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EFFECT OF A TERTIARY AMINE ON ALKALOID ACCUMULATION IN *CATHARANTHUS ROSEUS*

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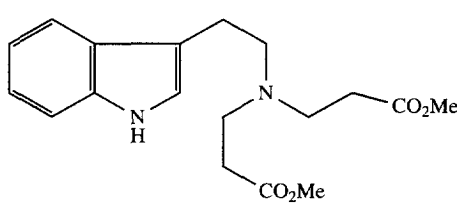
Key Word Index—*Catharanthus roseus*; Apocynaceae; alkaloids; biotransformation; tertiary amine.

Abstract—The Madagascar periwinkle, *Catharanthus roseus*, produces numerous indole alkaloids, several of which have important therapeutic activities. These secondary metabolites are among the most expensive drugs because of their low abundance in intact plants. We investigated the biotransformation of a synthetic product (*N*-(methoxycarbonylethyl)-*N*-[2-(1*H*-indol-3yl)-ethyl]-β-methyl alaninate) in the roots and aerial parts of *C. roseus* cultivated *in vitro*, in order to stimulate *de novo* the biosynthesis of biologically active indole alkaloids. The results showed a large increase (90%) of ajmalicine in the roots whilst serpentine and yohimbine levels were unchanged. In the aerial parts, the synthetic product was recovered and the time courses of various alkaloids were unchanged. No new molecule was observed. The four alkaloids tested increased with plant age whilst yohimbine gradually decreased. © 1997 Elsevier Science Ltd

INTRODUCTION

The Madagascar periwinkle, *Catharanthus roseus* produces numerous indole alkaloids. Many of them are used in human medicine, for example vincristine and vinblastine in cancer chemotherapy [1, 2]. Much research has been devoted to the improvement of alkaloid production in suspension culture of *Catharanthus* [3–7]. However, the results were not as successful as expected, due to somaclonal variation resulting from the genetic or epigenetic instability of such cultures [8, 9]. On the other hand, biological transformations by cell suspension cultures have served as important tools in the structural modification of compounds with therapeutic activity [10–12]. Surprisingly, only few studies concerned seedlings or young plants cultivated *in vitro* [13–15], so it seemed interesting to study the time course of indole alkaloids in seedlings of *C. roseus* cultivated *in vitro*, and the biotransformation of a foreign molecule. Our study concerned the ability of the entire plant to convert a synthetic molecule into biologically active indole alkaloids, which has not been previously reported. The synthetic tertiary amine: *N*-(methoxycarbonylethyl)-*N*-[2-(1*H*-indol-3yl)-ethyl]-β-methyl alaninate (MIA) was introduced into the nutritive medium in order to influence secondary metabolism in intact plants of *C. roseus* cultivated *in vitro*. The structure of the tertiary amine was close to that of tryptamine precursor, so it could be introduced into the indole pathway.



MIA

This paper reports the effects of the foreign molecule on the accumulation of the main indole alkaloids (ajmalicine, catharanthine, serpentine, vindoline and yohimbine) present in the roots and aerial parts of *C. roseus*.

RESULTS AND DISCUSSION

MIA was synthesized in the Laboratoire de Chimie Organique (Faculté de Pharmacie, Montpellier, France) by Michael addition of tryptamine on methylacrylate. Thirty-day-old seedlings of *C. roseus* cultivated *in vitro* were fed with MIA at 10^{-3} M. Various concentrations, 10^{-1} M, 10^{-3} M and 10^{-5} M had been previously tested, the intermediate being the highest that induced no apparent toxity in the plants. Our research was carried out over 35 days, to test the effect

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of the tertiary amine on the biosynthesis of indole alkaloid in both roots and aerial parts of *C. roseus*. Each experiment was carried out three times; each point for each experiment corresponded to the extraction of two culture pots (200 to 300 seedlings), in order to allow statistical tests.

Indole alkaloid accumulation in the roots

Figure 1(a) shows the influence of the synthetic molecule on the accumulation of ajmalicine. The control and stress curves increased rapidly but the stress curve was always above the control curve. In the fed seedlings, the ajmalicine rate increased from 0.15 to 0.55 mg g^{-1} dry weight (267%) between day 0 and day 35 of contact with MIA, whereas in the control, the rate ranged from 0.15 to 0.29 mg g^{-1} dry weight (93%). These results were confirmed with statistical tests which gave significant differences between stress and control. The ajmalicine accumulation increased with seedling age, contrary to cell suspension culture where this alkaloid decreases and its oxidative product, serpentine, increases [16]. MIA induced an important accumulation of ajmalicine (+90%) compared with the control.

