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# Putrescine priming effects on chlorophyll fluorescence, antioxidant enzyme activity, and primary metabolite accumulation in maize seedlings under water deficit

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## Abstract

This study aimed to evaluate the effect of putrescine priming on the initial growth, chlorophyll fluorescence, primary metabolites accumulation, and antioxidant enzyme activities in two maize hybrids with contrasting drought tolerances. Seeds of *Zea mays* L. hybrids DKB 390 (drought tolerant) and BRS 1030 (drought sensitive) were primed with putrescine (10 or 100 µM). Paper rolls moistened with distilled water or mannitol (-0.6 MPa) were maintened at 30°C for 7 d. The growth parameters were higher in the DKB hybrid than in the BRS hybrid. Putrescine priming (10 µM) promoted the root growth of BRS at levels similar to those of DKB and improved photochemical and non-photochemical quenching and maximum quantum efficiency of BRS seedlings. Higher levels of reducing sugars were found in DKB seedlings when compared to BRS in both roots and leaves, especially with 100 µM putrescine. Total soluble sugar and starch were lower in the maize roots under water deficit and with 10 µM putrescine for both hybrids. BRS seedlings showed higher starch content in the leaves in the control and 10 µM putrescine treatments. Superoxide dismutase was activated in BRS plants by the priming, especially in the roots, but this effect was not observed for catalase, ascorbate, or guaiacol peroxidase, although the DKB seedlings presented much higher guaiacol peroxidade activity than BRS seedlings in both the roots and shoots. In conclusion, putrescine priming (10 M) improved the morphological and biochemical responses of the drought sensitive maize hybrid BRS.

*Keywords*: antioxidant enzymes, chlorophyll fluorescence, photochemical quenching, polyamines, sugars.

## Introduction

Plants can be exposed to various environmental stresses that affect their growth and development. Water deficit resulting from drought is the abiotic stress that most reduces crop productivity (Islam *et al*. 2022a). These adverse conditions affect cereals, such as maize, from germination and seedling performance to grain filling, thereby negatively affecting agricultural sustainability. In addition, during sowing, water scarcity causes non-

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*Abbreviations*: ABA - abscisic acid; APX - ascorbate peroxidase; BOD - biochemical oxygen demand chamber; CAT - catalase; DNS - 3,5-dinitrosalicylic acid; EDTA - ethylenediaminetetraacetic acid; $F_0$' - light-adapted fluorescence; $F_m$ - maximum fluorescence; $F_m$' - maximum fluorescence emitted by the leaves; $F_s$ - constant fluorescence; GPX - guaiacol peroxidase; $H_2O_2$ - hydrogen peroxide; MCW - methanol:chloroform:water solution; NBT - nitroblue tetrazolium; NPQ - non-photochemical quenching; PMSF - phenylmethylsulfonyl fluoride; PS II - photosystem II; PUT - putrescine; PVP - polyvinylpyrrolidone; $q_p$ - photochemical quenching; SOD - superoxide dismutase; YII - effective photochemical quantum yield of PS II; ε - molar absorption coefficient.
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uniform germination, compromising the final stand of the area and generating losses and economic damage to farmers.

Plant responses to water stress are diverse and interconnected. Several reports have indicated reduced growth and changes in photosynthetic processes due to the loss of photochemical efficiency and damage to photosystem II (Xin *et al.* 2018, Zhang *et al.* 2018, AbdElgawad *et al.* 2020). These deleterious effects in the oxygen-evolving complex and reaction center of photosystem II lead to the accumulation of reactive oxygen species (ROS), which can cause photoinhibition and degradation of macromolecules, resulting in plant damage and decreased production (Huang *et al.* 2019). These molecules have a wide range of effects on plants that can be detrimental depending on their concentrations.

Seed priming is a controlled hydration technique that introduces molecules that initiate metabolic processes in germinating seeds, thus reconciling and standardizing the germination period, and providing an effective short-term treatment (Farooq *et al.* 2009, Voko *et al.* 2022). Studies conducted in the last decade indicate that priming is an efficient approach to stimulate cellular defense responses to biotic and abiotic stresses (Junges *et al.* 2013, Alcântara *et al.* 2015, Pallaoro *et al.* 2016).

In recent years, new methods that do not harm the environment are required to improve the performance of plants under adverse environmental conditions. Biostimulants are organic compounds and/or micro-organisms that can regulate plant growth behavior through molecular and physiological changes, modulations in metabolism and plant anatomy (Bhupenchandra *et al.* 2022, Meddich 2023). Sustainable biological practices, such as biostimulants that increase plant yield, quality, or tolerance to abiotic stresses should be explored to improve plant responses. The use of biostimulants has become a promising tool in the current climate change scenario. It is possible to use exogenous growth regulators, such as polyamines, as biostimulants in priming. Polyamines are small polycationic molecules essential for the growth and survival of all organisms. Putrescine (Put), spermidine, and spermine are the most abundant polyamines in plants. They are involved in various growth and developmental processes, including cell division stimulation, environmental stress responses, rhizogenesis regulation, embryogenesis, floral development, and senescence (Evans and Malmberg 1989, Kakkar and Sawhney 2002, Kusano *et al.* 2008). Put may play an essential role in plant growth and development by acting as a signaling molecule in cell proliferation and differentiation, or by regulating the auxin/cytokinin ratio (González-Hernández *et al.* 2022). Furthermore, Put can play a crucial role in rooting by increasing the quantity and quality of roots as described by Badawy *et al.* (2015).

