

Hypoxia Induced Changes in the Lipid Peroxidation, Membrane Permeability, Reactive Oxygen Species Generation, and Antioxidative Response Systems in *Zea mays* Leaves

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Abstract: The effects of hypoxia on the activities of some enzymes of antioxidative, non- enzymatic scavenging system, membrane permeability, lipid peroxidation, and some reactive oxygen species (ROS) in leaves of *Zea mays* were investigated. Samples were taken 48, 96, 144, and 192 h after the start of hypoxia treatment. A 192 h hypoxia treatment resulted in a significant rise in membrane permeability, lipid peroxidation (malondialdehyde level), and the production of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in maize leaves. A short duration of hypoxia enhanced the activity of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2), while further hypoxia significantly decreased the enzyme activity but increased the content of reduced glutathione (GSH) and ascorbic acid (AsA). It was observed that the reduction in SOD activity was greater than that in GR and APX (H_2O_2 scavengers). Our results showed that O_2^- induced membrane damage and lipid peroxidation, and that excessive accumulation of O_2^- is due to the reduced activity of SOD under hypoxia.

Key Words: Antioxidant enzymes, ascorbate, glutathione, hypoxia, malondialdehyde, membrane permeability, reactive oxygen species, *Zea mays*

Hipoksi, *Zea mays* (Mısır) Yapraklarında, Lipid Peroksidasyonu, Membran Geçirgenliği, Reaktif Oksijen Türlerinin Oluşumu ve Antioksidatif Cevap Sistemlerinde Değişiklikler Meydana Getirir

Özet: *Zea mays* (mısır) yapraklarında, antioksidatif bazı enzimlerin, enzimatik olmayan süpürücü sistemin, membran geçirgenliğinin, lipit peroksidasyonunun ve bazı reaktif oksijen türleri (ROS)'nin aktiviteleri üzerine hipoksinin etkileri araştırılmıştır. Hipoksi muamalesinden 48, 96, 144 ve 192 saat sonra örnekler alınmıştır. 192 saatlik hipoksi muamelesi, mısır yapraklarında membran geçirgenliğinde, lipit peroksidasyonunda (malondialdehit seviyesi), hidrojen peroksit (H_2O_2) ve süperoksit (O_2^-) oluşumunda önemli bir artış meydana getirmiştir. Kısa süreli hipoksi, süperoksit dismutaz (SOD; EC 1.15.1.1), katalaz CAT; EC 1.11.1.6), askorbat peroksidaz (APX; EC 1.11.1.11) ve glutatyon redüktazın aktivitesini artırırken, daha fazla hipoksi enzim aktivitesini önemli derecede azaltmıştır, fakat indirgenmiş glutatyon ve askorbik asitin içeriğini artırmıştır. SOD aktivitesindeki azalmanın, glutatyon redüktaz (GR) ve askorbat peroksidaz aktivitesindeki azalmadan daha büyük olduğu gözlenmiştir. Sonuçlarımız, süperoksitin (O_2^-) membran zararına ve lipit peroksidasyonuna sebep olduğu ve süperoksitin çok fazla birikiminin hipoksi altındaki SOD'un aktivitesinin azalmasından dolayı meydana geldiğini göstermiştir.

Anahtar Sözcükler: Antioksidant enzimler, askorbat, glutatyon, hipoksia, malondialdehit membran geçirgenliği, reaktif oksijen türleri, *Zea mays*

