Biological nitrogen fixation of *Biserrula pelecinus* L. under water deficit

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ABSTRACT

The present work studied the effects of water deficiency conditions on the biological nitrogen fixation of three native rhizobia (SafPt12, SafPt6, and AjuPt16) isolated from *Biserrula pelecinus* L., and a reference strain *Mesorhizobium ciceri* biovar *biserrulae*. In terms of plant-water status, *B. pelecinus* showed typical signs of drought avoidance strategies such as reducing the aboveground development (i.e. reduction in leaf surface area and increase in root/shoot ratio) in detriment of a better developed root system. Dry-matter production and nitrogen content of the aboveground biomass decreased with the increasing levels of drought stress, as well as nodulation and symbiotic nitrogen fixation, for all the tested isolates. The parameters investigated suggested that SafPt12 was the most successful native rhizobia to withstand severe water conditions without compromising nitrogen fixation demands.

Keywords: drought; *Mesorhizobium*; ^{15}N natural abundance; stress

Biserrula pelecinus L. is a self-regenerating annual legume from semi-arid oligotrophic pastures (Howieson et al. 1995), which forms an extremely close-relationship with *Mesorhizobium ciceri* biovar *biserrulae*, *M. opportuniscum* and *M. australicum* (Nandasena et al. 2009). *Biserrula* is considered an ideal legume for Mediterranean-type pastures since is widely found in sandy acid soils, and is able to subsist under water deficiency conditions (Howieson et al. 1995), a major abiotic stress that compromises legume growth and biological processes such as biological nitrogen fixation (BNF). BNF is highly sensitive to drought (Sinclair et al. 2007), where rhizobia are known to be more tolerant than their legume host (Zahran 1999); since they can survive in the water films surrounding soil particles. However, their growth and movement is limited under severe soil dehydration, reducing thus the number of free-living bacteria and threatening the first steps of symbiosis (Graham 1992). After infection, nodule formation also suffers from water deprivation mainly through morphological and physiological alterations, i.e. changes in nodule cortex structure, and increase of carbohydrates in the stressed nodules (Fellows et al. 1987). Indeterminate nodules with prolonged meristematic activity seem to be more resistant to low soil-water deficit in contrast to determine nodules, which have limited meristematic activity and with low recovery capacity from water deficits (Engin and Sprent 1973). Furthermore, drought accelerates nodule senescence, disrupting the control of oxygen exchanges in the bacteroids, and resulting in the interruption of the nitrogen fixation process (Esfahani and Mostajeran 2011). Moreover, the reduction in water absorption and consequent reduction in the water supply in tissues, promote damage in the nitrogen compound translocation (Serraj et al. 1999).

Rhizobial strains with different sensitivity to soil moisture can be selected (Zahran 1999, Esfahani and Mostajeran 2011). In this sense, the present work aims to study the effects of water deficiency in BNF of selected rhizobia isolates of *Biserrula pelecinus*, previously characterized (Vicente 2010), through (i) the study of general plant physiological parameters; and by (ii) the evaluation of different performances and potential of the selected iso-

Supported by the Portuguese Foundation for Science and Technology (FCT), Grant No. SFRH/BD/17252/2004.

lates to help plant host to withstand the stressful conditions.

MATERIAL AND METHODS

Greenhouse experiment. The experiment was carried out at the National Plant Breeding Station (Elvas, Portugal), under greenhouse conditions with an average temperature of 20 ± 2°C; 80% humidity; and a 14 h day-light and 10 h night-time photoperiod. Three water regimes, based on water holding capacity (WHC) of the substrate determined by gravimetric means (Hazelton and Murphy 2007), were tested: R1, control regime (70–90% WHC); R2, mild water stress regime (40–60% WHC); and R3, severe water stress regime (20–40% WHC). For each regime, five treatments were established: inoculation with rhizobial isolates (AjuPt16, SafPt6 and SafPt12), inoculation with *Mesorhizobium ciceri* biovar *biserrulae* reference strain, and non-inoculation (N-free treatment). Three blocks completely randomized with five plants per treatment and in the three water regimes (5 × 3 × 3) were used in the trial design.