Serpentine accumulations were identical for control and stress [Fig. 1(b)]. During the 35 day period of the experiment, serpentine quantities increased by 50 and 70% in control and fed seedlings, respectively. Statistical tests did not show any significant difference. It has been proposed that ajmalicine is converted into serpentine by basic peroxidases in the vacuoles [17]. However, in our experiments, the serpentine time course did not differ between fed and control plants, thus the ajmalicine conversion into serpentine may have been stopped. It has been postulated that tertiary amines act as oxygen scavengers, protecting from oxidative degradation indole alkaloids [18]. After addition of terpenoid precursors, an increase in the accumulation of ajmalicine and strictosidine, and a decrease of tryptamine level, were observed in suspension cell cultures [19]. In our experiments with addition of MIA, an increase of ajmalicine but no change in serpentine accumulation appeared. Tryptamine was not present in our plant extracts. MIA may also act as an enzyme inhibitor which could explain the accumulation of ajmalicine without serpentine conversion.

The time course of yohimbine is opposite to those of the two other alkaloids [Fig. 1(c)]. The decrease was from 0.24 to 0.30 mg g^{-1} dry weight (−55%) during the 35-day contact. No MIA influence was noted.

Indole alkaloid accumulation in the aerial parts

In our experiments, vindoline and catharanthine were the major alkaloids in the aerial parts of seedlings, whereas ajmalicine and serpentine were present only in small quantities. Vindoline was the main alkaloid in the aerial parts and was particularly abundant in the leaves [20]. Its biosynthetic enzyme was *N*-methyl transferase, which was located in chloroplasts [21]. Vindoline was also the predominant alkaloid in

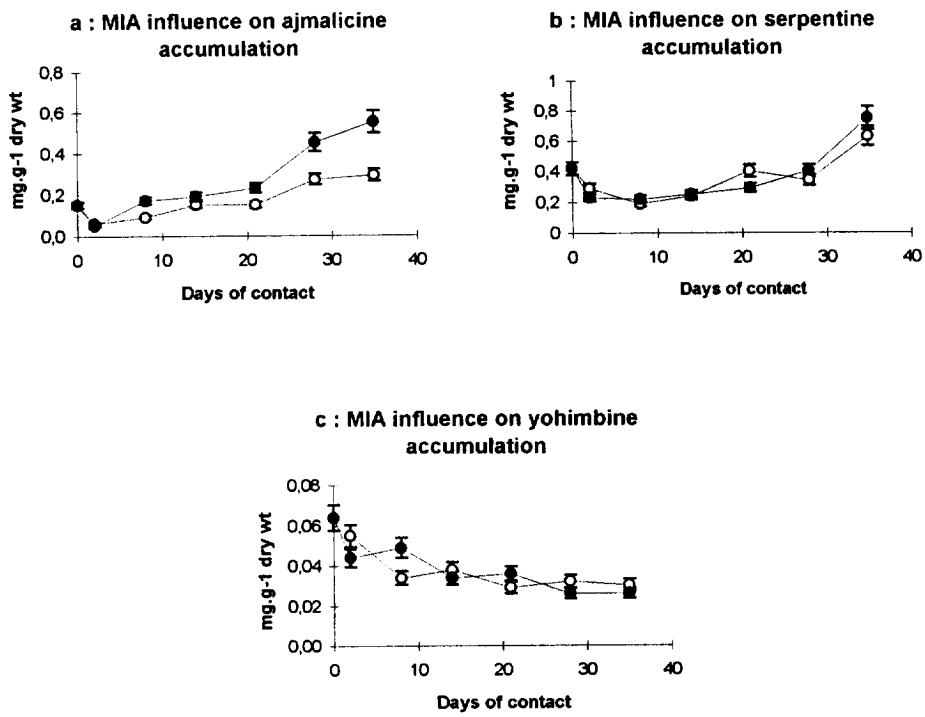

Fig. 1. MIA influence on the accumulation of alkaloids in the roots of *C. roseus*, –○– time course of alkaloids in the control plants of *C. roseus*, –●– time course of alkaloids in the fed plants of *C. roseus*.

hypocotyl and cotyledon. Vindoline levels were age-dependent, increasing by about 57% in 35 days [Fig. 2(a)]. On the other hand, catharanthine levels remained stable during this period of culture [Fig. 2(b)]. No MIA influence was noted.

Important increases in ajmalicine, serpentine and vindoline accumulation were age-dependent. Ajmalicine and serpentine accumulations were from 0 to 0.080 mg g^{-1} dry weight (100%) [Fig. 2(c)] and from 0.041 to 0.283 mg g^{-1} dry weight (600%) [Fig. 2(d)], respectively, in both fed and control plants. All of these alkaloids were unaffected by the absorption of MIA. It was noted that the minor alkaloids gave the strongest accumulation. The alkaloid increases in the aerial parts were simultaneous to those of the plant roots.