Thus, great interest has arisen in application of Put in the form of priming, with the ultimate goal of making plants more tolerant to water deficit in the early stages of maize growth. In addition, Put priming is expected to significantly improve the growth of maize seedlings through an increase in biomass and morphophysiological and biochemical responses.

Thus, we evaluated the effect of priming with Put on the seedling growth of two maize hybrids with contrasting drought tolerances. The purposes of this study were to: *1*) investigate whether Put priming has a positive or negative effect on seed germination, seedling growth, and chlorophyll fluorescence under water stress in the two hybrids and *2*) determine whether Put priming has an explicit impact on primary metabolite accumulation and antioxidant enzymes activity.

## Materials and methods

**Plants and germination assay:** Two maize (*Zea mays* L.) hybrids with contrasting drought tolerance were obtained: DKB 390 (tolerant) from Dekalb® and BRS 1030 (sensitive) from the Embrapa Breeding Program, Sete Lagoas, Minas Gerais (Souza *et al.* 2013, 2016). The seeds were soaked at room temperature for 20 h in 10 or 100 µM putrescine solutions for priming, and distilled water was used as a control. The experimental design was completely randomized, with five replications for each hybrid, total 125 seeds per treatment. Twenty hours was previously defined as the limit for priming, since after this period the seeds begin germinating. Subsequently, for the germination assay, 25 seeds were placed in rolls composed of three sheets of *Germitest*® paper moistened with distilled water or -0.6 MPa mannitol solution in a corresponding volume of 2.5 times the mass of the paper roll, according to the standardized methodology of Brazilian rules of seeds analyses (Brasil 2009). The paper rolls containing the seeds were placed in beakers closed with plastic bags to avoid water evaporation and kept in a bio-oxygen demand (BOD) chamber at a temperature of 30°C and a 12-h photoperiod wih an irradiance of 50 µmol $m^{-2}$ $s^{-1}$. Germination was monitored at 12-h intervals for 7 d and the seedlings were collected at the end of this period. Plant height, root length, and chlorophyll fluorescence were immediately measured and the seedlings were stored at -80°C for further analyses. For biometric and chlorophyll fluorescence analyses, 25 seedlings per treatment were used for each replicate. For the sugar content and antioxidant enzyme activity 10 seedlings were used for each replication.

**Chlorophyll fluorescence:** A *Mini-PAM* modulated fluorimeter (*Heinz Walz*, Effeltrich, Germany) was used to measure chlorophyll fluorescence parameters. The leaves were kept in the dark for 30 min, after which minimum fluorescence ($F_0$) was measured at a sufficiently low irradiance to avoid photochemical reactions. The maximum fluorescence ($F_m$) was determined using a saturating light pulse of 7 000 mol(photons) $m^{-2}$ $s^{-1}$ for 0.8 s. The leaves were then treated with actinic light at 1 500 µmol(photons) $m^{-2}$ $s^{-1}$. Subsequently, constant fluorescence ($F_s$) was determined, and another pulse of saturating light was applied for 1 s to obtain the maximum fluorescence emitted by the leaves ($F_m'$). The actinic light was

removed, and the leaves were irradiated with distant red light to obtain light-adapted $F_0$ ($F_0'$). The maximum photosystem II (PS II) efficiency was estimated using the $F_v/F_m$ ratio. Photochemical quenching was calculated as $q_p = (F_m' - F_s)/(F_m' - F_0')$, and non-photochemical quenching was calculated as NPQ = $(F_m - F_m')/F_m'$. The effective photochemical quantum yield of PS II was also evaluated as YII = $F_m' - F_s/F_m' = \Delta F/F_m'$ (van Kooten and Snel 1990).

**Extraction and analysis of sugars and starch:** Shoot and root samples (200 mg) were ground in 2 mL of methanol:chloroform:water (MCW, 12:5:3 v:v:v) solution and incubated at room temperature for 24 h. Subsequently, the samples were centrifuged for 30 min at 1 500 × *g*, and the supernatant was mixed with chloroform and water (4:1:1.5 v:v:v). The aqueous phase was collected after 24 h and used for the sugar analysis. Starch was extracted from the pellet after centrifugation by incubation with 30% perchloric acid and analyzed with the anthrone reagent, as described below.