Sowing and inoculation. *Biserrula pelecinus* cv. Casbah seed were scarified and surface sterilized over 2 min in 70% (v/v) ethanol, 20 min in 10% (v/v) sodium hypochlorite, followed by six rinses with sterile distilled water. Free-draining pots (0.2 dm^3), previously surface sterilized with 10% (v/v) sodium hypochlorite, were filled with 190 g of dry-substrate 1:1 mixture river sand and perlite steam-sterilised for 1 h at 121°C/1.5 atm. Each pot received one seedling pre-grown in water-agar plates, and was completely covered with sterilized polythene beads (Aulabor Industries, S.A. Barcelona, Spain) to prevent airborne contamination. Seedlings inoculation was conducted one week after sowing. Each rhizobial isolate was grown in YEM broth (yeast extract-mannitol, Sigma, Germany) at 28°C and 120 rev./min, till stationary phase. Each seedling received 8 mL of the appropriate bacteria culture and 20 mL of sterile N-free Jensen's nutritive solutions (Vincent 1970).

Irrigation and measurement of plant-water status. *B. pelecinus* seedlings grew without water restrictions for five weeks after germination. Water regimes began in the 6th week and endured until harvest (9th week). Twice a week, water regimes were monitored. The watering amount for each pot was determined according to the difference between the weight of a re-watered pot and the weight of the pot 48 h later. Once a week, plant-water status was checked by measuring leaf water potential (Ψ_w) and relative water content (RWC) in the most expanded leaves. Ψ_w, expressed in MPa, was determined using the Scholander pressure chamber (Turner 1986). Two leaflets, just below the youngest expanded leaf were cut, weighed (fresh weight, FW) and placed in distilled water at 4°C. After a 16 h period of saturation, leaves were weighed (turgid weight, TW) and dried at 65°C to constant mass (dry-weight, DW). RWC was calculated as:

$$RWC = (FW - DW)/(TW - DW) \times 100$$

Plant observations and harvesting. Leaf surface area (LSA) was measured using LI-3050A transparent belt conveyer accessory equipment (Nebraska, USA). Shoot, roots and nodules were oven-dried at 70°C during 48 h and then weighed. Dried shoot biomass was powdered with a mill ball (IKA Labortechnik, A10, Staufen, Germany) and analysed for shoot %N and $\delta^{15}N$ determination. Shoot N content was determined as:

$$Shoot\ N = \%N \times SDW$$

where: %N – the percentage of nitrogen of the sample.

Root/shoot ratio was calculated as: R/S = RDW/SDW.

^{15}N natural abundance. Dry shoot biomass samples were analyzed for isotopic composition ($\delta^{15}N$) through isotope ratio mass spectrometry (IRMS) at the UIB (University of the Balearic Islands, Islas Baleares, Spain). The fraction of N derived entirely from N_2 fixation (N_{dfa}) in the N_2-fixing plants (Högberg 1997) was calculated as:

$$\%N_{dfa} = (\delta^{15}N_{ref} - \delta^{15}N_{fix})/(\delta^{15}N_{ref} - B) \times 100$$

where: $\delta^{15}N_{ref}$ – the $\delta^{15}N$ from a non-fixing N_2 reference plant; B – the $\delta^{15}N$ from N_2 fixing-plant when totally dependent on N_2 as the only N source.

The B-value used for biserrula was –3.53‰ (Vicente 2010). The total amount of N in the plant derived from N_2 fixation (N_{fix}) was determined by:

$$N_{fix} = N_{dfa} \times N\ content$$

Statistical analyses. A multivariate general linear model was used to test the null hypothesis that there were no statistical differences between treatments under the three water regimes. When appropriate, one-way ANOVA was applied to determine the main effect of each source of variation. Tukey's test ($P < 0.05$) was used for multiple mean comparisons. All statistical analyses were performed using the software SPSS 15.0 for Windows (Windows version 7.0, USA).

RESULTS AND DISCUSSION

The effect of water stress in BNF was intensively studied in grain legumes such as *Cicer arietinum* L., *Glycine max* L. or *Phaseolus vulgaris* L. (Fellows et al. 1987, Purcell et al. 2004, Mnasri et al. 2007) and to a lesser extent, in tree (Mrema et al. 1997) and forage legumes (Engin and Sprent 1973). In this work, the effects of water deficiency in BNF of three native rhizobial isolates from forage legume *Biserrula pelecinus* (AjuPt16, SafPt6 and SafPt12) (Vicente et al. 2009) were investigated under three watering levels (R1, well-watered conditions; R2, mild-water stress; and R3, severe water-stress) applied during the *B. pelecinus* vegetative stage.

