

## RELATIONSHIP BETWEEN NITRIC OXIDE, ENZYMATIC ANTIOXIDANT SYSTEM AND ABA IN MAIZE UNDER LONG-TERM DROUGHT

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**ABSTRACT** - The relationship between nitric oxide (NO) and up-regulation of the antioxidant system ABA-mediated in maize was evaluated under long-term drought. Two maize genotypes with contrasting drought tolerance (BRS1010 – sensitive and DKB390 - tolerant) were exposed to two soil water levels, field capacity (FC) and water deficit (WD) at pre-flowering. After 12 days under these conditions, plants were irrigated and soil water level kept at FC until harvest. The plants BRS1010 under water deficit (WD) showed lower catalase activity and reduced levels of NO, whereas ABA and malondialdehyde (MDA) levels increased compared to plants under FC. Although, DKB390 plants under WD did not present changes on the activity of enzymatic antioxidant system and ABA compared to plants at FC, and cellular damage remain unaltered; this fact was associated with the increase in NO levels. As a result, DKB390 plants under WD showed harvest index values 50% higher than BRS1010 under the same condition.

**Key words:** DAF-2DA, catalase, mesophyll conductance, photoassimilate, harvest index.

## RELAÇÃO ENTRE ÓXIDO NÍTRICO, SISTEMA ANTIOXIDANTE ENZIMÁTICO E ABA EM MILHO SOB DÉFICIT HÍDRICO SEVERO

**RESUMO** – Avaliou-se a relação entre óxido nítrico (NO) e aumento na atividade de enzimas do sistema antioxidante induzido por ácido abscísico (ABA) em milho sob déficit hídrico severo. Para tanto, dois genótipos de milho contrastantes para tolerância à seca (BRS1010 - sensível e DKB390 - tolerante) foram cultivados sob irrigação e, ao atingirem o pré-florescimento, foram expostos a dois níveis de água no solo (CC - capacidade de campo e DH - déficit hídrico). Após 12 dias nessas condições, a irrigação foi restabelecida de modo a manter o nível de água no solo sob CC até a colheita. Plantas do BRS1010 sob DH tiveram a atividade da enzima catalase e os níveis de NO diminuídos, enquanto os níveis de ABA e de malonaldeído (MDA) foram aumentados em relação às suas respectivas plantas sob CC. Por sua vez, plantas do DKB390 sob DH não apresentaram variações na atividade de enzimas do sistema antioxidante, tampouco nos níveis de ABA em relação às suas respectivas plantas sob CC, e ainda assim os níveis de MDA não foram alterados; fato acoplado a aumentos nos níveis de NO. Consequentemente, sob DH, as plantas oriundas do DKB390 apresentaram valores 50% maiores de índice de colheita em relação às dos BRS1010.

**Palavras-chave:** DAF - 2DA, catalase, condutância do mesofilo, fotoassimilados, índice de colheita.

Maize (*Zea mays* L.) is a species that presents high water consumption, and stomatal closure is one of the first responses under water deficit (WD) condition, which limits the leaf gas exchange and, consequently, productivity (Souza et al., 2013). This fact occurs because reduction in soil water potential with the occurrence of WD induces the biosynthesis of signaling molecules in dehydrated roots, especially abscisic acid (ABA). This sign is emitted and transmitted to leaves where its accumulation prevents decrease in leaf water status via stomatal closure (Benesová et al., 2012), and thus induces the increase of the activity of enzymes of the antioxidant system (Souza et al. 2014).

Analysis of early-term drought in maize by Souza et al. (2014) showed that stomatal conductance to water vapor ( $g_s$ ) declined with the decrease of water status, which invariably coincided with the decrease in the photosynthetic rate ( $A$ ) and transpiration ( $E$ ). Furthermore, these authors proposed that  $A$  improvements in maize genotypes tolerant to WD occur, in a greater extent, by controlling the oxidative stress by increasing the activity of enzymes of the antioxidant system in leaves, being this process induced by the increase in ABA levels.

As drought progress, the levels of reactive oxygen species (ROS) potentially able to cause lipid peroxidation (Chugh et al., 2011) increases, impairing  $A$  and yield due to the decline in the enzyme activity of the antioxidant system and ABA levels (Souza et al. 2014). Several evidences suggest that nitric oxide (NO) is also an important signaling molecule involved in mechanisms of defense in response to long-term drought, by inducing ABA biosynthesis (Zhao et al., 2001; Xing et al., 2004; Zhang et al, 2011) and reducing damage at

membrane level and mesophyll cells (Jie et al, 2012; Zhuk & Musiyenko, 2012).

