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The Effects of Seed Treatment with Melatonin on Germination and Emergence Performance of Pepper Seeds under Chilling Stress

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ABSTRACT

Melatonin was first isolated from bovine pineal gland more than half a century ago as an important animal hormone and since then it was proved to be present in almost all forms of life including eukaryotic unicells, prokaryotes, fungi, algae, animals and plants. In this study, the effects of pre-sowing seed treatment with melatonin on germination and emergence performance of pepper seeds under chilling conditions were investigated. Seeds were immersed in 0 (distilled water), 1, 5, 10 or 25 µM melatonin solutions for 24 hours after which they were dried for one day and subjected to germination and emergence tests at optimum (25 °C) and chilling stress (15 °C) conditions. Untreated (dry) seeds were used as a control. Exogenous melatonin treatment promoted pepper seed germination and emergence under chilling conditions. Treatment of seeds with melatonin especially in 1 or 5 µM concentrations significantly improved germination and emergence percentage whereas control seeds and seeds treated with water exhibited the lowest germination and emergence performance. Melatonin application also reduced the MDA and H_2O_2 contents and elevated SOD and CAT enzyme activities. The improvement in germination and emergence performance of pepper under chilling stress conditions following melatonin treatment may therefore be due to reduced lipid peroxidation and elevated activities of antioxidant enzymes.

Keywords: Antioxidant enzymes; *Capsicum annuum*; Chilling stress; Melatonin; Seed treatment

Melatonin Uygulamalarının Üşüme Stresi Altındaki Biber Tohumlarının Çimlenme ve Çıkış Performansı Üzerine Etkisi

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ÖZET

Melatonin bir hayvansal hormon olarak ilk olarak sığır beyin üstü bezinden yarım yüzyılı aşkın bir süre önce izole edilmiş ve daha sonra tek hücreliler, mantarlar, algler, hayvanlar ve bitkiler gibi evrimsel olarak birbirlerinden çok

farklı organizmalarda varlığı kanıtlanmıştır. Bu çalışmada dışarıdan yapılan melatonin uygulamaları ile biberde (*Capsicum annuum* L.) çimlenme sırasında üşüme stresine karşı toleransın arttırılması hedeflenmiştir. Biber tohumları 24 saat süreyle farklı konsantrasyonlarda (0, 1, 5, 10 ve 25 µM) melatonin ile muamele edilmişler ve daha sonra bir gün kurutularak optimum (25 °C) ve üşüme stresi (15 °C) koşullarında çimlenme ve çıkış testlerine tabi tutulmuşlardır. Ekim öncesi tohum muamelesi şeklinde yapılan melatonin uygulamaları ile üşüme stresi koşulları altında biberin tohum çimlenmesi ve fide çıkış performansının olumlu yönde etkilenebileceği görülmüştür. En etkili melatonin konsantrasyonu olarak belirlenen 1 ve 5 µM melatonin uygulamaları sonucunda kontrol uygulamalarına kıyasla çimlenme ve çıkış yüzdeleri ile hızlarının arttığı saptanmıştır. Melatonin uygulamaları fidelerde H_2O_2 ve MDA içeriğini düşürmüş, buna karşılık SOD ve CAT enzim aktivitelerini arttırmıştır. Bu araştırma sonuçlarına dayanarak, biber tohumlarının çimlenme ve fide çıkış performanslarının arttırılmasında antioksidan enzim aktivitelerinin seviyelerindeki artışın neden olduğu dokulardaki lipitlerin peroksidayonunda gerçekleşen bozulmanın azalması olduğu söylenebilir.

Anahtar Kelimeler: Antioksidan enzimler; *Capsicum annuum;* Melatonin; Tohum uygulaması; Üşüme stresi

1. Introduction

Pepper is a warm season vegetable that requires relatively higher soil temperatures for rapid seed germination and seedling emergence and the optimum temperature for germination and emergence is between 25 and 30 ˚C (Lorenz & Maynard 1988). Even though pepper is a vegetable that's been cultivated via transplants, direct seeding is still used in some parts of pepper growing areas such as Kahramanmaraş province of Turkey. When direct seeded in cool soils in the early spring, pepper seed germination could be very erratic and non-uniform and seedling emergence may prolong for several weeks. This erratic and non-uniform seed germination and seedling emergence often results in non-uniform seedlings, poor crop stands, and may require crop replanting. Often, slow stand establishment leads to reduced yields and delayed harvest (losing early high market prices) (Watkins & Cantliffe 1983). Thus, achieving ideal plant stands necessitates rapid and uniform seedling emergence to evade these problems.

