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Salt tolerance of rhizobial populations from contrasting environmental conditions: understanding the implications of climate change

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Abstract It is predicted that global climate change may alter environmental parameters such as rainfall distribution which in turn may alter the salinity of soils with unpredictable effects upon soil microbial populations. In the present work the tolerance to salinity of rhizobia, isolated from locations with contrasting climatic conditions, and the potential of strains to fix nitrogen symbiotically under saline conditions were investigated. Since plasmids may encode key genes related to growth and survival under environmental stress conditions, which will reflect on protein synthesis, both the plasmid and protein profiles were analyzed. A multivariate statistical approach related salt tolerance to the origin of the isolates, identifying rainfall and water availability as a possible factor explaining the differences in salt tolerance displayed by rhizobia isolates. The classification analysis allowed the subdivision of isolates in terms of salt tolerance into extremely sensitive (≤0.15 %), sensitive (0.15–0.6 %), moderately tolerant (0.9–1.5 %), tolerant (2.1–3.6 %) and extremely tolerant (≥5.4 %). Taken all together it was shown that plasmids are involved in salt tolerance and that the impact of salinity on the protein profile and nitrogen fixation varied according to the salt tolerance of the strains, evidencing the susceptibility of rhizobial communities to changes in rainfall regimes.

Keywords Water availability · Rhizobia · Salinity · Plasmids · Nitrogen fixation

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Introduction

Salinization is one of the most crucial factors threatening agricultural land throughout the world. Approximately one third of the 260 million ha of irrigated land worldwide is affected by salinization (El-Akhal et al. 2013). Higher temperatures and altered rainfall distribution are alterations predicted for southern Europe (Connor et al. 2012). These alterations can increase agricultural salinization by increasing the rates of surface water evaporation and thus the water demand for irrigation. In consequence, nutrient acquisition decreases, biological nitrogen fixation is reduced, and nutrient cycling is disrupted (St.Clair and Lynch 2010).

Rhizobia are of considerable scientific, economic and ecological interest because of their ability to establish nitrogen-fixing nodules on leguminous hosts. This feature enables plant growth in soils with low nitrogen levels and good crop yields without high nitrogen fertilization, decreasing the contamination of water reservoirs by inorganic nitrogen compounds (Singh et al. 2011). Microorganisms, like rhizobia, can also impart some degree of tolerance to plants towards abiotic stresses like drought and salinity (Yang et al. 2009; Grover et al. 2011).

Salinity affects free-living rhizobia, since it imposes both ionic and osmotic stress, which can be extremely detrimental for microflora survival (Figueira 2000; Domínguez-Ferreras et al. 2006). Salinity also considerably

restrains the nodulation and symbiotic nitrogen fixation (Zahran 1999; Bolaños et al. 2003; Domínguez-Ferreras et al. 2006; Bianco and Defez 2009; Brígido et al. 2013). Therefore, it becomes imperative to find symbiotic partners that can fix nitrogen under stressful conditions. The selection and characterization of salt-tolerant rhizobia strains, able to fix nitrogen symbiotically under salt conditions, may constitute a strategy for improving legume symbiosis in adverse conditions and may constitute a better economic and sustainable alternative to chemical fertilization (Rejili et al. 2012). However, salt resistant strains can only be identified if the tolerance of rhizobia to salinity is investigated. Thus, studying the tolerance range of rhizobia to salinity is of prime relevance to determine if this range is wide enough to ensure the survival of selected strains to stressful conditions and to establish an efficient symbiosis in these adverse conditions.

Plasmids are considered important in the diversity and adaptation of bacterial populations. A general feature of rhizobia is the existence of a large amount of extrachromosomal DNA, located in large plasmids. Rhizobia plasmids carry genes involved in nodulation (*nod*) and symbiotic nitrogen fixation (*fix*). They may also contribute to saprophytic competence (Mercado-Blanco 1996; Domínguez-Ferreras et al. 2006) and to salt tolerance (Shamseldin 2008). Therefore, the study of the plasmid profile can be a useful tool in determining the plasmids that carry genes encoding for salt tolerance.

One approach for understanding the ability of rhizobia to tolerate salt has been to identify protein alterations induced by stress. A major consequence of salt-stress is the loss of intracellular water, which implies a water deficit and the consequent osmotic effects on a wide variety of metabolic activities (Fatnassi et al. 2011). Under stress, most of the rhizobacteria produce osmoprotectants to modulate their cytoplasmic osmolarity (Grover et al. 2011) and counteract the adverse effects of salinity on cellular processes (Domínguez-Ferreras et al. 2006). These adjustments represent metabolic changes that enable cells to tolerate saline conditions, and are the result of changes in protein synthesis. The study of protein profile alterations may allow assessing the degree of metabolic changes undergone by organisms in order to adapt to the new conditions.

Thus, the present work aimed to assess if strains from different locations present different tolerance to salinity and different efficiencies of N_2 fixation.

With this objective in mind, rhizobia were isolated from five sites with contrasting climatic conditions and geographic locations. The tolerance to salinity was screened and classified. Plasmid analysis was performed and changes in the protein profile induced by salinity were determined. The results were related to the strains tolerance level using a multivariate analysis approach. The influence of salinity on symbiotic N_2 fixation was also evaluated and related to the level of tolerance of the strains.

