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Polyploidy as a strategy to increase taxane production in yew cell cultures: Obtaining and characterizing a *Taxus baccata* tetraploid cell line

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ABSTRACT

Novel approaches to optimize the production of plant specialized metabolites are crucial to reach maximum productivity of plant biofactories. Plant polyploidization frequently enhances protein synthesis and thereby increases the biosynthesis of specialized metabolites. Paclitaxel is a valuable anticancer agent scarcely produced in nature. Therefore, plant biofactories represent a sustainable alternative source of this compound and related taxanes. With the aim of improving the productivity of *Taxus* spp. cell cultures, we induced polyploidy *in vitro* by treating immature embryos of *Taxus baccata* with colchicine. To obtain the polyploid cell lines, calli were induced from *T. baccata* plantlets previously treated with colchicine and ploidy levels were accurately identified using flow cytometry. In terms of cell morphology, tetraploid cells were about 3-fold bigger than the diploid cells. The expression of taxane pathway genes was higher in the tetraploid cell line compared to the diploid cells. Moreover, taxane production was 6.2-fold higher and the production peak was achieved 8 days earlier than in the diploid cell line, indicating a higher productivity. The obtained tetraploid cell line proved to be highly productive, constituting a step forward towards the development of a bio-sustainable production system for this chemotherapeutic drug.

1. Introduction

Polyploid plants typically exhibit enlarged parts, such as bigger flowers or fruits, and greater resistance to environmental stressors compared to their diploid counterparts (Kaensaksiri et al., 2011; Liu et al., 2011). It has also been demonstrated that the production of specialized metabolites is affected by polyploidization (Gantait and Mukherjee, 2021; Salma et al., 2017). Polyploidy or whole-genome duplication is a condition in which a normally diploid cell acquires one or more additional sets of chromosomes, which alters the metabolism of the plant. Polyploid plants usually present better qualities compared to diploid plants. Enhanced cellular activity increases the vigor, size and productivity of the plant, as the increase in cell size and nuclear activity alters gene expression and enzyme biosynthesis (Eng and Ho, 2019; Gantait et al., 2011; Moghbel et al., 2015). This alteration in metabolic activity ultimately leads to a higher production of alkaloids, terpenes and polyphenols, among other compounds (Gantait and Mukherjee, 2021; Madani et al., 2021; Majdi et al., 2014; Zahedi et al., 2014).

Chromosome doubling occurs spontaneously in some plant species but requires time. However, artificial systems have been developed to induce polyploidy in plant species in *in vivo*, *ex vitro* and *in vitro* conditions. The *in vitro* systems are the most widely used due to the shorter times required for polyploidy induction and their faster propagation in a limited space, with sterile and controllable environmental conditions (Eng and Ho, 2019). Moreover, with adventitious organ regeneration techniques, *in vitro* polyploidy induction has become the method of choice for several plant species because it increases the mutagenesis efficiency and decreases the occurrence of chimeras in comparison to *in vivo* methods (Rêgo et al., 2011).

The key step in the polyploidization process is the choice of suitable polyploidy inducers. To date, more than 60 studies have applied antimitotic agents for polyploidization using different plant tissues and organs and quantified the effects on specialized metabolite production

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(Gantait and Mukherjee, 2021). During polyploidization induction in *in vitro* conditions, several parameters can be manipulated to obtain the highest percentage of polyploids (Eng and Ho, 2019). The effectiveness of the antimitotic compound depends greatly on the applied concentration, the duration of treatment, the induction medium, the usage of shakers, the type of explant, and supplementation with DMSO (or other permeabilizers) to facilitate the penetration of the compound (Ascough et al., 2008).

Among the different antimitotic agents, colchicine has proved to be the most efficient and reliable in producing polyploids. It is a naturally produced alkaloid extracted from meadow saffron, which belongs to the *Liliaceae* family (Swarbrick, 1986). Its mechanism of action consists of binding to tubulin heterodimers, causing the failure of microtubule assembly during mitosis at the metaphase stage (Borisy and Taylor, 1967). This prevents chromosome separation, and therefore, by the end of the cycle, two sets of chromosomes will remain in a single nucleus and a polyploid cell will be formed.

Concentration and treatment duration are inter-dependent and considered the most crucial factors in the use of colchicine for the induction of polyploidization. In general, colchicine is applied at concentrations ranging from 0.01% (v/w) to 0.1% (v/w) for a time of 24–72 h. Low doses are usually unsuccessful whereas high doses can be lethal (Widoretno, 2016).

Colchicine has been widely used to induce polyploidy in medicinal plants, altering the production of specialized metabolites. An increment in the specialized metabolite levels of *Centella asiática* (Kaensaksiri et al., 2011; Thong-On et al., 2014), *Datura stramonium* (Al-Taweel et al., 2019), *Echinacea purpurea* (Abdoli et al., 2013; Yang et al., 2009), *Panax ginseng* (Kim et al., 2004), *Papaver somniferum* (Mishra et al., 2010), *Pogostemon cablin* (Widoretno, 2016; Yan et al., 2016), *Salvia miltiorrhiza* (Gao et al., 1996) and *Scutallaria baicalensis* (Gao et al., 2002), among other species, has been reported. However, polyploidization does not always benefit the production of specialized metabolites. Several studies have reported polyploid plants that are less productive or similar to diploid plants, such as *Solanum bulbocastarum* (Caruso et al., 2013) and *Miscanthus* × *ginganteus* (Ghimire et al., 2016).

Given the growing interest in increasing the supply of plant-derived bioactive compounds to treat human diseases, the induction of polyploidy plants is a potentially useful strategy to achieve enhanced production. The well-known anticancer drug paclitaxel (PTX) is produced in the inner bark of *Taxus* spp. trees, but in scarce quantities, so its current market demand cannot be met solely by extraction from natural sources. Therefore, the use of plant cell cultures of *Taxus* spp. is an attractive alternative for PTX production (Vidal-Limon et al., 2018). Different biotechnological approaches have been used to increase the *in vitro* production of PTX and related taxanes, both empirical (Cusido et al., 2014; Ramirez-Estrada et al., 2015; Escrich et al., 2021) and rational (Escrich et al., 2022; Sanchez-Muñoz et al., 2018). In this context, polyploidization has potential as an effective strategy to overcome the natural scarcity of taxanes by improving the growth, productivity, and yield of *Taxus* spp. *in vitro* cell cultures.

In vitro cultures of *Taxus* spp. can be established using different plant tissues and organs (Chee, 1995; Ewald, 2007; Xie and Guo, 1999). For polyploidy induction, it has been described that shoot apical meristems and seeds can be used as starting material to obtain polyploid plants (Salma et al., 2017), but young meristems have the highest efficiency of polyploidization (Gantait and Mukherjee, 2021). Nevertheless, depending on the explant type and plant species, cell permeability can be improved to facilitate the penetration of the mutagenic agents into cells. The addition of dimethyl sulfoxide (DMSO) in the process of polyploidy induction increases the efficiency of the mutagenic agent (Chen and Gao, 2007; Grouh et al., 2011; Yan et al., 2016). However, the study of cell viability is recommended when DMSO is added to the media due to its toxicity, which could compromise cell viability and influence plant growth (Eng and Ho, 2019).

