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ORIGINAL PAPER

Investigation of drought stress mitigation effects of different calcium (Ca^{+2}) applications on tomato seedlings

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Abstract

In the study, tomato (*Lycopersicon esculentum*) variety Adamset F1 seedlings were used. Plants were grown hydroponically in a controlled climate chamber with light intensity of 400 μmol $m^{-2}s^{-1}$, temperature of 20°C and humidity of 65%, with the dark/light photoperiod of 16/8 hours. 200 ppm, 250 ppm, 300 ppm, 350 ppm and 400 ppm Ca were applied to the Hoagland nutrient solution to determine whether it would increase the drought tolerance of the plants. When the plants had 5-6 leaves, 7% polyethylene glycol (PEG-6000) was added to the Hoagland solution. Total plant weight, chlorophyll, malondialdehyde (MDA) and enzyme (SOD, APX and CAT) activities of seedlings under drought stress were investigated on the 7th day of stress. In the study, while the total weight of the plants supplied with PEG+200 and 400 ppm Ca^{+2} dose was found to be the lowest, it was determined that the plants to which the same dose was applied were damaged morphologically on a 1-5 scale, chlorophyll was the lowest, and MDA was the highest. In terms of SOD and APX amount, the highest application was PEG + 200 and 400 ppm Ca^{+2}. On the other hand, when PEG+250 and 300 ppm Ca^{+2} doses were applied, the total plant weight was high, while the MDA and enzyme activities and scale values were low.

Keywords: tomato, drought, calcium, chlorophyll, malondialdehyde, enzyme activities

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INTRODUCTION

All kinds of factors that limit plant development are defined as "a stress" (Turkan 2008). Because a stressor negatively affects plant development, the plant yield decreases (Yasar et al. 2020, Maesaroh, Ozel 2021, Yasar, Uzal 2021). Plants that originate from natural and cultivated conditions face many stressors throughout their lives. A stress caused by certain soil components can last for several days; however, some environmental factors such as air temperature can lead to a short-term stress (Taiz, Zeiger 2010). Environmental pressures are the main factors limiting crop production in the world. Agricultural production declines by an average of 71% due to abiotic stresses and by 29% due to other stressors (Boyer 1982). Drought is the most important factor limiting crop production in the majority of the world's agricultural lands (Ozturk 1999). Drought is a physiological form of water deficiency in which the soil water available to the plant is insufficient and adverse effects on plant metabolism occur (Kumar et al. 2016, Yasar, Uzal 2021) – Figure 1.

When plants encounter drought stress, their initial response consists of changes in their external and internal structures. Plant growth deceleration is a significant consequence of water deficiency. An insufficient water supply during the vegetative period will especially cause such effects as fading of the leaf color, decrease in the number of leaves and reduction of the leaf area (Yang et al. 2021) – Figure 1. In studies on drought, the integrity of the membrane and its effect under stress were investigated while determining plant resistance. In wheat, cysteine is expressed in leaves,

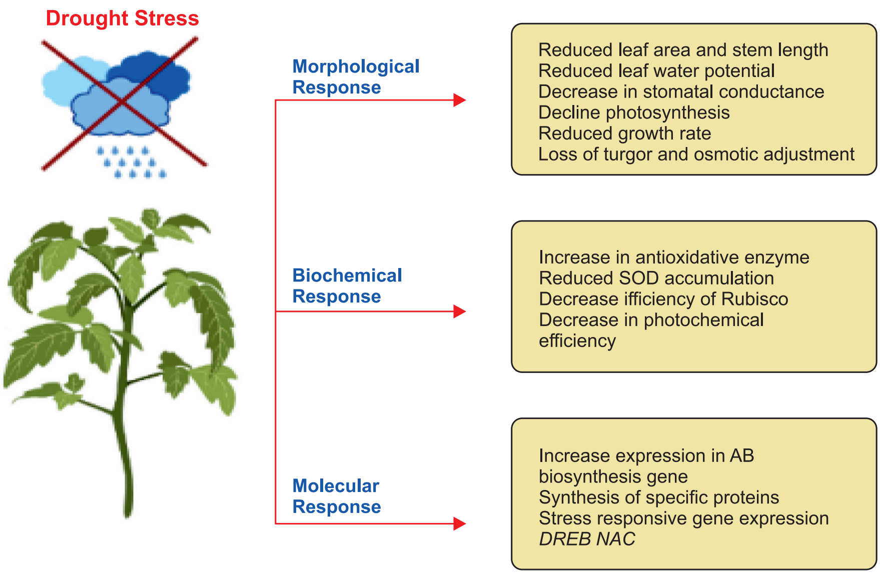

Fig. 1. Plants' responses to drought stress (Kumar et al. 2016)

