Long-Term Effect of a *Pythium* Elicitor Treatment on the Growth and Alkaloid Production of *Catharanthus roseus* Cell Suspensions

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Abstract

The treatment of a *Catharanthus roseus* cell suspension culture with a low concentration of *Pythium* elicitor stimulated the alkaloid production. When these pretreated cells were resuspended in a medium that did not contain the fungal extract, the positive effects of the treatment on alkaloid synthesis and excretion were lost and, moreover, the standard level of production was not recovered. A second treatment of these cells with *Pythium* elicitor at day 5 of the second culture cycle greatly impaired growth kinetics, but did not stimulate the alkaloid production observed with standard cultures. Repeated treatments with a low concentration of fungal elicitor seemed to have a negative long-term effect on both growth and alkaloid synthesis and did not appear to be a useful process for production purposes.

Key words

Alkaloids, *Catharanthus roseus* L. (G.) Don, Apocynaceae, cell suspension, fungal elicitor, *Pythium vexans*, re-elicitation.

Abbreviations

gEq:	Glucose-equivalent
2,4-D:	2,4 dichlorophenoxyacetic acid
HPLC:	high performance liquid chromatography
Py:	*Pythium vexans* elicitor

Introduction

Tissue culture techniques have been investigated with the aim to produce compounds of economic interest. In recent years efforts have been made to enhance the ability of these plant cell cultures to accumulate secondary products. Elicitor treatment appears to be one of the most successful techniques employed to stimulate alkaloid synthesis in *Catharanthus roseus* tissue culture. Both inorganic (1, 2) and organic compounds (3–6) have a positive effect on alkaloid production, but only on some of the cell lines tested (7, 8). Large increases in alkaloid level and metabolite excretion were observed when fungal extracts were used (7, 9). For each of these cell lines, the level of the response depended on the fungal elicitor used, the elicitor concentration, or the culture age of the treated cells (7, 10). Repeated treatments with fungal elicitor had not previously been investigated with *C. roseus* cells.

We have recently characterised a *C. roseus* cell suspension culture that reacted to the addition of a low concentration of *Pythium vexans* extracts by an induction of catharanthine synthesis and an increase in the production of the other alkaloids (10). This stimulation of the alkaloid production was accompanied by the release of both ajmalicine and catharanthine into the culture medium. Since growth was not altered by the treatment, we used these elicitor pretreated cells for another cycle of culture in order to observe alkaloid production in subsequent subcultures. In addition, we examined the alkaloid production of these elicitor pretreated *C. roseus* cells after a new treatment, at day 5 of subculture, by *Pythium* extracts.

Materials and Methods

Suspension cell cultures

The cell line was initiated from hypocotyl explants of *C. roseus* (L.) G. Don and characterised with respect to growth, mineral consumption, and alkaloid production (10). Stock suspension cultures were grown in 250 ml Erlenmeyer flasks containing 70 ml of a modified Murashige and Skoog (11) medium (lacking KI) supplemented with 2,4-D (0.45 μM), kinetin (4.5 μM), and 2 % (w/v) sucrose. The cells were transferred to fresh medium every week and maintained on a giratory shaker (80 rpm) at 28 °C under diffuse light (16 h day^{-1} at 20 W m^{-2}).

Fungal cultures and elicitor preparation

An isolated culture of *Pythium vexans* (de Bary) was maintained routinely on 2 % maltea medium. For the elicitor preparation, pieces of mycelium were used to initiate cultures in a minimum sucrose liquid medium. After 2 weeks, the culture was then autoclaved (20 min, 120 °C) and an ethanol-soluble extract was prepared using the method of Toppan and Esquerré-Tugayé (12). The dried residue was moistened with 3 ml of water and stored at −18 °C. Total sugar was determined by the anthrone method and expressed in μg glucose-equivalent (gEq) ml^{-1}.

Establishment of pretreated suspension cultures

Cells maintained 7 days in a standard medium were collected by filtration onto a sterilised nylon net (40 μm). Aliquots of filtered cells (6 g of fresh weight) were resuspended in 70 ml of fresh medium containing the fungal elicitor at a final concentration of 0.9 μg equivalent-glucose (gEq) ml^{-1} medium. After a complete culture cycle (7 days), these pretreated cells were filtered as indicated above and 6 g of fresh-weight cells were resuspended in 70 ml of a standard medium for subculture. For each culture cycle, 20 culture flasks were initiated and experiments were performed in triplicate.

