

FLUCTUATIONS IN MELATONIN CONTENT AND ITS EFFECTS ON THE AGEING PROCESS OF LETTUCE SEEDS DURING STORAGE

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ABSTRACT

This research was carried out to determine the changes in melatonin and tryptophan contents and the possible effects of melatonin on the ageing process of lettuce seeds stored for two years. For this purpose, seeds were treated with melatonin (0 or 250 µM) for one day after which they were stored for up to two years at two temperature (4°C or 25°C) regimes. The results showed that seed melatonin content varied significantly during storage reaching its peaks in October-December and lowest levels in August while tryptophan levels displayed an opposite trend with a strong peak in August and low levels in October. Similar seasonal changes were observed in seeds stored under both temperature conditions suggesting that endogenous melatonin could play a crucial role in seasonal rhythms independent of environmental conditions. Lettuce seed quality deteriorated fast when stored at 25°C but pre-treatment with melatonin reduced seed deterioration significantly by increasing the activities of antioxidant enzymes and restoring membrane properties indicating that melatonin could be used to slow the ageing process in seeds.

Key words: germination, melatonin, seed ageing, seed storage, tryptophan

INTRODUCTION

Seed quality is a broad and complex term used to express various seed attributes such as size, germination, vigor along with genetic and physical [Ellis 1992, Ajayi et al. 2001]. Seed quality starts to decline while the seeds are still on the mother plant and most of the quality loss occurs during storage. Seed deterioration is a natural process which is expressed as the loss of seed properties such as quality, viability and vigor [Jyoti and Malik 2013]. This process is cumulative, degenerative and irreversible and the best that can be done is to control its rate [Kapoor et al. 2011].

The identification of melatonin (N-acetyl-5-methoxytryptamine) from bovine pineal gland in 1958

was a major breakthrough [Lerner et al. 1958] and since then its existence in varying amounts has been proved in almost all life forms including eukaryotic unicells, prokaryotes, fungi, algae, bacteria, animals and finally in plants [Posmyk and Janas 2009, Tan et al. 2012]. Extensive research have documented that melatonin modulates seasonal reproductive physiology, sexual behavior, sleep in animals and circadian rhythm in all living forms including plants [Kolár and Macháčeková 2005, Reiter et al. 2015]. Melatonin is also known as a multi-signaling molecule in plants and the dual roles of melatonin in biotic and abiotic stress situations involve both its direct action as an antioxi-

dant and its role as a regulator of gene expression [Arnao and Hernández-Ruiz 2019a]. Examples of numerous factors and gene elements up- or downregulated by melatonin under various stress conditions are reported [Arnao and Hernández-Ruiz 2019b]. Extensive research indicate that melatonin is involved in enhancing the tolerance of plants to various environmental stresses such as high salinity [Li et al. 2012], heavy metals [Posmyk et al. 2008], drought [Cui et al. 2017], low temperature [Korkmaz et al. 2017a] and high temperature [Shi et al. 2015].

The notion of melatonin as a strong antioxidant in plants came mostly from the research carried out on whole plant studies but little is known about the involvement of melatonin on seed viability or ageing during storage and seasonal fluctuations of seed melatonin content during long term storage. Among the few published reports, Zhao et al. [2011] reported that treating *Rhodiola crenulata* calli with melatonin before placing them in liquid nitrogen significantly increased their survival rate. Melatonin treatment also significantly aided artificially-aged corn seeds to recover with improved germination by enhancing antioxidant enzyme activity and lowering lipid peroxidation [Deng et al. 2017]. Priming of waxy maize seeds with melatonin significantly improved germination under chilling stress via boosting antioxidant system and starch metabolism, which protected from oxidative damage [Cao et al. 2019]. Additionally, Kołodziejczyk et al. [2015] monitored melatonin content and the appearance of its potential metabolites in cucumber and maize seeds stored for one year and reported that melatonin levels fluctuated extensively during one year long storage, peaking in the winter months. However, there is no information available on changes in melatonin content of seeds stored for longer than one year. Therefore, our first objective was to identify the variations in melatonin and its precursor molecule-tryptophan contents in lettuce seeds stored for two years at two temperature regimes. Second objective was to determine the possible mitigating effects of melatonin on deterioration of lettuce seeds in storage.

MATERIALS AND METHODS

Plant material and melatonin treatments. Seeds of open pollinated ‘Yedikule’ romaine lettuce (*Lactu-*

ca sativa L.) cultivar produced in the summer of 2015 were purchased from Argeto Seed Company, Gaziantep, Turkey. Seed moisture content determination was carried out according to ISTA [2005] rules and found to be 5.93%. For melatonin application, single layers of lettuce seeds (in 100 g batches) were placed in 20 × 30 × 5 cm (l × w × h) trays between double layers of filter paper moistened with 250 µM melatonin solution and kept at 25°C in darkness for 24 hours. The concentration of melatonin treatment (250 µM) was selected based on the results of Kołodziejczyk et al. [2015] and our preliminary experiments (unpublished results). Following melatonin application, the seeds were washed for 1 min under running tap water and dried back to their original moisture content on paper towels in an incubator set at 25°C. After drying, they were sealed in individually laminated plastic bags covered with aluminum foil and stored in a refrigerator (4°C) or incubator (25°C) for two years from the beginning of December 2015 to December of 2017. Untreated seeds (0 µM) having the same moisture content were also packaged and stored under the same conditions as mentioned above.

