ACTA BIOLOGICA CRACOVIENSIA Series Botanica 65/2: Article ID: 6521
DOI: 10.24425/abcsb.2023.145595

KNOCK-OUT MUTATION OF *CAT2* DRASTICALLY REDUCED THE MELATONIN-MEDIATED RESISTANCE TO BORON TOXICITY IN *ARABIDOPSIS THALIANA*

AYKUT SAGLAM[1,2*], ASIYE SEZGIN MUSLU[2], CANSU ALTUNTAS[2], MEHMET DEMIRALAY[2] AND ASIM KADIOGLU[2]

[1]*Department of Molecular Biology and Genetics, Faculty of Science, Karadeniz Technical University, 61060, Trabzon, Turkey*

[2]*Department of Biology, Faculty of Science, Karadeniz Technical University, 61060, Trabzon, Turkey*

Received December 1, 2022; revision accepted July 19, 2023

The role of catalase in resistance to boron toxicity after melatonin application (MEL) was investigated in *Arabidopsis thaliana* plants. Col-0 and *cat2-2* plants were exposed to 50 µM MEL followed by boron toxicity (BT) in a medium containing 10 mM H_3BO_3. Pigment loss and accordingly chlorosis were reduced by melatonin under BT conditions, while they were more prominent in *cat2-2* mutants. Moreover, TBARS and H_2O_2 contents, which increased due to BT, decreased as a result of melatonin application and the levels of these parameters in *cat2*-2 mutants were higher than the values in Col-0. Antioxidant enzyme activity of SOD and *SOD1* gen transcript were induced by MEL under BT. Conversely, *APX4*, *PER10* and *CAT1* transcripts were down-regulated by MEL under BT. In addition, antioxidant enzyme activities and their transcript levels were lower than those of Col-0. Thus, we suggested that MEL scavenged ROS directly under BT. Melatonin also reduced the accumulation of boric acid in leaf tissues of Col-0, but not *cat2-2*. Finally, even though melatonin application provided a degree of endurance, the *cat2* mutation resulted in increased sensitivity to BT.

Keywords: *Arabidopsis*, boron toxicity, catalase, chlorosis, melatonin

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) has been shown to play a role in plant growth and development, influencing processes such as seed germination, shoot and root growth, and flowering. Moreover, melatonin (MEL) has been reported to delay leaf senescence and promote flower formation in some plant species (Pan et al., 2023). Apart from its role in plant development, MEL also plays a significant role in plant responses to stress. Plants can often encounter stressful environmental conditions, and MEL protects plants against damage caused by environmental stresses such as exposure to heavy metals (Tan et al., 2007), UV radiation (Afreen et al., 2006), and temperature changes (Lei et al., 2004). Besides, MEL (1µM) was found to eliminate the negative effects of boron toxicity (BT, 100 µM) on pepper plants (Sarafi et al., 2017). MEL-applied plants exhibit no visual symptoms of BT. Boron is essential for the growth of higher plants and agricultural production (Villordon and Gregorie, 2021). However, BT is a common problem for many field crops growing in soils with low precipitation and high levels of boron (Reid, 2013). Based on Keren and Bingham (1985), the maximum allowable concentrations of B in irrigation water are between 0.3 and 1.0 mg L^{-1} for susceptible plants such as pear, bean and walnut. It was between 1–2 mg L^{-1} for semi-tolerant plants (e.g.,

* Corresponding author, e-mail: saglama@ktu.edu.tr

PL ISSN 1898-0295

oat, maize and barley) and 2-4 mg L^{-1} for tolerant species (e.g., sugar beet, date palm and asparagus).

Cultivated species and cultivars have various ranges in which B is considered appropriate. For instance, in monocots, B concentrations range from 1 to 6 mg/kg, while in most dicots they range from 20 to 70 mg/kg. However, the B range required for optimum plant growth is very narrow (Princi et al., 2015). The absorption of boron by plants is also influenced by the rate of transpiration, which depends on the relative humidity and temperature of the air. Low humidity and higher temperatures result in increased absorption of boron by plants (Hu and Brown, 1997). High boron is known to trigger events such as plant stunting, burning of old leaves, loss of plant vitality and reduced fruit yield (Güneş et al., 2007). In addition, it is also involved in a wide variety of processes in vascular plants such as decreased stem cell division, decreased leaf chlorophyll content and photosynthetic ratio, and decreased lignin and suberin levels (Nable et al., 1997; Reid, 2007). Research reports on the influence of BT on the antioxidant system can also be found in the literature (Choudhary et al., 2020). High activity of antioxidant enzymes in BT-treated plants are extremely important for resistance to stress (Güneş et al., 2007). The effects of antioxidant enzymes on reducing the harmful effects of BT in certain plants have been shown (Güneş et al., 2006; Kayıhan et al., 2016).

One of the most important functions of melatonin is its contribution to the defence against internal and environmental oxidative stress factors (Nawaz et al., 2021). Melatonin has also been reported to have an antioxidant effect on many plant species (Murch and Erland, 2021). Furthermore, application of exogenous MEL under different stresses has been demonstrated to induce antioxidant enzyme activity and accumulation of antioxidants in different plants such as *Malus hupehensis* exposed to salt stress (Li et al., 2012), tomato subjected to cold (Ding et al., 2017), tea plant under different abiotic stresses (Li et al., 2019) and rice under continuous salt stress (Yan et al., 2021). Additionally, Al-Huqail et al. (2020) reported that melatonin (100 µM) alleviated the harmful effects of BT in wheat plants. They also showed that both enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and non-enzymatic (ascorbate and reduced glutathione) antioxidants were induced by application of MEL under BT. Activation of antioxidant defense in plants at various adverse growing conditions is interrelated. Simultaneous increase of the catalase activity is often observed. Enhanced catalase activity may be an expression of the adaptive response of plants to abiotic stress (Mhamdi et al., 2010). *Arabidopsis thaliana* contains three catalase proteins (CAT1, 2 and 3). Knockout of *CAT2* has a larger effect on the reduction in catalase activity than knockout of the other two catalase genes (Mhamdi et al., 2010). Therefore, *cat2* mutants become more susceptible to certain stresses, for example, knockout of the Arabidopsis *CAT2* gene, one of the major isoforms, results in a severe bleaching phenotype under photorespiratory conditions (i.e., ambient air with high light intensity) (Mhamdi et al., 2012). Furthermore, the level of *CAT2* expression in the leaves of *A. thaliana* plants exposed to Arsenic (108 µM) was upregulated compared to the control group, which was not treated with As (Pita-Barbosa et al., 2019). In our study, we hypothesized that *CAT2* might be associated with BT tolerance observed as a result of MEL application. Therefore, it was aimed to investigate this relationship between *CAT2* and MEL in detail by using *cat2-2* mutants in *A. thaliana* plants subjected to BT.

