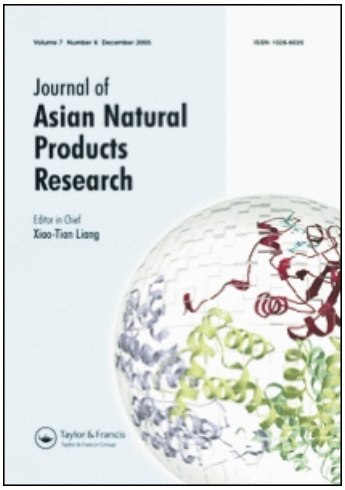

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The dynamic variation of several important taxane content in post-harvest *Taxus chinensis* clippings

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The dynamic variation of several important taxane content in post-harvest *Taxus chinensis* clippings

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The dynamic variations of several important taxanes, taxol, baccatin III (B-III), 1-acety-5,7,10-deacetyl-baccatin I (DAB-I) and baccatin VI (B-VI), were investigated in post-harvest clippings of *Taxus chinensis*. The clippings were preserved over 20 days at two different temperatures (4°C and room temperature), or by cuttage in the light and in the dark, or promptly dried. The accumulation of taxol in needles of the clippings was found increase in the initial stages of the stored period and then decreased gradually. The maximum accumulation of taxol occurred in the case of cold storing (4°C) at day 3, doubling the data on the day when the biomass was harvested. In contrast, in the cases of cold storage and cuttage the contents of the other three taxanes showed a sharp decrease at the beginning and then an increase from 3 to 6 days, and subsequently a drop until day 20. The similar variation of taxane contents was not found in the needles of immediately dried clippings as well as in the stem samples of clippings. These results indicated that the content variation of taxol and related taxanes of post-harvest clippings was related to the manner of preservation, timing and plant tissue. Moreover, the mechanism of the fluctuation of the taxane contents in post-harvest clippings is discussed, in particular taxol biosynthesis in response to mechanical wounding of harvest.

Keywords: *Taxus chinensiss*; Taxol; Taxanes; Accumulation; Post-harvest process; Phytomedicine

1. Introduction

Taxol is one of the most effective anti-cancer agents due to its unique mode of anti-cancer action, and has been highlighted since it was first isolated from the bark of *Taxus brevifolia* in 1971 [1]. The limited supply of the taxol in pharmacognosy has prompted intensive research efforts to develop an alternative source and way of producing taxol such as total synthesis [2,3], *Taxus* cell culture [4], and the structural modification of the abundant taxanes isolated from yew trees. The successful exploitation of Taxotere, another excellent anti-tumour drug derived from baccatin III and 10-deacetylbaccatin III with taxol-like structure [5,6], has spurred the isolation of many additional taxanes from the renewable parts such as needles and twigs of the plant to find either new compounds with even better activity than taxol, or

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potential taxanes for pharmaceutical use [7–10]. Equally important is the fact that the needles and twigs also contain a certain amount of taxol, although lower than that in barks, and can be harvested without killing the plant. Clearly, these renewable parts of the *Taxus* plant will continue to serve as a feasible and important source of taxol or its synthetic precursors. It is therefore of great interest to make use of the limited resources as wisely and completely as possible. This cannot be achieved without profiling the natural product content of the *Taxus* plant both *in vivo* and *in vitro*.

Previous works in our laboratory have established many methods to improve production of taxol in cell culture systems [11–13]. Also, we have isolated and identified several taxanes from needles of *Taxus chinensis* [14]. As a part of study on enhancing targeted taxane yields, the initial goal of this work was to elucidate the factors that effect on biosynthesis and accumulation of taxol at the level of whole plant. We therefore analyse various plant samples collected from different areas and seasons. We found the content of taxol and main taxanes, baccatin III (B-III), 1-acety-5,7,10-deacetyl-baccatin I (DAB-I), baccatin VI (B-VI), in the post-harvest plant materials showed considerable variation with storage time, ranging from less than 0.0001 to 0.01% of the needles of the plant dry weight. Interestingly, after the clippings were collected, an obvious but transient increase of taxol as well as the other taxanes accumulation took place. The maximum taxol accumulation was observed at post-harvest day 3, doubling the data on the day when the biomass was harvested. Further, it was found that the level of the increase or decrease depended on different treatments and plant tissues. To our knowledge, this observation in *Taxus sp.* described here is reported for the first time. This information should be useful for developing relational strategies, such as constituting proper processes in harvesting and storing to maximise the yields of taxol and related taxanes. Moreover, the dynamic variation of taxane content in post-harvest yew clippings provided chemical evidence to study the characteristics of taxol biosynthesis in response to mechanical wound, and may open the way for using plant tissues to study the production and biosynthesis of taxanes.

