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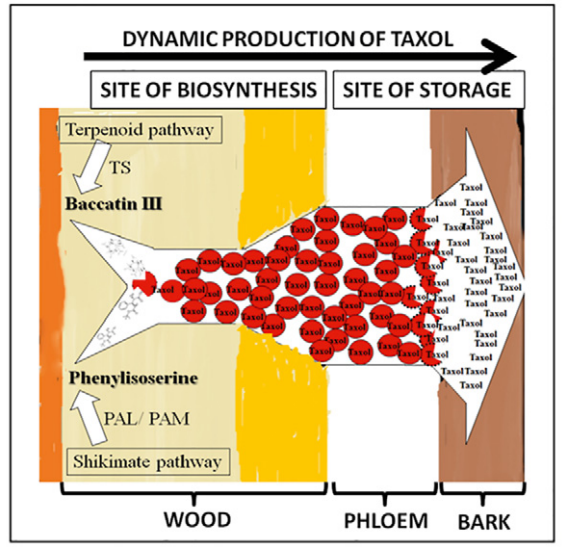

Taxol is potentially cytotoxic to its producing plant. However, a study of gene expression of the major Taxol biosynthetic genes and in situ immune-labelling in Taxus media reveals that taxol biosynthesis starts in the deep wood parenchyma cells from where it is transported in resin-like hydrophobic bodies to the needles, thus affording sufficient protection to the plant.

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Sites of biosynthesis and storage of Taxol in *Taxus media* (Rehder) plants: Mechanism of accumulation

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ABSTRACT

Taxol is a cytotoxic agent against various types of cancers. The cytotoxic activities of Taxol can be extended to its synthesizing plant. Here, Taxol is shown to have special synthesis, storage and transport mechanisms that avoid the toxic effects on its source plant. The sites of Taxol biosynthesis, transport and storage were revealed by quantification of plant Taxol, its intermediate baccatin III, the polyphenol side chain precursor , gene expression analysis of the major Taxol biosynthetic genes and *in situ* immuno-labeling. Although the biosynthesis of Taxol was limited by the expression of its biosynthetic genes and the presence of baccatin III, its presence did not correlate to baccatin III accumulation, nor to the expression of biosynthetic genes. However, Taxol presence positively correlated to polyphenol accumulation (late stage in Taxol assembly) and the resin-like hydrophobic bodies (HB, storage organelles). These results indicate that the presence of Taxol requires two complementary steps, biosynthesis followed by storage. Each step is limited by the availability of different precursors, which differ in their localization within the plant. Thus, the sites of biosynthesis, transport and storage of Taxol are different. *Taxus media* (Rehder) plant wood showed high concentrations of baccatin III and the expression of biosynthetic genes. However, the concentrations of Taxol, polyphenol and HB were very high in the plant outer layers including phloem and dead bark (rhytidome). Furthermore, *in situ* immuno-labeling showed that taxadiene synthase (the rate-limiting enzyme in Taxol biosynthesis) was mainly found in the wood, while Taxol primarily localized to the outer tissues. Conclusively, wood can be considered as the site of Taxol biosynthesis. Our data also propose that Taxol then accumulates into HB in order to permit its transport within the living plant tissues without causing toxic effects. This is followed by Taxol storage in the outer tissues including phloem and dead bark.

1. Introduction

Taxol is a microtubule-stabilizing drug used in the treatment of different types of cancers (Abal et al., 2003). However, Taxol's cytotoxic activities cannot distinguish between normal cells and cancer cells (Silvestrini et al., 1993). Similarly, Taxol cytotoxicity can be extended to its plant-producing cells (Soliman et al., 2015); and hence its synthesis in native plant tissues must be special and require special transport mechanisms. Therefore, it is important to know its synthesis and storage/sequestration mechanisms. Soliman et al. (2015) showed that Taxol is localized into resin-like hydrophobic bodies (HB) and released in response to pathogenic invasion by wood decaying fungi (Soliman et al., 2015). However, the sites of Taxol biosynthesis and storage are not clear.

Taxol is a taxoid diterpene and its biosynthesis requires two major metabolic pathways, the diterpenoid (Koepp et al., 1995) and the phenylpropanoid pathways (Fleming et al., 1993). The diterpenoid pathway provides the main taxane skeleton, baccatin III, and its biosynthesis is rate-limited by taxadiene synthase (TS) enzyme (Koepp et al., 1995). The phenylpropanoid pathway provides the phenylisoserine side chain and its biosynthesis is rate-limited by phenylalanine ammonia lyase (PAL) and phenylalanine amino mutase (PAM) (Fleming et al., 1993) (Fig. 1). The expression patterns of *TS*, *PAL* and *PAM* between different tissues and organs relative to the accumulation of major taxoids were studied in order to provide insight into the site of Taxol biosynthesis. The expression patterns of some taxoid biosynthetic genes were previously studied (Hao et al., 2011). The expression patterns of *taxadiene-5α hydroxylase*, *taxadien-5α-ol O-acetyltransferase*, *taxoid-2α*

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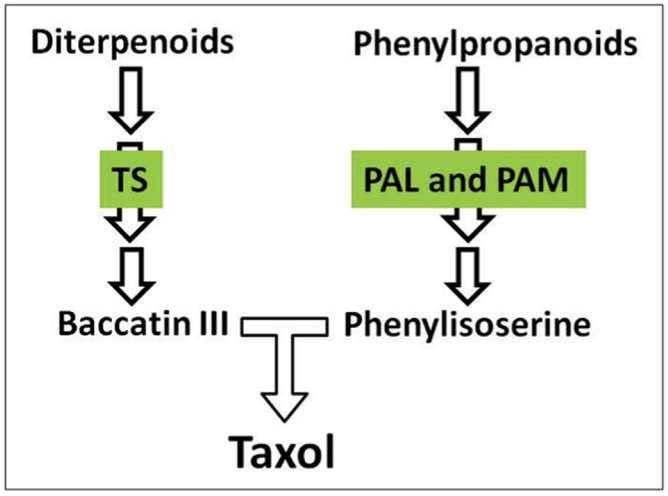

Fig. 1. Summarized Taxol biosynthetic pathway.

hydroxylase, *phenylpropanoyl transferase* and *PAM* were found to be upregulated in plant roots when compared to stems and needles (Hao et al., 2011). However, the expression of *TS* and *PAL* genes were not measured and the differences in the expression pattern of *PAM* between the stems and needles were also not measured . On the other hand, taxoid contents was observed at higher concentrations in the roots than needles (Hao et al., 2011). However, the differences in concentrations of taxoids within the tissues of the same organ including wood, phloem and outer dead bark (rhytidome) were not identified. Although PAL localization was identified in the vascular tissues of Poplar plants, another gymnosperm, where it was mainly expressed in older tissue in response to wound stimulus (de Jong et al., 2015; Ohl et al., 1990), its expression pattern in *Taxus* plants has not been reported. The PAM enzyme was isolated from the stem tissue of *Taxus* plants (Walker and Floss, 1998) but its expression pattern was never reported.