MATERIALS AND METHODS

PLANT GROWTH AND APPLICATIONS OF BORIC ACID AND MELATONIN

The seeds of *A. thaliana*, the ecotype Columbia (Col-0) and the *cat2-2* mutant used in this study were provided by Dr. Frank Van Breusegem (Gent University, Belgium). The surface of the seeds was sterilized in bleach. Twenty seeds of Col-0 and *cat2-2* were exposed to stratification by keeping them in petri dishes containing double-layer sterilized filter paper for 3 days at 4°C. Each petri dish contained 5 seeds. Subsequently, these seeds were transferred into sterile plastic containers containing MS medium (1/2) and grown for 15 days in a growing room with a light intensity (150 µmol m^{-2} s^{-1}), temperature (20/18°C day/night) and humidity (50%). Then the seedlings went through the following treatments: (1) MS medium (Control) called MOCK, (2) boron toxicity (BT), (3) melatonin (MEL) and (4) MEL+BT (Table 1). Three petri dishes were used for each treatment. A total of 60 seedlings were allocated to a particular treatment.

TABLE 1. Experimental setup.

	Treatments	Duration
1	MOCK	22 days in MS media
2	BT	15 days in MS media, then 7 days in MS media, including 10 mM of H_3BO_3
3	MEL	8 days in MS media, then 7 days in MS media, including 50 µM of MEL and then 7 days in MS media
4	MEL + BT	8 days in MS media, then 7 days in MS media, including 50 µM of MEL and then 7 days in MS media including 10 mM of H_3BO_3

FRESH SHOOT BIOMASS

Five plants from sterile containers subjected to the treatments were randomly weighed in three replicates and their weights were recorded.

DETERMINATION OF PHOTOSYNTHETIC PIGMENT CONTENT

The content of photosynthetic pigments was determined following Arnon (1949). Fresh leaf samples (0.25 g) were homogenized in 5 ml 80% acetone and the homogenate was centrifuged at 5000 g for 5 minutes at room temperature. The absorbance of the supernatant was measured at 663 and 645 nm using a spectrophotometer. The following formulas developed by Arnon (1949) were used to determine the pigment contents:

Chlorophyll a = 12.7 x A663 - 2.69 x A645
Chlorophyll b = 22.9 x A645 - 4.68 x A663

LIPID PEROXIDATION

Lipid peroxidation was measured based on the content of malondialdehyde, a lipid peroxidation product. The leaf sample (0.1 g) was homogenized in 1.8 ml of 0.1% trichloroacetic acid (TCA) by a tissue homogenizer. The homogenate was centrifuged at 15,000 g for five minutes. Four ml of 0.5% thiobarbituric acid (TBA) prepared with 20% TCA was added to 1 ml of the supernatant, then stored in an oven at 95°C for 30 minutes. The absorbance of the supernatant, which was cooled to room temperature, was recorded at 532 nm. The nonspecific absorption reading at 600 nm was removed from the calculation. The content of thiobarbituric reactive substances (TBARS) was calculated in accordance with the formula developed by Heath and Packer (1968): ΔAbs (Abs_{532}-Abs_{600}) = $\varepsilon \times C \times L$ (ε = 155 mM^{-1} cm^{-1}, L = 1 cm, C = mM, TBARS)

DETERMINATION OF HYDROGEN PEROXIDE

The hydrogen peroxide assay was carried out in accordance with Velikova et al. (2000). To achieve this, the extract obtained from the ground leaf samples (0.1 g) in trichloroacetic acid was centrifuged. 1 ml was taken from the supernatant, 10 mM of potassium phosphate buffer and 1M KI were added, and the absorbance was measured at 390 nm. The H_2O_2 content was determined using a standard curve with known H_2O_2 concentrations ranging from 0 to 100 µM.

DETERMINATION OF BORON CONTENT

The leaf samples (10 g) were ground after drying in an oven at 55°C for 12 hours, and 0.50 g of dry samples were weighed. Ten ml of nitric acid was added to Teflon tubes and digested using a microwave device. The ICP-OES technique was used for the determination of boron concentration (Derun et al., 2010). For the experiments a Perkin-Elmer Optima 2100 DV model Inductively Coupled Plasma Optical Emission Spectrometer equipped with an AS-93 Autosampler was used. The measurement conditions were adjusted at a power of 1.8 kW, a plasma flow of 15.0 L min^{-1}, an auxiliary flow of 0.8 L min^{-1} and a nebulizer flow of 1 L min^{-1}. The calibration was fit into a linear range. The standards used ranged between 0 and 600 mg L^{-1}.

DETERMINATION OF MELATONIN CONTENT

Melatonin was extracted from plant leaves by the acetone-methanol method of Pape and Lüning (2006). In summary, 1 g of leaf sample was ground with liquid nitrogen and transferred into 5 ml of extraction mixture (acetone: methanol: water = 89:10:1). After extraction, it was centrifuged at 4500 g for 5 minutes at 4°C. The supernatant was transferred into tubes containing 0.5 ml of 1% trichloroacetic acid for protein precipitation and centri-

fuged at 12 000 g for 10 min at 4°C. This supernatant was used for melatonin level measurement by Melatonin ELISA Kit (EK-DSM; Buhlmann Laboratories AG, Schonenbuch, Switzerland) according to the manufacturer's instruction. The kit used a self made melatonin standard varying from 0.1 to 2.7 pg/well. The melatonin standard (100 pg/µL) was diluted with methanol at a concentration of 0.025 pg/µL and 10, 30, 90, 180 or 270 µL were pipette-fed into the microplate wells. Following evaporation of methanol in a vacuum concentrator, 250 µL reconstitution buffer was added to each container and melatonin was allowed to dissolve for 30 minutes. After the stop solution was added, the optical density at 450 nm was measured using a microplate reader (Rayto, RT-2100C, China).

DETERMINATION OF CHANGES IN ENZYMATIC ANTIOXIDANTS

The plant leaf (0.5g) was weighed and ground into liquid nitrogen, then homogenized into 5 ml extraction buffer (50mM K_2HPO_4, 1 mM EDTA pH 7.0, 1% PVPP). The extract was centrifuged at 20000 g at 4°C for 20 minutes. The obtained supernatant was used for determination of the enzymatic activity.

PEROXIDASE

The peroxidase activity (POD, EC 1.11.1.7) was determined in accordance with Urbanek et al. (1991). The enzyme activity was determined by measuring 2 ml of the reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 and 50 µl enzyme extract at 470 nm for 1 min. The POD activity was calculated using the extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

CATALASE

The catalase activity (CAT, EC 1.11.1.6) was determined following Aebi (1983). The enzyme activity was determined by measuring 1 ml of the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2 and 20 µL enzyme extract at 240 nm for 5 min. The catalase activity was calculated using the extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ for H_2O_2.

