Role of Indoleamines in Regulation of Morphogenesis in In Vitro Cultures of St. John's wort (*Hypericum perforatum* L.)

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Abstract

The presence of indoleamines and their potential role in morphogenesis in vitro was investigated in axenic cultures of St. John's wort (*Hypericum perforatum* L. New Stem). De novo shoot regeneration was induced on etiolated hypocotyl explants after 9 days of exposure to thidiazuron (5 µmol/L). Melatonin, a tryptophan-derived mammalian neurohormone was quantified in leaf, flower, stem, and etiolated hypocotyls. Radiolabel from ^{14}C-tryptophan was recovered as ^{14}C-melatonin in sterile plantlets indicating endogenous synthesis of this compound in St. John's wort. Application of inhibitors of mammalian neuro-processes including: p-chlorophenylalanine, d-amphetamine, Prozac™, Ritalin™ and hydralazine modulated regeneration and levels of endogenous auxin and indoleamines. Although there is no known role for melatonin in plant tissues, these data provide an indication that the balance of the endogenous concentrations of serotonin and melatonin may play a role in in vitro plant morphogenesis.

INTRODUCTION

Melatonin and serotonin are mammalian neurohormones that have been implicated in the regulation of hormonal and neurological processes and responses to environmental stimuli in a wide range of species. The mammalian neurohormone melatonin were recently discovered in the medicinal plants St. John's wort (*Hypericum perforatum* L.), feverfew (*Tanacetum parthenium*) and Huang-qin (*Scutellaria baicalensis* Georgi) (Murch et al., 1997, 2000a; Murch and Saxena, 2002a). Manchester et al., (2000) suggested that "the extremely high levels (µg/g dry plant tissue) of melatonin in feverfew and St. John's wort…, plants that thrive best in northernmost latitudes of America, might be related to melatonin's importance in retarding or blunting environmental stressors such as extremes in cold or heat, prolonged episodes of drought as well as protection against environmental chemical pollutants which may cause damage to the germ tissue residing in the seed or flower."

Although the specific function of melatonin in higher plants remains undefined, it has also been hypothesized that the role of melatonin in plants may be analogous to its function in mammals as a chemical messenger of light and dark, calmodulin binding factor or an antioxidant (Balzer and Hardeland, 1996). Melatonin is a metabolite of tryptophan, biochemically related to auxin and other alkaloids and indoles (Murch et al., 2000a; Radwanski and Last, 1995). Radiolabel was incorporated into the endogenous pools of melatonin and its precursor serotonin following incubation with ^{14}C-tryptophan thereby establishing one potential biosynthetic route for the indoleamines in St. John's wort (Murch

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et al., 2000a). In in vitro studies of root organogenesis, the relative balance of melatonin to serotonin was found to mediate the morphogenic responses such that decreased melatonin reduced root organogenesis and increased serotonin increased shoot organogenesis in the presence of exogenous auxin (Murch et al., 2001). There have been no previous studies to investigate a potential role for melatonin in cytokinin-induced shoot organogenesis.

A model system was established that utilizes thidizuron [TDZ: N-phenyl-N'-(1,2,3-thidiazol-yl)urea] for the induction of de novo shoots on etiolated hypocotyl or mature stem segments of St. John's wort plants (Murch et al., 2000b). Thidiazuron is a potent regulator of plant morphogenesis both in vitro and in vivo and has been found to induce de novo shoot foramtion in a wide range of plant species (Murthy et al., 1998). Although the precise mode of action of TDZ is not clear, accumulations of endogenous auxins and cytokinins have been observed in TDZ-exposed tissues (Murthy et al., 1995; Hutchinson et al., 1996). The TDZ-induced regeneration system offered unique opportunities for the investigation of the role of indoleamines in shoot regeneration since prolific shoot organogenesis was induced on all explants, endogenous synthesis of melatonin and serotonin had been established and a series of inhibitors of indoleamine action and transport were found to effectively mediate regenerative responses in St. John's wort tissues (Murch et al., 2001). Therefore, the overall objective of this study was to use the TDZ-induced regeneration system and inhibitors of indoleamine action and transport to investigate the role of serotonin and melatonin in cytokinin-induced shoot regeneration.