2. Results and discussion

2.1 Determination of the related taxanes

In order to investigate the profile of interested taxane content in the needles of *Taxus chinensis*, HPLC analysis of both sample and standard mixture solutions, including 10-deacetyl-baccatin III (10-DAB-III), (1-acety-5,7,10-deacetyl-baccatin I (DAB-I), baccatin III (B-III), baccatin VI (B-VI) and taxol, were performed, and the chromatograms are shown in figure 1. Compared the corresponding t_R in sample chromatograms with the standards, the four peaks could be assigned to be DAB-I, B-III, B-VI and taxol, respectively. LC-ESI-MS and MS-MS spectra of the samples were subsequently employed to confirm the assignment of the peaks. Figure 2 shows the results of LC-ESI-MS, MS-MS spectra and the calculated structures. The mass data are as follows. DAB-I, *m/z* 586 ($M + NH_4^+$); 551 ($M—H_2O + H^+$); 431 ($M—H_2O—2AcOH + H^+$); 371 ($M—H_2O—3AcOH + H^+$); 293 ($M—H_2O—3AcOH—2CH_2CO + H^+$); B-III, 604 ($M + NH_4^+$); 587 ($M + H^+$); 465 ($M—BzOH + H^+$); 415 ($M—BzOH—AcOH + H^+$); 345 ($M—BzOH—2AcOH + H^+$); 287 ($M—BzOH—2AcOH—CO—CH_2O + H^+$); 123 (BzOH); B-VI, 670 ($M + NH_4^+$); 653 ($M + H^+$); 611 ($M—CH_2CO + H^+$); 593 ($M—AcOH + H^+$); 575 ($M—AcOH—H_2O + H^+$); 533 ($M—AcOH—H_2O—CH_2CO + H^+$);