where it affects leaf development (Poddar et al. 2022). Another morphological feature that occurs in leaves under drought is rolling. This is due to potential internal water failure from the upper epidermis of the leaf (Yang et al. 2021). In a way, this is a strategy to avoid drought because when the leaf area decreases, water loss by transpiration will be lower. Drought stress leads to a higher risk of protein damage due to cellular instability/imbalances. Therefore, several proteins with protective mechanisms show increased levels (for example HSPs, namely HSP70, HSP90). Disturbed cellular metabolism leads to oxidative damage, and hence an increase in ROS scavenging enzymes has also been observed (SOD, GST, CAT, APX, and others), which is a common and practical developments in plants under drought stress (Havrlentová et al. 2021) – Figure 1. When plants are exposed to any stress factor, non-enzymatic antioxidants (carotenoids, ascorbate and tocopherol) and enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) act as protectors of the system and show their effects (Yasar et al. 2014*a*, 2016, Uzal 2017, Yasar, Uzal 2021).

Calcium (Ca^{+2}) is highly immobile in plants, and its uptake in drought conditions is affected by limited water availability (Naeem et al. 2017). Limited water is one of the main causes of insufficient Ca uptake from roots (Adams, Ho 1993). A low Ca concentration in plant tissues is the main cause of various physiological disorders (Alexander, Clough 1998). Ca deficiency which occurs in plants under drought stress impairs osmoregulation in the plant, prevents the activation of enzymes and, by reducing the biosynthetic activities of plants, it inhibits the normal functions of physiological processes, being able to eventually cause permanent damage that leads to death (Jenne et al. 1958, Yasar et al. 2013a, b, 2014a, b, 2016, Uzal 2017)

Calcium is a vital macronutrient for the normal growth and development of plants because it plays an important role in balancing membrane structures, increases the intake of nutrients and activates metabolic processes (Tuna et al. 2007, Sarwat et al. 2013). Another role of calcium is to maintain the integrity of the cell wall and to provide bonding between cells (Marschner 1995). Calcium also reduces harmful stress effects by regulating antioxidant metabolism (Ahmad et al. 2015).

Based on the information that when plants are exposed to drought stresses there is a decrease in the intake of Ca nutrient elements causing metabolic deterioration, this study was conducted to find out whether external Ca^{+2} applications to plants under drought stress can protect the plants from the stress, and to understand how the plants develop a resistance strategy to drought.

MATERIAL AND METHODS

Plant material and growing conditions

This study was carried out in the Plant Physiology Laboratory of Van Yuzuncu Yil University. Adamset F1 tomato (*Solanum lycopersicum*) variety was used as plant material.

The experiment was carried out in water culture, in a split air-conditioned climate chamber, where normal atmosphere is maintained. Tomato seeds were sown in 40x25x5 cm foamed germination pots filled with sieved small-grain pumice and irrigated with tap water. Each of the foamed germination containers has a total of 18 holes in three containers with a diameter of 0.5 cm, and drainage of irrigation water is provided. After the excess irrigation water was filtered out, the foamed germination pots were placed in a climate chamber with temperature of 25±2^{o}C and humidity of 70-80%, covered with A4 sheets of paper, regularly checked every day, and continued to be watered in a way that maintained the humidity of the pumice. Irrigation was continued with Hoagland nutrient solution in order for the seedlings to develop better into seedlings whose cotyledon leaves became horizontal and the first true leaves began to appear (Hoagland, Arnon 1938). The seedlings which also formed the second true leaves in the pumice medium were transferred to water culture in 25x25x18 cm plastic tubs filled with Hoagland nutrient solution. Tomato seedlings were placed on perforated plastic trays by wrapping each with a small sponge. The trays were placed on tubs so that the plant roots were immersed in the nutrient solution. Aeration was ensured by immersing thin plastic hoses connected to an aquarium air pump in the nutrient solution. At the stage of drought stress application, plants were grown in hydroponic culture using Hoagland nutrient solution and maintaining light intensity of 400 µmol m^{-2} s^{-1}, temp. of 20°C, and 65% humidity, in a controlled climate room with a light/dark photoperiod of 16/8 hours. 200 ppm, 250 ppm, 300 ppm, 350 ppm and 400 ppm doses of Ca were applied to the nutrient solution. When the plants grew 5-6 leaves, 7% of polyethylene glycol (PEG-6000) was added to Hoagland nutrient solution. After the plants were under drought stress for 7 days, the total plant weight, the effect of the stress on the plants expressed on a 1-5 scale, chlorophyll content, MDA and enzyme (SOD, APX and CAT) activities were examined.

