



Investigation of The Roles of Hydrogen Peroxide and NADPH Oxidase in The Regulation of Polyamine Metabolism in Maize Plants under Drought Stress Conditions

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ABSTRACT

The relationship between hydrogen peroxide and the metabolism of polyamines and the role of NADPH oxidase (NOX) in that relationship under drought conditions remains unclear. To reveal the relationship, expression levels of the genes in polyamine metabolism, such as arginine decarboxylase, agmatine aminohydrolase, spermidine synthase, S-adenosyl methionine decarboxylase, diamine oxidase, and polyamine oxidase were determined by RT PCR under drought stress combined with exogenous hydrogen peroxide (H₂O₂) and diphenyleneiodonium chloride (DPI) treatments in maize seedlings. In addition, some basic stress

parameters (leaf water potential, lipid peroxidation), levels of polyamines (putrescine, spermidine, and spermine), and gene expression of NOX were measured under drought stress. Exogenous H₂O₂ induced the polyamine content by up-regulating polyamine-synthesizing genes and downregulating polyamine oxidizing genes. When the NOX enzyme was inhibited by DPI, the polyamine pathway tended towards degradation instead of production. Exogenous H₂O₂ regulated the metabolism of polyamines to promote their synthesis, and NOX played a key role in that regulation.

Keywords: Antioxidant enzymes, Arginine decarboxylase, Diphenyleneiodonium chloride, Polyamine oxidation, Reactive oxygen species

1. Introduction

Polyamines, similar to hormones, have been considered to function as regulatory molecules in several basic cellular and physiological processes such as cell division, differentiation, and proliferation, cell death, membrane stabilization, DNA replication, protein synthesis, flower formation and development, leaf senescence, fruit development and maturation, biotic and abiotic stress responses (Alcazar et al. 2006a; Kusano et al. 2008). Recent studies of transgenic and mutant plants with excess or less polyamine suggest that polyamines play a protective role against abiotic stress (Alcazar et al. 2006b; Kusano et al. 2008; Gill & Tuteja 2010). For instance, the excessive level of putrescine in the Arabidopsis plant by overexpression of the Arginine decarboxylase 2 (ADC2) gene has probably stimulated stomatal closure, and this decrease in water loss increased drought tolerance (Alcazar et al. 2010a). In a study by Kusano et al. (2007), Arabidopsis *acl5/spms* double mutant plants, which are unable to produce spermine, became hypersensitive to drought and saline stress. In the same study, however, the externally applied spermine mitigated the effects of the stresses mentioned in the plant. Besides, Yamaguchi et al. (2006; 2007) reported that the susceptibility to drought and salt stresses increased in the studies with double mutants of *spms1/acl5-1* of Arabidopsis to investigate the protective role of spermine against abiotic stress.

Drought stress is known to increase the generation of reactive oxygen species (ROS) in cell compartments such as chloroplasts, peroxisomes, and mitochondria. Be exposed to environmental stresses, such as drought, chilling, heat, or high light irradiation, is considered to lead to high concentrations of ROS, such as superoxide, H₂O₂, single oxygen, and hydroxyl radical (Das & Roychoudhury 2014; Akyol et al. 2020). If drought stress is prolonged, ROS production becomes dominant over the scavenging capacity of the antioxidant system, resulting in extensive cellular damage and death (Cruz de Carvalho 2008). On the other hand, ROS function as signal molecules in plants (Waszczak et al. 2018), controlling growth, development, responses to biotic and abiotic environmental stimuli, and programmed cell death (Bailey-Serres & Mittler 2006). The production of H₂O₂ is strongly associated with the catabolic processes of polyamine. Because H₂O₂ is not only produced by the of NADPH oxidases

(NOX), but also by amine oxidases (Cona et al. 2006a; 2006b). Apoplastic polyamine oxidase in tobacco and polyamine oxidase encoded by the Arabidopsis polyamine oxidase 3 (*AtPAO3*) gene have been documented to contribute to the production of H₂O₂ (Moschou et al. 2008a; Wu et al. 2010). Polyamine oxidation has been reported to be regulated by polyamine oxidases at different levels of expression in *Arabidopsis thaliana*, thus rendering ROS levels is quite complex (Fincato et al. 2011). Many studies confirm that NOX homologs are essential for ROS accumulation in plants (Kwak et al. 2003; Sagi et al. 2004; Torres et al. 2005). Mutants of the NADPH oxidase genes have been employed in most of these studies. ROS accumulation, especially H₂O₂, decreases in these plants. Papadakis & Roubelakis-Angelakis (2005) have suggested that the cross-linking between polyamines and NOX regulates protoplast regeneration in a tobacco plant. Andronis et al. (2014) have found that exogenously applied polyamines stimulate NADPH oxidase-dependent oxygen consumption in Arabidopsis. In the study, the amount of oxygen in the mutant plants carrying the overexpressed *AtPAO3* gene decreased compared to the mutant *Atpao3* mutant plants with incomplete function. In the *Atpao3* mutants, the amount of H₂O₂ was low. In the case of *AtPAO3* over-expression, the opposite was the case. Andronis et al. (2014) stated that there could be a very strong relationship between polyamines and H₂O₂, and this relationship could also be associated with NOX. Furthermore, Liu et al. (2019) determined that exogenously applied putrescine contributed to the development of resistance against pathogenic bacteria in *Arabidopsis thaliana*. The resistance was found to be dependent on hydrogen peroxide and NOX (*RbohD* and *RbohF*). In addition, Seo et al. (2019) found out that *NtRbohD* and *NtRbohF* genes were downregulated in *SAMDC* overexpressing *Nicotiana tabacum* under salt stress conditions. Consequently, they concluded that polyamines inhibited the production of ROS. However, the relationship between NADPH oxidase and polyamines has not been fully elucidated under drought stress conditions yet. In the current study, the hypothesis that NOX acts as a regulator in the metabolism of polyamines under drought stress conditions has been tested. For this purpose, the biosynthesis pathway of polyamines in drought-stressed maize plants was studied in detail after the application of H₂O₂ and a NOX inhibitor.

