Acta Societatis Botanicorum Poloniae

ORIGINAL RESEARCH PAPER Acta Soc Bot Pol 84(1):125–132 DOI: 10.5586/asbp.2014.038
Received: 2014-04-07 Accepted: 2014-11-17 Published electronically: 2015-01-13

Identification and quantitative determination of pinoresinol in *Taxus* ×*media* Rehder needles, cell suspension and shoot cultures

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Abstract

The aim of our study was to investigate the presence and quantitative contents of lignans in the tissues of *Taxus* ×*media*. The presence of the lignans: pinoresinol, matairesinol and secoisolariciresinol was assessed in needles, shoots cultures and suspension culture. Pinoresinol was the only lignan found in the tissue of *T.* ×*media*. The total pinoresinol content in the needles and in the shoots was 1.24 mg/g dry weight (dw) and 0.69 mg/g dw, respectively. Most of the pinoresinol identified was appeared glycosidically bound. In needles, the amount of glycosidically bound pinoresinol (0.81 mg/g dw) was about twice as high as that of free pinoresinol (0.43 mg/g dw). The content of free and glycosidically bound pinoresinol showed the level of 0.18 mg/g dw and 0.51 mg/g dw, respectively in the in vitro shoot cultures. In the cell culture, no pinoresinol was found.

Keywords: pinoresinol; *Taxus* ×*media*; shoot culture; suspension culture

Introduction

The species of *Taxus* genus are noted due to their anticancer properties of taxane diterpenoids, mainly paclitaxel [1,2]. Nowadays, there is a growing interest in other groups of secondary metabolites found in *Taxus* species, including lignans with a considerable pharmacological and hence clinical potential. Lignans are a class of phenylpropanoids that present a large structural and biological variety as they have antitumor, antiviral, hepatprotective, antioxidant, antiallergic and antiosteoporotic properties [3]. They are generally detected in vascular plants from different families [4]. Their primary physiological role in plants is defensive, particularly in heartwood and seed-forming tissues [5]. Important developments have been reached in elucidating the biosynthesis and chemistry of lignans [4–6]. Lignans are formed of two units of hydroxycinnamoyl alcohol, mostly coniferyl alcohol, via C8-C8′ linkage. The connection of coniferyl alcohol forms pinoresinol. The twice reduction of the product leads to the formation of secoisolariciresinol. Next, the lactone ring is closed to provide matairesinol, which can be the initial point for all lactone ring lignan paths [7–11] (Fig. 1). Topcu and Demirkiran [12] published a review of isolation and structural clarification studies of *Taxus* lignans, with their biological properties. About 50 lignans have been found in eight *Taxus* species. The most common lignans of *Taxus* species are α-coniferin, taxiresinol, secoisolariciresinol, isolariciresinol and lariciresinol.

The highest lignan diversity was observed in *T. mairei*, with over 35 lignans described in this species [13–15], followed by *T. baccata*, with 18 lignans identified [16–22]. The most studies of the lignans, inclusive *Taxus* species, have concentrated on the bioactivities, to ensure the future lead drugs. The results of the studies imply the use of isotaxiresinol in postmenopausal osteoporosis treatment, mainly in the prevention of estrogen deficiency induced bone fracture [23].

Cytotoxic activity analyzes conducted on α-conidendrin, secoisolariciresinol, isotaxiresinol and taxiresinol, proved efficient cytotoxicity against KB-16, A-549 and HT-29 tumor cell lines [24].

Secoisolariciresinol, taxiresinol and isotaxiresinol, the main compounds obtained from the wood of *T. yunnanensis* tree, were assessed for the antiproliferative properties against human fibrosarcoma cell lines and murine colon carcinoma [25]. Taxiresinol, isolated from *T. wallichiana*, showed noteworthy in vitro anticancer action against colon, ovarian, liver and breast cancer [26]. Matairesinol and pinoresinol were proved to have antileukemia activity and the ability to inhibit cAMP [27], as well as they were found to be an anti-HIV agents [28].

The aim of our study was to verify the presence and quantitative contents of lignans in the tissues of the *T.* ×*media* tree with the help of already developed by Schmitt and Petersen [29] method.

