Plant Physiology and Biochemistry 94 (2015) 191–196

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Melatonin in *Arabidopsis thaliana* acts as plant growth regulator at low concentrations and preserves seed viability at high concentrations

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ARTICLE INFO

Article history:
Received 9 April 2015
Received in revised form
4 June 2015
Accepted 8 June 2015
Available online 12 June 2015

Keywords:
Arabidopsis thaliana
Germination
Melatonin
Senescence
UHPLC-MS/MS

ABSTRACT

Since the discovery of melatonin in plants, several roles have been described for different species, organs, and developmental stages. *Arabidopsis thaliana*, being a model plant species, is adequate to contribute to the elucidation of the role of melatonin in plants. In this work, melatonin was monitored daily by UHPLC-MS/MS in leaves, in order to study its diurnal accumulation as well as the effects of natural and artificial light treatments on its concentration. Furthermore, the effects of exogenous application of melatonin to assess its role in seed viability after heat stress and as a regulator of growth and development of vegetative tissues were evaluated. Our results indicate that melatonin contents in Arabidopsis were higher in plants growing under natural radiation when compared to those growing under artificial conditions, and its levels were not diurnally-regulated. Exogenous melatonin applications prolonged seed viability after heat stress conditions. In addition, melatonin applications retarded leaf senescence. Its effects as growth promoter were dose and tissue–dependent; stimulating root growth at low concentrations and decreasing leaf area at high doses.

1. Introduction

Melatonin (MT, N-acetyl-5-methoxytryptamine) was primarily known as a vertebrate pineal secretory molecule (Reiter, 1991) and was first isolated from bovine pineal gland by Lerner et al. (Lerner et al., 1958). In 1991, melatonin was detected in *Lingulodinium polyedrum* and, later, in other dinoflagellates and green algae (Hardeland and Fuhrberg, 1996; Balzer and Hardeland, 1991). In 1995, Dubbels et al. and Hattori and colleagues submitted reports showing the presence of melatonin in plants (Dubbels et al., 1995; Hattori et al., 1995).

To date, the presence of melatonin has been demonstrated in more than 20 dicotyledonous and monocotyledonous plant families (Posmyk and Janas, 2009). Nearly 60 commonly used Chinese medicinal herbs contain melatonin with a wide range of concentrations from nanograms to micrograms per gram of plant tissue (Chen et al., 2003). There is vast evidence that the presence of melatonin in plants helps to protect them from oxidative damage and from adverse environmental conditions (Hardeland and Fuhrberg, 1996; Kolar and Machackova, 2005; Wang et al., 2013; Lee et al., 2014). Alpine and Mediterranean plants exposed to UV radiation showed an increase in melatonin contents, suggesting that melatonin antagonizes the damage caused by light-induced stress, probably through its antioxidant properties (Pandi-Perumal et al., 2006).

Melatonin is rhythmically secreted by the pineal gland in vertebrates and is involved in regulation of circadian and, sometimes, seasonal rhythms (Reiter, 1993). Circadian rhythms with nocturnal maxima have also been described for insects and for *L. polyedrum* (Hardeland and Poeggeler, 2003; Poeggeler et al., 1991). Contrary to these findings, the studies of daily variations of melatonin contents in plants have displayed contrasting results. While experiments did not show any clear pattern of changes in a 12 h light/12 h dark regime in *Pharbitis nil* and *Solanum lycopersicum* (Van Tassel et al.,

Abbreviations: MT, melatonin; MS/MS, tandem mass spectrometry; UHPLC, ultra high performance liquid chromatography; Fv/Fm, maximum quantum efficiency of photosystem II photochemistry; PSII, photosystem II.

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http://dx.doi.org/10.1016/j.plaphy.2015.06.011
0981-9428/

2001), a daily rhythm with a sharp maximum at night and very low levels during the day was found in shoots of *Chenopodium rubrum* (Wolf et al., 2001). Melatonin levels also fluctuate during the day in grapes and *Eichornia crassipes*, showing a higher concentration level at pre-dawn in grapes and a peak during the late light phase in *Eichornia* (Boccalandro et al., 2011; Tan et al., 2007).