Materials and methods

Soil description

Agricultural soils with different salinities, water contents, and geographical locations were used to isolate rhizobia: Costa Nova (40°35′15.52″ N, 8°45′21.03″ W)—a sandy soil with maritime influence (1.12 mg Na^+ g^{-1} dry soil, 0.11 %) and water availability throughout the year; Vagos (40°32′39.94″ N, 8°42′29.08″ W)—a sandy loam soil with low salinity (0.23 mg Na^+ g^{-1} dry soil, 0.023 %) and high water availability throughout the year; S. Bernardo (40°36′25.89″ N, 8°37′12.92″ W)—a silt loam soil with low salinity (0.29 mg Na^+ g^{-1} dry soil, 0.029 %) and water availability throughout the year; and Alentejo (south of Portugal, 38°55′01.96″ N, 7°19′10.89″ W)—a clay loam soil with low water availability and high temperatures during part of the year. In the Alentejo area, soils were collected in late spring (0.36 mg Na^+ g^{-1} dry soil, 0.036 %) and late autumn (0.32 mg Na^+ g^{-1} dry soil, 0.032 %).

Rhizobia isolates

Pisum sativum L. plants cultivar "Resal" (previously described as a salt tolerant pea cultivar—(Figueira 2000; Figueira and Caldeira 2005) were grown for 30 days in a greenhouse with natural light, at 22/15 °C day/night temperature and 65–75 % relative humidity, in containers filled with the soils described above. Nodules were detached from roots and washed in tap water to remove soil particles. Washed nodules were sterilized in 2.5 % sodium hypochlorite for 2 min followed by 95 % ethanol and washed thoroughly in six changes of sterile water. Surface-sterilized nodules were streaked onto the surface of yeast extract–mannitol (YEM) agar containing Congo Red (YEM-CR). After 5 days growing at 26 °C, the colonies that do not absorbed the dye (white or light-rose colonies) were transferred to YEM-CR and simultaneously to YEM supplemented with bromothymol blue (YEM-BTB) and PGA media (peptone, glucose agar) supplemented with bromocresol purple (PGA-BCP). After 5 days of growth, isolates that simultaneously showed low absorption of Congo red, a yellow ring surrounding colonies growing in YEM-BTB and poor growth in PGA-BCP medium, were presumably accepted as rhizobia isolates (Somasegaran and Hoben 1994). To confirm isolate authenticity, inoculation under controlled conditions was performed for all

isolates according to Somasegaran and Hoben (1994). Twenty two isolates were obtained from Costa Nova (CN isolates), thirty one from Vagos (V isolates), sixteen from S. Bernardo (SB isolates), twenty three from Alentejo during late spring (ALE isolates), and thirty seven in Alentejo in late autumn (AL isolates).

Screening for salt tolerance

The salt tolerance of the 129 rhizobia isolates was screened in YEM (Somasegaran and Hoben 1994) added with different NaCl concentrations: 0, 0.075, 0.15, 0.3, 0.6, 1.2, 1.8, 2.4, 3, 3.6, 4.2, 4.8, 5.4, 6, 7.2, 8.4, 9.6 and 10.8 %. The selection of these concentrations was based on the tolerance displayed by the strains. For each isolate and salt concentration at least four replicates were performed. Inoculated tubes were incubated at 26 °C in an orbital shaker (200 rpm) for 72 h. For growth measurement, optical density (620 nm) was determined (Figueira et al. 2005).These results were used to calculate IC_{50} values, i.e. the concentration that inhibited growth by 50 %.

A sub-set of 24 isolates, defined according to the range of IC_{50} values, was chosen for screening nitrogen fixation, plasmid profiling and protein analysis. These isolates were identified by 16S rDNA (Pereira 2010) as *Rhizobium leguminosarum* biovar *viceae*.

Screening for nitrogen fixation potential

The nitrogen fixation potential of the 24 *R. leguminosarum* strains was determined both in the absence (0 %) and presence of salt stress (0.54 %). With this purpose, seeds of *P. sativum* cultivar Resal (previously shown to tolerate 0.54 % NaCl by Figueira (2000) and Figueira and Caldeira (2005) were surface-sterilized in 30 % (w/v) H_2O_2 for 2 min, rinsed with sterile water and germinated in moist autoclaved vermiculite and sand (1:1) at 26 °C for 48 h. The young seedlings were sown in Leonard jars (one per jar) with nutrient solution (Somasegaran and Hoben 1994). Nutrient solution was renewed twice a week. Each seedling was inoculated with 1 mL of *R. leguminosarum* fresh culture (10^9 cell mL^{-1}), and grown in a greenhouse with natural light, at 22/15 °C day/night temperature and at a relative humidity of 65–75 %. For screening nitrogen fixation under salt stress, the same procedure was followed, but NaCl (0.54 %) was added to the nutrient solution. In both conditions (with and without salt) two different controls were included, C0 (no inoculation and no addition of inorganic nitrogen) and CNO_3 (no inoculation and 5 mmol L^{-1} NO_3). For each strain and for controls three replicates (three pots with three plants each) were performed. The shoots were harvested 30 days after sowing, dried at 60 °C for 48 h, and the dry weights recorded. Root nodules were detached, counted and their colour recorded. Symbiosis effectiveness was evaluated by the presence of pink nodules and by shoot dry weight, as described by Somasegaran and Hoben (1994) and El-Akhal et al. (2013). Both controls were used as minimum (C0) and maximum (CNO_3) reference for plant nitrogen nutrition, since the only nitrogen source for plants grown in the C0 condition is the nitrogen present in the seed and for plants grown in CNO_3 condition is the presence of 5 mmol L^{-1} NO_3, which is easily available for uptake (Figueira and Caldeira 2005).