Introduction

In plants, a deficiency in oxygen leads to altered cellular metabolism and can dramatically reduce crop productivity (1). Photoreduction of molecular oxygen (O_2) in higher plants is an unavoidable process that induces O_2^- generation (2). Once generated, O_2^- will be rapidly dismutated to yield hydrogen peroxide (H_2O_2), which in turn reacts with O_2^- to form the highly reactive hydrogen radical ($\cdot OH$) that may attack and damage many molecules in the living cell within a short time (3). These reactive oxygen species (ROS) are all very reactive and cause severe damage to membranes, DNA, and proteins (4,5). There are some indications that waterlogging can induce oxidative stress, causing increased production of toxic oxygen species (6). The plant cells' defense against the damaging effects of oxidative injury involves both enzymatic (superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2)) and non-enzymatic components (ascorbic acid (AsA), reduced glutathione (GSH), phenolic compounds, and tocopherols) (7). Under normal conditions, the production and scavenging of these ROS are regulated well. However, when the accumulation of ROS under stressful conditions exceeds the removing capacity of the antioxidant system, the effects of oxidative damage arise. Changes in the lipid content and composition (8), increase in peroxidation of membrane lipids (9), and a decrease in membrane integrity (10) are symptoms of injury when plants are subjected to flooding. Waterlogging of rape plants *Brassica napus* at the seedling and stem stages resulted in a significant accumulation of leaf malondialdehyde (MDA) and ethylene production, and decreases in SOD and CAT activities (11). Vantoai and Bolles (12) have reported that the postanoxia damage associated with short duration of anoxia decreased SOD activity in soybean roots but was alleviated by AsA. Ushimaru et al. (13) reported reduced activities of CAT, APX, SOD, and GR in anoxia-tolerant rice *Oryza sativa* seedlings grown under water in comparison with aerobically germinated seedlings. While the effects of hypoxia on plant growth and metabolism as well as plant adaptation to hypoxia stress are well documented (14,15), data explaining the relationship between hypoxia and oxidative stress are still scarce. Moreover, not enough information is available about *Zea mays*. The aim of the present study was to investigate the influence of different

periods of hypoxia on activated oxygen (O_2^- and H_2O_2) production and their scavenging system, as well as factors related to stress injury such as levels of lipid peroxidation and membrane permeability in *Zea mays* leaves.

Materials and Methods

Plant material and treatment conditions

Maize (*Zea mays* L. cv. 704) seeds were obtained from the Agricultural Research Center of Iran, West Azarbayjan, Iran. Seeds were surface sterilized in 1% NaOCl (w/v) for 20 min, rinsed with distilled water, and imbibed for 12 h. After imbibition, the seeds were placed in petri plates containing moist filter paper in the dark at 27 °C in an incubator for 3 days. Then, 10 seedlings were sown in each plastic pot (12 cm in diameter) containing equal amounts (1 kg) of a homogeneous mixture of sand and clay soil (1:1 w/w). Seedlings were then left to grow in a growth chamber under normal day/night conditions (at 27/20 °C day/night, with a 12-h photoperiod, 300-350 $\mu mol.m^{-2}.s^{-1}$ photon flux density, and relative humidity of 60%). For hypoxia treatment, after 5 days of planting half the number of pots were subjected to excess moisture conditions (standing water up to 2 cm). Control plants remained well watered (60% soil moisture) during the period of the experiment. The upper leaves of treated and control plants were harvested 48, 96, 144 and 192 h after the start of the hypoxia treatment, frozen in liquid N_2 , and stored at -80 °C until analysis.

Determination of superoxide generation rate

O_2^- generation rate was estimated based on the method described by Schneider and Schlegel (16). Washed leaves (1 g) were homogenized in 3 ml of 65 mmol/l phosphate buffer (pH 7.8). The homogenate was centrifuged at 5000 $\times g$ for 10 min. The supernatant was used for measuring O_2^- production rate. Then 1 ml of the supernatant was mixed with 0.9 ml of phosphate buffer and 0.1 ml of hydroxylammonium chloride (10 mM). After keeping at 25 °C for 20 min, 1 ml of the above reaction mixture was added to 1 ml of 18 mM 4-aminobenzenesulphonic acid and 1 ml of 7 mM α -naphthylamine. The components were mixed and then separated into 2 layers using ether to eliminate the interference caused by the pigment. The absorption of the pink water-phase of the lower layer was measured at 530 nm. O_2^- content was calculated according to Bors et al. (17).

Determination of the hydrogen peroxide content

H₂O₂ was extracted from plant tissues as described by Patterson et al. (18). Fresh leaves (0.5 g) were frozen in liquid nitrogen and ground to powder using a mortar and pestle, together with 5 ml of 5% TCA and 0.15 g of activated charcoal. The mixture was centrifuged at 10,000 ×g for 20 min at 4 °C. The supernatant was adjusted to pH 8.4 with 17 M ammonia solution and then filtered. The filtrate was divided into aliquots of 1 ml. To one of these, the blank was added, 8 µg of catalase, and then kept at room temperature for 10 min. To both aliquots with and without catalase, 1 ml of colorimetric reagent was added. The reaction solution was incubated for 10 min at 30 °C. Absorbance at 505 nm was determined spectrophotometrically. The colorimetric reagent contained 10 mg of 4-aminoantipyrine, 10 mg of phenol, and 5 mg of peroxidase (150 U/mg), dissolved in 50 ml of 100 mM acetic acid buffer (pH 5.6) (19).

