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Effects of Nitric Oxide Application on Antioxidant Enzyme Activities of Pepper Plants under Drought Stress

Abstract

The purpose of the study was to determine the relationship between the messenger molecule Nitric oxide (NO) and antioxidative enzyme (SOD: Superoxide Dismutase; CAT: Catalase; APX: Ascorbate Peroxidase) activities in some metabolic changes that occur under the effect of drought stress in plants, to determine the possible roles of Nitric Oxide and to obtain complementary information. The experiment conducted in a controlled environment, and plant were cultured in containers containing Hoagland nutrient solution. For drought stress application, 10% Polyethylene Glycol (PEG 6000) was added to the nutrient solution, which is equivalent to -0.40 MPa osmotic potential. Before the drought stress is applied, pepper seedlings of Demre cv were pre-treated with different doses of Sodium Nitroprusside (SNP) and Carboxy-PTIO (potassium salt) (cPTIO) (SNP 0.01, SNP 1, SNP 100 and SNP 0.01 + cPTIO, SNP + cPTIO, SNP 100+ cPTIO). On the 10th day of the drought application, the growth parameters of the plants; the plant fresh weights and their Antioxidative Enzyme Activities (SOD, CAT, APX) were determined. In terms of plant growth parameters, both plant growth and antioxidant anzyme activities of plants pretreated with 0.01 and 1 doses of SNP were lower than the high dose of SNP and the PEG application without pretreatment. The reason for the low enzyme activities in these applications can be attributed to factors such as the excess accumulation of organic acids such as proline in the cells of the plants and the decrease in H₂O₂ and O⁻² levels in the presence of SNP.

INTRODUCTION

The most exposed organisms to environmental conditions change and adverse conditions are plants due to their inability to move. Abiotic stress due to climatic changes such as drought, salinity, excessive rainfall, temperature or cold throughout plant life cycles directly affect their growth and development (Taiz and Zeiger, 2010; Erman et al. 2021). Plants can stretch their growth and development mechanisms within their genetic capabilities so as to minimize damage from these changes that may occur in environmental conditions and even adapt to the conditions of the ecology they are in when they grow in the same ecology for a long time. The distribution of plants belonging to the same species in regions of the world with changing climatic characteristics is the best indicator that they can adapt to very different environmental conditions (Dolfeus, 2014). The reason why our world has so much vegetative diversity is that there are regions with very different ecological characteristics. In this context, it is not difficult to predict that not only physiological but also metabolic changes may occur in a plant that is exposed to drought stress. When plants encounter arid conditions, depending on the duration and severity of the stress, they can restructure their metabolism in a way that will make radical and dramatic changes in their metabolic and genetic structures due to their desire to survive and continue their generations due to their genetic structure (Yasar et al., 2010; Berber and Yasar, 2011; Yasar et al., 2014; Taş and Öktem, 2019).

Plants are confronted with the phenomenon of synthesis of various reactive oxygen species (ROS) by reducing the molecular oxygen in the cell as a result of all the metabolic activities they encounter in the processes of photosynthesis, respiration, growth and development (Yasar, 2003, Yasar et al., 2008a; Çulha and Çakırlar, 2012). Active radicals such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2 \cdot^-$) and hydroxyl radical ($OH \cdot$),

which are reactive oxygen species, are produced in plant cells as a result of oxidative reactions occurring in chloroplasts, mitochondria and peroxisomes. With the effect of these oxygen derivatives, lipids, proteins and nucleic acids suffer oxidative damage and as a result, serious problems occur in metabolism (Demiral, 2003; Yasar et al., 2016). Plants under any abiotic stress develop various antioxidative defense mechanisms that provide control and detoxification of ROS in response to oxidative stress in order to survive and cope with stress. In cases where this protection system does not work or is insufficient, death occurs in plant cells (Büyük et al., 2012; Yasar et al., 2013b; Yaşar et al., 2020). It is possible to categorize the antioxidants they develop against the harmful effects of free oxygen radicals in plants under stress conditions in two groups; enzymatic and non-enzymatic antioxidants. The main protective enzymatic antioxidants in the plant cell are superoxide dismutase, peroxidase, catalase, ascorbate peroxidase and glutathione reductase. Superoxide dismutase (SOD) enzyme is an enzyme that catalyzes the conversion of superoxide radical to hydrogen peroxide and oxygen (Alscher et al., 2002). Catalase (CAT) uses H_2O_2 as an electron trap, to oxidize the substrate and convert H_2O_2 to O_2 and H_2O . This enzyme is found mostly in the peroxisome in higher plants (Jamei et al., 2009). Another of some metabolic changes that occur under the effect of drought stress in plants is nitric oxide (NO), which has a messenger molecule feature. NO is a colorless, inorganic molecule consisting of a nitrogen and an oxygen atom, lipophilic, gaseous, easily diffusible without being dependent on the receptor, very short half-life, containing unpaired electrons, characterized as free radicals (Olson and Garban, 2008). NO alone does not harm cells even at high concentrations, but the uncontrolled production of NO in cells reacts with super oxide anions and causes

