INFLUENCE OF EXTRACT FROM SHOOTS OF *TAXUS BACCATA* VAR. 'ELEGANTISSIMA' ON MITOTIC ACTIVITY OF MERISTEMATIC CELLS OF *ALLIUM CEPA* L. ROOTS

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ABSTRACT

We investigated the influence of different concentrations of alcoholic extract from young shoots of *Taxus baccata* var. 'Elegantissima' (TbE), containing paclitaxel (81.6 µmg/g fresh mass), on mitotic activity and DNA content in meristematic cells of *Allium cepa* adventitious root tips. During 24 hours of incubation we obtained different level of inhibition of mitotic activity: ranging from slight changes (dilution 1:32), through complete inhibition reversible in postincubation (dilution 1:8), to whole inhibition irreversible in postincubation (dilution 1:4). Inhibition of mitotic activity was connected with an increase of prophase and metaphase and changes in morphology of chromosomes (thick chromosomes of prophase with diluted karyolymph and c-metaphases). During the experiment, the number of aberrations and micronuclei didn't increase but the number of cells with replicated DNA (4C) blocked in G2 phase was higher. This study was done by a flow cytometry method. The optimal concentration of the TbE extract, which inhibited mitotic activity, did not damage the genetic material (chromosomes) visibly.

KEYWORDS: *Taxus baccata* var. 'Elegantissima', paclitaxel, mitotic activity, phase indices, DNA content.

INTRODUCTION

Yew-trees belong to the most poisonous plants of Polish flora. Extracts from yew bark were used in folk medicine as antiseptic and abortion agent and also against skin cancer (Appendino 1993). In 1971, active agent taxol (now called paclitaxel), was isolated from bark of *Taxus brevifolia* and it was found that this compound killed in vitro cultured cells of leukaemia (Wani et al. 1971). Paclitaxel was soon found to be useful in cancer therapy (breast, lung and ovarian cancer) and in 1996 it was introduced to Polish therapeutics as a drug Taxol® produced by the Bristol-Myers Squibb company. This compound is a diterpen pseudoalkaloid, the anticancer properties of which consist in stabilisation of microtubules (MTs) by inhibition of microtubule depolymerisation in opposition to well known cytostatics (colchicine, vinblastine, vincristine), which block polymerisation of tubulin and MTs formation (Shiff et al. Horwitz 1980). Under the influence of pa-

clitaxel, MTs don't undergo depolymerisation, which prevents formation of normal mitotic spindle and thus inhibits of cell divisions (Shiff et al. Horwitz 1980).

The problem with application of Taxol® is its insolubility in water (Latini J. 1993). As liquid solvents of this drug the following substances are used: 'Cremophor El', applied in medicine, but noxious to health, and ethyl alcohol and dimethyl sulfoxide (DMSO), used in laboratories.

The main obstacle in wide application of paclitaxel in anticancer therapy is the small content of this compound in plant. From 3 kilograms of bark of a one hundred year-old yew tree, only 300 mg of paclitaxel can be obtained, which is sufficient for one therapeutic dose (Appendino 1993). Therefore new sources of this compound are still searched for, both between different varieties of yew trees, their cultivation in ground and also with use of biotechnological methods of its production from different organs and tissues in in vitro cultures. Such studies, with positive results, are carried out in Poland

(Furmanowa et al. 1997, 1998, 1999b; Wawrzynowicz et al. 1999).

Another problem limiting application of paclitaxel are drug-induced side effects, resulting from its high toxicity (Rowinsky et al. 1993). Although Taxol® was thoroughly therapeutically studied, there are no sufficient data on cytological aspects of its influence both on animal and plant cell. Such studies were carried out by Rowinsky et al. (1988), Schiff and Horwitz (1980), Furmanowa et al. (1999a), Bednara et al. (1998). The aim of our work was to investigate the content of paclitaxel in shoots of *T. baccata* var. 'Elegantissima' and to compare our data with the results of other authors (Vidensek et al. 1990) obtained for other varieties of yew trees. Our next aim was the evaluation of effect of TbE extract on plant experimental system of actively dividing meristematic cells of *Allium cepa* L. root tips. The results could be a reference point for further studies on the effect of paclitaxel on human carcinoma cells cultured in vitro.

