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INDUCTION, GROWTH AND PACLITAXEL CONTENT OF NEEDLE- AND PETIOLE-DERIVED CALLI IN HIMALAYAN YEW (*TAXUS WALLICHIANA* ZUCC.) UNDER LIGHT-EMITTING DIODES

DUONG TAN NHUT[1*], PHAN LE HA NGUYEN[1], NGUYEN TRINH DON[1], N.T.T. HIEN[1], NGUYEN PHUC HUY[1], NGUYEN BA NAM[1], BUI THE VINH[2], AND TRAN CONG LUAN[2]

[1]Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, 116 Xo Viet Nghe Tinh, Dalat city, Lam Dong Province, Vietnam,
[2]Ho Chi Minh City Research Center of Ginseng and Medicinal Materials, 41 Dinh Tien Hoang, District 1, Ho Chi Minh City, Vietnam

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To achieve a better understanding of accumulation of paclitaxel and related taxanes in Himalayan yew (*Taxus wallichiana* Zucc.) callus mass, induction and growth of calli from needles and petioles under light-emitting diodes (LEDs) and production of paclitaxel and baccatin were investigated. Calli were induced from needles and petioles on Gamborg's B5 medium supplemented with 1 $mg \cdot l^{-1}$ kinetin and 4 $mg \cdot l^{-1}$ 2,4-D under different combinations of blue and red LEDs. Calli were further cultured in that medium under the same lighting conditions. Callus induction from needles and petioles and growth was better under blue LEDs than under red LEDs and fluorescent light. The combinations of blue and red LEDs produced variable results. The paclitaxel content of these calli was quantified by high-performance liquid chromatography. The paclitaxel content of calli derived from needles was 0.00628% and the 10-DAB (10-Deacetylbaccatin III) content was 0.00366%. The paclitaxel content of calli derived from petioles was 0.00412%, and no 10-DAB was detected.

Key words: 10-Deacetylbaccatin III, callus, high-performance liquid chromatography, light-emitting diodes, paclitaxel, *Taxus wallichiana* Zucc.

INTRODUCTION

The structurally complex polyoxygenated diterpenoid alkaloid paclitaxel, widely known under the brand name Taxol, has been well studied for its bioactivity of stopping cell division by stabilizing microtubules (Bestoso et al., 2006) and triggering apoptosis (Rodi et al., 1999). Paclitaxel is commonly used as a potential component in treatments for various cancers (e.g. breast, ovarian, lung) and AIDS (Ojima et al., 2002). Conventionally, paclitaxel production is based mainly on extraction from bark and needles of Pacific yew (*Taxus brevifolia*), and is very limited due to slow growth of *Taxus*. The complex chemical structure of paclitaxel hinders economically feasible production of via chemical synthesis, despite several efforts to synthesize paclitaxel from its precursors such as baccatin III and 10-deacetyl baccatin III (10-DAB) (Nicolaou et al., 1994; Holton et al., 1994a,b). Alternatively, recent research has focused on production of paclitaxel via plant tissue culture of *Taxus* species, including *T. cuspidata* and *T. media* (Fett-Neto et al., 1992, 1994a,b; Wickremesinhe et al., 1993; Tachibana et al., 1994; Ketchum et al., 1995; Wang et al., 1997; Son et al., 2000; Furmanowa et al., 2000; Zhang and Xu, 2001) and fermentation of *Taxus* endophytic fungi (Stierle et al., 1993; Strobel et al., 1996). Callus culture of *Taxus* species is a promising approach for paclitaxel production and has been well studied with the use of various plant growth regulators and medium components (Enaksha et al., 1993; Mayumi et al., 2002; Nhut et al., 2007). The influence of single-wavelength light on *Taxus* calli is yet to be determined.