peroxynitrite, resulting in toxic effects (Stöhr and Ullrich, 2002). NO is synthesized and released in small amounts in plant cells under normal conditions. In plants, NO is synthesized by two different metabolic pathways, enzymatic and non-enzymatic. NO synthesis varies depending on the plant species, texture and growing conditions. The NO production site in plant cells is the cytosol, nucleus, peroxisome matrix and chloroplasts (Pedroso et al., 2000). Also, non-enzymatic processes play a role in the formation of NO in plants. In an acidic or light environment, NO₂ can be converted to NO (Cooney et al., 1994). Nitric oxide is an important signaling molecule with various physiological functions in plants. It is thought to play an important role in the growth and development of plants from seed to flowering stage, and the ripening of fruits. In addition, in case of danger caused by environmental stress caused by abiotic and biotic factors, NO can be produced in different plant species and organs. Nitric oxide is a very active molecule that has been proven to protect plants by various biological means against the damage caused by oxidative stress conditions (Carlos and Lorenzo, 2001). Nitric oxide can have harmful effects as well as beneficial in plant cells. This situation depends on the amount of nitric oxide. The aim of the current study is to determine the relationship of Nitric oxide (NO), which has a messenger molecule property, to antioxidative enzyme activities (SOD: Superoxide Dismutase; CAT: Catalase; APX: Ascorbate Peroxidase) in some metabolic changes occurring under the effect of drought stress in pepper plants and to identify possible roles of Nitric Oxide and to obtain complementary information.

MATERIAL and METHODS

Plant material. This study, which aims to investigate the relationship between drought stress and nitric oxide (NO) in pepper (*Capsicum annuum*) plant, was carried out in a controlled climate room using the Demre variety (pointed pepper).

Method. Pepper seeds were planted in plastic germination containers filled with perlite, then irrigated and left to germinate. Irrigation was started with Hoagland nutrient solution (Hoagland and Arnon, 1938) for seedlings which their first true leaves started to be seen. The pre-treatment of the seedlings in the perlite medium with the second true leaves was prepared for 2 days in brown bottles, the NO donor sodium nitroprusside (SNP) and the NO catcher 1 μ M c-PTIO at 0.01, 1, 100 μ M concentrations prepared in 1/2 Hoagland solution [2- (4) -carboxy-phenyl) -4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazole-1-oxy-3-oxide] + 1 μ M SNP. Then, the pre-treated and untreated seedlings were taken into the aquaculture medium. For the aquaculture, 25x25x18 cm plastic tubs filled with Hoagland nutrient solution were used. The nutrient solutions were refreshed at weekly intervals, while the locations of the tubs were changed to ensure that all plants get an equal lighting condition.

Application of drought stress

After the seedlings were grown in water culture for a week, drought application was started (Table 1). At this stage, it was observed that the seedlings had 3-4 true leaves. Seedlings were determined as 15 plants with three replications from each genotype. 10% Poly Ethylene Glycol (PEG 6000) was added to the Hoagland nutrient solution (Türkan et al., 2005). Samples were taken from the plants harvested after the drought application.

Table 1: Applications to pepper plants.

1- Application: Control (Hogland)
2- Application: PEG group + 10% Poly Ethylene Glycol (PEG 6000)
3- Application: 1 μ M C-PTIO + 0,01 μ M SNP+% 10 Poly Ethylene Glycol (PEG 6000)
4- Application: 1 μ M C-PTIO + 1 μ M SNP + % 10 Poly Ethylene Glycol (PEG 6000)
5- Application: 1 μ M C-PTIO +100 μ M SNP+% 10 Poly Ethylene Glycol (PEG 6000)
6- Application: 0,01 μ M SNP + % 10 Poly Ethylene Glycol (PEG 6000)
7- Application: 1 μ M SNP + % 10 Poly Ethylene Glycol (PEG 6000)
8- Application: 100 μ M SNP + % 10 Poly Ethylene Glycol (PEG 6000)

