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TERPENOID INDOLE ALKALOID BIOSYNTHESIS AND ENZYME ACTIVITIES IN TWO CELL LINES OF *TABERNAEMONTANA DIVARICATA*

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Key Word Index—*Tabernaemontana divaricata*; Apocynaceae; suspension cultures; terpenoid indole alkaloids; biosynthesis; enzyme activities; precursor feeding.

Abstract—The possible limitation of the rate of biosynthesis of terpenoid indole alkaloids by low enzyme levels was investigated in two cell lines of *Tabernaemontana divaricata* with different terpenoid indole alkaloid biosynthetic capacities. The activities of tryptophan decarboxylase (TDC), strictosidine synthase (SSS), strictosidine glucosidase (SG), isopentenyl pyrophosphate isomerase (IPP isomerase) and geraniol 10-hydroxylase (G10H) of both cell lines were compared. The activities of TDC, SSS and IPP isomerase did not show a direct relationship with the biosynthetic capacity but SG and G10H might be limiting. In order to test whether the availability of the terpenoid precursor limits the biosynthesis of the terpenoid indole alkaloids, loganin was fed to the cultures. Loganin-feeding did not influence any of the measured enzyme activities but increased the terpenoid indole alkaloid accumulation of both cell lines to similar levels. A five-fold increase was observed for the accumulating line and a more than 100-fold increase for the low-accumulating one. Strictosidine accumulated mainly in the low-accumulating cell line which has high TDC and low SG activity; the amounts and types of the other terpenoid indole alkaloids which accumulated were similar in both lines. From this it can be concluded that the biosynthesis of terpenoid indole alkaloids in both cultures is limited by the availability of terpenoid precursors; this pathway is not saturated with substrates under normal culture conditions.

INTRODUCTION

There are several ways in which cells may control the flux of metabolites through a biosynthetic pathway. Attempts are being made to try to understand the control mechanisms that determine the amount of secondary metabolites accumulated by plant cell suspension cultures; this would allow the manipulation of the levels of compounds of interest. Control mechanisms of biosynthetic pathways have been mainly investigated at the enzymatic level [1, 2]. Enzymes of several pathways have now been isolated and characterized [3]. Environmental modifications or direct genetic manipulation have been carried out in order to modify enzyme levels and compare their effects on metabolite accumulation. Studies concerning substrate/coenzyme limitations [4], the eventual effect of inhibitors and compartmentation [5] have been less frequent.

To investigate whether enzyme levels in suspension cultures of *Tabernaemontana divaricata* have a direct relationship with terpenoid indole alkaloid biosynthesis, two closely related cell lines were compared with respect to alkaloid biosynthesis and accumulation, and the activities of some enzymes belonging to the early steps of this pathway. The two cell lines used for the experiment were originally obtained from the same suspension culture and have always been maintained under the same culture conditions. Nevertheless, they differ in their alkaloid accumulating capacity; one accumulates the terpenoid indole alkaloids *O*-acetylvallesamine, vallesamine and voaphylline while these compounds are barely detectable in the other. Previous isotope dilution experiments, in which *O*-acetyl [^{15}N] vallesamine and [^{15}N] voaphylline were fed to the cultures, demonstrated that the ability of the cell lines to catabolize these alkaloids was similar and that the difference in accumulating capacity was due to their different biosynthetic abilities [6]. Since this difference was observed for the two terpenoid indole alkaloids of different biosynthetic classes, it was concluded that the limiting step in alkaloid biosynthesis by the low-accumulating line probably occurs before the bifurcation of the two pathways.

In the present study, the activities of some of the enzymes of the early terpenoid indole alkaloid biosynthetic pathway (Fig. 1) of the two cell lines were com-

