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Alleviation of *Phytophthora capsici*-induced oxidatıve stress by foliarly applied proline in *Capsicum annuum* L.

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Abstract: *Phytophthora capsici* is a highly destructive pathogen of pepper. To examine whether proline modifies the levels of plant defense compounds produced in response to *P. capsici*-induced stress, pepper seedlings were infected with *P. capsici-22* in the presence of proline (1 mM, 10 mM) or in its absence. Proline was sprayed on the leaves of CM-334 and Kekova pepper cultivars prior to inoculation. CM-334 was more resistant to *P. capsici-22*, while the Kekova cultivar exhibited a sensitive reaction. *P. capsici-22* increased the total phenolic compound and H_2O_2 levels, as well as phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase activities in pepper seedlings. The application of exogenous proline further increased the activities of phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase, as well as the total levels of phenolic compounds and the fresh and dry weights of the plants on the 5th and 7th days post treatment. After proline application, the highest catalase activity was found in both cultivars on the 5th day of the 10 mM proline + *P. capsici* application. On all days of the experiment, the applications caused a decrease in disease severity, necrosis length and H_2O_2 levels in both cultivars. In addition, proline decreased the colony growth of *P. capsici* and the number of zoospores. This finding indicates that enzymes and total phenolic compound levels protect the pepper seedlings against stress-related damage. Moreover, proline has the potential to directly scavenge free radicals and promote enzyme activity in pepper seedlings under *P. capsici* stress. These results suggest that foliar application of proline is an effective way to improve the stress tolerance of pepper to *P. capsici*.

Key words: antioxidants; *Capsicum annuum*; *Phytophthora* root rot; proline; oxidative stress tolerance

INTRODUCTION

P. capsici is an important oomycete plant pathogen that results in significant losses worldwide. Phytophthora root rot caused by *P. capsici* is one of the most destructive soilborne diseases of pepper (*Capsicum annuum* L.) production worldwide [1]. *P. capsici* is detected in many plant species and is known to infect more than 45 cultivated species, including cucurbits, pepper, tomato, eggplant, watermelon, cocoa, pumpkin, squash, snap and lima beans [2-3]. Phytophthora root rot is found on the roots, stems, leaves and fruits of the plant. *P. capsici* has been reported at locations worldwide, including North and South America, Asia, Africa and Europe [4]. *P. capsici* results in significant product loss in the Marmara, Aegean, Mediterranean, Black Sea and southeastern regions of Turkey, which are all areas with significant pepper cultivation [5].

P. capsici was first described on chili pepper in New Mexico [2]. As reported in previous pepper breeding studies, it is unfortunately not possible to obtain a pepper culture resistant to all *P. capsici* isolates. Studies on pepper have also shown that the use of chemicals against pepper root rot is not significantly effective [6]. These chemicals also have a negative impact on human health, they pollute the environment, decrease plant quality and increase the cost of the product. Therefore, the use of resistant varieties represents the safest way to grow pepper.

The amino acid proline plays a significant role in plants exposed to environmental stresses such as drought, salinity and temperature extremes. In addition to its role as an osmolyte, proline also plays two major roles during stress – as a metal chelator and as a signaling molecule [7]. *In vitro* studies have shown that proline is a free radical scavenger that protects

macromolecules from denaturation [8]. Proline can also protect cellular and subcellular membranes from oxidative stress [9]. The regulation of proline metabolism in plants under growth-related and abiotic stress conditions has been undertaken, and several reports indicate that the exogenous application of proline can play an important role in enhancing plant tolerance to abiotic stresses. While these studies mostly focused on abiotic stress, there is little information on the effects of exogenous proline application to plants exposed to biotic stress. Reviewing the literature has revealed that there are no studies or data concerning the effect of exogenous proline application to pepper seedlings exposed to *P. capsici* stress, or of the effect of proline on *P. capsici* colony growth and numbers of zoospores.

Thus, the aim of this study was to determine to the extent to which exogenous application of proline could change certain physiological parameters, such as phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX) and catalase (CAT) activities, the total amounts of phenolic compounds and hydrogen peroxide (H_2O_2), as well as the fresh and dry weights, disease severity and the length of necrosis. These parameters are accepted as indicators of disease resistance and of responses to pathogen infection in two pepper cultivars with different sensitivities to *P. capsici*. In addition, the effect of proline application on *P. capsici* colony growth and number of zoospores was investigated. It is expected that the obtained data will stimulate novel approaches to *P. capsici* control and open the way for similar applications in other species.