Determination of measurements and analysis

Total plant weight (g)

The fresh weight of all parts of the plants, including the root, was determined by weighing on a balance with an accuracy of 0.001 g.

1-5 scale

A scale was created to assess the degree of morphological damage in young tomato plants. The plants were scored from 1 to 5 according to the symptoms stated below, manifesting the degree of damage (Kusvuran 2010).

– no influence (control plants),
– slowdown in growth,
– paleness of lower leaves,
– upper leaves curling (closing) and wilting,
– severe wilting and yellowing of the leaves, and the beginning of drying on the lower leaf edges.

Measurement of chlorophyll amounts

The third leaf from the top was taken from the plants, and these samples were stored in a deep freezer at -84°C until analysis. 200 mg of leaf samples frozen at -84°C were placed in 80% ethanol and kept in a water bath at 80°C for 20 min, after which the absorbance values at 654 nm were read spectrophotometrically (Luna et al. 2000). At the end of these measurements, the total amount of chlorophyll in the fresh leaf sample was determined as g/mg fresh weight using the formula below:

total chlorophyll = absorbance values 1000=39.8.

Determination of lipid peroxidation (MDA) content

Determination of the amount of MDA, which is a product of the oxidation of lipids induced by damage to cell membranes, was achieved according to Lutts et al. (1996). Namely, an amount of 150-200 mg was taken by weighing from leaf samples. Thereupon, 5 ml of 0.1% trichloroacetic acid (TCA) was added and this mixture was centrifuged at 12500 rpm for 20 min. 3 ml of the supernatant were taken from 5 ml of the extract. Then, 3 ml of 0.1% TCA containing 20% thiobarbutyric acid (TBA) were added, and the resulting mixture was kept in a hot water bath at 95°C for 30 min. The prepared samples were read in a spectrophotometer, at A532 and A600 nm absorbance values.

Antioxidant enzyme activities

In order to understand how drought stress affects plants, antioxidant enzyme activities were examined. Approximately 1 g of fresh leaf sample was crushed in liquid nitrogen in a porcelain mortar and then homogenized with 50 mM, 10 ml phosphate buffer solution (pH:7.6) containing 0.1 mM Na-EDTA. The centrifuged material obtained after the homogenized samples had been centrifuged at 15000 g for 15 min was used for enzyme analysis. The samples in which enzyme activities were determined were kept in snow in order to keep them at +4°C until measurement. Measurements were carried out with a spectrophotometer. Superoxide dismutase (SOD) activity, according to the method of reduction of NBT (nitrobluete trazolium chloride) by O_2 under light, ascorbate peroxidase (APX) activity, oxidation of ascorbate at 290 nm (E = 2.8 mM cm^{-1}), catalase activity (CAT) measurements were based on the degradation rate of H_2O_2 at 240 nm (E = 39.4 mM cm^{-1}) (Cakmak and Marschner 1992).

Statistical analysis

In order to evaluate the research data, intended to determine the effects of Ca application on chlorophyll, MDA, and enzyme activities in tomato plants, variance analysis was performed in Statgraphics statistical analysis package program. The trial objects that were found to be statistically significant were grouped with the Duncan test at the 5% significance level.

RESULTS

In this study, the morphological and physiological characteristics of tomato seedlings submitted to different calcium applications under drought stress were investigated. At the end of the study, changes in total plant weight, MDA, chlorophyll, and some enzyme (SOD, CAT, and APX) activities were determined in the samples taken.

Total plant weight (g)

At the end of the 7th day, the total plant weight of the tomato plants was measured, and the values obtained are given in Figure 2. When plant growth parameters were evaluated, statistically significant effects on total plant weight were found.

Significant decreases were observed in the mean total plant weight of the plants compared to the control. As a result of the 7-day drought stress, it was observed that the total plant weight decreased significantly in PEG + Ca 400 ppm application compared to the control group, and there was a very slight decrease in PEG + Ca 250 ppm and PEG + Ca 300 ppm applications compared to the control group (Figure 2).

In order to determine the morphological damage in the plants, the seedlings were assessed on the 1-to-5 scale chosen (Figure 2).

