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Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures

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Abstract

Precursors from the terpenoid and tryptophan branches were fed to *Catharanthus roseus* to determine which of the two branches limits metabolic flux to indole alkaloids. The feeding of tryptophan at 17 days of the culture cycle produced auxin-like effects. Addition of low levels of auxin or tryptophan resulted in significant increases in flux to the indole alkaloids. Conversely, feeding higher levels of auxin or tryptophan resulted in increased branching and thickening of the hairy root cultures. A dramatic reduction in flux to the alkaloids was also observed. However, feeding tryptamine or terpenoid precursors had no effect. Therefore, neither pathway tested revealed to be rate-limiting during the late growth phase. Feeding of either geraniol, 10-hydroxygeraniol, or loganin at 21 days each resulted in significant increases in the accumulation of tabersonine. The addition of tryptophan or tryptamine had no effect during the stationary phase of the growth cycle. Thus, during the early stationary phase of growth the terpenoid pathway appears to be rate-limiting. Combined elicitation with jasmonic acid and feeding either loganin or tryptamine did not further enhance the accumulation of indole alkaloids.

Keywords: *Catharanthus roseus*; Indole alkaloids; Geraniol; Indole acetic acid

1. Introduction

Catharanthus roseus (L.) G. Don, the Madagascar periwinkle, synthesizes numerous terpenoid indole alkaloids. Notably, the dimeric alkaloids vincristine, vinblastine and synthetic derivatives of these natural products are powerful anti-cancer agents. The amounts of these alkaloids produced by the plant are extremely low, hence leading investigators to examine plant cell and tissue cultures as alternative means of production. Ajmalicine and serpentine are also medicinally valuable *C. roseus* alkaloids that have use as anti-hypertension agents. Plant cell and tissue cultures of *C. roseus* have been studied extensively for the intended purpose to enhance production of these valuable indole alkaloids (Verpoorte et al., 1993).

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Tissue differentiation plays a significant role in the types of alkaloids produced. For example, the synthesis of vindoline is restricted to the leaves of the plant (De Luca et al., 1988), while ajmalicine and serpentine are the major alkaloids found in roots of the plant as well as cell suspension cultures (van der Heijden et al., 1989). However, in hairy roots of *Catharanthus* species, tabersonine, lochnericine, and hörhammericine are major products in addition to ajmalicine and serpentine (Davioud et al., 1989; Shanks et al., 1998). Because the biosynthesis and accumulation of specific metabolites are influenced by tissue type, rate limitations in hairy roots may be different than in cell suspension cultures.

All of the terpenoid indole alkaloids are derived from a central intermediate, strictosidine (Fig. 1). The indole portion of the molecule is derived from tryptamine, which is formed by the decarboxylation of tryptophan. The terpenoid portion of strictosidine, secologanin, is known to be derived from geranyl pyrophosphate, which is subsequently converted to geraniol, 10-hydroxygeraniol, and loganin by multiple steps (Meijer et al., 1993).