Total soluble sugars and starch were determined colorimetrically after reaction with anthrone (Yemm and Willis 1954). Briefly, the samples were mixed with water to a final volume of 1 mL and then mixed with 2 mL of anthrone reagent (20 mg anthrone, 500 µL water, and 10 mL concentrated $H_2SO_4$). The samples were shaken and incubated at 100°C for 5 min. The absorbance was determined at 620 nm and quantified using a glucose standard curve.

Reducing sugar content was determined using 3,5-dinitrosalicylic acid (DNS). The samples were mixed with water (final volume of 1.1 mL) and 1 mL of the DNS reagent. The mixture was shaken and incubated at 100°C for 5 min. Absorbance was determined at 540 nm and quantified using a glucose standard curve (Miller 1959).

**Antioxidant enzyme activity determinations:** Shoots and root samples (300 mg) were homogenized in four volumes of 50 mM phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 1 200 × *g* for 30 min and the supernatant was used to determine the enzymatic activity. Protein content was determined using the Bradford method (Bradford 1976) using bovine serum albumin as the standard and enzymatic activity was determined according to García-Limones *et al.* (2002).

Superoxide dismutase (SOD) activity was determined using the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture was formed by 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 2 M riboflavin, and different volumes of the enzyme extract. The reaction was initiated by adding riboflavin and the absorbance at 560 nm was measured after 12 min of incubation at room temperature under continuous light.

Guaiacol peroxidase (GPX) activity was determined using the increase in absorbance at 470 nm caused by the oxidation of guaiacol (ε = 26.6 $mM^{-1}$ $cm^{-1}$). The reaction mixture consisted of 100 mM phosphate buffer (pH 6.5), 15 mM guaiacol, 0.05% (v/v) $H_2O_2$, and the enzyme extract.

Catalase activity (CAT) was determined by measuring the decrease in absorbance at 240 nm. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM $H_2O_2$, and different volumes of enzyme extract. The reaction was initiated by the addition of $H_2O_2$ (ε = 36 $mM^{-1}$ $cm^{-1}$).

Ascorbate peroxidase (APX) activity was evaluated by the oxidation of ascorbate (ε = 2.8 $mM^{-1}$ $cm^{-1}$) measured at 290 nm. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.25 mM sodium ascorbate, 5 mM $H_2O_2$, and different volumes of the enzyme extract. The reaction was initiated by the addition of $H_2O_2$.

**Statistical analysis:** The experimental design was completely randomized, with five replicates of 25 seeds per treatment. The data obtained were subjected to analysis of variance (*ANOVA*), and the means were compared using *Tukey*'s test at 5% significance using *Sisvar* software, *version 5.6* (Federal University of Lavras, Lavras, MG, Brazil).

## Results and discussion

The growth parameters differed between the two hybrids (Fig. 1). Surprisingly, the fresh mass was higher in the drought-sensitive hybrid BRS 1030, but the root and shoot lengths were shorter than those of the drought-tolerant hybrid DKB. As pointed out by Kränzlein *et al.* (2022) plants have different response mechanisms in relation to the decline of water in the soil and can be categorized as isohydric or anisohydric. Isohydric plants maintain water potential while anisohydric plants are characterized by large fluctuations in leaf water potential. It seems to be the case of BRS 1030 and DKB 390 respectively. However, the performance of plants with different water regulation modes depends on the intensity and duration of water deficit. However, germination percentage did not differ between the treatments. Additionally, only BRS was affected by the mannitol treatment in biomass and shoot and root length. Put priming at 10 µM in the presence of mannitol at -0.6 MPa promoted root growth to be similar to that of DKB plants (Fig. 1*C*). Although -0.6 MPa does not cause excessive osmotic stress to DKB plants, this value of osmotic pressure was chosen based on previous experiments when BRS plants were extremely affected by higher values. Xin *et al.* (2018) reported a wide range of physiological and biochemical changes in maize seedling exposed to -0.8 MPa. In our case, the shoot length of BRS plants was negatively affected at -0.6 MPa, and the leaves withered excessively. However, in the presence of 10 µM Put, the shoot length was recovered (Fig. 1*D*). Putrescine plays several roles, including scavenging of reactive oxygen species, osmotic balance adjusting, and increased cell division (Tyagi *et al.* 2023), which results in shoot growth. However, the difference in results between the concentrations may be related to several factors, such as the species under study, conditions of the experiments,

