# IMPROVED TAXANES PRODUCTION IN CALLUS CULTURES OF *Taxus baccata* L.

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***Abstract:*** *In this study, callus cultures from leaves and young shoots of Taxus baccata L.were established in Gamborg's B5 medium supplemented with 2,4-dichlorophenoxiacetic acid (2 mg/L), kinetin (0.5 mg/L) and gibberellic acid (0.25 mg/L). Callus growth and taxane production were evaluated using two culture media: Woody Plant Medium and Gamborg's B5 supplemented with picloram (2 mg/L), kinetin (0.1 mg/L) and gibberellic acid (0.5 mg/L). The effect of the inoculum size (50, 100 and 150 g FW/L) and culture media (Woody Plant Medium and Gamborg's B5) with and without the presence of methyl jasmonate (100 μM) on T. baccata cell suspensions was assessed. Taxane analysis revealed that the calus in Gamborg's B5 produced taxol (50 μg/g DW), baccatin III, 10-deacetyl baccatin III and 10-deacetyl taxol. Woody Plant Medium also induced the production of taxol, although to a lesser extent. The optimum inoculum size was 50 g FW/L. In cell suspension cultures, both media had a significant effect on taxane production when supplemented with methyl jasmonate. In WPM, at day 14, a total concentration of 185.35 μg/L of taxol, 172.98 μg/L of baccatin III, 658.97 μg/L of 10-deacetyl baccatin III and 259.75 μg/L 10-deacetyl taxol were obtained, with total excretion of baccatin III and 10-deacetyl taxol to the culture medium. The best culture conditions for producing taxol were found to be WPM supplemented with MeJ. The taxol level achieved in these conditions was 3.4 higher than in the same medium without elicitation and over 9 times higher than in the cultures grown in B5, elicited or not.*

***Key words****: anticancer agent, in vitro cultures, methyl jasmonate, taxanes, Taxus baccata L.*

## INTRODUCTION

*In vitro* cultures are an attractive alternative for obtaining high added value metabolites when these are produced in plants at low concentrations, their chemical synthesis is economically unfeasible, or their semi synthesis is also difficult and expensive (ARIAS *ET AL*., 2009). One of the strategies to consider for in vitro plant secondary metabolite production is the selection of productive species or explants that naturally accumulate the target compounds (EXPOSITO *ET AL*., 2010). The diterpenoid compound Paclitaxel (Taxol) has important anticarcinogenic properties and is produced mainly by species of the *Taxus* genus (SCHIFF AND HORWITZ, 1979). Production is variable among species and generally occurs at low concentrations, so the most viable option is *in vitro* culture of callus and cell suspension cultures (VAN ROZENDAAL *Et Al*., 2000). Various biotechnological strategies have been evaluated in *in vitro* cultures of different *Taxus species*, including the type of media, supplementation of culture medium with different growth regulators, biotic and abiotic elicitation, and different types of culture techniques such as two-phase culture, immobilized cells and scaling up to bioreactor level (MALIK *Et Al*., 2011). *Taxus baccata* or the European yew is distributed throughout the temperate zones of the northern hemisphere. It is a smallto medium-sized evergreen tree that historically has been used for weapon-making and medicine, and is poisonous except for the fruit. The genus *Taxus* belongs to the Class *Pinopsida*, the Order *Taxales* and the Family *Taxaceae*. As the species are highly similar, they are often easier to separate geographically than morphologically. Typically, eight species are recognized: *T. baccata* (European or English yew), *T. brevifolia* (Pacific yew or Western yew), *T. canadensis* (Canadian yew), *T. chinensis* (Chinese yew), *T. cuspidata* (Japanese yew), *T. floridana* (Florida

yew), *T. globosa* (Mexican yew) and *T. wallichiana* (Himalayan yew). There are also two recognized hybrids: *Taxus* × *media* = *T. baccata* × *T. cuspidata* and *Taxus* × *hunnewelliana* = *T. cuspidata* × *T. canadensis* (COPE, 1998).