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Handling Editor: Jan Rybczyński

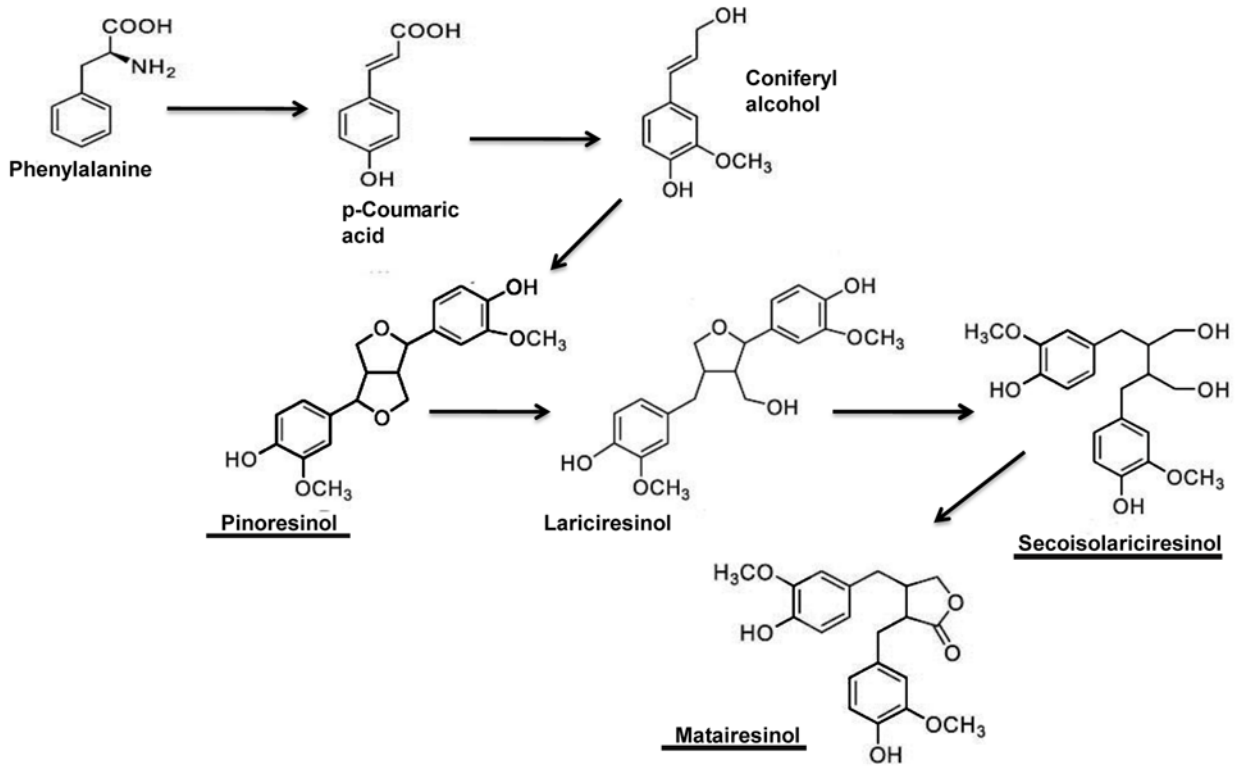

Fig. 1 Biosynthetic pathway of pinoresinol, secoisolariciresinol and matairesinol.

Material and methods

Plant material

The experimental plant material originated from *T. ×media* plant growing in The Botanical Garden of Warsaw University, Warsaw, Poland. Needles for lignans determination and establishment of cell suspension culture were excited from the plant. Shoot cultures were established from shoot of this plant. The initial plant material both needles and shoots were harvested in May 2009.