Recent studies have suggested that NO is also involved in enhancing the enzyme activity mediated by ABA antioxidant system such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Zhou et al., 2005; Shi et al., 2014). However, it has been observed the action of NO inhibiting the expression and activity of these enzymes (Clark et al., 2000; Murgia et al., 2004). Further evidences are required to find out the involvement of NO in the increasing of the activity of enzymes of the antioxidant system ABA-mediated. Apparently, increases in ABA levels when plants are exposed to long-term drought occur at the expense of basal levels depletion of NO in plant tissues (Lozano-Juste & Leon, 2010), or in the absence of other tolerance mechanism.

The objective of this study was to evaluate the relationship between NO and the up-regulation of enzymes of the antioxidant system ABA-mediated in maize under long-term drought.

## Material and Methods

### Plant material, cultivation conditions and experimental design

The experiment was carried out in a greenhouse at National Maize and Sorghum Research Center (19°28' S, 44°15'08' W, 732 m), and the plant material consisted of two maize genotypes, one tolerant (DKB 390) and one sensitive (BRS 1010). The choice of genotypes was based on previous results from a field experiment carried out by researchers from the breeding program of National Maize and Sorghum Research

Center, which has accumulated experience in maize phenotyping for drought tolerance.

Plants were grown in plastic pots containing 20 kg typical dystrophic Red Latosol. The water content in the soil was daily monitored between 09:00 a.m. and 03:00 p.m., with a moisture sensors model GB Reader N1535 (Measurement Engineering, Australia) installed in the center of each pot with the aid of a screw auger, to a depth of 20 cm. These sensors detect the water content in the soil based on electrical resistance and are coupled to digital meters. Water replacement by irrigation was based on the data obtained with the sensor and was added to reach FC during the period preceding the imposition of the treatments. The water replacement calculations were performed with the aid of a spreadsheet, based on soil water retention curve. The required cultivation and phytosanitary treatments were performed.

At the pre-flowering growth stage, half of each initial treatment was subjected to water deficit (WD); the other half continued to receive daily irrigation in order to maintain soil moisture close to field capacity (FC), with soil water tension of -18 kPa. WD occurred by daily providing 50% of the total available water until the soil water tension reaches at least -138 kPa. After twelve days under this condition, physiological evaluations were performed as mentioned below.

Gas exchange and the fluorescence of chlorophyll *a* were measured in leaves corresponding to the ear insertion with an infrared gas analyzer (IRGA - Infrared Gas Analyzer) Model 6400 LI (LI-COR, Lincoln, NE, USA) equipped with a fluorometer (LI 6400-40, LI-COR Inc.) (Souza et al., 2013). Samples of leaves were collected and stored in liquid nitrogen for determination of

antioxidant enzymes activity, levels of abscisic acid (ABA) and nitric oxide (NO), as well as cellular damage based on malondialdehyde (MDA) accumulation. Then, the water supply was restored and maintained at optimum levels by the end of the cycle, when agronomic parameters associated with productivity were measured. The experimental unit was the pot containing two plants, with six replications per treatment.

Preliminary statistical tests were used to adjust the results of the analysis of variance (ANOVA). The Scott-Knott test at 5% probability was used to test any contrast.

### Enzymatic assays

The activity of the enzymes of the antioxidant system, named dismutase superoxide (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11), was determined from the plant material extracted in a medium containing potassium phosphate buffer 0.1M (pH 6.8), 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1% PVPP (w/v). Total SOD activity was determined by measuring the ability to inhibit the photochemical reduction of *p*-nitro-blue-tetrazolium chloride at 560 nm. The activity of CAT was estimated by measuring the rate of decomposition of H<sub>2</sub>O<sub>2</sub> at 240 nm, while total APX activity was determined by monitoring the decline in absorbance at 290 nm. The levels of ABA quantification was performed using immunoenzymatic assay kits (Phytodetec ABA Enzyme Immunoassay Test Kit—Sigma-Aldrich). The MDA accumulation was estimated as the content of total 2-thiobarbituric acid-reactive substances. Additional details are described in Souza et al. (2014).

