The Arbuscular Mycorrhizal Fungus *Rhizophagus Clarus* Improve Physiological Tolerance to Drought Stress in Soybean Plants

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Abstract

Soybean (*Glycine max* L.) is an economically important crop worldwide. However, increasingly long periods of drought have reduced the productivity of this crop. Studies have shown that inoculation with arbuscular mycorrhizal fungi (AMF) provides a potential alternative strategy for mitigating drought. In the present study, we measured the physiological and morphological performance of two soybean cultivars under drought in symbiosis with *Rhizophagus clarus*. Soybean plants Anta82 and Desafio, were grown in pots previously inoculated with *R. clarus*. Water deficit (WD) was imposed at the V3 development stage and maintained for 7 days. A control group was performed in parallel with well-irrigated plants in the absence of *R. clarus* in a greenhouse. Three and seven days after the WD imposition the analysis were performed. Cultivar Anta82 showed a higher percentage of colonization, N and K leaf content, whereas Desafio, showed higher water potential, water-use efficiency under WD, and thermal dissipation that allowed higher values for F_v/F_m , A , and PH under WD+AMF. The Principal Components Analysis results were able to demonstrate that both cultivars in water deficit with AMF colonization clustered together with well-watered plants. These findings suggest that AMF had an effect on plants in order to reduce drought physiological impairment.

Introduction

Soybean [*Glycine max* (L.) Merrill] is the most important economic legume in the world, mainly because it is an abundant source of vegetable oil and protein¹, as the principal ingredient in several products and byproducts. Soybean grains can be used as food and animal feed²; in chemical industries, as well as for the production of biofuel production³.

Soybean cultivation has been increasing over the years. Nevertheless, frequent periods of water deficit have caused significant losses in crop productivity because of increasing climate instability^{4–6}. Climate changes are attributed to rising global surface temperatures of 1.8 to 3.6°C by the end of this century, making them one of the greatest challenges facing society⁷.

This abiotic stress directly interferes with primary metabolism, where it usually causes inhibition of photosynthesis as a result of reduced CO₂ assimilation due to stomatal closure^{8–10}, compromising plant growth and yield^{11,12}. The impacts caused by the water deficit depend on the intensity and frequency of stress, in addition to the plant tolerance.

To mitigate water deficit, different strategies have been sought, among which, the association of arbuscular mycorrhizal fungi (AMF) with plants may protect the latter against the effects of water deficit. AMFs are linked to soil properties favoring the porosity that are important for nutrient and water flow^{13,14}. The formation of arbuscular structures inside the roots of host plants provides pathways for nutritional exchange between the fungi and host¹⁵; in addition, the hyphae modulate water transport between the

soil and the plant^{16,17}. It increases the efficiency of water use as well as the maintenance of plant metabolism, even in environments with low water availability^{18–21}.

Higher plant productivity has been reported when associated with AMF in soil under water deficit^{22–24}. The use of these organisms is a potential strategy for increasing crop productivity in agricultural production and also for ecosystem sustainability^{25–26}. The objective of this work was to evaluate the morphophysiological and nutritional performance of soybean cultivars under water deficit in symbiosis with FMA *Rhizophagus clarus* (= *Glomus clarum*²⁷) as an alternative to mitigate the drought effects in Brazilian Cerrado and ensure food security.

Material And Methods

Plant material and experimental conditions. The experiment was carried out in an acclimatized greenhouse (~27 °C and ~75 % of relative humidity) at the Laboratory of Ecophysiology and Plant Productivity at the Goiano Federal Institute of Science and Technology, Rio Verde Campus, Goiás, Brazil. Soybean plants (*Glycine max* (L.) Merrill), cultivar Anta 82 RR (Anta82; Geneze Seeds, São Paulo, Brazil) and Desafio 8473 RSF (Desafio; Brasmax Sementes, Cambé, PR, Brazil) were grown in 3-dm³ pots containing a mixture of Red Latosol (LVdf) soil and sand (2:1) previously corrected with dolomitic limestone PNRT 100 for 60 % of base saturation. The substrate was fertilized based on the substrate chemical analysis (Supplementary Table S1) and recommendation for Cerrado soils²⁸.

The strain of the AMF *Rhizophagus clarus* was obtained by a donation from the germplasm bank of the Soil Microbiology Laboratory at the São Paulo State University, Ilha Solteira campus, Ilha Solteira, São Paulo, Brazil. (UNESP). For inoculum multiplication, the substrate composed of a mixture of soil with sand (2: 1) was initially sterilized in cotton bags in an autoclave by 121 °C at 1.5 atm, in two periods of two hours, on alternate days. Then, the substrate was dried in an oven at 105 °C for 24 h and moistened for the same period with distilled and sterilized water^{29–30}. Plastic pots (1 dm³) containing sterilized substrate received approximately 10 g inoculum and *Sorghum bicolor* were sowed as host plants. After three months, the soil was collected and stored in ultrafreezer for further use in the experiment. The number of spores was determined in a spore sample that was counted on an acrylic plate with concentric rings, under a stereomicroscope (SteREO Discovery.V8, Zeiss, Göttingen, Germany) according to Gerdemann and Nicolson³¹ and Jenkins³². The spores were stored at ultrafreezer.

