PRELIMINARY RESULTS OF INDOLE ALKALOIDS PRODUCTION IN DIFFERENT ROOTS OF *CATHARANTHUS ROSEUS* CULTURED IN VITRO

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ABSTRACT

Six groups of untransformed and hairy root cultures of *Catharanthus roseus* (L.) G. Don were established. *Agrobacterium rhizogenes* strains: ATCC 15834, LBA 9403, and TR 105 were used for infection of the 3-week old rooted plantlets of *C. roseus*. The highest contents of examined indole alkaloids were found in: roots of intact plants – yohimbine and serpentine; in hairy roots – catharanthine. Vinblastine and ajmalicine were detected in untransformed roots of plants regenerated in vitro, and transferred to the soil for 5 months.

KEY WORDS: root cultures, indole alkaloids, *Catharanthus roseus*, *Agrobacterium rhizogenes*.

INTRODUCTION

Some of the indole alkaloids produced by *Catharanthus roseus* – periwinkle (Apocynaceae) show a pharmacological activity and are very important in the pharmaceutical industry. Serpentine is useful for treatment of hypertension, ajmalicine is cardiovascular drug. Vindoline and catharanthine are the obvious precursors in the biosynthetic pathway of the most important bisindole alkaloids, i.e. vinblastine and vincristine which are used in medicine as anticancer drugs. This plant does not occur, however, in central Europe, so an alternative source of indole alkaloids are the plants and its organs cultivated in vitro or transformed root cultures (Pietrosiuk et al. 1995; Pietrosiuk and Furmanowa 1997). The establishment of some transformed root cultures of *C. roseus* has been reported (Parr et al. 1988; Toivonen et al. 1989; Jung et al. 1992; Bhadra et al. 1993; Ciau-Uitz et al. 1994; Sim et al. 1994; Vázquez-Flota et al. 1994). The growth indices of these cultures and the content of some indole alkaloids (Table 1) among them were different. There was no comparison with untransformed root culture except for Ciau-Uitz et al. (1994) study. The highest accumulation of ajmalicine (4.0 mg g^{-1} DW), catharanthine (2.0 mg g^{-1} DW), and serpentine (2.0 mg g^{-1} DW) was obtained in transformed root culture by Bhadra et al. (1993). Ajmalicine, serpentine, vindolinine and catharanthine were prominent components in transformed roots of *C. roseus* obtained by Parr et al. (1988). The authors (l.c.) detected also vinblastine at a level 0.5 μg g^{-1} dry weight, using combination of HPLC and radioimmunoassay. In the present study, untransformed and transformed roots of *C. roseus* were established and the content of some indole alkaloids were compared in it.

Abbreviations:
B5 – medium of Gamborg et al. 1968; NN – medium of Nitsch et Nitsch, 1969; IBA – indole-3-butyric acid; SA – adenine sulfate; NAA – naphthaleneacetic acid; MCPP – 2-(2-methyl-4-chlorophenoxy)-propionic acid, TLC – thin-layer chromatography, HPLC – high pressure liquid chromatography, DW – dry weight, FW – fresh weight

MATERIALS AND METHODS

Untransformed root cultures

The untransformed root cultures were established from roots excised from 10-days old seedlings obtained through in vitro germination of immature seeds and roots excised from 2-months old plants regenerated in vitro on NN medium supplemented with IBA 0.5 mg l^{-1}, kinetin 0.1 mg l^{-1}, and SA 10.0 mg l^{-1} (Olszowska et Furmanowa 1987). Seedling roots were transferred to B5 and 1/2 B5 solid or liquid medium supplemented with NAA in concentration of 0.5 mg l^{-1}, and 0.25 mg l^{-1}. The roots derivating from 2-months old plants regenerated in vitro were placed in liquid 1/2 B5 medium supplemented with NAA in concentration of 0.5 mg l^{-1}, and 0.25 mg l^{-1} and in liquid B5 medium supplemented with MCPP in concentration of 0.5 mg l^{-1}, and 1.0 mg l^{-1}. The initial inoculum was 0.5 g fresh weight (FW) and it was incubated in the dark at 40 rpm and 25° ± 3°C.

