



## **Photosynthetic and Production of *Urochloa ruziziensis* Inoculated with *Azospirillum brasilense* under Drought**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Authors LGB and VFG prepared the study project. Authors LGB, AMI and AGB performed the analysis with IRGA, statistics and managed the writing of the manuscript. Authors RCJ and ADS managed the field and driving analyzes. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** The objective of this study was to evaluate the photosynthetic activity and production of *Urochloa ruziziensis* when inoculated with *Azospirillum brasilense* in the presence and absence of drought.

**Study Design:** Randomized block design and factorial 2x2.

**Methodology:** The first factor was the presence or absence of seed inoculation with *A. brasilense* strains AbV5 + AbV6; the second factor was the presence or absence of drought. The variables evaluated were: relative water content (RWC), soil gravimetric moisture, net assimilation rate of CO<sub>2</sub>, response in function of active photon flux density, apparent quantum efficiency, light compensation point, absolute integrity of membrane, damage to membranes, dry mass aborted leaves, and total dry mass of aerial part.

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**Results:** Results demonstrate that plants maintained in drought presented a reduction in all evaluated variables. Under conditions of drought the use of *A. brasilense* promoted smaller variations in RWC, net assimilation rate of CO<sub>2</sub>, apparent quantum efficiency, light compensation point, absolute integrity of membrane, and damage to membranes; no variations were observed for dry mass, aborted leaves or total dry mass of aerial part.

**Conclusion:** The inoculation of *U. ruziziensis* seeds with *A. brasilense* mitigates drought damage in plant physiology, but it does not mitigate leaf losses or plant productivity.

**Keywords:** Gas exchange; plant growth promoting bacterium; membrane damage; dry mass; net assimilation rate of CO<sub>2</sub>.

## 1. INTRODUCTION

Water is a resource essential for plant development, the abiotic factor that restricts crop productivity. Drought conditions are characterized by periods when plants can't replace the water lost to the atmosphere [1], resulting in lower turgor and a deficit in biochemical and physiological functions, such as photosynthesis [2].

Perennial crops are affected by droughts over the years. Grassland, besides being cultivated perennially, presents the aggravation of being grown on degraded soils with low fertility and without mineral supplementation [3]. Among the various genres of pasture, *Urochloa* is distinguished by high dry matter production [4], satisfactory levels of protein and fiber, and high adaptability to various soils and climates. Among the climatic factors, the drought demands quickly and momentary adaptation by plants [5].

Genetic improvement is the main tool used for expression of drought tolerance. However, it is a slow and expensive process restricting the higher value-added crops, such as corn and soybeans. Nevertheless, reports indicate that management practices may increase the tolerance of plants to drought, stimulating soil water maintenance [6], higher land capacity use [7], and better nutrition and hormonal balance of plants [8].

Based on the above, the use of growth promoting bacteria is widely studied, mainly the genus *Azospirillum*, which stimulates the action of active mechanisms in tolerance to drought [9,10]. Inoculation with *A. brasilense* promotes increases in the plant root system [11], production of dry matter [12], crop production [11], gas exchanges [13], and hormone regulation. Recent literacy indicates that this bacterial species also maximizes the plant's ability to tolerate saline environments [14].

Another aggravating point in drought conditions is the high incident light which leads to increased leaf temperature accompanied by stomatal closure, causing degradation of chlorophyll and reducing the saturation point of photosynthetic activity [15].

Thus, the present study had as objective to evaluate the photosynthetic activity and production of *Urochloa ruziziensis* when inoculated with *Azospirillum brasilense* in the presence and absence of drought.

## 2. MATERIALS AND METHODS

The experiment was conducted in a greenhouse utilizing pots with a nominal capacity of 8.7 L and using as soil a substrate from horizon A Hapludox eutrophic soil, under a randomized block design and factorial 2x2 with five replications. The first factor was the presence or absence of seed inoculation with *A. brasilense* strains AbV5 + AbV6; the second factor was the presence or absence of drought.

