Systematic and Applied Microbiology 42 (2019) 232–239

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: http://www.elsevier.com/locate/syapm

The rhizosphere of the halophytic grass *Sporobolus robustus* Kunth hosts rhizobium genospecies that are efficient on *Prosopis juliflora* (Sw.) DC and *Vachellia seyal* (Del.) P.J.H. Hurter seedlings

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ARTICLE INFO

Article history:
Received 28 August 2018
Received in revised form 18 October 2018
Accepted 19 October 2018

Keywords:
Nurse plant
Salt tolerance
Nitrogen fixation
Phylogeny
Bioremediation

ABSTRACT

The aim of this study was to survey the abundance and genetic diversity of legume-nodulating rhizobia (LNR) in the rhizosphere of a salt-tolerant grass, *Sporobolus robustus* Kunth, in the dry and rainy seasons along a salinity gradient, and to test their effectiveness on *Prosopis juliflora* (SW.) DC and *Vachellia seyal* (Del.) P.J.H. Hurter seedlings. The results showed a significant decrease in LNR population density and diversity in response to salinity, particularly during the dry season. A phylogenetic analysis of the 16S-23S rRNA ITS region clustered the 232 rhizobium isolates into three genera and 12 distinct representative genotypes: *Mesorhizobium* (8 genotypes), *Ensifer* (2 genotypes) and *Rhizobium* (2 genotypes). Of these genotypes, 2 were only found in the dry season, 4 exclusively in the rainy season and 6 were found in both seasons. Isolates of the *Mesorhizobium* and *Ensifer* genera were more abundant than those of *Rhizobium*, with 55%, 44% and 1% of the total strains, respectively. The abundance of the *Mesorhizobium* isolates appeared to increase in the dry season, suggesting that they were more adapted to environmental aridity than *Ensifer* genospecies. Conversely, *Ensifer* genospecies were more tolerant of high salinity levels than the other genospecies. However, *Ensifer* genospeciesproved to be the most efficient strains on *P. juliflora* and *V. seyal* seedlings. We concluded that *S. robustus* hosts efficient rhizobium strains in its rhizosphere, suggesting its ability to act as a nurse plant to facilitate seedling recruitment of *P. juliflora* and *V. seyal* in saline soils.

Introduction

Soil salinization occurs naturally and/or as a consequence of mismanaged irrigation, especially in arid and semi-arid areas of the world. Around 20% of all cultivated lands in the world are affected by salinity [43]. In Senegal, 45% of agricultural lands are salt-affected [21]. Soil salinity affects the growth and development of plants, causing a loss of natural vegetation [12,18]. Despite the loss of vegetation, *Sporobolus robustus* Kunth, a halophyte grass, forms a perennial herbaceous cover in which seedlings of *Prosopis juliflora* (SW.) DC and *Vachellia seyal* (Del.) P.J.H Hurter, two non-salt-tolerant legume trees, grow. The ability of *P. juliflora* and *V. seyal* to become established in poor soils can be partly attributed to their symbiotic association with legume-nodulating rhizobia (LNR) [22,3].

LNR are symbiotic nitrogen-fixing bacteria able to convert N_2 atmospheric nitrogen into nitrogen compounds [36]. They contribute not only to the N sources of legumes, but also to the N content of the soil [36]. LNR have been found to be essential components of *P. juliflora* and *V. seyal* [14,1]. Molecular studies of LNR that develop symbiosis with *V. seyal* and *P. juliflora* have shown that these species are associated with a broad range of species belonging to the genera *Mesorhizobium*, *Ensifer*, *Rhizobium* and *Bradyrhizo-*

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https://doi.org/10.1016/j.syapm.2018.10.006
0723-2020/

bium [12,42,3]. Nevertheless, preferential nodulation by strains of *Bradyrhizobium*, *Mesorhizobium* and those of *Ensifer,* rather than strains of *Rhizobium,* are clearly reported [44,42]. LNR have been shown by many researchers to promote plant growth and salinity tolerance [4]. They promote salinity tolerance by enhancing nutrient uptake [36]. Diouf et al. [15] and Lal and Khanna [27] demonstrated that inoculation with salt-tolerant strains of rhizobia can enhance the nodulation and nitrogen-fixing ability of the *Vachellia* genus growing under saline conditions. However, *V. seyal* and *P. juliflora* rhizobium populations differ in their ability to tolerate salinity and in their nitrogen-fixing ability, as shown by Diouf et al. [12] and Benata et al. [3].