The quantification of Taxol, baccatin III (Taxol major intermediate), HB (Taxol storage organelles) in different plant organs and within the tissues of the same organ may also provide insight into the storage site (s) of Taxol. Taxol, baccatin III and other taxoid derivatives have been isolated from almost all *Taxus* plant tissues (Mukherjee et al., 2002), but the concentrations are very variable and the variation affected by seasonal (Vance et al., 1994). Most taxoids have been found at higher concentrations in the stems compared to the needles, while baccatin III concentrations in the needles sometimes reach the level found in stems or higher (Vance et al., 1994). Taxol has been found to mainly accumulate in the outer dead bark (Vance et al., 1994). However, the differences in the concentration of taxoids (Taxol to baccatin III ratio) within the same organ tissues such as wood, phloem and bark have never been reported. On the other hand, the accumulation of Taxol, baccatin III and other taxoids in plant tissue culture is very variable (Bruňáková and Košuth, 2009) and some cell lines are completely devoid of Taxol (Roberts, 2007). The reasons underlying this variability have not been explained.

In this research study, the concentration of the taxoid intermediate, baccatin III, and Taxol as a final end product, were linked to the expression levels of biosynthetic genes and the density of storage organelles. The results obtained help to assign the sites of biosynthesis and storage of Taxol and hence its mechanism of accumulation. The present study can be considered as part of understanding the evolutionary

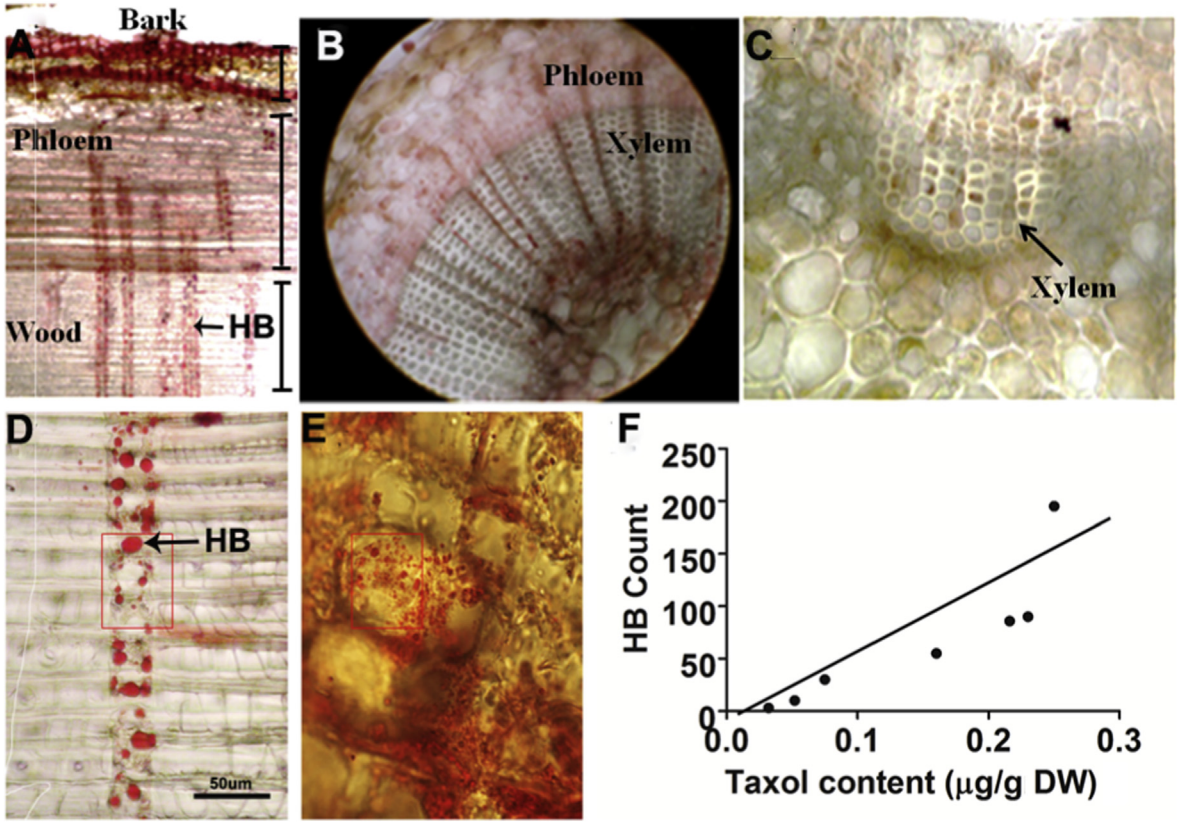

Fig. 2. Taxol localization and storage. (**A**) Transverse section of a *Taxus* plant stem showing the location of Taxol-containing HB (red-stained droplets) within the wood and phloem in comparison to (**B**) close up view in the vascular bundle (VB) of a root section and (**C**) close up view in the vascular bundle (VB) of a needle (*Taxus* leaf) section. (**D**) and (**E**) Close up views in the wood and phloem, respectively, to show the distribution of Taxol-containing HB within *Taxus* stem sections. (**F**) Pearson's correlation between Taxol content and the number of HB within different plant samples (Pearson's correlation, $r^2 = 0.77$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

production of Taxol in nature.

2. Results

2.1. Storage mechanism of Taxol: Taxol is localized to resin-like hydrophobic bodies (HB)

We confirmed that Taxol is localized to HB within the plant parenchyma rays using immuno-histochemistry (Soliman et al., 2015). Furthermore, the HB, stained red with Sudan IV, was found mainly in the plant stems and at a lesser extent in both roots and needles (Fig. 2A–C). Within the plant stems, HB was located in the wood (xylem) parenchyma rays (Fig. 2D) and phloem (Fig. 2E) but not in the dead outer bark (rhytidome). The number of HB accumulated in the plant tissues positively correlated to Taxol accumulation (Pearson's correlation, $r^2 = 0.77$) (Fig. 2F). This confirms that Taxol is stored in HB in the living plant tissues, and that HB is a good indicator for the presence of Taxol within *Taxus* plant tissues.

2.2. The biosynthetic mechanism of Taxol

As mentioned earlier, the biosynthesis of Taxol requires both the diterpene and phenylpropanoid pathways. Below the importance of both pathways are described.