Determination of lipid peroxidation level

The level of lipid peroxidation was measured by the amount of malondialdehyde (MDA), a product of unsaturated fatty acid peroxidation. MDA content was determined using a 2-thiobarbituric acid reaction (20). Thus 0.5 g of fresh leaves was homogenized in 10 ml of 5% TCA and the homogenate was then centrifuged at 4000 ×g for 10 min. The supernatant was mixed with an equal volume of 2-thiobarbituric acid (0.5% in 20% TCA), and then boiled at 100 °C for 20 min followed by centrifugation at 7000 ×g for 5 min. The absorbance was read at both 532 nm and 600 nm. The amount of MDA was calculated with the extinction coefficient of 155 mmol/cm.

Determination of membrane permeability

Membrane permeability of a leaf was assayed by electrolyte conductivity (EC). The washed fresh leaves were cut into 1 cm pieces and placed in a beaker containing 10 ml of deionized water. The leaf samples were immersed at 30 °C for 3 h, and then the EC of the solution was determined. After boiling the samples for 2 min, their conductivity was measured again when the solution was cooled to room temperature. The percentage of EC was calculated as follows: $EC\% = C_1/C_2 \times 100$; C_1 and C_2 are the electrolyte conductivities determined before and after boiling, respectively (10).

Assay of antioxidant enzymes activities

Fresh leaves (0.5 g) were homogenized under ice-cold conditions in 10 ml of 50 mmol/l phosphate buffer (pH

7.0), 1.0% (w/v) polyvinylpyrrolidone (PVP), and 10 mmol/l of AsA. The homogenate was centrifuged at 15,000 ×g for 20 min. The supernatant was used for the enzyme assay.

SOD activity was assayed by recording the decrease in absorbance (560 nm) of superoxide-nitro blue tetrazolium complex by enzyme. Activity was taken as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzyme. The reaction mixture contained 75 mmol/l of sodium phosphate (pH 7.0), 45 mmol/l of H₂O₂, and 0.01 ml of enzyme extract (21).

APX activity was measured by recording spectrophotometrically the decrease in ascorbate content at 290 nm, 1 min after addition of H₂O₂. The assay was carried out at 25 °C in a reaction mixture containing 50 mmol/l of potassium phosphate (pH 7.0), 0.5 mmol/l of ascorbate (extinction coefficient 2.8 mmol/cm), 0.1 mmol/l of H₂O₂, and 0.05 ml of enzyme extract (22).

GR activity was assayed by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mmol/cm). The assay mixture contained 100 mmol/l of Tris-HCl (pH 7.8), 0.5 mmol/l of oxidized GSH (GSSG), 0.05 mmol/l of NADPH, 2 mmol/l of EDTA, and 0.01 ml of enzyme extract at 25 °C (23).

CAT activity was determined by monitoring H₂O₂ consumption (extinction coefficient 39.4 mmol/cm) at 240 nm in 3 ml of reaction mixture containing 50 mmol/l of phosphate buffer (pH 7.0), 15 mmol/l of H₂O₂, and 0.01 ml of enzyme extract (24).

Determination of GSH and AsA

Fresh leaves (0.5 g) were ground in 5 ml of 5% TCA, and then the homogenate was centrifuged at 16,000 ×g for 10 min. The supernatant was used for GSH and AsA assays. GSH concentration was measured using 5,5'-dithio-2,2'-dinitrobenzoic acid as a reagent (25). AsA content was assayed at 520 nm using the 2,4-dinitrophenylhydrazine method (26).

Statistical analysis

All experiments were performed with at least 4 replicates. One-way ANOVA was applied to determine the significance of results between different treatments and then Tukey's multiple range tests ($P < 0.05$) were performed. All the statistical analyses were performed using SAS v.7 for Windows.

Results

The effects of hypoxia on malondialdehyde amount and electrolyte conductivity

Hypoxia led to increases with time (hours) in MDA amount and EC (Figure 1). The changes in EC at the earlier stage of hypoxia (96 h) were slight, but were more pronounced after 96 h. By the 144 and 192 h EC increased to 133% and 148% of the controls, respectively. MDA content in control plants was not changed, whereas this parameter was significantly enhanced in treatments. The highest effect was found 192 h after hypoxia and MDA amount was about 368% higher than that in the control plants.

