Inoculation of plant-growth-promoting rhizobacteria in *Myracrodruon urundeuva* Allemão supports in tolerance to drought stress

Douglas Moreira de Oliveira, André Luiz Alves de Lima, Nathália Bandeira Diniz, Carolina Etienne de Rosália e Silva Santos, Sérgio Luiz Ferreira da Silva and Adriano do Nascimento Simões

Department of Plant Production, Federal Rural University of Pernambuco, Serra Talhada, Brazil; Department of Soil Science, Federal Rural University of Pernambuco, Recife, Brazil

ABSTRACT

This study evaluated the influence of *Azospirillum lipoferum* on the growth of *Myracrodruon urundeuva* (Anacardiaceae) plants under drought stress, by means of biometric, physical–chemical and biochemical parameters. The association of *A. lipoferum* with the roots of the plants provided increases of 30% root length, 50% root dry weight, 34% shoot dry weight and 10% soluble protein content. The inoculated plants still maintained 5% higher leaf water potential than those not inoculated and lower membrane damage. Furthermore, the inoculated plants showed less leaf fall and dark green leaves, confirmed by maintenance of the highest levels of chlorophyl a; b and total. On the other hand, superoxide dismutase activity was significantly lower in the inoculated plants, possibly due to the induction of a non-enzymatic protective feature. In this way, the inoculation of PGPR in *M. urundeuva* can be an alternative for the production of plants that are more tolerant to drought stress.

1. Introduction

Dry tropical forests (DTF) are distributed in seasonally dry regions characterized by arid and semi-arid climates, and represent 40% of tropical forests (Beuchle et al. 2015). Among the DTF, the Caatinga stands out, which is a typical forest at the semi-arid region of Brazilian Northeast characterized by high temperatures, with an annual average of 25°C, and a low annual average rainfall of approximately 450 mm (Moura et al. 2007). In this biome remains high biodiversity, however, over the years the intense anthropic activity has resulted in a great degradation of this environment, leading to a reduction in plant diversity (Beuchle et al. 2015).

The Ministry of the Environment (2008) emphasizes the risk of loss of plant diversity, once about 48 species of plants from Caatinga are in threat of extinction, including *Myracrodruon urundeuva* Allemão (Anacardiaceae), popularly known as *Aroeira do Sertão*. This species has great economic, medicinal and ecological importance, its wood is commercially appreciated (Lorenzi 2014), it presents a high tannin concentration (Calou et al. 2017) and may interact with diverse microorganisms still unknown. In this way, its extinction can represent immeasurable losses.

Plants under drought stress change their metabolism to minimize the adverse effects of drought (Hayat et al. 2012). Plants under drought stress rapidly increase the production of hormones such as abscisic acid (ABA) and ethylene, which are involved in stomatal closure, thus minimizing water loss (Barna et al. 2012; Oskabe et al. 2014) and foliar abscission that represents one of the strategies of plants to tolerate drought stress (Sakamoto et al. 2008).

Plant-growth-promoting rhizobacteria (PGPR) compose a group of bacteria present in the rhizosphere, which can minimize drought stress by inducing the production of antioxidant enzymes, as well as hormones and metabolites that play important roles in reduction of adverse effects caused by abiotic stresses such as shortage of water (Figueiredo et al. 2008; Kohler et al. 2008; Yang et al. 2009; Wang et al. 2012).

In the last decades several studies about the influence of PGPR species aimed the minimization of oxidative stress and/or the increase of productivity of crops (Mayak et al. 2004; Sottero et al. 2006; Walker et al. 2011; Karawal and Kumar 2012; Stefan et al. 2013; Lucas et al. 2014; Yadav and Verma 2014 and Kang et al. 2014). However, fewer studies have been published about the effects of PGPR inoculation in native tree species from arid and/or semi-arid areas and under drought stress. In addition, studies that have evaluated the interaction of PGPR with native DTF plants are not representative in relation to the geographical distribution of these plants, such as Mediterranean (*Pinus halepensis*; *Quercus coccifera*) (Rincón et al. 2008) and China (*Platycladus orientalis*) (Liu et al. 2013). This studies did not address oxidative damage in plants, although they presented results that suggest the minimization of the effects of water stress. It is believed that inoculation with a PGPR, *Azospirillum*
The inoculated and no inoculated (control) seeds were sown into the containers and transferred to a climatic growth chamber (Fitotron – SGC 120) under the following conditions: photoperiod of 12 h at 28°C and 12 h in the dark at 26°C; Constant RH of 50%; Luminous intensity 450 μmol photon m⁻² s⁻¹ for 81 days.

