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# *Azospirillum brasilense* affects the antioxidant activity and leaf pigment content of *Urochloa ruziziensis* under water stress<sup>1</sup>

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## ABSTRACT

Water stress leads to the formation of reactive oxygen species, resulting in degradation of leaf pigments and cell death. This study aimed at assessing the oxidative enzyme activity and photosynthetic pigment content in seeds and/or leaves of *Urochloa ruziziensis* (syn. *Brachiaria*) inoculated with *Azospirillum brasilense* under water stress. Assessments of soluble proteins, chlorophylls *a* and *b* and carotenoid contents, as well as the activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) enzymes, were conducted at the beginning of the water stress process and also under severe water stress and during plant rehydration. Seed inoculation showed a reduction in the action of SOD, under water stress, with an increase after rehydration. POD exhibited an activity greater than CAT in all the assessments, but it did not differ statistically under severe water stress. CAT activity increased under severe stress in all treatments, particularly for leaf inoculation. Chlorophyll *a* was slightly degraded, maintaining the levels of the irrigated control, while the chlorophyll *b* and carotenoid contents, in plants subjected to leaf inoculation with *A. brasilense*, were higher under water stress. It was concluded that the leaf inoculation of *U. ruziziensis* with *A. brasilense* makes the plant more efficient at removing reactive oxygen species and protecting chlorophyll *a*.

KEY-WORDS: Superoxide dismutase; catalase; peroxidase; chlorophyll; plant growth-promoting bacteria.

## RESUMO

*Azospirillum brasilense* afeta a atividade antioxidante e o teor de pigmentos foliares de *Urochloa ruziziensis* sob déficit hídrico

O déficit hídrico leva à formação de espécies reativas de oxigênio, que resultam em degradação de pigmentos foliares e morte celular. Objetivou-se avaliar a atividade de enzimas oxidativas e o teor de pigmentos fotossintéticos em sementes e/ou folhas de *Urochloa ruziziensis* (syn. *Brachiaria*) inoculada com *Azospirillum brasilense*, sob déficit hídrico. As avaliações do teor de proteínas solúveis, clorofilas *a* e *b* e carotenoides e da atividade das enzimas superóxido dismutase (SOD), peroxidase (POD) e catalase (CAT) foram realizadas no início do déficit hídrico, no estágio de déficit hídrico severo e na reidratação das plantas. A inoculação das sementes demonstrou redução na ação da SOD sob déficit hídrico, sendo incrementada após a reidratação. A POD apresentou maior atividade que a CAT em todas as avaliações, contudo, não se diferenciou estatisticamente sob déficit severo. A CAT teve atividade aumentada sob déficit severo em todos os tratamentos, com destaque para a inoculação foliar. A clorofila *a* foi pouco degradada, mantendo os níveis do controle irrigado, enquanto os conteúdos de clorofila *b* e carotenoides nas plantas inoculadas com *A. brasilense* via foliar foram superiores, sob déficit hídrico. Conclui-se que a inoculação foliar de *U. ruziziensis* com *A. brasilense* torna a planta mais eficiente na remoção de espécies reativas de oxigênio e na proteção da clorofila *a*.

PALAVRAS-CHAVE: Superóxido dismutase; catalase; peroxidase; clorofila; bactérias promotoras de crescimento vegetal.

## INTRODUCTION

Plants need water for growing and sustaining vital activities. Their metabolism changes under water stress, which is one of the greatest challenges in agriculture, especially for crops predisposed to adverse weather conditions.

The formation of reactive oxygen species in plants occurs in oxygen-dependent processes, such as photosynthesis. However, in plants subjected to environmental stresses, such as drought, the free

radical formation increases due to stomatal closure. The limited availability of CO<sub>2</sub> in chloroplasts increases the formation of the superoxide anion radical (O<sub>2</sub><sup>•-</sup>) (Barbosa et al. 2014). This free radical accumulation results from reduced NADPH oxidation, meaning that the electron transferred from ferredoxin to NADP is transferred to molecular oxygen (O<sub>2</sub>), forming O<sub>2</sub><sup>•-</sup> (Ahmad et al. 2008).

