Plant Cell Rep (2007) 26:1481–1490
DOI 10.1007/s00299-007-0357-0

CELL BIOLOGY AND MORPHOGENESIS

The mode of action of thidiazuron: auxins, indoleamines, and ion channels in the regeneration of *Echinacea purpurea* L.

Maxwell P. A. Jones · Jin Cao · Rob O'Brien · Susan J. Murch · Praveen K. Saxena

Received: 1 December 2006 / Revised: 21 March 2007 / Accepted: 23 March 2007 / Published online: 5 May 2007

Abstract The biochemical mechanisms underlying thidiazuron (TDZ)-induced regeneration in plant cells have not been clearly elucidated. Exposure of leaf explants of *Echinacea purpurea* to a medium containing TDZ results in undifferentiated cell proliferation and differentiated growth as mixed shoot organogenesis and somatic embryogenesis. The current studies were undertaken to determine the potential roles of auxin, indoleamines, and ion signaling in the dedifferentiation and redifferentiation of plant cells. *E. purpurea* leaf explants were found to contain auxin and the related indoleamine neurotransmitters, melatonin, and serotonin. The levels of these endogenous indoleamines were increased by exposure to TDZ associated with the induction of regeneration. The auxin-transport inhibitor 2,3,5-triiodobenzoic acid and auxin action inhibitor, *p*-chlorophenoxyisobutyric acid decreased the TDZ-induced regeneration but increased concentrations of endogenous serotonin and melatonin. As well, inhibitors of calcium and sodium transport significantly reduced TDZ-induced morphogenesis while increasing endogenous indoleamine content. These data indicate that TDZ-induced regeneration is the manifestation of a metabolic cascade that includes an initial signaling event, accumulation, and transport of endogenous plant signals such as auxin and melatonin, a system of secondary messengers, and a concurrent stress response.

Keywords *Echinacea purpurea* · Regeneration · Thidiazuron · Serotonin · Melatonin · Calcium · Sodium

Communicated by P. Lakshmanan.

M. P. A. Jones · J. Cao · P. K. Saxena
Department of Plant Agriculture,
University of Guelph, Guelph, ON,
Canada N1G 2W1

J. Cao · R. O'Brien · S. J. Murch (✉)
Department of Chemistry, I. K. Barber School of Arts
and Sciences, University of British Columbia Okanagan,
3333 University Way, Kelowna, BC, Canada V1V 1V7
e-mail: susan.murch@ubc.ca

Introduction

A recent literature search found more than 825 reports of the use of the synthetic cytokinin-like plant growth regulator thidiazuron (TDZ: *N*-phenyl-*N*′-[(1,2,3-thidiazol-5-yl)urea]) for the induction of plant regeneration in vitro. TDZ is a substituted phenylurea that found original application as a defoliant for the mechanical harvest of cotton bolls (Arndt et al. 1976). In excised plant tissues in culture, both traditional cytokinin and auxin-type responses have been reported including examples of bud break, shoot organogenesis, and somatic embryogenesis (reviewed in Murthy et al. 1998). TDZ-exposure stimulated ethylene production concurrent to accumulations of auxin and Ca^{2+} in mungbean hypocotyls (Yip and Yang 1986). TDZ-induced tissues undergo a stress response that includes accumulation of abscisic acid, proline, and γ-aminobutyrate (Murch et al. 1997a). In peanut seedlings cultured on a medium containing TDZ, proline accumulated to 6% of the dry weight (Murch and Saxena 1997). Endogenous auxin metabolism and transport were accelerated in plant tissues exposed to TDZ (Murthy et al. 1995; Hutchinson et al. 1996; Murch and Saxena 2001). More recently, the auxin-related compounds serotonin and melatonin were found to be important in TDZ-induced regeneration (Murch and Saxena 2005). However, in spite of the popularity of TDZ

as a plant growth regulator and more than three decades of research, the specific biochemical mode of action underlying the TDZ-induced responses remains undiscovered.

Thidiazuron-induced regeneration has been reported in various explants of the medicinal species *Echinacea purpurea* L. (Jones et al. 2007). In *E. purpurea* leaf explants, regeneration responses are manifested as a mixture of callus and differentiated regenerants, some of which appear to be somatic embryos while others are de novo shoots (Jones et al. 2007). This interesting continuum of redirected development offers new opportunities to investigate the biochemical mechanism of TDZ-induced morphogenesis. The current experiments were undertaken to examine the developmental and biochemical changes in *Echinacea* leaves in response to TDZ. The specific objectives of the research were (a) to determine whether *E. purpurea* tissues contain high levels of auxin or the related compounds melatonin and serotonin, (b) to determine whether auxin, melatonin or serotonin are involved in TDZ-induced regeneration in *E. purpurea*, and (c) to investigate the potential role of ion signaling in TDZ-induced regeneration.

Materials and methods

Chemicals

2,3,5-Triiodobenzoic acid (TIBA; Sigma St Louis, MO, USA), *p*-chlorophenoxyisobutyric acid (PCIB; Sigma), Lidocaine, (Sigma) and (S)-Bay K8644 (*S*-(–)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester; AG Scientific, CA, USA) solutions were prepared by dissolving the compounds in 14 ml of 95% ethanol. Certified analytical standards of melatonin, serotonin, and indole-3-acetic acid were also obtained from Sigma Chemical Co (St Louis, MO, USA). Solvents included HPLC-grade methanol and acetonitrile (VWR Scientific, Mississauga, ON, USA), AR grade formic acid (Sigma) and AR grade HCl (Sigma).

Plant cultures

Plant material was obtained from a clonally propagated population of *E. purpurea* plants maintained in vitro. The plants were multiplied by crown divisions and maintained on a modified MS (Murashige and Skoog 1962) basal medium containing MS salts, B_5 vitamins (Gamborg et al. 1968), 30 g/l sucrose, 3 g/l Gellam gum (Sigma, St Louis, MO, USA), as described earlier (Jones et al. 2007). Leaf lamina explants (6 mm diameter disks) were cultured with their abaxial surface in contact with the culture medium in Petri dishes (Fisher, Ottawa, Canada) each containing 20 ml of the MS culture medium. The medium was supplemented with 1 μM TDZ (*N*-phenyl-*N*′-[(1,2,3-thidiazol-5-yl)urea]) (Riedel-de Haën, Seelze, Germany) prior to adjusting the pH to 5.7 and autoclaving (20 min at 121°C at 1.4×10^4 kg/m/s^2). TIBA, PCIB, Lidocaine or Bay K8644 (0, 20, 40, 60 μM) were filter sterilized (0.20 μm filter, Fisher, Ottawa, ON, Canada) and added to the autoclaved TDZ-media once it had cooled to approximately 55°C.