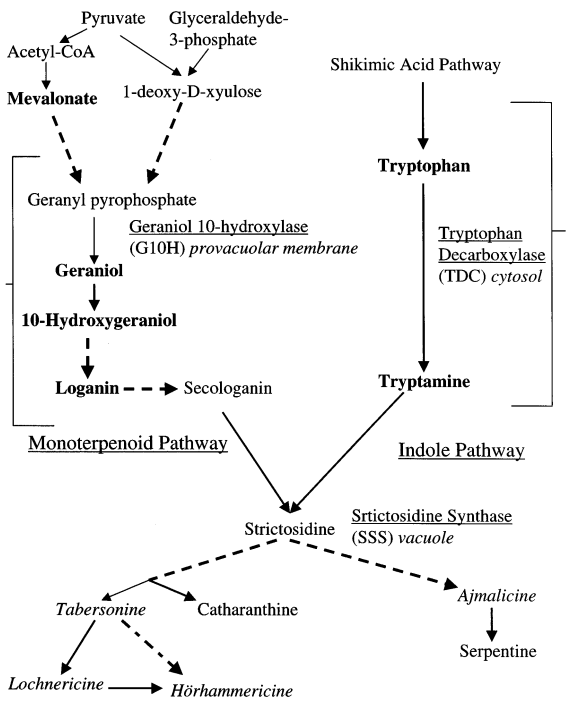

Fig. 1. Proposed biosynthetic pathway leading to the indole alkaloids in *C. roseus* hairy roots. Dashed lines represent multiple or uncharacterized reactions in the pathway. Compounds in bold were fed as precursors in this study. Italicized compounds were quantified from the extracts of hairy roots.

Based upon precursor feeding and enzyme activity studies, a current hypothesis is that precursors from the terpenoid pathway are a flux limitation in cell suspension cultures (Schiel et al., 1987; Moreno et al., 1993). However, previous feeding studies have reported contradictory results. For example, tryptophan feeding has resulted in increased growth and specific yields of serpentine in one cell line (Zenk et al., 1977), reduced alkaloid content in different study (Knobloch and Berlin, 1980), and no effect in another study (Kargi and Ganapathi, 1991). In other examples, addition of tryptophan to cells increased tryptamine levels, but had no effect on indole alkaloid levels (Merillon et al., 1986; Facchini and DiCosmo, 1991). Similarly, addition of tryptamine stimulated alkaloid production in one study (Krueger and Carew, 1978), while inhibiting alkaloid accumulation in another (Döller et al., 1976).

The addition of loganin or secologanin to cell suspension cultures increased indole alkaloid levels (Merillon et al., 1989; Moreno et al., 1993), which suggests the monoterpenoid pathway may be rate limiting. Feeding of geraniol was found to have no effect on alkaloid production (Krueger and Carew, 1978). Even earlier in the pathway, the feeding of mevalonic acid had no effect on indole alkaloid production levels, which indicates the rate limiting enzyme may lie between mevalonic acid and loganin (Krueger and Carew, 1978; Moreno et al., 1993). However, recent evidence indicates that mevalonic acid is not the only precursor of the strictosidine. Instead, a novel triose phosphate/pyruvate pathway is implicated as the major provider of carbon for the monoterpenoid pathway leading to the indole alkaloids (Contin et al., 1998).

The goal of this study was to determine the presence of flux-limitations in the pathways leading to the formation of indole alkaloids by pre-

cursor feeding. The unique aspects of this study are the system used, as well as the wide range of precursors fed and alkaloids quantified.

2. Materials and methods

2.1. Chemicals

The precursors were of highest available purity. Geraniol (Fluka, Ronkonkoma, NY) and 10-hydroxygeraniol (Aldrich, Milwaukee, WI) were added directly to the culture media as liquids. Mevalonic acid was prepared by mixing an equal molar solution of $NaHCO_3$ and mevalonic acid lactone (Sigma Chemical, St. Louis, MO). Loganin (Apin Chemical Ltd., Oxon, UK), tryptamine (Fluka), pyruvic acid, and L-tryptophan (Sigma) were dissolved in filter sterilized water before adding to the cultures. The hormones 1-napthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) were added from stock solutions (Sigma). When necessary a few drops of HCl were added to help dissolve the precursor. Equal amounts of distilled water, with and without HCl were added to control cultures.

2.2. Methods

Earlier work characterized the growth of the hairy roots into exponential and stationary phases when modeled by a simple exponential growth model (Bhadra and Shanks, 1997). Comparing biomass accumulation data with the model, the exponential growth phase ends at 21 days for this culture. The timing of the precursor addition was designed to explore alkaloid productivity in late growth phase (17–21 days) as well as early stationary phase (21–24 days). Precursors were added at 17 and 21 days with the cultures harvested at 21 and 24 days, respectively. In the elicitation experiments, jasmonic acid (JA) (50 mg l^{-1}) was added either on day 20 or 22 with tryptamine (20 mg l^{-1}) and loganin (40 mg l^{-1}) added on day 21. Cultures were harvested on day 24.