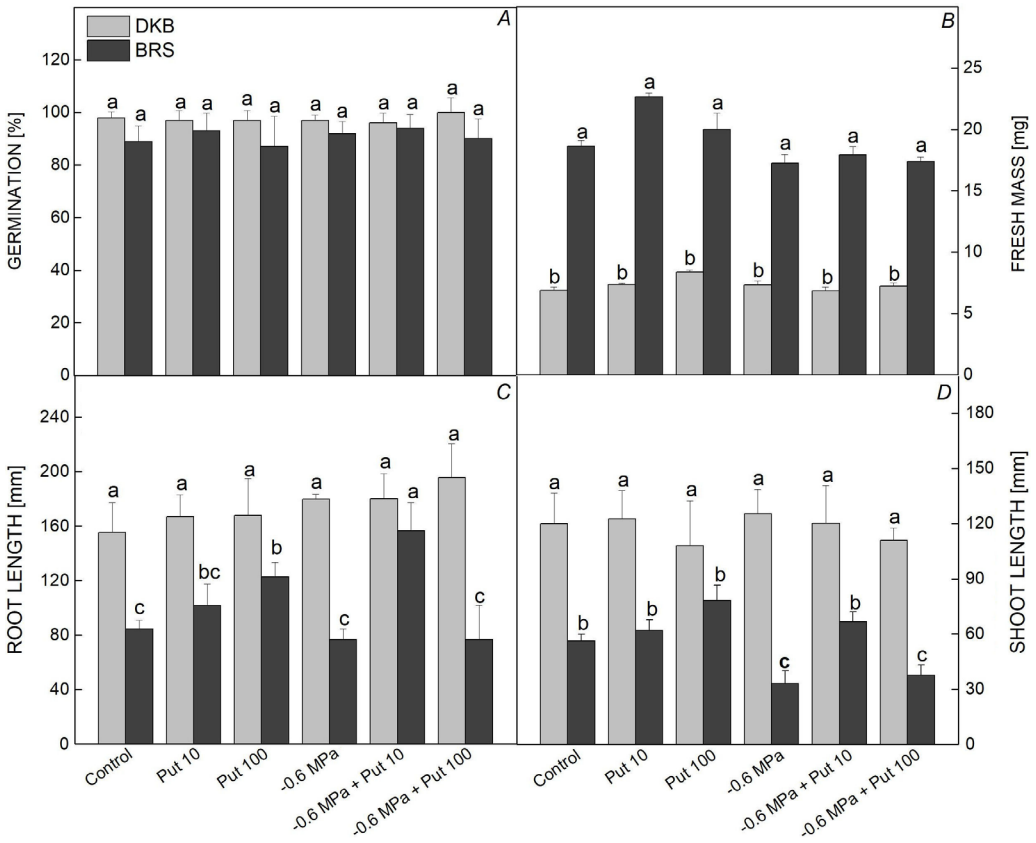


Fig. 1. Effect of water deficit and putrescine priming on growth parameters of maize hybrids with contrasting drought tolerances. Means ± SDs, $n = 25$, *different letters* indicate statistical differences by the *Tukey*'s test at 5% significance level.

intensity of the water deficit, among others. Hussein *et al*. (2023) found positive effects on the growth of wheat plants under water deficit with 1 mM Put. Doneva *et al.* (2021) also found better growth of the wheat shoots under water deficit after priming in 0.5 mM Put. These results show that putrescine can be effective over a wide range of concentrations. Furthermore, in the case of this work, it is observed that the concentration of 100 µM was less effective than 10 µM but did not cause deleterious effects.

Regarding chlorophyll fluorescence measurements, as expected, DKB plants were only slightly affected by treatments. In contrast, there was a considerable effect on BRS plants (Fig. 2). The effective photochemical quantum yield (YII) of DKB plants remained unchanged in all treatments, but the BRS plants showed a decrease in the presence of water deficit (-0.6 MPa). However, when primed with 10 µM of putrescine, the YII of the BRS samples recovered (Fig. 2*A*). Similarly, photochemical ($q_p$) and non-photochemical (NPQ) quenching as well as the maximum quantum efficiency ($F_v/F_m$) of BRS were restored to the same levels as those of DKB when primed with putrescine at 10 µM (Fig. 2*B-D*). During the light reaction, Put accumulates in the thylakoid lumen, acting as a permeable buffer and osmolyte and minimizing the possibility of chloroplast damage, chlorophyll degradation, and photoinhibition in plants under oxidative stress (Islam *et al.* 2022a). As reviewed by Lopes *et al.* (2011), many changes have been observed in the photosynthetic apparatus of $C_4$ plants in response to drought stress. Many strategies have been used to ensure the proper functioning of the photosynthetic apparatus to maintain the water use efficiency of these plants. The NPQ of DKB plants increased when they were subjected to water deficit (Fig. 2*B*). To corroborate these results, polyamines have been demonstrated to increase the fluorescence quenching of isolated LHC II from green algae, while *in vivo*, spermine and spermidine induced NPQ in higher plants under low light conditions (Ioannidis and Kotzabasis 2007, Ioannidis *et al.* 2011). NPQ is an important process used by plants to dissipate excess of absorbed light energy and protect the photosynthetic apparatus from damage caused by abiotic stresses (Li *et al.* 2018). These authors also verified that NPQ increased in both sensitive and tolerant maize plants when subjected to drought stress and that this effect was potentiated by spermidine in a concentration-dependent manner. In the present study, the NPQ of the BRS plants did not increase in the presence of drought stress without Put priming. Interestingly, photochemical quenching ($q_p$) in stressed BRS plants primed with 10 µM Put was higher than that in DKB seedlings under the same conditions (Fig. 2*C*), which may be explained by the lower capacity of BRS to cope with damages caused by water deficit, as $q_p$ is a non-regulated process (van Amerongen and Chmeliov 2020). In ginseng seedlings under salt stress, the action of Put improves chlorophyll fluorescence parameters,