MATERIALS AND METHODS

Plant material

Criollo de Morelos 334 (CM-334) and Kekova pepper (*Capsicum annuum* L.) cultivars were used. The cultivar CM-334 is resistant to the *P. capsici* pathogen [10]. CM-334 was supplied by the French National Institute for Agriculture Research (INRA) (Montfavet, France). Kekova is a highly productive cultivar that is commercially grown in Turkey, but is susceptible to the disease. The Kekova pepper cultivar was supplied by the Antalya Agricultural Production and Marketing Consulting Corporation (Antalya, Turkey). After germination, pepper seedlings were sown in a plastic pot containing a steam-sterilized soil/fertilizer/sand mix (at a ratio of 1/1/1, v/v/v). The plants were grown in a growth chamber under controlled environmental conditions (25±2°C and 16-h light, 8-h dark photoperiods). At the end of two months, the seedlings were collected once they reached the six-leaf stage.

Preparation of *Phytophthora capsici* Leon.-22 zoospore suspension

P. capsici-22 was obtained from the fungal culture collection of Ankara University, Faculty of Agriculture, Ankara, Turkey, and was grown on V8-juice agar plates at 25°C in the dark [11]. Zoospores were produced from the mycelia. Zoospore production and spore concentrations were determined as described previously [12-13]. The concentration of 10^4 zoospores mL^{-1} was the desired inoculum concentration and the optimal zoospore concentration for causing the disease in pepper. This concentration was obtained by diluting with sterile distilled water that had several drops of Tween-20 (to collect spores) per L.

Proline treatment and plant inoculation

The roots of the seedlings were washed with tap water and disinfected with sodium hypochlorite (0.75%) for 1-2 min and then washed with sterile distilled water several times. Five seedlings of similar size with six true leaves were bunched together and wrapped in aluminum foil 3-4 cm above the root. Ten seedlings were placed into a sterile glass bottle containing 400 mL of Hoagland solution. The plants were then incubated for three days at 22±3°C, 60% humidity and a 14-h light period, so that they could acclimatize. The following four treatments were applied on the two pepper cultivars: control (without *P. capsici* and proline); *P. capsici* alone; 1 mM proline + *P. capsici*; 10 mM proline + *P. capsici*. For both cultivars, each treatment was repeated three times (three bottles were used for each repetition). A total of 30 seedlings were used for each repetition of each application. Proline was applied using the superficial spraying method on the leaves of pepper seedlings prior to inoculation; 30 mL of either 1 mM or 10 mM proline were sprayed on the leaves per single repetition; proline spraying was performed once. Sterile distilled water was applied to the control groups; the plants were then transferred

back to the growth chamber and incubated for three days at 22±3°C, 60% humidity and a 14-h light period. The inoculation procedure [13] was carried out 72 h after proline application. A 100 mL zoospore suspension (10^4 zoospores mL^{-1}) was placed into 250-mL beakers; sterile water served as the control. The seedling bunches (only the roots) were dipped in the solutions inside the beakers for 1 h, and then returned to the glass bottles which were placed in the growth chamber. Hoagland's solution was renewed at an interval of two days to avoid excessive depletion of any particular ion, and continuously aerated by an air pump throughout the duration of the experiment. Under the same conditions, random samples were taken on the 3rd, 5th and 7th days according to the random blocks trial pattern model. The leaves were separated, frozen in liquid nitrogen and ground in a pre-chilled mortar, put into nylon bags, labeled and stored at -70°C until analysis.

Disease severity and necrosis length

The two pepper cultivars' responses to the *P. capsici*-22 isolate were examined under controlled conditions. For each measurement, a separate experiment assembly was prepared. The two pepper cultivars were exposed to three different treatments: *P. capsici* alone; 1 mM proline + *P. capsici*; 10 mM proline + *P. capsici*. Each treatment was replicated three times, and each replicate had 15 plants (in a glass bottle containing 400 mL of Hoagland solution). The plants were incubated for three days at 22±3°C, 60% humidity and a 14 h light period so that they could acclimatize. The 1 mM and 10 mM proline solutions were sprayed before inoculation. Distilled water was applied to the control groups. The inoculation procedure [13] was carried out 72 h after the application of proline. The disease severity caused by a 10^4 zoospore mL^{-1} concentration on the six leaf pepper cultivars subjected to a pretreatment with proline (either 1 mM or 10 mM proline) was determined based on a 0 to 5 scale (0 – no visible disease symptoms; 1 – leaves slightly wilted with brownish lesions beginning to appear on the stems; 2 – stem lesions extending to the cotyledons, defoliated first and second leaves; 3 – stem lesions extending to the second leaves, yellowing or defoliation of some upper leaves; 4 – long, brownish lesions on stems extending up to 10 cm, all leaves except the uppermost leaf defoliated, seedling tissues collapsing and wilted shoots; 5 – plant dead) [14]. Following the treatment and inoculation, average disease severity and necrosis lengths were determined on the 3rd, 5th and 7th days. The disease severity index was calculated according to the following formula:

$$\Sigma \text{ (number of plants x scale value)} \\ \text{/total number of plants.}$$

Measurement of fresh and dry weights of leaves

The fresh weights of the leaves of the pepper cultivars were determined using a precision balance (±0.1 g). The dry weight of the leaves was determined by placing the leaves in a drying oven set to 60°C until they reached a constant weight, and measuring their dry weights with a precision balance (±0.1 g). The measurements were carried out in three repetitions; five seedlings were used in each repetition.

Determination of phenylalanine ammonia-lyase (PAL: EC 4.3.1.5) activity

PAL was extracted from the leaves as described [15]. The supernatant was collected and used as the enzyme extract. The assay mixture was incubated for 1 h at 37°C and the reaction was terminated by adding 0.5 mL of 6 M HCl. The increase in absorbance over a 1-min period was recorded with a spectrophotometer at 290 nm [16]. The calibration curve was constructed using cinnamic acid.

Determination of polyphenol oxidase (PPO: EC 1.10.3.1) activity

PPO was extracted from the leaves, and the increase in absorbance over a 1-min period was recorded with a spectrophotometer at 420 nm [17].

Determination of peroxidase (POX: EC 1.11.1.7) activity

POX was extracted from the leaves, and the enzyme activity was determined by measuring the increase in absorbance at 470 nm, extinction coefficient 26. 6 $mM^{-1}cm^{-1}$ [18].

Determination of catalase (CAT: EC 1.11.1.6) activity

CAT was extracted from the leaves, and the enzyme activity was determined using the extinction coefficient (39. 4 $mM^{-1}cm^{-1}$) of H_2O_2 [19].

Analysis of phenolic compounds

Phenolic compounds were extracted by applying 80% methanol in a water bath (80°C) for 15 min, and the extracts were centrifuged for 10 min at 500xg, after which the pellets were re-extracted [20]. The Folin-Ciocalteu method was used to determine the level of phenolic compounds [21]. The phenolic compound content was calculated using gallic acid as a standard, and expressed per g of fresh weight.

Determination of the H_2O_2 content

Samples were homogenized in 5 mL of 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12000×g for 15 min, and then 0.5 mL of the supernatant was added to 10 mM of potassium phosphate buffer (pH 7.0), followed by 1 mL of 1M KI. The absorbance of the supernatant was measured at 390 nm. The content of H_2O_2 was calculated from a standard calibration curve previously made using different concentrations of H_2O_2 [22].

Statistical analysis

The normal distribution of the data obtained in the study was checked using the Anderson-Darling test. The variance homogeneity of the subgroups was checked using the Levene test. Variance analysis (ANOVA-factorial design: cultivar×day×treatment) was conducted using a test arrangement in which data analysis was completely random. The trials were arranged to create an experimental design with three repetitions in randomized blocks. A 5% significance level was used in Tukey tests and in the interpretation of the result. All calculations were performed using the Minitab 16 package software. Based on the variance analyses conducted for all the properties, the cultivar×day×treatment triple interactions were found to be statistically significant ($p<0.01$).

RESULTS

Disease severity index and stem necrosis length

Disease severity was determined according to the index of disease scale values for the 15 seedlings (for each repetition). These were evaluated according to the 0-5 scale and are presented in Fig. 1 for the 3rd, 5th and 7th days of observation. Based on variance analysis, the cultivar×treatment×day interaction for the disease severity index values and necrosis length of the two pepper cultivars were found to be statistically significant ($p<0.01$) (Fig. 1).