The inoculation was performed with 1 mL inoculum ( $2 \times 10^8$  CFU mL<sup>-1</sup>) to 1000 seeds of *U. ruziziensis* (R.Germ. & C.M.Evrard), or  $2 \times 10^5$  CFU per seed. The seeds were homogenized by agitating them with the inoculant, keeping them in the shade for 30 minutes and sowing them. After they emerged, two plants of *U. ruziziensis* were kept in each pot, with daily replacement of water until field capacity. The plants were constantly monitored to ensure adequate development and that mineral supplementation wasn't necessary.

After 45 days of sowing, drought conditions were started. Therefore, all pots were previously irrigated to field capacity and the treatments with drought conditions had their irrigation suspended. Drought conditions were maintained until at least one of the treatments showed

inhibition of photosynthetic activity, which occurred on the sixth day of drought imposition.

Relative water content (RWC) and gravimetric soil moisture were evaluated. Known leaf area segments were collected and the RWC was determined by the difference between the wet mass, full content of water mass after 6 hours at 25 °C, and dry mass after 48 hours at 65°C [16]. Substrate samples were collected and the gravimetric soil moisture was determined by the difference between the mass at the time and the dry mass after 24 hours at 105°C.

The net assimilation rate of CO<sub>2</sub> (A) as a function of the light level, was determined using an IRGA (Infra-Red Gas Analyzer) model LI-6400XT (Licor Inc. Lincoln, NE). The readings were performed in the morning using a concentration of CO<sub>2</sub> of 380 μmol mol<sup>-1</sup>, a flow rate in the chamber of 500 μmol s<sup>-1</sup>, and a block temperature of 25°C. Evaluations were performed on fully developed leaves photosynthetically active and with no injuries located in the middle canopy, which were chosen randomly before drought imposition and marked, so that the same leaves would always be evaluated at the following photosynthetic photon flux density values (PPFD – Q) 0; 20; 40; 80; 150; 300; 400; 700; 900; 1200; 1600; 2000 and 2400 μmol m<sup>-2</sup> s<sup>-1</sup>, using the variable net CO<sub>2</sub> assimilation rate.

To calculate the apparent quantum efficiency (Φ [μmol CO<sub>2</sub>/μmol photons]) the following concentrations were used: 0; 20; 40; 80 and 150 μmol m<sup>-2</sup> s<sup>-1</sup> photon adjusting the equation (A = a + ΦQ) where a and Φ are coefficients, and Q represents the PPFD, where Φ is the inverse of the angular coefficient of the line. At the intersection of the line in the X-axis, the value of the light compensation point [Γ (μmol m<sup>-2</sup> s<sup>-1</sup>)] was established. The response curve of A as a function of PPFD was adjusted by the rectangular hyperbolic function (A = A<sub>max</sub>Q/“a” + Q), where maximum net assimilation rate of CO<sub>2</sub> (A<sub>max</sub>) “a” was an adjusted coefficient of the equation, and Q represents the PPFD.

In order to determine the percentage of absolute integrity of membrane and damage to membranes, leaf segments of 1.5 cm<sup>2</sup> were collected and washed with deionized water. Afterwards, the leaf segments were immersed in 50 mL of distilled and deionized water, conditioned in B.O.D. at 25 ° C for 24 hours, to then determine the electric conductivity. They were then sealed with aluminum foil and taken to

a water bath at 100°C for 1 hour, allowed to cool at room temperature to 25°C, and a new electrical conductivity reading [17] was taken.

The dry mass evaluation of aborted leaves and total dry mass of aerial part of plants were determined at the end of the evaluations on the sixth day. The dry mass of leaves aborted was determined by the collection of all the leaves that presented less than 30% of green area, located in the middle and lower third of the plants. In the end, the rest of the parts of the plants were collected to determine the total dry mass of aerial part. The samples were oven dried in forced air circulation at 65°C for 72 hours.

The RWC, soil moisture, absolute membrane integrity, membrane damage, dry mass of aborted leaves and total dry mass of aerial part were submitted to analysis of variance by the F test at 5% probability and when a significant difference was found, the data was compared using the Tukey test at 5% probability.

### 3. RESULTS AND DISCUSSION

Results showed an interaction between inoculation and drought for relative water content (RWC) and soil moisture. For the RWC the presence of drought led to a reduction of 39.9%, while the application of *A. brasilense* increased the RWC by 8.9%. Soil moisture was reduced in treatments with drought in 56%, and among the inoculated treatments none significant differences were observed (Table 1).