In the Sine-Saloum Delta, *S. robustus* is a virtually ubiquitous grass species and forms a perennial herbaceous cover in which *P. juliflora* and *V. seyal* grow. Seedlings of *P. juliflora* and *V. seyal* often grow in the tussocks of *S. robustus* when soil salt levels are low. A recent study has described the ability of *S. robustus* to accumulate significant amounts of Na, making this halophyte promising as a desalinization tool in saline soils [20]. *S. robustus* could be a "nurse plant" by facilitating *P. juliflora* and *V. seyal* establishment by ameliorating the soil's biological and chemical environment. The improvement of seedling growth by pioneer shrubs, also called the "nurse plant effect", is a general facilitative process [31]. Nurse plants facilitate vegetation growth by ameliorating the physical environment and by increasing soil fertility [7,41]. We put forward the hypothesis that *S. robustus* can host adapted LNR communities in its rhizosphere. These could form effective symbiosis with *P. juliflora* and *V. seyal.* The objectives of this study were therefore to determine the abundance and genetic diversity of legume-nodulating rhizobium populations in the rhizosphere of *S. robustus* in relation to salinity and seasons, and their efficiency on *P. juliflora* and *V. seyal* seedlings.

Material and methods

Study site, soil sampling and analysis

The study site, Niamdiarokh, is located in the Sine-Saloum Delta, in the region of Fatick, in Senegal (14°17′N; 16°46′W). The climate there is semi-arid and characterized by a short rainy season (July–October) and a long dry season (November–June). The mean annual temperature is 27.1 °C and the mean annual rainfall is 634 mm. Soils in this area are ferruginous leached, and the soil texture is mainly sandy. Saltwater intrusion has induced soil salinization. In addition, the high dry season temperatures result in increased evapotranspiration, leading to markedly elevated soil salinity at the end of the dry season. Many plants in this area are halophytes that can survive on saline soils with low nutrient loads. The dominant plants of the vegetation in Niamdiarokh are the grasses *S. robustus* and *Leptochloa fusca* growing on saline soils, whereas the perennial species *Tamarix senegalensis*, *P. juliflora* and *V. seyal* occur on less saline soils.

A plot of 1000 m^2 was chosen along a transect starting on a seaward edge and taking a direction with increasing elevation up to a forested zone. The sampling area was divided into four plots measuring 25 × 10 m each and located 1 m away from each other. Each plot had a mean salinity level where the soil samples from the rhizosphere of *S. robustus* and the bulk soil had approximately the same salinity. The average salinity levels of the plots were 2.10‰, 4.73‰, 14.86‰ and 30.66‰ in the dry season, and 2.14‰, 3.17‰, 8.48‰ and 10.85‰ in the rainy season. In each plot, soil samples were taken from around three *S. robustus* plants. Three soil samples (approximately 500 g in dry weight) were separately collected from the rhizosphere and bulk soil of each plant. Soils were sampled in the dry (June 2014) and rainy (August 2014) seasons. In all, we collected 24 soil samples per plot and per season (12 from the rhizosphere and 12 from bulk soil). In order to compare rhizobium populations inside and outside the root rhizosphere using the Most Probable Number (MPN) assay, rhizosphere and bulk soils were separately pooled per plot and per season. For the physico-chemical analyses, the remaining soil samples from the rhizosphere and bulk soils were pooled per plot and per season. Soil samples were carefully collected from the top 0–25 cm, passed through a coarse sieve (2 mm mesh) and then stored at 4 °C in plastic bags. Physico-chemical analyses were carried out at the LAMA analytical laboratory in Dakar, Senegal (www.lama.ird.sn).

Determining the Most Probable Number of legume-nodulating Rhizobia

The numbers of indigenous soil LNR populations able to nodulate legumes were estimated by the MPN assay [6] using *V. seyal* and *P. juliflora* as the trap hosts. *V. seyal* and *P. juliflora* seeds were collected at Niamdiarokh. They were scarified, surface-sterilized and germinated as described by Fall et al. [19]. Plants were grown in an incubation chamber as previously described by Diouf et al. [16]. After 1 week of growth, seedlings were inoculated with the 16 diluted soil samples. Soil inocula were obtained with 10 g of each soil sample, stirred for 1 h at room temperature (25 °C) in 90 ml of sterile buffered saline solution, pH 7 (NaCl, 0.15 mol.l-1; KH_2PO_4, 0.002 mol l-1; Na_2HPO_4, 0.004 mol l-1). The resulting suspension was serially diluted tenfold from 10^{-1} to 10^{-6}. An aliquot of 1 ml of diluent was then used to inoculate each seedling in Gibson tubes. All the treatments, including control seedlings, were replicated four times. Six weeks after inoculation, the number of nodulated plants was recorded for each dilution. The mean data on the LNR MPN counts were calculated using McGrady tables [35]. Nodules obtained from soil dilution 10^{-1} were then removed from each plant for further isolation of LNR.