For the experiment, AMF *Rhizophagus clarus* was inoculated into the seeding hole at 10 g of *R. clarus* containing 3.5 g spores in 3 dm³-pot during the soybean sown³³. Water treatments were imposed when the plants reached the vegetative developmental stage (V3) at 60 % of holding water capacity, using the gravimetric method. The treatments were: well-watered plants (WW); well-watered plants inoculated with *R. clarus* (AMF); water deficit (WD); and WD-treated plants inoculated with *R. clarus* (WD+AMF). Evaluations were performed on the 3rd and 7th days after treatment imposition. The experimental design was in randomized blocks, with four replicates.

Mycorrhizal colonization rate. Mycorrhizal colonization rate was determined according to Koskey and Gemma³⁴. Root samples (~0.4 g) were collected and stored in a 50 % ethanol solution. For the analysis, the samples were initially immersed in 2 % KOH, heated in a stove at 90 °C for 120 minutes, and washed in distilled water. Subsequently, the samples were immersed in 1 % HCl for 30 minutes, water-washed, and stained with 0.05% Trypan blue in lactoglycerol solution (1:1:1 lactic acid, glycerol, water) at 90 °C for 10 min³⁵. Roots fragments were mounted on microscope slides and observed at a magnification of 200x using a microscope Olympus (BX61, Olympus, Tokyo, Japan) to determine the percentage of roots colonized by the fungus³⁶.

Water potential. The predawn leaf water potential (Ψ_w) was measured using a Scholander pressure chamber (3005-1414, Soil moisture Equipment Corp., Goleta, CA, USA).

Physiological traits. Gas exchange was measured in fully expanded leaves to determine the net photosynthetic assimilation rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and the ratio between internal and external CO_2 concentrations (C_i/C_a). The measurements were performed using an infrared gas analyzer (IRGA; LI-6400xt, Licor, Lincoln, NE, USA). The instantaneous water-use efficiency (WUE, $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$) was calculated as the ratio between A and E . The measurements were conducted under constant photosynthetic photon flux density (PPFD, $1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and at environmental atmospheric CO_2 concentration (C_a , $\sim 400 \mu\text{mol mol}^{-1}$), temperature ($\sim 25 \text{ }^\circ\text{C}$), and relative air humidity ($\sim 50 \%$).

Chlorophyll *a* fluorescence traits were evaluated using a 6400-40 LCF fluorometer coupled to the IRGA. The leaves were initially dark-acclimated for 40 min to obtain the minimal (F_0) and maximal chlorophyll fluorescence (F_m) values and to calculate the maximum quantum yield of photosystem II (PSII) [$F_v/F_m = (F_m - F_0)/F_m$]. Then, the leaf tissues were exposed to actinic light and a saturating pulse to obtain the steady-state fluorescence (F) and the maximum fluorescence in a light-adapted state (F_m'), respectively. This allowed determination of the effective quantum yield of PSII [$Y_{II} = (F_m' - F)/F_m'$] and non-photochemical quenching [$\text{NPQ} = (F_m - F_m')/F_m'$]. The Y_{II} was used to calculate the electron transport rate ($\text{ETR} = Y_{II} \cdot \text{PPFD} \cdot \text{Leaf}_{\text{ABS}} \cdot 0.5$), where PPFD is the photon flow ($\mu\text{mol m}^{-2} \text{ s}^{-1}$) on the leaves, Leaf_{ABS} is the fraction of incident light that is absorbed by the leaves, and 0.5 is the excitation energy fraction directed to the PSII.

Photosynthetic pigments were extracted from leaf discs ($\sim 2\text{-cm}^2$) in dimethyl sulfoxide solution saturated with calcium carbonate in a $65 \text{ }^\circ\text{C}$ water bath. After 24 h, the solution absorbance was read at 480, 649.1, and 665.1 nm using a UV-VIS spectrophotometer (Evolution 60S, Thermo Fisher Scientific, Madison, WI, USA). Chlorophyll *a* ($\text{Chl}a = 12.4 \cdot A_{665.1} - 3.62 \cdot A_{649.1}$), chlorophyll *b* ($\text{Chl}b = 25.06 \cdot A_{649.1} - 6.50 \cdot A_{665.1}$) and total carotenoid ($\text{Carot} = [1000A_{480} - 1,29 \text{ Chl}a - 53.78 \text{ Chl}b]/220$) were expressed as $\mu\text{g cm}^{-2}$.

Morphological traits and nutrient content. The plants were measured to determine the plant height (PH, cm) and stem diameter (SD, mm). Leaves were separated to obtain the leaf area (LA, cm²). Leaves and roots were dried in a forced-air circulation oven at 65 °C until constant weight to obtain the leaf dry matter (LDM, g) and root dry matter (RDM, g).

To quantify the nutrient contents in leaves and roots, the dry plant material (~500 mg) was extracted with nitric-perchloric (3:1) digestion according to Embrapa³⁷. Nitrogen (N) was measured via titration by Kjeldahl method using a nitrogen distiller (TE-0364, Tecnal, Piracicaba, Brazil). Phosphorus (P) and sulfur (S) were determined via molybdenum blue and turbidity with barium chloride methodologies, respectively, using molecular absorption spectrophotometry (SP1105, Tecnal, Piracicaba, Brazil). Potassium (K) was analyzed using flame photometry (B462, Tecnal, Piracicaba, Brazil) and Ca and Mg using atomic absorption spectrophotometry (SavantAA, GBC Scientific Equipment, Braeside, Australia).