Treatment at day 5

After 5 days of culture, 150 μl of a sterilised elicitor solution (2.8 μg gEq ml^{-1} in final concentration) were added to culture flasks containing pretreated cells or standard cultures. Controls consisted of pretreated suspension subcultures and standard cultures. Experiments were performed in duplicate.

Analytical procedures

Three culture flasks were combined for each experiment. After pH measurement of the whole suspension, cells and medium were separated by filtration under partial vacuum through a glass fiber filter. Growth was estimated by measuring the total fresh weight and the dry weight (a 500 mg aliquot of fresh cells was dried at 60 °C for 24 h). Alkaloids were extracted following the method of Roustan et al. (13). Quantification was performed by HPLC using a 250 mm C_8 (5 μm) reverse phase column. The flow rate of the isocratic eluent system (30 % acetonitrile with HCl/KCl 40 mM) was set to 1 ml min^{-1} and the column eluent was monitored at 254 and 280 nm with a UV detector. Retention times of ajmalicine, catharanthine, and serpentine were 13.3, 15.5, and 17.2 min, respectively, in this system.

Results

Growth kinetics after one or two treatments with Pythium *elicitor*

Standard cell cultures treated at day 0 of a first culture cycle exhibited a rather similar growth rate compared to control (Fig. 1A). The growth kinetics were not significantly modified by the treatment and the dry weights obtained after 7 days of culture were almost identical for the two cultures. In the same way, a treatment applied to these standard cultures at day 5 of a second culture cycle did not greatly impair culture growth (Fig. 1B). At the end of the culture cycle, equal dry weights were measured in control and treated cells. On the other hand, a great difference in the growth kinetics was observed when cells treated at day 0 of the first culture cycle were transferred to a standard medium free of elicitor (Fig. 1B). For these pretreated cells, significant growth was only observed after 3 days of subculture. The dry weight was then 45 % lower than in the control after 8 days of culture. Moreover, a renewed treatment of these pretreated cells 5 days after their subculture markedly reduced culture growth as soon as the treatment was applied. Microscopic observations did not reveal abnormal cell death. However, a cell swelling was visible in pretreated cultures from day 5 of the first culture cycle that induced a dry weight difference between standard and pretreated cultures at subculturing. In addition, the pH kinetics of the cell suspensions were affected by adding elicitor. The pH of treated cultures were generally slightly more acidic but a difference of 0.7 pH units was observed after 5 days of treatment at day 0 (data not shown).

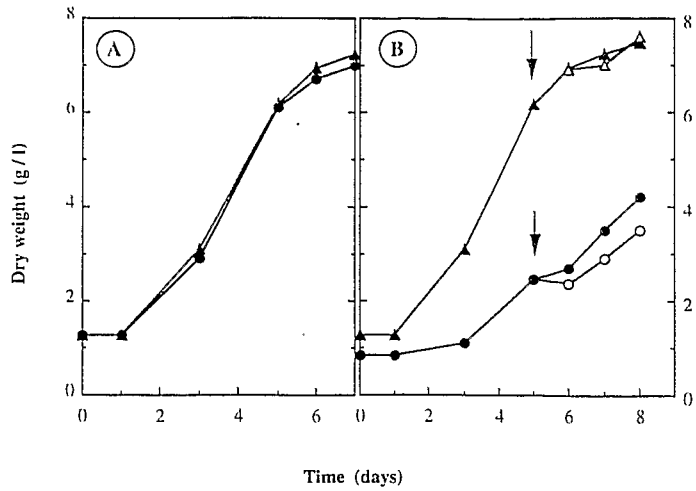

Fig. 1 Growth kinetics of standard (–▲–) and pre-treated (–●–) cultures of *C. roseus* cells with *Pythium* elicitor extract. **A**: first culture cycle with a treatment at day 0; **B**: second culture cycle with treatment at day 5 (indicated by an arrow) (treated cells = open symbols).