Light is a critical factor affecting several growth and development processes in plants, including photosynthesis, photomorphogenesis and phototro-

*e-mail: duongtannhut@gmail.com

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pism (Taiz and Zeiger, 1991). Fluorescent light is made up of visible wavelengths from 320 to 800 nm and is the major lighting source used in plant propagation. Since this combination also includes short-wavelength light that is unfavorable for plant growth (Kim et al., 2004), novel lighting systems that use single-wavelength light such as that from LEDs have been investigated and considered for large-scale use; advantages of LEDs include their small size, extended longevity, and easy control of light spectra (Bula et al., 1991; Brown et al., 1995; Tamulaitis et al., 2005). LEDs have also been used to study the effect of light intensity and wavelength on the growth and development of several species. This work has included improvement of in vitro photoautotrophic growth of *Cymbidium* (Tanaka et al., 1998), improvement of in vitro growth of strawberry (Nhut et al., 2003), and study of morphogenesis and growth of *Lilium* bulb scales (Lian et al., 2002) under different combinations of blue and red LEDs. Other examples include studies of growth and petiole morphogenesis in *Zantedeschia* under LEDs (Jao et al., 2005), and improvement of growth and carbohydrate biosynthesis of grapes exposed to LEDs (Heo et al., 2006). Previous studies have found that light quality influences callus induction, proliferation and production in plants cultured in vitro (Morini et al., 2000; Le and Tanaka, 2004) and secondary metabolite biosynthesis (Jie et al., 2003, Kee-Won et al., 2005; Stijn et al., 2012; Gang et al., 2014). To examine the effect of single-wavelength light on Himalayan yew callus yield and biosynthesis of paclitaxel and related taxanes, in this study we investigated callus induction, growth, and the content of paclitaxel and its precursor 10-DAB in calli derived from petioles and needles under different combinations of blue and red LEDs compared to fluorescent light.

MATERIALS AND METHODS

PLANT MATERIALS, CULTURE MEDIA AND LIGHTING CONDITIONS

Calli were induced from needles and petioles of 7-year-old Himalayan yew (*Taxus wallichiana* Zucc.) grown at the Tay Nguyen Institute for Scientific Research in natural temperate climatic conditions (Da Lat city, Lam Dong Province, Vietnam). Needles and petioles were washed under running tap water for 30 min and then disinfected with 70% ethanol for 30 sec and 0.1% mercury chloride for 10 min, followed by three washes with sterile distilled water.

B5 medium (Gamborg et al., 1968) supplemented with 2,4-D at various concentrations (2.0, 3.0, 4.0, 5.0 $mg \cdot l^{-1}$) with or without 1.0 $mg \cdot l^{-1}$ kinetin (KIN) was used for callus induction. All culture media contained 8 $g \cdot l^{-1}$ agar and 20 $g \cdot l^{-1}$ sucrose, and were adjusted to pH 5.7–5.8 before autoclaving. Callus induction under LEDs was assessed (Super Bright LEDs Inc., St. Louis Missouri, USA) in different red:blue ratios (100:0, 75:25, 50:50, 25:75, 0:100), with fluorescent light as the control. Callus induction and callus growth were evaluated after eight weeks.

CULTURE CONDITION AND STATISTICAL ANALYSIS

All treatments were made in triplicates of 20 explants each. All the cultures were under a 16 h photoperiod (60 $\mu mol/m^2/s$ light intensity) at 24 ± 1°C and 55–60% relative humidity.

The data were analyzed by ANOVA and the means were compared using Duncan's multiple range test (Duncan, 1995) using SPSS ver. 16.0 at $\alpha = 0.05$.

ANALYSIS OF PACLITAXEL AND 10-DAB CONTENT IN CALLI

To determine the content of Paclitaxel and 10-DAB, 8-week-old calli were subcultured three times at 8-week intervals and then harvested. The calli were dried at 60°C in an MOV-212 Drying Oven (SANYO, Japan) to constant weight. Dried calli (1.0 g) were extracted with methanol (5 × 10 ml) under sonication at 60°C. The methanol extract was dried, resuspended in 10 ml distilled water, and shaken with *n*-hexane (5 × 5 ml). The aqueous phase was collected and extracted with dichloromethane (5 × 5 ml). The dichloromethane extract was then dried and replaced with 10 ml acetonitrile, passed through a 0.45 μl filter membrane, and 20 μl filtrate was injected into the Shimadzu 20AD HPLC system coupled with a SPD-M20A photodiode array detector (set at 228 nm for paclitaxel and 232 nm for 10-DAB) using a Supelco C18 column (250 × 4.6 mm i.d., 5 μm) at 25°C. We used an acetonitrile:water gradient solvent system at 1 ml/min flow, previously developed by Tram et al. (2008) as follows: acetonitrile at 28% for the first 16 min, increased to 40% until 17 min, held at 40% until 29 min, followed by an increase to 45% until 60 min.