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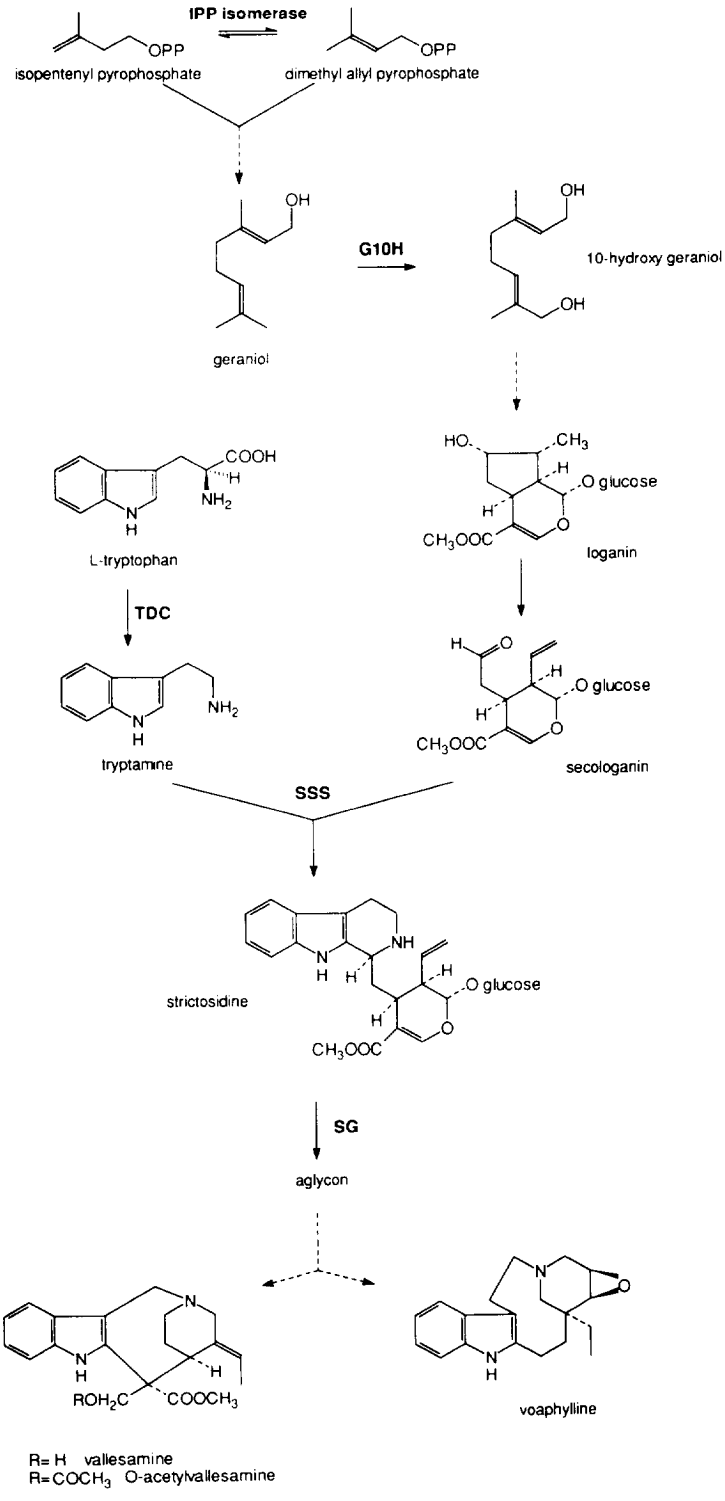

Fig. 1. Biosynthesis of the main alkaloids accumulated by *T. divaricata* suspension cultures (no competing pathways are indicated). IPP isomerase: isopentenyl pyrophosphate isomerase; G10H: geraniol-10-hydroxylase; TDC: tryptophan decarboxylase; SSS: strictosidine synthase; SG: strictosidine glucosidase.

pared. The enzyme activities measured were those of tryptophan decarboxylase (TDC, EC 4.1.1.28), strictosidine synthase (SSS, EC 4.3.3.2), strictosidine glucosidase (SG), isopentenyl pyrophosphate isomerase (IPP isomerase) and geraniol 10-hydroxylase (G10H).

RESULTS AND DISCUSSION

The growth curves and alkaloid accumulation patterns of the cell lines were compared (Fig. 2). Both lines accumulated dry weight in a similar way. However, alkaloid

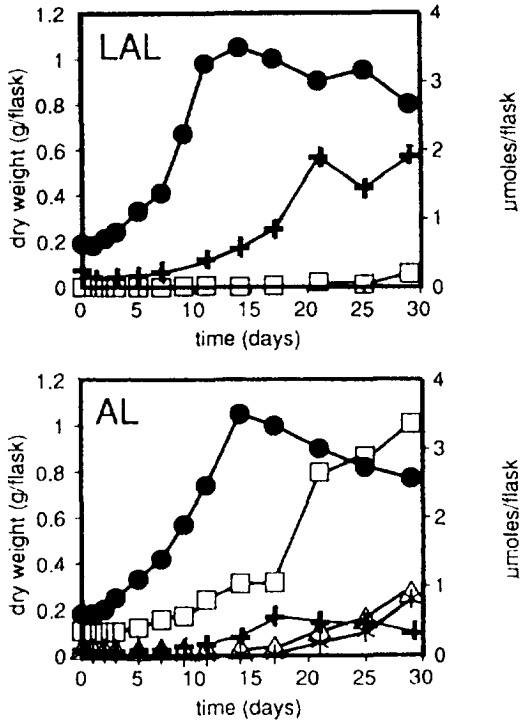

Fig. 2. Biomass and indole alkaloid accumulation time curves for the accumulating (AL) and low-accumulating (LAL) cell lines. ●—●, dry wt; + — +, tryptamine; □—□, *O*-acetylvallesamine; * –*, vallesamine; Δ—Δ, voaphylline. This experiment was carried out in 250-ml Erlenmeyer flasks originally containing 50 ml of culture medium and using 4 g fr. wt as inoculum. No vallesamine or voaphylline could be detected in the cultures of the low-accumulating cell line.

accumulation differed considerably. One cell line (the accumulating cell line) accumulated the terpenoid indole alkaloids *O*-acetylvallesamine, voaphylline and vallesamine while in the other these compounds were barely detectable. The precursor of these compounds, tryptamine, which is found mainly in the biomass of both cell lines, was accumulated in larger amounts in the low-accumulating line.

The difference in the terpenoid indole alkaloid biosynthetic capacity of the two cell lines found previously [6] was confirmed by an isotope dilution experiment. *O*-Acetyl [^{15}N] vallesamine was added to the culture medium before inoculation and the amount of labelled compound and dilution of the label were measured after 20 days (Table 1). Degradation of *O*-acetylvallesamine by the culture of the two cell lines was proportional to the amount present in the cultures since the final levels of labelled compound were similar (143 nmol/flask of *O*-acetyl [^{15}N] vallesamine). The accumulating and low-accumulating lines diluted the original labelled compound 2.6 and 1.2 times, respectively This result shows clearly that the difference in accumulating capacity of the cell lines was due to differences in their biosynthetic ability, reconfirming what had been found previously for the terpenoid indole alkaloids voaphylline and *O*-acetylvallesamine [6].

