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SIGNALLING MOLECULES AND THE SYNTHESIS OF ALKALOIDS IN *CATHARANTHUS ROSEUS* SEEDLINGS

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Abstract—The Madagascar periwinkle, *Catharanthus roseus*, produces numerous alkaloids, several of which have important pharmaceutical uses. *Catharanthus* seedlings rapidly accumulate the monomeric alkaloids, vindoline, catharanthine and tabersonine, during germination. Various plant signalling molecules were tested for their ability to enhance alkaloid synthesis in *Catharanthus* seedlings. The compounds tested included plant hormones, fatty acid-derived messengers and agents that can induce systemic-acquired resistance in plants. Of these compounds, only methyl jasmonate (MeJa) enhanced the synthesis of monomeric alkaloids. However, feeding of a MeJa biosynthetic precursor, or inhibition of the lipoxygenase pathway, had no effect on alkaloid production in the seedlings. We conclude that, although MeJa is able to enhance alkaloid synthesis when supplied exogenously, the lipoxygenase pathway probably does not play a role in the regulation of alkaloid synthesis during normal germination of *Catharanthus*. Furthermore, it was found that *Catharanthus* seedlings accumulate small quantities of the dimeric alkaloid, vinblastine, a valuable antitumour drug. Contrary to its effect on the accumulation of monomeric alkaloids, MeJa did not influence the accumulation of vinblastine.

INTRODUCTION

The Madagascar periwinkle, *Catharanthus roseus*, is the source of several pharmaceutically important alkaloids. In particular the alkaloid vinblastine is an extremely valuable antineoplastic agent [1, 2]. However, vinblastine accumulates only in trace amounts in the plant and much research has been devoted to the improvement of alkaloid production in *Catharanthus* [3]. The direct precursors for vinblastine biosynthesis are derived from the monomeric alkaloid stemmadenine (Fig. 1; [4]). Via two separate branches of the pathway, stemmadenine is transformed into the monomeric alkaloids, catharanthine, tabersonine and vindoline. The dimeric alkaloid, vinblastine, is finally formed by coupling of vindoline to catharanthine, whereby modifications in the catharanthine moiety occur (Fig. 1).

Alkaloids often accumulate as part of the developmental programme of plants [5–7]. *Catharanthus* seedlings rapidly accumulate catharanthine, tabersonine and vindoline during germination. The alkaloid content of the seedlings reaches a plateau a few days after the onset of germination [8]. External application of the fatty acid-derived plant signalling molecule, methyl jasmonate (MeJa), dramatically enhances the accumulation of monomeric alkaloids in seedlings. These effects were shown to be most pronounced when MeJa was applied early during the germination process [8]. Jasmonate is produced in the so-called 'lipoxygenase pathway', and increases in the activities of some enzymes in this pathway have been observed in germinating seedlings [9–11]. Rapid synthesis of lipoxygenase pathway products has been implicated in some defence reactions *in planta*, for instance, the production of proteinase inhibitors in tomato leaves upon wounding [12]. Since alkaloids are also considered to be defence compounds, this raises the possibility that induction of the lipoxygenase pathway and accumulation of endogenous MeJa may regulate alkaloid synthesis during germination of *Catharanthus*. We tested this hypothesis in two ways: by investigating the effects on alkaloid synthesis of feeding 13-hydroperoxylinolenic acid (13-HPLA), a precursor to MeJa in the lipoxygenase pathway, and by monitoring the effects of treatment of seeds with lipoxygenase pathway inhibitors.