Since the isolation from bovine pineal gland for the first time in the late 1950s (Lerner et al 1958), melatonin (N-acetyl-5-methoxytryptamine) was identified in evolutionary distant life forms including bacteria, animals and higher plants (Posmyk & Janas 2009; Tan et al 2012). Although some preliminary findings had been reported (Van Tassel et al 1993; Kolár & Macháckova 1994), the first evidence that melatonin was indeed present in plants independently came from two different group of researchers (Dubbels et al 1995; Hattori et al 1995). In the following years, melatonin in varying quantities has also been detected in different organs of a variety of fruits, cereals, vegetables, and in medicinal herbs (Chen et al 2003; Reiter et al 2007a; Paredes et al 2009; Posmyk & Janas 2009; Korkmaz et al 2014).

Even though the physiological functions of melatonin in plants are still to be definitively established, some functional roles have already been proposed (Tan et al 2012). Recent studies have documented that melatonin is a proven powerful free radical scavenger and a broad spectrum antioxidant in plants (Paredes et al 2009; Tan et al 2012), and provides significant protection against such environmental stresses as cold (Posmyk et al 2009), salinity (Li et al 2012), water (Zhang et al 2013) and excess UV light (Afreen et al 2006). Additionally, melatonin is known to promote growth of roots (Arnao & Hernández-Ruiz 2007) and leaves (Okazaki et al 2010; Wang et al 2013), and it may also serve as the regulator of circadian rhythm and photoperiodic reactions (Kolár et al 1999; Kolár & Macháckova 2005) in plants. In our ongoing research, we previously established the presence of melatonin in different organs of two pepper cultivars and its variation during various growth stages (Korkmaz et al 2014). In this research, our objective was to investigate whether pre-sowing treatment with melatonin would enhance pepper seed germination and subsequent seedling emergence at chilling temperatures.

2. Material and Methods

2.1. Plant material and treatments

Seeds of 'Sena' red pepper (*Capsicum annuum* L.), all from the same seed lot, were obtained from Agricultural Research Institute, Kahramanmaras, Turkey. Seeds were disinfested in 1% (active ingredient) sodium hypochlorite for 15 minutes to eliminate seed-borne microorganisms. Following disinfestation, they were rinsed under running tap water for one minute and surface dried by placing them between paper towels for 30 minutes at room temperature.

Single layer of pepper seeds (5 g), placed in covered transparent polystyrene boxes (10x10x4 cm) on double layers of filter paper and wetted with 20 mL of 0 (distilled water), 1, 5, 10, and 25 µM melatonin (Sigma-Aldrich, MO, USA) solutions, were kept at 20 ˚C in darkness for 24 hours (Karaca 2013). The seeds then were rinsed for 1 minute under running water and left to dry on paper towels for 24 hours under room conditions (20-22 ˚C and 50-60% relative humidity). Due to light sensitive nature of melatonin, all experiments were always carried out under dim light.

2.2. Germination test

Germination test was carried out in darkness in temperature-controlled incubators held at 15±1 ˚C (chilling stress) or 25±1 ˚C (optimum conditions, ISTA 2007). Fifty seeds were placed on two layers of filter paper moistened with 5 mL of distilled water in covered 10 cm petri dishes. To prevent fungal contamination, 1 mL of 0.5% Captan (Koruma, Turkey) was added to the water. Treatments were arranged in completely randomized design with four replications. Untreated dry seeds were taken as dry control. Radicle protrusion to 2 mm was scored as germination. Germination was recorded daily until the numbers stabilized and germinated seeds were removed from the petri dishes. From the total number of seeds germinated, final germination percentage (FGP) and days to 50% of FGP (G_{50}) (Farooq et al 2005), which is an inverse measure of germination rate, were calculated.