Statistical Analysis. The data were subjected to analysis of variance (ANOVA) and the means were compared by the Tukey test ($p < 0.05$) using SISVAR software (v. 5.6, Lavras, MG, Brazil).

Principal Components analyses (PCA) was performed in the dataset using both cultivars and separated by cultivar. These analyses used the *FactoMineR*³⁸ and *factoextra*³⁹ packages in software R⁴⁰. First, data was scaled using the *scale* function and the analyses were performed using *PCA* function. Eigenvalues were evaluated to determine the number of dimensions to be evaluated. We considered the graphical approach based on the screen plot, using Cattell's rule that states that components corresponding to eigenvalues to the left of the straight line (eigenvalues corresponding to random variation lie on a straight line) should be retained⁴¹. To better understand the variables representation in each component, we used the *fviz_contrib* and *dimdesc* functions, which provides the variable's contribution to each component and correlation with each component, respectively.

Ethics approval and consent to participate. We all declare that manuscripts reporting studies do not involve any human participants, human data, or human tissue. So, it is not applicable.

Complies with international, national and/or institutional guidelines. Experimental research and field studies on plants (either cultivated or wild), comply with relevant institutional, national, and international guidelines and legislation.

Results

Mycorrhizal Colonization. The cultivar Anta82 WD+AMF-treated plants showed higher difference in the percentage of colonization between treatments at 3 days (6.66 %) ($F_{\text{Anta3.15}} = 9.22$; $p = 0.0019$) (Fig. 1A). At seven days ($F_{\text{Anta3.15}} = 84.25$; $p < 0.0001$), a colonization percentage of 14.67 % (WW+AMF) and 24.0 % (WD+AMF), higher than WW (4.34 %) and WD (3.34 %) treatments. In cultivar Desafio, there was a tendency for a slight increase in mycorrhizal colonization in DW+AMF plants at 7 days (12.0 %)

($F_{\text{Desafio3.15}} = 4.64$; $p = 0.0223$) (Fig. 1B). The presence of arbuscules by mycorrhizal colonization in the root cortex is shown in figure 2.

Water relations. Soybean plants of cultivar Anta82, under water deficit (WD), showed water potential of -0.94 MPA after 3 days of treatments ($F_{\text{Anta3.15}} = 10.51$; $p = 0.0011$). Anta82 WW-, AMF- and WD+AMF-treated plants showed Ψ_w of -0.19 , -0.19 and -0.31 MPa, respectively (Fig. 3). Seven days after water treatment imposition ($F_{\text{Anta3.15}} = 13.53$; $p = 0.0004$), WD+AMF plants showed Ψ_w (-0.51 MPa) similar to AMF (-0.22 MPa) and WW (-0.26 MPa) plants, and with higher values for WD-treated plants (-0.95 MPa) (Fig. 3).

Desafio cultivar WW-plants after 3 days ($F_{\text{Desafio3.15}} = 38.85$; $p = 0.0001$) showed Ψ_w (-0.27 MPa) similar to that of AMF (-0.23 MPa) and WD (-0.27 MPa) plants, differing only from WD plants (-0.24 MPa). After 7 days ($F_{\text{Desafio3.15}} = 32.12$; $p = 0.0001$), WD+AMF treatment showed Ψ_w of -0.54 MPa, and WD treatment -0.79 MPa (Fig. 3B).

Photosynthetic traits. Photosynthetic rates (A) were significantly altered in the Anta82 cultivar in relation to the treatments with the same behavior at 3 days ($F_{\text{Anta3.15}} = 4.214$; $p = 0.0298$) and 7 days ($F_{\text{Anta3.15}} = 4.80$; $p = 0.0201$). After 3 days WW+AMF showed ($7.19 \mu\text{mol m}^{-2} \text{s}^{-2}$), WW ($5.37 \mu\text{mol m}^{-2} \text{s}^{-2}$), WD+AMF ($5.14 \mu\text{mol m}^{-2} \text{s}^{-2}$) e ($4.22 \mu\text{mol m}^{-2} \text{s}^{-2}$) (Fig. 4A). The stomatal conductance (g_s) was lower in WD-treated plants than in WW plants at three days (Fig. 4C). Transpiration rate (E) was significantly altered at 7 days ($F_{\text{Anta3.15}} = 18.93$; $p = 0.0001$). After 7 days WD treatment showed lower values ($0.86 \text{ mmol m}^{-2} \text{s}^{-2}$) and WW ($3.43 \text{ mmol m}^{-2} \text{s}^{-2}$) showed higher values followed by WW+AMF ($2.93 \text{ mmol m}^{-2} \text{s}^{-2}$) and WD+AMF treatment ($2.08 \text{ mmol m}^{-2} \text{s}^{-2}$) (Fig. 4E). The ratio C_i/C_a (~ 0.79) were not affected by the treatments. WUE was significantly altered at 7 days ($F_{\text{Anta3.15}} = 4.42$; $p = 0.0258$). The WD treatment showed higher value (5.66), followed by the WW+AMF (2.61) and WD+AMF (2.76), the WW treatment showed lower value (1.77) (Fig. 4G).

