Plant Cell Tiss Organ Cult (2012) 108:535–539
DOI 10.1007/s11240-011-0060-2

RESEARCH NOTE

L-tryptophan decarboxylase activity and tryptamine accumulation in callus cultures of *Vinca minor* L.

Olga Molchan · Svetlana Romashko · Vladimir Yurin

Received: 7 April 2011 / Accepted: 18 September 2011 / Published online: 8 October 2011
© Springer Science+Business Media B.V. 2011

Abstract L-tryptophan decarboxylase (TDC, EC 4.1.1.28) catalyses the formation of tryptamine from tryptophan, and therefore it plays a role in terpenoid indole alkaloids biosynthesis. In this study, TDC activity and tryptamine accumulation were monitored in callus cultures of important medicinal plant *Vinca minor* L. Callus cultures, established from leaf tissues, were incubated on Murashige and Skoog (MS) medium supplemented with 4.4 μM kinetin and different concentrations (0.44, 1.1, 2.2, 4.4 and 6.6 μM) of naphthaleneacetic acid (NAA), and grown either in the dark or under 16 h photoperiod. When the basal enzyme activity of TDC was determined in these cultures, it was 0.5–0.7 nmol tryptamine mg^{-1} prot. min^{-1}. Moreover, this activity remained linear over time and over protein concentrations, and with optimum pH levels between 6.5 and 7.5, and an optimum temperature of 35°C. The Michaelis–Menten constant (K_m) for L-tryptophan was 1.3 mM. TDC cofactor, pyridoxal-5′-phosphate (1 mM), increased the enzyme activity. During later stages of callus culture growth cycle, an increase in TDC activity was observed, and this activity depended on culture conditions and age of callus cultures. In addition, TDC activity and tryptamine accumulation in callus cultures were strongly enhanced by light treatment.

Keywords Callus · Tryptophan decarboxylase · Tryptamine · *Vinca minor*

Abbreviations

K_m	Michaelis-Menten constant
NAA	α-naphthaleneacetic acid
TIA	Terpenoid indole alkaloids
TDC	Tryptophan decarboxylase

O. Molchan (✉) · S. Romashko · V. Yurin
Plant Physiology and Biochemistry Department, Faculty of Biology, Belarusian State University, Minsk, Belarus
e-mail: olga_molchan@mail.ru

Vinca minor L., a member of *Apocynaceae*, is an important medicinal plant producing terpenoid indole alkaloids (TIA). The major alkaloid of *V. minor* leaves is the monoterpenoid indole alkaloid vincamine (Smeyers and Smeyers 1991). Vincamine and its derivatives are used as cerebral vasodilators.

L-tryptophan decarboxylase (aromatic L-amino acids decarboxylase, EC 4.1.1.28) is the key enzyme, which is involved in the early stages of indole alkaloid biosynthesis (Facchini et al. 2000). Tryptophan decarboxylase (TDC) catalyses transformation of the L-tryptophan to tryptamine. TDC is a cytosolic protein (115 kDa) with two identical subunits. TDCs have critical regulatory functions because they link a number of primary and secondary metabolic reactions. It is known that a tryptamine-dependent IAA biosynthetic pathway may operate in some plants (Woodward and Bartel 2005). High level of TDC activity has been shown to correlate with plant pathogen resistance (Yao et al.1995; Facchini et al. 2000). TDCs are also involved in serotonin (5-hydroxytryptamine) and β-carbolyne alkaloids biosynthesises (Berlin et al. 1994; Kang et al. 2007; Facchini et al. 2000). The most research has focused on the role of TDC in the biosynthesis of pharmaceutically important TIA in *Catharanthus roseus* plants (Noé et al. 1984; Facchini 2001; El-Sayed and Verpoorte 2007; Pati et al. 2011).

Plant cell cultures are commonly used as systems for production of important TIAs and for investigating biosynthesis of metabolites (Pietrosiuk et al. 2007). To date, TIA biosynthesis in *V. minor* remains poorly understood (Tanaka et al. 1995); and there is no information on levels

of TDC activity and endogenous tryptamine content both in plants and cultures of *V. minor*. In this study, TDC activity and tryptamine accumulation in callus cultures of *V. minor* are investigated and monitored, particularly in response to different culture conditions.

