Paclitaxel and cephalomannine in *in vitro* cultures of *Taxus cuspidata* Sieb. et Zucc. shoots and plantlets

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Summary

Micropropagation of a mature female *Taxus cuspidata* tree with paclitaxel and cephalomannine content in the needles of 457.6 μg g^{-1} DW and 340.9 μg g^{-1} DW, respectively, was elaborated using shoot tips as explants. A new yew clone derived from a seed of the mother plant was also obtained. Single shoots developed from shoot tips on WP mineral basal medium with B5 vitamins (WP-B5) supplemented with 20 mg l^{-1} BAP and 5 mg l^{-1} activated charcoal were propagated by 1 cm long segments cultured on hormone-free medium. When WP-B5 medium with 0.3 mg/l 2-iP and 0,1 mg/l IAA was used 92% of shoots rooted within three months. Paclitaxel content in shoots growing on solid medium and in a mist trickling bioreactor was 45.6–86.5 μg g^{-1} DW. Plantlets from *in vitro* culture, grown in pots contained 99.0–213.1 μg g^{-1} paclitaxel and 558.0 μg g^{-1} DW cephalomannine.

Key words: *Taxus cuspidata, yew, micropropagation, rooting, shoot culture, mist trickling bioreactor, paclitaxel, cephalomannine*

INTRODUCTION

The diterpenoid paclitaxel originally isolated from *Taxus brevifolia* bark (0.01% dry weight, DW) is a well known antitumor agent used in the treatment of various cancers. The sources of palitaxel are very limited. Nowadays, paclitaxel is produced semisynthetically from 10-deacetylbaccatin III isolated from the needles of different *Taxus* species. Tissue cultures of yew seem to be another promising

source of paclitaxel and other taxanes [1]. Cephalomannine is a natural congener of paclitaxel and it can be used for semisynthesis of paclitaxel and novel taxane derivatives with antitumor activity [2]. Taxane content of yew trees varies, depending on a number of factors such as the population, tissue type, and season of collection, but the genetic factor seems by far the most important [3-5]. Development of efficient *in vitro* methods to propagate selected genotypes with high taxane content would be a source of valuable crops. Several methods of *in vitro* propagation of some *Taxus* species have been reported, however, significant variation in the *in vitro* culture response among different yew genotypes was found [4, 6, 7].

Zygotic embryos at various stages of development were the most frequently used explants to start the micropropagation procedure of some *Taxus* species. Plant regeneration occurred *via* direct and indirect organogenesis from zygotic embryos of yew [7].

Majada et al. [4] developed a high-yield procedure for the *in vitro* propagation of juvenile material of *Taxus baccata* involving a combination of seed handling and induction of shoot proliferation from nodal explants obtained from 2-month old plantlets raised from seedlings. This procedure allowed the fast screening of individuals for their taxane content.

Micropropagation of *Taxus mariei* was achieved using bud explants derived from approximately 1,000-year-old field grown trees by Chang et al. [6].

The objective of this study was to develop a regeneration procedure for a mature female *Taxus cuspidata* tree. Paclitaxel content in the needles of the tree collected in September 2005 was 457.6 μg g^{-1} dry weight (DW). Shoot tips were used as explants. A new *T. cuspidata* clone was also established from a zygotic embryo isolated from a seed of the mother tree. Paclitaxel and cephalomannine content in shoots growing *in vitro*, in plantlets from *in vitro* culture and in the needles of the mother plant collected at different seasons was estimated.

MATERIAL AND METHODS

Plant material and establishment of aseptic shoot culture

A female individual of *T. cuspidata* growing in the Botanical Garden of the Polish Academy of Sciences in Powsin was the source of shoot tips and seeds used for establishment of *in vitro* culture. Preliminary experiments had shown that September was the best time to start *in vitro* culture from shoot tips of this genotype. The WP-B5 medium consisting of Lloyd and McCown [8] WP basal mineral culture medium with Gamborg B5 medium [9] vitamins was the most suitable. Shoot tips 0.5 cm in length collected in 2005, 2006 and 2007 were sterilized by 15 min treatment with 70° ethanol, followed by 0.5% sodium hypochlorite for 3 min and then rinsed three times with sterile distilled water. The sterilized explants were transferred into 100 ml Erlenmeyer flasks containing WP-B5 medium supplemented

with 6-benzylaminopurine (BAP) at different concentrations (0.25–20 mg l^{-1}), sucrose 30 g l^{-1}, activated charcoal 5 g l^{-1}, Bacto agar 7g l^{-1}. The pH was adjusted to 5.6 before autoclaving at 121°C for 20 min. The explants were incubated at 25°C under 40 μmol m^{-2} s^{-1} light for 12 h a day.

