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Physiological responses of *Urochloa ruziziensis* inoculated with *Azospirillum brasilense* to severe drought and rehydration conditions

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Abstract

This study evaluated the tolerance of *U. ruziziensis* under severe water stress conditions and its recovery when rehydrated, based in responses of relative leaf water content and gas exchange of the crop. The experiment was conducted in green house in Oxisol. The experimental design was a randomized block design, totaling five treatments, was represented by inoculation of *A. brasilense* strains AbV5 + AbV6, as follow: seed inoculation, foliar spray application, seed inoculation + foliar spray application, a non-inoculated control with water deficit treatment and a irrigated control without inoculation. Drought was imposed 45 days after sowing, after the vessel was irrigated until saturation of the substrate, by total removal of irrigation for six days, and evaluated three days after the resumption of water, evaluating gravimetric soil moisture, relative leaf water content and gas exchange. Soil moisture showed non variation to treatments non irrigated, but the relative leaf water content was lower in the control without water. The control and the treatment inoculated with *A. brasilense* in seeds exhibited lower rates of gas exchange. Recovery full of photosynthetic activity occurred three days after rehydration in all treatments. *A. brasilense* foliar sprayed is efficient to increase the tolerance of *U. ruziziensis* to water deficit and presented higher photosynthetic rates even under conditions of severe water deficit.

Keywords: Growth promoting bacteria, Forage grasses, Relative water content, Gas exchange.

Abbreviations: *A* _ CO_2 net assimilation rate; *E* _ transpiration; g_s _ stomatal conductance; *Ci* _ internal CO_2 concentration; WUE _ efficiency of water use; iWUE _ intrinsic efficiency of water use; RWC _ relative water content.

Introduction

Water is of utmost importance in productive systems, and may represent about 90% fresh weight in plants (Azevedo et al., 2005). In major crops and pastures, this representation varies according to the developmental stages, ranging from 40% to 80% (Lima et al. 2012). Thus, the water deficit has a paramount impact in productivity systems.

Water deficit takes place when the plant transpiration rate exceeds the water uptake rate (Taiz and Zeiger 2013), leading to a physiological imbalance (Fioreze et al. 2013), triggering a series of reactions including the reduction of yields, grains and, in extreme cases, death of organs or the whole plant (Souza et al., 2014).

Grasses have peculiaritys that ensure resistance to drought, through morphological and physiological adaptations (Souza et al., 2014). In Brazil, pastures occupy most of the agricultural area, 173.2 millons hectares, and are responsible for maintaining the national herd. In this scenario, grasses, especially belonging to the genus *Urochloa* (syn. *Brachiaria*), represent about 80% of planted and/or reformed pastures (Lang et al., 2011). However, they are usually grown in soils of low fertility, in hilly areas and with limited mechanization procediments, making them dependent on natural rainfall, which are irregular (Maranhão et al., 2010)

Thus, the effect of water deficit has been shown in several grasses, causing reduction of development, mainly because leaf expansion restriction, increasing leaf senescence and reducing the emergence of tillers. Mattos et al. (2005) reported the effect of drought on *Urochloa* pastures, reducing the growth of plants and dry matter. It was also demonstrated reduction in the development of *Cynodon* grown under drought conditions (Silva et al., 2005; Zhou et al., 2013).

Several studies investigated ways to increase tolerance to water deficit, primarily via genetical enhancement. Nevertheless, some studies indicate that the use of plant growth promoting bacteria increases the tolerance to water deficit under field conditions. Among these bacteria, *Azospirillum brasilense* has been widely used in many crops (Dartora et al., 2013; Quadros et al., 2014), aiming the production of plant hormones (Kuss et al., 2007), stimulation of root development and increase shoot dry matter (Dartora et al., 2013), intensifications in photosynthesis (Inagaki et al., 2015) and in crop yields (Quadros et al., 2014). Much of these results are associated with better use of productive resources, such as water.