Quantitative analysis was carried out with authentic paclitaxel and 10-DAB standards (Sigma-Aldrich). The yields of paclitaxel and 10-DAB (percentage of dry weight) were determined using the following formula.

$$Yield\ (\%) = \frac{C \times a}{100 \times m}$$

where C is the concentration of 10-DAB or paclitaxel quantitated based on standards ($\mu g \cdot ml^{-1}$), a is purity (0.9840 for 10-DAB and 0.9835 for paclitaxel), and m is the sample's dry weight (mg).

RESULTS AND DISCUSSION

EFFECT OF PLANT GROWTH REGULATORS ON CALLUS INDUCTION

Many media have been used in callus culture of *Taxus* species, such as MS, B5, SH, Harvey, and Anderson and White (Bringi et al., 1993; Zhu et al., 1991; Gan and Zheng, 1994). B5 medium was found to be the most effective medium among them, and was also used as the induction medium in this study.

Callus fresh weight on media with different concentrations of 2,4-D and KIN was evaluated at the sixth week after setting aseptic culture (Fig. 1). Callus derived from needles reached the highest fresh weight on media containing 2,4-D at 3.0 and 4.0 mg·l^{-1} in combination with 1.0 mg l^{-1} KIN (64 and 68 mg respectively). However, on media containing 3.0 mg·l^{-1} 2,4-D in combination with 1.0 mg l^{-1} KIN, blackening of callus was observed due to the release of phenolic compounds (data not shown).

Medium containing 4.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} KIN was shown to be suitable for callus induction of *Taxus wallichiana* Zucc.; the fresh weight of callus derived from petioles was highest (460 mg).

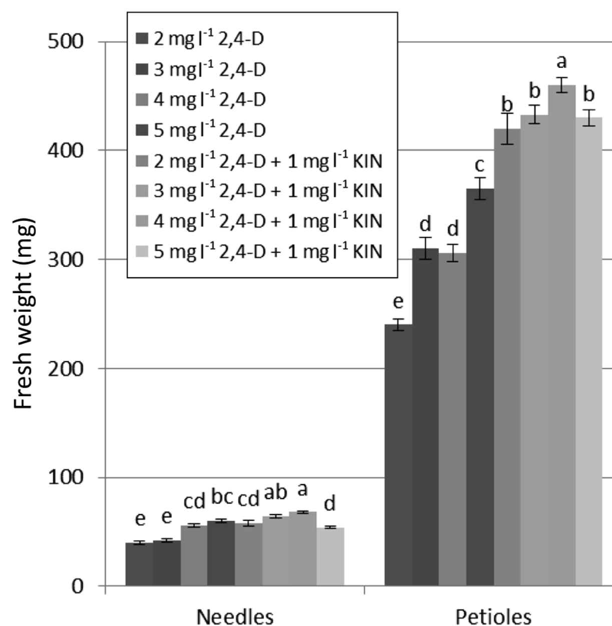

Fig. 1. Himalayan yew callus induction on B5 media supplemented with various concentrations of 2,4-D with and without KIN after 6 weeks of culture.

EFFECT OF LIGHTING CONDITIONS ON CALLUS INDUCTION AND GROWTH

The best callus induction (Fig. 3) and growth (Fig. 4) were recorded under 100% blue LEDs.

