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Tryptophan is a precursor for melatonin and serotonin biosynthesis in in vitro regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants

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Abstract Evidence of a pathway for the biosynthesis of the mammalian neurohormones melatonin and serotonin in in vitro regenerated plantlets of St. John's wort (*Hypericum perforatum* cv. Anthos) is presented. Isotope tracer experiments were performed on plantlets regenerated from thidiazuron-induced stem explants and grown on MS basal medium for 2 months. Radiolabel from ^{14}C-tryptophan was recovered as ^{14}C-indoleacetic acid, ^{14}C-tryptamine, ^{14}C-5-hydroxytryptophan, ^{14}C-serotonin and ^{14}C-melatonin in the treated St. John's wort plantlets. Chromatographic peak identity was confirmed by high performance liquid chromatography-mass spectrometry-mass spectrometry and quantification of melatonin by radioimmunoassay. Significantly more radiolabel was recovered in serotonin relative to melatonin under low light conditions with this ratio being reversed under increased lighting, indicating that the rate of flow through this biosynthetic pathway is regulated, at least in part, by light.

Key words Tryptophan · Indoleacetic acid · Melatonin · Serotonin · St. John's wort

Abbreviations *CID*: Collision-induced dissociation *ECD*: Electrochemical Detection · *IAA*: Indoleacetic acid · *LC-MS-MS*: High Performance Liquid Chromatography-Mass Spectrometry-Mass Spectrometry · *MRM*: Multiple reaction monitoring · *RIA*: Radioimmunoassay · *TDZ*: Thidiazuron

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Introduction

In plant tissues, the primary metabolites of tryptophan are auxin, glucosinolates, phytoalexins, alkaloids and indoles (Radwanski and Last 1995). Of these, the most highly characterized metabolite of tryptophan is the plant hormone indoleacetic acid (IAA; Bartel 1997), which exerts a strong biological activity at very low concentrations and is essential for maintenance of physiological processes in plants in both in vitro and in vivo. Although the primary precursor for auxin biosynthesis is thought to be tryptophan, it has been difficult to unequivocally identify a single biosynthetic pathway for IAA, since there may be several precursors and conjugated forms of IAA (Bartel 1997). Also, results generated from various plant species indicate that in some species or stages of development, IAA may be produced from an alternate indole biosynthetic pathway (Michalczuk et al. 1992; Östin et al. 1999).

We previously reported significantly higher levels of melatonin, used in the treatment of neurological disorders, in several medicinal plants including St. John's wort (Murch et al. 1997). Although there is currently no known role for melatonin in plant morphogenesis or physiology, Balzer and Hardeland (1996) hypothesized that melatonin in plants may have an analogous role to that in mammals, acting as a chemical messenger of light and dark, a calmodulin binding factor or as an antioxidant. In this way, the relative ratios of melatonin and serotonin may be involved in regulation of light : dark responses, seasonality and circadian rhythms in plants (Balzer and Hardeland 1996; Kolar et al. 1997). However, a biosynthetic pathway for serotonin and melatonin has not been established in a higher plant species. In mammals, yeast and bacteria, melatonin is synthesized from tryptophan via 5-hydroxytryptophan and serotonin (Fig. 1) and the rate of flow of metabolites through this pathway in the mammalian system is

