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The effects of melatonin application on the drought stress of different citrus rootstocks

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Abstract: Drought is among the most crucial limiting factors for crop production and is considered a major threat to food security. Rootstock selection is an important approach for drought management. This research was aimed to study the responses of important rootstocks against drought stress and to examine the impacts of melatonin application on the drought stress of these rootstocks. Carrizo citrange, fying dragon, and sour orange were selected for the current study. To test the drought stress, three different irrigation regimes were applicated separately to each rootstock as 100%, 75%, and 50% of water requirement. The melatonin was then tested on each rootstock*irrigation plot with four different doses (0.5 mM, 1.0 mM, 1.5 mM, and 2 mM) and an untreated control application. Results suggested that melatonin application significantly increases the root and shoot growth of the test rootstocks. Besides that, the highest root length was noted from a Flying dragon, while the Carrizo citrange had the highest shoot height. As a response to the drought stress, the leaf membrane permeability of rootstocks increased and the relative water contents decreased. Both traits were noted to be improved by the melatonin application. In terms of the leaf relative water content and membrane permeability, the Flying dragon rootstock was observed to be more tolerant to drought as compared with the Carrizo citrange and sour orange. External melatonin application was also observed to reduce the proline content in the leaves under drought stress, which is a sign of improvement of the crop's tolerance to drought.

Key words: Carrizo citrange, Flying dragon, sour orange, root growth, membrane permeability, leaf area

1. Introduction

Insufficient precipitation, high temperatures, and excessive use of water cause loss of water resources and a prolonged shortage of available water is defined as drought (Bullock et al., 2018). One of the most important reasons for drought is global warming (Magadza, 2000). Drought is among the most crucial limiting factors for crop production which is considered a major threat to food security on the earth (Kang et al., 2009). Drought stress slows the crop root/shoot growth, delays development & maturation, and significantly decreases the crop yield (Lesk et al., 2016). Management of drought has to start well before the occurrence of drought (Reddy et al., 2021). Adaptive approaches have to be incorporated in agricultural activities to reduce the impacts of climate change and help to ensure sustainability in production (Kumar, 2016). There are several approaches for managing drought. Selection of resistant crops, selection of drought-tolerant rootstocks, crop breeding, use of growth retardants, use of antitranspirants, and proper irrigation & nutrition are among the important approaches for drought management (Parray et al., 2017; Kahramanoğlu et al., 2020).

Drought stress has the largest share (about 26%) among the stress factors of the crops (Maggio et al., 2000). Drought stress may cause drastic changes in the physiological activity and biochemical compositions of plants (Okunlola et al., 2017). As an initial response to drought, most of the plants produce reactive oxygen species (ROS) mainly hydrogen peroxide (H_2O_2) (Bhargava and Sawant, 2013). A prolonged shortage of water causes destruction in membrane structure and increases permeability (Cheng et al., 2018). "Drought avoidance" and "drought tolerance" are the two separate mechanisms which help plants to adapt to drought conditions. The ability of plants to

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regulate some morphological structures for reducing water loss or increasing water uptake is known as "drought avoidance" (Mansuri et al, 2018). Moreover, the enzymatic and nonenzymatic protection mechanism of plants which help to alleviate the negative impacts of ROS is known as the "drought tolerance". Improved osmotic regulators (increase in prolin content), antioxidant enzymes (such as superoxide dismutase (SOD) and ascorbate peroxidase (APX)) and secondary metabolites (i.e. flavonoids (FC) and total phenolic contents (TPC)) can protect the membranes and encourage water uptake of the plants (Cheng et al., 2018; Gao et al., 2020). Moreover, abscisic acid (ABA) also has an important role in drought resistance, which regulates the stomatal closure of the leaves (Ban et al., 2017).

Drought tolerant rootstocks have an important role in the management of drought in horticultural crops (Tworkoski et al., 2016). Rootstock can help to regulate the carbon and water fluxes of scion leaves and improve vegetative growth by regulating stomatal openings and improving the absorption of water and nutrients (Han et al., 2019; Cao et al., 2019). Rootstocks can change tolerance, resistance, or susceptibility to biotic and abiotic stress conditions, and also affect traits like vigor, productivity, fruit quality, and plant longevity (Vacante and Gerson, 2012). One of the most important and widely consumed fruits crops on the earth is citrus fruits, which are grown all over the world but well suited to be cultivated in Brazil, China, the USA, South Africa, India, and Mediterranean countries. The unique flavour and high nutritional contents make it a very important fruit for the human diet (Albertini et al., 2006).

There are significant differences in both physiological and morphological characteristics of the root system of rootstocks used for citrus trees (Robles et al., 2017). These factors, especially in the case of soil water scarcity, affect the uptake of water and mineral matter, thus determining the viability of the scion grafted on the rootstock and its tolerance to water stress (Pérez-Pérez et al., 2008; Robles et al. 2017). The most commonly used method in citrus cultivators for the improvement of tree growth, fruit quality, and fruit yield is the use of different rootstocks. Flying dragon, Carrizo citrange, and sour orange are among the most important rootstocks for citrus species. Flying dragon (*Poncirus trifoliate* var. Monstrosa) rootstock (synonym: hardy orange) is one of the few rootstocks with very good results in terms of tree growth, fruit quality, and yield (Mademba-Sy et al., 2012). Flying dragon improves tissue hardiness in drought conditions and provides better performance under drought stress (Gonçalves et al., 2016). Flying dragon rootstock is a rootstock that provides good yield and fruit quality with good size control. Sour orange (*Citrus aurantium* L.) was the most common rootstock, until the observation of its sensitivity to tristeza and destroy of more than 85 million citrus trees (until 2000) grafted on sour orange by citrus tristeza virus (CTV) (Cambra et al., 2000). Citrus rootstock is a deep, moderately branching, less dense rootstock (Morales Alfaro et al., 2021). It is well adapted to different soil types, including calcareous soils (Morales Alfaro et al., 2021). High-quality fruit (good flavor, desired size, and thin peel) is obtained in varieties grafted on sour orange rootstock (Morales Alfaro et al., 2021). As a consequence of the hazard caused by CTV, several studies had been performed and Carrizo citrange (*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osb.) was considered as resistant to CTV (Pina et al., 2000), and have been widely used since then. Carrizo citrange rootstock provides good performance than many other rootstocks in terms of plant growth, yield, and fruit quality, under normal conditions. However, it is more sensitive than sour orange against drought and salinity (Ribeiro et al., 2014).