Voaphylline and *O*-acetylvallesamine belong to different biosynthetic classes (Fig. 1); since the low-accumulating cell line had a low rate of biosynthesis for each of the different biosynthetic classes of alkaloids it seemed probable that the limiting step in the biosynthesis occurred before the bifurcation of the two pathways. Another indication that enzymes further down the biosynthetic pathway were present and were not limiting was provided by the fact that a previous study had shown that feeding tabersonine (a supposed precursor of voaphylline) to the low-accumulating line led to a high accumulation of voaphylline [7]. It was possible, therefore, that the low level of biosynthesis in this cell line might be owing to low levels of enzymes of the early terpenoid indole alkaloid biosynthetic pathway. This assumption was tested by comparing the activities of enzymes of this pathway: TDC, SSS, SG, IPP isomerase and G10H of the accumulating and low-accumulating cell lines (Fig. 3).

Tryptophan decarboxylase. Clear differences between the TDC activities of the different cell lines were detected. The low-accumulating cell line showed a sharp increase in activity between the 2nd and 5th day in culture. The increase in activity in the other cell line was much less pronounced. The high terpenoid indole alkaloid accumulating line showed the lowest activity throughout the culture cycle. The TDC activity levels were not related to terpenoid indole alkaloid accumulation. Tryptamine was accumulated mainly by the low-accumulating cell line; curiously during the period of highest TDC

Table 1. Comparison of the dilution of *O*-acetyl[^{15}N]vallesamine added and the amount of labelled compound recovered after 20 days of culture of the accumulating and low-accumulating cell line with and without the addition of loganin

	Loganin (μmol flask^{-1})		*O*-Acetyl[^{15}N]vallesamine (μmol flask^{-1})		Dilution of label (fold)
	Added	Recovered	Added	Recovered	
AL*	6	—	240	143	2.6
	10400	3600	240	104	18.9
LAL†	0	—	240	143	1.2
	10400	3000	240	132	20.0

*Accumulating cell line.
†Low-accumulating cell line.

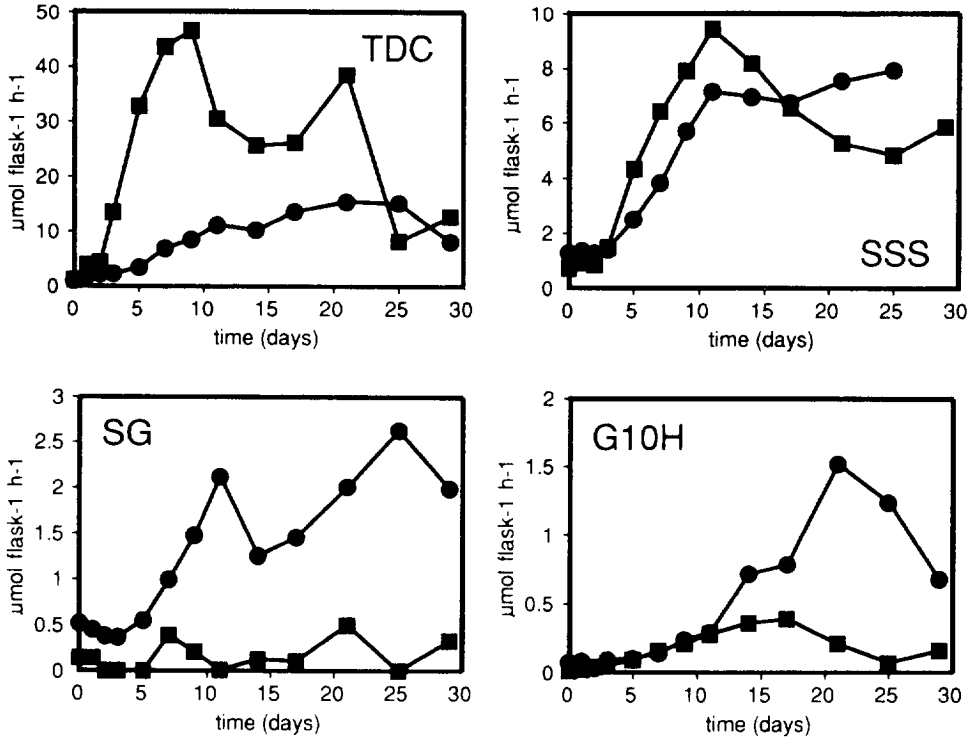

Fig. 3. Time courses of amount of substrate/product expected to be consumed/formed per flask per hour if all the enzymes present in the biomass of the accumulating (●) and low-accumulating (■) cell line were operating at maximum velocity (at substrate saturation). G10H, amount of 10-hydroxygeraniol formed; TDC, amount of tryptamine formed; SSS, amount of strictosidine formed; and SG, amount of strictosidine consumed. The experiment was carried out as in Fig. 2.

activity, between the 4th and 9th day of culture, only a minor increase in tryptamine amounts was observed. It is possible that no tryptamine was formed because of the unavailability of its precursor, tryptophan. Tryptamine could always be found in the biomass of the two cultures (Fig. 2). Nevertheless, in the accumulating cell line the total amount of terpenoid indole alkaloids (which necessarily need tryptamine as a precursor) was about two times higher than the amount of tryptamine accumulated by the low-accumulating cell line.