### Detection of •NO by confocal laser scanning microscopy (CLSM)

•NO was detected in lateral root tips with 10 M 4-aminomethyl 2,7 difluorofluorescein diacetate (DAF-2 DA, Sigma, St. Louis, MO, USA) prepared in 10 mM Tris-HCl (pH 7.4). The samples were examined with a confocal laser scanning microscope (Leica TCS SL, Leica Microsystems, Heidelberg GmbH, Wetzlar, Germany).

### Photosynthetic gas exchange measurements

The photosynthetic rate ( $A$ ), stomatal conductance to water vapor ( $g_s$ ), substomatal  $CO_2$  concentrations ( $C_i$ ) and transpiration rate ( $E$ ) were measured simultaneously with chlorophyll *a* fluorescence parameters from 10:00 a.m. to 1:00 p.m., when  $A$  is at its maximum, under artificial *PPFD* of  $1500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  at the leaf level,  $400 \text{ mol } CO_2 \text{ mol air}^{-1}$  and 21%  $O_2$ . During the measurements, the leaf-to-air vapor pressure deficit was ca 1.0 kPa and leaf temperature of 25°C. The equipment was programmed to make curves  $A/C_i$  varying sequentially  $CO_2$  partial pressure: 40, 30, 20, 10, 5, 40, 60, 80, 100, 120, 140 and 160 Pa. Estimations of  $g_m$  were performed using the combined gas exchange/fluorescence data (Harley et al., 1992).  $A-C_i$  curves were converted into  $A-C_c$  curves to estimate the maximum rate of carboxylation of Ribulose 1,5 bisphosphate (Rubisco,  $V_{cmax}$ ) and phosphoenolpyruvate (PEPc,  $V_{pmax}$ ), as well as the maximum rate of carboxylation limited by electron transport ( $J_{max}$ ) (Massad et al., 2007). Rates of ATP and NADPH consumption, as well as  $H^+$  requirement were estimated based on von Caemmerer (2000). Additionally, nitrogen (N) allocated in the

photosynthetic machinery was accessed as Niinemets et al. (1997).