Furthermore, we decided to test other types of plant

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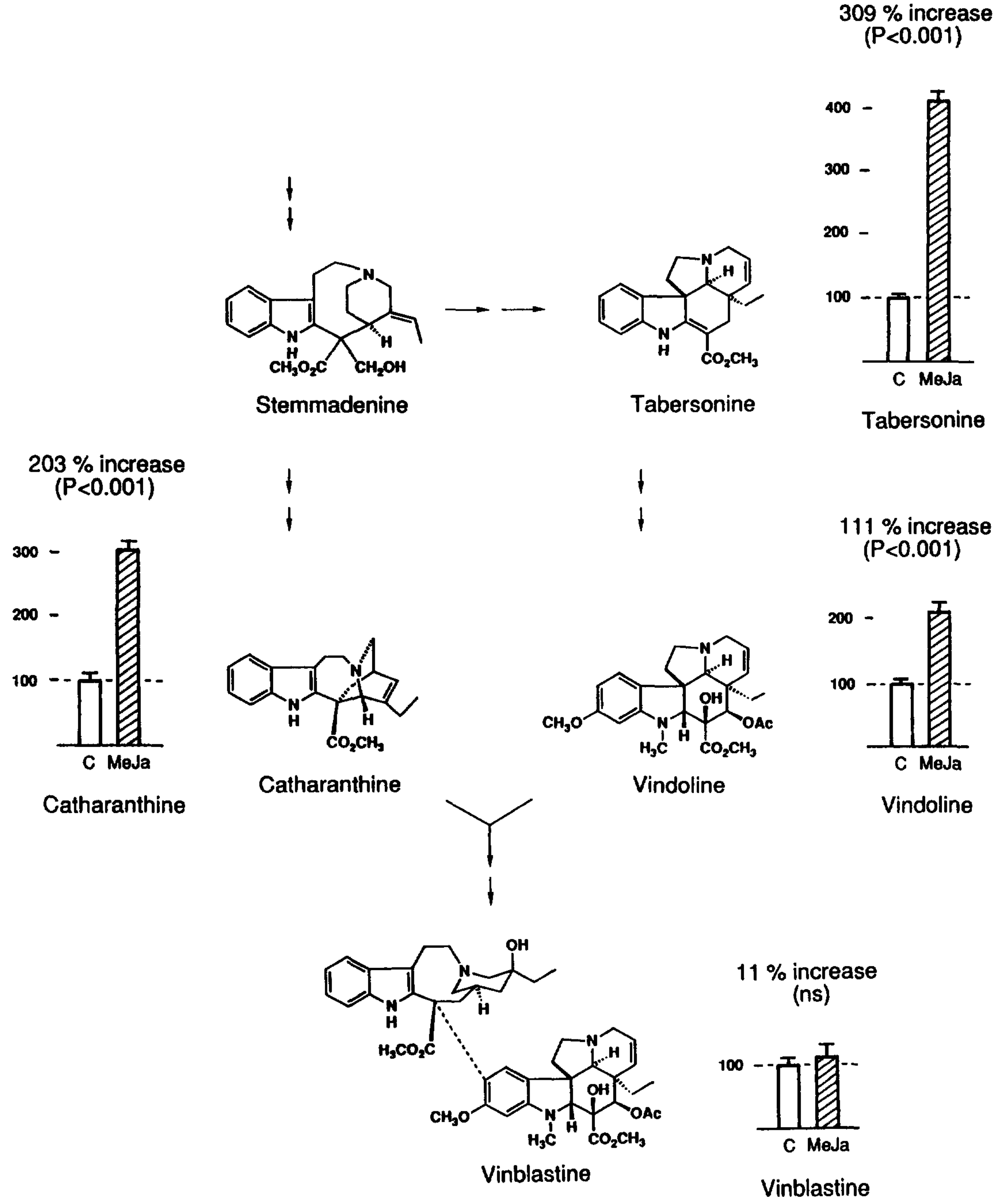

Fig. 1. Pathway of alkaloid biosynthesis in *Catharanthus* seedlings and effects of methyl jasmonate (MeJa) on alkaloid accumulation. Germinating seeds were exposed to 6 ppm of MeJa or to an equivalent amount of solvent (controls: C). Seedlings were harvested 9 days later and analysed for alkaloid content. Control levels (pmol seedling^{-1}) correspond to 413 for tabersonine, 729 for vindoline, 884 for catharanthine, and 16.8 for vinblastine. Statistical significances of the differences between treatments and controls are indicated [t-test (ns = not significant, $\alpha = 0.05$)]. Means ±s.e. of three to six separate experiments are shown.