Measurements and analyses. During 24-month long storage, samples were taken every two months (every 6 months for enzyme analysis) and seasonal changes in melatonin and tryptophan contents and the effects of melatonin application on seed ageing during storage were established. At the end of each storage time, seed moisture content was determined and following analyses were conducted.

Germination test was carried out at 20°C in darkness for 14 days. Fifty seeds in four replicates were germinated in 9 cm petri dishes lined with double layers of filter paper and from the total number of seeds germinated final germination percentage (FGP) was calculated.

For melatonin and tryptophan analyses, extraction procedures were always initiated at the same time of the day (around 3:00 pm) since melatonin levels may exhibit a daily circadian rhythm in plant tissues. Extraction and analysis of melatonin and tryptophan were carried out under dim light by slightly modified method reported in Korkmaz et al. [2014] and Korkmaz et al. [2017b]. Therefore, 0.5 g seeds from each treatment in 4 replicates were placed in test tubes containing ethyl acetate (3 mL) following homogeniza-

tion with liquid nitrogen. After leaving them overnight (17 h) at 4°C in darkness with shaking, the tubes with plant tissues were centrifuged at 6,000 g and 4°C for 20 min. The supernatant was transferred to another tube and the remaining plant residue was washed with 0.5 mL ethyl acetate. The extract and wash from each sample were evaporated to dryness using a vacuum concentrator (Labconco, Kansas City, MO). The residue was re-dissolved in methanol (1.0 mL), transferred to a C18 solid phase extraction (SPE) cartridges (Waters, Milford, MA, USA) and analyzed using HPLC with fluorescence detection. Shimadzu, Prominace UFLC equipment utilizing an Inertsil ODS-2 column (GL Sciences, 5 µm, 150 × 4.6 mm) was used to simultaneously measure melatonin and tryptophan levels. An excitation wavelength of 280 nm and an emission wavelength of 350 nm were used. The mobile phase was constituted of methanol: 0.1 mM Na₂HPO₄/H₃PO₄ buffer (40:60, v/v) pH 4.5 at a flow rate of 0.6 mL min⁻¹. Twenty µL extracted samples were injected for the analyses and column oven temperature was set at 35°C. The data were analyzed using the LC Solutions Software and the concentrations of melatonin and tryptophan in each sample were calculated from the integrated chromatographic peak area on the basis of standard calibration curve, and expressed as ng g⁻¹ fresh weight (FW).

Half g of seeds in four replicates were homogenized in 3 mL of 10% trichloroacetic acid and centrifuged at 10,000 g for 15 min and seed MDA and H₂O₂ contents were determined by the methods reported in Zhang et al. [2005] and Özden et al. [2009], respectively. Extractions for enzyme analysis were performed as described by Seçkin et al. [2010]. Total soluble protein contents of the enzyme extracts were calculated using bovine serum albumin (BSA) as a standard and the protein concentration was determined from a BSA standard curve. Peroxidase (POX, EC 1.11.1.7) and ascorbate peroxidase (APOX, EC 1.11.1.11) activities were determined by the methods of Dolatabadian et al. [2008] and Nakano and Asada [1981], respectively.

RESULTS AND DISCUSSION

Seed melatonin and tryptophan levels exhibited significant seasonal fluctuations. To our knowledge, there is no information available related to long-term

variations in tryptophan and melatonin contents in seeds. Herein, changes in the levels of these molecules in lettuce seeds stored for 2 years were established. Melatonin content of untreated seeds and seeds treated with exogenous melatonin showed similar significant seasonal changes regardless of storage temperature; they reached their peaks and lowest levels around October–December period and August, respectively (Fig. 1A). Additionally, in general, seeds stored at low temperature (4°C) exhibited higher melatonin levels than those stored at room temperature (25°C) at all storage times except in the month of August where their melatonin contents were at their lowest levels. Endogenous melatonin content of untreated seeds were quite high (around 900 ng g⁻¹ FW) at the beginning of the experiment but continuously declined, reaching their lowest levels (25–30 ng g⁻¹ FW) of the year regardless of storage temperature. However, seed melatonin content increased significantly to their highest levels (450–550 ng g⁻¹ FW) of the year in October 2016 and remained high during December after which they declined again to their lowest values (5–10 ng g⁻¹ FW) in August of 2017. A similar trend in melatonin levels was also observed in the winter months of 2017 which coincided with the end of the experiment. Seeds that were treated with exogenous melatonin exhibited almost the same seasonal variation pattern during the course of storage, reaching peak levels in October–December period and lowest levels in August. Moreover, although melatonin-treated seeds exhibited the same seasonal change in melatonin content as untreated seeds, they always had considerably higher melatonin levels at all storage periods with the differences between them getting progressively smaller as the time in storage progressed.

