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Effects of Melatonin on Tomato Infected with Root-knot Nematode *Meloidogyne incognita*

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ABSTRACT
This study was conducted in 2020 to investigate the influence of melatonin (10, 50 and 100 μM) given in three methods (immersion, irrigation, and foliar spraying) on some physiological aspects of tomato seedlings inoculated with root-knot nematode *Meloidogyne incognita* [(Kofoid and White) Chitwood]. The seedlings were inoculated with 1000 second-stage juveniles of *M. incognita*. Dualex® optic sensor was used to in situ measure total chlorophylls, flavonols, anthocyanins contents and nitrogen balance index (NBI). Results indicated that while no significant effects were observed on chlorophyll content, melatonin ameliorated the adverse effects of *M. incognita* on chlorophyll depending on the concentration and mode of application. Flavonols were at the highest in the irrigated plants and the lowest in the immersed ones. NBI was affected by the method the melatonin was applied, and immersing boosted it while irrigation caused a significant decrease. Irrigation resulted in significantly higher anthocyanins compared to the other two methods. One observation was that applying melatonin in the low and medium concentrations to the soil containing nematode increased the anthocyanin content of the plant. Melatonin merits a value in developing a response against the nematode but needs further elucidation.

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Melatoninin Kök-ur Nematodu *Meloidogyne incognita* ile İnfekteli Domates Üzerindeki Etkileri

ÖZET
Bu çalışma 2020 yılında üç yöntem ile (daldırma, sulama ve spreyleme) verilen melatoninin (10, 50 ve 100 μM) kök-ur nematodu *Meloidogyne incognita* [(Kofoid and White) Chitwood] uygulanan domates fidelerinin bazı fizyolojik yönleri üzerindeki etkisini araştırmak için yürütülmüştür. Fidelere 1000 adet ikinci dönem *M. incognita* larvası inoküle edilmiştir. Toplam klorofil, flavonol ve antosiyanin içerikleri ile azot balans indeksini (NBI) ölçmek için Dualex® klorofilmetre kullanılmıştır. Sonuçlar, klorofil içeriği üzerinde önemli bir etki olmadığını, melatoninin konsantrasyona ve yönteme bağlı olarak *M. incognita*'nın klorofil üzerindeki olumsuz etkilerini iyileştirdiğini göstermiştir. Flavonoller, sulama ile verilen bitkilerde en yüksek, daldırma ile verilenlerde ise en düşük olmuştur. NBI, melatoninin uygulanma yönteminden etkilenmiş, daldırma ile artış, sulama ile ciddi bir düşüş göstermiştir. Sulama, diğer iki yönteme kıyasla önemli ölçüde yüksek antosiyaninlere sebep olmuştur. Nematodlu bir toprağa düşük ve orta konsantrasyonlarda melatonin uygulanmasının antosiyaninleri arttırdığı gözlemlenmiştir. Melatonin, nematodlara karşı tepki geliştirmede önemli olup daha fazla çalışmaya ihtiyaç duyulmaktadır.

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INTRODUCTION

Pathogen attacks are one of the growth-and quality-limiting factors plants face throughout their lives. Ameliorating or completely evading these stress factors rely on plant's innate resistance capacity as well as the favorable support from environment. Nematodes, especially root-knot nematodes (RKN) are one of the major pests that use hundreds of plants as hosts. Four predominant species of *Meloidogyne* species are *M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*. Symptoms shown by plants after nematode infection are loss of chlorophyll in leaves, nutrient deficiency leading to growth loss, and plant death if persisted. Due to damage in vascular tissues of root, water and nutrients can fail to move through the plant (Ralmi et al., 2016).

Despite that RKNs could be managed with the use of chemical nematicides, increasing crop production in an ecologically friendly manner is both a necessity and a challenge in a world with increasing awareness in human and environment health (Collange et al., 2011). In the recent years, melatonin, a naturally existing compound in plants, has attracted an interest in sustainable crop production due to its beneficial effect on human health and environment (Reiter et al., 2015; Qiao et al., 2019). Melatonin functions as an antioxidant against reactive oxygen and nitrogen species (Moustafa-Farag et al., 2020).