SUPEROXIDE DISMUTASE

The activity of superoxide dismutase (SOD, CE 1.15.1.1) was determined using Beauchamp and Fridovich (1971) method. 2 µM riboflavin was added to 1 ml reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 µM nitro blue tetrazolium, and 50 µL extract. In addition to reaction mixture, absorbance of the enzyme-free mixture called as blank is measured. The nitro-blue tetrazolium chloride reacts with superoxide anions produced during riboflavin illumination to produce formazan, a blue complex. The reduction in formazan formation is directly proportionate to the amount of SOD in the sample. A 50% decrease of formazan formation is considered as a unit of SOD. The reaction started and ended by turning the light on and off. Following exposure of this mixture to white light of 375 µmol m^{-2} s^{-1} for 10 minutes, the absorbance values at 560 nm were determined. The SOD activity was calculated according to the following formula:

(absorbance of blank- absorbance of tested sample) ÷ absorbance of blank
÷ (50% × volume of reaction mixture) ÷ protein concentration in enzyme extract

ASCORBATE PEROXIDASE

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined by decreasing absorption to 290 nm (Nakano and Asada, 1981). The enzyme activity was determined by measuring 1 ml of the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 250 µM ascorbate (ASC), 5 mM H_2O_2 and 20 µl enzyme extract. The ascorbate peroxidase activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} for ASC at 290 nm.

DETERMINATION OF PROTEIN CONTENT

Protein determination was carried out by spectrophotometry in accordance with Bradford (1976). The standard graph was created using Bovine Serum Albumin (BSA) standards (0-100 µg/ml). The complex of Coomassie Brilliant Blue G250 dye and protein was measured at 595 nm. Protein concentration was calculated as mg and used to express enzymatic activities.

RNA ISOLATION AND cDNA SYNTHESIS

For qPCR studies, leaf samples (100 mg) were frozen using liquid nitrogen and stored in a freezer at -80°C. Total RNA was isolated from frozen samples using a Qiagen RNeasy Plant mini-kit (Cat. No: 74904). RNA samples were measured for quantity and purity using a nanophotometer (Thermo Scientific, Nanodrop 2000, USA). RNA samples were stored at -80°C for cDNA synthesis.

The cDNA was obtained from the isolated Total RNA samples at 2000 ng per group. An Applied cDNA Synthesis Kit (High Capability cDNA Reverse Transcription Kit 4368814, Applied Biosystems) was used for the cDNA synthesis. Purity of cDNA recorded at absorbance of A_{260} /A_{280} nm was 1.87. The synthesized cDNAs were stored at -20°C until qPCR analysis.

QUANTITATIVE PCR (qPCR) MEASUREMENT

The cDNAs were used in qPCR analyzes to determine gene expressions. 5x HOT FIREPol Eva Green qPCR Supermix (08-36-00008, Solis Biodyne) and CFX Connect Real Time PCR Detection System (BioRad) were used for the analysis. qPCR process steps were modified by Solis Biodyne instructions as follows: 12 minutes at 95°C, 15 seconds at 95°C 45 cycles, 30 seconds at 60°C, 30 seconds at 72°C. 0.5°C increments were carried out for a melt curve from 65°C to 95°C. Each biological replication was analyzed as 3 technical replicates and the mean technical error was accepted as 0.5 (±1) Cq values.

Gene-specific primers were used to examine the levels of expression for *SOD1*, *CAT1*, *APX4* and *PER10* (Table 2). Beta Actin gene was used as a control. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression (Bookout and Mangelsdorf, 2003).

STATISTICAL ANALYSIS

All experiments were repeated with three biological replicates. The variance analysis of the data obtained as a result of the extraction and analysis performed in triplicate (technical replicates) was determined according to the Duncan Multiple Comparison Test included in the Statistical Package for Social Sciences (SPSS for Windows 23.0).

RESULTS

FRESH SHOOT BIOMASS

The fresh weight of *cat2-2* was 1.3 and 1.2 times higher than Col-0 in the MOCK and MEL treatments, respectively. Besides, the fresh weight (FW) of the shoots was decreased in Col-0 and *cat2-2* under BT compared to MOCK. Moreover, the FWs of Col-0 was 1.5-fold higher than *cat2-2* under BT. In addition, the application of MEL+BT increased the fresh weight of shoots in Col-0 and *cat2-2* in comparison with BT (Fig. 1a).

PHOTOSYNTHETIC PIGMENTS

The chlorophyll a (chl a) content of Col-0 was 1.3-fold higher than *cat2-2* in the MOCK treatment (Fig. 1b). On the contrary, the chl a content was observed to decrease in Col-0 and *cat2-2* exposed to BT compared to MOCK. Moreover, chl a

TABLE 2. The sequences of specific primers used for qPCR analysis.

Target gene	NCBI accession no.	Primer sequences
Superoxide dismutase 1 (SOD1)	NM_100757.3	AtSOD1F:5'-ACAATGGCGAAAGGAGTTGC-3' AtSOD1R:5'-AAGGCCAGAAACTGTTCCAC-3'
Ascorbate peroxidase 4 (APX4)	NM_116970.3	AtAPX4F:5'-TCCTGATCAAGCTGCAACAG-3' AtAPX4R:5'-ACGGTTTCACGGCTTCTTTG-3'
Catalase 1 (CAT1)	NM_101914.3	AtCAT1F: 5'-ACGACAAGCTGCTTCAAACC-3' AtCAT1R: 5'-TCATGGTGGTTGTTGTGGTG-3'
Peroxidase super family protein (PER10)	NM_103845.3	AtPER10F: 5'-TCATGCGCTGACATAGTTGC-3' AtPER10R: 5'-TTTGTATTCGCCGCTTGCTC-3'
Actin 2 (ACT2)	NM_112764.3	AtACT2F: 5'-TGCTGTTGACTACGAGCAGG -3' AtACT2R: 5'-CGAGGGCTGGAACAAGACTT-3'

Fig. 1. Effects of boron toxicity and melatonin on fresh shoot biomass (**a**), chlorophyll a (**b**), chlorophyll b (**c**) and appearance of plants (**d**,**e**). The bars show the standard deviation. The difference between columns with the same letter is insignificant at the 5% ($P < 0.05$) level.

content of Col-0 was 1.3-fold higher than *cat2-2* under BT. Melatonin application induced the chl a content under the BT (MEL+BT) compared to BT only. However, there was no difference in the pigment content between MOCK and MEL under stress-free conditions.

Chl b content of Col-0 was 1.3-fold higher than *cat2-2* in MOCK treatment (Fig. 1c). Similar to chl a, the chl b content also decreased as a result of BT compared to MOCK groups (Fig. 1c). In addition, the chl b content of Col-0 was 1.43-fold higher than *cat2-2* under BT. Following the application of melatonin under the BT (MEL+BT), the chl b contents of all plants increased compared to BT alone. However, there was no difference in the chl b content between the MOCK and MEL treatments.

Under boron toxicity conditions, chlorophyll degradation was observed in both Col-0 and *cat2-2* plants compared to the MOCK group (Fig. 1d,e). In addition, chlorosis was quite pronounced in *cat2-2* mutants (Fig. 1e). In contrast, chlorophyll synthesis was stimulated by the application of melatonin under BT conditions (MEL+BT) in comparison with BT alone.