Strictosidine synthase. The time courses of SSS activity of the cell lines were similar. Between the 3rd and 10th day the low-accumulating line showed higher activity. Both lines showed maximum activity during the growth phase, around the 8th day of culture. No strictosidine could be detected in the cultures, so most of the product formed through this reaction must have been further metabolized. During this period other terpenoid indole alkaloids accumulated only in the accumulating cell line.

Strictosidine glucosidase. The two cell lines showed clearly distinct levels of activity. The activity was related to the biosynthetic pattern and the accumulating cell line exhibited the highest activity throughout the culture cycle. Indeed, in the low-accumulating cell line hardly any activity could be detected. In *T. divaricata*, no other non-specific glucosidases were found to influence the SG assay carried out with crude enzyme extracts (L. H. Stevens, pers. comm.).

Isopentenyl pyrophosphate isomerase. The IPP isomerase activity of both cell lines increased from the first days of culture until the 14th day. The levels of activity of both cell lines were very similar (increasing from around 0.3 µmol to 1.5 µmol dimethylallyl pyrophosphate formed flask^{-1} hr^{-1}) and no relationship was found between activity and alkaloid production. The levels of prenyltransferases, which can compete with the terpenoid indole alkaloid pathway for precursors, were also similar for both cell lines (results not shown).

Geraniol 10-hydroxylase. The enzyme activities of the two cell lines were similar from the 2nd until the 11th day of culture. During this time, a gradual increase in activity occurred. Terpenoid indole alkaloid accumulation was observed only in the accumulating cell line. Differences in activity were must pronounced during the stationary phase and were positively related to terpenoid indole alkaloid accumulation. Maximum activity was found in the accumulating cell line during the stationary phase.

By simply comparing the various *in vitro* enzymatic activities of the two cell lines, it can be concluded that the levels of TDC and SSS present in the cells did not limit the biosynthetic capacity of the low-accumulating cell line. In earlier experiments carried out with *Catharanthus roseus* suspension cultures, in which the culture medium composition was altered so as to obtain maximum alkaloid yields, TDC and SSS amounts showed no direct relationship with alkaloid accumulation capacity [8, 9].

However, it is better to compare the rate of biosynthesis (rather than levels of accumulation) since indole alkaloids can be metabolized by suspension cultures [10, 11] and as a consequence the levels of accumulation and of biosynthesis are not always necessarily related.

The amount of G10H might have been limiting alkaloid accumulation, although its lower level probably does not completely explain the lower accumulating capacity of the low-accumulating cell line, since the similar activities in both lines at the beginning of the culture cycle did not result in similar terpenoid indole alkaloid accumulation patterns. In *C. roseus* suspension cultures increased terpenoid indole alkaloid accumulation has been correlated to increased G10H levels [12]. Another possibility is that SG was limiting accumulation, since the levels of activity of this enzyme were low compared to the accumulating line though this probably would have resulted in some strictosidine accumulation (SSS amounts in both cell lines were similar) in case the terpenoid precursors were available. Since no strictosidine was detected in this cell line, it is improbable that the low SG amounts were responsible for the low biosynthetic levels found.

Enzyme activities measured *in vitro* are related to the amount of enzyme present at the time of harvest, but *in vivo* the activity can be lower due to various factors such as substrate/coenzyme limitation, presence of inhibitors, etc. From the enzyme activities measured *in vitro* it is possible to calculate the biosynthetic potential of the cell lines; namely how much product would be formed by the cultures if all of the enzymes were catalysing the reactions at maximum speed. For example, the enzyme activity of IPP isomerase, G10H, TDC, SSS and SG present in the biomass of the terpenoid indole alkaloid accumulating cell line on the 9th day of culture (8 g fr. wt) would allow the formation of 0.7 μmol hr^{-1} of dimethylallyl pyrophosphate, 0.2 μmol hr^{-1} of 10-hydroxygeraniol, 8 μmol hr^{-1} of tryptamine, 6 μmol hr^{-1} of strictosidine and the consumption of 1 μmol hr^{-1} of strictosidine, respectively. The total amount of alkaloid accumulated by this cell line during the 9 days of culture was 0.8 μmol. This represents most of the biosynthesized compound since hardly any biological degradation occurs until the stationary phase [10]. This is an extremely small amount when compared to the catalytic capacity calculated above; thus it is unlikely that the amounts of these enzymes were limiting terpenoid indole alkaloid accumulation. The conclusions discussed above concern only the enzymes assayed. The possibility that other enzymes not assayed will limit terpenoid indole alkaloid accumulation cannot of course be ruled out.

To investigate whether a biosynthetic pathway is saturated with respect to substrate availability, precursors can be fed. Thus the feeding of loganin and secologanin to suspension cultures of *C. roseus* was found to enhance terpenoid indole alkaloid accumulation [13, 14]. To test whether the addition of terpenoid precursors would enhance terpenoid indole alkaloid accumulation by the low-accumulating cell line, loganin was fed to the cultures of both cell lines. The uptake of loganin by both cultures was similar (Table 1) and this compound could not be detected in the culture medium after the 11th day of culture (data not shown). The biomass accumulation pattern was not affected by the addition of loganin and the maximum dry weight accumulation according to the dissimilation curves occurred after 10 days of culture for both cell lines (data not shown).