A naturally-occurring compound, melatonin (N-acetyl-5-methoxytryptamine), which was first discovered at the bovine pineal gland in 1958 (Lerner et al., 1958) is now very famous in plant science studies. Although its impacts on the plants had not been studied well (Wei et al., 2015), it is reported to improve the plant performance under several abiotic and biotic stress conditions including heavy metals (Tan et al., 2007), salinity (Li et al., 2012), cold (Lei et al., 2006), high temperature (Baghurst and Coghill, 2006), drought (Zhang et al., 2013; Arnao and Hernandez-Ruiz, 2014; Zhang et al., 2015; Imran et al., 2021) and pathogens (Yin et al., 2012). Exogenous melatonin application was also reported to improve lateral root growth and development (Zhang et al., 2013; Meng et al., 2014). External melatonin treatment was also noted to induce antioxidant defense systems of grafted Chinese hickory plants and improve drought tolerance (Sharma et al., 2020). In another study, it was reported that the melatonin application induces root regeneration, chlorophyll content, biomass, and proline content of cherry rootstock (Sarropoulou et al., 2012a). Very few studies exist on the impacts of melatonin on citrus trees. In one of these studies, melatonin was noted to induce a defensive response against Huanglongbing disease (Nehela and Killiny, 2020). Therefore, the present study aimed to investigate the effects of four different melatonin doses (0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM), under three different irrigation regimes (50%, 75%, and 100%) on the three different citrus rootstocks (Carrizo citrange, Flying Dragon and sour orange). This study is novel as it cross-tested melatonin doses under different irrigation regimes and different rootstocks.

2. Materials and methods

2.1. Experimental plot and materials

The study was carried out in black single pots with a volume of 5 L in a 500 m^2 plastic-covered, unheated greenhouse belonging to Ortaca Vocational School, located in Ortaca District of Muğla Province, Turkey. Carrizo citrange, Flying Dragon, and sour orange rootstocks of citrus were selected for current work. Sour orange has been highly used in the Mediterranean region, Carrizo citrange has been newly introduced into the region and the Flying Dragon has several advantages which makes it an important alternative for the region. The soil of the growing mediums was obtained from the surrounding agricultural areas. The soil had a pH of 7.67, an EC of 0.71 ds/m, 1.63% of organic matter, a medium lime content (6.26%), and is slightly alkaline. The salt content of loamy texture trial soil was only 0.02%, which makes it to be classified as nonsalty. The N, P, Mg, Ca, Zn, Cu, Fe and Mn contents of the experimental soil were, 0.24%, 10.53 kg/da, 64.05 kg/da, 5155.5 mg/kg, 170 mg/kg, 0.82 mg/kg, 1.28 mg/kg, 3.75 mg/kg and 13.55 mg/kg, respectively.

2.2. Study design and applications

Split-split plot design with three factors (rootstock, irrigation regime, and melatonin dose) and three replications were used in the present study. A total of 5 plants were used in each replication. Before planting, 100 g of 10–10–20 compose fertilizer was applied for 1 tonne of soil. The rootstocks were about 20–25 cm each at the time of planting and they were 70 days old. After the rootstocks were planted in pots and kept under normal growing conditions for 60 days, drought stress was created by reducing irrigation. The drought stress was performed for 60 more days after the normal growing conditions. Three different irrigation regimes were examined. Irrigation was performed weekly with 7-days of interval. Full irrigation (100%) was applied under the control group. In the other two groups, 75% and 50% of the irrigation were applied separately. To do so, measured water was applied until 20% of the drain water was collected from the pot base. Moreover, 75% and 50% of this amount was calculated and applied to produce drought stress on the plants. At the same time of the beginning of drought stress, melatonin treatments took place. One group was not treated with melatonin as control and the other four groups were treated with different doses of melatonin (0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM).

2.3. Data collection

Studies were continued from April 2017 to October 2018. During the studies, the leaf numbers were counted on day 15 and day 30, and their averages were used in further evaluations. Leaf area was determined with a portable digital area meter (CI 202) sensitive to 0.01 cm^2. Five full-size leaves from the mid-point of the shoot were used for leaf area determination. The leaf area determination was performed 60 days after the beginning of drought stress (Güleryüz and Aslantaş, 1998).

Leaf relative water content (RWC) of the samples was determined according to the method of Yamasaki and Dillenburg (1999). The leaf RWC was also determined after 60 days after the beginning of drought stress. Disc-shaped samples were taken from the leaves of 3 plants from each experimental unit and their fresh weight (FW) was determined with a digital balance sensitive to 1/1000. Then, the samples were kept in distilled water for 4 h under low light to make them turgorized. At the end of the period, they were roughly dried and their turgid weight (TW) was determined. Finally, the dry weights (DW) of the samples were measured after drying in an oven set at 70 °C for 24 h. Leaf relative water content of the leaves was then calculated with the following formula:

$$LRWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$$

The membrane permeability was determined 60 days after the beginning of drought stress according to the method of Lutts et al. (1996). A total of 9 leaf discs were taken from each experimental unit, and the surfaces of the leaf discs were washed 3 times with distilled water. Leaf discs were shaken for 24 h in containers containing 10 mL of distilled water at 25 °C. The electrical conductivity (L_t) of the shaken samples was measured, then the samples were kept in an autoclave at 120 °C for 20 min and the final measurement (L_0) was made when the temperature was 25 °C. The membrane permeability was then determined with the following formula:

$$Membrane\ permeability\ (\%) = (L_t/L_0) \times 100$$

Leaf proline content (μmol/gr) was assessed according to the method of Bates et al. (1973). Samples were collected 60 days after the beginning of drought stress and stored at –24 °C for 2 months before the analysis. Fresh leaf samples (0.5 g) were taken and crushed by mixing with 3% sulfosalicylic acid in a water bath at 100 °C for 30 min and then filtered. The filtered sample (2 mL) was taken and 2 mL of acetic acid and 2 mL of ninhydrin reagent were placed on it. Then, the samples were placed in the tubes and kept in a water bath at 100 °C for 1 h and the reaction was terminated on ice. Toluene (6 mL) was added to the cooled samples, vortexed, and read in the spectrophotometer at 520 nm. Leaf proline content was determined by calculation with proline standards.

The method of Strain and Svec (1966) was then used for the determination of the leaf chlorophyll (Chl) and carotenoid contents. Samples were collected 60 days after the beginning of drought stress and stored at –24 °C for 2 months before the analysis. Samples (0.25 g) were taken from the leaves of 3 plants in each experimental unit, CaCO3 was added with the tip of a spatula and homogenized with 15 mL of 80% acetone. The mixture was made up to 20 mL with acetone and centrifuged for 5 min. The upper phase (4 mL) was withdrawn from the sample, 12 mL of acetone was added, and it was read in the spectrophotometer at 645 and 663 nm wavelengths. For the determination of carotenoids, readings were made at 450 nm. The obtained values were calculated as mg/kg according to the following formulas:

$$Chl_a = [(11.64 \times A663) - (2.16 \times A645)] \times 1000$$

$$Chl_b = [(20.97 \times A645) - (3.94 \times A663)] \times 1000$$

$$Total\ Chl = Chl_a + Chl_b$$

$$Carotenoid = \frac{[(1000 \times A470) - (2.27 \times Chl_a) - (81.4 \times Chl_b)]}{227} \times 1000$$

After 135 days of transplanting (75 days after the beginning of drought stress), plants were uprooted from the pots. After removing the samples for analysis, root lengths and shoot heights were measured with a ruler in cm. Afterwards, the plants were cleaned, roots and shoots were separated, broken down into simple pieces and placed in Petri dishes. Firstly, fresh weights of the samples were determined and noted; and then the samples were dried in a drying oven at 65 °C for 48 h and their dry weights were determined (Kacar, 1972).