Plasmid profiling

Plasmid analysis was performed for the 24 strains selected. Plasmids were separated on horizontal agarose gels using an in-well lysis method (Hynes et al. 1985). Gels were prepared in Tris-borate-EDTA (TBE) buffer (pH 8.0) with 0.75 % agarose and 1 % (w/v) SDS. Approximately 0.1 mL of fresh culture (OD 620 nm = 0.2) was mixed with 0.5 mL of a 3 % N-lauroylsarcosine solution in a sterile microtube on ice. The cells were pelleted by centrifugation at 14 000 *g* for 5 min at 4 °C, resuspended in 20 µL of a freshly prepared lysis solution (10 % sucrose, 100 µg mL^{-1} lysozyme, and 10 µg mL^{-1} RNase in TBE), and immediately loaded into the wells in the gel. Electrophoresis was carried out at 10 V for 60 min and then at 80 V for 6 h. Gels were stained for 30 min in ethidium bromide solution (0.5 mg mL^{-1}). Densitometric readings were performed using a Bio-Rad densitometer (Model GS-800). Quantity-One Software (Bio-Rad) was used to estimate the molecular weight of plasmids using strains USDA 1021 (155, 185, 600, 800 and 1,000 MDa), USDA 1025 (430 and 700 MDa) and USDA 2101 (150, 220 and 500 MDa) as markers (Hashem and Kuykendall 1994). USDA strains were kindly provided by Peter van Berkum from the United States Department of Agriculture.

Protein extraction and SDS-PAGE electrophoresis

The selected strains were grown until late logarithmic phase in YEM medium (control) and in YEM supplemented with NaCl concentrations inducing 50 % growth inhibition. Cells were harvested by centrifugation at 2,100 *g* for 15 min at 4 °C, washed with dH_2O, pelleted by centrifugation at 15,000 *g* for 20 min at 4 °C, and resuspended in 200 µL of sample buffer (Hames and Rickwood 1981). Samples were then sonicated 1 min and submitted to 95 °C for 3 min. Lysates were centrifuged at 15,000 *g* for 20 min at 4 °C to remove cell debris. The supernatant was collected and used for protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), carried out in bis-Tris precast gels

(4–12 %) 0.75 mm thick (Bio-Rad) following the system of Laemmli (1970). Molecular weight standards used were purchased from Bio-Rad (Prestained SDS-PAGE standard-Broad Range). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). Densitometric readings were performed using a Bio-Rad densitometer (model GS 710). The molecular weight and relative amount of proteins corresponding to each band were calculated using Quantity-One software (Bio-Rad) (Figueira et al. 2005).

Statistical analysis

The IC_{50} matrix data, for all isolates, was transformed into a Euclidean distance similarity matrix using the average-clustering algorithm. This similarity matrix was submitted to classification analysis.

Data on symbiosis efficiency (evaluated by shoot dry weight) were submitted for hypothesis testing using permutation multivariate analysis of variance, following the calculation of Euclidean distance matrix among samples. A one way hierarchical design was followed, with the tolerance level as the main fixed factor. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among different salinities. The null hypotheses tested were: (a) for a given tolerance level, no significant differences exist between conditions (0 and 0.54 % NaCl); (b) for a given condition (0 and 0.54 % NaCl), no significant differences exist between tolerance levels. The similarity matrix was simplified through the calculation of the distance among centroids matrix based on the tolerance level, which was then submitted to ordination analysis, performed by non-metric multidimensional scaling (NMDS).

Data on plasmid profiles, for all strains, were transformed into a Euclidean distance similarity matrix using the average-clustering algorithm. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on the tolerance levels, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson's correlation vectors corresponding to the total plasmids number and to the plasmids identified were superimposed on the top of the PCO graph.

Data on protein profile alterations, for all strains, were transformed into a Euclidean distance similarity matrix using the average-clustering algorithm. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on the tolerance levels, which was then submitted to ordination analysis, performed by PCO. Pearson's correlation vectors corresponding to the total number of alterations and to the proteins whose synthesis was altered by salinity were superimposed on the top of the PCO graph. The Euclidean distance similarity matrix, based on the proteins profile alterations, was submitted to hypothesis testing using permutation multivariate analysis of variance. A one way hierarchical design was followed, with the tolerance level as the main fixed factor. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistic in the pairwise comparisons was evaluated in terms of significance among different salinities. The null hypothesis tested was: no significant differences exist between tolerance levels.

All analyses were performed using the PRIMER v6 software, with PERMANOVA + add-on (Anderson et al. 2008).

