Induction of catharanthine synthesis and stimulation of major indole alkaloids production by Catharanthus roseus cells under non-growth-altering treatment with Pythium vexans extracts

C. Nef 1,2, B. Rio 1, and H. Chrestin 1

1 Laboratoire de Physiologie et Biotechnologie Végétales, ORSTOM-IRSDA, B.P. V-51, ABIDJAN 01. Côte d’Ivoire, France
2 Present address: Lab. du Métabolisme, INRA, Route de Saint-Cyr, 78026 Versailles Cedex, France

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ABSTRACT

A Catharanthus roseus cell line was selected that synthesised catharanthine exclusively under elicitation.

From the first day of culture, treatment with very low concentrations of a Pythium extract did not alter the growth of the suspension but, within 24 hours, induced the synthesis of catharanthine and stimulated the production of ajmalicine. Kinetic analysis showed that serpentine then began to accumulate and that all of these effects lasted more than 7 days. Elicitation also induced changes in the cell/medium distribution of the alkaloids. Higher, although non-lethal, concentrations of the fungal elicitor were shown to impair alkaloid production. This cell line will serve as a model to study the conditions for the expression of catharanthine synthesis at the molecular level.

ABBREVIATIONS

gE : glucose-equivalent,
MS : Murashige and Skoog medium,
2,4-D : 2,4-Dichlorophenoxyacetic acid.

INTRODUCTION

Elicitors have been extensively used to induce or stimulate secondary metabolism in plant cell suspension cultures. Fungal elicitors have been tested in various plant suspension cultures (Eilert et al. 1985; Leguay et al. 1988), in particular in Catharanthus roseus cell cultures. Principal results have been recently summarized in review articles (Van der Heijden et al. 1989; Ganapathi and Kargi 1990).

C. roseus cells, treated at the beginning of, or during the growth phase, exhibited a very high increase in alkaloid production (Di Cosmo et al. 1987), sometimes accompanied by the excretion of some of these compounds into the culture medium (Eilert et al. 1986). These effects, observed within a relatively short period (a maximum of 72 hours following elicitation), were generally associated with browning of the culture, due to an excessive production of phenolics. It has been demonstrated that the accumulation of these compounds resulted from the induction of enzymes of the alkaloid and phenolic pathways (Eilert et al. 1987; Seitz et al. 1989).

In this work, we characterize a new C. roseus cell line which exhibits (1) G. Don. Stock suspension cultures were grown in 250 ml Erlenmeyer flasks containing 70 ml of a modified MS (Murashige and Skoog 1962) medium (lacking KI) supplemented with 2,4-D (0.45 μM), kinetin (4.5 μM) and 2% (w/v) sucrose. The suspensions were subcultured weekly and maintained on a gyratory shaker (80 rpm) at 28°C under diffuse light (16 hours/day; 20 W.m-²).

Preparation of the crude fungal elicitor. Mycelium of Pythium vexans (de Bary), isolated from necrotic leaves of C. roseus plants grown in the field, were maintained routinely on 2% Malteia medium. For preparation of the elicitor extracts, pieces of mycelium were cultured for 3 weeks in a minimum sucrose culture medium. An ethanol-soluble extract was obtained, using the method of Toppan and Esquerré-Tugayé (1984). The dried residue was moistened with 3 ml of H2O and stored at -18°C (crude elicitor extract).

Treatments with elicitors. In treatments begun on day 0 (the first day of subculture), aliquots of the crude elicitor extract, containing 1.5 mg/ml glucose equivalent (gE) as determined by the anthrone method, were added to the culture medium of each flask before autoclaving to reach a final concentration ranging from 0.87 to 870 μg glucose equivalent (gE)/ml medium. For treatments applied during the cell culture, aliquots of a sterile preparation of the crude fungal elicitor extract were added to the cell culture at day 5 and 8 to reach a final concentration of 120 μg gE/g fresh weight.

Growth and indole alkaloid analysis. After pH measurement of the whole suspension, cells and

Offprint requests to: C. Nef
medium were separated by filtration under partial vacuum through a glass fibre 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 48 h).
Glucose, fructose and sucrose consumption were estimated by enzymatic determination (Bernt and Bergmeyer 1974), while \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) were measured by the fluorimetric methods (Smith and Snell 1949; Solorzano 1969). The alkaloids were extracted following the method of Roustan et al. (1982), qualitatively screened by TLC (silica gel 60F254 plates, Merck) and quantitatively analysed by HPLC (Column Merck CB, 5um, 250mm; eluent: acetonitrile/(HCl:KC1) 40mM, pH 2.5 (30/70); flow rate 1ml/min; U.V. detection at 254 and 280 nm).

RESULTS AND DISCUSSION

Characterization of the cell culture
Growth and some nutritional characteristics.
This cell line, grown on a medium containing a high cytokinin concentration with respect to auxin (ratio 10/1), exhibited a rather low growth rate compared to those obtained on classical culture medium (Courtois and Guern 1980). Fresh and dry weights (Fig.1) increased only about 6-fold during the first 7 days of culture. However, the consumption kinetics of carbon and nitrogen sources (Fig.2) fit well with those described elsewhere (Van der Heijden et al. 1989), showing that the hormonal composition of the medium did not modify the typical kinetics of growth and nutritional assimilation of \( C. \text{roseus} \) cell suspension cultures.

Production of alkaloids.
The total production (cells+medium) of the two major alkaloids is presented in figure 3. In spite of a similar hormonal composition of the medium, it can be seen that, as reported by Doireau et al. (1987) for three other strains of \( C. \text{roseus} \), the cell line described here produced ajmalicine at the end of the growth phase (day 7-8), while the production of serpentine essentially took place during the stationary phase (days 10-12). No trace of catharanthine was ever detected in these routine culture conditions.

Effects of elicitor concentrations and cell culture age on the response to elicitor.
For this study, only concentrations of the fungal elicitor that neither altered growth nor resulted in browning of the cell suspensions were considered. When the treatment was applied at the first day of culture, it can be seen (Fig.4) that the lowest concentrations of elicitor (0.87 to 8.7 \( \mu \)g gE/ml) induced an increase in the production of ajmalicine (x 5 and 1.5 respectively) within the first 24 hours, but either had no effect or exhibited a depressive effect on the production of serpentine. One of the most interesting responses is a de novo synthesis of catharanthine, the production of which can reach that of serpentine. Higher concentrations of elicitor (from 26 \( \mu \)g gE/ml) induced negative effects, decreasing by 8-fold the production of ajmalicine compared to the control, and inhibiting the synthesis of catharanthine.

Although the effects noticed after 24 hours of treatment on day 0 were minor, we can confirm that, as observed by Eilert et al. (1986), \( C. \text{roseus} \) cell suspensions exhibit a dose-dependent increase in alkaloid production in response to fungal elicitor.