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Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase over-expressing transgenic cell line T22 of *Catharanthus roseus*

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Abstract

To obtain more insight into the regulation of terpenoid indole alkaloid (TIA) biosynthesis in *Catharanthus roseus* (L.) G. Don cell cultures and particularly to identify possible rate limiting steps, a transgenic cell line over-expressing tryptophan decarboxylase (*Tdc*), and thus having a high level of tryptamine, was fed with various amounts of precursors (tryptophan, tryptamine, loganin and secologanin) in different time schedules and analyzed for TIA production. When these precursors were added to this culture it was found that the optimal time for supplying the precursors was at inoculation of the cells into the production medium. Alkaloid accumulation by line T22 was enhanced by addition of loganin or secologanin; however, the secologanin feeding was less effective. Tryptamine or tryptophan alone had no effect on TIA accumulation. The over-expression of *Tdc* causes this cell line to produce quite large quantities of alkaloids after feeding loganin or secologanin. However, in combination with tryptophan or tryptamine, feeding of these precursors resulted in an even further increase of alkaloid accumulation and under optimal conditions line T22 accumulated around 1200 μmol l^{-1} of TIAs whereas the control cultures accumulated less than 10 μmol l^{-1} TIAs.

Keywords: Cell culture; Genetic engineering; Secondary metabolism; Strictosidine synthase; Terpenoid indole alkaloids; Tryptophan decarboxylase

1. Introduction

Catharanthus roseus (L.) G. Don (Apocynaceae) produces a wide range of terpenoid indole alkaloids (TIAs) and among them ajmalicine, serpentine, vinblastine and vincristine are of pharmaceutical interest. Field grown plants of *C. roseus* are still the only commercial source for the production of these drugs (DiCosmo and Misawa,

Abbreviations: GM, growth medium; MS, Murashige and Skoog (1962); NAA, naphthalene acetic acid; PM, production medium; STR, strictosidine synthase; TDC, tryptophan decarboxylase; TIA, terpenoid indole alkaloid.

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1995). Cell and tissue cultures of *C. roseus* have been investigated as an alternative source of TIA production. The optimization of culture conditions such as medium composition, including adding biosynthetic precursor(s) to the medium, may enhance alkaloid production where the productivity is limited by lack of that particular precursor. Results have been limited, and stability is a major problem for high producing strains. Genetic engineering seems to offer a new interesting possibility for improving the production of alkaloids in plants or plant cell cultures (Verpoorte et al., 1997).

Two convergent metabolic pathways supply the indole and the iridoid precursor for the biosynthesis of TIAs. The amino acid L-tryptophan is derived from the shikimate pathway, and tryptophan decarboxylase (TDC) catalyzes its conversion to tryptamine, which provides the indole moiety. Secologanin, a mono-terpenoid glucoside, is derived from loganin via the MEP pathway (Contin et al., 1998). Tryptamine and secologanin are condensed by STR to form 3α(*S*)-strictosidine, the general precursor of a multitude of diverse TIAs (Fig. 1).

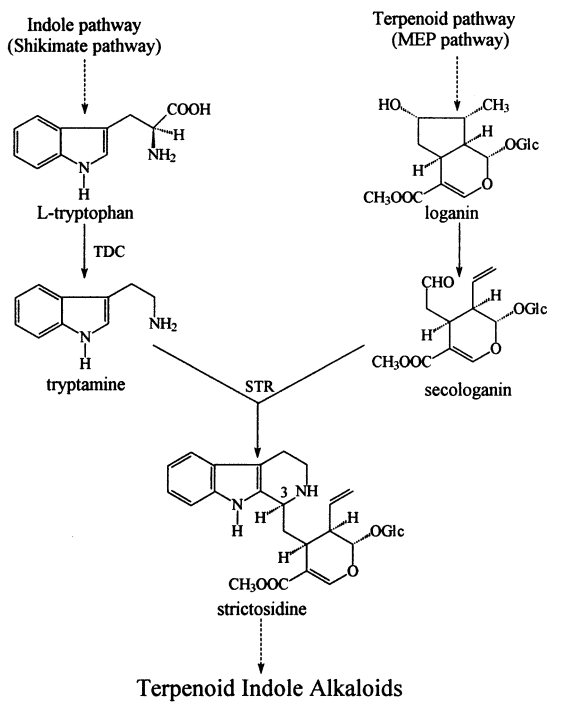

Fig. 1. Biosynthesis of TIAs in *C. roseus*. STR, strictosidine synthase; TDC.

