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Araştırma Makalesi

Changes in Germination, Antioxidant Enzyme Activities and Biochemical Contents of Safflower (*Carthamus tinctorius* L.) Under Different Salinity Levels

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Abstract: The present study was carried out using Dinçer and Olas safflower varieties at 5 different salt (NaCl) concentrations (0, 50, 100, 150, and 200 mM) for 14 days. The germination percentages of the cultivars under salt conditions as well as the activities of antioxidant enzymes (SOD, CAT, POD and APX) and biochemical changes (protein and MDA) in the seedlings were determined. The germination percentage decreased with increased salt concentrations, and the greatest decrease in germination percentage was observed at a 200 mM salt concentration by 34% in both cultivars. The activity of superoxide dismutase (SOD) increased at low salt concentrations, but decreased after 100 and 150 mM salt concentrations, respectively. Catalase (CAT) and ascorbate peroxidase (APX) activities, as well as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents, increased with increasing salt concentrations at Dinçer and Olas, but total soluble protein content decreased with increasing salt concentration. Peroxidase (POD) activity was not significantly affected by salt stress in safflower. Germination percentage showed negative correlations with CAT, MDA and H_2O_2 levels, and showed a positive correlation with soluble protein content under salt stress in safflower. The present results may be useful to identify mechanisms of salt tolerance involving antioxidant enzyme activities and biochemical changes in safflower seedlings.

***Key words**:* Germination, Lipid peroxidation, Oxidative stress, Protein, Salt stress

Farklı Tuzluluk Seviyelerinde Aspir'de (*Carthamus tinctorius* L.) Çimlenme, Antioksidan Enzim Aktiviteleri ve Biyokimyasal Bileşenlerin Değişimi

Öz: Mevcut çalışma Dinçer ve Olas aspir çeşitleri kullanılarak 5 farklı tuz (NaCl) konsantrasyonunda (0, 50, 100, 150 ve 200 mM) 14 gün boyunca yapılmıştır. Çeşitlerin tuzluluk şartları altında çimlenme oranları ve fideciklerdeki antioksidan enzim aktiviteleri (SOD, CAT, POD ve APX) ve biyokimyasal değişiklikler (protein and MDA) belirlenmiştir. Tuz konsantrasyonu arttıkça çimlenme oranı azalmış ve her iki çeşit için çimlenme oranındaki en yüksek düşüş %34 ile 200 mM tuz konsantrasyonunda gözlenmiştir. Süperoksit dismutaz (SOD) aktivitesi düşük tuz konsantrasyonlarında artmış fakat Dinçer çeşidinde 100 mM ve Olas çeşidinde 150 mM tuz konsantrasyonlarında SOD aktivitesinin azaldığı gözlenmiştir. Dinçer ve Olas çeşitlerinde katalaz (CAT) ve askorbat peroksidaz (APX) aktivitesi, malondialdehit (MDA) ve hidrojen peroksit (H_2O_2) miktarlarının tuz konsantrasyonlarına bağlı olarak arttığı fakat toplam çözünebilir protein içeriğinin ise azaldığı belirlenmiştir. Peroksidaz (POD) aktivitesi aspirde tuz stresine bağlı olarak önemli bir değişim göstermemiştir. Aspirde tuz stresi sonucu çimlenme yüzdesindeki azalma CAT, MDA ve H_2O_2 ile negatif, çözünür protein miktarı ile pozitif korelasyon göstermiştir. SOD aktivitesindeki değişim ise POD, CAT, MDA ve H_2O_2 ile pozitif korelasyonları göstermiştir. Mevcut sonuçlar, aspirin tuzluluk toleransını regüle etmek için fideciklerdeki antioksidan enzim aktiviteleri ve biyokimyasal içeriklerdeki değişimler hakkında yararlı olacaktır.