Different functional roles for melatonin have been described in both organs and developmental stages of several plant species. For example, it has been described that melatonin protects *Brassica oleracea rubrum* seeds and young seedlings against toxic concentrations of copper (Posmyk et al., 2008). Melatonin also plays a function in cold resistance, improving cucumber germination during chilling stress (Posmyk et al., 2009) and protecting cold-stressed wheat (Turk et al., 2014) and rice seedlings (Kang et al., 2010).

Previous studies have suggested that melatonin could act as a growth promoting compound, probably increasing auxin levels or showing an auxin-like activity (Chen et al., 2009). When etiolated hypocotyls from *Lupinus albus* were treated with a range of melatonin and indole-3-acetic acid (IAA) concentrations, both compounds elicited plant growth at micromolar concentrations, but repressed the growth at higher levels (Hernández-Ruiz et al., 2004). It was also confirmed that melatonin acts as a growth promoter in coleoptiles of wheat, barley, canary grass, and oat (Hernández-Ruiz et al., 2005). Melatonin also affected the regeneration of lateral and adventitious roots and the expansion of cotyledons in etiolated seedlings of *L. albus*, and in *Brassica juncea* young seedlings. Lower concentrations of melatonin have been found to stimulate the root growth and to raise the endogenous levels of IAA, but higher concentrations have inhibitory effects (Chen et al., 2009; Hernández-Ruiz et al., 2004).

Twenty years after the initial finding of melatonin in higher plants, accurate determinations still represent a major challenge because of plant (matrix)-specific problems (Kolar and Machackova, 2005; Pape and Luning, 2006). Chemical complexity of plant's extract can interfere with melatonin determinations, giving false positive results if methods from vertebrate's melatonin research are directly adopted, for example, because of coelution in LC or cross-reactivity with antibodies of immunological methods like RIA or ELISA (Pape and Luning, 2006). Setting-up more reliable analytical methods for melatonin extraction, detection, and quantification is a basic requirement to get more insight into the melatonin roles in plant physiology and ecology (Caniato et al., 2003).

In view of the molecular-genetic studies in plants, *Arabidopsis* is the best resource to dissect the functions of genes responsible for melatonin synthesis (Byeon et al., 2014; Lee et al., 2015). In this work, optimal conditions for extracting melatonin from *Arabidopsis thaliana* leaves prior to its determination by UHPLC-MS/MS were established. Furthermore, melatonin levels were monitored during the day as well at different growth conditions. In addition, the effects of exogenous application of melatonin to assess its role on seed viability and as a regulator of growth and development of plants were studied.

2. Materials and methods

2.1. Reagents and solutions

Melatonin was purchased from Sigma Chemical (St Louis, MO, USA). Acetonitrile, dichloromethane, chloroform, ethyl acetate, methanol, and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Formic acid, 98%, was obtained from Fisher Scientific (Loughborough, UK). HakaphosTM was obtained from Compo (Spain). Ultrapure water (18 MΩ cm) was obtained from EASY pure (RF Barnstead, IA, USA).

2.2. Plant materials and growth conditions

A. thaliana plants used for melatonin determination during the day were cultivated at a greenhouse under natural radiation with a 12/12 h dark/light cycle, with a maximum light intensity of 800 μmol/m^2/s, at a constant temperature of 23 ± 2 °C. Leaves were harvested every three hours and immediately frozen in liquid nitrogen and then lyophilized in darkness. Before the extraction, lyophilized material was homogenized with liquid nitrogen with a mortar and a pestle.

The concentrations of exogenous applied melatonin were selected based on preliminary studies performed in our lab as well as previous studies (Pelagio-Flores et al., 2012; Wang et al., 2012).

Plants used for the determination of leaf area and chlorophyll fluorescence were grown in a growth-chamber with a photoperiod of 12-h light/12-h darkness, light intensity of 180 μmol/m^2/s, and temperature of 23 °C. Plants were treated with 1 mL of the different melatonin concentration solutions (10, 50, and 500 μM) and with distilled water (control) three times a week.