Results

Salt tolerance

Growth in media with different concentrations of NaCl allowed determining the tolerance levels of the 129 isolates.

Figure 1 shows the affinity groups obtained from the classification analysis based on the IC_{50} data. When including all the isolates in the analysis, two main groups were separated in the classification diagram: group A, constituted by the isolates with IC_{50} lower than 1.5 % NaCl; and group B, characterized by the isolates with IC_{50} higher than 1.5 % NaCl. Both groups were subdivided into subgroups: A.1 and A.2; B.1 and B.2. The subgroup B1 includes six isolates with IC_{50} between 1.5 and 3.6 % NaCl, all from AL, and these isolates were classified as tolerant (T). The subgroup B2 comprises thirteen isolates with IC_{50} higher than 5.7 % NaCl, all from ALE, being considered extremely tolerant (ET). The subgroup A2 includes ten isolates, with IC_{50} between 0.9 and 1.5 % NaCl, nine from ALE and one from AL, classified as medium salt tolerant (MT). Finally, the subgroup A1 includes most of the isolates (78 %) and was further divided into two subgroups. Subgroup A.1.1 includes 47 isolates with IC_{50} lower than 0.15 % NaCl, considered extremely sensitive (ES), and A1.2 subgroup comprises 53 isolates with IC_{50} between 0.15 % and 0.65 % NaCl, classified as sensitive to salinity (S).

The provenance of rhizobia revealed a strong influence in the observed salt tolerance of the isolates. Isolates from Vagos, Costa Nova and S. Bernardo are all included in group A1 (cf. Fig. 1). However, differences between these locations were noticed, since all the Vagos isolates were classified as ES (Fig. 2), whereas the Costa Nova and S. Bernardo isolates included both ES and S isolates, although

Fig. 1 Rhizobia tolerance affinity groups, based on IC_{50} of the 129 isolates ($n = 4$), obtained by classification analysis. Five affinity groups were obtained: *A.1.1* isolates with $IC_{50} \leq 0.13$ % NaCl—extremely sensitive (ES); *A.1.2* isolates with $0.13 \% < IC_{50} \leq 0.6$ % NaCl—sensitive (S); *A.2* isolates with $0.9 \% < IC_{50} \leq 1.5$ % NaCl—moderately tolerant (MT); *B.1* isolates with $2.1 \% < IC_{50} \leq 3.6$ % NaCl—tolerant (T); and *B.2* isolates with $IC_{50} \geq 5.4$ % NaCl—extremely tolerant (ET)

in different proportions (cf. Fig. 2). In Costa Nova approximately 55 % of the isolates were classified as S, while in S. Bernardo the percentage of S isolates was 81 %. Isolates from Alentejo (ALE, spring isolates and AL, autumn isolates) exhibited salt tolerance. Interestingly, AL isolates showed lower salt tolerance than ALE isolates, since more than half of the ALE isolates presented IC_{50} higher than 5.7 % NaCl (ET) and none of the AL isolates were able to grow at such salinity. However, AL was the location where isolates displayed higher diversity of tolerance to salt, including all classes of tolerance except ET.

Nitrogen fixation potential

In the absence of salt all strains established effective symbiosis, as confirmed by the formation of pink nodules; however growth responses varied greatly (Fig. 3). Comparative analysis of shoot dry weight revealed that: (i) except for plants inoculated with the strains V7, ALE20-7, ALE20-8, and V30, all the remaining plants grew more than C0 ($p < 0.05$), in the absence of NaCl; (ii) all plants grew less than CNO_3 ($p \leq 0.05$), in the absence of NaCl; (iii) the plants inoculated with the strains ALE20-8, ALE1-2, ALE2-1, ALE4-2, ALSI2-1, ALNI-3 grew more than C0 ($p \leq 0.05$), in the presence of NaCl; (iv) all plants grew less than CNO_3 ($p \leq 0.05$), in the presence of NaCl. Thus, under 0.54 % NaCl, plants inoculated with most of the strains exhibited a low number of small white nodules, indicating that an efficient symbiosis was not established. In fact, the results obtained showed that shoot growth was severely affected by salinity. Nevertheless, some strains (25 %) were able to establish an efficient symbiosis under salt conditions, being the most effective strains from AL.

Figure 4a shows the symbiotic effectiveness of strains for each tolerance level. The results revealed that under non saline conditions the performance of all strains was similar regardless the level of salinity tolerance, except ET strains which displayed significantly ($p \leq 0.05$) lower symbiosis efficiency. Under moderate salt stress (0.54 % NaCl), all strains decreased significantly ($p \leq 0.05$) the symbiosis efficiency for all levels of salt tolerance. Nevertheless, differences were noticed. Reduction in shoot dry weight was higher (approximately 70 %) in the sensitive (ES and S) than in the tolerant (MT, T and ET) strains (50 % in MT and T strains and 40 % in ET strains) (cf. Fig. 4a). The NMDS ordination plot supported the influence of external salinity in symbiosis efficiency, showing two affinity groups that represent non exposed (group A) and exposed strains (group B) to NaCl. In group B the sensitive strains (ES and S) are very close to each other and quite distant from the tolerant strains (T and ET).