In recent years, melatonin research have focused on its effects on plant growth and development along with its protective role against abiotic stress factors (Sharif et al., 2018; Yu et al., 2018). However, its effects on helping plants cope with biotic stress factors have not been explored in as much detail. The effects of melatonin against fungal pathogens in apple, banana, potato, strawberry, and tomato were reported (Yin et al., 2013; Wei et al., 2017; Zhang S. et al., 2017; Aghdam et al., 2017; Liu et al., 2019). It was reported that protection comes from increase in defense gene expression, scavenging reactive oxygen species, raise in nitric oxide production and cell wall thickening (Shi et al., 2015; Wei et al., 2018; Zhao et al., 2019). However, to our best knowledge, there is not a study that involves influence of melatonin against root-knot nematode attacks on plants.

Early assessment of stress symptoms is possible with non-destructive monitoring of crops using optical or thermal sensors. *In situ* and *de novo* changes in the physiological aspects of plant development can enable to have the results quickly and periodically with ease (Padilla et al., 2014). Among these sensors is Dualex® (Orsay, France), a leaf-clip chlorophyll-meter which estimates chlorophyll (CHL) and leaf epidermal flavonoids as well as anthocyanins using CHL fluorescence screening method (Agati et al., 2016). Non-destructive optical tools use spatial and temporal dimensions to assess adaptability levels of plants under stress conditions (Barnes et al., 2015). Dualex® have been used for many abiotic stress related studies for early detection of symptoms, for instance, heat (Zhou et al., 2017), chilling (Oustric et al., 2017) and UV (dos S. Nascimento et al., 2020). In more recent years, it has been also utilized for grapevine leaf stripe disease (Di Gennaro, 2016), root-knot nematodes in eggplant (Silva-Sánchez et al., 2019), reniform nematode in cotton (Singh et al., 2020) and wheat stripe rust (Emebiri et al., 2020).

This study was conducted to assess the effects of melatonin applied by immersion, irrigation, and spraying on some physiological characteristics of tomato plants inoculated with *M. incognita.*

MATERIALS and METHODS

Seedlings of commercially tomato (*Lycopersicon esculentum* L.) cv. H2274 with 3-4 leaves were used as the plant material. Melatonin purchased from Merck (M2250) was prepared in three concentrations (10, 50 and 100 μM).

Designing the Experiment

Seedlings were separated in three groups according to the application method of the melatonin solutions (root-immersion, root-irrigation, and foliar spraying). The trial also had one negative control (distilled water), and one positive control (nematode inoculation only). Details regarding the methods and melatonin applications were given below.

Root-immersion: The roots of the seedlings were washed free of peat under tap water and dried with a paper towel. Then, the roots were immersed in a container with melatonin solution for 10 min. In control groups, distilled water was used instead of melatonin.

Root-irrigation: The seedlings were irrigated with the melatonin solutions at an amount of 10 ml/pot. Second (20 ml/pot) and third (40 ml/pot) applications were done 1 week apart. Control groups were irrigated with distilled water.

Foliar-spraying: After transplanting, the leaves of the seedlings were sprayed with 10 ml melatonin solution. Second (20 ml/pot) and third (40 ml/pot) applications were done 1 week apart. Distilled water was used as control.

The plants in 1.4-liter plastic pots contained sterilized soil and sand mixture (approx. 450 g) and were placed in a growth chamber under the conditions of 27±2°C and 18/6 h photoperiod for 8 weeks. Plants were irrigated as needed (50 ml per pot).

Nematode Inoculation

Second stage juveniles (J2s) of *M. incognita* were inoculated as 1000 J2s/pot in all applications immediately after melatonin applications in the spray and irrigation methods. Root-immersed plants were inoculated 24 hours after the transplantation.

Optical Measurements

At 56 days after nematode inoculation, optical measurements with the Dualex© Scientific+ Chlorophyll and Polyphenol-Meter (Force-A, Centre Universitaire Paris-Sud, France) were performed on the third youngest leaf. The portable meter Dualex® allowed simultaneous readings from the abaxial and adaxial surface of the leaves and provided a mean value for each reading. Three readings per leaf away from the midrib were made. Features measured were chlorophyll (μg per cm^2), relative absorbance units of flavonols (0 to 3) and anthocyanins (0 to 1.5), and the nitrogen balance index, determined by the relationship between chlorophyll and flavonols.