2. Material and Methods

2.1. Plant material and treatments

Maize seeds (*Zea mays* L cv Akpınar sensitive to drought, (Güven 2013) provided from Karadeniz Agricultural Research Institute, Samsun, Turkey, were sterilized with 0.1% HgCl₂ for 3 min and washed with sterilized distilled water (Terzi et al. 2014). Ten seeds were grown in pots containing peat and sand (5:1) in a growth chamber, which was set to 16 h light (PPFD on the leaf surface per 400 µmol.m⁻².s⁻¹), 8 h dark photoperiod at 25 °C ± 2 and ambient humidity 60% ± 5. Four-week-old seedlings were cut from near bottom then immersed distilled water for alleviating injury stress, and subsequently, plants were divided three groups as followed i) Control (3% PEG 6000 only, -0.5 MPa), ii) 3% PEG 6000 + 10 mM H₂O₂ (Terzi et al. 2014) and iii) 3% PEG 6000 + 100 µM DPI (NOX inhibitor diphenyleneiodonium chloride, Potocký et al. 2007). All treatments were done on the excised seedlings immersed in the test solutions mentioned. The seedlings were kept in the test solutions for 6 hours. At the end of this period, the third leaves from the base of the seedlings were selected and used in the experiments.

2.2. Leaf water potential

Leaf water potential (MPa) was measured by using a C52 thermocouple psychrometer (Wescor Inc.). Leaf disks approximately 6 mm in diameter were placed in the C52 sampling chamber and monitored for one hour with the PSYPRO data logger (Saglam et al. 2011).

2.3. Lipid peroxidation

The quantity of thiobarbituric acid reactive substances (TBARS) was measured to determine lipid peroxidation according to Heath & Packer (1968). Leaf samples (0.25 g) were homogenized with 20% trichloroacetic acid (TCA) including 5% thiobarbituric acid (TBA) and centrifuged at 10000 g for 10 minutes at 4 °C. TBARS content was expressed as nmol g⁻¹ dry weight.

2.4. Endogenous H₂O₂ content

The endogenous content of H₂O₂ was measured according to Velikova et al. (2000). Frozen leaf samples (0.25 g) were homogenized with 1% TCA and centrifuged at 15000 g for 15 minutes at 4 °C. The resultant supernatant was added to the reaction mixture, including 10 mM of potassium phosphate buffer (pH 7), and 1 M of potassium iodide (KI). Absorbance was measured at 390 nm. The H₂O₂ content was given as nmol g⁻¹ dry weight.

2.5. Enzyme activities

Leaf samples (0.1 g) were pulverized in liquid nitrogen and extracted with 1.8 ml of extraction buffer (100 mM K₂HPO₄, 0.1 mM EDTA pH 7.0, 0.1% Triton). The extracts were centrifuged at 15000 g for 20 minutes at 4 °C. The resultant supernatant was used to determine enzyme activity.

The activity of NADPH oxidase (EC 1.6.3.1) was measured by Cakmak & Marschner (1988). NADPH oxidase activity was determined based on NADPH's oxidation at 340 nm ($\epsilon = 6.2 \text{ mM cm}^{-1}$). Enzyme activity was determined based on the decrease in absorbance for 3 minutes in the reaction medium consisting of 100 mM of potassium phosphate buffer (pH 7.0), 0.1 mM of EDTA, and 100 μM of NADPH.

Catalase (EC 1.11.1.6) activity was determined according to Aebi (1983). Enzyme activity was determined by measuring a 1 mL reaction mixture containing 50 mM of potassium phosphate buffer (pH 7.0), 30 mM of H_2O_2 , and 20 μL of enzyme extract at 240 nm for 5 minutes. The catalase activity was calculated by using the extinction coefficient $\epsilon = 39.4 \text{ mM cm}^{-1}$ for H_2O_2 .

Peroxidase (EC 1.11.1.7) activity was determined according to Urbanek et al. (1991). The enzyme activity was determined by measuring the 2 mL reaction mixture containing 100 mM of potassium phosphate buffer (pH 7.0), 0.1 mM of EDTA, 5 mM of guaiacol, 15 mM of H_2O_2 , and 50 μL of enzyme extract for 1 minute at 470 nm. The peroxidase activity was calculated using the extinction coefficient ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

The activity of superoxide dismutase (EC 1.15.1.1) was determined according to Beauchamp & Fridovich (1971). The reaction was started by adding 2 μM of riboflavin to 1 mL reaction medium containing 50 mM of potassium phosphate buffer (pH 7.8), 0.1 mM of EDTA, 13 mM of methionine, 75 μM of nitro blue tetrazolium and 50 μL of extract. Following exposure to white light at $375 \mu\text{mol m}^{-2} \text{ s}^{-1}$, absorption values were determined at 560 nm.

2.6. Polyamine content

The determination of polyamines was performed as outlined in Terzi et al. (2014). Briefly, the fresh leaves (5g) were homogenized with 10 mL of perchloric acid 0.4 M. The homogenate was centrifuged at 3000 g for 4 minutes at 4 °C. The supernatant was collected and perchloric acid (10 mL of 0.4M) was added to the supernatant. This mixture was then re-centrifuged. NaOH (1 M) and sodium hydrogen carbonate were added to 1 mL of the supernatant. The mixture was vortexed for 30 seconds. Subsequently, 2 mL of dansylchloride chloride was added (10 mg mL^{-1} in acetone). The reaction was stopped with 0.1 mL of 25% ammonium hydroxide. An HPLC device with UV/VIS detector (Shimadzu, LC 20 AT / Prominence, Japan) read the polyamine content. The mobile phase consisted of 0.1 M ammonium acetate and 19 M acetonitrile (65:35 v/v). The flow rate was 0.70 mL min^{-1} and the column temperature was 50 °C. The 20 μL sample was injected into the C18 column ($4.6 \times 250 \text{ mm}$ Supelco, Bellefonte, USA). Polyamines were determined at 254nm wavelength. Peak fields of the polyamines were recorded, then putrescine (put), spermine (spm), and spermidine (spd) concentrations were calculated using LabSolutions software. The concentrations were expressed in $\mu\text{g g}^{-1}$ dry weight.

2.7. Protein content

Protein determination was performed spectrophotometrically according to Bradford (1976). Thirty microliters of sample were used from the extracts prepared for enzymatic activity. Bovine serum albumin (BSA) standards were prepared, and the complex of proteins with Coomassie Brilliant Blue G250 was measured at 595 nm. The protein concentration was determined in mg and used for enzyme activities.