2.3. Culture conditions

The hairy root line LBE 6-1 was used for all experiments (Bhadra et al., 1993). The media was a filter-sterilized solution of 30 g l^{-1} sucrose, half strength Gamborg's B5 mineral salts and full strength Gamborg's vitamins. The initial pH of the media was adjusted to 5.7. Cultures were initiated every 3 weeks by placing five root tips (35–40 mm) into 50 ml of media. The cultures were shaken at 100 rpm, and incubated at 26°C in the dark.

2.4. Alkaloid extraction

The fresh weight of the cultures was measured after thorough blotting of excess media. Cultures were then immediately frozen at -70°C, and dry weight was measured after lyophilization. Dried roots were finely ground, and ~ 0.15 g were extracted with 45 ml of MeOH in a sonication bath for 5 h. The supernatant was clarified by centrifugation of the cellular debris for 15 min.

2.5. Alkaloid analysis

The crude MeOH extract was evaporated to a volume of ~ 4 ml, and then passed through a 0.22 μm, 13 mm filter, of which 10 μl were injected on a Bondclone 10μ C18 HPLC column (300×3.9 mm) (Phenomenex, Inc., Torrance, CA). The mobile phase consisted of a 32:32:36 mixture of MeOH:MeCN:5 mM $(NH_4)_2PO_4$ buffer. An initial flow rate of 1 ml min^{-1} was maintained for 20 min, and ramped linearly for 10 min to 1.4 ml min^{-1}. The flow rate was returned to 1 ml min^{-1} over the next 5 min, where it was held for 5 min. Alkaloids were identified by photodiode array detection, and were quantified as reported previously (Shanks et al., 1998). The alkaloids quantified in this study were ajmalicine, serpentine, tabersonine, lochnericine, and hörhammericine.

2.6. Statistical analysis

The effect of a treatment was analyzed by using a Student's *t*-test to compare means (Microsoft Excel 4.0, Redmond, WA).

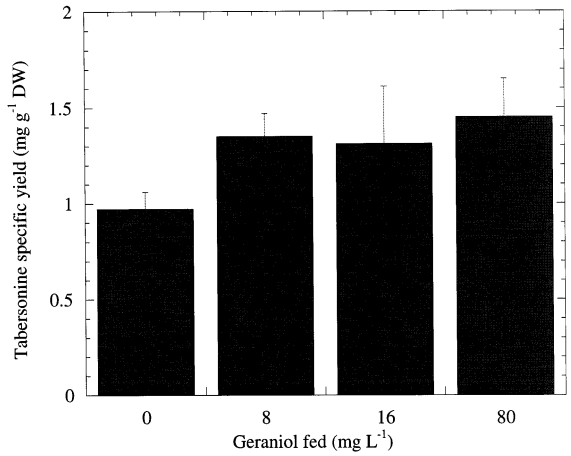

Fig. 2. The effect of feeding various levels of the precursor geraniol on the specific yield of tabersonine. Geraniol was fed at 21 days and the cultures were harvested after 72 h. There was no statistical difference between the treatments. The difference between feeding geraniol and the control was statistically significant ($P < 0.05$) for the 8 and 80 mg l^{-1} treatments.

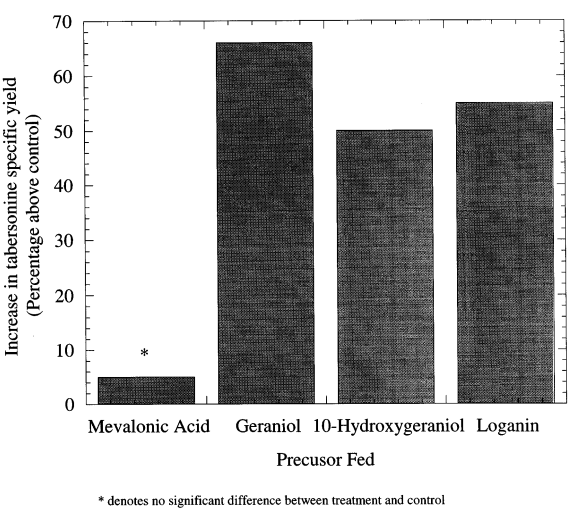

Fig. 3. Feeding the terpenoid precursors, geraniol, 10-hydroxygeraniol and loganin significantly ($P < 0.05$) increased the specific yield of tabersonine in hairy root cultures. The precursors were added at 21 days and the cultures were harvested at 24 days. *, indicates no significant difference between treatment and control.