Leaf explant cultures were prepared as described previously (Jones et al. 2007). Three explants were cultured per Petri dish (60 mm × 15 mm; Fisher, Ottawa, ON, Canada) containing 20 ml of media and sealed using parafilm. The cultures were maintained in the dark at 25°C for 4 weeks. After 4 weeks the number of regenerants formed on each explant was quantified and the amount of callus formation, tissue expansion, and color of the tissues were recorded as a qualitative estimate compared to the controls. Samples were collected from the cultures, immediately frozen in liquid nitrogen and stored at –80°C until analysis.

Quantification of indole-3-acetic acid, serotonin, and melatonin

Frozen plant tissues were completely dried under nitrogen gas, accurately weighed and each was transferred to a 50 ml beaker. A 10 ml aliquot of 100% methanol was added to each sample and the solution was sonicated in an ultrasonic bath (Bransonic 1510R-MT, 42 KHZ) for 45 min. The supernatant was decanted and centrifuged (Hamilton bell Co Inc, model 1500; 10 min at [4,200×*g*]). The resulting extract was filtered (hydrophilic PTFE, 0.45 μm, Millipore) and combined with the filtered supernatant of two subsequent methanol washes of the tissue residue. Each extract was completely dried in a 25 ml beaker covered with aluminum foil, and kept under nitrogen gas in a fume hood. Dried extracts were subsequently re-suspended in 1 ml methanol in preparation for injection into an LC-MS/MS apparatus. For quantification, repeated injections of 50 μl were made into the LC-MS/MS apparatus under the optimized detection conditions for each compound (Cao et al. 2006).

Compounds were separated by an Alliance series HPLC (Waters Inc. Mississauga, ON, USA) coupled with a Premier LCT MS/MS (Waters) and controlled with an Empower data system (Waters) as described previously (Cao et al. 2006). Compounds were detected within the MS/MS using optimized parameters for each metabolite (Table 2). Melatonin, serotonin, and IAA were separated across an Xterra C_{18} HPLC column (2.1 mm × 1,000 mm, 3.5 μm; Waters) heated to 30°C with a gradient of 0.45% formic acid:acetonitrile (0–5 min, 95:5%, v/v; 5–6 min, 95:5–0:100%, v/v; 6–16 min, 0:100%, v/v). Compounds were

eluted at 0.25 ml/min over 16 min followed by a 14 min column re-equilibration period. Detection parameters were optimized for each metabolite as described previously (Cao et al. 2006). In brief, melatonin was detected and quantified in the ESI +ve mode as the parent compound with a molecular mass of 233 and principle daughter ion with a mass of 174. Serotonin was detected and quantified in the ESI +ve mode as a parent compound with mass 177 and principle daughter ion with mass 160 while indole-3-acetic acid was also detected in the ESI +ve mode with mass of the parent compound of 176 and a principle daughter compound with a mass of 130.

Statistical analysis

The experiment was arranged in a completely randomized design. Each treatment consisted of eight plates containing three explants each. The experiment was conducted twice. All statistical analyses were conducted using SAS version 8.02 (SAS Institute, Carey, NC, USA) with type 1 error rate of 0.05. Means between replicate experiments were pooled after contrast statements determined that they were not significantly different between experiments. Significance was determined by comparison of means with a Student–Newman–Keuls means separation test.

Results and discussion

TDZ and auxin metabolism

Leaf explants of *E. purpurea* cultured on a basal medium devoid of plant growth regulators remained green but demonstrated no signs of expansion, growth, dedifferentiation or morphogenesis (Fig. 1a). The supplementation of culture medium with the plant growth regulator TDZ (1 μM) resulted in the induction of regeneration with an average of 42.7 ± 1.97 regenerants per explant (Fig. 1b). These TDZ-induced regenerants are capable of growth to maturity forming whole plants (Jones et al. 2007). Serotonin and melatonin were found in *E. purpurea* explants with average concentrations of 118 ± 5.8 ng/g and 131 ± 6.5 ng/g, respectively. Phytochemical analysis of the tissue explants following the expression of regeneration revealed the accumulation of significantly higher amounts of IAA, serotonin, and melatonin in the TDZ-exposed tissues (Fig. 1c, d, e).

Inclusion of TIBA, an auxin-transport inhibitor, in the TDZ-supplemented culture medium reduced the TDZ-induced regeneration significantly at 20 μM (Fig. 2a, d) and completely inhibited the TDZ-induced morphogenesis at concentrations greater than 40 μM TIBA (Fig. 2b, c, d).