2.8. RNA isolation and cDNA synthesis

Fresh leaf samples (0.1 g) were used for total RNA isolation. Total RNA isolation was carried out using a total RNA isolation kit (RNeasy Plant Mini Kit 74904, Qiagen Netherlands). The samples frozen with liquid nitrogen were ground by tissue homogenizer (TissueLyser LT, Qiagen Germany). The amount and the purity of the RNA samples were measured using a nanodrop spectrophotometer (Thermo Scientific, Nanodrop 2000, America). A c-DNA reverse transcription kit (4368814, Applied Biosystems America) was used for the synthesis of c-DNA from the isolated total RNA. The cDNA concentration was adjusted to 2000 ng in each group. The synthesized cDNAs were stored at -20 °C until Real-Time PCR analyses were performed.

2.9. Quantitative Real-Time PCR analysis

The c-DNAs from the previous step were used in the Real-Time PCR analysis to determine gene expression. 5x HOT FIREPol Eva Green qPCR Supermix (08-36-00008, Solis Biodyne, Estonia) was used to analyze gene expression through CFX Connect Real-Time PCR system (BioRad, USA). RT PCR protocol modified according to Solis Biodyne instructions was as follows 12 minutes at 95 °C, 45 cycles at 95 °C, 30 seconds at 60 °C, 30 seconds at 72 °C. The melt curve was held in 0.5 °C increments from 60 to 95 °C. The primers were designed with Primer 3 plus (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>). As a reference for gene expression, primers belonging to the Actin1 gene were used. In the study, the genes targeted for expression and primers from those genes were given in Table 1.

Table 1- The sequences of specific primers used for qRT-PCR analysis

Target gene	NCBI accession no.	Primer names and their sequences
<i>Actin 1</i>	NM_001155179.1	ACT1Zm_F: “GAAGATCACCTGTGCTGCT” ACT1Zm_R: “ACCAGTTGTTGCGCCACTAG”
<i>Arginine decarboxylase (ADC)</i>	EU968980.1	ADCZm_F: “GACATCACCTGCGACAGTGA” ADCZm_R: “GAACAGGTTGTGCTTGCCAG”
<i>Spermidine synthase (SPDS)</i>	EU976300.1	SPDSZm_F: “TTCCCCTGTTCGATTACCG” SPDSZm_R: “GCAGAACCGTTTCTTGCGTC”
<i>Agmatine iminohydrolase (AIH)</i>	NM_001156428.1	AIHZm_F: “GACGAGGATACAAACGGCCA” AIHZm_R: “ACGGACTGGGAGAGAACAGA”
<i>S-Adenosylmethionine decarboxylase (SAMDC)</i>	EU968400.1	SAMDCZm_F: “GGAGGCGTGAAGAAGTTCCA” SAMDCZm_R: “TTATCAGGAAGCAGCAGGCC”
<i>Diamine oxidase (DAO)</i>	NM_001152492.1	DAOZm_F: “ACAGCAAGTCCGAGAAGTGG” DAOZm_R: “TGTACCACAGCAGCATGTCC”
<i>Polyamine oxidase 1 (PAO)</i>	NM_001111636.1	PAOZm_F: “CGCTACGAATACGACCAGCT” PAOZm_R: “TGGGCGCAGTTGATGAGAAT”
<i>NADPH oxidase B</i>	DQ890023.1	NOXZm_F: “GGGCCAGTACTTCGGTGAAA” NOXZm_R: “AAGCTTCACCAGGCTACGAC”

2.10. Statistical analysis

The variance analysis of means obtained as a result of the analyzes with three replications with 10 samples was performed according to the one-way ANOVA (Duncan Multiple Comparison Test) included in the package of the Statistical Package for Social Sciences (SPSS 16.0 for Windows) at $P < 0.05$. The relative gene expression level was analyzed by the Bio-Rad CFX Manager 3.1. Expression levels were also assayed by SPSS software. Variance analysis of mean values was carried out by one-way ANOVA ($P < 0.05$).

3. Results and Discussion

Water potential measurements were performed to determine the effect of H_2O_2 on the water content of maize plants subjected to drought stress conditions. Leaf water potential has been regarded as a good indicator of drought stress (Shaw et al. 2002). Sharma & Dubey (2005) reported in their study that the water potential of rice plants exposed to drought stress could be reduced from -0.5 MPa to -2 MPa. Similarly, drought stress in wheat reduced leaf water potential from -0.63 MPa to -2 MPa (Siddique et al. 2000). Unlike the above studies, Terzi et al. (2014) determined that the water potential of the leaf increased in drought stress conditions with the application of H_2O_2 in maize plants compared to control (PEG only). Likewise, in the current study, the leaf water status was improved by the H_2O_2 application (Figure 1a). The water potential of the H_2O_2 -treated group was 1.2-fold higher than that of the control group (PEG only), and the levels of polyamine increased along with this improvement. Following the use of DPI to reduce the endogenous H_2O_2 content, there was a decrease in the leaf water potential and the polyamine content. The water potential of the DPI group was 1.3-fold lower than that of the control group. The water potential value of the DPI group was also less than that of the H_2O_2 group. The water potential of the DPI group was 1.65-fold lower than that of the H_2O_2 -treated group. DPI was found to influence the water status of the plant by decreasing the water potential relative to the control group (PEG application). The reduction suggested that applying H_2O_2 could affect water potential by regulating polyamine metabolism. As is known, polyamines play a role as an osmotic regulator in drought stress (Sequera-Mutiozabal et al. 2017).