Loganin feeding increased the accumulation of all the terpenoid indole alkaloids not only in the low-accumulating but also in the accumulating cell line (Fig. 4). The compounds accumulated were mostly the same as those normally found in the accumulating line so it seems that, qualitatively, the pattern of alkaloid accumulation in cultures of *T. divaricata* is strictly controlled. The only other terpenoid indole alkaloid that accumulated was strictosidine (found only in the biomass), the precursor of all the terpenoid indole alkaloids.

When loganin was fed to the cultures, tryptamine accumulation was considerably reduced while terpenoid indole alkaloids were accumulated. Furthermore, the amount of terpenoid indole alkaloids accumulated by both lines by far exceeded the amount of tryptamine that the cultures accumulated in the absence of the terpenoid precursor, which makes the possibility that limitation of tryptophan might limit tryptamine biosynthesis in normal culture conditions unlikely. The *in vitro* TDC activity levels of control and fed cultures were very similar (see below). This might mean that the rate of tryptamine biosynthesis was the same for both types of cultures but that part of this tryptamine in non-fed cultures was channelled into other metabolic pathways. Among the other possibilities was that factors other than enzyme levels could be influencing enzyme activity *in vivo*. Tryptamine has been shown to be a competitive inhibitor of TDC from *C. roseus* [15]. Storage of tryptamine is accepted to occur mainly in the vacuole while TDC should be present in the cytosol [16]. Nevertheless, such product inhibition could decrease TDC activities *in vivo*. Thus feeding of loganin to the cultures would allow a more rapid removal of tryptamine by the subsequent enzymatic reaction; i.e. its coupling to secologanin in the vacuole by SSS.

The final levels of terpenoid indole alkaloid accumulation were similar for both cell lines but the relative increase in the accumulation differed for each alkaloid. From this it becomes clear that biosynthesis in the low-accumulating cell line with respect to the accumulating one was mainly limited by the availability of terpenoid precursors. Whether this low availability was caused by low levels of G10H activity is still uncertain. Thus any other enzyme of this pathway could have been limiting the supply of precursors. It is also clear that the availability of the terpenoid precursors was in fact also limiting the biosynthesis of terpenoid indole alkaloids by the accumulating cell line.

The activities of the enzymes TDC, SSS, SG and G10H in the cultures to which loganin was added were compared with the usual activities found in the cultures (Fig. 5). No significant differences were observed. Although the flux of substrates through this pathway must

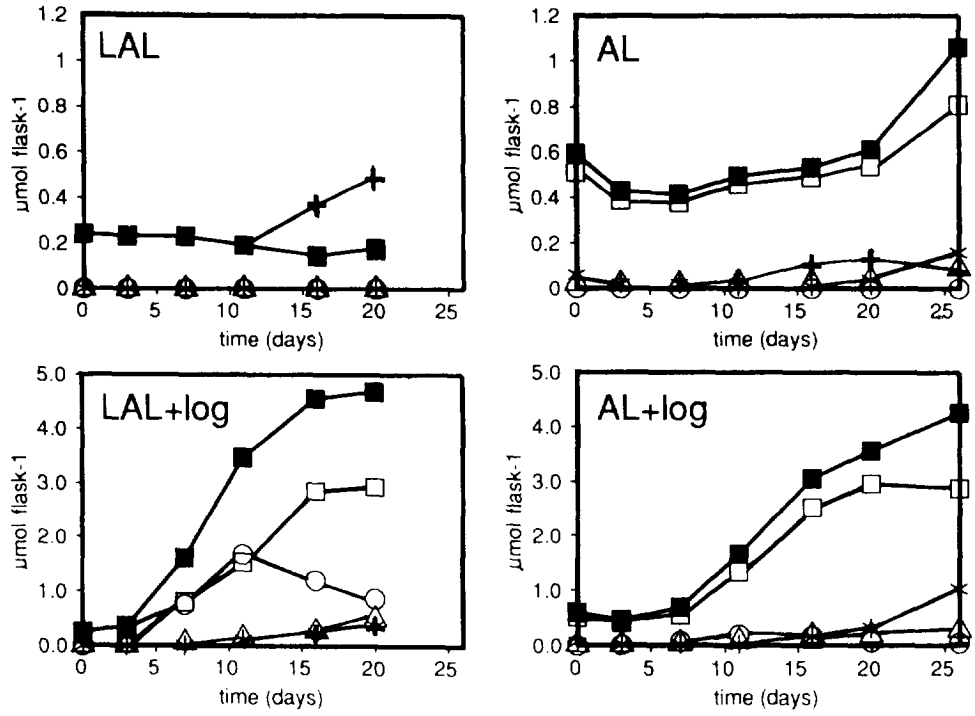

Fig. 4. Indole alkaloid accumulation time courses of the accumulating (AL) and low-accumulating (LAL) cell lines with and without feeding the terpenoid precursor loganin + – +, tryptamine; ○—○, strictosidine; □—□, O-acetylvallesamine *—*, vallesamine; Δ—Δ, voaphylline (Δ), and ■—■, total terpenoid indole alkaloids. This experiment was carried out in 100-ml Erlenmeyer flasks originally containing 20 ml of culture medium and using 1.6 g fr. wt as inoculum.