3. Results and discussion

3.1. Precursor dosage determination

The first issue examined was the proper amount of precursor to add to the cultures. Feeding studies with different dosages of geraniol revealed that at and above 160 mg l^{-1} there were toxic effects on the growth of the cells. Fig. 2 shows that lower dosages of geraniol resulted in the similar effects upon the accumulation of tabersonine. To feed a constant amount of precursor per unit biomass, 8 mg l^{-1} of geraniol was the dosage selected for feeding at 17 days, and 16 mg l^{-1} at 21 days. For the other precursors, equal molar (52 μM at 17 days or 104 μM at 21 days) amounts were fed. At these levels, no precursor had a significant effect on biomass accumulation (data not shown). Therefore, specific yields of alkaloid accumulation were used for comparison of treated and control cultures.

3.2. Early stationary phase feeding

The timing of feeding had significant effects on the accumulation of alkaloids. Results from the precursor feeding at 21 days will be presented first, followed by the results from feeding at 17 days. Neither tryptophan nor tryptamine had a positive effect on alkaloid accumulation. The only statistically significant effect ($P < 0.05$) due to tryptophan feeding was a 29% reduction in the accumulation of tabersonine. Feeding the terpenoid precursors also significantly affected tabersonine. In contrast to tryptophan, adding geraniol, 10-hydroxygeraniol, and loganin all significantly increased the accumulation of tabersonine by more than 50% above the control (Fig. 3). There was no statistically significant difference between the three treatments. Loganin was found to be removed completely from the media by 48 h, and no intracellular pools of loganin were detected. Surprisingly, the specific yield of hörhammericine was reduced by 24% when either geraniol or loganin was fed ($P < 0.05$). However, the increase in tabersonine total yield was larger than the decrease in hörhammericine (data not shown). The addition of mevalonic acid had no effect on

alkaloid accumulation, which is similar to previous reports (Krueger and Carew, 1978; Moreno et al., 1993).

The evidence for a limitation in precursor flux occurring in the terpenoid pathway is rather limited, as only the specific yield of only one alkaloid was found to be increased in response to feeding, and in some cases the specific yield of hörhammericine was reduced. To support these findings, further experiments were performed. Linalool, an analog of geraniol, known not to be incorporated into the indole alkaloids was fed at the same level as geraniol. Contrary to the positive effect of geraniol, the addition of linalool had no effect on alkaloid accumulation. Thus, the increase in tabersonine from geraniol feeding does not appear to be due to non-specific elicitation. The question why only tabersonine accumulated was investigated by feeding tabersonine (40 mg l^{-1}) at 21 days and harvesting cultures at 24 days. The added tabersonine was taken up completely during this period, but only a small amount was transformed to other products (Fig. 4). This implies that the roots may have a limited capacity to transform tabersonine at this stage of the growth cycle. However, it could also be that tabersonine did not reach the correct cellular or subcellular location necessary for transformation. Interestingly, the specific yield of hörhammericine was significantly reduced in response to tabersonine feeding (Fig. 4). One possibility for this decrease is that tabersonine is an inhibitor of hörhammericine formation. As a whole there is convincing evidence that the terpenoid pathway limits flux during this stage of growth for hairy root cultures.