2.2.1. Taxol production is limited by the presence of the phenolic side chain, phenylisoserine, in differentiated cells

The accumulated amount of Taxol in plant tissues did not correlate with baccatin III accumulation (Pearson's correlation, $r^2 = 0.012$) (Fig. 3A) but was strongly positively correlated with total polyphenols (Pearson's correlation, $r^2 = 0.95$) (Fig. 3B). These results indicate that the presence of Taxol is limited by the production of polyphenols but not by baccatin III. This is consistent with the literature since baccatin III is an intermediate not only in the biosynthesis of Taxol but other taxoid derivatives (Croteau et al., 2006; Fleming et al., 1994; Kucukboyaci and Sener, 2000). On the other hand, gene expression analysis of TS, the rate-limiting enzyme in the biosynthesis of the diterpene skeleton (taxadiene) in the baccatin III/Taxol biosynthetic pathway (Koksal et al., 2011), showed no correlation with Taxol accumulation (Pearson's correlation $r^2 = 0.07$) (Fig. 3C), but strong positive correlation with baccatin III accumulation (Pearson's correlation $r^2 = 0.80$) (Fig. 3D). This pattern of correlation can be explained since baccatin III (but not Taxol) is the major intermediate in the biosynthesis of diverse taxoids including Taxol, and Taxol is not the only final end product as long as other taxoid derivatives are produced from baccatin III (Croteau et al., 2006; Fleming et al., 1994; Kucukboyaci and Sener, 2000). Taxol accumulation showed no correlation with *PAL* and *PAM* gene expression (Pearson's correlation $r^2 = 0.1569$ and 0.018, respectively).

2.2.2. Taxol production is limited by the presence of its intermediate precursor, baccatin III, in undifferentiated cells

Taxol content (Fig. 4A) and the number of HBs (Fig. 4B) in *T. media* callus culture were coincidently very low compared to its original source plant (Fig. 4A, C, respectively). Similarly, baccatin III accumulation (Fig. 4A) and *TS* expression (Fig. 4D and E) were very low in the

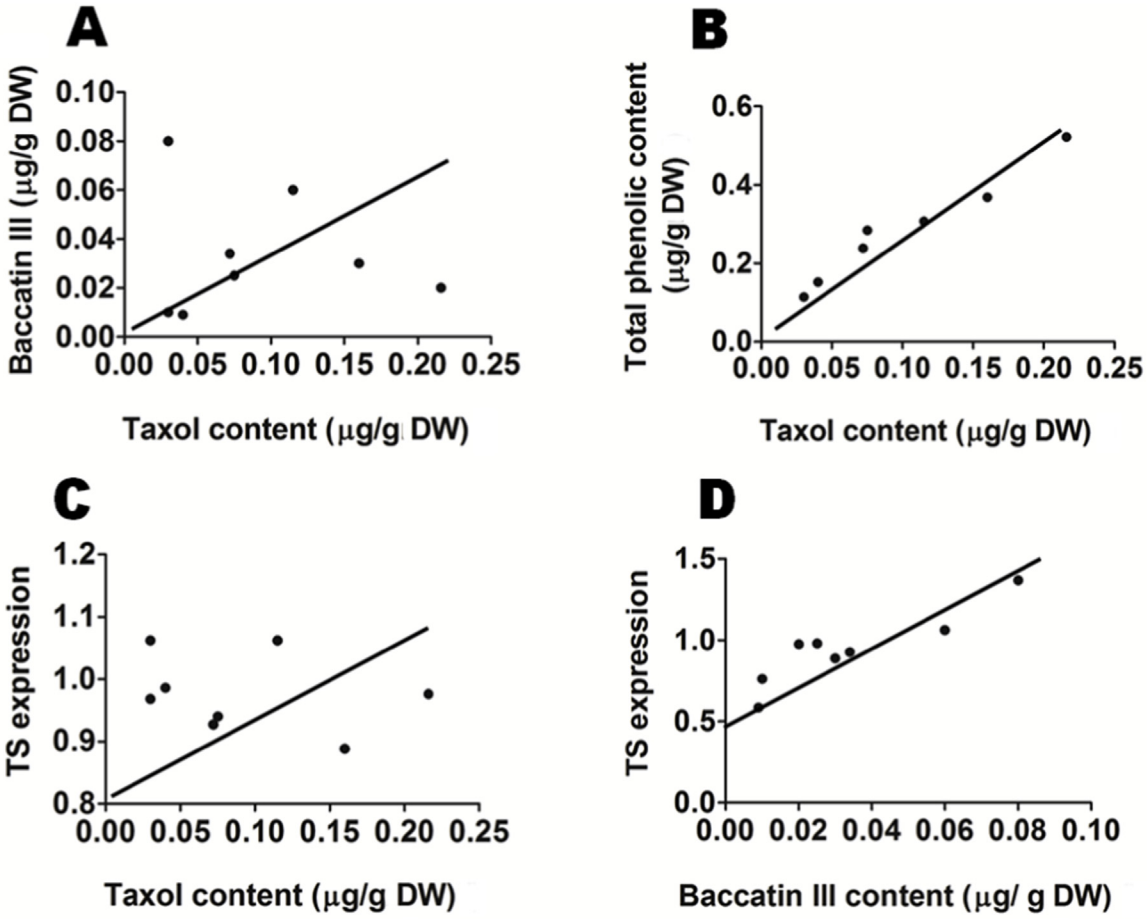

Fig. 3. Correlation of Taxol accumulation to (**A**) baccatin III accumulation (Pearson's correlation, $r^2 = 0.12$), (**B**) polyphenol accumulation (Pearson's correlation, $r^2 = 0.95$) and (**C**) TS gene expression (Pearson's correlation, $r^2 = 0.07$). (**D**) Correlation of baccatin III to TS gene expression (Pearson's correlation, $r^2 = 0.80$). The data were analyzed using Pearson's correlation and the data were considered to have a normal distribution. *P* value < 0.05 was considered as significant.