There are several reports about precursor feeding experiments with cell suspension cultures of *C. roseus*. The reported effects of exogenous tryptophan and tryptamine on TIA production are contradictory (Döller et al., 1976; Zenk et al., 1977; Krueger and Carew, 1978; Bongaerts et al., 1998). In addition, Naudascher et al. (1989a,b) studied the utilization of secologanin and loganin by *C. roseus* cell cultures in time–course experiments. They hypothesized a different compartmentation for exogenous and endogenous secologanin and reported that unlike exogenous secologanin, exogenous loganin is used in toto by *C. roseus* cells. In several studies (Zenk et al., 1977; Naudascher et al., 1990; Facchini and DiCosmo, 1991; Moreno et al., 1993; Morgan and Shanks, 2000), however, the addition of both loganin and secologanin to the medium has been found to enhance TIA accumulation. We also observed that feeding of the iridoid loganin, the immediate precursor of secologanin, consistently resulted in enhanced TIA accumulation by transgenic *C. roseus* cell lines S10 (transgenic for *Str*; Whitmer et al., 1998) and S1 (transgenic for *Str*; manuscript in print). Considering our previous results, in the present study we investigated precursor feeding to the transgenic cell line T22 which carries a recombinant, constitutively over-expressed version of the endogenous *Tdc2* gene (Canel et al., 1998). Line T22 is the only transgenic cell line with over-expression of the *Tdc* gene which does produce TIAs in our laboratory. The alkaloid production profile of line T22 is strictosidine, ajmalicine, catharanthine, and serpentine.

Here, we report the significance of supplying the biosynthetic precursors secologanin, loganin, L-tryptophan, and tryptamine in various concentrations and combinations under constitutively elevated TDC activity. The influence of the feeding schedule, feedings with single or double precursor(s), and the effect of addition of different concentrations of precursor(s) on accumulation of tryptamine, strictosidine, ajmalicine, catharanthine and serpentine in production medium by line T22 were monitored in order to identify rate-limiting steps in the TIA pathway.

2. Materials and methods

2.1. Culture media and components

GM (MS58) (l^{-1}): Murashige and Skoog salts (Murashige and Skoog, 1962), 0.1 g *myo*-inositol, 0.4 mg thiamine, 2 mg NAA, 0.2 mg kinetin, and 30 g sucrose; PM (l^{-1}): Murashige and Skoog salts devoid of phosphate and nitrate, 0.1 g *myo*-inositol, 0.4 mg thiamine, and 80 g sucrose. GM was supplemented with 50 mg l^{-1} of hygromycin B for selection. Sucrose and hygromycin B were purchased from Duchefa (Haarlem, The Netherlands), salts, vitamins, and hormones from Merck (Darmstadt, Germany).

2.2. Cell lines and culture conditions

The transgenic cell line T22 was generated by *Agrobacterium*-mediated transformation of seedlings leaves explants of *C. roseus* 'Morning Mist' (Kieft, The Netherlands), as described previously (Canel et al., 1998). Wild type cell line CRPM was established from seeds of *C. roseus* in 1983. Lines were maintained by periodic subculture every week into 250-ml Erlenmeyer flasks with silicone foam stoppers (Shin Etsu, Tokyo, Japan) containing 50 ml of liquid medium in a 1:3 ratio. Cultures were placed on a gyratory shaker (New Brunswick Scientific, Edison, USA) at 110 ± 5 rpm and at 25 ± 1 °C, with light 2100 ± 100 lux, 24 h per day. To generate sufficient biomass for inoculation of a large number of flasks, cell suspension cultures were sequentially scaled up to 0.5–l and 2–l flasks containing 100 and 500 ml of MS58 after 7-day growth periods. For the experiments, 5 ± 0.1 g cells, 7-day-old, were inoculated into 50 ml of PM in each 250-ml flask. Aqueous solutions of secologanin, loganin, L-tryptophan and tryptamine-HCl were added from filter-sterilized 100 mM or 200 mM stock solutions to the suspension cultures. In all combination feedings, the concentration of each fed precursor was the same as the concentration used in single feedings (in all feedings the quoted feeding amount always represents a final concentration in the culture medium of the fed precursor(s)). Loganin was purchased from Extrasynthese (Genay, France), L-tryptophan from Merck, tryptamine-HCl from Aldrich (Zwijndrecht, The Netherlands). Secologanin with a purity of 96% was obtained from crude extracts of berries of *Symphoricarpus* sp. as described previously (Whitmer et al., 1998). Treated and control cultures were harvested in duplicate; extraction and analysis were carried out after pooling the replicate samples.

2.3. Enzyme assays

Soluble proteins were extracted from 350 mg of frozen biomass by homogenization in 350 μl of extraction buffer (0.1 M sodium phosphate, pH 7, 2 mM EDTA, 4 mM DTT), in the presence of 17.5 mg of polyvinylpyrrolidone. Homogenization was performed in 1.5-ml microfuge tubes using hand-held plastic micropestles (Van Oortmerssen, Rijswijk, The Netherlands). A clear supernatant containing the enzymes of interest was obtained by centrifugation of the homogenate at $14\,000 \times g$, 4 °C, for 30 min. Protein concentration was determined using Bio-Rad's staining reagent and 3550-UV Microplate Reader (Bio-Rad, Veenendaal, The Netherlands), and bovine serum albumin as standard. The procedures to assay the activities of TDC, and STR have been described (Pennings et al., 1987, 1989). The enzyme activities are reported in pkat mg^{-1} where 1 katal (kat) is the amount of the enzyme transforming 1 mol of substrate per s (forming 1 mol product per s) under defined incubation conditions.

