds being released in their entirety from the cells to the culture medium. In contrast, cephalomannine production (91.43 μg L^{-1}) was only induced in cell cultures growing in Gamborg's medium (B5) (Gamborg et al. 1968).

Further work is still necessary to reach a competitive biotechnological taxane production in the Mexican yew comparable with that achieved in other *Taxus* species. In the current study, we evaluated for the first time in Mexican yew cell cultures the effect of simultaneous cell immobilization in alginate beads and methyl jasmonate (100 μM) elicitation in two culture media supplemented with different plant growth regulators. The experiments were performed in a two-phase culture system, in which first the biomass production was stimulated and, then, in the second phase, the biosynthesis and accumulation of taxol and other taxanes were promoted.

Materials and methods

Plant material

We used a *T. globosa* cell line previously established as described in Tapia et al. (2013) and characterized by having a doubling time of 2.32 days and a taxol productivity of 4.26 μg L^{-1} day^{-1}. An inoculum of 50 g wet weight L^{-1} was used in all the experiments and cells were cultured in Woody Plant Medium (WPM) (McCown and Lloyd 1981) supplemented with the plant growth regulators (PGR) picloram (PIC) (2 mg L^{-1}), kinetin (KN) (0.1 mg L^{-1}) and gibberellic acid (GA_3) (0.5 mg L^{-1}). Cultures were placed in a growth chamber under a controlled temperature (25 ± 2 °C) in darkness. Flasks were shaken at 110 rpm in an IKA orbital shaker. The cell suspension was routinely subcultured every 12 or 15 days to obtain enough biomass for further experiments.

Cell immobilization in alginate beads

T. globosa cell cultures growing in WPM medium were immobilized in alginate beads. Five alginate concentrations (2.5, 3, 3.5, 4 and 5 %) were evaluated and cells were immobilized according to the protocol described by Gillet et al. (2000) and modified by Osuna et al. (2008). Fresh weight of free cells (1 ± 0.5 g) was transferred to culture flasks with 20 mL WPM medium containing sodium alginate at the concentrations described above. The cell suspension was dripped into 100 mL of a sterile 0.2 M $CaCl_2$ solution to form the biocatalytic beads by ionotropic gelation of alginate. Alginate beads were maintained for 30 min in a 0.2 M $CaCl_2$ solution and then washed with culture medium and filtered. The immobilized cell suspension was cultured for 20 days in the same conditions as free cells in WPM supplemented with PIC (2 mg L^{-1}), KN (0.1 mg L^{-1}) and GA_3 (0.5 mg L^{-1}). At the end of this period, the

growth medium was replaced by the production medium. B5 medium (Gamborg et al. 1968) supplemented with two different PGR combinations was used: 2,4-dichlorophenoxyacetic (2,4-D) (2 mg L^{-1}), benzylaminopurine (BAP) (0.1 mg L^{-1}) and GA_3 (0.5 mg L^{-1}) in Treatment 1 (T1), and PIC (2 mg L^{-1}), KN (0.1 mg L^{-1}) and GA_3 (0.5 mg L^{-1}) in Treatment 2 (T2), both with and without methyl jasmonate (MeJ). The phytohormones were added to the media before autoclaving. MeJ (Aldrich) (100 μM) (Expósito et al. 2010) was dissolved first in ethanol (Yukimune et al. 1996) and then in the culture medium. It was sterilized by filtering through 0.22 μm sterile acrodisc (PALL) and added at the beginning of the culture.

The flasks were shaken (110 rpm) in a growth chamber at 25 ± 2 °C in darkness for 15 days. Triplicate samples were taken every 3 days. To dissolve the alginate, beads were immersed in 20 mL of an EDTA-phosphate solution (0.2 M), stirred for 15 min and then filtered by Whatman No. 42 filter paper. The fresh cell biomass (FW) was weighed. Biomass production was measured as the growth index (GI) and average doubling time (dt) was based on dry weight (DW) (Brunakova et al. 2004).

Cell viability was determined by the fluorescein diacetate staining technique, as described by Duncan and Widholm (1990).