Induction of H_2O_2 and O_2^- generation by hypoxia

The alterations in H_2O_2 content and O_2^- generation rate are similar to the changes in EC and MDA after hypoxia (Figure 2). H_2O_2 and O_2^- levels in controls were enhanced slightly during the experiment, but, compared with the controls, hypoxia enhanced the generation of O_2^- and H_2O_2 , especially after 48 h of treatment. The increase

in O_2^- was higher than that in H_2O_2 , with increases of 201%, 298%, and 481% for O_2^- (Figure 2A) and 175%, 199%, and 201% for H_2O_2 (Figure 2B) at 96, 144, and 192 h, respectively. O_2^- generation rate was linearly correlated with EC ($R^2 = 0.9677$), MDA ($R^2 = 0.9425$), and H_2O_2 ($R^2 = 0.9306$) (Figure 3).

The effects of hypoxia on the activities of antioxidant enzymes

Figure 4 shows the time courses of antioxidant enzymes in leaves of *Zea mays* treated with or without hypoxia. In general, the activities of SOD, CAT, APX, and GR were characterized by the gradual reduction in the controls and treatments. However, hypoxia enhanced CAT activity after 48 h and the activities of the SOD, APX, and GR after 96 h of treatment compared with the control plants. A greater increase was observed in SOD (113%) after 96 h of hypoxia, but the activity of SOD after 144 and 192 h of hypoxia was insignificantly higher than that of the controls. CAT activity decreased significantly ($P < 0.05$) after 96, 144, and 192 h of hypoxia. The highest

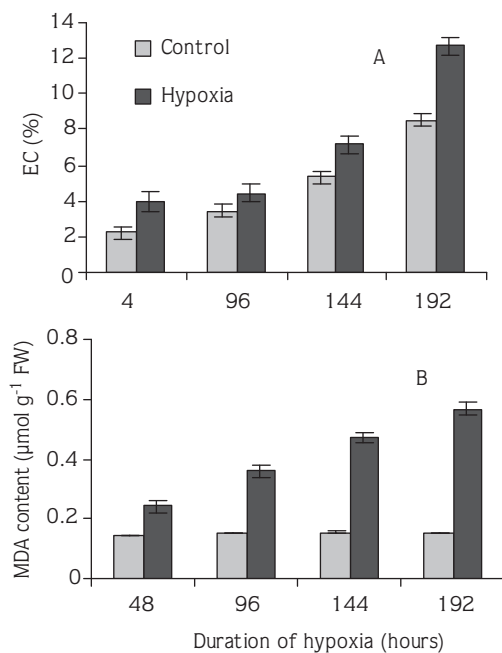


Figure 1. Changes in (A): relative electrolyte conductivity (EC) and (B): malondialdehyde (MDA) content in *Zea mays* leaves subjected to hypoxia. Eight-day-old seedlings were subjected to excess moisture conditions (standing water up to 2 cm). Samples were taken 48, 96, 144, and 192 h after the start of hypoxia. Means of 4 replicates with standard errors, $P < 0.05$.

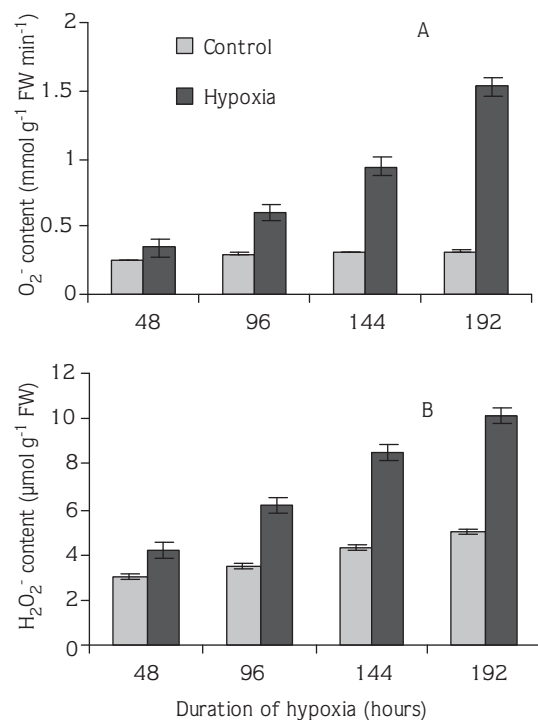


Figure 2. Effect of hypoxia on (A): superoxide (O_2^-) generation rate and (B): hydrogen peroxide (H_2O_2) content in *Zea mays* leaves (mean \pm SE, $n = 4$), $P < 0.05$.