The genus *Taxus* has generated considerable interest due to its content of diterpene alkaloids, particularly taxol (known also as the generic drug paclitaxel and by the registered trade name Taxol® BMS [Bristol-Myers Squibb]). The anticancer properties of taxol were discovered in *T. brevifolia* extracts in 1971 (WANI *et al*., 1971), while in 1979 Schiff, working with *T. baccata*, found that the cellular target of taxol was tubulin (SCHIFF - HORWITZ, 1979). Since the discovery of taxol, considerable energy has been invested in trying to increase its extraction. A serious obstacle to overcome is the low concentration (0.001–0.05%) of taxol found even in the most productive species, *T. brevifolia*. Since it is necessary to take 10,000 kg of *Taxus* bark or 3000 yew trees to produce only one kilogram of the drug and a cancer patient needs approximately 2.5–3 g of paclitaxel (GANEM *et al*., 2007) the treatment of each patient consumes about eight 60-year-old yew trees. Additionally, extraction of taxol from yew trees requires a complex system and specific purification techniques using advanced and expensive technology. Taking into account the above facts, together with the seasonal variation in taxane concentration in *Taxus* (CAMERON *et.al.*, 2008) and the high demand for the drug, there is an urgent need to find other alternative sources of taxol production.

More studies on the *in vitro* production of taxanes in cell cultures of *T. baccata* are, therefore, required. In the work presented here, we first evaluated the nutritional impact of two culture media, Woody Plant Medium (WPM) and Gamborg medium (B5), and elicitation with methyl jasmonate (MeJ) (100μM) on *T. baccata* cell growth, with the aim of increasing the in vitro production of taxol and related taxanes and their excretion from the producer cells to the culture medium.

## MATERIALS AND METHODS

### Plant material

Establishment of *T. baccata* in vitro cultures Explants were obtained from leaves and young stems of wild *T. baccata* in October 2015. The explants were surfacesterilized and after stripping the needles, longitudinally halved stem sections (length 0.8–1.0 cm) and leaves were inoculated onto hormone-free Gamborg medium (B5) (GAMBORG *et al*., 1968) for 20 days to verify the absence of bacteria and fungi. To obtain calli from the aseptic explants, leaves and stems were then cultured for 2 months in B5 supplemented with 2,4-dichlorophenoxiacetic acid (2,4-D) (2 mg/L), kinetin (KN) (0.5 mg/L), gibberellic acid ($GA_3$) (0.5 mg/L) and sucrose (3 %), and solidified with 0.3 % phytagel (Sigma) (TI), or B5 supplemented with picloram (PIC) (2 mg/L), KN (0.1 mg/L), $GA_3$ (0.5 mg/L) and sucrose (3 %), and solidified with 0.3 % phytagel (TII). The cultures were maintained at 25 ± 2 $^0$C in the dark. The established calli were then subcultured every 10 or 12 days on B5 supplemented with 2,4-D (2 mg/L), benzyl aminopurine (BAP) (0.1 mg/L), $GA_3$ (0.5 mg/L) and sucrose (3 %), and solidified with 0.3 % phytagel (TIII) until a high biomass of friable calli was obtained (CUSIDO´ *et al*., 2002).

### Measuring growth and taxane production in *T. baccata* callus

To determine the kinetics of growth and production in two different basal media, 500 mg FW of callus was inoculated onto petri dishes containing 20 mL of WPM (MCCOWN AND LLOYD, 1981) with sucrose (1%) + fructose (1 %), or B5 with sucrose (3 %), both media being supplemented with the growth regulators PIC (2 mg/L), KN (0.1 mg/L) and $GA_3$ (0.5 mg/L), and solidified with 0.3 % phytagel. To determine the growth measured as fresh weight (FW), 20–25 callus pieces grown in each culture media were harvested every week during a culture period of 6 weeks, and weighed separately. Dry weight (DW) was recorded from lyophilized

callus pieces. The growth parameters of growth index (GI) (KETCHUM *et al.*, 1995) and average doubling time (dt) were established based on the FW (BRUNAKOVA *et al.*, 2004). The dry plant material was extracted to analyze the taxane content following the methodology described below.

**Establishing *T. baccata* cell suspension cultures**

*T. baccata* cell suspension cultures were established by inoculating 10 g FW calli into a 250-mL Erlenmeyer flask containing 100 mL of WPM supplemented with PIC (2 mg/L), KN (0.1 mg/L) and $GA_3$ (0.5 mg/L). The cultures were maintained in a rotary shaker at 100 rpm in the dark at 25 $^0$C for 12 or 15 days, until sufficient biomass was obtained for subsequent studies.