signalling molecules for their ability to influence alkaloid synthesis in germinating *Catharanthus* seedlings. Thus, the following fatty acid-derived messengers were tested: traumatic acid, which is another product of the lipoxygenase pathway [13], and lysophosphatidylethanolamine, a plant metabolic regulator [14]. In addition, we tested the plant hormones, abscisic acid (ABA), whose effects are often similar to those of MeJa [15, 16], and indolebutyric acid (IBA), which has been shown to enhance the activity of one of the key enzymes (tryptophan decarboxylase) leading to alkaloid synthesis in *Catharanthus* seedlings [17]. Finally, evidence indicates that systemic-acquired resistance (SAR) to pathogens in plants is accompanied by an induction of the lipoxygenase pathway [18]. Various agents that can induce SAR were tested for their effects on alkaloid accumulation in *Catharanthus* seedlings, viz. salicylic acid (SA), chlorinated SA derivatives, and 2,6-dichloro-*iso*nicotinic acid (INA).

Vinblastine accumulates in mature tissues of *Catharanthus* plants, but it has also been found in juvenile tissues. Thus, the presence of vinblastine was reported in a multiple shoot culture initiated from seedlings and in a callus culture [19, 20]. In this paper, we report for the first time that *Catharanthus* seedlings accumulate small quantities of vinblastine. The influence of MeJa treatment on vinblastine accumulation in the seedlings was investigated.

RESULTS AND DISCUSSION

Effects of signalling molecules

At the onset of germination, *Catharanthus* seeds were treated with the test substances and the effects on alkaloid synthesis were assessed by HPLC and TLC a few days later. The substances were all tested in broad concentration ranges. Except for MeJa, none of the test substances was able to increase alkaloid accumulation in the seedlings (Table 1). MeJa exerts its effect on alkaloid accumulation through both an enlargement of the alkaloid precursor pool and an enhancement of several enzyme activities in alkaloid biosynthesis [8]. The lack of effect with IBA suggests that artificial enhancement of the activity of tryptophan decarboxylase, an enzyme linking primary with secondary metabolism, is not sufficient to enhance alkaloid contents in *Catharanthus* [17].

Table 1. Effects of various signalling molecules on alkaloid accumulation in germinating *Catharanthus* seedlings*

Test substance	Concentration	Effect on alkaloid content
ABA	0.5–25 μM	None
	50–200 μM	Decrease
IBA	40 μM	Decrease
Salicylic acid (SA)	0.5–1.25 mM	None
	2.5–5.0 mM	Decrease
4-ChloroSA	33–500 μM	None
	1000 μM	Decrease
5-ChloroSA	33 μM	None
	100–1000 μM	Decrease
3,5-DichloroSA	33 μM	None
	100–1000 μM	Decrease
INA	0.1–10 μM	None
	100–400 μM	Decrease
Traumatic acid	1–750 μM	None
	1500–3000 μM	Decrease
Lysophosphatidyl-ethanolamine	10–30 mg l^{-1}	None
	170–335 mg l^{-1}	Decrease
MeJa	0.06–6 ppm	Increase

*In each experiment, besides untreated controls, MeJa-treated seedlings were included as positive controls.