Statistical Analysis

The experiment was arranged in a completely randomized design with three replications. The data of chlorophyll, flavonols, anthocyanins and nitrogen balance index (NBI) were implemented on R statistical package program (version 4.0.2; 2020-06-22) (Pallmann and Hothorn, 2016). In R analyses the differences between the applications are made according to the general mean. The groups that are out of the upper and lower limits are statistically more important than the other groups.

RESULTS and DISCUSSION

Melatonin used in three application modes (i.e., root-immersion, root-irrigation, and foliar-spraying) resulted different responses in chlorophyll, flavanols, anthocyanins and nitrogen balance index in tomato plants inoculated with *M. incognita.*

Although application methods of melatonin were not affected the CHL amounts (Figure 1), the plants that applied root-immersion had more CHL than the irrigated or sprayed ones. The addition of nematodes resulted in 40-50% reduction in positive control plants compared to negative controls.

Plants treated with Melatonin resulted in as much CHL, depending on the concentration, as in the distilled water treated ones. It was observed that Melatonin in the nematode inoculated plants ameliorated the adverse effects on CHL depending on the concentration and mode of application, for instance immersing in 100 μM Melatonin or spraying with 50 μM Melatonin, provided increased levels of CHL. It is believed that primary site for melatonin production is chloroplasts (Martinez et al., 2018). Weeda et al. (2014) stated that melatonin can act as a protectant of CHL content. Wang et al. (2013) also reported decreased levels of chlorophyll-degrading enzyme, pheide-a-oxygenase with melatonin. Zhang et al. (2014) expressed that melatonin, when applied exogenously, protected CHL in the apple leaves exposed to abiotic stress. Yin et al. (2013) showed that apple plants irrigated with melatonin had comparably close contents of chlorophyll to the control plants when infected with *Marssonina* apple blotch. Sun et al. (2019) indicated that melatonin treated cucumber against downy mildew had increased levels of chlorophyll. Similar activity was observed in this research where tomato plants exposed to nematode damage.

Plants respond to stress factors by synthesizing flavonols (Brunetti et al., 2013). R analysis indicated that the application mode of melatonin had a significant influence on flavonol production in the tomato plants (Figure 2). They were the highest in the irrigated plants, ranging from 0.9 to 1.5 and the lowest in the immersed ones, between 06-1.1. Flavonoids are formed when plants are exposed to pests (Brunetti et al., 2013). Bali et al (2018) stated that flavonoid contents rose in the seedlings of tomato plants treated with jasmonic acid against *M. incognita*. Although no significant effects of melatonin were detected in the present study, how it is received by the intact plants showed a clear importance.

The anatomical structure of the leaves (i.e., trichomes and undulations on the surface) might be a possible reason for the lowest amount of flavonols in spraying. The studies have shown that flavonoids are placed in epidermal layers or in the cuticle of leaves which might have been a barrier for the solution to infuse (Tattini et al., 2004).

In the current study, NBI level was affected by the application method of that melatonin, and immersing increased it while irrigation caused a significant decrease (Figure 3 2b). It is considered a general acceptance that plant nitrogen status of a plant can be estimated through chlorophyll and flavonoid contents (Agati et al., 2016). Because nitrogen is incorporated in chlorophyll (Evans et al., 2001), and flavonoids contents act oppositely to N contents in the plant, the ratio of CHL to flavonols is shown to be a more sensitive indication of plant nitrogen status (Longchamps and Khosla, 2014; Padilla et al., 2014). Kautz et al. (2014) indicated that under saline conditions the tomato leaves had elevated levels of NBI. Sun et al. (2019) reported increased levels of enzymes in N metabolism in cucumber seedlings treated with melatonin against *Pseudoperonospora* *cubensis*. The plots of both CHL and NBI show that root-immersion and spraying were suppressed the adverse effects of *M. incognita*. Scientific evidence indicates that growth and development in plants are closely regulated by auxin and melatonin sharing a precursor with auxin might aid same processes (Arnao and Hernandez-Ruiz, 2007). Direct contact with melatonin in the roots through immersion for 10 min. might have induced level of auxin and improved root activity (Chen et al., 2009) and efficiency in nitrogen uptake and metabolism (Zhang et al., 2017).