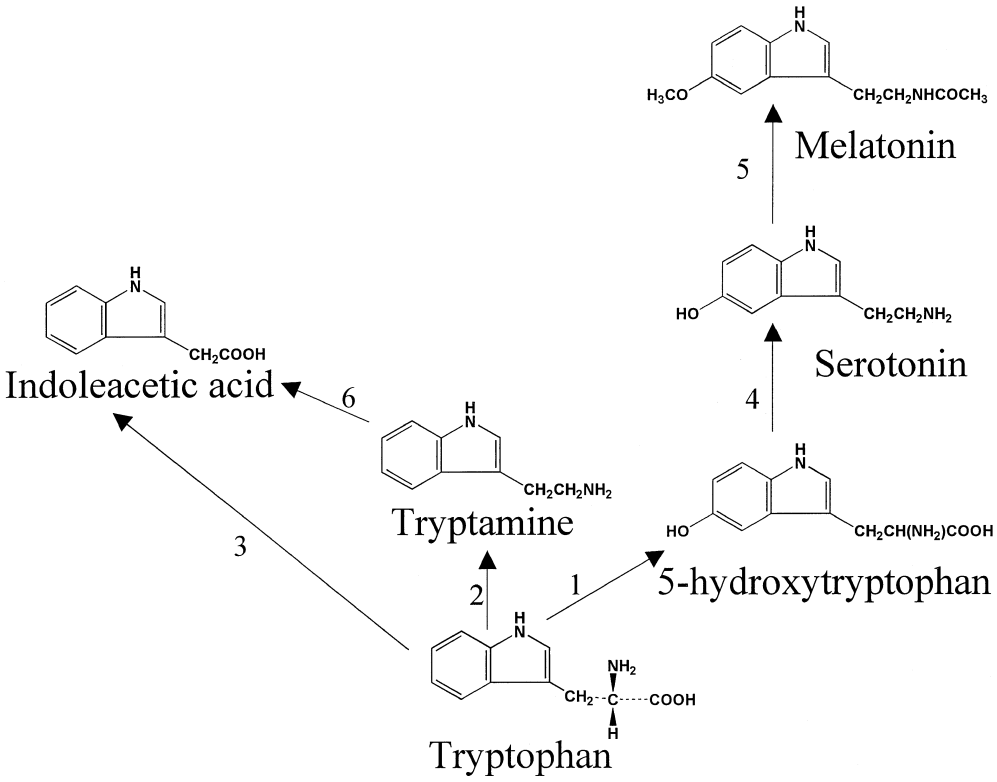

Fig. 1 Metabolic pathways of tryptophan in mammals and plants (adapted from Yu and Reiter 1993 and Salisbury and Ross 1992). *1* Tryptophan 5-hydroxylase EC 1.14.16.4, *2* tryptophan decarboxylase EC 4.1.1.28, *3* L-tryptophan transaminase and decarboxylase EC 1.4.1.19; 4.1.1.43; 1.2.3.7, *4* L-amino acid decarboxylase EC 4.1.1.28, *5* serotonin *N*-acetyltransferase EC 2.3.1.5, *6* tryptamine deaminase EC 1.13.11.11

responsive to both metabolic and environmental factors (Yu and Reiter 1993; Balzer and Hardeland 1996). The objective of this study was to investigate the potential occurrence of the melatonin biosynthetic pathway in St. John's wort and to quantify the potential incorporation of radiolabel from tryptophan into auxin and indoleamine metabolites under low and supplemental light conditions.

Materials and methods

In vitro culture of St. John's wort

Seeds of St. John's wort (*Hypericum perforatum* L. cv Anthos) were germinated as described previously (Murch et al. in press). Nine stem cuttings, approximately 1 cm long, were cultured on a medium containing MS salts (Murashige and Skoog 1962) with B5 vitamins (Gamborg et al. 1968), 30 g l^{-1} sucrose (hereafter referred to as MSO), supplemented with varying levels (0, 5, 10, 15 and 20 µmol l^{-1}) of the growth regulator thidiazuron (TDZ: *N*-phenyl-*N′*-(1,2,3-thidiazol-yl)urea; Sigma, St. Louis, Mo.). The pH was adjusted to 5.7 and 3 g l^{-1} gellan gum (Gelrite, Schweitzerhall, South Plainfield, N.J.) was added before the medium was autoclaved. The optimal time of exposure to TDZ was determined in explant cultures transferred onto MS after 3, 6, 9 and 12 days of incubation. All cultures were incubated in a growth cabinet with a 16 h photoperiod under cool white light at 40–60 µmol m^{-2} s^{-1} (model F40/CW/RS/EW-II Philips, Scarborough, Ontario, Canada). The effect of TDZ on regeneration of stem cuttings was quantified after 18 and 23 days of culture.