MATERIAL AND METHODS

Extract preparation

One and two-year old shoots of *Taxus baccata* var. 'Elegantissima' (TbE) were obtained from Botanical Garden of Warsaw University. 25 g portions of fresh material (collected in March) were cut into small fragments and treated with 300 ml of 70% ethanol. Extraction was carried out for 24 hours with use of rotary shaker at 25°C. Water evaporated completely from the extract at 40°C, and the obtained residue was diluted in water solution of dimethyl sulphoxide (DMSO). Its final concentration did not exceed 0.4%. Such concentration of DMSO reportedly did not cause structural changes in the cell (Rowinsky et al. 1988). This was also confirmed in our studies. In the initial solution the content of paclitaxel was determined by the HPLC method. This solution was also used for preparation of further dilutions in proportions of 1:32, 1:16, 1:8, 1:4, which contained 2, 4, 8 and 16 mg/ml of paclitaxel respectively.

HPLC determination of paclitaxel

Phytochemical analysis of extract containing taxoids – structural analogues of paclitaxel and also other various substances of different polarity as chlorophylls and waxes began from TLC (thin layer chromatography) method which allowed to separate a band of paclitaxel (Wawrzynowicz et al. 1999). The quantitative analysis of paclitaxel was done using the HPLC method. The following conditions were applied: A Hewlett-Packard (Palo Alto, CA) Model 1050 liquid chromatograph, equipped with a stainless-steel 200 × 4.6 mm i.d., Hypersil ODS 5 µm column (Shandon, UK), was used for quantification of paclitaxel in fresh shoots of *Taxus baccata* var. 'Elegantissima'. The mobile phases consisted of mixtures of acetonitrile in water 50:50 v/v for paclitaxel. In each case, 10 µl aliquots after TLC were injected into the chromatograph. The analyses were made at 200 nm. An external standard HPLC method was used for quantitative determination of the analysed paclitaxel.

The HPLC chromatogram of paclitaxel is presented in Fig. 9.

Plant material and treatment

Adventitious root tips of onion (*Allium cepa* L.) were used as experimental material for studying the influence of TbE extract on plant cell. The onions were obtained from Plant

Genetic Resources Laboratory of Research Institute of Vegetable Crops in Skierniewice. The roots were grown in distilled water, in darkness and at room temperature. When they acquired the length of 4 cm (± 1 cm), the onions were transferred to the TbE extract in dilutions of 1:32, 1:16, 1:8, 1:4 for 3, 6, 9, 12, 24 hours of incubation (three onions for each dilution). The roots were then rinsed several times in distilled water for 30 min. and were left in fresh water for 2, 4, 6, 8, 12 i 24 hours of postincubation. After each experiment 2 mm long root tips were collected to study the effect of TbE extract on mitotic activity of meristematic cells, on their phase index and DNA content.

Microscopic preparations and mitotic and phase indices

After each of the incubation and postincubation periods 2 mm root tips were cut off and fixed for 2 hours in Carnoy's fixer (1886). Then the material was rinsed in 96% and 70% ethyl alcohol and stored in it (no longer than 48 hours). Fixed root tips were macerated in 2% acetoorceine at room temperature and then squashed on microscopic slides. The mitotic index and phase indices were counted according to Lopez-Saez and Fernandez-Gomez (1965) method. For each variant of experiment, five roots from each of three different onions were collected. The indices were counted and microphotographs were taken with light microscope (Nikon).

Flow cytometry

Root tips about 2 mm long were cut off after 3, 12 and 24 hours of incubation in 1:8 dilution of the extract as well as after 12 and 24 hours of postincubation in water. For each treatment two onions were used. Samples of three root tips from each onion were prepared according Galbraith et al. (1983), with some minor modifications. Plant material was fragmented into small pieces in a Petri dish with 250 µl nucleus-isolation buffer (0.1 M Tris, 2.5 mM MgCl₂ × 6 H₂O, 85 mM NaCl, 0.1% Triton X-100; pH 7), supplemented by 4' 6- diamino-2-phenylindole (DAPI; 2 µg/ml). After fragmentation the suspension was passed through 50 µm mesh nylon filter. For each sample 3000-6000 nuclei were with Partec CCA (Munster, Germany) flow cytometer. Obtained histograms were analysed using DPAC v. 2.2 computer programme and G2/G1 ratio was calculated.

RESULTS

Analysis of mitotic activity in root tips after treatment with extract from *Taxus baccata* var. 'Elegantissima'

Root tips of onion were treated with the TbE extract, containing 81.6 µg/g (fresh mass) of paclitaxel (as shown by HPLC analysis), (Fig. 9).