This study aimed to obtain polyploid *in vitro T. baccata* cell cultures for the first time by treating immature embryos with colchicine and in this way to increase the taxane yield. A combination of three concentrations of colchicine and three exposure times were applied to assess the best conditions to obtain polyploid *T. baccata* cell lines. Therefore, the present work evaluates the effectiveness of colchicine in inducing polyploidy in *T. baccata*. In the polyploid cell cultures, both the production of taxanes and the expression of PTX biosynthetic genes were assessed to determine the effect of polyploidization on the specialized metabolism of *T. baccata*. Overall, an in-depth characterization of morphology, taxane production, and gene expression was performed to determine the effects of polyploidy in *T. baccata* cell lines.

2. Materials and methods

2.1. Plant material

Seeds containing immature embryos were collected from *T. baccata* trees. They were sterilised by washing with sterile distilled H_2O, 70% ethanol for 30 s, rinsed 2 times with sterile distilled H_2O, 2% mercury chloride ($HgCl_2$) for 30 min, rinsed with sterile distilled H_2O, 30% bleach with 10 drops of Tween-20 for 30 min in an ultrasonic bath, and finally, rinsed 2 times with sterile distilled H_2O. Sterilized seeds, isolated embryos and the new plantlets were cultured on Gupta and Durzan medium (G&D) (Gupta and Durzan, 1985) supplemented with 3% sucrose and 5 g/L of phytoagar. Isolated embryos from sterilized seeds were cultured in darkness for six weeks, and then maintained in an 8–16 h light-dark cycle at 25ºC. *T. baccata* plantlets were cultured in the same temperature and photoperiod conditions.

2.2. Colchicine treatment

Embryos were treated with different concentrations of colchicine (0.01%, 0.05% and 0.1%) for three different exposure times (24 h, 48 h and 72 h) in G&D liquid media. 2% (v/v) dimethyl sulfoxide (DMSO) was added to the treatments as a permeabilizer. Sterile distilled H_2O was added to the medium instead of DMSO for the negative controls. The incubation with colchicine was carried out using an orbital shaker at 115 rpm. Once the exposure time was over, embryos were cultured in solid fresh G&D media without colchicine in darkness at 25ºC.

2.3. Callus induction and cell suspension cultures

For callus induction, B5 medium (Gamborg et al., 1968) was supplemented with 3% sucrose, 4 mg/L of 2,4-dichlorophenoxyacetic acid, 1 mg/L of kinetin and 0.5 mg/L of gibberellic acid, pH 5.8. For callus maintenance, B5 medium was supplemented with 0.5% sucrose, 0.5% fructose, 2 mg/L of 1-naphthaleneacetic acid, 0.1 mg/L of 6-benzylamino purine and 0.5 mg/L of gibberellic acid, pH 5. 8. All calli were maintained at 25ºC in darkness and were subcultured biweekly. Suspension cell cultures were generated and two-stage cultures were established as described by Cusido et al. (2002), as this system has proved effective for the stimulation of taxane production.

2.4. Morphological analysis

A magnifier colour camera (Optronics, Goleta, CA) mounted on a Leica MZFLIII stereomicroscope (Leica Microsystems, Bannockburn, IL) was used to acquire pictures of embryos. For calli, images were taken by Leica THUNDER DMi8 microscope (Leica Microsystems, Bannockburn, IL) using LAS X 3.0.1 software. ImageJ software (Wayne Rasband, National Institutes of Health-NIH, Maryland, USA) was used to measure cell size and set the scale bar.

2.5. Flow cytometric analysis

Nuclei were extracted from calli using the CyStain™ PI Oxprotect kit

(Sysmex, Gorlitz, Germany). 200 mg of callus was incubated in 500 μL of staining buffer for 1 min. Samples were filtered using disposable filters CellTrics™ 50 μm (Sysmex, Gorlitz, Germany) and 1.5 mL of staining buffer was added to the filtrate. Then, the filtrate containing the released nuclei was stained with 2.5 μL of 4′,6-diamidino-2-phenylindole (DAPI) (1 mg/mL). All extraction steps were carried out on ice to maintain nuclei integrity. Samples were analysed by flow cytometry using a Gallios flow cytometer (Beckman Coulter, Brea, CA, EEUU.). The DAPI stain was excited at 405 nm and the emission was collected from 430 to 470 nm. The figures from the flow cytometer analysis were obtained using FlowJo v10 (Ashland OR 97520).

2.6. Taxane determination

Taxanes were extracted from the culture media and lyophilized cells were harvested at 0, 8, 16 and 24 days as described by Cusidó et al. (1999) and quantified by UPLC (Bonfill et al., 2003) with a Water Acquity Ultra Performance LC system (Waters, Milford, MA, USA). Target taxanes, 10-deacetylbaccatin III (DABIII), baccatin III (BACIII), 10-deacetyltaxol (DAT), cephalomannine (CEPH) and paclitaxel (PTX), were provided by Abcam (Cambridge, UK).

2.7. Quantitative real-time PCR (RT-qPCR)

For gene expression analysis, total RNA was isolated from 100 mg of lyophilized cells using the Real Plant RNA Kit (REAL, Valencia, Spain) following the manufacturer's instructions. cDNA was prepared from 1 μg of RNA with SuperScript IV reverse transcriptase (Invitrogen, CA, USA) and an RT-qPCR assay was performed with SYBR Green (Biorad, Hercules, CA, USA) using gene-specific primers designed with Primer3 (v 0.4.0; Table S1), in a 384-well platform system (LightCycler ® 480 Instrument, Roche, USA). The expression of genes encoding geranylgeranyl diphosphate synthase (*GGPPS*), taxadiene synthase (*TXS*), 10-deacetylbaccatin III-10β-*O*-acetyltransferase (*DBAT*), baccatin III 13-*O*-(3-amino-3-phenylpropanoyl) transferase (*BAPT*) and 3′-N-debenzoyl-2′-deoxytaxol-N-benzoyltransferase (*DBTNBT*) was quantified. *TBC41* was used as a housekeeping gene for expression normalization. For each gene, relative expression levels were normalized relative to time 0 (reference value = 1). Samples for determining taxane biosynthetic gene expression were taken at 0, 4, 8, 12, 24, 48 and 72 h.

2.8. Statistical analysis

For the statistical analyses, Shapiro's and Levene's tests were used to test the normality and variance of each dataset. A two-factor ANOVA was used as a parametric test and the Krustal-Wallis rank sum test followed by Dunn's Multiple Comparison test were used as non-parametric tests. A p-value of < 0.05(*), < 0.01(**) and < 0.001(***) were assumed for significant differences. All the analyses were performed in R.

3. Results

3.1. Effect of colchicine on embryo germination and development

T. baccata embryos were treated with colchicine and DMSO was used as a permeabilizer in all samples. Consequently, the effects of DMSO were determined in terms of embryo development. The embryos were cultured for 24, 48 and 72 h with 2% DMSO or H_2O (control conditions). Of the 200 embryos treated with DMSO or H_2O, 70% and 71%, respectively, had a normal development (Fig. S1). Therefore, 2% (v/v) DMSO did not affect the germination and development of embryos during the three exposure times (24, 48 and 72 h).

The embryos treated with colchicine displayed a lagged germination, suggesting the colchicine concentration was a more important variable than the exposure time (data not shown). Consequently, we focused our attention on the different colchicine concentrations.

