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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin and serotonin in flowers and fruits of *Datura metel* L.

Abstract: *Datura metel* is a plant that contains several different neurologically active phytochemicals which affect human health. On-going research has examined the potential role of the human neuroindoles, melatonin and serotonin, in medicinal plants with neurological efficacy. In this report, we describe the quantification of melatonin and serotonin in flowers and developing fruits of *Datura metel* and the effects of cold stress on the levels of these neuroindoles in the reproductive tissues of this plant. Melatonin and serotonin were found at the highest levels in the least developed flower buds with decreasing concentrations as the flower buds matured. Cold stress significantly increased the concentration of melatonin in young flower buds. In the developing fruit, melatonin was present at relatively stable, high concentrations for the first 10 days after anthesis. After 10–15 days, the ovule had grown to a sufficient size for excision and analysis and melatonin was found to be at the highest concentrations in the developing ovule with minimal concentrations of the neuroindoles in the fleshy fruit. Together, these data indicate that melatonin may play a role in protecting the reproductive tissues during flower and seed formation in a *Datura* species.

Susan J. Murch[1], Ali R. Alan[2], Jin Cao[3] and Praveen K. Saxena[4]

[1]Chemistry, Earth & Environmental Science, University of British Columbia Okanagan, Kelowna, BC, Canada; [2]Department of Biology, Pamukkale University, Denizli, Turkey; [3]Research Centre, Xiyuan Hospital, Beijing, China; [4]Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada

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Address reprint requests to Praveen K. Saxena, Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada N1G 2W1. E-mail: psaxena@uoguelph.ca

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Introduction

Datura metel L., commonly known as 'Devil's Trumpet' or 'Angel's Trumpet' is a narcotic plant with a long history of use as a medicine by many cultures from around the world. In Traditional Chinese Medicine, the flowers of *D. metel* are known as 'baimantuoluo' and prescribed for skin inflammation and psoriasis [1]. In Ayurvedic medicine, seeds of *D. metel* are used to treat skin rashes, ulcers, bronchitis, jaundice and diabetes [2]. In Brazil, seeds are prepared in a tea as a sedative and flowers are dried and smoked as cigarettes [3]. The anticholinergic activity of flowers and seeds of *D. metel* has been described in several cases of accidental poisoning with symptoms such as delirium, drowsiness, agitation, hallucination, ataxia, myoclonus jerking, convulsion, coma, dilated pupils, dry mouth, dry skin, hyperthermia, sinus tachycardia, cardiac conduction abnormalities, dysrhythmia, urinary retention and paralytic illnesses [4]. Chemically, *D. metel* is a source of tropine alkaloids such as hyoscyamine, scopolamine, anisodamine, and anisodine as well as megastigmane sesquiterpenes [5] and recently withanolides [6, 7].

Melatonin has been found to be a potentially active constituent in medicinal plants [8, 9] and previous preliminary evidence indicated that melatonin may be present in *Datura* species [10] but comprehensive studies have not been conducted. Recent reviews have highlighted the importance of melatonin from the perspectives of the physiology of the plants and the activity of phytomelatonin in human physiology [11–13]. The known physiological functions of melatonin in animals include the timing of circadian rhythms and signaling of environmental changes, neurotransmission, and detoxification of reactive oxygen species (ROS) and other free radicals [14–16] but the biochemical role(s) of melatonin in plant physiology have yet to be clearly elucidated [13, 17]. Further, recent research has indicated that the melatonin metabolite AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine) and similar related metabolites may provide plant tissues with additional protection from oxidative damage through direct radical scavenging [13, 18–20]. Previous workers have hypothesized that melatonin may serve as an antioxidant [18, 21, 22] or a plant growth regulator [23, 24] in flower and seed development of plants.

The current study was undertaken to determine the presence of melatonin and serotonin in the flower and fruit tissues of *D. metel*, to understand the changes in melatonin content with the maturity of the fruit and flower tissues and to determine whether environmental factors could affect the melatonin and serotonin content of *D. metel* tissues.