Taxane extraction

Taxane extraction was carried out as previously described (Tapia et al. 2013). Lyophilized biomass (50 mg) was cleaned with 2 mL of hexane for 12 h and then centrifuged at 2500 rpm for 20 min. The pellet was extracted with 1 mL of MeOH: CH_2Cl_2 (1:1) by sonication at room temperature and centrifuged at 2500 rpm for 20 min. The extracts were dried at 25 °C under a low pressure system. The dried extract was fractionated by bipartition with 2 mL of a mixture of dichloromethane:water (1:1), and centrifuged at 2500 rpm for 20 min. The organic fraction was made to dry and finally redissolved in 500 μL of MeOH for quantification by HPLC. For taxane extraction from the culture medium, dichloromethane was added to a final concentration of 25 %. The mixture was vortexed for 2 min and the organic phase was dried on a rotary evaporator at 60 °C. The dried residue was redissolved in 500 μL of MeOH for high-performance liquid chromatography (HPLC) determination (Bentebibel et al. 2005; Bonfill et al. 2007; Osuna et al. 2008).

Quantification of taxanes by liquid chromatography (HPLC)

The content of taxol and other taxanes was determined by HPLC analysis, which was conducted on a 2695 separation module (Waters, USA), equipped with a Waters 2996 photodiode array detector. Separation was carried out using an RP-18 Superspher (Merck) column (120 × 4 mm; 5 μm particle size). The mobile phase consisted of a gradient mixture of solvent A (water) and solvent B (acetonitrile): A:B = 100:0 (0–1 min); 55:45 (2–6 min); 50:50 (7–10 min); 0:100 (11–12 min); and 100:0 (13–14 min). The sample injection volume was 20 μL with a 1.0 mL/min flow rate. The detection wavelength was scanned at 220 nm. Quantification of all the taxanes was achieved using calibration curves that were separately constructed with pure standards (Sigma) and every sample was analyzed with three replications.

Determination of carbohydrate uptake

Carbohydrate uptake (sucrose, glucose and fructose) was determined according to the method of De Vries and Egberg (1979) with some modifications. The culture medium (1 mL) was filtered through 0.3 μm, and 80 μl samples were directly injected into the HPLC system. Quantification was performed on a Waters 2695 HPLC, with a Refractive Index Detector Waters 2414. The stationary phase was an amino column ($NH_2$100) and the elution solvent was composed of CH_3CN: H_2O (80:20) at a flow rate of 1.5 mL min^{-1}. Calibration curves were performed using the external standard method with standards from Sigma.

Statistical analysis

All data were analyzed using R statistical software: Language and Environment for statistical computing and graphics. ANOVA and Tukey tests were performed to determine significant differences between treatments.

Results and discussion

Cell growth of free and immobilized cell cultures of *T. globosa*

As mentioned above, we cultured the *T. globosa* cells in a two-phase system. After 20 days in the growth medium, the cells were transferred to two types of production media: B5 medium supplemented with either 2 mg L^{-1} 2,4-D, 0.1 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 (T1), or 2 mg L^{-1} PIC, 0.1 mg L^{-1} KN and 0.5 mg L^{-1} GA_3 (T2). In these conditions, the cells were elicited with 100 μM of MeJ. In the first phase, the *Taxus* cells grew actively, achieving a cell biomass of 3.3 g L^{-1} measured as DW (Fig. 1a). When transferred to the T2 medium, cells maintained the high growth rate until day 29. In the T1 medium, the cells