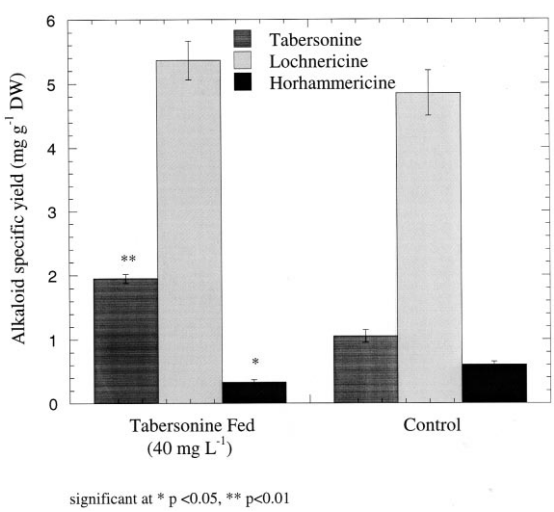

Fig. 4. The addition of tabersonine (40 mg l^{-1}) to hairy root cultures at day 21 resulted in the increased specific yield of tabersonine ($P < 0.01$) and the decreased specific yield of hörhammericine ($P < 0.05$).

Other feeding studies with different tissue types had generally similar results. In cell suspensions fed at a similar time in the growth phase of the cultures, the terpenoid precursors, loganin and secologanin significantly enhanced ajmalicine accumulation (Moreno et al., 1993). Later work on a cell line genetically engineered to constitutively overexpress the strictosidine synthase gene, showed that feeding loganin and tryptamine together enhanced the flux late in the growth cycle (Whitmer et al., 1998a). On the contrary, in our experiments, combined feeding of tryptamine and loganin produced the same results as feeding loganin alone (data not shown).

If the terpenoid pathway is limiting upstream of geraniol, the next step would to examine which steps are responsible. The phosphorylated intermediates IPP, DMAPP, and GPP are not amenable to feeding studies since they are unlikely to be able to cross the cell membrane. In primary metabolism, pyruvate is a precursor that leads to both the mevalonate pathway or the deoxyxyulose phosphate pathway. Hence, 200 mg l^{-1} of pyruvate was added to cultures at 21 days. After 3 days, 50% of the pyruvate remained in the media. Not surprisingly, pyruvate feeding had no significant effect on alkaloid accumulation. As more details arise from the deoxyxyulose phosphate pathway perhaps precursors leading to IPP will become available for future feeding studies.

3.3. Late growth phase feeding

The results from precursor feeding at 17 days were both quantitatively and qualitatively different. Contrary to feeding at 21 days, tryptophan enhanced accumulation of the alkaloids tabersonine, hörhammericine, and serpentine (Table 1). This is significant in that the enhancement was in alkaloids in two downstream pathways (Fig. 1).

Table 1
Percent change compared with control in alkaloid specific yield cultures fed during late growth phase (day 17)[a]

Precursor fed alkaloid	Indole precursors		Monoterpenoid precursors		Loganin
	Tryptophan	Tryptamine	Geraniol	10-Hydroxy-geraniol	
Tabersonine	41[b]	23	13	5	+3
Lochnericine	11	−1	31[b]	−5	+7
Hörhammericine	22[b]	−8	−5	−2	−29
Ajmalicine	11	36	8	−14[b]	−1
Serpentine	50[b]	−14	−8	2	−23[b]

[a] Cultures were harvested 96 h after the addition of the precursor.
[b] Indicates statistically significant difference ($P<0.05$) between treatment and control ($n=3$).

However, tryptamine feeding did not have any statistically significant effect on alkaloid accumulation as would be expected if tryptophan was limiting. No tryptamine pools were detected in the cultures.

To further test the enhancement of alkaloid accumulation, a larger amount of tryptophan (100 mg l^{-1}) was fed to cultures at 12 days in the growth cycle. The morphology of the roots began to change after 4 days, with pronounced thickening of the roots and proliferation of root branching. However, even though there was accelerated growth, the total yields of alkaloids were found to be significantly lower. Based on these findings, an explanation for the influence of tryptophan on growth and alkaloid accumulation was sought. We hypothesized that the effects may be auxin mediated as the morphology of the roots was similar to that reported when exogenous auxin is added (Aerts et al., 1992). Indeed feeding NAA (1 μM) at 14 days and harvesting the root cultures at 21 days enhanced growth compared to controls grown over the same period and also reduced the total yield of the predominant alkaloid of the roots, lochnericine (Fig. 5). Furthermore, the roots displayed the characteristic thickened morphology.