It has been reported that, in hormone-free medium, the growth was suppressed and the contents of vindoline and catharanthine were significantly greater than those of the medium containing benzyladenine (BA) [22]. This could explain the important rates of vindoline and catharanthine found in our experimental seedlings, but we observed normal development when the first leaves appeared in 26- to 30-day-old seedlings. No major difference in alkaloid content between culture systems have been reported in the literature. The differences in alkaloid synthesis in natural roots, root cultures and cell suspension cultures appeared to be essentially quantitative rather than qualitative [23].

MIA amounts in the roots and aerial parts

Intact MIA was recovered in the roots, which was proof of its root absorption [Fig. 3(a)]. MIA amounts were variable from day 2 to day 21. Maximum MIA levels appeared at day 14 followed by a decrease, perhaps as the result of detoxification or catabolism. Low levels were recovered from aerial parts [Fig. 3(b)] compared with roots, indicating that the MIA was not only transferred to superior parts, but introduced into a metabolic pathway. These levels were 7.6 times higher in the roots than in the leaves and hypocotyls. Thus, a small portion was transferred to aerial parts via conducting tissue such as xylem.

The foreign molecule may be absorbed then metabolised and/or transported to aerial parts. The time course of this foreign molecule may be explained by the results obtained in the aerial parts, where MIA was surprisingly present.

Product accumulation by plant cell cultures has been known to be stimulated by stress factors such as UV irradiation, osmotic shock, fatty acids, inorganic salts, heavy metal ions and fungal wall components

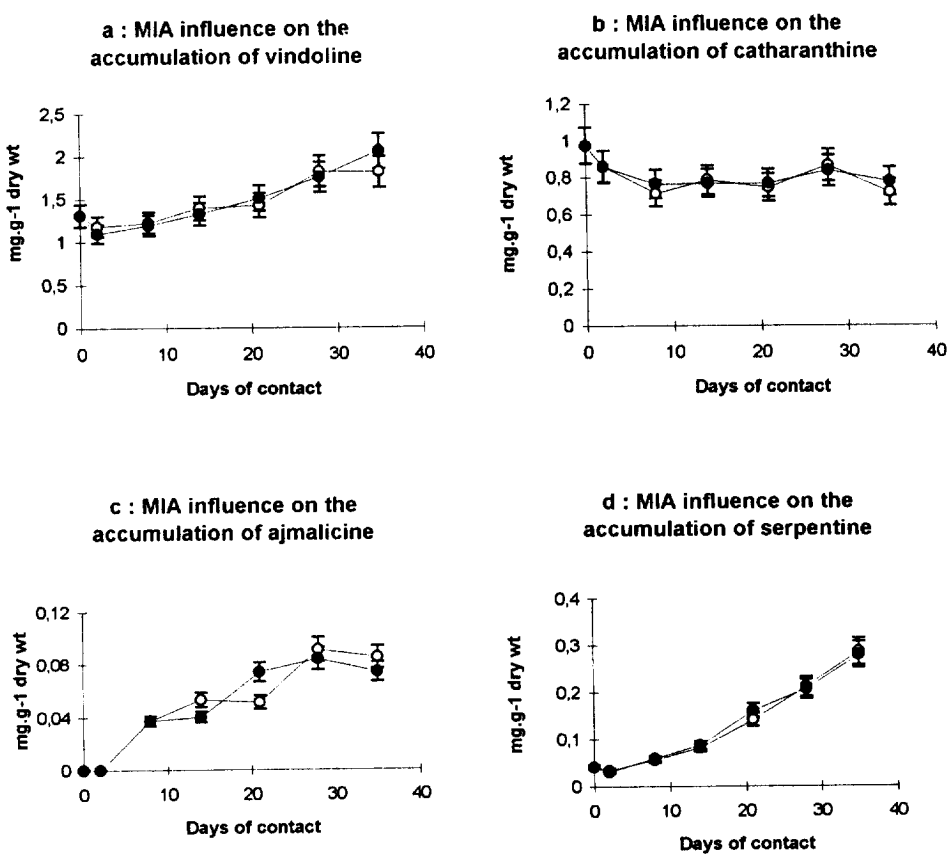

Fig. 2. MIA influence on the accumulation of alkaloids in the aerial parts of *C. roseus*, –○– time course of alkaloids in the control plants of *C. roseus*, –●– time course of alkaloids in the fed plants of *C. roseus*.