Lipoxygenase pathway and alkaloid synthesis

At concentrations up to *ca* 1×10^4-fold higher than those used with MeJa, no effect of 13-HPLA on alkaloid levels in *Catharanthus* seedlings could be observed (Fig. 2). For comparison, the same level of induction of proteinase inhibitor I in tomato leaves has been reported with a *ca* 50-fold higher concentration of 13-HPLA than that of jasmonic acid [12]. The same lack of effect as with 13-HPLA was observed with 9-hydroperoxylinolenic acid (9-HPLA), an intermediate in the lipoxygenase pathway related to 13-HPLA, but one feeding into a different branch and not leading to MeJa (Fig. 2). Furthermore, all the lipoxygenase pathway inhibitors tested did not block alkaloid accumulation in the seedlings (Table 2). The inhibitors were applied up to toxic concentrations, which were similar to, or higher than, the concentrations used in other studies [21–23]. Thus, although external application of MeJa can exert a striking effect on alkaloid synthesis in *Catharanthus* seedlings (Fig. 2), taken together, our results indicate that the lipoxygenase pathway may not play a role in the normal regulation of alkaloid synthesis in developing *Catharanthus* seedlings, but that alkaloid synthesis is controlled by other endogenous signals of the developmental programme. In this respect, alkaloid synthesis in *Catharanthus* seedlings seems to differ from the wound-induced synthesis of proteinase inhibitors in tomato leaves and the elicitor-induced synthesis of various secondary compounds in cultures of undifferentiated plant cells [12, 24, 25]. A difference in the effects of MeJa on plant cell cultures and *Catharanthus* seedlings is also evident from the fact that alkaloid biosynthesis in the seedlings is influenced by MeJa only during a narrow time-window, after which MeJa hardly affects alkaloid contents [8].

Accumulation of vinblastine

When extracts of 11-day-old, light-grown *Catharanthus* seedlings were analysed by HPLC, a compound with the same retention time and UV-absorption spectrum as the authentic vinblastine standard was found. The retention time of the vinblastine peak in the extracts changed in concert with that of authentic vinblastine when the gradient applied in the HPLC system was altered so that the retention time changed over a broad range (14–60 min). The identification of vinblastine in the seedlings was confirmed by electrospray-ionization mass spectrometry (ESI-MS). In the mass spectrum of the purified vinblastine sample from the seedlings, an ion with *m/z* 812 ($[M+H]^+$) was observed. The spectrum of authentic vinblastine showed the same ion. Furthermore, the fragmentation pattern was studied both of the purified vinblastine sample from the seedlings and of authentic vinblastine