Thus, as *A. brasilense* maximizes the use of productive resources, like water, it was aimed to evaluate the tolerance of *Urochloa ruziziensis* inoculated with *Azospirillum brasilense* under water stress conditions and rehydration, by means of the responses of relative leaf water content and gas exchange of the crop.

Results

Soil mosture and relative leaf water content (RWC)

Was verified that only the irrigated control differed from the other treatments for the evaluations performed in moderated and severe water deficit (Fig. 1a). After the rehydration, no significant differences were observed among all the treatments.

There were not significant effects on relative leaf water content (RWC) in plants from different treatments on moderate water stress and rehydration (Fig.1b). Variations were obtained on severe water stress, where the control has less relative leaf water content (49.47%) in relation to *A. brasilense* foliar spraying, while those theatments that received inoculation with *A. brasilense* in seed presented RWC of 62.37%, foliar spray 65.05% and in seed + foliar spraying 62.96%, representing a superiority of 26.07%, 27.26% and 31.49% compared with the control, respectively (Fig. 1b). In turn, the plants kept under irrigation had a mean RWC of 93.79%, promoting the highest average.

Gas exchange

The net assimilation rate of CO_2 (*A* rate) under conditions of moderate water stress showed that the treatments kept in water restriction didn't differentiate between them. In severe water stress, control non irrigated and seed inoculation of *A. brasilense* had their rates of *A* rate close to zero, promoted values of 0.015 µmol CO_2 m^2 s^{-1} and 0.018 µmol CO_2 m^2 s^{-1}, respectively. On the other hand, the plants that received *A. brasilense* seed inoculation + foliar spraying maintained higher *A* rates, followed by foliar spray of *A. brasilense*. In rehydration, seed inoculation promoted lower values than control and foliar spray of *A. brasilense*, however, all plants showed complete recovery of their photosynthetic rate (Fig.2a).

The internal CO_2 concentration (*Ci*) under moderate deficit conditions presented lower mean in control non irrigated and seed inoculation, the same treatments in severe deficit presented higher values of *Ci*, similar to C_3 plants, evidencing the deficiency in the CO_2 net assimilation. The other treatments maintained in water deficit presented variations among themselves according to *A* rates, while foliar spray of *A. brasilense* show greater *Ci* than seed inoculation + foliar spray *A. brasilense*. In rehydration, the values of *Ci* were normalized, and only seed inoculation + foliar spraying *A. brasilense* differed from the other non-irrigated treatments (Fig.2b).

For leaf transpiration (*E*) and stomatal conductance (*gs*) differences were obtained in moderate water deficit, linked to lower soil moisture (Fig.1a). At that moment the control non irrigated and seed inoculation of *A. brasilense* presented lower *E*, while the foliar spray of *A. brasilense* and seed inoculation + foliar spraying maintained greater *E* and stomatal opening (Fig. 3a and 3b). These results suggest that the control non irrigated and seed inoculated of *A. brasilense* felt the lack of water in advance, they began the closing of the stomata to avoid greater losses of water in longer time. While the plants with foliar spray of *A. brasilense,* seed inoculation + foliar spraying *A. brasilense* and irrigated control kept their stomata open and increased transpiration rate.

In severe water stress, no significant differences were observed between treatments maintained in water deficit for *E*, being only the irrigated control superior. In rehydration for *gs* and *E*, seed inoculation of *A. brasilense* presented lower values, differing only from control irrigated in *E*, and irrigated control, control non irrigated, and foliar spray of *A. brasilense* in *gs*.

In severe water stress, control non irrigated and seed inoculation *A. brasilense* had values of *WUE* and *iWUE* near zero, while *A. brasilense* seed inoculated + foliar spraying elevated *WUE* and *iWUE*, surpassing the average of the irrigated control plants. Foliar spray of *A. brasilense* presented high averages, similar to irrigated control in *WUE*, and surpassed in *iWUE* (Fig. 4a and 4b).