After eight weeks on callus induction medium, callus formation on needles was induced under all lighting conditions examined. Callus induction under blue LEDs differed significantly from that under red LEDs or fluorescent light. The fresh weight of callus derived from needles under blue LEDs (Fig. 2a) was 2.1-fold higher than under red and 3.1-fold higher than under fluorescent (Fig. 3). Soft brown and green calli were obtained under blue LEDs. Only brown calli were observed under fluorescent light and under combined red and blue LEDs. However, greener calli were recorded under red LEDs. After eight weeks on callus growth medium the fresh weight of needle-derived calli under blue LEDs was 1.5-fold higher than under red LEDs and 1.3-fold higher than under fluorescent light (Fig. 4). The 75:25 blue:red LED combination also gave a relatively high amount of callus: 852±57.19 mg on growth media, higher than in the other lighting treatments except for 100% blue LEDs.

The results were similar for callus derived from petioles (Fig. 2b): 100% blue LEDs were the optimal lighting condition for callus induction and growth, with fresh weight of callus at 580±24.70 and 1900±38.60 mg respectively (Figs. 3, 4). However, calli derived from petioles were soft and transparent white or pale yellow under blue and fluorescent

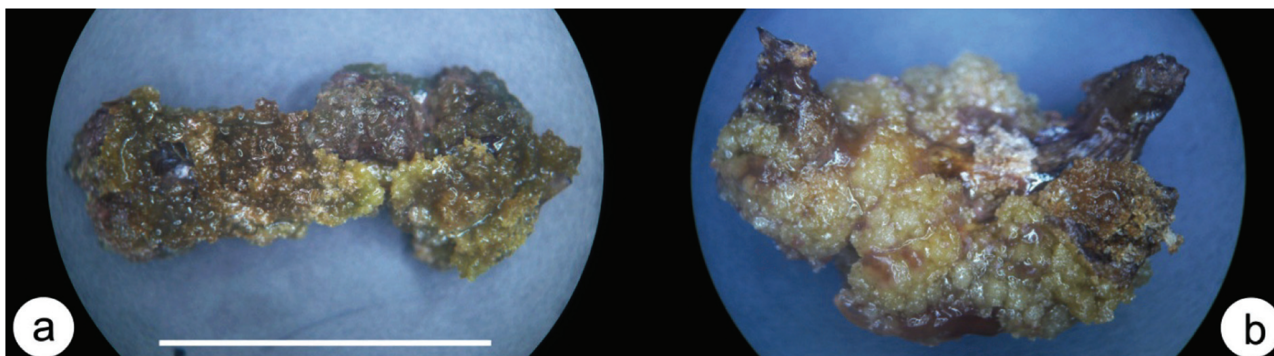

Fig. 2. Himalayan yew callus induction under blue LEDs. (**a**) Needle, (**b**) Petiole. Bar = 1 cm.

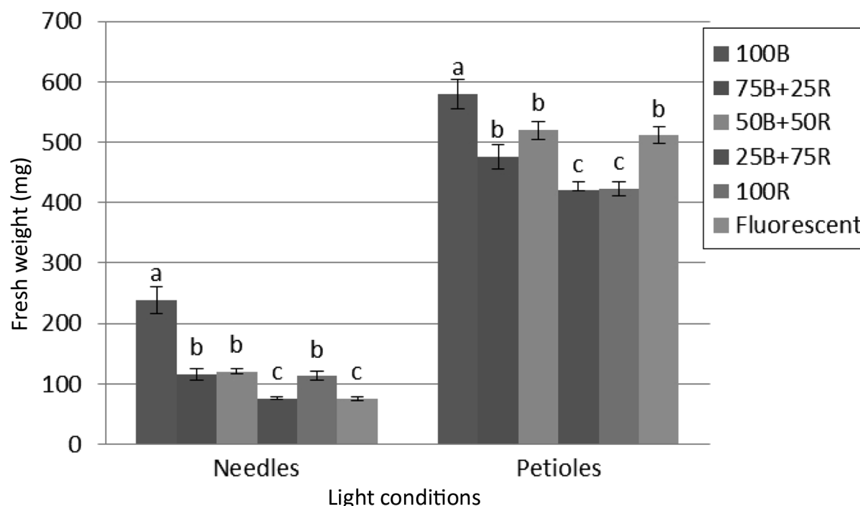

Fig. 3. Himalayan yew callus induction from needles and petioles of Himalayan yew under different lighting conditions after 8 weeks of culture.