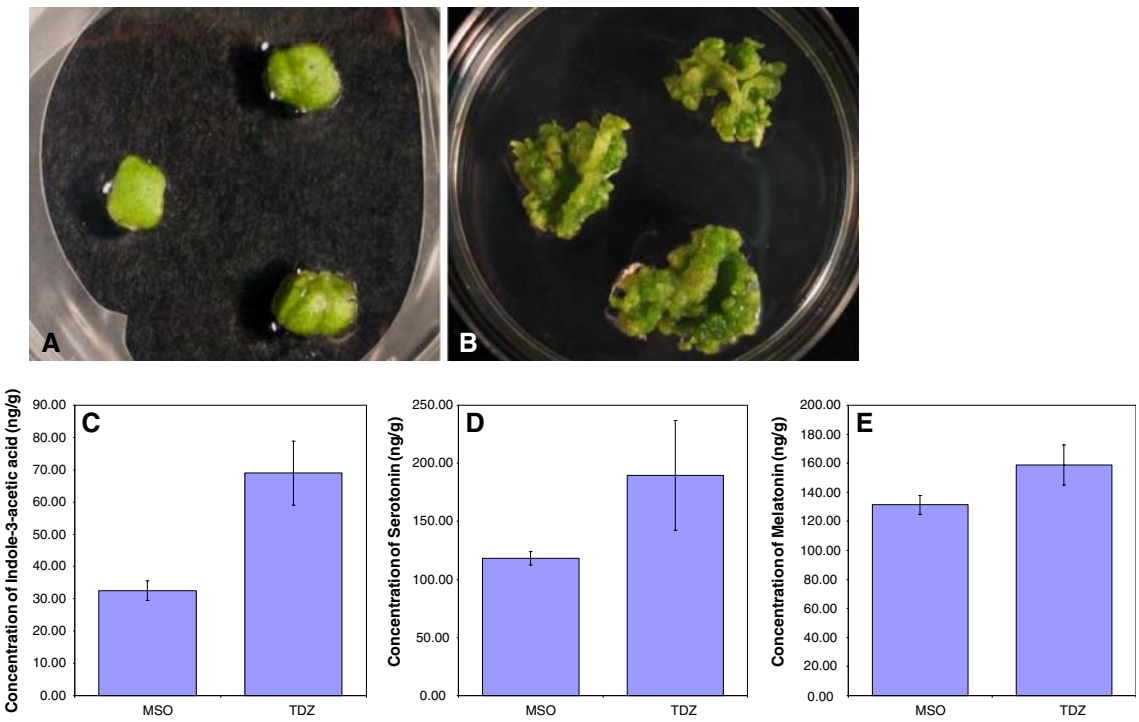

Fig. 1 Effect of thidiazuron (TDZ) on regeneration, auxin, and indoleamine content of *Echinacea purpurea* leaf explants. **a** Leaf explants cultured on basal medium. **b** Leaf explants cultured on medium supplemented with 1 μM TDZ. **c** Indole-3-acetic acid content of leaf explants of basal medium or medium containing 1 μM TDZ. **d** Serotonin content of leaf explants of basal medium or medium containing 1 μM TDZ. **e** Melatonin content of leaf explants of basal medium or medium containing 1 μM TDZ

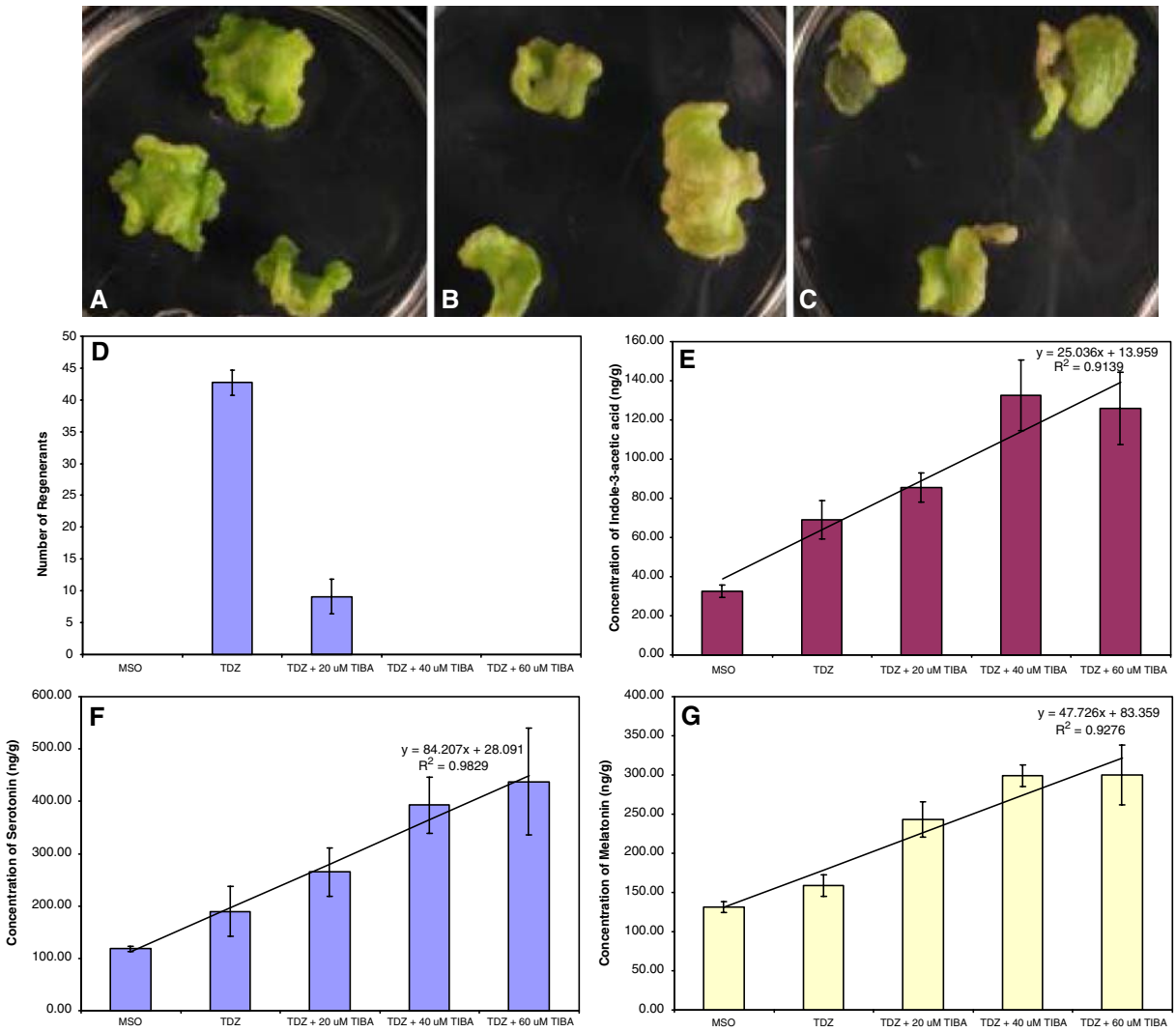

Fig. 2 Effect of TIBA on thidiazuron (TDZ)-induced regeneration, auxin, and indoleamine content in leaf explants of *Echinacea purpurea*. **a** Explants cultured on medium containing 1 μM TDZ + 20 μM TIBA. **b** Explants cultured on medium containing 1 μM TDZ + 40 μM TIBA. **c** Explants cultured on medium containing 1 μM TDZ + 60 μM TIBA. **d** Number of regenerants formed on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM TIBA. **e** Auxin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM TIBA. **f** Serotonin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM TIBA. **g** Melatonin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM TIBA

The inhibition of regeneration was accompanied by a significant linear increase in concentration of endogenous auxin (Fig. 2e), serotonin (Fig. 2f) and melatonin (Fig. 2g). A similar significant reduction in regeneration was observed on explants cultured on the medium supplemented with TDZ and the auxin activity inhibitor PCIB (Fig. 3a, b, c, d). The explants retained their green color despite the inhibition of callus formation and regeneration in the presence of PCIB. However, significant differences were not observed in the auxin content of explants exposed to TDZ and PCIB. *Echinacea* leaf explants cultured on the medium containing TDZ and 20 μM or 40 μM PCIB were found to have significantly higher levels of serotonin and melatonin in the plant tissues (Fig. 3e).