Mature *T. cuspidata* seeds collected from the *T.cuspidata* tree were rinsed for 5 weeks in running tap water [10]. The seeds were sterilized as described above. The embryos were excised aseptically from the surrounding gametophyte tissue and placed on hormone-free solid WP-B5 medium. The embryos were incubated at 25°C in darkness for 2 weeks and thereafter at 25°C under 40 μmol m^{-2} s^{-1} light for 12 h a day. After 8 weeks of germination shoot tip segments of the seedlings were excised and placed on the same medium and kept under the same conditions as shoot tips from the mature tree.

Shoot culture

Shoots developed from shoot tips originated from the mature plant and from shoot tips of seedlings were cut into segments about 1 cm in length and subcultured every 6 weeks using hormone-free WP-B5 medium with 5 g l^{-1} activated charcoal.

Rooting of the shoots

For *in vitro* rooting, shoots about 5 cm in length were transferred to either half strength WP-B5 (1/2WP-B5) medium with 2.5 mg l^{-1} indole-3-butyric acid (IBA), sucrose 30 g l^{-1}, or full-strength WP-B5 medium supplemented with 0.3 mg l^{-1} 6-(dimethylallylamino)-purine (2iP) and 0.1 mg l^{-1} indole-3-acetic acid (IAA). The rooted plantlets were transferred to sterilized soil.

Bioreactor experiment

The shoots of zygotic embryo origin after the 10th subculture were used in the experiment. A mist trickling bioreactor of 5 l in volume was used [11]. It was made of glass and stainless steel and furnished with a spraying nozzle made of polypropylene. The bioreactor consisted of one vessel. The WP-B5 medium (volume 1.5 l) without BAP, supplemented with sucrose 30 g l^{-1} was situated at the bottom of the vessel. The peristaltic pump provided recirculation and dosage of the nutrient medium through a polypropylene dispersal nozzle situated 90 mm from the bottom of the vessel. The operating time of the pump was 10 s and the breaks in nutrient medium supply were 50 s. The shoots were supported on a stainless steel wire mesh (with 3 mm pore size) situated 14 cm above the nozzle. The bioreactor was inoculated with 10 shoots. Shoots grew at 25º C under 40 μmol m^{-2} s^{-1} light 12 h a day and were harvested after 5 weeks.

Analytical methods

Freeze-dried plant material was powdered and extracted with methanol according to previously reported method [12]. The sample was cleaned according to the method used by Theodoridis et al. [13]. Paclitaxel and cephalomannine were determined using the HPLC method elaborated by Theodoridis et al. [14]. Statistical analysis was performed using the StatSoft® *STATISTICA* PL programme.

RESULTS AND DISCUSSION

The WP-B5 medium, used by Majada et al. [4] for micropropagation of *T. baccata* proved the best for *in vitro* culture of the studied *T. cuspidata* tree. Among several concentrations of BAP tested in our study, shoot development from shoot tips excised from the mother plant occurred only on the medium with 20 mg l^{-1} BAP. After 6 weeks of incubation single shoots 1.5–3 cm long developed from about 100% of non contaminated explants collected in September and cultivated on the WP-B5 medium with 20 mg l^{-1} BAP. The ranges of contamination among shoot tips collected in three successive years were 5–7%. When lower BAP concentrations were used shoot tips did not grow. BAP at concentrations up to 2.5 mg l^{-1} is the cytokine most commonly used for shoot development in yew [7]. Chang et al. [15] found that increasing concentrations of BAP up to 20 mg l^{-1} induced multiple shoots from shoot tips of *T. mairei*. Proliferation of multiple shoots occurred when basal explants from 2-month old *T. baccata* plantlets were cultured on medium with 5 mg l^{-1} BAP [4]. In our studies, after initiation of shoot culture from shoot tips on the medium with 20 mg l^{-1} BAP, subsequent multiplication of shoots was achieved by cutting them into 1 cm long segments which were placed on hormone-free medium. Within six weeks of culture shoots about 5 cm in length developed from shoot segments; the mean dry weight of 10 shoots was 0.68 g and the average micropropagation rate was 5.

Only about 10% of the excised embryos derived from seeds of the *T. cuspidata* tree germinated on hormone-free WP-B5 medium and a well growing shoot culture of one independent clone was established with the micropropagation rate the same as that for shoot culture started from shoot tips of the mother plant.

As early as week 6, about 30% of the shoots rooted on ½WP-B5 medium with 2.5 mg l^{-1} IBA and 92% of shoots rooted on WP-B5 medium with 0.3 mg l^{-1} 2-i P and 0.1 mg l^{-1} IAA within three months.

In the literature there is little information concerning successful *in vitro* rooting of yew shoots. Majada et al. [4] noted that most *T. baccata* shoots showed spontaneous root formation after 6 months of culture. *T. wallichiana* microshoots rooted at 40% frequency on the modified Murashige and Skoog [16] medium after four months of culture [7].