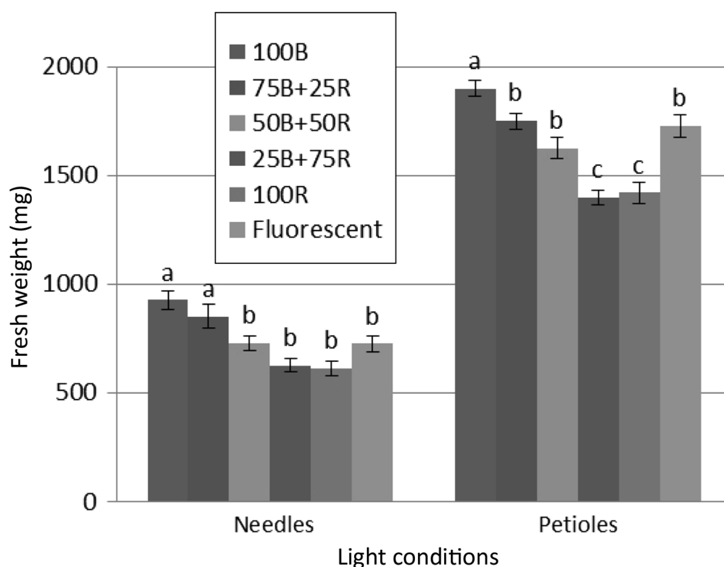

Fig. 4. Growth of Himalayan yew callus under different lighting conditions after 8 weeks of culture.

light. Interestingly, white friable calli were obtained when calli derived from petioles were cultured under combinations of red and blue LEDs.

The effect of blue light on plant growth and development is mediated by the phytochrome system and/or certain blue light receptors (Rajapakse et al., 1999), and it plays a critical role in the formation of chlorophyll during chloroplast development (Akoyunoglou and Anni, 1984), explaining the greener color of calli under blue LEDs. Slower callus growth under red LEDs might be due to unbalanced distribution of energy in photosystems I and II (Tennessen et al., 2004). These data further support the long-recommended use of LEDs for plant tissue culture and improved plant growth (McCree, 1972).

ANALYSIS OF PACLITAXEL AND 10-DAB IN THE CALLI

Long-term maintenance of calli and successful production of paclitaxel from them have been studied in *T. baccata, T. brevifolia, T. cuspidate*, and *T.* × *media* (Wickremesinhe and Arteca, 1993). In this study we investigated production of paclitaxel and its precursor 10-DAB in cultures of *T. wallichiana*. Enaksha et al. (1993) demonstrated that *Taxus* spp. callus could be maintained only 7–8 weeks without subculture. In the present study we harvested,