Regarding the effect of colchicine on embryo germination, the survival rate was measured for eight weeks after the treatment, and non-germinated and brown embryos were considered dead or aborted. The three treatments of colchicine applied to the immature embryos showed significant differences in terms of viability (Fig. 1A). 0.1% colchicine caused the maximum mortality rate, resulting in 72% of embryos being non-viable, followed by the 0.05% treatment, which caused 68% mortality. On the other hand, the germination rate of the control treatment was 70%, followed by the 0.01% colchicine treatment with a germination rate of 63% (Fig. 1A).

The colchicine concentration also affected the development of the embryo. In the early stages of embryo development aerial parts and roots are produced in a coordinated process. However, it was observed that the treated embryos often did not develop roots. In some cases, they only developed aerial parts and had a lower survival rate compared to the diploid cells (Fig. 1). The negative control conditions produced the highest rate of well-developed embryos (close to 80%), followed by the 0.01% colchicine treatment (50%) (Fig. 1B). In contrast, the 0.05% and 0.1% colchicine treatments resulted in semi-developed embryos (80% and almost 90%, respectively), which had aerial parts but not roots (Fig. 1B). Apart from the alterations in development, early-stage growth was also slower in the treated embryos than in the control samples. For that reason, 0.01% colchicine seemed the best concentration for embryo growth and development (Fig. 1).

A morphological study was then performed based on the developmental differences between the treated and untreated embryos. Fig. 2 shows the structural differences between the control embryos (Fig. 2A) and those of the different colchicine treatments (Fig. 2B-D). Whereas control embryos had correctly developed roots with numerous absorbent hairs (Fig. 2A), the embryos treated with 0.01% colchicine had

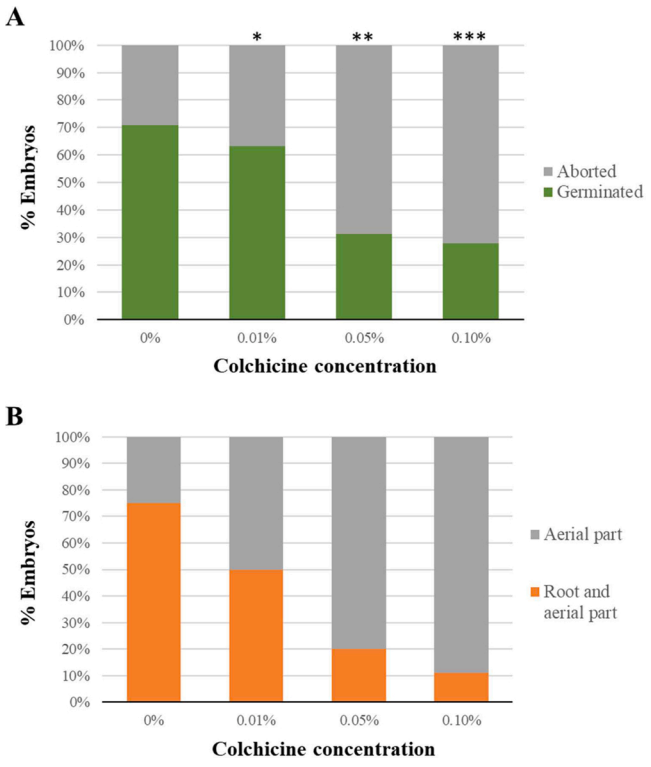


Fig. 1. A) Germination (green) and mortality (grey) rate of embryos. B) Early steps of embryo development at different colchicine concentrations indicating the presence of only an aerial part (grey) or both root and aerial parts (orange). Untreated embryos (control conditions; 0% colchicine) and the different colchicine concentrations (0.01%, 0.05% and 0.1%) are depicted in the x-axis. A p-value of < 0.05 (*), < 0.005 (**), and < 0.001 (***) was used for statistically significant differences.

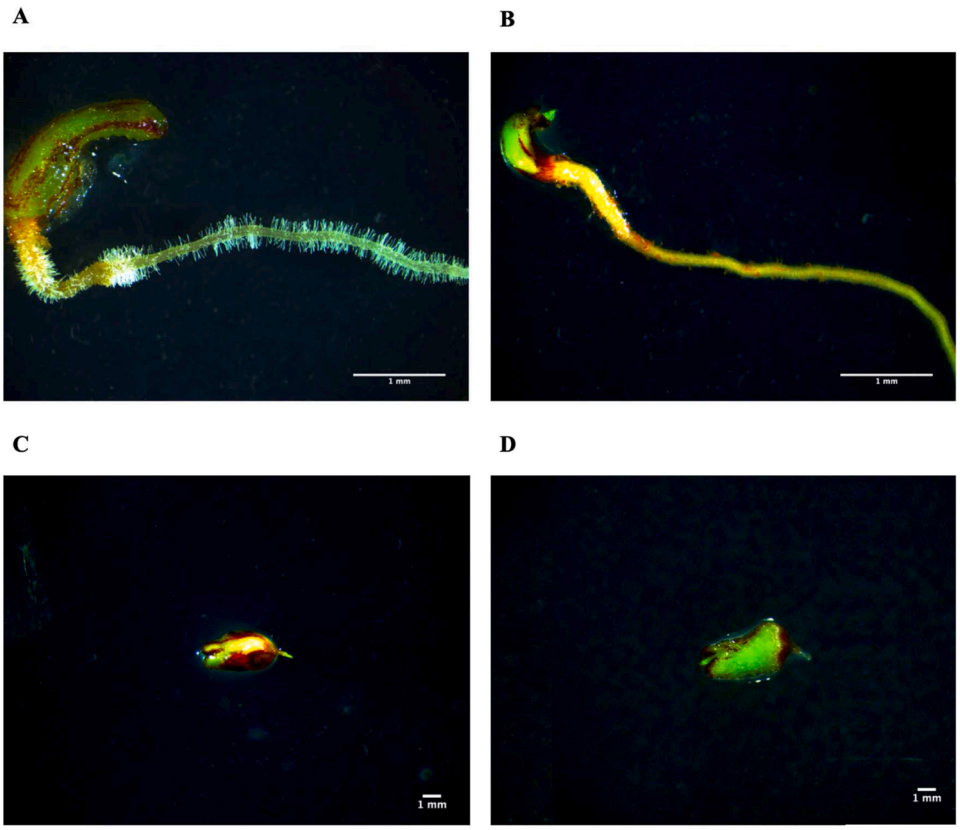


Fig. 2. Early stage of embryo development. Embryos treated with different concentrations of colchicine: **A)** 0% (negative control), **B)** 0.01%, **C)** 0.05%, **D)** 0.1%. Scale bar = 1 mm.

roots with scarce and burned absorbent hairs (Fig. 2B). Moreover, embryos treated with 0.05% and 0.1% colchicine did not develop roots, presenting only the radicle (Fig. 2C and D, respectively).

From the obtained embryos, the ones exhibiting notable phenotypic differences and proper growth, all of them treated with 0.05% or 0.1% colchicine, were selected for culture (Fig. 2). As shown in Fig. 3, the putative polypoid *T. baccata* plants presented an abnormal growth, whereas control plants (diploid) had a slim, well-defined stem and clear gravitropic growth (Fig. 3A). The putative polyploid plant obtained with 0.1% colchicine presented a stem that was not completely developed, and its growth was not phototropic (Fig. 3C). Moreover, it had smaller leaves and shorter internodal distances than the diploid plant. The control plants had a deep green coloration in contrast with the yellowish-green of putative polyploid plants obtained with 0.05% and 0.1% colchicine (Fig. 3 A-C). The morphology associated with 0.05% colchicine treatment differed from the normal development of the control plant in that the treated plants had two shoots and greater leaf length and nodal distances, although the number of leaves was the same (Fig. 3B).