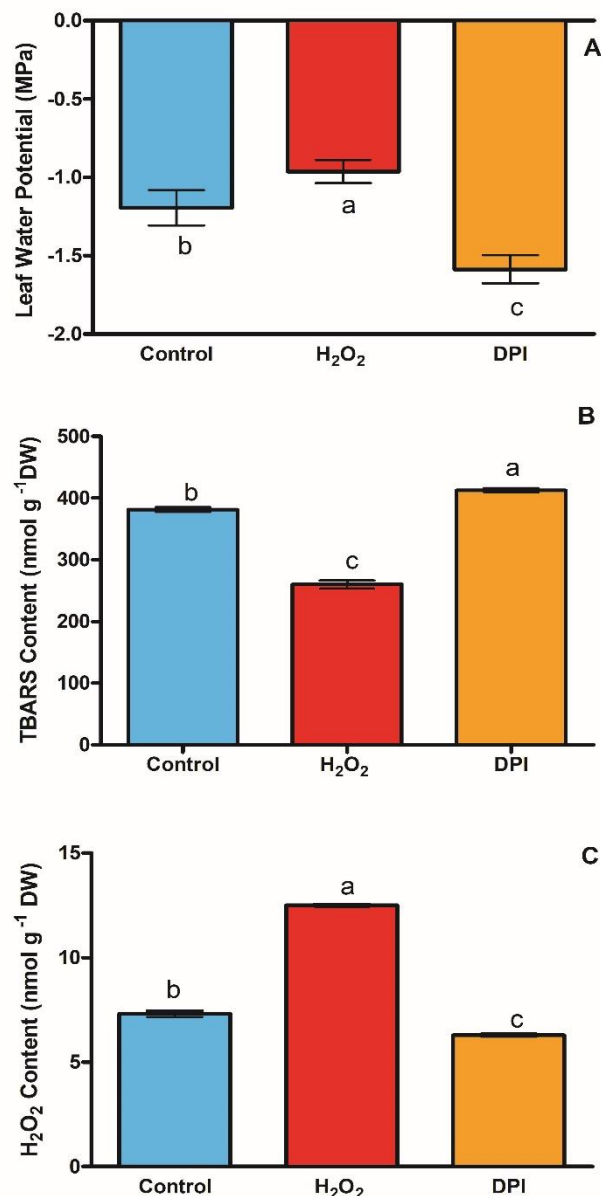


Figure 1- Effect of exogenous H₂O₂ and DPI on leaf water potential (A) TBARS (B) and endogenous H₂O₂ contents in the leaves of detached maize seedlings under PEG-induced drought stress conditions. The seedlings were submitted to three treatments: Applied with PEG (Control); applied with H₂O₂ and PEG (H₂O₂); applied with DPI and PEG (DPI). Vertical bars are standard deviations. Different letters indicate significant differences among different treatments at P<0.05

In the present study, lipid peroxidation was measured to determine the effects of H₂O₂ and DPI on membrane damage under drought stress. ROS is known to cause high levels of lipid peroxidation, resulting in damage to cell membranes (Munne-Bosch et al. 2001). However, in our study, the 10 mM of H₂O₂ was determined to reduce lipid peroxidation under drought stress conditions (Figure 1b). The TBARS content of the H₂O₂-treated group was 1.5-fold lower than that of the control group. The amount of TBARS measured in the DPI group was significantly increased concerning the control and the H₂O₂-treated groups. The TBARS content of the DPI group was 1.1 and 1.6-fold higher than the control and the H₂O₂-treated groups respectively. Furthermore, the application of 10 mM H₂O₂ significantly increased the endogenous level of H₂O₂ compared with the control group. The endogenous H₂O₂ content of the H₂O₂-treated group was 1.7-fold higher than that of the control group. Following DPI treatment, the level of endogenous H₂O₂ decreased significantly in comparison with the control and H₂O₂ groups (Figure 1c). The endogenous H₂O₂ content of the DPI group was 1.2 and 2.0 times lower than the control group and the H₂O₂ treated group, respectively. Here, H₂O₂ applied exogenously may have caused the endogenous hydrogen peroxide to act as a signal molecule and stimulate antioxidants. The stimulation of antioxidants in response to a non-lethal dose of hydrogen peroxide allows cells to adapt to exposure to a much larger dose and thus survive (Rodríguez-Rojas et al. 2020).

Similarly, in our study, the application of H₂O₂ increased catalase (CAT) and peroxidase (POD) activities, but the activity of superoxide dismutase (SOD) declined. The catalase activity of the H₂O₂-applied group was 1.3 and 1.4-fold higher than that of

the control and the DPI groups, respectively (Figure 2a). In the DPI group, the CAT activity was found to be lower than that of the control group. The CAT activity of the DPI group was 1.1-fold lower than that of the control group. POD activity was determined as the highest in the H_2O_2 group compared to the control and the DPI groups (Figure 2b). The peroxidase activity of the H_2O_2 applied group was 1.3 and 1.4-fold higher than the control and the DPI groups, respectively. Furthermore, because of the DPI application, the activity decreased by 1.1 times compared to the control group. In terms of superoxide dismutase, the highest activity was identified in the control group (Figure 2c). The SOD activity of the control group was 1.5 and 1.6-fold higher than that of the H_2O_2 and DPI groups, respectively. There was no difference between the H_2O_2 and DPI groups in terms of the SOD activity.