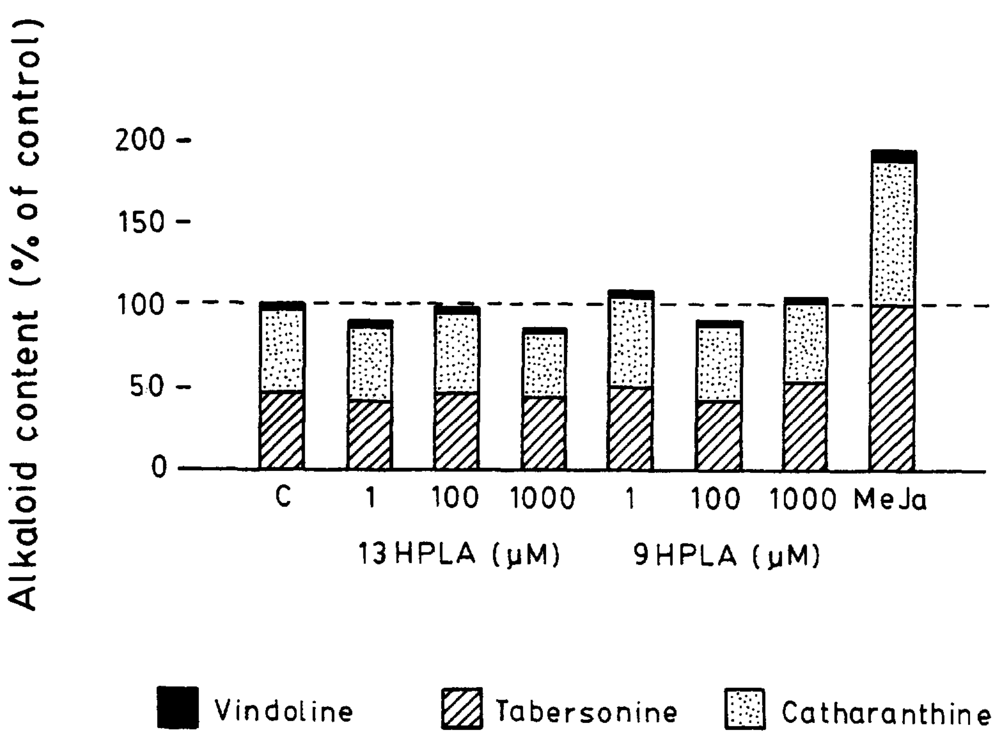

Fig. 2. Effects of 13- and 9-hydroperoxylinolenic acid on alkaloid accumulation in *Catharanthus* seedlings. Germinating seeds were exposed to various concentrations of 13-HPLA or 9-HPLA or to 1.5 ppm of MeJa; seedlings were harvested 4 days later and analysed. At the highest concentrations of 13- and 9-HPLA, deformation of the seedlings was observed. Control level corresponds to 2670 pmol alkaloid seedling^{-1}. Means of two separate experiments are shown.

upon electrospray tandem mass spectrometry (ESI-MS/MS). The main degradation peak was in both cases an ion with *m/z* 355, corresponding to the velbanamine (catharanthine-derived) moiety of the molecule. In addition, both spectra contained peaks with *m/z* 457, corresponding to the vindoline moiety. This indicates that vinblastine was fragmented into its original building moieties [26].

Dry *Catharanthus* seeds have been reported to contain only trace amounts of the major alkaloids [27, 28]. The alkaloids present in the seedlings are synthesized during the germination process. Just like the other alkaloids, vinblastine was barely detectable in dry seeds, indicating that the vinblastine present in the seedlings accumulates concomitantly with the other alkaloids during germination. As far as we are aware,

Table 2. Effects of lipoxygenase pathway-inhibitors on alkaloid accumulation in germinating *Catharanthus* seedlings

Inhibitor	Concentration (µM)	Effect on alkaloid content
Aspirin	375–1500*	None
n-Propyl gallate	30–750†	None
Salicylhydroxamic acid	30–750†	None
Ibuprofen	30–100†	None
Mefenamic acid	30–100†	None
Antipyrine	30–100	None
	750†	Decrease in catharanthine but not in tabersonine‡
Phenylbutazone	30–100	None
	750†	Decrease in tabersonine but not in catharanthine‡

*Toxic at concentrations of 1500 µM or higher.

†Toxic at higher concentrations.

‡Observed effects could not be reversed by simultaneous MeJa treatment, showing that effects are not related to lipoxygenase pathway.

this is the first report of the presence of vinblastine in seedlings of *Catharanthus*. The amount of vinblastine in seedlings is lower than that reported for mature plants [29].

Effect of MeJa on vinblastine accumulation

The effect of 6 ppm of MeJa vapour on alkaloid accumulation in 11-day-old, light-grown seedlings is shown in Fig. 1. Treatment with MeJa resulted in a striking enhancement of the levels of the monomeric alkaloids. Vinblastine accumulation, however, was not significantly enhanced by MeJa. As expected, the vinblastine content was very low in the controls (*ca* 2% of that of vindoline and catharanthine). Interestingly, although contents of the two parent molecules were increased by MeJa treatment, this did not improve the coupling of these precursors to make more vinblastine.

There is precedent for the fact that in young tissues high vindoline and catharanthine levels do not necessarily result in a relatively high vinblastine content. Thus, in a shoot culture the amounts of vindoline and catharanthine were found to be comparable to or even higher than those in the mature plant, but vinblastine concentrations were lower [20].

Although it cannot be excluded that vinblastine is an artefactual compound, the difference between the effects of MeJa on the accumulation of the monomeric alkaloids and on the accumulation of vinblastine suggests that the biosynthesis of the former alkaloids is under different regulatory controls from that of the latter. This is also evident from the fact that, in contrast to the monomeric alkaloids, the accumulation of dimeric alkaloids seems to be linked to ageing of tissues in *Catharanthus* [26, 28, 30].