MATERIAL AND METHODS

All plant material was cultured as described previously (Murch et al., 2000a &b). Briefly, St. John's wort seeds were surface sterilized by immersing in a 70 % ethanol solution for 5 s, followed by an immersion in a 30 % solution of 5.4 % sodium hypochlorite (Lilo Products, Hamilton, ON) in water with two drops of Tween 20 per 100 mL for 20 min., and rinsed three times in sterile distilled water. Surface sterilized seeds were germinated on water agar (8 $g \cdot l^{-1}$) in a growth cabinet in darkness at 24°C for 16 days. Hypocotyl sections were excised from the etiolated seedlings and cultured on a medium containing MS salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg et al. 1968), 30 $g \cdot l^{-1}$ sucrose, and 0 or 5 $\mu mol \cdot l^{-1}$ thidiazuron (TDZ). The pH was adjusted to 5.7 and 3 $g \cdot l^{-1}$ gellan gum (Gelrite, Schweitzerhall Inc., South Plainfield, NJ) was added prior to autoclaving the media.

Regenerated shoots were excised from the hypocotyl explants after 21 days and subcultured onto the same medium described above devoid of plant growth regulators for at least 2 months. Stem segments were excised from the sterile seedlings and cultured on the same medium supplemented with 0 or 5 $\mu mol \cdot L^{-1}$ TDZ and varying levels of 2,3,5-triiodobenzoic acid (TIBA), p-chlorophenoxyisobutyric acid (PCIB), p-chlorophenylalanine (p-CPA), d-amphetamine, fluoxetine (Prozac™) and methylphenidate (Ritalin™), (0, 20, 40, 60, 80 100, 200 $\mu mol \cdot l^{-1}$). All cultures were incubated in a growth cabinet with a 16 h photoperiod under cool white light @ 40-60 $\mu mol \cdot m^{-2} \cdot ls^{-1}$ and regeneration was quantified after 18 days of culture.

Sterile stem sections, approximately 15 cm long, were excised from the 2-month old seedlings grown as described above and placed vertically in eppendorf tubes containing 200 µL of the ^{14}C-Trp infusate solution (370 $kBq \cdot l^{-1}$; ^{14}C -(3-sidechain)-tryptophan, Dupont/ New England Nuclear, MA) in ½ strength liquid MSO with or without 5 $\mu mol \cdot l^{-1}$ TDZ. Samples were collected at 10 min intervals for a period of 60 min. The relative rate of incorporation of label was determined as described previously (Murch et al., 2000a). Briefly, frozen samples were mixed with 100 µl of Tris buffer (1 M Tris-HCl, pH 8.4) and homogenized with 300 µl of buffer (0.4 $mol \cdot l^{-1}$ perchloric acid, 0.05 % sodium meta-bissulfate, 0.1 % EDTA; Poeggeler et al., 1994). After a 15 min incubation, samples were centrifuged at 12,000 x g for 15 min and 200 µl of the resulting supernatant was injected into the high performance liquid chromatography (HPLC) system. Tryptophan, indoleacetic acid, serotonin and melatonin were quantified on a Waters HPLC system (LCM1; Waters Chromatography Canada Inc., Mississauga, ON) 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 as described previously (Murch et al., 2000). Radioactivity was quantified in the effluent fractions collected at 1.0 min intervals (Model 2128 Fraction Collector, BioRad, CA) and counted to 2σ (Beckman LS Counter, Beckman, CA) with Cytoscint (ICN Biologicals, Costa Mesa, CA) liquid scintillation counting fluid. HPLC peak identification was confirmed by LC-MS-MS and the concentration of melatonin was confirmed by radioimmunoassay as described previously (Murch et al., 2000a).

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

RESULTS

Exposure to thidiazuron in the culture medium has previously been shown to result in the accumulation of endogenous auxins and cytokinins (Hutchinson et al., 1996; Hutchinson and Saxena, 1996). Here, we observed, a significant increase in the rate of incorporation of radiolabel from tryptophan into auxin in TDZ-exposed tissues (Table 1). Conversely, there was a significant decrease in the rate of appearance of radiolabel from tryptophan in 5-hydroxytryptophan and melatonin in stem sections exposed to TDZ (Table 1) Sterile stem sections cultured on a medium supplemented with TDZ formed masses of *de novo* shoots within 18-23 days. De novo root formation was not observed on the TDZ-exposed sections while spontaneous root formation was observed on explants cultured on the same medium devoid of TDZ (Figs. 1-5). Supplementation of the TDZ-culture medium with the auxin activity inhibitor PCIB significantly decreased de novo shoots formation (Fig. 1). Similarly, the auxin transport inhibitior TIBA was found to significantly inhibit TDZ-induced shoot organogenesis in stem explants (Fig. 2). A chemical inhibitor of the conversion of serotonin to melatonin, p-chlorophenylalanine (pCPA) significantly reduced TDZ-induced shoot organogenesis in stem sections (Fig. 3). Supplementation of the TDZ-containing culture medium with a different inhibitor of melatonin biosynthesis, d-amphetamine, significantly increased the number of de novo shoots at lower concentrations and significantly decreased shoot organogenesis at higher levels (Fig. 4). Selective serotonin reuptake inhibitors previously shown to effect morphogenesis in St. John's wort were investigated in the presence of TDZ. There was no significant difference in the TDZ-induced formation of de novo shoots on stem segments as a result of incorporation of Prozac™ (results not presented). However, incorporation of Ritalin™ into the TDZ-containing medium resulted in a significant decrease in shoot organogenesis (Fig. 5).