This result demonstrates that the plants used the water available in the vessels in a similar way; hence, the variations obtained for the RWC could possibly be linked to the better utilization of the water contained in the foliar tissue. These results can be explained in the literature, which reports that plants inoculated with *A. brasilense* use the water contained in the foliar tissues better in order to remain hydrated for a longer period [13,18,19], qualifying this condition as an alternative treatment to increase the tolerance of plants to drought in relation to their physiological variations. This condition is reported in the literature as a signaling effect, as a result of the inoculation with plant growth promoting bacteria. The process occurs via the stimulation of production and the release of compounds such as, the plant's hormones, particularly auxin and abscisic acid, but also compounds such as osmolytes betaines, proline and amino acid [20], that signal the lack of water.

**Table 1. Relative leaf water content (RWC) and soil moisture with *Urochloa ruziziensis* inoculated with *Azospirillum brasilense* in the presence or absence of drought**

	RWC (%)			Soil moisture (%)		
	With drought	Without drought	Means	With drought	Without drought	Means
Control	49.48 bB	92.58 aA	71.03 b	15.00 aB	36.52 aA	25.76 a
<i>A. brasilense</i>	62.37 aB	93.58 aA	77.98 a	17.10 aB	36.42 aA	26.78 a
Means	55.92 B	93.08 A		16.05 B	36.47 A	
C.V. (%)	7.20			7.58		

\*Means followed by the same lowercase letter in the column and upper case in the row do not differ from each other by the Tukey test 5% probability

Although, these effects are not completely elucidated, several papers have been reporting such condition. In corn plants, inoculation of *A. lipoferium* increased the concentration of amino acids, proteins, proline and sugars, and the use of plant growth promoting bacteria showed promising effects and may help the crop tolerate the lack of water [21].

Therefore, the plants inoculated with *A. brasilense* presented higher RWC in comparison with the control plants. This condition allowed these plants to maintain higher values of net assimilation rate CO<sub>2</sub> ('A') when compared to the control. The results of 'A' obtained in the dry control did not adjust to the proposed response curve, presenting an average net assimilation rate of CO<sub>2</sub> of -0.035 μmol m<sup>-2</sup> s<sup>-1</sup>; that is, the plants were consuming photo assimilates to survive the condition of drought, being that the net assimilatory rate of CO<sub>2</sub> (*A*<sub>max</sub>) obtained in the different luminosities reached 0.21 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 1a). In turn, the plants inoculated with *A. brasilense* reached a greater value, *A*<sub>max</sub> 4.57 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 1b); which means that, even though, the plants showed a reduction in the net assimilation rate of CO<sub>2</sub> when compared to the irrigated treatments, which presented a control value of 27.36 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 1c) and when inoculated, a value of 26.14 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 1d), the inoculation of seeds with *A. brasilense* under conditions of drought and maintaining a greater photosynthetic activity was possible.

In drought conditions, photosynthesis becomes limited due to the need to break the water molecule to electronic excitation, releasing electrons which are used as initial acceptors in the production of ATP [1]. Another condition that leads to the reduction of *A*<sub>max</sub> is associated to the limitation of CO<sub>2</sub> in the substomatal chamber caused by stomatal closure. Therefore, plants with higher RCW can maintain greater diffusion of CO<sub>2</sub> [22].

The high photosynthetic rates obtained for the irrigated plants are related to the photosynthetic efficiency of *U. ruziziensis*, it being a C<sub>4</sub> with high photosynthetic capacity and very responsive to the elevation of luminosity when under adequate conditions.

Obviously, plants grown under water limited conditions reduced their photosynthetic rate, but the use of inoculation of seeds with *A. brasilense* resulted in the mitigation of the effects of drought. Results showed that the control plants had a reduction of *A*<sub>max</sub> in 99.23% and 99.18% compared to the irrigated control plants and the irrigated plants with seed inoculation, respectively. In turn, the inoculation of the seeds in water deficit decreased by *A*<sub>max</sub> in 83.3% and 82.5% compared to irrigated control plants and irrigated plants with seed inoculation, respectively. When comparing the treatments kept in drought, controls presented *A*<sub>max</sub> 95.4% less than the plants with inoculated with *A. brasilense*.