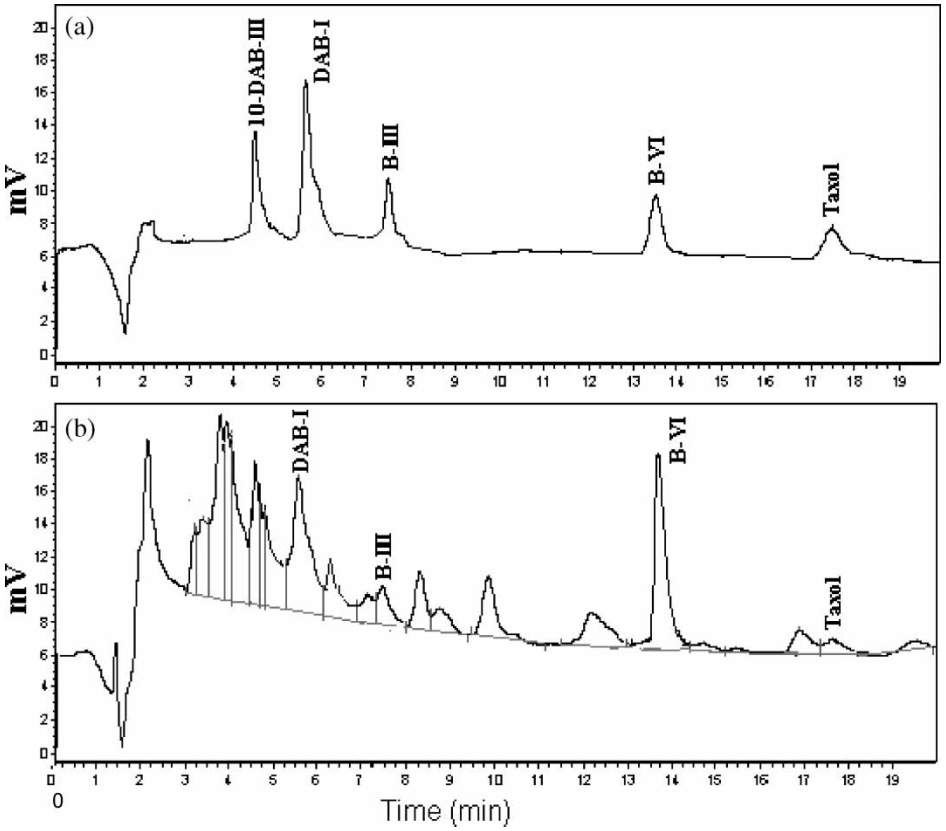


Figure 1. HPLC chromatograms of the standards (a) and a sample (b). The sample was an extract of needles of *Taxus chinensis*. Chromatographic conditions were as given in Section 3. t_r values for DAB-I, B-III, B-VI and taxol were 4.70, 5.67, 7.40, 13.56, 17.59 min, respectively.

311 (M—AcOH—H_2O—CH_2CO—BzOH + H^+); 293 (M—AcOH—$2H_2O$—CH_2CO—BzOH + H^+); 123 (BzOH); taxol, 871 (M + NH_4^+); 854 (M + H^+); 569 (M—ScOH + H^+); 327 (M—ScOH—2AcOH—BzOH + H^+); 286 (ScOH + H^+); 123 (BzOH). We noticed that 10-DAB, one important precursor for Taxotere semisynthesis, did not give a baseline resolution in the LC conditions. Thus, the four taxane components were targeted as DAB-I, B-III, B-VI and taxol for tracing the variation of their content in post-harvest clippings.

2.2 Profiles of variation of taxane contents in the needles of post-harvest clippings

To profile the taxane content as well as the effects of the conditions under conventional preservation on taxane content upon harvesting, the harvested clippings were divided into five groups. Group **1** was stacked at room temperature (25°C ± 5°C), group **2** was refrigerated at 4°C, group **3** was cuttaged in pots in the dark, group **4** was cuttaged in the light (3.5 μmol m^{-2} s^{-1} for 8 h), and group **5** was placed at room temperature after immediate drying. When sampling was needed, the clippings were separated into needles and stems. The effect of delayed period for sampling on taxane contents was overcome by immediately storing plant samples at −80°C and grinding with the help of liquid nitrogen at −70°C