Materials and methods

Plant material

The seeds of *D. metel* var. *fastuosa* were obtained from the Kampong Botanic Garden (Coral Gables, FL, USA) and aseptic plant cultures were established by germinating surface sterilized seeds in vitro. Seeds were surface sterilized by dipping in 70% ethanol for 30 s followed by sterilization in 20% Clorox with 0.1% Tween-20 for 30 min and three washes in sterile water. Four seeds were placed in a Magenta box containing 65 mL of MSO

medium consisting of MS salts [25], B5 vitamins [26], 2.5 g/L gelrite (Sigma Chemical Co., St Louis, MO, USA), 30 g/L sucrose, and pH adjusted to 5.75 before sterilization. The cultures were maintained in a culture room set for 16 h light at 28°C and 8 h dark at 24°C. The individual plants were clonally maintained through subculturing of shoot cuttings. Shoot tips with 2–3 nodes were excised from the in vitro-maintained plants and transferred to Magenta boxes with fresh MS0 medium. Three plants from these cultures were transplanted into pots filled with Promix soil-less mix (Promix BX, Premier Horticulture Ltd, QC, Canada). The pots were covered with plastic bags and placed in a growth chamber set for 16 h light at 24°C and 8 h dark at 20°C and 80% relative humidity. The plastic bag covers were removed after 1 wk and plants were kept in the growth chamber for another 3 wks before transfer to a regular greenhouse where they were kept throughout the experiment. Plants were watered with 20–8–20 fertilizer (Plant Products Co. Ltd, Brampton, ON, Canada) daily and supplemented with 16 h light (between 6:00 and 22:00 h) in the greenhouse. Plants started blooming about 2 months after transplantation.

Collection of flower buds and fruits for chemical analysis

All flower buds used for the chemical analyses were collected between 06:30 and 08:30 h. Flower buds collected were grouped into 10 classes based on their length (1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, and 8 cm). About half of the flowers were immediately frozen in liquid nitrogen while the other half was placed in plastic bags and kept at 4°C in the dark for 3 days (cold treatment) before freezing in liquid nitrogen. Fruits were collected at various stages of development following anthesis (day 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45 after anthesis and dried fruit). Fruits from 10 day and later stages were cut in half and one half was dissected to flesh and seeds. Whole ovary and pistils were used from day 10 and earlier stage fruits. Fruit tissues from all stages were frozen in liquid nitrogen. All frozen flower buds and fruit tissues were stored in a freezer at −80°C until analyzed.

Chemical extraction and analyses

Samples of *D. metel* flowers and fruits were analyzed for melatonin and serotonin content using a slightly modified version of the previously described method [10]. Briefly, frozen samples were accurately weighed, cut into smaller pieces and transferred to a 50 mL beaker for extraction. Methanol (80%) was added on a weight to volume ratio of 1 g to 10 mL and samples were sonicated in an ultrasonic bath (Bransonic 1510R-MT, 42KHZ) for 45 min. Sample extracts were then filtered (hydrophilic PTFE, 0.45 μm syringe filters, Millipore Inc). The extraction was repeated and the filtrates combined with the final volume recorded and 10 μL of the final extract was separated and quantified by HPLC-MS/MS using the MRM mode.

Chromatography/mass spectrometry

Auxin and indoleamines were identified by comparison to certified analytical standards of melatonin (N-acetyl-5-methoxytryptamine), serotonin (5-hydroxytryptamine), and indole-3-acetic acid (Sigma Chemical Co.). All separations were performed with an Alliance series High performance liquid chromatographic system (Waters Inc., Mississauga, ON, Canada) coupled with a Premier series LCT MS/MS (Waters Inc.) and controlled with a Masslynx V4.0 Data Analysis System (Waters Inc.) described previously [10]. Compounds were separated with an Xterra C18 HPLC column (2.1×1000 mm, 3.5 μm; Waters Inc.) 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). The indoleamines were eluted at 0.25 mL/min over a 20 min period and detected within the MS/MS in ESI positive mode using the MRM mode and optimized parameters for each metabolite (Fig. 1B; [10]). The method detection limit (LOD) for melatonin, serotonin and IAA was defined as the analyte concentration producing a signal of at least 2 times higher than noise while the limit of quantification was defined as the inflection point at the bottom of the linear range. For melatonin, an LOD was determined as 0.01 ng/mL with an LOQ of 2 ng/mL while serotonin had an LOD of 0.05 ng/mL and an LOQ of 10 ng/mL. The LOD for IAA was 0.01 ng/mL with an LOQ of 2 ng/mL. Recovery of melatonin, serotonin and IAA from the extraction and chromatography was 92%, 92% and 91%, respectively ($n = 5$).