Extraction and quantification of melatonin in plant tissues is more difficult than in animal tissues [Arnao and Hernández-Ruiz 2009, Arnao and Hernández-Ruiz 2018, Reiter et al. 2007]. Endogenous melatonin levels in plants were found to vary significantly among plant species and even within different varieties of the same species. [Arnao 2014, Hattori et al. 1995, Posmyk and Janas 2009]. Additionally, time of sampling during the day, the growth stage of plants and the cultivation conditions along with environmental factors also exert a strong influence in melatonin content of plant tissues [Arnao and Hernández-Ruiz 2015, Reiter et al. 2007].

The current findings revealed that sampling time within a year is also highly important due to differences of several orders of magnitude reported depending on the time of the year. Thus, it is essential to compare the melatonin content of various plant materials collected at the same time of the year and inform the readers the time at which the measurements were made [Li et al. 2020]. Melatonin levels in the blood of mammals display a rhythm with higher levels in darkness (night time) and lower values under light (day time). Melatonin is also known as chemical expression or hormone of darkness because exposure to light at night time blocks melatonin production [Reiter 1991, Paul et al. 2015]. In plants, the possible role of melatonin as a daily circadian rhythm regulator has been examined, with the investigations producing variable results. For example, Wolf et al. [2001] reported maximum melatonin levels in the shoots of *Chenopodium rubrum* at night while several studies demonstrated dual melatonin peaks in sweet cherry fruits [Zhao et al. 2012], grape berries [Boccalandro et al. 2011], lupin seedlings [Arnao and Hernández-Ruiz 2013] and eggplant seedlings [Korkmaz et al. 2017b]. The changes in the melatonin content of plants regarding its possible role as a regulator of light/dark-mediated rhythms were associated with external factors-photoperiod or temperature [Li et al. 2020]. However, our results clearly indicate that seed melatonin levels showed significant seasonal changes over the course of experiment and that the changes are regulated by factors independent of environmental conditions since seeds were stored in darkness and constant temperature regimes. Although similar seasonal changes have been reported in corn and cucumber [Kołodziejczyk et al. 2015], to our knowledge, this is the first report that indicates an existing circannual rhythm in melatonin levels in seeds; this rhythm possibly persists over the life of the seed albeit in a diminishing fashion. These results are in close agreement with those of Kołodziejczyk et al. [2015] who suggested that there is a possible independent 'chemical memory' in plants which is not influenced by external factors and that significant seasonal variations in seed melatonin content may be indicative of an internal clock operating as a biochemical calendar (possibly acquired through an evolutionary process) precisely regulated by melatonin. Lately, a new but unconfirmed model has been proposed where core

biological clock, melatonin and the newly-discovered phytomelatonin receptor PMRT1 are integrated in the coordination and response to the redox network [Arnao and Hernández-Ruiz 2020]. If this model is confirmed, involvement of melatonin in transferring biological clock oscillations to the redox network and acting as a plant master regulator provides the adequate response to reach redox homeostasis in stress situations, very similar to animal melatonin.

Similar seasonal changes in melatonin levels, with higher and lower levels in winter summer respectively, have been reported in humans [Morera and Abreu 2006]. Longer nights during the winter months promote more melatonin production in humans and whether this has implications for human physiology remains unknown. Human newborns do not start to produce melatonin for three to five months after birth during which time they get their melatonin through the mother's milk [Engler et al. 2012]. Melatonin production reaches its highest nocturnal levels between the ages of four to seven years, declining gradually afterwards over the life-span of humans [Karasek 2004]. Obviously, the seeds do not have a connection with the mother plant after shedding and they have to utilize the resources they have at the time of shedding throughout their lives. Judging from the results obtained here, it is clear that they have the maximum capacity to produce melatonin during their first winter with diminishing levels in the following year. Since melatonin is a powerful free radical scavenger [Reiter et al. 2017], higher melatonin levels are essential for enhanced antioxidant activity in newly-separated seeds. By this way, the seeds could have significant protection against ageing and/or harsh environmental conditions, allowing them to reproduce the next generation.

Contrary to fluctuations in melatonin content, seed tryptophan content exhibited an opposite trend as shown in Figure 1B where it reached its peaks and lowest levels during the months of August and October, respectively. This change is expected since tryptophan is the precursor of melatonin and at times where melatonin content reached its peaks (October), tryptophan reserves were likely to be depleted. After December, the tryptophan content started to increase steadily until August when seed melatonin content was its lowest level of the year. Similar diurnal and

seasonal changes were reported in eggplant leaves and roots and an inverse relationship was observed between tryptophan and melatonin levels [Korkmaz et al. 2017b]. Additionally, seeds treated with exogenous melatonin exhibited significantly lower tryptophan content than the untreated seeds regardless of storage temperature during a major portion of the experiment. This may be because seeds with higher melatonin content due to exogenous application did not need to produce melatonin which, in turn, might have reduced the need for the precursor molecule, tryptophan.