Statistical analyses

The design for all regeneration experiments was a complete randomized block and all treatments consisted of five replications. All the experiments were repeated at least twice and the data were analysed using SAS Version 6.12 (SAS 1995). Significant differences between means were assessed by a Student-Neuman-Keuls means separation test at $P \leq 0.05$.

^{14}C-tryptophan metabolism

Regenerated plantlets were subcultured in Magenta boxes containing 50 ml of MSO 2 months prior to radioisotope tracer studies. To ensure that there was no residual bacterial or fungal population in the cultured plantlets, four plantlets were homogenized in 1.0 ml of phosphate buffered saline and the resulting slurry was incubated on nutrient agar (Bacto-Difco) at 30 °C for 7 days.

Individual plantlets, about 15 cm long, were separated from the culture boxes and acclimatized to experimental conditions in the culture vessels for 3 h prior to infusion. Plantlets were transferred to Eppendorf tubes containing 200 µl of the ^{14}C-tryptophan infusate solution (370 kBq l^{-1} ; ^{14}C-(3-sidechain)-tryptophan, Dupont/New England Nuclear, Mass.) in half-strength liquid MSO. Over the 60-min infusion period, samples were collected at 10-min intervals, immediately immersed in liquid nitrogen and stored at −80 °C until analysis. The relative rate of incorporation of label was determined with ^{14}C-radioisotope infusion studies under low light (6 µmol m^{-2} s^{-1}) and supplemental light (40 µmol m^{-2} s^{-1}) conditions provided by cool white fluorescent tubes.

Sample preparation

Frozen samples were ground in 100 µl of Tris buffer (1 M Tris-HCl, pH 8.4) and prepared as described by Poeggeler and Hard-

eland (1994). Briefly, a 300 mg sample was mixed with 300 µl of buffer (0.4 mol l^{-1} perchloric acid, 0.05 % sodium metabissulfate, 0.1 % EDTA). An internal standard, 3,4-dihydroxybenzyl amine (DHBA), was added to each sample for quantification and samples were incubated in the dark at room temperature for 15 min. Particulate matter was removed by centrifugation at 12,000 × g for 15 min and 200 µl of the resulting supernatant was injected into the high performance liquid chromatography system.

High performance liquid chromatography analysis

Tryptophan, indoleacetic acid, serotonin and melatonin were quantified on a Waters HPLC system (LCM1; Waters Chromatography, Mississauga, Canada) with concurrent electrochemical [Waters 460 electrochemical detector (ECD); 2 namp, 0.85 V] and UV (Waters 484 variable UV detector; 278 nm) detection. The separation was performed on a Catecholamine column (3.9 × 150 mm; Waters) at room temperature with a flow rate of 0.8 ml min^{-1} and an isocratic elution buffer consisting of 0.1 mol l^{-1} sodium acetate, 0.1 mol l^{-1} citric acid, 0.5 mmol l^{-1} sodium octanyl sulfonate, 0.15 mmol l^{-1} EDTA, pH 3.7 and 5 % methanol. Radioactivity was quantified in the effluent fractions collected at 1.0 min intervals (model 2128 Fraction Collector, BioRad, Calif.) and counted to 2σ (Beckman LS Counter, Beckman, Calif.) with Cytoscint (ICN Biologicals, Costa Mesa, Calif.) liquid scintillation counting fluid.

Liquid chromatography/mass spectrometry

A 100 µl aliquot of each sample was injected by an autosampler (Hewlett Packard 1090 Series II/L, Palo Alto, Calif.) into the HPLC. The chromatography was performed using a Prodigy 5 ODS-2 column (150 × 3.2 mm i.d. with a 5 µm particle size, Phenomenex, Torrance, Calif.) and eluted with a mobile phase, consisting of 90 % NANOpure water (Barnstead, Dubuque, Canada) containing 0.1 % trifluoracetic acid (TFA, reagent grade, Caledon, Georgetown, Canada) and 10 % acetonitrile (HPLC grade, Caledon, Georgetown, Canada), delivered using a quaternary step gradient program [10 % CH_3CN in 90 % acidified water (0.1 % TFA) for 5 min; 50 % CH_3CN for 10 min] by binary LC pumps (Hewlett Packard 1090 Series II/L, Palo Alto, Calif.). The eluent was passed through the sample injection valve (model 7010, Rheodyne) to an atmospheric pressure chemical ionization (APcI) source configured with a corona discharge pin.