In our preliminary tests, we have optimised growth conditions for *V. minor* calluses, e.g. the combination of plant growth regulators, nutrients, agar, temperature, illumination, etc. (data not shown). Primary callus culture has been obtained from sterilised leaf explants using Murashige and Skoog medium (MS; Murashige and Skoog 1962) and different concentrations of 6-furfurylaminopurine (kinetin) and NAA. After 35–40 days of "primary cultivation", induced calli (five pieces; weight of each piece was 200–300 mg) have been transferred to fresh medium. This procedure has been repeated every 35–40 days during 2 years. 10-cm round glass Petri dishes have been used. MS medium has been supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 1 mg l^{-1} pyridoxine, 1 mg l^{-1} thiamine, 1 mg l^{-1} glycine, 4.4 μM kinetin and NAA (0.44, 1.1, 2.2, 4.4 and 6.6 μM). Callus has been cultivated in growth chamber with controlled environment (25°C) in the following conditions: (1) 16-8-h light–dark cycle (light intensity 150 μmol m^{-2} s^{-1}), (2) absence of light period (dark conditions). Viability of cultures has been tested using the technique based on the reduction of 2.3.5-triphenyltetrazolium chloride as described elsewhere (Duncan and Widholm 1990). The growth index of callus has been determined as follows: $I = (W_f–W_i)\ W_i^{-1}$, wherein W_f is final fresh weight of callus and W_i is initial fresh weight. The TDC activity has been assayed as described by Sangwan et al. (1998). Callus was homogenised in the presence of 100 mM sodium phosphate buffer (pH 7.5) containing 5% (w/v) polyvinyl polypyrrolidone, 1 mM EDTA, 5 mM *β*-mercaptoethanol and 5 mM thiourea. The homogenate was centrifuged at 10,000×*g* (30 min). All operations were carried out at 4°C. The supernatant has been used as 'enzyme extract'.

The standard reaction mixture (1 ml) contained 100 mM sodium phosphate buffer (pH 8.5), 3.5 mM *β*-mercaptoethanol, 0.25–2 mM pyridoxal-5′-phosphate, 0.25–2.5 mM L-tryptophan and enzyme extract. The reaction mixture was incubated in a shaking water bath (temperature was controlled as required; 20–45°C). To terminate reaction, 2 ml of 4 M NaOH (termination solution) was added to the reaction mixture. This treatment inhibits TDC but it does not affect tryptamine concentration. Tryptamine has been extracted by addition of 3.5 ml of ethylacetate and its concentration has been determined (280/350 nm excitation/emission) using fluorescence spectrophotometer Varian Cary Eclipse (Varian Ltd, Australia). To establish the basal ('zero') fluorescence level (also used for determination of tryptamine concentrations in calluses) termination solution has been added simultaneously with 'enzyme extract'. Tryptamine concentrations in extracts have been determined using calibration curve. The protein content has been measured as described elsewhere (Bradford 1976). To test the effect of pH, different buffers (bis–Tris, Hepes or Tris) have been added to the reaction mixture as required. Agar, EDTA, glycine, kinetin, *β*-mercaptoethanol, MS medium salts, NAA, pyridoxine, sucrose, thiamine, tryptophan, tryptamine, were purchased from Sigma (USA). All data are mean±SE; five independent replicates.

Our results have shown that the rate of tryptamine formation linearly increased with protein concentration and time of incubation (up to 1 h; Fig. 1a, b). TDC activity increased with concentration of substrate (L-tryptophan), demonstrating classical hyperbolic 'saturation' curve (for 0.5–2.5 mM tryptophan; Fig. 1c). The values of apparent K_m and V_{max} (1.3 mM and V_{max} was 0.6 nmol tryptamine mg^{-1} prot. min^{-1}, respectively) have been determined using graphical analysis of Lineweaver–Burk plot (data not shown). K_m values (found in out study) were similar to those that have been measured in *C. roseus* and *L. esculentum* (0.075 mM and 3 mM respectively; Facchini et al. 2000). This suggests that TDC functional characteristics have been conserved over different plant species producing TIA. Pyridoxal-5′-phosphate is required as a cofactor by many plant TDCs (Facchini et al. 2000). Our experimental data indicate that TDC activity was stimulated significantly in the presence of 1 mM pyridoxal-5′-phosphate (Fig. 1d). Higher concentrations of pyridoxal-5′-phosphate produced the inhibition of activity. The influence of incubation medium pH is shown in Fig. 1e. Enzyme activity was highest at pH 6.5–7.5 with 40–50% of maximum activity as low as pH 5.0 and as high as pH 9.0. Fig. 1f summarises the effect of incubation temperature on TDC activity. TDC activity displayed a maximum at 35°C.