2.4. Analysis of alkaloids and precursors

For the determination of TIAs in the culture medium, the medium was centrifuged at $14\,000 \times g$ for 5 min and 150 μl of the supernatant were injected directly into the system and analyzed by HPLC (Van der Heijden et al., 1987). For analyses of tryptamine, 50 mg freeze dried biomass were extracted with 5 ml of dichloromethane (Schripsema and Verpoorte, 1992), and quantified by HPLC (Van der Heijden et al., 1987). TIAs, tryptophan, loganin and secologanin were extracted from 100 mg of freeze dried cell material with 15 ml ethanol using a Ystral homogenizer.

Table 1
Feeding plan used in the first experiment

Feeding type	Fed precursor(s)	Fed on day(s)	Harvest days
Single	Loganin	0	1, 5, 10, 15
Single	Loganin	5	6, 10, 15
Single	Loganin	10	11, 15
Single	Loganin	0	1, 5, 10, 15
Single	Loganin	5	6, 10, 15
Single	Loganin/ tryptamine	10	11, 15
Multiple	Loganin/ tryptamine	0, 5	6, 10, 15
Multiple	Loganin/ tryptamine	0, 5, 10	11, 15

The cultures were fed with loganin or loganin/tryptamine to a concentration of 0.2 and 0.4 mM only once, single feeding, and two or three times, multiple feeding.

After centrifugation at $3500 \times g$ for 30 min, 10 ml of the extract was taken to dryness under reduced pressure. One milliliter of 1 M phosphoric acid was added to the dried extract and the suspension was homogenized using a Vortex mixer. After centrifugation of the acidic alkaloid solution at $14\,000 \times g$ for 5 min, 50 μl of supernatant were analyzed by HPLC (Van der Heijden et al., 1987). The identity of the analytes was established by photodiode array detection of their UV spectra and ESP LC–MS. ESP LC–MS was performed on a Finnigan MAT TSQ-70 triple quadruple mass spectrometer equipped with a custom-made electrospray interface. The gradient HPLC (Giroud et al., 1991) runs were carried out using a system consisting of two LC pumps (LKB, Bromma, Sweden), a Waters model 440 UV detector with 280 nm filter (Waters Associates, Milford, MA, USA), a model 7125 injection valve (Rheodyne, Berkeley, CA, USA) and Waters μBondapak Phenyl column with a C_{18} precolumn (Millipore B.V., Etten-Leur, The Netherlands).

3. Results and discussion

In all the experiments, the addition of various concentrations and combinations of precursors did not affect the culture growth, as determined by dry weight (data not shown), except with 12.8 mM loganin/tryptamine feeding (see the third experiment). The dry weight to fresh weight ratio was in the range of 0.11–0.15 in all cultures.

In the first experiment, single and multiple feedings with loganin and a combination of loganin/tryptamine, with the final concentrations of 0.2 and 0.4 mM in the medium, were carried out. Sets of cultures were fed once (single feeding) or two and three times (multiple feeding), and harvested on different days (Table 1). In control cultures of line T22 small amounts of tryptamine and TIAs were accumulated (Fig. 2).

Feeding with all combinations a positive effect on the TIA accumulation on the day after the feeding was observed, however, the excess accumulation of TIAs was reduced to nearly zero over the culture period. The later the feeding during the experiment period, the less accumulation of TIAs is observed.

Single loganin feedings on day 0 caused largest TIA accumulation, and the addition of tryptamine along with loganin in the single feedings did not have any further positive effect on TIA accumulation. Doubling the precursor concentrations reflected differently on accumulation of each alkaloid (Fig. 2). Serpentine (Fig. 2 S) accumulation was not affected while ajmalicine (Fig. 2 A) and catharanthine (Fig. 2 C) accumulation increased about 2-fold. In the case of strictosidine (Fig. 2 Str), doubling precursor concentration in the single feedings either with loganin or loganin/tryptamine from 0.2 to 0.4 mM increased strictosidine accumulation roughly 4-fold on day 1, and 3-fold on day 6. The conversion percentage of loganin into TIAs was around 14, 12 and 6% the day after the feeding, respectively, for day 0, 5 and 10 feedings. No loganin, secologanin, tryptamine and TIAs were found in the culture medium upon harvesting. With none of the 0.2 mM feedings did either loganin or secologanin accumulate in the T22 cells. The results of the 0.2 and 0.4 mM feeding are summarized in Table 2. These results suggest that part of the loganin was used for other pathway(s), which became saturated at higher loganin concentration resulting in higher incorporation into alkaloids. The rapid disappearance of strictosidine in the

first 5 days after day 0 feedings did not result in an increased levels of ajmalicine. Further feeding on day 5 and 10 kept ajmalicine accumulation at the same level (Fig. 2 A). An effect of feeding for catharanthine accumulation was seen only for feedings on days 0 and 5 (Fig. 2 C). This might be explained by a very rapid conversion of catharanthine into other products that are not detected in our system. The rapid disappearance of catharanthine was also observed in *C. roseus* line S1 over-expressing *Str* (manuscript in print). Apparently, breakdown also occurred for the other TIAs as can be concluded by comparing the amount of TIAs on day 1 with the single loganin feeding (about 80 μmol l^{-1}) with the level on day 5 (36 μmol l^{-1}). Both The TDC and STR activities of the control cultures showed a decline starting on day 1–6 and then followed by an increase back to about the beginning level on day 15. In all type of feedings, TDC activities in fed cultures were lower than the control cultures.