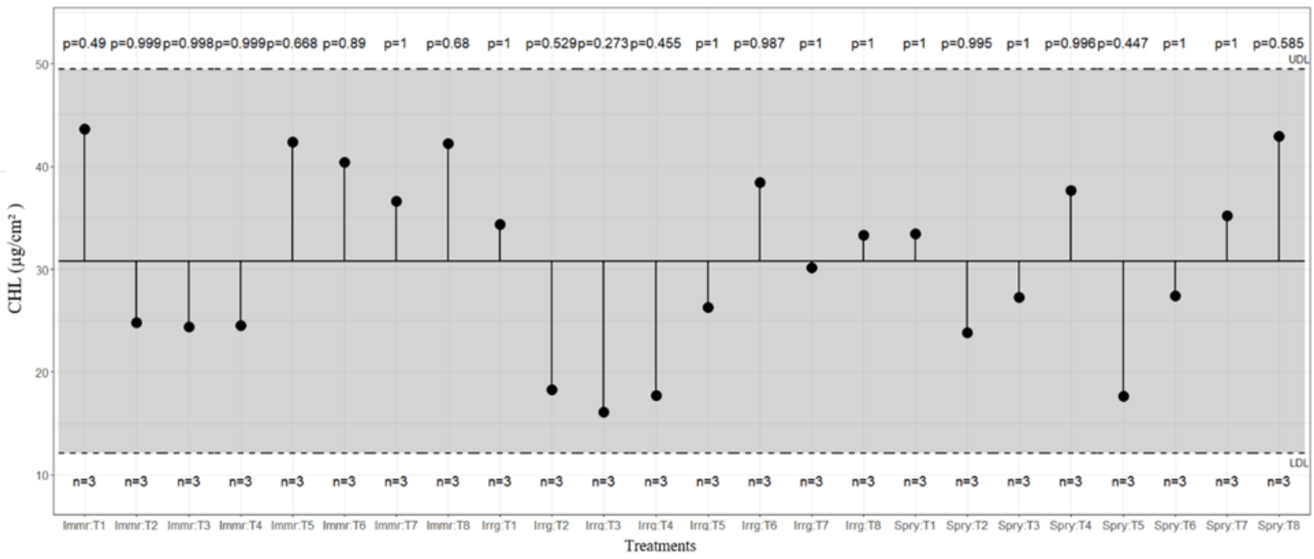

Figure 1. Effects of melatonin concentrations on chlorophyll content (CHL) of tomato plants inoculated with *Meloidogyne incognita* (Immr: immersion, Irrg: irrigation, Spry: foliar spraying, T1: distilled water, T2: distilled water+nematode, T3: 10 µM melatonin+nematode, T4: 50 µM melatonin+nematode, T5: 100 µM melatonin+nematode, T6: 10 µM melatonin, T7: 50 µM melatonin, T8: 100 µM melatonin)

Şekil 1. Melatonin konsantrasyonlarının Meloidogyne incognita uygulanan domates bitkilerindeki klorofil içeriğine olan etkileri (Immr: daldırma, Irrg: sulama, Spry: spreyleme, T1: saf su, T2: saf su+nematod, T3: 10 µM melatonin+nematod, T4: 50 µM melatonin+nematod, T5: 100 µM melatonin+nematod, T6: 10 µM melatonin, T7: 50 µM melatonin, T8: 100 µM melatonin)