TABLE 1. Indole alkaloids in transformed roots of *Catharanthus roseus*.

Species and determination method	Identified indole alkaloids	Authors
C. roseus (1, 2, 3, 4)	Ajmalicine (0.200 mg g^{-1} FW) Serpentine (0.051 mg g^{-1} FW) Catharanthine (0.045 mg g^{-1} FW) Vindolinine (0.091 mg g^{-1} FW) Vinblastine (0.003 µg/g^{-1} FW)	Parr et al., 1988
C. roseus (1)	O-acetylovallosamine, Tabersonine Tetrahydroalstonine, Horhammericine, Lochnericine, Venalstonine 19-epi-windolinine, Perikalline Yohimbine, Ajmalicine (1.1 mg g^{-1} DW), Catharanthine (1.4 mg g^{-1} DW)	Toivonen et al., 1989
C. roseus (1)	Catharanthine (1.9 mg g^{-1} DW) Ajmalicine (0.8 mg g^{-1} DW)	Jung et al., 1992
C. roseus (1, 3)	Ajmalicine (4.0 mg g^{-1} DW) Serpentine (2.0 mg g^{-1} DW) Catharanthine (2.0 mg g^{-1} DW) Vindoline (0.4 mg g^{-1} DW)	Bhadra et al., 1993
C. roseus (5)	Ajmalicine (1.33 mg g^{-1} DW) Serpentine (16.4 mg g^{-1} DW) Catharanthine (0.42 mg g^{-1} DW)	Ciau-Uitz et al., 1994
C. roseus (1)	Catharanthine (20.5 mg l^{-1}) Ajmalicine (1.7 mg l^{-1})	Sim et al., 1994
C. roseus (5)	Ajmalicine (0.57 mg g^{-1} DW) Catharanthine (0.36 mg g^{-1} DW)	Vázquez-Flota et al., 1994
C. roseus (1, 2)	Ajmalicine (1.801 mg g^{-1} DW) Catharanthine (1.523 mg g^{-1} DW) Yohimbine (0.847 mg g^{-1} DW) Serpentine (0.098 mg g^{-1} DW) Vinblastine (0.048 mg g^{-1} DW)	Pietrosiuk, 1997

1 – HPLC; 2 – TLC; 3 – GC/MS; 4 – cross reaction with appropriate antibody; 5 – densitometric method

Transformed root cultures

Hairy root cultures of *Catharanthus roseus* were established using three strains of *Agrobacterium rhizogenes*: ATCC 15834, LBA 9403, and TR 105. The 3-week old, rooted plantlets, regenerated in vitro (using the Furmanowa et al. 1994 method), were directly wounded and contaminated by the same bacterial strain. Infected plantlets were grown on hormone-free B5 medium and kept in darkness for 12 to 24 hours. The hairy roots which appeared at wounded site of stem or leaf (Fig. 1) were transferred for one passage (4 weeks) to the solid hormone-free 1/2 B5 medium containing 500 mg Claforan l^{-1} to kill bacteria. The hairy root cultures were initiated from a single root in liquid hormone-free 1/2 B5 medium (40 ml for 250 Erlenmeyer flask) and transferred to fresh medium every 4 weeks. One root tip (2 cm long) cut from branches was used as an inoculum and was incubated in the dark at 40 rpm and 25° ± 3°C. Transformation was confirmed in dried root tissue extracts by detection of mannopine after electrophoresis (Petit et al. 1983). Hairy roots in 11th passage (line 8) were elicited with 100 µM methyl jasmonate added to the medium after autoclaving for one passage.