2.3. Emergence test

Pepper seeds were treated with melatonin as described above and 40 seeds from each treatment were planted into 1.0 cm depth in 18x9x4 cm (length x width x height) plastic cups filled with growth medium consisting of peat and perlite in the ratio of 4:1. After watering the cups, half of them were placed in a growth room at 15±1 ˚C (chilling stress) while the other half was placed at 25 ˚C (optimum conditions) under cool fluorescent lamps providing a photosynthetic photon flux density of 250 µmol m^{-2} s^{-1} for 16 h day^{-1} at the seedling level. Relative humidity levels varied between 60% and 75% in the growth rooms. The treatments were replicated four times and all the cups were arranged in completely randomized design in the growth chamber. Emergence counts (hypocotyl arch visible) were made daily until the percentage of emerging seedlings had stabilized in all treatments and final emergence percentage (FEP) and days to 50% of FEP (E_{50}) were calculated. When the percentage of emergence had stabilized in all treatments, seedlings were sampled for H_2O_2, malondialdehyde (MDA) and antioxidant enzyme analysis. The seedlings were cut at the medium surface and their fresh weights were recorded.

H_2O_2 content was determined according to the method suggested by Özden et al (2009). Shoot (cotyledons and hypocotyls) samples of 0.25 g were homogenized in 3 mL of 1% (w v^{-1}) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 *g* and 4 ˚C for 10 minute. Subsequently, 0.75 mL of the supernatant was mixed with 0.75 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1.5 mL of 1 M KI. H_2O_2 content of the supernatant was determined by comparing its absorbance at 390 nm to a standard calibration curve. The content of H_2O_2 was calculated from a standard curve plotted in the range from 0.1 to 100 µmol mL^{-1}. H_2O_2 concentration was expressed as µmol g^{-1} FW.

The MDA concentration was determined according to the method of Zhang et al (2005) with some modification. Fresh shoot samples (0.25 g) were homogenized in 3 mL of 10% TCA and centrifuged at 10,000 *g* for 15 minute. The supernatant was collected and 1 mL was mixed with 1 mL of 0.6% thiobarbituric acid. The mixture was boiled at 100 ˚C for 20 min, cooled quickly, and centrifuged at 10,000 *g* for 10 minute after which its absorbance

was measured at 532, 600, and 450 nm. The MDA concentration was calculated by Equation 1.

$$MDA\,(\mu mol\ g^{-1}\ FW) = 6.45\times(A_{532}-A_{600})-0.56\times A_{450} \quad (1)$$

Enzyme extractions were performed as described in Seckin et al (2010). Fresh leaf samples (0.5 g) were rapidly extracted in a pre-chilled mortar on an ice bath with 1.5 mL of ice cold 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA-Na_2 and 2% (w v^{-1}) PVPP. Samples were centrifuged at 14,000 *g* for 20 min, and supernatants were used for the determination of protein content and enzyme activities. Total soluble protein contents of the enzyme extracts were calculated according to Bradford (1976) using bovine serum albumin (BSA) as a standard and the protein concentration was determined from a BSA standard curve. The specific enzyme activity for all enzymes was expressed as in unit mg^{-1} protein.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined using the slightly modified method of Xu et al (2008). One hundred µL of the enzyme extract was added to 2.465 mL of 100 mM phosphate buffer (pH 7.8), 75 µL of 55 mM methionine, 300 µL of 0.75 mM nitroblue tetrazolium (NBT) and 60 µL of 0.1 mM riboflavin in a test tube. The test tubes containing the reaction solution were placed under 2 fluorescent light tubes (40 µmol m^{-2} s^{-1}) for 10 min and their absorbance were measured at 560 nm with a UV/visible spectrophotometer. Blanks and controls were run in the same manner but without illumination and enzyme, respectively. One unit of SOD activity was defined as the amount of enzyme that would inhibit 50% of NBT photo reduction.

Catalase (CAT, EC 1.11.1.6) activity was determined by the method of Çakmak & Horst (1991), which measures the initial rate of disappearance of H_2O_2 at 240 nm. The reaction mixture contained 70 µL crude enzyme extract, 930 µL 50 mM Na-phosphate buffer (pH 7.0) with 0.1 mM EDTA and 3% H_2O_2. The decrease in the absorption was followed for 3 minute and 1 µmol H_2O_2 mL^{-1} min^{-1} was defined as 1 unit of CAT.