Plant-water status. The parameters Ψ_w and RWC were used as indicators of plant-water status. Statistical differences ($P < 0.01$) were found between the three water levels (Table 1, Figure 1), as seen in *P. vulgaris* (Martínez et al. 2007) and *Trifolium* sp. (Iannucci et al. 2002). In general, Ψ_w lowered significantly from –0.68 MPa in R1, to –0.74 MPa in R2, and –0.97 MPa in R3, while RWC decreased only by 10%. However, the effect of rhizobia inoculation was not seen ($P > 0.05$) (Table 1), indicating that the inoculation with the three selected isolates had no influence in the plant-water status parameters (Figure 1). Plant host is generally more sensitive to water stress than its bacterial symbiont (Zahran 1999). These results helped to see that *Biserrula pelecinus* also present a degree of tolerance to water stress conditions, avoiding tissue dehydration. This feature was expected to be so, since biserrula develops a deep root system which enables its persistence during dry autumns and springs (Howieson et al. 1995). Biserrula seedlings maintained a high RWC as Ψ_w decreased, a reflection of biserrula tolerance to dehydration. Nunes et al. (2008) observed also for the plant model *Medicago truncatula* L. that leaf RWC was maintained in mild-water conditions, decreasing only in severe drought.

Although the inoculation effect was not consistent to biserrula plant-water status in the three water-levels, rhizobial isolates obtained from biserrula nodules were able to withstand soil water potentials as low as –1.80 MPa (in the case of SafPt6). According to Zahran (1999), the survival and activity of microorganisms may depend on their distribution among microhabitats and changes in soil moisture. In accordance to this, it is not surprising that SafPt12 was able to present as good performance as that of *Mesorhizobium*, since its origin was from the southeast part of Alentejo region, Safara (Moura) where the annual precipitation does not exceed 450 mm (Costa 2008). The same was observed by Swaine et al. (2007), which described that *Bradyrhizobium elkanii* strains in *Albizia adianthifolia* in Ghana evolved in response to local differences in seasonal water availability.

Growth and productivity. Plant growth (LSA and R/S) and productivity (SDW and N content) was compromised by water stress conditions (Table 2). Reduction in biserrula above-ground biomass and increase in R/S, as seen in *Amaranthus* spp. (Liu and Stützel 2004), were indicatives of drought avoidance strategies. The LSA reduced significantly from R1 to R3, nearly about 70% for plants inoculated with SafPt12, SafPt6 and *M. ciceri* biovar *biserrulae*. Biserrula seedlings inoculated with AjuPt16 only suffered a reduction of 56% in the LSA from R1 to R3 regime. Both LSA and R/S were only statistically affected ($P < 0.01$) by the water regime. Biomass production and N content in *B. pelecinus* also decreased with increasing levels of drought stress. SDW was significantly influenced ($P < 0.01$) by the water-level conditions and the different treatments. The best SDW was obtained in the well-watered conditions (R1 regime) whereas, for all treatments, a reduction in 50 to 60% was observed in R3 regime. Seedlings inoculated with SafPt12 and *M. ciceri* biovar *biserrulae* showed the higher SDW than the other rhizobial isolates, 146 and 150 mg/plant in R1, and 82 and 81 mg/plant in R3, respectively. Seedlings from the control treat-

Table 1. Results of multivariate analysis comparing plant-water status parameters (Ψ_w and RWC) in seedlings of *Biserrula pelecinus* grown under three water regimes (R) and treatments (SafPt12, SafPt6, AjuPt16; *Mesorhizobium ciceri* biovar *biserrulae*, N-free)

Source	Variable	*df*	MS	*F*	*P*
Water regime (R)	Ψ_w	2	1.911	14.393	0.000**
	RWC	2	0.222	17.187	0.000**
Treatments (T)	Ψ_w	4	0.089	0.671	0.613^{ns}
	RWC	4	0.038	2.939	0.021*
R × T	Ψ_w	8	0.142	1.067	0.386^{ns}
	RWC	8	0.016	1.223	0.286^{ns}
Error	Ψ_w	253	0.133	–	–
	RWC	253	0.013	–	–
Total	Ψ_w	268	–	–	–
	RWC	268	–	–	–