Analysis of growth parameters

The time course of growths of hairy roots and untransformed roots of *C. roseus* were examined during 28-days of culture. Approximately 0.5 g fresh weight of roots (transformed and untransformed) were inoculated into 50 ml of the appropriate medium in 300 ml Erlenmeyer flasks. Every 3 days the roots from 3 flasks were harvested. The fresh and dry weights (after lyophilization) were determined. The pH, specific conductivity and sugar content of the media were measured. Results are presented on Table 2. For the purpose of phytochemical analysis six groups of roots of different origin were used.

Analytical methods

The extraction of plant material and the samples preparation for chromatographic analysis were performed as described earlier (Furmanowa et al. 1994; Pietrosiuk et al. 1999). The final alkaloid fractions were redissolved in 1 ml of methanol for TLC and HPLC analysis. TLC was carried out using conditions presented by Farnsworth et al. (1964) and Ruszkowska et al. (1994). HPLC analysis was performed by the method of Jung et al. (1992), which employed a µBondapak C_{18} reverse phase column and methanol/acetonitrile/aqueous $(NH_4)_2HPO_4$ as a mobile phase.

Statistical analysis

Statistical analysis was performed using the StatSoft® STATISTICA PL programme.

Fig. 1. Hairy roots of *C. roseus* which appeared at wounded site of stem or leaf 7-10 days after transformation with *A. rhizogenes* ATCC 15834. Bar = 1 cm.

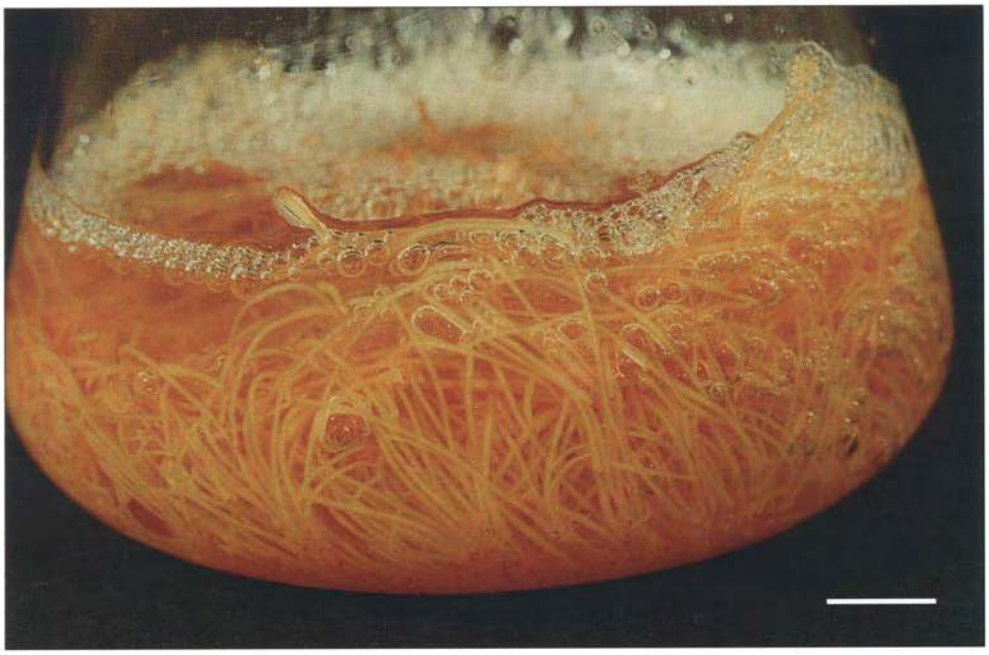

Fig. 2. Hairy roots of *C. roseus* (after transformation of ATCC 15834 *A. rhizogenes* strain) cultured in liquid 1/2 B5 medium without growth regulators (8 line, 11 passage). Bar = 1 cm.