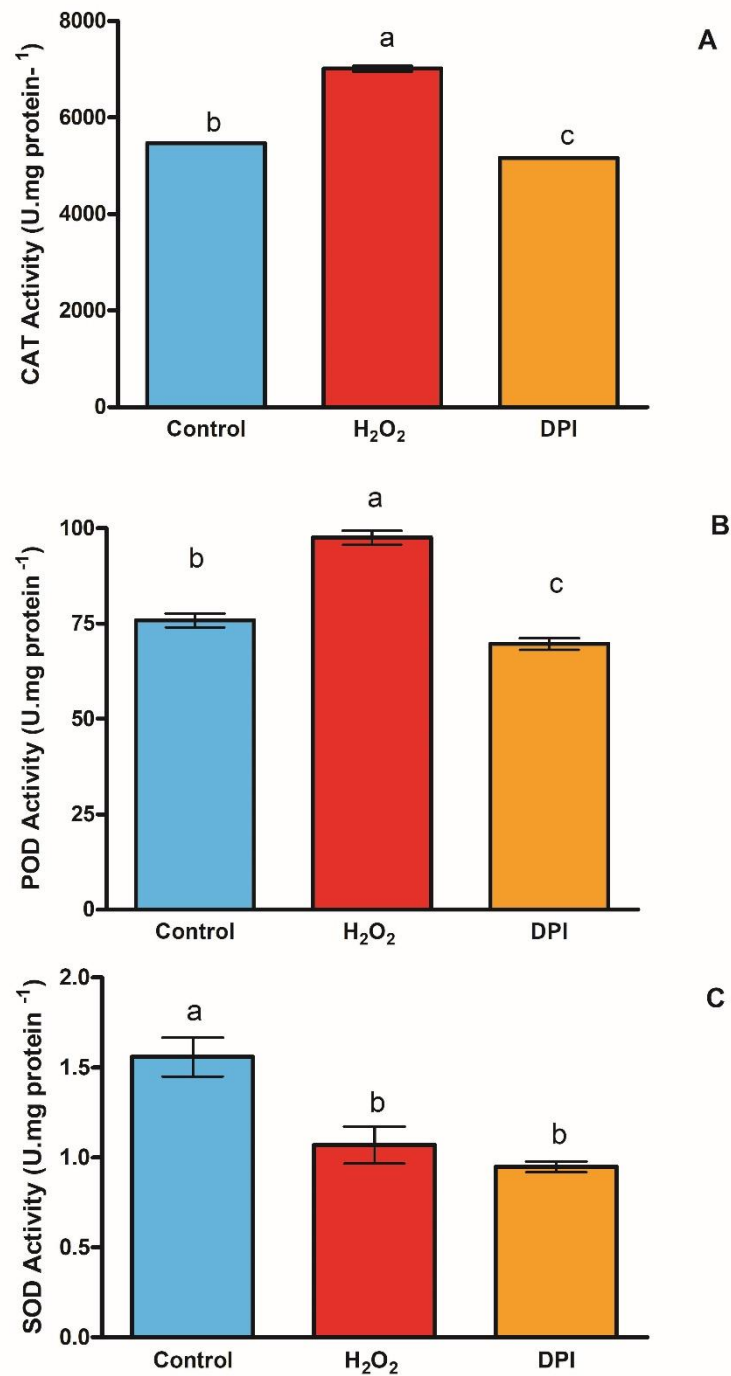


Figure 2- Effect of exogenous H_2O_2 and DPI on antioxidant enzyme activities in the leaves of detached maize seedlings under PEG-induced drought stress conditions. Catalase (A), Peroxidase (B) and Superoxide dismutase (C).

The enzyme catalase is known to scavenge excess H_2O_2 by reducing it to water and oxygen (Chaudiere & Ferrari-Iliou 1999). This enzyme has also been reported to play a role in the prevention of potential damage (such as lipid peroxidation) of H_2O_2 in membranes (Chaudiere & Ferrari-Iliou 1999). Similarly, peroxidases have also been reported to be responsible for scavenging H_2O_2 under stress and maintaining cell membrane integrity (Parida & Das 2005). We concluded that the application of H_2O_2 protected the integrity of the cell membrane by inducing catalase and peroxidase activities and therefore reduced TBARS levels. Similar findings were reported in wheat plantings under drought conditions and in cucumber and corn plants under osmotic stress conditions (He et al. 2009; Liu et al. 2010; Terzi et al. 2014). In our study, following the application of DPI, the endogenous H_2O_2 level, NADPH oxidase activity, and NOX expression level decreased significantly compared to other groups. The lowest NOX activity was observed in the DPI-treated group (Figure 3a). On the other hand, exogenously applied H_2O_2 significantly increased NOX activity compared to the control. The NOX activity of the H_2O_2 treated group was 1.1 and 1.3 higher than that of the control and DPI groups, respectively. In addition to NOX activity, the DPI reduced the level of expression of the NOX gene in comparison to the control group (Figure 3b). The NOX expression level of the DPI applied group was 1.3 times lower than that of the control group; however, the highest NOX expression level was found in the H_2O_2 group (Figure 3b). The NOX expression of the H_2O_2 applied group was 1.4 and 1.9-fold higher than the control and the DPI groups.

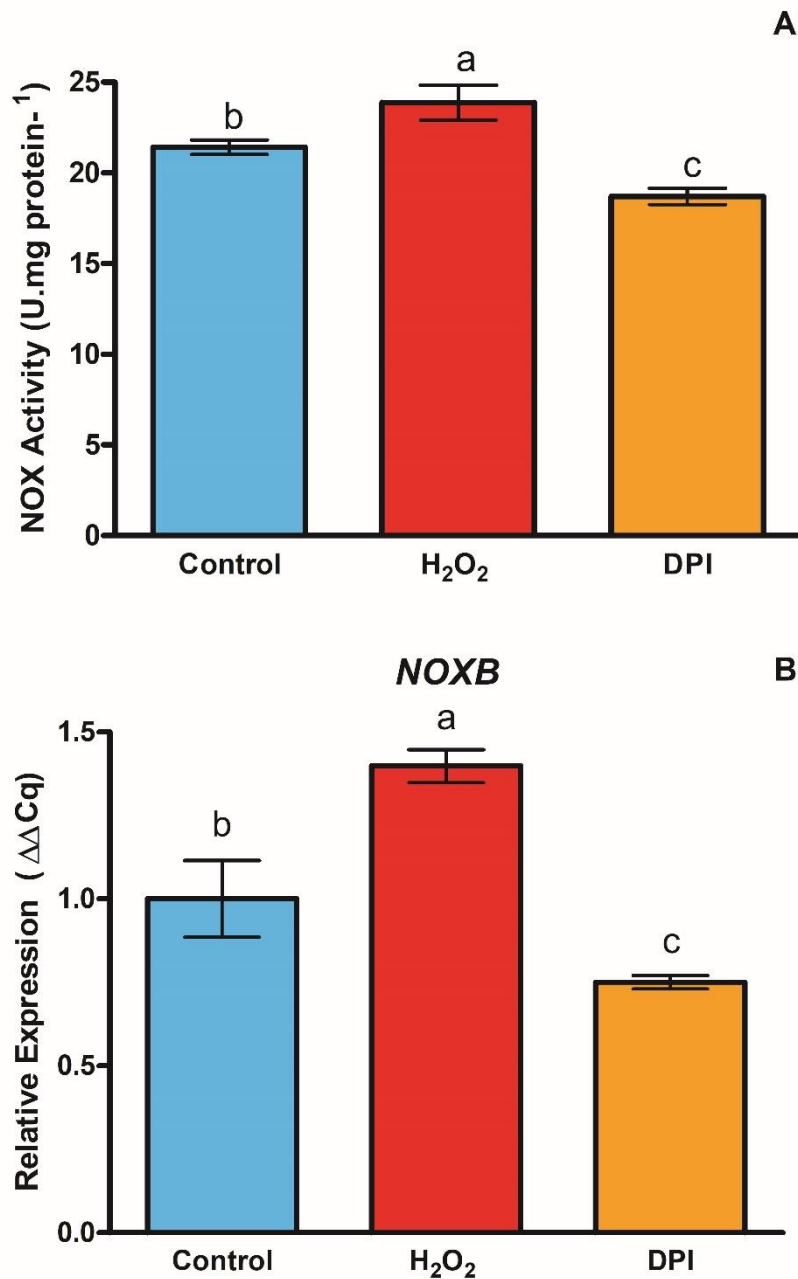


Figure 3- Effect of exogenous H_2O_2 and DPI on NADPH oxidase (NOX) activity (A) and NOXB gene expression (B) in the leaves of detached maize seedlings under PEG-induced drought stress conditions.