Establishment of shoot culture

Shoots of *T. ×media* were soaked for 15 min in tap water with a detergent, then rinsed several times with distilled water, incubated for 15 min in 70° EtOH, followed by 30 min in 2.5% chlorine disinfectant solution. After repeated rinsing of the plant material in sterile water, the shoots were cut into 2 cm long pieces and placed onto 30 ml of solid Gupta and Durzan (DCR) medium [30] and woody plant medium/Gamborg medium (WPM/B5) medium in 100 ml conical flasks. WPM/B5 medium is the combination of mineral salts of woody plant medium (WPM) [31] and vitamins of B5 medium [32]. WPM/B5 medium was supplemented with 1 mg/l NAA (1-naphthaleneacetic acid) and 0.1 mg/l BA (6-benzyladenine) and 1.0 mg/l active charcoal. DCR medium was supplemented with 0.8 mg/l NAA and 1.0 mg/l BA. Both media contained 30 g/l sucrose. The shoot culture was maintained at 25°C in the day-light (50 μM $m^{-2}s^{-1}$ and 14 h photoperiod) and subcultured every four weeks on the same fresh media (Tab. 1).

Establishment of cell suspension culture

The needles were cut along the main nerve and placed onto solid Rangaswany medium (WR) [33] in 100 ml conical flasks for callus initiation. WR medium was supplemented with 80 mg/l KNO_3, 500 mg/l casamino acids, 30 g/l sucrose and 5 μM picloram. The callus culture was maintained at 25°C in the day-light light intensity. After three months of culture 2 g of developed callus excised and transferred into 250 ml conical flasks containing 50 ml WPM /B5 liquid medium supplemented with 500 mg/l casamino acids, 10 mg/l glycine-betaine (GB–Green Steem®, Finnsugar

Tab. 1 Growth parameters of *Taxus ×media* shoot cultures on DCR (0.8 mg/l NAA and 1.0 mg/l BA) and WPM/B5 (1 mg/l NAA, 0.1 mg/l BA and 1.0 mg/l active charcoal) solid media*.

Medium	Contamination (%)		Necrosis (%)		Survival (%)	Shooting (%)**	
DCR	1 month	15	1 month	7	73	2 months	32
	3 months	5	3 months	-		3 months	58
	Total	20	Total	7		4 months	79
WPM/B5	1 month	13	1 month	9	74	2 months	25
	3 months	5	3 months	-		3 months	55
	Total	18	Total	9		4 months	69

* The experiment was performed for 120 shoot explants in 120 flasks on DCR solid medium as well as on WPM/B5 solid medium.
** The shooting was calculated as the percentage ratio of the number of developed side shoots to the total number of survivors shoot explants.

Bioproducts), 10 mg/l adenine sulphate, 5 μM picloram, 20 g/l sucrose and phosphoric buffer according to Tóth et al. [34]. The culture was maintained at 25°C in the continuous light (50 μM $m^{-2}s^{-1}$) on a Gyrotory Shaker (New Brunswick Scientific Co.) at 110 rpm. The established cell suspension culture consisted of 30 flasks, was subcultured every three weeks by transferring about 2 g fresh weight of 21 day old cell culture into 50 ml fresh medium in 250-ml conical flasks. Growth parameters and lignans contents of *T. ×media* cell suspension culture were determined over a 3 passages. The culture of the three conical flasks was harvested every 7 days. Fresh and dry weight were determined separately for each sample and then presented as average value (Tab. 2).

Tab. 2 Development of fresh weight (fw), dry weight (dw) of *Taxus ×media* suspension culture in WPM/B5 liquid medium supplemented with 500 mg/l casamino acids, 10 mg/l glycine-betaine, 10 mg/l adenine sulphate, 5 μM picloram.

	Passage 1		Passage 2		Passage 3	
Growth (days)	Fresh weight (g)*	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
7	2.75 ±0.30	0.42 ±0.40	2.54 ±0.24	0.45 ±0.61	2.32 ±0.17	0.47 ±0.18
14	4.28 ±0.22	0.85 ±0.42	5.00 ±0.28	0.76 ±0.24	4.99 ±0.78	0.67 ±0.15
21	4.00 ±0.56	0.55 ±0.20	4.04 ±0.23	0.58 ±0.19	3.90 ±0.52	0.53 ±0.11

* Values are means of three samples ±*SD*.