Plants have removal mechanisms for these radicals via antioxidants, in a non-enzymatic system, and through an enzymatic system linked to oxidative

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stress (Sharma et al. 2012). The enzymatic system is performed by superoxide dismutase (SOD), the primary enzyme responsible for plant detoxification, whose function is dismutating  $O_2^{\bullet-}$ , transforming it into hydrogen peroxide ( $H_2O_2$ ) and  $O_2$  (Ahmad et al. 2008). However,  $H_2O_2$  is also harmful to plants that have peroxidases (POD) and catalases (CAT) for their degradation. The reactive oxygen species production occurs on a larger scale in peroxisomes and chloroplasts, leading to chlorophyll degradation in plants.

Increased enzyme activity linked to oxidative stress under drought is reported in a wide range of crops, including corn (Talaat et al. 2015), rice (Kumar et al. 2015), wheat (Huseynova 2012), grass of the *Cynodon* genus (Liu & Chan 2015) and other  $C_4$  plants (Aimar et al. 2014). However, when water stress is severe, the enzyme activity is limited. In *Cynodon dactylon*, SOD displays high activity after 7 days without irrigation, but becomes limited at 14 days, due to restricted synthesis of the enzyme, caused by water stress (Shi et al. 2012, Liu & Chan 2015). Thus, treatments that allow the plant to maintain an active detoxification metabolism for a longer period of time are needed to minimize damage and prevent cell death.

Given that the production of reactive oxygen species and maintenance of antioxidative enzyme activity are largely modulated by the plant water content, techniques that improve the plant ability to maximize the use of water could be beneficial. The use of plant growth regulators and growth-promoting bacteria are alternatives to increase water use efficiency, since they promote better root development (Zakikhani et al. 2012). These symbiotic bacteria may also act as signalers, identifying water shortage in advance in the soil, thereby limiting water losses and allowing the maintenance of enzyme activity (Zakikhani et al. 2012, Zhou et al. 2013).

The present study aimed at assessing the oxidative enzyme activity and photosynthetic pigment content in seeds and/or leaves of *U. ruziziensis* (syn. *Brachiaria*) inoculated with *A. brasilense*, under water stress.

## MATERIAL AND METHODS

The experiment was conducted from February 2 to April 26, 2015, in a greenhouse with an arch-shaped roof covered with 150  $\mu$ -thick low-density polyethylene film with UV protection and sides

protected by 40 % white shade cloth. The pots used had a nominal capacity of 8.7 liters and contained soil substrate from the horizon A of an eutrophic Oxisol. The soil had a base saturation of 60.2 %, pH of 5.5, organic matter content of 24.61 g  $dm^{-3}$ , P content of 3.08 mg  $dm^{-3}$ , K content of 0.28 cmol  $dm^{-3}$  and a natural diazotrophic population of  $4 \times 10^5$  CFU  $g^{-1}$  of soil.

A randomized blocks design, with five treatments and four replications, was used. The treatments were: T1: control under water stress; T2: leaf inoculation with *A. brasilense* under water stress; T3: seed inoculation with *A. brasilense* under water stress; T4: seed and leaf inoculation with *A. brasilense* under water stress; T5: irrigated control.

Two seeds of *U. ruziziensis*, with a pure live seed percentage of 60 %, were planted per pot. Seeds were inoculated with *A. brasilense* strains AbV5 and AbV6 (commercial product), with a dose of 1 mL of inoculant ( $2 \times 10^8$  CFU  $mL^{-1}$ ) for every 1,000 seeds. After planting, the pots were kept at field capacity and watered daily. Leaf application of both *A. brasilense* strains (AbV5 and AbV6) was performed when the plants were in the early tillering stage (15 days after emergence), using a dose of 300  $mL ha^{-1}$  of inoculant and spray volume of 300  $L ha^{-1}$ . A  $CO_2$  pressurized backpack sprayer, with Magno 11002 ADGA nozzles spaced 0.5 m apart and constant pressure of 40  $kgf cm^{-2}$ , was used. After applications, the plants were constantly monitored to ensure proper growth, and no mineral nutrients were applied. Once the plants reached the recommended leaf growth for grazing (45 days after planting), water stress was imposed. Irrigation was suspended in the treatments that included water stress and withheld for six days, at which time the plants exhibited a net photosynthetic rate close to zero (conditions monitored daily with a LI-6400XT infrared gas analyzer). Next, the plants were rehydrated until they reached field capacity.