Plants used for root elongation determinations were germinated and grown on Petri dishes containing MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524, purchased from Sigma Chemical (St. Louis, MO, USA)). Plants were placed in a growth-chamber with a photoperiod of 12-h light/12-h darkness, light intensity of 180 μmol/m^2/s, and temperature of 23 °C.

2.3. Germination tests

Seeds were sterilized by chlorine fumes for 8 h, and air-dried prior to melatonin treatment. Sterilized seeds were placed on 9-cm diameter Petri dishes with 0.7% agar (Britania®) with and without the addition of melatonin. The following melatonin concentrations were evaluated; 0 (control), 100, 300, 500, and 1000 μM. Seeds were stratified for three days. After stratification, seeds were placed in an oven at a constant temperature of 35 °C for three days and then germinated at 23 °C in a growth chamber with a 12-h light/12-h dark photoperiod. Ten replications of 25 seeds were arranged in a completely randomized design. Seeds were considered germinated when the seed coat was broken and the radicle was visible.

2.4. Viability tests

For tetrazolium tests, seed coats were removed and then the embryos were submerged in a 0.5% tetrazolium solution for 24 h at 35 °C protected from light (Kristof et al., 2008). Red-stained embryos were scored as viable embryos under an optical microscope (Nikon Eclipse E200).

2.5. Leaf area and root length determinations

For measurements of leaf area, images of entire plants were taken every seven days, from week 2–5, and the area was calculated using the program Measure (Datinf®). Root length was determined using images taken at the end of the experiment (14 days).

2.6. Chlorophyll fluorescence

The chlorophyll fluorescence was measured using a MINIPAM portable chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The maximum quantum efficiency of PSII

photochemistry (Fv/Fm), the maximal fluorescence in the light-adapted state (Fm), the nonphotochemical quenching of chlorophyll fluorescence (NPQ), the non-photochemical coefficient (qN), and the photochemical quenching coefficient (qP) were the variables analyzed. Twenty minutes of dark adaptation with a leaf clip was used to allow various photosynthetic and photoprotective mechanisms and state transitions to relax.

2.7. Melatonin extraction

Melatonin extraction from leaves was carried out under dim green light (2 μmol/m^2/s) to prevent analyte degradation. Homogenized tissues were accurately weighted (0.2 g), and transferred to a 15 mL glass tubes. After that, 1 mL of 50% (v/v) methanol–water was added to each sample and then, tubes were vortexed during 15 s. Ultrasonication was employed to assist and accelerate the extraction of melatonin from vegetal tissues in an ultrasonic bath (200 W, 15 °C; Cleanson 1106, Buenos Aires, Argentina) filled with cold water for 20 min. The supernatant was decanted and centrifuged for 5 min at 3500 rpm (1852.2 g). The glass tubes were stored at 4 °C for 20 min and then 1 mL of 50% (v/v) methanol – water was added to each sample. The tubes were vortexed until the pellet was resuspended and then, centrifuged again for 5 min at 3500 rpm. The resulting supernatant was filtered through a 0.22 μm syringe filter (Osmonics®) and stored in an amber vial suitable for UHPLC-MS/MS analysis.

2.8. Chromatographic conditions

An AcquityTM Ultra High Performance LC system (UHPLC) (Waters, Milford, USA) equipped with autosampler injection and pump systems (Waters, Milford, USA) was used. The autosampler vial tray was maintained at 15 °C. The needle was washed with proper mixtures of acetonitrile and water. The separation was performed by injecting 10 μL of sample onto ACQUITY UHPLC®, C8 columns (Waters, Milford, USA) with 2.1 mm internal diameter × 50 mm length, and 1.7 μm particle size. A mobile phase gradient program with solvent A (formic acid, 0.1% (v/v)) and B (acetonitrile, 0.1% (v/v) of formic acid) was applied at a flow rate of 0.25 mL min^{-1}. The gradient program started with 10% B, followed by a linear increase of B to 50% in 3.0 min. Then the mobile phase B was reduced to the initial conditions within 0.2 min, where it was held for 0.8 min. Thus, the total chromatographic run time was 4.0 min. The column was kept at a 35 °C temperature.