Peroxidase (POX, EC 1.11.1.7) activity was measured according to the method described by Herzog & Fahimi (1973). The reaction mixture contained 75 µL crude enzyme extract and 925 µL 3,3-diaminobenzidine-tetra hydrochloride dihydrate solution containing 0.1% (w v^{-1}) gelatine and 150 mM Na-phosphate-citrate buffer (pH 4.4) and 0.6% H_2O_2. The increase in the absorbance at 465 nm was followed for 3 min and one unit of POX activity was defined as µmol H_2O_2 decomposed mL^{-1} min^{-1}.

2.4. Statistical analysis

Data from all experiments were subjected to analysis of variance and mean separation was performed by Fisher's least significant difference (LSD) test if F test was significant at P= 0.05. Germination and emergence percentage data were arcsine transformed before statistical analysis. Experiments were repeated twice and since there was no significant difference between the results of two experiments, data from both experiments were pooled and the mean values are presented (n= 8).

3. Results

3.1. Seed germination

Pre-sowing seed treatment with melatonin significantly improved pepper seed germination under chilling stress conditions compared to dry seeds and seeds treated with 0 µM melatonin which had FGP of 45% and 61%, respectively (Figure 1A).

Even though all melatonin treatments had similar effect on FGP of pepper seeds, 25 µM treatment resulted in the highest FGP (93%). Under optimum conditions, however, melatonin had little or no effect on germination percentage of pepper seeds. Additionally, application of melatonin improved the germination rate of pepper seeds under chilling stress compared to dry seeds (G_{50}= 20.3 days) and 0 µM melatonin treatment (G_{50}= 17.0 days); and the highest germination rate was obtained from seeds treated with 1 µM melatonin (G_{50}= 15.6 days) (Figure 1B). Moreover, application of 1 µM and 5 µM melatonin were also effective in improving germination rate compared to the both control treatments under optimum conditions.

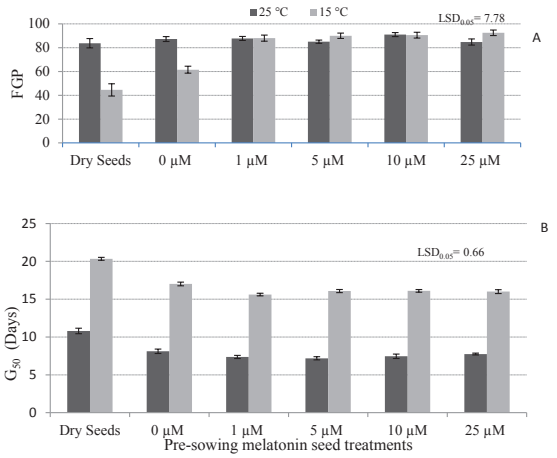

Figure 1- Effect of pre-sowing seed treatment with melatonin on pepper seed final germination percentage (FGP, A) and germination rate (G_{50}, B), under optimum (25 ˚C) and chilling (15 ˚C) conditions. Vertical bars represent mean±SE (n= 8)

Şekil 1- Ekim öncesi tohuma yapılan melatonin uygulamalarının optimum (25 °C) ve üşüme stresi (15 °C) koşullarında biber tohumlarının çimlenme yüzdesi (FGP, A) ve çimlenme hızı (G_{50}, B) üzerine etkileri. Dikey barlar ortalama±standart hatayı temsil eder (n= 8)

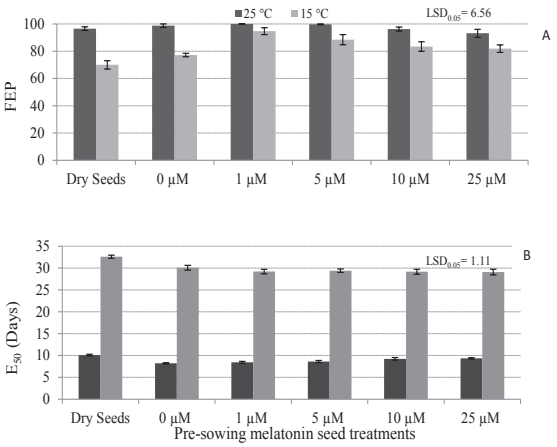

Figure 2- Effect of pre-sowing seed treatment with melatonin on pepper seedling final emergence percentage (FEP, A) and emergence rate (E_{50}, B) under optimum (25 ˚C) and chilling (15 ˚C) conditions. Vertical bars represent mean±SE (n= 8)