***Anahtar kelimeler**:* Çimlenme, Lipid peroksidasyon, Oksidatif stres, Protein, Tuz stresi

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1. Introduction

Safflower (*Carthamus tinctorius* L.) belongs to the Asteraceae family and it is cultivated worldwide for its oil and flowers, which are used in food, cosmetics and paint industries. Due to Its tolerance to drought and salinity, it is suitable for cultivation in semi-arid and saline soils where other oliferous plants cannot be grown economically. Salinity is an important abiotic stress factor, and plays an important role in limiting the production of various crop species [1]. More than 20% of the world's agricultural land and 33% of irrigated land are affected by salinity [2], and salinity could affect about 50% of arable land by 2050 [3].

Salinity is an important problem that limits plant growth and reduces yields. Sodium chloride (NaCl) is among salts that cause salinity, and salinization caused by sodium chloride accounts for most of the affected land by salinity [4]. Salt stress reduces efficiency of the electron transport system and various detoxification reactions, and increases photorespiration and oxidation in mitochondria and chloroplasts [5]. In addition, high Na^+ concentration in tissues leads to disruption of ion homeostasis, membrane damage, and reduction of metabolic activity, which further reduces plant growth and yield [6]. Salinity causes water deficit stress due to high solute concentrations in soil and disruption of K^+/Na^+ and Na^+/Cl^- balance in plant tissues [7]. Water stress caused by salinity produces reactive oxygen species (ROS), such as singlet oxygen (O_2), hydroxyl ($OH^•$) and superoxide (O_2^-) radicals and hydrogen peroxide (H_2O_2), resulting in oxidative stress in cells [8].

Plants have both non-enzymatic and enzymatic antioxidant defense mechanisms for detoxification of ROS, such as superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11). Many studies revealed that these antioxidant enzymes have important roles in the defense mechanisms of various plants under salt stress [9, 10]. Free radicals adversely affect the function and structure of organelles and macromolecules by inducing lipid peroxidation in cells [11]. CAT, APX and SOD maintain stable levels of hydroxyl and superoxide radicals in cells. CAT and SOD work together because the product of the reaction catalyzed by SOD is the substrate (H_2O_2) of CAT [12]. One of the most commonly used assays to determine oxidative damage is the measurement of malondialdehyde (MDA), the product of lipid peroxidation, an indicator of stress-induced damage at the cellular level [13]. Hydrogen peroxide could act as an intracellular signal molecule by passing through cell membranes [14]. However, metal ions produced under stress conditions react with H_2O_2, leading to the formation of hydroxyl molecules and oxidative damage in the cell. Detoxification of H_2O_2 occurs through CAT and APX, and SOD scavenges superoxide radicals and prevents the formation of hydroxyl radicals [4].

Germination, seedling development, flowering, and seed maturation are sensitive developmental stages that are critically affected by stress conditions in plants. Therefore, the aim of the present study was to investigate seed germination, the antioxidant enzyme activities and biochemical changes in seedlings of safflower under different salt concentrations.

2. Material and Method

2.1 Seed material and stress treatments

Safflower cultivars, Dinçer and Olas, were used as plant materials. Surface sterilization of the seeds was performed by soaking the seeds in sodium hypochlorite solution (1%) for 10 min. Then all seeds were rinsed with distilled water and dried overnight at room

temperature. Seeds for each treatment were sown in 15 cm wide Petri dishes covered with two layers of Whatman No. 1 filter paper moistened with 15 ml solution. Five different concentrations (0, 50, 100, 150, and 200 mM) of sodium chloride solutions were used to assess the germination percentage of the seeds. Seeds were incubated at 25±1 °C for 14 days in a germination cabinet [15]. Each petri dish contained 50 seeds, and each treatment contained four replications. After 14 days, the germination percentage was calculated using the following formula [16].

$$\text{Germination percentage} = (N_p / N_t) x\, 100 \quad (1)$$

N_p: the number of germinated seeds, N_t: the total number of seeds

Seedling samples were frozen in liquid nitrogen and stored at -80 °C until biochemical and antioxidant enzyme analysis.