Discussion

The lowest RWC obtained in the control non irrigated plants is linked to the fact that these treatment promoted higher mass accumulation in the moment of the imposition of water deficit; in this moment, the control non irrigated plants had a shoot dry matter mass of 6.79 g $plant^{-1}$, while plants inoculated with *A. brasilense* in seed inoculation, on foliar spray application and *A. brasilense* seed inoculation + foliar spraying had 5.41, 4.59 and 4.95 g $plant^{-1}$, respectively.

As already mentioned, the water content in plants has no direct relationship with soil moisture (Pimentel et al., 2002), such condition occurred in the present study when the soil moisture gradualy decreased, the RWC showed an abruptly decreased (Fig. 1a and 1b).

Although the genus *Urochloa* is resistant to drought (Souza et al., 2014), these have a daily evapotranspiratory rate considered high, reaching 1.2 mm day^{-1} $plant^{-1}$ in sand soil, reaching 3.0 mm day^{-1} $plant^{-1}$ for larger leaf area (Silva et al., 2014) and 2.6 day^{-1} $plant^{-1}$ in the Brazilian cerrado soil (Meirelles et al., 2011). These values are close soybean (Casaroli et al., 2007) and bean (Fernandes et al., 2015), before the reproductive stages.

One of the causes for the sudden reduction in RWC is related to the adaptability of forage grasses: when it detect the lack of water by the increase in the content of abscisic acid (Taiz and Zeiger, 2013), initiating a process of leaf senescence in the lowest part of the canopy (Hu et al., 2010), it starts to prioritize water to the upper leaves. This fact occurred in compliance with this study, where the control non irrigated plants presented senescence of 1.20 g $plant^{-1}$ of leaves, while plants with foliar spray application of *A. brasilense* promoted 0.96 g $plant^{-1}$ of leaves. The mean was 0.84 g $plant^{-1}$ of leaves, suggesting that the foliar spray *A. brasilense* increases the plant tolerance to water deficit. However, under reduction of water in the substrate, the RWC quickly decreased and photosynthetic rate, being necessary to perform the rehydration.

This condition was also observed for *Cynodon dactylon* in shallow soil, where the cultivars possessing leaf senescence and increase abscicic acid resisted for until 20 days to the lack of water (Zhou et al., 2013). The same authors also report that *gs* is reduced in the plant in different ways, and while the canopy presents stomata closed at the top they are partially open. Water restriction also influenced reducing the rate of leaf elongation, total length of leaf and number of green leaves and the development of forage grasses: *Brachiaria purpurascens*, *Hermarthria altissima* and *Acroceras macrum*, (Silva et al., 2005). In *U. humidicola, U. decumbens* and *U. mutica* are also reported on water deficit effects, including leaf senescence and reduction in leaf elongation rate (Mattos et al., 2005).

In other grasses there are reports that the RWC gradually decreases, as shown in wheat (Santos et al., 2012) and barley (Thameur et al., 2012), attributed to the improvement process

Table 1. Chemical and physical characteristics of the soil used for the implementation of the experiment.

P	O.M.	pH $CaCl_2$	H+Al	Al^{+3}	K^+	Ca^{+2}	Mg^{+2}	SB	CTC	V	Al
mg dm³	g dm³	mol L^{-1}		---------------------	cmol$_c$ dm^{-3}	--------------------------				-----%------	
5,56	17,77	4,67	3,44	0,2	0,13	3,82	1,03	4,98	8,42	59,14	3,86
Cu	Zn	Mn	Fe		Argila			Silte		Areia	
----------------mg dm^{-3}----------------					----------------------------g kg^{-1}----------------------------						
26,40	11,60	578,00	348,58		578,00			348,58		3,42	

O.M: Organic matter; H + Al (potential acidity), SB (base sum), CTC (cation exchange capacity), C (organic carbon), V% (base saturation).