Lignan extraction and determination

For free and glycosidically bound lignans Schmitt and Petersen [29] method was used. Needles harvested in May 2009, side shoots from 4 months old in vitro shoot culture on both solid media (Tab. 1) and biomass from cell suspension culture (Tab. 2) were lyophilized and ground in a mortar. A portion of 0.1 g plant material was suspended in 1 ml MeOH and twice extracted in a ultrasonic bath for 30 s with indirect cooling on ice. After adding of 4 ml H_2O (adjusted to pH 5 with 1M H_3PO_4), the obtained extract was mixed carefully for 30 min with 5 ml CH_2Cl_2. The organic phase, containing lignan aglyca, was removed, evaporated to dryness and resuspended 3 times in 250 μl MeOH. After evaporation of the solvent, the residue was redissolved in 150 μl 40% MeOH for HPLC analysis. The remaining H_2O phase, containing lignan glucosides, was incubated with 0.5 mg β-glucosidase (1000 U mg^{-1}, Roth, Karlsruhe, Germany) per 5 ml for 3.5 h at 35°C and then again extracted with 5 ml CH_2Cl_2 as described above. By this procedure, lignan glucosides are hydrolyzed and then extracted as aglyca. Samples of all H_2O and CH_2Cl_2 phases were analyzed by HPLC [29]. We also extended the duration of plant material MeOH-extraction up to 24 h according to Theodoridis et al. [35] method used for paclitaxel determination. A portion of 0.1 g freeze-dried, powdered material was suspended in 1 ml MeOH, mixed, sonicated for 15 min, and next centrifuged on the Gyrotory Shaker at 110 rpm for 24 h. The plant sample was centrifuged at 5°C, 15 500 rpm for 15 min, then the methanolic extract was collected and the residue was again extracted with 1 ml MeOH, mixed, sonicated for 15 min and centrifuged as above. A second methanolic extract was collected and combined with the first one. After evaporation of the solvent, the residue was redissolved in 1 ml MeOH and treated according to Schmitt and Petersen [29] method.

HPLC-DAD analysis of pinoresinol

HPLC analysis was performed on the Shimadzu system consisting of LC-10A pump, UV ASD-10A detector, CBM-20A integrator and LC Solution program. The reversed phase, a Nova-Pak Phenyl 3.9 × 150 mm C18 column (Waters) was used with the following gradient program: 0 min 0% B, 5 min 35% B, 25 min 70% B, 40 min 100% B at a flow rate of 1 ml min^{-1} (solvent A: H_2O plus 0.01% H_3PO_4 and solvent B: 50% acetonitrile plus water). Eluted substances were monitored at 280 nm. The peak areas and retention times were compared to authentic pinoresinol, matairesinol (PhytoLab), and secoisolariciresinol (ChromaDex) standards for identification and quantification. To check the identity and purity of lignans from *T. ×media* tissues, the samples were subjected to HPLC analysis with diode array detection on the DIONEX system consisting of P580 pump, UVD 340S detector, automated sample injector ASI-100 and Dionex Data System – Chromeleon Version 6.1. For this purpose, the same column, analytical parameters and solvents were used as above. The content of pinoresinol aglyca and pinoresinol glucosides were calculated from three samples of the plant material with every sample measurement performed in triplicate and presented as mean values.

Results

Shoot and suspension culture

To determine and compare lignans content in shoot cultures WPM/B5 and DCR solid media were chosen. Both WPM/B5 and DCR media affected shoot survival and growth (data not shown; Fig. 2a). We analyzed the shooting percentage of explants derived from the yew tree on WPM/B5 and DCR media over a culture of period of 4 months. After three months of culture up to 80% of explants on both solid media did not show any signs of contamination. The necrosis of explants during the first month of culture was mainly caused by damage during surface disinfection (Tab. 1). The suspension culture, obtained from callus on WR medium (Fig. 2b) and maintained in WPM/B5 liquid medium, which consisted of green cells aggregates, was growing fast and required the subculture every 3 weeks (Fig. 2c). The development of the biomass of the suspension culture is shown in Tab. 2 for three consecutive passages.