**Inoculum size**

Growth kinetics was measured in the *T. baccata* cell suspensions established to determine the inoculum size for an optimum growth. We evaluated the following inoculum sizes: 50, 100, and 150 g FW/L. Cells were cultured in WPM supplemented with PIC (2 mg/L), KN (0.1 mg/L) and $GA_3$ (0.5 mg/L) for 30 days. The flasks were shaken (110 rpm) at 25 ± 2 $^0$C in the dark. Triplicate samples were taken every 3 days. The measured variables were: fresh and dry weight, GI, viability, final volume of the medium and high resolution liquid chromatography (HPLC) quantification of taxanes in the biomass and culture medium (ZHANG et al., 2002).

**Effect of methyl jasmonate (MeJ) elicitation of *T. baccata* cell suspension cultures**

The effect of two basal media, WPM and B5, both supplemented with PIC (2 mg/L), KN (0.1 mg/L) and $GA_3$ (0.5 mg/L), on cell growth and taxane production, with and without MeJ, was evaluated. The previously established *T. baccata* cells were filtered and those with the optimized inoculum size (50 g FW/L) were placed into 125 mL Erlenmeyer flasks with 10 mL of the indicated media. MeJ (Aldrich) (100 μM) (EXPOSITO *et al*., 2010).

**Cell viability**

The cell viability in all the culture conditions was studied using the method described by DUNCAN AND WIDHOLM (1990). The results are expressed as the percentage of living cells from the total cells.

**Quantification of taxanes by high resolution liquid chromatography (HPLC)**

The production of taxol and other taxanes was determined following the methodology described by RICHHEIMER *et al*. (1992).

## RESULTS AND DISCUSSION

**Establishment of callus cultures**

The sterilization process used in this experiment was more effective for leaves than stems, 97 and 89 % of respective explants being without contamination after 15 days. Two different auxins (2,4-D and PIC) at the same concentration, Media TI and TII, were assayed for callus development. Calli were obtained after 20 days in both media, but with varying success. In TI (Figure 1), the percentage of leaves and stem forming calli was 100 and 80 %, respectively, while in TII it was no higher than 40 %. The callus obtained from leaves were more light-cologreen and friable than those established from stems. It is possible that the dedifferentiation of leaf explants was due to the effect of the auxin 2,4-D on meristematic areas of petioles and the apex of the leaves, which increased cell division by inhibiting morphogenesis and resulted in an early callus formation. In contrast, the induction of calli from stems was lower.

The synthetic auxin 2,4-D is known to be a general promoter of biomass formation since it induces cell dedifferentiation leading to callus development (MIHALJEVIC *et al*., 2002).

BA was used as the cytokinin because it has proven very effective for the growth of cell cultures of several *Taxus* species: *T. baccata* (PALAZO´N *et al.*, 2003), *T. cuspidata* and *T. canadiensis* (FILOVÁ *et al.*, 2011), *T. cuspidata* (NIMS *et al.*, 2006), and *T. brevifolia* (KHOSROUSHAHI *et al.*, 2011) as well as other plant cell cultures.