When the scale values are examined, it is seen that the plants most affected by stress are from the PEG+Ca 400 ppm application variant. This is followed by PEG+Ca 350 ppm and PEG+Ca 200 ppm applications, consecutively. It was observed that the plants which received PEG+Ca 250 ppm and PEG+Ca 300 ppm had the least morphological damage compared to the control (Figure 2).

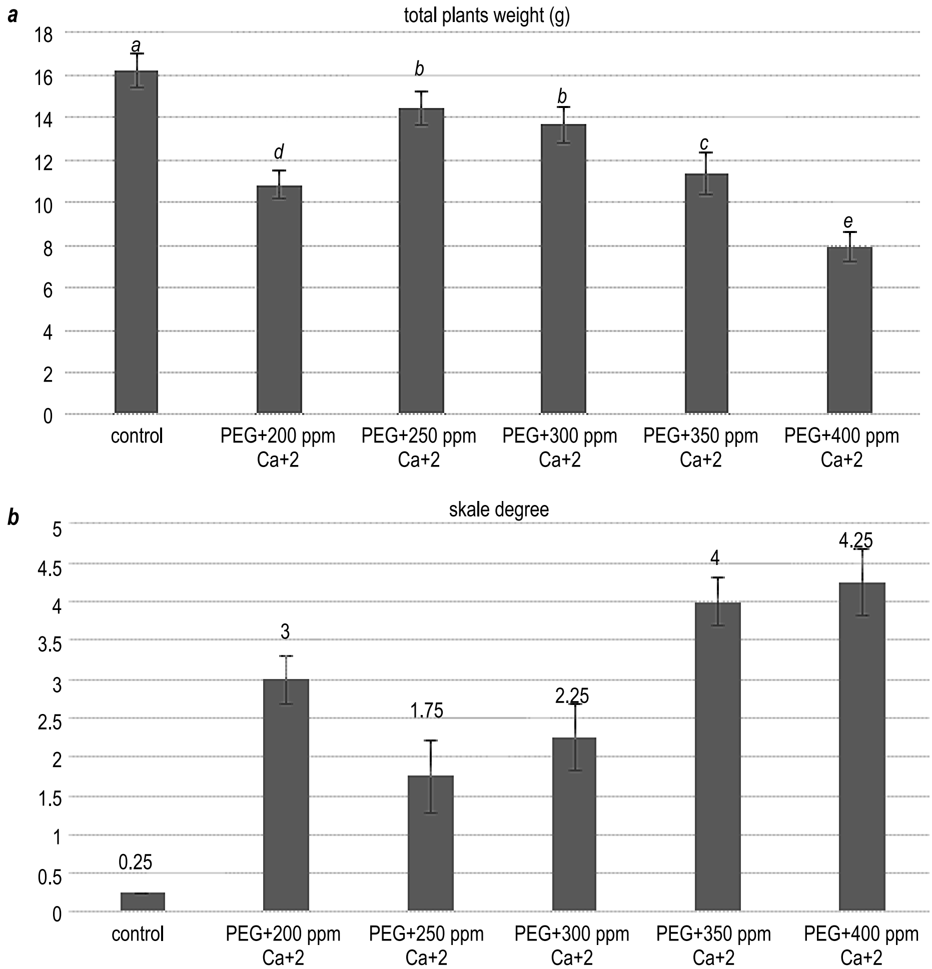

Fig. 2. Total plant weight (g) of seedlings under drought stress treated with calcium (Ca^{+2}): (*a*) and scores on the resistance scale according to symptoms of stress; (*b*) the difference between the means with the same letter is not significant according to $p \leq 0.05$

The amounts of chlorophyll and lipid peroxidation determined in the leaves of plants treated with calcium (Ca^{+2}) at the end of the 7^{th} day are given in Figure 3.

When the amount of chlorophyll was examined, the differences between the applications were found to be statistically significant. The chlorophyll content of drought-treated plants increased compared to the control. The highest amount of chlorophyll was measured in 300 ppm Ca^{+2} and 350 ppm Ca^{+2} applications in the same statistical group. When the damage to the cell membrane, which is a sign of drought stress-induced oxidative damage, or the amount of MDA, which is a by-product of lipid peroxidation, were examined, it was determined that there was an increase in levels