Overall, our experiments have shown that the following conditions were optimal for determination of TDC activity: 50-min incubation at 35°C, protein concentration 100 μg ml^{-1}, in presence of 1 mM pyridoxal-5′-phosphate. The basal TDC activity was 0.5–0.7 nmol tryptamine mg^{-1} protein min^{-1}. This value was much higher (10-100-fold) than the value obtained for *Oryza sativa* leaf tissues and suspension culture of *C. officinalis*. However, it was similar to values obtained for suspension culture of *C. roseus* and *Camptotheca acuminate*, and poplar leaves (De Luca et al. 1989; Skinner et al. 1987; Canel et al. 1998; Silvestrini et al. 2002; Gill and Ellis 2006).

TDC activity has been examined during the growth cycle (5, 10, 15, 20, 25, 30, 35 and 40 days of cultivation; these tests have been combined with measurements of fresh weight). With 1.5 g inoculums (fresh weight), the culture cycle completed in approximately 35 days (maximal weight). A typical

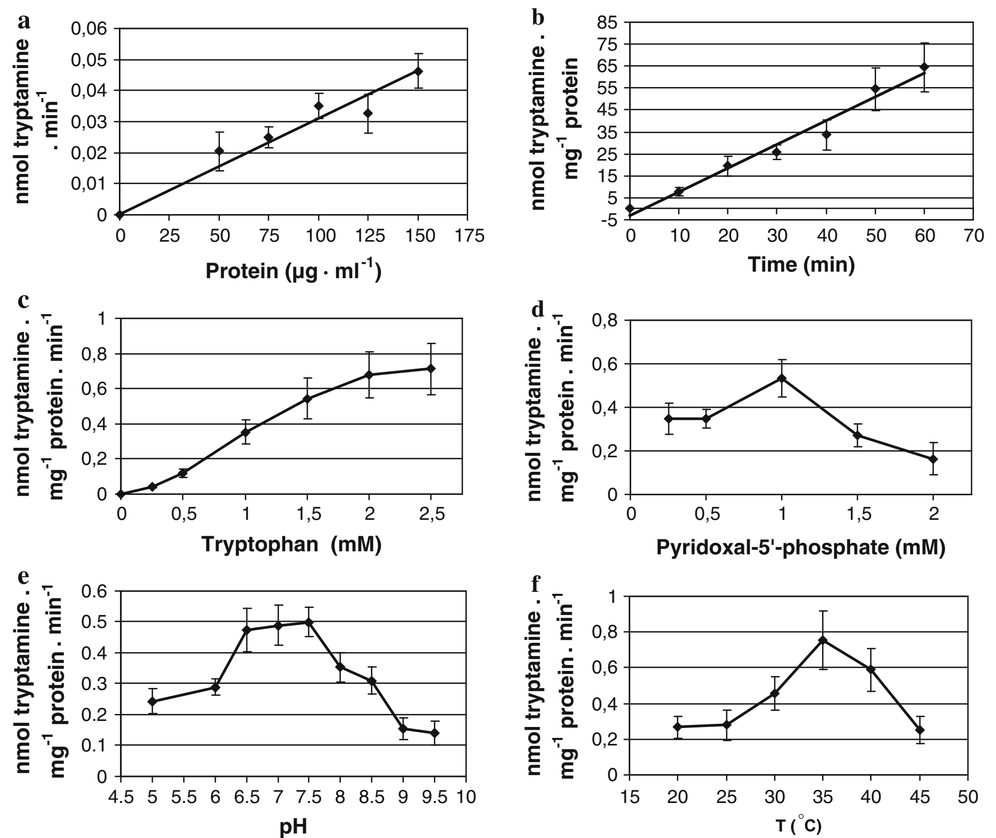

Fig. 1 Changes in TDC activity in *V. minor* callus induced by different of protein content (**a**), incubation time (**b**), substrate (L-tryptophan) concentration (**c**), pyridoxal-5′-phosphate concentration (**d**), pH (**e**) and temperature (**f**)