RESULTS AND DISCUSSION

Untransformed roots of *C. roseus* excised from 10-days old seedlings were grown both on the solid and liquid B5 and 1/2 B5 media supplemented with NAA 0.5 and 0.25 mg l^{-1}. First, the callus tissue was formed from the roots, and than the roots were differentiated from the callus. The rhizogenesis process was decaying during the following passages. The best results in untransformed root culture were obtained on 1/2 B5 medium supplemented with NAA 0.5 and 0.25 mg l^{-1} in the second passage. The growth rates were high and amounted to 3672.50 and 3008.52% respectively (Table 2). The root suspension culture consisting of pieces of callus and small agregates of differentiated roots were formed from untransformed roots of *C. roseus* derivating from 2-months old plants regenerated in vitro and placed in liquid 1/2 B5 medium supplemented with NAA in concentration of 0.5 and 0.25 mg l^{-1}, and in liquid B5 medium supplemented with MCPP in concentration of 0.5 and 1.0 mg l^{-1}.

Hairy roots were obtained 7-10 days after infection of 3-weeks old rooted plants of *C. roseus* with the agropine-type of *Agrobacterium rhizogenes* strains: ATCC 15834, LBA 9402 and TR105. Transformation was proven by the detection of opines (agropine and mannopine) using paper electrophoresis according to the modified method of Petit et al. (1983). The 2 cm long roots obtained in the place of infection were excised and cultured individually in 1/2 B5

TABLE 2. Growth rates of different *C. roseus* roots.

The investigated material	Growth rate
Hairy roots after second passage in 1/2 B5 medium	3798.90 ± 1919.17
Untransformed roots from in vitro culture after second passage in liquid 1/2 B5 medium with NAA 0.5 mg l^{-1}	3672.50 ± 1392.12
Untransformed roots from in vitro culture after second passage in liquid 1/2 B5 medium with NAA 0.25 mg l^{-1}	3008.52 ± 194.33

medium. The differences in growth and morphological structure between each clone of roots derived from three various strains of bacteria were observed already in the first passage. The hairy roots obtained after transformation of LBA 9402 and TR105 bacterial strains did not grow and became thick and brown. The hairy roots after transformation of ATCC 15834 *A. rhizogenes* strain (Fig. 2) showed the highest growth and were yellow, long, and had many lateral branches which formed root hairs typical for transformed root culture of several species. And these roots were selected for further investigations. The roots grew well in liquid 1/2 B5 medium without growth regulators and they were subcultured monthly on the fresh medium. The level of sucrose of the medium was 3% and the pH value was 5.7. The conductivity of the medium at the beginning of culture was 2 mS^{-1} and it started to decline as from 7th day while the pH started to increase. The content of sucrose started to decline at the same time as conductivity. The fast increase of fresh weight was observed from 11th day of culture and it is closely connected with the sugar consumption in the medium.

C. roseus hairy roots produce alkaloids characteristic for native plant (Table 3) what was proven by TLC and HPLC analysis. Ajmalicine (1.801 mg g^{-1} DW), catharanthine (1.523 mg g^{-1} DW) yohimbine (0.847 mg g^{-1} DW) and serpentine (0.098 mg g^{-1} DW) were detected in extracts from transformed root. Much more serpentine was obtained (16.4 mg g^{-1} DW) by Ciau-Uitz et al. (1994). An amount of vinblastine (0.048 – 0.111 mg g^{-1} DW), bisindole alkaloid typical for green part of plants was also observed in *C. roseus* hairy roots. This alkaloid was also found in extract from untransformed roots in amount of 0.449 mg g^{-1} DW. These alkaloids were determined in the HPLC method by comparison of peaks retention time to the standards. Vindoline was not detected in group of investigated roots however Bhadra et al. (1993) showed the presence of vindoline in two clones of hairy roots of *C. roseus*. Comparing with the results of other investigations (Table 1) so high level (0.111 mg g^{-1} DW) of vinblastine was detected in this extracts from hairy roots for first time, it is much more to compare with Parr et al. (1988). It can be connected with the origin of seeds which were used for our investigation. The seeds were obtained from India – the place of their natural occurrence. Also the two steps of culture of plants – in tissue culture and in green house – could have some influence. Methyl jasmonate did not induce production of indole alkaloids in hairy roots of *C. roseus* (Table 3). In addition, the amount of investigated alkaloids in this line of roots was less than in adequate line cultured in medium without methyl jasmonate. But, it was observed that this elicitor is active in cause of another species, i.e. it increased the accumulation of paclitaxel in hairy root cultures of *Taxus* × *media* var. *Hicksii* Rhed. (Furmanowa and Sykłowska-Baranek 2000). These results and results from literature data suggest that hairy roots of *C. roseus* can be a potential source of indole alkaloids significant in therapy, especially catharanthine which was produced in the content three times higher than in untransformed roots.