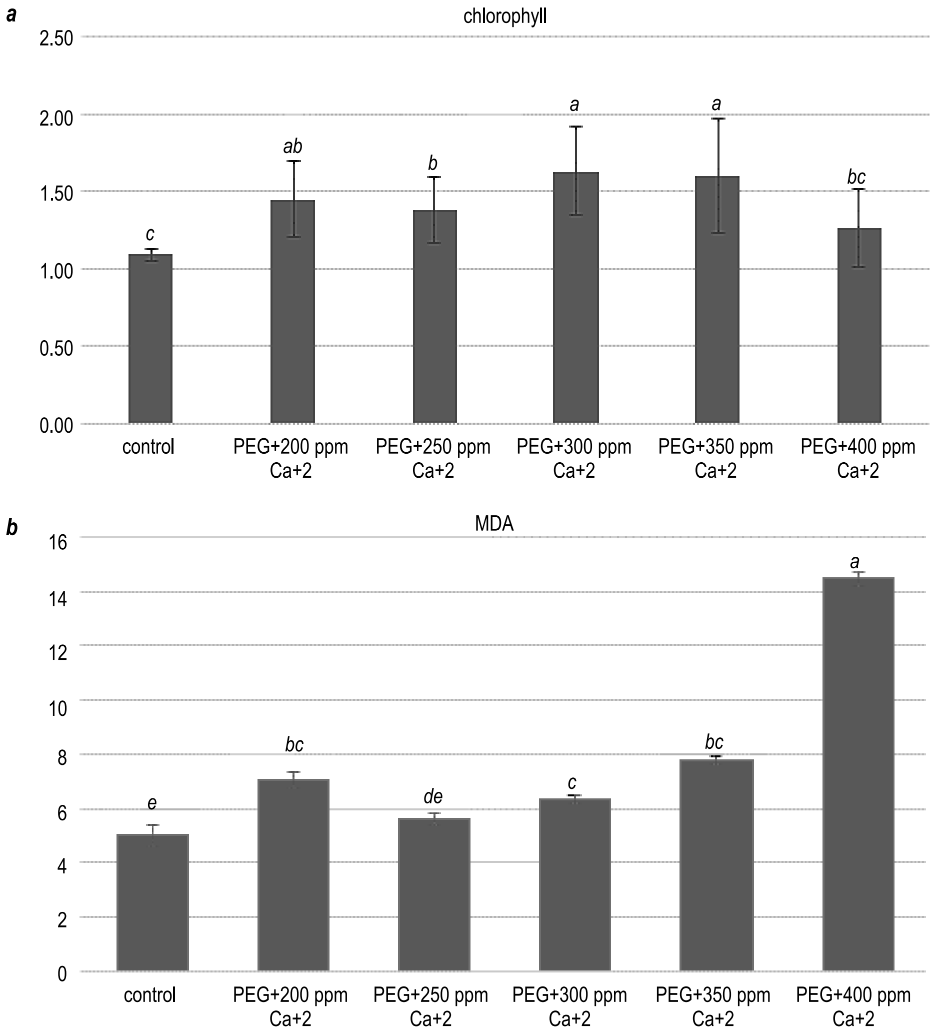

Fig. 3 Chlorophyll and MDA contents (μ mol g^{-1} F.W.) of leaves under drought stress treated with calcium (Ca^{+2}). The difference between the means with the same letter is not significant according to $p \leq 0.05$

of these properties in all drought stress applications compared to the control. Compared to the control, the highest increase was observed at 400 ppm Ca^{+2}, and the lowest value was observed at 250 ppm Ca^{+2}.

Antioxidant enzyme activities

The activities of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) (mol min^{-1} mg^{-1} F.W.) were examined in leaves of tomato plants treated with different doses of Ca^{+2} and growing under drought stress, and the data obtained are given in Figure 4.