The sowing was performed on 30 January 2016, and seed germination occurred after three days. After 7 days, irrigation treatments corresponding to 100%, 75%, 50% and 25% of the FC were restored every 48 h, representing irrigations levels of 180, 135, 90 and 45 mm, respectively, during 71 days. The irrigation regime of 180 mm was considered the control, 135 and 90 mm were considered moderate drought stress and 45 mm was considered severe drought stress. The plants were watered with the nutrient solution of Hoagland and Arnon (1950) every seven days (Bogino et al. 2006).

Plants of M. urundeuva remained in climatic plant growth chamber during 81 days. The 81 days were composed by 3 days until the beginning of seed germination, then 7 days until the beginning of the application of the irradiation treatments, and finally 71 days of application of irrigation regimes. At the end of the 81 days, the inoculation was confirmed, and biometric, physicochemical and biochemical analyzes were performed.

2.4. Confirmation of Azospirillum lipoferum inoculation

Confirmation of inoculation was performed using the semi-solid nitrogen-free bromothymol (NFB) blue culture medium (Sucrose: 10 g L⁻¹, K₂HPO₄: 0.6 g L⁻¹, MgSO₄: 0.20 g L⁻¹ NaCl: 0.2 g L⁻¹, K₃SO₄: 0.1 g L⁻¹, CaCO₃: 2.0 g L⁻¹, pH 6.8, 2.0 ml of 0.5% bromothymol blue solution). This culture medium is selective for Azospirillum spp. growth (Ramanathan et al. 2015).

The plants were removed from the containers and the substrate adhered to the roots was washed with distilled and sterilized water, and stored in sterile plastic pots. Subsequently, aliquots of 1 mL of the washing water were distributed in flasks containing the semi-solid NFB culture medium. These flasks were then incubated in BOD at 30°C for four days. After this period, the color change of the NFB medium was determined. The inoculated and non-inoculated (control) flasks had slightly different colors. The plants were watered with the nutrient solution of Hoagland and Arnon (1950) every seven days (Bogino et al. 2006).

2.5. Biometric analysis

After 81 days, biometric parameters such as plant height (from the collar up to the apical bud), the length of roots and plant girth, were measured using a digital pachymeter (Stainless Hardened). The foliar area and the density (green) were determined by scanning the leaflets in a commercial scanner and the generated image was processed.
using the Image-Pro Plus software version 4.5.0.29 for Windows® (Media Cybernetics). Dry mass was established after drying the plant material (one plant of each treatment; shoot and roots) dried at 65°C for 72 h.

2.6. Physicochemical analyses

2.6.1. Leaf water potential (Ψ)
The leaf water potential was measured at 8 a.m. in a plant of each treatment, using the third branch with leaflets fully expanded, but not senescent, using Scholander pressure chamber (Model 3005F01) (Oliveira et al. 2014).

2.6.2. Electrolyte leakage
Electrolyte leakage was performed according to Shanahan et al. (1990). Ten millimeters diameter leaf discs were collected and immersed in test tubes containing deionized water, incubated at 30°C for eight hours and then determined the electrical conductivity (C1). Membrane damage (DM) was estimated using the formula: DM = (C1 ÷ C2) × 100. Where the electrical conductivity (C2) was measured after the samples were submitted to 100°C for 1 h.

2.7. Biochemical analysis
Approximately three grams of fresh mass of leaflets were collected and frozen in liquid nitrogen. They were then kept in ultrafreezer at −80°C until the beginning of biochemical analyzes.