Isolation of legume-nodulating Rhizobia

In all, 320 fresh nodules from cultured *V. seyal* and *P. juliflora* were used for isolations. Twenty nodules were chosen for each soil (two plants for each soil, with ten nodules per plant). Nodules were analyzed separately. Nodules were surface-sterilized by immersion in 30% H_2O_2 for 6 min and rinsed four times with sterile distilled water. The nodules were individually crushed in a drop of sterile yeast-mannitol (YEM) broth. The nodule suspensions were then placed on YEM agar medium [47]. Plates were incubated at 28 °C for three days to one week, and pure cultures of rhizobium strains were obtained after several subculture steps. The purified isolates were stored at −80 °C in 1.5 ml tubes containing 30% (v/v) glycerol in YEM broth for further molecular characterization.

PCR characterization of legume-nodulating Rhizobium isolates

The partial sequence of the 16S–23S rDNA ITS region of fresh rhizobia obtained as described above was investigated. PCR amplifications were performed directly on 2 μL of the supernatant of a single colony re-suspended in 20 μl of sterile water. Primers Br5; 5′-CTT-GTA-GCT-CAG-TTG-GTT-AG-3′ [48] and 23S-38; 5′-CCG-GGT-TTC-CCC-ATT-CGG-3′ [33] were used for PCR amplification. All PCR amplifications were performed with Go-Taq polymerase (Promega) following the manufacturer's instructions. PCR amplification was carried out in a Gene Amp PCR System 2400 thermal cycler adjusted to the following temperature profile: initial denaturation at 94 °C for 5 min, 35 amplification cycles (denaturation at 94 °C for 30 s, hybridization of primers at 55 °C for 30 s and extension at 72 °C for 1 min), and final extension at 72 °C for 7 min. PCR products were purified and sequenced by Genoscreen Inc. The ITS rRNA was sequenced with the Br5 primer used for PCR.

Phylogenetic analysis

Codoncode Aligner version 3.7 was used to partition ITS sequences into operational taxonomic units (OTUs) defined as a group of sequences sharing at least 97% similarity. Representative sequences for each OTU were compared with the National Center for Biotechnology Information Genbank databases using the BLASTn search program (Basic Local Alignment Search Tool nucleotidic) and any closely related sequences found were included in our phylogenetic analyses. Multiple nucleotide alignments for the test isolates, the type strains in the genera *Mesorhizbium*, *Ensifer* and *Rhizobium* and the reference species were carried out using the CLUSTAL W program [46] from MEGA version 6 [45]. The phylogenetic tree was constructed based on the maximum likelihood (ML) method using MEGA version 6 [45]. The Tamura Nei model was used in the ML analysis for the ITS gene. The robustness of the topology of the ML tree was calculated from a bootstrap analysis with 1000 replications. *Bradyrhizobium japonicum* LMG 6138T and *Bradyrhizobium elkani* LMG 6134T were used as outgroup references

The relative abundance of each rhizobium genotype was calculated as the ratio of the abundance of each rhizobium genotype to the total abundance of rhizobium genotypes.

Authentication and determination of symbiotic efficiency

The rhizobium isolates chosen as representing the ITS genotypes (sequence similarity $\geq$ 97%) were tested for their ability to nodulate their original host (*V. seyal* and *P. juliflora*). Germination and plant cultivation were conducted as described above. Each strain was grown in YEM medium [47] for 2–3 days at 28 °C with rotary shaking at 150 rpm. One week after transfer into Gibson tubes, the seedlings were inoculated with 1 ml of approximately log-phase 10^8 cells of each isolate. Four replicates were tested for each rhizobium strain. Controls, inoculated with 1 ml of sterile YEM medium, were included. Nodulation was assessed after 6 weeks of growth. The symbiotic efficiency of rhizobium isolates was evaluated from their effects on plant growth, nodule numbers and nitrogen fixation.