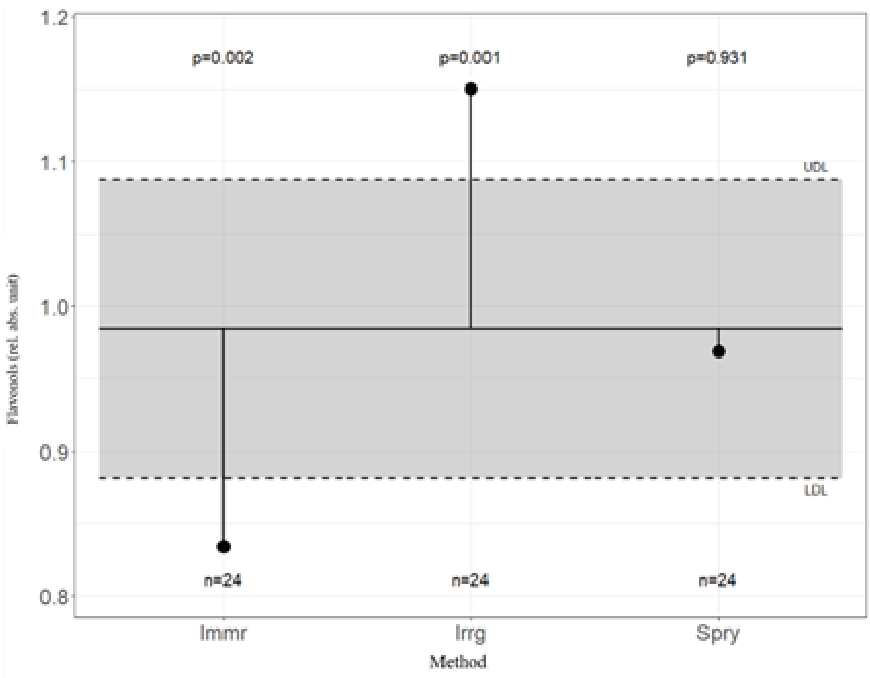

Figure 2. Effects of melatonin application methods on flavonols of tomato plants inoculated with *Meloidogyne incognita* (Immr: immersion, Irrg: irrigation, Spry: foliar spraying)

Şekil 2. Melatoninin veriliş yöntemlerinin Meloidogyne incognita uygulanan domates bitkilerindeki flavonoller üzerindeki etkileri (Immr: daldırma, Irrg: sulama, Spry: spreyleme)

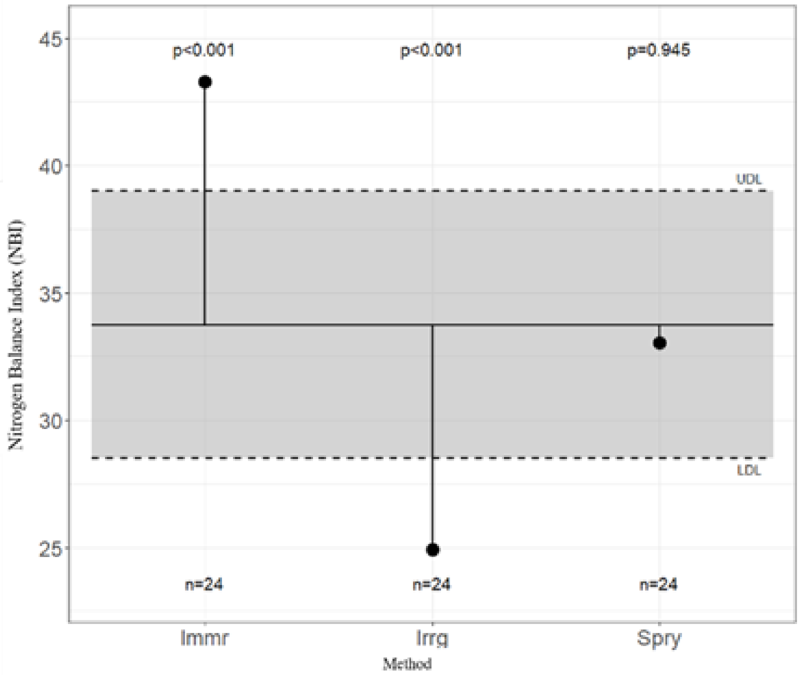

Figure 3. Effects of melatonin application methods on Nitrogen Balance Index (NBI) of tomato plants inoculated with *Meloidogyne incognita* (Immr: immersion, Irrg: irrigation, Spry: foliar spraying)