3.2. Callus induction

Plantlets (control and treated with colchicine) were used to induce callus lines (Fig. 3D-F) to obtain polyploid cell cultures for the taxane production studies. The induced calli from the control plantlet (untreated with colchicine) had brown cells forming aggregates (Fig. 3D). In contrast, the putative polyploid calli derived from the 0.05% colchicine-treated plantlets were characterized by dark brown tissue and large aggregates, whereas the calli derived from the 0.1% colchicine-treated plantlets had light-brown friable tissue (Fig. 3E and F, respectively).

3.3. Ploidy determination

Calli obtained from *T. baccata* plantlets were analyzed by flow cytometry to reveal the ploidy levels of each cell line. The callus induced from the untreated embryo (0% colchicine) was used as the negative control (diploid).

In the flow cytometric histograms, the peak of the diploid *T. baccata* callus (2x) was set at channel 222 K (Fig. 4 A). Therefore, the peak of tetraploid (4x) nuclei was expected at channel 444 K, and a peak corresponding to 4x appeared at 455 K (Fig. 4B). The mix of diploid (2x) and tetraploid (4x) is represented in Fig. 4 C. This mix was performed to corroborate the polyploidy determination, where the tetraploid peak had approximately twice the fluorescence intensity in proportion to the square root of ploidy of the diploid peak (Fig. 4 C). Moreover, the statistics (mean, median and mode) between the diploid *T. baccata* cell line and the induced tetraploid cell line were proportional and the difference between each measurement was approximately 2-fold (Fig. 4 F).

Regarding the morphology produced by 0.1% colchicine, a peak at 299 K was obtained in the cytometry analysis (Fig. 4D), which did not correspond to a 4x peak. To determine if this was due to analytical effects, such as aggregations of DAPI, a mixture of that sample and the diploid control was analyzed. Two different peaks were obtained (Fig. 4E), one at channel 222 K belonging to 2x, and another one at channel 299 K belonging to an incomplete polyploidization.

The statistics regarding the unusual peak found in the IP cell line showed that the difference in mode is approximately 1.4-fold compared to the control (Fig. 4F). Therefore, it could not be considered either a tetraploid or a diploid, and it was described as an incomplete or intermediate ploidy.

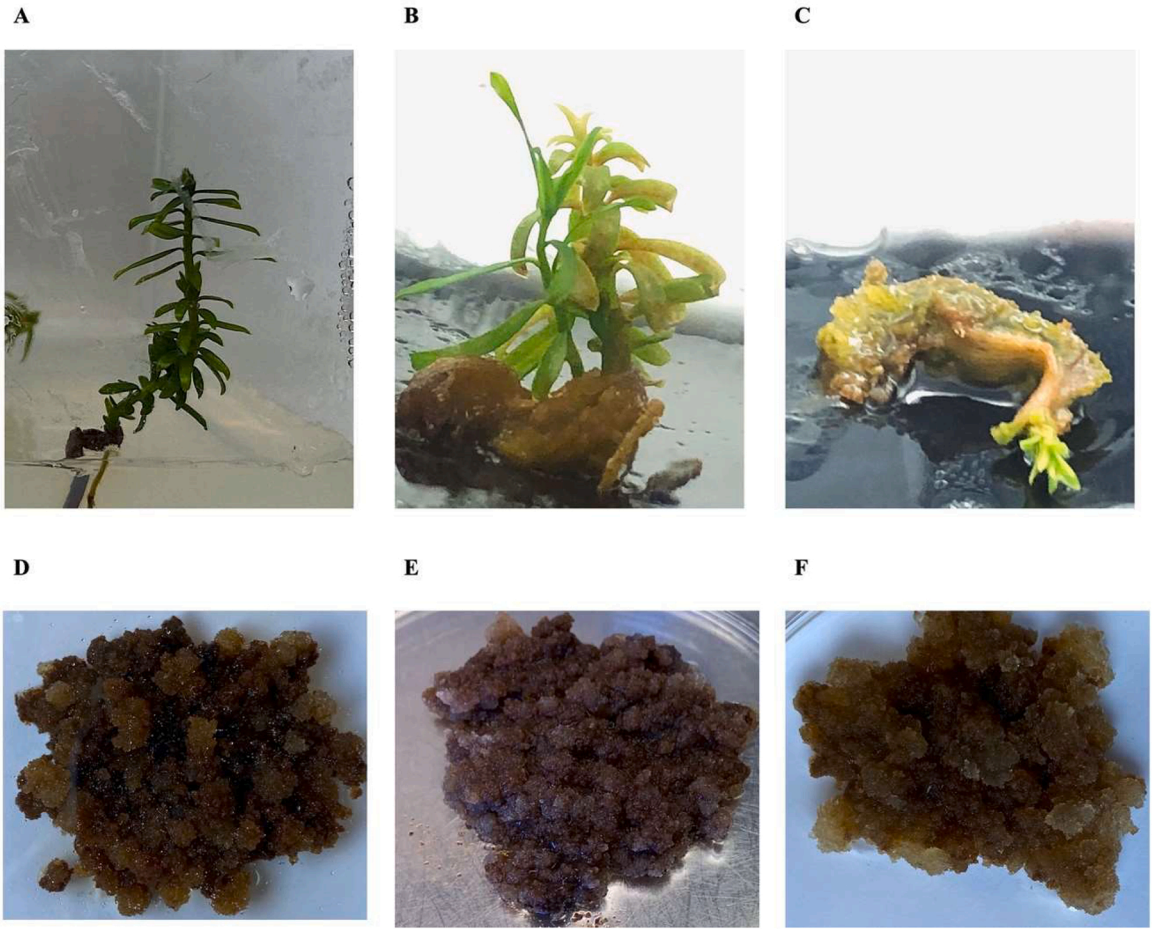


Fig. 3. Candidate plantlet polyploids: **A)** Control 2x (Diploid), **B)** Morphology associated with 0.05% colchicine **C)** Morphology associated with 0.1% colchicine. Calli induced from A, B and C plantlets used as explants: **D)** Control 2x callus, **E)** Calli from plantlets treated with 0.05% colchicine and **F)** Calli from plantlets treated with 0.1% colchicine.

3.4. Cell size determination

Two types of cells were observed in the diploid line of *T. baccata*, either elongated or rounded. The same cell morphologies were found in the cell cultures obtained from colchicine-treated embryos. In Fig. 5, it can be observed that the diploid (2x) cell culture was a mixture of elongated and rounded cells (Fig. 5A). The cell culture with intermediate ploidy (treated with 0.1% colchicine) looked like the diploid one (Fig. 5B). Finally, the tetraploid cell line (treated with 0.05% colchicine) also presented a mixture of elongated and rounded cells (Fig. 5C and D).

However, in the line with incomplete ploidy (IP), cell size was similar to the control and did not show significant differences (Table 1). Meanwhile, in the tetraploid (4x) cell culture, the cell size was 3.33- and 2.99-fold higher for the elongated and rounded cells, respectively, compared to the diploid cells (Table 1).

3.5. Effect of ploidy on taxane production

The total taxane content of the three cell lines, diploid (2x), IP and tetraploid (4x) was determined by UPLC as the sum of DABIII, BACIII, DAT, CEPH and PTX at different time-points (0, 8, 16 and 24 days).