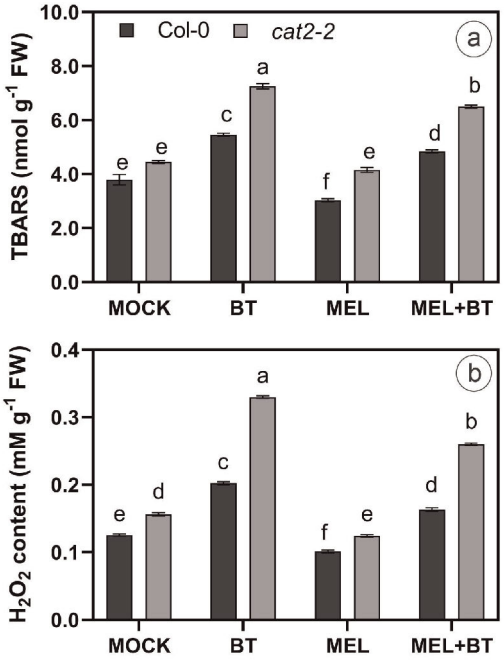

Fig. 2. Effects of boron toxicity and melatonin on TBARS (**a**) and H_2O_2 (**b**). The bars show the standard deviation. The difference between columns with the same letter is insignificant at the 5% ($P < 0.05$) level.

TBARS AND H_2O_2

TBARS levels increased significantly in Col-0 and *cat2-2* plants under boron toxicity conditions in comparison with the MOCK group (Fig. 2a). In addition, under BT conditions, the TBARS content in *cat2-2* plants was 1.3 times higher than that of Col-0 plants. However, melatonin applica-

tion mitigated lipid peroxidation in both Col-0 and *cat2-2* under stressful conditions (MEL+BT) compared to BT.

Under boron toxicity conditions, the hydrogen peroxide content increased in Col-0 and *cat2-2* plants compared to the MOCK group. This increase was significant in *cat2-2* plants (Fig. 2b). In addition, the H_2O_2 content in *cat2-2* plants was 1.6 times higher than that of Col-0 plants under BT conditions. Similar to lipid peroxidation, melatonin application also reduced the H_2O_2 content in Col-0 and *cat2-2* under both stressful (MEL +BT) and stress-free conditions (MEL) compared to their respective controls (BT and MOCK). However, under MEL+BT conditions, the H_2O_2 content of *cat2-2* plants was 1.6 times higher than that of Col-0 plants.

ENZYME ACTIVITIES

The SOD activity of Col-0 was higher than that of *cat2-2* in all treatment groups. Additionally, the SOD activity was induced by BT. Under BT conditions, the activity was 1.6 times higher for Col-0 and 1.4 times higher for *cat2-2* in comparison to MOCK (Fig. 3a). The MEL application also increased the SOD activity of Col-0 relative to the MOCK application but made no difference in the activity of *cat2-2*. The MEL+BT treatment increased the SOD activities of Col-0 and *cat2-2* 1.1 times compared to the BT application.

The CAT activity of Col-0 was also higher than the CAT activity of *cat2-2* in all treatment groups, like the SOD activity. In addition, BT promoted the CAT activity, compared to MOCK (Fig. 3b). The BT application resulted in 1.5-fold and 1.4-fold increase in CAT activities of Col-0 and *cat2-2* relative to MOCK, respectively. The MEL +BT application caused a 1.1-fold reduction in CAT activities of Col-0 and *cat2-2* compared to BT.

The APX activity of *cat2-2* was higher than Col-0 in all treatment groups (Fig. 3c). In addition, BT stimulated APX activity, compared to MOCK. The activity increased 1.3 times in Col-0 and 1.2 times in *cat2-2*. The MEL treatment did not alter the APX activity of Col-0 relative to MOCK,

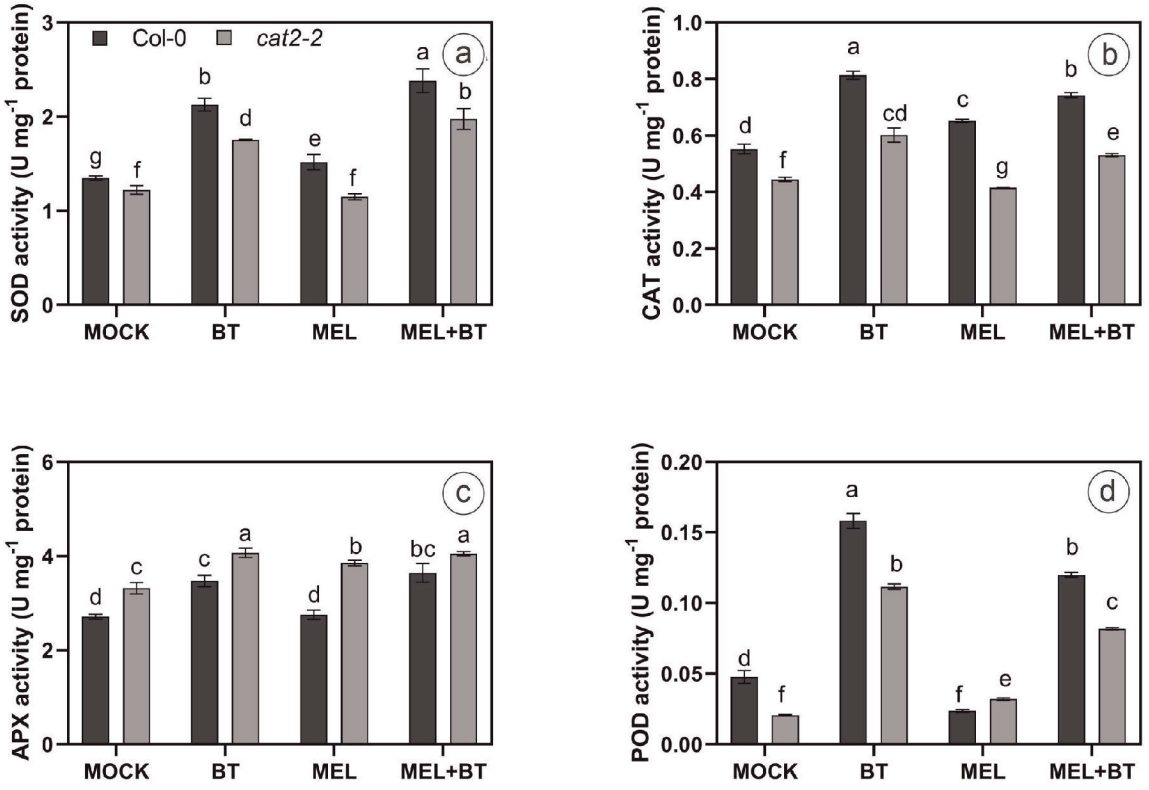

Fig. 3. Effects of boron toxicity and melatonin on antioxidant enzyme activities. SOD (**a**), CAT (**b**), APX (**c**) and POD (**d**). The bars show the standard deviation. The difference between columns with the same letter is insignificant at the 5% ($P < 0.05$) level.

but did stimulate the APX activity of *cat2-2*. Moreover, no statistically significant differences were found between BT and MEL+BT applications.

Significant increases in POD activities of Col-0 and *cat2-2* were determined under BT conditions compared to MOCK (Fig. 3d). However, the POD activity of Col-0 was 1.4 times higher than that of *cat2-2* under BT conditions. The MEL+BT application reduced POD activity 1.3 times in Col-0 and 1.4 times in *cat2-2* relative to BT conditions. However, the POD activity of Col-0 was 1.5 times higher than that of *cat2-2* under BT conditions.