2.4. Data analysis

Raw data of the experiments were subjected to analysis of variance test by using the SPSS 22.0 software. The significance of difference among the mean values was examined by Tukey's HSD test at $p < 0.05$.

3. Results and discussions

3.1. Melatonin impacts on the root growth under drought stress

The ability of plants to absorb water and nutrients from the growing environment is closely related to the root structure of the crops and is highly related to the plants' ability to tolerate drought stress (Markesteijn and Poorter, 2009). Considering the statistical effect of applications on root length; maximum root length (36.2 cm) was observed on Flying dragon rootstock, and the lowest root length was observed on Carrizo citrange rootstock (26.3 cm) (Figure 1). Under drought stress, the root length was decreased, and the minimum root length was observed from 50% in the irrigation regime (28.18 cm). The highest root length was found with 32.86 cm in a 100% irrigation regime. The highest dose of melatonin (2 mM) application increased the root length at the highest rate (33.2 cm).

Moreover, the interactions of rootstocks, irrigation amount, melatonin, rootstock * irrigation, rootstock * melatonin, rootstock * irrigation * melatonin were all found to significantly affect the root dry weight. Among the tested rootstocks, it was observed that the maximum weight (7.03 g) was from the sour orange rootstock. Root dry weight decreased with the restriction of irrigation amount. The lowest root dry weight was determined as 4.9 g from the 50% irrigation regime and the highest value was observed as 6.91 g at the 100% irrigation regime. Melatonin application increased root dry weight, with the highest melatonin dose (2.0 mM) the highest increase (7.05 g). The lowest dry weight was observed in the control and followed by the lowest dose of melatonin (0.5 mM).

According to the results obtained, root length reached the maximum level at the highest melatonin doses (1.5 mM and 2 mM). Root length increased by 17.9% compared to control with 2 mM melatonin application. Roots directly affect the drought stress tolerance/resistance of plants. Roots are very sensitive to changes in pH, salinity, oxygen availability, toxic elements, and water potential. A strong root system resists the negative effects of drought stress. The function of melatonin as a rooting agent has been demonstrated many times (Zhang et al., 2015). However, current results include a comprehensive test among the melatonin doses under different irrigation regimes. It was clearly observed that the melatonin doses provide better performance on the root growth under drought stress conditions.

It was also stated by Arnao and Hernandez-Luiz (2007) that the application of melatonin increases the number and length of adventitious roots. Zhang et al. (2013) evaluated the effect of melatonin application on root formation in cucumber and indicated that it significantly stimulated root growth. Melatonin was noted to have a significant increase in the root length and root biomass of cherry rootstock when applied with 0.5 mM concentration (Sarropoulou et al., 2012a). The increase in the root biomass of cherry rootstocks with the melatonin application was also suggested by Sarropoulou et al. (2012b).

3.2. Melatonin impacts on the shoot growth under drought stress

Considering the statistical effect of the applications on the shoot height; rootstock, irrigation, melatonin dose, rootstock * irrigation, irrigation * melatonin, rootstock * irrigation * melatonin interactions were all found to be significant. Among the tested rootstocks, the maximum shoot height (93.4 cm) was observed in Carrizo citrange rootstock, and the minimum shoot height was observed from the sour orange rootstock (76.1 cm). Under the drought stress (50% irrigation regime), the shoot height decreased the most (78.0 cm) (Figure 2). The highest shoot height was then determined with 90.7 cm in 100% irrigation regime. The highest doses of melatonin (2 mM and 1.5 mM) provided a higher increase in the shoot height. The shoot height increased by 14.30% compared to the control at the highest dose of melatonin (2 mM). Similar to the shoot height results, it was observed that all factors and their interactions have a significant influence on the shoot dry weight. Among the examined rootstocks, the maximum shoot dry weight (14.1 g) was observed in Flying dragon rootstock. Under the drought stress conditions, the shoot dry weight decreased and the lowest dry weight was determined as 10.1 g in 50% irrigation