Şekil 2- Ekim öncesi tohuma yapılan melatonin uygulamalarının optimum (25 °C) ve üşüme stresi (15 °C) koşullarında biber fidelerinin çıkış yüzdesi (FEP, A) ve çıkış hızı (E_{50}, B) üzerine etkileri. Dikey barlar ortalama±standart hatayı temsil eder (n= 8)

3.2. Seedling emergence

Seed application of melatonin in various concentrations enhanced the FEP of pepper seedlings under chilling conditions (Figure 2A). Treating the seeds with 1 µM (95%) and 5 µM (88%) melatonin increased the emergence percentage significantly under stress conditions compared to dry seeds (70%) and seeds treated with 0 µM (77%), while the FEP of 10 µM and 25 µM melatonin treatments were not statistically different than that of 0 µM melatonin treatment. Under optimum conditions, however, melatonin application prior to sowing did not have any major effect on seedling emergence performance and all treatments exhibited 93% or higher FEP. Moreover, all melatonin treatments enhanced the emergence rate of pepper seedlings compared to seedlings obtained from dry seeds under chilling conditions, while there was no difference in term of emergence rates of melatonin treatments in comparison with 0 µM treatment (Figure 2B). Additionally, no differences in emergence rates were observed between the melatonin treatments and control treatments under optimum conditions.

Imbibing of pepper seeds before sowing in 1 µM melatonin significantly affected seedling shoot fresh weight under optimum conditions while 5 µM melatonin treatment increased shoot fresh weight under chilling stress conditions compared to dry seeds (Figure 3). On the other hand, the fresh weight of seedlings obtained from 0 µM melatonin treatment was similar to those of the rest of melatonin treatments.

Even though all melatonin applications as pre-sowing seed treatment affected the H_2O_2 content of seedlings under chilling stress conditions, 1 µM melatonin application was the only treatment to significantly reduce H_2O_2 content under chilling stress conditions compared to both control

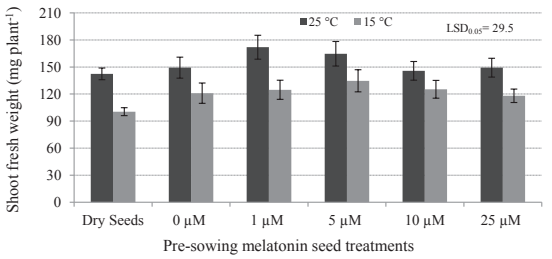

Figure 3- Effect of pre-sowing seed treatment with melatonin on pepper seedling shoot fresh weight under optimum (25 ˚C) and chilling (15 ˚C) conditions. Vertical bars represent mean±SE (n= 8)

Şekil 3- Ekim öncesi tohuma yapılan melatonin uygulamalarının optimum (25 °C) ve üşüme stresi (15 °C) koşullarında biber fidelerinin taze ağırlığı üzerine etkileri. Dikey barlar ortalama±standart hatayı temsil eder (n= 8)

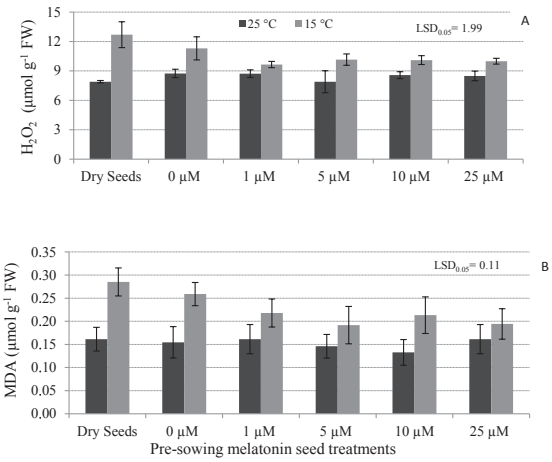

Figure 4- Effect of pre-sowing seed treatment with melatonin on H_2O_2 content (A) and MDA concentration (B) of pepper seedlings under optimum (25 ˚C) and chilling (15 ˚C) conditions. Vertical bars represent mean±SE (n= 8)