Effect of melatonin on seed ageing and germination performance. The moisture content of the seeds rose considerably during 2 year storage even though they were stored in sealed plastic packages after drying back to their original moisture content (Fig. 2). Because the seeds were not stored hermetically, it is normal that their moisture content increased over time in storage. Seed moisture changed slightly during the first 6 months, rose sharply by the end of the first year and continued to increase steadily until the end of the experiment, finally reaching 7.05%. Neither melatonin application nor storage temperature had any significant effect on seed moisture content since there was no difference among the treatments.

Germination of seeds from all treatments did not change during the first year of storage and remained above 80% (Fig. 3). Storage temperature significantly affected seed germination and seeds stored at 4°C maintained higher FGP until the end of the experiment. However, starting from the end of the first year, FGP of seeds stored at 25°C began to decline and seeds not treated with melatonin were almost dead by the end of the storage as indicated by very low germination percentage (3%). On the other hand, seeds treated with melatonin exhibited significantly higher FGP during the second year of the storage indicating that melatonin treatment slowed the ageing process.

Genetics is one of the main factors determining the longevity of seeds and some species are inherently short-lived while others have a long life span owing to differences in their genetic makeup [Jyoti and Malik 2013]. It is also known that seed moisture and storage temperature must be low in order to extend the longevity. Lettuce seeds are known to deteriorate fast especially when they are stored at unfavorable conditions such as high temperature and humidity [Demir and

Ozcoban 2007, Nagel and Börner 2010]. For example, it was reported that lettuce seeds with 7% moisture content were no longer viable after 20 months of storage at ambient conditions with yearly average temperature of 18°C while those with the same moisture content stored at an average yearly temperature of 14°C exhibited 50% viability after 24 months [Demir et al. 2016]. The persistent rise in seed moisture content finally exceeding 7% along with higher storage temperature may explain the reason why the seeds not treated with melatonin lost their viability completely after 24 month of storage. In contrast, seeds treated with melatonin exhibited 30% germination after 24 months of storage at 25°C indicating that melatonin slowed seed deterioration.

Peroxidation of lipids in cellular membranes due to generation of free radicals such as O_2^- , $\cdot OH$ and H_2O_2 results in impaired membrane functions in ageing seeds [Bailly et al. 2008, Schwember and Bradford 2011]. The decomposition of membrane fatty acids by peroxidation generates a toxic byproduct- MDA, thus, estimating the free radical levels and MDA contents are valuable tools. Seed MDA (Fig. 4A) and H_2O_2 (Fig. 4B) contents exhibited similar trends and increased significantly over the course of the experiment. However, it is interesting that strong peaks in the levels of these two variables were observed in August of both years, coinciding with the lowest levels of melatonin. Additionally, seeds treated with exogenous melatonin always had lower H_2O_2 and MDA concentrations than the untreated seeds and storing the seeds at 25°C resulted in significant rises in the amount of H_2O_2 and MDA present compared to storage at 4°C. The fact that higher seed MDA and H_2O_2 contents and lower melatonin levels observed in August were followed by the lower MDA and H_2O_2 and the higher melatonin levels in October support the findings of Zhao et al. [2012] who reported that oxidative stress caused by high temperature resulted in the accumulation of MDA in cherry fruits, triggering melatonin synthesis.

Damage caused by peroxidation of lipids in membranes is reduced by such protective mechanisms as boosting the activities of free radical and peroxide-scavenging enzymes [Mansouri-Far et al. 2015]. Melatonin is known to play a vital role in the control – both direct (as scavenger) and indirect (as gene