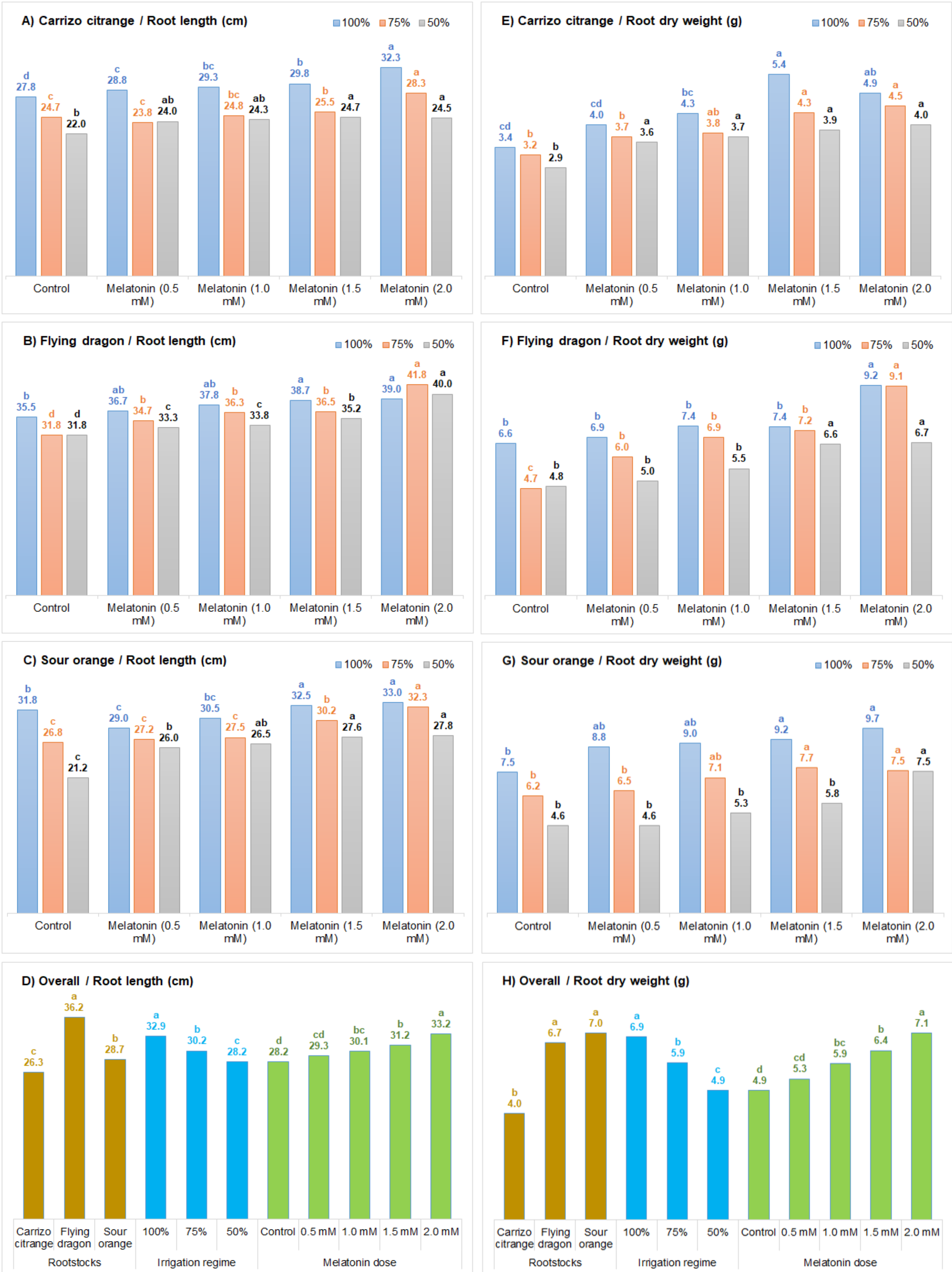

Figure 1. Change in root length and root dry weight of Carrizo citrange (A, E), Flying dragon (B, F), sour orange (C, G), and overall (D, H) as a response to melatonin application under different irrigation regimes. Different letters over the columns of the same trait (with the same color) represent significant difference according to Tukey's test at 5% significance.

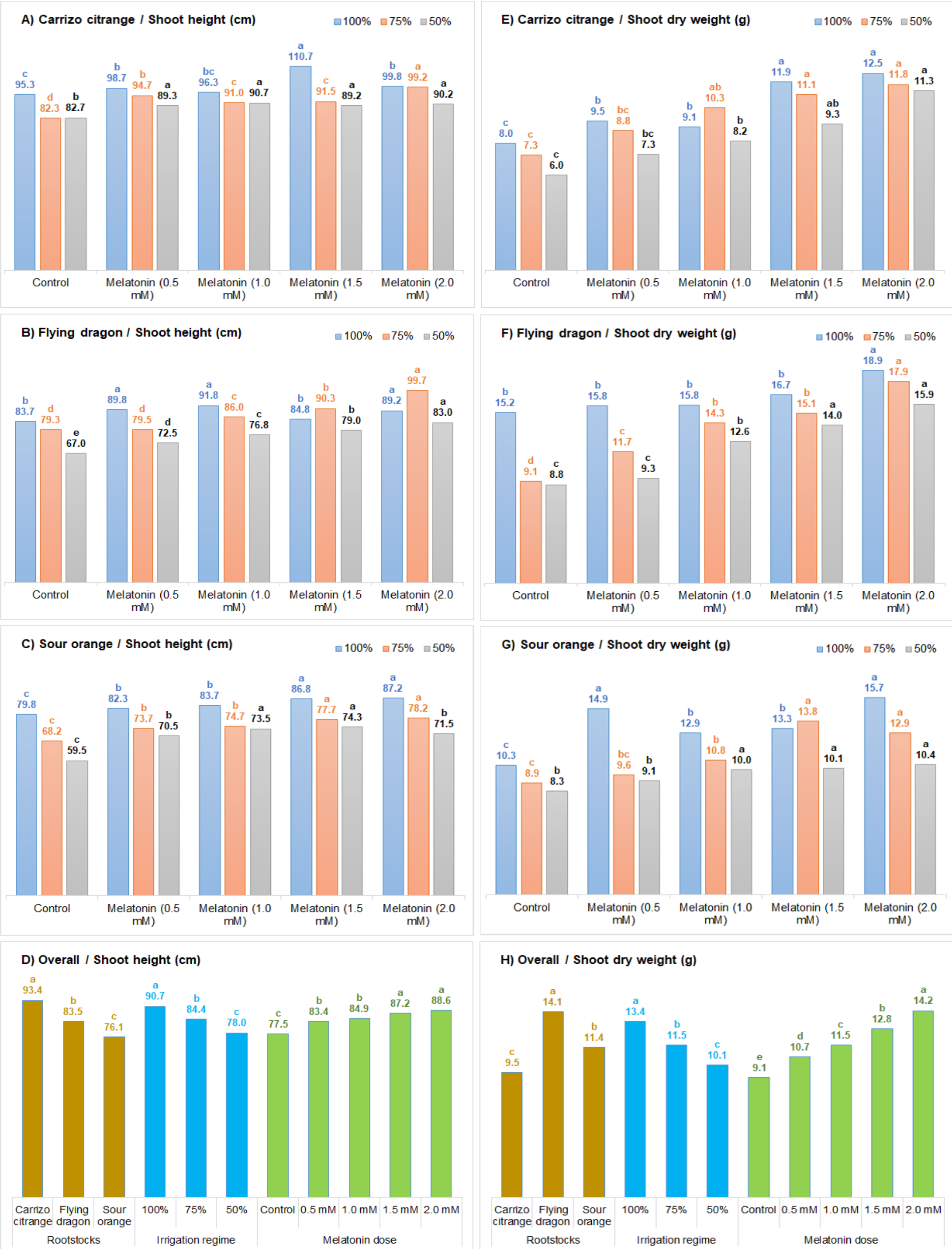

Figure 2. Change in shoot length and shoot dry weight of Carrizo citrange (A, E), Flying dragon (B, F), sour orange (C, G), and overall (D, H) as a response to melatonin application under different irrigation regimes. Different letters over the columns of the same trait (with the same color) represent significant difference according to Tukey's test at 5% significance.

regime. Moreover, the highest shoot dry weight was observed as 13.4 g in 100% irrigation regime. Melatonin application increased the shoot dry weight and the highest dry weight (14.2 g) occurred at the highest melatonin dose (2.0 mM). In the control, the lowest shoot dry weight was observed as 9.1 g.

Evaluation of the shoot dry weight results showed that the rootstocks had about 16.26% higher dry weight under 100% irrigation regime as compared with those with 50% irrigation regime. Therefore, drought conditions reduced plant growth. Recently, the roles of melatonin in plant growth and development have become an important research topic for science (Kanwar et al., 2018).