2.2 Biochemical analyses and enzyme assays

For the extraction of SOD, POD, and MDA, samples (5 g) were homogenized in 12.5 mL of 100 mM ice-cold sodium phosphate (pH 6.4) containing 0.5 g of polyvinylpolypyrrolidone. For CAT extraction, 12.5 mL of 50 mM ice-cold sodium phosphate (pH 7.0) containing 0.5 g of polyvinylpolypyrrolidone was used, and for APX extraction, 12.5 mL of 50 mM potassium phosphate buffer (pH 7.3) containing 1 mM EDTA and 2 mM DTT was used. The homogenate was centrifuged at 27.000 x *g* for 50 min at 4 °C, and the supernatants were used to measure enzyme activity. The protein content of the extracts was determined following Bradford method [17].

The SOD enzyme activity was assayed according to Constantine and Stanley [18] with slight modifications. The reaction mixture contained 3 mL of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitroblue tetrazolium (NBT), 10 µM EDTA, 2 µM riboflavin and 0.1 mL of the enzyme extract. Glass tubes containing the mixture were illuminated with light (60 µmol/m^2/s) for 10 min and absorbance was then determined at 560 nm. Identical solutions, which were not illuminated served as blanks. One unit of SOD activity was defined as the level of enzyme activity that caused a 50% decrease in SOD-inhibitable NBT reduction. The SOD enzyme activity was expressed as U mg^{-1} protein.

The POD activity was performed following the method of Jiang et al. [19]. The reaction mixture contained 0.5 mL of the enzyme extract, 2 mL of buffered substrate (100 mM sodium phosphate, pH 6.4 and 8 mM guaiacol). The reaction was initiated with the addition of 1 mL of H_2O_2 (24 mM). The increasing absorbance was recorded at 460 nm for 120 s. The specific activity was expressed as U mg^{-1} protein.

The CAT enzyme activity was determined according to the method of Beers and Sizer [20]. The CAT reaction mixture contained 0.5 mL of enzyme extract, 2 mL of 50 mM sodium phosphate buffer (pH 7.0), and 0.5 mL of 40 mM H_2O_2. The reaction was initiated with the addition of H_2O_2. Decreasing absorbance for H_2O_2 (ε_{240}= 43.6 mM^{-1} cm^{-1}) was recorded at 240 nm for 120 s. One unit of CAT was defined as H_2O_2 decomposition per minute. The CAT specific activity was expressed as U mg^{-1} protein.

The APX enzyme activity was assayed by the procedure of Nakano and Asada [21]. The reaction mixture contained 900 µL of 0.05 M Na-phosphate buffer (pH 7.0), 1.2 mM H_2O_2, 0.1 mM EDTA-Na_2, 0.5 mM ascorbate and 0.1 mL of the enzyme extract. The reaction was initiated with the addition of H_2O_2. The decreasing absorbance was recorded at 470 nm for

180 s. The specific activity was expressed as U mg^{-1} protein. One unit of APX was defined as the level of enzyme activity causing a change of 0.01 in absorbance per minute. The APX specific activity was expressed as U mg^{-1} protein.

MDA analysis was performed as described by Jiang et al. [19]. Two mL of 0.5% thiobarbituric acid (TBA) in 15% trichloroacetic acid (TCA) was added to 1 mL of the solution. The mixture was incubated at 95 °C for 20 min and centrifuged at 12.000 x *g* for 10 min. The absorbance at 532 nm was measured and subtracted from the absorbance at 600 nm. The amount of MDA was calculated using an extinction coefficient of 155 mM cm^{-1}.

H_2O_2 content was determined according to the method of Velikova et al. [22]. Samples (100 mg) were homogenized in 5 mL of 0.1% TCA and centrifuged at 15.000 x *g* for 15 min at 4 °C. The reaction mixture contained 0.5 mL of 10 mM phosphate buffer (pH 7.0), 1 mL potassium iodide (1 M) and 0.5 mL of the extract. The reaction mixtures were incubated in the dark for 1 h, and absorbance was measured at 390 nm. The H_2O_2 content was expressed as μmol g^{-1}.