Only two of four studied dilutions of the TbE extract, namely 1:16 and 1:8 (Fig. 1), caused reduction or complete inhibition of cell divisions after 24 hours of incubation. During 24 hours of postincubation, renewal of cell divisions took place. These dilutions were recognised as sublethal for the meristematic cells. Higher dilutions of the TbE extract (1:32) did not decrease mitotic activity after 24 hours of incubation, whereas after treatment with weaker extract dilution (1:4) mitotic activity was inhibited completely and we did not observe its renewal during postincubation (Fig. 1). This dilution was confirmed as lethal.

In root tips, treated with the extract in 1:16 dilution, mitotic activity was gradually decreasing to achieve its lowest value of 5% after 9 hours of incubation (Fig. 1). During postincuba-

Fig. 1. Mitotic index in control root tips of *A. cepa* and after treatment with different dilutions of TbE extract.

tion the mitotic index increased quickly and exceeded the control value in 4 hours. After next 2 hours of postincubation it reached 25%, which was almost twice as high as in control (Fig. 1).

In root tips treated with the extract in 1:8 dilution, the mitotic index was decreasing gradually and after 9 hours of incubation it reached 4% (Fig. 1 and 6 B). After 24 hours of incubation cell divisions were completely inhibited (Fig. 1 and 6 C). During postincubation the mitotic activity was gradually coming back; after 6 hours the value of the mitotic index amounted to about 2%, after 8 hours to 12%, whereas after 24 hours of postincubation the mitotic index exceeded the control value and amounted to 22% (Fig. 1 and 6 D).

Under the influence of both dilutions of extract, apart from changes of mitotic activity, phase indices were altered and differed from the control.

During incubation in 1:16 dilution, the prophase index exceeded considerably the level of control whereas indices of the remaining phases were below control (Fig. 2). Accumulation of prophases was connected with changes in structure of chromosomes, which were thicker and shortened with diluted karyolymph between them (Fig. 7B). The number of such prophases increased between the 3rd and 9th hour of incubation and slightly oscillated in consecutive hours (Fig. 3). Meanwhile, as soon as after 3 hours of incubation, changed metaphases appeared. Commonly they were metaphases with distinct shortened chromosomes, which did not form regular metaphase plates, but typical c-metaphases (Fig. 7C). The

Fig. 2. Phase indices in control root tips of *A. cepa* and after treatment with TbE extract in dilution of 1:16.

Fig. 3. Proportion of normal to changed prophase and metaphases after treatment with TbE extract in dilution of 1:16.

percentage of changed metaphases increased until 9 hours of incubation, when they consisted of 89% of all metaphases (Fig. 3). After 12 and 24 hours of incubation their percentage decreased gradually, and at the beginning of postincubation (24/2) (Fig. 3) metaphases returned to their normal look.

In root tips treated with the extract in dilution of 1:8, the prophase index was higher in the control already at the beginning of incubation and it gradually decreased during next hours of incubation (Fig. 4). At the same time the metaphase

Fig. 4. Phase indices in control root tips of *A. cepa* and after treatment with TbE extract in dilution of 1:8.

Fig. 5. Proportion of normal to changed prophase and metaphases after treatment with TbE extract in dilution of 1:8.

Fig. 6. Microphotographs of changes in mitotic activity in root tips of *A. cepa* resulting from incubation and postincubation in the TbE extracts. Fig. 6A. Control; Normal cell divisions. Bar = 20 µm.

Fig. 6B. Few changed prophases (arrows) and changed metaphase (arrowhead); 9 hours of incubation in the TbE extract in dilution of 1:8. Bar = 20 µm.

Fig. 6C. Inhibited cell divisions; 24 hours of incubation in the TbE extract in dilution of 1:8. Bar = 20 µm.

Fig. 6D. Renewal of mitotic activity. Normal structure of mitotic phases; 24 hours of postincubation of root tips which were earlier treated with TbE extract in dilution of 1:8.

Fig. 7. Microphotographs of changes in mitotic activity in root tips of *A. cepa* after treatment with TbE extract.

Fig. 7A. Prophase (arrow) and metaphase (arrowhead) in control root tip. Bar = 20 μ m.

Fig. 7B. Changed prophase with shortened and thick chromosomes (arrow) and with light kariolymph visible (arrowhead) in root after 9 hours of incubation in the TbE extract in dilution of 1:16. Bar = 20 μ m.