Mass spectrometry detection was achieved using a VG Quattro II (Fisons, Altrincham, UK) triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APcI) source configured with a corona discharge pin. Liquid N_2 (Praxair, Kitchener, Canada) was used as drying and sheath gas. The drying and sheath gas flow rates were 200 and 50 l/h, respectively. The instrument was operated in positive ion mode with an ion source temperature of 150 °C, an APcI probe temperature of 450 °C, a corona discharge pin potential of + 3.10 kV and a cone voltage of 12 V. Instrument control, data acquisition and data processing were carried out using the MassLynx software package.

Daughter ion spectra were obtained by tandem mass spectrometry (MS/MS), which was performed by transmitting the protonated molecular ion ($[M+H]^+$) through the first quadrupole and into the second quadrupole containing ultrapure argon (Praxair, Kitchener, Canda) gas at a pressure of 2.0×10^{-3} mbar. The fragment ions resulting from collision-induced dissociation (CID) of the selected protonated molecule were detected by scanning the third quadrupole from m/z 45 to 700 in 1.2 s. The collision energy was varied between 20 and 50 eV to optimize fragmentation of the selected protonated molecular ion. Data was acquired in MS/MS mode using multiple reaction monitoring (MRM).

In the MRM experiments, the protonated molecular ion was selected and the most intense daughter ion was monitored to obtain a maximum sensitivity. The MRM was performed with a dwell time of 0.5 s, a span of 0.2 Da and an inter-channel delay of 0.02 s for each parent/fragment ion pair viz. tryptophan, 205188; tryptamine, 161144; indoleacetic acid, 176130; 5-hydroxytyptophan, 221204; serotonin, 177160; melatonin, 233174.

Immunoassay for melatonin

Methods for the determination of melatonin concentration in plant extracts were modified from Yie et al. (1993). Plant extracts, prepared with dichloromethane, were assayed with the commercial kit (CIDtech Research, Mississauga, Canda) with [^{3}H]melatonin as the quantitative tracer.

Results and discussion

In vitro regeneration

De novo shoot organogenesis was induced on stem segments of sterile St. John's wort seedlings cultured on an induction medium supplemented with TDZ, while one or two shoots originating from the node were observed on sections cultured on the same medium devoid of TDZ. The optimal concentration of TDZ supplementation to the induction medium was either 5 µmol l^{-1} or 10 µmol l^{-1} for a period of 6 days with subsequent subculture onto MSO (Fig. 2). At these optimal levels, the average number of shoots/explant was 29.4 or 27.2, respectively. Visual examination of the stem segments revealed that the epidermis split and the regenerants originated from the endodermal layers (Fig. 3A, arrowheads). Regenerants developed further and red-coloured hypericin glands were visible on the developing regenerants by day 18 (Fig. 3B). Explants, transferred to Magenta boxes containing MSO, rooted and formed whole plantlets within 1–2 months (Fig. 3C).

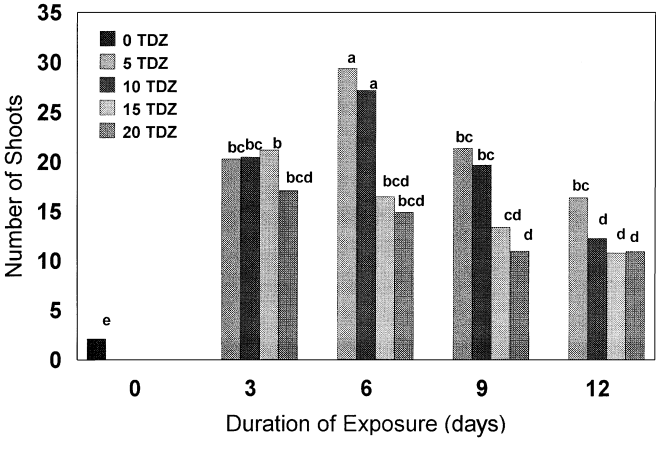

Fig. 2 Effects of different concentrations and exposure duration of TDZ on regeneration of St. John's wort stem explants cultured for 23 days. Statistical differences between means were assessed by the Student Newman-Keuls mean separation test