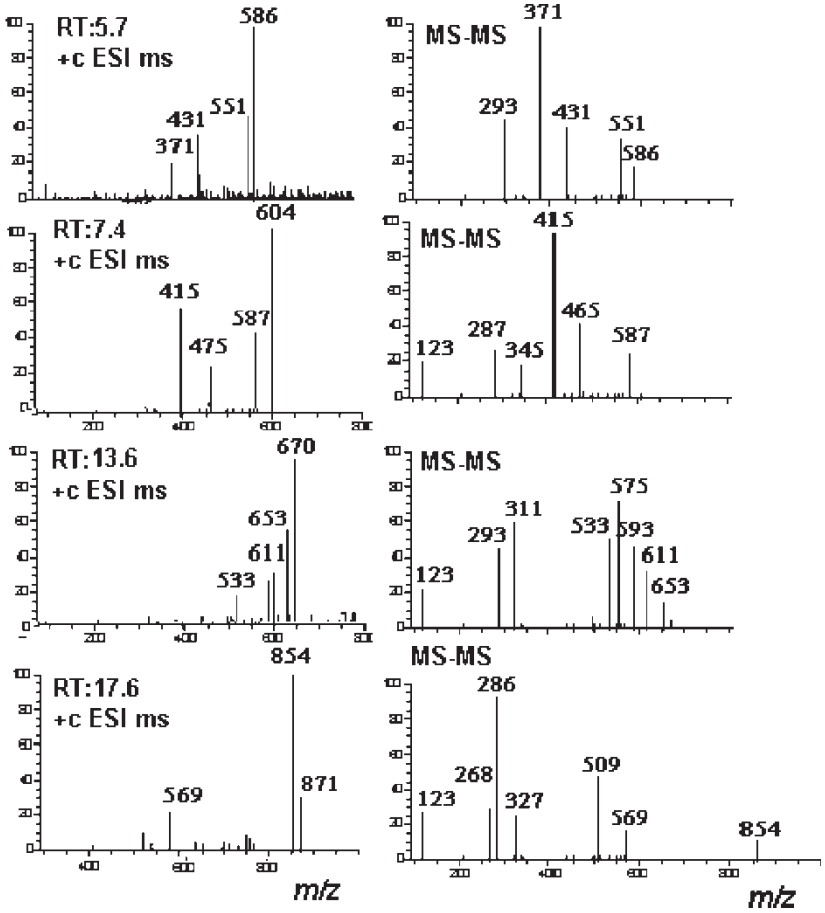


Figure 2. LC-positive ion ESI-MS spectra of peak t_R at 5.7, 7.4, 13.6, 17.6 min and MS-MS spectra of $[M + NH_4^+]$ or $[M + H^+]$ ions at *m/z* 586, 587, 653 and 854 in the sample from the needles of *Taxus chinensis* and the structure of related taxanes.

before extracting three times with methanol. The starting data were based on the extracts from just harvested plant materials, which taken from *T. chinensis* trees of 30 years old over three different sampling. The samplings were followed after 3,6, 10, 15 and 20 days in every experimental group. The values presented in this paper are the average of three replicates. The quantitative methodology was validated to evaluate linearity range, accuracy and precision (presented in Section 3). The data from the needles are displayed in figure 3 and partly shown in table 1.

The content of taxol in needles tended to decline during 20 days except that a significant increment was obtained from 1 to 3 days under the treatment of groups **1**–**4**. The fluctuation of taxol level differed slightly under the four treatments. For the cold storage, the content of taxol within the post-harvest 3 days increased from 25.3 to 53.2 $\mu g\, g^{-1}$, doubling its starting content. Then, the enhanced content declined till the end of the test. On the 20th day the taxol content approximately equalled or was slightly less than its starting content. For the treatment **1**, the lowest taxol content among the other three treatments was observed during 20 days in the needles from clippings. For example, there was about 98–100% loss of taxol on day 20 under the treatment. But it is interesting that, in spite of the lowest level, there was

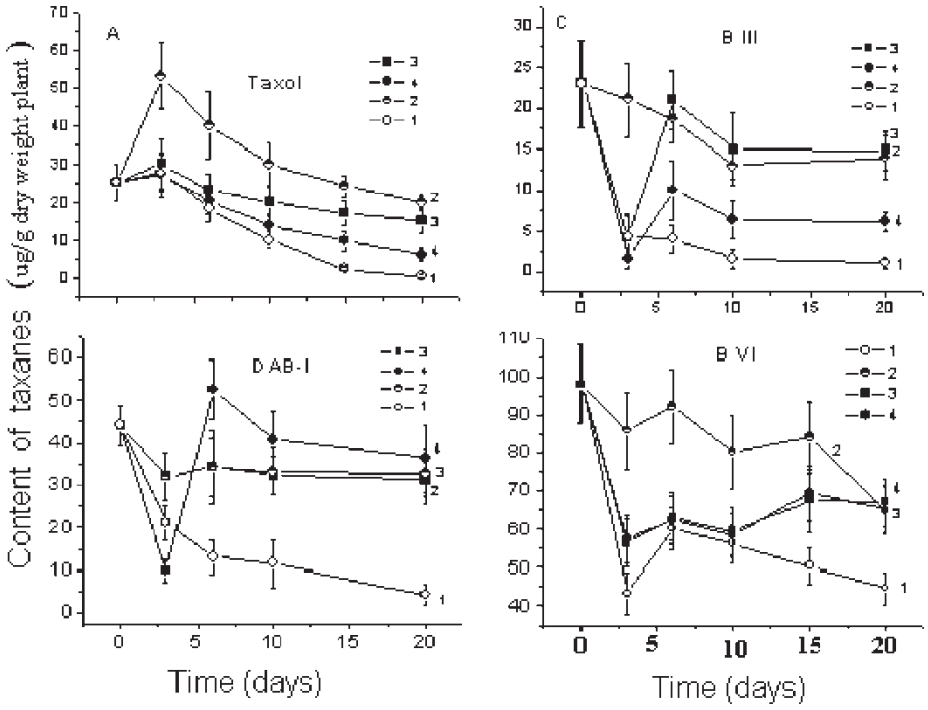


Figure 3. The profile of variation in taxane content in needles of clippings in the case of the various preservation processes. 1, stacked at room temperature; 2, stored at 4°C; 3, cuttaged in the dark; 4, cuttaged in the light.

also a rise in the taxol content on day 3. For the treatment by cuttage, the taxol levels were between the levels in the samples handled by groups **2** and **1**. Moreover, there were about 40% and 76% taxol level losses in the treatments **3** (dark) and **4** (light) corresponding to in the dark and light, respectively, after 20 days.