In the first experiment, we observed that the multiple loganin/tryptamine feedings kept ajmalicine accumulation constant resulting in highest TIA accumulation at the end of the culture period. We thus decided to study the effect of continuous feeding in more detail. Two sets of test cultures were fed either 0.4 mM loganin on day 0

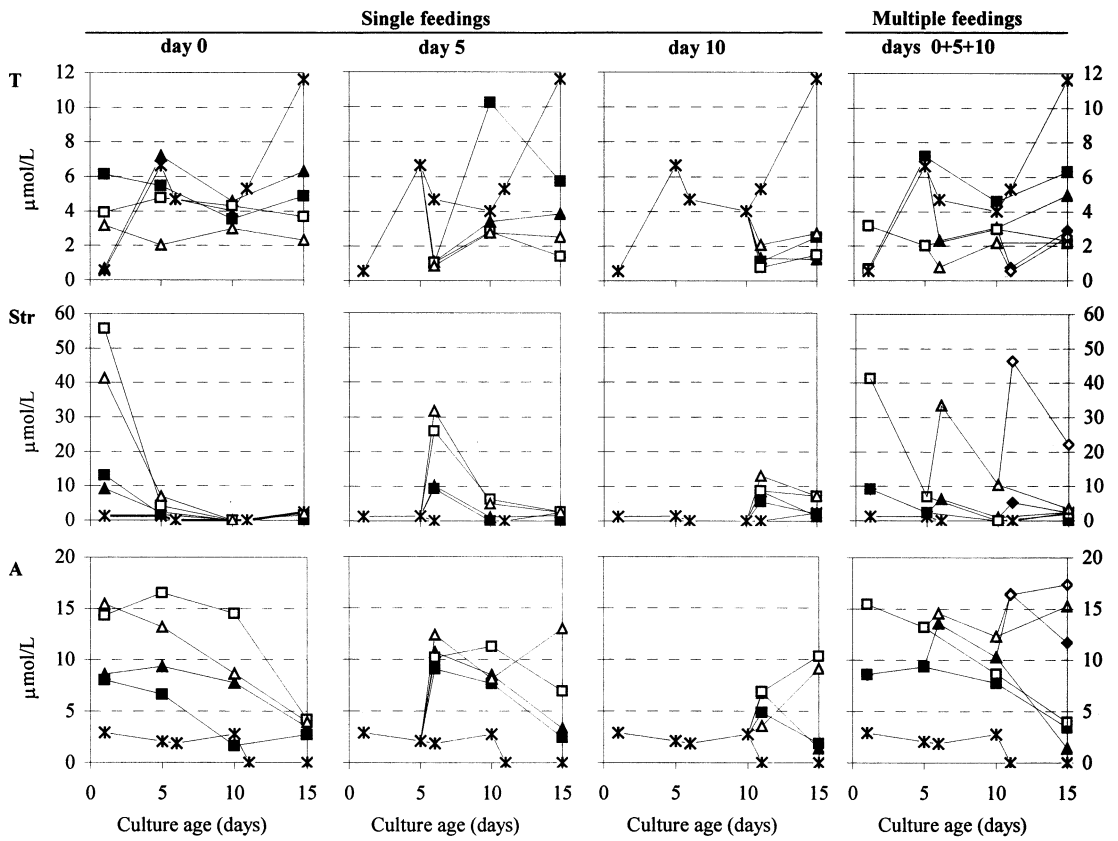

Fig. 2. Intracellular accumulations of tryptamine (T), strictosidine (Str), ajmalicine (A), catharanthine (C), serpentine (S) and total TIAs (Str + A + C + S) by the cultures of line T22 (transgenic for *Tdc*) with the single and multiple feedings of a concentration to 0.2 mM and 0.4 mM loganin (L) and loganin/tryptamine (LT). In the single feedings: Cultures were fed on day 0, 5 or 10. (*) Control; (■) 0.2 mM L; (▲) 0.2 mM LT; (□) 0.4 mM L; (△) 0.4 mM LT. In the multiple feedings cultures were fed with LT: (*) Control; (■) 0.2 mM and (□) 0.4 mM day 0; (▲) 0.2 mM and (△) 0.4 mM days 0 and 5; (◆) 0.2 mM and (◇) 0.4 mM days 0, 5, and 10. Duplicate cultures were pooled upon harvesting and analyzed as single.