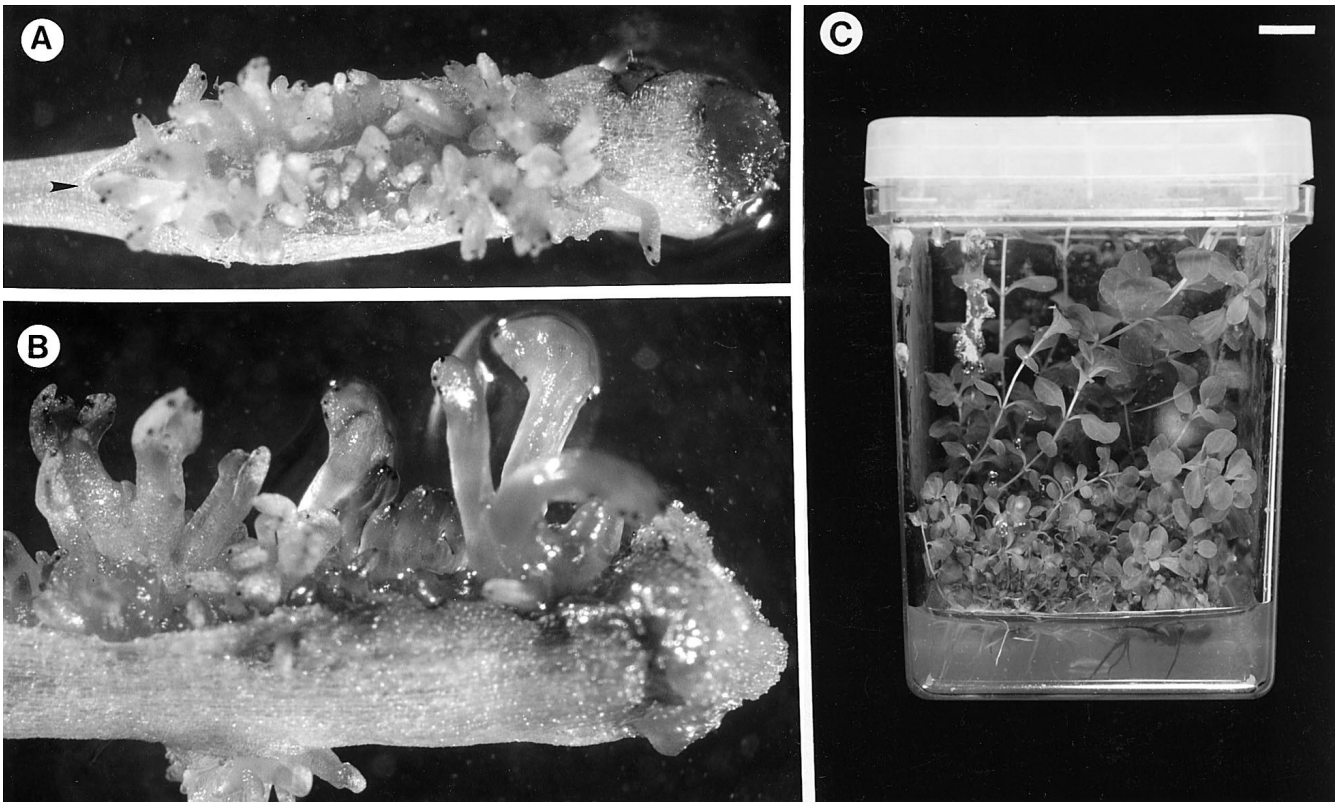

Fig. 3A-C Thidiazuron-induced regeneration on sterile stem segments of St. John's wort (*Hypericum perforatum* cv. Anthos). **A** Shoot organogenesis developing at a split in the epidermis in response to culture on a medium supplemented with thidiazuron for 6 days (*bar* 0.03 cm). **B** Further development of regenerants and appearance of hypericin glands after 18 days (*bar* 0.015 cm). **C** Whole St. John's wort plantlets grown in Magenta boxes on basal medium for 2 months prior to radioisotope tracer analysis (*bar* 1.2 cm)