2.7.1. Total soluble protein content
Samples of fresh leaves (0.1 g) were macerated in a mortar with liquid nitrogen, then potassium phosphate buffer (100 mM; pH 7.0) was added. Soluble protein content was measured according to Bradford (1976) and quantified based on the standard curve of (BSA).

2.7.2. SOD activity
The activity of SOD(EC: 1.15.1.1) was determined according to the methodology described by Gianopolitis and Ries (1977). Briefly, 0.1 ml aliquots of the protein extract were transferred to reaction medium in light-protected tubes illuminated by a 30-watt lamp (30 s). The transfer of the tubes, without light protection, to a chamber was initiated by the addition of 2 mM riboflavin and rapid pure oxygen (a/c). Twenty mg of fresh leaf tissue containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine and 75 μM NBT. The reaction was initiated by the addition of 2 mM riboflavin and rapid transfer of the tubes, without light protection, to a chamber illuminated by a 30-watt lamp (30 μmol of photons m⁻² s⁻¹) for six minutes. The reaction was interrupted by the shutdown of the light, the tubes were coated with dark film and readings were taken at 540 nm.

The activity was estimated based on the inhibition of NBT reduction, defining a unit of activity as the amount of enzyme required to inhibit 50% of the photosynthesis (Beauchamp and Fridovich 1971). The activity was expressed in U.A. g⁻¹ FW min⁻¹.

2.7.3. Proline content
Proline content was measured using the acid ninhydrin method (Bates et al. 1973); Twenty mg of fresh leaf tissue were immersed into test tubes containing 10 mL of deionized water, then heated at 100°C in a water bath (TECNAL – TE 056 mag) for one hour. Aliquots of 1 mL of the extract were transferred to threaded tubes and then added 1 mL of the acid ninhydrin reagent an 1 mL of glacial acetic acid, and shaken vigorously (PHOENIX, AP 56). The tubes were once again heated at 100°C in a water bath for 1 hour and then placed in an ice bath until they reached room temperature. Finally, 2 mL of toluene (P.A.) was added and stirred for 15 s.

The readings were taken at 520 nm in the spectrophotometer. The proline content was quantified based on the standard curve of L-proline.

2.7.4. Chlorophyl content
The levels of chlorophyl a, b and total were quantified according to Lichtenhaler and Buschmann (2001). Eighty mg of leaflets were macerated in 2 mL of acetone (80%) with CaCO₃. The macerate was filtered and read in a spectrophotometer (biochrom) at 645, 652 and 663 nm. In addition, a non-specific absorbance of 710 nm was recorded, which chlorophyl a, b and totals do not absorb, for color correction, turbidity, and contaminant compounds (Oliveira et al. 2014).

2.7.5. Experiment design and statistical analysis
The data were submitted to the normality test (Shapiro-Wilk) and the homoscedasticity test (Bartlett). The experiment was carried out in a Completely Randomized Design and the analyzes of variance and regression were performed in a 2 × 4 factorial scheme (with and without inoculum x four irrigation levels), thus, eight treatments were obtained, with three replications.

Twenty-four containers (vessels) were used during the experiment. The means ± standard deviation were compared by the least significant difference test with P<0.05. The statistical tests were performed by the ExpDes and Agricolae packages of the R software (www.R-project.org) version 3.0.2. The graphs were generated using the Sigma Plot software version 12.

3. Results
The effectiveness of Myracroduon urundeuva colonization by the PGPR, Azospirillum lipoferum, was evidenced by a color change from yellowish green to blue of NFB culture medium. While control, the color of NFB medium remained unchanged (Figure 1).

All plants showed a small decrease in height in relation to the control, c.a. 1.10 cm, when the irrigation regime was reduced from 180 to 135 mm (Figure 2). Whereas the irrigation level was reduced from 135 to 90 mm and from 90 to 45 mm, the decrease of height means was relatively proportional, approximately 3.0 cm (Figure 2). On the other hand, apparently, the inoculated plants and the control showed no perceptible visual differences (Figure 2). However, the inoculated plants maintained a greater number of leaflets with green tonality, while the control plants presented more leaflets with purple tonality (Figure 3). This difference in tonality was significant for the irrigation level of 180 mm (Figure 6(B)). In addition, it was observed that the reduction in irrigation levels resulted in a lower number of purplish leaflets for the plants without inoculum (Figure 3).