The decreases in endogenous H_2O_2 level, NADPH oxidase activity, and *NOX* expression levels and the increased amount of TBARS following the DPI application could be explained by decreasing catalase and peroxidase activities. These changes in enzyme activities showed that hydrogen peroxide acted as a signaling molecule, activated the antioxidant system and protected corn plants from the effects of stress.

Significant increases in levels of endogenous polyamines due to drought stress have been noted in some studies (Erdei et al. 1996; Galston et al. 1997; Groppa & Benavides 2008). In this study, the application of H_2O_2 under drought stress increased the levels of polyamines such as put and spm in comparison with the control group. The maximum put concentration (in all groups) was determined in group H_2O_2 (Figure 4). The putrescine level was found to be the lowest in the DPI among all groups. The put content of the H_2O_2 treated group was 1.3 and 2.1-fold higher than the control and DPI groups, respectively. The concentration of (spd) in the control group was highest among all groups. The spd content was significantly reduced by the H_2O_2 and DPI treatments relative to the control group. The spd content in the control group was 1.2 and 1.6-fold higher than the H_2O_2 and DPI groups, respectively. Furthermore, the spd content of the H_2O_2 group was found to be greater than that of the DPI group. As for the spm content, the application of H_2O_2 resulted in the accumulation of spm. The spm content of the H_2O_2 -applied group was 1.4 and 2.0-fold higher than the control and DPI groups, respectively. Moreover, the lowest spm concentration was determined in the DPI group (Figure 4).

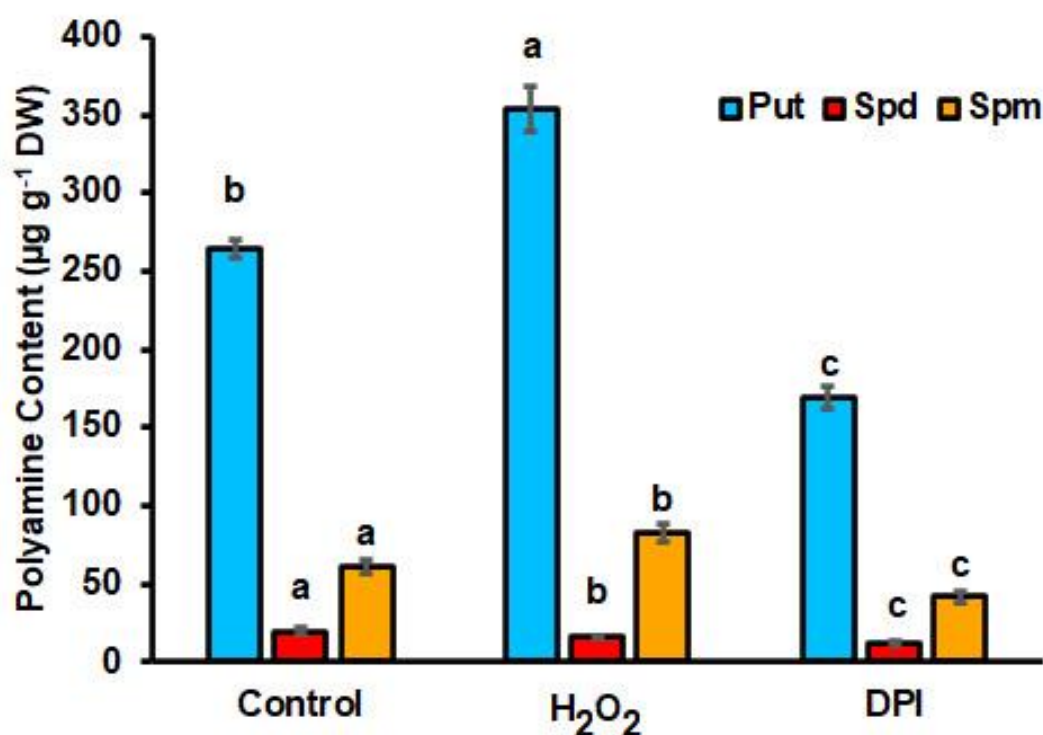


Figure 4- Effect of exogenous H_2O_2 and DPI on endogenous polyamine contents in the leaves of detached maize seedlings under PEG-induced drought stress conditions.

Abass & Mohamed (2011) suggested that the application of H_2O_2 to bean seeds stimulated polyamine content under drought conditions. Similarly, Terzi et al. (2014) exposed maize seedlings to osmotic stress after performing pre-treatment of H_2O_2 and found significant increases in the put, spm and spd contents. Besides, in our study, the decrease in polyamine level due to DPI application supported the idea that *NOX*-induced H_2O_2 may have a stimulating effect on the polyamine synthesis pathways.

The level of expression of the *ADC* gene, located in the first stage of the put synthesis, increased with the application of H_2O_2 in comparison to the other groups in our study. The *ADC* expression of the H_2O_2 -treated group was 1.2 and 1.3-fold higher than the control and DPI groups, respectively (Figure 5a). The put pool is mainly under the control of the *ADC* gene and shows an increase in drought stress (Urano et al. 2004; Alcazar et al. 2010b). For this reason, the increase in the putrescine level in the experimental group with H_2O_2 -applied may be related to the increased expression level of the *ADC* gene. Indeed, Urano et al. (2004) reported that the increase in putrescine levels in *Arabidopsis* plants under drought conditions was positively correlated with the stimulation of *ADC* transcripts. This positive relationship between *ADC* and putrescine, which we found in our study, was similar to the one obtained by Urano et al. (2004). Abass & Mohamed (2011) and Terzi et al. (2014), in their drought-related stress studies, determined that the application of H_2O_2 increased the polyamine content. Our findings were consistent with the

data from these researchers. In addition, the RT PCR analyses of the present study confirmed our opinion that H_2O_2 promoted the accumulation of polyamines.

In contrast to H_2O_2 , DPI decreased the expression level of the *ADC* gene by 1.1-times compared to the control group (Figure 5a). The downregulation of the *ADC* gene through DPI also supported the idea that H_2O_2 derived from NOX may be necessary for the regulation of polyamine biosynthesis. The transcript level of the agmatine aminohydrolase (*AIH*) gene, which is another enzyme involved in putrescine synthesis, was enhanced with the application of H_2O_2 (Figure 5b). The *AIH* expression of H_2O_2 -applied group was 2.2 and 3.3-fold higher than that of the control and the DPI groups, respectively. Because of the DPI application, the *AIH* expression level was reduced by 1.5 times compared to the control group.