In the *P. capsici*-22 isolate, the two pepper cultivars were compared in terms of the severity of the infection on the 3rd, 5th and 7th days. Following infection, the highest level of disease severity and necrosis length were observed in the Kekova cultivar ($p<0.05$). Compared with the sample exposed to *P. capsici* alone,

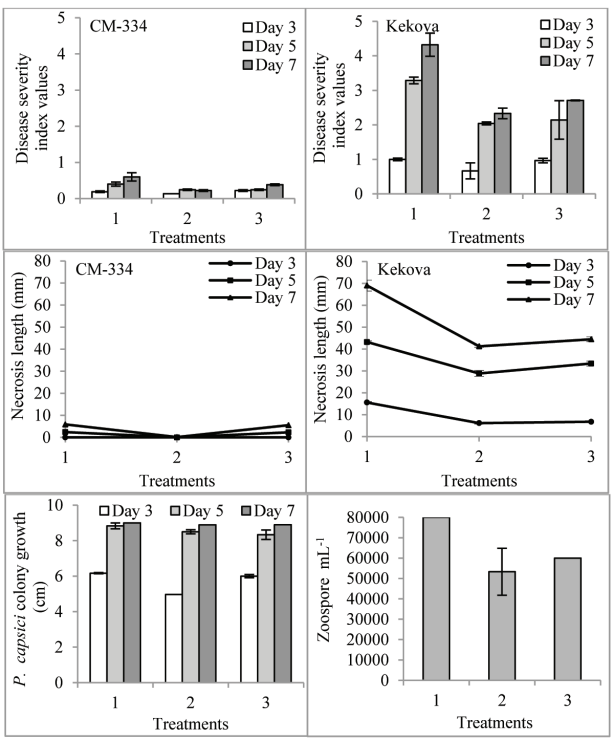

Fig. 1. Effect of proline application prior to exposure of pepper seedlings to *P. capsici* on the disease severity index and necrosis length (n=3) ($p<0.05$). The effect of different proline concentrations on colony growth after 3, 5 and 7 days of incubation, and on the amount of *P. capsici* zoospores (cultivated for 7 days). All values are the mean of three replications (n=3). Vertical bars represent standard errors ($p<0.05$). Type of treatment: **1** – samples treated with *P. capsici* alone; **2** – treated with 1 mM proline + *P. capsici*; **3** – 10 mM proline + *P. capsici*.

proline application before inoculation decreased the disease severity index and the necrosis length in two pepper seedlings. The difference between them was found to be statistically significant. The 1 mM proline + *P. capsici* treatment was the most effective treatment in both cultivars ($p<0.05$) (Fig. 1).

The effect of proline application on the *P. capsici*-22 isolate-treated samples was determined, and the *P. capsici* colony growth was measured. The measurements were performed by exposing mycelial plugs to two concentrations of proline. To this end, the *P. capsici*-22 isolate was grown on V8 agar at 25±2°C in the dark. Mycelium plugs (2 mm in diameter) on the agar from the edge of 10-day-old *P. capsici* cultures were then placed in the center of Petri plates (9 cm in diameter), filled with sterile medium (V8 agar) containing either 1 mM or 10 mM proline. There was no proline in the control group. The Petri dishes were incubated for 7 days at 25±2°C in the dark. The colony diameter of *P. capsici* (colony growth) was measured after the 3rd, 5th and 7th days of incubation (Fig. 1). The obtained results demonstrated that, compared to *P. capsici* alone, the most effective treatment was with 1 mM proline; this treatment decreased *P. capsici* growth on the 3rd, 5th and 7th days ($p<0.05$) (Fig. 1). The effect of proline on zoospores was also examined. Zoospores were harvested from the *P. capsici* plates and incubated with two concentrations of proline in sterile medium (V8 agar) on Petri plates. Zoospore suspensions were then obtained from the Petri plates, including from the 7-day cultivar in order to determine the effect on the number of zoospores. Counting was performed using a hemocytometer [12]. Compared to the samples treated with *P. capsici* alone, the application of 1 mM and 10 mM proline decreased the number of zoospores. The most effective concentration was found to be 1 mM proline ($p<0.05$) (Fig. 1).