The positive impacts of melatonin on plant growth and development were previously reported for different stress conditions, like salt, cold, and drought (Shi et al., 2015; Antoniou et al., 2017). Zhang et al. (2015) stated that under drought/arid conditions, plants growth decrease and a significant decrease is being observed in net photosynthesis rates and chlorophyll content. Melatonin application significantly reduces growth inhibition, thus allowing plants to maintain a robust root system and improve photosynthetic capacity (Zhang et al., 2015). Sarropoulou et al. (2012a) found a positive correlation between low melatonin doses and root fresh weight. In the present study, the highest root fresh/dry weight was observed in the low dose melatonin (0.5 mM) application. Exogenous application of melatonin was also reported to improve the shoot height and shoot dry weight of *M. hupehensis* under UV-B stress conditions (Wei et al., 2019).

3.3. Melatonin impacts on the leaf growth under drought stress

In the present study, a significant difference was observed among the rootstocks' leaf numbers (Figure 3). The highest number of leaves was determined as 18.0 per plant in Carrizo citrange rootstock, and the lowest number of leaves was observed as 15.1 in Flying dragon rootstock. The effect of irrigation conditions on the number of leaves was also found to be significant in the statistical evaluation. As the amount of irrigation decreased, the number of leaves also decreased. Therefore, the highest number of leaves was determined as 18.9 per plant under 100% irrigation conditions, while the number of leaves decreased by 15.0% with the decrease in irrigation level to 16.1 per plant in the 75% irrigation regime. With the reduction of irrigation to 50%, an average of 14.0 leaves per plant was observed, and this value is 26.2% lower than that of the 100% irrigation regime. Melatonin application had a positive effect on the number of leaves. In parallel with the increase in the dose of melatonin, the average plant leaf number also increased. In the application of the highest dose of melatonin (0.5 mM), the highest leaf number was determined as 17.8 per plant. Statistical comparison of the 2.0 mM and 1.5 mM doses of melatonin resulted in being in the same grouping, but both of them differed from the control plants. Except for rootstock * irrigation * melatonin interaction, all other bilateral interactions and single factors were found to be statistically significant. Flying dragon rootstock was found to be tolerant against drought stress and gives good results to melatonin application.

Although the number of leaves of this rootstock is less than other rootstocks, the loss rate is also less with drought stress. The irrigation regime of 75% showed a 24.7% loss in the number of leaves as compared to 100% irrigation regime, while the 50% irrigation regime showed a 46.5% loss in sour orange. A similar situation was observed as 10.7% and 22.9% for the Flying dragon rootstock under 75% and 50% irrigation regimes as compared to 100% irrigation regime. The loss in the number of leaves of Carrizo citrange under the same conditions were 20.7% and 30.2%. Responses of the same rootstocks with reduced irrigation at 2 mM melatonin dose, Carrizo citrange leaf loss rates decreased by 11.8% in 75% irrigation and 24.9% in 50% irrigation conditions. In the Flying dragon rootstock, which seems to be resistant, the loss in leaf number was only 4.6% and 6.8% at a dose of 2 mM melatonin.

A significant difference was determined among the leaf area of test rootstocks. The maximum leaf area was 24.9 cm^2 per plant and was determined on Carrizo citrange rootstock. Moreover, the lowest leaf area was 15.2 cm^2 which was noted on the Flying dragon rootstock. In the sour orange rootstock, the observed leaf area was 15.5 cm^2. The effect of irrigation regime and drought conditions on leaf area is significant, and as the amount of irrigation decreases, a decrease in leaf area was observed. The highest leaf area was determined as 20.7 cm^2 under 100% irrigation regime, which showed an 11.5% decrease and measured as 18.3 cm^2/plant under the 75% irrigation regime. Moreover, with the reduction of the irrigation regime to 50%, an average of 16.5 cm^2 leaf area per plant was observed, which equals about a 19.9% decrease when compared to 100% irrigation regime.

Melatonin application positively affected the leaf area of the rootstocks. In parallel with the increase in the dose of melatonin, there was an increase in the average plant leaf area. In the application of the highest dose of melatonin (2 mM), the highest leaf area was determined as 19.5 cm^2/plant. While the 2 mM dose and the 1.5 mM dose of the applications were included in the same grouping in the statistical evaluation, both were found to be different from the control rootstocks. It has been observed that especially the Flying dragon rootstock is generally tolerant to drought stress conditions and can struggle with drought levels. Although the leaf area of this rootstock is less than the other rootstocks, the loss rate was also found to be less with drought stress conditions. In the control application of Carrizo citrange rootstock, 75% reduction in irrigation caused 18.1% in the leaf area as compared to

