CYTOLOGICAL CHANGES IN MERISTEMATIC CELLS OF *ALLIUM CEPA* L. ROOT TIP TREATED WITH EXTRACTS FROM CALLUS OF *CATHARANTHUS ROSEUS* (L.) G. DON.

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ABSTRACT

The effect of an ethanolic extract from callus of *Catharanthus roseus* on *Allium cepa* root cells divisions was investigated. Two lines: white and green callus, were established on solid B5 medium with IAA 1 mg/l and kinetin 0.1 mg/l. The HPLC analysis of callus extracts showed the presence of indole alkaloids, however not known pharmacologically active alkaloids or derivatives used in semisynthesis (vinblastine, ajmalicine, serpentine, yohimbine, vindoline and catharanthine) have been found. The ethanolic extract of *C. roseus* callus inhibited the number of mitoses in *Allium cepa* root tip cells. Short (1-3 hours) treatment resulted in an increase in the index of late prophase, with characteristic light spaces, and the index of metaphases with twisted chromosomes forming an equatorial plate or irregular structures of c-metaphases. At the same time, the percentage of anaphases and telophases decreased significantly. Longer treatment of the root tip inhibited mitotic activity, stopping it completely already after 12 hours. Interphase nuclei became at first denser and homogeneous, eventually their structure became partitioned into zones and formation of chromatin territories, with distinct large nucleoli has been observed. Electron microscope observations revealed well developed rough endoplasmic reticulum and thick, invaginating cell membrane.

KEYWORDS: *Catharanthus roseus*, callus, alkaloids, mitotic activation, cell ultrastructure.

INTRODUCTION

Catharanthus roseus (L.) G. Don – periwinkle (Apocynaceae) is a medicinal plant which produces a wide variety of indole alkaloids as monomers and bisindoles, many of which show pharmacological activity. Antineoplastic bisindole alkaloids – vinblastine and vincristine – are used as chemotherapeutic agents against cancers such as acute leukaemia and Hodgkin's lymphoma (Hirata et al. 1994).

Some monomers, for example catharanthine and vindoline, can be used in biosynthesis of the bisindoles via chemical or enzymatic coupling (Kutney 1990). Other monomeric alkaloids used in medicine are ajmalicine, serpentine, yohimbine.

The formation of indole alkaloids in cells and tissues of *C. roseus* has been investigated in many laboratories. These studies have been focused mainly on establishing different kinds of cultures such as callus, suspension, transformed and untransformed root cultures, followed by phytochemical analysis. Usually the callus tissue is a starting material for suspension culture or induction of plant organs like shoots or roots, but it can be also used as a source of secondary metabolites.

Vinblastine and vincristine were observed in white and green lines of callus of *C. roseus* during testing of the effect of various growth regulators on induction of green callus in

3-year-old cell lines. The green line had about twice as much of the above mentioned alkaloids as the white one (Loyola-Vargas et al. 1986).

Alkaloid metabolism in cultured leaf tissue and primary callus of *C. roseus* under a variety of culture conditions was also studied (Morris 1987). Rapid growth of callus was observed after 10-20 days of culture. The two main leaf alkaloids, catharanthine and vindoline, were rapidly metabolised and were found neither in primary callus nor in the original leaf tissue after 40 days of culture. During the callus induction and growth period the alkaloids serpentine and ajmalicine accumulated in the leaf tissue but were predominantly present in the callus. Serpentine was the major alkaloid accumulating in the light whereas ajmalicine was synthesized in the dark.

The callus from young leaf segments of *C. roseus* was initiated on Murashige-Skoog medium supplemented with naphthalenacetic acid (NAA) and kinetin in the dark. The callus was brown and compact, formed many roots and contained vinblastine, whose presence was confirmed by high performance liquid chromatography and mass spectrometry (Miura et al. 1987).

The eight callus lines of *C. roseus* callus culture on Lin-smaier-Skoog (LS) medium supplemented with 2,4-dichloro-

phenoxyacetic acid (2,4-D), BAP and coconut milk had been established by other authors (Marfori et al. 1993). They used cultures derived from roots, leaves and flowers of white and pink-purple varieties of *C. roseus*. Callus was then transferred to LS medium without auxins and cultured for a year. The yield of alkaloids was higher in callus derived from roots of pink-purple variety of periwinkle than that of the white one, where it depended on the part of the plant from which the callus originated.