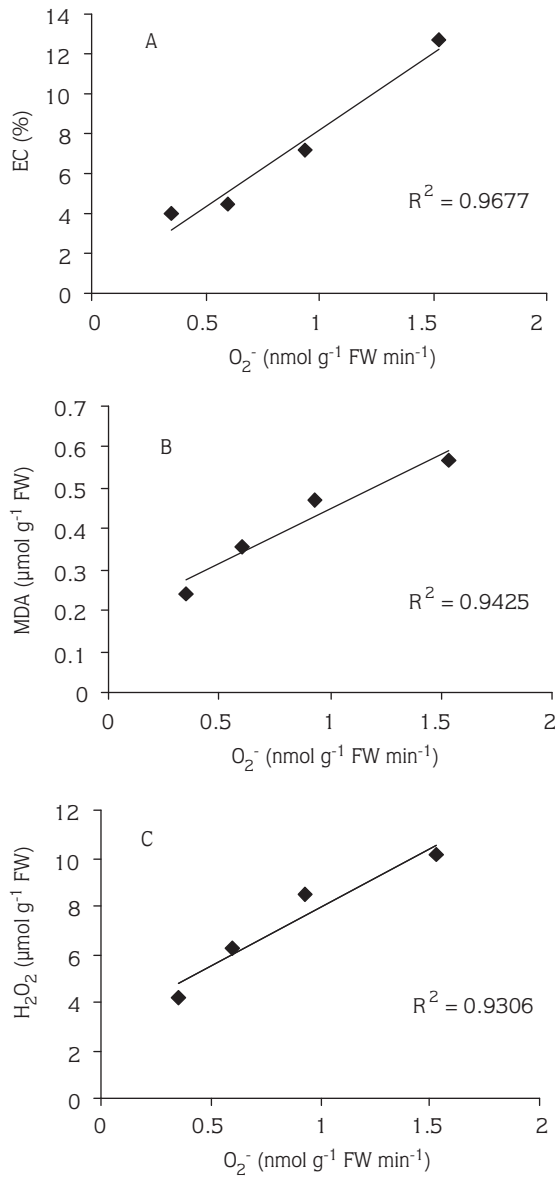


Figure 3. Effect of hypoxia on superoxide (O_2^-) generation rate with (A): relative electrolyte conductivity (EC), (B): malondialdehyde (MDA) content, and (C): hydrogen peroxide (H_2O_2) content in *Zea mays* leaves subjected to hypoxia.

effect was found 192 h after hypoxia: the activity was about 74% lower than that in the control plants (Figure 4B). The activity of APX decreased slightly after 144 h of hypoxia, but the reduction in APX activity reached the highest level (80%) 192 h after treatment (Figure 4C). Similarly, the decrease in GR activity was also lower than the control levels 192 h after hypoxia (87%) (Figure 4D).