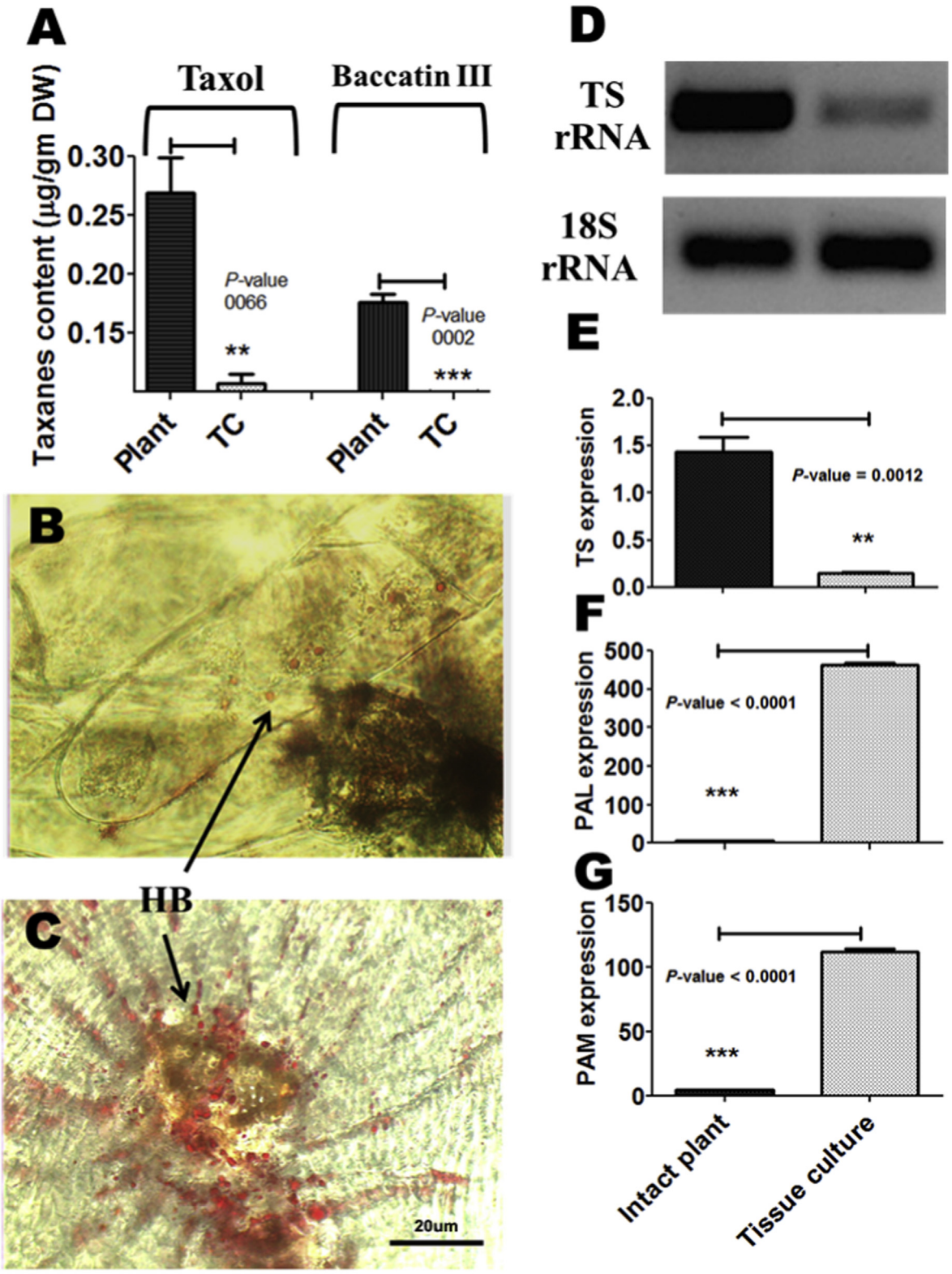

Fig. 4. Comparison between intact plant and plant callus culture (TC) in terms of (**A**) taxoid contents (Taxol versus baccatin III), number of HB (indicated as red droplets) in (**B**) TC and (**C**) intact plant stem sections, and expression of (**D** and **E**) TS, (**F**) PAL and (**G**) PAM genes. The data are displayed as the mean ± standard error of the mean. The statistical significance was calculated with the Student-t-test and the significance level indicated by asterisks. *P*-value < 0.05 was considered significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

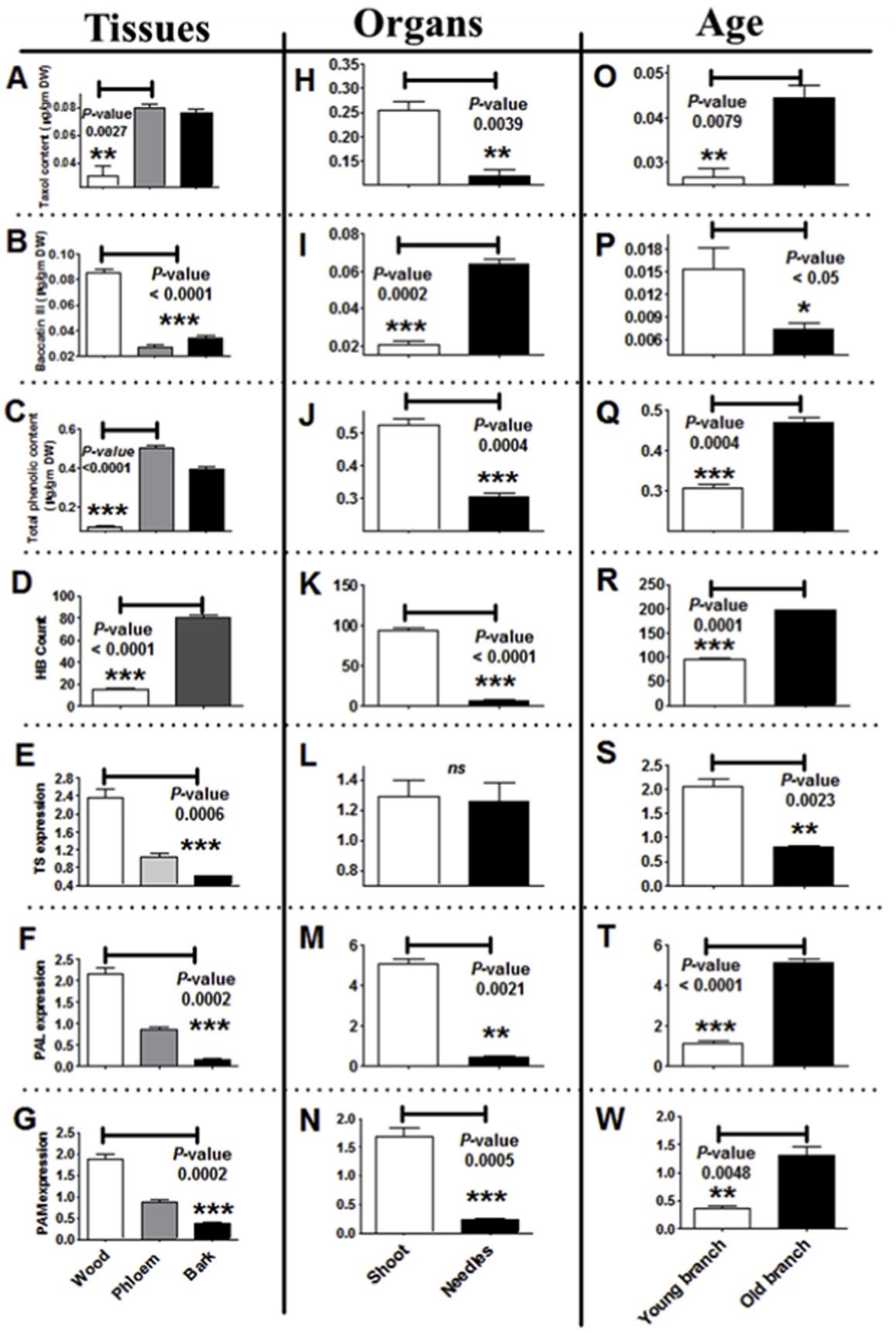

Fig. 5. Comparison between *Taxus* plant (**A-G**) tissues, (**H–N**) organs and (**O–W**) age on the contents of (**A, H, O)** Taxol (**B, I, P**) baccatin III, (**C, J, Q**) polyphenols, (**D, K, R**) HB and the expression of (**E, L, S**) TS, (**F, M, T**) PAL and (**G, N, W**) PAM genes. In case of (**D**) the comparison was between wood and phloem only, since the bark did not show HB. The data are displayed as the mean ± standard error of the mean. The statistical significance was calculated with a Student-T-test and one-way ANOVA, and the significance level indicated by asterisks. *P*-value < 0.05 was considered significant.