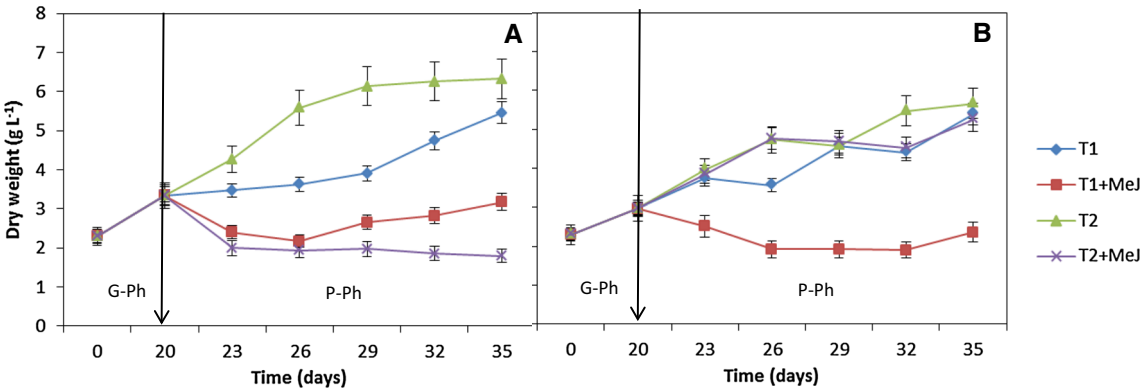

Fig. 1 Time course of the biomass production measured as dry weight (DW) of the free (**a**) and immobilized (**b**) *T. globosa* cell cultures. G-Ph, growth phase: cells were grown in: WPM with PIC (2 mg L^{-1}), KN (0.1 mg L^{-1}) and GA_3 (0.5 mg L^{-1}); P-Ph, production phase: (T1) [B5 with 2,4-D (2 mg L^{-1}), BAP (0.1 mg L^{-1}) and GA_3 (0.5 mg L^{-1})] and (T2) [PIC B5 medium (2 mg L^{-1}), KN (0.1 mg L^{-1}) and GA_3 (0.5 mg L^{-1})], with or without MeJ (100 μM). Each value is the average of three replicates ±SD. *$p < 0.0001$. The *arrow* indicates the medium change

showed a low growth ratio during this period; only in the last part of the culture (days 29–35) was a significant increase in the biomass production observed. In both treatments, elicitation with 100 μM MeJ dramatically decreased the biomass production of the system, measured as DW (Fig. 1). Elicitor growth inhibition throughout the experiment was on average 63 % in the T1 medium and 37 % in T2. The inhibitory effect of MeJ at this concentration has been previously reported in other *Taxus* species, such as *T. media* (Cusido et al. 2002) and *T. baccata* (Onrubia et al. 2011).

To optimize the sodium alginate concentration for the cell immobilization beads, 2.5, 3, 3.5, 4 and 5 % alginate concentrations were tested. The best results were obtained with the 3.5 % solution, which provided uniformly sized and solid beads of suitable hardness for the immobilization experiments (data not shown). After immobilization, the alginate beads were cultured in the same conditions as the free cell suspension and also elicited with MeJ (100 μM). As with the free cells (Fig. 1b), the biomass production of the immobilized culture, measured as DW, was higher in the T2 than T1 medium (Fig. 1b). Throughout the culture, immobilized cells showed a slightly lower growth than the free cell suspensions, as previously observed by Bentebibel et al. (2005) in immobilized *T. baccata* cell cultures, but the inhibitory effect of MeJ on growth was apparent only in the T1 medium. Thus, in the conditions of this experiment, cell entrapment in alginate beads did not significantly inhibit the growth capacity of the system, and previous reports describing the inhibitory effect of 100 μM MeJ on the biomass production in other *Taxus* species were corroborated (Furmanova et al. 2000; Baebler et al. 2005). In contrast, several authors have reported that MeJ does not negatively affect cell growth (Ketchum et al. 1999; Bonfill et al. 2006; Dong and Zhong 2001). A possible explanation for these conflicting observations is that the effect of MeJ depends not only on factors related to the elicitor, such as concentration, which in this case did not vary, but also on the state of the culture development, which is regulated by the PGR supplement (Bonfill et al. 2003).

Carbohydrate uptake of the *T. globosa* cell cultures

Figure 2 shows the time course of the carbohydrate uptake of the *T. globosa* cell cultures in T2 medium during the second phase of the experiment (days 23–35). In free cell suspensions, at the beginning of the second phase, we detected a high level of sucrose in the cell culture medium, as well as a small quantity of fructose and glucose, which probably arose from a partial hydrolysis of sucrose during the autoclaving. During the culture period, sucrose levels decreased significantly, almost disappearing at day 35. In contrast, levels of glucose and fructose remained practically constant, even increasing toward the end (Fig. 2a), thereby indicating the preference of *Taxus* cells for sucrose as a source of energy. The scarcity of sucrose did not cause cell death, since cell viability remained high (about 81 %) at the end of the culture period (data not shown).

In the immobilized cell suspension, sucrose contents decreased along the experiment, but in contrast with the free cells, 11.833 g sucrose L^{-1} remained in the culture medium at the end (Fig. 2b). Since the growth capacity of both systems (free and immobilized cells) was similar (Fig. 1), our results suggest carbohydrate resources were used more efficiently in the immobilized cultures. Additionally, the growth in the immobilized cells was more regular than in the free cell cultures, which showed a clearer exponential phase. This fact could also explain the more constant, but incomplete uptake of sucrose.