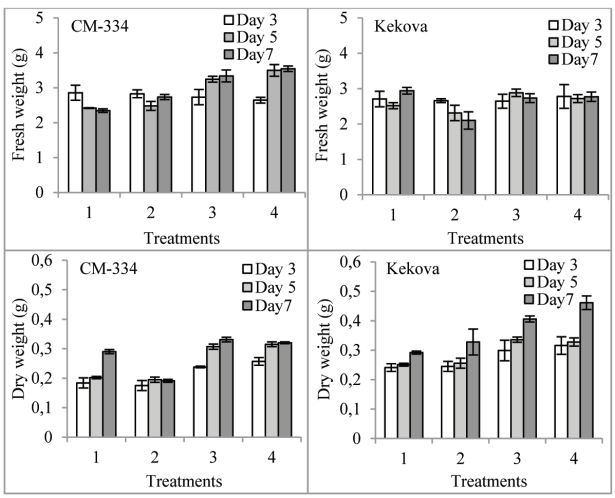

Fig. 2. Effect of proline application prior to exposure of pepper seedlings to *P. capsici* on fresh and dry weights of leaves. All values are the mean of three replications (n = 3). Vertical bars represent standard errors ($p<0.05$). Type of treatment: **1** – control (no proline, no *P. capsici*); **2** – samples treated with *P. capsici* alone; **3** – treated with 1 mM proline + *P. capsici*; **4** – 10 mM proline + *P. capsici*).

Fresh and dry weights of the leaves of pepper cultivars

Compared with the control, the fresh weight of *P. capsici*-infected Kekova cultivar leaves decreased during the experiment ($p<0.05$). When samples treated with 1 mM proline + *P. capsici* and 10 mM proline + *P. capsici* were compared with the *P. capsici*-treated sample, it was found that the pretreatment with proline increased the fresh and dry weights of the leaves in both pepper cultivars. The difference between the cultivars was found to be statistically significant ($p<0.05$). The application of 10 mM proline + *P. capsici* caused the highest increases in the fresh and dry weights of leaves of both cultivars ($p<0.05$) (Fig. 2).

PAL, PPO, POX and CAT activities in the leaves of pepper cultivars

PAL activity in *P. capsici*-infected leaves increased during the experimental period in the two pepper cultivars (Fig. 3). Compared with the control, the maximum increase in PAL was observed on the 7th day in the leaves of CM-334 seedlings infected with *P. capsici*. When the proline + *P. capsici* treatments were compared with the treatment with *P. capsici* alone, the pretreatments with proline were found to increase PAL activity in the leaves of the two pepper cultivars. Compared to the treatment with *P. capsici* alone, the highest enzyme activity was observed in the 1 mM proline + *P. capsici*-treated samples on the 5th day in both the CM-334 and Kekova pepper cultivars; the increases in enzyme activities for these two cultivars were 145% and 163%, respectively ($p<0.05$) (Fig. 3.).

When PPO activity in the two cultivars treated with *P. capsici* alone was compared on the 3rd, 5th and

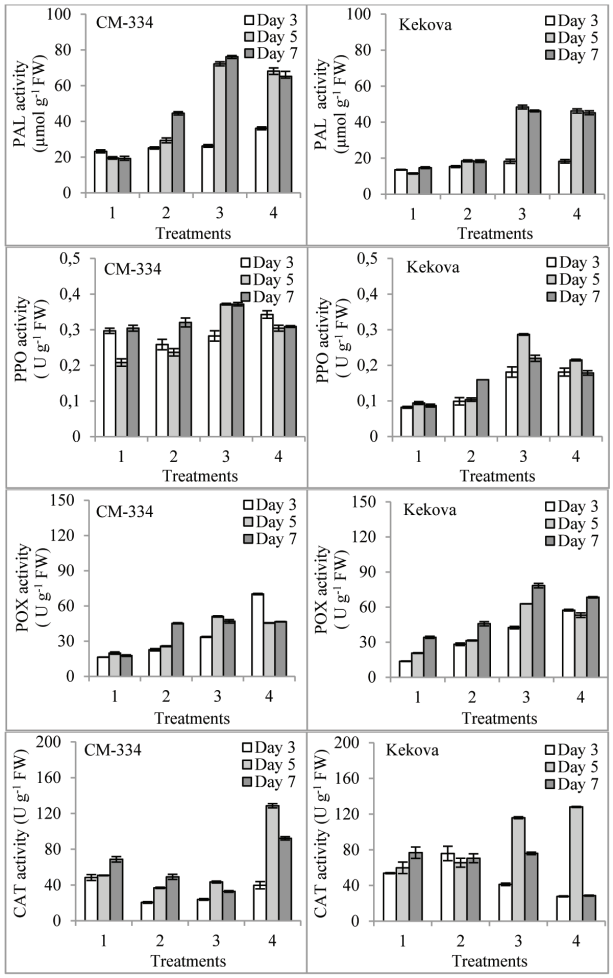

Fig. 3. Effect of proline application prior to exposure of pepper seedlings to *P. capsici* on PAL, PPO, POX and CAT activities in leaves. All values are the mean of three replications (n = 3). Vertical bars represent standard errors (p<0.05). Type of treatment: **1** – control (no proline, no *P. capsici*); **2** – samples treated with *P. capsici* alone; **3** – treated with 1 mM proline + *P. capsici*; **4** – 10 mM proline + *P. capsici*).