Phytochemical analysis

Pinoresinol was the only lignan present in the extracts of *T. ×media* needles as well as in shoot cultures on WPM/B5 and DCR medium. No lignans were detected in the suspension culture. The retention time (Rt) for the pinoresinol standard was 10.7 min (Fig. 3a). The UV spectrum of the pinoresinol standard is presented in Fig. 3b. Sample chromatogram of needles is shown in Fig. 4a before hydrolysis and in Fig. 4b after β-glucosidase hydrolysis. Based on the chromatogram of secoisolariciresinol and matairesinol

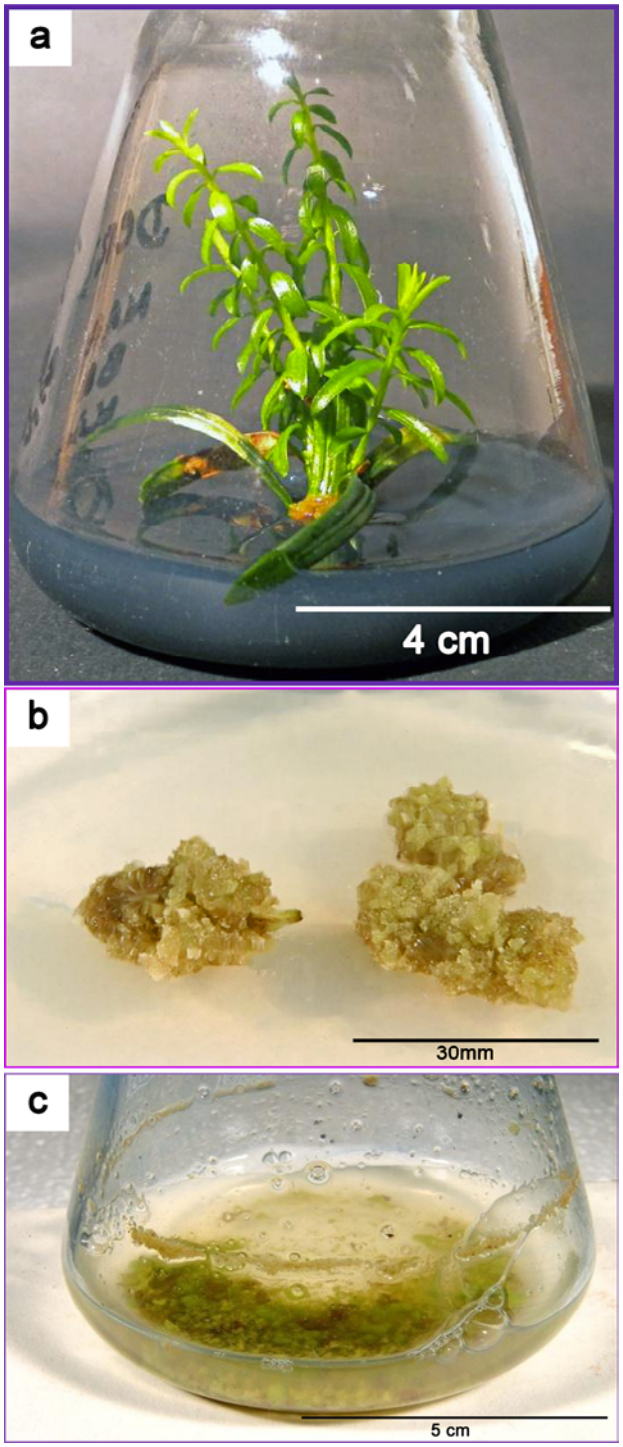

Fig. 2 **a** *Taxus ×media* shoot culture on WPM/B5 solid medium supplemented with 1 mg/l NAA, 0.1 mg/l BA and 1.0 mg/l active charcoal after 3 months of cultivation. **b** The callus culture of *T. ×media* on WR solid medium supplemented with 80 mg/l KNO_3, 500 mg/l casamino acids, 30 g/l sucrose and 5 μM picloram after 2 months of cultivation. **c** Cell suspension culture of *T. ×media* in WPM/B5 liquid medium supplemented with 500 mg/l casamino acids, 10 mg/l glycine-betaine, 10 mg/l adenine sulphate, 5 μM picloram after 3 weeks of cultivation.