Chlorophyl a and b concentrations were significantly higher in leaflets of plants that were inoculated and submitted to irrigation regimes of 180 and 135 mm in relation to the control (Figure 4(A,B)), resulting in the increase of total chlorophyls, in the 180 and 135 mm levels, and in the a / b ratio only at the maximum irrigation (180 mm) (Figure 4).
(C,D)). In contrast, the inoculation did not maintain chlorophyll levels a, b, total and a/b ratio higher than the control at the maximum drought stress (45 mm) (Figures 4(A–D)).

As irrigation levels decreased, the number of loose leaflets was lower in control plants (Figure 6(A)). On the other hand, the inoculated plants clearly showed smaller percentages of untied leaflets, besides maintaining all leaflets in the maximum irrigation level 180 mm (Figure 6(A)).

The inoculated plants had significantly higher root lengths than those not inoculated, with significant increases of 30% for those submitted to 90 and 180 mm irrigation levels (Figure 5(A)). In addition, root growth was highest when the inoculated plants were irrigated with 180 mm (Figure 5(A)). Consequently, root dry mass was significantly higher for those inoculated plants (c.a. 50%) and for those plants irrigated with 90 mm (Figure 5(C)).

When irrigated with 45 and 90 mm, in the shoot of inoculated plants had significantly larger plant girth, c.a. 13% (Figure 5(B)); the height and the leaf area were not
Figure 4. Chlorophyll a (A), b (B), total (C) and chlorophyll a/b ratio (D) of *Myracroduon urundeuva* plants, with and without inoculation (*Azospirillum lipoferum*) under four irrigation levels (180, 135, 90 and 45 mm). The values of averages followed by the same letter are not significantly different in $P \leq 0.05$.

Figure 5. Root length (A), plant girth (B), dry mass of root (C) and shoot (D) of *Myracroduon urundeuva* plants, with and without inoculum (*Azospirillum lipoferum*) under four irrigation levels (180, 135, 90 and 45 mm). The values of averages followed by the same letter are not significantly different in $P \leq 0.05$. 
significantly influenced by inoculation (Table 1). On the other hand, decreases in irrigation levels resulted in gradual decreases of these parameters (Table 1). In addition, the shoot dry mass was significantly higher after inoculation, c.a. 34%, for the plants irrigated with 135 mm (Figure 5(D)).

The soluble protein content was 10% higher for inoculated plants compared to control (Table 2). In addition, although the amount of water supplied for the plants was also significant for the soluble protein content, the mean contents remained statistically the same for all irrigation levels (Table 1). Furthermore, the irrigation regimes and the inoculation were not significant for the proline content of *M. urundeuva* plants.

The foliar water potential remained 5% higher in the plants that were previously inoculated in relation to the control (Table 2). In addition, little variations were observed with no water effect as the water was restricted, except for the 90 mm irrigation level (Table 1). Moreover, the inoculation significantly reduced membrane damage for plants under 90 mm irrigation regime (Figure 6(C)). Although this was not observed for 180, 135 and 45 mm irrigation levels (Figure 6(C)).

Leaflets of control plants showed SOD activity significantly higher than the inoculated plants (Figure 6(D)). In addition, the continuous reduction in water supply up to 90 mm resulted in a gradual increase of the SOD activity in the leaflets of the no inoculated plants. While in the inoculated plants there was a gradual decrease of the SOD activity, also up to the 90 mm irrigation level (Figure 6(D)). In addition, water reduction from 90 to 45 mm resulted in increased SOD activity in leaflets of inoculated plants and reduction in leaflets of control plants (Figure 6(D)).