Determination of Antioxidant Enzyme Activities

In order to examine the change in enzyme activities that may occur in plants under drought stress, approximately 1 gr of fresh leaf sample was crushed in porcelain mortars in liquid nitrogen, and then homogenized with 50 mM, 10 ml phosphate buffer solution (pH: 7.6) containing 0.1 mM Na-EDTA. The centrifuges obtained after the homogenized samples were centrifuged at 15000 rpm for 15 minutes were used in enzyme analysis. Samples in which enzyme activities will be determined will be kept at +4 °C until the measurement is made. Measurements were carried out using spectrophotometer (Analytic Jena 40 model). Superoxide Dismutase (SOD), Catalase (CAT) and Ascorbate Peroxidase (APX) activity were determined at 290 nm ($E = 2.8 \text{ mM cm}^{-1}$) according to the method of reduction of NBT (nitro blue tetrazolium chloride) by O_2 - oxidation of ascorbate, catalase activity (CAT) was measured based on the fragmentation ratio of H_2O_2 at 240 nm ($E = 39.4 \text{ mM cm}^{-1}$) (Cakmak and Marschner, 1992).

Making evaluations

The treatments were arranged according to the complete randomized design with 3 replications in which 15 plants were in each

repetition. The statistical analyzes of the plant growth parameters and the data obtained were performed according to Duncan multiple comparison test ($P < 0.05$) using the SAS Insiue, (1985) package program.

RESULTS

As a result of the application of PEG 6000 to pepper plants, when we examined the total plant age weights, it was seen that there were differences among the applications. Applications in the same statistical range with the control group were SNP 0.01+ PEG, SNP 1+ PEG. Other applications had decreased compared to the control. The highest decrease seen was in C.PTIO + SNP 100+ PEG application (Table 2). When we examined the SOD activities of the plants as a result of the application of PEG 6000 to pepper plants, it was seen that there were differences among the applications. While the SOD activities of all applications increased compared to the control, the highest increase was observed in C.PTIO + SNP100, PEG, SNP 100, C.PTIO + SNP1, respectively. SOD enzyme activities of the plants in the applications of SNP 1 + PEG, SNP 0.01 + PEG were found to be at the lowest values, respectively.

Table 2. The antioxidant enzymes CAT, APX (mmol / min / mgY. A.) and SOD (Unit / mg protein) activities and total plant wet weight (gr) from drought-stressed pepper leaves.

APPLICATION	SOD	CAT	APX	TOTAL PLANT WEIGHT
CONTROL	53.33 F	239.39 G	12.69 E	23.88 A
PEG	168.67 B	469.25 B	27.90 B	16.98 C
SNP 0.01+ PEG	131.33 D	262.17 F	18.81 D	23.79 A
SNP 1+ PEG	91.33 E	196.53 H	14.02 E	23.09 A
SNP 100+ PEG	159.33 BC	378.08 D	31.70A	15.98 D
C.PTIO+SNP 0.01+ PEG	146.33 C	319.39 E	19.22 D	18.91 B
C.PTIO+SNP 1+ PEG	158.00 BC	434.95C	25.65 C	15.48 C
C.PTIO+SNP 100+ PEG	215.33A	509.08A	29.54 B	9.77 E

The difference between the averages that get the same capital letter in the same column is insignificant according to $P \leq 0.05$.

Likewise, when we examined the CAT activity of pepper plants, it was seen that there were differences among applications, C.PTIO + SNP100 + PEG, PEG, C.PTIO + SNP1 + PEG, SNP100 + PEG, C.PTIO + SNP0.01 + PEG and SNP0.01 + PEG, while CAT activity increased compared to control, SNP1 + PEG decreased compared to control. However, the highest increase was seen in the application of C.PTIO + SNP100 + PEG in CAT activity as well as in SOD activity (Table 2). When we examined the APX activities of the plants as a result of the application of PEG 6000 to pepper plants, it was seen that there were differences among the applications. Although APX activities in SNP100 + PEG, C.PTIO + SNP100 + PEG, PEG, C.PTIO + SNP1 + PEG, C.PTIO + SNP0.01 + PEG and SNP 0.01 + PEG applications increased compared to control, SNP 1+ PEG application was found in the same statistical group range with the control. As in other enzyme activities, SNP 1+ PEG and SNP 0.01+ PEG applications were found to be the closest to the control in APX enzyme activity (Table 2).