The variation in the contents of DAB-I as well as B-III and B-VI was generally similar to each other but different slightly compared with variation of taxol. The fluctuation of the taxane level with the time was also observed in the treatments **3** and **4**. But in the treatments **1** and **2**, there was only decline without increment within 20 days. In the treatments **3** and **4** (cuttage), the content of both DAB-I and B-III was found to decline for the first 3 days of the 20-day period, and then an increase. In particular, the increase was more pronounced in the case of the treatment **3**. The optimum time, in which the content of these taxanes reached the highest compared with other periods, was day 6. In treatment **1**, the contents decreased sharply so that there was 60% and 70% loss of DAB-I and B-III, respectively, after 20 days. In treatment **2**, after 20 days the harvested needles still contained a significant

Table 1. Contents of taxanes in *Taxus chinensis* clippings before and after drying.

	Undried fresh clippings	*Dried on Day 1*	*Day 3*	*Day 5*	*Day 10*	*Day 20*
Taxol	25.3 ± 4.6	18.4 ± 3.2	18.5 ± 3.3	18.0 ± 2.6	18.3 ± 2.5	17.6 ± 3.0
DAB-I	44.5 ± 5.0	38.4 ± 4.2	37.6 ± 3.2	38.6 ± 3.6	37.4 ± 4.5	36.4 ± 4.4
B-III	23 ± 4.5	20 ± 3.2	19.0 ± 4.2	19.4 ± 3.2	18.5 ± 3.6	19.6 ± 4.6
B-VI	98.6 ± 6.8	89.6 ± 7.6	88.6 ± 4.6	89.6 ± 6.6	89.4 ± 5.8	88.6 ± 6.8

Values are the average of three replicates ± absolute deviation (AD). The contents of taxanes are calculated as $\mu g \cdot g^{-1}$ plant dry weight.

amount of the DAB-I, B-III and B-VI, although their increments of contents were not obvious during the 20 days.

As for treatment **5**, there were no significant changes ($p \geq 0.05$) for all the detected taxane quantities during the storage process after the clippings were dried at 60°C for 2 h (table 1). Only 27%, 13%, 13% and 9.1% loss of taxol, DAB-I, B-III and B-VI, respectively, resulted from the drying process.

2.3 Profiles of variation of taxane contents in the stems of post-harvest clippings

For the stems of the clippings, the profile of the content of DAB-I, B-III and taxol is shown in figure 4. Although the twigs were neither heated nor dried upon harvesting, the three determined taxane levels basically held the line. In other words, there was no obvious fluctuation up and down in the content of taxol as well as in DAB-I and B-III. This result indicates little effect of different preservation process on the taxane content in *in vitro* stems. Therefore, the factors that influence the variation of taxane content in the post-harvest plant materials mainly occurred in the needles.

2.4 The meanings of the variation of taxane contents in post-harvest clippings of Taxus

First of all, the results of this investigation indicate the importance of the proper process for preservation and the optimum time for the extraction of taxanes. It is clear from these variations of taxol content that the best treatment and period in which the maximum amount of taxol could be captured should be under the condition of refrigerated storage within 3 days after harvesting if a further process (e.g. extraction) is needed for the clippings. More DAB-I and B-III exist at lower temperature storage and cuttage; in particular, an additional amount of them can be obtained if the time for the extraction process is selected properly. The light and unforced drying at room temperature result in a greater loss of the taxanes. Therefore, the preservation should be performed at a lower temperature and away from direct light. The data stored at 4°C indicate that the condition of routine refrigerated storage can be a reasonable method for the maintenance of taxane content. Comparing the optimum times at which taxol, DAB-I and B-III achieve their the highest contents, it is apparent that the most practical