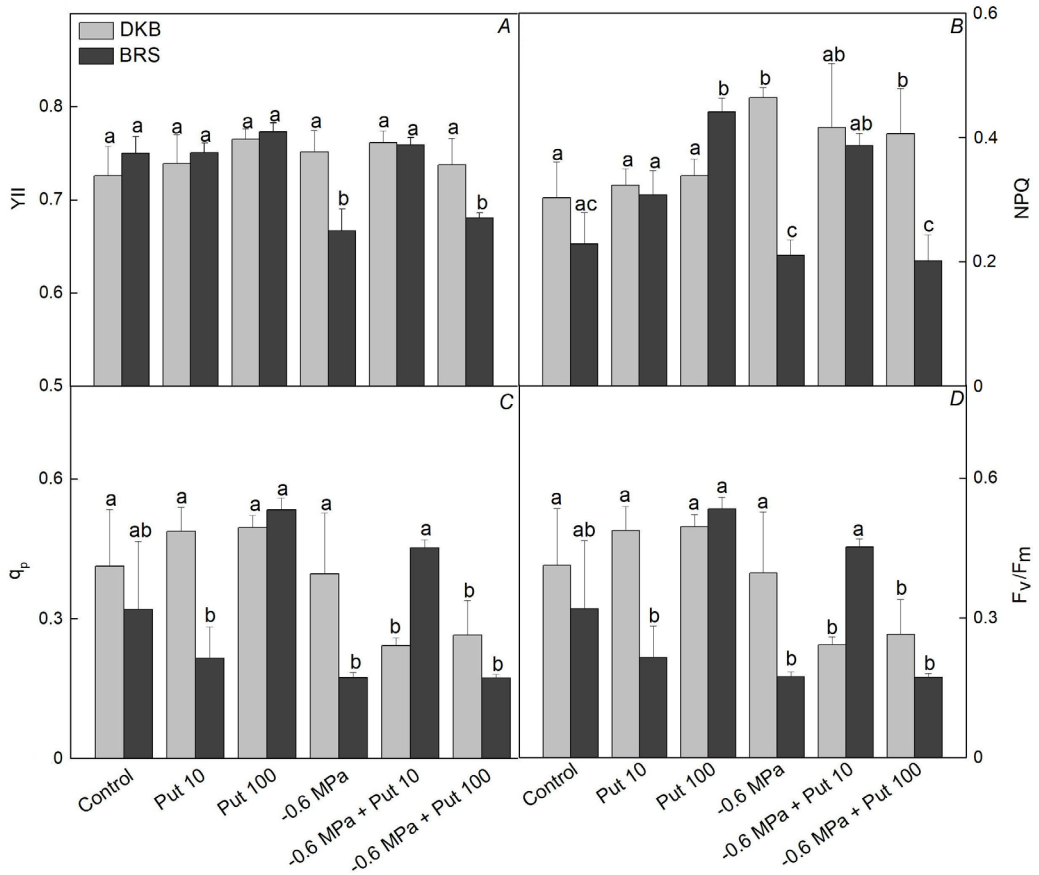


Fig. 2. Effect of water deficit and putrescine priming on the chlorophyll fluorescence parameters of maize hybrids with contrasting drought tolerances. YII - effective photochemical quantum yield of PS II; $q_p$ - photochemical quenching; NPQ - non-photochemical quenching; $F_v/F_m$ - variable to maximum fluorescence ratio (the maximal effciency of PS II photochemistry). Means ± SDs, $n$ = 25, *different letters* indicate statistical diferences according to *Tukey*'s s test at 5% significance level.

thus protecting the plants from stress-induced damage and restoring morphophysiological activities (Islam *et al.* 2021). However, the concentration of 100 µM of Put did not change the fluorescence parameters for the BRS hybrid under deficit. Under these conditions, it was observed that the lower concentration of Put (10 µM) was better and induced greater efficiency in the photosystems.