The in vitro grown plantlets used for all of the indoleamine metabolite analyses were selected for uniform height and vigour. There was no evidence of bacteria, fungus or insect contamination detected either visually in the boxes or in extracts of the plantlets cultured on bacterial culture medium.

Verification of analytical data

The identity and concentration of the metabolite peaks identified in HPLC chromatograms was confirmed by LC-MS-MS spectra generated from commercial standards, eluted fractions and the same plant extracts. Melatonin was quantified from the standard and isolated fractions but the concentration of melatonin in the St. John's wort extracts was close to the detection limits of the LC-MS-MS. Doubling of the injection volume of the extract resulted in a twofold increase in the melatonin peak identified by LC-MS-MS but melatonin could not be reliably quantified by this method; therefore, quantification of melatonin was performed by HPLC-ECD. The standard for 5-hydroxytryptophan gave a peak in the LC-MS-MS spectra but the concentration of this metabolite was below detection limits in the extracts of St. John's wort plants.

A second method was used to verify the accuracy of the quantification of melatonin by HPLC-ECD. A commonly used radiommunoassay (RIA) protocol was used to quantify melatonin in the same extracts of St. John's wort plantlets, HPLC fractions and standards. The RIA analysis verified the concentration as determined by HPLC quantification with a 91 % degree of accuracy.

^{14}C-tryptophan metabolism

The excised plantlets were incubated with a solution of ^{14}C-tryptophan for quantification of indoleamine metabolism. There was a gradual increase in the total radiolabel recovered under the tryptophan peak in extracts of plantlets (Fig. 4). The total amount of radiolabel increased sevenfold over the 60 min incubation. There was a significant increase in the total concentration of tryptophan in the plantlet extracts during the 60 min incubation period (Fig. 4).

Radiolabel from ^{14}C-tryptophan was detected by HPLC-ECD in peaks co-eluting with tryptophan, tryptamine, 5-hydroxytryptophan, IAA, serotonin and melatonin throughout the 60 min incubation period. Significant differences were observed in the allocation

Table 1 Effect of different light levels on the rate of appearance of radiolabel in auxin and indoleamine metabolites in St. John's wort plantlets

Metabolite	Rate of appearance of radiolabel[c]	
	Low light	Supplemental light
Tryptophan	1401[a]	1174[a]
Tryptamine	15.2[b]	210.9[a]
5-Hydroxytryptophan	40.2[b]	164.5[a]
Indoleacetic acid	37.2[b]	174.3[a]
Serotonin	38.5[a]	4.3[b]
Melatonin	2.5[b]	96[a]

[ab] Values within a row with the same superscript are not statistically different ($P < 0.05$)
[c] Rates calculated from means of four replicate samples collected at 10-min intervals between 30 and 60 min. of the incubation period expressed as Bq μmol^{-1} min^{-1}

of radiolabel under ambient and supplemental lighting conditions (Table 1). There was no significant difference in the rate of radiolabel appearance in the tryptophan amino acid pool as a result of the alteration in light level (Table 1). In general, there was more radiolabel recovered in the auxin and indoleamine metabolites at the higher light level, except for serotonin, where the rate of recovery of radiolabel was significantly higher under low light than under supplemental light conditions (Table 1). The amount of ^{14}C from tryptophan incorportated into IAA and melatonin increased over the first 30 min under supplemental light conditions. However, after 30 min, significantly more label was incorporated into IAA than melatonin (Fig. 5). In contrast, plantlets incubated under the low lighting conditions had no significant changes in the rate of incorporation of ^{14}C from tryptophan into IAA or melatonin in the first 30 min of incubation. After 30 min the amount of label incorporated into IAA increased significantly while the label incorporated into melatonin remained unchanged (Fig. 6).