DISCUSSION

Radiolabel tracer studies have shown that ^{14}C-tryptophan was metabolized to ^{14}C melatonin in St. John's wort tissues (Murch et al., 2000a). The results of the current study suggest that this process is modulated by exposure of the tissues to thidiazuron. These results provide the first indication that the morphological processes induced by TDZ that ultimately result in *de novo* shoot organogenesis may also be mediated by the relative ratio of melatonin to serotonin. Thidiazuron has been found to modify the concentration and activity of other plant growth regulators including endogenous auxins and cytokinins (Murthy et al., 1995; Hutchinson et al., 1996). An optimal concentration and transport of endogenous auxin was found to be required for induction of regeneration and cell proliferation in thidiazuron-exposed cells (Hutchinson et al., 1996). St. John's wort explants exposed to PCIB had significantly lower endogenous concentrations of IAA, tryptamine and melatonin while the explants cultured on the medium containing TIBA did not have significantly altered metabolite concentrations (Murch et al., 2001). Interestingly, there was also a reduction of TDZ-induced shoot formation in explants exposed to pharmaceuticals that modulate serotonin and melatonin metabolism. Although the mechanism for this inhibition of development is unclear, explants exposed to p-CPA or Ritalin™ also had significantly lower melatonin concentrations (Murch et al., 2001) and together these data may suggest a role for

melatonin in the competence of a cell to accept the inductive stimuli of thidiazuron and other plant growth regulators.

Medicinal plants provide unique challenges for the development of systems commonly used in agriculture. Efficient regeneration systems are essential for genetic engineering, mass-propagation studies and optimized plant production. The discovery of human neurohoromes in medicinal plants used in the treatment of neurological disorders (Murch et al., 1997) and the demonstration of a role for these compounds in the regulation of plant development (Murch et al., 2001 an d herein) stimulated our interests in further investigations of the role of melatonin in higher plant physiology and biochemistry. Recently, we have reported the accumulation of melatonin in flower tissues of St. John's wort at specific stages of development (Murch & Saxena, 2002b). The results of the current studies coupled with the findings of this study provide further evidence of a role for alternate metabolites of tryptophan in the regulation of regenerative processes. It seems likely that the further investigation of the specific roles of melatonin and serotonin in morphogenic model systems will provide a new understanding of the basic processes of plant growth and developmental pathways. The discovery of mammalian neurohormones in plants used in the treatment of human ailments provides new avenues for investigation of medicinally active compounds.

ACKNOWLEDGEMENTS

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Tables

Table 1. Effect of thidiazuron on the rate of incorporation of radiolabel from tryptophan into auxin and indoleamines.

Metabolite	MS Medium	5 μmol·L^{-1} TDZ
Indoleacetic acid	56.84^{b}	144.32^{a}
Tryptamine	23.69^{a}	39.29^{a}
5-Hydroxytryptophan	78.42^{a}	36.99^{b}
Serotonin	3.12^{a}	6.4^{a}
Melatonin	39.06^{a}	18.09^{b}

^{ab} Values within a row with the same superscript are not significantly different ($P<0.05$)

