0273–2289/87/1402–0101$02.00

Increased Synthesis of Ajmalicine and Catharanthine by Cell Suspension Cultures of *Catharanthus roseus* in Response to Fungal Culture-Filtrates

F. DiCosmo,*[1,2,3] A. Quesnel,[4] M. Misawa,[4] and S. G. Tallevi[1]

[1]*Centre for Plant Biotechnology,*[2] *Department of Botany, and* [3]*Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario, M5S 1A1, Canada; and* [4]*Allelix Inc., 6850 Goreway Drive, Mississauga, Ontario, L4V 1P1, Canada*

Received May 12, 1986; Accepted September 8, 1986

ABSTRACT

The ammonium sulfate-precipitated fraction from mycelia and culture-filtrates and the crude, cell-free culture filtrates from the growth medium of the fungi *Chrysosporium palmorum, Eurotium rubrum, Micromucor isabellina,* and *Pythium aphanidermatum* when aseptically added to cell suspensions of *Cantharanthus roseus* caused a rapid and dramatic increase in indole alkaloid biosynthesis. Up to 400 μg/L ajmalicine and 600 μg/L catharanthine were detected in *C. roseus* cell suspension grown in the presence of the *M. isabellina* fungal culture filtrate for 3 d. Untreated cells produced only trace levels of ajmalicine and catharanthine per liter of cell suspension after 15 d of culture.

Index Entries: *Catharanthus roseus;* fungal elicitation; ajmalicine; catharanthine.

INTRODUCTION

The rapid development of cultured plant cell techniques has allowed for penetrating investigation of secondary metabolism, including enzy-

*Author to whom all correspondence and reprint requests should be addressed.

mology of indolic alkaloids in suspension cultures of *Catharanthus roseus* (*1–8*). However, the main obstacle often faced by researchers is the low levels of indolic alkaloids produced by *C. roseus* cell suspensions compared to the intact plant. The manipulation of culture media, culture conditions, hormone regimes, and cell selection has not yet allowed for profitable, commercial production of the *Catharanthus* alkaloids using cell culture systems. This failure must certainly reflect our lack of understanding of metabolic regulation in cultured plant cells. Until a more complete understanding is realized, methods that allow for increased biosyntheses should be explored. One method under investigation is the induction of indolic alkaloids in *C. roseus* cell cultures through microbial insult (*9*). For example, Tallevi and DiCosmo (*10*) showed that 2-yr-old *C. roseus* callus cultures normally producing low levels of tryptamine and ajmalicine could be stimulated to produce dramatically increased levels of these in rapid response to contact with crude culture filtrates derived from several fungi. The greatest increase in ajmalicine production was noted in response to contact with crude culture filtrates derived from *Micromucor isabellina* and *Pythium aphanidermatum*.

Microbe-derived molecules that stimulate secondary metabolism are called "elicitors." DiCosmo and Misawa (*11*) reviewed the use of elicitors and other inducers as modulators of secondary metabolism in cultured plant cells. The technique showed great promise for allowing for the induction and accumulation of commercially valuable phytochemicals, including indolic alkaloids from *C. roseus* cell suspensions. This implies that the activities of the alkaloid biosynthetic enzymes are also being altered in response to various inducers. The technique should allow us to alleviate the problem pointed out by Stockigt (*12*) that "for successful cell-free studies . . . cultures capable of synthesizing high quantities of these alkaloids" are required.

We have treated cell suspensions and callus cultures of *C. roseus* normally producing trace levels of ajmalicine and catharanthine with crude culture filtrates derived from the fungi *Chrysosporium palmorum, Eurotium rubrum, M. isabellina,* and *P. aphanidermatum* and found rapid, dramatic increases in the biosyntheses of these products and other alkaloids.

MATERIALS AND METHODS

Plant Cell Cultures

Cell suspensions of *C. roseus* were initiated from callus cultures (cell line FD/ST-84/07) and were 2 yr old. Cell suspensions were propagated in dark at 27°C on a gyratory shaker (120 rpm) in 500-mL Erlemeyer flasks with 100 mL of amended Schenk and Hildebrandt (SH) medium (13) containing 4.0 mg/L α-napthalene acetic acid as the sole growth regulator. Cultures were transferred into new medium every 14 d using 20 mL of

cell suspension as inoculum. Callus cultures were grown on 30 mL of amended SH medium solidified with 0.8% agar.

Fungal Elicitor Preparation

Cultures of *C. palmorum, E. rubrum, M. isabellina,* and *P. aphanidermatum* were grown in 250-mL flasks with 50 mL of SH medium devoid of growth regulators. Cultures were kept on an orbital shaker (120 rpm) at room temperature (25 ± 4°C) in light for 20 d.