The level of expression of the *AIH* gene has been reported to increase in Arabidopsis plants exposed to drought stress (Alcazar et al. 2006b). According to the results of our study, another reason for the increase in putrescine due to the application of H_2O_2 was the positive regulation of the *AIH* gene. At the same time, the decrease in the *AIH* transcript level with DPI application indicated that H_2O_2 could regulate the expression level of the gene in question, and the level of putrescine increased. Alcazar et al. (2011) and Do et al. (2014) found elevated levels of transcripts of spermidine synthase (*SPDS*) genes in Arabidopsis and rice plants, respectively, under drought stress conditions. In our study, the amount of spd was found to be decreasing in the H_2O_2 group compared to the control group. This decrease might be caused by the recovery of spermidine into the putrescine pool through PAO activity or by the reduction of transcripts of the *SPDS* gene because the *SPDS* gene in the control group was more expressed than the H_2O_2 group in our study. The expression level of *SPDS* in the control group 1.8-fold higher than H_2O_2 group (Figure 5d). Furthermore, the low level of expression of *PAO* in the H_2O_2 group compared to the control group may help explain the fluctuations in spermidine levels.

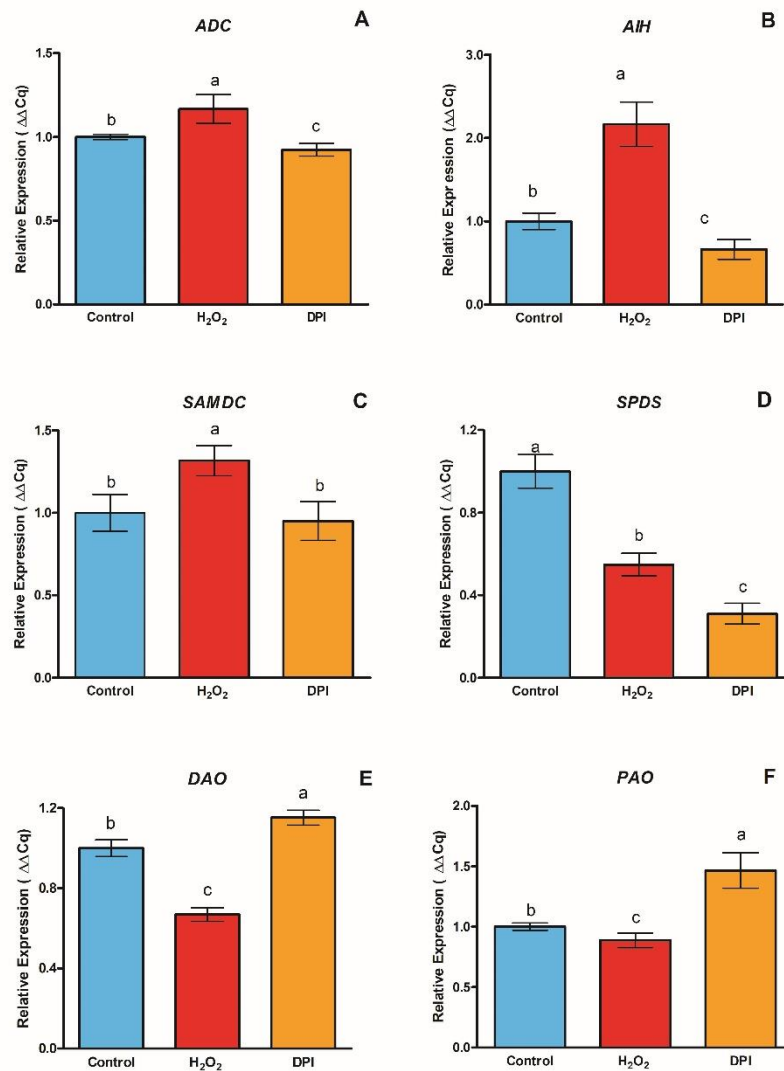


Figure 5- Effect of exogenous H_2O_2 and DPI on gene expression levels of Arginine decarboxylase (A), Agmatine iminohydrolase (B), S-adenosylmethionine decarboxylase (C), Spermidine synthase (D), Diamine oxidase (E), Polyamine oxidase (F) in the leaves of detached maize seedlings under PEG-induced drought stress conditions.

In conclusion, according to the control group, the decrease in endogenous spd level in our study due to the H₂O₂ application was consistent with the reduction in the expression level of the *SPDS* gene. In our study, the expression level of the S-adenosyl methionine decarboxylase (*SAMDC*) gene, which is involved in the synthesis of spd and spm, was determined. The level of expression of *SAMDC* was significantly enhanced by H₂O₂ treatment. The expression level of *SAMDC* in the H₂O₂-applied group was 1.3 and 1.4-fold higher than the control and the DPI groups, respectively (Figure 5c). The measurement of the degree of expression of this gene provided valuable information for the regulation of spd and spm concentrations. A limited number of studies reported increases in *SAMDC* expression levels under the stress of drought. For example, under drought stress conditions, there has been an increase in *SAMDC* transcripts in Arabidopsis and rice plants (Li & Chen 2000; Alcazar et al. 2011). In addition, the level of spm was reported to increase in rice plants subject to drought stress (Capell et al. 2004). In our study, under drought conditions, the *SAMDC* transcription level when H₂O₂ was applied was higher than other experimental groups. This was consistent with the increased spermine level with hydrogen peroxide application. As in our study, Terzi et al. (2014) reported increases in spm levels as a result of H₂O₂ treatment in maize seedlings subjected to osmotic stress. Furthermore, increased expression of a *SAMDC* by H₂O₂ in our study supported the idea that H₂O₂ plays an important role in the regulation of polyamine biosynthesis.