Results

Fruit and flowers of *D. metel* are used for the preparation of many different types of traditional medicines and served as the source material for these experiments. Flowers appear as vase-shaped, purple blooms on a black stem in both greenhouse-grown and garden-grown *D. metel* (Fig. 1A). Melatonin, serotonin and the plant growth regulator indole-3-acetic acid were analyzed by HPLC with tandem MS/MS and MRM monitoring (Fig. 1B). Auxin recovery in flower tissues was not sufficient for analytical quantification whereas serotonin and auxin were not detected in the seed tissues (Fig. 1B,C).

Flowers were collected during the various stages of development ranging from a 1 cm long bud to an 8 cm long, fully developed flower (Fig. 2A). Both melatonin and serotonin were found throughout the development of the flowers and were found at the highest concentrations in young flower buds approximately 1 cm in length (Fig. 2B,C). Melatonin and serotonin concentrations declined as the flower buds matured and the levels of both of these neuroindoles dropped below detection limits when the flowers reached a length of 7–8 cm. At this size, the flower buds were fully developed, open and mature (Fig. 2A). Fruit and developing seeds of *D. metel* were collected beginning 1 day after anthesis and collection continued for 45 days (Fig. 3). At 10 days after anthesis, the fruit appeared soft, fleshy and purplish (Fig. 3A). Seeds developed in the fruit around day 20–25 (Fig. 3B). After about 45 days, the fruit became desiccated and split open

