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Phenotypic and genetic diversity of Moroccan rhizobia isolated from *Vicia faba* and study of genes that are likely to be involved in their osmotolerance

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ABSTRACT

Rhizobia are symbiotic nitrogen-fixing bacteria in root nodules of legumes. In Morocco, faba bean (*Vicia faba* L.), which is the main legume crop cultivated in the country, is often grown in marginal soils of arid and semi-arid regions. This study examines the phenotypic diversity of rhizobia nodulating *V. faba* isolated from different regions in Morocco for tolerance to some abiotic stresses. A total of 106 rhizobia strains isolated from nodules were identified at the species level by analysing 16S rDNA. Additionally, for selected strains *recA*, *otsA*, *kup* and *nodA* fragments were sequenced. 102 isolates are likely to belong to *Rhizobium leguminosarum* or *R. laguerreae* and 4 isolates to *Ensifer meliloti*. All strains tolerating salt concentrations of 428 or 342 mM NaCl as well as 127 or 99 mM Na2SO4 were highly resistant to alkaline conditions (pH 10) and high temperature (44 °C). Three strains: RhOF4 and RhOF53 (both are salt-tolerant) and RhOF6 (salt-sensitive) were selected to compare the influence of different levels of salt stress induced by NaCl on growth and on trehalose and potassium accumulation. We find a direct correlation between the trehalose contents of the rhizobial strains and their osmotolerance.
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Introduction

In the Mediterranean agrosystems, legumes are the second most important crops after cereals, being of important agronomic and economic interest and a valuable source of proteins, iron, carbohydrates, magnesium and zinc [40]. The ecological interest in these crops relies on their ability to fix atmospheric nitrogen in symbiosis with rhizobia inside nodules [13]. Rhizobia are Gram-negative soil bacteria, which have the ability to establish nitrogen-fixing symbioses with legume plants under conditions of nitrogen deficiency. During this process, a set of signal exchange occurs between the plant and bacteria leading to root nodule formation, where biological nitrogen fixation takes place [25]. This complex process is strongly influenced by various soil parameters, such as drought, extreme pH, salinity, high or low temperature [4,32,60]. Salinity is one of the major factors responsible for deterioration of soil and making it unfit for agriculture. Nearly 5% of the Moroccan land surface can be classified as endangered by salinity [5]. It has been shown in many studies that soil salinity may affect the establishment of functional symbiosis at different steps: growth and survival of rhizobia in soil, root colonization, infection and nodule development processes and nodule functioning [21,23]. Consequently, the non-adaptability of both symbionts may be an important limiting factor. Although rhizobia are often more tolerant to salt stress than their leguminous hosts [1,32], an efficient rhizobium-legume symbiosis under salt stress requires the selection of salt tolerant rhizobia [15]. It has been observed that inoculation by salt tolerant rhizobia strains increases the tolerance and the production of several legumes [17,35,53]. For instance, Suárez et al. [46] reported an increase in root nodule number and nitrogen fixation by *Phaseolus vulgaris* inoculated with a trehalose-6-phosphate synthase-overexpressing strain of *Rhizobium etli*. In addition, Bertrand et al. [7] showed that alfalfa inoculated with a salt-tolerant rhizobial strain maintained higher water content under salt stress.

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These findings emphasize the importance of studying the adaptation mechanisms of rhizobia to salt stress. Indeed, rhizobia adapt to the fluctuations in salinity by either changing the composition of the cell envelope [10,27] or accumulating osmolytes such as trehalose and potassium ions [60]. These compatible solutes confer protection against the deleterious effects of salinity stress, protect intracellular macromolecules and maintain the appropriate cell volume [11]. Trehalose is a non-reducing glucose disaccharide; it protects numerous biological structures against abiotic stress including heat, cold, dehydration, and hyperosmotic conditions [54]. It is therefore of interest to study the effect of salinity stress on trehalose and potassium bioaccumulation in rhizobia. The purposes of the work described here were threefold. First, genotypic and phenotypic characterization of 106 rhizobia strains isolated from nodules of faba bean grown in different regions of Morocco. Second, screening of salt tolerant strains and identification of the genes *otsA* and *kup* known to be involved in osmotic stress tolerance. Third, we focused on trehalose and potassium bioaccumulation in selected tolerant strains. This approach may lead to a wider use and an increased production of Moroccan faba bean using significantly less input of synthetic nitrogen fertilizer, thus making its cultivation economically and environmentally more attractive.

Materials and methods

Isolation and purification of rhizobial strains

Nodules of *Vicia faba* plants were disinfected with sodium hypochlorite (4 °chl) for 15 min and washed several times with sterile physiological water. Nodules were crushed in a sterile tube. The suspension was streaked on Petri dishes containing YEM medium agar with Congo red [52]. After incubation for 48 h–72 h at 28 °C, colonies of rhizobia characterized by a gluey aspect and not taking up Congo red were isolated on YEM medium. The rhizobial strains were purified by repeated streaking on YEM medium agar with Congo red. All purified strains were used in inoculation experiments and all strains nodulated *V. faba* seedlings grown in sterile sand. After identification of RhOF96, the strain was used for reinfection of *V. faba*. Bacteria were reisolated from nodules and the identity of RhOF96 was confirmed again by sequencing of 16S rDNA. Strains were stored at −25 °C in 30% glycerol.