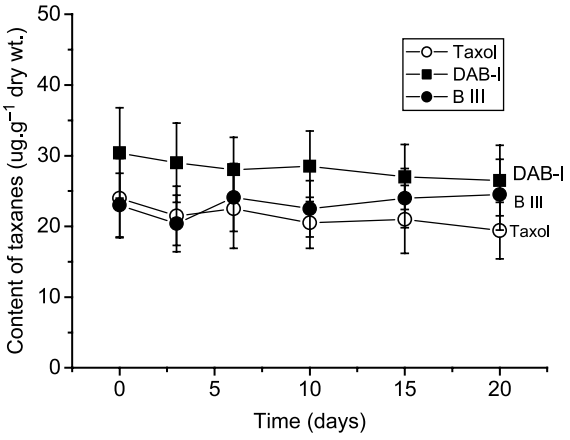


Figure 4. The contents of taxanes in young stems of *T. chinensis* clippings stored at room temperature, 25°C.

period for all the determined taxanes among the post-harvest needles with near optimum contents is from 3 to 6 days.

Plant post-harvest biochemical process has been well studied in tuberous roots of Cassava and is called post-harvest physiological deterioration (PPD) [16,17], which is an abiotic stress response and often leads to the accumulation of some secondary metabolites. For example, Wheatley [18] described a 150- to 200-fold increase of scopoletin in post-harvest *Cassaca* during the first 24–48 h, and assumed that this phenomenon occurred due to the involvement of PPD. Nevertheless, the symptoms and susceptibilities of the deterioration among various plant species and substances towards PPD differ.

As regards the factors that influence taxol biosynthesis and accumulation at the level of the whole plant, the effects of plant living environment on taxane contents have been noticed [19,20]. There also were a few reports on the effect of preservation on the yield of taxanes from *Taxus* plant cuttings [21,22]. However, there is little information on what profiles of the variation in taxane (including taxol) content will respond to PPD. To our knowledge, the observation in *Taxus sp.* described here is reported for the first time.

As a group of secondary metabolite of *Taxus*, taxol is assumed to be produced by plants as a response to pathogenic attack and other abiotic stress [23]. An example for this supposition is that oxidative stress [12] and the use of fungal elicitors [11] could improve taxol production in cell culture systems. The quantitative fluctuation of the taxanes in the present study indicates that taxol as well as other taxanes not only can be degraded by *Taxus* upon harvesting but also, like flavonoids, coumarin and flavonoid-glycosides, they may serve as 'stress metabolites' involved in PPD.

The investigation by Brincat et al. [24] speculated that the phenylpropanoid pathway critically controlled by phenylalanine ammonia lyase may be a divarication into taxol biosynthesis. A transient higher quantity of taxol observed here may suggest the involvement of the related enzymes, which is probably caused by a rapid and direct response to wounding. Except for phenylalanine ammonia lyase, other enzymes such as GGPPs, which is a first key enzyme in the formation of taxane skeleton, may respond to wounding slowly. This may be employed to explain the phenomenon why the maximum accumulations of the other three taxanes appeared 5 to 7 days post-harvest, rather than being consistent with taxol. Furthermore, although there is no direct evidence to prove whether B-III, DAB-I or B-VI is biosynthesised before taxol, the period during which the decline of contents of the three taxanes arrived at their rock bottom was just the time when the taxol content reached its maximum (see figure 3), suggesting that these taxanes may be the biological precursors of taxol.

For stems, however, the profile of variation in taxane content did not agree with needles in the same treatment. The transient increases of the taxane contents were not observed in the stem extracts. This suggests that the biosynthesis and accumulation involved in the related taxane production may be tissue specific. ElSohly et al. [20] noticed that drying the biomass with the needles attached to the clippings significantly preserves the needles' taxol content, because the average taxol content of the needles from intact clippings dried at room temperature was higher than the value of the stripped needles dried at room temperature. These phenomena can serve as indirect evidence to support our observation. Perhaps *Taxus* needles have more activity than the stems for biosynthesising related taxanes, including taxol and its precursors, and the stems may facilitate accumulation of the taxanes.