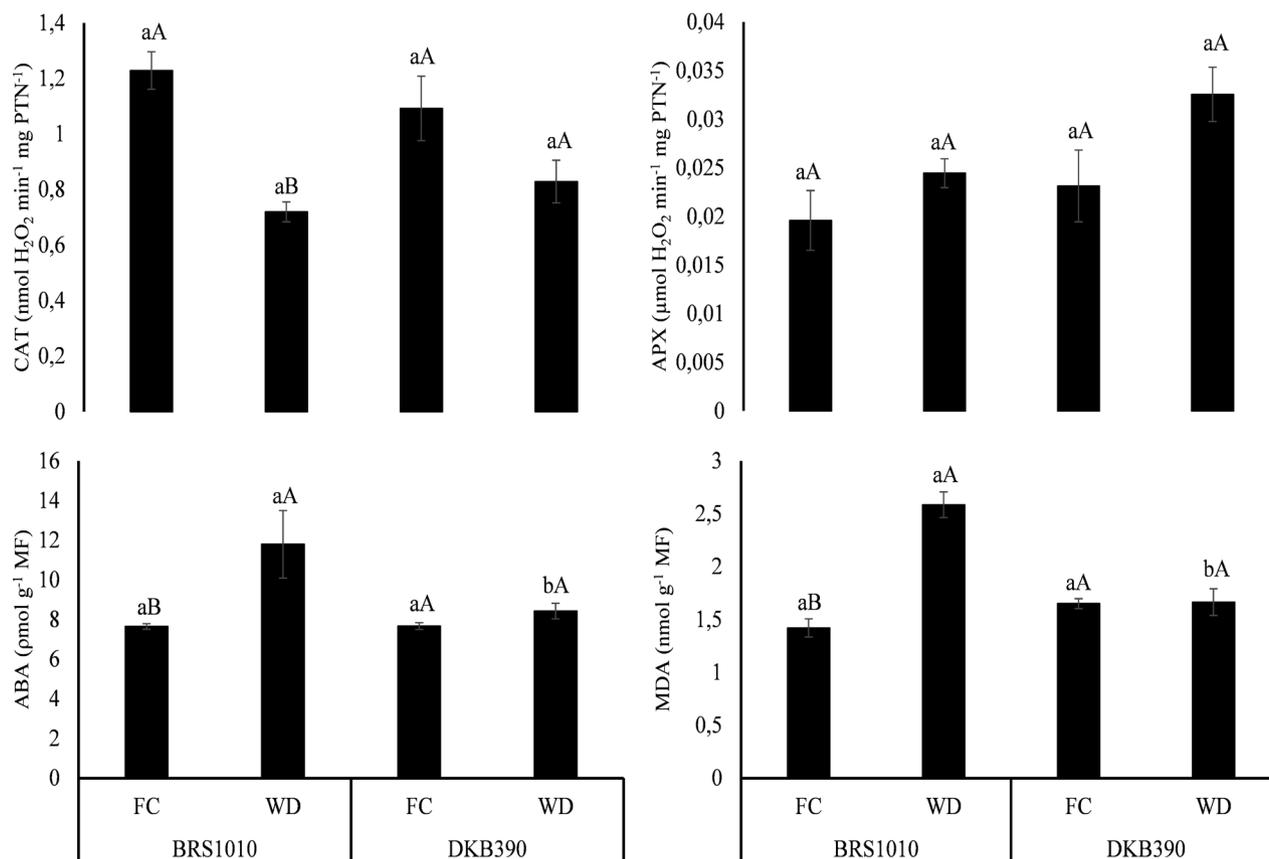
### Agronomic parameters

At harvesting, plants were partitioned into roots, stems, leaves, tassels, ears (cob, straw and grains), and dried in oven with forced air circulation at 70°C for 72 h. It was evaluated the harvest index, number of grains per ear and weight of 100 grains (Souza et al., 2013).

Additionally, a group of 50 grains was subjected to morphometric characterization, by measuring the dimensions of the grains (length, width and thickness) using a digital caliper rule, with three replications per treatment. Subsequently, these grains were soaked overnight in ethylenediamine (10%, w/v) and longitudinally cut by a knife to evaluate possible changes in embryo size, depending on the treatment. Photographs were obtained using a stereoscopic microscope and the *ImageJ* program was used to calculate the ratio between the areas of the endosperm and the embryo.

### Results and Discussion

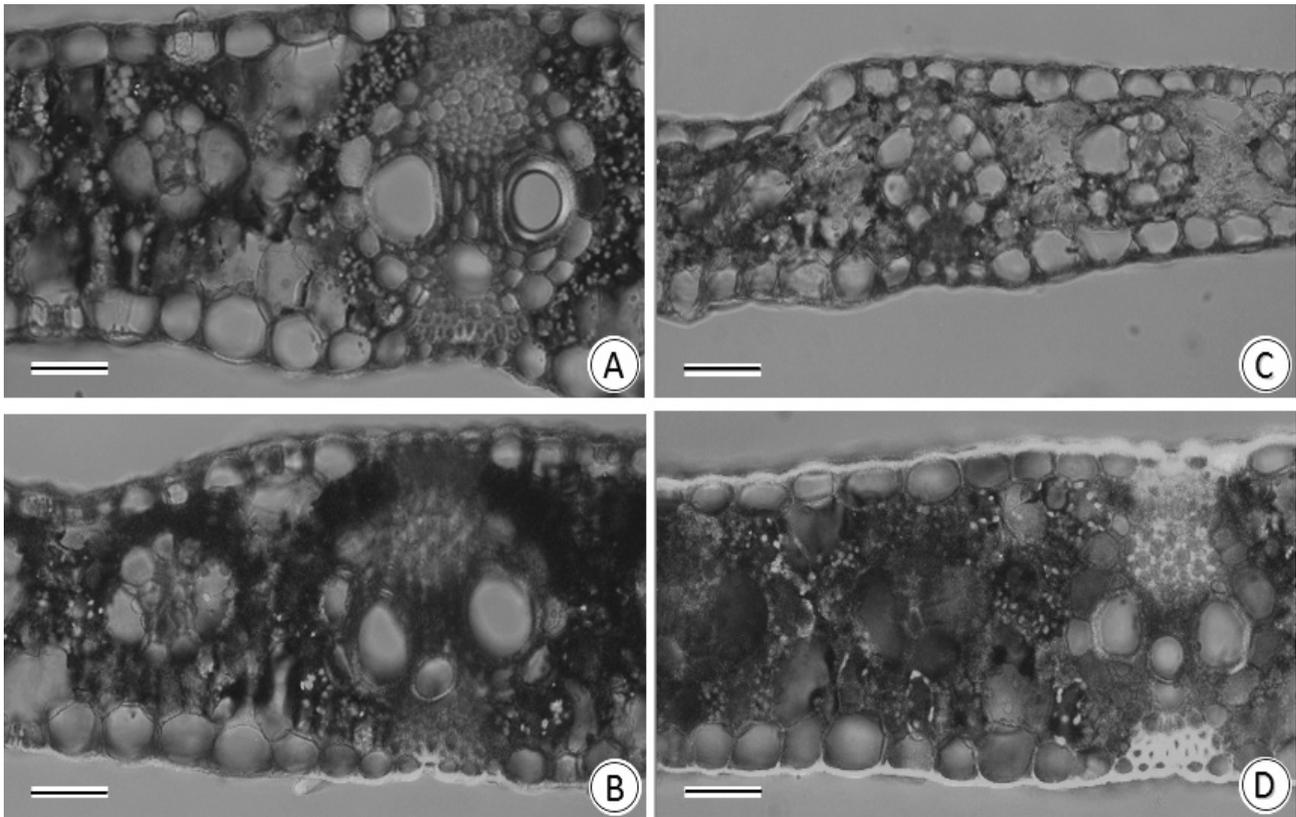
The activity of SOD was not significantly different between the genotypes and soil water levels (data not shown). With exposure to WD, APX activity also remained unchanged, while CAT decreased only in BRS1010 genotype; in this genotype the decrease in CAT activity was followed by significant increase in the levels of ABA and MDA (Figure 1). Plants DKB390 under WD had no significant changes in the activity of enzymes of the antioxidant system, either in ABA levels related to their respective plants in FC, and still MDA levels were not increased (Figure 1).