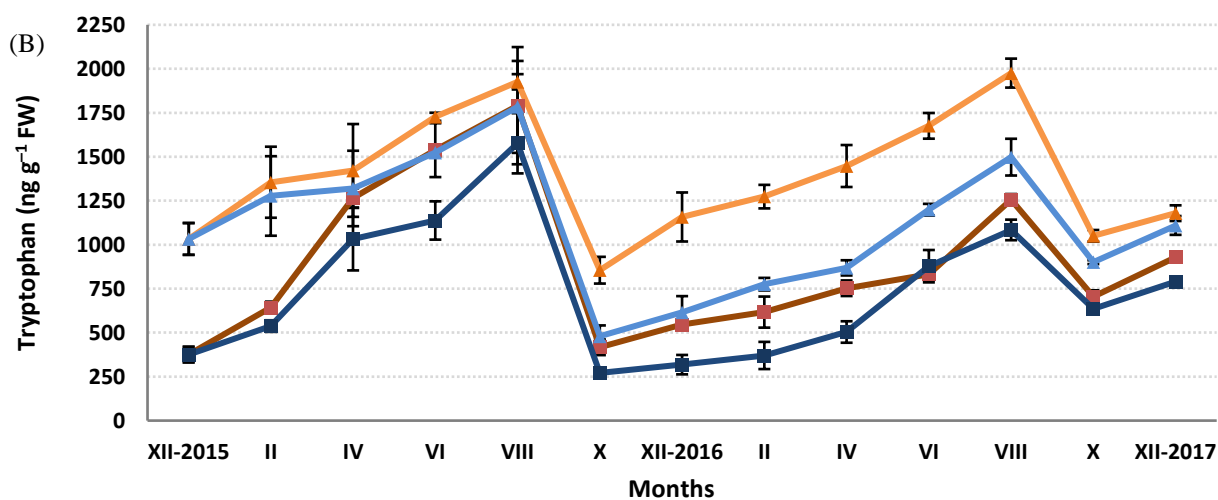
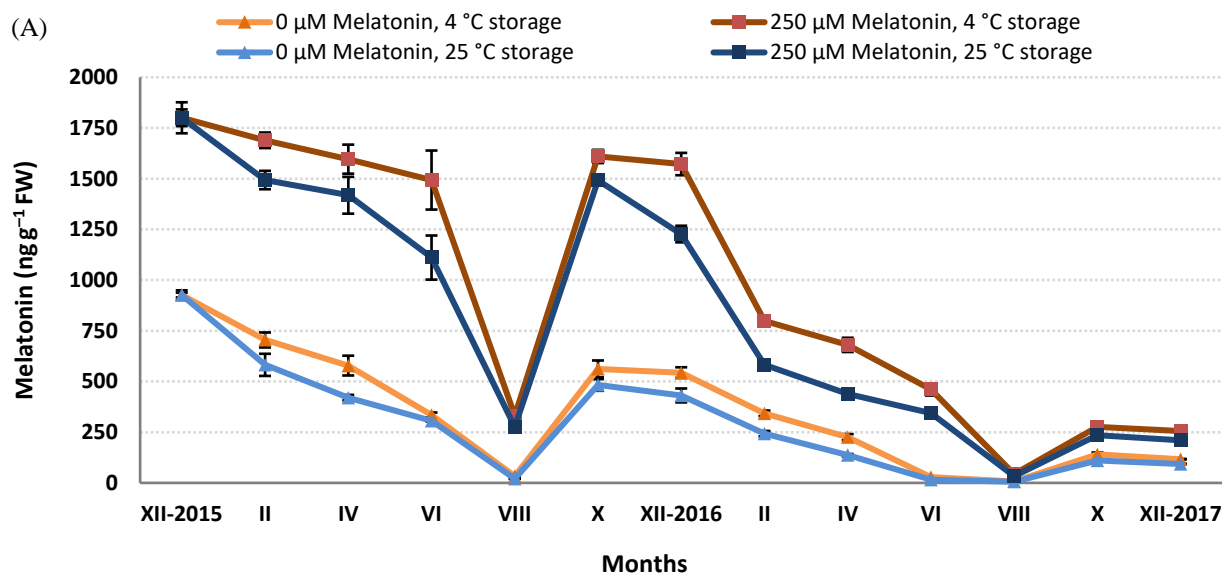


Fig. 1. Changes in melatonin (A) and tryptophan (B) levels of lettuce seeds during 24-month storage. Seeds were treated with melatonin (0 and 250 μM), stored at 4°C and 25°C for two years and sampled for melatonin and tryptophan analysis at 2-month intervals starting from December of 2015

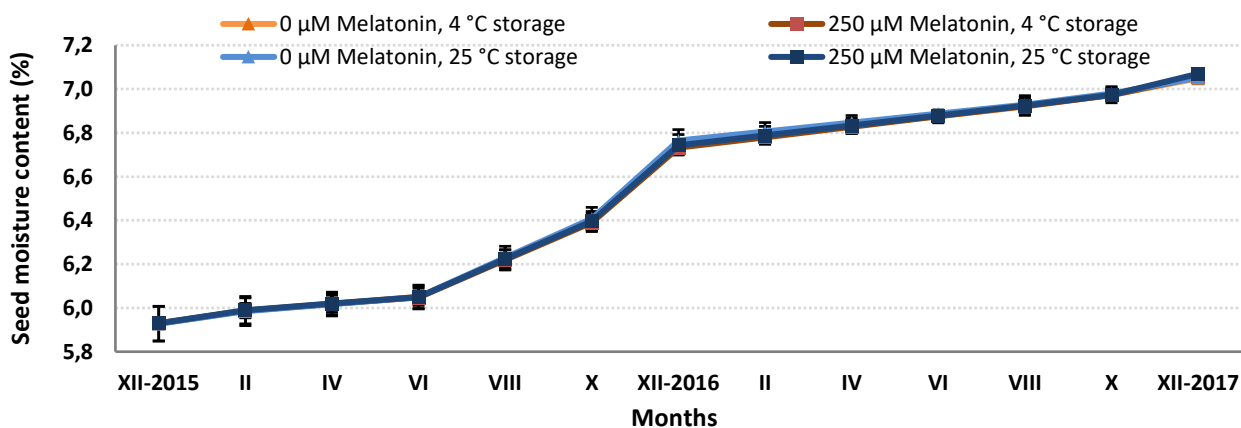


Fig. 2. Changes in moisture content of lettuce seeds during 24-month storage. Seeds were treated with melatonin (0 and 250 μM), stored at 4°C and 25°C for two years and sampled for moisture content analysis at 2-month intervals starting from December of 2015