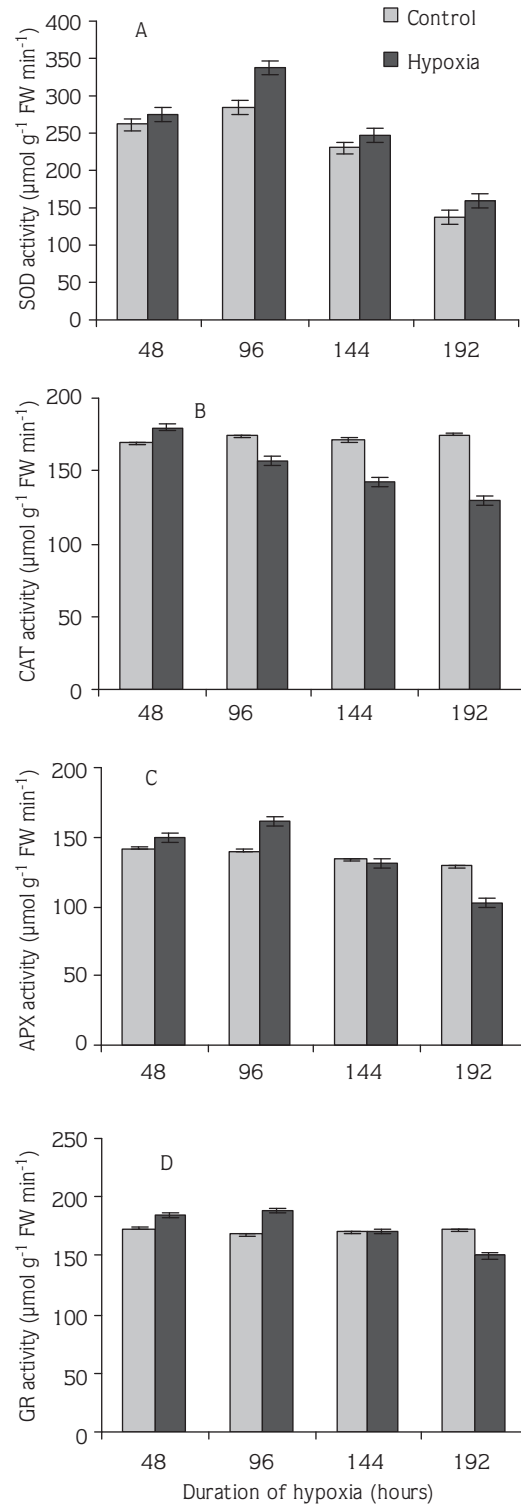


Figure 4. Effect of hypoxia on the activity of (A): superoxide dismutase (SOD), (B): catalase (CAT), (C): ascorbate peroxidase (APX), and (D): glutathione reductase (GR) in *Zea mays* leaves (mean \pm SE, $n = 4$, $P < 0.05$).

The effects of hypoxia on the non-enzymatic scavenging system

The pattern of alternations in AsA and GSH contents were different from those of the enzymatic scavenging system (Figure 5). The changes in AsA in treated plants were greater than those in control plants after 96, 144, and 192 h of hypoxia. Hypoxia lowered AsA content after 48 h of hypoxia. The highest increase (122%) was observed 192 h after hypoxia (Figure 5A). GSH level in treated leaves was higher than that in the controls during the entire duration of the experiment. The amount of GSH in plants under hypoxia conditions increased to 128% of the control plants after 192 h (Figure 5B).

Discussion

Hypoxia treatment of 192 h resulted in a significant increase in the level of lipid peroxidation (as indicated by MDA accumulation) and membrane permeability in *Zea mays* leaves (Figure 1). These results suggest that as they

mature leaves become more sensitive to hypoxia and that oxidative injuries may be associated with this sensitivity. Therefore, the present research indicates that the speed of hypoxia damage is consistent with that of MDA accumulation and membrane deterioration. For this reason, these parameters can serve as indexes for evaluating hypoxia damage in *Zea mays*. Enhanced EC is considered a criterion of membrane injury or deterioration (27). Peroxidation of unsaturated fatty acids in their membrane phospholipids may deteriorate the membrane (8). In the case of lipid peroxidation, hypoxia damage occurred at the cellular membrane. In general, as MDA had a greater percentage increase than EC, it appears likely that lipid peroxidation results in membrane damage and so could be one of the early causes of hypoxia injury (Figure 1). In agreement with our results, an increase in membrane permeability in barley plants (10) and activation of lipid peroxidation (9) have been reported. The increased contents of both H_2O_2 and O_2^- facilitate the metal catalyzed formation of high $\cdot OH$ via the Haber-Weiss cycle (28). $\cdot OH$ generally is held to be the most likely ROS to initiate lipid peroxidation with consequent membrane damage. The present research showed that the elevated level of O_2^- was much higher than that of H_2O_2 . A positive correlation between O_2^- concentration and EC, MDA, and H_2O_2 was observed (Figure 3). Hence, it seems reasonable to propose that hypoxia first induced the O_2^- increase, and then O_2^- in turn initiates lipid peroxidation through the formation of $\cdot OH$, which results in membrane injury in *Zea mays* leaves. Plant tissues generate O_2^- , which is removed by SOD. As SOD may control other ROS (H_2O_2 and $\cdot OH$), it is defined as a key antioxidant enzyme in the system (29). When O_2^- contents were increased, the activities of SOD and other antioxidant enzymes were also elevated during early hypoxia (Figure 4). Similar results have been reported under chilling (30), drought (31), and low temperature (32). One probable explanation is that substrate O_2^- induces SOD (33). It is possible that elevated contents of active oxygens stimulate the cellular protective mechanism to mitigate injury. Hence, early hypoxia did not have harmful effects on *Zea mays* but with the progress of hypoxia, particularly when significant hypoxia damage occurred (96 h), SOD activity decreased significantly and content of O_2^- was enhanced 1.2-fold. Increased O_2^- could be related to the inhibition of SOD activity. Restricted capacity of the scavenging system to eliminate excessive active oxygen formed during hypoxia