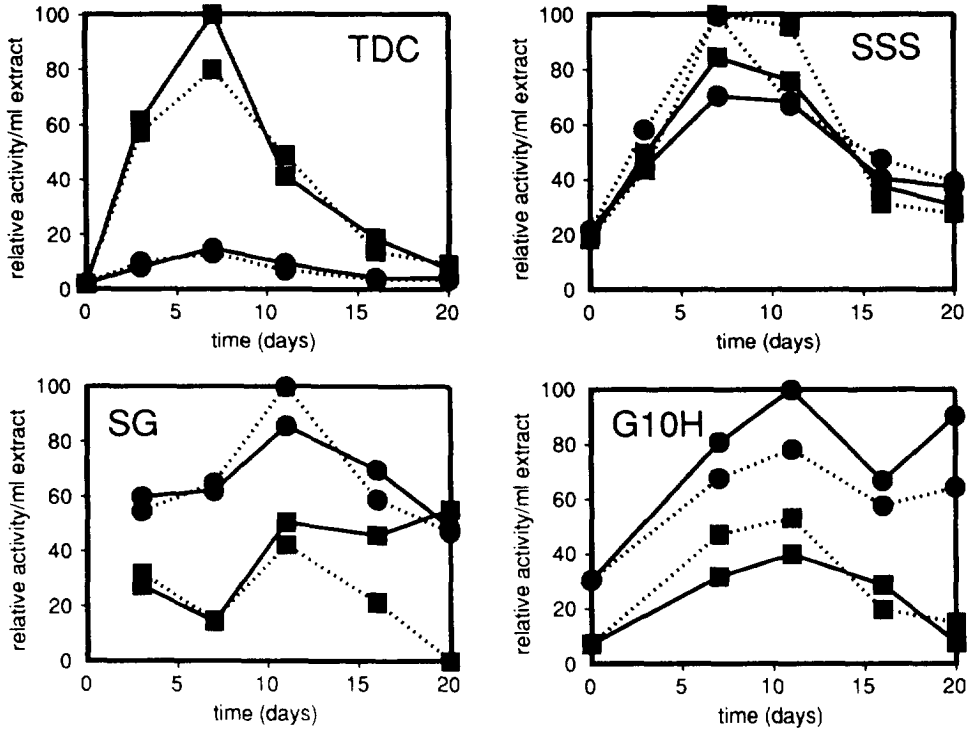

Fig. 5. Comparison of enzyme activities of the accumulating (●) and low-accumulating (■) cell line in cultures maintained in the usual culture medium (—— —) and in cultures to which loganin was added (...). Abbreviations as in Fig. 1.

have been greatly enhanced this did not lead to the induction of enzyme accumulation, as shown by the higher accumulation of terpenoid indole alkaloids obtained with the same enzyme activity levels. This indicated that this part of the pathway, in cultures maintained under normal culture conditions, was, as expected, not saturated with respect to substrate availability. The lower SG activity in the low-accumulating cell line did not seem to limit the accumulation of the terpenoid indole alkaloids *O*-acetylvallesamine, vallesamine or voaphylline since the amount of these compounds accumulated by the two cultures after feeding of loganin was similar. Nevertheless, strictosidine, the precursor of these compounds, was accumulated in much higher amounts in the low-accumulating cell line when loganin was fed. In fact, the rate of accumulation of terpenoid indole alkaloids after feeding loganin was much higher in the low-accumulating cell line, mainly owing to the rapid accumulation of strictosidine. By comparing the TDC activities of the two cell lines, it is clear that, in principle, the capacity of the low-accumulating cell line to biosynthesize tryptamine was much higher than the accumulating one. The faster ability of the low-accumulating cell line to accumulate strictosidine after feeding of loganin might have been owing to the fact that this cell line was able to biosynthesize higher amounts of tryptamine when terpenoid precursors were available. Furthermore, tryptamine has been found to inhibit SSS from *C. roseus* [17] and *Rauvolfia serpentina* [18]. The fact that tryptamine accumulation was reduced after feeding loganin would decrease the inhibition of SSS activities *in vivo*. The similar accumulation of the latter indole alkaloids by the two cultures might point to a limitation in strictosidine transport from the vacuole, where it is formed [19], to the outside of the tonoplast where SG is most likely located [16]. To our knowledge, strictosidine transport through membranes has not yet been investigated. Nevertheless, the fact that this molecule is more polar than most other terpenoid indole alkaloids suggests that its diffusion across membranes should be more difficult. *O*-Acetylvallesamine, the main terpenoid indole alkaloid accumulated by the cultures, is constantly exchanged between culture medium and biomass [10]. Strictosidine, in contrast to all other terpenoid indole alkaloids accumulated by the cultures, cannot be detected in the culture medium. From previous experiments with *T. divaricata* suspension cultures, it has become clear that levels of accumulation are not always directly related to levels of biosynthesis but that catabolism and/or chemical stability of the terpenoid indole alkaloids also influence these levels. It is thus possible that the similar levels of accumulation of these two cell lines was being regulated by catabolism.