"growth curve" is shown in Fig. 2a. TDC showed significant activity during 40 days of measurements. TDC activity increased during the exponential phase of growth curve (Fig. 2a). The greatest enzyme activity has been observed at the beginning of the stationary growth phase (Fig. 2a). After achieving maximum, TDC activity decreased (Fig. 2a). TDC activity directly correlated with tryptamine accumulation (Fig. 2b). The values of tryptamine content (0.15–0.5 µmol g^{-1}, fresh weight) were similar to those that were found in poplar and rice leaves as well as *C. roseus* callus (Gill and Elis 2006; De Luca et al. 1989). These data are consistent with previous findings, which indicated that the activity of TDC is different in different preparations and stages of plant development (Facchini et al. 2000; El-Sayed and Verpoorte 2007; Lucumi et al. 2002; Whitmer et al. 1998).

Environmental and hormonal signals can regulate physiological processes in plant cultures (Lee et al. 2011; Magyar-Tabori et al. 2010; Garcia et al. 2011; Lin et al. 2011). The effect of auxin on TDC activity has already been tested in alkaloid-producing species (Facchini et al. 2000; Kang et al. 2007; De Luca et al. 1988). Addition of auxin to cell suspension cultures of *Catharanthus roseus* resulted in induction of cell division and transcriptional down regulation of *TDC* gene (Facchini et al. 2000; De Luca et al. 1989). Here, we have investigated the effect of NAA on TDC activity,

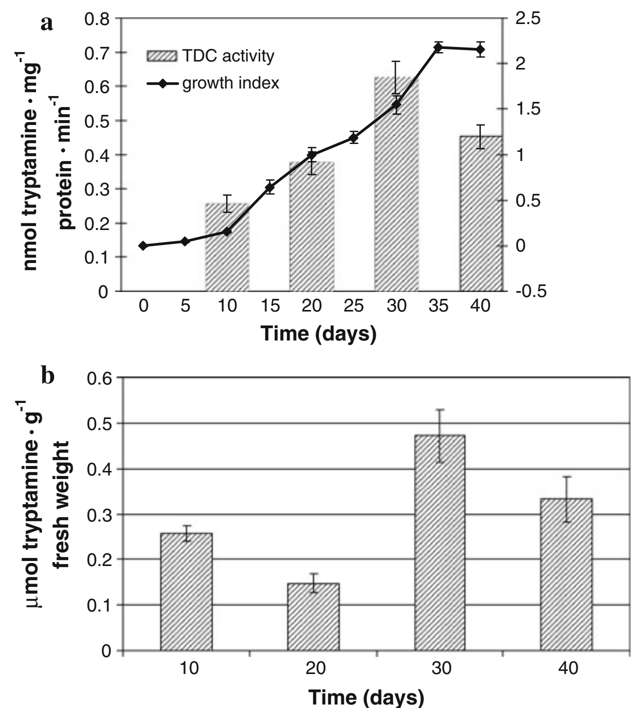

Fig. 2 Growth (**a**, *line*), TDC activity (**a**, *bars*) and tryptamine accumulation (**b**) in callus cultures of *V. minor* grown in the dark on MS medium supplemented with 4.4 µM kinetin and 1.1 µM NAA

tryptamine synthesis and growth of *V. minor* callus (Fig. 3). These experiments have been carried out in 'light' and 'dark' conditions (Fig. 3). Figure 3a shows that 0.44–4.4 µM NAA stimulates callus growth, while higher concentration of NAA (6.6 µM) caused growth inhibition. Thus, 4.4 µM NAA has been used for callus cultivation (causing maximal growth stimulation) (Fig. 3a). NAA caused similar action on TDC activity (Fig. 3b). 0.44–2.2 µM NAA stimulated TDC activity while 4.4–6.6 µM had an adverse effect (inhibition by 25–50%). All tested NAA concentrations (0.44–6.6 µM) did not change significantly the tryptamine content in the callus (Fig. 3c).