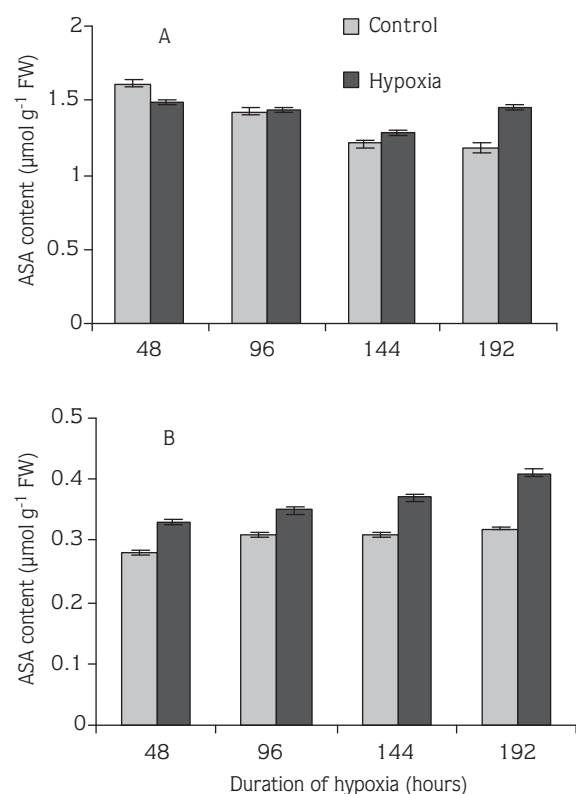


Figure 5. Changes in the contents of (A): ascorbic acid (AsA) and (B): reduced form glutathione (GSH) in *Zea mays* leaves subjected to hypoxia (mean \pm SE, $n = 4$), $P < 0.05$.

(especially decreased SOD activity in chloroplasts) together with decreased photosynthetic activity may be responsible for the damaging effect of this kind of stress (34). The activity of CAT decreased significantly after 96 h of hypoxia (Figure 4B). CAT, which is present in peroxisomes, acts in the removal of H_2O_2 , which is also produced outside the chloroplasts by H_2O_2 generating oxidases in the peroxisomes (35). In spite of its restricted localization, it may play an important role in detoxifying H_2O_2 , but H_2O_2 can readily diffuse across the membranes and enter the chloroplast (4). AsA-GSH-NADPH system catalyzed by GR and APX can remove H_2O_2 . Although SOD, APX, and GR, H_2O_2 scavengers, showed a similar reduction, it seems that the depletion of APX and GR activities was slower than SOD (Figure 4). Moreover, hypoxia could increase the level of non-enzymatic scavengers AsA and GSH. This indicated that leaves of treated plants maintain a certain level of H_2O_2 scavengers, which quench a part of H_2O_2 . Therefore, the elevated H_2O_2 in treated leaves is far less than O_2^- (Figure 2). Excessive accumulation of O_2^- caused by restricted SOD activity could be one of the reasons responsible for *Zea mays* hypoxia damage. In other words, the reduction in SOD activity occurred before the H_2O_2 scavenging system in chloroplasts was decomposed. It could be concluded that the generation and scavenging of ROS are interactive

and relatively stable (36). Plants are well adapted for minimizing radical damage using their natural defense mechanisms. Thus, the equilibrium between the formation and detoxification of ROS is critical to cell survival during hypoxia. At the early stage of hypoxia the removal capacity of ROS in *Zea mays* plants exceeds the oxyradical production rate and result in less damage to the plants. However, with prolonged hypoxia, the balance between free radical production and defense reactions was changed in favor of free radical generation. The reduction in the activities of antioxidative enzymes can be seen as a weakening in the scavenger mechanism of plants. Excessive production of ROS induces irreversible hypoxia damage in *Zea mays* plants.

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