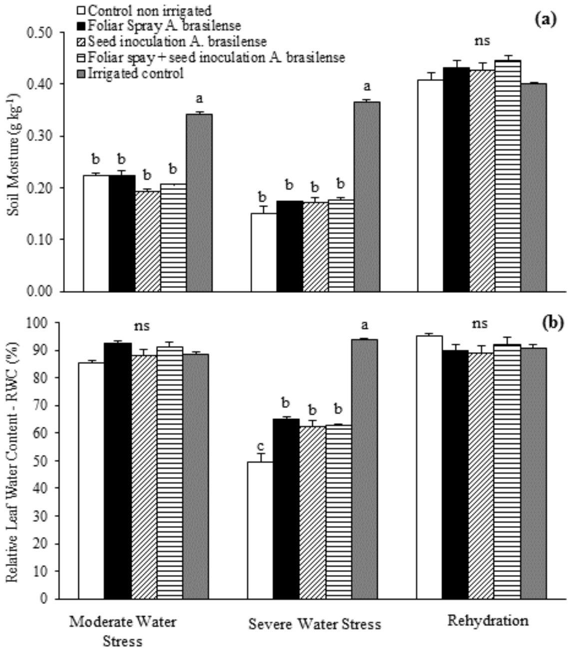

Fig 1. Soil Mosture (a) and Relative leaf water content (b) in *U. ruziziensis* under different inoculations with *A. brasilense* subjected to controlled drought. * Error bars indicate the SE. Moderate and Severe water stress assessed 4 and 6 days after withdrawal of water, respectively. Rehydration evaluated 3 days after water recovery. ns: not significant; Lowercase letters do not differ from each other by the Tukey test p ≤0.05.

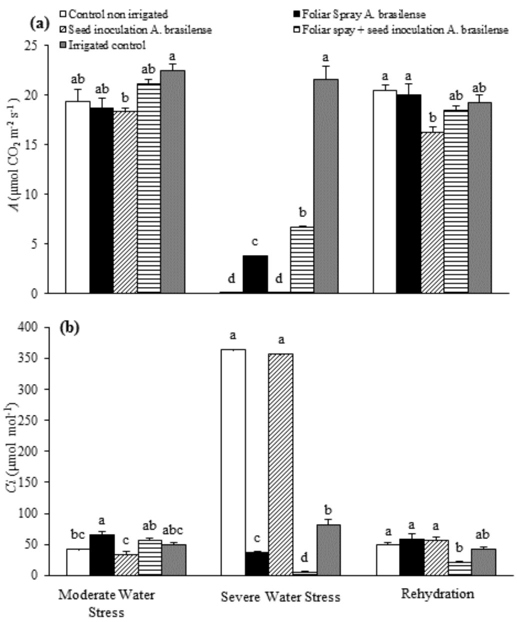

Fig 2. Net assimilation rate of CO_2 - *A* (a) and internal CO_2 concentration - *Ci* (b) *U. ruziziensis* subjected to different forms of inoculation of *A. brasilense* subjected to controlled drought. * Error bars indicate the SE. Moderate and Severe water stress assessed 4 and 6 days after withdrawal of water, respectively. Rehydration evaluated 3 days after water recovery. Lowercase letters do not differ from each other by the Tukey test p ≤0.05.