DISCUSSION and CONCLUSION

Seedlings belonging to the Demre long pepper variety used in the study were grown in Hoagland nutrient solution and stress was sustained by applying 10% PEG 6000 to these plants to apply drought stress. In addition, nitric oxide (NO) donor sodium nitroprusside (SNP) at 0.01, 1, 100 μM concentrations prepared in $\frac{1}{2}$ Hoagland solution for 2 days before drought stress is applied to 6-day-old seedlings and 1 μM c-PTIO [2- (4-carboxy) -phenyl) -4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazole-1-oxy-3-oxide] + 1 μM SNP, drought stress was applied. The growth of pepper plants under drought stress with and without pretreatment was compared in terms of metabolic responses. In terms of total plant weight, it was observed that the pre-applications of SNP and c.PTIO + SNP were in the same range as the control plants with 0.01 and 1 μM doses without drought, and they developed much better than the PEG application without pretreatment. It has been observed that the 100 μM dose of SNP and c.PTIO + SNP does not have a positive effect, and it

is more stressful on the growth of the plants compared to PEG without pretreatment. Ekinci et al., (2020) NO mitigated the negative effects of drought stress on fresh and dry weights of leaf and root, chlorophyll content, gas exchange parameters and electrical leakage in all doses, especially for doses of 100 and 150 μM for both s and sf applications. Sekmen et al. (2005), in their study by applying salt stress in tomato plants, found that the root and stem weights and lengths of pre-treated plants on the 28th day increased compared to the plants treated with salt stress without pretreatment. However, by losing the pretreatment effect on the 43rd day of the stress, the plants reported the same reaction as the untreated plants. Likewise, Tuna and Eroğlu (2017) examined the root stem and leaf weights of the plants under stress by applying NO pretreatment to pepper plants under salt stress. While root, stem and leaf growth of plants decreased compared to control, it was determined that they developed better than salt application without pretreatment. Many researchers who conducted studies similar to our study obtained similar results. Kausar et al. (2013), on the other hand, stated that nitric oxide pre-application in wheat (*Triticum aestivum*) plant, on which salt stress was applied, positively affected the growth and yield of the plants. When we evaluated the effects of drought stress on pepper plants in terms of antioxidative enzyme activities, which is one of the most important biochemical criteria, very different results were encountered in this study. In this study, in which we found that SNP increased with 0.01 and 1 doses and decreased with SNP 100 and cPTIO + SNP 100 doses, enzymes showed the opposite response. SOD, CAT and APX enzymes were found at the lowest level in the control application, followed by 0.01 and 1 doses of SNP. The highest enzyme activities (SOD, CAT and APX) were observed in cPTIO + SNP 100 + PEG application and non-pretreatment PEG application. However, it was seen that cPTIO application had a

negative effect on the other parameters mentioned above. In previous studies conducted by many different researchers with different plants, it has been observed that when stress is applied to plants, there is an increase in antioxidant enzyme activities depending on the genetic structure of the species and variety (Yasar, 2003; Türkan et al., 2005; Yasar et al., 2008a,b; Yasar et al., 2010, Yasar et al., 2013a, Yasar et al., 2016). After pre-application with SNP, we can say that there is no increase in enzyme activities as a result of the treatment of 0.01 and 1 μM , especially as a result of the treatment of SNP at 0.01 and 1 μM , because the plants on which these applications are applied have better developed in plant weight. As in the study mentioned, in cases where the regulation of ion leaks, regulation of carbonic contents and reduction of H_2O_2 and O^{2-} levels with SNP application, in cases where the amount of MDA, which is an oxidative damage product, decreases (Çelik and Eraslan, 2015), the plant does not undergo oxidative stress and reactive oxygen derivatives (ROT) there is an opinion that it does not occur. In this case, the activities of antioxidative enzymes are low and they are expected to be at values close to the control plants. In such cases, it is not expected to be high. In a different study by Tian and Lei (2006); the effects of SNP, which is a nitric oxide donor, on drought stress caused by 15% PEG in wheat germs were investigated. It has been reported that drought stress causes an increase in lipid peroxidation with H_2O_2 accumulation, while SOD, CAT and L-phenylalanine ammonia lyase (PAL) activities increase under mild stress conditions and decrease under severe stress conditions. They stated that 0.2 mM SNP application increased the growth of the shoots and provided a high water content. In addition, the researchers reported that the addition of 0.2 mM SNP reduced oxidative damage. As can be seen, 0.2 mM SNP application increased plant growth as in our study. We can say that the reason for this may be the increase of organic acids in the

plant cell and especially the increase in potassium ion uptake and most importantly, it may prevent ROS formation.

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