Total production and distribution of the alkaloids between cells and medium

A single treatment of standard cultures, at day 0 of a first culture cycle (Fig. 2A) or at day 5 of a second culture cycle (Fig. 2B), was immediately accompanied by a modification in the production of alkaloids and their distribution between cells and medium (Table 1). The total synthesis (within the cells and in the culture medium) of the two major compounds, ajmalicine and serpentine, was greatly increased by the treatment and the presence of a new alkaloid, catharanthine, was noticed. Except for ajmalicine production, these effects were maintained throughout the time of treatment, when the treatment was applied at day 5. However, no significant response was obtained when pre-treated cells were treated at day 5 of their subculture (Fig. 2B). These cells, elicited during the first culture cycle, exhibited greatly modified production kinetics compared to standard cultures when they were transferred to a standard medium. Ajmalicine was poorly produced (2- to 6-fold less than in the control) and serpentine more largely synthesized. Except on day 6, serpentine was the major alkaloid, with a production on average 5-fold higher than ajmalicine and 3-fold higher than in the control. Moreover, these cells had lost the ability to produce catharanthine, but a transient production of this compound could be observed at day 5 or 6 of culture. The re-elicitation of these pretreated cells on day 5 did not greatly modify the alkaloid synthesis. The only positive effect noticed was a weak transient stimulation of ajmalicine synthesis that gave, after 24 hours of treatment, an ajmalicine level 1.5-fold higher than in pre-treated cells not re-elicited at day 5 of the second culture cycle. The variations in the production of the two other alkaloids, serpentine and catharanthine, were not significantly different between pre-treated cells re-elicited or not.

Alkaloid		Day of culture: First cycle 0	1	3	5	7	Second cycle 0	1	3	5	6	7	8
Ajmalicine	T	24	17	59	12	18	29	18	59	11	10	21	42
	+ Py	–	–	–	–	–	–	–	–	–	86	70	88
	E	24	95	84	92	89	75	45	15	87	14	37	43
	+ Py	–	–	–	–	–	–	–	–	–	71	21	54
Serpentine	T	0	39	17	15	9	0	25	21	22	9	15	7
	+ Py	–	–	–	–	–	–	–	–	–	7	3	5
	E	0	0	9	22	12	15	6	4	30	34	6	6
	+ Py	–	–	–	–	–	–	–	–	–	17	8	3

Table 1 Percentage (external/total production) of each major alkaloid released in the culture medium during two consecutive cycles of culture in the presence or absence of *Pythium* elicitor. (T = standard culture, E = culture pre-treated with *Pythium* extract, + Py = treatment at day 5 of the second culture cycle with *Pythium* extract).

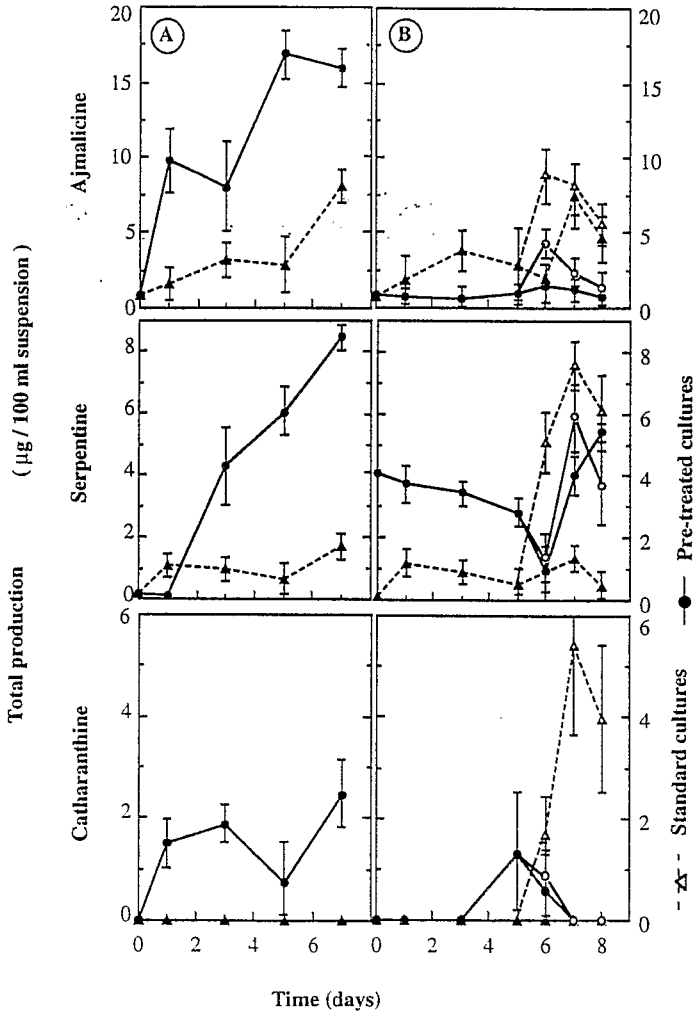

Fig. 2 Total production (internal and external) of major alkaloids by standard (–▲–) and pre-treated (–●–) cultures during two consecutive culture cycles. **A:** first culture cycle; **B:** second culture cycle without (closed symbols) or with (open symbols) a treatment at day 5 with *Pythium* elicitor extracts.