The aim of this work was to ascertain whether there were indole alkaloids present in two green and white lines of *C. roseus* callus cultured in our laboratory for eight years, we were particularly interested in finding vinblastine, a potential inhibitor of cell division. It acts by inhibiting the assembly of microtubular proteins into spindle microtubules, which produces a metaphase arrest.

The second aim was to investigate the biological activity of an ethanolic extract from these lines of callus.

MATERIALS AND METHODS

Initiation of callus

Callus culture was established eight years ago from the hypocotyls of seedlings of *C. roseus* developed from seeds received from the Institute of Medicinal Plants in Poznań (Poland). The seeds were surface sterilized by immersion in 70% (v/v) ethanol for 1 min followed by 5% sodium hypochlorite for 15 min, then rinsed several times with sterile water and allowed to germinate at 25°C in the dark in Petri dishes on wet sterile filter paper.

Callus was initiated from hypocotyl fragments on two modifications of solid Gamborg medium (Gamborg et al. 1968) supplemented with: 1) indolyloacetic acid (IAA) 1 mg/l, kinetin 0.1 mg/l; 2) 2,4-D 1 mg/l, kinetin 0.1 mg/l described earlier by Morris (1986).

The cultures were incubated in a culture chamber with a photoperiod of 12 h (40 $\mu\text{mol}/\text{m}^2/\text{s}^{-1}$) and 25° $\pm$ 1°C temperature. After 3 weeks of incubation callus was transferred to fresh medium and then subcultured every 4 weeks. Callus taken for cytological studies was eight years old.

Cytological studies

The 70% ethanol extracts of 100 ml were made from 5 g of fresh green callus tissue and 5 g of white callus both cultured on Gamborg (B5) medium with IAA and kinetin. The extraction was performed for 24 hours at room temperature after an initial 5 min boiling to inactivate the enzymes. The extract was evaporated and redissolved in 1 ml ethanol to which 99 ml of water had been added. The extract was evaporated again and redissolved in water (100 ml). The roots of three onions (*Allium cepa* L.) were grown in distilled water (250 ml flasks) in the dark at room temperature. When they reached 3 cm ($\pm$ 0.5 cm) of length they were placed in a prepared extract of *C. roseus* callus solution for incubation.

Before incubation (control) and after 1, 3, 12 and 24 hours of incubation, 5 root tips (5 mm) (from every 3 bulbs) were fixed (2 h) in glacial acetic acid: absolute ethanol mixture (1:3). For squash preparation for light microscopy the fixed root tips were macerated and stained in 2% acetorceine for 2 hours in room temperature by the procedure of Kuraś and Malinowska (1978), Zhang et al. (1996).

The average mitotic index for every fixed root (like above) was characterized by the percent of divisions per thousand meristematic cells per squash preparation.

The phase indices were obtained by counting 200 meristematic cells (of the same squash preparation) being in the process of cell division according to Lopez-Saez and Fernandez-Gomez (1965).

Additionally two roots were always fixed for electron microscopy in a mixture of 2.5% glutaraldehyde with 4% paraformaldehyde, pH 7.2 (phosphate buffer) (Karnovsky 1965).

After dehydrating in a series of alcohols and propylene oxide the samples were embedded in Spurr (1969) resin. Semi-thin (1-2 μm) and ultra-thin (about 80 nm) sections were cut with an LKB ultramicrotome. Semi-thin sections were stained with 0.1% toluidine blue and used for light microscopy (Nikon). The ultra-thin sections were stained for contrast with uranyl acetate and lead citrate according to Reynolds (1963) and analyzed with BS 500 transmission electron microscope.

Statistical evaluation was done by the T-Student test.