Protein extraction was carried out as described in Tonguç et al. [23]. The total soluble protein content was determined according to the method of Lowry et al. [24]. The reaction mixture contained 0.9 mL of 7 mM potassium-sodium tartrate, 0.81 M sodium carbonate, 0.5 N sodium hydroxide and 1 mL of the extract. The reaction mixtures were incubated in a water bath at 50 °C for 10 min. Then, 0.1 mL of the solution containing 70 mM potassium-sodium tartrate, 40 mM copper sulfate and 3 mL Folin-Ciocalteu reagent was mixed with the reaction mixture and incubated in a water bath at 50 °C for 10 min. After cooling, absorbance of the samples was determined at 650 nm and bovine serum albumin (BSA) was used to obtain the standard curve.

2.3 Statistical analysis

The germination experiments were performed with 4 replications and biochemical and enzyme analyses were performed with three replicates. Results were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test ($p \leq 0.05$) was used to discriminate the differences between the means. To show the relationship between the parameters, Pearson's linear correlation analysis (heat map correlation) was calculated using OriginPro software (version 2021, OriginLab, Northampton, MA). All values were expressed as mean ± standard deviation (SD).

3. Results and Discussion

The germination percentages of Dinçer and Olas decreased with increasing salt concentration. At a concentration of 200 mM NaCl, the germination percentage of both cultivars decreased by 34% compared with the control (Fig. 1A). At 100 and 150 mM NaCl concentrations, the germination percentage in Dinçer decreased to 78% and 76%, respectively, although reduction in germination percentage between the treatments was not significant. However, the reduction in germination percentage for the same treatments for Olas was 79% and 71%, respectively, and was significant between the treatments. Germination is one of the first and most critical events in the life cycle of plants and it is very sensitive to environmental conditions [25]. Salinity is one of the most important environmental factors adversely affecting germination, seedling growth and plant development in crops. The germination of 10 safflower cultivars at 5 different NaCl concentrations (0, 60, 120, 180 and 240 mM) decreased with increasing salt concentration,

and with the greatest decrease in germination at 180 and 240 mM salt concentrations [16]. It was also reported that increased salt concentrations decreased germination of safflower and sunflower [26, 27]. Salinity inhibits germination by interfering with water uptake, metabolic activity, protein structure and function, resulting in ion toxicity and oxidative stress in plants [28, 29].

Antioxidant molecules and enzymes are involved in the detoxification of ROS produced in cells under stress conditions by catalyzing the degradation and recycling of ROS [6]. Due to the type of stress applied, the timing and duration of application, age of the plants, intensity of stress conditions, type of tissue sampled, and genotypic differences between the used, different results could be obtained for antioxidant enzyme levels in plants under stress conditions. Therefore, it is difficult to provide definitive evidence and modeling to explain how antioxidant enzyme activities change during stress within and between plant species. In the present study, activities of four antioxidant enzymes that are part of the detoxification mechanism in plants under different salt conditions were investigated. SOD activity in Dinçer increased and reached its highest level up to 100 mM salt concentration (15.65 U mg^{-1} protein). SOD activity in Dinçer decreased at 150 and 200 mM salt concentrations (Fig. 1B). SOD activity reached the highest level at 150 mM (10.76 U mg^{-1} protein), and decreased at 200 mM salt concentrations (9.70 U mg^{-1} protein). Similar to our observation, Erdal and Cakirlar [4] reported that the activity of SOD initially increased at low salt concentrations, but decreased at higher salt concentrations in safflower. From the published reports, it may be concluded that salt stress inhibits SOD activity at high concentrations, leading to accumulation of ROS.

POD is involved in cell wall biosynthesis, lignification, suberization, ethylene biosynthesis, and protects cells from the effects of H_2O_2 by catalyzing H_2O_2 to water [30, 31]. The salt treatments increased POD activity in Dinçer compared to control and the highest activity was observed at 100 mM salt treatment (Fig. 1C), but the differences between the other salt treatments for POD activity were not significant to each other. In Olas, similar to Dinçer, there was a significant increase only at 150 mM salt concentration. POD activity was the same between control and 50 mM treatment. Jabeen and Ahmad [26] reported that POD activity increased with increasing salt concentration in sunflower compared to control, while the POD activity increased in safflower up to a salinity level of 8.6 dS m^{-1} and remained unchanged at higher concentrations.