Not all the 10.4 μmol of loganin added to the culture medium of each flask was accumulated as terpenoid indole alkaloids by the cultures. Part of the loganin added could still be found unchanged in the biomass after 20 days of culture (3.6 and 3.0 μmol flask^{-1} in the accumulating and low-accumulating cell line, respectively). Most of the secologanin that must have been formed from loganin so that terpenoid indole alkaloid accumulated, was metabolized, since only low levels could be detected in the cells. The total amount of terpenoid indole alkaloid accumulated corresponded to *ca* 40% of the added loganin and to *ca* 60% of the loganin which could not be recovered. In *C. roseus*, also, not all loganin was incorporated [13]. The lower incorporation found for *T. divaricata* suspension cultures was not due to incomplete uptake by the cells since no loganin could be found in the medium after 7 days in culture. Similarly, it was not due to the chemical instability of the molecule in the culture medium, since no chemical decomposition could be detected. Catabolism and/or the chemical instability of the terpenoid indole alkaloids that normally accumulate may explain the lack of a molar relationship between the amount of loganin that could not be recovered and the amount of terpenoid indole alkaloid accumulated.

It has been demonstrated before that catabolism influences the levels of terpenoid indole alkaloid accumulation in suspension cultures of *T. divaricata* [10]. As was the case for the biosynthetic part of the pathway, the catabolic part might not be saturated under normal culture conditions and an increase in the amount of terpenoid indole alkaloids biosynthesized could lead to an increase in the amounts of compound catabolized. Thus precursor feeding to enhance alkaloid yields might not always be very efficient.

EXPERIMENTAL

Two cell lines of *Tabernaemontana divaricata* (L.) R. Br. ex Roem & Schult were chosen for the experiment. Both were obtained from the same suspension culture which was originally maintained in a modified [20] MS culture medium [21]. Both cell lines have always been kept under the same culture conditions (MS culture medium [21] containing no growth regulators; gyratory shaker 100–120 rpm, 25° and 1500 lux).

Experiment 1. *Comparison of enzyme activities and alkaloid accumulation of the two cell lines.* Either the accumulating or low-accumulating line (each 4 g fr. wt) was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of MS culture medium [21] containing no growth regulators. During the experiment the cultures were maintained as described above except that the Erlenmeyer flasks were closed with silicon stoppers (T-32, Shin-Etsu Polymer Co., Ltd) instead of cotton plugs to allow determination of the dissimilation curves [22]. *Harvesting*: Biomass was sepd from the culture medium by filtration through a sintered glass filter and the culture medium stored at $-15°$. The biomass was stored at the same temp. before and after it had been freeze dried.

Indole alkaloid extraction and quantification. For quantification purposes the apolar alkaloids were extracted from the freeze-dried biomass with CH_2Cl_2 as previously described [10]. The culture medium was injected directly into the HPLC system. The conditions used were: Merck LiChrospher 60 RP-select B (5 μm, 125 × 4 mm) column, eluent 0.05 M NaH_2PO_4–MeCN–

2-methoxyethanol (16:4:1); pH adjusted to 3.9 with phosphoric acid, flow rate 1 ml min^{-1}, detection diode-array UV-visible detector.

Strictosidine was extracted from the biomass, previously extracted with CH_2Cl_2, with MeOH (*ca* 50 mg of freeze-dried biomass extracted with 2 × 5 ml MeOH). The extract was dried under red. press. and redissolved in 1 M H_3PO_4. The filtered H_3PO_4 soln was injected into the HPLC system; because of interfering compounds in the extract it was not possible to quantify strictosidine with the HPLC system described above and so the eluent was slightly modified by adjustment of the pH to 4.7 instead of 3.9.

Protein extraction procedure. For the determination of TDC, SSS, SG and G10H activities, cells were extracted directly after harvesting by mixing 2 g fr. wt of cells and 2 ml 50 mM KPi buffer, pH 7, containing 1 M glucose, 1 mM EDTA, 2 mM DTT, 10 μM leupeptin and 100 mg PVPP with a Potter homogenizer at maximum rpm (16 vertical movements, the whole procedure carried out in an ice bath). This extract was centrifuged for 20 min at 5000 rpm. The supernatant was then centrifuged at 13 000 rpm for 1 hr, and the pellet kept for measurement of G10H activity. The supernatant (1 ml) was desalted on a PD-10 column according to manufacturer's instructions. The desalted extract was used for assaying TDC, SSS and SG.

For the determination of IPP isomerase activity, biomass which had been stored at − 80° was used. Protein extraction was carried out as described by A. Ramos-Valdivia. (unpublished work); 1 ml of 100 mM TRIS buffer, pH 7.5, containing 2 mM EDTA, 2 mM DTT, 10 μM leupeptin and 5% glycerol was added to 1 g fr. wt of cells and an extract prepared as described above. Desalted extracts were used to determine the enzyme activities.