2.9. Mass spectrometry instrumentation and MS/MS conditions

Mass spectrometry analyses were performed in a Quattro PremierTM XE Micromass MS Technologies triple quadrupole mass spectrometer (MS/MS). ZSprayTM electrospray ionization (ESI) source (Waters, Milford, USA) was operated in a positive (ES+) mode at 350 °C with N2 as the nebulizer gas and the source temperature was kept at 150 °C. The capillary voltage was maintained at 3.3 kV and the extractor voltage was set at 3.0 V. Ultrapure nitrogen was used as desolvation gas with a flow of 800 L h^{-1}. Argon was used as collision gas at a flow of 0.19 mL min^{-1}.

After optimization, detection was performed in MRM mode of selected ions at the first (Q1) and third quadrupole (Q3). To choose the fragmentation patterns of m/z (Q1) → m/z (Q3) for the analyte in MRM mode, direct infusions (via syringe pump) into the MS of melatonin standard solution in methanol was performed and the product ion scan mass spectra was recorded. Thus, the transitions: 233 > 174 and 233 > 216 were assessed. Quantification of MT was done by measuring the area under the specific peak using MassLinx Mass Spectrometry Software (Waters, Milford, USA).

2.10. Statistical analysis

Analysis of variance (ANOVA) and Bonferroni's post tests were performed in order to assess minimum differences between means, with a significance level of $p \leq 0.05$. The analysis was carried out with GraphPad Prism 5 software.

3. Results and discussion

3.1. Melatonin levels are not diurnally regulated in Arabidopsis leaves

The UHPLC-MS/MS analyses were carried out following the methodology previously developed in our lab (Gomez et al., 2013). An optimization of the extraction strategy for melatonin in *Arabidopsis* leaves was carried out for this work. Once the conditions for extraction, separation, and quantification were optimized and established, the methodology was applied to the determination of melatonin by UHPLC-MS/MS in *A. thaliana* leaves that were harvested every three hours. Surprisingly, the melatonin content in leaves did not show a significant variation during the day, and its concentration was within 80–120 ng/g dry weight (Fig. 1). This pattern was also observed and reported for morning glory (*P. nil*) and tomato (*S. lycopersicum*) by Van Tassel et al. (Van Tassel et al., 2001). On the contrary, there are some scientific studies reporting daily variations in melatonin contents (Wolf et al., 2001; Boccalandro et al., 2011; Tan et al., 2007; Kolar et al., 1997), but showing dissimilar results. While Wolf et al. (Wolf et al., 2001) couldn't associate the increased levels of MT with a direct action of light in *Chenopodium*, Boccalandro et al. (Boccalandro et al., 2011) observed a light-induced depletion of MT levels in *Vitis*, and Tan et al. (Tan et al., 2007) reported high levels of MT near sunset in *Eichornia* due to a promotion of MT synthesis by light. It is important to mention that these results have been obtained not only in different species, but also under several growth conditions.

3.2. Melatonin contents depends on growth conditions

The levels of melatonin in 5- week old plants growing both under natural and artificial light conditions were quantified. It has

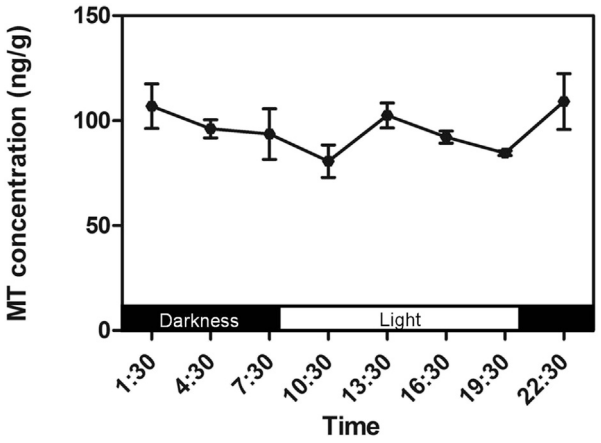

Fig. 1. Melatonin concentration (expressed as ng/g dry weight) at different times in *Arabidopsis thaliana* leaves that were harvested every three hours. Data are shown as the mean values of three replicates ± SE.