Exposure of leaf explants to TDZ resulted in cell dedifferentiation and redifferentiation culminating in regeneration that was accompanied by significant increases in endogenous auxin, melatonin, and serotonin contents. The application of inhibitors of auxin transport and biosynthesis inhibited the regeneration and significantly increased the endogenous concentrations of auxin, melatonin, and serotonin. Interestingly, both the auxin transport (TIBA) and auxin activity inhibitor (PCIB) inhibited regeneration equally effectively, but the inhibition with TIBA occurred with distinct elevation in endog-

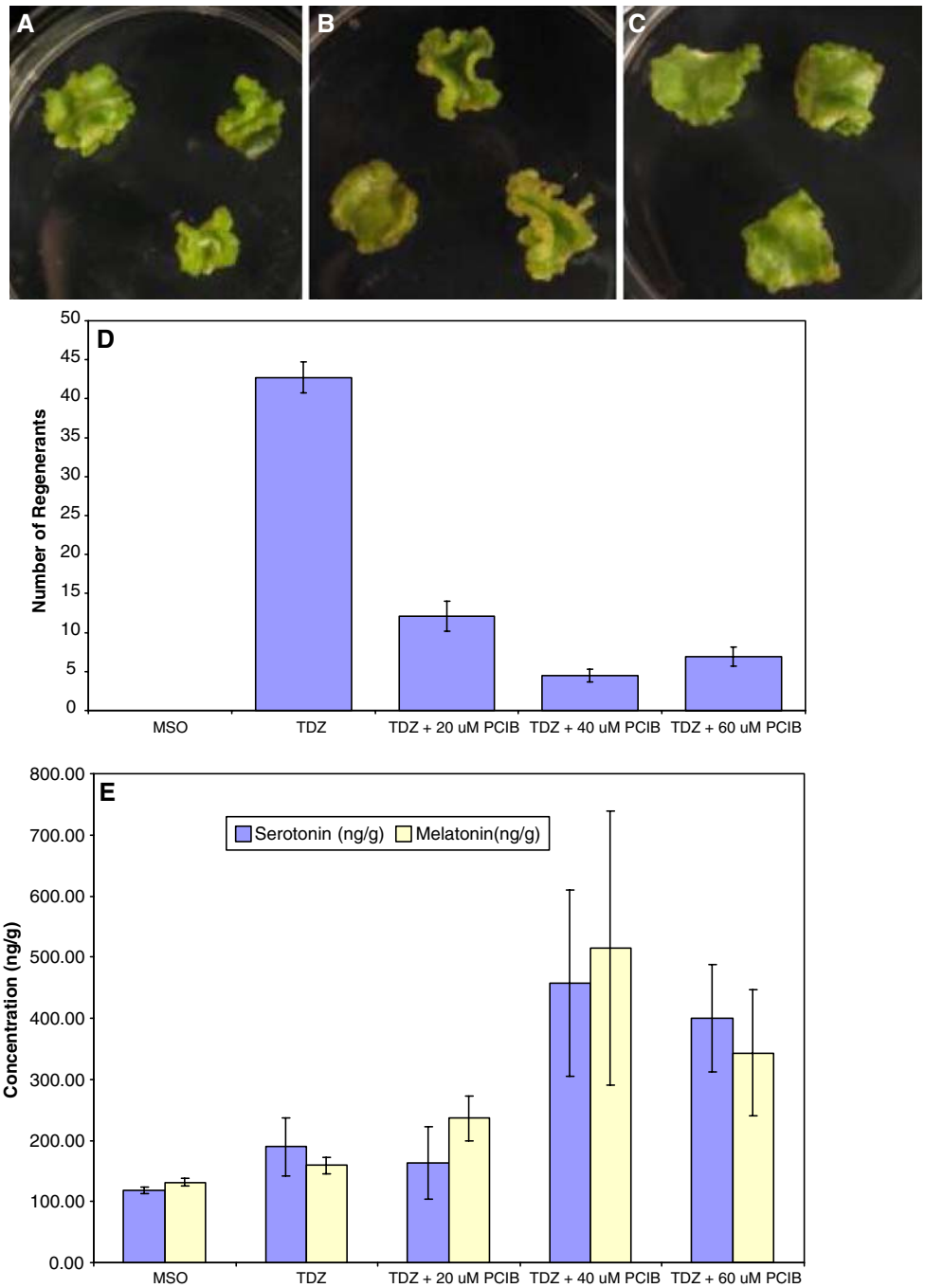

Fig. 3 Effect of PCIB on thidiazuron (TDZ)-induced regeneration and indoleamine content in *Echinacea purpurea*. **a** Explants cultured on medium containing 1 μM TDZ + 20 μM PCIB. **b** Explants cultured on medium containing 1 μM TDZ + 40 μM PCIB. **c** Explants cultured on medium containing 1 μM TDZ + 60 μM PCIB. **d** Number of regenerants formed on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM PCIB. **e** Serotonin and melatonin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM PCIB

enous IAA content. The observation that auxin concentrations were not different from controls when the auxin activity inhibitor PCIB was used to inhibit regeneration indicates the interplay of multiple factors beyond an optimum concentration dependency of TDZ-induced regeneration. Suppression of TDZ-induced regeneration by inhibitors of the auxin biosynthesis, transport, and action and simultaneous modulation of endogenous IAA in this study together with previous studies of different regeneration systems (Hutchinson et al. 1996; Murch and Saxena 2001, 2004; Murch et al. 2003) further strengthen the evidence of auxin involvement in TDZ-mediated morphogenesis.