Determination of alkaloids

Lyophilized *C. roseus* callus lines (white and green 2 g), previously cultured on solid Gamborg medium (Gamborg et al. 1968) supplemented with IAA 1 mg/l and kinetin 0.1 mg/l after 96 passages (8 years old), were separately used for chemical investigations. The essence of this method is the extraction of dry plant material by a neutral hydroxylic solvent, twofold purification of the alkaloid fraction by salting it out of the water phase at the second stage of extraction, and then alkaloid determination by various chromatographic techniques (Ruszkowska et al. personal information). In our experiment a citrate extract of *C. roseus* alkaloids (pH 2) was extracted with ethylene dichloride. The remaining water phase was adjusted to pH 6 and then extracted with benzene. The above mentioned extracts were then evaporated to dryness and 2 mg of each dry residue, dissolved in an appropriate volume of methanol, was investigated by high performance liquid chromatography.

Chromatographic separation was performed on a Shimadzu LC-10AD liquid chromatograph equipped with SPD-10A UV spectrophotometric detector and integrator Chromatopak C-R3A. The conditions of isocratic HPLC analysis were: column: (3.9 x 300) $\mu\text{Bondapak C}_{18}$; eluent: methanol/acetonitrile/diammonium hydrogen phosphate solution (pH 6) (3:4:3); flow rate: 1 ml min^{-1} ; detection: wavelength 295 nm (Jung et al. 1992).

RESULTS

Maintenance of callus

Two types of callus were obtained from the hypocotyl of *C. roseus* after four weeks of incubation on two modifications of Gamborg (B5) medium. The green, compact callus with small roots disappearing after 4 passages was observed on the first (1 B5) modification and very soft, brown, watery callus on the second (2 B5) modification. Green callus was subcultured every 4 weeks on fresh 1 B5 medium and was maintained throughout on this medium. Conglomerates of white cells were formed on the surface of green callus after the 9th passage. During subsequent passages white cell conglomerates were separated and cultured on 1 B5 medium. In contrast to the green line of callus the white line callus was flossy and loose (Fig. 1A and 1B).

Cytological observation in light and electron microscope

The green callus passaged several times on Gamborg (B5) medium supplemented with IAA 1 mg/l and kinetin 0.1 mg/l

Fig. 1A. Callus of *C. roseus* – white line growing on Gamborg B5 medium with IAA (1 mg/l), and kinetin (0.1 mg/l).

Fig. 1B. Callus of *C. roseus* – green line growing on Gamborg B5 medium with IAA (1 mg/l), and kinetin (0.1 mg/l).

Fig. 1A and 1B. Bar 1 cm

contained well developed parenchymal cells with vessel elements in the middle (Fig. 3A stars).

An alcohol extract of this callus, dissolved in water, induced significant changes in the cellular structure of cells of the root tip in onion after only 1 hour of treatment. After this time the mitotic index was reduced from 12.0 to 5.5% (indicating inhibition of mitoses by almost 55% in comparison to the control) (Fig. 6 and Fig. 2A and 2B). The phase index also changed. In comparison to the control, the index of prophase significantly increased, the metaphases index remained almost unchanged, and the index of anaphases and telophases slightly decreased (Fig. 7).

Among the observed prophase, besides normal ones the same as in control (marked in the Fig. 7 as normal prophase), there were numerous prophase (about 35%) in which the chromosomes were thicker and more condensed (compare Fig. 2A, arrows, 2B, asterisk and 3B, black star, 3C, white star). Light spaces (Fig. 3C, thick arrow) and the outlines of nucleoli (Fig. 3C, arrowhead) were visible among chromosomes of abnormal prophase.

Metaphases were also changed. Although some of them appeared normal, as in the control (Fig. 3B, arrow), at least half of them had after treatment shortened chromosomes, either forming a slanted equatorial plate (Fig. 2B, right arrow) or

not forming it at all. Chromosomes were observed in characteristic structures of c-metaphases (Fig. 2B, left arrow).

All changes observed after 1 hour of treatment became more distinct after 3 hours of treatment. The mitotic index decreased to 2.3% (Fig. 6 and Fig. 2C). Chromosomes of prophase and metaphases were more condensed (Fig. 3D, arrowhead and arrow). The phase index underwent further changes: prophase constituted more than 80%, of which 50% were abnormal (Fig. 7 and Fig. 3D, arrowhead). The metaphases index was less than 10%, and all metaphases were abnormal, with c-metaphase chromosomes significantly shortened (Fig. 3D, arrow). This resulted in disturbances of the process of anaphases and telophases, leading to aberration, thickening of chromosomes and formation of several nuclei in a single cell (Fig. 3E, thick arrows).