![Figure 6. Percentage of fallen leaflets (A), density (green) of leaflets (B), membrane damage (C) and SOD activity (D) of *Myracroduon urundeuva* plants, with and without inoculum (*Azospirillum lipoferum*) under four irrigation levels (180, 135, 90 and 45 mm). The values of averages followed by the same letter are not significantly different in $P \leq 0.05$.](image_url)
4. Discussion

Native plants of semi-arid climate regions are often exposed to the damaging effects caused by drought stress, the main adversity for these plants, primarily in their initial growth stage, highlighting the importance of microbial–plant association such as PGPR for early tree development. According to Suárez-Vidal et al. (2017) the seedling stage is the most critical, while survival depends on germination, with fast rooting during the rainy season. Thus, the purpose of inoculation of *A. liporeum* in *M. urundeuva* seeds was to present an alternative for the production of these plants, which are more tolerant to drought stress at the initial growth stage.

In the present study, a reduction in water supply for the plants caused visual morphological changes in leaves and roots (Figure 2). These data are confirmed by biometrical measurements that showed gradual decreases in plant height, root length, plant girth and foliar area, both in previously inoculated and in control plants (Figure 5(A,B) and Table 1). These changes are related to a reduction in the turgidity pressure of plants subject to water restrictions (Bartlett et al. 2012).

The appearance of the plants, when comparing those inoculated with the control (not inoculated), it was not possible to observe visual changes in size (Figure 2). However, when root length was measured, the plants inoculated and irrigated with the maximum and intermediate irrigation levels, 180 and 90 mm, respectively, roots presented increases of approximately 30% (Figure 5(A)). These higher root lengths of the inoculated plants also reflected significant increases of 50% in their dry mass (Figure 5(C)).

This PGPR induced root expansion under drought stress also occurred in maize (Vardharajula et al. 2011; Naseem and Bano 2014). This suggests that the induction of greater root length may be related to the production of indoleacetic acid and/or other yet unknown metabolites produced by PGPR (Mantelin and Touraine 2004; Adesemoye et al. 2008; Yu et al. 2017). The benefit of inoculation on root length and plant girth is possibly due to the proximity of the site of interaction between PGPR and plants (Dal Cortivo et al. 2017). The increase of these measures, in the moderate and/or severe drought stress (90 and 45 mm), probably favored the absorption and conduction of water in the inoculated plants (Noumavo et al. 2016).

The inoculation had no significant effect on plant height (Table 1). However, the plants that were inoculated presented significant increases in the plant girth, when submitted to the intermediate and minimum irrigation levels, 90 and 45 mm, respectively (Figure 5(B)), resulting in an increase of approximately 13%. However, roots of inoculated plants and submitted to the intermediate irrigation level, 135 mm, showed a dry mass 34% higher than the control (Figure 5(D)), proving the efficiency of PGPR, *A. liporeum*, in the increment of dry mass under moderate drought stress. This fact was mainly reflected by plant girth (Figure 5(B)) and greater number of leaflets (Figure 6(A)) of the plants with inoculum. In addition, these data converge with significant increases of dry mass in several inoculated crops, under drought stress (Naseem and Bano 2014; Prudent et al. 2015; Ullah et al. 2016).

The leaf water potential (Ψ) of *M. urundeuva* adult plants located in *Caatinga* area presents little variation, close to –0.3 MPa, independent if it is during a rainy or dry season (Trovão et al. 2007). This is related to the presence of tuberous roots of this species, since they are capable of storing nutrients and water, helping the plant to maintain its high leaf water potential under adverse conditions of its natural habitat (Feliciano et al. 2008). These data may explain the findings in the present study, where Ψ values had small variations in this plant, with averages around −0.23 MPa (Table 1). These values are relatively high when compared with other plants from *Caatinga* that are on average −1.0 MPa (Trovão et al. 2007). In addition, Ψ of the plants inoculated with the PGPR was 5% higher than those not inoculated (Table 2). The Ψ is directly related to the integrity of the cytoplasmatic membrane (Rincón et al. 2008). Thus, higher values of Ψ promoted by the association between plants and PGPR may have influenced lower membrane damage when plants were submitted to maximum (180 mm) and moderate (90 mm) irrigation (Figure 6(C)), as also verified by Ullah et al. (2016).