*$P < 0.5$; **$P < 0.01$; ^{ns}not significant

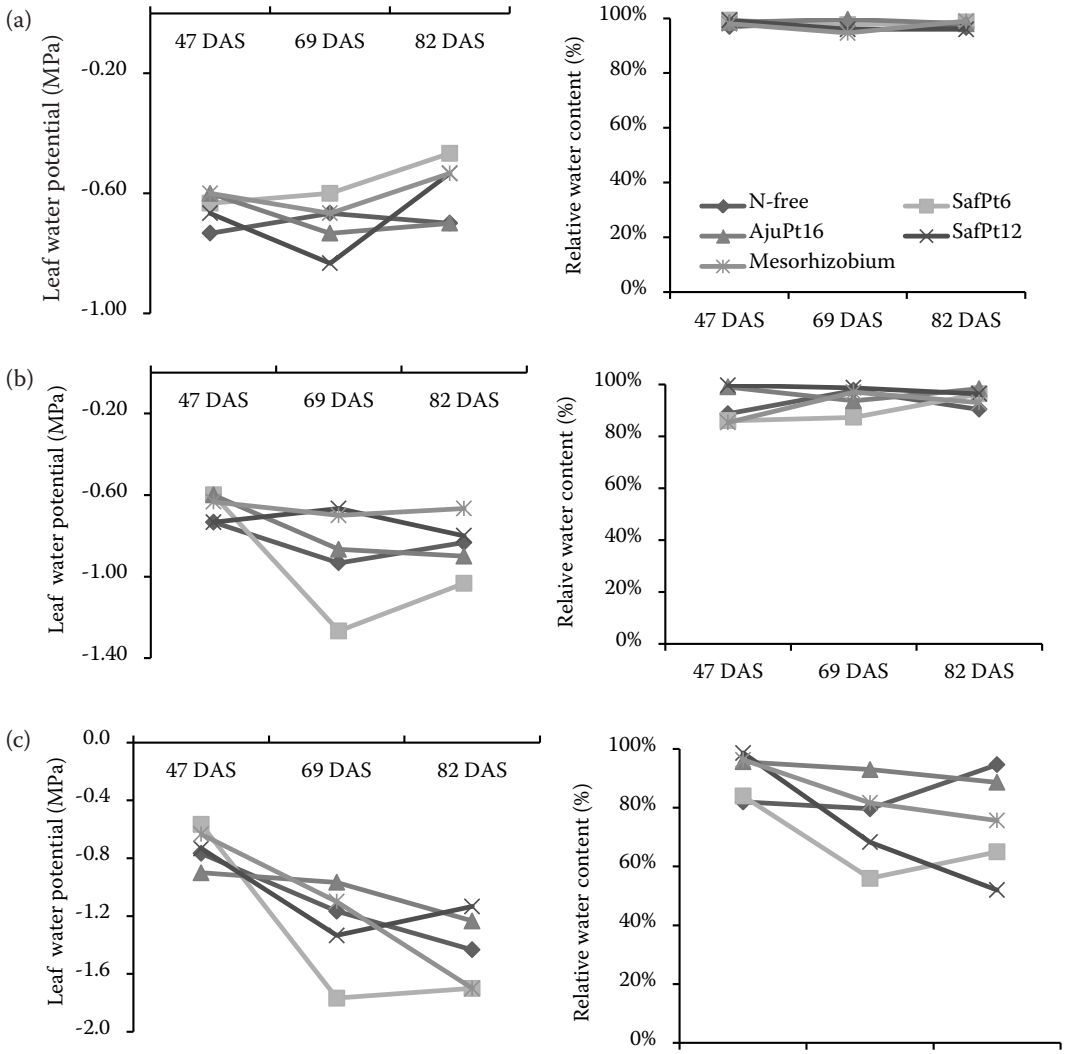

Figure 1. Effect of water stress regimes (R1 – control (a); R2 – mild stress regime (b); R3 – severe stress regime (c)) in leaf water potential and relative walter content in *B. pelecinus* seedlings grown under treatments (SafPt12, SafPt6, AjuPt16; *Mesorhizobium ciceri biovar biserrulae*, N-free)

ment presented lower SDW in all water regimes. Shoot N content decreased significantly ($P < 0.01$) with water deprivation, and was statistically influenced ($P < 0.05$) by different treatments. In *Vigna unguiculata*, differences between performance of *Bradyrhizobium* strains were observed in terms of shoot biomass, which was the clue to select strains resistant to water stress (Figueiredo et al. 1998). In R1, seedlings inoculated with SafPt12, AjuPt16 and *M. ciceri* biovar *biserrulae* recorded approximate N content, without statistical differences ($P > 0.05$). In R2, plants from *M. ciceri* biovar *biserrulae* inoculation treatment continued to present the highest N concentration in shoot biomass, decreasing considerably, about 50%, in R3 regime. In R3, shoot N content from SafPt12 and *M. ciceri* biovar *biserrulae* plants were statistically similar ($P > 0.05$). The response to inoculation with SafPt12 in mild stress and severe stress were almost indistinguishable, possibly indicating that this isolate may present tolerance to severe water stress.