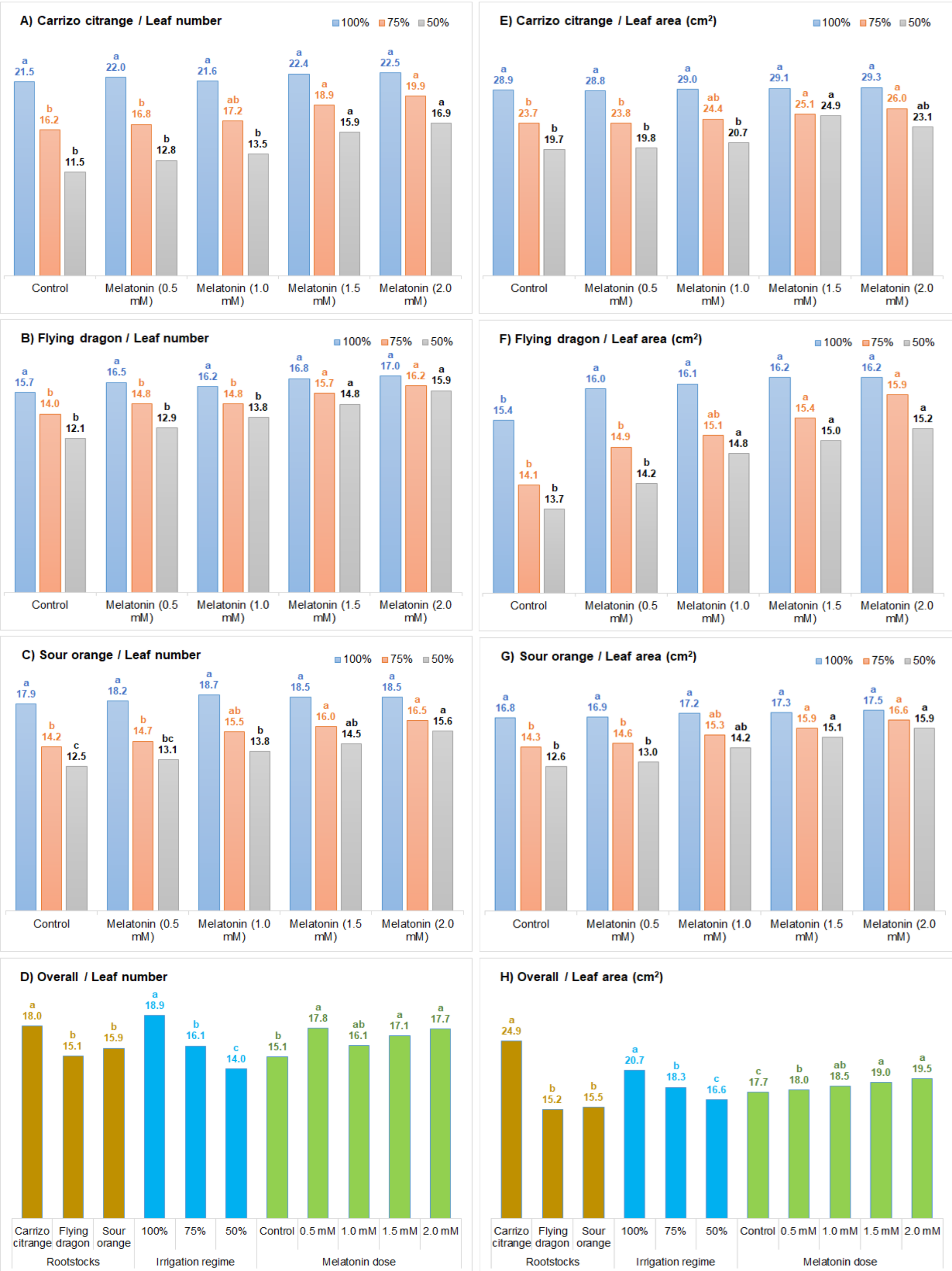

Figure 3. Change in number of leaves and leaf area of Carrizo citrange (A, E), Flying dragon (B, F), sour orange (C, G), and overall (D, H) as response to melatonin application under different irrigation regimes. Different letters over the columns of the same trait (with the same color) represent significant difference according to Tukey's test at 5% significance.

100% irrigation conditions, and 31.9% reduction in the leaf area under the 50% irrigation regime. The responses of the Flying dragon rootstock to the same conditions were observed as 8.3% and 11.4%, respectively. Lei et al. (2006) stated that plants develop a drought tolerance mechanism by forming high root-to-shoot ratios, fewer and smaller leaves in arid conditions. In the present study, there was a decrease in leaf area and the number of leaves, and root fresh and dry weights in plants with the most drought and the least melatonin applied plants, which supports the basic information provided by Lei et al. (2006). Therefore, the increase in the number of leaves and leaf areas under high doses of melatonin can be accepted as a sign of the increment in the tolerance of these rootstocks. The impact of melatonin on the number of leaves was also observed by Wei et al. (2019) under the UV-B stress conditions at the *M. hupehensis* plant.

3.4. Melatonin impacts on the leaf water content and membrane permeability under drought stress

In the present study, rootstocks, irrigation, and melatonin factors alone and rootstock * irrigation, rootstock * melatonin and irrigation * melatonin interactions were all found to have a significant influence on the leaf relative water content (Figure 4). Evaluation of the rootstocks resulted in the highest leaf relative water content (72.2%) from the Flying dragon rootstock and was followed by the Carrizo citrange rootstock with 71.1% RWC. In the statistical evaluation, Carrizo citrange and Flying dragon rootstocks were included in the same statistical group. The lowest leaf RWC was observed in sour orange rootstock with 68.4%. Considering the effect of irrigation regimes, the RWC of the leaves decreased with the increase in the drought stress. Each application took place in different statistical groups, reducing the irrigation by 50% decreased the RWC of the leaves by 26.3% as compared to the 100% irrigation application. Parallel to the increase in melatonin doses, the RWC of the leaves increased. Leaf RWC was found to be the lowest value (65.4%) in control plants. The highest leaf RWC was observed in the plants applied with 2 mM melatonin with 76.3%, which is 16.6% higher than the control plants. In the 50% reduction of the irrigation amount in the Flying dragon rootstock, the leaf RWC decreased by 19.9% as compared to the control plants, while the decrease in the sour orange rootstock was 29.0%. This shows that Flying dragon rootstock is more tolerant to drought conditions than orange rootstock. Melatonin application positively affected the proportional water content of leaves in all rootstocks. The highest dose of melatonin (2 mM) was found to improve the RWC as 10% in sour orange, and nearly 17% in the Flying dragon rootstock. This result reveals that melatonin application is effective according to the tolerance of the rootstock in terms of the leaf RWC. According to the research findings, rootstock difference affected the membrane permeability.

The lowest membrane permeability was observed in Flying dragon rootstock, while the other two rootstocks formed membrane permeability values close to each other. In terms of rootstocks, two groups were formed in the statistical evaluation, while Carrizo citrange and sour orange rootstocks were included in the same group, and Flying dragon rootstock was in a separated group. The low permeability of the membrane is one of the most important parameters showing the tolerance of the rootstock and it is evidence showing the tolerance of the Flying dragon rootstock to drought stress conditions. The decrease in the amount of irrigation (drought stress) also negatively affected the membrane permeability, and the membrane permeability increased under drought stress. The lowest membrane permeability value was observed as 15.9% in 100% irrigation regime, it increased to 24.1% by reducing irrigation to 75% and to 26.0% by reducing irrigation to 50%.

Rootstocks without melatonin treatment were found to have higher membrane permeability, while the treated plants had lower values. The decrease in membrane permeability was parallel to the dose increase. The highest membrane permeability value was observed at the level of 23.4% in the control plants, and it decreased to 20.3% in the plants treated with a 2 mM melatonin dose. No significant difference was observed between the 2 mM and 1.5 mM doses of melatonin. The lowest membrane permeability was noted from the Flying dragon rootstock. Among the melatonin doses tested, the maximum leaf RWC and minimum membrane permeability of Flying dragon rootstock under the highest drought stress (50%) were observed from the 2 mM melatonin dose applied plants. This result suggested that the melatonin dose of 2 mM is effective in improving the tolerance of Flying dragon rootstock against drought stress. Foliar melatonin application was previously noted for the soybean to protect cell membrane structure and improve crops physiology against drought stress by improving photosynthesis capacity and inhibiting ROS activity (Zou et al., 2021).

3.5. Melatonin impacts on the leaf chlorophyll and carotenoids under drought stress

Chlorophyll pigment is a crucial indicator for the photosynthetic activity of plants (Carvalho et al., 2001). Moreover, the chlorophyll contents play an important role in plants' ability to adopt stress conditions. Each and every single factor (melatonin, rootstock, and irrigation regime) and their interactions were found to have a significant influence on the chlorophyll content of the leaves (Figure 5). In terms of rootstocks, Carrizo citrange and Flying dragon rootstocks were in the same statistical grouping and were separated from the sour orange rootstock. The highest total chlorophyll value was found in Flying dragon rootstock with 3.10 mg/kg.