In order to determine the enzyme activity, fresh *U. ruziziensis* leaves (0.5 g) were collected at predawn at the water stress onset (first day after water restriction), under severe water stress (six days after water restriction) and after rehydration (one day after rehydration). Soluble protein contents were measured using the Bradford assay. SOD activity was determined by the enzymes ability to inhibit superoxide radicals from reducing nitroblue tetrazolium (NBT) (Giannopolitis & Ries 1977).

POD activity was measured at 30 °C by direct spectrophotometry, based on the guaiacol oxidation (Hammerschmidt et al. 1982). CAT activity was determined by monitoring the variation in hydrogen peroxide absorption (Havir & McHale 1987).

In order to measure leaf pigment contents, leaf segments with fresh mass of 0.5 g were placed in covered flasks filled with 80 % acetone solution and stored under refrigeration without light for 72 h. Next, spectrophotometer readings were conducted, using 665 nm for chlorophyll *a*, 649 nm for chlorophyll *b* and 480 nm for carotenoids (Lichtenthaler & Buschmann 2001).

Statistical analysis was performed at each assessment via the F-test and, when appropriate, means were compared by the Tukey test at 5 %, using the Sisvar statistical software.

## RESULTS AND DISCUSSION

The highest soluble protein content at the water stress onset was obtained in seed inoculation with *A. brasilense*, followed by the irrigated control. It is important to underscore that, up to this point, the plants exhibited only the inoculation effects. Under severe water stress, seed inoculation with *A. brasilense* once again showed the highest soluble protein levels, with no difference between the remaining treatments. It was observed that protein contents tend to increase under drought, likely due to dilution, since the presence of less water in the leaf tissue means that proteins are more concentrated. This is confirmed by the fact that soluble protein contents in the irrigated control remained constant. After rehydration, both controls showed a rise in soluble protein contents, reaching higher means, whereas low soluble protein levels were recorded for inoculation, regardless of the method (Figure 1).

The rise in protein contents under severe water stress and the dilution effect are associated with increased enzyme activity, protecting them from oxidation and degradation. The results obtained in the present study corroborate those found in cowpea grown under saline conditions, in response to increased POD activity (Maia et al. 2012). Previous results from hypocotyls of corn seedlings also indicate that proteins were protected by increased CAT activity (Rosa et al. 2005).

In the assessment of SOD activity, leaf application of *A. brasilense* induced greater activity,

followed by the irrigated control (Figure 2). High daytime temperatures (maximum temperature of 37 °C) may have led the plant to experience temporary water stress.

Under severe water stress, low SOD activity was observed for seed inoculation, whereas leaf application and leaf and seed inoculation had similar results (Figure 2). The decline in SOD activity, except for combined seed and leaf inoculation of *A. brasilense*, occurred as a result of the plants limited recovery ability with less water available in the soil. SOD activity decreased as the water shortage in the substrate increased (Campos et al. 2011). This decline is associated with the rise in the H<sub>2</sub>O<sub>2</sub> concentration, which reduces SOD activity (Gill & Tuteja 2010).