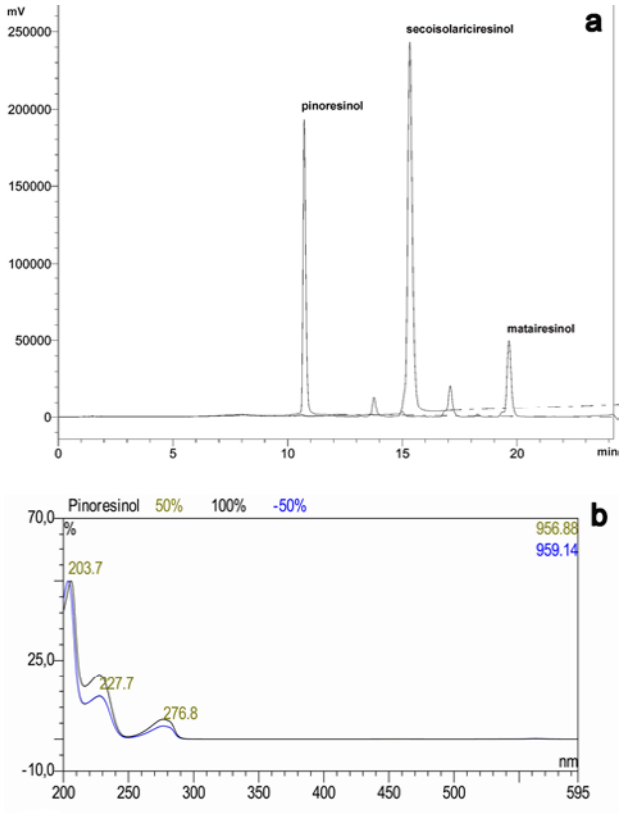

Fig. 3 **a** Chromatogram of the pinoresinol, secoisolariciresinol and matairesinol standards. **b** UV spectrum of the pinoresinol standard.

standards (Fig. 3a), we did not find any of these lignans in the analyzed samples. The highest total content of pinoresinol was obtained from the needles 1.24 mg/g dry weight (dw) and was about twice as high as that that found in the shoot culture on WPM/B5 and DCR media. Most of the pinoresinol found was glycosidically bound and recovered after enzymatic hydrolysis with β-glucosidase. The amount of glycosidically bound pinoresinol was about twice as high as that of free pinoresinol in needles and about four times higher than in shoots (Tab. 3).

Discussion

The most studies of the lignans, along with *Taxus* species, have concentrated on the bioactivities, to provide the future lead drugs. Based on the effects of numerous biological research, it was established that pinoresinol is the potential protecting agent of human health. The carried out investigations indicated its analgesic and local anesthetic properties [36]. Pinoresinol is an effective inhibitor of cAMP [37] and also showed selective inhibitory activity against NF-kB mediated transcription of HIV-1 [38]. The inhibitory ability of the inflammatory responses in lipopolysaccharide (LPS)-activated microglia, attenuation mRNA and protein levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) as well as proinflammatory cytokines in LPS-activation suggest that pinoresinol could be potentially useful in modulation of inflammatory status in brain disorders [39]. Pinoresinol was also recognized as a