Figures

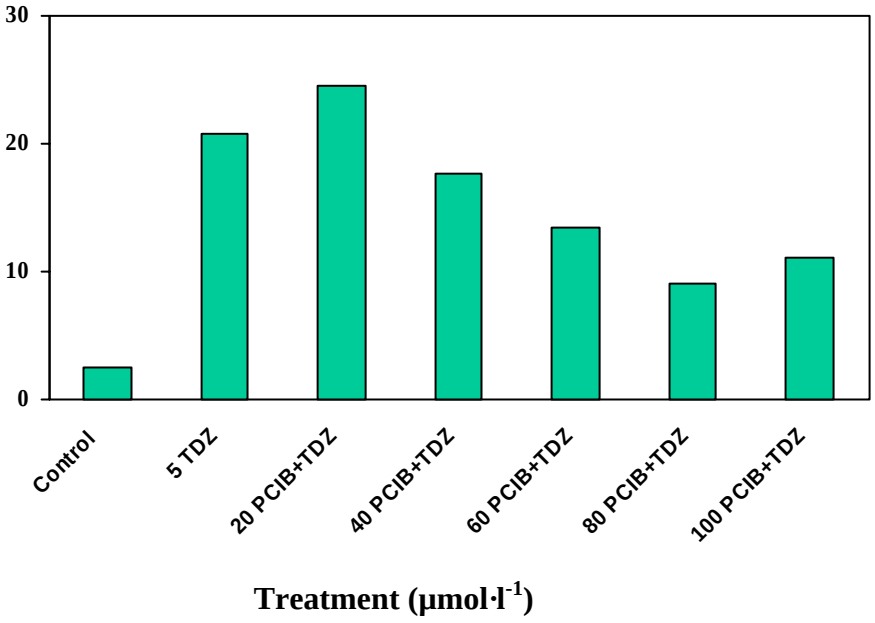

Fig. 1. Effect of p-chlorophenoxyisobutyric acid (PCIB) on TDZ-induced shoot organogenesis in stem explants of St. John's wort.

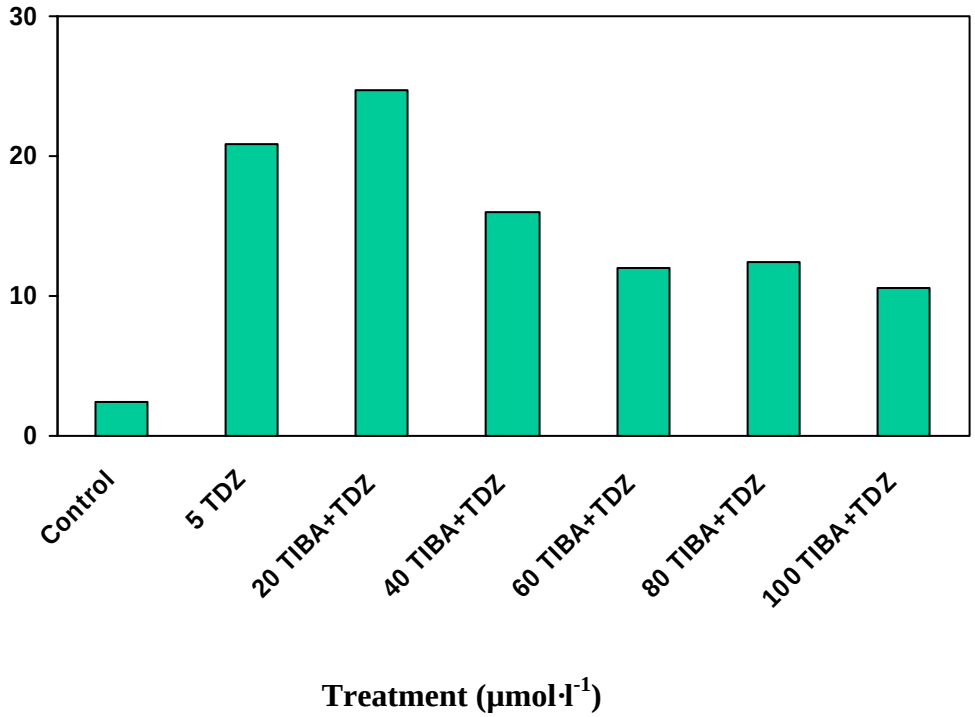

Fig. 2. Effect of 2,3,5-triiodobenzoic acid (TIBA) on TDZ-induced shoot organogenesis in stem explants of St. John's wort.