TDZ and ion channels

The calcium channel agonist (S)-Bay K8644 also inhibited TDZ-induced regeneration in explants of *E. purpurea* (Fig. 4a, b, c, d) and (S)-Bay K8644 supplementation of the TDZ medium significantly increased the endogenous

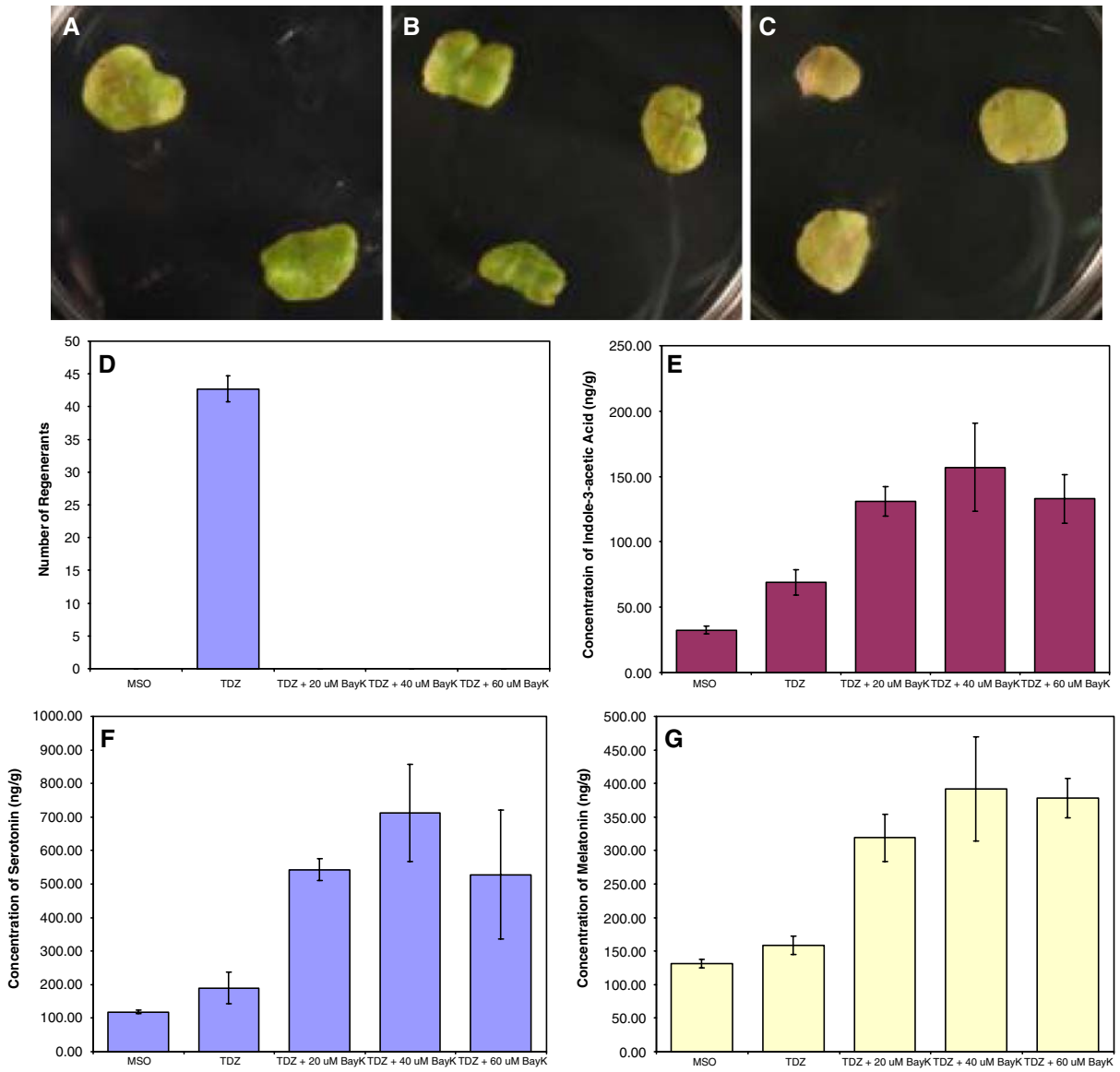

Fig. 4 The effect of the calcium channel agonist (S)-Bay K8644 on thidiazuron (TDZ)-induced regeneration, auxin, and indoleamine metabolism in *Echinacea purpurea.* **a** Explants cultured on medium containing 1 μM TDZ + 20 μM (S)-Bay K8644. **b** Explants cultured on medium containing 1 μM TDZ + 40 μM (S)-Bay K8644. **c** Explants cultured on medium containing 1 μM TDZ + 60 μM (S)-Bay K8644. **d** Number of regenerants formed on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM (S)-Bay K8644. **e** Auxin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM (S)-Bay K8644. **f** Serotonin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM (S)-Bay K8644. **g** Melatonin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM (S)-Bay K8644

concentrations of indole-3-acetic acid (Fig. 4e), serotonin (Fig. 4f) and melatonin (Fig. 4g). The treatment with (S)-Bay K8644, a calcium channel activator that changes cell polarity (Ferrante et al. 1989), completely inhibited TDZ-induced callus induction and regeneration and increased the concentration of auxin, serotonin, and melatonin in the explants. In general, calcium channels open in response to multiple inductive stimuli such as TDZ or auxin, and transmit intermittent signals via transient elevations in cytosolic calcium thereby initiating a cascade of metabolic responses (Trewavas 1999; White and Broadley 2003). Calcium agonists change the frequency, amplitude, duration, and number of Ca^{2+} oscillations in plant cells (Sathyanarayanan and Poovaiah 2004 and references therein). Previous researchers have hypothesized that the mode of action of TDZ may involve activation of a cytosolic calcium cascade (Mundhara and Rashid 2002; Murch and Saxena 2005). In African violets, the Ca^{2+} agonist changed

the pattern of regeneration and the responding cell type but did not decrease the number of regenerants (Murch et al. 2003). Hosseini-Nasr and Rashid (2002) reported that the addition of lanthanurn, a calcium-uptake inhibitor, reduced the frequency of shoot formation from *Albizia julibrissin* root cultures in response to TDZ. Similarly, the regeneration of hypocotyl explants of *Linum usitatissimum* required the presence of cytosolic Ca^{2+} (Jain and Rashid 2001). However, a prolonged increase in cytosolic Ca^{2+} can be lethal to plant cells and has been implicated in apoptosis (White and Broadley 2003).