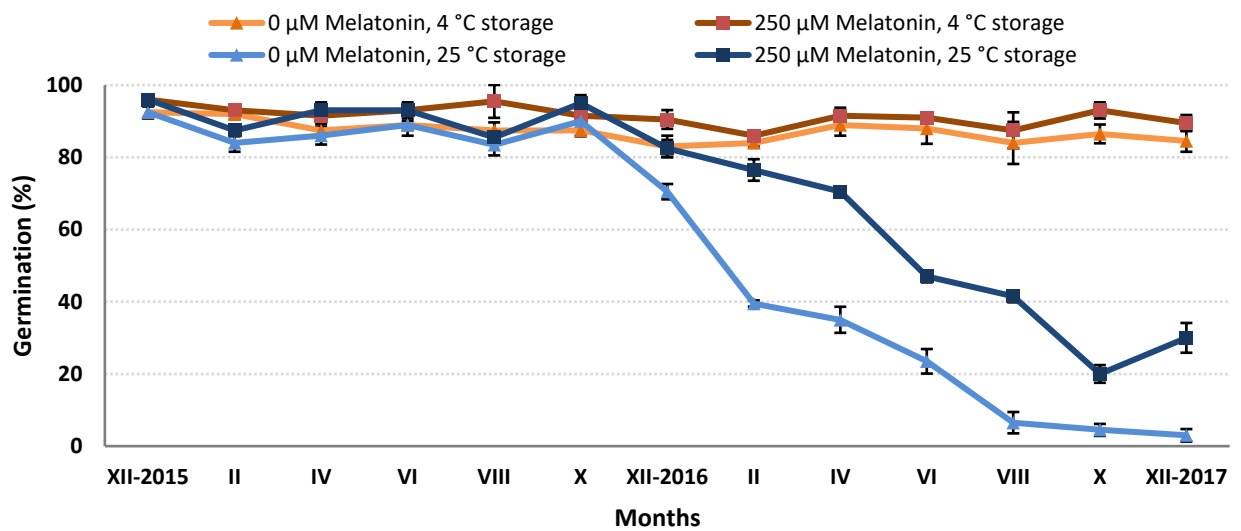


Fig. 3. Changes in final germination percentage (FGP) of lettuce seeds during 24-month storage. Seeds were treated with melatonin (0 and 250 μM), stored at 4°C and 25°C for two years and subjected to germination test at 2-month intervals starting from December of 2015