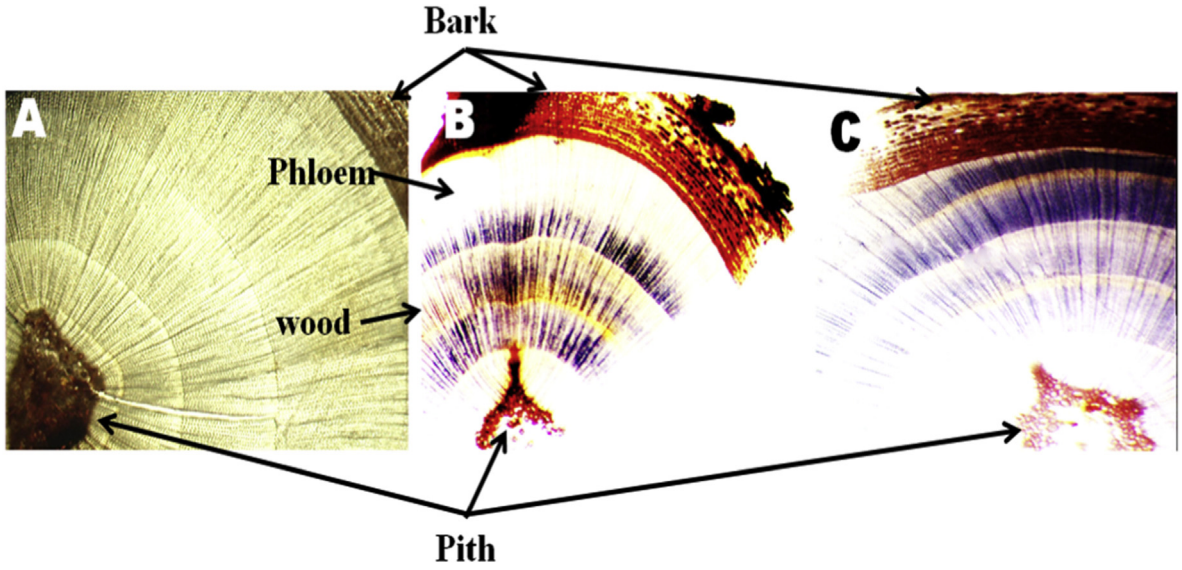

Fig. 6. *In situ* immuno-labelling of TS enzyme and Taxol in *Taxus* stem transverse sections. (**A**) Control stem section received no primary Abs compared to (**B**) a section that received anti-TS Ab and (**C**) a section that received anti-Taxol Ab.

callus culture, however polyphenolaccumulation and *PAL/PAM* gene expression were very high (Fig. 4F and G). The results from callus culture indicated that Taxol production is limited by the production of its intermediate baccatin III, since the side chain precursors were very high in callus cultures when compared to baccatin III.

The results from both intact plants (differentiated cells) and plant callus culture (undifferentiated cells) indicated that there should be certain concentrations of both the intermediate and the side chain compounds in the tissue in order to produce a sufficient amount of Taxol. Furthermore, the presence of HB is critical for the occurrence of Taxol in plant tissues. These observations suggest that there could be another factor that may contribute to controlling the production process of Taxol and its storage organelles (HB).

2.3. The complementary production mechanisms of Taxol

2.3.1. Taxol is produced in the wood and stored in the phloem and dead bark: horizontal transport

To assess the synthesis mechanism of Taxol by plants, we compared the accumulation of Taxol, baccatin III (major intermediate in Taxol biosynthesis), polyphenols (Taxol side chain), and HB (storage organelle) and the expression of *TS* (rate-limiting in baccatin III/Taxol biosynthesis), *PAL* and *PAM* (rate-limiting in the phenolic side chain biosynthesis) within different *Taxus* plant tissues, organs and plant ages. At the tissue level, Taxol (Fig. 5A, wood versus phloem and bark), polyphenols (Fig. 5C, wood versus phloem and bark) and HB (Fig. 5D, wood versus phloem) showed greater accumulation in the phloem and lower accumulation in the wood. However, baccatin III (Fig. 5B, wood versus phloem and bark) showed the reverse: greater accumulation in the wood and very low accumulation in the phloem. Similarly, the expression *TS* (Fig. 5E), *PAL* (Fig. 5F) and *PAM* (Fig. 5G) genes were very high in the wood followed by phloem and to a lower extent in the outer bark (Fig. S1). The higher expression levels of *TS*, *PAL* and *PAM* genes, and the presence of higher level of baccatin III in the plant wood, leads to the conclusion that plant wood is the site of Taxol precursor biosynthesis. On the other hand, the higher accumulation of Taxol and polyphenols, and the presence of higher number of HB in the phloem, indicates that phloem is the site of storage. In the dead outer bark, Taxol and polyphenol accumulation were very high, but HB was not detectable. This can be attributed to Taxol being cytotoxic, thus it is stored in HB to avoid its cytotoxic effect on the living phloem tissue, while this storage barrier mechanism is not required in the case of dead outer bark. Mechanistically, it can be proposed that once the Taxol precursors are biosynthesized, they are immediately assembled in HB in order to avoid the Taxol toxic effects on the living plant tissues including phloem and wood parenchyma rays. Taxol-containing HBs are then transported to the dead bark, where Taxol is released from HB possibly for defense purposes (Soliman et al., 2015).

2.3.2. Taxol is produced in the stems and transported to the needles (Taxus leaves): vertical transport

Regarding the plant organs, the accumulation of Taxol (Fig. 5H), polyphenols (Fig. 5J) and HB (Fig. 5K) were coincidently greater in the stems and lower in the needles; however the needles showed higher accumulation of baccatin III (Fig. 5I) than the stems. On the other hand, *TS* gene expression was similar in the stems and needles (Fig. 5L), while *PAL* (Fig. 5M) and *PAM* (Fig. 5N) geneexpression were higher in the stems when compared to the needles (Fig. S1). This could be explained by baccatin III biosynthesis occurring in both stems and needles. However, the enzymes employed for the conversion of baccatin III to other taxoids including Taxol are highly expressed in the stems. On the other hand, the expression of these enzymes may be very minimal in the needles, resulting in accumulation of baccatin III. Thus, the needles are considered as an excellent source of baccatin III as reported before (Denis et al., 1988; Fu et al., 2009). Furthermore, the plant needles are not dead and hence the existence of Taxol in the needles is possibly because of its transport from the stems in its final storage form (Taxol-containing HBs).