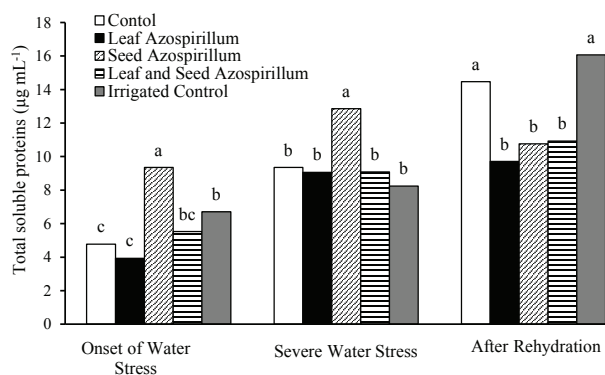


Figure 1. Total soluble protein contents in *Urochloa ruziziensis* leaves submitted to inoculation with *Azospirillum brasilense* under water stress. \* Different letters indicate a difference between treatments, according to the Tukey test at 5 %.

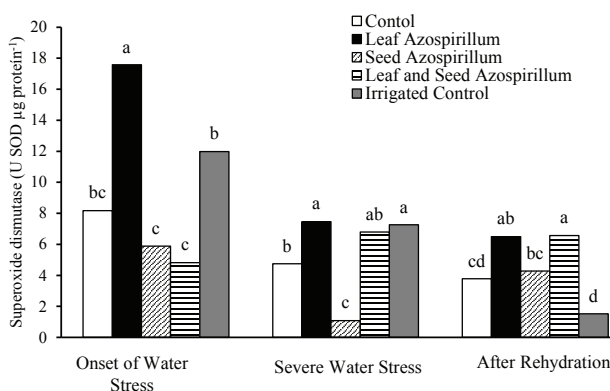


Figure 2. Superoxide dismutase (SOD) activity, in *Urochloa ruziziensis* leaves submitted to inoculation with *Azospirillum brasilense* under water stress. \* Different letters indicate a difference between treatments, according to the Tukey test at 5 %.

After rehydration, combined seed and leaf inoculation with *A. brasilense* produced the highest SOD activity, with no difference in relation to leaf application. The maintenance of SOD activity levels indicates that the plant is still suffering the effects of water stress, and still eliminating reactive oxygen species. This is a standard response, since plants try to balance their physiological and biochemical systems, what is considered a critical moment, given the stress imposed.

It is important to point out that some of the results observed are difficult to explain, such as the decrease in SOD activity observed in the irrigated control. This is possibly linked to uncontrollable variations, among which stands out the genetic variation inherent of some grasses. *Urochloa ruziziensis* plants have great genetic variability, which may have led to the inconsistencies found between evaluations. Another possible explanation may lie in environmental changes between evaluations.

CAT activity prior to imposed water stress was higher in the leaf application of *A. brasilense*, but differed only from the irrigated control (Figure 3a). Similar results were recorded under severe water stress and during rehydration, with leaf inoculation exhibiting the highest CAT activity, differing from the combined seed and leaf applications. The lowest CAT activity levels were obtained in the irrigated control.

POD assessment indicated that irrigated control plants initially displayed the lowest activity (Figure 3b). No significant difference was observed for POD activity under severe water stress. After rehydration, control plants maintained under water stress exhibited higher POD activity, with no difference in relation to irrigated controls, which, in turn, did not differ from *A. brasilense* inoculated plants.

The high expression of POD throughout the experiment indicates that this was an important enzyme for the removal of  $H_2O_2$  radicals in all the assessment days (Figure 3b). CAT activity was the highest one under severe water stress (Figure 3a), corroborating findings in plants grown under drought stress.

The behavior of these enzymes may be explained by the oxidation mechanism used. POD depends on a reducing agent, such as guaiacol, a phenolic compound whose formation declines under water scarcity. Plants constantly produce reactive oxygen species, and prior to water stress,

they occur mainly in the chloroplasts, as a result of photoinhibition, which leads to greater POD activity.

In turn, CAT operates without the need of a reducing agent, resulting in increased activity. As such, this enzyme becomes more important in adverse conditions, such as severe water stress (Esteves & Suzuki 2008, Barbosa et al. 2014). Another advantage of CAT is its  $H_2O_2$  removal ability of approximately 6 million molecules per minute. Moreover, the enzyme acts primarily in peroxisomes (Gill & Tuteja 2010, Sharma et al. 2012), the main sites of reactive oxygen species formation under severe adverse conditions (Sharma et al. 2012, Barbosa et al. 2014).