Among all the tested in this work groups of roots, the highest contents of vinblastine and ajmalicine were presented in untransformed roots excised from plants cultured in NN medium with IBA 0.5 mg l^{-1} and kinetin 0.1 mg l^{-1} and than transferred to the soil for 5 months, yohimbine and serpentine – in roots of intact plants and catharanthine in hairy roots.

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TABLE 3. Alkaloid level in different roots of *C. roseus* (concentration in mg g^{-1} D.W.).

Material	Yohimbine	Ajmalicine	Catharanthine	Serpentine	Vinblastine
Hairy roots cultured on 1/2 B5 medium (6 passage)	0.847 ± 0.034	1.801 ± 0.107	**1.523** ± 0.076	0.098 ± 0.002	0.048 ± 0.001
Hairy roots – line 8, cultured on 1/2 B5 medium (11 passage)	0.292 ± 0.075	0.296 ± 0.050	0.877 ± 0.029	0.272 ± 0.058	0.111 ± 0.003
Hairy roots – line 8, cultured during 1 passage on 1/2 B5 medium with methyl jasmonate (11 passage)	0.132 ± 0.010	0.108 ± 0.006	0.083 ± 0.006	0.062 ± 0.004	0.045 ± 0.006
Untransformed roots cultured on 1/2 B5 medium with NAA 0.5 mg l^{-1} (6 passage)	0.042 ± 0.016	0.053 ± 0.017	0.464 ± 0.016	0	0
Untransformed roots excised from plants cultured in NN medium with IBA 0.5 mg l^{-1} and kinetin 0.1 mg l^{-1} and transferred to the soil for 5 months	1.485 ± 0.016	**1.881** ± 0.262	1.507 ± 0.241	0	**0.449** ± 0.015
Untransformed roots excised from plants growing only in the soil for 5 months	**1.685** ± 0.015	1.787 ± 0.021	0.544 ± 0.047	**0.475** ± 0.014	0.093 ± 0.014

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WSTĘPNE WYNIKI WYTWARZANIA ALKALOIDÓW INDOLOWYCH W KORZENIACH *CATHARANTHUS ROSEUS* Z RÓŻNYCH KULTUR IN VITRO

STRESZCZENIE

Badano korzenie *Catharanthus roseus* (L.) G. Don obejmujące korzenie normalne i transgeniczne pochodzące z hodowli in vitro. W celu uzyskania korzeni transgenicznych, procesowi transformacji poddano trzytygodniowe ukorzenione in vitro roślinki *C. roseus*, które zakażano trzema szczepami *Agrobacterium rhizogenes*: ATCC 15834, LBA 9403 i TR 105. Badano także korzenie roślin niezmienionych genetycznie. W uzyskanych wszystkich grupach korzeni poszukiwano winblastyny, windoliny, serpentyny, katarantyny, ajmalicyny i johimbiny. Najwyższą zawartość badanych alkaloidów indolowych – johimbiny i serpentyny oznaczono w korzeniach roślin pochodzących z gruntu; katarantyny w korzeniach trangenicznych; ajmalicyny i winblastyny w normalnych korzeniach roślin zregenerownych in vitro a następnie przeniesionych do gruntu na pięć miesięcy. Windoliny nie wykryto w żadnej z sześciu grup badanych korzeni.

SŁOWA KLUCZOWE: kultury korzeniowe, alkaloidy indolowe, *Catharanthus roseus*, *Agrobacterium rhizogenes*.