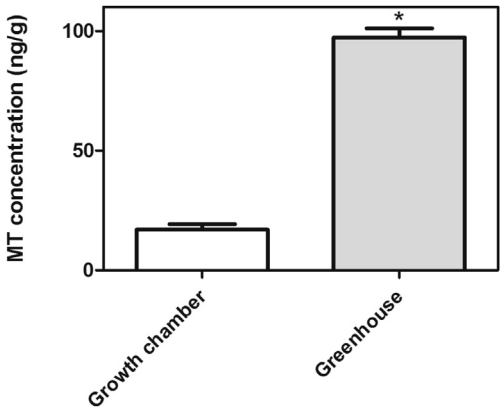

Fig. 2. Melatonin concentration (expressed as ng/g dry weight) in *Arabidopsis thaliana* plants that were cultivated under different growth conditions. Data are shown as the mean values of at least six replicates + SE. Asterisk (*) indicates significant differences ($P < 0.0001$).

been reported that melatonin levels vary not only between different species, but also within the various organs of the same plant (Posmyk and Janas, 2009; Van Tassel et al., 2001). Melatonin levels can also be affected by growth conditions (Tan et al., 2007; Arnao and Hernández-Ruiz, 2013). In this study, it was found a six-fold decrease of melatonin concentration in plants that were cultivated in a growth-chamber with a 12-h light/12-h darkness photoperiod, photosynthetically active radiation (PAR) of 180 μmol/m^2/s, and temperature of 23 °C when compared to plants cultivated in a greenhouse under natural radiation with a maximum PAR of 800 μmol/m^2/s (at similar photoperiod and temperature to those grown in chambers) (Fig. 2).

This significant difference in melatonin contents may be due to the light intensity as well as the difference in spectral composition between natural and artificial light treatments.

3.3. Melatonin maintains high levels of germination post heat stress

To investigate the effect of the application of melatonin on the germination of seeds under stress, the germination percentage of non treated and treated seeds with various melatonin concentrations was evaluated. Melatonin treatment increased seed germination after the application of a heat stress up to a 60% when compared to the control treatment (Fig. 3a). The highest level of germination post stress was obtained with the 1000 μM MT treatment, reaching germination percentages of 92.8 ± 2.1%. To check whether the reduction in germination after heat stress was due to the promotion of seed dormancy by warm temperatures or to a decrease in seed viability, we analyzed seed viability of non-germinating seeds by tetrazolium tests (Kristof et al., 2008). This test revealed that most of the control seeds that had suffered heat stress had lost their viability compared with melatonin treated seeds (Fig. 3b). The latter demonstrates that melatonin can be used to maintain high viability and germination of seeds that had suffered heat stress, and this is probably due to its powerful antioxidant capacity, which has been extensively verified in previous reports (Poeggeler et al., 2002).

3.4. Melatonin maintains high levels of quantum efficiency of PSII in post-flowering plants

The analysis of chlorophyll fluorescence has become one of the most powerful and widely used methods for obtaining information about the state of the photosystem (PSII). This technique reveals the