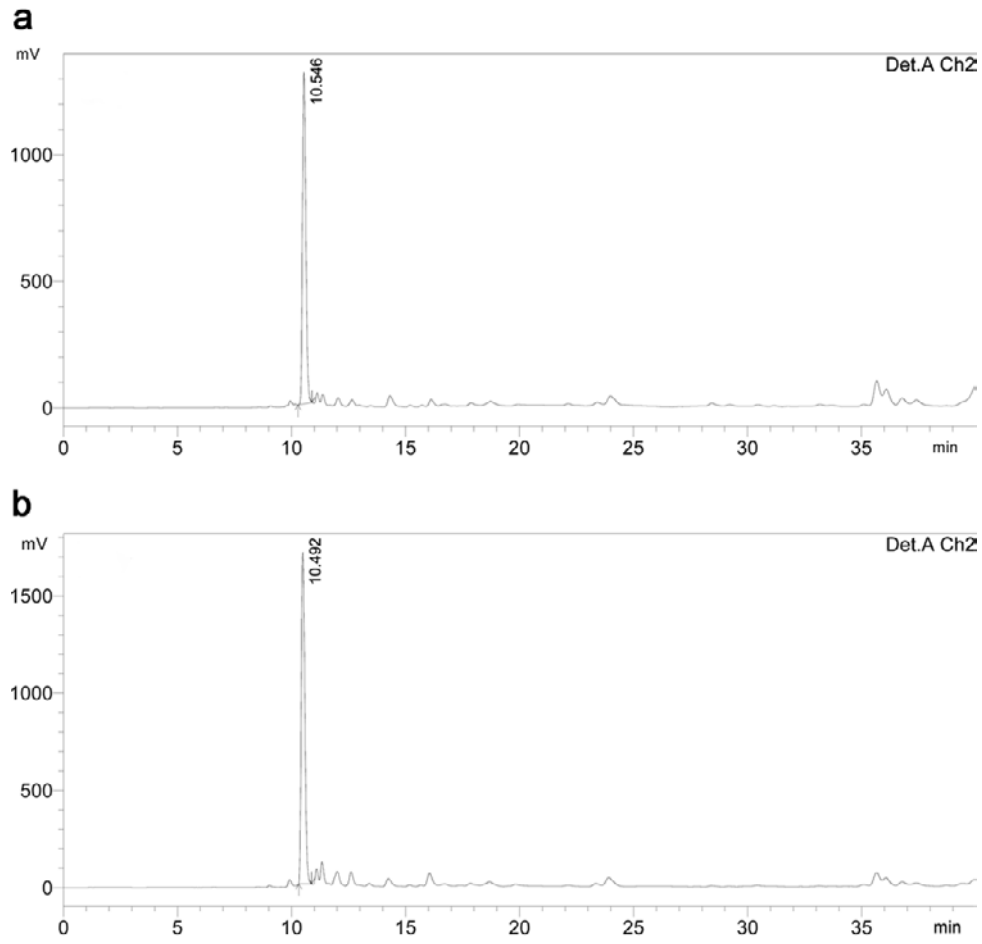

Fig. 4 Chromatogram of sample of *Taxus ×media* needles extract before (**a**) and after (**b**) enzymatic hydrolysis.

Tab. 3 Pinoresinol content in *Taxus ×media* needles and side shoots after 4 months of the in vitro cultivation.

Plant material	Schmitt and Petersen [29] method of extraction	Free pinoresinol (mg/g dw)	Glycosidically bound Pinoresinol (mg/g dw)	Total pinoresinol content (mg/g dw)
Needles	Original	0.34 ±0.03*	0.65 ±0.01	0.99 ±0.01
	With extended MeOH extraction	0.43 ±0.06	0.81 ±0.05	1.24 ±0.01
Shoots on WPM/B5	Original	0.14 ±0.03	0.43 ±0.07	0.57 ±0.05
	With extended MeOH extraction	0.18 ±0.05	0.51 ±0.01	0.69 ±0.01
Shoots on DCR	Original	0.11 ±0.05	0.40 ±0.04	0.51 ±0.05
	With extended MeOH extraction	0.15 ±0.05	0.53 ±0.02	0.67 ±0.06

* Values are means of three samples ±*SD*. Variations among accumulation of free and glycosidically bound pinoresinol between original and extended MeOH extractions and between media used for shoot culture (Tukey–Kramer post-hoc test.) do not differ statistically at $P < 0.05$.

putative hypoglycemic agent that inhibited intestinal maltase. The investigations proved that an assorted-type inhibition mechanism showed pinoresinol as an agent responsible for delaying enzyme work by straight binding to maltase and interfering maltase-moltose intermediate [40]. Due to the maltase inhibitory activity of pinoresinol by noncompetitive way, it would demonstrate synergistic impact with antidibetic drugs such as acarbose in extinguishing level of blood

glucose, consequently intensely reducing the applying dose of acarbose [41]. Furthermore pinoresinol is metabolized in human digestive tract to enterolactone and enterodiol, the principal lignans related with decreased risk of peculiar cancers and cardiovascular diseases [42]. The studies carried out on pinoresinol-rich extra virgin olive oil exhibited its ability to decrease the cell viability, induces cell cycle arrest and apoptosis by specifically upregulation the ATM-p53 cascade. It indicates that pinoresinol-rich extra virgin olive oil might be an effective agent in the chemoprevention colorectal cancer cells [43].