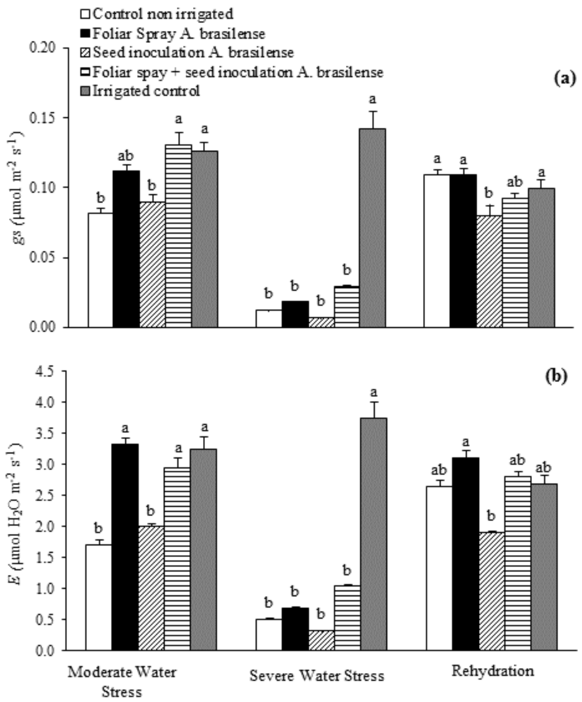

Fig 3. Stomatal conductance - *gs* (a) and transpiration leaf - *E* (b) *U. ruziziensis* subjected to different forms of inoculation of *A. brasilense* subjected to controlled drought.* Error bars indicate the SE. Moderate and Severe water stress assessed 4 and 6 days after withdrawal of water, respectively. Rehydration evaluated 3 days after water recovery. Lowercase letters do not differ from each other by the Tukey test p ≤0.05.

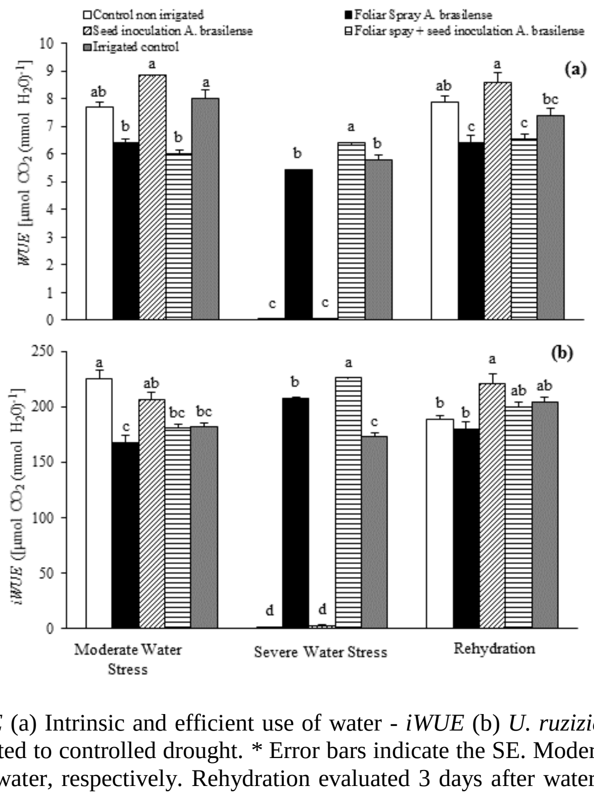

Fig 4. Efficient Water Use – *WUE* (a) Intrinsic and efficient use of water - *iWUE* (b) *U. ruziziensis* subjected to different forms of inoculation of *A. brasilense* subjected to controlled drought. * Error bars indicate the SE. Moderate and Severe water stress assessed 4 and 6 days after withdrawal of water, respectively. Rehydration evaluated 3 days after water recovery. Lowercase letters do not differ from each other by the Tukey test p ≤0.05

that aims the maximum crop yield, not resulting in efficient process of leaf abortion in forage grasses. Among the adaptations of forage grasses to water deficit there are the physiological modifications, rapid responses not always linked to the evolutionary factor, but to adaptation to the environment where the plant develops (Sherrard et al. 2009; Souza et al. 2014), being the photosynthetic rate, stomatal conductance and transpiration, changes that firstly respond to water deficiency.Stomatal conductance presents variation more rapidly with the reduction of water available in the soil, as observed in several studies independent from the photosynthetic physiology of plants, reported for C_3 (Santos et al., 2012; Yousfi et al., 2012; Fernandes et al., 2015) and C_4 plants (Hu et al., 2010; (Mattos et al., 2005; Hu et al., 2010; Zhou et al., 2013). In the present study, this condition also occurs being observed the reduction of *gs* (Fig.3a). These results show that control non irrigated plants and seed inoculated *A. brasilense* were earlier sensitive to lack of water and initiated the stomata closure process to avoid further loss of water. On the other hand, plants inoculated foliar spray *A. brasilense* and control non irrigated, hold their stomata opening and resulting in greater transpiration.