2.3.3. Taxol production is limited by specialized elements

A comparison between old and young stems showed that accumulation of Taxol (Fig. 5O), polyphenols (Fig. 5Q) and HB (Fig. 5R) and the expression of *PAL* (Fig. 5T) and *PAM* (Fig. 5W) were significantly higher in the old stems (Fig. S1). In contrast, baccatin III accumulation (Fig. 5P) and expression of *TS* (Fig. 5S) were higher in the young stems. Similarly, this can be explained by the presence of specialized elements in the production process of Taxol from baccatin III in the older but not the younger tissues. For example, old mature trees have more heartwood which contains very large amounts of resinous extractives and polyphenols (Zobel and Buijtenen, 2012).

2.4. Sites of biosynthesis and storage of Taxol are not coincident

In situ immunolocalization of the TS enzyme using polyclonal anti-

TS Ab and Taxol using anti-Taxol Ab showed that in comparison to control samples (Fig. 6A), TS highly accumulated within the wood parenchyma ray cells (Fig. 6B), while Taxol mainly accumulated to the outer tissues including phloem and outer dead bark (Fig. 6C). Because the accumulation of baccatin III (Fig. 5B) and localization of the TS enzyme (Fig. 6B) were greater in wood compared to the phloem, one can conclude that wood parenchyma rays are the sites of biosynthesis. However, the prominent localization of Taxol in the phloem and dead bark (Figs. 5A and 6C) suggest that phloem and dead bark are the sites of storage.

3. Discussion

The localization of plant Taxol to hydrophobic bodies (HB) was in agreement with the discovery by us (Soliman et al., 2015) that Taxol-containing HBs are located in the living wood parenchyma rays and phloem. The purpose of Taxol localization in HB is to protect the living cells (phloem and wood parenchyma rays) from the cytotoxic effects of Taxol, which is not required in the case of dead outer bark (Soliman et al., 2015). This could explain why HBs are not found in the dead outer bark. Thus we assume that Taxol is released from the HBs to the dead outer bark as a response to factors including pathogen invasion and herbivore attack (Wagner and Flores, 1994). *Taxus* is an evergreen tree (Thomas and Polwart, 2003) and the persistence of the plant is mainly dependent on the roots and shoots (Souza et al., 2009). While the roots are minimally exposed to herbivores, shoots are not and this may explain why Taxol and HBs are mainly present within the plant shoots (Gratani, 2014), but at lower concentrations in the roots and needles (Vidensek et al., 1990). These results were confirmed by the positive correlation between Taxol content and the number of HBs present within different plant tissues (Fig. 2F). On the other hand, localization of Taxol to HBs could afford special transport mechanism as previously suggested (Naill et al., 2012; Soliman et al., 2015).

The absence of correlation between Taxol and baccatin III was coincident with the fact that baccatin III is an intermediate in the biosynthesis of not only Taxol but also other taxoid derivatives (Croteau et al., 2006; Fleming et al., 1994; Kucukboyaci and Sener, 2000). However, the positive correlation between Taxol and polyphenol accumulation was coincident with the fact that polyphenol production is considered as late stage in the biosynthesis of Taxol (Fleming et al., 1994). Similar to Taxol, polyphenol production in the plant superficial layers is considered as a protection mechanism (Kusumoto and Suzuki, 2003). Similarly, the positive correlation between *TS* gene expression and baccatin III accumulation but not between *TS* gene expression and Taxol accumulation can be explained by *TS* being critical to baccatin III biosynthesis. While, baccatin III is an intermediate precursor in Taxol biosynthesis, Taxol is not the only final product from baccatin III (Walker and Croteau, 2001). These results were confirmed by the *in situ* immunolocalization comparison between Taxol and TS enzyme within a transverse section in the *Taxus* plant stems (Fig. 6). TS enzyme was mainly located in the wood tissues, in the form of rays, similar to the accumulation of baccatin III, while Taxol was mainly located outward in the phloem and dead bark tissues. Future study on baccatin III immunolocalization within plant tissues could be a further important piece of information. The absence of correlation between Taxol and *PAL/PAM* gene expression can be explained as PAL and PAM are also general enzymes required in the biosynthesis of several polyphenols (Hahlbrock and Scheel, 1989 and β-amino acids (Szymanski et al., 2009), respectively, other than the biosynthesis of the Taxol side chain. One can conclude that although baccatin III biosynthesis is limited by *TS* expression, Taxol production itself is limited by the presence of specific phenolic and PAL/PAM biosynthetic pathways in intact plants.

In the case of plant tissue culture, the concentrations of Taxol, HB andbaccatin III, and *TS* gene expression were lower when compared to the concentration of polyphenols and *PAL/PAM* gene expression. This result indicates that Taxol production is limited by baccatin III production in plant tissue culture.

The variable results from both intact plants and tissue culture may be attributed to not only the availability of precursors that control the production of Taxol, but also other factors. These factors may include the source plant organ used to establish callus culture (Bestoso et al., 2006; Brukhin et al., 1996) and/or the presence of endophytic fungi (Li and Tao, 2009; Soliman et al., 2013). In our study, we used the needles to establish callus culture which also showed absence of Taxol-producing fungus *Paraconiothyrium* SSM001 (data not shown). Since, Taxol-producing endophytic fungi are considered as important partner sin Taxol production, comparing the amount of Taxol-producing endophytic fungus between callus culture and its source plant could help in future explanations of this phenomenon.

The coincident greater concentration of baccatin III and higher *TS* gene expression in wood tissues compared to phloem and bark, was in agreement with the previously published data showing the presence of a higher concentration of baccatin III in the heartwood (Kucukboyaci and Sener, 2000; Phu et al., 2013). This leads to the conclusion that the wood is the site of biosynthesis. The higher expression of *PAL* in the plant wood is coincident with the special presence of polyphenic cells within the wood of conifer trees (Bedon et al., 2010) and its main localization within the xylem of willow and poplar trees (de Jong et al., 2015; Osakabe et al., 1996). On the other hand, the presence of a higher level of Taxol and polyphenols in the outer superficial layers including phloem and bark can indicate that phloem and bark are the sites of storage. This can explain why Taxol is mainly extracted from the outer bark and phloem of *Taxus* plants (Kelsey and Harmon, 1989; Kusumoto and Suzuki, 2003; Vance et al., 1994; Vidensek et al., 1990).