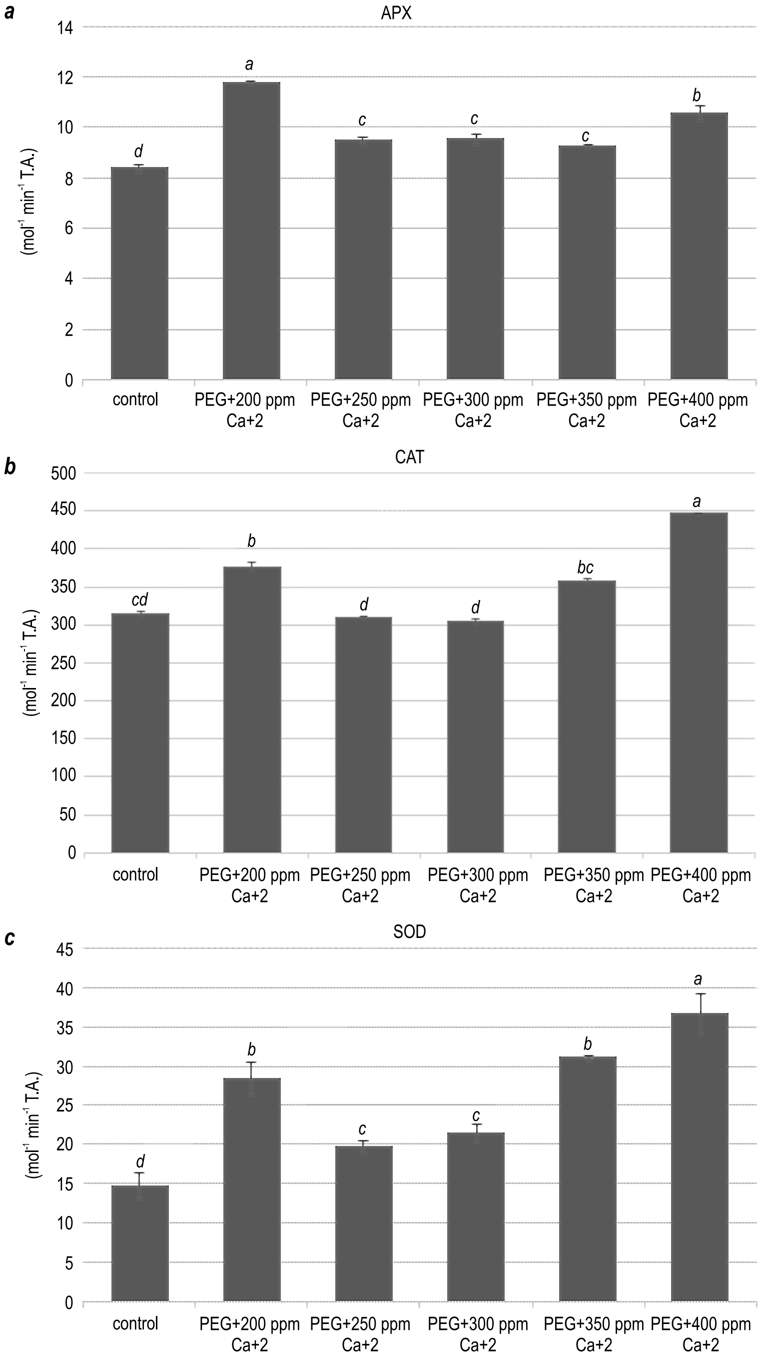

Fig. 4 The activities of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) (mol min^{-1} mg^{-1} F.W.) enzymes. The difference between the means with the same letter is not significant according to $p \leq 0.05$

When the enzyme activity of superoxide dismutase in the tomato leaves sampled on the 7th day after the Ca^{+2} application is examined, it is seen that there is an increase in all drought-treated plants compared to the control. The highest value of superoxide dismutase activity was measured in the 400 ppm Ca^{+2} application, while the lowest value was measured in the 250 ppm Ca^{+2} application. When the applications are examined in terms of ascorbate peroxidase enzyme activity, it is seen that there is a partial increase in all drought-treated plants compared to the control. Compared to the control group, the highest APX activity value was measured in plants given the 200 ppm Ca^{+2} application, while the lowest value was measured after the application of 350 ppm Ca^{+2}. It was determined that the results caused by the 250, 300, and 350 ppm doses of calcium were in the same statistical group. Statistical differences in terms of catalase enzyme activity were determined between the applications of calcium versus the control. Compared to the control group, catalase enzyme activity increased in all applications except the 200 and 250 ppm Ca^{+2} doses. The catalase enzyme activity following these two treatments was in the same statistical group as in the control plants. While the highest CAT value was measured in plants treated with 400 ppm Ca^{+2}, the lowest value was observed in plants given PEG+ 250 Ca^{+2} and PEG+ Ca^{+2} 300 ppm doses.