The *A* rates values, found before the fourth day are consistent with those described in the literature for forages, since values between 15 and 20 µmol m^2 s^{-1} are found for *Urochloa* (Mattos et al., 2005). The drop of *A* rates obtained in the severe water (Fig. 2a) is associated with the low water availability, since this is an initial electron acceptor for the photosystem II. There is also an increase in the abscisic acid content produced in the root by low water availability that is transported to the aerial part (Taiz and Zeiger, 2013).

The increase in *Ci* points to two conditions related to *A* rates, being beneficial or undesirable to the plant. Thus, when the maintainance of the photosynthesis is observed, the increase in *Ci* is desirable, showing that the photosynthetic apparatus is functional. However, when the photosynthesis is near zero, as the obtained for the control non irrigated and the seed inoculation with *A. brasilense*, it represents a deficiency in the photosynthetic apparatus of the plant, because with the stomatal closure caused by the low water availability, the CO_2 content in the substomatal chamber increases. However, the Rubisco is not fully activated due to the lack of water, thus the CO_2 is not carboxylated and incorporated into the Krebs cycle, therefore increasing the *Ci*.

The evaluations of *WUE* and *iWUE* were performed to confirm the direct relationships between *A* rates, *gs* and *E*, showing behavior similar to those found for each variable individually. Even under conditions of water deficit, *WUE* and *iWUE* can remain high, which was found in treatments of foliar spraying of *A. brasilense* and *A. brasilense* seed inoculation + foliar spraying (Fig. 4a and 4b). This fact was also registered in soybean crops (Hossain et al., 2014), which represents resistence of plant to water deficit. The *WUE* and *iWUE* were also used to differentiate cultivars of *Cynodon* spp. (Zhou et al., 2013) and barley (Thameur et al., 2012), resistant and susceptible to drought. In this sense, we can consider that the foliar application of *A. brasilense* makes the plant more resitant to water deficit. As mentioned above, when rehydration occurs, the plant rapidly recovers its content of water in the leaves. However, the photosynthetic process does not recover at the same rate (Slama et al., 2015). This is related to the abscisic acid level, which acted limiting photosynthesis. During the recovery, the plant can respond by two ways, gradually increasing gas exchange until restoration, which is desirable from the point of view of energy cost and use of water, which ocurred in the control and inoculation in the seed. Or even, occuring exchanges in an unregulated form, with large increases and subsequent reductions, condition managed by the hormonal lack of control of the abscisic acid that still acts in the plant, as in the treatments that received foliar spraying *A. brasilense*. The classic study of Mansfield and Davies (1985) evidenced that the effect of water deficit can remain in the plant for a long period, what they called "residual effect of water deficit", interfering in crops gas exchanges, being represented mainly by the restriction in the CO_2 uptake. Thus, when we evaluate *Ci* (Fig. 2b), could be observed that it recover his concentration similarly to the initial condition, indicating that *U. ruziziensis*, after three days from the rehydration, functioning of the photosynthetic apparatus similar to initial condition. Unlike the results of this study, Mattos et al. (2005), also under greenhouse conditions, mentioned that even after nine days of rehydration, *U. brizantha* and *U. mutica* were not able to retake gas exchanges at the original levels observed before the introduction of stress.

Materials and methods

Plant materials

For the development of the study we used pelletized seeds of *Urochloa ruziziensis*, with a cultural value of 60%, being a grass of tropical climate, with average height of 1.5 m, a habit of growing decaceous rook and high capacity of dry mass.

Experimental design and Treatments

The work was conducted under a randomized block design, consisting of five treatments and four replications. The treatments were represented as follows: Irrigated control without *A. brasilense* inoculation; Non-irrigated control without inoculation*; A. brasilense* seed inoculation without irrigation; *A. brasilense* foliar spraying without irrigation; *A. brasilense s*eed inoculation + folair spraying without irrigation.