The similar level of *TS* gene expression in the stems and needles with the predominant presence of Taxol in the stems and baccatin III in the needles (Denis et al., 1988; Fu et al., 2009) can be explained by the occurrence of factors used in the biosynthesis of Taxol in the stems but not in the needles including polyphenols (Kelsey and Harmon, 1989) and endophytic fungi (data not shown). These results were confirmed by the higher expression of *PAL* and *PAM* genes in the stems, the key switch effect of PAL in metabolite biosynthesis (Osakaba et al., 2007), and the presence of a higher number of HBs in the stems. As the plant gets older, it does require more protection for its persistence and thus adapts several factors for the maximum production level of Taxol (Cheng et al., 2005; Mukherjee et al., 2002) including but not limited to polyphenols and its related genes, *TS* gene expression, and the existence of HBs. However, baccatin III biosynthesis showed lower levels in old tissues, since it is continuously used up in the biosynthesis of Taxol when the plant becomes older.

In summary as described in the Taxol dynamic release model (Fig. 7), Taxol biosynthesis starts in the deep wood parenchyma cells and then Taxol is transported by HBs either horizontally to the superficial layers or vertically to the needles to afford sufficient protection to the plant; thus helping plant survival and persistence.

4. Experimental

4.1. Materials and reagents

The following reagents were from Sigma (USA), including Taxol (# T7402) and baccatin III (#B8154). All solvents used for extraction, TLC and HPLC were HPLC grade and obtained from Fisher Scientific. *Taxus media* (Rehder) plant samples were collected during the Fall from the University of Guelph Main Campus, ON, Canada. Taxol monoclonal antibody (#SC-69899) was commercially obtained from Santa Cruz Biotechnology Inc., USA. The anti-taxadiene synthase (TS) antibody was a gift from Prof. Rodney Croteau (Washington State University).

4.2. Light microscopy and histochemistry

Freshly hand-made plant sections were obtained according to

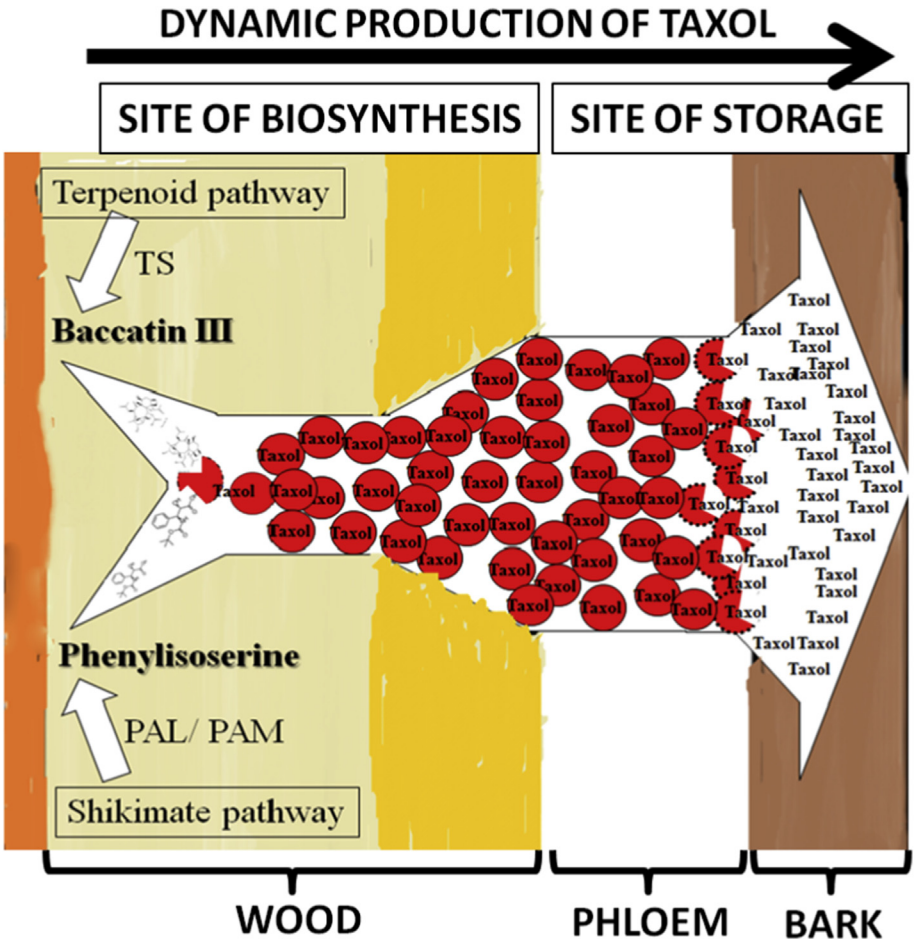

Fig. 7. Model summarizes the dynamic production of Taxol in *Taxus* plant stem sections suggesting wood as the site of Taxol biosynthesis, and phloem and outer bark as the sites of storage.

Soliman et al. (2015). Both plant sections and callus culture cells were stained by Sudan III/IV in 50% ethanol for examination of resin-like hydrophobic bodies. The stained plant sections were then washed with ethanol prior to examination under light microscopy.

4.3. *In situ* immuno-labelling of TS enzyme and Taxol

Taxus plant stem pieces were fixed overnight at 4 ˚C in phosphate buffered saline (PBS; 10 mM sodium phosphate, pH 7.2, 150 mM NaCl) and 4% paraformaldehyde. The tissues were then washed five times in 0.1% Tween 20 in PBS (PBT). Hand-made cross sections of the stem were subjected to immunolabelling using protocol adapted from Reid et al. (2001) . All prehybridizations, hybridizations and subsequent washes were performed at room temperature. Briefly, plant sections were incubated for 2 h in prehybridization buffer consisting of 50% formamide in 0.75 M NaCl, 75 mM sodium citrate (5 X SSC, pH 4.5), 0.1% Tween 20, and 5 nM disodium ethylenediamine tetra-acetic (EDTA) acid. The prehybridization buffer was replaced with fresh buffer containing antibodies and incubated for 4 h. The samples were subsequently washed for 10 min in 2 X SCC at room temperature, and twice in 0.2 X SSC for 30 min. The samples were then incubated for 1 h at room temperature in alkaline phosphatase conjugated anti-rabbit antibody (Roche) diluted 1:200 in 0.2 X SSC buffer. Samples were washed five times for 45 min each, in 5X SSC buffer at room temperature to remove the excess unbound antibody. The buffer was then replaced with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate substrate solution (NBT/BCIP, Roche, prepared according to manufacturer's instructions) and the specimens were incubated in the dark until the colored reaction product was visible. Specimens were then rinsed six times with PBT to stop the colorimetric reaction and to remove excess substrate, and then stored in PBT with 100 mM EDTA at 4 ˚C in the dark. Images were captured from a Zeiss photo microscope using a Sony Power HAD digital camera and Northern Eclipse Image Analysis software (Empix Imaging, Inc., Mississauga, Canada).