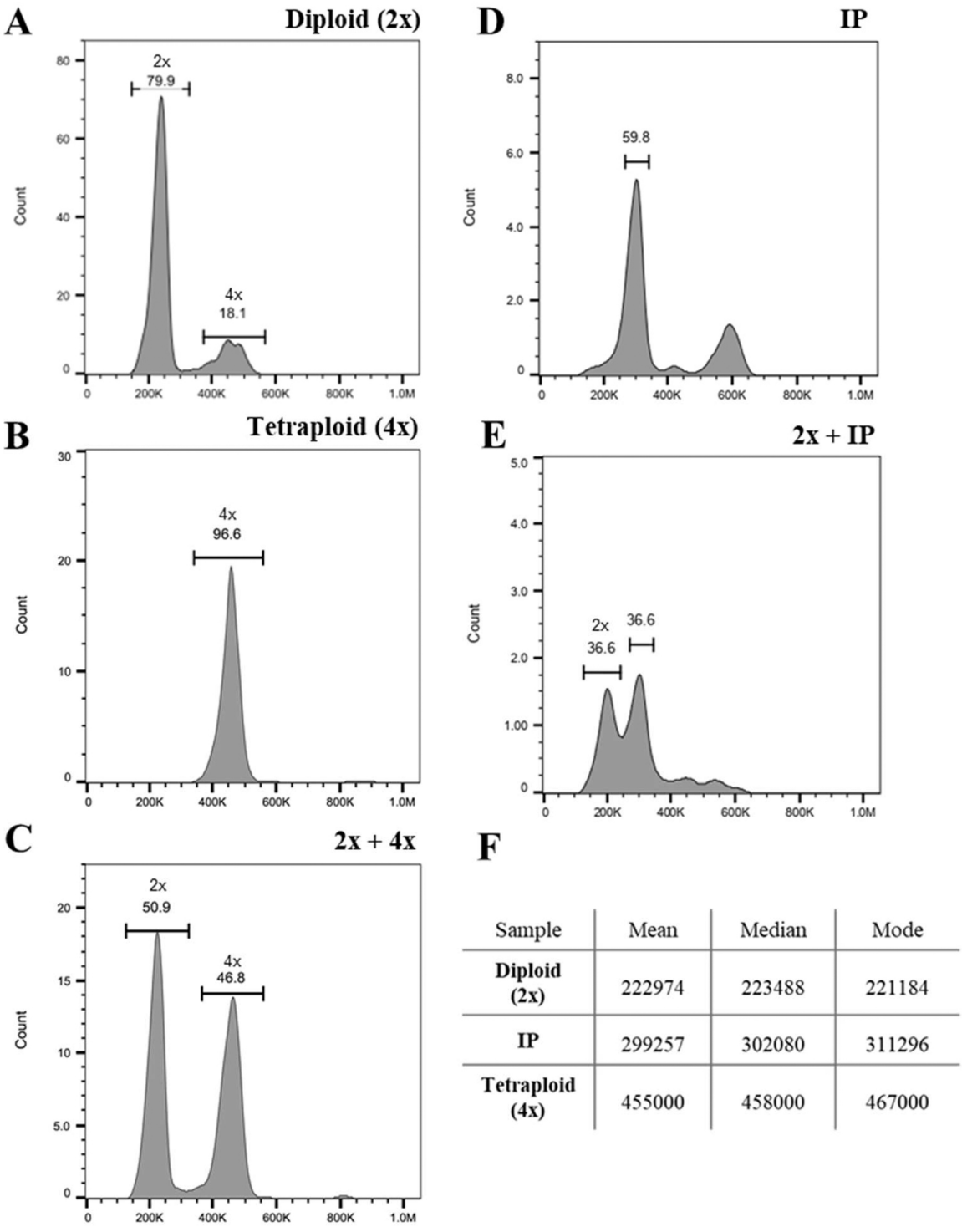
Fig. 6 shows the extracellular, intracellular and total taxane content. The extracellular taxane content oscillated between 0.7 mg/L and 19 mg/L (Fig. 6A). The highest content was obtained by the tetraploid cell line (4x) at day 8, which was 10.8-fold higher compared to the diploid cell line on the same day, indicating the tetraploid cell line had a higher productivity (Fig. 6A). On the other hand, the maximum intracellular taxane content of approximately 2.5 mg/L was observed in the 2x cell line (Fig. 6B). Although no significant differences were found in the intracellular production of taxanes between the three cell lines, the taxane content in the tetraploid cell line was 2.5-fold lower than in the diploid line at the end of the experiment.

Regarding the total taxane content (Fig. 6C), the maximum production in the diploid cell culture was 13.7 mg/L and reached at day 16. On the other hand, the taxane production levels remained very low in the IP cell line throughout the experiment, oscillating between 1.8 and 2.5 mg/L. Finally, the highest taxane production was achieved by the tetraploid cell (4x) line at day 8, when it was 6.2-fold (21.64 mg/L) higher than in the diploid cell line at this time, indicating a higher productivity and greater taxane yield.

The profile of the taxanes produced was similar between the three cell lines, with DAT produced in higher quantities than PTX. The taxane composition of the diploid cell line was more uniform compared to the tetraploid line, as all intermediates were converted into DAT. Regarding taxane excretion, the studied taxanes mainly accumulated extracellularly rather than intracellularly.

3.6. Taxane gene expression

The transcription profile of the taxane biosynthetic genes in the cell lines was assessed by RT-qPCR (Fig. 7). The relative expression of target genes belonging to early (*GGPPS* and *TXS*), intermediate (*DBAT*) and late steps (*BAPT* and *DBTNBT*) of the taxane biosynthetic pathway was analyzed. The results showed that all the target genes were



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Sample	Mean	Median	Mode
Diploid (2x)	222974	223488	221184
IP	299257	302080	311296
Tetraploid (4x)	455000	458000	467000

Fig. 4. Flow cytometric histograms of DAPI-stained nuclei from *Taxus baccata* calli maintained in *in vitro* conditions. **A)** Control, diploid (2x). **B)** Treated with 0.05% colchicine, tetraploid (4x). **C)** Mixture of control and treated with 0.05% colchicine (2x + 4x). **D)** Treated with 0.1% colchicine, unusual peak, incomplete ploidy (IP). **E)** Mixture of control and treated with 0.1% colchicine. X-axis represents arbitrary units of fluorescence. Y-axis represents the number of nuclei analyzed. **F)** Mean, median and mode for the three samples (diploid (2x) cell line, IP cell line and tetraploid (4x) cell line) were used as statistics for the flow cytometric analysis.

overexpressed in the tetraploid line compared to the diploid line. The basal transcription levels of the tetraploid cell line (4x) compared to the control (2x) were between 1.84- and 12.83-fold higher (Fig. 7).

Regarding the transcript levels of the early step genes, maximum *GGPPS* expression was observed in the 4x cell line at 48 h, being 1.76- and 4.7-fold higher than the diploid and IP cell lines, respectively. For *TXS*, the tetraploid line showed two peaks of expression, at 12 and 72 h, which were 2.84- and 2.66-fold higher than the diploid line, respectively (Fig. 7). The expression of *TXS* in the IP cell line was 1.45- and 1.77-fold higher at 4 and 24 h compared to the diploid line (Fig. 7).