The supplementation of the TDZ medium with Lidocaine, a sodium channel blocker, resulted in elevated levels of serotonin and melatonin but not auxin and significantly decreased the rate of TDZ-induced regeneration in the *E. purpurea* explants (Fig. 5a, b, c, d). Lidocaine treatment did not significantly alter the endogenous concentrations of auxin but low concentrations of Lidocaine significantly increased the concentrations of serotonin and melatonin in *E. purpurea* tissues (Fig. 5e). Lidocaine has been previously shown to inhibit regeneration in African violets (*Saintpaulia* ssp.) (Murch et al. 2003) and *Kalanchoe pinnata* (Sawhney and Sawhney 2002) as well as to inhibit polar movement of ions in Mimosa pudica (Milne and Beamish 1999) but evidence of a biochemical mechanism for Lidocaine activity in plants has not been discovered.

Melatonin and serotonin in TDZ-induced regeneration

Thidiazuron-induced morphogenesis has been the subject of numerous studies in *E. purpurea* (Jones et al. 2007), geraniums (Hutchinson et al. 1996), peanuts (Murthy et al. 1995) and many other species (reviewed in Murthy et al. 1998). In some plant systems such as geranium, there is a relatively short induction period of TDZ-exposure followed by a period of expression of morphogenesis in the absence of any exogenous growth regulators (Hutchinson et al. 1996) but in other species, longer incubations on TDZ-supplemented media are necessary for induction of regeneration. TDZ-induced regeneration in *E. purpurea* requires an induction period of 4 weeks of exposure in total darkness for optimal regeneration (Jones et al. 2007) and regeneration is expressed as concurrent development of de novo shoots and somatic embryos at several stages of maturity (Jones et al. 2007). The development of a TDZ-induced regeneration system with a 4-week induction period and an interaction with light offered a new opportunity for study of endogenous plant growth regulators including auxins and indoleamines. Previous studies have shown TDZ-induced accumulation of melatonin and serotonin associated with shoot organogenesis in St John's wort (Murch and Saxena 2005) and accumulation of melatonin in dark adapted plant tissues (Murch et al. 2001; Murch and Saxena 2005). However, the cumulative effects of TDZ-exposure and dark adaptation have not previously been investigated in any species. Therefore, the current studies were undertaken to determine the relative accumulations of auxin and related compounds at the end of the regeneration induction phase.

Melatonin and serotonin were significantly elevated in all instances of inhibition of TDZ-induced regeneration caused by TIBA, PCIB, and the ion channel activator/blockers. The explants retained their green color, remained turgid, and expanded similar to the TDZ-exposed explants except for the induction of regenerative response. The absolute concentrations of melatonin and serotonin were also higher in the inhibited tissues compared to those induced by the TDZ alone. Melatonin is a ubiquitous, highly conserved molecule found in humans and many other vertebrates (Pandi-Perumal et al. 2006). The presence of melatonin has been detected in nearly all organisms including bacteria, fungi, invertebrates, and plants. The known physiological functions of melatonin in animals include the timing of circadian rhythms and signaling of environmental changes, neurotransmission, and detoxification of oxygen free radicals (Hardeland et al. 2006). The high antioxidant efficiency of melatonin allowing the survival of the cells is mediated through a direct binding or indirectly by enhancing the activity of other free radical scavenging antioxidants and associated enzymes (Pandi-Perumal et al. 2006; Hardeland et al. 2006).

Melatonin has now been found in more than 130 plant species to date (Murch and Saxena 2002; Chen et al. 2003; Kolar and Machackova 2005) and relatively higher levels of melatonin have been reported in some medicinal plants (Murch et al. 1997b, 2000; Chen et al. 2003). However, research on plant melatonin and serotonin is still in its infancy and little is understood of their roles in plant growth and development. The results of the present study suggest several possibilities of the mode of melatonin action in TDZ-induced regeneration. First, elevated levels of melatonin and serotonin in the TDZ treated tissues may indicate that melatonin acts as a hormone independently or in concert with auxin and its own precursors and metabolites. We have previously shown that the exposure of plant tissues to TDZ results in an immediate and prolonged increase in endogenous auxin (Murthy et al. 1995; Murch and Saxena 2001) that is essential for the regeneration (Hutchinson et al. 1996; Murch et al. 2003). Hernandez-Ruiz et al. (2004) have shown an auxin-like activity of melatonin in elongation of hypocotyls *Lupinus albus* and the high endogenous concentration of auxin and melatonin in the hypocotyl tissues supported the notion of a hormonal function of melatonin. As well, research with axenic plantlets of *Hypericum perforatum* has demonstrated that auxin, serotonin, and melatonin are synthesized in plants