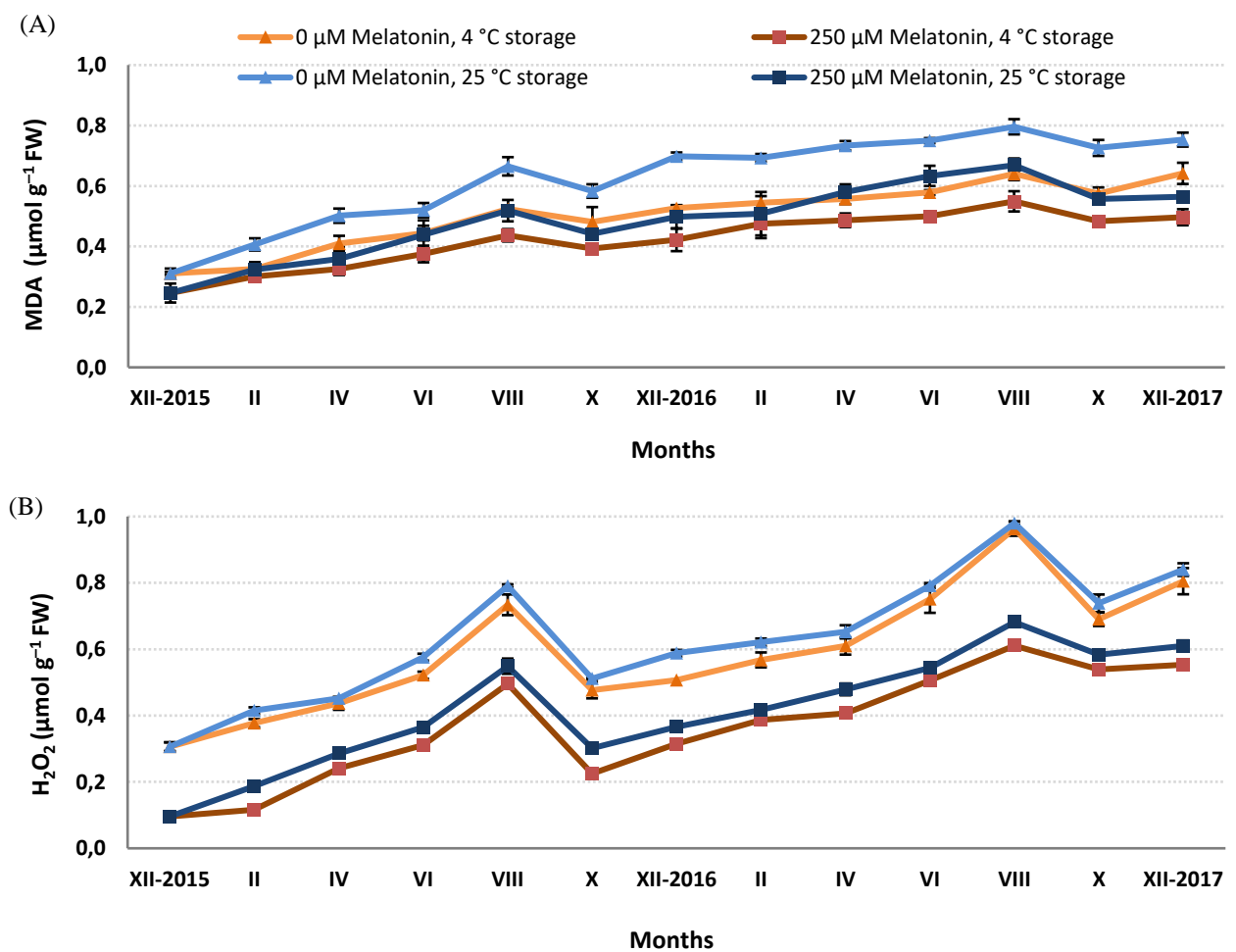
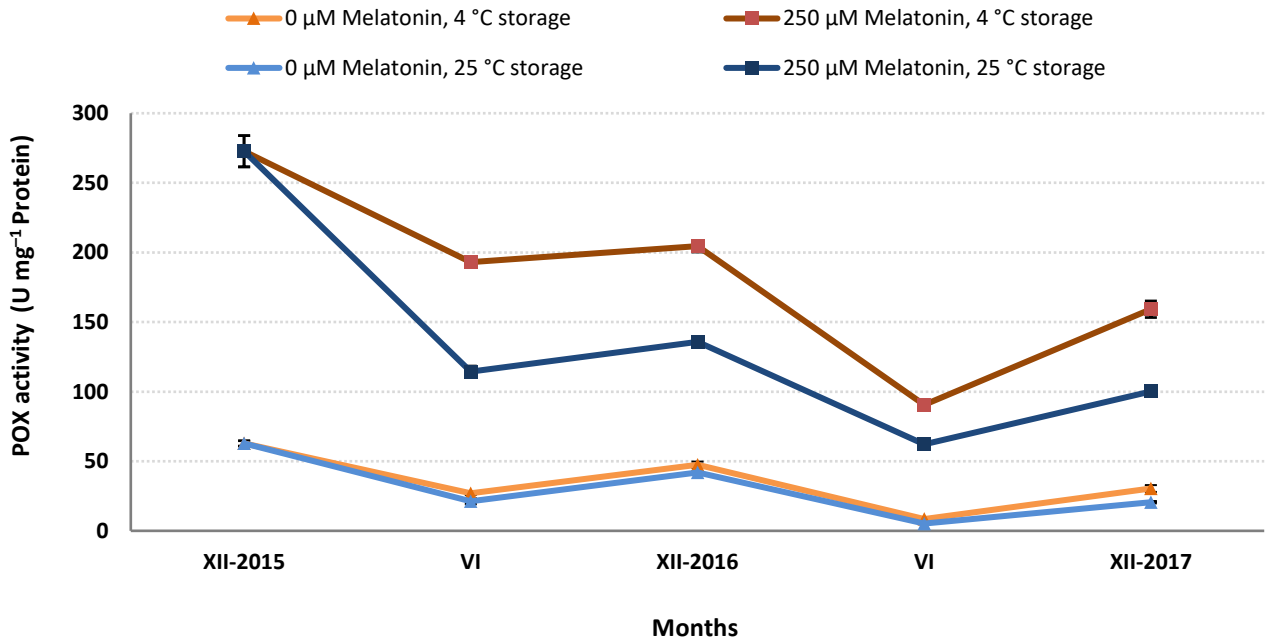


Fig. 4. Changes in MDA (A) and H₂O₂ (B) content of lettuce seeds during 24-month storage. Seeds were treated with melatonin (0 and 250 μM), stored at 4°C and 25°C for two years and sampled for MDA and H₂O₂ analysis at 2-month intervals starting from December of 2015

(A)



(B)