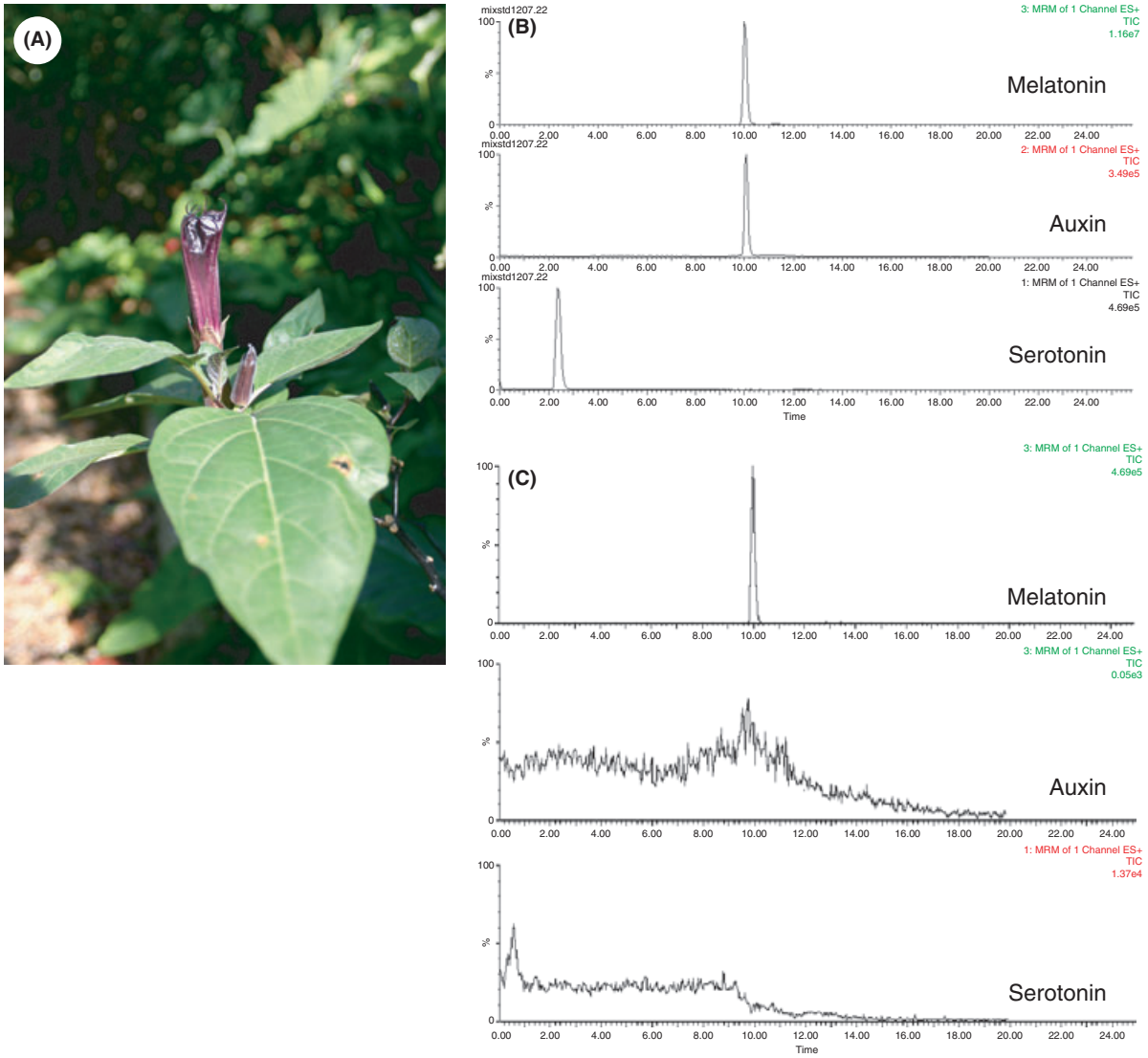

Fig. 1. Detection and quantification of melatonin, serotonin and auxin (indole-3-acetic acid) in flowers of *D. metel*. (A) The flower of *D. metel* is vase shaped and grows upward from the apex of the black stem of the plant. (B) Chromatogram showing the separation and identification of standards of melatonin, auxin and serotonin in *D. metel* by HPLC-MS/MS with MRM monitoring. (C) Chromatogram showing the detection of melatonin, auxin and serotonin in a seed of *D. metel*. Note: Serotonin and auxin were not detected in the seed tissues and auxin recovery in flower tissues was not sufficient for analytical quantification.

allowing the seeds to disperse (Fig. 3C). Melatonin was quantified at several stages in the developing fruits, seeds and embryos (Fig. 3D). Initial high levels of melatonin in the fruit declined after the first 10–15 days following anthesis (Fig. 3D). High levels of melatonin were found in the developing ovules after 10–15 days corresponding to the early stages of seed development (Fig. 3D). Very little melatonin was quantified in the fleshy part of the fruit and melatonin levels in the intact fruits were not significantly different from melatonin levels in isolated ovules of the same age between 30–45 days (Fig. 3D). Serotonin was not detected in the developing fruit.

The hypothesis that melatonin may function as a mechanism to protect reproductive plant tissues from environmental stresses was tested with a 4°C cold treatment for 3 days. The smallest flower buds of *D. metel* had the highest concentrations of both melatonin and serotonin (Fig. 4A,B). The exposure of the flower buds to a cold stress significantly increased the concentrations of both serotonin and melatonin in the youngest buds at the most sensitive stage of reproductive development (Fig. 4A,B).