The results using high levels of tryptophan and auxin, led us to test if lower levels of auxin feeding would resemble lower levels of tryptophan feeding. With the addition of 10 nM IAA, an increase in alkaloids from the two downstream pathways was seen without an effect on growth. In cell suspensions of *C. roseus*, ajmalicine and serpentine contents were enhanced by the addition of low concentrations of IAA or NAA (Merillon et al., 1989). However, it is generally found in cell suspensions that auxin represses alkaloid accumulation (Whitmer et al., 1998b). Similar to the results presented here, alkaloid production in transformed root cultures of *Hyoscyamus muticus* was enhanced significantly by the addition of auxin without affecting the root growth. At least for hairy roots, it appears that endogenous auxin production is not optimal for alkaloid accumulation.

Interestingly, auxin has been reported to affect the activity of tryptophan decarboxylase (TDC), a key enzyme in alkaloid biosynthesis, in opposite

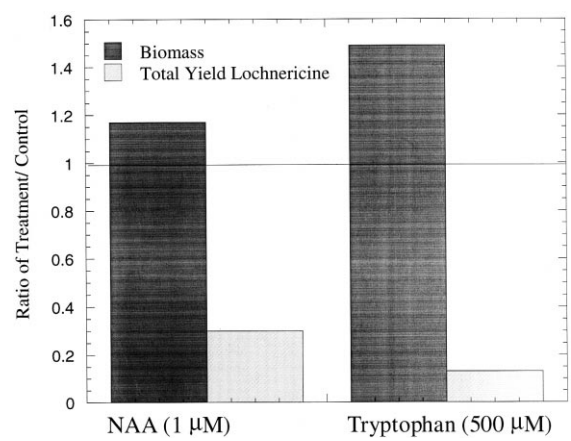

Fig. 5. A comparison of the effects feeding NAA (1 μM) and tryptophan (500 μM) on the accumulation of biomass and the total yield of lochnericine. NAA was added at 2 weeks and tryptophan was fed at 12 days. Each treatment and corresponding control cultures were harvested at 21 days.

Table 2
The effect of combined elicitation with JA (50 mg l^{-1}) on day 20 and addition of either loganin (40 mg l^{-1}) or tryptamine (20 mg l^{-1}) on day 21[a]

Treatment	Alkaloid specific yield (mg g^{-1} DW)				
	Serpentine	Ajmalicine	Tabersonine	Hörhammericine	Lochnericine
Control	0.42 ± 0.04	0.72 ± 0.07	0.90 ± 0.06	0.64 ± 0.06	3.81 ± 0.28
JA only	1.10 ± 0.06	1.13 ± 0.05	0.92 ± 0.10	6.40 ± 0.75	6.50 ± 0.43
JA + loganin	0.66 ± 0.17[b]	0.90 ± 0.13[b]	0.79 ± 0.08[b]	5.36 ± 0.82	6.00 ± 0.74
JA + tryptamine	0.70 ± 0.03[b]	0.91 ± 0.13[b]	0.77 ± 0.09[b]	5.67 ± 0.47	5.40 ± 1.00

[a] Cultures were harvested 72 h after addition of precursor (day 24).
[b] Indicates statistically significant difference ($P < 0.05$) between JA + precursor fed and JA only treatment ($n = 3$).

directions. In a hairy root culture of *C. roseus*, TDC activity was down-regulated by auxin at the transcriptional level (Goddijn et al., 1992). The levels of auxin used by Goddijn et al. were higher than those used in the portion of this study which resulted decreased alkaloid yields. In another study, TDC activity was enhanced in radicles of seedlings (Aerts et al., 1992). The levels of auxin used in this study were similar to the study by Goddijn et al. (1992) but the difference in results could be due to the different tissues examined. Since it is known that hairy roots are more sensitive to auxin than non-transformed tissues perhaps the response of TDC activity is a function of the tissue type and hormone concentration (Shen et al., 1990).