Plasmid analysis

Figure 5 presents the centroids PCO ordination graph, based on the plasmids data. The centroids representation

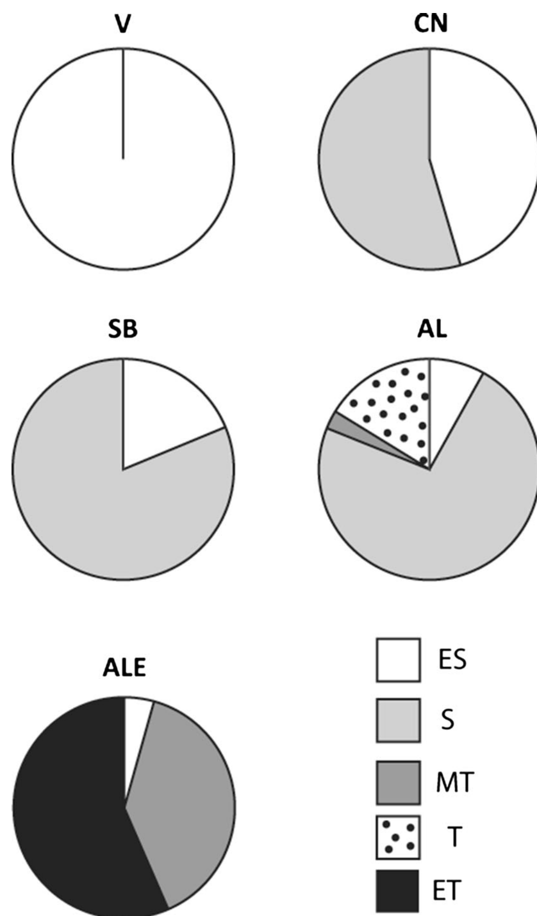

Fig. 2 Percentage of rhizobia isolates tolerance level (ES, S, MT, T and ET) in the five study sites: *V* Vagos, *CN* Costa Nova, *SB* S. Bernardo, *AL* Alentejo winter, *ALE* Alentejo spring

was based on the tolerance level of each strain. Results showed that the two first principal components accounted for nearly 90 % of the overall variability of the data. The PCO1, which accounted for 74.7 % of the variability of the data, clearly separates the strains taking into account their tolerance to salt, with ES and S strains in the positive side of axis 1 and ET, MT and T strains in the negative side of axis 1. The total number of plasmids and the plasmids P170, P427, P201, P343 and P275 (cf. Fig. 5) presented high correlation with axis 1 ($r > 0.80$), while the plasmids P820, P734 and P147 showed high negative correlation with axis 1 ($r < -0.80$). These results highlight that the presence of the plasmids P820, P734 and P147 are clearly associated with the ET, MT and T strains, while the presence of the plasmids P170, P427, P201, P343 and the total number of plasmids are closely related to the sensitive strains (cf. Fig. 5).

Protein profiles

The electrophoretic profiles of soluble proteins in the presence and absence of salt stress were used to evaluate the effect of salt stress on protein synthesis. Figure 6 represents the PCO ordination graph, based on the protein profile alterations matrix. Centroids were obtained grouping strains according to their tolerance level. The results evidenced that the two principal components explained 92.6 % of the total variance. PCO1, which explained 84.1 % of the total variation, was associated with the total number of alterations, separating the strains according to their tolerance level. Indeed, in ET strains the protein