Noticeably, the uniform decrease in all the determined taxane content occurred in nearly all the designed treatments in this investigation after 6–7 days. This can be explained in

terms of degradation of taxanes or further metabolic transformation. Generally, degradation of a compound is mainly caused by two factors, chemical and biological. The fact that a more stable taxane level following treatment **5** and the higher taxane contents were obtained at lower temperature (storage at 4°C vs at 25°C) indicates that the effect of biological factors on the taxane levels is significant. Previous research found that taxol was susceptible to hydrolysis and epimerisation in animal culture media [25]. Similarly, taxol can be degraded by the enzymes occurring in plants. Other biological factors including unknown specific enzymes, endogenesis and environmental microbes also may contribute to the degradation. Activities of both enzymes and microbes often decline at a lower temperature and under light. This may explain the observation that there was a higher taxane level at 4°C than at 25°C and when *Taxus* clippings were stored in the dark rather than in the light. It is still unclear why the cuttage treatment could yield more taxane than that of unforced drying. This needs further studies to determine whether the variation in taxane content post-harvest involves a defensive response to the invasion of micro-organisms.

3. Experimental

3.1 Plant material

Lateral braches of *Taxus chinensis* were harvested in the summer of 2003 from a copse located Xian Ning, Hubei province in China. The age of the trees calculated on the basis of bole radius was 30 years old. The clippings were harvested from five trees of 30 years old.

3.2 Reagents and treatment procedures

Standards of taxol, 10-deacetylbaccatin III (10-DAB-III) and baccatin III (B-III) were a gift from NCI in the USA; The referent substances of DAB-I and B-VI were previously isolated from our laboratory and identified by NMR [14]. Methanol used for HPLC analysis was of chromatographic grade; other reagents used were of analytical grade for the extraction and solution.

Harvested lateral branches were cut into 4–6 cm long sections, and the needles the limbs were preserved intact. To investigate the contents of taxanes on the starting day upon harvesting, taxanes must be extracted on the harvesting day or the cuttings have to be stored at -80°C for taxane sampling analysis.

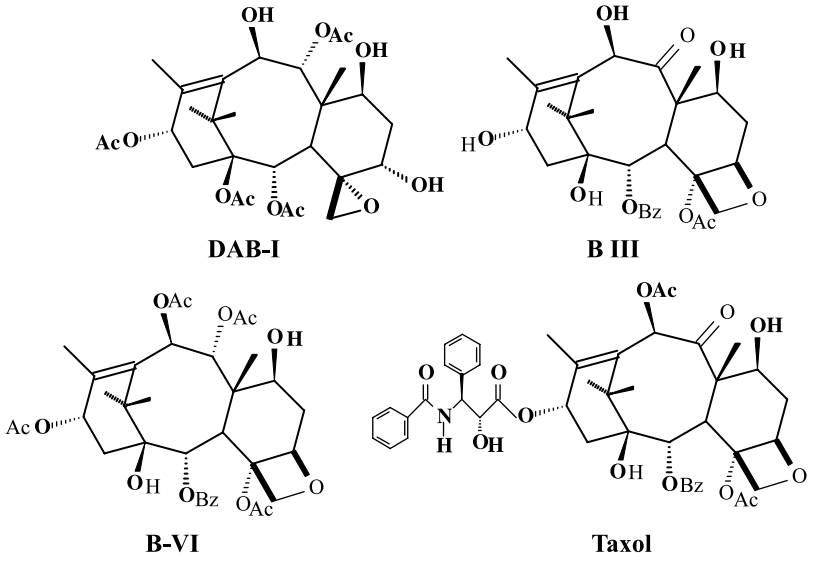
To analyse the variation of taxane *in vitro*, the harvested plants of three 30-year-old trees were divided into five parts for different treatments. Group **1** was stacked at ambient temperature (25°C $\pm$ 5°C). Group **2** was stored in a refrigerator at 4 $\pm$ 1°C. Group **3** was cuttaged in flowerpots with humus in the dark at ambient temperature (25 $\pm$ 5°C). We repeated the treatment of group **3** but placed the pots in the light (3.5 μmol m^{-2} s^{-1} for 8 h) as group **4**. The last part (group **5**) was heated immediately at 60°C in a dryer for 2 h. After the drying run was completed, samples were stored at room temperature (25°C).The contents of the taxanes in the dried samples were determined on days 1, 3, 5, 10 and 20.

3.3 Extraction and sample preparation

The cuttings were sampled for taxane analysis on days 1, 3, 5, 10, 15 and 20. Each time point in the tests was sampled over three data. The data given in this paper are the average values of the samples.