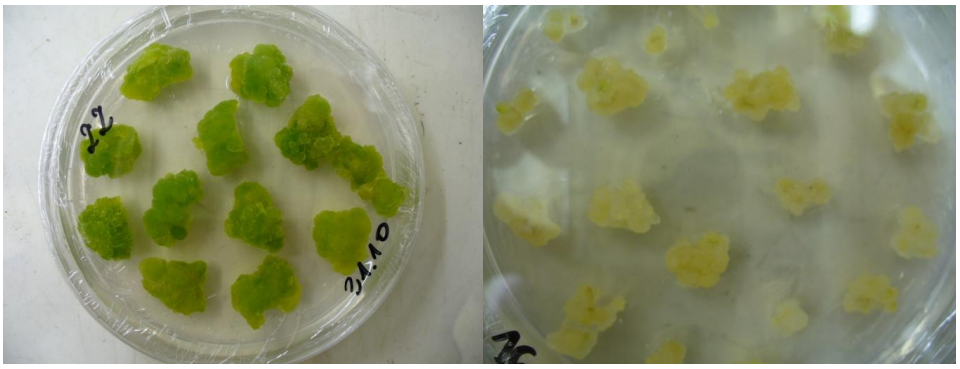

Figure 1: Callus cultures of *T. baccata* (a) WPM medium and (b) in B5 medium

**Growth and taxane production of *T. baccata* L. callus**

Friable calli obtained as described above were selected with the aim of determining their capacity for growth and taxane production in the two different basal media. In addition, after assaying 48 different media, we have previously shown that optimum improvement of taxol production in *T. baccata* cell cultures. As shown in Figure 2, *T. baccata* calli grown in WPM achieved the highest growth after 4–6 weeks of culture, with a growth index (GI) of 0.70 and an average doubling time (dt) of 13.2 days. When calli were grown in B5 medium, the highest growth was observed at week 4. After this point, the cells entered the stationary growth phase, which lasted 1 week, decreasing thereafter. In both assayed media, the lag phase lasted at least 2 weeks. In B5 medium, the GI was 0.44 and the dt was 13.8 days. The average dt obtained in the callus cultures was within the range reported for other *Taxus* species: *T. globosa* (14 days) (BARRADAS *et al.*, 2010), *T. media* and *T. baccata* (13 days) (WICKREMESINHE AND ARTECA, 1993).

The calli grown in both media produced taxanes throughout the time period studied. Total taxane content at the end of the experiment (6 weeks of culture) in calli grown in B5 was more than two times higher than in WPM-cultured callus. The main taxane found in calli grown in B5 was 10-DAT followed by taxol. After phytochemical screening of the calli, it was observed that taxol production occurred in cycles, and not at a constant rate during the culture period. Taxol production and accumulation in WPM-cultured calli occurred mainly in the first week (16 μg/g DW) and week 6 (24 μg/g DW). In contrast, the production of 10-deacetyl taxol in these cultures remained constant throughout the culture, accumulating at levels similar to taxol (23.87 μg/g DW). Meanwhile, taxol production and accumulation in B5-cultured callus peaked in the first week (50 μg/g DW) (Table 1).

*Table 1*

Taxane contents (expressed as mg/g DW) of *T. baccata* calli grown in B5 or WPM, both supplemented with growth regulators: PIC (2 mg/L), KN (0.1 mg/L) and $GA_3$ (0.5 mg/L), after 6 weeks of culture

| Medium | Taxol | BIII | 10-DAB III | 10-DAT | Total |
|---|---|---|---|---|---|
| **B5** | 50 | 38.06 | 8.73 | 80.82 | 177.61 |
| **WPM** | 24 | 22.03 | 16.02 | 23.87 | 85.92 |

BIII baccatin III, 10-DAB III 10-deacetyl baccatin III, 10-DAT 10-deacetyl taxol,

The production of taxol and related taxanes obtained in *T. baccata* callus cultures was double that reported by BARRIOS *et al*. (2009), and significantly higher than that reported by FILOVÁ *et al*. (2011).

**Optimizing the inoculum size in *T. baccata* cell suspensions**

To establish *T. baccata* cell suspensions with good growth, three different inoculum sizes (50, 100 and 150 g FW/L) were assayed in WPM. During 30 days, the color of the cultures was observed to change from light yellow to reddish. This effect was especially noticeable when the inoculum was 100 and 150 g FW/L, together with the formation of green-brown cell aggregates. In cultures started with the higher inocula (100 and 150 g FW/L), the biomass increased very little during the 30-days period, the highest growth being only 18.5 % of the maximum achieved by cell cultures inoculated with 50 g FW/L. The GI corresponding to cell suspensions inoculated with 50 g/L of cells was 6.5, with a dt of 3.9 days.

All three cultures showed a capacity to produce taxol and related taxanes. Table 2 presents the highest levels obtained of each taxane and their respective volumetric productivities. In cell cultures inoculated with 50 g FW/L of biomass, volumetric productivities were higher (except for 10-deacetyl baccatin III and 10-deacetyl taxol) than those of the other inoculum sizes studied and the 5 target taxanes were only found with this inoculum (Table 2). Moreover, when using 50 g of cells, the highest taxane content was generally obtained earlier than when starting with 100 and 150 g FW/L of cells (data not shown). Taxane excretion, although always very low, was also higher with the inoculum of 50 g FW/L (data not shown).