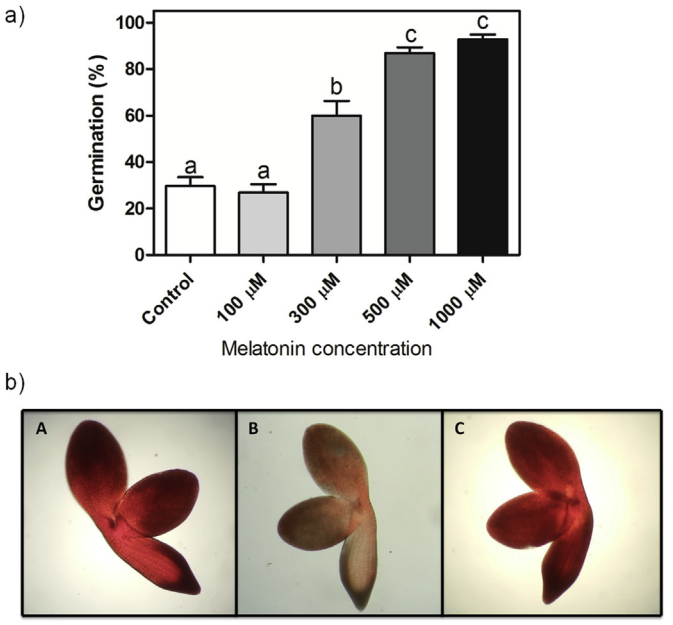

Fig. 3. a Effect of exogenous melatonin on *Arabidopsis thaliana* seeds germination after heat stress. Data are shown as the mean values of ten replicates + SE. Different letters indicate significant differences between treatments according to Bonferroni's multiple comparison tests ($P < 0.05$). b Embryos of *Arabidopsis thaliana* seeds after tetrazolium treatment observed under a microscope where A) non stressed seed (control); B) stressed seed without the addition of melatonin and C) stressed seed embedded with melatonin (1000 μM). Experimental conditions as described in Section 2.3.

extent to which PSII uses the energy absorbed by chlorophyll and how much it is damaged by an excess of light. The flow of electrons through PSII is indicative of the overall rate of photosynthesis under many conditions. The PSII is the part of the photosynthetic apparatus most vulnerable to light-induced damage, which is often first reflected in leaf status. In this study, maximum quantum efficiency of PSII, estimated as the ratio of variable fluorescence (Fv) over the maximum fluorescence value (Fm) for dark-adapted leaves, was strongly reduced in non melatonin treated leaves after flowering. The application of 500 μM melatonin solution, enabled plants to maintain higher Fv/Fm than the controls (Fig. 4). This data suggests that MT can delay natural senescence of *A. thaliana* leaves in post-flowering plants, probably due to its antioxidant effect. Similar results had been reported in barley (Arnao and Hernández-Ruiz, 2009) and apple (Wang et al., 2012).

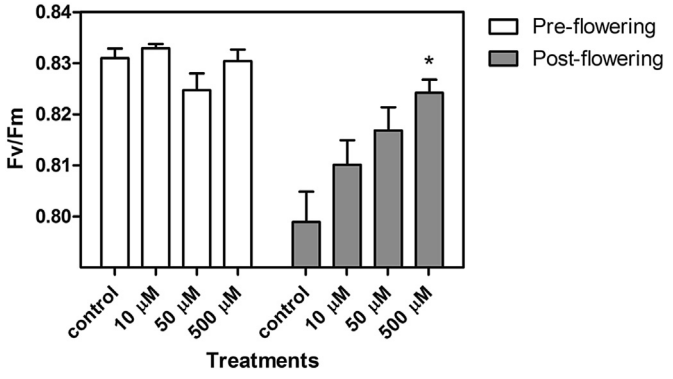

Fig. 4. Effect of melatonin on Fv/Fm in pre and post-flowering *Arabidopsis thaliana* leaves. Data are shown as the mean values of ten replicates + SE. Asterisk (*) indicates significant differences between treatments and control according to Bonferroni's multiple comparison test ($P < 0.05$).

3.5. Melatonin regulates root length

A possible role of melatonin as a root growth stimulator was also studied. A significant difference in the effect of different concentrations of melatonin on the root growth of 14-d seedlings was found. Treatment carried out with 0.1 μM melatonin increased root elongation, while melatonin concentrations of 500 μM and 1000 μM had a significant inhibitory effect (Fig. 5). Similar results have been reported in *B. juncea* (Chen et al., 2009), where it has also been observed a root length promotion at 0.1 μM melatonin concentration and a significant inhibitory effect in 2-d-old etiolated seedlings at concentrations of 100 μM. Pelagio-Flores also reported an increase of rooth growth in *A. thaliana*, although these effects were observed at higher concentrations than the ones reported here (150 μM) and in different growth conditions: photoperiod of 16-h light/8-h darkness, light intensity of 100 μmol/m^2/s, and temperature of 22 °C, without noticing any inhibitory effect (Pelagio-Flores et al., 2012). However, inhibitory effects at low concentrations of melatonin has also been reported in some monocots species (Hernández-Ruiz et al., 2005).