After longer times of incubation (12 and 24 hours), mitotic activity was totally inhibited (Fig. 6 and Fig. 2D and 3F). Although nuclei of interphase cells were still oval, they were distinctly smaller and their structure, previously homogeneous, became more dense, and eventually partitioning occurred leading to formation of characteristic chromatin territories, with very large nucleoli preserved (Fig. 2D and Fig. 3F, stars).

In the electron microscope (Fig. 4 and 5) the structure of chromatin was dispersed without visible euchromocenters

Fig. 2A. Control, usual frequency of stages of mitosis, normal structure of phase of mitosis: prophase (black arrows) and metaphase (white arrow). Acetoalcohol, acetoalcohol

Fig. 2B. One-hour treatment, decreased the number of mitoses with abnormal prophases (asterisk) and changed metaphases (black arrows). Acetoalcohol, acetoalcohol.

Fig. 2C. Three-hour treatment. A few mitoses only visible: c – metaphases (black arrow) Acetoalcohol, acetoalcohol.

Fig. 2D. Twelve-hour treatment. No divisions. Smaller nuclei, compare Fot. 2A and 2D

Changes in chromatin; more dense with heterochromatin and big nucleoli. Acetoalcohol, acetoalcohol.

Fig. 2A-2D. Bar 20 μ m

Fig. 3A. Green callus with well developed veins containing lignified vessel elements (stars), paraformaldehyde, semithin section, safranina and fast green. Bar 20 μ m

Fig. 3B. Control with normal prophase (black star) and metaphase (black arrow) Acetoalcohol, acetorceine.

Fig. 3C. One-hour treatment. One normal prophase (black star) and one with abnormal condensed chromosoms (white star) with many areas of light spots (thick arrow). Outline of nucleolus (arrowhead). Acetoalcohol, acetorceine.

Fig. 3D. Three-hour treatment. Abnormal prophase with thick and condensed chromosomes with many light spots (arrowhead) Slanted metaphase with shorter chromosomes (arrow) Acetoalcohol, acetorceine.

Fig. 3E. Three-hour treatment. Lost anaphase/telophase chromosomes (thick arrows) which may form later four nuclei. Acetoalcohol, acetorceine.

Fig. 3F. Twelve-hour treatment. Inhibition of divisions. Condensation of chromatin. Big nucleoli (black stars). Acetoalcohol, acetorceine.

Fig. 3B- 3F. Bar 20 μ m

Fig. 4A. Three hour treatment. Ultrastructure of four cells. Nuclei contain dispersed chromatin and heterogeneous structure of nucleolus (star). Second cell from the top contains one large telophase group of chromosomes and three small groups which are formed possibly from chromosome pieces (arrows), Karnovsky, Reynolds. Bar 1 μ m.

Fig. 4B. Three hour treatment. Invagination in the nucleolemma (black arrow) with three mitochondria (white arrows), Karnovsky, Reynolds. Bar 1 μ m.

Fig. 4C. Three hour treatment. Numerous organelles, mitochondria with round cristae (black arrow) and electron dense plastids (empty arrows); Karnovsky, Reynolds. Bar 1 μ m.

Fig. 4D. Three hour treatment. Junction of two cells with cell wall (asterisk) in the center with numerous plasmodesmata. Cell membrane is thick (double arrows). Peroxisome (thick arrow), Karnovsky, Reynolds Bar 0.5 μ m.

Fig. 5A. One-hour treatment. Numerous areas of electron dense heterochromatin (arrowhead) and heterogenous nucleolus (star). Many mitochondria (white arrow) and electron dense plastids are dividing (black arrow) Karnovsky, Reynolds. Bar 1 μ m.

Fig. 5B. Three-hour treatment showing froth-like structure of heterochromatin (arrow) and surrounding it dispersed euchromatin. Rough ER (short arrow). Karnovsky, Reynolds. Bar 0.5 μ m

Fig. 5C. Three-hour treatment. Numerous rough ER sheets (thin black arrows) mitochondria showing areas of lighter colour in the matrix (thick arrows) Karnovsky, Reynolds. Bar 1 μ m

Fig. 5D. Control with froth-like structure of heterochromatin dispersed (arrows). Karnovsky, Reynolds. Bar 1 μ m.