Due to the changes fostered in 'A' the apparent quantum efficiency (Φ) in dry control was elevated, 169.49 μmol photons μmol CO<sub>2</sub>, while in seed inoculated treatments this value was 37.78 μmol photons μmol CO<sub>2</sub>, that is, a mitigation of 77.7%. When comparing Φ of the plants with and without drought, it was observed that the dry control had an elevation of 1147% and 1186% compared to the irrigated control plants and the irrigated plant with seed inoculation, respectively, while the use of seed inoculation resulted in an elevation of 228.7% and 236.5% when compared to the irrigated control plants and the irrigated plants with seed inoculation, respectively.

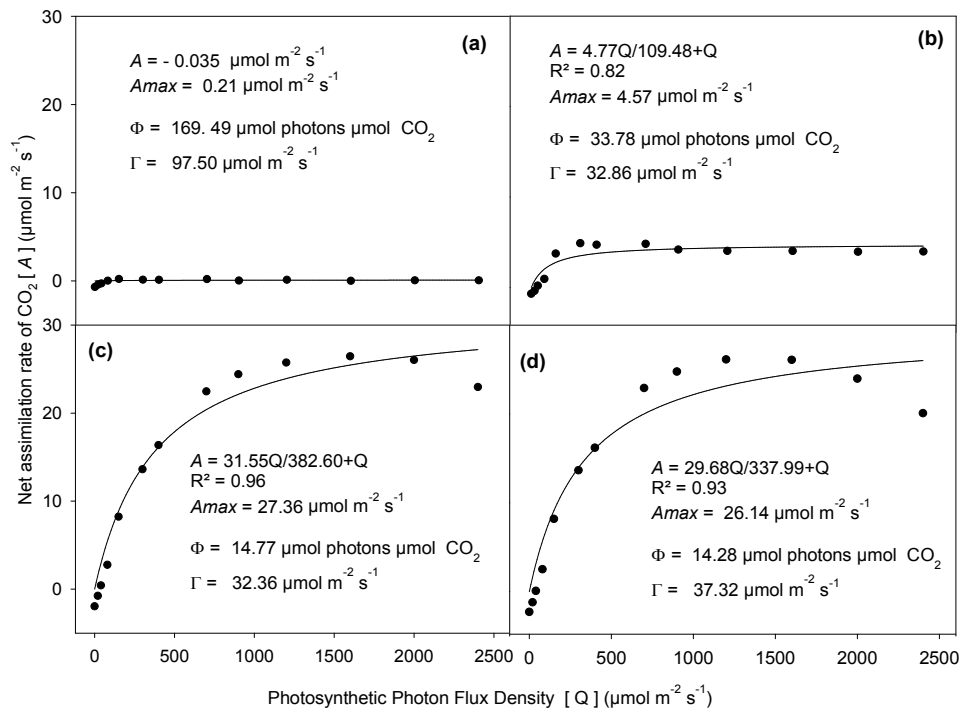
The elevation of Φ in plants evidences that the photosynthetic apparatus is disordered, so that the efficiency in the use of ATP and NADPH in

the Calvin cycle is impaired. In this sense, [23], results report that the elevation of  $\Phi$  also represents a deficiency in the use of light energy in the photochemical reactions of photosynthesis. The elevation of  $\Phi$  is a consequence of the limitation of  $\text{CO}_2$  by stomatal closure [24], resulting in a lower efficiency in fixing the  $\text{CO}_2$  with greater light energy expenditure to fix a molecule of  $\text{CO}_2$ , as a result of the drought increasing the photorespiration.