Şekil 3. Melatoninin veriliş yöntemlerinin Meloidogyne incognita uygulanan domates bitkilerindeki Nitrogen Balance Indeksi (NBI) üzerindeki etkileri (Immr: daldırma, Irrg: sulama, Spry: spreyleme)

Anthocyanins were affected both by the concentrations and the application methods (Figure 4, 5). Irrigation resulted in significantly higher anthocyanins compared to the other treatments (Figure 4). Plants having nematode but no melatonin (T2) produced the highest amounts of anthocyanins (Figure 5). The rest of the treatments found in the same group showing similar values. One observation was that applying melatonin in the low and medium concentrations to the nematode-inoculated plants increased the anthocyanins. On the other hand, Melatonin alone seemed not supportive of the anthocyanin production. Although response of anthocyanin accumulation under stress conditions differed in plants, melatonin appeared to have an increasing effect on nematode-inoculated tomato in the current study compared to the uninoculated ones. However, anthocyanin accumulation decreased when its concentration was increased. similar observation was confirmed by Zhang et al. (2016) on cabbage seeds. Bali et al. (2018) reported increased levels of anthocyanins in the tomato plants after jasmonic acid application, indicating that signaling molecules have a stimulating effect on antioxidative defense system.

CONCLUSION

Root-knot nematodes have been a subject of plant survival studies due to their countless number of hosts and above ground symptoms costing a loss in yield and in extreme cases, plant's life. Using synthetic chemical-based compounds for protection has raised environmental and public health safety, therefore a tendency to utilize more friendly approaches increases. Melatonin, being a safe molecule both present in humans and plants, is becoming a center of studies for its regulatory and supporting roles in plant's growth and development.

Results of this study showed that there was no significant effect on the chlorophyll content, but melatonin ameliorated the adverse effects of *M. incognita* on chlorophyll depending on the concentration and method. Flavonols were highest in plants given melatonin by irrigation and lowest in plants given by immersion. NBI was affected by the application method of melatonin, increased by immersion, and decreased by irrigation. Irrigation resulted in significantly higher anthocyanins compared to the other two methods. It has been observed that the application of low and medium concentrations of melatonin to nematode-infested soil increases anthocyanins.

Briefly, findings of this research indicate that it merits a value in developing a response against the nematode but needs further elucidation. This response might come from protection of chlorophyll content, therefore preserving nitrogen balance in the plant, as well as stimulating defense mechanisms. Different concentrations with varying exposure time are needed to elucidate these responses.