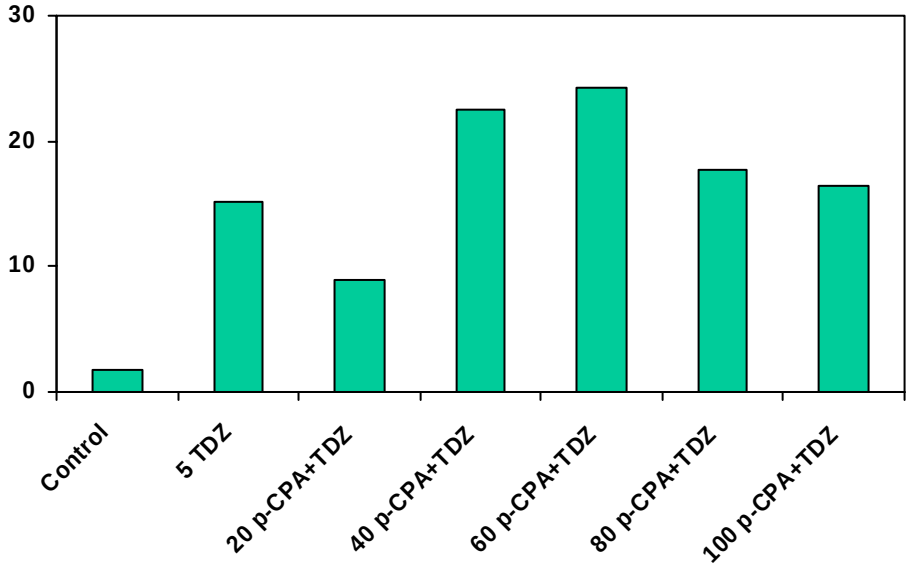

Fig. 3. Effect of p-chlorophenylalanine on TDZ- induced regeneration in St. John's wort stem explants.

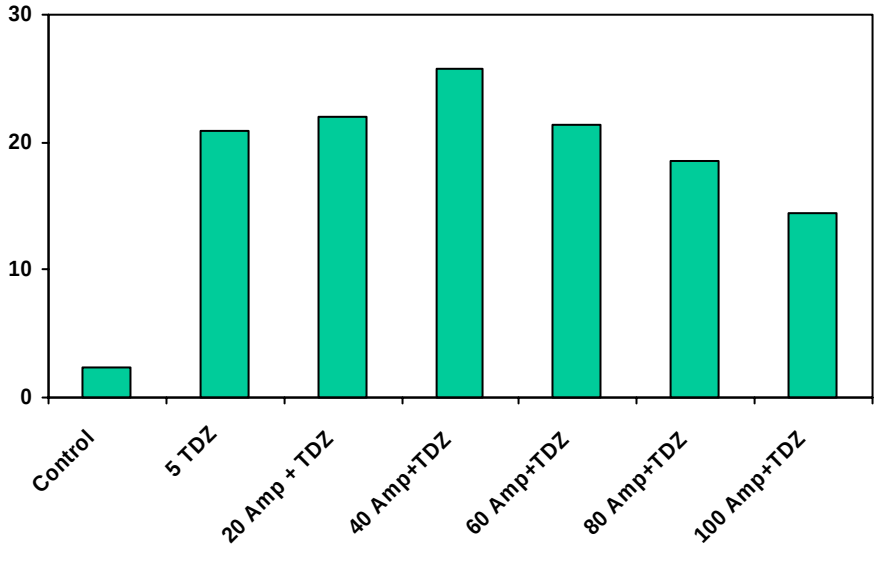

Fig. 4. Effect of d-amphetamine on TDZ-induced organogenesis in stem explants of St. John's wort.

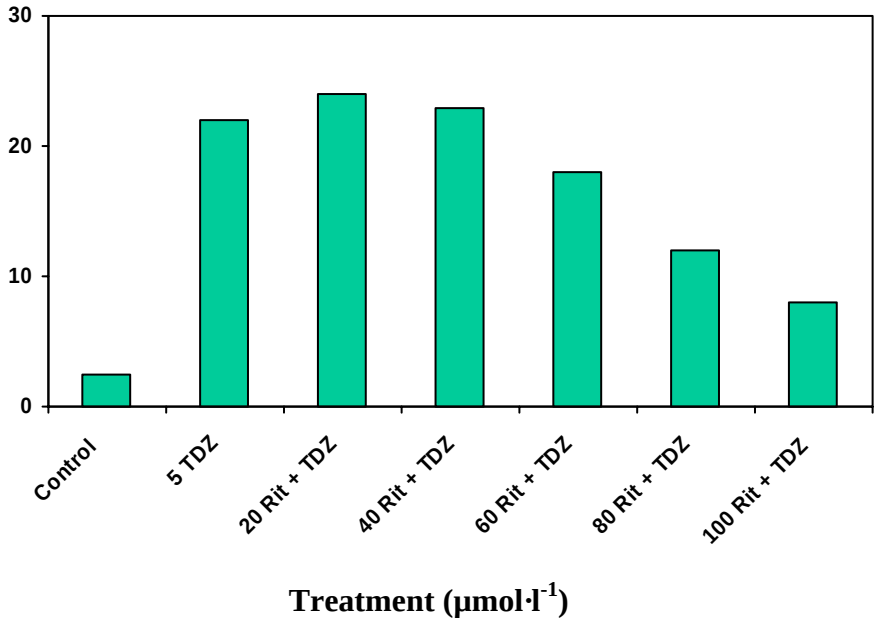

Fig. 5. Effect of the selective serotonin reuptake inhibitor methylphenidate (Ritalin™) on TDZ-induced morphogenesis in St. John's wort stem explants.