The data shown in Fig. 3b indicate that the TDC activity and tryptamine content were very sensitive to light regime (they increased up to three-fold under light, as compared to the 'dark' conditions). A number of studies have previously demonstrated that light can stimulate TIA biosynthesis (Facchini 2001; El-Sayed and Verpoorte 2007; De Luca et al. 1988). Our results show that tryptamine synthesis in *V. minor* callus culture is also under control by light/dark regimes. This suggests that light may have a critical role in TIA biosynthesis and probably in the production of the vincamine (commercially important cerebral vasodilator).

The main conclusion of this study is that *V. minor* can be cultivated in vitro. Callus of *V. minor* demonstrates significant TDC activity and tryptamine content and can potentially be used for commercial production of tryptamine derivatives, such as vincamine. Light and auxin major factors regulating TDC activity and tryptamine content in *V. minor*.

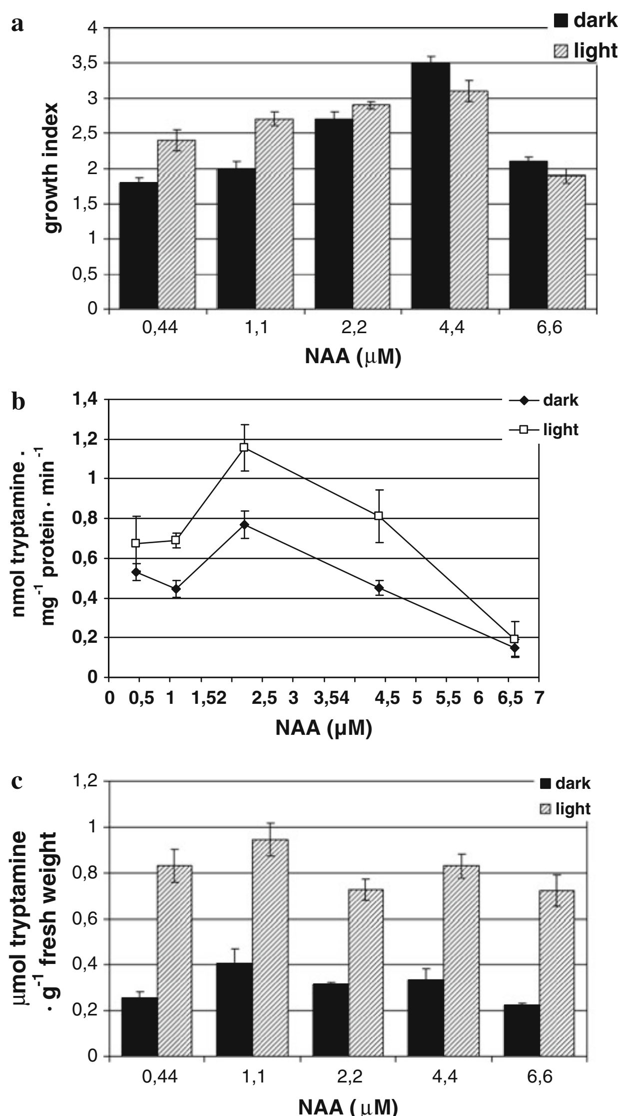

Fig. 3 Effect of NAA and light on growth (**a**) TDC activity (**b**) and tryptamine accumulation (**c**) in *V. minor* callus cultures

References

Berlin J, Rügenhagen C, Kuzovkina IN, Fecker LF, Sasse F (1994) Are tissue cultures of *Peganum harmala* a useful model system for studying how to manipulate the formation of secondary metabolites? Plant Cell Tiss Org Cult 38:289–297

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Canel C, Cardozo IL, Whitmer S, Fits L, Pasquali G, van der Heijden R, Hoge JHC, Verpoorte R (1998) Effect of over-expression of strictosidine syntase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta 205:414–419

De Luca V, Fernandez J, Campbell D, Kurz W (1988) Developmental regulation of enzymes of indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol 86:447–450

De Luca V, Marineau C, Brisson N (1989) Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: comparison with animal dopa decarboxylase. Proc Natl Acad Sci USA 86:2582–2586