Photosynthetic rates (A) were significantly altered in the Desafio cultivar in relation to the treatments with the same behavior at 3 days ($F_{\text{Desafio3.15}} = 14.206$; $p = 0.0003$) and 7 days ($F_{\text{Desafio3.15}} = 18.06$; $p = 0.0001$) (Fig. 4B). At seven days there was an increase in inoculated plants WW+AMF ($8.53 \mu\text{mol m}^{-2} \text{s}^{-2}$) and WD+AMF ($7.03 \mu\text{mol m}^{-2} \text{s}^{-2}$) compared to that of WW ($4.82 \mu\text{mol m}^{-2} \text{s}^{-2}$) and WD ($3.31 \mu\text{mol m}^{-2} \text{s}^{-2}$) (Fig. 4B). The g_s showed differences between treatments with the same behavior after 3 days ($F_{\text{Desafio3.15}} = 13.59$; $p = 0.0004$) and 7 days ($F_{\text{Desafio3.15}} = 14.97$; $p = 0.0002$) (Fig. 4D). After 7 days WW+AMF treatment showed higher value ($0.26 \text{ mmol m}^{-2} \text{s}^{-2}$) followed by WW ($0.21 \text{ mmol m}^{-2} \text{s}^{-2}$), WD+AMF ($0.17 \text{ mmol m}^{-2} \text{s}^{-2}$), the WD treatment showed lower values ($0.09 \text{ mmol m}^{-2} \text{s}^{-2}$) (Fig. 4D). The E rate showed the same behavior as the g_s parameter at 3 days ($F_{\text{Desafio3.15}} = 8.67$; $p = 0.0025$) and 7 days ($F_{\text{Desafio3.15}} = 12.427$; $p = 0.0005$) (Fig. 4F). After 7 days WD plants showed lower value ($1.19 \text{ mmol m}^{-2} \text{s}^{-2}$) than in the other treatments (Fig. 4F). The C_i/C_a ratio was lower in WD ($0.63 \text{ mmol m}^{-2} \text{s}^{-2}$) plants than in WW ($0.78 \text{ mmol m}^{-2} \text{s}^{-2}$) plants and WW+AMF ($0.77 \text{ mmol m}^{-2} \text{s}^{-2}$) and WD+AMF ($0.72 \text{ mmol m}^{-2} \text{s}^{-2}$)

²) after 7 days (Fig 4H) The WUEs were higher in inoculated plants after 7 days ($F_{\text{Desafio3.15}} = 7.47$; $p = 0.0044$), WD+AMF (4.81) WW+AMF (4.02), than in WD (2.89) and WW (1.89), respectively (Fig. 4J).

Chlorophyll a fluorescence. The minimum fluorescence (F_0) was significantly altered in the Anta82 cultivar in relation to the treatments with the same behavior at 3 days ($F_{\text{Anta3.15}} = 4.160$; $p = 0.0309$) and 7 days ($F_{\text{Anta3.15}} = 11.19$; $p = 0.0008$). After 7 days the WW showed higher value (311.25) followed by WD+AMF (230), WW+AMF (221) and WD (197.3) (Fig. 5A). The maximum quantum yield of PSII (F_v/F_m) were not changed in cultivar Anta82 at three days of evaluation ($F_{\text{Anta3.15}} = 4.160$; $p = 0.0309$). At seven days ($F_{\text{Anta3.15}} = 11.195$; $p = 0.0008$), reductions in F_0 were observed in WD (0.65) the others treatment showed higher values as WW (0.75) and WW+AMF (0.76) followed by WD+AMF (0.72) treated plants (Fig. 5B). The effective quantum yield of PSII (Y_{II}) was significantly altered at 3 days ($F_{\text{Anta3.15}} = 6.731$; $p = 0.0065$) and 7 days ($F_{\text{Anta3.15}} = 7.818$; $p = 0.0037$), the electron transport rate (ETR) was significantly altered at 3 days ($F_{\text{Anta3.15}} = 7.358$; $p = 0.0047$) and 7 days ($F_{\text{Anta3.15}} = 7.341$; $p = 0.0437$). After 7 days the plant inoculated with AMF showed Y_{II} higher value WW+AMF (0.15), WD+AMF (0.14) followed by WW treatment (0.09), WD (0.08) (Fig. 5 C). After 7 days the plant inoculated with AMF showed ETR higher value WW+AMF (67.88), WD+AMF (62.33) followed by WW treatment (43.45), WD (39.10) (Fig. 5D).

The minimum fluorescence (F_0) was not significantly altered in Desafio cultivar at at 3 days ($F_{\text{Desafio3.15}} = 0.120$; $p = 0.9469$) and 7 days ($F_{\text{Desafio3.15}} = 1.107$; $p = 0.3845$). The maximum quantum yield of PSII (F_v/F_m) was not changed at seven days of evaluation. At 3 days ($F_{\text{Desafio3.15}} = 0.120$; $p = 0.9469$) reductions in F_v/F_m were observed in WD (0.59) and WW (0.64) and the others treatment showed higher values as WD+AMF (0.74) and WW+AMF (0.74) treated plants (Fig. 5D). The effective quantum yield of PSII (Y_{II}) was not significantly altered at 3 days ($F_{\text{Desafio3.15}} = 2.071$; $p = 0.1577$) and 7 days ($F_{\text{Desafio3.15}} = 0.240$; $p = 0.8668$). The electron transport rate (ETR) was significantly altered only at 7 days ($F_{\text{Desafio3.15}} = 6.078$; $p = 0.0093$) (Fig. 5H). After 7 days the plant inoculated with AMF showed ETR higher value WD+AMF (59.20), WW+AMF (54.53) followed by WW treatment (50.46) and then WD (33.45) (Fig. 5H). Non-photochemical quenching (NPQ) was higher in WD at 3 days and 7 days than in WW at three days for both cultivars ($F_{\text{Anta3.15}} = 71.326$; $p = 0.0001$) ($F_{\text{Desafio3.15}} = 9.428$; $p = 0.0018$) plants inoculated with AMF to both cultivars showed lower NPQ (Fig. 5E, F).