Fig. 7C. Metaphase with c-chromosomes (arrow) in root after 9 hours of incubation in the TbE extract in dilution of 1:16. Bar = 20 μ m.

index was increasing continuously and it exceeded the control value after 12 hours of incubation (Fig. 4). Among prophases and metaphases, changed prophases and metaphases were observed. Their percentage increased with the progress of incubation (Fig. 5 and 6B). As soon as after 3 hours of incubation, 46% of prophases were changed. After 6 hours they increased to 94%, and after 9 and 12 hours of incubation, all prophases were changed (Fig. 5). After 3 hours, 75% of metaphases were c-metaphases whereas after 6 hours they reached 100% and such percentage was observed until 12 hours of incubation (Fig. 5). After 24 hours of incubation, cell divisions ceased and only sometimes single changed prophases and c-metaphases were observed.

After 6 hours of postincubation of root tips, which had earlier been treated for 24 hours with the extract in 1:8 dilution, new cell divisions caused an increase of prophase index (Fig. 4 and 6D). After 12 hours of postincubation, the prophase index decreased whereas the telophase index increased (Fig. 4). This tendency altered after 24 hours of postincubation.

In postincubation of roots, which had earlier been under the influence of both dilutions of the extract, changed prophases and c-metaphases did not appear.

After treatment with both dilutions of the extract, neither chromosomal aberrations nor micronuclei were observed.

Flow cytometric analysis of the nuclear DNA replication stages

Flow cytometry is a very accurate method for studying nuclear replication stages and quantitative measurement of DNA in G1, S and G2 phases of cell cycle (Śliwińska 1996).

The majority of the cells of untreated root tips were arrested in G1/G0 phase of the cell cycle (2C DNA content). However, a considerable number of the cells was arrested in G2 phase of the cell cycle (4C DNA content; Fig. 8). The frequency of G2 cells increased with time of incubation in the extract. The G2/G1 ratio increased from 0.600 in untreated roots to 1.457 in roots incubated for 24 hours in the dilution of the extract 1:8. During postincubation in water, the share of G2 cells decreased gradually, and the G2/G1 ratio reached 0.788 after 24 hours.

DISCUSSION

Anticancer diterpen compounds derived from yew, such as paclitaxel and its semisynthetic derivative docetaxel, are now the best functioning components of anticancer drugs. In spite of its great effectiveness, the application of those drugs is limited. One of the problems is the availability of paclitaxel caused by the limited amounts of this compound in plants (0.01%). Therefore investigations are being carried out, aimed at finding more productive sources of paclitaxel by studying its content in not yet analysed varieties of yews, in particular parts of the plant and in the tissues and cells from in vitro cultures.

This explains our attempt to study the content of paclitaxel in extract derived from shoots of *Taxus baccata* var. 'Elegantissima' and to define its influence on mitotic activity and DNA content in the root tips of *Alium cepa* L.

HPLC analysis showed 81.6 μ g/g (fresh mass) of paclitaxel in the shoots, what in terms of 3-kg shoots gave 245 mg of paclitaxel. Such quantity is comparable to the studies of Appendino (1993) who obtained 300 mg of paclitaxel from 3 kg of bark. On the other hand, the studies of Videnesek et al. (1990) showed that the shoots of yew contain considerably less paclitaxel in comparison with the bark. We suppose, that such a large quantity of paclitaxel obtained in our studies was connected with the variety of the yew *Taxus baccata* var. 'Elegantissima' and with good condition of trees cultivated in the Botanical Garden of Warsaw University. Also the season of shoots' collection favoured an increased content of this compound. The material was collected at the end of winter when yews contain the highest amount of paclitaxel.

Our results show also that the extract from shoots of *Taxus baccata* var. 'Elegantissima' used in different dilutions functioned similarly as other taxanes, including paclitaxel and plant cells reacted to this extract similarly to human cells; that is, cell divisions were less numerous or inhibited in G2/M phases. In lymphoblastic leukaemia cells, Rowinsky et al. (1988) observed aster like microtubule arrangement in G2/M phases as a result of treatment with Taxol® dissolved in 0.1% DMSO and applied in 10, 1 and 0.1 μ M doses during 2, 4 and 22 hours.

Fig. 8. Histograms of DNA content in nuclei of control meristematic cells and in nuclei of cells treated with TbE extract in dilution of 1:8. Fig. 8A. Control.

Fig. 8B-D. The results of different times of incubation in TbE extract.

Fig. 8E-F. The effects of postincubation length in roots incubated earlier for 24 h in TbE extract.

Fig. 9. HPLC separation of paclitaxel – T in TbE extract.