Discussion and conclusion

Drought negatively affects plant growth, development, dry matter production, and potential yield (Zhang et al. 2018). At the end of the study, despite the slight regression in plant growth and development, tomato plants generally continued to develop under drought stress. It is seen that there are much more significant differences between Ca doses in terms of total plant weight. In their salinity studies on different species, Yasar et al. (2008*a*, 2013, 2016) also showed that total plant weight is an important parameter in determining the response to salt stress. In this study, while the 1st dose of Ca could not reduce the negative effects of drought, especially the 2^{nd} and 3^{rd} doses and partially the 4th dose produced positive effects. The results we obtained in our study showed that under drought stress without increasing a Ca dose, the growth and development of plants decrease due to slowdowns in respiration. Hormonal disorders occur in the plant as a result of a decrease in stomatal motility in the respiratory system. Consequently, there is a decrease in the photosynthesis of the plant, thus the assimilation rate declines. As a result of all these, plant growth and development decrease (Yasar 2003). However, the decrease due to drought stress is lower in plants treated with an increased Ca dose because Ca acts as a signal molecule in different physiological and biochemical processes in the plant that can increase stress resistance, and it also acts as an important macronutrient and second messenger (White, Broadley 2003). As a matter of fact, there are studies proving the protective role of exogenous calcium applications against drought stress (Khushboo et al. 2018, Hosseini, 2019, Birgin et al. 2021). Calcium plays an important role in cell membrane stabilization, nutrient intake, as well as enzymatic and hormonal regulation (Ahmad et al. 2015). Because of these properties, Ca may enable plants to continue their development under drought stress. Calcium is one of the essential macronutrients for plant growth and development, participating in many processes such as cell wall stabilization, regulation of ion transport and selectivity, regulation of ion exchange behavior and enzyme activation, and structural and functional integrity of the plant membrane (Rengel 1992). Therefore, Ca is not only a mineral nutrient for the plant, but it also mediates the plant response to different stress conditions by regulating cell and plant development processes and many physiological and cellular aspects (White, Broadley 2003). For these reasons, it enables plants to continue their development with very little loss under drought stress.

In this study, having applied drought and Ca to tomato plants and measuring the results on a 1-to-5 scale, which is a morphological observation carried out to control all the growth and biochemical parameters we examined, it was found that the most damaged plants were seen at the 5^{th}, 4^{th} and 3rd doses of calcium, respectively. The least damage occurred at the 2^{nd} and 3^{rd} doses. The evaluation of plants on the scale created according to the degree of damage from drought stress showed the morphological signs of stress damage and their grading, and the accuracy of the biochemical analyses made in the study was tested by comparing it with other parameters examined. Aktas (2002) used the scale they created in their studies on pepper, Yasar (2003) – on eggplant, and Oztas (2018) - on pepper. All these researchers stated that the scale value was in very high correlation with total plant weights and especially with antioxidant enzyme activities and MDA.

The amount of chlorophyll is one of the photosynthetic properties strongly affected by water deficiency. In the case of water deficiency, the opening and closing of the plant's stomata directly affect the amount of chlorophyll (Havrlentová et al. 2021). The decrease in chlorophyll causes a reduction in plant productivity (Bijalwan et al. 2022). The chlorophyll amount was measured during the seven-day drought application. For this analysis, mature leaves were used. In this study, Peg + Ca applications showed an increase in the amount of chlorophyll compared to the control. The reason for this may be the decrease in leaf areas of plants under drought stress and the increase in the number of pigments per unit area under stress. At the 400 ppm Ca dose, a decrease was observed in the amount of chlorophyll compared to the other ca treatments, and it was close to the control, and the plants were stressed at that dose, showing an extreme regression in plant development. At the same time, an increase in the amount of MDA was observed. Therefore, it may be possible to say that there is an increase in the amount of ROS, and they cause damage to the chlorophyll pigments (Yasar et al. 2008, Yasar et al. 2010).

Drought stress impacts the electron transport chain, which initiates ROS production, which is harmful to cell organelles like mitochondria and chloroplasts. ROS reduces the amount of chlorophyll in leaves and decomposes the chlorophyll in the leaves, causing a decrease in photosynthetic pigmentation. In addition, the increase in drought and the reduction of N_2 fixation are associated with raised oxygen resistance caused by ROS (Farooqi et al. 2020). Most of the total protein and lipid composition of leaf tissue is found in chloroplasts. Therefore, lipids, one of the main components of the membrane, are affected by drought (Ishaku et al. 2020). ROS that is formed in plants under stress cause peroxidation of membrane lipids and damages the cell membrane (Sreenivasulu et al. 2000, Yasar et al. 2008a). In addition, the determination of the amount of malondialdehyde, a product of lipid peroxidation, is used as the simplest indicator of oxidative damage (Yasar 2003, Yasar et al. 2008*a*,*b*, 2010, Uzal 2017).

In order to understand whether the gradual increase of Ca applied to the drought-stressed tomato plants would reduce the damage effect of drought, the MDA amounts in the plants were examined. In the measured MDA amounts, the Ca dose closest to the control and correlated with the least damage were 250 and 300 ppm doses. The Ca dose with the highest MDA accumulation and thus the greatest damage was 400 ppm. As can be seen, it has been observed that the appropriate calcium dose protects plants from the harmful effects of drought stress.