Regarding the intermediate steps, *DBAT* transcript levels in the tetraploid line were higher than in the 2x and IP cell lines, the relative expression value at 4 h being 9.1-fold higher than in the control.

The genes involved in the last steps, *BAPT* and *DBTNBT*, are known

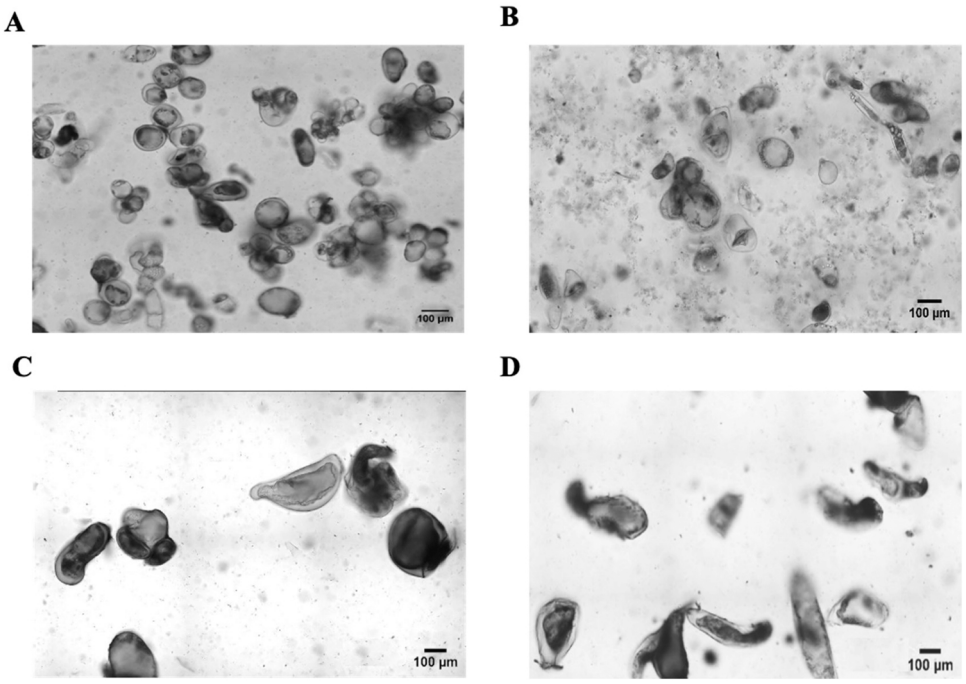

Fig. 5. Morphological comparison between: **A)** diploid cells (2x), **B)** incomplete ploidy cells (IP), **C)** rounded tetraploid (4x) cells and **D)** elongated tetraploid (4x) cells. Scale bar = 100 µm.

Table 1
Mean cell size (µm) of the different *Taxus baccata* cell lines: diploid (2x), incomplete ploidy (IP), and tetraploid (4x).

Sample	Elongated cells (µm)	Rounded cells (µm)
2x	170.62 ± 45.78	105.33 ± 18.48
Incomplete ploidy (IP)	160.33 ± 20.24	112.33 ± 13.34
4x	569.22 ± 95.46***	315.70 ± 83.32***

Statistical analysis was performed comparing 2x (control) samples with the IP and 4x cell lines (*p*-value: * < 0.05; ** < 0.01; *** < 0.001).

to be most regulated genes of the pathway. Compared to the diploid line, where the transcripts of *BAPT* and *DBTNBT* genes were almost undetectable, the transcriptional peaks in the tetraploid cell line were 7.17-fold higher at 4 h for *BAPT* and 53.2-fold higher at 48 h for *DBTNBT*, showing the highest increase of all the studied genes (Fig. 7).

To sum up, the tetraploid cell line showed an enhanced expression of all the genes studied, which correlates with its high taxane production rates. On the other hand, the IP line presented a non-homogenous expression profile that clearly differs from the 2x profile. The expression rates of *GGPPS*, *DBAT* and *DBTNBT* genes were lower in the IP than the diploid cell line. In contrast, the expression values for the *TXS* and *BAPT* genes in the IP line were similar to those of the tetraploid cell line (Fig. 7).

4. Discussion

To the best of our knowledge, this is the first report of the successful induction of tetraploidy in *Taxus* spp. by treating diploid embryos with colchicine in *in vitro* conditions. Although polyploidy is relatively common in angiosperm trees, and perhaps all angiosperms have experienced polyploidy during their evolutionary history (Wendel, 2000), it is rather infrequent among gymnosperms (Adams and Wendel, 2005; Ahuja, 2005).

It has been demonstrated that the *in vitro* treatment of nodal segments with colchicine successfully induces polyploidy (Iannicelli et al., 2016; Inthima and Sujipuli, 2019; Javadian et al., 2017). In this study, *T. baccata* embryos were used due to the lack of efficient protocols for plant regeneration and organogenesis of *Taxus* spp. Therefore, *T. baccata* embryos treated under different conditions were cultured to obtain plantlets, which were transferred to an induction medium to obtain polyploid calli. Finally, polyploid cell cultures of *Taxus* spp. were generated from callus tissue and, after their morphological characterization, their taxane content and the expression of several genes involved in taxane biosynthesis were determined.

The effect of colchicine on the embryo survival rate and germination capacity depended mainly on the concentration rather than the exposure time. As the colchicine concentration increased, the rate of live and well-developed embryos decreased. The untreated embryos (control) mostly developed normally, and only 20% did not develop roots, attributed to potential injuries in the manual process of embryo isolation. On the other hand, the rooting of treated embryos occurred later than in the untreated ones. This delay in rooting when using colchicine treatments has also been reported by other authors (Gantait et al., 2011; Tavan et al., 2015). In addition, in the treatments with 0.05% and 0.1% colchicine, 80% and 90% of the embryos, respectively, did not develop roots. The embryos that only had aerial parts and radicles were non-viable, since root development is necessary for nutrient and water uptake from the culture medium, as well as for the proper physiological regulation of the growing process (Jones and Dolan, 2012). Nevertheless, due to the inhibitory effect of colchicine on cell division (Ascough et al., 2008) we can expect an increase in mortality and abnormal growth associated with chemical damage by the mutagenic agent.

In mature stages, polyploidy plantlets were yellowish green, which could be due to worthless stems failing to transport water and nutrients to the aerial parts (Pate and Dieter Jeschke, 1995). Furthermore, the lack of apical dominance and changes in tropism could be correlated with the worthless polyploidy stem development (Ahuja, 2005). Moreover, although gymnosperm seeds, such as those of *Taxus* spp., only develop one shoot, two shoots developed simultaneously in the tetraploid plants. These observations could be related to the growth abnormalities of the two plant morphologies selected, the morphology of IP showing more growth abnormalities than tetraploid plantlets (4x). Furthermore, the growth aberrations in the early stages of plantlet