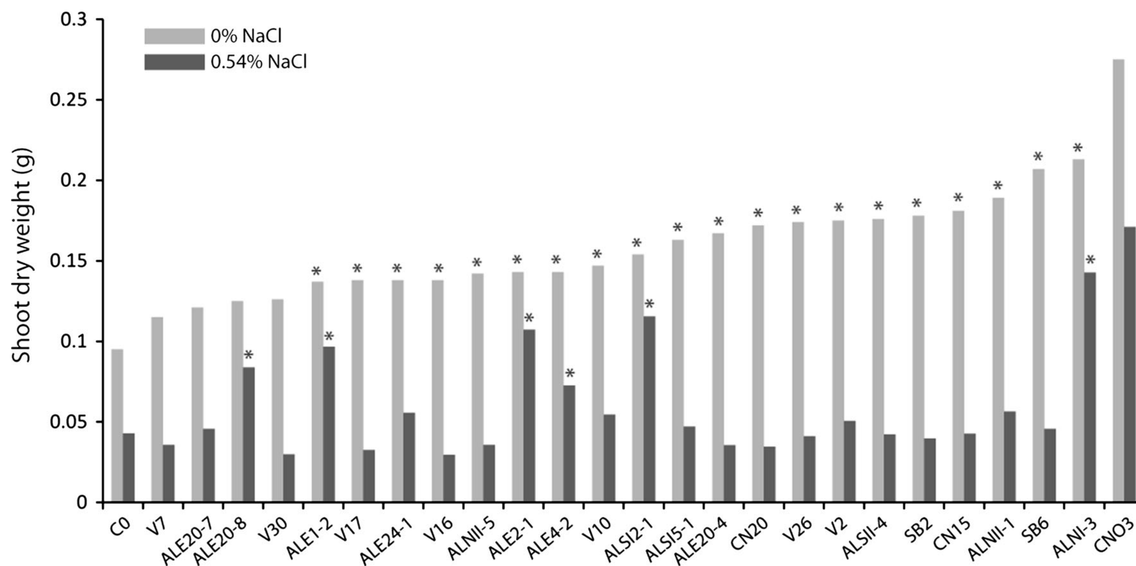

Fig. 3 Symbiotic effectiveness (measured by shoot dry weight) of *Rhizobium* strains from different locations (V, CN, SB, AL and ALE) in the absence (0 %) and presence (0.54 %) of NaCl. Letters (S and N) and numbers correspond to different plant individuals and isolates. *Values* are the mean of 3 replicates, with standard deviation ranging between 5 and 18 %. *Asterisks* represent significant differences ($p \leq 0.05$) between isolates and Control condition (C0), both for the absence (0 %) and presence (0.54 %) of NaCl

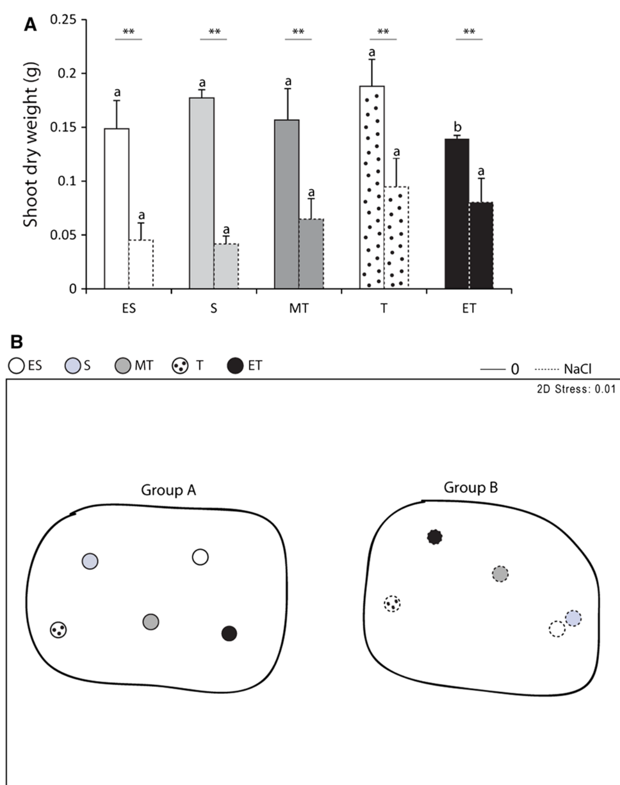

Fig. 4 **a** Symbiotic effectiveness (measured by shoot dry weight) of *Rhizobium* strains with the same tolerance level (ES, S, MT, T or ET) in the absence (0 % NaCl—*solid line*) or presence (0.54 %—*dashed line*) of NaCl. *Values* are the average of strains with the same level of tolerance (±standard deviation). **b** Non metric dimensional scaling (NMDS) diagram for symbiotic effectiveness, in the absence (*solid line*) or presence (*dashed line*) of NaCl, represented by the tolerance level: group A, strains under 0 % NaCl; group B, strains under 0.54 % NaCl

profile was marginally affected compared to the less tolerant, especially S, strains. PCO2 accounted for 8.5 % of the total variation. Alterations in the expression of polypeptides with 13.4, 15.5, 62.9, 90 and 155.9 kDa were only noticed in S and ES strains. Polypeptides P34 and P29.7 altered their expression in S, MT and T strains and the expression of P59.7 was altered in strains with different salt tolerances, except in ET strains.

Discussion

The salt tolerance of rhizobia from different locations and their N_2 fixation efficiency under salt conditions were studied. Five affinity groups were identified based on the growth inhibition data of 129 isolates. Salt tolerance was clearly associated with location; tolerant isolates (MT, T and ET) were all from Alentejo (AL and ALE isolates) and were able to grow under 0.9 and 10.8 % NaCl. To our knowledge 10.8 % is the highest salt tolerance recorded for rhizobia (Zahran 1999; Bouhmouch et al. 2005; Bianco and Defez 2009; Brígido et al. 2012), which was displayed by 60 % of ALE isolates. On the other hand, all northwest isolates (CN, SB and V) were sensitive to salinity (ES and S) and 0.15 % NaCl concentration was enough to inhibit growth by 50 % in ES isolates. Brígido et al. (2012) reported that isolates from Madeira, Beira Baixa, Ribatejo and Baixo Alentejo were less tolerant to 1.5 % NaCl than Algarve, Beira Alta and Beira Litoral isolates, showing an association between salt tolerance and provenance of the isolates.