This is the first report on shoot culture of *T. cuspidata* in a bioreactor. Within 5 weeks shoots elongated without branching, and hyperhydricity phenomena were observed. The dry weight increase was low and the dry weight of 10 shoots collected from the bioreactor was 0.82 while the dry weight of the corresponding 10 shoots used as inoculum was 0.80 g.

Paclitaxel and cephalomannine content found in different *T. cuspidata* organs is given in table 1.

Table 1.

Paclitaxel and cephalomannine in needles of female *T. cuspidata* tree and in shoots and plantlets from *in vitro* culture

plant material	paclitaxel content [μg g^{-1} DW]	cephalomannine content [μg g^{-1} DW]
mother plant needles collected in:		
September 2005	457.64±24.62*	nt**
March 2007	455.77±24.35	nt
June 2007	375.78±52.46	nt
September 2007	410.53±31.46	nt
January 2008	398.89±21.74	nt
September 2008	350.00±12.44	340.90±21.36
shoots obtained from shoot tips of the mother plant after 4th passage	58.00±14.33	nt
plantlets of the mother plant clone after 3 months of growth in pots	213.15±5.01	nt
shoots of the new clone of zygotic embryo origin after 16th passage	86.5±12.69	0
plantlets of the new clone after 3 months growth in pots	99.03±12.86	nt
plantlets obtained *in vitro* after 12 months grow in pots	184.05±13.32	558.00±8.16
shoots after 5 weeks of growth in mist trickling bioreactor	45.65±5.85	nt

*Data represent the mean value ± standard deviation from triplicate determinations.

**nt – not tested

Rozendal et al. [3] found that paclitaxel and cephalomannine content in the needles of different yew species ranged from 50 μg g^{-1} to 300 μg g^{-1} DW and from 0 μg g^{-1} to 480 μg g^{-1} DW, respectively. Paclitaxel content in needles of the female *T. cuspidata* tree tested in this study was 350.0–457.6 μg g^{-1} DW depending on the season (tab. 1). Cephalomannine content in the needles collected in September 2008 was 340.9 μg g^{-1} DW. Cephalomannine was not found in shoots growing *in vitro*. Paclitaxel content in shoots grown on solid medium (58.0–86.5 μg g^{-1} DW) was higher than that in shoots from mist trickling bioreactor (45.6 μg g^{-1} DW). Paclitaxel content increased when the shoots were rooted *in vitro* and transferred to pots. Very young plants of the maternal genotype contained 213.1 μg g^{-1} DW paclitaxel. Plantlets of the new clone developed from one seedling originated from a seed of the mother plant also contained high amounts of paclitaxel (184.0 μg g^{-1} DW) and cephalomannine (558.0 μg g^{-1} DW, see tab. 1). The new clone can be expected to give valuable plants when grown in soil.

Our studies have shown that propagation of selected valuable mature yew plants from existing meristems could be useful for mass production of plants as an alternative source of paclitaxel.

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PAKLITAKSEL I CEFALOMANNINA W KULTURACH *IN VITRO* PĘDÓW *TAXUS CUSPIDATA* SIEB. ET ZUCC. ORAZ W ROŚLINKACH

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S t r e s z c z e n i e

Opracowano sposób mikrorozmnażania dorosłego żeńskiego krzewu *Taxus cuspidata* z pączków szczytowych, a także uzyskano kulturę nowego klonu pochodzącego z nasienia tego okazu. Pojedyncze pędy, które wyrosły z pączków szczytowych na pożywce składającej się z soli mineralnych pożywki WP oraz witamin z pożywki B5 (WP-B5), uzupełnionej 20 mg l^{-1} BAP i 5 g l^{-1} węgla aktywnego, dzielono na fragmenty o długości 1 cm i przenoszono na pożywkę bez regulatorów wzrostu w celu dalszego mnożenia. Pędy ukorzeniały się na pożywce WP-B5 z 0,3 mg l^{-1} 2-iP i 0,1 mg1^{-1} IAA w ciągu 3 miesięcy. Zawartość paklitakselu w pędach rosnących na pożywce stałej i w bioreaktorze rozpyłowym wynosiła od 45,6 do 86,5 μg g^{-1} sm. Roślinki otrzymane *in vitro* rosnące w doniczkach zawierały od 99,0 do 213,1 μg g^{-1} paklitakselu i 558,0 μg g^{-1}sm. cefalomanniny, podczas gdy w igłach rośliny macierzystej było 457,6 μg g^{-1} sm paklitakselu i 340,9 μg g^{-1} sm cefalomanniny.

***Słowa kluczowe**: Taxus cuspidata, cis, mikrorozmnażanie, ukorzenianie, kultura pędów, bioreaktor rozpyłowy, paklitaksel, cefalomannina*