During germination, the mobilization of complex polymers from storage tissues, such as endosperm or cotyledons provides energy and building blocks for seedling growth (Sánchez-Linares *et al.* 2012). Post-germination events occur during the utilization and transport of these compounds. Fig. 3 illustrates some of these activities. Higher contents of reducing sugars were observed in DKB plants than in the BRS hybrids, both in the roots and leaves (Fig. 3*A*,*B*). The accumulation of organic osmolytes, such as soluble sugars, is a common response to drought stress (Prazeres and Coelho 2020). Therefore, this result is expected for drought-tolerant plants such as DKB. There was an increase in the content of reducing sugars in the roots of BRS plants in the presence of stress. However, this effect was not observed in the shoots. This result corresponds to the damage observed in the photosynthetic apparatus of the BRS plants. Although Put priming treatment recovered the chlorophyll fluorescence parameters in the BRS samples, it did not result in the accumulation of reducing sugars. The contents of soluble sugars and starch were similar between the plants. In fact, BRS plants presented a higher content of starch in the shoots in the absence of stress and this content decreased with the treatments because of the mobilization to produce smaller osmotically active molecules. However, the content of reducing sugars remained low. Working with DKB 390, Queiroz and Cazetta (2016) found that trehalose content did not increase in response to different (-0.3, -0.6, -0.9, and -1.2 MPa) osmotic potentials. Those authors argued that this molecule was used more as an energy source than as an osmoprotectant. Li *et al.* (2017) observed a significant increase in total soluble sugar content in a stress-dependent manner. Similarly, Prazeres and Coelho (2020) reported increased content of soluble sugars in response to water deficit, which may be related to drought tolerance in high-vigor seeds. In the present study, there was no clear increase in sugar content owing to stress. However, the intrinsically high content of reducing sugars in DKB plants are highly suggestive of their importance in drought tolerance.

Sugar and starch content was similar between plants. In general, the BRS hybrid accumulated starch and soluble sugars at the same levels as DKB in the presence of stress.