Fig. 6. Mitotic index in root tips of control and treated with extract from *C. roseus* (mean of 15 roots from 3 different onion bulbs).

Fig. 7. The phase index in root tips treated as Fig. 6.

(Fig. 4A). Some dividing cells after 3 hours of treatment showed lost pieces of chromosomes (Fig. 4A, arrows) micronuclei or at least an unusual shape of the nucleus with invaginations of its envelope (Fig. 4B, black arrow). Many mitochondria were observed in the vicinity of such invaginations (Fig. 4B, white arrows). Numerous plastids contained electron-dense matrix (Fig. 4C, empty arrows). Peroxisomes were oval, electron-compact and with rough surface. The plasma-lemma was denser and thicker (Fig. 4D, double arrows) than in the control, and the endoplasmic reticulum (ER), mostly rough, showed a darker grey lumen ER (Fig. 5B, thick arrow). Numerous mitochondria and plastids were dividing (Fig. 5A, arrow), and the nucleolus (Fig. 5A, star) and the nucleus seemed active, showing a heterogeneous structure (Fig. 5A, arrowhead; Fig. 5B, arrow) nearly like that of the control (Fig. 5D, thick arrows). Very often several sheets of rough ER were located in the vicinity of one another (Fig. 5C, arrows) and the mitochondria showed areas of lighter colour in the matrix (Fig. 5C, thick arrows).

Alkaloids of callus

Good separations of standard substances (ajmalicine, vindoline, catharanthine, serpentine, vinblastine) used for comparative studies were obtained (Fig. 5A). An isocratic elution system was employed. Under the same conditions HPLC analysis of dried *C. roseus* callus extracts dissolved in methanol was performed. The analysis did not reveal the above mentioned alkaloids in the investigated material. Some other dis-

tinct peaks of alkaloids were observed; they were higher in extracts from white than from green callus (Fig. 5B and 5C).

HPLC analysis showed that compounds from the green callus extract differed only a little from the phytochemicals in white callus, but neither of them contained the active anti-cancer alkaloids vinblastine and vincristine.

DISCUSSION

Two lines: white and green callus of *C. roseus* have been investigated. As the tissue grew rapidly on solid Gamborg medium supplemented with IAA 1 mg/l and kinetin 0.1 mg/l, this medium appeared to be convenient for starting and maintenance of callus culture.

According to the literature, callus of *C. roseus* can be a source of indole alkaloids. Loyola-Vargas et al. (1986) observed vinblastine and vincristine in white and green lines of three year-old callus of *C. roseus* while testing the effect of various growth regulators on the induction of callus tissue.

Morris (1987) showed that two main leaf alkaloids, catharanthine and vindoline, were rapidly metabolised and were found neither in primary callus nor in the original leaf tissue after 40 days culture. The alkaloids serpentine and ajmalicine accumulated in leaf tissue but were located predominantly in callus.

The callus of *C. roseus* initiated on Murashige-Skoog medium supplemented with naphthalenacetic acid (NAA) and kinetin, and growing in the dark (Miura et al. 1987), contained vinblastine, whose presence was confirmed by high performance liquid chromatography and mass spectrometry. Marfori et al. (1993) also found the alkaloids in the eight callus lines of *C. roseus*.

In our work, indole alkaloids used as standard substances: yohimbine, ajmalicine, vindoline, catharanthine, serpentine and vinblastine (important for the pharmacy) have not been found in alkaloid fractions, from white and green lines of callus of *C. roseus*. However, we observed other unidentified peaks on the chromatograms after HPLC separation of callus extracts (Fig. 5B and 5C). The absence of such important alkaloids may be caused by the age of the investigated tissue (96 passage – 8 years old). Our observation showed that young callus of *C. roseus* cultured for less than one year did not lose its ability to produce some alkaloids. In an earlier investigation Pietrosiuk (1997) determined the presence of ajmalicine in four-month-old callus extracts. This observation was in accordance with that of Loyola-Vargas et al. (1986) who found pharmacologically active alkaloids in young callus of *C. roseus*.