Discussion

Despite a wide spread occurrence of melatonin in plants, the knowledge of its physiology and functions in plants remains limited. In general, melatonin in plants has been shown to mediate photoperiodic responses, root development mimicking auxin, and stress caused by environmental and chemical factors through scavenging the free radicals [20, 21, 24, 27–30]. Previous studies have shown that

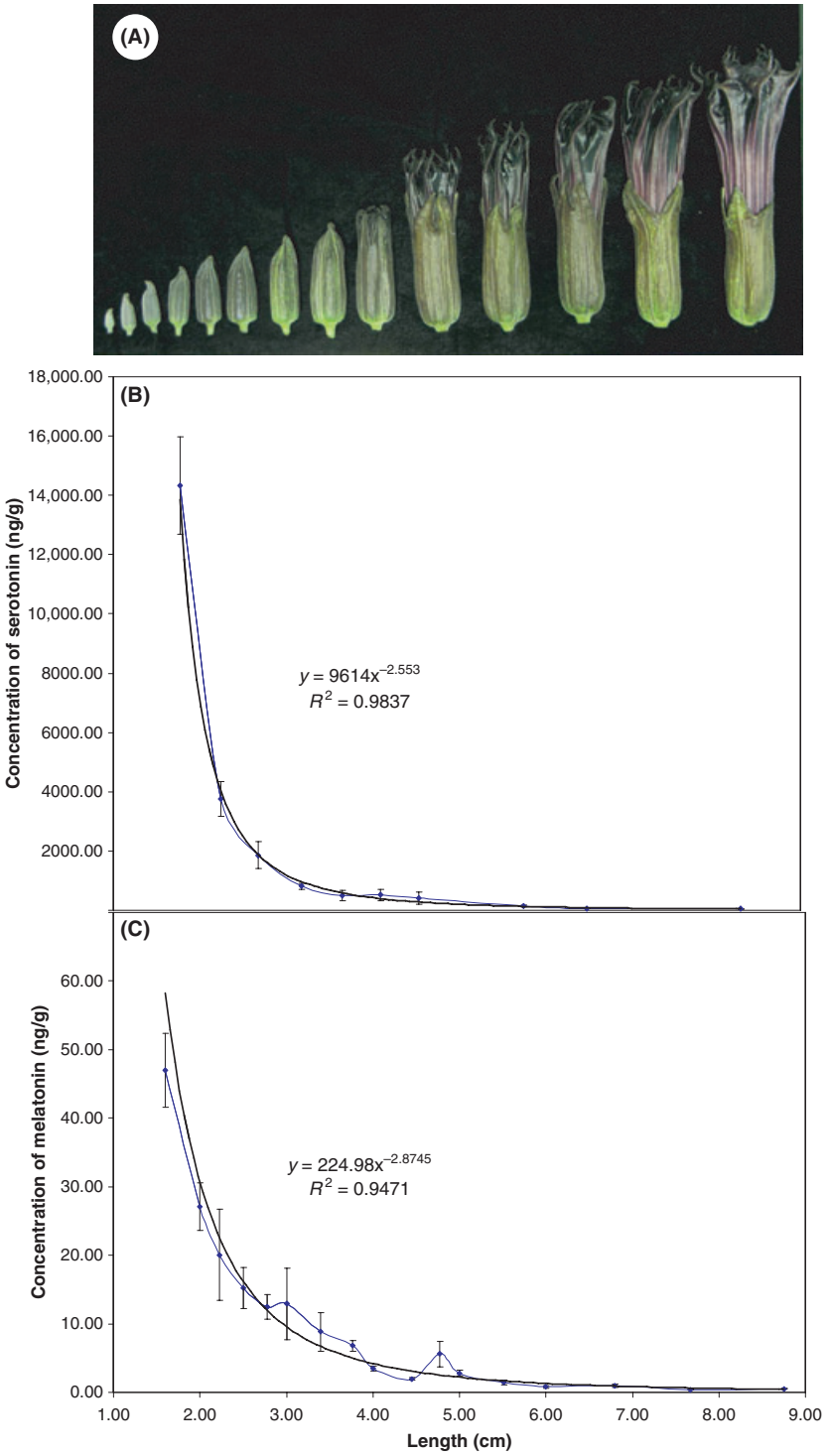

Fig. 2. Melatonin and serotonin concentrations during flower development of *D. metel*. (A) Flower buds were harvested throughout the development cycle and separated by size. The exact length of each individual flower bud was recorded and flowers were grouped by size for chemical analysis. (B) Quantification of serotonin in the flower buds. (C) Quantification of melatonin in the flower buds.

melatonin may act as a plant growth regulator to direct the differentiation of plant cells into tissues and organs [27–30] but specific mechanisms of action remain to be discovered.