CAT activity increased significantly with increasing salt concentrations in both cultivars (Fig. 1D). The highest CAT activity (144.98 U mg^{-1} protein) was observed at 200 mM salt concentration in Olas, whereas the highest CAT activity (128.85 U mg^{-1} protein) was observed at 100 mM salt concentration in Dinçer. However, CAT activity was not significantly different at 100 and 150 mM salt concentration, and the lowest CAT activity was observed in control treatments of both cultivars in the study. Similar to our results, Jabeen and Ahmad [26] reported that CAT activity increased with increasing salt concentration in safflower and sunflower compared to control, and Alasvandyari and Mahdavi [32] found that CAT activity increased in safflower under 50, 100 and 150 mM salt concantrations compared to control. Salt stress increases ROS in plants and triggers oxidative damage in membranes [33]. Enzymes, such as CAT are part of the detoxification mechanism to remove and reduce free radicals, thus maintaining homeostasis between ROS production and reduction to limit their effects within cells [34].

APX converts H_2O_2 to water and oxygen. APX activity in both safflower cultivars increased at all salt concentrations compared to control (Fig. 1E). At a concentration of 200 mM NaCl,

APX activity in Dinçer and Olas increased 5- and 6.5-fold, respectively, compared with the control. It was reported that APX activity increased in sesame [35], canola [10], and safflower [4, 36] under saline conditions, as was also observed in the present study.

It is well known that an increase in ROS causes lipid peroxidation, which leads to oxidative damage [37-39]. Lipid peroxidation is the main cause of membrane damage and ion leakage, and is distinguished by increased MDA levels, as they are the products of oxidation of polyunsaturated fatty acids [40, 41]. In the present study, MDA levels increased 3.4- and 2.5-fold with higher salt concentrations in Dinçer and Olas, respectively, at 200 mM salt concentration (Fig 1F). MDA content was reported to increase in alfalfa [42], safflower [4, 36], tomato [43] and chickpea [44] under salinity. MDA accumulation depends on the activities of CAT and APX, which in turn reduces membrane damage by detoxifying ROS and H_2O_2 in cells [26].