7th days following infection, the differences in activities between the cultivars were statistically significant (p<0.05). When proline was applied prior to the inoculation with *P. capsici*, PPO activity increased, with the highest increase observed after treatment with 1 mM proline + *P. capsici* on the 5th day in both the CM-334 and Kekova pepper cultivars. The increases in the activities were 57.3% and 176.3%, respectively (p<0.05) (Fig. 3).

Compared with the control, the activity of POX increased on the 3rd, 5th and 7th days following infection of the two pepper cultivars with *P. capsici* (Fig. 3). In the leaves of the CM-334 cultivar, maximum POX activity was observed after treatment with 10 mM proline + *P. capsici* as compared to both the control and samples treated with *P. capsici* alone (p<0.05) (Fig 3). In the Kekova cultivar, the highest POX activity was measured in the 0.1 mM proline + *P. capsici*-treated sample on the 7th day, and in the 10 mM proline + *P. capsici*-treated sample on the 3rd day (p<0.05) (Fig. 3).

Compared with the control, CAT activity increased on the 3rd, 5th and 7th days in the Kekova cultivar infected with *P. capsici*, but not in the CM-334 cultivar. A decrease in CAT activity when compared to the control (p<0.05) was observed on all days in the CM-334 cultivar infected with *P. capsici* (Fig. 3). In the proline-treated samples, the highest enzyme activity in both pepper cultivars was observed on the 5th day in the 10 mM proline + *P. capsici*-treated sample as compared to the sample treated with *P. capsici* alone. The increase in enzyme activity was 249.3% in the CM-334 cultivar and 95.3% in Kekova cultivar; the difference between the two was significant (p<0.05) (Fig. 3).

Total phenolics and H_2O_2 levels in the leaves of pepper cultivars

The application of *P. capsici* alone increased the total phenolic level in the two cultivars on the 3rd, 5th and 7th days when compared with matching controls. The application of proline prior to inoculation increased PPO activity on the 5th and 7th days in both pepper cultivars; the difference between them was significant (p<0.05). In the CM-334 cultivar, the highest increase in the level of phenolics was observed on the 5th day in the 10 mM proline + *P. capsici*-treated sample in comparison to both the control and sample treated with *P. capsici* alone. The increase was 9.3% as compared to the sample treated with *P. capsici* alone (p<0.05) (Fig. 4). Compared to both the control and samples treated with *P. capsici* alone, the maximum level of phenolics was observed in the Kekova cultivar after treatment with 1 mM and 10 mM proline + *P. capsici* on the 5th day. Compared with samples treated with *P. capsici* alone, the increase in phenolic levels were approximately 62.9% and 57.8%, respectively (p<0.05) (Fig. 4.).

Treatment with *P. capsici* alone increased the H_2O_2 level in the two pepper cultivars on the 3rd, 5th and 7th

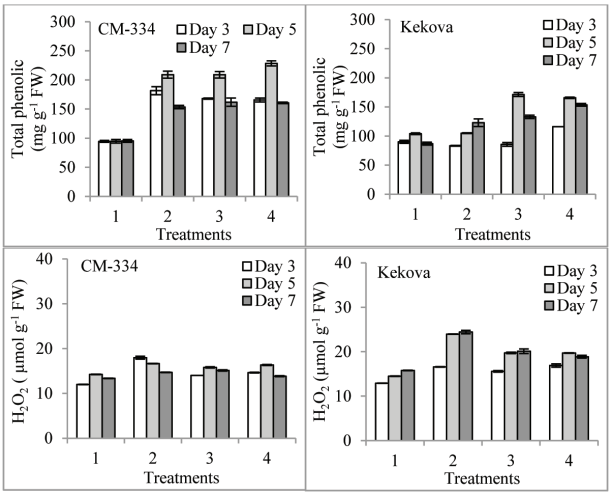

Fig. 4. Effect of proline application prior to exposure of pepper seedlings to *P. capsici* on total phenolic and H_2O_2 levels in leaves. All values are the mean of three replications (n=3). Vertical bars represent standard errors ($p<0.05$). Type of treatment: **1** – control (no proline no *P. capsici*); **2** – samples treated with *P. capsici* alone; **3** – treated with 1 mM proline + *P. capsici*; **4** – 10 mM proline + *P. capsici*).