Estimation of BNF. The effects of water depletion in the BNF of *Biserrula pelecinus* are also frequently seen in other leguminous species (Woldeyohannes 2007, Lobato et al. 2009). Limiting water availability decreased nodulation (NN and NDW) (Table 2) and nitrogen fixation (Figures 2). NN and NDW were significantly affected ($P < 0.01$) by water regimes and their interaction with the treatments applied. NDW of the four rhizobia inoculated decreased more significantly under severe drought. The average NN have slightly changed from R1 to R3 for all rhizobial isolates,

Table 2. Growth (leaf surface area (LSA) and root/shoot ratio), productivity (shoot dry weight (SDW) and N content) and nodulation (number of nodules (NN) and nodules dry weight (NDW)) parameters (means and s.d., n = 5) of *Biserrula pelecinus* seedlings at different water regimes (R1, R2, R3) and treatments (SafPt12, SafPt6, AjuPt16; *Mesorhizobium ciceri* biovar *biserrulae*, N-free)

Regimes	Treatments	LSA (mm^2)	Root/Shoot	SDW (mg/plant)	N (mg N/SDW)	NN	NDW (mg/plant)
	SafPt12	40.74 (9.78)^{a}	1.18 (0.26)^{a}	146.17 (30.85)^{a}	5.77 (1.26)^{a}	6.50 (2.35)^{ab}	5.65 (1.22)^{a}
	SafPt6	35.14 (9.79)^{a}	1.07 (0.24)^{a}	96.2 (12.71)^{b}	4.62 (1.17)^{ab}	4.33 (2.81)^{b}	3.44 (1.50)^{b}
R1	AjuPt16	34.92 (16.69)^{a}	1.18 (0.17)^{a}	139.92 (59.88)^{ab}	5.37 (2.22)^{a}	6.31 (3.40)^{ab}	4.36 (2.09)^{ab}
	Mesorhizobium	44.29 (19.78)^{a}	1.11 (0.27)^{a}	149.69 (69.74)^{a}	5.98 (2.82)^{a}	6.93 (2.77)^{a}	3.77 (1.50)^{a}
	N-free	34.02 (11.92)^{a}	1.09 (0.16)^{a}	117.46 (25.43)^{ab}	3.82 (0.54)^{b}		
	SafPt12	25.41 (9.53)^{b}	1.33 (0.26)^{ab}	85.13 (10.03)^{c}	3.20 (1.43)^{c}	5.94 (3.20)^{b}	2.94 (1.17)^{ab}
	SafPt6	17.66 (4.54)^{c}	1.41 (0.24)^{a}	117.54 (37.19)^{b}	4.34 (1.22)^{b}	7.95 (4.31)^{a}	4.55 (1.71)^{ab}
R2	AjuPt16	23.26 (8.99)^{bc}	1.15 (0.26)^{ab}	104.72 (31.45)^{bc}	3.97 (1.29)^{bc}	5.74 (3.85)^{ab}	3.45 (1.94)^{a}
	Mesorhizobium	33.72 (14.76)^{a}	1.17 (0.22)^{ab}	152.03 (40.80)^{a}	5.82 (1.50)^{a}	10.02 (3.88)^{a}	4.49 (1.94)^{b}
	N-free	28.26 (7.17)^{ab}	1.03 (0.27)^{b}	117.46 (25.43)^{ab}	3.13 (0.74)^{c}		
	SafPt12	11.47 (7.38)^{a}	1.52 (0.26)^{a}	82.47 (20.85)^{a}	3.00 (0.74)^{a}	5.74 (1.61)^{ab}	2.70 (0.75)^{ab}
	SafPt6	10.07 (5.13)^{a}	1.61 (0.29)^{a}	64.62 (30.37)^{ab}	2.33 (1.06)^{ab}	4.50 (2.10)^{b}	2.65 (2.24)^{ab}
R3	AjuPt16	15.23 (7.39)^{a}	1.31 (0.36)^{b}	74.09 (27.80)^{ab}	2.67 (1.06)^{ab}	6.62 (3.17)^{a}	3.18 (1.42)^{a}
	Mesorhizobium	12.98 (7.18)^{a}	1.56 (0.26)^{a}	80.69 (27.89)^{ab}	2.94 (0.96)^{a}	7.48 (3.05)^{a}	2.11 (0.93)^{b}
	N-free	14.84 (6.86)^{a}	1.21 (0.16)^{b}	60.57 (24.37)^{c}	2.15 (0.98)^{b}		
F_{regime}		102.07**	31.80**	47.90**	61.25**	5.32**	17.13**
$F_{treatments}$		4.41**	7.52**	8.06**	9.13**	75.60**	57.91**
$F_{regime \times treatments}$		1.72	2.74	3.69**	3.64*	3.53**	5.71**