We analyzed the melatonin and serotonin levels in developing flower buds and ovules of *D. metel* and recorded three interesting observations in this study: (i) the presence of higher melatonin and serotonin concentrations in young flower and tissues of *D. metel*; (ii) enhanced melatonin and serotonin levels in cold treated, young flower buds; and (iii) decline in melatonin and serotonin content in progressively differentiated tissues of the fruit. Interestingly, only the young buds and the early stages of ovule and fruit development showed a high melatonin and serotonin content and mature buds or fruit had little of both compounds. Previous studies with tomatoes have found higher concentrations of melatonin in the later stages of fruit development [24] but the mature fruits of *D. metel* are desiccated rather than ripe and fleshy. Together these

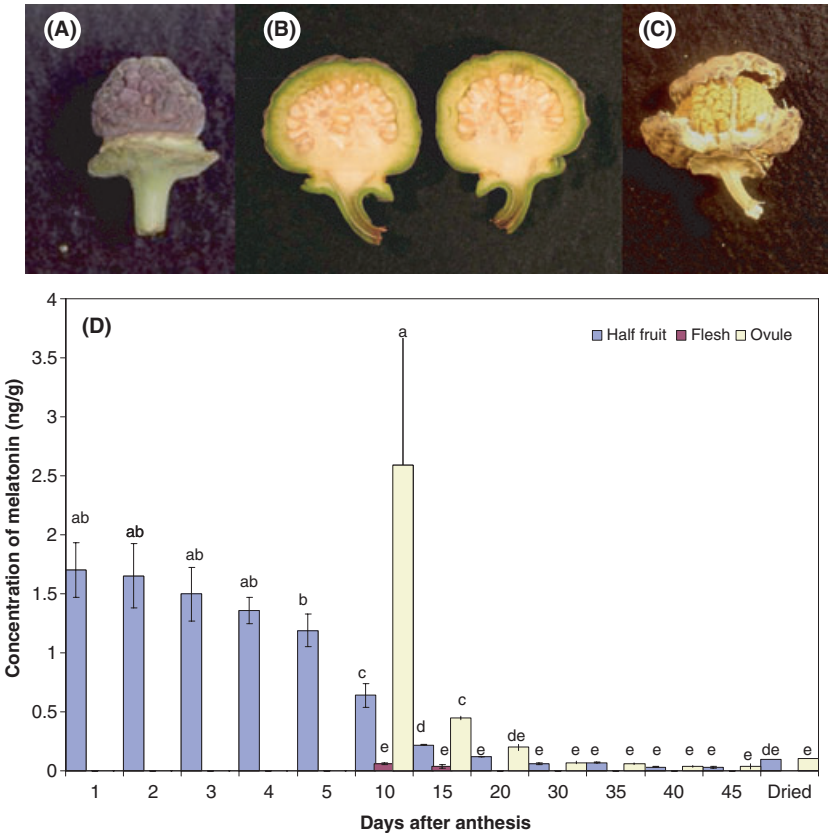

Fig. 3. Melatonin in fruit and seed development of *D. metel*. (A) Developing fruit at 10 days after anthesis. (B) Fruit and seed development at 25 days after anthesis. (C) Desiccated fruit and seed development at 45 days after anthesis. (D) Melatonin content of developing fruits and ovules throughout the 45 day period following anthesis. Note: Standard error bars represent the error of each data point and bars with the same letters are not significantly different by a Student-Newman-Kuels means separation test ($P < 0.05$).

results provide support for the hypothesis that the balance of melatonin and its metabolites may play a protective role in early stages of the development of reproductive cells and embryonic tissues [15, 19–21]. This hypothesis is further supported by the observation that an exposure of buds to a cold stress resulted in elevated levels of both melatonin and serotonin, but only in younger buds with insignificant changes in older buds which contained relatively mature reproductive tissues.