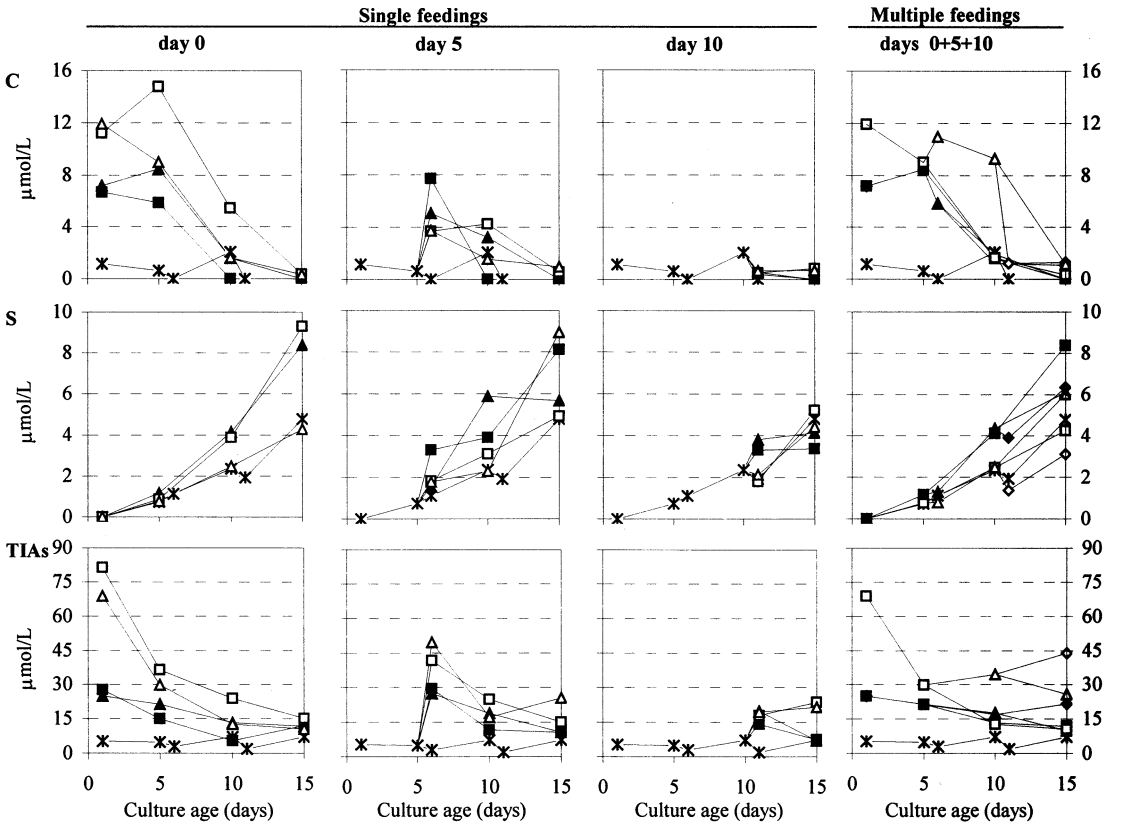

Fig. 2. (*Continued*)

Table 2
Intracellular loganin and secologanin accumulation by line T22 (transgenic for *Tdc*) with loganin (L) or loganin/tryptamine (LT) to a concentration of 0.4 mM

Feeding type	Fed on day(s)	Harvest on day	Loganin[a]	Secologanin[a]
Single L	5	6	27.3	55.9
	10	11	71.0	–
Single LT	5	6	16.4	24.8
	10	11	135.7	–
Multiple LT	0 and 5	6	18.9	36.0
	0, 5 and 10	11	112.7	–

The table consists of days on which either loganin or secologanin accumulation was detected. In all types of 0.2 mM feedings and day-0 0.4 mM feedings neither loganin nor secologanin accumulated. Duplicate cultures were pooled upon harvesting on day 2 and analyzed as single samples.

[a] Concentrations are expressed in µmol l^{-1} (–, not detected or it was too small to quantify).

and harvested every day for 5 days, or fed every day starting on day 0 for 10 days with 0.4 mM loganin/tryptamine and harvested every day. As a control, another set of the culture flasks were harvested every day for 10 days. With the single loganin feeding, we determined that the largest excess TIA accumulation was on day 2 (Fig. 3A). In the continuous loganin/tryptamine feeding,

TIAs accumulated at a rate of about 200 μmol l^{-1} per day for the first 5 days and reached a maximum of 1250 μmol l^{-1} on day 8 (Fig. 3A). However, about 85% of the total TIAs was strictosidine and only 8% was in the form of ajmalicine. Apparently, continuous feeding had the most influence on strictosidine accumulation. With multiple feeding the cultures accumulated loganin and tryptamine but no secologanin. It might be that the turnover rate of secologanin into strictosidine was higher than the turnover rate of loganin into secologanin. The level of TDC activity in the fed cultures was about half of that in the control (Fig. 3B). STR activities of the control and fed cultures are shown in Fig. 3C.