GENE EXPRESSIONS

SOD1 expression level in Col-0 was higher than that of *cat2-2* in all groups (Fig. 4a). In addition, the BT up-regulated gene expression level 6 times in Col-0 and 2 times in *cat2-2* compared to MOCK. MEL treatment stimulated gene expression in Col-0 relative to MOCK, but decreased it in *cat2-2*. MEL+BT application increased the *SOD1* expression level under BT conditions: 1.8 times in Col-0 and 1.4 times in *cat2-2*.

CAT1 expression was induced by BT compared to MOCK (Fig. 4b). The *CAT1* expression level increased 14.9 times in Col-0 and 4.3 times in *cat2-2* under BT compared to MOCK. MEL stimulated the gene expression in Col-0 relative to MOCK, but decreased it in *cat2-2*. MEL+BT treatment resulted in a decreased expression level compared to BT. The gene expression decreased 2.1 times in Col-0 and 3.3 times in *cat2-2* when plants were subjected to MEL+BT.

The *APX4* gene expression level increased under BT conditions compared to MOCK (Fig. 4c). The *APX4* gene was up-regulated 11 times in Col-0 and 1.9 times in *cat2*-2 under BT compared to MOCK. MEL stimulated the *APX4* expression in Col-0 relative to MOCK, but down-regulated it in *cat2-2*. Moreover, MEL+BT caused to decrease in APX4 expressions of both Col-0 and *cat2-2* com-

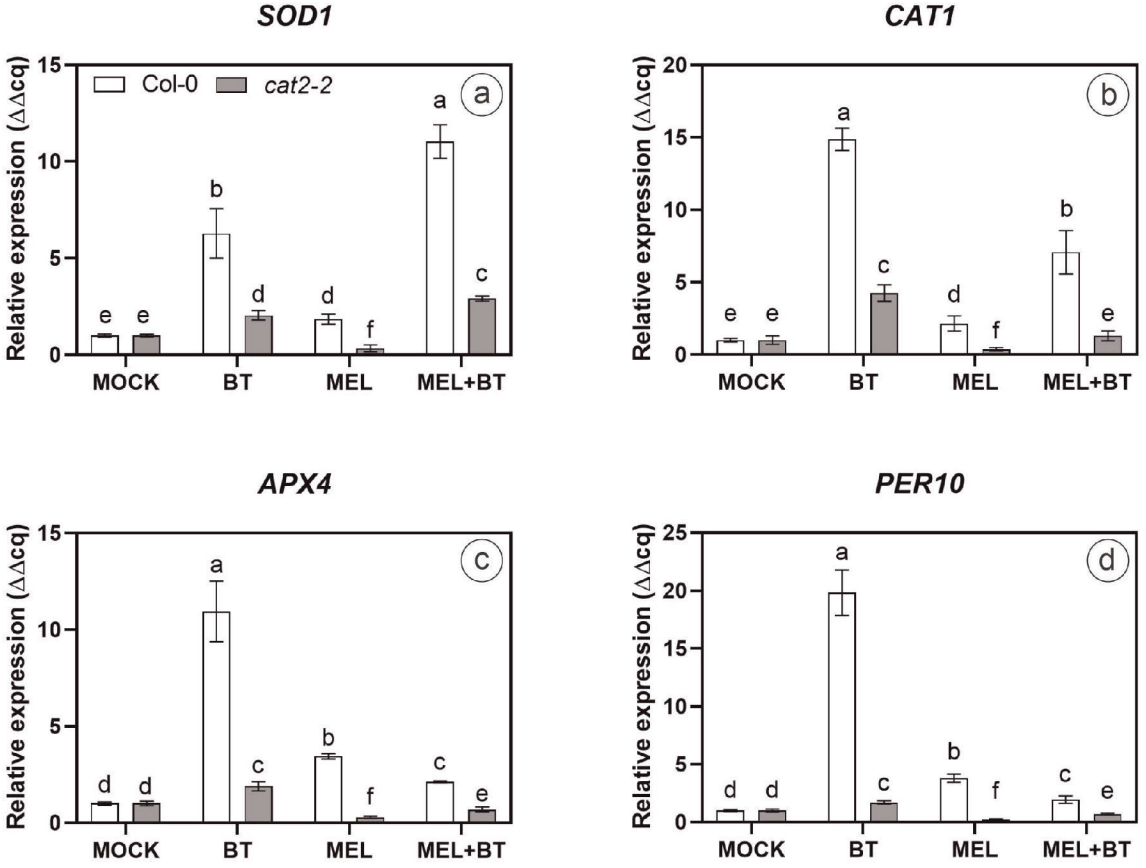

Fig. 4. Effects of boron toxicity and melatonin on transcriptions of genes coding antioxidant enzymes. *SOD1* (**a**), *CAT1* (**b**), *APX4* (**c**) and *PER10* (**d**). The bars show the standard deviation. The difference between columns with the same letter is insignificant at the 5% ($P < 0.05$) level.

pared to BT. The gene expression was diminished 5.2 times in Col-0 and 2.8 times in *cat2-2* plants exposed to MEL+BT compared to BT.

PER10 expression was up-regulated under BT compared to MOCK (Fig. 4d). The expression increased 19.8 times in Col-0 and 1.7 times in *cat2*-2 compared to MOCK when the plants were subjected to BT. MEL induced the APX4 expression in Col-0 relative to MOCK, but down-regulated it in *cat2-2*. The *PER10* expression was diminished 10.1 times in Col-0 and 2.4 times in *cat2-2* plants exposed to MEL+BT compared to BT.

MELATONIN AND BORON CONTENTS

Endogenous MEL contents of Col-0 and *cat2-2* plants were decreased under BT compared to MOCK (Fig. 5a). Under BT, the MEL content ascended 1.4 times in Col-0 and 1.3 times in *cat2-2* compared to MOCK. MEL application resulted in an increasing MEL content in Col-0 and *cat2-2* compared to MOCK. The increases were 1.1 times in Col-0 and 6.5 times in *cat2-2* when exogenous MEL was applied to the plants. MEL+BT treatment also induced the MEL content in Col-0 and *cat2-2* compared to BT treatment.

The boron content of Col-0 was higher than that of *cat2-2*, except for MEL+BT application (Fig. 5b). Moreover, BT significantly increased the boron content in Col-0 and *cat2-2* compared to MOCK. MEL application also increased the boron content in Col-0 and *cat2-2* compared to MOCK. While MEL+BT application decreased the boron content of Col-0, it did not change that of *cat2-2* compared to BT.