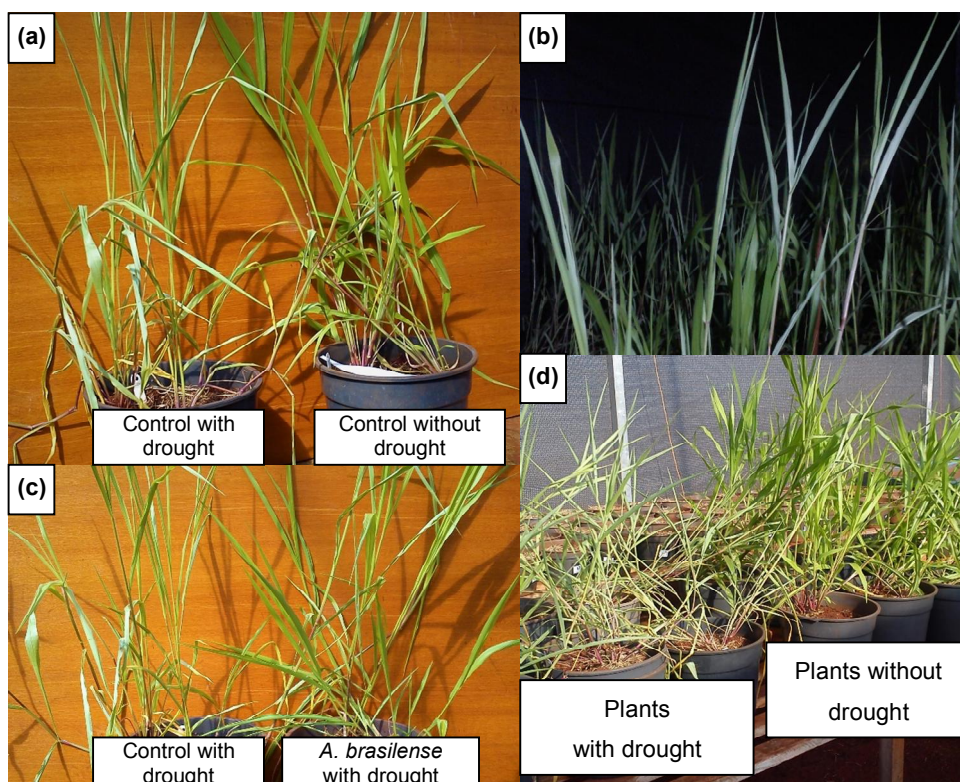
The evaluation of the light compensation point ( $\Gamma$ ) confirms the results obtained when studying 'A', which indicated that dry control plants were consuming stored photo assimilates. Accordingly, [25] results reported that the increase of  $\Gamma$  indicates that for plant respiration to overcome dry conditions of drought, control plants and seed inoculated plants needed to use the accumulated photo assimilates, which explains why they did not differ in dry mass production per plant (Table 3). Thus, dry control plants showed a  $\Gamma$  of  $97,50 \mu\text{mol m}^{-2} \text{s}^{-1}$ , while those plants inoculated with *A. brasilense* had values of  $32.86 \mu\text{mol m}^{-2} \text{s}^{-1}$ , similar to those of plants irrigated, that is, a reduction of 66.3%.

The occurrence of water deficiency associated with the reduction of the net assimilation rate of  $\text{CO}_2$  leads to an inadequate functioning of the biochemical and physiological systems of the plants, resulting in the degradation of membranes. Thus, when absolute membrane integrity was assessed (AMI) it was observed that the plants inoculated with *A. brasilense*, independently of the presence or absence of drought, promoted higher values, surpassing the control plants in 3.45%. When observing the treatments kept in water limitation, plants inoculated were higher than the control by 6.9% (Table 2).

Similarly, to the AMI, membrane damage showed that plants maintained in water deficit suffered greater damage (31%) than those absent from water limitation. When observing the treatments in drought, greater damages to the membrane were observed in the control, surpassing the inoculated plants by 22.8%. The control plants presented lower AMI and greater membrane damage in the presence of water deficiency, while those inoculated with *A. brasilense* did not differ among themselves in the presence or absence of drought.



**Fig. 1. Net assimilation rate of  $\text{CO}_2$  *Urochloa ruziziensis* inoculated with *Azospirillum brasilense* in the presence or absence of drought. (a) Control with drought; (b) Seed inoculation *A. brasilense* with drought; (c) Control without drought; (d) Seed inoculation *A. brasilense* without drought**



**Fig. 2. Visual comparison between plants evaluated under conditions of severe drought. (a) Control with drought and control without drought; (b) Absence of nocturnal recovery in plants with drought preceding the evaluation; (c) Control with drought and inoculation *A. brasilense* with drought; (d) Overview of plants under drought conditions and without drought**