and the NDW decreased considerably in R3 regime, indicating that, in water-stressful conditions, there was formation of small-sized nodules. Williams and Mallorca (1984) showed that under mild water stress conditions, the NN of *Glycine max* was reduced, while moderate and severe water stress reduced both the number and size of nodules. BNF parameters (%N_{dfa} and N_{fix}) also decreased significantly from R1 to R3. This result was also seen by Khadka and Tatsumi (2006) in *Phaseoli vulgaris*, *Vignia unguiculata*, and *Glycine max* and by Kurdali and Al-Shamma's (2010) in *Lens culinaris*

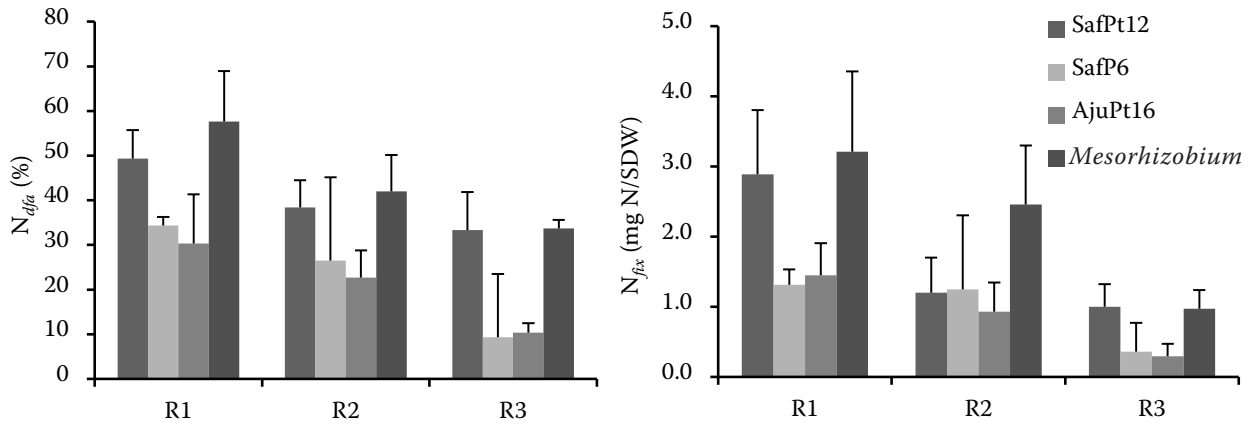

Figure 2. Effect of water stress regimes (R1 – control; R2 – mild stress regime; R3 – severe stress regime) in N_{dfa} and N_{fix} analyzed in *B. pelecinus* seedlings grown under three inoculation treatments (inoculation with SafPt6, AjuPt16, SafPt12, and *Mesorhizobium ciceri biovar biserrulae*)

plants. Biserrula plants inoculated with reference strain presented an estimated reduction of 58% in N_{dfa} and a 30% reduction in N_{fix}. No statistical differences ($P > 0.05$) were observed in seedlings inoculated with SafPt12 and *Mesorhizobium* under mild (R2) and severe (R3) water stress conditions. Plants inoculated with SafPt6 and AjuPt16 recorded lower values of $\%N_{dfa}$ and N_{fix} from R1 to R3 regimes when compared with the other two rhizobial isolates. Among rhizobial isolates tested, *Mesorhizobium* reference strain and SafPt12 were superior to the others in their resistance to water stress, forming associations of greater symbiotic efficiency and helping the biserrula plants to better resist water stress.

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Received on December 30, 2011

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