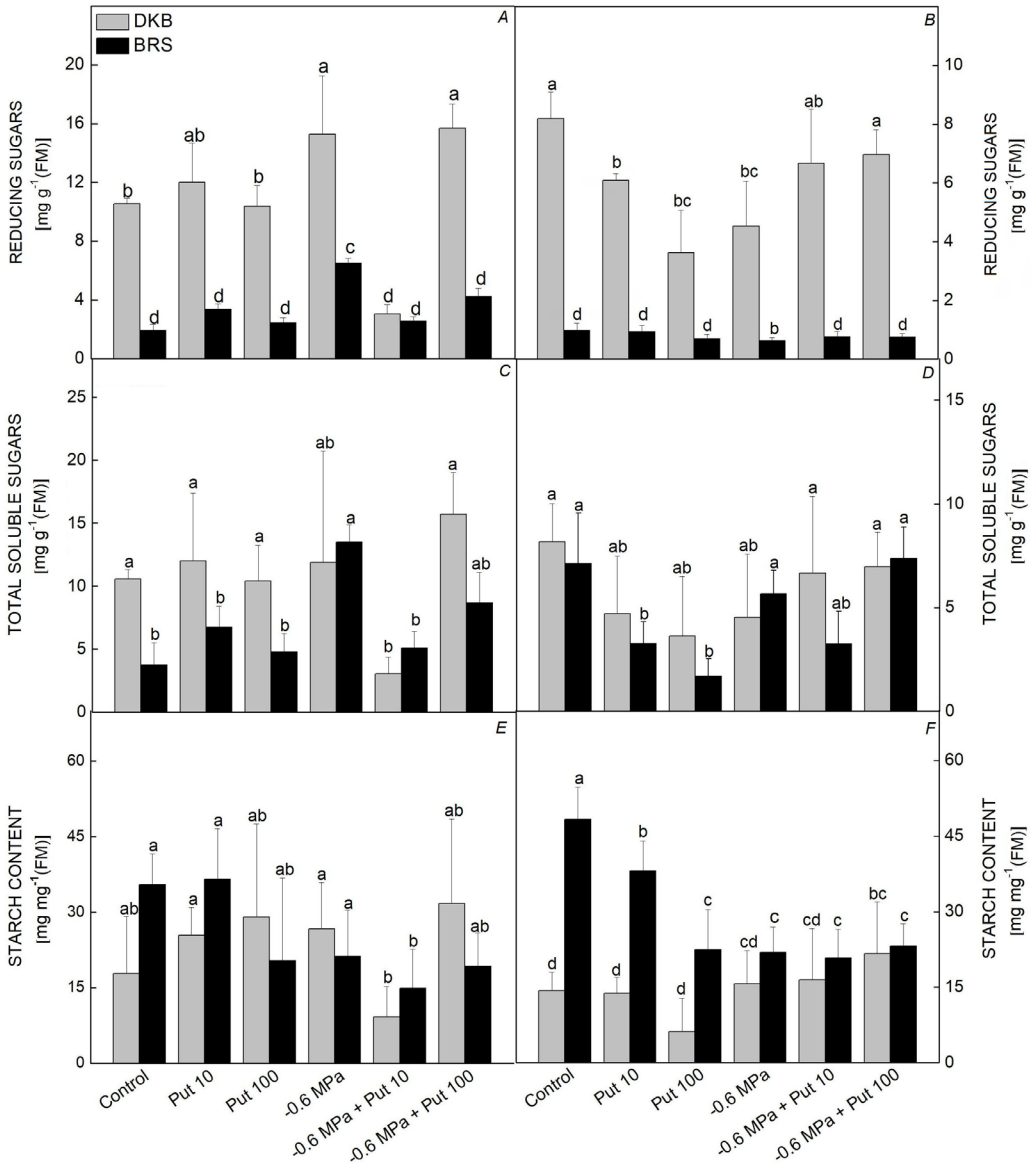


Fig. 3. Effect of water deficit and putrescine priming on reducing sugars, soluble sugars, and starch contents in roots (*A*, *C*, and *E*) and shoots (*B*, *D*, and *F*) of maize hybrids with contrasting drought tolerances. Means ± SDs, $n = 10$, *different letters* indicate statistical differences according to *Tukey*'s test at 5% significance level.

Therefore, the main difference appeared to be related to the accumulation of reducing sugars. The greater amount of reducing sugars in DKB may be related to the ability to mobilize reserves since the starch content in this hybrid is lower than in BRS in the absence of stress (Fig. 3).

As indicated by several authors (Alcântara *et al.* 2015, Avramova *et al.* 2017, Namjoyan *et al.* 2020), redox status is closely linked to plant responses to abiotic stress. In general, water deficit causes an increase in oxidative stress, which leads to a response by the plant. Priming favored SOD activity in the roots of the two hybrids in the absence of stress (Fig. 4*A*). However, the BRS plants were more responsive. In the presence of stress, there was an increase in SOD activity only in BRS plants, whereas that in DKB plants was not significantly different. In shoots, only Put priming in the absence of stress increased SOD activity. All other treatments did not significantly differ (Fig. 4*B*).

The GPX activity in the roots was much higher in DKB plants than in BRS plants, both in the absence and presence of stress (Fig. 4*C*). No clear patterns were observed in the roots or shoots for catalase or ascorbate peroxidase (Fig. 5). In general, DKB and BRS seedlings showed similar behavior, which could relate to the wide variation in the data. In the absence of stress, priming caused an increase in GPX activity in DKB plants. However, when subjected to water deficit, there was no increase in enzyme activity. In the case of BRS, there was no difference between treatments. According to Chugh *et al.* (2013), the activation of peroxidases may be a protective response

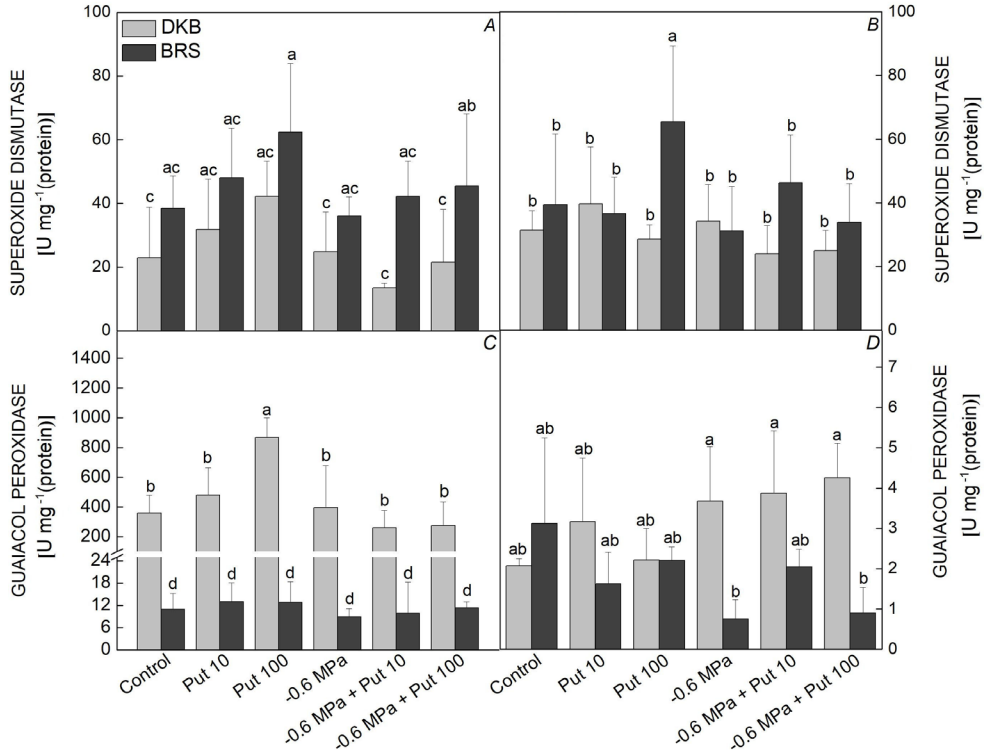


Fig. 4. Effect of water deficit and putrescine priming on superoxide dismutase and guaiacol peroxidase activities in the roots (*A* and *C*) and shoots (*B* and *D*) of maize hybrids with contrasting drought tolerances. Means ± SDs, *n* = 10, *different letters* indicate statistical differences according to *Tukey*'s test at a 5% significance level.