Plants tend to recover under rehydration. However, enzyme activity may be maintained low, since recovery is gradual. This is evident in *Panicum virgatum*, in which a slight variation in enzyme activity was recorded 12 h after rehydration, increasing after 24 h (Aimar et al. 2014). A similar behavior has been observed in citrus plants (Campos et al. 2011), in which SOD and POD activities showed no variation during the rehydration of *Portulaca oleracea* for 72 h (Jin et al. 2015).

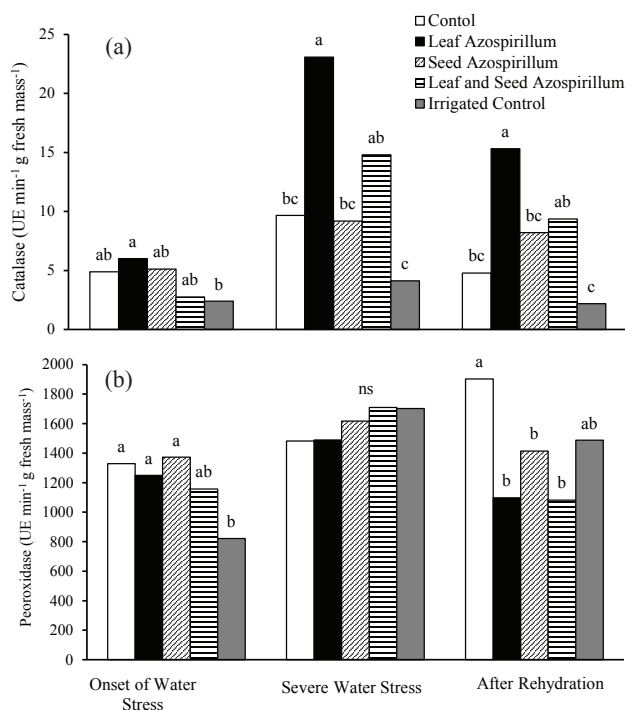


Figure 3. Catalase (a) and peroxidase (b) activity, in *Urochloa ruziziensis* leaves submitted to inoculation with *Azospirillum brasilense* under water stress. \* Different letters indicate a difference between treatments. ns not significant, according to the F-test at 5 %.



Thus, an increase in enzyme activity is a drought tolerance mechanism in plants. As such, leaf inoculation with *A. brasilense* may be used to improve the drought tolerance of *U. ruziziensis*, since POD and CAT activities increased under moderate and severe water stress, respectively.

Chlorophyll, found primarily in chloroplasts (Streit et al. 2005), is divided into two types: chlorophyll *a*, responsible for photochemistry, and chlorophyll *b*, which captures light energy (Taiz & Zeiger 2013). Chlorophyll molecules are predisposed to the action of free radicals formed within chloroplasts (Uzilday et al. 2012).

The assessment of chlorophyll *a* content in *U. ruziziensis* leaves resulted in no significant inter-treatment differences prior to the water stress onset. Under severe water stress, a decline in chlorophyll *a* levels was observed in the treatment with combined leaf and seed application of *A. brasilense*. No significant inter-treatment variations were recorded after rehydration (Figure 4a). This is related to the increase in oxidative enzyme activity (Figures 2 and 3), which effectively prevented chlorophyll degradation.

Lack of chlorophyll degradation is also reported for  $C_4$  plants, such as *U. ruziziensis* (Abdelgawad et al. 2015). Low chlorophyll *a* degradation under water stress is desirable, because water scarcity inhibits the chlorophyll synthesis (Jin et al. 2015). Thus, plants are dependent on chlorophyll preservation, in order to resume photosynthesis after rehydration. This explains the absence of new chlorophyll synthesis after rehydration (Figures 4a and 4b), since the plant was still stabilizing its metabolic status and photosynthetic apparatus (data not shown).