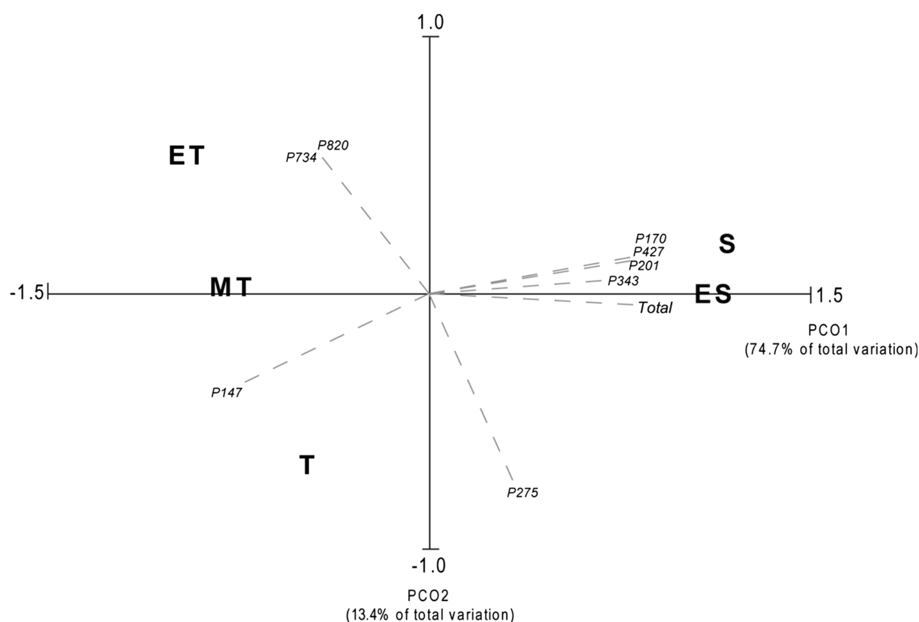

Fig. 5 Centroids ordination diagram (PCO) based on *Rhizobium* strains tolerance level (ES, S, MT, T or ET). Pearson correlation vectors are superimposed using plasmids (82–820 MDa molecular weight) and total number of plasmids data as supplementary variables (r > 0.95)

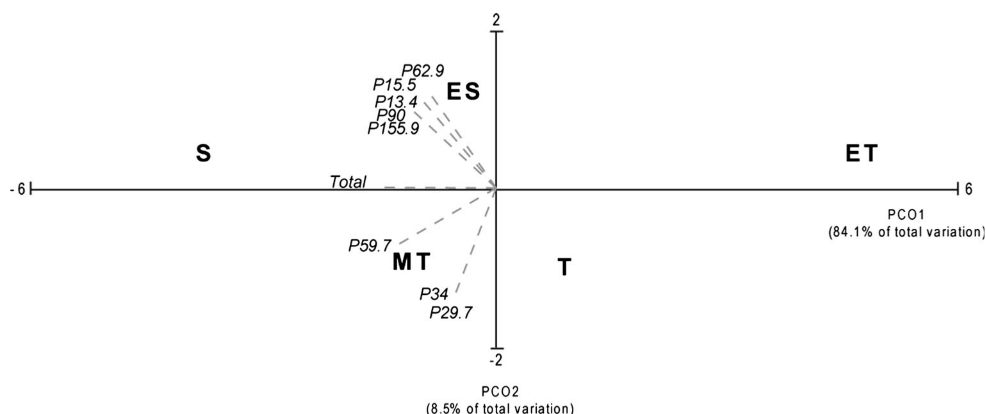

Fig. 6 Centroids ordination diagram (PCO) based on *Rhizobium* strains tolerance level (ES, S, MT, T or ET). Pearson correlation vectors are superimposed using polypeptides (6.6–155.9 kDa molecular weight) and total number of polypeptides whose synthesis was altered by salinity data as supplementary variables ($r > 0.95$)

The five locations in our study had different types of soils, sandy (CN), sandy loam (V), silt loam (SB) and clay loam soil (AL and ALE) and different distances from the sea, 0.2 km (CN), 4.5 km (Vagos), 10 km (S. Bernardo) and around 200 km (AL and ALE). The tolerance of isolates to salinity shows that proximity to the sea did not have a strong influence on the isolates salt tolerance, since CN population, located closer to the sea, presented high susceptibility to salinity. The type of soil also did not prove to be a crucial parameter to bestow salinity tolerance. Sandy, sandy loam and silt loam soils harbored rhizobial populations with high salt sensitivity and rhizobia isolated from the same soil in different seasons displayed differences in salt tolerance.

Rainfall regimes were quite different between northwest and southeast isolates: the annual rainfall in the southeast was around 750 mm and there was almost no rain in late spring (0.7 mm); in the northwest the annual rainfall was over 1,300 mm and in the late spring the precipitation was 102 mm (146 fold higher than in southeast). The high availability of water in northwest locations was not linked with salt tolerance in rhizobia. In particular the population from Vagos, a location crossed by a permanent freshwater stream, presented extreme sensitivity (ES) to salt. Isolates displaying higher salt tolerance were all from the southeast. The variation in water availability between northwest and southeast locations seems to be determinant for the onset of salt tolerance. Differences were also noted between seasons. ALE isolates are more tolerant to salinity than AL isolates, showing that rainfall differences occurring between late autumn (165 mm) and late spring (0.7 mm) are a crucial factor in the tolerance of rhizobia populations to salinity.