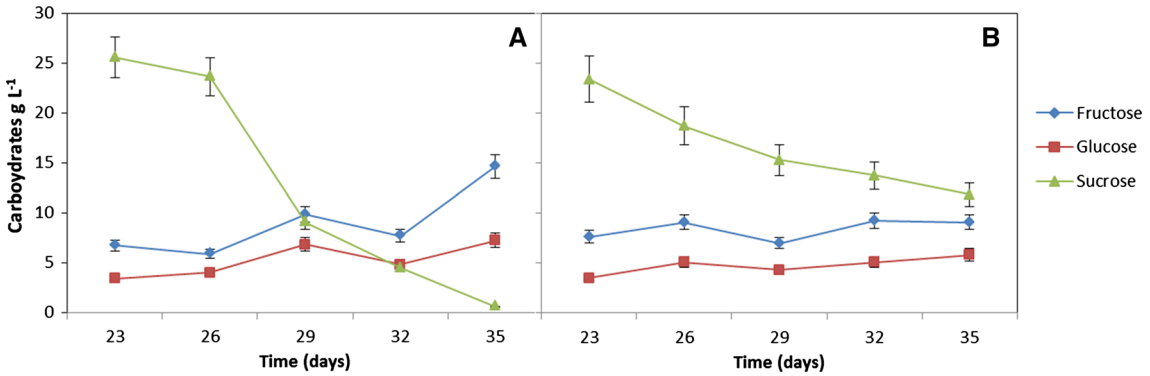

Fig. 2 Time course of the carbohydrate uptake of the free (**a**) and immobilized (**b**) *T. globosa* cells during the second phase of the culture. Each value is the average of three replicates ±SD. *$p < 0.0001$

Taxane contents in free and immobilized cell cultures of *T. globosa*

In the second phase of the culture (production phase), taxol and its precursors baccatin III and 10-deacetylbaccatin III, as well as 10-deacetyltaxol and cephalomannine were determined in the *T. globosa* free cell suspensions. In control conditions (without elicitation), total taxane production was low throughout the experiment, although it increased from day 23 to 32, decreasing thereafter until the end of the culture period; additionally, total taxane production in the T1 medium was higher than in T2 throughout the experiment (Fig. 3a). The addition of MeJ (100 μM) induced a dramatic increase in taxane production in the free cell cultures. This was observed in both media, but particularly in T2, where the taxane contents of *T. globosa* free cells reached similar or even greater levels than in elicited T1 cells from day 29. The elicitor-induced increase of total taxanes was on average twofold in the T1 medium and si-fold in T2. Thus, the response of the cell suspensions to the presence of the elicitor was generally higher in the lower-producing cell cultures (T2 medium) than in those with more capacity to form and accumulate taxanes (T1 medium). In previous studies, we have obtained similar results when the taxane production was measured in cell lines with variable productivity, always treated with MeJ at the same concentration (Bonfill et al. 2006).

The taxane profiles of the cultures differed according to the treatment. In MeJ-elicited cells in the T1 medium, the main taxane accumulated was cephalomannine, while a high level of taxol was found under T2 conditions (Fig. 3a). When studying the individual taxanes in T1 cultures, the maximum production (intra- and extracellular) of baccatin III was observed at day 15 (123 μg L^{-1}); 10-deacetylbaccatin III and 10-deacetyltaxol at day 12 (67.4 and 38.2 μg L^{-1}, respectively); and cephalomannine at day 6 (295.6 μg L^{-1}) after elicitation. The production of taxanes bearing the lateral chain formed from baccatin III and β-phenylalanoylCoA was clearly activated. Baccatin III production was also enhanced in the T1 medium, but to a far lesser extent. In the case of taxol, production peaked at day 35 (503 μg L^{-1}) in the cells grown in the T2 medium (Fig. 3a). The maximum taxol productivity (53.3 μg L^{-1} d^{-1}) (Table 1) was 37 times higher than that previously reported for this line (1.434 μg L^{-1} d^{-1}) in the same conditions (Tapia et al. 2013).

The *Taxus* free suspension cultures showed a variable capacity to release taxanes from inside the cells to the culture medium and the % of excretion depended on the taxane type and day of culture, reaching 84 % for baccatin III at the moment of its maximum productivity (Table 1). Studies carried out by Seki et al. (1997) with free and immobilized cell cultures of *T. cuspidata* showed that taxol concentrations in the medium increased, but with marked fluctuations during the growth period.