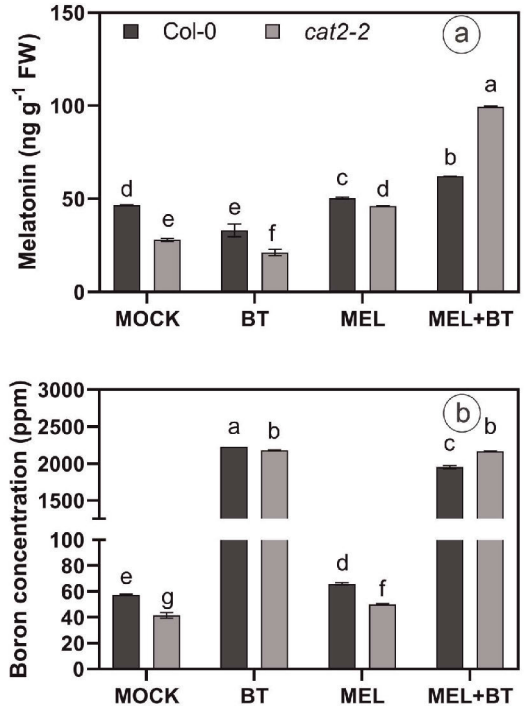

Fig. 5. Effects of boron toxicity and melatonin on endogenous melatonin (**a**) and boron (**b**) contents. The bars show the standard deviation. The difference between columns with the same letter is insignificant at the 5% ($P < 0.05$) level.

DISCUSSION

Boron toxicity, which is one of the abiotic stresses affecting agricultural areas around the world, has been accepted as one of the most important problems that influence productivity by restricting plant growth (Brdar-Jokanovic, 2020). Therefore, it has become important to make plants resistant to BT conditions. In our study, we found that external application of melatonin made *A. thaliana* resistant to BT and that catalase 2 was also important for this resistance. The main effects of metal toxicity include suppression of stem and root growth (Angulo-Bejanaro et al., 2021). Suppression of seedling growth under a variety of metal toxicity conditions may be reduced through external application of melatonin. For instance, Posmyk et al. (2008) found that application of 1 and 10 µM of melatonin favored germination and seedling growth of red cabbage seeds under copper stress. Similarly, it has been reported that the weights of red cabbage seedlings treated with 10 µM melatonin under copper stress were significantly higher than those of the control (only copper treated) seedlings (Posmyk et al., 2009). Similarly to our study, Al-Huqail et al. (2020) observed increased wheat plant weight and height under BT conditions with external MEL application. The positive effects in vegetative development are due to the fact that MEL acts as a plant growth regulator and biostimulant (Arnao and Hernández-Ruiz, 2015; Siddiqui et al., 2019). In addition, it promotes photosynthesis rates, particularly in stressful conditions (Xu, 2010). Previous studies showed that the chlorophyll content was elevated in plants with a high melatonin content or in plants with external melatonin application, or that chlorophyll loss was slowed or avoided. For example, Zhang et al. (2022) determined a significant increase in the total chlorophyll content as a result of MEL application to

tomato plants under copper toxicity conditions compared to the group exposed to copper only. Chlorophyll biosynthesis was also significantly enhanced in wheat seedlings treated with 50 μM melatonin under boron toxicity compared to BT only (Al-Huqail et al., 2020). In addition, Jahan et al. (2020) reported that chlorophyll synthesis genes such as Protochlorophyllide oxidoreductase, Chlorophyllide a oxygenase, and Chlorophyll synthase were up-regulated in MEL-treated tomato seedlings under Ni stress. In the present study, the application of MEL was found to increase the chlorophyll content of plants subjected to boron toxicity. This finding was consistent with previous studies mentioned above. Recovery of impaired chl synthesis may be linked to MEL because of its role in the biosynthesis of porphyrins, glycine and succinyl-CoA by regulating the activity of δ-aminolevulinate synthase (Sarropoulou et al., 2012). MEL also induces ferredoxin regulating GSH accumulation, which prevents the degradation of chl (Hoque et al., 2021). In fact, chloroplasts appeared to be the main production site for melatonin. A significant amount of melatonin is necessary to maintain its structure and functionality. Therefore, absorbed and synthesized melatonin can work together to mitigate BT-induced ROS and help cope with stress. It is known that metal-induced free radicals increase MDA and disrupt biological membranes (Dutta et al., 2018). Exogenous MEL improves the stress tolerance to heavy metals such as Al, As, Cd, Cr, Cu, Fe, Ni, and Pb and metalloids such as B of different plant species through scavenging ROS directly, and increasing antioxidant enzyme activities (Hodžić et al., 2021) and related gene expression such as Cu/Zn-SOD, POX, GPX, and MDHAR and by scavenging excessive reactive species. Here we established that TBARS and H_2O_2 contents of Col-0 and *cat2-2* were stimulated by BT, however MEL decreased both of them under BT. Under boron toxicity conditions, antioxidant enzyme activities (SOD, CAT, APX and POD) and their transcript levels were also induced to compensate for the increase in H_2O_2 and TBARS relative to MOCK. In the case of MEL+BT application, the activities of other antioxidant enzymes, except SOD, decreased compared to BT conditions. This decrease in antioxidant enzyme activity (CAT and POD) and gene expression (*CAT1*, *APX4* and *PER10*) may indicate that MEL may have directly eliminated ROS and TBARS, because the reductions in hydrogen peroxide and TBARS were in agreement with those under MEL+BT conditions. However, SOD activity significantly increased under MEL+BT conditions compared to BT conditions. This showed that MEL directly stimulates antioxidant enzyme activity as well as ROS scavenging. In addition, the endogenous melatonin concentration was significantly decreased in Col-0 and *cat2-2* plants under BT conditions compared to MOCK, while a remarkable increase in endogenous melatonin concentration was observed when external MEL was given (MEL+BT) compared to BT application. This change in the endogenous melatonin content supported the idea that melatonin was synthesized to scavenge reactive oxygen (H_2O_2) and carbon (TBARS) derivatives in the case of MEL+BT applications where antioxidant enzyme activities and transcription levels of related genes were decreased compared to BT. Moreover, SOD and CAT are the primary defence of the antioxidant enzyme system among ROS antioxidant enzymes. Furthermore, the intracellular level of H_2O_2 is regulated by several enzymes, the most important of which are CATs and peroxidase (Dumanović et al., 2021). For instance, the activity of CAT was determined to be stimulated in duckweed plants exposed to arsenic, a metalloid element (Duman et al., 2010). Besides, CAT activity and CAT2 gene expression were induced in germinating maize embryos subjected to arsenic (Mylona et al., 1998). In addition, CAT activity was reported to increase in wheat, citrus and chickpea plants subjected to BT (Landi et al., 2019). Therefore, we suggested that CAT is important in metalloid element toxicity, including BT. For this reason, we preferred to study the CAT mutant under BT conditions.

In our study, MEL treatment significantly improved the level of B in leaves compared to MOCK. However, application of MEL induced resistance to B by suppressing its accumulation in the leaves under MEL+BT conditions compared to BT, particularly in Col-0, but not *cat2-2* (no differences detected). Similary, Al-Huqail et al. (2020) observed reduced boron levels when melatonin was applied externally to wheat plants under boron toxicity conditions. Futhermore, Sarafi et al. (2017) reported that melatonin application to *Capsicum annuum* reversed the toxic effect of B by regulating B accumulation in leaves and fruits, increasing photosynthetic activity, and improving dry weight. Similarly, in the case of watermelon, application of melatonin increased nitrogen concentration in roots by improving root elongation, root diameter and root surface area subjected to limited availability of nitrogen (Nawaz et al., 2016). However, when

MEL was applied, no evidence of the uptake of B under BT conditions has yet been identified, which requires further research.