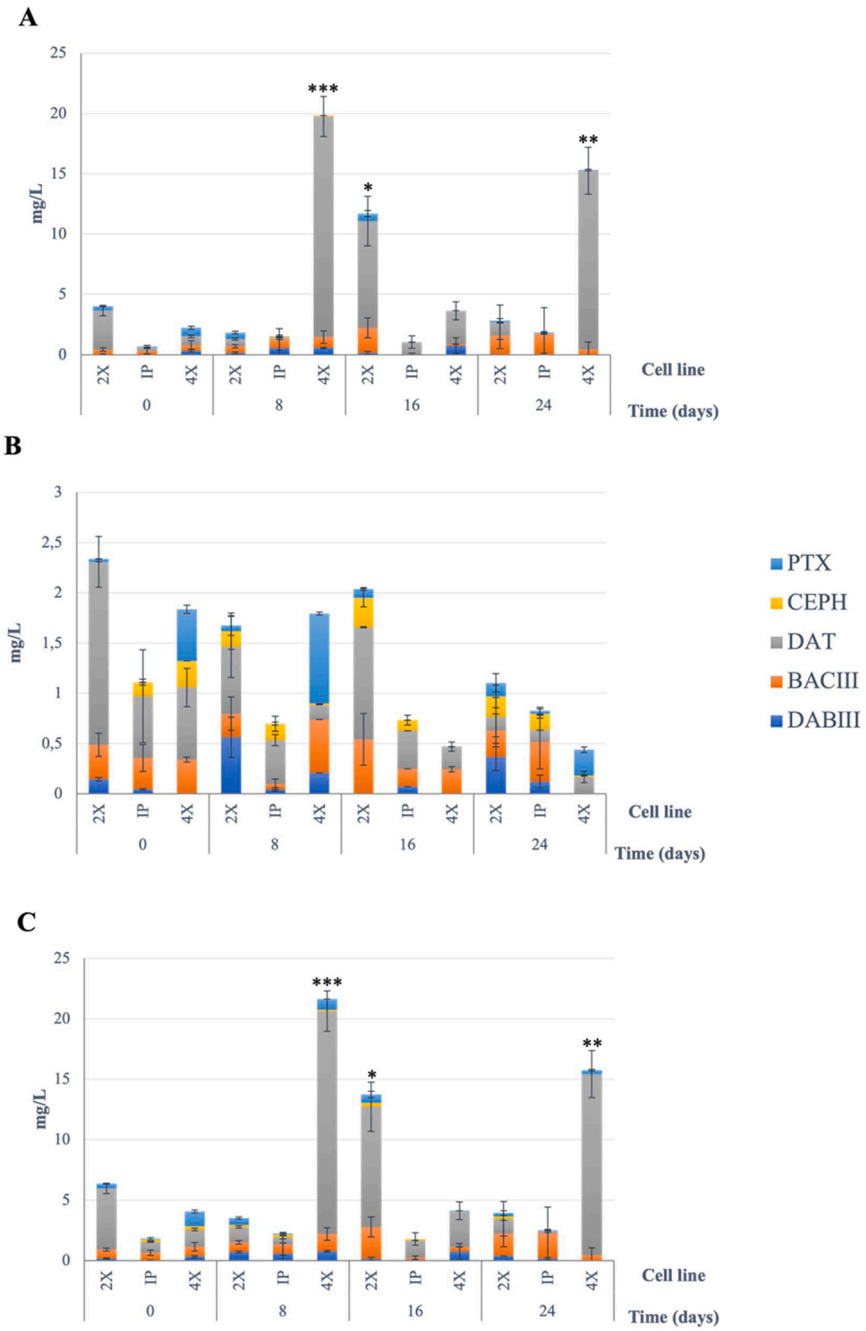

Fig. 6. Taxane production of the *Taxus baccata* cell cultures: diploid (2x), incomplete ploidy (IP) and tetraploid (4x). **A)** Extracellular taxane content. **B)** Intracellular taxane content. **C)** Total taxane content. A *p*-value of < 0.05 (*), < 0.005 (**), and < 0.001 (***) was used for significant differences. 10-Deacetylbaccatin III (DABIII), baccatin III (BACIII), 10-Deacetyltaxol (DAT), cephalomannine (CEPH) and paclitaxel (PTX).

growth could be explained by the fact that polyploidy is rare in gymnosperms, and induced polyploids in conifers are prone to exhibit growth abnormalities (Ahuja, 2005).

Due to the limited embryo germination and seedling development, calli were induced from plantlets to provide enough plant material for the study. The calli corresponding to the 0.1% colchicine treatment (IP) had a similar appearance to those of the diploid control, whereas those of the 0.05% colchicine treatment (4x) were dark brown and had large aggregations of cells. This callus phenotype has been correlated with high taxane production rates (Patil et al., 2013; Sanchez-Muñoz et al., 2018). The ploidy level of the callus was determined by flow cytometry, a commonly used method for ploidy determination because it is fast and reliable (Eng and Ho, 2019; Gantait and Mukherjee, 2021). The nuclei fluorescence peak of the tetraploid cell line was twice as intense as that of the diploid, representing a double quantity of DNA. However, in 0.1% colchicine-treated samples, an intermediate peak between the diploid and tetraploid peaks was observed (Fig. 4D). This peak, however, is unlikely to match triploid nuclei because it is not equidistant from the diploid and tetraploid peaks, appearing to be the result of an incomplete ploidy. In a study with *Watsonia lepida,* an unusual peak was also found in the flow cytometric analysis of seedlings treated with colchicine (Ascough et al., 2008). Colchicine may have caused a disruption in cell division in some cells, so that DNA replication was not fully accomplished, leading to an incomplete doubling of the chromosomes and an intermediate polyploid cell culture. Although uncommon, incomplete doubling and aneuploid and triploid production can occur when

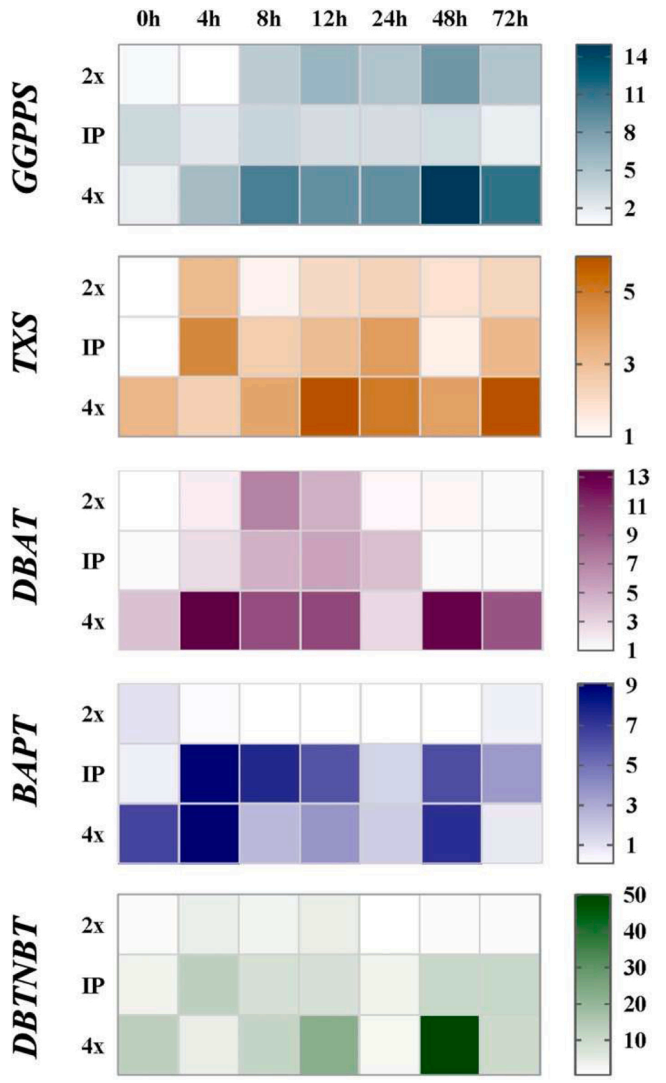

Fig. 7. Transcript profile of taxane pathway genes in the three cell lines: diploid (2x), incomplete ploidy (IP) and tetraploid (4x). Colors in the expression profile represent each gene of the taxane pathway, with dark colors for high relative expression and light colours for low relative expression. Geranylgeranyl diphosphate synthase (*GGPPS*): turquoise blue; taxadiene synthase (*TXS*): orange; 10-deacetylbaccatin III-10β-*O*-acetyltransferase (*DBAT*): purple; baccatin III 13-*O*-(3-amino-3-phenylpropanoyl) transferase (*BAPT*): dark blue, and 3′-N-debenzoyl-2′-deoxytaxol-N-benzoyltransferase (*DBTNBT*): green. Color scale ranges represent the relative expression of each gene.

attempting to induce tetraploid plants, as reported in previous studies (Zlesak et al., 2005).