*Table 2*

Effect of inoculum size (FW) on taxane production in *T. baccata* cell suspension cultures

| **Inoculum g FW/L** | **Taxol μg/L** | **Baccatin III μg/L** | **10-DAB III μg/L** | **10-DAT μg/L** |
|---|---|---|---|---|
| **50** | 62.98 | 125.57 | 515.39 | 135.25 |
| **100** | 50.76 | 149.58 | 454.75 | 879.56 |
| **150** | 35.59 | 64.24 | 689.45 | 1008.25 |

10-DAB III 10-deacetyl baccatin III, 10-DAT 10-deacetyl taxol

**Effect of methyl jasmonate (MeJ) elicitation on *T. baccata* cell suspensions**

To study the effect of the elicitor methyl jasmonate (MeJ) (100 μM) on growth and taxane production and the interaction between the elicitor and the basal medium contents, *T. baccata* cell cultures were established in WPM and B5, with and without MeJ. The WPM and B5 media used for the control cultures turned green-brown, which lasted throughout the

experiment, whereas those of the elicited cultures remained pale yellow. The biomass corresponding to the WPM cultures turned brown with time in all the conditions assayed, in contrast with cells maintained in B5, which did not darken in either control or elicited conditions. This change in the color of the cultures, also reported in other *Taxus species*, was mainly due to the intracellular accumulation of phenolic compounds, which was provoked by submitting the cultures to nutritional stress. In our case, the cell suspension cultures grown in WPM produced the highest quantity of phenolic compounds, which were then excreted to the medium. Cell suspensions in WPM without elicitation presented a lag phase of 3 days and continued growing until the end of the experiment, whereas the presence of the elicitor dramatically inhibited the increase of biomass. The dt in control cultures was 2.32 days and the GI was 6.14, whereas in the MeJ-treated cultures the dt was 3.06 days and the GI 0.77. Cultures grown in B5 medium did not present significant differences between the control and elicited conditions, the increase of biomass being very low throughout in both cases.

**Taxane production in cell suspensions of *T. baccata***

Table 3 shows the contents and productivity of the total taxanes studied (cell-associated plus extracellular) in *T. baccata* cell cultures grown in WPM or B5, with or without MeJ. As can be seen, taxol production was most induced by the effect of WPM supplemented with MeJ. In WPM the highest total taxol content was observed at day 14: 185.35 μg/L, with a productivity of 13.239 μg/L $day^{-1}$. The taxol precursors 10-deacetyl baccatin III (658.97 μg/L) and baccatin III (172.98 μg/L) also accumulated at the highest concentration at day 20, together with 10-deacetyl taxol (259.75 μg/L), which was excreted in its entirety into the medium. In summary, productivity in control conditions was higher in WPM than in B5 for all the taxanes studied except baccatin III. The elicitation of taxane production by MeJ in vitro has been extensively reported for other *Taxus* species (BONFILL *et al.*, 2006).

*Table 3*

Influence of the culture conditions on taxane production in *T. baccata* cell suspension cultures

| Culture conditions | Time[a] (days) | Concentration (□g/L) | Productivity (□g/L $d^{-1}$) |
|---|---|---|---|
| **Taxol** | | | |
| **WPM-C** | 14 | 68.25 | 4.875 |
| **WPM-MeJ** | 14 | 185.35 | 13.239 |
| **B5-C** | 14 | 17.75 | 1.268 |
| **B5-MeJ** | 20 | 31.56 | 1.578 |
| **Baccatin III** | | | |
| **WPM-C** | 20 | 138.56 | 6.928 |
| **WPM-MeJ** | 20 | 172.98 | 8.649 |
| **B5-C** | 14 | 83.46 | 5.961 |
| **B5-MeJ** | 14 | 104.72 | 7.480 |
| **10-Deacetyl baccatin III** | | | |
| **WPM-C** | 14 | 499.99 | 35.714 |
| **WPM-MeJ** | 14 | 658.97 | 47.069 |
| **B5-C** | 20 | 70.59 | 3.529 |
| **B5-MeJ** | 20 | 38.95 | 1.948 |
| **10-Deacetyl taxol** | | | |
| **WPM-C** | 14 | 216.25 | 15.446 |
| **WPM-MeJ** | 14 | 259.75 | 18.554 |
| **B5-C** | 14 | 91.45 | 6.532 |
| **B5-MeJ** | 14 | 33.85 | 2.417 |