Chloroplastidic pigments. The total chlorophyll concentration was significantly altered in the Anta82 cultivar in relation to the treatments with the same behavior at 3 days ($F_{\text{Anta3.15}} = 11.16$; $p = 0.0009$) and 7 days ($F_{\text{Anta3.15}} = 14.39$; $p = 0.0003$) (Fig. 6A). After 7 days plants inoculated with AMF, WW+AMF ($33.01 \mu\text{g cm}^{-2}$), WD+AMF ($32.27 \mu\text{g cm}^{-2}$) showed the same behavior that WW plants ($30.37 \mu\text{g cm}^{-2}$); the plants without AMF submitted deficit water showed lower value ($14.36 \mu\text{g cm}^{-2}$) (Fig. 6A). The carotenoid concentration was significantly altered in the Anta82 cultivar in relation to the treatments at 3 days ($F_{\text{Anta3.15}} = 15.84$; $p = 0.0002$) and 7 days ($F_{\text{Anta3.15}} = 4.25$; $p = 0.0289$) (Fig. 6B). After 7 days plants WD+AMF showed higher value ($6.91 \mu\text{g cm}^{-2}$) followed by WW+AMF ($6.75 \mu\text{g cm}^{-2}$) and WW (6.35), plants without AMF submitted deficit water showed lower value ($5.47 \mu\text{g cm}^{-2}$) (Fig. 6B).

The total chlorophyll concentration was significantly altered in the Desafio cultivar in relation to the treatments with the same behavior at 3 days ($F_{\text{Desafio}3.15} = 31.95$; $p = 0.0002$) and 7 days ($F_{\text{Desafio}3.15} = 15.05$; $p = 0.0002$) (Fig. 6C). After 7 days plants WW showed higher value ($23.93 \mu\text{g cm}^{-2}$) followed by WW+AMF ($22.38 \mu\text{g cm}^{-2}$) the plants WD ($15.70 \mu\text{g cm}^{-2}$) and WD+AMF ($15.18 \mu\text{g cm}^{-2}$) showed the same behavior with lower chlorophyll concentration (Fig. 6C). The carotenoid concentration was significantly altered in the Desafio cultivar in relation to the treatments at 3 days ($F_{\text{Desafio}3.15} = 31.95$; $p = 0.0002$) and 7 days ($F_{\text{Desafio}3.15} = 15.05$; $p = 0.0002$) (Fig. 6D). After 7 days plants inoculated with AMF showed higher value WW+AMF ($7.13 \mu\text{g cm}^{-2}$), WD+AMF ($7.15 \mu\text{g cm}^{-2}$) compared with plants without AMF inoculation, WW ($4.77 \mu\text{g cm}^{-2}$) and WD ($3.90 \mu\text{g cm}^{-2}$) (Fig 6D).

Morphological traits. The plant height, stem diameter, leaf dry matter, and root dry matter was not significantly altered in the Anta82 cultivar in relation to the treatments with the same behavior at 3 days (Fig. 7A, C, G, I). After 3 days the parameter leaf area was significantly altered in the Anta82 ($F_{\text{Anta}3.15} = 5.02$; $p = 0.017$), plants WW+AMF showed higher value ($385.06 \mu\text{g cm}^{-2}$) followed by WD+AMF ($269.8 \mu\text{g cm}^{-2}$) and plants WW ($288 \mu\text{g cm}^{-2}$), plants WD showed the lower value (193.42) (Fig. 7E).

The plant height, stem diameter, leaf dry matter, and root dry matter was not significantly altered in the Desafio cultivar in relation to the treatments at 3 days (Fig. 7B, D, H, J). After 3 days the parameter leaf area was significantly altered ($F_{\text{Desafio}3.15} = 15.20$; $p = 0.0002$), plants WW+AMF showed higher value ($407.89 \mu\text{g cm}^{-2}$) followed by WW ($310.19 \mu\text{g cm}^{-2}$), WD ($231.37 \mu\text{g cm}^{-2}$) and plants WD+AMF (235.56) (Fig. 7F).

Principal Components analyses. The first two components explained 43.4 % and 46.9 % of the variance on Anta82 and Desafio data, respectively. PCA biplot of the first two dimensions was able to cluster WD from the other treatments (Fig. 8) for Anta data (Fig. 8A).