Enzyme assays. Assays to determine TDC, SSS, SG and G10H were carried out immediately on completing the extraction procedure. Assays for IPP isomerase were carried out the day after extraction. All assays were carried out in duplicate. Blanks for the reactions consisted of the cocktails containing the enzyme extract where the reaction was stopped at time zero. *TDC*—The assay was carried out by slightly modifying a method previously described [23]. Desalted extract (50 μl) was added to 0.15 M TRIS buffer (50 μl), pH 7.5, containing 20 nmol pyridoxal phosphate, 0.2 μmol tryptophan and 0.2 μmol DTT. After incubation for 1 hr at 30°, the reaction was stopped by adding 200 μl MeOH. The amount of tryptamine formed was measured by HPLC-fluorescence detection. *SSS*—This assay was carried out by slightly modifying a method previously described [24]. Desalted extract (50 μl) was added to 0.1 M NaPi buffer (50 μl), pH 6.8, containing 0.1 μmol tryptamine, 0.4 μmol secologanin, 10 μmol gluconic acid lactone and 10 μmol TRIS. The cocktail was incubated for 1 hr at 30° and the reaction stopped by the addition of 200 μl 5% TCA. The amount of strictosidine formed was measured by HPLC-UV with the same system used for the quantification of alkaloids. *SG*—This assay was carried out by slightly modifying a method previously described [16]. Desalted extract (50 μl) was added to 0.1 M NaPi buffer (75 μl), pH 6.3, containing 50 nmol strictosidine. After a 1 hr incubation at 30°, the reaction was stopped by adding 200 μl 5% TCA. The decrease in the amount of strictosidine was measured by HPLC-UV. *IPP isomerase*—This assay was carried out according to A. Ramos-Valdivia, (unpublished work). Protein extract (10 μl) was added to the cocktail that contained 2 mM DTT, 50 mM KF, 0.02% BSA, 1.5 mM $MgCl_2$ and 1.5 mM $MnCl_2$ in 100 mM TRIS-HCl buffer, pH 7.5. The reaction was started by adding 4 μl of a 1 mM [1 − ^{14}C] IPP soln (10 Ci mol^{-1}). The reaction was stopped after incubating for 10 min at 37° by adding 200 μl 25% HCl in MeOH. The product of the enzyme reaction, dimethylallyl pyrophosphate, was then left to hydrolyse to dimethylallyl alcohol for 15 min at 37°. The aq. phase was saturated with NaCl and extracted twice with toluene. The combined toluene extracts were dried with Na_2SO_4. The radioactivity of the extract was determined by liquid scintillation counting. Activities were determined up to and including the 14th day of culture. *G10H*—The pellet obtained during the protein extraction procedure was resuspended in 200 μl 50 mM KPi buffer, pH 7.5, containing 1 mM EDTA. The assay was carried out by adding 50 μl of this suspension of the cocktail described before [25]. The reaction was started by adding 210 nmol geraniol to the preincubated (5 min at 30°) cocktail; it was stopped after incubating for 30 min at 30°. The reaction mixt. was extracted with EtOAc and the solvent evapd to dryness under a stream of N_2. The extracts were redissolved in 50 μl MeOH and 5 μl of this soln injected directly into the GC system; no derivatization procedure was used. Quantification of 10-hydroxygeraniol was carried out by GC-FID on a CP Sil 5cb capillary column (10 m × 0.22 mm i.d., film thickness 0.13 μm). Analysis conditions were: injector temp. 230°, detector temp. 290°, initial oven temp. 100°, oven rise from 0 to 4 min 10° min^{-1} and from 4 to 9 min 49° min^{-1} up to 280°, N_2 was used as carrier gas (50 kPa) and the injection split ratio was 1 to 50. Quantification of 10-hydroxygeraniol was carried out by means of a calibration curve prepared with geraniol.

Experiment 2. Comparison of terpenoid indole alkaloid biosynthesis and accumulation, and enzyme activities of the two cell lines with or without feeding the precursor loganin. *O*-Acetyl [^{15}N] vallesamine and loganin were dissolved in MS culture medium containing no growth regulators. *O*-Acetyl [^{15}N] vallesamine soln or both *O*-acetyl [^{15}N] vallesamine and loganin solns were added by filter sterilization to a larger amount of the same culture medium. Either of these culture media (20 ml) was added to a 100-ml Erlenmeyer flask. About 1.6 g fr. wt (accurately weighed) of one of the cell lines was used as inoculum for each 100-ml Erlenmeyer flask. The cultures were maintained as described above except that silicon stoppers T-22 were used to close the flasks. The stability of loganin in the culture medium was determined by leaving fresh culture medium to which this compound had been added under the same culture conditions described above. Indole alkaloid extraction and quantifica-

tion procedures, protein extraction and determination of enzyme activities were carried out as described above.

Determination of labelling percentages. GC-MS analysis to obtain labelling percentages of biomass and culture medium extracts was carried out on a Finnigan MAT 900 mass spectrometer, source operating conditions electron impact, electron energy 70 eV, source temp. 373 K. Measurements were carried out with the multiple-ion detection technique. The GC conditions used were: column DB-1 10 m × 0.25 mm i.d., film thickness 0.1 μm. injector temperature 220°, initial oven temperature 175° then rising from 175° to 280° at 10° min^{-1}.

Loganin extraction and quantification procedure. Loganin in the biomass was measured in the MeOH extract used for strictosidine determination (procedure described above). The biomass extracts or culture medium (each 10 μl) were quantitatively analysed by HPLC-UV (235 nm; diode array detection). The conditions used were: Serva DEAE = SI 100 column, (250 × 4.6 mm) eluent MeCN–H_2O (100:3), flow rate 2 ml min^{-1}. For loganin R_t *ca* 11 min; detection limit was below 2 μmol g^{-1} dry wt and 100 nmol ml^{-1} culture medium. With this system secologanin had a R_t of 7 min.

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