Besides the alterations in the polyploid development, the cell size of the IP line was very similar to the control line. In contrast, the tetraploid cell size was significantly higher than the diploid cell line. Similar alterations in cell size have been reported by other authors (Kondorosi et al., 2000; Robinson et al., 2018; Zhang et al., 2019). Regarding specialized metabolite production, it has been demonstrated that polyploids usually produce more metabolites than diploids (Salma et al., 2017). Higher levels of phytopharmaceutical compounds have been reported in tetraploid plants, such as a 50% increase in the morphine content of *Papaver somniferum* (Mishra et al., 2010) and an 11% increment of triterpenes of *Centella asiatica* (Kaensaksiri et al., 2011), as well as an increased production of anticancer compounds such as podophyllotoxin in *Linum album* or beta-asarone in *Acorus calamus* (Madani et al., 2021). However, to date few studies have examined the effects of ploidy in cell cultures; for example, production of the antioxidant baicalin was studied in cell cultures of *Scutellaria baicalensis* (Gao et al., 2002).

In the present study, not only was the production of taxanes in the tetraploid cell line of *T. baccata* significantly increased, being 10.8-fold higher compared to the control, but the peak of production was obtained earlier, at day 8, thus demonstrating an improved productivity and taxane yield. The taxane production of 21.64 mg/L achieved in the tetraploid cell line is significantly higher than previously reported yields in diploid cell lines of *T. baccata*. For example, Perez-Matas et al., (2022) obtained a maximum taxane production of 5 mg/L at day 12 of culture, and Sanchez-Muñoz et al. (2018) reported total taxane contents of 2.48 mg/L in an old *T. baccata* cell line with low productivity and 5.36 mg/L in a high-producing line after 24 days of culture. Considering these data, the results obtained in the present study with the polyploid *T. baccata* cell line are highly encouraging, as the treatment not only improved yields, but also shortened the time required to reach optimal production.

Another biotechnological approach to taxane production is based mainly on elicitation. For instance, the taxane yield of *T. media* cell cultures was 140 mg/L after elicitation with methyl jasmonate and cyclodextrins (Sabater-Jara et al., 2014), and 85 mg/L after treatment with coronatine and calix[8]arenes (Escrich et al., 2021). In both cases, the taxane yield was greater than in the polyploid cell line (21.64 mg/L), but the high economic cost of these treatments hinders their industrial application.

Moreover, the taxane content inside the cells of the tetraploid line was 2.5-fold lower compared to the diploid line, indicating that the polyploid line had a higher capacity to excrete taxanes to the medium. The facilitation of taxane excretion from cell cultures constitutes another advantage of a polyploidy system for the biotechnological production of taxanes. Therefore, future studies could focus on the use of elicitors such as coronatine or methyl jasmonate, alone or in combination, with permeabilizers such as cyclodextrins or calix[8]arenes to optimize the production of PTX in the tetraploid cell line, following the approach we have previously used in *Taxus* spp. diploid cell lines (Escrich et al., 2021; Onrubia et al., 2013; Ramirez-Estrada et al., 2015). Nevertheless, due to the physiological and morphological differences between the diploid and tetraploid cell lines, the elicitation conditions will need to be careful optimized.

In turn, the intermediate ploidy cell line was less productive than the diploid control in the period of time studied. Lavania et al. (2012) demonstrated that cytosine methylation in genomic regions in tandem with ploidy upliftment diminished biometabolite production in *Cymbopogon* spp., and it has also been demonstrated that cytosine methylation modulates the production of taxanes (Escrich et al., 2022; Sanchez-Muñoz et al., 2018). Therefore, the reduction in taxane production of the intermediate ploidy cell line could be due to the stress generated by an incomplete ploidy linked to epigenetic events. On the other hand, the study carried out by Manz et al. (2022) links the cell shape with its productive capacity, concluding that rounded cells are more productive than elongated ones. In our study, the majority of cells in the IP cell line were elongated, which could be another reason for its low production profile. In contrast, the tetraploid line had more rounded than elongated cells, in line with the high productivity of our polyploid cell line.

Chromosome doubling often results in a higher expression of genes in comparison with diploid cells (Madani et al., 2021; Majdi et al., 2014). Based on the transcription profile analysis, our results support that the whole-genome duplication in the tetraploid cell line caused an increase in the expression levels of the target taxane-related genes compared to the diploid control. The genes involved in the last steps of the taxane pathway, *BAPT* and *DBTNBT*, are the most regulated ones (Cusidó et al., 2002; Exposito et al., 2010; Onrubia et al., 2013; Sabater-Jara et al., 2014). Their transcription levels in the tetraploid cell line were 7.17-fold and 53.2-fold higher compared to the diploid line, increasing the flux of metabolites to the production of final taxanes. This enhanced expression

might be correlated with the increase in transcription factors and *cis*-elements. Last but not least, the transcription levels of all the target genes tested increased, representing an up-regulation of the taxane pathway.

5. Conclusion

In conclusion, in this study an *in vitro* tetraploid cell line derived from immature embryos of *T. baccata* was successfully induced for the first time. 0.05% colchicine proved to be the most efficient concentration for the induction of polyploidy in *Taxus* spp. The polyploid line showed a significant increase in cell size, the elongated and rounded cells being 3.33- and 2.99-fold larger, respectively, than in the diploid cell line. The maximum total taxane production of the tetraploid cell line was 6.2-fold higher and occurred 8 days earlier compared to the diploid line, showing an improved productivity and taxane yield. In addition, the extracellular taxane content in this new tetraploid cell line was 10.8-fold higher compared to the diploid line. These results were accompanied by an increase in the transcription levels of all the taxane-related genes studied, particularly the *BAPT* and *DBTNBT* genes, which are active in the last steps of the pathway. Based on these results, polyploid cell lines of *T. baccata* represent another step forward in the development of optimal *Taxus* spp. biofactories for the production of PTX and related taxanes. Further research should focus on determining the effect of elicitors on the tetraploid cells. To sum up, this promising new biotechnological strategy for taxane production offers the possibility of achieving higher yields than currently available methods.

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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.

Data Availability

No data was used for the research described in the article.

Acknowledgments

JP and EM conceived the project and designed the research plan. AE performed the cell cultures. AE and DH performed data analysis. AE, R S-M and EM interpreted the data and wrote the draft of the manuscript. MB and JP supervised and supplemented the writing. All authors reviewed and approved the final version for publication. JP acquired the funds for the project.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111776.

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