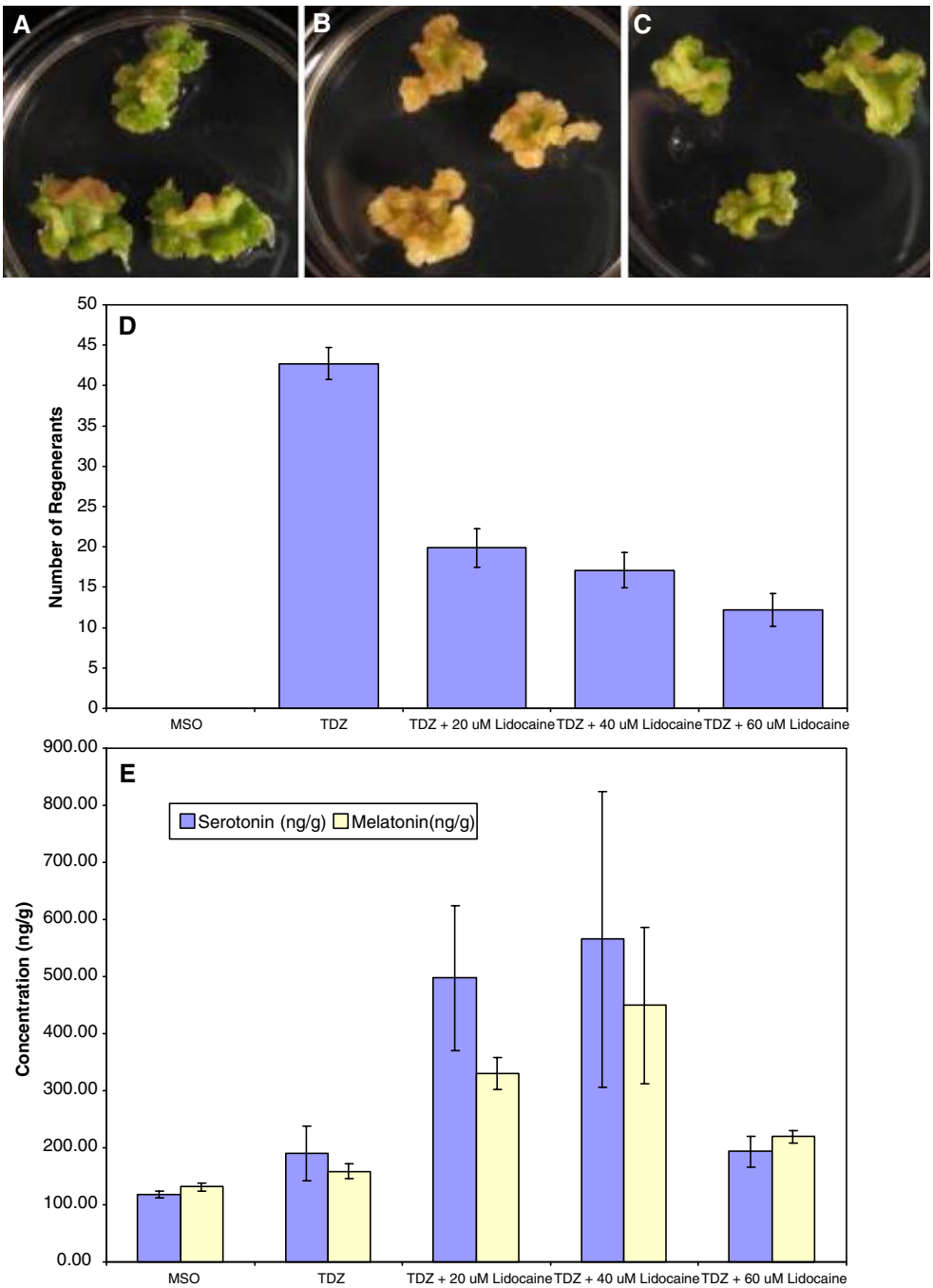

Fig. 5 The effects of the sodium channel antagonist Lidocaine™ in thidiazuron (TDZ)-induced regeneration and indoleamine metabolism in *Echinacea purpurea*. **a** Explants cultured on medium containing 1 μM TDZ + 20 μM Lidocaine. **b** Explants cultured on medium containing 1 μM TDZ + 40 μM Lidocaine. **c** Explants cultured on medium containing 1 μM TDZ + 60 μM Lidocaine. **d** Number of regenerants formed on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM Lidocaine. **e** Serotonin and melatonin content of leaf explants cultured on basal medium and media containing 1 μM TDZ with 0, 20, 40, or 60 μM Lidocaine

from a common precursor tryptophan and that this pathway may be involved in organogenesis (Murch et al. 2000; Murch and Saxena 2004). The metabolism of tryptophan under different conditions including in vitro culture stress is likely to result in different rates of biosynthesis, interconversions, and catabolism of auxin and melatonin. Serotonin, a precursor of melatonin, is thought to mediate several physiological functions in animals including cell communication, division, and growth (Buznikov and Shmukler 1981; Banasr et al. 2001). Further, melatonin binding and interactions with Ca^{2+}-calmodulin have been shown to regulate many calcium-dependent cellular functions in animal cells. Melatonin action in plants via Ca^{2+}-calmodulin is therefore a potential mechanism of signaling in plants particularly in view of the Ca^{2+} dependent activity of auxin in several physiological responses. Secondly, melatonin may balance the oxidative stress generated by explanting and exposure to growth regulators such as TDZ during the culture process. Plants utilize reactive oxygen species as second messengers in signal transduction

cascades in diverse physiological functions including cell division, senescence, and tropisms (Foyer and Noctor 2005). Oxidative stresses have earlier been shown to stimulate cell division, differentiation, and morphogenesis analogous to auxins (Pasternak et al. 2002). Concurrent with the TDZ-induced regeneration is the accumulation of indoleamines and stress related molecules (Murch et al. 1997a, b, 1999, 2003; Mundhara and Rashid 2006). Melatonin and its metabolites in animal cells are known to trigger a cascade of free radical scavenging activities and a similar mode of action in plants is conceivable. Onset of regeneration in vitro is a complex process involving acquisition of competence and the expression of induced morphogenesis and may implicate stage-specific accumulation and detoxification of free radicals in TDZ-induced regeneration. In many plants, only a brief exposure to TDZ is sufficient for inducing regeneration and prolonged exposure leads to abnormal development suggesting differential oxidative stress sensitivity and requirements of the developmental program. Finally, the commonality of suppression of regeneration by a range of inhibitors and elevated melatonin and serotonin may indicate that they are byproducts of regenerative stress.

Conclusion

In conclusion, a model for TDZ-induced morphogenesis is emerging that involves a metabolic cascade including an initial signaling event, accumulation, and transport of endogenous plant signals such as auxin and melatonin, a system of secondary messengers and a concurrent stress response that may or may not be manifested as morphogenesis. In view of the known multiple functions of melatonin and serotonin, the possibility of their direct or indirect role in more than one way cannot be ruled out and further research is needed to elucidate their interactions and roles as signaling molecules in regenerative processes. This is the first report of the presence of these human neurotransmitters in *E. purpurea* and as such the TDZ-induced regeneration system opens several possibilities of investigating the physiological functions of melatonin and serotonin in plant development.