Several studies have reported that genes involved in the destruction of polyamines encode diamine oxidase (DAO) and PAO enzymes (Cohen 1998; Angelini et al. 2010; Fincato et al. 2012). In our study, the expression of the *DAO* gene was significantly decreased by H₂O₂ application in drought conditions compared to other groups. The exogenous H₂O₂ treatment reduced the expression level of the *DAO* gene as 1.5-fold than the control group (Figure 5e). This reduction may help explain the high level of put in the H₂O₂ group compared with other groups. DPI application was the group with the highest level of *DAO* transcript. The gene expression level of *DAO* in the DPI group was 1.2 and 1.7-fold higher than the control and the H₂O₂-treated groups, respectively (Figure 5e). This was confirmed by examining putrescine rates. Regulation of the *DAO* gene supported the idea that there could be a crosstalk between H₂O₂ and polyamine levels during drought stress. In our study, the levels of *PAO* transcripts in the control and DPI groups were higher than the H₂O₂ group under drought conditions. The level of expression of the *PAO* gene in the DPI group was 1.5 and 1.7 times higher than that of the control and H₂O₂ groups, respectively. On the other hand, the expression level measured in the H₂O₂ group was 1.1-fold lower than the control group (Figure 5f).

Alcazar et al. (2011) reported a rise in *PAO* expression in drought-exposed Arabidopsis plants. In another study, *PAO* transcripts were also found to increase significantly, while put and spd levels decreased in grape varieties exposed to drought stress (Hatmi et al. 2015).

On the other hand, the application of H₂O₂ which we carried out in our study resulted in a decrease in *PAO* transcripts and an increase in polyamine levels. This was evidenced by the rise in the putrescine rate in the H₂O₂ group. The increased expression level of the *PAO* gene by DPI also supported that NOX-derived H₂O₂ had a regulatory role in polyamine degradation.

Superoxide (O₂^{•-}) and H₂O₂, the most studied ROS derivatives, have critical roles in many processes (Pitzschke et al. 2006; Dikalov et al. 2011; Suzuki et al. 2013). In our study, DPI reduced the H₂O₂ accumulation by blocking the NADPH oxidase enzyme, which resulted in significant reductions in polyamine levels. Furthermore, the levels of expression of *DAO* and *PAO* responsible for polyamine degradation increased. On the other hand, the model of polyamine oxidation initiated by the H₂O₂ signal cascade has been accepted because H₂O₂ is a direct product of polyamine oxidation (Moschou et al. 2008b). In summary, the literature indicates that polyamine metabolism can be regulated by H₂O₂, which is derived from the activities of *DAO* and *PAO* enzymes. However exogenously applied H₂O₂ in our study decreased the gene expression level of the *DAO* and *PAO*, while leading to an increase in expressions of *ADC*, *AIH*, *SPDS*, and *SAMDC*. So, according to our study, it concluded that H₂O₂ was capable of positive regulation on polyamine metabolism. Consequently, in our study, not only H₂O₂ due to polyamine oxidation, but also H₂O₂ derived from NADPH oxidase and externally applied H₂O₂ affects polyamine signaling. Because when NOX is inhibited, the metabolic pathway has shifted from synthesis to breakdown.

4. Conclusions

Based on the data obtained in this study, a model diagram showing the potential H₂O₂-polyamine relationship was established (Figure 6). The model of polyamine metabolism controlled by hydrogen peroxide can be summarized as follows. H₂O₂ which was applied externally in drought conditions, probably induced both *NOX* gene expression and enzyme activity. Then, H₂O₂ induced the expression of *ADC*, *AIH*, *SPDS*, and *SAMDC*, one of the polyamine synthesis genes, directly or indirectly, by other possible metabolic pathways. Due to the increased level of gene expression, the amount of endogenous polyamine has increased. Besides, H₂O₂ has been suggested to reduce the production of polyamine oxidation enzymes by directly or indirectly suppressing the expression of polyamine degradation genes. In conclusion, H₂O₂ plays an essential regulation role in the metabolism of polyamines by adjusting the expressions of genes for the synthesis and degradation of polyamines.

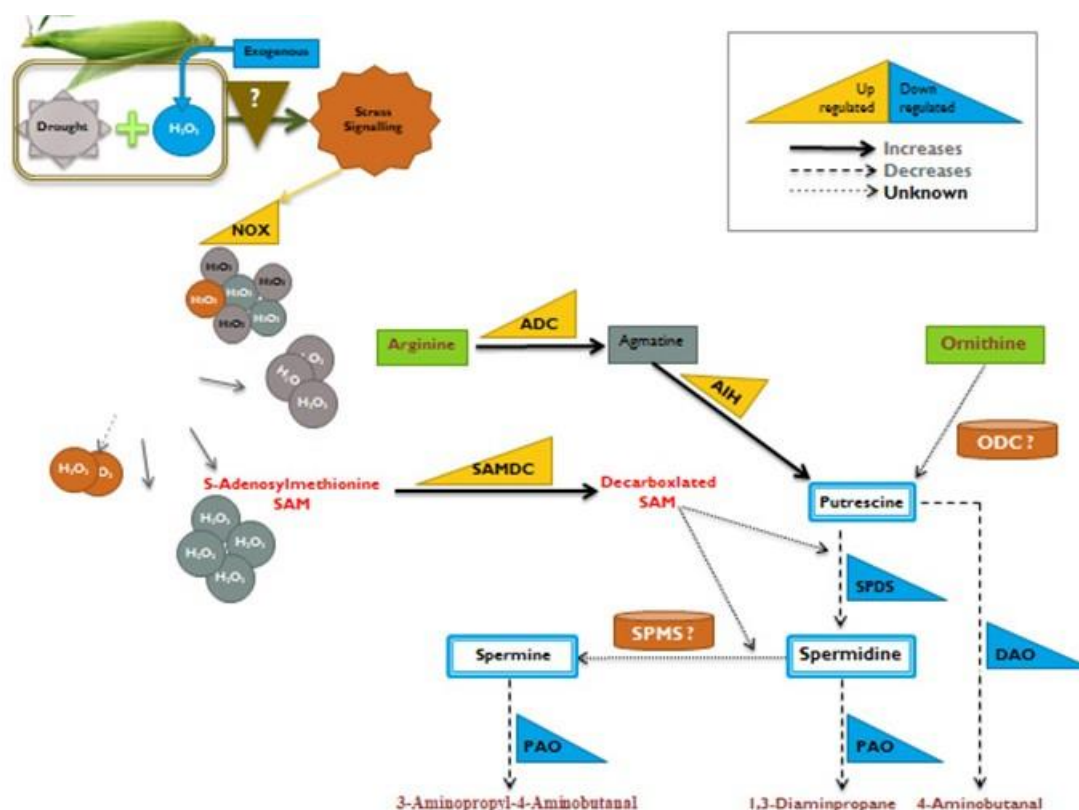


Figure 6- Putative H₂O₂-related polyamine metabolism under drought stress conditions

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