The process of formation of reproductive tissues is highly sensitive to potential environmental damage from abiotic stressors. Stress inducers such as cold temperatures, UV irradiation or exposures to metal ions that can induce generation of reactive oxygen species (ROS) leading to the phenomenon of oxidative stress [31, 32] which may impair the normal development of plant tissues and organs. A few days of cold treatment of rice anthers at the young microspore stage induced severe pollen sterility [33]. In a cytoplasmic male sterile line of rice the oxidative stress originating in mitochondria was found to be associated with premature abortion of microspores in the early stage of pollen development [34]. As well, the treatment of maize anthers with the ROS progenitors resulted in abnormal cell divisions and progeny cell degradation during the development of the microspores [35]. Similarly, the development of fertilized egg to an embryo has been found to be sensitive to a multitude of environmental factors. *Arabidopsis* plants under stress showed accumulation of ROS in very early stages of embryo development beginning with the egg or central cell and subsequently spreading into other regions [36]. Given the sensitivity of plant reproductive tissues during the development of anthers, microspores, ovules and seeds, the presence of melatonin at relatively high levels at these early stages may serve to protect the developing seed [21, 22] or to guide the normal reproductive process [23]. A recent study [37] with transgenic rice has suggested that serotonin may also play a protective role against ROS in delaying the process of senescence. It is likely that serotonin in *D. metel* acts as an antioxidant in protecting the young reproductive tissues either independently or synergistically with melatonin. Absence of serotonin in fruits is suggestive of a relatively lesser sensitivity of mature tissues to environmental stress.

The original work to investigate melatonin in medicinal plants hypothesized that the presence of high levels of melatonin in plants traditionally used in the treatment of neurological disorders may contribute to the medicinal or pharmacological efficacy in humans [8]. In the last decade, many plant species that are neurologically active in humans have been found to contain melatonin and serotonin as well as a variety of other neurotransmitters [13, 17]. Recent research has determined that melatonin consumed in medicinal plants, nuts and vegetables [38–40] is absorbed through the human digestion system and metabolites of plant-based melatonin have been found in urine samples [39]. Such data indicates that melatonin in our diets and plant-based medicines can affect our health and may have an impact in several chronic diseases.

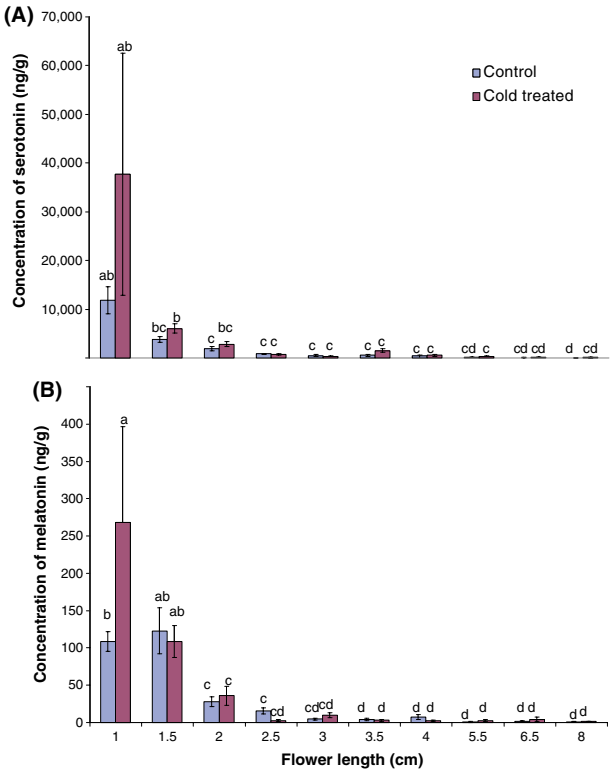

Fig. 4. Effect of a 4°C cold treatment for 3 days on the melatonin and serotonin content of developing flower buds of *D. metel*. (A) Serotonin. (B) Melatonin. Note: Standard error bars represent the error of each data point and bars with the same letters are not significantly different by a Student-Newman-Kuels means separation test ($P < 0.05$).

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