The first dimension of PCA with Desafio data, which explain 24.6 % of the variance, is not related to the treatments (Fig. 8B). And the second component was able to cluster WD at the negative side, WD_AMF and WW in the middle and AMF at the positive side. Variables (LDM, E , Ψ_w , Chl, Carot, S_{root} , g_s , A, ETR, RDM, Y_{II} , F_v/F_m , Mg_{leaf} , Nleaf, MCR, C_i/C_a , SD, PH, Ca_{leaf} , Ca_{root}) had positive and significant ($p < 0.05$) correlation with the first dimension of PCA with Anta data. Therefore, these variables decrease with the water deficit treatment. On the opposite, WUE, Mg_{root} , K_{root} , N_{root} and NPQ had negative significant correlation with PC1. Thus, these variables increase with water deficit in Anta cultivar. The Ψ_w , Carot, LDM, E , F_v/F_m , C_i/C_a , g_s , PH, LA, Nroot, ETR, Caleaf, WUE, Mg_{leaf} , and RDM had positive significant correlation with PC2 with Desafio data, while S_{root} and NPQ had negative correlations. Therefore, the first group of variables increase with AMF and decrease in WD treatments, respectively. Meanwhile S_{root} and NPQ increase with WD treatment and decrease with AMF in Desafio cultivar.

The third component explained 10.3 % and 9.3 % of the variance for Anta82 and Desafio data, respectively. PC3 was able to differentiate WW from the other treatments in both cultivars. For Anta82

data, PC3 also clustered WD+AMF. The K_{leaf} , Y_{II} , ETR, P_{leaf} , WUE, Carot, and N_{root} was positively correlated with PC3 using Anta data. Thus, these variables increase with WD+AMF and decrease with WW. F_0 , E and PH were negatively correlated with PC3 using Anta data. Therefore, these variables had the opposite pattern, increasing with WW and decreasing with WD+AMF. F_v/F_m , Carot, NPQ, M_{groot} , WUE, and M_{gleaf} were positively correlated with PC3, and K_{leaf} , RDM, and N_{leaf} were negatively correlated for Desafio data. As WW treatment was positioned at the negative side of the third dimension, the first set of variables decreased and the second one increases with WW treatment.

In general, PCA results were able to demonstrate that plants in water deficit with AMF colonization clustered together with well-watered plants for both cultivars, showing that AMF had an effect on plants in order to reduce drought physiological impairment. Moreover, drought impairment seems to be stronger in Anta than Desafio cultivar. Looking further in the third component for Anta82 data and the second component in Desafio data, it is possible to understand the physiological impact of AMF on plants independently of watering treatments.

Discussion

The Anta82 and Desafio soybean cultivars presented differential responses to the water treatments imposed. Inoculation by arbuscular mycorrhizal fungi (AMF) *Rhizophagus clarus* favored the maintenance or increase of water status and/or metabolic activities in soybean plants, mainly in the cultivar Desafio.

Mycorrhizal colonization is the main means of symbiosis with the plant⁴², favoring both organisms involved. The colonization potential depends on the fungus and the plant used, in addition to the cultivation conditions and exposure time^{43, 19, 44,45}. The cultivar Anta82 is more sensitive to water deficit and showed an increase in mycorrhizal colonization, especially under drought stress at 3 and 7 experimental days (Fig. 1A). However, the Desafio cultivar showed a pronounced increase in mycorrhizal colonization after 7 days of drought (Fig. 1B). Plants of the drought-sensitive *Citrullus lanatus* variety (Y34) with ~5 true leaves and 12 days of water stress inoculated with *Glomus vesiform* showed 75 % mycorrhizal colonization⁴⁶, while *Lycium barbarum* plants incubated with *Rhizophagus irregularis* showed 50 % of colonization after 3 weeks of exposure to water deficit⁴⁷. According to these authors, the increase in mycorrhizal colonization was sufficient to mitigate water stress in plants of *C. lanatus* and *L. barbarum*. Also, AMF inoculation has been described as a potential tool used to mitigate the effects of water deficit and increase plant resistance in bean⁴⁸, rice⁴⁹, tomato⁵⁰, wheat⁵¹, and trees^{52,53}.

Mechanisms associated with maintaining water status are rapidly triggered under limiting water conditions, as water deficit is one of the major factors affecting plant growth and production^{54,5}. Among these mechanisms, stomatal regulation responds rapidly to water stimuli^{9,55}. In addition, the association with mycorrhizal fungi has been shown to be a potential ally for maintaining plant water status, as root-bound hyphae allow for greater soil water absorption⁴³. The results of the water potential under drought

stress showed that the AMF mitigated the adverse effects of drought stress for the two cultivars (Fig. 3). Similarly, *Poncirus trifoliata* plants increased their water potential by 20 % by inoculating mycorrhizal fungi under deficit water conditions⁵⁶.

Stomatal closure, in an attempt to maintain plant turgor, may limit photosynthetic processes and compromise crop yield⁵⁷⁻⁵⁹. Low photosynthetic yield due to stomatal limitation was observed in plants exposed to water deficit. Plants with AMF under drought stress increased stomatal conductance compared to non-AMF plants (Fig. 4 C and D). In addition, photosynthesis (Fig. 4A and B) and transpiration (Fig. 4E and F) were higher in the presence of mycorrhiza under water deficit conditions, for both cultivars, suggesting that plants with AMF can improve water status under water deficit with the ability to maintain greater water-use efficiency (Fig. 4I and J). These findings suggest that AMF showed a very active role in this process, allowing greater stomatal opening and higher photosynthetic rates associated with higher turgor as recently observed in *Olea europaea*⁶⁰, eggplant²², and tomato⁵⁰. This was possible because AMF assisted with water uptake, even under limiting conditions^{61,62} inducing changes in critical substrate water potential and increasing the water transport within colonized substrates²⁰, which allow for greater water-use efficiency. Besides that, the maintenance of water status favors a better fixation of atmospheric CO₂ and increases the movement of photoassimilates ("sink effect") from the aerial parts to the roots¹⁹.