Figure 4. Change in relative water content and leaf membrane permeability of Carrizo citrange (A, E), Flying dragon (B, F), sour orange (C, G), and overall (D, H) as response to melatonin application under different irrigation regimes. Different letters over the columns of the same trait (with the same color) represent significant difference according to Tukey's test at 5% significance.

The total chlorophyll content of the leaves decreased with the increase in the drought stress. Therefore, the lowest chlorophyll content was determined as 3.0 mg/kg in 50% irrigation regime, and the highest value was observed as 3.2 mg/kg in 100% irrigation regime. Melatonin application was then noted to increase the chlorophyll content. The rate of increase varied depending on the dose of melatonin. A similar situation exists in terms of carotenoid content. However, in terms of rootstocks, all were found to be in the same group, although the Carrizo citrange has a little higher carotenoid content. The carotenoid content of the Carrizo citrange was noted as 0.24 mg/kg, and was followed by 0.22 mg/kg both in sour orange and Flying dragon rootstocks. The total carotenoid content of the leaves decreased with the increase in the drought stress. The lowest carotenoid content was found at 0.20 mg/kg in 50% irrigation regime, the highest value was found as 0.25 mg/kg in 100% irrigation regime.

Drought stress is known to damage the photosynthesis system by decreasing the chlorophyll content and reducing the sun-light absorption (Flexas and Medrano, 2002). Similar results were observed in the current study, where the melatonin treatment increased the chlorophyll content and helped the plants to fight against drought stress. Similar results were reported by Liang et al. (2019) for kiwifruits. In another study, foliar application of melatonin was suggested to have a slightly positive impact on the leaf chlorophyll contents of tomato seedlings (Jahan et al., 2020).

External application of melatonin was previously reported to increase the chlorophyll content of the cherry rootstocks (Sarropoulou et al., 2012). The positive impacts of melatonin on the chlorophyll concentration were also noted for *Malus hupehensis* under UV-B stress conditions (Wei et al., 2019). Foliar melatonin treatment was suggested to regulate the concentrations of β-carotene and lycopene in tomato (Sun et al., 2020), which is in agreement with the findings of the current study. In a different study, not under drought stress, but under salt stress, the melatonin treatment was again noted to provide a significant improvement in the concentrations of chlorophyll and carotenoids in basil crops and improve crops tolerance against salinity (Bahcesular et al., 2020).

3.6. Melatonin impacts on the leaf proline content under drought stress

Each and every single factor (melatonin, rootstock, and irrigation regime) and their interactions were found to have a significant influence on the leaf proline content of the rootstocks (Figure 6). Among the rootstocks, the highest proline content was determined as 20.5 µmol/gr in the sour orange rootstock, while the lowest value was determined as 17.5 µmol/gr in Carrizo citrange rootstock. Decreased irrigation and increase in drought stress had been observed to cause an increase in proline accumulation. This is a general response of the plants to the drought stress conditions. Decreasing the irrigation from 100% to 50% caused an increase in the proline content from 13.2 µmol/gr to 24.4 µmol/gr. The increase in proline, which emerged as a symptom of stress, became evident with the formation of drought stress conditions.

Melatonin application had a significant influence on the proline content of the leaves. The lowest proline content was determined as 16.9 µmol/gr at 2 mM melatonin dose, while the highest proline content was observed as 20.7 µmol/gr in leaf samples of control plants. There was no statistical difference between the untreated control plants and the 0.5 mM dose of melatonin. Maximum leaf proline content was observed in sour orange rootstock (20.5 µmol/gr), 50% irrigation regime (24.4 µmol/gr) and control plants (20.7 µmol/gr) & lowest (0.5 mM) melatonin dose (20.51 µmol/gr) applications. On the other hand, the lowest leaf proline content was observed in Carrizo citrange (17.5 µmol/gr), 100% irrigation (13.19 µmol/gr), and 2 mM melatonine dose (16.88 µmol/gr). Rootstocks and applications with low membrane permeability and low proline content show higher tolerance to drought stress. When the proline content and membrane permeability are examined, it is concluded that Flying dragon and Carrizo citrange rootstocks are more tolerant to drought than sour orange rootstock.

Contrary to present results, the higher concentrations of melatonin (5 and 10 mM) on cherry rootstocks was noted to increase the levels of proline in leaves (Sarropoulou et al., 2012). However, in the same study researchers reported a decrease in the proline content at the 0.05 mM, 0.1 mM, 0.5 mM, and 1.0 mM melatonin doses, which are in agreement with the current study. Proline, as an N storage compound, is recognized as an important compound against stresses (Sarropoulou et al., 2012).

4. Conclusions

Drought stress significantly affected the physiological and morphological properties of citrus rootstocks and caused different behaviors under drought stress conditions. The results showed that, as the drought stress increases the root and shoot growth & development of the rootstocks are negatively affected, and the number of leaves & leaf areas are decreased. Moreover, as a result of drought stress, the leaf membrane permeability of the plants increased, and in response to this, the relative water content of the leaves decreased. Likewise, the chlorophyll and carotenoid contents of plants decreased under drought stress. Root length, root dry weight, shoot height, shoot dry weight, leaf number, and leaf area values were measured as maximum under 100% irrigation application, while the minimum values were observed in arid conditions where 50% irrigation was applied. Moreover, leaf membrane permeability of rootstocks increased and the relative water