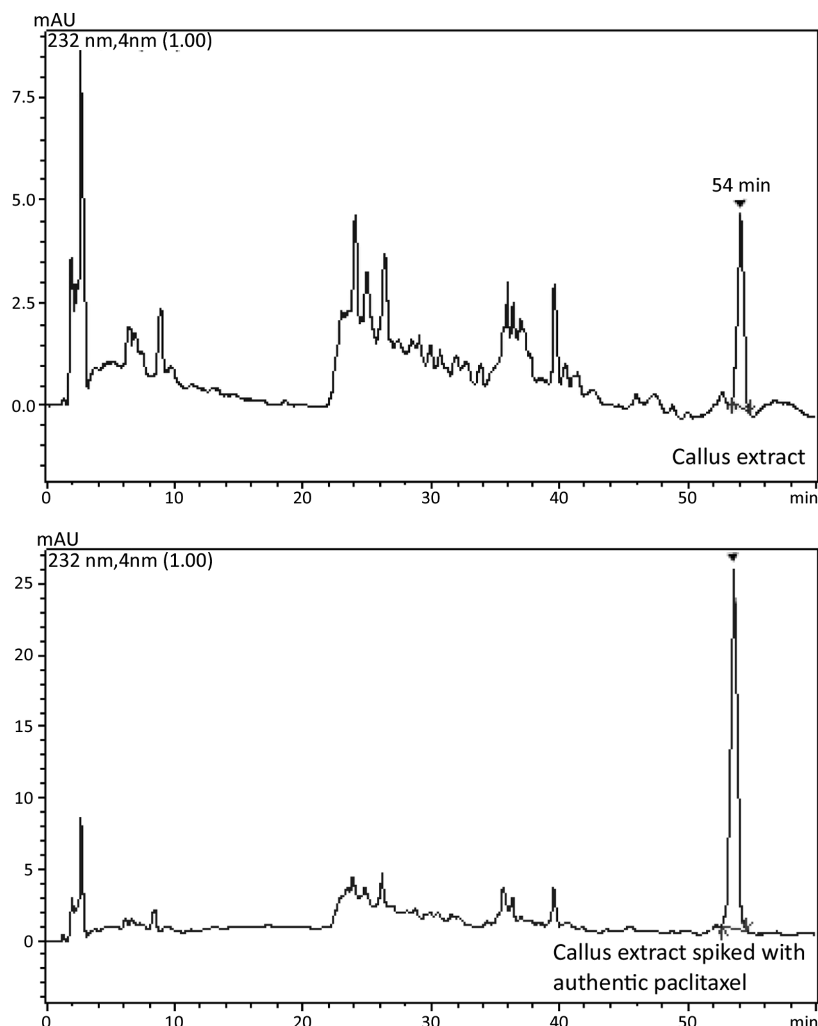

Fig. 5. HPLC analysis of metabolites extracted from Himalayan yew (*T. wallichiana*) calli (from petioles). Triangles indicate peaks of paclitaxel.

dried and extracted 8-week-old calli after three subcultures at 8-week intervals. HPLC analysis of dried calli revealed a peak at 54 min, the same retention time as for the authentic paclitaxel standard (Fig. 5). When paclitaxel was spiked into the test sample only a single peak was observed at 54 min, indicating the presence of paclitaxel in the test sample.

Quantitative analysis showed 0.00412% paclitaxel in petiole-derived and 0.00628% paclitaxel in needle-derived dried Himalayan yew calli (Figs. 5, 6, 7). These yields are comparable to the paclitaxel yield extracted from the bark of *T. brevifolia* (0.001–0.006% in September) (Wheeler et al., 1992) and considerably higher than those from calli of *T. media* and *T. baccata* (0.00017–0.00142%) (Wickremesinhe et al., 1993) but slightly lower than the content from the bark of *T. cuspidata* (0.011–0.031%) (Fett-Neto and Dicosmo, 1992).

The 10-DAB content of calli derived from needles, revealed as a peak at 12 min, was 0.00366% (Fig. 7). Interestingly, no 10-DAB was detected in Himalayan yew calli derived from petioles (Fig. 7). As 10-DAB is an intermediate in the biosynthetic pathway of paclitaxel (Walker and Croteau, 2000), nondetection of 10-DAB suggests that almost all of the 10-DAB was efficiently directed to paclitaxel formation in in vitro-grown Himalayan yew calli, and our system could be further optimized to improve extraction of relatively pure paclitaxel without intermediates.

CONCLUSION

Our results support the use of light-emitting diodes in callus culture of *Taxus wallichiana* Zucc. A simple and effective procedure using B5 media supple-