**Table 2. Absolute membrane integrity and membrane damage *Urochloa ruziziensis* inoculated with *Azospirillum brasilense* in the presence or absence of drought**

	Absolute membrane integrity (%)			Membrane damage (%)		
	With drought	Without drought	Means	With drought	Without drought	Means
Control	0.81 bB	0.86 aA	0.84 b	18.37 aA	13.52 aB	15.95 a
<i>A. brasilense</i>	0.87 aA	0.88 aA	0.87 a	14.96 bA	11.93 aA	13.44 a
Means	0.84 A	0.87 A		16.67 A	12.72 B	
C.V. (%)		3.10			26.29	

\*Means followed by the same lowercase letter in the column and upper case in the row do not differ from each other by the Tukey test 5% probability

The degradation of membranes in plants occurs among other factors by the formation of free radicals, basically in the form of reactive oxygen species (ROS) which are formed in plants when exposed to stress, in this case drought [1]. The production and inactivation of ROS in the plant occurs steadily, keeping the balance. When the plant is under drought it loses the capacity of inactivation due to the deregulation of the enzymatic activity, leading to the ROS degrading the membranes of the cell with extravasation of

the cellular content and consequently causing the death of the cell. The variations exposed in this study suggest that the use of *A. brasilense* can positively interfere with membrane inertness. This result is a reflection of the hormonal stimuli. Since this characteristic in stressed plants is regulated by the activity of abscisic acid and cytokinin [8,26], the cytokinin produced by *A. brasilense* [27,28], as well as the abscisic acid [29] results in lower membrane degradation.

**Table 3. Dry mass of aborted leaves and total dry mass of aerial part *Urochloa ruziziensis* inoculated with *Azospirillum brasilense* in the presence or absence of drought**

	Dry mass of aborted leaves			Total dry mass of aerial part		
	(g plant <sup>-1</sup> )					
	With drought	Without drought	Means	With drought	Without drought	Means
Control	1.20 aA	0.81 aB	1.005 a	9.43 aB	10.03 aA	9.73 a
<i>A. brasilense</i>	1.20 aA	0.89 aB	1.045 a	8.71 aB	10.63 aA	9.67 a
Means	1.20 A	0.85 B		9.07 B	10.33 A	
C.V. (%)		18.09			7.98	

\*Means followed by the same lowercase letter in the column and upper case in the row do not differ from each other by the Tukey test 5% probability

The variations observed in the physiological system and the damage caused to the membranes of the plants of *U. ruziziensis* come as a result of the response to the morphological levels in the form of abortion of plant organs and consequently the reduction in the production of dry mass.

The plants kept in water restriction had a greater leaf abortion, being this value 41.2% superior compared to the irrigated plants. In a similar way, the total dry mass of aerial part was superior without drought in 12.2%. When considering the interaction of the factors no significant differences in the treatments were observed, and it was verified that, regardless of the treatment being in the presence of drought, the leaf abortion was superior and the total dry mass of aerial part was lower (Table 3).

Although, inoculation with *A. brasilense* promoted beneficial effects on RWC, net assimilation rate of CO<sub>2</sub> and cell membrane preservation, such effects were not able to mitigate leaves abortion and reduction in dry plant mass.

This condition is possibly due to the aggressiveness of the effects of drought in plant physiology. The incidence of drought leads to a morphological response in the plants that culminate, in advanced stages, in the abortion of plant organs, causing a reduction in the production of dry mass of aerial part and an increase in the mass of aborted leaves. In this sense, it is demonstrated in a study with forage plants that the incidence of water deficiency leads to a reduction in dry mass productivity due to leaf abortion and lower physiological plant activity, which results in a reduction of green leaf blades [4], condition also observed in the present study.

In this way, the inoculation of the seeds of *U. ruziziensis* is shown as an option to mitigate the damages caused by the occurrence of drought on the physiological system, however, not alleviating the losses in dry mass production of aerial part.

#### 4. CONCLUSION

The inoculation of seeds of *Urochloa ruziziensis* with *Azospirillum brasilense* raises the relative water content, with smaller reduction in the net assimilation rate of CO<sub>2</sub> and a decrease of the cellular membranes damage due to the incidence of water deficit.

However, it does not reduce the abscission of plant organs or increase the production of dry mass of aerial part.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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