Şekil 4- Ekim öncesi tohuma yapılan melatonin uygulamalarının optimum (25 °C) ve üşüme stresi (15 °C) koşullarında biber fidelerinin H_2O_2 (A) ve MDA (B) içerikleri üzerine etkileri. Dikey barlar ortalama±standart hatayı temsil eder (n= 8)

treatments (Figure 4A). Moreover, though not statistically significant, melatonin pre-treatments also considerably lowered the MDA content of seedlings subjected to chilling stress, and of the melatonin concentrations tested, pre-treatment of the seeds with 5 µM reduced the MDA content of the seedlings the most (Figure 4B). Additionally, there were no significant differences among the treatments in terms of H_2O_2 and MDA contents under optimum conditions.

Moreover, pre-sowing seed treatment with increasing concentrations of melatonin significantly increased SOD enzyme activity under chilling stress conditions (Figure 5A). Seedlings obtained from 1 µM, 10 µM and 25 µM melatonin treatments exhibited higher SOD enzyme activity than the seedlings of 5 µM melatonin pre-treatment which had similar enzyme activity as those of dry seed and 0 µM melatonin treatments. Similarly, pre-treatment of seeds with 5 and 25 µM melatonin also significantly increased the CAT enzyme activity in seedlings compared to two control treatments under chilling stress conditions (Figure 5B). In the plants raised under optimum conditions, however, melatonin application as seed treatment did not alter the activities of SOD and CAT enzymes except that seedlings obtained from 1 µM melatonin treatment exhibited significantly higher SOD enzyme activity than both control treatments. Additionally, even though chilling stress enhanced the POX enzyme activity of pepper seedlings compared to optimum conditions, there were no significant differences among treatments in terms of POX enzyme activity of seedlings raised under both conditions (Figure 5C).

4. Discussion

Chilling stress is one of the most important abiotic stresses hindering seed germination and seedling emergence of crops that are native to tropics or subtropics. In this research, we found that germination performance of pepper seeds was found to be adversely affected by chilling stress and chilling-induced impairment of pepper seed germination has already been reported previously by a number of studies (Sachs et al 1980;

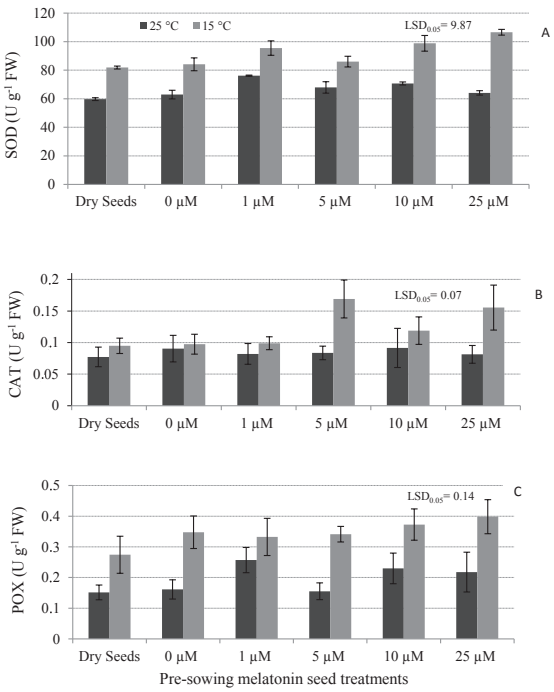

Figure 5- Effect of pre-sowing seed treatment with melatonin on SOD (A), CAT (B), and POX (C) enzyme activity of pepper seedlings under optimum (25 ˚C) and chilling (15 ˚C) conditions. Vertical bars represent mean±SE (n= 8)

Şekil 5- Ekim öncesi tohuma yapılan melatonin uygulamalarının optimum (25 °C) ve üşüme stresi (15 °C) koşullarında biber fidelerinin SOD (A), CAT (B) ve POX (C) enzim aktiviteleri üzerine etkileri. Dikey barlar ortalama±standart hatayı temsil eder (n= 8)

Korkmaz & Korkmaz 2009). Recently, Posmyk et al (2009) found that pre-treatment of seeds with melatonin during priming significantly enhanced the germination of cucumber seeds during chilling stress. Similarly, Tiryaki & Keles (2012) reported that melatonin application markedly reversed the inhibitory effects of light and high temperature in *Phacelia tanacetifolia* seed germination. Our results also showed strong evidence that melatonin has the ability to relieve the adverse effects of chilling stress on pepper seed germination. Pre-sowing seed treatment with melatonin improved significantly germination (Figure 1A and 1B) and emergence (Figure 2A and B) performance along with seedling growth (Figure 3) under chilling stress conditions.