In spite of the absence of known pharmacologically active alkaloids, the ethanolic extract of callus caused retardation of mitoses and influenced all stages of mitosis when investigated after 1 and 3 hours. This was a short enough time to ensure that the cell at the particular stage we observed had been at another stage one hour or three hours earlier, thus enabling conclusions to be drawn about the influence of the extract at a particular moment of the cell cycle. Longer periods, 12 and 24 hours of treatment, allowed considering of what had happened when synthesis and other G₁, or G₂ phases were influenced, as well as, the longest period of treatment affected the whole cell cycle.

After the initial shock to the chromatin, which appeared to form light-coloured areas, three hours later it looked similar to the control, and even the cytoplasm was not very much vacuolized. Divisions were retarded after 3 hours and inhibited after 12 hours. Electron microscope observations revealed in-

Fig. 8. HPLC chromatogram of:

A – original samples of 1 – yohimbine, 2 – vindoline, 3 – ajmalicine, 4 – catharanthine, 5 – vinblastine, 6 – serpentine;
 B – ethylene dichloride extract of *C. roseus* green callus;
 C – ethylene dichloride extract of *C. roseus* white callus.

tensively developed rough endoplasmic reticulum and thick invaginating cell membrane. Those facts and the existence of numerous mitochondria suggest that the cells were at an active stage of protein production with a high level of energy supply.

The inhibition of mitoses gives hope that such extracts used as antimetotics would cause few chromosomal aberrations. Our observation showed that even if some extracts of *C. roseus* do not contain known active compounds the presence of several other compounds, even in very low concentration, could be antimetotic.

So far the extracts of *Catharanthus roseus* callus have not been biologically investigated. The results of our study show

that this tissue could be interesting for further cytological and even immunological studies.

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ZMIANY CYTOLOGICZNE W KOMÓRKACH MERYSTEMATYCZNYCH *ALLIUM CEPA* L.
POD WPLYWEM EKSTRAKTÓW ETANOLOWYCH
Z TKANKI KALUSOWEJ *CATHARANTHUS ROSEUS* (L.) G. DON.

STRESZCZENIE

Badano wpływ etanolowych ekstraktów z tkanki kalusowej *Catharanthus roseus* (L.) G. Don na podziały komórek merystematycznych *Allium cepa*.

Wychodowano dwie linie białą i zieloną kalusa *C. roseus* na stałej pożywce Gamborga (B5) uzupełnionej IAA 1 mg/l i kinetyną 0,1 mg/l. Analiza metodą HPLC ekstraktów z kalusa wykazała obecność alkaloidów indolowych lecz nie było wśród nich związków o znanym działaniu farmakologicznym lub ich pochodnych stosowanych w semisyntezie (winblastyna, ajmalicyna, johimbina, windolina i katarantyna).

Etanolowe ekstrakty z kalusa *C. roseus* hamowały liczbę mitoz w komórkach korzeni *Allium cepa*. Krótkie (1-3 h) traktowanie powodowało stopniowe wyhamowanie aktywności mitotycznej, zwiększanie indeksu późnych profaz oraz indeksu metafaz ze skróconymi chromosomami ułożonymi w płycie równikowej albo tworzącymi nieregularne układy c-metafaz. W tym samym czasie zmniejsza się wyraźnie procent anafaz i telofaz. Przedłużone traktowanie wierzchołków całkowicie hamowało aktywność mitotyczną zatrzymując ją całkowicie już po 12 godzinach. Podczas inkubacji struktura jąder interfazowych początkowo stawała się gęstsza i homogenna a później dochodziło do strefowego jej pogrubienia i wyodrębnienia chromatynowych terytoriów z wyraźnie zachowanymi dużymi jąderkami.

Obserwacje w mikroskopie elektronowym ujawniły intensywny rozwój szorstkiego retikulum endoplazmatycznego oraz pogrubienie i uwypuklenie plazmalemy.

SŁOWA KLUCZOWE: *Catharanthus roseus*, kalus, alkaloidy, aktywność mitotyczna, ultrastruktura.