CONCLUSION

To sum up, application of melatonin improved the performance of *A. thaliana* by inducing the resistance against BT. Melatonin is thought to scavenge free radicals by itself or by increasing antioxidant enzyme activities, especially SOD. In addition, we suggest that application of melatonin to reduce the accumulation of boric acid in plant tissues (Col-0) may also have reduced the effect of oxidative stress due to lower metal accumulation. However, lower fresh weight, chl b content, enzyme activities and gene expression levels, higher chlorosis, TBARS and H_2O_2 contents and unchanged B contents of *cat2-2* mutants under MEL+BT conditions compared to Col-0 indicated that *cat2* mutation resulted in increased sensitivity to BT. The susceptibility to boron toxicity observed in the *cat2*-2 mutants relative to Col-0 supports our assumption that the CAT2 is associated with resistance to BT.

AUTHORS' CONTRIBUTIONS

A.S. wrote the research paper and conducted the research. A.S., A.S.M., C.A. and M.D. planned the research, analyzed the data, and helped to draft the manuscript. AK revised the manuscript. AS and AK supervised the research work. The final manuscript was read and accepted by all the authors. The authors declare no conflict of interest related to this article.

ACKNOWLEDGEMENTS

The research was supported by the Karadeniz Technical University Scientific Research Projects Coordination Unit through the project FHD-2016-5556.

REFERENCES

Aebi HE. 1983. Catalase. In: Bergmeyer HU [ed.], *Methods Of Enzymatic Analysis*, 273–286. Verlag Chemie, Weinhem, Germany.

Afreen F, Zobayed SM, and Kozai T. 2006. Melatonin in *Glycyrrhiza uralensis*: Response of plant roots to spectral quality of light and UV-B radiation. *Journal of Pineal Research* 41: 108–115.

Al-Huqail AA, Khan MN, Ali HM, Siddiqui MH, Al -Huqail A, Al-Zuaibr FM, Al-Muwayhi MA, Marraiki N, and Al-Humaid L. 2020. Exogenous melatonin mitigates boron toxicity in wheat. *Ecotoxicology and Environmental Safety* 201: 110822.

Angulo-Bejarano PI, Puente-Rivera J, and Cruz-Ortega R. 2021. Metal and metalloid toxicity in plants: An overview on molecular aspects. *Plants* 10: 635.

Arnao MB, and Hernández-Ruiz J. 2015. Functions of melatonin in plants: a review. *Journal of Pineal Research* 59: 133–150.

Arnon DI. 1949. Copper enzyme polyphenoloxides in isolated chloroplast in *Beta vulgaris*. *Plant Physiology* 24: 1–15.

Beauchamp C, and Fridovich I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* 44: 276–287.

Bookout AL, and Mangelsdorf DJ. 2003. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nuclear Receptor* Signaling 1: e012.

Bradford MM. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

Brdar-Jokanović M. 2020. Boron toxicity and deficiency in agricultural plants. *International Journal of Molecular Sciences* 21: 1424.

Choudhary S, Zehra A, Naeem M, Khan MMA, and Aftab T. 2020. Effects of boron toxicity on growth, oxidative damage, antioxidant enzymes and essential oil fingerprinting in *Mentha arvensis* and *Cymbopogon flexuosus*. *Chemical and Biological Technologies in Agriculture* 7: 8.

Derun E, Kipçak AS, and Özdemir O. 2010. The determination of the boron amounts of teas that are sold in Turkey by using the ICP-OES technique. *Proceedings of the World Congress on Engineering*, June 30 - July 2, 2010, 2277–2279. London.

Ding F, Liu B, and Zhang S. 2017. Exogenous melatonin ameliorates cold-induced damage in tomato plants. *Scientia Horticulturae* 219: 264–271.

Duman F, Ozturk F, and Aydin Z. 2010. Biological responses of duckweed (*Lemna minor* L.) exposed to the inorganic arsenic species As(III) and As(V): effects of concentration and duration of exposure. *Ecotoxicology* 19: 983–993.

Dumanović J, Nepovimova E, Natić M, Kuca K, and Jaćević, V. 2021. The significance of reactive oxygen species and antioxidant defense system in plants: A concise overview. *Frontiers in Plant Science* 11: 552969.

Dutta S, Mitra M, Agarwal P, Mahapatra K, De S, Sett U, and Roy S. 2018. Oxidative and genotoxic damages in plants in response to heavy metal stress and maintenance of genome stability. *Plant Signaling and Behavior* 13: e1460048.

Güneş A, Inal A, Bagci EG, Çoban S, and Pilbean DJ. 2007. Silicon mediates changes to some physiological and enzymatic parameters symptomatic for oxidative stress in spinach (*Spinacia oleracea* L.) grown under B toxicity. *Scientia Horticulturae* 113: 113–119.

Güneş A, Söylemezoğlu G, Inal A, Bağci EG, Çoban S, and Sahin O. 2006. Antioxidant and stomatal responses of grapevine (*Vitis vinifera* L.) to boron toxicity. *Scientia Horticulturae* 110: 279–284.

Heath RL, and Packer L. 1968. Photoperoxidation in isolated chloroplast. I. kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemisty and Biophysics* 125: 189–98.

Hodžić E, Galijašević S, Balaban M, Rekanović S, Makić H, Kukavica B, and Mihajlović D. 2021. The protective role of melatonin under heavy metal-induced stress in *Melissa officinalis* L. *Turkish Journal of Chemistry* 45: 737–748.

Hoque MN, Tahjib-Ul-Arif M, Hannan A, Sultana N, Akhter S, Hasanuzzaman M, Akter F, Hossain MS, Sayed MA, Hasan MT, Skalicky M, Li X, and Brestič M. 2021. Melatonin modulates plant tolerance to heavy metal stress: morphological responses to molecular mechanisms. *International Journal of Molecular Sciences* 22: 11445.

Hu H, and Brown PH. 1997. Absorption of boron by plant roots. *Plant and Soil* 193: 49–58.

Jahan MS, Guo S, Baloch AR, Sun J, Shu S, Wang Y, Ahammed GJ, Kabir K, and Roy R. 2020. Melatonin alleviates nickel phytotoxicity by improving photosynthesis, secondary metabolism and oxidative stress tolerance in tomato seedlings. *Ecotoxicology and Environmental Safety* 197: 110593.

Kayihan DS, Kayihan C, and Çiftçi YÖ. 2016. Excess boron responsive regulations of antioxidative mechanism at physio-biochemical and molecular levels in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* 109: 337–345.

Khan MK, Pandey A, Hamurcu M, Avsaroglu ZZ, Ozbek M, Omay AH, Elbasan F, Omay MR, Gokmen F, Topal A, and Gezgin S. 2021. Variability in physiological traits reveals boron toxicity tolerance in Aegilops species. *Frontiers in Plant Science* 12: 736614.

Keren R, and Bingham FT. 1985. Boron in water, soils, and plants. In: Stewart, BA [ed.], *Advances in Soil Science*, 229– 276. Springer, New York.

Landi M, Margaritopoulou T, Papadakis IE, and Araniti F. 2019. Boron toxicity in higher plants: an update. *Planta* 250:1011–1032.