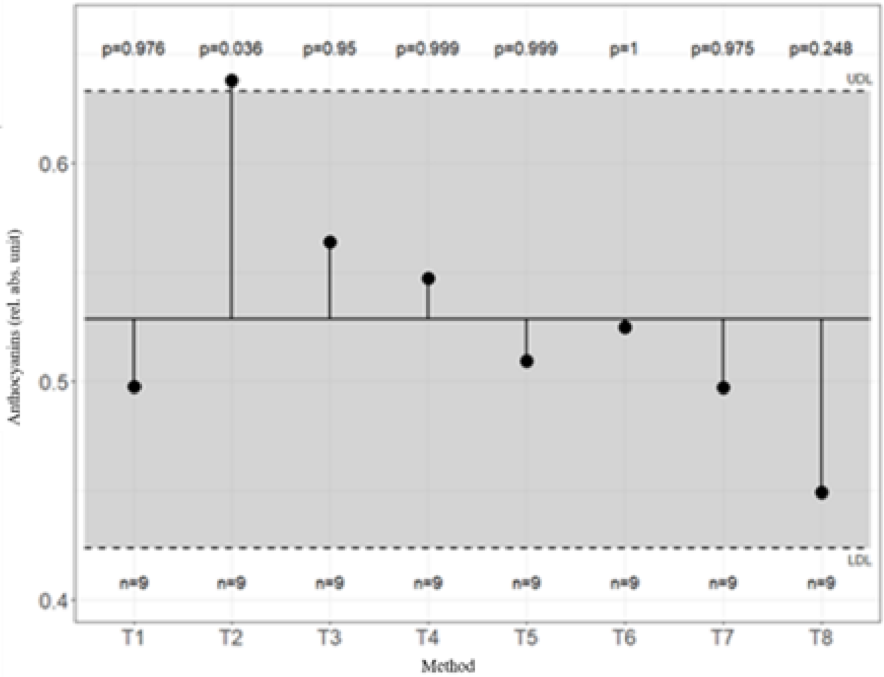

Figure 4. Effects of melatonin concentrations on anthocyanins of tomato plants inoculated with *Meloidogyne incognita* (Immr: immersion, Irrg: irrigation, Spry: foliar spraying, T1: distilled water, T2: distilled water+nematode, T3: 10 µM melatonin+nematode, T4: 50 µM melatonin+nematode, T5: 100 µM melatonin+nematode, T6: 10 µM melatonin, T7: 50 µM melatonin, T8: 100 µM melatonin)

Şekil 4. Melatonin konsantrasyonlarının Meloidogyne incognita uygulanan domates bitkilerindeki antosiyaninler üzerindeki etkileri (Immr: daldırma, Irrg: sulama, Spry: spreyleme, T1: saf su, T2: saf su+nematod, T3: 10 µM melatonin+nematod, T4: 50 µM melatonin+nematod, T5: 100 µM melatonin+nematod, T6: 10 µM melatonin, T7: 50 µM melatonin, T8: 100 µM melatonin)

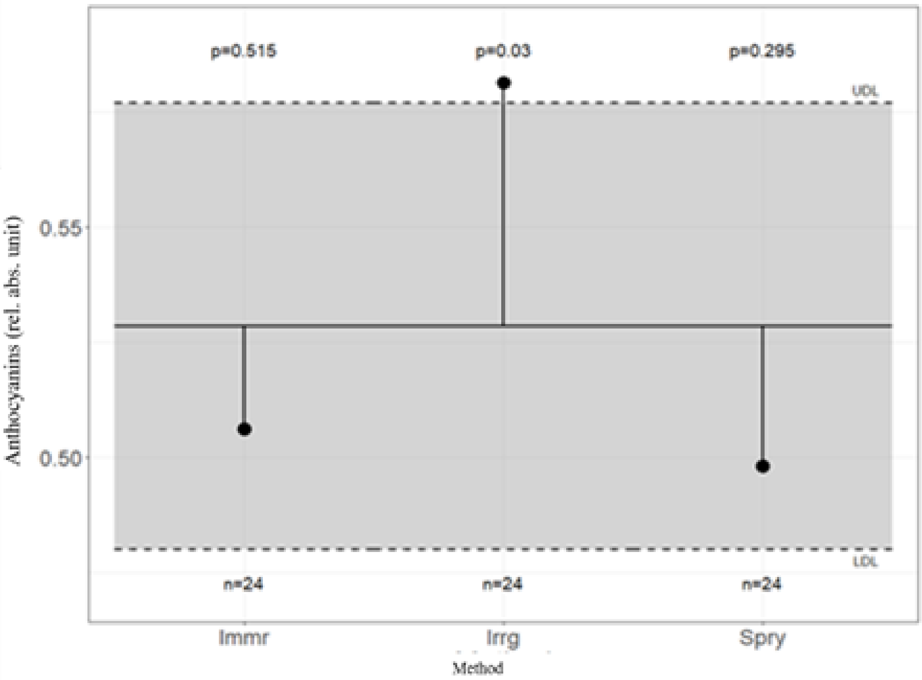

Figure 5. Effects of melatonin application methods on anthocyanins of tomato plants inoculated with *Meloidogyne incognita* (Immr: immersion, Irrg: irrigation, Spry: foliar spraying)

Şekil 5. Melatoninin veriliş yöntemlerinin Meloidogyne incognita uygulanan domates bitkilerindeki antosiyaninler üzerindeki etkileri (Immr: daldırma, Irrg: sulama, Spry: spreyleme)

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Researchers Contribution Rate Declaration Summary

The contribution of the authors is equal.

Conflicts of Interest Statement

Authors have declared no conflict of interest.

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