Despite the high salt tolerance of isolates from Alentejo, populations from both seasons include isolates with different salt tolerances, even ES. It is intriguing how sensitive isolates are able to survive during late spring and summer, when temperature is high and water availability is low. These bacteria may have survived in microsites able to maintain the soil moisture at levels sufficient to assure their survival, as suggested by Sprent and Zahran (1988) and Zerhari et al. (2000). The survival of isolates with diverse salt tolerance may support fluctuations in the average tolerance of rhizobial population to salinity over the year, as the environmental conditions change, since populations adapt to the prevailing conditions at a particular time (St.Clair and Lynch 2010), as observed in the present work.

Results showed that the salt tolerance can be highly explained by the presence of certain plasmids. Strains possessing the P147 are all tolerant and those with P734 or P820 are extremely tolerant. No relationship between salt tolerance and the presence of plasmids was found in mesorhizobia by Brígido et al. (2012), but Shamseldin (2008) found that all salt-tolerant strains of common bean rhizobia contained a 250 kb plasmid with the exception of one strain, suggesting that this plasmid may play a role in the salt tolerance mechanism. Domínguez-Ferreras et al. (2006) obtained evidence that salt stress and hyperosmotic stress have similar effects on gene transcription in *Sinorhizobium meliloti*, causing induction of a large number of genes (mainly on plasmids). Altogether the results presented revealed the importance of plasmid genes for the activation of biochemical responses to osmotic and salt stresses, conferring osmotic adaptation when rhizobia are exposed to salt stress, such as intracellular accumulation of low-molecular weight organic solutes or inorganic ions, namely K (Miller and Wood 1996; Zahran 1999; Grover et al. 2011). These responses have to be achieved by changes in the metabolic pathways and thus in the protein profiles, evidencing the impact of salinity on cell metabolism. The pathways of osmoprotectants synthesis (glutamate, trehalose, proline, glycine betaine, proline betaine and ectoine) are upregulated, and the synthesis of

macromolecules is inhibited, as well as many metabolic pathways of the central metabolism and energy production (Domínguez-Ferreras et al. 2006). Thus, under salt conditions increases and decreases in the expression of polypeptides occur and the protein profiles are altered. Usually, the higher the impact the greater the number of changes. Our work showed a strong association between alterations in the expression of polypeptides and the less tolerant strains (ES, S and MT). In fact, ET strains presented few changes and S and MT isolates exhibited a higher number of changes. The reduced impact of salinity on the protein profile revealed by most tolerant strains may indicate that the cellular metabolism of these strains was not greatly affected by the imposed saline conditions, suggesting the presence of constitutive mechanisms conferring tolerance to NaCl. Interestingly, ES strains did not undergo many changes in protein expression, presumably because they are facing difficulties for adapting to salinity, even at low concentrations (0.15 %), and this lack of adaptation could have led to ES.

The high sensitivity of the legume-rhizobia symbiosis to salinity has long been recognized. Our study showed that salinity severely affected the efficiency of N_2 fixation. Bolaños et al. (2003) also found that salt stress (0.45 %) inhibited N_2 fixation and the development of *P. sativum* cultivar Argona plants, and Brígido et al. (2013) reported a considerable inhibition of nodulation in *Cicer arietinum* plants grown at 0.15 % NaCl. Additionally our work showed that the salt tolerance of strains impacted differently the symbiosis efficiency, under salt conditions. Inoculation with sensitive strains (S and ES) had higher impact (70 %) on symbiosis efficiency than tolerant strains, especially with ET strains (40 %). These results prove that the salt tolerance of the microsymbiontic partner influences the symbiosis performance, suggested by Brígido et al. (2013) and Mhadhbi et al. (2004).

It is generally accepted that the most sensitive symbiotic partner to salinity is the host, i.e. the legume (Zahran 1999; Bouhmouch et al. 2005; Bianco and Defez 2009; Brígido et al. 2012). The results of our work are not in accordance to these reports. All isolates from the three locations in northwest Portugal displayed higher sensitivity than the pea cultivar used in this work. Noteworthy are the salinities tolerated by all the isolates from Vagos and almost half of the isolates from Costa Nova, which are well below (three to four times lower) the salinity tolerated by the plant host, showing that in the northwest of Portugal rhizobia is the symbiotic partner more vulnerable to salinity. These results highlight that alterations of the present climate conditions leading to salinity or osmotic increase in the soil may severely affect these rhizobial populations either in free-living or in symbiosis with a legume host.

Conclusion

In summary, the tolerance of rhizobial populations to salinity is wide, changing spatial (different locations) and temporally (Alentejo late spring and late autumn). The environmental conditions selecting for osmotic tolerance, also select for salinity tolerance. The salinity tolerance can only be achieved by metabolic changes, which are reflected in protein profile alterations.

Our results suggest that rhizobial populations from southeast Portugal can withstand changes in soil water availability, but the populations from northwest Portugal cannot, being more susceptible to changes in soil water availability, if the predicted scenario of dry summers, described as inevitable in the context of global changes for southern Europe materializes. Our work illustrates the impact of the climate change predicted (lower rainfall, increased variability in water supply, increased salinity) on ecological systems with different salt sensitivities, highlighting the need to consider not only the nature of the climate change, but also the vulnerability of any given system when predictions of climate change impact are made.

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Conflict of interest The authors declare that they have no conflict of interest.

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