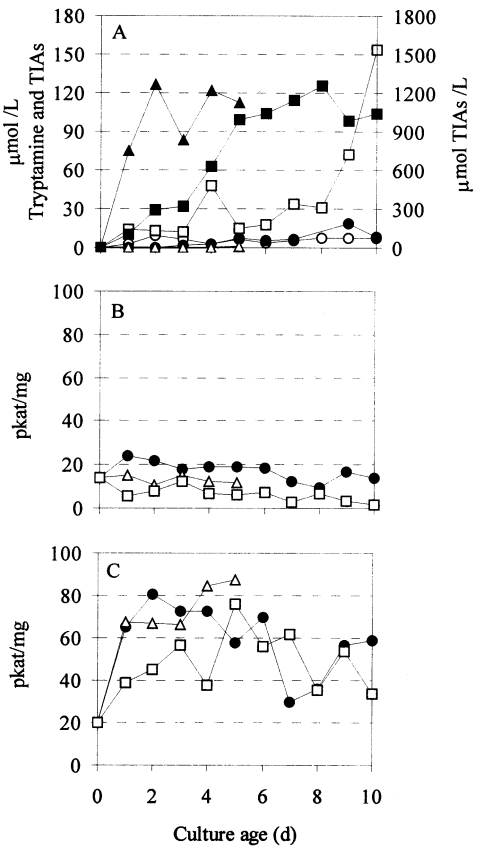

Fig. 3. Ten-day time–course experiments with loganin (L) and loganin/tryptamine (LT) feedings to a concentration of 0.4 mM on TIA and tryptamine accumulation (A) and TDC (B) and STR (C) enzyme activities in line T22 (transgenic for *Tdc*). The control cultures were not fed, the L-fed cultures were fed at inoculation whereas the LT-fed cultures were fed daily. From each set, duplicate cultures were pooled upon harvesting and analyzed as single samples. In A: Control (●) TIA and (○) T; day-0 L-feeding (▲) TIA, (△) T; continuous LT-feeding (■) TIA—with values represented on the right *y*-axis-, (□) T accumulation. In B and C, TDC and STR, respectively, (●) Control, (△) day-0 L feeding and (□) continuous LT-feeding.

From the previous experiments, it seemed that for line T22, tryptamine was not the limiting factor for TIA biosynthesis. In a third experiment, the cultures were fed with various concentrations of loganin, and loganin/tryptamine to determine the cells' capacity to supply tryptamine. The control and fed cultures were harvested on day 2. Line T22 was able to supply tryptamine up to 1.6 mM final concentration of fed loganin. Above 0.8 mM loganin, the cells started accumulating loganin and secologanin; tryptamine could not be detected (Table 3). TIA accumulations of the loganin/tryptamine fed cultures were similar to the loganin fed cultures up to 1.6 mM. Above this level with loganin no further increase in TIAs was observed, because, endogenous tryptamine has become limiting. The STR, however, was capable of even higher TIA production, as with the loganin/tryptamine feedings up to 6.4 mM, TIA accumulation rose to 1100 μmol l^{-1} ($\sim$ 575 mg l^{-1}) (Fig. 4). Strictosidine was the major alkaloid accumulated. Under similar conditions *C. roseus* line S1 (transgenic for *Str*) accumulated about 600 μmol l^{-1} of TIAs (manuscript in print). The 12.8 mM loganin/tryptamine feeding lead to a sharp decline in TIA accumulation, which is accompanied by a decrease in cell yield (Fig. 4). This indicates that the cell viability is affected at high concentration of precursors. With continuous feeding, cell line T22 accumulated 1250 μmol l^{-1} ($\sim$ 625 mg l^{-1}) of TIAs in which about 45 mg l^{-1} was ajmalicine and serpentine while the control culture's accumulation was less than 10 μmol l^{-1}. The total TIA level of T22 after feeding is larger than the reported value of 565 mg l^{-1} of heteroyohimbine alkaloids (serpentine and ajmalicine) accumulated by the MT79 suspension cell cultures of *C. roseus* (Deus-Neumann and Zenk, 1984) obtained by extensive selection. Furthermore, the cultures of line T22 accumulated about 80% more TIAs than the cultures of line S1

Table 3
Utilization of exogenous loganin [a] and tryptamine [a] by line T22 (transgenic for *Tdc*) in the third experiment (EC, extracellular; IC, intracellular)

	Tryptamine[b]		Loganin[b]		Secologanin[b]	TIAs[b]	TDC[c]	STR[c]
	EC	IC	EC	IC	IC	IC		
Control	–	23.1	–	–	–	9.9	24.1	76.8
0.4 L	–	6.5	–	–	–	100.0	27.2	53.8
0.8 L	–	1.1	–	–	–	251.7	21.8	49.1
1.6 L	–	–	–	++	84.7	498.8	28.4	73.1
3.2 L	–	–	30.8	++	190.4	511.2	20.6	48.9
6.4 L	–	–	97.7	++	++	519.6	14.2	48.5
0.4 LT	–	18.0	–	15.6	–	90.7	21.2	41.3
0.8 LT	–	21.1	–	7.2	–	191.5	22.1	45.8
1.6 LT	118.5	37.8	–	175.9	–	541.3	23.1	75.6
3.2 LT	1432.1	++	–	++	–	902.4	41.6	60.7
6.4 LT	++	++	–	++	–	1103.8	89.6	88.8
12.8 LT	++	++	++	++	–	246.8	ND	ND

[a] Feedings consisted of addition of loganin (L) and loganin/tryptamine (LT) in a series of concentrations from 0.4 mM up to 6.4 mM at time of inoculation. Duplicate cultures were pooled upon harvesting on day 2 and analyzed as single samples.