**FIGURE 1.** Activity of antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX), and levels of abscisic acid content (ABA) and malondialdehyde (MDA) in leaves of maize genotypes with contrasting drought tolerance (BRS1010-sensitive and DKB390-tolerant), cultivated under two different soil water levels (FC-field capacity and WD-water deficit). Means followed by the same letter are not statistically different from each other. Lowercase letters denote comparisons between genotypes within the same soil water level, and uppercase letters denote comparisons between the soil water levels within the same genotype. Means were compared by Scott-Knott test at 5% probability.

Through the analysis of cross-sections of the leaf with confocal microscopy to laser using a fluorescent probe sensitive to NO (DAF-2DA) it was possible to detect the presence of NO in xylem vessel elements, sclerenchyma and epidermal cells, despite the genotype and soil water level.

However, under WD the production of NO derived from green fluorescent labeling with DAF-2DA in BRS1010 genotype decreased. In turn, plants DKB390 under WD showed increased fluorescence of DAF-2DA in the leaves tissues when compared to plants under FC (Figure 2).



**FIGURE 2.** Confocal laser scanning microscope analysis for NO using probe DAF-2DA in leaves of maize genotypes with contrasting drought tolerance (BRS1010-sensitive and DKB390-tolerant), cultivated under two different soil water levels (FC-field capacity and WD-water deficit). A) BRS1010 under FC; B) DKB390 under FC; C) BRS1010 under WD; D) DKB390 under WD. Barr: 50  $\mu\text{m}$ .

Under FC, the values of  $A$ ,  $g_s$ ,  $V_{p,\max}$ ,  $V_c$ ,  $J_{\max}$  and  $J_{\max}$  were higher in DKB390 compared to BRS1010, while the values of  $C_i$  and  $C_c$  were smaller. The  $F_v/F_m$ ,  $E$ ,  $g_m$  and  $PR$  values did not change (Table 1).

Irrespective of genotype, there was a significant reduction of  $A$ ,  $g_s$ ,  $E$ ,  $F_v/F_m$ ,  $V_{p,\max}$ ,  $V_{c,\max}$ ,  $J_{\max}$  and  $A/E$  in plants exposed to the stress caused by WD compared to those who had the soil moisture

maintained close to FC, whereas  $C_i$ ,  $C_c$  and  $PR$  values increased. However, plants DKB390 under WD showed  $A$ ,  $g_s$ ,  $E$ ,  $g_m$ ,  $PR$ ,  $V_{p,\max}$ ,  $V_{c,\max}$ ,  $J_{\max}$  and  $A/E$  values higher than BRS1010 under the same condition (Table 1). Only plants BRS1010 showed very low  $A$  values under WD due to additional  $g_m$  limitation, which associated with a low  $E$  value resulted in extremely low efficiency in water use ( $A/E$ ) (Table 1).