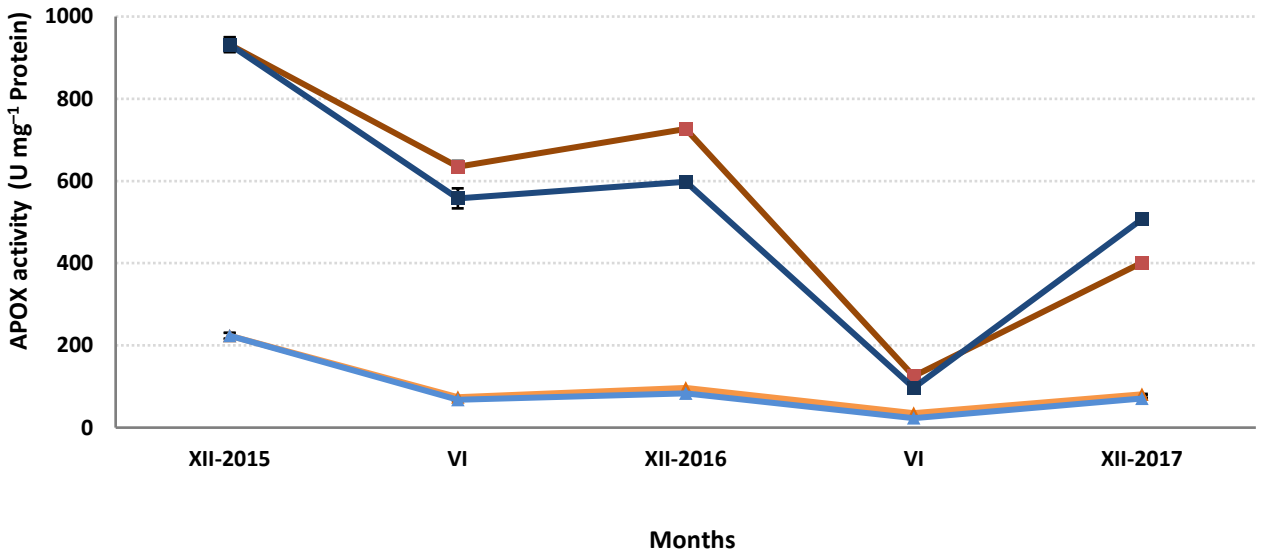


Fig. 5. Changes in the activities of POX (A) and APOX (B) enzymes of lettuce seeds during 24-month storage. Seeds were treated with melatonin (0 and 250 μM), stored at 4 $^{\circ}\text{C}$ and 25 $^{\circ}\text{C}$ for two years and sampled for enzyme analysis at 6-month intervals starting from December of 2015

regulator) – in the levels and flux of reactive oxygen and nitrogen species, which will act as messengers in many cellular and physiological responses. [Arnao and Hernández-Ruiz 2019a]. We observed significant reduction in MDA (Fig. 4A) and H₂O₂ levels (Fig. 4B) after melatonin application, which was resulted from enhanced antioxidant enzyme activities and direct free radical scavenging by melatonin. Moreover, even though the activities of antioxidant enzymes were not measured as frequently as the other variables, it is still clear that activities of POX (Fig. 5A) and APOX (Fig. 5B) followed the similar pattern as the melatonin content. Significantly higher POX and APOX activities in December accompanied the highest melatonin levels, strongly indicating that melatonin was involved in regulation of the activities of these enzymes. Additionally, POX and APOX activities in seeds treated with melatonin were higher than those of untreated seeds, a clear sign that exogenously applied melatonin promoted the activities of these enzymes. In agreement with our results, it was reported that melatonin pre-treatment of waxy maize [Simlat et al. 2018] and pepper [Korkmaz et al. 2017a] seeds germinated under chilling stress conditions provided significant protection of membrane structures against peroxidation and MDA accumulation. Moreover, melatonin application improved germination performance of cotton [Xiao et al. 2019] and *Limonium bicolor* [Li et al. 2019] seeds by boosting the antioxidant enzymes activities thereby reducing the accumulation of MDA and regulating the levels of key plant hormones such as gibberellic acid and abscisic acid during germination.

CONCLUSION

In conclusion, long-term seasonal changes in melatonin and tryptophan contents in lettuce seeds stored for 2 years were established. The results indicate that seed melatonin content fluctuated considerably during two year-long storage, reaching its peaks in October and December and lowest levels in August. Conversely, tryptophan levels displayed an opposite trend with a strong peak in August and low levels in October. Higher levels of MDA and H₂O₂ detected in seeds in August coincided with the lowest levels of melatonin present in the seeds indicated that melatonin was directly involved in regulation of lipid peroxidation in

seeds during the ageing process. Observation of similar seasonal changes in seeds stored both temperature conditions (4°C and 25°C) suggests that endogenous melatonin plays a vital role in seasonal rhythms independent of environmental conditions. However, even though we identified the seasonal fluctuations in melatonin content during long-term storage, we still do not know what triggers or governs these changes in seed tissues. Thus, additional research is necessary to determine the factors regulating melatonin biosynthesis specifically the effects of presence and amount of the pre-cursor molecules in seed tissues at different times of the year and to identify long-term seasonal fluctuations in the seeds of different species. Only after by doing so, will we have a better understanding the physiological roles of seasonal variations in melatonin content of stored seeds. Results also confirmed the previous studies that lettuce seeds were short-lived when stored at unfavorable conditions; however, melatonin application significantly slowed the ageing process by protecting the membrane structures against peroxidation and MDA accumulation through enhancing the activities of antioxidant enzymes. The fact that pre-treatment with melatonin could be used as a valuable tool for slowing seed ageing may have significant practical applications especially when storing the seeds of endangered species or precious breeding material.

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