4.4. *Semi-quantitative RT-PCR*

RNA was prepared using the RNeasy Mini Kit (QIAGEN, Mississauga, ON, Canada). Genomic DNA was eliminated by loading RNase-free DNaseI onto the filter column (QIAGEN). Nucleic acid extraction was performed according to the manufacturer's instructions. First-strand cDNA was synthesized using oligo-dT primers with M-MuLV reverse transcriptase according to the manufacturer's instructions (Fermentas, USA). Two hundred nanograms of total RNA were used for cDNA synthesis; the synthesized cDNA was subsequently diluted with nuclease-free water (QIAGEN) to 100 ng/μL. Semi-quantitative RT-PCR amplification mixtures (20 μL) contained 100 ng template cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1X buffer (Green GoTaq Flexi Buffer, Promega, USA, #M8911), 0.5 U Taq DNA polymerase (New England BioLabs, USA), and 0.2 μM of each primer set. RT-PCR was performed using the following primers: for phenylalanine ammonia lyase (*PAL*), primers PalF2 (5′-GTGTCGCACTTCAGA-3′) and PalR2 (5′-GGCTTGTT TCTTTCGTGCTT-3′) were used; for phenylalanine amino mutase (*PAM*), primers PamF1 (5′- GCGCTTCCTCCGTCGACGAG -3′) and PamR1 (5′-CTTGAATGGCCGCAGCCC-3′) were used; for taxadiene

synthase (*TS*), primers TSinF (5′-GGTTTGCTCCAAATCAGGGC-3′) and TSinR (5′-TAACATTGTGGTGCCACAGA-3′) were used. The following conditions were used for PCR amplification: 94 °C for 2 min, followed by 40 cycles of 94 °C for 45 s, 56.6 °C for 1 min and 72 °C for 2 min, and then a final extension cycle of 1 min at 72 °C in a PTC200 thermocycler (MJ Research). The produced amplicons from the PCR were applied onto a 2% agarose gel stained with ethidium bromide. *Taxus 18S* rRNA was used as an internal standard for normalization using the following primers (Soliman et al., 2013): Tax18SF2 (5′-TTTTCCCTTTGCAATGCC-3′) and Tax18SR2 (5′-TCGCCCTTGTAATAACCCG-3′).

4.5. Quantitative real-time PCR

RNA was isolated and PCR reaction efficiencies were determined by a series of ten-fold dilutions using plant 18S rRNA specific primers (Soliman et al., 2013): Tax18SF2 (5′-TTTTCCCTTTGCAATGCC-3′) and Tax18SR2 (5′-TCGCCCTTGTAATAACCCG-3′). Amplification conditions were 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 56.6 °C for 30 s and extension at 72 °C for 1 min. The specificity of the reaction is given by the detection of the T_m values of the amplification products immediately after the last reaction cycle. Results were analyzed with the melting curve StepOne analysis software (Applied BioSystems) (Pfaffl et al., 2002). The relative expression ratio of each gene was analyzed based on real-time PCR efficiency and the crossing point differences of the samples versus the plant 18S rRNA (Tax 18S). The results were verified using REST (Relative Expression Software Tool) (Pfaffl et al., 2002).

4.6. Callus culture initiation protocol

Needle sections of *T. media* were surface sterilized with 3% Clorox solution containing two drops of Tween 20 for 20 min with shaking. The sterilized explants were dipped into 70% ethanol for approximately 5 min and then washed five times with sterile deionized H_2O. The tissues were then cut into small explants (0.5–1 cm^3) prior to being placed onto B5CA culture medium. B5CA consisted of Gamborg's B5 medium (Gamborg et al., 1968) supplemented with 0.2% casamino acids (CA), 1% sucrose, 1 mg/L 2,4-D, 0.75% agar, with pH adjusted to 5.5 prior to autoclaving (Gibson et al., 1993). Cultures were initiated in the dark at 25 °C. The callus cultures were sub-cultured every 1–2 months after the removal of explants on the same media.

4.7. Taxane analysis in plant tissues and callus cultures

Fresh *Taxus* plant tissues were ground with a mortar and pestle and then extracted with ethyl acetate 3 times. Similarly ground callus cultures were extracted with ethyl acetate three times. The filtered and washed organic layers were then evaporated at 45 °C under reduced pressure. The residue was then subjected to HPLC analysis against standard Taxol and baccatin III. HPLC analysis was performed according to a previous study (Soliman et al., 2011). To quantify the amounts of Taxol and baccatin III within different samples, two sets of serial dilutions of standard Taxol and baccatin III (0, 20, 40, 60, 80, 100 and 120 μg/mL) were used to plot a linear calibration curve. The peak area of each sample injected was measured by a UV detector at λ_{max} 233 nm and then factored against the calibration curve generated from injecting different standard concentrations. For each treatment, two sets of plant samples were prepared; one set was injected directly into HPLC and the other set was spiked with 200 ng standards of both Taxol and baccatin III prior to injection. The spiked peak area was then calibrated and subtracted from the spiked amount of standard Taxol.

4.8. Phenolic assays

For measuring plant total phenolics, the Folin-Ciocalteau method (Duval et al., 1999; Mossor and Schramm, 1972) was applied. Briefly, 1.0 mL plant phenolics aqueous extract was transferred to glass test tubes along with 1.0 mL of 95% EtOH, 5.0 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteau reagent (#F9252, Sigma, USA). The solution was incubated for 5 min, after which 1.0 mL of 5% Na_2CO_3 was added, then vortexed and placed in the dark for 1 h. The absorbance of the resulting dark blue colour was measured using an Ultraspec 2100 pro UV/Visible spectrophotometer at 725 nm and plotted against a standard curve generated using pure cinnamic acid. One mL of each sample in triplicate was read using a clear cuvette.

4.9. Statistical analysis

The data obtained was plotted using Graph Pad 5.0. The measurement of Taxol, baccatin III, polyphenollevels and the gene expression of *TS*, *PAL* and *PAM* were analyzed by the Student-T-Test and one-way analysis of variance (ANOVA) using Dunn's Multiple Comparison Test. *P*-value < 0.05 was considered as significant.

The relationship between Taxol or baccatin III and gene expression and/or HB and polyphenols was generated using Graph Pad 5.0 to produce what is referred to as the coefficient value (Zar, 1984). Pearson's correlation attempts to draw a line of best fit through the data of two variables. The Pearson's correlation coefficient, *r*, indicates how far away all these data points are to this line of best fit. A value of -1 represents a perfect negative linear relationship, while $+1$ represents a perfect positive linear relationship.

Author contributions

SS planned and conducted all experiments to investigate the original hypothesis. SS and MR wrote and revised the manuscript.

Declaration of competing interest

All authors declared there is no financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112369.

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