Highest taxane concentration (cell-associated plus extracellular taxane contents) and productivity, days of culture when the highest taxane production was achieved WPM-C Control WPM, WPM-MeJ WPM supplemented with MeJ (100 μM), B5-C Control B5 medium, B5-MeJ B5 medium elicited with MeJ (100 μM)

[a] Time in which occurs the greater production of the different taxanes, by the effect of each culture conditions

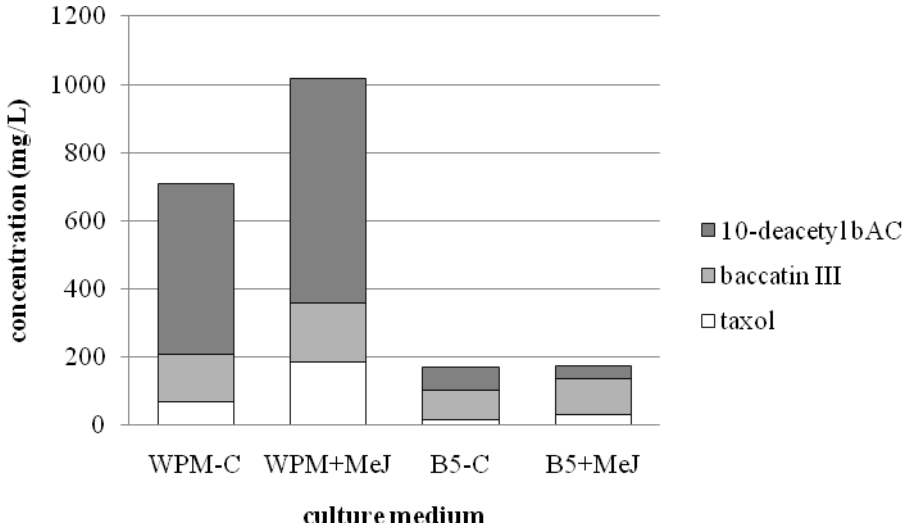


Figure 2**:** Effect of culture media WPM and B5 and MeJ elicitation on taxol production in *T. baccata* cell suspensions. WPM-C, B5-C: Cultures grown in WPM and B5 medium without elicitation; WPM MeJ and B5-MeJ: Cultures grown in WPM and B5 medium supplemented with MeJ (100 μM). Data represent average values
from three separate experiments ± SD, *p\0.0001.

The best culture conditions for producing taxol (Figure 2) were found to be WPM supplemented with MeJ. The taxol level achieved in these conditions was 3.4 higher than in the same medium without elicitation and over 9 times higher than in the cultures grown in B5, elicited or not. Moreover, in WPM MeJ elicitation increased the productivity of taxol and baccatin III, but not the other 3 taxanes. However, the taxol biosynthesized by cells grown in WPM was not excreted to the medium. In contrast, cells grown in B5 excreted a high percent of all taxanes to the medium (Table 3), although their productivity was generally lower than in WPM medium (except for baccatin III). Briefly, the productivity increase of taxol and baccatin III was achieved using WPM-MeJ and of 10-deacetyl taxol and 10-deacetyl baccatin III using WPM without elicitation. Taxol production in *T. baccata in vitro* cultures has been studied by three other research groups. Furthermore, we increased the concentration of taxol 3.96-fold in cell suspensions, as well as that of baccatin III (4.22-fold), 10-deacetyl baccatin III (39.5-fold) and 10-deacetyl taxol (13.93-fold), mainly due to the use of WPM in combination with MeJ. Our taxol production was also similar to that reported for other *Taxus* species: 550 μg/L $day^{-1}$ in *T. baccata* (HIRASUNA *et al.*, 1996).

A rational approach might provide new insight into how the taxol biosynthetic pathway is regulated, with genetic and metabolic engineering techniques, differential genetic expression, transcription factors and key genes leading to higher taxol yields. One aspect to take into account is the mechanism of taxol excretion from cells, which could be enhanced by employing a two-phase culture system, so far not assayed in *T. baccata* cell suspensions (FILOVÁ, 2013). Future perspectives could be focused on the simultaneous use of empirical and rational approaches and assaying the two-phase culture system in order to develop a biotechnological system for high taxol production.

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