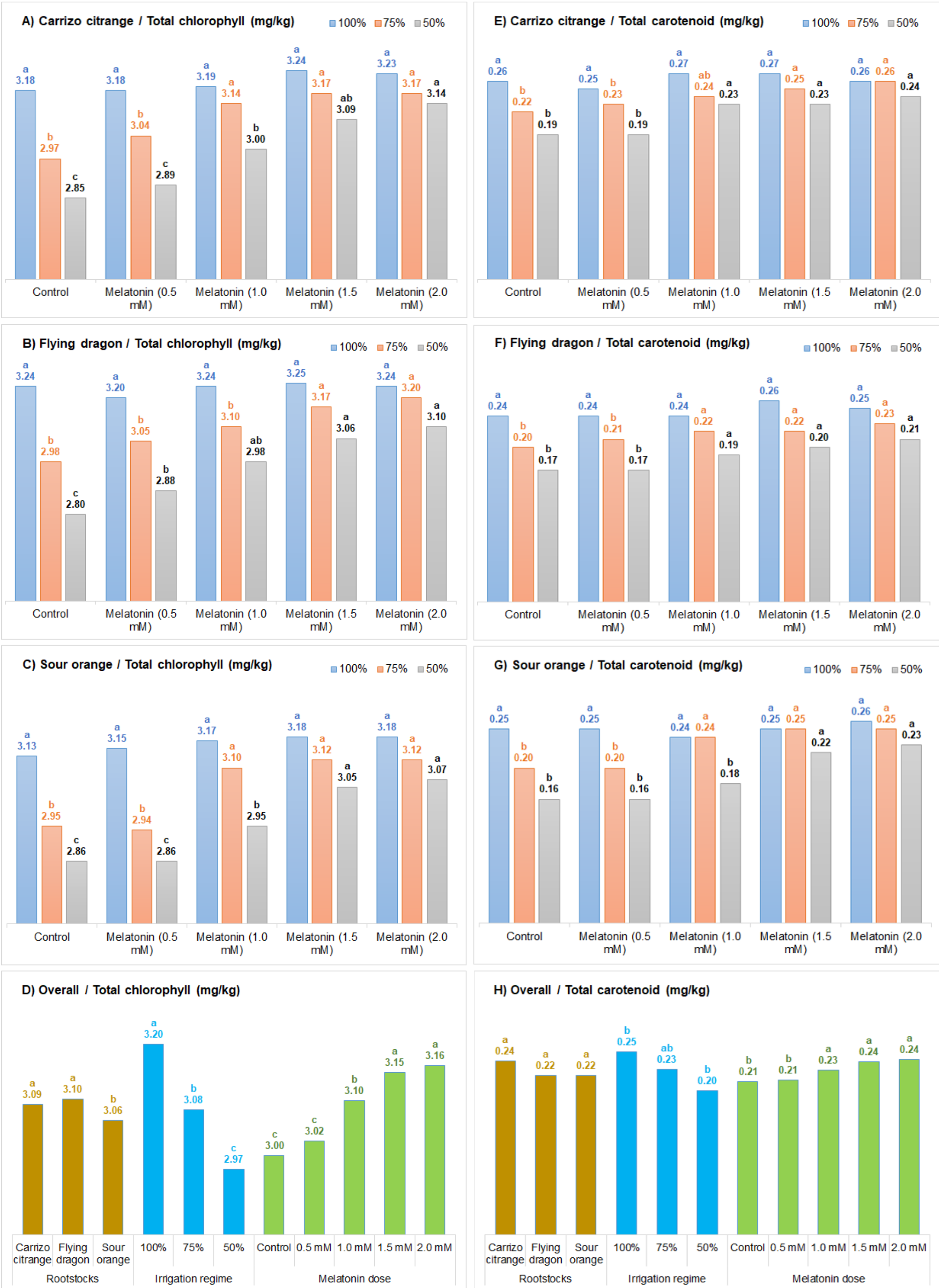

Figure 5. Change in chlorophyll and carotenoid contents of Carrizo citrange (A, E), Flying dragon (B, F), sour orange (C, G), and overall (D, H) as response to melatonin application under different irrigation regimes. Different letters over the columns of the same trait (with the same color) represent significant difference according to Tukey's test at 5% significance.

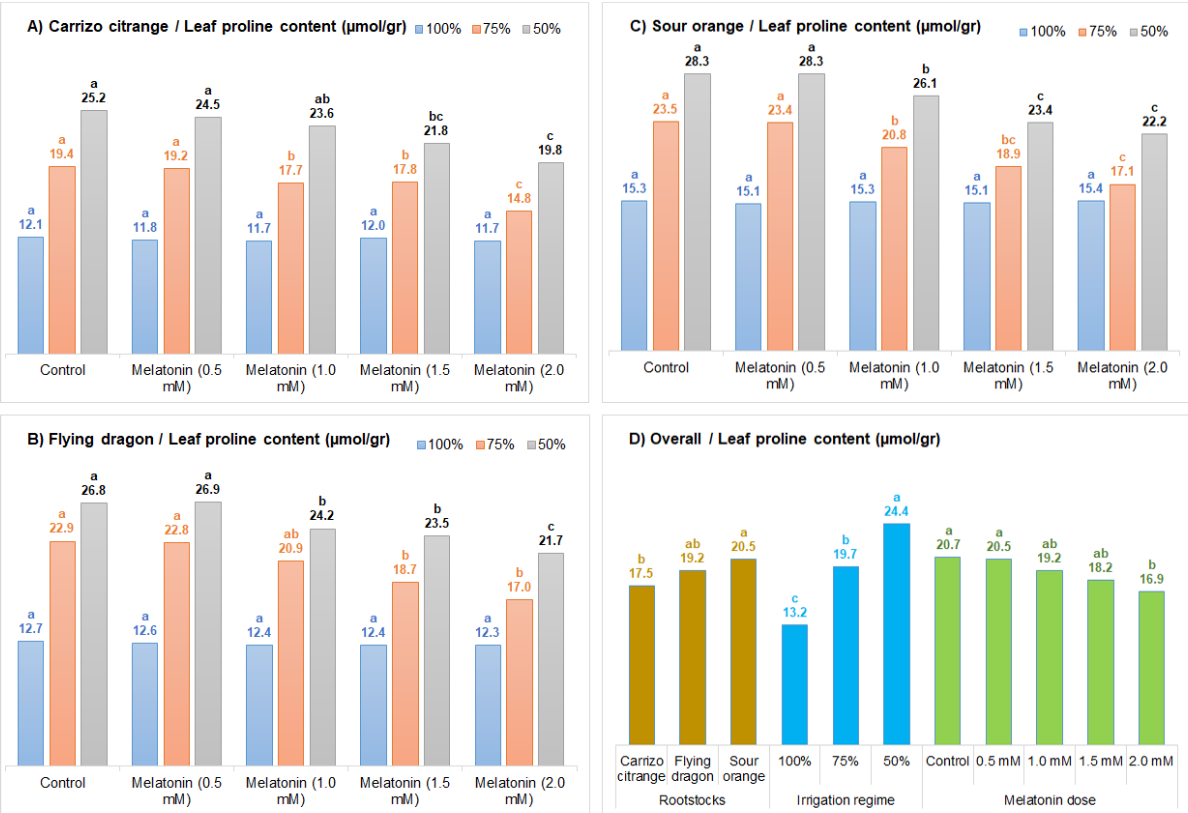

Figure 6. Change in leaf proline content of Carrizo citrange (A), Flying dragon (B), sour orange (C), and overall (D) as response to melatonin application under different irrigation regimes. Different letters over the columns of the same trait (with the same color) represent significant difference according to Tukey's test at 5% significance.

contents decreased as a response to the drought stress (50% irrigation regime). All these parameters showed improvement in rootstocks treated with melatonin. Among the tested rootstocks, the Flying dragon was observed to be more tolerant to drought than the other test rootstocks in terms of the leaf relative water content and membrane permeability. Consequently, it was determined that melatonin application contributed to the preservation of physiological and morphological characteristics of plants under drought stress, and decreased the amount of proline released as a result of stress.

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