days ($p<0.05$) (Fig. 4). Proline application before inoculation decreased the level of H_2O_2 in both pepper cultivars. The difference between the two treatments was found to be statistically significant ($p<0.05$), with the highest decrease observed in the 1 mM proline + *P. capsici*-treated CM-334 and Kekova pepper cultivars. Compared with samples treated with *P. capsici* alone, the levels of decrease in H_2O_2 in the CM-334 and Kekova pepper cultivars were 9% and 21.3%, respectively ($p<0.05$) (Fig. 4).

DISCUSSION

In this study, the Kekova cultivar presented a sensitive reaction to the pathogen. The disease proceeded so rapidly in the Kekova cultivar that most of the seedlings were severely damaged by the 7th day; there was an increase in the disease severity index values and the necrosis lengths. In this study, it was observed that CM-334 was the most resistant to *P. capsici-22*. In time, the difference between the cultivars increased in terms of resistance. When the effects of the treatments were compared on the 3rd, 5th and 7th days in the two cultivars, pretreatment with proline decreased disease severity and necrosis. These data show that the treatments were effective against *P. capsici*, and that they reduce pathogen levels under stress conditions.

Reactive oxygen species (ROS) are produced as normal products of plant cellular metabolism. Despite their destructive activity, they also function as second messengers in cellular signaling pathways, including the development of tolerance to various environmental stresses. Abiotic and biotic stresses lead to excessive production of ROS, causing membrane damage, chlorophyll destruction and oxidation of several important metabolites in the cell, and ultimately cell death [23]. The electron transport chain in photosystems I and II (PSI, PSII (respectively) are the main sources of ROS in chloroplasts. ROS can cause a Mehler reaction during energy transfer from the chlorophyll in the thylakoid membrane of chloroplasts, and they can initiate lipid peroxidation that leads to a disturbance in the membrane structure and its functions and adversely affect the transport and maintenance of membrane proteins. In this study, the treatment with 1 and 10 mM proline + *P. capsici* increased the fresh and dry weights of plant leaves. This can be related to the antioxidant properties of proline that protects pigments against ROS under *P. capsici* stress, which in turn decreases leaf pigment loss while also increasing photosynthetic efficiency and neutralizing the toxic effects of the fungus. Exogenously applied proline likely plays a role in protecting the photosynthetic apparatus from the adverse effects of *P. capsici* stress. These assumptions are also supported by data from studies conducted with various stress factors that indicated exogenous proline application preserves the photosynthetic mechanism [24,25]. Exogenous proline application improved the photosynthetic capacity of salt-stressed olive plants by increasing photosynthetic activity and the level of the photochemical efficiency of PSI and PSII [24]. It was observed that leaf photosynthetic pigments were maintained by proline under salt stress [25]. The increase in fresh and dry weights could be associated with proline's functions, which include the protection of the thylakoid membranes of chloroplasts (and hence of photosynthetic processes) against ROS attacks (proline decreases the production of singlet oxygen and reacts with hydroxyl radicals to generate nontoxic hydroxyproline). Proline also enhances general adaptation to negative environmental conditions.

Pathogen stress-induced excess generation of ROS, as well as the subsequent enhanced levels of defense compounds and the increased activities of many enzymes during stress, has been reported in many plant species [23]. The present study indicates that the application of *P. capsici* alone increased PAL, PPO and POX activities and the total phenolic compound content and H_2O_2 levels when compared with the control. However, CAT activity responded differently in the two pepper cultivars under pathogen stress. CAT protects plants from oxidative stress-induced damage [26]. In a study investigating oxidative stress in eggplants, it was determined that *Ralstonia solanacearum* infections caused an increase in total phenolic and H_2O_2 levels, and in CAT activity [26]. The antioxidant potential of CAT in the tissues of *Capsicum annuum* is not sufficient for preventing the oxidative damage in some conditions. Therefore, if CAT becomes inactive under some stress conditions, the toxic property of H_2O_2 is prevented with the increase in POX activity. These findings from previous studies supported the results of the present study. In the present study, a decrease in CAT activity was observed in comparison to the control as a result of the *P. capsici* infection in CM-334, while on the other hand, POX activity increased in the same cultivar. POX stimulates the formation of compounds such as lignin and suberin, strengthening the cell wall and increasing the production of phytoalexin, thus forming a physical barrier that prevents the spread of the pathogen during the life cycle of the plant.