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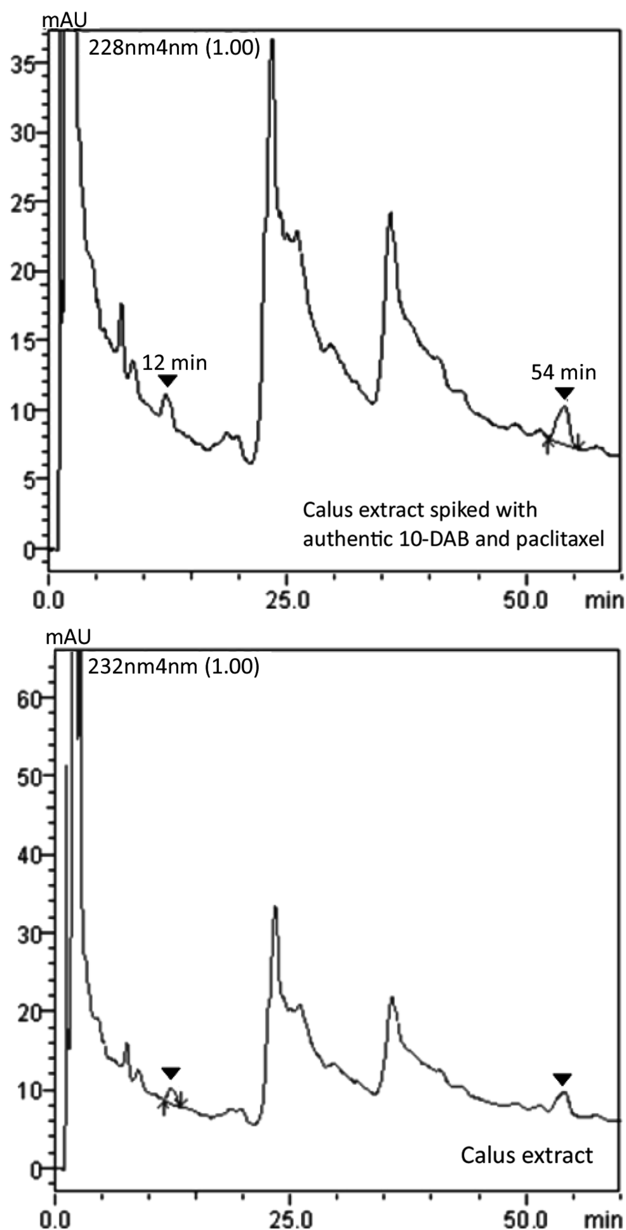

Fig. 6. HPLC analysis of metabolites extracted from Himalayan yew (*T. wallichiana*) calli (from needles). Triangles indicate peaks of 10-DAB at 12 minutes and paclitaxel at 54 minutes.

mented with 4 mg·l^{-1} 2,4-D and 1 mg·l^{-1} KIN for callus induction and growth under blue LEDs was developed.

The HPLC results showed differences in paclitaxel and 10-DAB content between calli derived from needles and petioles. Calli derived from petioles showed higher induction and growth rates than those derived from needles but produced less paclitaxel and 10-DAB.

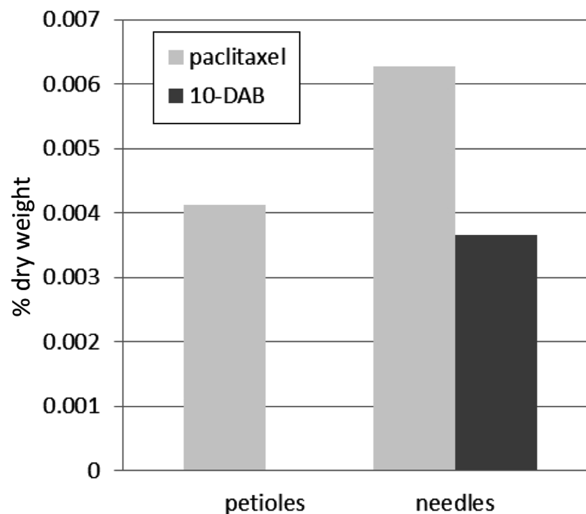

Fig. 7. Content of paclitaxel and 10-DAB in needle-derived and petiole-derived calli of Himalayan yew.

AUTHORS' CONTRIBUTIONS

DTN and TCL formulated the original idea and supervised the research; PLHN and BTV designed and performed the experiments and wrote the manuscript; NTD, NPH, NTTH and NBN critically read and revised the manuscript.

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