Compared with free cells, taxane production in immobilized *T. globosa* cell cultures was generally lower, although it was also higher in the T1 media than in T2 (Fig. 3b) under control conditions (unelicited cells). Total taxane contents (intra + extracellular) remained practically constant throughout the culture period or increased slowly. Contrary to the results achieved in free cells, in the alginate-immobilized cultures, MeJ elicitation was ineffective in increasing taxane levels, which remained similar or even lower than in the unelicited T1 cell cultures. A high taxol production was only reached in T2 cell cultures at days 23 and 35, when it was 6.5 (391.041 μg L^{-1}) and 7.3 (436.336 μg L^{-1}) times higher, respectively, than the original production achieved by the same *T. globosa* cell line (59.6 μg L^{-1}) (Tapia et al. 2013). Also, MeJ had a far greater effect on the taxane production in the lower-producing conditions of the T2 cultures than in the T1 medium. The high amount of taxol produced by the cells so soon after elicitor treatment (3 days) explains why the maximum productivity in the immobilized cells (130.34 μg

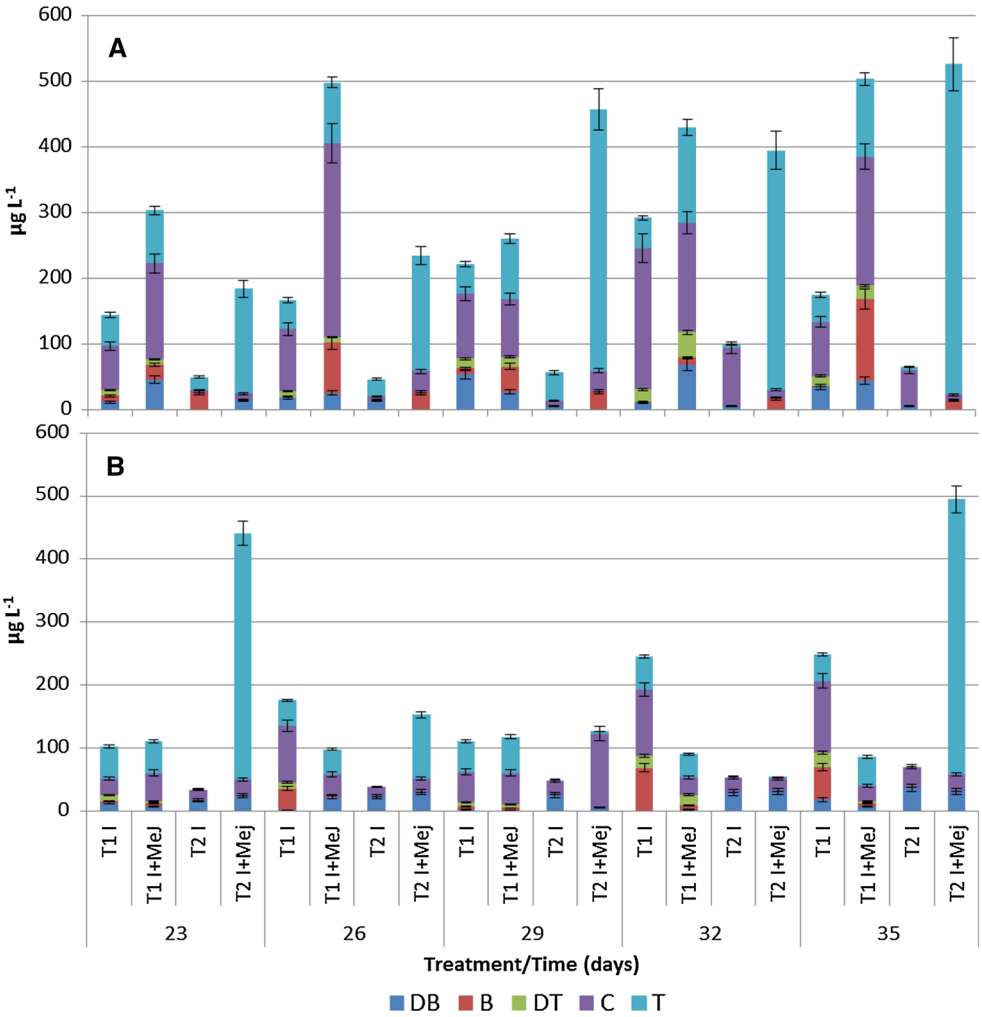

Fig. 3 Time course of the total taxane (intra- and extracelular) production in free (**a**) and immobilized (**b**) *T. globosa* cells during the second (production) phase of the culture in (T1) and T2 medium with or without elicitation with MeJ 100 µM. *DB* 10-deacetylbaccatin III, *B* baccatin III, *DT* 10-deacetyltaxol, *C* cephalomannine, *T* taxol. Each value is the average of three replicates ±SD. *$p < 0.0001$

L^{-1} d^{-1}) was two times higher than in the free cell cultures (53.32 µg L^{-1} d^{-1}) growing in the same conditions (Table 1).