The hypothesis that tryptophan feeding increases the levels of IAA, was not completely unexpected. In hairy roots derived from agropine *Agrobacterium* strains, the genes for the synthesis of IAA from tryptophan are inserted (Cardarelli et al., 1985). LBE 6-1 is derived from *A. rhizogenes* 15834 (Bhadra et al., 1993) which is an agropine strain. Furthermore, even in untransformed roots of *Arabidobsis thaliana*, a model dicotyledon plant, tryptophan was reported to be the precursor of IAA in roots (Müller et al., 1998). The low levels of auxin that were found to affect metabolism are likely due to hairy roots enhanced sensitivity to auxin (Shen et al., 1990).

The earlier studies and the results reported herein show the importance of considering differences in tissue type and amount of hormone applied when interpreting results from hormone treatments. Furthermore, since tryptophan has multiple biochemical fates, such as protein, alkaloid, and hormone synthesis, the effects of metabolic manipulations on all of these pathways must be considered. For example, *C. roseus* cell lines engineered with high levels of TDC were found to be detrimental to normal growth of the cultures (Canel et al., 1998). It was hypothesized that tryptophan necessary for growth was diverted into tryptamine, thus causing these negative effects. Another example of how converting tryptophan to tryptamine has interactive effects is the participation of tryptophan in the regulation of the phenylpropanoid pathway (Yao et al., 1995).

3.4. Elicitation and precursor feeding

Earlier work from our laboratory has shown that JA increases significantly the flux to alkaloids when hairy root cultures were elicited at 3 weeks (Rijhwani and Shanks, 1998). To understand which precursors are limiting under elicited conditions, an experiment analyzed the combination of elicitation with JA with precursor feeding. Elicitation alone produced a 2.5-fold increase in the specific yield of the five quantified alkaloids (Table 2). Adding the precursors a day before elicitation resulted in no significant difference between this treatment and only JA treated cultures. Surprisingly, adding the precursors a day after elicitation significantly reduced the specific

yields of tabersonine, ajmalicine, and serpentine (Table 2). This finding was consistent whether tryptamine or loganin was added. Similarly, earlier work on combined elicitation and precursor feeding found that elicitation and feeding secologanin or loganin resulted in lower increases in ajmalicine than precursor feeding alone (Moreno et al., 1993). One difference between that study and the present work was for the elicitation only control, we observed large increases in flux to several alkaloids (Table 2), while in the work of Moreno et al. ajmalicine levels remained unchanged (Moreno et al., 1993). This is likely due to the use of different elicitors. In this study, we used JA a known potent elicitor of secondary metabolism, while Moreno et al. used a cell free extract from a *Pythium* (Moreno et al., 1993) fungal culture.

The issue of which precursor pathway has more control over metabolic flux during the late growth phase is still unresolved. Because feeding tryptamine had no positive effect on alkaloid accumulation, one possibility are limitations in the terpenoid branch after loganin, such as secologanin. However, this would be in contrast to what was found with cell suspension cultures. During the growth phase of the SSS overexpressing cell line, loganin feeding alone enhanced flux to total alkaloids (Whitmer et al., 1998a).

In conclusion, the amount of increase in flux to total indole alkaloids due to precursor feeding was moderate compared to elicitation with JA. Several reasons may account for the limited increases in alkaloid accumulation. First, the low flux increase simply reaffirms the observation that it is difficult to enhance flux without changing the levels of several enzymes (ap Rees and Hill, 1994). Elicitation, which does change enzyme levels was more effective at enhancing overall flux to indole alkaloids from hairy roots (Rijhwani and Shanks, 1998). Although all of the precursors are known to be taken up, it is not known if there was preferential uptake by certain regions of the roots. Furthermore, the subcellular distribution of precursors was not examined. In the future, more precise studies using genetic means will be necessary to test metabolic control of flux in *C. roseus* hairy root cultures.

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