Crude fungal culture filtrate preparations were obtained using the method of DiCosmo et al. (*14*). Fungal filtrates were reduced to about 5 mL by vacuum distillation and were dialyzed against 2 L distilled water for 36 h at 5°C with three changes of water, 1, 6, and 12 h after the start of dialysis. The dialyzed fraction was centrifuged at 40,000*g* for 30 min (5°C). The supernatant was collected and stored at −20°C. Alternatively, mycelium from the fungi was washed with 1 L of cold (5°C), distilled water in a Buchner funnel and collected on Whatman no. 1 filter paper. The mycelial mat was cooled to 5°C and homogenized in 100 mL cold TRIS–EDTA, pH 7.6, containing 1 m*M* dithiothreitol. The homogenate was centrifuged at 40,000*g* for 1 h at 5°C. The supernatant was brought to 70% saturation with $NH_4(SO_4)_2$, the precipitate was collected by centrifugation (40,000*g* for 1 h) and dialyzed against 2 L distilled water for 24 h at 5°C, with three changes, 1, 3, and 6 h after start of dialysis. The dialyzed material is the precipitated mycelium protein (MPE).

The culture filtrate protein extract (CFPE) was prepared by bringing the freshly collected, spent culture filtrate to 70% saturation with $NH_4(SO_4)_2$. The precipitate was collected by centrifugation at 40,000*g* for 1 h at 5°C. The pellet was taken up in the minimum amount of TRIS–EDTA buffer, pH 7.6, and dialyzed as previously described. All preparations were stored at −20°C.

Protein Determination

The protein content of each fungal preparation was determined using the method of Kalb and Bernlohr (*15*).

Elicitation

The crude fungal culture filtrate derived from *M. isabellina* was added aseptically to 12-d-old *C. roseus* suspension cultures using a Millex-GV 0.22-μm membrane filter to give a final concentration of 7.5% fungal-filtrate/flask (v/v). Callus cultures received amounts of proteinaceous elicitors as described in text. Control cultures received an equal volume of sterile, distilled water or buffer. Cultures were incubated in dark for 72 h at 27°C on a gyratory shaker (120 rpm).

Alkaloid Extraction and Identification

After incubation, elicited cells were harvested and their alkaloid content extracted by standard techniques. High-performance liquid chromatography (HPLC) analysis of alkaloid extracts was performed using a Waters 840 system equipped with a Hewlett-Packard 1040A UV-detector set at 226 nm. Elution was accomplished within 30 min using a Brownlee RP-8 Spheri-5 column (22 cm × 4.6 mm); the initial condition of methanol/water (55/45, with 2.5 m*M* tetrabutylammonium phosphate) was maintained for 2 min followed by a convex (curve 5) solvent gradient [55/45 (MeOH/H_2O) to 90:10] for 18 min. The HPLC was also performed with a Waters 660 solvent programmer equipped with a μ Bondapak C_{18} reverse-phase column using methanol/water (60/40–100/0, convex gradient curve 7, 20 min) and 0.5% triethylamine at a flow-rate of 1.5 mL/min. Quantiation was by the use of external standard curves fitted by linear regression correlation coefficient > 0.990. Thin-layer chromatography (TLC) was performed using preparative silica-gel-coated plates (Baker Si500F), Kodak 13181 analytical silica gel plates or Kieselgel $60F_{254}$ (Merck) developed with ethyl acetate/methanol, 90:10 and methanol/ethylacetate, 90:10, with 0.5% triethylamine. Alkaloids were detected with ceric ammonium sulfate (CAS) spray reagent or TLC plates were examined using a Shimadzu TLC scanner. Areas corresponding to tryptamine, ajmalicine, and catharanthine (determined as R_f-values of authentic standards) were scraped off and eluted with methanol. The physical and UV-spectral data of the induced products conformed in all respects with those of authentic standards.

RESULTS AND DISCUSSION

A *C. roseus* cell line, designated FD/ST-84/07, that normally produces trace levels of indole alkaloids was used in these experiments. We treated callus cells by aseptically adding crude culture filtrates to the surface of the cells and incubating the treated cells for 36 h at 27°C in the dark. All treated callus showed rapid and increased levels of tryptamine and ajmalicine. For example, when 1 mL of culture filtrate protein extract from *P. aphanidermatum*, containing 120 μg of protein, was added to our cells, up to 80–100 μg of ajmalicine was produced. A similar response was noted when a similar *M. isabellina* elicitor preparation that contained 333 μg protein was used. Only traces of ajmalicine were found in untreated cells. We found *M. isabellina* elicitor preparation to yield the best and most reproducible results. Subsequently, we used crude culture filtrates of *M. isabellina* and *C. roseus* cell suspensions.

Culture filtrates from *M. isabellina* were aseptically added to 12-d-old *C. roseus* suspension cultures. After a 3-d incubation, cells were harvested and assayed for their alkaloid content. Fungal culture filtrate-treated cultures accumulated ajmalicine and catharanthine (Table 1).

TABLE 1
Increased Production of Ajmalicine and Catharanthine in Suspension Cultures[a]

Treatment	Fresh weight, g	Dry weight, g	Ajmalicine, µg/gdw[b]	Ajmalicine, µg/L[c]	Catharanthine µg/gdw[b]	Catharanthine µg/L
Control	24.88	2.49	TRACE		2.33	5.8
Culture, filtrate-treated	16.02	1.60	250.0	400.0	375.0	600.0

[a]Cultures were *C. roseus* treated with the crude culture-filtrate of *M. isabellina* for 72 h.
[b]µg/g dry weight.
[c]µg/L.