We checked N_2 fixation in each of the inoculated plants using the acetylene reduction activity (ARA) method [23]. The ARA measurements were carried out on fresh nodules using a gas chromatograph (Agilent 6850 Series GC System) to determine nitrogenase activity. Plants were individually harvested 60 days after planting and placed in 150 ml serum bottles. After sealing, 15 ml of air was removed from each bottle and replaced by 15 ml of pure acetylene gas (C_2H_2) so that a final 10% acetylene concentration was obtained. A volume of 1 ml of gas samples was removed after 3-h incubation periods for ethylene analysis. Acetylene reduction activity was calculated as μmol of ethylene produced per hour per plant. After ARA analysis, nodules were removed and counted. The samples (nodules and plants) were oven dried at 65 °C for 72 h before weighing. Specific acetylene reduction activity (SARA) was calculated as the ratio between ARA and nodule dry weight.

Statistical analysis

For species richness, the Margalef index was used [$R = (S - 1)/\ln(n)$], where S is the number of groups and n is the number of taxa in all groups. The genetic diversity of the isolates was estimated by the Shannon index (H’) and the Pielou index (J). These indexes of biodiversity were implemented in the Vegan package (version 2.0–10) and were calculated using R statistical language (version 3.1.0).

Data were subjected to a one-way analysis of variance (ANOVA), and the mean values were compared by the Newman–Keuls test at $P < 0.05$. ANOVA tests were carried out using XLSTAT software, version 2010.

Results

Soil properties and legume-nodulating Rhizobium populations

The ranges of chemical characteristics for the studied soils are given in Table 1. Soil salinity ranged from 2.10 to 30.66‰ in the dry season and from 2.14 to 10.85‰ in the rainy season. The data showed that the soil pH, total P, available P and pH increased with salinity in both seasons. The soil samples from Niamdiarokh were classed as sandy.

Table S1 (supplemental) indicates the estimated LNR populations in the soils sampled at 4 saline levels from the rhizosphere of *S. robustus* and bulk soil in the dry and rainy seasons. The results showed that the estimates of LNR populations decreased as salinity increased. Overall, the estimates of LNR populations were higher in soils sampled in the rainy season compared to soils sampled in the dry season. Furthemore, a higher LNR population density was detected in the rhizosphere of *S. robustus* compared to the bulk soil.

PCR analysis of the amplified 16S-23S rDNA region

Of the soils sampled, 232 rhizobium isolates were collected from the root nodules of cultured *V. seyal* and *P. juliflora*. Twelve rhizobium isolates were chosen as representing the ITS genotypes (sequence similarity $\geq$ 97%). PCR analyses of the ITS rRNA region grouped the 12 representative LNR isolates into 8 *Mesorhizobium* spp., 2 *Rhizobium* spp. and 2 *Ensifer* spp. genotypes (Table 2). They were given an accession number in the LCM (Joint Microbiology Laboratory) LNR culture collection: LCM 4574 to LCM 4578, LCM 4580, LCM 4583 and LCM 4584 for *Mesorhizobium* spp., LCM 4573 and LCM 4582 for *Rhizobium* spp., LCM 4571 and LCM 4579 for *Ensifer* spp.

Two of the twelve ITS rRNA genotypes were exclusively found in the dry season (Table 2), four were specific to the rainy season and six were distributed in both seasons. The data showed that the strain genotypes belonging to the *Mesorhizobium* and *Ensifer* genera were more abundant and amounted to 55% and 44% of the isolates, respectively, while the *Rhizobium* genus amounted to approximately 1% of the isolates. *Mesorhizobium* sp. (LCM 4578) and *Ensifer* sp. (LCM 4579) were the major rhizobium species isolated in the rhizosphere of *S. robustus* and in bulk soil in the dry (38%) and rainy season (42%), respectively.

Distribution and diversity of legume-nodulating Rhizobia along the saline gradient

The distribution and diversity of rhizobia associated with cultured *V. seyal* and *P. juliflora* differed in the soils sampled along the saline gradient in the rhizosphere of *S. robustus* and in the bulk soil (Figs. 1 and 2).

Eight rhizobium genotypes were found in the soils sampled in the dry season (Fig. 1). In contrast to the members of the *Mesorhizobium* genus, which were frequent in low salinity, the *Ensifer* genus (with the representatives LCM 4571 and LCM 459) was the most frequent rhizobium genus at the soil saline level of 30.66‰. For the bulk soil, *Mesorhizobium* spp. were the only genospecies up to the saline level of 14,86‰, gradually being replaced thereafter by *Ensifer* sp. LCM 4579.