[b] Concentrations are expressed in μmol l^{-1} (–, not detected or it was too small to quantify; ++, for tryptamine > 300 μmol l^{-1} and for loganin and secologanin > 400 μmol l^{-1}; ND, not determined). Neither secologanin nor TIAs were detected in the medium.

[c] TDC and STR activities are expressed in pkat mg^{-1} of soluble protein.

(transgenic for *Str*) under similar conditions (manuscript in print).

Taking into the account the results of this study and those previously reported in the literature we wanted to further investigate the effects of addition of ultimate and penultimate precursors on tryptamine and TIA accumulation in the fourth experiment. The cultures were fed on day 0 with 1 mM of loganin, secologanin, tryptamine, L-tryptophan, or the combinations of loganin/tryptamine, loganin/tryptophan, secologanin/tryptamine, and secologanin/tryptophan. The control and fed cultures were harvested on days 2 and 14. The control cultures' TIA accumulation on day 2 was too small to quantify, however, the fed cultures' TIA accumulation was similar (Table 4) to that found by feeding 0.8 mM in the previous experiment (Table 3). Of the single precursor feedings, the largest accumulation was observed with loganin feeding. Also the combination loganin/tryptamine, and loganin/tryptophan feedings increased the alkaloid accumulation (Fig. 5). Secologanin enhanced accumulation only slightly, tryptophan and tryptamine did not affect alkaloid accumulation. Feedings with secologanin-combinations, with either tryptamine or tryptophan, affected TIA accu-mulation only slightly. This proves again the poor utilization of secologanin in feeding experiments (Moreno et al., 1993; Whitmer et al., 1998). On day 2 the cells still

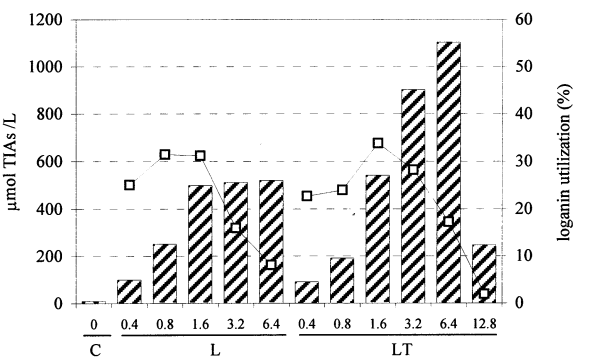

Fig. 4. Utilization of various concentrations of loganin (L) and loganin/tryptamine (LT) by line T22 (transgenic for *Tdc*). Feedings consisted of addition of L and LT in a series of concentrations from 0.4 mM up to 12.8 mM at inoculation (C, control). Right hand *y*-axis represents percentages of loganin (□) utilized into TIAs. Duplicate cultures were pooled upon harvesting on day 2 and analyzed as single samples.

Table 4
Utilization of exogenous iridoid and indole precursors [a] by line T22 (transgenic for *Tdc*) in the fourth experiment (EC, extracellular; IC, intracellular)

Harvestday		Tryptophan[b]		Tryptamine[b]		Loganin[b]		Secologanin[b]		TIAs[b]	TDC[c]	STR[c]
		EC	IC	EC	IC	EC	IC	EC	IC	IC		
2	C	–	–	–	9.1	–	–	–	–	–	33.4	57.8
	L	–	–	–	0.4	–	–	–	–	251.4	38.3	70.6
	S	–	–	–	6.8	–	–	–	–	15.3	33.4	60.1
	T	–	–	–	++	–	–	–	–	0.7	121.3	60.4
	Trp	206.7	++	–	57.7	–	–	–	–	0.2	112.2	79.9
	LT	–	–	–	14.8	–	++	–	–	297.7	57.4	48.9
	LTrp	326.5	++	–	1.7	–	++	–	–	197.5	42.8	40.8
	ST	–	–	–	++	–	–	–	–	6.9	116.3	63.6
	STrp	++	++	–	–	–	–	–	–	11.6	78.6	70.8
14	C	–	–	–	4.0	–	–	–	–	1.4	131.5	92.5
	L	–	–	–	4.2	–	–	–	–	47.6	56.9	140.7
	S	–	–	–	5.5	–	–	–	–	5.7	89.2	123.6
	T	–	–	–	52.7	–	–	–	–	–	161.2	142.1
	Trp	–	36.2	–	67.0	–	–	–	–	–	260.9	235.0
	LT	–	–	–	8.2	–	–	–	–	39.1	116.9	80.7
	LTrp	–	4.1	–	8.5	–	–	–	–	27.3	83.6	296.4
	ST	–	–	–	28.7	–	–	–	–	6.7	131.8	275.7
	STrp	–	3.7	–	++	–	–	–	–	4.2	275.9	281.3

[a] Feedings consisted of addition of loganin (L), secologanin (S), tryptamine (T), L-tryptophan (Trp) and their combination to a concentration of 1 mM at time of inoculation (C, control). Duplicate cultures were pooled upon harvesting on days 2 and 14, and analyzed as single samples.