Quantitative analysis of alkaloid extracts by HPLC revealed that after 15 d of growth our control cultures produced only trace quantities of ajmalicine and catharanthine. Treated cultures, however, showed a large increase in the levels of both these alkaloids, accumulating up to 400 µg/L ajmalicine and 600 µg/L catharanthine.

The accumulated alkaloids ajmalicine and catharanthine belong to the Coryanthe-type and Iboga-type alkaloids, respectively, and represent branches of metabolic pathways. It seems reasonable, therefore, to suggest that treatment of *C. roseus* cell suspensions with fungal culture filtrates that stimulate alkaloid synthesis is not a simple process. Much more careful and penetrating studies are required to identify the mechanics of the process.

It is well recognized that cultured plant cells represent a potential rich source of commercially valuable secondary metabolites. However, the recalcitrant nature of cultured plant cells indicates that methods aimed at breaking the strict metabolic control must be developed. For example, the use of amino acid analogs may increase the enzymes of secondary metabolism several-fold, resulting in increased product yield (*16*). Alternatively, high-yielding cell lines can be selected (*17*). However, the problem remains that profitable commercial exploitation of cultured plant cell secondary metabolism has not been generally realized because of a lacuna in our understanding of the biochemistry and regulation of secondary metabolism.

Our approach has been to treat *C. roseus* cell cultures with fungal culture filtrates. Similar approaches have been used to cause increased synthesis of alkaloids, coumarins, flavonoids, acetylenes, terpenoids, and a steroid [cited in ref. (*11*)]. In our system, we have been able to cause an increase in the synthesis of several indolic alkaloids, including ajmalicine and catharanthine. We have also found that the time during the growth cycle of cells at which the elicitor is added is critical. The general trend seems to suggest that as cells age they become less responsive. Cell cultures that received no fungal filtrate failed to show this dramatic response. This is important because enzymes for ajmalicine and catharanthine synthesis are enriched in the system, and the rapid, increased

synthesis should allow for decreased fermentation time. The results also suggest that cultured cells possess the biosynthetic potential of the plant from which they were derived and need only the proper stimulus for expression of this biosynthetic capability. This has obvious practical application for the production of rare or fine chemicals.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge stimulating discussions with Drs. A. Goodbody and J. Smith of Allelix Inc. Dr. R. Sommerbell (University of Toronto) kindly supplied the fungal cultures. Research was funded by a NSERC operating grant, a NSERC Biotechnology Strategic grant, and generous equipment funding from the University of Toronto and the Bickell Foundation made to F. DiCosmo.

REFERENCES

1. Hemscheidt, T., and Zenk, M. H. (1985), *Plant Cell Reports* **4,** 216.
2. Sasse, F., Buchholz, M., and Berlin, J. (1983), *Z. Naturforsch.* **38(C),** 916.
3. Knoblock, K. -H., Hansen, B., and Berlin, J. (1981), *Z. Naturforsch.* **36(C),** 40.
4. Hemscheidt, T., and Zenk, M. H. (1980), *FEBS Lett.* **110,** 187.
5. Treimer, J. H., and Zenk, M. H. (1979), *FEBS Lett.* **97,** 159.
6. Treimer, J. H., and Zenk, M. H. (1979), *Eur. J. Biochem.* **101,** 225.
7. Mizukami, H., Nordlov, H., Lee, S.-L., and Scott, A. I. (1979), *Biochemistry* **18,** 3760.
8. Stockigt, J., Treimer, J., and Zenk, M. H. (1976), *FEBS. Lett.* **70,** 267.
9. DiCosmo, F., and Tallevi, S. G. (1985) *Tibech.* **3,** 110.
10. Tallevi, S. G., and DiCosmo, F. (1985) *1st Canadian Plant Molecular Biology Workshop,* Abstract B18, May 26–28, Guelph, Ontario.
11. DiCosmo, F., and Misawa, M. (1985) *Tibech.* **3,** 318.
12. Stockigt, J. (1980), in *Indole and Biogenetically Related Alkaloids* (Phillipson, J. D., and Zenk, M. H., eds.), pp. 113–141, Academic London.
13. Schenk, R. U., and Hildebrandt, A. C. (1972) *Can. J. Botany* **50,** 199.
14. DiCosmo, F., Norton, R., and Towers, G. H. N. (1982), *Naturwiss.* **69,** 550.
15. Kalb, V. F., and Bernlohr, R.W. (1977), *Anal. Biochem.* **82,** 362.
16. Widholm, J. M. (1980), in *Plant Tissue Culture as a Source of Biochemicals* (Staba, E. J., ed.), pp. 99–113, CRC, Boca Raton, FL.
17. Zenk, M. H., El-Shagi, H., Arens, H., Stockigt, J., Weiler, E. W., and Deus, B. (1977), in *Plant Tissue Culture and its Bio-technological Application* (Barz, W., Reinhard, E., and Zenk, M. H., eds.), pp. 27–43, Springer-Verlag, Berlin, New York.