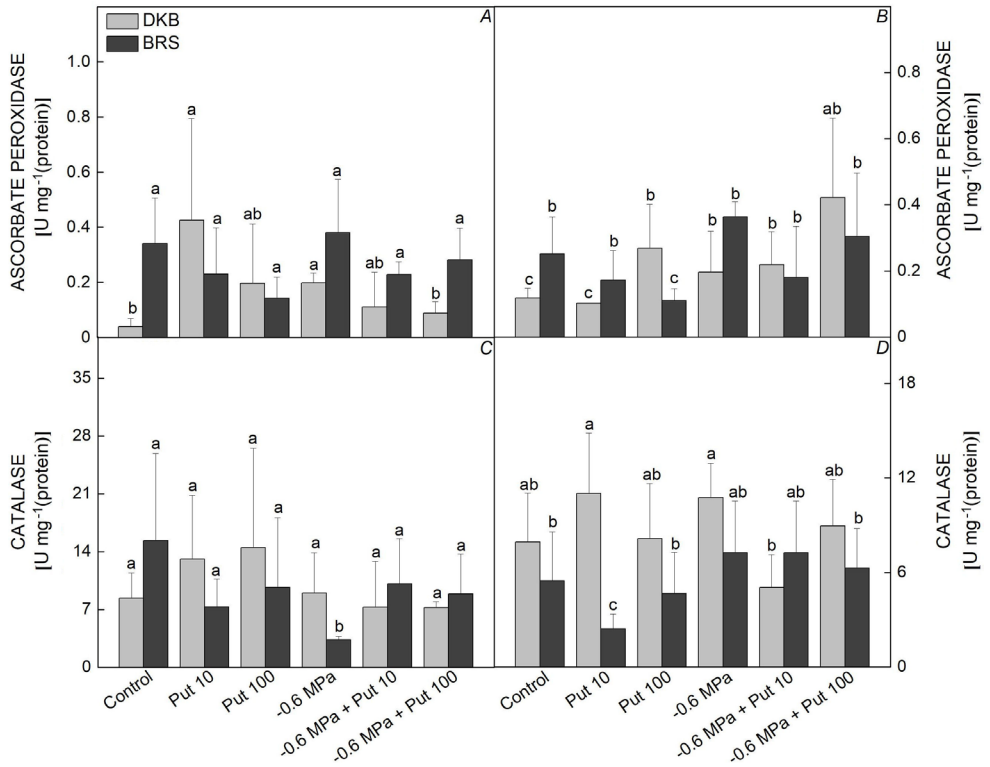


Fig. 5. Effect of water deficit and putrescine priming on ascorbate peroxidase and catalase activities in the roots (*A* and *C*) and shoots (*B* and *D*) of maize hybrids with contrasting drought tolerances. Means ± SDs, *n* = 10, *different letters* indicate statistical differences according to *Tukey*'s test at 5% significance level.

to overcome unfavorable environmental conditions including drought. Thus, the higher peroxidase activity of the DKB hybrids may contribute to increased tolerance. As previously demonstrated by Avila *et al.* (2016), DKB plants can invest in roots when subjected to drought. Based on our data, this anatomical response can be related, at least in part, to higher peroxidase activity since this class of enzymes is involved in several functions in the plant life cycle, such as cell wall metabolism, lignification, suberization, ROS metabolism, and wound healing among others (Pandey *et al.* 2017). The shoots showed less pronounced effects, but the DKB plants showed greater GPX activity under stress conditions, whereas 100 µM Put priming resulted in partial recovery of GPX activity (Fig. 4*D*). Under water deficit conditions, exogenous application of Put increases drought tolerance, ROS scavenging, and protects cells through various morpho-physiological and biochemical processes (Islam *et al.* 2022b).

Put had a protective effect only in the sensitive maize genotype (BRS). This result may be related to preventing protein denaturation, facilitating protein folding, activating the stress response, promoting defense reactions, inducing growth and development processes, and initiating the biosynthesis of secondary metabolite precursors (Nandy *et al.* 2022). In addition, under environmental stress, the mechanisms of Put are associated with the elimination of free radicals, regulation of abscisic acid (ABA) content, prevention of lipid peroxidation, maintenance of cellular pH and ionic balance, and regulation of cationic channels (Gill and Tuteja 2010). Thus, it is possible that there were multiple effects on BRS seedlings, with the action of Put as a growth regulator and the protective effect of priming, helping in the initial development of maize.

In conclusion, priming with 10 µM Put has a protective effect on maize seedlings under water deficit. The drought-sensitive genotype (BRS) benefits from Put priming by promoting increased root and shoot growth, photosystem efficiency (YII), and guaiacol peroxidase activity. Put priming is a promising strategy for protecting the initial growth of maize seedlings under water deficit conditions.

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