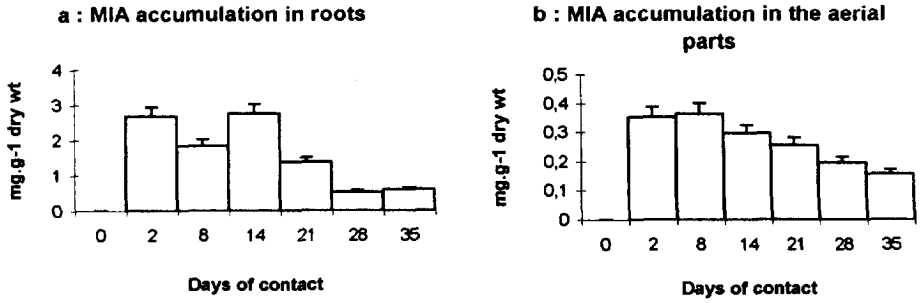

Fig. 3. MIA accumulation in roots and aerial parts of *C. roseus*.

(elicitors). Plant cell suspension cultures have the ability to respond to external stimuli with changes in gene expression that result in altered secondary metabolism. The induction of tryptophan decarboxylase (TDC) and strictosidine synthase (SS) enzyme activities has been followed by the accumulation of indole alkaloids [24]. This production of secondary metabolites was considered to be part of the defence mechanism of the plant cells in response to an offending foreign substance [18]. MIA may stimulate the defence process of the plant and one result was an increase (+90%) in the accumulation of ajmalicine in the roots.

According to our results, we suggest that MIA could be incorporated into the alkaloid pathway, acting, directly or indirectly, as a peroxidase blocker, or working as protective agent of ajmalicine catabolism. Further studies with [^{13}C]MIA should be carried out in order to follow its incorporation into the indole alkaloid pathway.

EXPERIMENTAL

Plant material and growth conditions. Seeds of *Catharanthus roseus* (L.) G. Don cv. Little Pinkie (Ducrettet, Thonon-les-Bains, France) were surface sterilized by successive immersion in a soln of EtOH 70° for 30 s then immersion in a soln of 3.6% NaClO (commercial dilution) for 30 min followed by thorough rinsing in sterile H_2O. Seeds were placed on MS medium [25] supplemented with 3% sucrose and 11 g l^{-1} agar but without phytohormone (=day 0). They were grown in the dark for 10 days before being placed under a regular diurnal 12 hr light/dark regime.

Feeding method. 30-day-old shoots of *C. roseus* were transferred to MS medium without phytohormone, supplemented with 3% sucrose, 15 g l^{-1} agar and 10^{-3} M of *N*-(methoxycarbonylethyl)-*N*-[2-(1*H*-indol-3yl)-ethyl]-*β*-methyl alaninate (MIA) dissolved in DMSO. The final culture DMSO concn was 1%; control contained DMSO as appropriate.

Alkaloid extraction and separation. Every 7 days, the plants were extracted. The roots and the aerial parts were cut and lyophilized separately. The freeze-dried material was ground in a mortar and suspended in 5% HOAc (200 ml per g) for 1 hr, then sonicated for 30 min. The soln was filtered on glass fiber (1.6 μm) and the residue suspended in 5% HOAc for 30 min. After sonication for 15 min, the filtrates were combined and alkalinized with 30% NH_4OH to pH 9.5, then extracted ×3 with the same vol. of CH_2Cl_2. After drying over Na_2SO_4, filtration and evapn to dryness, the residue was dissolved in 0.4 ml of CH_2Cl_2 and placed on the top of a small column containing 0.7 g of silica gel (chromagel 60 Å, 35–70 μm) and eluted successively with CH_2Cl_2, CH_2Cl_2–MeOH (9:1) and CH_2Cl_2–MeOH–Et_2NH (15:4:1) Five frs were obtained, evapd to dryness and investigated for alkaloids.

Alkaloid analysis. The root frs and aerial parts were suspended in 0.2 ml and 0.5 ml of MeOH, respectively, and alkaloid identification was performed using TLC and HPLC. TLC was performed on silica gel plates (Macherey Nagel) using 3 mobile phases: A: C_7H_8–Me_2CO–MeOH–NH_4OH 28% (49:140:10:1); B: CH_2Cl_2–MeOH (4:1) and C: EtOAc. Identification was made using authentic samples of ajmalicine, catharanthine, serpentine, vindoline and yohimbine after colour reaction with $(NH_4)_4Ce(SO_4)_4$ reagent [26] and fluorescence at 254 and 366 nm.

The frs were analysed by HPLC using an analytical reversed-phase Nova-Pak C_{18} column (250 × 4.6 mm, 5 μm). The column was eluted with an isocratic solvent system MeOH–$(NH_4)_2HPO_4$ 5mM (pH 7.3) (7:3) with a flow rate of 1 ml min^{-1}. The effluent from the column was monitored using a photodiode array detector (Waters 990). Identification was carried out by comparing R_f and UV spectra with those of authentic samples.

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