Glycosidically bound pinoresinol: pinoresinol 4′-O-β-D-glucopyranoside and pinoresinol di-O-β-D-glucopyranoside, has been shown to possess anti-hypertensive effects, could increase luciferase activity in both estrogen receptor (ER) ERβ and ERα, they were equally potent in eliciting transactivation through the two ER subtypes [44]. Blood vessels express ERs, and ERβ plays an essential role in the regulation of vascular function and blood pressure [45]. Activating ERs reinforces their anti-hypertensive effect.

The method developed by Schmitt and Petersen [29] for the quantitative estimation of free and glycosidically bound pinoresinol, matairesinol and secoisolariciresinol in *Forsythia ×intermedia* cell suspension culture appeared effective in our experiments concerning preparation of extracts of *T. ×media* tissues. Using the Schmitt and Petersen [29] method, pinoresinol was the only lignan found and quantified in needles of *T. ×media* tree as well as in shoots growing in vitro. *Taxus ×media* suspension culture did not contain any of the lignans in question. After extended duration of methanolic extraction according to Theodoridis et al. [35] method used for paclitaxel determination, the pinoresinol content was about 20 percent higher comparing Schmitt and Petersen [29] method employed (Tab. 1).

So far, the presence of pinoresinol has been determined solely in *T. mairei* [15] and *T. cuspidata* [46]. Pinoresinol in wood, twigs (0.02 mg/g dw) and roots (0.01 mg/g dw) was presented. The needles did not contain the lignan in question [46].

Our studies indicated that examined *T. ×media* is a rich sourse of pinoresinol, the amount determined in needles (1.24 mg/g dw) and shoot culture (0.69 mg/g dw) is comparable to that of pinoresinol isolated from knots of *Abies alba* (Pinaceae) which ranged from 0.36 mg/g dw to 1.0 mg/g dw [47,48].

In the flaxseed (*Linum usitatissimum* L.), the richest known source of precursors of phytoestrogens (enterolactone and enterodiol), the content of pinoresinol was 0.03 mg/g dw [49]. Only a few of a large variety of plant lignans are converted into the enterodiol and enterolactone by the intestinal microflora. It was initially considered that only secoisolariciresinol and matairesinol were enterolignan precursors, but later identified precursors lariciresinol and pinoresinol have a high degree of conversion [50].

The presence of lignans has been confirmed in almost all parts of the plant: bark, heartwood, needles and roots. Taking into account the large amount of lignans isolated from *Taxus* species, the explanation of great structural variety of Taxaceae family lignans is problematic.

To date, two lignans 7-hydroxymatairesinol and epinortrachelogenin have been found in *T. ×media* [51]. In *T. cuspidata* [46] with the considerable amount of pinoresinol, the presence of taxiresinol (0.37 mg/g dw) and secoisolariciresinol (9.5 mg/g dw) in the needles was also determined.

Examinations of gymnosperms and angiosperms exhibited the presence of a general lignan biosynthetic pathway where it performs as enzymes, especially pinoresinol/lariciresinol reductase [52] and secoisolariciresinol dehydrogenase [53].

The absence of secoisolariciresinol and matairesinol in the needles and shoot cultures of the *T. ×media* tree might indicate that pinoresinol/lariciresinol reductase and secoisolariciresinol dehydrogenase involved in the next steps of lignan biosynthesis, are not active in these organs. In the suspension culture, no products of biosynthetic pathway of the lignans in questions were found. The issue of lignan biosynthesis of the *T. ×media* tree requires further study.

Acknowledgments

We would like to thank Agnieszka Pietrosiuk, Phar D, PhD for her help in performing the phytochemical analysis and Ireneusz Rudnicki for photographs of the in vitro cultures.

Authors' contributions

The following declarations about authors' contributions to the research have been made: concept of the study: PM, OO; laboratory research and data analyses: PM, HCM, AKK, WJS; writing of the manuscript: PM, AKK, OO, WJS.

Competing interests

No competing interests have been declared.

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