Photosynthetic limitation is directly related to the photochemical responses of plants demonstrated by the chlorophyll *a* fluorescence variables⁶³. Drought stress may compromise the functionality of the chloroplast electron chain^{8,64,65} and limit the energy production and reduce the power required for the completion of the photosynthetic process. In the present study, the inoculation of soybean plants with AMF under water deficit allowed the maintenance of the maximum quantum yield of PSII (F_v/F_m) compared to well-watered plants (Fig. 5C e D), as previously observed in rice⁶⁶, watermelon⁴⁶, wheat⁶⁷ and maize⁶⁸. By contrast, from 3 days of evaluation, plants under water deficit used a thermal dissipation mechanism (NPQ) to avoid excess energy (Fig. 5I and J). Thermal dissipation by the xanthophyll cycle, activated in response to the pH gradient formed by the cyclic electron cycle^{69,70}, has been an initial response to abiotic stress and allows for the regulation of the amount of excitation energy directed to the reaction centers of photosystems^{71,72}. Higher NPQ values are important early protection mechanisms for the photosystem, as they avoid photooxidative stress on photosynthetic photochemical complex proteins. The increase in NPQ was efficient, especially in plants of cultivar Desafio, even after 7 days of exposure to water deficit in the presence of AMF (Fig. 5J).

Plant exposure to water deficit can cause an excess of energy not directed to the photosynthetic process. This results in increased production of reactive oxygen species (ROS) that promote lipid, protein and chloroplast pigment peroxidation^{73,74}. There was a reduction in the concentration of photosynthetic pigments, mainly chlorophyll *a*, which also acts on the photosystem reaction centers. In the present study, the soybean plants under drought stress (Fig. 6 A and B) contributed to the photosynthetic reduction. Nevertheless, it was possible to observe the mitigation of damage caused by water deficit in plants

inoculated with AMF (Fig. 6). These results corroborate those of Baslam and Goicoechea⁷⁵ who found that AMF *Glomus intraradices* and *Glomus mosseae* associated with *Lactuca sativa* plants increased the content of chlorophylls and carotenoids even after exposure to water deficit.

Impairment of the photochemical stage, associated with oxidative stress due to water deficit, may limit plant growth. Initially, under unfavorable water conditions, plants tend to increase the root system to allow more area of contact with the soil^{76,11,77}. These compromises shoot growth, such as the development of new leaves and shoots⁷⁸. Even in a short period of stress, it was possible to observe a reduction in vegetative growth of soybean plants exposed to water deficit that was slightly increased in plants inoculated with AMF *Rhizophagus clarus* (Fig. 7). The contribution of inoculation with *Funneliformis geosporus* and *F. mosseae* on the growth of *Fragaria ananassa* plants under water deficit was observed by Boyer et al.⁷⁹. Oliveira et al.⁸⁰ found an increase in *Cicer arietinum* yield inoculated with AMF *Rhizophagus irregularis* and the bacteria *Mesorhizobium mediterraneum* in the absence of water deficit. However, under water deficit, the authors observed an increase in plant biomass and content of crude proteins of grains, favoring the maintenance of productivity in relation to control plants. In crops, Bernardo et al.⁸¹ found that plants of *Triticum spp.* inoculated with *Glomus mosseae* obtained higher dry matter accumulation in the area compared to plants without AMF under water deficit. Plant growth occurs with higher nutrient accumulation, as promoted by soybean-AMF interaction. Under water deficit, nutrients may be accumulated or adsorbed to the roots, because of the reduction in mass flow promoted by transpiration. In association with AMF, plants increase water use efficiency, allowing nutrient translocation to the shoots. The symbiosis of AMF and legume plants can be relevant to N₂-fixing nodulation rhizobia bacteria. This interaction could be responsible for nutrient recycling and favor to nutrient uptake⁶¹ and improve tolerance of abiotic stress⁸².

Overall, *Rhizophagus clarus* increased soybean plant tolerance under water stress treatments on the metabolism may allow the maintenance and/or increase of their development under moderate water deficit conditions. The potential mitigating effect of water deficit by AMF *Rhizophagus clarus* allows the design of new field experiments to verify the response of plants under natural conditions, with the potential occurrence of combined abiotic stresses, for potential use in agricultural areas subject to moderate water stress.

Conclusion

The application of arbuscular mycorrhizal fungi *Rhizophagus clarus* allowed for the maintenance of the water status of Anta82, a drought-sensitive cultivar, mitigating the negative effects on the photosynthetic apparatus of these soybean plants. In Desafio, a moderately drought-tolerant cultivar, higher colonization of *Rhizophagus clarus* increased the concentration of photosynthetic pigments and improved physiological performance and plant growth. These data suggest that *Rhizophagus clarus* is an important tool for maintaining soybean yield, especially in regions with low precipitation and drought.