**TABLE 1.** Leaf gas exchange obtained in situ and derived from A-Cc curves in leaves of maize genotypes with contrasting drought tolerance (BRS1010-sensisitive and DKB390-tolerant), cultivated under two different soil water levels (FC-field capacity and WD-water deficit). Each data were obtained from the leaf above the ear (n=6).<sup>1</sup>

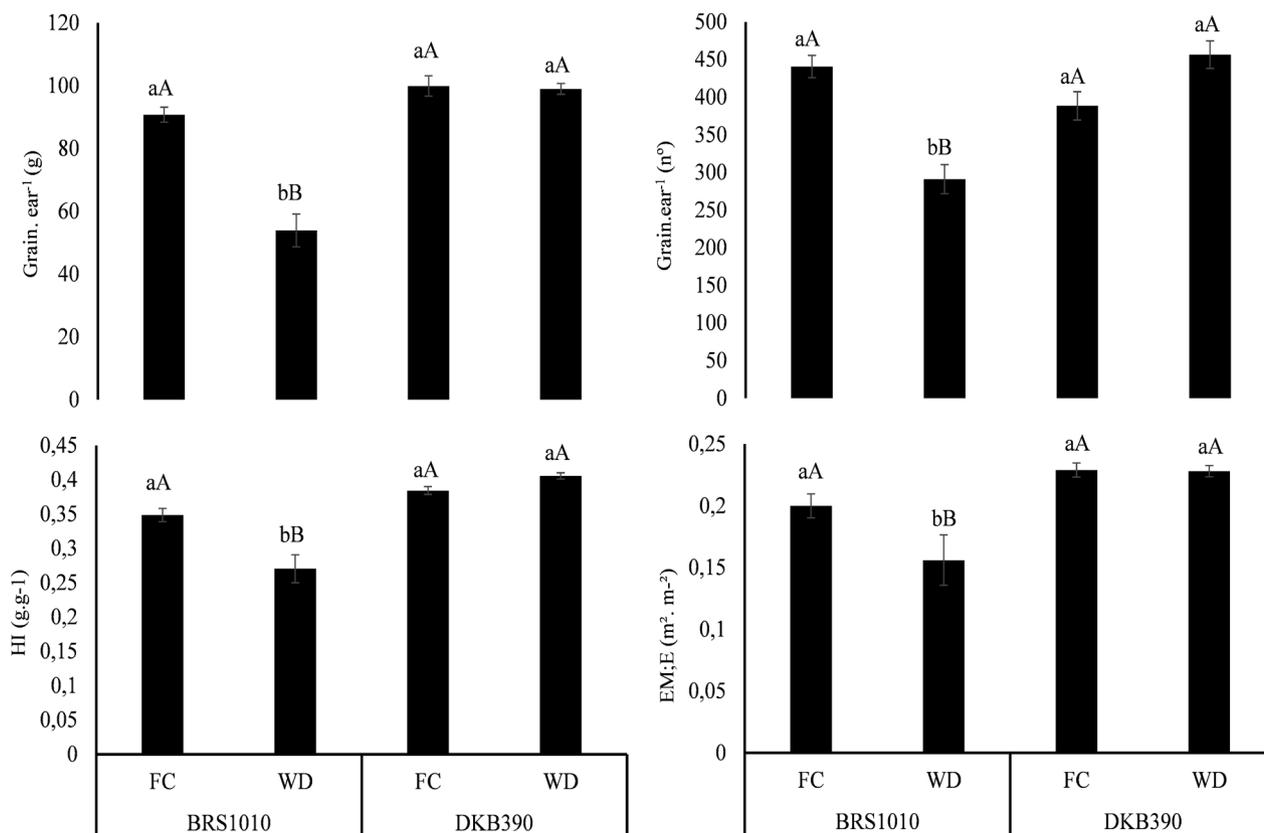
Parameter	BRS1010		DKB 390	
	FC	WD	FC	WD
A <sup>2</sup>	23,20 bA <sup>2</sup>	0,152 bB	27,72 aA	2,257 aB
F <sub>v</sub> /F <sub>m</sub>	0,803 aA	0,762 aB	0,800 aA	0,757 aB
g <sub>s</sub>	0,102 bA	0,007 bB	0,138 aA	0,023 aB
E	2,107 aA	0,088 bB	2,347 aA	0,499 aB
C <sub>i</sub>	75,27 aB	357,8 aA	43,38 bB	215,5 bA
g <sub>m</sub>	55,17 aA	0,830 bB	61,62 aA	83,79 aA
C <sub>c</sub>	63,67 aB	357,8 aA	29,52 bB	214,4 bA
PR	-1,91 aB	0,619 bA	-2,63 aB	1,122 aA
V <sub>pmax</sub>	38,26 bA	12,54 bB	42,60 aA	14,94 aB
V <sub>cmax</sub>	64,84 bA	50,66 bB	67,60 aA	52,03 aB
J <sub>max</sub>	231,8 bA	71,29 bB	260,0 aA	81,56 aB
A/E	11,99 aA	1,645 bB	11,81 aA	4,592 aB