Conduction of experiment

The experiment was conducted in pots with nominal capacity of 8.7 liters, which contained soil from the Oxisol A horizon (Table 1), with a natural population of diazotrophs at $4x10^5$ CFU g^{-1} soil (Dödereiner et al., 1995).

The application of *A. brasilense* strain AbV5 + AbV6 on seeds of *U. ruziziensis* was performed at a dose of 1 mL inoculum ($2x10^8$ CFU mL^{-1}) for 1000 seeds, which were homogenised and kept in the shadow for about 30 minutes before sowing. After sowing, pots were kept under field conditions, with daily replenishment of water. Two plants were kept per pot, without nutrient application throughout the experiment. The *A. brasilense* foliar spraying on was carried out when the plants were at the early tillering, approximately 20 days after sowing, with a dose of 300 mL ha^{-1} of inoculum and spray volume of 300 L ha^{-1}, with the aid of a CO_2 sprayer with constant pressure 40 kgf cm^{-2}.

At 45 days after sowing (DAS) was imposed water deficit. All pots were watered before to start drought, to reaching the field condition. Treatments of water deficit had their irrigation suspended, and kept under water restriction for six days when the plants, in at least one treatment, reached the photosynthetic rate near zero, after this all out rehydrated (Supplementary Fig 1, 2, 3 and 4). The plants were rehydrated by watering the pots until they reached the field capacity, similar to initial moisture content of the experiment

(Supplementary Fig. 1a). The evalution perform in the moderate water stress (plants suffer from water deficit during the day and recover slowly at night) , in the severe water stress (plants suffer from water deficit during the day and do not fully recover overnight) and rehydration was evaluated until plants showed photosynthetic rate close to the initial condition, 3 days after irrigation (Supplementary Fig 2, 3 and 4). In the treatment without water deficit (control irrigated), pots were periodically irrigated with adequate water supply, based in gravimetric moisture initial (Fig 1), replacing water to maintain the gravimetric moisture level of the previous day. Was determined daily, at predawn, a sample of the soil profile contained in each pot was taken, weighed at the time of collection (U1), and dried in a forced air circulation oven at 105 °C for 24 h, and then weighed again (U2). The gravimetric moisture was obtained by: UG (kg kg^{-1})=U1-U2/U2.

Determination of relative leaf water content (RWC)

The determination of RWC was performed daily at predawn by collecting 1.5 cm² leaf samples located in the middle of the plant. This, which were weighed at the time of collection, after they were taken for hydration for at 25 °C for six hours, to obtain turgid fresh weight and subsequently taken to a forced air circulation oven at 65ºC until constant weight to determine dry matter (DM) (Slavik, 1974).

Gas exchange

To examine the stress imposed about gas exchange, were determined daily using the equipment IRGA LI-6400XT (*Infra Red Gas Analyzer*) (*Licor Inc. Lincoln, NE*). Readings were taken in the morning between 09h00min and 11h00min using CO_2 ambiente content of 380 µmol mol^{-1}, in fully developed leaves, photosynthetically active and intact, located in the middle third.

We also determined net CO_2 assimilation rate (*A*), leaf transpiration rate (*E*); stomatal conductance (*gs*), internal CO_2 concentration (*Ci*), and the following ratios: *WUE* = *A*/*E*, *iWUE* = *A*/*gs* (Zhang et al., 2001), wherein *WUE* is the efficiency of water use, *iWUE* is the intrinsic efficiency of water use.

Statistical analysis

Statistical analysis was performed by F-test, and the means were compared by Tukey's test at 5% probability, using the software SISVAR.

Conclusion

A. brasilense foliar spraying and *A. brasilense* seed inoculation + foliar spraying is efficient to increase the tolerance of *U. ruziziensis* to water deficit. The plants that received *A. brasilense* on foliar spray, isolated or combined with seed inoculation, presented higher photosynthetic rates even under conditions of severe water deficit.

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