Duncan DR, Widholm JM (1990) Measurements of viability suitable for plant tissue cultures. In: Pollard JP, Walker JM (eds) Methods in Molecular Biology. Plant cell and tissue organ culture, vol 6. The Humana Press, New Jersey, pp 29–37

El-Sayed M, Verpoorte R (2007) *Catharanthus* terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev 6:277–305

Facchini PJ (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Plant Mol Biol 52:29–66

Facchini PJ, Huber-Allanach KL, Tari LW (2000) Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation, and metabolic engineering applications. Phytochemistry 54:121–138

Garcia R, Pacheco G, Falcao E, Borges G, Mansur E (2011) Influence of type of explant, plant growth regulators, salt composition of basal medium, and light on callogenesis and regeneration in *Passiflora suberosa* L. (Passifloraceae). Plant Cell Tiss Org Cult 106:47–54

Gill R, Ellis B (2006) Over-expression of tryptophan decarboxylase gene in poplar and its possible role in resistance against *Malacosoma disstria*. New Forests 31:195–209

Kang S, Kang K, Lee K, Back K (2007) Characterization of rice tryptophan decarboxylases and their direct involvement in serotonin biosynthesis in transgenic rice. Planta 227:263–272

Lee Y, Lee D, Lee H et al (2011) Influence of auxins, cytokinins, and nitrogen on production of rutin from callus and adventitious roots of the white mulberry tree (Morus alba L.). Plant Cell Tiss Org Cult 105:9–19

Lin Y, Li J, Li B, He T, Chun Z (2011) Effects of light quality on growth and development of protocorm-like bodies of *Dendrobium officinale* in vitro. Plant Cell Tiss Org Cult 105:329–335

Lucumi E, Vera A, Hallard D, van der Heijden R, Verpoorte R (2002) Alkaloid formation in cell suspension cultures of *Tabernaemontana elegans* after feeding of tryptamine and loganin or secologanin. Plant Cell Tiss Org Cult 68:293–299

Magyar-Tabori K, Dobranszki J, Silva TJ, Bulley S, Huda I (2010) The role of cytokinins in shoot organogenesis in apple. The Plant Cell Tiss Org Cult 101:251–267

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Noé W, Mollenschott C, Berlin J (1984) Tryptophan decarboxylase from *Catharanthus roseus* cell suspension cultures: purifcation, molecular and kinetic data of the homogenous protein. Plant Mol Biol 3:281–288

Pati P, Kaur J, Singh P (2011) A liquid culture system for shoot proliferation and analysis of pharmaceutically active constituents of *Catharanthus roseus* (L.) G. Don. Plant Cell Tiss Org Cult 105:299–307

Pietrosiuk A, Furmanova M, Lata B (2007) *Catharanthus roseus*: micropropagation and in vitro techniques. Phytochem Rev 6:459–473

Sangwan RS, Mishra S, Kumar S (1998) Direct fluorometry of phase-extracted tryptamine-based fast quantitative assay of L-tryptophan decarboxylase from *Catharanthus roseus* leaf. Anal Biochem 255:39–46

Silvestrini A, Pasqua G, Botta B, Monacelli B, van der Heijden R, Verpoorte R (2002) Effects of alkaloid precursor feeding on a *Camptotheca acuminata* cell line. Plant Physiol Biochem 40:749–753

Skinner NJ, Robins RJ, Rhodes MJC (1987) Tryptophan decarboxylase, strictosidine synthase and alkaloid production by *Cinchona ledgeriana* cell suspension cultures. Phytochemistry 26:721–725

Smeyers YG, Smeyers NJ (1991) A structural and pharmacological study of alkaloids of Vinca minor. Mol Eng 1:153–160

Tanaka N, Takao M, Matsumoto T (1995) Vincamine production in multiple shoot culture derived from hairy roots of *Vinca minor*. Plant Cell Tiss Org Cult 41:61–64

Whitmer S, Canel C, Hallard D, Goncalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. Plant Physiol 116:853–857

Woodward AW, Bartel B (2005) Auxins: regulation, action, and interaction. Ann Bot 95:707–735

Yao K, De Luca V, Brisson N (1995) Creation of metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to Phytophthora infectans. Plant Cell 7:1787–1799