Lei X, Zhu R, Zhang G, and Dai Y. 2004. Attenuation of cold-induced apoptosis by exogenous melatonin in carrot suspension cells: The possible involvement of polyamines. *Journal of Pineal Research* 36: 126–131.

Li C, Wang P, Wei Z, Liang D, Liu C, Yin L, Jia D, Fu M, and Ma F. 2012. The mitigation effects of exogenous melatonin on salinity-induced stress in *Malus hupehensis*. *Journal of Pineal Research* 53: 298–306.

Li JH, Yang YQ, Sun K, Chen Y, Chen X, and Li XH. 2019. Exogenous melatonin enhances cold, salt and drought stress solerance by improving antioxidant defense in tea plant (*Camellia sinensis* (L.) O. Kuntze). *Molecules* 24: 1826.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. In: Douce R and Packer L [eds.], *Methods in Enzymology*, 350–382. Academic Press Inc., New York, USA.

Mhamdi A, Noctor G, and Baker A. 2012. Plant catalases: peroxisomal redox guardians. *Archives of Biochemistry and Biophysics* 525: 181–194.

Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F, and Noctor G. 2010. Catalase function in plants: a focus on *Arabidopsis* mutants as stress-mimic models. *Journal of Experimental Botany* 61: 4197–4220.

Mylona PV, Polidoros AN, and Scandalios JG. 1998. Modulation of antioxidant responses by arsenic in maize. *Free Radical Biolology and Medicine* 25: 576–85.

Murch SJ, and Erland LAE. 2021. A systematic review of melatonin in plants: An example of evolution of literature. *Frontiers in Plant Science* 12: 683047.

Nable RO, Bañuelos GS, and Paull JG. 1997. Boron toxicity. *Plant and Soil* 193: 181–198.

Nakano Y, and Asada Y. 1981. Hydrogen peroxide is scavenged by ascorbate-spesific peroxidase in spinach chloroplasts. *Plant and Cell Physiology* 22: 867–880.

Nawaz K, Chaudhary R, Sarwar A, Ahmad B, Gul A, Hano C, Abbasi BH, and Anjum S. 2021. Melatonin as master regulator in plant growth, development and stress alleviator for sustainable agricultural production: Current status and future perspectives. *Sustainability* 13: 294.

Nawaz MA, Huang Y, Mu X, and Bie Z. 2016. Melatonin application alters the root morphology and nitrogen uptake of watermelon. *Second Asian Horticultural Congress*, 26-28 September 2016, 28-29. Chengdu, China.

Pan Y, Xu X, Li L, Sun Q, Wang Q, Huang H, Tong Z, and Zhang J. 2023. Melatonin-mediated development and abiotic stress tolerance in plants. *Frontiers in Plant Science* 14: 1100827.

Pape C, and Lüning K. 2006. Quantification of melatonin in phototrophic organisms. *Journal of Pineal Research* 41: 157–165.

Pita-Barbosa, A, Williams, T.C.R, and Loureiro, ME. 2019. Effects of short-term arsenic exposure in *Arabidopsis thaliana*: Tolerance versus toxicity responses. *Biologia Plantarum* 63: 43–53.

Posmyk MM, Kuran H, Marciniak K, and Janas KM. 2008. Presowing seed treatment with melatonin protects red cabbage seedlings against toxic copper ion concentrations. *Journal of Pineal Research* 45: 24–31.

Posmyk MM, and Janas KM. 2009. Melatonin in plants. *Acta Physiologiae Plantarum* 31: 1–11.

Princi M, Lupini A, Araniti F, Longo C, Mauceri A, Sunseri F, and Abenavoli RM. 2015. Boron toxicity and tolerance in plants: recent advances and future perspective. In:

Ahmad P [ed.], *Plant Metal Interaction: Emerging Remediation Techniques*, 115– 147. Elsevier, Amsterdam.

Reid R. 2007. Update on boron toxicity and tolerance in plants. In: Xu F, Goldbach HE, Brown PH, Bell RW, Fujiwara T, Hunt CD, Goldberg S, and Shi L [eds.]. *Advances in Plant and Animal Boron Nutrition*, 83–90. Springer, Dordrecht.

Reid R. 2013. Boron toxicity and tolerance in crop plants. In: Tuteja N, and Gill S [eds.], *Crop Improvement Under Adverse Conditions*, 333–346. Springer, New York.

Sarafi E, Tsouvaltzis P, Chatzissavvidis C, Siomos A, and Therios L. 2017. Melatonin and resveratrol reverse the toxic effect of high boron (B) and modulate biochemical parameters in pepper plants (*Capsicum annuum* L.). *Plant Physiology and Biochemistry* 112: 173–182.

Sarropoulou VN, Therios IN, and Dimassi-Theriou KN. 2012. Melatonin promotes adventitious root regeneration in in vitro shoot tip explants of the commercial sweet cherry rootstocks CAB-6P (*Prunus cerasus* L.), Gisela 6 (*P. cerasus* × *P. canescens*), and MxM 60 (*P. avium* × *P. mahaleb*). *Journal of Pineal Research* 52: 38–46.

Siddiqui MH, Alamri S, Al-Khaishany MY, Khan MN, Al-Amri A, Ali HM, Alaraidh IA, and Alsahli AA. 2019. Exogenous melatonin counteracts NaCl-induced damage by regulating the antioxidant system, proline and carbohydrates metabolism in tomato seedlings. *International Journal of Molecular Sciences* 20: 353.

Tan DX, Manchester LC, Di Mascio P, Martinez GR, Prado FM, and Reiter RJ. 2007. Novel rhythms of N1-acetyl-N2-formyl-5-methoxykynuramine and its precursor melatonin in water hyacinth: importance for phytoremediation. *FASEB Journal* 21: 1724–1729.

Urbanek H, Kuzniak-Gebarowska E, and Herka K. 1991. Elicitation of defense responses in bean leaves by *Botrytis cinerea* polygalacturanase. *Acta Physiologiae Plantarum* 13: 43–50.

Velikova V, Yordanov I, and Edreva A 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective roles of exogenous polyamines. *Plant Science* 151: 59–66.

Villordon A, and Gregorie JC. 2021. Variation in boron availability alters root architecture attributes at the onset of storage root formation in three sweet potato cultivars, *HortScience* 56: 1423–1429.

Xu XD. 2010. Effects of exogenous melatonin on physiological response of cucumber seedlings under high temperature stress. Dissertation, Northwest A&F University, Yangling Shanxi, China.

Yan F, Wei H, Ding Y, Li W, Liu Z, Chen L, Tang S, Ding C, Jiang Y, and Li G. 2021. Melatonin regulates antioxidant strategy in response to continuous salt stress in rice seedlings. *Plant Physiology and Biochemistry* 165: 239–250.

Zhang T, Wang Y, Ma X, Ouyang Z, Deng L, Shen S, Dong X, Du N, Dong H, Guo Z, Meng G, Piao F, and Sun K. 2022. Melatonin alleviates copper toxicity via improving ros metabolism and antioxidant defense response in tomato seedlings. *Antioxidants* 11: 758. https://doi.org/10.3390/antiox11040758