<sup>1</sup>Abbreviations: photosynthetic rate ( $A$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ ), maximal efficiency of photosystem II ( $F_v/F_m$ ), stomatal conductance to water vapor ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2}\text{s}^{-1}$ ), transpiration rate ( $E$ ,  $\text{mol H}_2\text{O m}^{-2}\text{s}^{-1}$ ), internal  $\text{CO}_2$  concentration ( $C_i$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ ), mesophyll conductance ( $g_m$ ,  $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ),  $\text{CO}_2$  concentration in chloroplast ( $C_c$ ,  $\mu\text{mol mol}^{-1}$ ), photorespiratory rate ( $FR$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), maximum rate of carboxylation limited by 1,5 bisphosphateRubisco ( $V_{c,max}$ ,  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), maximum rate of carboxylation of phosphoenolpyruvatecarboxylase ( $V_{p,max}$ ,  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), maximum rate of carboxylation limited by electron transport ( $J_{max}$ ,  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), water use efficiency ( $A/E$ ).

<sup>2</sup>Means followed by the same letter are not statistically different from each other. Lowercase letters denote comparisons between genotypes within the same soil water level, and uppercase letters denote comparisons between the soil water levels within the same genotype. Means were compared by Scott-Knott test at 5% probability.

Under WD plants DKB390 showed, higher mass and number of grains per ear (Figure 3), compared to BRS1010, without alteration in total biomass (data not shown). Consequently, the HI in plants DKB390 under FC was 50% higher than

in BRS1010 (Figure 3). Only BRS1010 plants under WD presented declined EM:E values compared to FC (Figure 3), corroborating a lower photoassimilates flux in this maize genotype under WD.



**FIGURE 3.** Agronomic performance in maize genotypes with contrasting drought tolerance (BRS1010-sensitive and DKB390-tolerant), cultivated under two different soil water levels (FC-field capacity and WD-water deficit). Means followed by the same letter are not statistically different from each other. Lowercase letters denote comparisons between genotypes within the same soil water level, and uppercase letters denote comparisons between the soil water levels within the same genotype. Means were compared by Scott-Knott test at 5% probability.

The tolerance to oxidative stress associated with WD, is greatly due to the induction of increased SOD activity, which converts  $\bullet\text{O}_2$  into  $\text{H}_2\text{O}_2$ , and subsequent increase in the activity of other enzymes such as CAT and APX, removing the formed product; the ABA induction controls such increase (Bano et al, 2012; Souza et al, 2014; Cai et al, 2015). In this study, the level of oxidative damage associated with WD was evident only in the BRS1010 genotype. In previous studies,

the genotype DKB390 behaved like tolerant WD (Souza et al., 2014) and it was expected on plants of this genotype increases in ABA levels and activity of enzymes of the antioxidant system, and a smaller content of MDA, indicating an increase in the antioxidant capacity and little damage at the membrane level. Surprisingly, plants DKB390 under WD showed no significant peroxidative damage in the membrane, without the need for adjustments in the activity of antioxidant enzymes ABA-mediated.