The effects of different calcium levels on plants exposed to drought stress are seen in the cells of living plants, increasing the cytoplasm ratio (Knight 2000). The proline content is related to the water status of the plant and its accumulation has been found to increase under different abiotic stress conditions (Hasanuzzaman et al. 2018). In addition, exogenous Ca has been shown to improve plant water status by regulating a balanced proline content (Rahman et al. 2016). In addition, Ca prevented salt-induced damage from cellular dehydration by regulating the osmotic strength of the cytoplasm. In tomato plants sprayed with calcium carbonate, it causes a reduction in respiratory activities and leaf transpiration compared to tomato plants not sprayed with calcium carbonate. Accordingly, the development of the plant continues without a decrease in cell turgor (Patane et al. 2018).

There have been significant changes in the antioxidant enzyme activities of the plants due to the increase in the Ca doses in the nutrient media of the drought-stressed tomato plants. Five different doses of Ca were applied to the plants, and CAT, APX and SOD enzyme activities at all doses increased compared to the control, but the enzyme activities of the plants at the doses of 250 to 300 ppm Ca were found to be close to the control. However, it was observed that all three enzyme activities of the plants applied 200 and 400 ppm Ca were significantly higher than the control and other doses. These results showed parallelism with total plant weight, MDA, chlorophyll and scale values. The total plant weights of the plants treated with 250 and

300 ppm Ca were found to be higher than the others, and at the same time, the amount of MDA resulting from stress-induced cell damage was lower. Again, the enzyme activities of the plants to which these doses were applied were low. Considering all these results, the antioxidant enzyme activities were found to be low, since the drought-related stress severity was low in the plants to which these doses were applied. Yasar et al. (2020) investigated the CAT, APX and SOD enzyme activities in the leaves of pepper plants grown under salt stress and applied different doses of calcium in the nutrient media. It was observed that there were statistical differences between the applications in all three enzyme activities. The enzyme activities of the plants under salt stress increased compared to the control, but as the Ca doses increased, the enzyme activities started to decrease and approached the control according to the dose increase. Similarly, Yaşar and Üzal (2021) investigated the growth performance and antioxidant enzyme activities of the plants by applying different doses of potassium to the nutrient solutions of tomato plants under salt stress. In the study, the researchers evaluated the results of antioxidant enzyme activities and total plant wet weights, and concluded that potassium protected plants from the toxic effect of salt. In this context, they stated that the activities of antioxidant enzymes decreased because the plants were not stressed or were exposed to very little stress. In addition, in their study on tomato plants, Tuna et al. (2016) found that CAT, SOD and APX enzyme activities were increased at low doses of potassium, while there was a decrease in the activities of these enzymes at high potassium doses.

Under biotic and abiotic stresses, oxidative stress occurs in plants, resulting in increased production of reactive oxygen species ROS (Qu et al. 2013, Uzal et al. 2019). Mullineaux et al. (2000) reported that HO functions as a signaling molecule during abiotic stresses as well as during pathogen-induced responses. Superoxides (O_2) are toxic by-products of oxidative metabolism and react with H_2O_2 to form highly reactive hydroxyls (–OH), which are primarily responsible for the appearance of oxygen radicals in cells (Yasar 2003, Miller et al. 2010). Dismutation of O_2 and H_2O_2 to oxygen is the first step in protecting the cell (Azevedo-Neto et al. 2005). Antioxidant enzymes act as ROS (reactive oxygen species) scavengers to mitigate oxidative damage. SOD (superoxide dismutase) reacts with superoxide radical to produce H_2O_2, and H_2O_2 is swept away by peroxidases, primarily APX (ascorbate peroxidase) and CAT (catalase) Yasar (2003), Yasar et al. (2010), Miller et al. (2010). An increase in the enzyme activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) in four tomato genotypes exposed to drought stress was noticed, and this rise was found to be associated with a protective role against reactive oxygen species (ROS).

As a result, we can say that the plants can be protected from stress to a significant level, which is demonstrated by the results obtained in our study when the appropriate doses of Ca are applied to tomato plants experiencing drought stress. When 250 and 300 ppm Ca were added to the nutrient solution supplied to tomato plants treated with 7% (PEG 6000) polyethylene glycol, the MDA amounts and enzyme activities of the plants were low, and the total plant weights were high. For this reason, it was concluded that the stress levels of the plants to which these doses were applied were low.

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