References

Arndt FJ, Rusch R, Stilfried HV (1976) SN 49537, a new cotton defoliant. Plant Physiol 57:99

Banasr M, Hery M, Brezun JM, Daszuta A (2001) Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus. Eur J Neurosci 14:1417–1424

Buznikov GA, Shumkler YB (1981) Possible role of pre-nervous neurotransmitters in cellular interactions of early embryogenesis—a hypothesis. Neurochem Res 6:55–68

Cao J, Murch SJ, O'Brien R, Saxena PK (2006) Rapid method for accurate analysis of melatonin, serotonin and auxin in plant samples using liquid chromatography-tandem mass spectrometry. J Chromatogr A 1134:333–337

Chen G, Huo Y, Tan D-X, Liang Z, Zhang WB, Zhang YK (2003) Melatonin in Chinese medicinal herbs. Life Sci 73:19–26

Ferrante J, Luchowski E, Rutledge A, Tringgle DJ (1989) Binding of a 1,4-dihydropyridine calcium channel activator, (–)S-Bay K8644 to cardiac preparations. Biochem Biophys Res Commun 158:149–154

Foyer CH, Noctor G (2005) Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ 28:1056–1071

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hardeland R, Pandi-Perumal SR, Cardinali DP (2006) Melatonin. Int J Biochem Cell Biol 38:313–316

Hernandez-Ruiz J, Cano A, Arnao MB (2004) Melatonin: a growth-stimulating compound present in lupin tissues. Planta 220:140–144

Hosseini-Nasr M, Rashid A (2002) Thidiazuron-induced shoot-bud formation on root segments of *Albizzia julibrissin* is an apex-controlled, light-independent and calcium-mediated response. Plant Growth Regul 36:81–85

Hutchinson MJ, Murch SJ, Saxena PK (1996) Morphoregulatory role of thidiazuron: evidence of the involvement of endogenous auxin in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium* × *hortorum* Bailey). J Plant Physiol 149:573–579

Jain P, Rashid A (2001) Stimulation of shoot regeneration on *Linum* hypocotyl segments by thidiazuron and its response to light and calcium. Biol Plant 44(4):611–613

Jones MPA, Yi Z, Murch SJ, Saxena PK (2007) Thidiazuron-induced regeneration of *Echinacea purpurea* L.: micropropagation in solid and liquid culture systems. Plant Cell Rep 26:13–19

Kolar J, Machackova I (2005) Melatonin in higher plants: occurrence and possible functions. J Pineal Res 39:333–341

Milne A, Beamish T (1999) Inhalation and local anesthetics reduce tactile and thermal responses in *Mimosa pudica*. Can J Anaesth 46:287–289

Mundhara R, Rashid A (2002) Stimulation of shoot-bud regeneration on hypocotyl of Linum seedlings, on a transient withdrawal of calcium, cytokinin and thidiazuron. Plant Sci 162:211–214

Mundhara R, Rashid A (2006) TDZ-induced triple-response and shoot formation on intact seedlings of *Linum*, putative role of ethylene in regeneration. Plant Sci 170:185–190

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

Murch SJ, Saxena PK (1997) Modulation of mineral and fatty acid profiles during thidiazuron mediated somatic embryogenesis in peanuts (*Arachis hypogaea* L.). J Plant Physiol 151:358–361

Murch SJ, Saxena PK (2001) Molecular fate of thidiazuron and its effects on auxin transport in hypocotyls tissues of *Pelargonium* × *hortorum* Bailey. Plant Growth Regul 35(3):269–275

Murch SJ, Saxena PK (2002) Melatonin: a potential regulator of plant growth and development? In Vitro Cell Dev Biol Plant 38:531–537

Murch SJ, Saxena PK (2005) Role of indoleamines in regulation of morphogenesis in in vitro cultures of St. John's wort (*Hypericum perforatum* L.). Acta Hortic 629:XXVI International horticultural congress: The future for medicinal and aromatic plants

Murch SJ, KrishnaRaj S, Saxena PK (1997a) Thidiazuron-induced morphogenesis of regal geranium (*Pelargonium domesticum*): a potential stress response. Physiol Plant 101:183–191

Murch SJ, Simmons CB, Saxena PK (1997b) Melatonin in feverfew and other medicinal plants. Lancet 350:1598–1599

Murch SJ, Victor JMR, KrishnaRaj S, Saxena PK (1999) The role of proline in thidiazuron-induced somatic embryogenesis of peanuts. In Vitro Cell Dev Biol Plant 35:102–105

Murch SJ, KrishnaRaj S, Saxena PK (2000) Tryptophan is a precursor for melatonin and serotonin biosynthesis in in vitro regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. Plant Cell Rep 19:698–704

Murch SJ, Campbell SSB, Saxena PK (2001) The role of serotonin and melatonin in plant morphogenesis: Regulation of auxin-induced root organogenesis in *in vitro* cultured explants of St. John's wort (*Hypericum perforatum* L.). In Vitro Cell Dev Biol 37:786–793

Murch SJ, Victor JMR, Saxena PK (2003) Auxin, calcium and sodium in somatic embryogenesis of African violet (*Saintpaulia ionantha* Wendl. Cv. Benjamin). Acta Hortic 625:XXVI. International Horticultural Congress: Biotechnology in horticultural crop improvement: achievements, opportunities and limitations

Murthy BNS, Murch SJ, Saxena PK (1995) Thidiazuron induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea* L.): endogenous growth regulator levels and significance of cotyledons. Physiol Plant 94:268–276

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev Biol Plant 34:267–275

Pandi-Perumal SR, Srinivasan V, Maestroni GJM, Cardinali DP, Poeggeler B, Hardeland R (2006) Melatonin: nature's most versatile biological signal? FEBS J 273:2813–2838

Pasternak TP, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, Van Onckelen HA, Dudits D, Feher A (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol 129:1807–1819

Sathyanarayanan PV, Poovaiah BW (2004) Decoding Ca2+ signals in plants. Crit Rev Plant Sci 23:1–11

Sawhney N, Sawhney S (2002) Local anaesthetic lidocaine modulates epiphyllous bud differentiation in *Kalanchoe pinnata*. Plant Growth Regul 38:45–49

Trewavas A (1999) Le Calcium, C'est la vie: calcium makes waves. Plant Physiol 120:1–6

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92:487–511

Yip W-K, Yang SF (1986) Effect of thidiazuron, a cytokinin-active urea derivative, in cytokinin-dependent ethylene production systems. Plant Physiol 80(2):515–519