Souza et al. (2014) observed that the activity of antioxidant enzymes is high during the first days under WD, but after ten days under such stressful condition the activity of antioxidant enzymes decreases, confirming the results of this study in which the plants were exposed to WD for 12 days. Possibly, not only the CAT enzyme but also the ABA have been disabled due to long exposure to WD, and other mechanisms of tolerance to WD, except the increase in enzyme activity of the antioxidant system mediated by ABA, were activated to control oxidative processes only in plants DKB390 under WD. Only plants BRS1010 under WD presented increased ABA levels compared to the respective control under FC. However, due to inactivation of the enzyme antioxidant defense system and other protection mechanisms against oxidative damage, MDA levels increased greatly. In fact, the increase in ABA levels occurs only if there is a depletion of basal levels of NO (Lozano-Juste and Leon, 2010).

The role of NO in oxidative stress tolerance mechanism under WD has not been elucidated (Xing et al., 2004; Zhang et al, 2011). Several authors have shown that both NO and ROS induce ABA biosynthesis during WD (Zhao et al., 2001; Xing et al, 2004), but the signaling pathway for NO is different from that widely accepted for ROS (Xing et al., 2004). Recently, Jie et al. (2012) showed that increased NO level reduces damage to the membrane by decreasing  $E$  in leaves of rice plants under WD. Notably, the plants BRS1010 under WD closed stomata to preventing loss of latent heat via  $E$ , and did not invest in the production of NO, causing an increase in leaf temperature followed by damage to the photochemical step of photosynthesis. With photochemical damage, the availability of NADPH and ATP for use by carbon reduction and phosphorylation enzymes at biochemical step were affected, thereby explaining the increase in  $C_i$  and

the very low  $A$  values compared to plants of that genotype under FC. The low  $A$  values, associated with also lower  $E$  values resulted in lower  $A/E$ . In addition, only BRS1010 plants under WD showed  $g_m$  limitation.

According to Souza et al. (2013), the maintenance of a high yield in drought tolerant genotype under WD is related to increase in  $E$  which, in turn, occurs at the expense of higher water consumption. By releasing more water via stomatal opening, there would be a leaf cooling, which would prevent photochemical damage. Plants DKB390 under WD also limited  $E$  and  $g_s$  showing photochemical damage; however,  $g_m$  was not limited suggesting that  $g_m$  offset reductions in  $g_s$  in plants DKB390 under WD. Hommel et al. (2014) showed the importance of  $g_m$  in the optimization of resources in water restriction periods, and Zhuk & Musiyenko (2012) proposed that NO reduces the destructive effects of oxidative stress in the mesophyll cells in wheat plants under WD, although the role of  $g_m$  has not been considered.

In previous studies conducted by the research group, it was observed that the N invested in the photosynthetic apparatus for carboxylation enzymes, electron transport chain and the complex collector of light in the photosystem was reduced in plants of BRS1010 and DKB390 under WD. Only plants of BRS1010 genotype under WD had the  $g_m$  limited by low investment in N. Perhaps the DKB390 under DH only decreased the  $g_s$  by the need to limit  $E$  and prevent embolism in the xylem which, in turn, favored the N deviation from enzymes invested in the photosynthetic apparatus not only for the construction of aquaporins, and other proteins that contribute to  $g_m$ , but also for costs associated with its maintaining (Cowan, 1986).

The increase in gene expression and abundance of aquaporins in bean leaves under WD are correlated with the decline in  $E$  due to stomatal closure induced by ABA and increased  $g_m$  (Aroca et al., 2006). Parent et al. (2009) suggested that ABA affects hydraulic properties of plants by increasing the abundance of aquaporins, which contribute to the maintenance of leaf water status. Some aquaporins, however, are little affected by ABA (Xu et al., 2013), and NO induces increases in the expression of genes encoding aquaporins (Liu et al., 2007; Di Pietro et al., 2013). It is suggested therefore that NO induces the expression of genes encoding aquaporin in DKB390 under WD which, in turn, regulates  $g_m$  to avoid damage at the membrane level, at least when the antioxidant enzyme system defense mediated by ABA is disabled. Analysis of agronomic characters confirmed tolerance and sensitivity, respectively, of DKB390 and BRS1010 under WD.

### Conclusion

The tolerance to drought in DKB390 genotype is associated with increased levels of NO, offsetting the loss in enzyme activity of the antioxidant system mediated by ABA.

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