These results reflect the inverse relationship between cell growth and secondary metabolite accumulation in plant cell cultures (Bentebibel et al. 2005). In a study on the kinetics of growth and paclitaxel yield in cell suspension cultures of *T. cuspidata*, Fett-Neto et al. (1994) observed that the taxane levels decreased with the enhancement of the respective cell biomass.

Conclusion

Taxane production has been widely studied in different *Taxus* spp. cell cultures under various elicitation conditions, but very few studies have been carried out with cell suspensions of the Mexican yew (*T. globosa*). The two types of culture systems considered in this work were tested for growth and taxane production capacities in the *T. globosa* cell line studied. Whereas the taxane production achieved was generally higher in the T1 medium, the biomass formation was greater in T2. In both conditions studied (T1 and T2 media), the main taxanes accumulated in the unelicited free cell cultures were taxol and cephalomannine. This capacity to produce taxanes with the phenylisoserine lateral chain attached to the C13 position was enhanced by the addition of MeJ to the cultures. In the T1 medium, after elicitation, the cephalomannine and taxol yield of the experiment represented, on average, 44 and 27.6 %, respectively, of the total taxane production, while in T2 conditions taxol constituted more than 87 %. This pattern was also observed in the immobilized cultures, where the main taxane at the peak of production was taxol.

Additionally, although alginate immobilization did not generally increase taxane production in the *T. globosa* cell line studied, a very high productivity was achieved due to the high total taxane levels obtained only 3 days after elicitation. Notably, the main taxane accumulated was

Table 1 Total taxane concentration, productivity, and excretion of the *T. globosa* free and immobilized cell cultures

Day[a]	Treatment	Concentration ($\mu g\ L^{-1}$)	Free cells		Concentration ($\mu g\ L^{-1}$)	Immobilized cells	
			Productivity ($\mu g\ L^{-1}\ d^{-1}$)	Excretion (%)		Productivity ($\mu g\ L^{-1}\ d^{-1}$)	Excretion (%)
Taxol							
3	T2 + MeJ	159.95 ± 0.99	53.32 ± 1.47	2	391.04 ± 7.83	130.35 ± 9.68	1
Baccatin III							
6	T1 + MeJ	76.73 ± 0.01	12.79 ± 0.59	84	33.91 ± 0.003	5.65 ± 0.10	100
	T1						
10-Deacetylbaccatin III							
3	T1 + MeJ	45 ± 0.001	15.00 ± 0.75	0	30.35 ± 0.11	5.06 ± 0.12	19
6	T2 + MeJ						
10-Deacetyltaxol							
6	T1	38.16 ± 0.002	3.18 ± 0.09	63	10.08 ± 0.005	1.68 ± 0.07	45
12	T1 + MeJ						
Cephalomannine							
6	T1 + MeJ	295.63 ± 0.003	49.27 ± 1.68	9	89.22 ± 0.003	14.86 ± 0.89	22
	T1						

T1: B5 + [2,4-D (2 mg L^{-1}), BAP (0.1 mg L^{-1}), GA_3 (0.5 mg L^{-1})]. T2: B5 + [PIC (2 mg L^{-1}), CN (0.1 mg L^{-1}), GA_3 (0.5 mg L^{-1})]. Each value is the average of three replicates ±SD

[a] Day of maximum productivity

taxol, which represented more than the 88 % of the total taxanes measured.

Taking all the results as a whole, we can infer that *T. globosa* cell cultures constitute a promising biotechnological system for taxane production and that the total and individual taxane productions were modulated by the culture conditions and the addition of the elicitor MeJ in the culture medium.

Author contribution statement This paper is the second part of the Ph.D. dissertation of Nadia Tapia, who obtained the line cells, studied the biomass changes in free and immobilized cells of *T. globosa*, and prepared samples for HPLC analysis. Alejandro Zamilpa performed HPLC analysis. Rosa Cusidó, Mercedes Bonfill, and Javier Palazón participated in in vitro metabolomic study, gene expression, immobilization of cell cultures of *Taxus globosa*, and participated in revising the manuscript. Javier López-Upton provided the Mexican yew plant material and participated in revising the manuscript. Francisco Cruz-Sosa coordinated the study and provided study material and facilities for the experiments. Lidia Osuna provided the idea and participated in the design and development of research. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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