Proline is known to act as an enzyme protectant during stress conditions [7,27]. Proline can protect cellular and subcellular membranes from oxidative stress by enhancing the activities of various defense enzymes [27-29]. This is because the 3-D structure of proteins is governed by hydrophobic/hydrophilic, ionic interactions and interactions between side chains of the constituent amino acids. Proline could interfere with these side chain bonds and induce conformational changes in enzymes and thus affect their activity [7]. In the present study, the exogenous application of proline improved the enzyme activities of *P. capsici*-stressed pepper plants. When proline was administered before inoculation, it was observed that it caused further increases in PAL, PPO and POX activities and total phenolic compound levels in both pepper cultivars. The highest CAT and POX activities were observed in both pepper samples exposed to 10 mM proline + *P. capsici*. CAT and POX activities increased in both pepper varieties subjected to *P. capsici* stress, and exogenous proline enhanced the activities further, pointing to the H_2O_2 scavenging role of proline. The high activities of enzymes coinciding with the lower accumulation of H_2O_2 in *P. capsici* stress induced a significant increase in H_2O_2 in the two pepper cultivars. The exogenous application of proline resulted in a reduced H_2O_2 level and hence in lower oxidative stress in plant cells. This positive effect of proline may be related to its antioxidant property [7].

Some of the important phenolic compounds are derivatives of the phenylpropanoid pathway. PAL is the enzyme at the entry-point of the phenylpropanoid pathway. There are many reports describing changes in PAL and PPO activities which support the notion that PAL and PPO are involved in the synthesis of phenolics. In the present study, pretreatment with proline increased PAL and PPO activities and the level of total phenolics on the 5th day when compared with *P. capsici*-treated pepper cultivars. A positive connection was observed between PAL activity and phenolic compounds in the two cultivars. PAL is a key enzyme of phenolic biosynthesis in plants. PPO has antipathogen effects, including general toxicity, which is provided by PPO-generated quinones to pathogens and plant cells that accelerate cell death, alkylation and reduce the bioavailability of cellular proteins to the pathogen, cross-link quinones with proteins or other phenolic compounds, forming a physical barrier to pathogens in the cell wall [30]. Phenolic compounds help to strengthen the cell wall against pathogens, inhibit fungal growth, and act as free radical scavengers, thereby overcoming oxidative stress [30].

Despite the beneficial effects of its exogenous application, proline can also have toxic effects when applied at high concentrations. The negative effects of exogenous proline were observed in tomato plants. Proline applied exogenously at low concentrations was found to decrease the adverse effects of salt stress in tomato plants, while higher concentrations of proline resulted in toxic effects and poor plant growth [31]. Low levels of exogenous proline application caused an increase in PAL activity and stimulated PAL gene expression in rosemary callus culture [32]. In both pepper cultivars, the highest PAL and PPO activities were determined in the 1 mM proline + *P. capsici*-treated

seedling, while the highest POX and CAT activities were found in the 10 mM proline + *P. capsici*-treated seedlings. The results of this study indicate that the application of 1 and 10 mM proline has a positive effect on pepper under *P. capsici* stress.

The findings presented here show that proline is a signaling molecule capable of activating PAL, PPO, POX and CAT enzymes, thereby limiting free radical generation and preventing membrane peroxidation and denaturation of biomolecules, resulting in improved seedling growth under *P. capsici* stress. Similar effects have also been observed in several other recent studies [24-33].

The importance of proline in enhancing plant stress tolerance through exogenous application reported here is supported by reports describing its beneficial effects in plants under different stress conditions [7,25,34]. In studies conducted during the last decade related to the control of fungal plant diseases, proline application has been suggested as a new strategy for regulating plant stress tolerance. Thus, the exogenous application of proline may be an efficient approach to ameliorate the adverse effects of *P. capsici*-induced stress. Nevertheless, the effectiveness of proline applied as a foliar spray depends on the type of cultivars, stress level, age of plant, time of application and concentration. In this study, the different defense responses are linked to different genotypes. These findings have provided a clue to the role of proline application in plant defense and show that determining the appropriate concentrations of exogenously applied proline may be an effective way of protecting pepper plants against *P. capsici* infection.

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