Oxidative stress is considered as the main detrimental factor in seeds or plants subjected to a variety of abiotic stresses including chilling stress. Free radical accumulation and peroxidation of lipids in cellular membranes results in impaired membrane function, reduced fluidity, and inactivation of membrane-bound enzymes and receptors (Bewley et al 2013). Numerous studies have demonstrated that tolerance to chilling in various species results from an elevated antioxidant defense system (Hodges et al 1997; Kang & Saltveit 2002; Gill & Tuteja 2010). Damage caused by peroxidation of lipid portions of biological membranes might be decreased or avoided by protective mechanisms involving free radical and peroxide-scavenging enzymes such as SOD, CAT and POX. SOD is the enzyme that is involved in dismutation (or partitioning) of the superoxide ($O_2^{\cdot-}$) radical into either ordinary oxygen (O_2) molecule or hydrogen peroxide (H_2O_2) (Bowler et al 1992) whereas CAT along with POX catalyzes the decomposition of hydrogen peroxide to water and oxygen (Scandalios et al 1997; Dey et al 2007). Thus, the induction of a protective mechanism to reduce oxidative damage triggered by stress through boosting the activities of antioxidative enzymes could be characteristics of elevated levels of tolerance to abiotic stress conditions (Gill & Tuteja 2010).

Since the discovery of melatonin in plants, extensive research has been carried out to identify its physiological roles in plants. It has been postulated that melatonin may serve as a photoperiodic and circadian rhythm regulator as well as a universal antioxidant because of its wide distribution in fungi, algae, bacteria, animals and plants (Paredes et al 2009; Tan et al 2012). Melatonin and its wide variety of metabolites are known as endogenous free radical scavengers and potent broad-spectrum antioxidants, and may directly scavenge H_2O_2, maintaining intracellular H_2O_2 concentrations at constant levels (Tan et al 1993; Tan et al 2000; Reiter et al 2007b). We observed considerable

reduction of H_2O_2 concentration (Figure 4A) and MDA accumulation (Figure 4B) by melatonin application, which may have resulted from direct free radical scavenging by melatonin and enhanced antioxidant enzyme activities. Similarly, Posmyk et al (2009) reported that seed application of melatonin in cucumber seeds germinated under chilling conditions provided significant protection of membrane structures against peroxidation and MDA accumulation. Our results also clearly indicate that melatonin is involved in boosting the activities of the antioxidant enzymes, signifying their essential role in providing antioxidative defense under chilling stress conditions. Melatonin was involved in free radical scavenging in chilling-stressed pepper seeds and seedlings since SOD, CAT and to some degree POX showed significant rises in activity with the melatonin treatment under chilling conditions (Figure 5A-C). Confirmatory findings have also been reported by Zhang et al (2013) who showed increased levels and activities of the antioxidant free radical scavenging enzymes, i.e., SOD, CAT, and POX by melatonin application in cucumber seeds germinated under drought stress conditions.

In summary, the result of the present study revealed that seed application of 1 µM or 5 µM melatonin significantly improved pepper seed germination and seedling emergence at chilling temperatures compared to seeds not treated with melatonin. Seed treatment with melatonin may be an effective way to shorten the time of emergence and improve the stand establishment in pepper during early spring sowings. The fact that melatonin, a broad spectrum antioxidant, could be used to prevent crop losses due to chilling temperatures may have a significant practical application. It is also possible to deduce from the results of current study that melatonin application enhanced the germination and emergence performance of pepper seeds by protecting the membranes against free radical destruction through enhancing antioxidant enzyme activities and by stabilizing the membranes during chilling stress.

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