Drought stress implies in the reduction of the quality of the plants, resulting in loss of leaf turgor (Ψ reduction) (Bartlett et al. 2012), and causing increased foliar senescence (Das et al. 2015). However, previously inoculated plants presented high Ψ, maintaining the turgor pressure, avoiding the increase in membrane damages (Table 2 and Figure 6(C)), minimizing the leaflet loose (Figure 6(A)), as well as maintaining the green tonality of these plants (Figure 6(B)). This was verified by the higher concentration of chlorophyll *a*, *b* and total (Figure 4(A–C)), suggesting that inoculation under moderate drought conditions contributed to the longevity of *M. urundeuva* leaflets.

Some studies reported that one of the pillars for plant tolerance to stress situations is the increase in enzymatic activity that suppresses ROS, which is initiated by SOD (Møller et al. 2007). In the present study, this behavior occurred in leaflets of plants that were not inoculated when irrigation was restricted up to 90 mm (Figure 6(D)). On the other hand, in the leaflets of the inoculated plants, this water restriction caused a decrease in SOD activity (Figure 6(D)). This suggests that under these conditions, SOD was not a good biochemical marker, since the tolerance of these plants to conditions of water restriction may have occurred by non-enzymatic factors (Osakabe et al. 2014), which were not evaluated in the present study.

The results found in the present study show that the soluble protein contents were 10% higher in the leaflets of the inoculated plants compared to the leaflets of the control (Table 2). This reveals that turnover is favorable for the synthesis of new proteins or preexisting proteins involved in the drought stress tolerance induced by PGPR, as evidenced by Gagné-Bourque et al. (2015). Although in the present study, no such proteins have been identified.

Therefore, inoculation with *A. liporeum* presumably conferred to *M. urundeuva* plants a certain tolerance to moderate drought stress (135 mm and 90 mm irrigation levels). This fact can be confirmed in root features such as an increase in length and dry mass. While in the aerial part, the size changes were less pronounced, except for the increase of the plant girth, an increase of the dry mass, reduction of leaflet loose, as well as, the increase of the green tonality, confirmed in the chlorophyll content. Additionally, the physical–chemical and biochemical parameters studied, such as soluble protein concentration, leaf water potential and individual and total chlorophylls, were higher in inoculated plants, submitted to moderate drought stress (135 mm and 90 mm). The opposite was observed in SOD activity, in
which the inoculated plants had less activity of this enzyme; this fact could evidence the relative protection by non-enzymatic factors.

The presence of the PGPR minimized the damage to plants caused by drought stress; this fact was more evident in the moderate drought imposed (90 mm). However, the mechanisms of action of PGPR that signal the defenses of these plants, such as hormones, should be the focus of future researches. The present study showed that proline was not a metabolite that mediates the signaling of drought stress in the inoculated plants. It is believed that the regulators ABA, cytokine, ethylene and ACC may be the key to the mechanisms of water stress tolerance. Further studies are needed to identify metabolites and enzymes that mediate the signaling and transduction of signaling that confer greater tolerance to drought stress to *M. urundeuva* plants inoculated with PGPR.

In conclusion, the use of a PGPR, *A. lipoferum*, conferred greater tolerance to drought stress in plants of *M. urundeuva*. Currently, this plant species is threatened with extinction and its natural habitat, the *Caatinga*, suffers numerous environmental pressures. Given these circumstances, there is a latent need for recovery of this environment; however, there is not much information that helps in the elaboration of management plans and recovery of degraded areas. In this sense, the present study showed potential advances with the inoculation of PGPR in *M. urundeuva* as an alternative to subsidize the preparation of programs for the recovery and reforestation of degraded areas.

Acknowledgments

We thank the National Council for the Improvement of Higher Education (CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), the State of Pernambuco Research Council (FACEPE – Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco) and the Pro-Rectory of Research and Postgraduate of UFRPE (Pró-Reitoria de Pesquisa e Pós-Graduação da UFRPE) for their financial support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Council for the Improvement of Higher Education (CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), the State of Pernambuco Research Council (FACEPE – Fundação de Amparo à Ciência e Pesquisa de Pernambuco) and the Pro-Rectory of Research and Postgraduate of UFRPE (Pró-Reitoria de Pesquisa e Pós-Graduação da UFRPE).

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