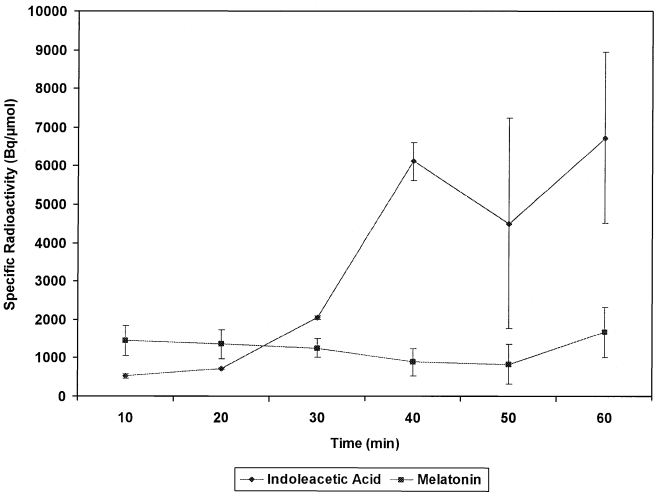

Fig. 5 Accumulation of radiolabel from tryptophan in indoleacetic acid and melatonin in plantlets incubated under supplemented lighting conditions

Medicinal plants provide a unique opportunity for the investigation of biochemical pathways since the medicinal efficacy, in many instances, can be attributed to elevated levels of primary and secondary metabolites. St. John's wort, a medicinal plant traditionally used in the treatment of neurological disorders and depression (Cott 1997; Evans and Morgenstern 1997), was found to have significantly higher levels of mammalian indoleamine neurohormones than had previously been reported (Murch et al. 1997), thereby making this plant an ideal candidate for investigations of metabolites of tryptophan.

As a prerequisite for this investigation, an efficient system was developed for induction of regeneration from St. John's wort stem sections that provided a reliable source of genetically consistent, sterile plant material for the biochemical analysis. The regenera-

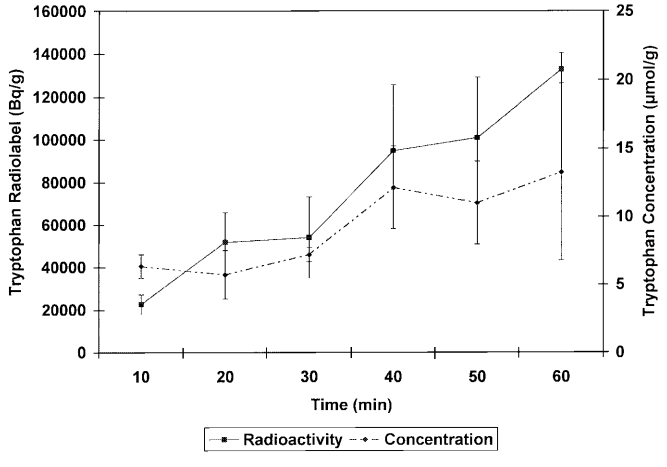

Fig. 4 Rate of accumulation of radiolabel eluted under the tryptophan peak and tryptophan concentration determined from HPLC profiles of the extracts of St. John's wort plantlets

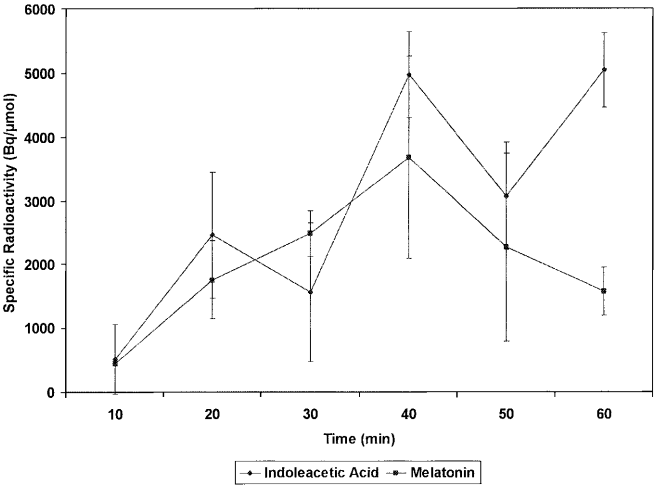

Fig. 6 Accumulation of radiolabel from tryptophan in indoleacetic acid and melatonin in plantlets incubated at low light