In standard culture conditions, alkaloids accumulated within the cells (Table 1). A slight release to the medium was routinely observed throughout cell culture, except for the ajmalicine on day 3 of both culture cycles where 59% of the total production was excreted in the medium. At the end of each culture cycle, serpentine was much more accumulated than ajmalicine. The treatment at day 0 of the first culture cycle or at day 5 of the second cycle promoted ajmalicine excretion, 70 to 95% of the total production being released into the culture medium. Serpentine concentrations were also greatest in the culture medium of treated cells but excretion was not increased by the treatment, the ratio of concentrations between cells and medium being similar for untreated and treated cells. Conversely, this compound was accumulated more within the cells in the presence of *Pythium* elicitor, especially when the treatment was applied at day 5. When the elicitor pre-treated cells were resuspended in standard culture medium, the distribution of each alkaloid between cells and medium was not constant. Great variations in the ratio external/total production were noticed from day to day of sampling not only for ajmalicine but also for serpentine. Whereas serpentine seemed to be essentially accumulated in the cells, ajmalicine was sometimes more present in the extracellular medium (at day 0 or day 5 of the subculture). For that reason, the effects of re-elicitation on the alkaloid distribution of pre-treated cells did not appear clearly. The over-excretion of ajmalicine observed after one treatment with *Pythium* elicitor was not obtained.

Discussion

Our results show that *Catharanthus roseus* cells that had never been treated with *Pythium vexans* extracts responded to a first elicitation at day 0 or at day 5 by a stimulation of their alkaloid production and the release of some of these alkaloids into the culture medium. This response agrees with results obtained in short-term treatments (7). In our experiments the low concentration of elicitor used did not immediately alter growth and allowed us to observe that elicitor effects were maintained throughout one cell culture cycle. Ajmalicine was largely released into the culture medium from the first day of both treatments, whether this was day 0 or 5 (Table 1). However, serpentine was accumulated within the cells, despite its over-production. The different distribution of these two indole alkaloids may be related to the nature of each compound. Indeed, ajmalicine is a weakly basic alkaloid (pK = 6.3) which can diffuse across plant cell membranes and accumulate in the acidic vacuole (14) where it is then trapped (15, 17). Since the mechanisms of transport of ajmalicine through tonoplast and plasmalemma are dependent on pH gradients (15, 18), the external medium acidified by the fungal elicitor treatment could constitute a new acidic compartment where ajmalicine would be trapped or degraded. On the other hand, the higher pK value of serpentine leads this alkaloid to accumulate in the acidic vacuole whatever the variations of external pH in the medium obtained by elicitor treatment. Moreover, the vacuolar pool of serpentine

may be enlarged by the serpentine resulting from the conversion of ajmalicine by basic vacuolar peroxidases (16).

Nevertheless, preliminary experiments upon re-elicitation of pre-treated cells showed that none of these modifications in alkaloid production and distribution were obtained. In fact, these pre-treated cells did not have the usual characteristics of standard cultures with respect to growth and alkaloid production when they were transferred to a standard culture medium. The modifications in growth kinetics were clearly visible on subculturing. They indicate alterations in cell metabolism that were probably induced during the first culture cycle by the long duration of the pre-treatment. Indeed, ultrastructural changes have been observed with long-term treatment of *Papaver bracteatum* cell cultures (19). The long-term treatment could act on the cell wall structure, the ionic equilibrium or the membrane permeability that found expression at the same time in the change in cell water content visible only upon subculture and in the loss of a constant distribution of alkaloids between cells and medium. Moreover, the removal of the stimulation of alkaloid synthesis upon re-elicitation suggested undesirable long-term effects of the fungal elicitor and the emergence of a negative response of cell cultures to repeated treatments, even though cells were subcultured in standard medium before re-elicitation. However, these preliminary data on re-elicitation have to be confirmed by new experiments where variations in time of pre-treatment and elicitor concentration are made, associated with microscopic observations and cell physiology studies. Actually, we applied, to the pre-treated cultures, the dose of elicitor that was the most effective for fast growing standard cultures (10). This concentration appeared to be ineffective for these subcultured pre-treated cells which presented a low cell density after 5 days of culture. Thus, in contrast to results obtained on *Papaver somniferum* cell suspension cultures (20), repeated use of elicitation, in the experimental conditions presented here, did not appear to be a useful process for production purposes with our *Catharanthus roseus* cell line submitted to *Pythium vexans* extracts.

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