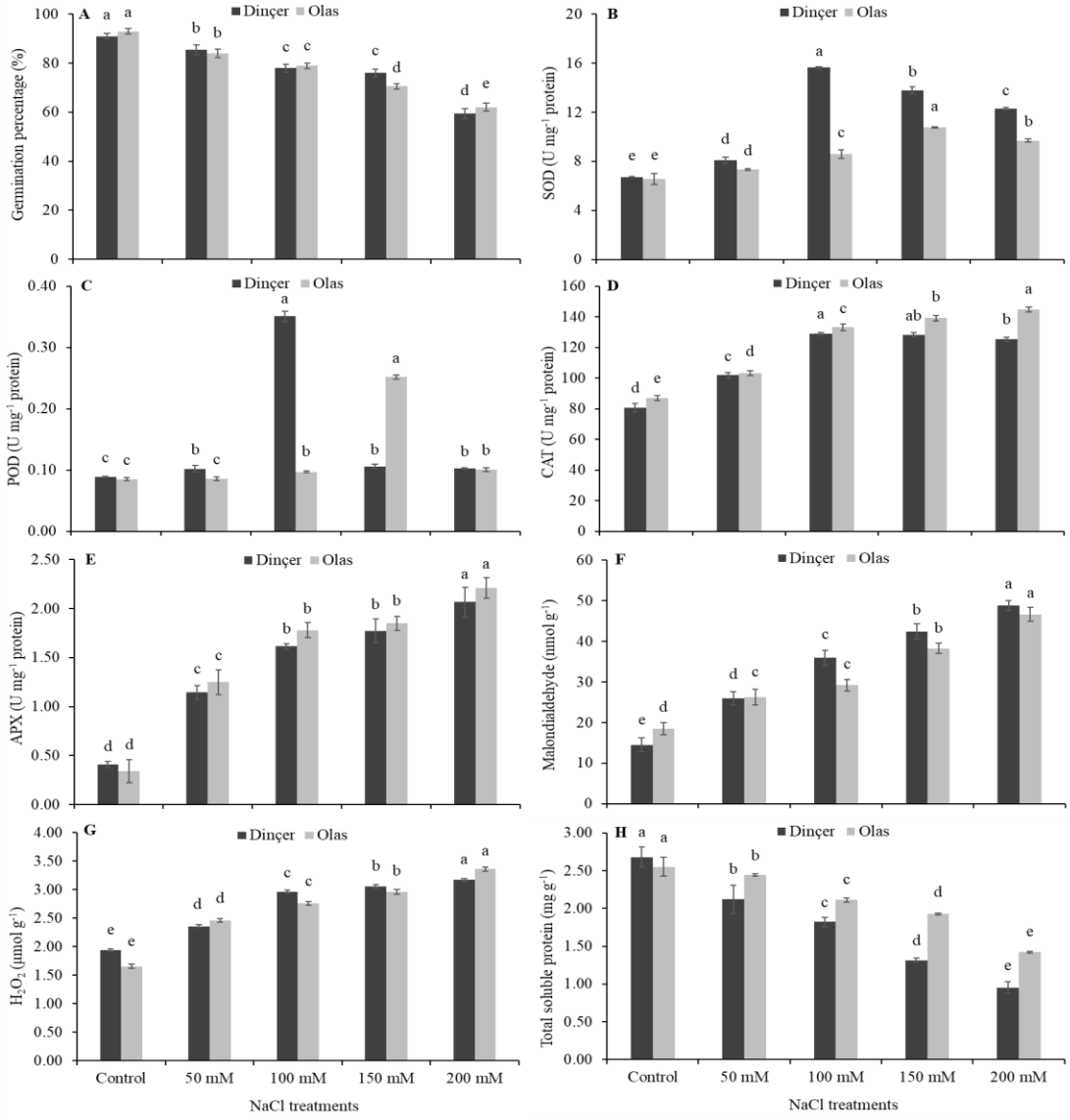

Figure 1. Figures showing effects of 5 different salt concentrations on (A) germination percentage; (B) superoxide dismutase (SOD); (C) peroxidase (POD); (D) catalase (CAT); (E) ascorbate peroxidase (APX); (F) malondialdehyde (MDA); (G) hydrogen peroxide (H_2O_2); and (H) total soluble protein for the safflower cultivars. Values represent the means ± SE from three replicates. Different letters indicate significant differences between treatments at 0.05 level.

The most commonly found reactive oxygen species are superoxide and hydroxyl radicals, H_2O_2 and singlet oxygen [38, 39]. Metabolic processes in plant cells consume oxygen,

which is converted to ROS as a byproduct, and ROS is removed by the antioxidant defence system [45]. Salt stress causes excessive ROS production by disrupting the balance between accumulation and detoxification of ROS in plants. The H_2O_2 content, associated with the ROS accumulation, increased with increasing salt concentration (Fig. 1G). The highest H_2O_2 content was observed at 200 mM salt concentration for Dinçer (3.17 µmol g^{-1}) and Olas (3.36 µmol g^{-1}). Shaki et al. [36] also reported that the H_2O_2 content in safflower increased with increasing salinity compared to the control.

The accumulation of organic compounds in the cytoplasm plays an important role in osmotic regulation of plants by reducing water potential and alleviating stress damage. Total soluble protein content in Dinçer and Olas decreased with increasing salinity compared to controls (Fig. 1H). The total soluble protein content of Dinçer and Olas decreased by 65% and 44%, respectively, at 200 mM salt concentration compared with the control. Salinity decreases amino acid production, denatures enzymes involved in protein synthesis, decreases protein synthesis and protein content in plants [46]. Jabeen and Ahmad [26] reported that the protein content of safflower and sunflower decreased by 46% and 42%, respectively, with increasing salt concentration compared to the control. Shaki et al. [47] showed that at 100 and 200 mM salt concentration, the soluble protein content decreased as a function of increased salt concentration in safflower.