Conclusions

Of the various hormones, fatty acid-derived messengers and SAR-inducing agents tested in this study, only treatment with MeJa was able to enhance monomeric alkaloid contents in *Catharanthus* seedlings, indicating the specificity of the mode of action of MeJa. However, we have not been able to find evidence for a role of the lipoxygenase pathway, as such, in the regulation of alkaloid biosynthesis during normal germination of *Catharanthus*. Finally, our results indicate that MeJa treatment predominantly influences regulatory controls leading to monomeric alkaloid synthesis, but less so those leading to vinblastine accumulation in young *Catharanthus* seedlings.

EXPERIMENTAL

Growth and treatment of seedlings. Seeds of *C. roseus* (L.) G. Don (*Vinca* dwarf little mixt., Sakata Seed Corp.) were surface-sterilized, allowed to imbibe H_2O and placed in sterile Petri dishes on paper tissues wetted with sterile H_2O (=day 0) as described in ref. [8]. On day 2, seeds were treated with the test substances in broad concn ranges (see Fig. 2; Tables 1 and 2). Most substances were tested in two solvents. Substances dissolved in aq. EtOH (0.5%): ABA, INA (formulation CGA 41396), lysophosphatidylethanolamine (mainly stearic and palmitic acids), *n*-propyl gallate, salicylhydroxamic acid, ibuprofen, mefenamic acid, antipyrine, phenylbutazone; (0.25%): IBA; (0.25–0.5%): SA, 4-chloroSA, 5-chloroSA, 3,5-dichloroSA); in aq. DMSO (5%): ABA, INA, lysophosphatidylethanolamine; (3–5%): traumatic acid; (2%): 13-HPLA, 9-HPLA, aspirin, *n*-propyl gallate, salicylhydroxamic acid, ibuprofen, mefenamic acid, antipyrine, phenylbutazone); in sterile H_2O: 13-HPLA and 9-HPLA. Controls were exposed to 0.25–0.5% EtOH, 2–5% DMSO or to sterile H_2O. Treatment with MeJa was as described in ref. [8]. Seeds were germinated at 26° in the dark and, on day 6, harvested (expts shown in Tables 1 and 2, and Fig. 2), or on day 6 placed in the light (45 μmol m^{-2} s^{-1}, 13:11 hr light:dark), and harvested on day 11 (expt shown in Fig. 1). The seedlings were stored frozen at −70° prior to further processing. Morphological development of the seedlings was under all conditions identical to that of controls, except at high, toxic concentrations of the substances, where inhibition of germination and deformation of seedlings were observed.

Determination of alkaloids. Monomeric alkaloids were extracted from batches of 20–25 seedlings as described in ref. [8]. For vinblastine determinations, extraction was performed in the same way, but larger batches of 70 seedlings were taken and amounts of solvent increased accordingly. Additionally, batches of 70 dry seeds were extracted after homogenization in liquid N_2 with a pestle and mortar. Tabersonine, vindoline and catharanthine were analysed by TLC or HPLC [8, 27]. Vinblastine was analysed with the same HPLC system, except that the eluent was 29% MeCN in H_2O (0.2% Et_3N), with, after 10 min, a linear rise over 60 min to 36% MeCN, followed by a further rise to 65% over 15 min. Quantification and identification of alkaloids was by means of ref. compounds.

Isolation and identification of vinblastine. For confirmatory identification of vinblastine, extracts of *ca* 2000 seedlings were pooled, vacuum-dried and the combined extract again acid-base purified. Subsequently, half of the sample was injected into the HPLC system, the vinblastine fr. collected, vacuum-dried and dissolved in MeOH. The purified vinblastine fr. was analysed by ESI-MS and ESI-MS/MS (29 eV).

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