Phenotypic characterization of rhizobial strains

The tolerance of rhizobia to some environmental stresses was determined on yeast extract mannitol (YEM) agar medium. The following treatments were applied: salt 0–428 mM NaCl and 0–127 mM Na_2SO_4; temperature 28–44 °C; pH 5, 6, 8, 9 and 10. A strain was considered tolerant if its growth was similar to that observed on the control plate (3.42 mM NaCl, 0 mM Na_2SO_4, temperature 28 °C and pH 7). Three replicates were included per treatment.

DNA extraction, PCR, sequencing, amplified ribosomal DNA restriction analysis (ARDRA) and accession numbers

For genomic DNA extraction, rhizobia were cultivated in liquid YEM medium at 28 °C for 48 h and 4 mL of the bacterial culture were collected by centrifugation. The bacterial biomass was washed with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8) and resuspended in 300 μL of Tris-EDTA buffer. 100 μL of 5% SDS and 100 μL pronase E (2.5 mg/mL in TE buffer) were added. After mixing, the solution was incubated over-night. The solution was then mixed with 300 μL of phenol-Tris. After centrifugation at 15,000 rpm for 3 min, DNA was further purified by mixing with 300 μL of chloroform-isoamyl alcohol (24:1, v/v). After centrifugation at 15,000 rpm for 5 min, DNA from the aqueous phase was precipitated with 2.5 volumes of absolute ethanol. The samples were centrifuged for 10 min at 14,000 rpm at 4 °C. The resulting DNA pellet was washed with 70% ethanol, vacuum dried, and solubilized in 100 μL of sterile Milli-Q water. The purity and the quantity of extracted DNA were determined using a NanoDrop.

PCR amplification was carried out in a total volume of 50 μL. The reaction mixture contained 100 ng of rhizobial DNA, 5 μL of 10× Dream Taq buffer, 1 μL of dNTP (10 mM), 100 pmol of each primer (Table S1), 1.25 U of DreamTaq polymerase and sterile Milli-Q water (up to 50 μL). The conditions for amplification were: an initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s; annealing temperatures (for 30 s) and extension times (at 72 °C); and a final extension at 72 °C for 10 min. The PCR products were checked by horizontal gel electrophoresis in a 1% (w/v) agarose gel in Tris-Acetate-EDTA (TAE) buffer. The PCR products were purified by "MEGA quick-spin™ Total Fragment DNA Purification Kit" (HiSS Diagnostics, Freiburg). Nucleotide sequencing was done by GATC Biotech (Konstanz) on both strands using the same primers that were used for PCR. For long fragments, internal primers were used in addition (Table S2). Phylogenetic analysis was conducted with MEGA version 6 [48].

For ARDRA, the purified fragments of 16S rDNA were digested with the restriction enzyme *Hpa*II. The restriction patterns were resolved by electrophoresis in 3% agarose gels (130 V for 3 h in Tris–borate-EDTA buffer).

Nucleotide sequences from DNA fragments are deposited in Genbank. Accession numbers for 16S rDNA, *recA*, *nodA*, *kup* and *otsA* are in Table S3.

Extraction and measurement of intracellular trehalose and potassium

To investigate if there is a correlation between salinity tolerance and compatible solutes accumulation, three rhizobia strains (RhOF6, RhOF4 and RhOF53) displaying a different salt tolerance were selected.

The strains were grown in modified rhizobium defined medium (RDM) containing glutamate (1.1 g/L), mannitol (10 g/L), biotin, thiamine, mineral salts [K_2HPO_4 (0.3 g/L), $MgSO_4$ $7H_2O$ (0.15 g/L), $CaCl_2$ $2H_2O$ (0.05 g/L), $FeCl_3$ (0.006 g/L) and NaCl (0.05 g/L)]. The medium was supplemented with different levels of NaCl (0, 50, 100 and 200 mM).

Liquid cultures were started by inoculation with an initial absorbance of 0.04 (A600 nm) and incubated in a gyratory shaker at 30 °C for four days. Growth was registered every 24 h by measuring the absorbance at a wavelength of 600 nm using a spectrophotometer.

In order to determine the intracellular trehalose level in rhizobia, the cells were harvested by centrifugation at 13.000 rpm for 20 min at 4 °C. Cell pellets from 15 mL of early stationary phase cultures in modified RDM medium were washed with isotonic carbon-free medium and resuspended in 1 mL of the same medium. Cells were lysed by incubation at 95 °C for 30 min. After centrifugation, trehalose was assayed in a 2.54 mL reaction volume containing 200 μL of the supernatant according to the commercial kit protocol (Megazyme, Wicklow, Ireland).

To determine the potassium content for each treatment, 10 mL of cultured cells were harvested by centrifugation at room temperature (10,000 rpm, 10 min) and the pellet was washed with 10 mL of physiological water. Samples were resuspended in 1 mL of perchloric acid (0.5 M) and heated for 10 min at 70 °C. Cell debris was removed by centrifugation at 10,000 × g for 10 min. The potassium concentrations of the supernatant was determined using a flame photometer (Jen Woy PFP7).

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