Declarations

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Author contributions

G.C.M, C.M, E.L.S and J.S.R.C. designed and supervised the research. Material preparation and data collection were performed by T.C.O, J.S.R.C., L.R.S., G.G.T., L.D.S.S., G.C.M and C.M. Analyses were performed by T.C.O, J.S.R.C and T.P.P. A.C.C. and F.G.S. cooperated in the physiological analyses of plants. The first draft of the manuscript was written by T.C.O and was revised by G.C.M, C.M., J.S.R.C, T.P.P. and E. L. S. All authors commented on previous versions of the manuscript. The work was substantially revised by G.C.M., C.M., T.C.O. and J.S.R.C. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Mycorrhizal colonization (MCR) in soybean Anta82 (A) and Desafio (B) cultivars with or without AMF inoculation under well-watered (WW) and water deficit (WD) for 3 (WD-3days) and 7 (WD-7days) days. WW, well-watered without inoculation, WD, water deficit without inoculation, AMF, inoculated well-watered plants, WD+AMF, inoculated plants under water deficit. Bars represent mean $\pm$ sem ($n = 4$). Means followed by the same letter do not differ ($p > 0.05$) among themselves by the Tukey test.

Figure 2

Root cortex of soybean Anta82 (A, B, C, D) and Desafio (E, F, G, H) cultivars with or without AMF inoculation under well-watered (WW) and water deficit (WD) for 7 days. WW, well-watered without

inoculation, WD, water deficit without inoculation, WW+AMF, inoculated well-watered plants, WD+AMF, inoculated plants under water deficit. Black arrows indicate colonization of mycorrhizal fungi. Bar scale 200 μm (A, B, C, E, F, G, H) and 1 μm (D).

Figure 3

Water potential (Ψ_w) in soybean Anta82 (A) and Desafio (B) cultivars with or without AMF inoculation under well-watered (WW) and water deficit (WD) for 3 (WD-3days) and 7 (WD-7days) days. WW, well-watered without inoculation, WD, water deficit without inoculation, WW+AMF, inoculated well-watered plants, WD+AMF, inoculated plants under water deficit. Bars represent mean $\pm$ sem (n = 4). Means followed by the same letter do not differ (p > 0.05) among themselves by the Tukey test.

Figure 4

Photosynthetic rate (A), stomatal conductance (g_s), transpiration (E) and water use efficiency (WUE) in soybean Anta82 (**A, C, E, G**) and Desafio (**B, D, F, H**) cultivars with or without AMF inoculation under well-watered (WW) and water deficit (WD) for 3 (WD-3days) and 7 (WD-7days) days. WW, well-watered without inoculation, WD, water deficit without inoculation, WW+AMF, inoculated well-watered plants, WD+AMF,

inoculated plants under water deficit. Bars represent mean $\pm$ sem (n=4). Means followed by the same letter do not differ ($p > 0.05$) among themselves by the Tukey test.

Figure 5

Minimal fluorescence (F_0), maximum quantum yield of the PSII (F_v/F_m) and non-photochemical quenching (NPQ) in soybean Anta82 (A, C, E) and Desafio (B, D, F) cultivars with or without AMF

inoculation under well-watered (WW) and water deficit (WD) for 3 (WD-3days) and 7 (WD-7days) days. WW, well-watered without inoculation, WD, water deficit without inoculation, WW+AMF, inoculated well-watered plants, WD+AMF, inoculated plants under water deficit. Bars represent mean $\pm$ sem ($n = 4$). Means followed by the same letter do not differ ($p > 0.05$) among themselves by the Tukey test.

Figure 6

Total chlorophylls (Chl_{tot}) and total carotenoids ($\text{Carot}_{\text{tot}}$) in soybean Anta82 (A, C) and Desafio (B, D) cultivars with or without AMF inoculation under well-watered (WW) and water deficit (WD) for 3 (WD-3days) and 7 (WD-7days) days. WW, well-watered without inoculation, WD, water deficit without inoculation, WW+AMF, inoculated well-watered plants, WD+AMF, inoculated plants under water deficit. Bars represent mean $\pm$ sem ($n = 4$). Means followed by the same letter do not differ ($p > 0.05$) among themselves by the Tukey test.

Figure 7

Plant height (PH), leaf area (LA), leaf dry matter (LDM) and root dry matter (RDM) of soybean Anta82 (A, C, E, G, I) and Desafio (B, D, F, H, J) cultivars with or without AMF inoculation under well-watered (WW) and water deficit (WD) for 3 (WD-3days) and 7 (WD-7days) days. WW, well-watered without inoculation, WD, water deficit without inoculation, WW+AMF, inoculated well-watered plants, WD+AMF, inoculated

plants under water deficit. Bars represent mean $\pm$ sem ($n = 4$). Means followed by the same letter do not differ ($p > 0.05$) among themselves by the Tukey test.

Figure 8

PCA biplot of the first four dimensions (first and second at top and third and fourth at bottom) using Anta data at left (A) and Desafio data at right (B). WW, well-watered without inoculation, DW, water deficit without inoculation, WW+AMF, inoculated well-watered plants, DW+AMF, inoculated plants under water deficit.

Supplementary Files

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- [SupplementaryMaterial.docx](#)