The result of Pearson's linear correlation analysis for germination percentage, biochemical analyses, and antioxidant enzyme activities were given in Fig. 2. Of the 28 coefficients, 13 were significant. Of the 13 coefficients, 8 were positively correlated and 5 were negatively correlated. Germination percentage was negatively correlated with CAT (-0.83), MDA (-0.95), and H_2O_2 (-0.92) and positively correlated with total soluble protein (0.86). SOD had positive correlations with POD, CAT, MDA, and H_2O_2.

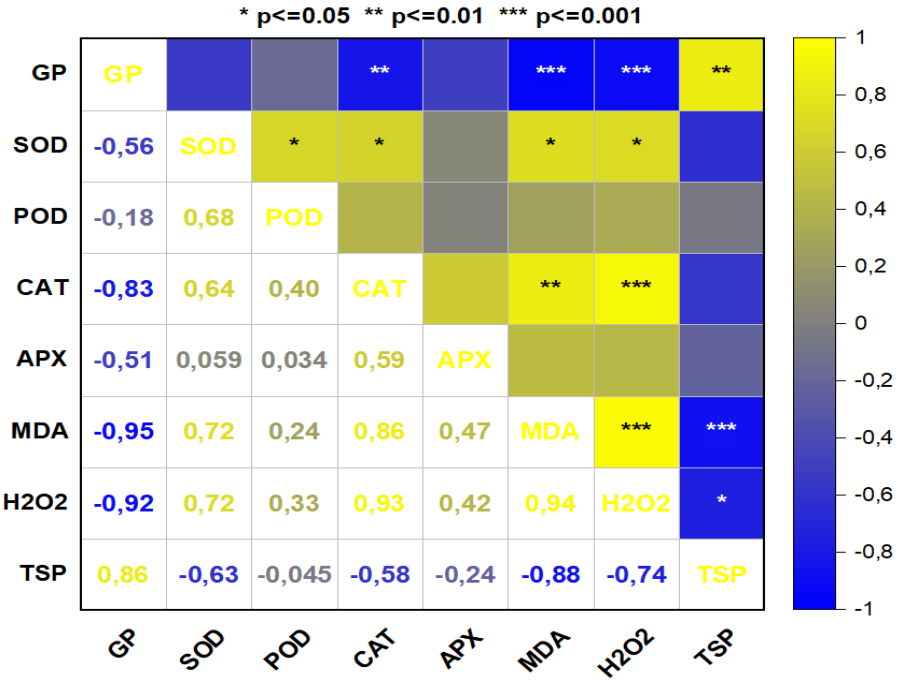

Figure 2. Relationships and correlations obtained for safflower under different salt stress by Heat map using the mean values. (GP, Germination percentage; SOD, Superoxide dismutase; POD, Peroxidase; CAT, Catalase; APX, Ascorbate peroxidase; MDA, Malondialdehyde; H_2O_2, Hydrogen peroxide; TSP, Total soluble protein)

4. Conclusion and Comment

Development of new salt-resistant crop varieties depends on elucidating the underlying genetic and physiological mechanisms of resistance. To understand these mechanisms, it is necessary to screen diverse germplasm sources to characterize their resistance levels and to study the underlying mechanisms. In the present study, the germination capacity of safflower cultivars was greatly reduced at 150 and 200 mM salt concentrations. In addition, plants accumulated increased amounts of H_2O_2 and MDA, byproducts of lipid peroxidation, under salt stress. SOD, CAT and APX activities showed a close relationship with salt stress in the study, but POD activity did not show any significant correlation to other parameters. Germination capacity was positively correlated with protein content and negatively correlated with H_2O_2, MDA and CAT. The present study will enhance our understanding of salinity tolerance mechanisms in safflower during germination and seedling development stages.

Author Statement

Sercan ÖNDER: Investigation, Original Draft Writing, Review and Editing
Ebru DAYAN: Investigation.
Yaşar KARAKURT: Supervision.
Muhammet TONGUÇ: Original Draft Writing, Review and Editing.

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Conflict of Interest

As the authors of this study, we declare that we do not have any conflict of interest statement.

Ethics Committee Approval and Informed Consent

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

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