3.6. High concentrations of melatonin reduce leaf area per plant

Leaf area is an important physiological feature for certain edible plants such as lettuce, spinach, tea, tobacco, etc. Leaf area differences can be attributed to photosynthetic, water or nutrient demands. Our results indicate that the application of exogenous melatonin (500 μM) significantly reduced leaf area, when compared to controls (Fig. 6). These data may suggest a decrease in growth-related gene expression according to the data obtained recently by Weeda et al. (Weeda et al., 2014).

4. Conclusions

Several conclusions arise from our findings. We demonstrated that melatonin contents in *Arabidopsis* depend on growth conditions, but they are not diurnally regulated. This information could be used for food industry (i.e. to improve food quality and plant traits of agronomic interest such as an increment of aerial biomass of green edible plants, or to improve the conditions for seed storage). Exogenous melatonin is a potential plant bio-stimulator, prolonging seed viability after heat stress as well as delaying post-flowering leaf senescence. Thus, its application in agriculture could result in a good, feasible and cost-effective method, preventing stress and improving plant growth and development under different conditions.

This paper provides useful data for further related research on melatonin role in plants. However, taking into account that melatonin effects as growth promoter are dose and tissue–dependent; more studies including different species at several developmental stages are needed to establish its optimal concentrations to enhance plant production.

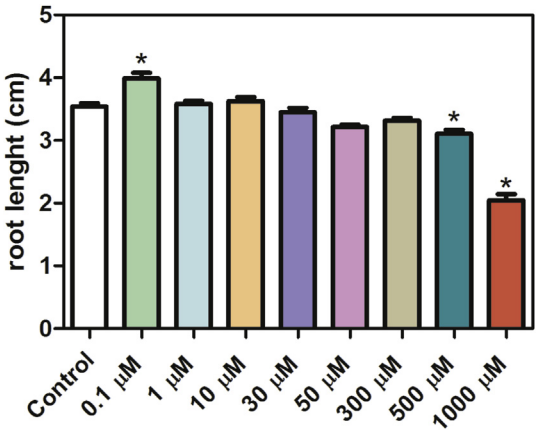

Fig. 5. Effect of melatonin on root length of 14 days old *Arabidopsis thaliana* seedlings. Data are shown as the mean values of at least thirty replicates + SE. Asterisks (*) indicate significant differences between treatments and control according to Bonferroni's multiple comparison test ($P < 0.05$).

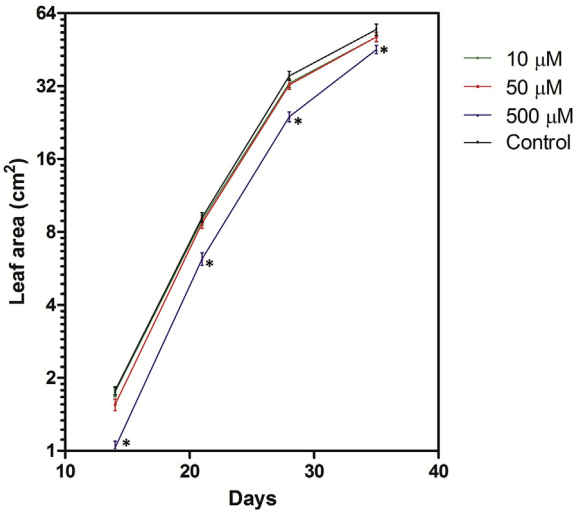

Fig. 6. Effect of melatonin on leaf area of *Arabidopsis thaliana* enriched with 1 mL of melatonin solutions three times a week. Data are shown as the mean values of at least ten replicates ± SE. Asterisks (*) indicate significant differences between treatments and control according to Bonferroni's multiple comparison test ($P < 0.05$).

Contributions

IGH, FJVG, SC, MVA and MFS designed the research. IGH and FJVG performed most of the experiments. IGH, SC and MFS conducted the data analysis. IGH, FJVG and MFS wrote the manuscript. All the authors contributed to improving the paper and approved the final manuscript.

Acknowledgments

The authors would like to gratefully acknowledge the financial support received from CONICET (PIP 309), University of Cuyo (6/A445), and National University of San Luis (INQUISAL) (PROICO 21512).

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