In our experiment the cytostatic effect of the extract depended on concentration and time of incubation. In treatment with 1:16 dilution, cell divisions were not totally inhibited, but the mitotic index decreased to 5% after 9 hours of incubation. During postincubation, the mitotic index was increasing and it exceeded the control value. This proved that the influence of the extract was not irreversibly toxic. In root tips treated with the extract in 1:8 dilution, mitotic activity was completely inhibited after 24 hours of incubation, which was connected with an increased content of paclitaxel.

The decrease of mitotic activity was correlated with changes of phase indices. During incubation of root tips in both the sublethal dilutions of the extract, the prophase index exceeded the control value. Under the influence of extract in 1:8 dilutions, the metaphase index also increased. With increasing time of incubation in both dilutions, the number of changed prophases and c-metaphases increased. This indicates that, the cells were halted at this stage of cell cycle. It was undoubtedly connected with prevention of MTs depolymerisation (stabilisation) appearing in stages G2 and M, causing disturbances in kariokinetic spindle formation and inhibition of cell divisions (Schiff and Horwitz 1980).

Studies of Rieder et al. 1994 showed that microtubules stabilised by paclitaxel (particularly by its high concentrations) do not attach to kinetochores of chromosomes, which causes their chaotic distribution in the cell and formation of c-metaphases.

Under the influence of 1:8 and 1:16 dilutions of extract, chromosomal aberrations and micronuclei did not appear, which indicates safe effect of the extract.

In accordance with our observations, c-metaphases chromosomes changed into c-anaphase chromatydes by partial separation of chromatydes; however, instead of separation and

shifting to the poles of the spindle, they just underwent despiralisation and interphases nuclei with 4C DNA content were formed.

This was confirmed by analysis of cell cycle made by flow cytometry method after treatment with the extract from TbE. This study showed that, during incubation of roots in the extract, participation of cells in G2/M phase of the cell cycle increased whereas participation of the cells in G0/G1 phase decreased.

This was also confirmed by studies on animals cells, where cell divisions were stopped in G2/M phase of the cell cycle and ploidy of the cells (Schiff and Horwitz 1980) was changed. Simultaneously these data explained the accumulation of cells in prometaphases during incubation in the extract.

The results of our findings showed that the extract from young shoots of *Taxus baccata* var. 'Elegantissima' contained a high amount of paclitaxel (81.6 µg/g) and that it stopped the mitotic activity, but did not cause aberrations and formation of micronuclei. We suggest that if similar effects in treatment of human cancer cells are obtained, the extract from this variety of yew might be safely used in anticancer therapy. This has to be confirmed by additional tests.

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WPLYW EKSTRAKTU Z PĘDÓW *TAXUS BACCATA* VAR. 'ELEGANTISSIMA'
NA AKTYWNOŚĆ MITOTYCZNĄ KOMÓREK MERYSTEMATYCZNYCH
W WIERZCHOŁKACH KORZENI *ALLIUM CEPA* L.

STRESZCZENIE

Badano wpływ różnych stężeń alkoholowego ekstraktu z młodych pędów *Taxus baccata* var. 'Elegantissima' (TbE), zawierającego paklitaksel (81.6 µg/g świeżej masy) na aktywność mitotyczną i zawartość DNA w komórkach merystematycznych wierzchołków korzeni przybyszowych cebuli (*Allium cepa*). Podczas 24 godz. inkubacji uzyskiwano różny stopień zahamowania aktywności mitotycznej: począwszy od niewielkiej zmiany (rozcieńczenie 1:32), poprzez całkowite zahamowanie ale odwracalne w postinkubacji (rozcieńczenie 1:8), aż do całkowitego zahamowania, nieodwracalnego w postinkubacji (rozcieńczenie 1:4). Inhibicji aktywności mitotycznej towarzyszyło procentowe zwiększenie profaz i metafaz oraz zmiana morfologii chromosomów (grube chromosomy profazowe z rozrzedzoną kariolimfą i c-metafazy). W trakcie eksperymentu nie dochodziło do zwiększenia liczby aberracji i mikrojąder natomiast wzrósł natomiast wzrost liczby komórek o zreplikowanej ilości (4C) DNA zablokowanych w fazie G2. Badanie to wykonano metodą cytometrii przepływowej. Optymalne stężenie ekstraktu TbE, które hamowało aktywność mitotyczną nie powodowało widocznego uszkodzenia materiału genetycznego (chromosomów).

SŁOWA KLUCZOWE: *Taxus baccata* var. 'Elegantissima', paclitaxel, aktywność mitotyczna, indeks fazowy, zawartość DNA.