tion system is similar to a protocol for TDZ-induced de novo shoot regeneration on St. John's wort etiolated hypocotyl explants (Murch et al. in press). The optimal duration of exposure of hypocotyl explants to TDZ was 9 days in contrast to the 6-day optimal duration for stem segments. The more rapid response of the stem segments to TDZ exposure potentially occurred as a result of the maturity of the tissue or different profiles of endogenous growth regulators. The rate of development of regenerants may have been influenced by the presence of a node in the stem explants; however, visual observations indicated that the regeneration originated from the endodermal cell layers of the stem segments. The high frequency of regeneration achieved in our protocol provides a new technology with the potential to alleviate some of the problems associated with the commercial manufacture of St. John's wort. A 17-fold difference in the concentration of the marker compound hypericin indicating a high degree of variability in the quality of commercial preparations of St. John's wort was recently reported (Consumer Safety Symposium on Dietary Supplements and Herbs 1998). Adulteration of field-grown plant material with insects, fungi, bacteria and other *Hypericum* species has also been observed (St.John's Wort Monograph 1997; Hobbs 1989).

The most significant finding of this research was the recovery of radiolabel from tryptophan in auxin, serotonin and melatonin. The importance of auxin in growth and development of plant tissues is well established. A large body of evidence exists demonstrating the synthesis of IAA from tryptophan (Bandurski et al. 1995; Bartel 1997) and the tryptophan pool of various plant species has been shown to be 3–4 orders of magnitude larger than the pool of IAA, thereby providing the precursor for the biosynthesis of several important classes of metabolites (Bandurski et al. 1995). Our data indicate that there is an active biosynthetic pathway for IAA from tryptophan in St. John's wort. Additionally, the data generated in the current study provides the first evidence of the incorporation of carbon skeleton from tryptophan into serotonin and melatonin in a higher plant species.

It has been hypothesized that melatonin may act as a chemical messenger to the circadian system or may be involved in the mediation of photoperiodic responses in higher plants (Balzer and Hardeland 1996). The differential synthesis of indoleamines in light and dark observed in the current study suggests that, analogous to the mammalian system, the relative ratio of serotonin to melatonin may play a role in light-mediated responses in plants. The indoleamine biosynthetic pathway and the relative roles of serotonin and melatonin have been well characterized in yeast, bacteria and mammals (Yu and Reiter 1993) and the physiological role of these metabolites has been primarily associated with the regulation of circadian rhythms (Balzer and Hardeland 1996) and control of free radicals in the cell (Dubbels et al. 1995). In vertebrates, the rate of flow through the indoleamine biosynthetic pathway was found to oscillate with a circadian rhythm in the photoreceptive pinealocytes of the pineal gland and within the rods and cones of the retinas (Cassone and Natesan 1997). Similarly, in the short-day plant *Chenopodium rubrum*, Kolar et al. (1997) demonstrated diurnal fluctuations in the concentration of melatonin, with the highest levels of melatonin quantified after 4–6 h in the dark.

In conclusion, our studies have established a model system for the investigation of auxin and indoleamine biosynthesis from tryptophan in sterile, consistent plantlets under controlled conditions. There is the potential for the application of St. John's wort regeneration as a model system for the biochemical characterization of *Hypericum* sp. and for investigations into the physiological role of indoleamines in plants. The results of these studies provide the first evidence for the potential role of melatonin in the response of plants to changes in the environment, especially changes in light. The quantification of the indoleamine biosynthetic pathway across a wide range of animal, bacteria and plant species is indicative of a high degree of conservation of this pathway through divergent evolution. Further studies of indoleamine biosynthesis and metabolism in St. John's wort will enable us to elucidate the physiological role of serotonin and melatonin in plant development.

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