[b] Concentrations are expressed in μmol l^{-1} (–, not detected or it was too small to quantify; ++, for tryptamine and tryptophan > 300 μmol l^{-1} and for loganin and secologanin > 400 μmol l^{-1}).

[c] TDC and STR activities are expressed in pkat mg^{-1} of soluble protein.

contained loganin but no secologanin when fed with loganin, loganin/tryptamine and loganin/tryptophan (Table 4). There was no precursor in the medium, except for tryptophan that was present on day 2 (Table 4). On day 14, TIA accumulation in the loganin, loganin/tryptamine, and loganin/tryptophan-fed cultures was still high compared with control cultures; nevertheless, TIA accumulation was more than 5-fold smaller than those accumulated on day 2, probably due to breakdown. Under the same feeding regimen, line S1 (transgenic for *Str*) accumulated more TIAs on day 14 than day 2. Apparently, the breakdown rate in line T22 is larger than in line S1 (manuscript in print). Moreover, the productivity of the line T22 was moni-tored over a period of 30 months with our transgenic cell lines over-expressing the *Tdc* and *Str* genes (manuscript in preparation).

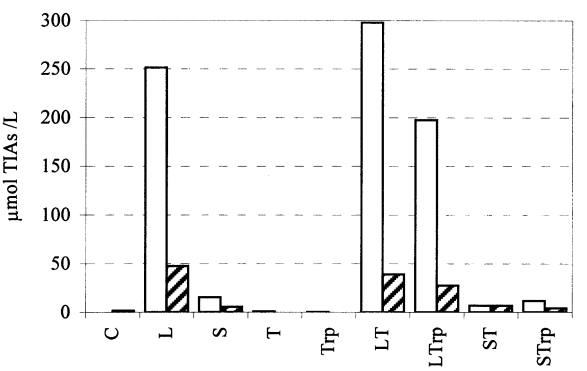

Fig. 5. Utilization of exogenous iridoid and indole precursors by line T22 (transgenic for *Tdc*). Feedings consisted of addition of loganin (L), secologanin (S), tryptamine (T), L-tryptophan (Trp) and their combination to a concentration of 1 mM at time of inoculation (C, control). Duplicate cultures were pooled upon harvesting on days 2 (white bars) and 14 (hatched bars), and analyzed as single samples (C, control).

When 1 mM loganin/tryptamine is fed to the wild type line CRPM at inoculation, the cultures accumulated 114 and 587 μmol l^{-1}, on days 2 and 14, respectively. CRPM does not normally accumulate TIAs. It is notable that the cultures of CRPM only accumulated strictosidine on day 2 and its level was smaller than the level in the cultures of T22. However, the cultures of CRPM accumulated much higher amounts of TIAs (strictosidine and ajmalicine) on day 14 (Table 4) pointing to a slower rate of strictosidine synthesis and catabolism.

4. Conclusion

As we observed a pattern of increased TIA accumulation the day after the feeding in the first experiment, we conclude that TIA biosynthesis in line T22 can be readily enhanced by precursor addition and it is more effective soon after the cultures are inoculated into fresh medium. Our results obtained from the first and third set of experiments show that one of the limitations on TIA production in line T22 is in the terpenoid pathway (Fig. 1) similar to findings in the other *C. roseus* cell cultures (Whitmer et al., 1998). However, we also met other limitations. Precursor feeding at high concentrations results mostly in high strictosidine accumulation either in transgenic or wild type cell lines. The conversion into further downstream alkaloids such as ajmalicine, however, is very slow at high strictosidine levels. At high level loganin feeding, the conversion of loganin into secologanin also becomes a limiting step. From low level feedings, it is clear that there is a continuous catabolism of the alkaloids downstream. Their level can only be kept up by continuous feeding of precursors. The alkaloid production capacity of the cells is apparently quite high as can be seen from the high concentration feedings. In this *Tdc* over-expressing cell line no direct correlation between TDC activity and TIA accumulation is found anymore as generally found in non-transformed (Knobloch and Berlin, 1983; Mérillon et al., 1986; Eilert et al., 1987; Facchini and DiCosmo, 1991; Islas et al., 1994) and also in *Str* over-expressing (Whitmer et al., 1998) cell cultures of *C. roseus*. The constitutive TDC levels present due to the over-expression line T22 can produce sufficient amounts of tryptamine to produce up to 500 μmol l^{-1} of TIAs with high concentration of loganin feedings. At this level of production of strictosidine synthase is still not a limiting factor. The tryptophan–tryptamine pathway is capable of considerably increasing the flux if compared with the control where tryptamine levels are quite low. It is not clear how this is regulated. Is loganin an inducer of this pathway and/or tryptamine an inhibitor and for both, at what levels? Another possibility is that the tryptamine in the control cultures is also subject to a rapid catabolism. Apparently, when a large amount of precursors is supplied, even a non-TIA accumulating wild type cell line like CRPM can accumulate high amounts of TIAs.

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