Plant cuttings were ground in a grinder assisted with liquid nitrogen at − 70°C and exacted three times with methanol. After centrifugation, the supernatant was evaporated to dryness under reduced pressure. The residue was resolved with a certain amount of methanol. Then, taxanes were isolated by the preparative TLC method as described by Srinivasan et al. [15].

The silica gel GF254 plates were prepared in our laboratory. Solutions of 50 μl were spotted, and plates were developed in $CHCl_3$:CH_3OH (95:5). The bands of DAB-I, DAB-I, B-III, B-VI and taxol were roughly estimated visually by comparing them with those of authentic standards exposed to UV light (254 nm). Subsequently, the substance of the presumed taxanes detected under UV was scraped into glass vials and eluted with methanol. After filtration through the organic filter (Φ0.45), the filtrates were further isolated and analysed with HPLC.



3.4 The identification and quantification of taxanes

In this investigation, LC with UV was used for isolation and quantification of taxanes, and LC with MS was used for structural identification. HPLC analysis was performed in a Waters HPLC system consisting of a 510 pump, 486 UV detector and Chemstation N2000 (Zhe Jiang University, China). Separation was conducted in a Kromasil C18 column (5 μm × 4.6 mm × 250 mm) with acetonitrile/methanol/water (30:25:45) as the mobile phase. The flow rate was 1 ml min^{-1} and the detection was done at 227 nm. The column oven temperature was 35°C. Three 10-μl injections were made for each sample.

The structure identification of the taxanes was performed in the LC-ESI-MS system platform LCQ DUO MS-MS (Finnigan Co., USA) including a TSP P4000 pump separation module HPLC system and a ZMD Micromass quadruped mass spectrometer equipped with an ESI source. The HPLC procedures were maintained under the same conditions mentioned above. Mass detection of taxanes was made with electrospray ionisation (ESI) in the positive ion mode. The source temperature was 200°C, ion energy 0.5 V and capillary voltage 4.5 kV. The drying gas was N_2 at flow rate of 40 psi.

The ESI-MS spectrum of chromatographic peak was obtained by scanning at presumed taxane retention time. The taxanes were quantified by means of an external standard curve in HPLC analysis at 227 nm. Linearity of the detector response was determined with triplicate analysis of DAB-I, B-III, B-VI and taxol standard solutions of eight concentration points ranging from 0.35 to 136, 0.69 to 189, 0.55 to 250 and 0.53 to 250 $\mu g\ ml^{-1}$, respectively.

3.5 Statistical analysis of data

Each test was performed with three samples. Data were analysed using two-way analyses of variance. The statistically significant differences of treatments were analysed using Tukey's test with a family error rate of 5%.

3.6 Validation of quantitative method

The quantitative method was validated to evaluate linearity range, accuracy and precision. The Linearity of detection was determined by triplicate analysis of standard mixture solutions of each concentration point. The regression equations of DAB-I, B-III, B-VI and taxol were determined to be $Y = 62388X - 15067.5$, $R^2 = 0.9945$; $Y = 14732X - 40786.6$, $R^2 = 0.9937$; $Y = 15436X - 39787.6$, $R^2 = 0.9921$; $Y = 27970X - 42190.8$, $R^2 = 0.9993$, respectively. In the equation, Y refers to the peak area, X the concentration of taxanes ($\mu g\ ml^{-1}$), and R^2 the correlation coefficient. The linearities of DAB-I, B-III, B-VI and taxol obtained were in the range of 1.0–130, 6.0–170, 7.0–200 and 2–180 $\mu g\ ml^{-1}$, respectively. The accuracy and precision were assessed by recovery and repeatability. The known quantities of the four reference substances were added to crude methanol extracts of *Taxus* needles at low, medium and high levels, and the recovery was calculated by comparing the found amount of standards to those added, being 97.6 ± 1.2% for taxol, 95.6 ± 2.3% for B-III,100.4 ± 1.32% for B-VI and 89.4 ± 4.5% for DAB-I ($n = 9$), respectively. For the repeatability of HPLC analysis, the intra- and interprecisions obtained from repeatable injection of the standard mixture solution at the same concentration were the within-day relative standard deviation (RSD) of 0.85% ($n = 9$) and the between-day RSD of 2.45% (an injection was carried out every 3 days of over 15 days, $n = 5$), indicating a good precision of the quantitative method.

Acknowledgements

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