The results demonstrate that chlorophyll *a* was preserved under water stress. This is consistent with the antioxidant enzyme activity and increased carotenoid content observed, which reached maximum levels with the foliar application of *A. brasilense*. This was the treatment that promoted the greatest protection in this forage crop.

With respect to leaf chlorophyll *b* content prior to water stress, leaf inoculation with *A. brasilense* showed the highest mean, and no difference in relation to the control. Under severe water stress, seed inoculation resulted in the highest chlorophyll *b* content, with no difference when compared to leaf application (Figure 4b). Regarding the carotenoid content before water stress, the irrigated control

exhibited the highest levels, with no difference in relation to the leaf application of *A. brasilense*. Under severe water stress, leaf inoculation showed the highest mean, whereas seed application obtained the greatest mean after rehydration, with no difference from the irrigated control.

The increase in chlorophyll *b* under severe water stress for leaf application of *A. brasilense* (Figure 4b) is related to the 'stay-green' effect promoted by inoculation (Quadros et al. 2014). This is also a photoprotective response associated with the increased carotenoid content (Figure 4c). Carotenoids are pigments that sequester  $O_2^-$ , preventing chlorophyll degradation (Gill & Tuteja 2010).

Our results demonstrate the potential for using the growth-promoting bacteria *A. brasilense* to increase the antioxidant activity in *U. ruziziensis*. However, further research is needed to determine

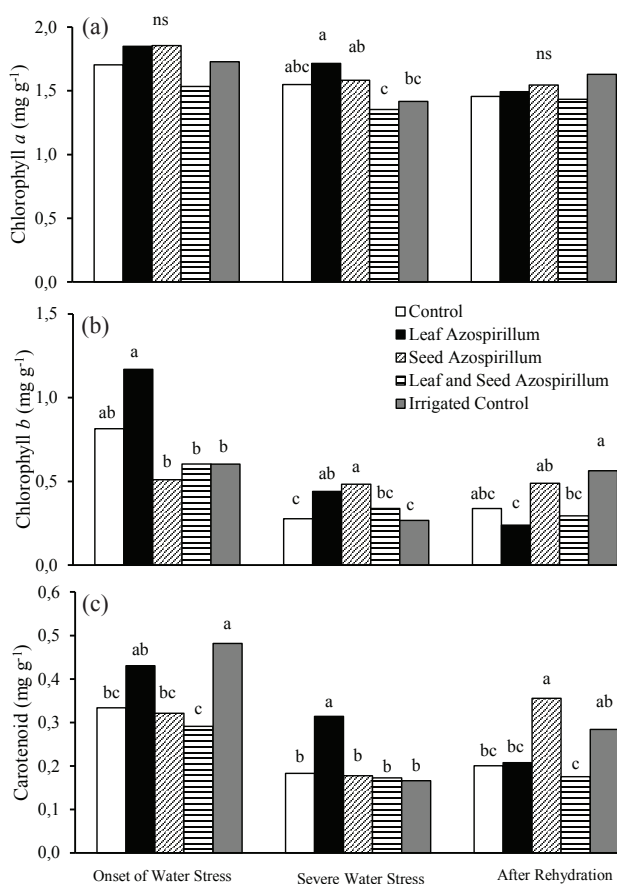


Figure 4. Concentrations of chlorophyll *a* (a), chlorophyll *b* (b) and carotenoid (c), in *Urochloa ruziziensis* leaves submitted to inoculation with *Azospirillum brasilense* under water stress. \* Different letters indicate a difference between treatments. ns not significant, according to the F-test at 5 %.

which of the bacteria actions interfere directly in this response, since the antioxidant system of plants varies with a series of environmental and ontogenetic factors.

## CONCLUSIONS

1. Leaf inoculation with *Azospirillum brasilense* is effective in raising SOD and CAT activities under severe water stress;
2. Leaf application of *A. brasilense* increases SOD activity;
3. No decline in chlorophyll *a* content was observed under water stress.
4. Leaf inoculation with *A. brasilense* exhibited the highest carotenoid and chlorophyll *a* and *b* concentrations, and could therefore be used as a management tool for *U. ruziziensis*, during the dry season.

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