The richness index and Shannon diversity index (H’) were lowest in the soil sampled at 30.66‰ salinity among the four saline grades

Table 1

Chemical properties of soil samples from the rhizosphere of *Sporobolus robustus* and bulk soils at 4 salinity levels in the dry and rainy seasons. The values in each column represent the mean values of the soil samples from the rhizosphere of *Sprobolus robustus* and the bulk soil at each salinity level in the dry and rainy seasons.

Season	Salinity (‰)	pH_{H20}	P, total	P, available	N, total %	$N, NO_3 + NH_4$	C, total %
			mg/kg	mg/kg		mg/kg	
Dry							
	2.10	6.85	108	6.50	0.11	8.02	1.20
	4.73	6.89	101	6.00	0.12	6.02	1.53
	14.86	7.75	174	11.00	0.11	9.24	1.37
	30.66	8.08	310	26.00	0.13	7.66	1.57
Rainy							
	2.14	7.16	93	4.50	0.10	2.40	1.17
	3.17	7.83	156	13.00	0.12	1.46	1.43
	8.48	8.09	131	8.00	0.13	0.33	1.50
	10.85	8.58	181	17.00	0.08	1.53	1.08

Table 2

Representative isolates obtained by PCR analysis of the 16S-23S rRNA gene ITS, proposed taxonomic position, locality of isolation, host of isolation and abundance of rhizobium isolates in the rhizosphere of *S. robustus* and in bulk soils, in relation to the season. The abundance data shown combine the results for the rhizosphere and bulk soils.

Representative isolate	Locality of isolation	Host of isolation	Closest relative species on the basis of the ITS gene	Similarity (%)	Abundance of rhizobium strains in the dry and rainy seasons	
					Dry	Rainy
LCM 4571	Senegal	*V. seyal* and *P. juliflora*	*Ensifer fredii* LMG 6217^T	92	6	18
LCM 4573	Senegal	*V. seyal*	*Rhizobium pisi* DMS 30132^T	85	1	0
LCM 4574	Senegal	*V. seyal* and *P. juliflora*	*Mesorhizobium plurifarium* LMG 11892^T		20	19
LCM 4575	Senegal	*V. seyal* and *P. juliflora*	*Mesorhizobium plurifarium* LMG 11892^T	92	11	16
LCM 4576	Senegal	*V. seyal*	*Mesorhizobium plurifarium* LMG 11892^T	92	0	2
LCM 4577	Senegal	*V. seyal*	*Mesorhizobium plurifarium* LMG 11892^T	93	3	3
LCM 4578	Senegal	*V. seyal* and *P. juliflora*	*Mesorhizobium plurifarium* LMG 11892^T	90	43	7
LCM 4579	Senegal	*V. seyal* and *P. juliflora*	*Ensifer fredii* LMG 6217^T	90	28	50
LCM 4580	Senegal	*V. seyal*	*Mesorhizobium* sp. ORS 3359	99	1	0
LCM 4582	Senegal	*V. seyal*	*Rhizobium pisi* DMS 30132^T	85	0	1
LCM 4583	Senegal	*V. seyal*	*Mesorhizobium* sp. ORS 3657	99	0	2
LCM 4584	Senegal	*P. juliflora*	*Mesorhizobium plurifarium* LMG 11892^T	93	0	1

A representative isolate corresponds to samples that presented at least 97% of 16S-23S rRNA gene ITS sequence similarity. The proposed taxonomic position is based on the 16S-23S rRNA gene ITS sequence analysis. LCM culture collection of the IRD/ISRA/UCAD joint microbiology laboratory, Dakar, Senegal.

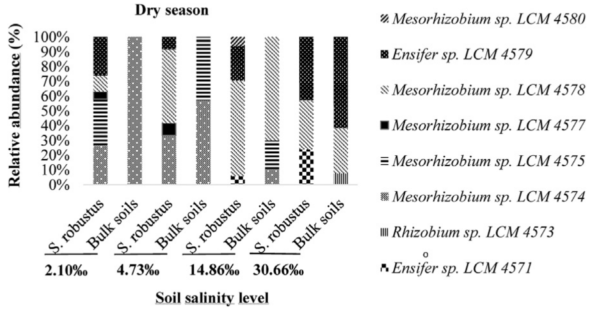

Fig. 1. Diagram of the relative abundance of 8 representative rhizobium genotype isolates in soil collected from the rhizosphere of *Sporobolus robustus* and the bulk soils at 4 salinity levels in the dry season. Abundance is presented as a percentage of the total rhizobium sequences in a sample.

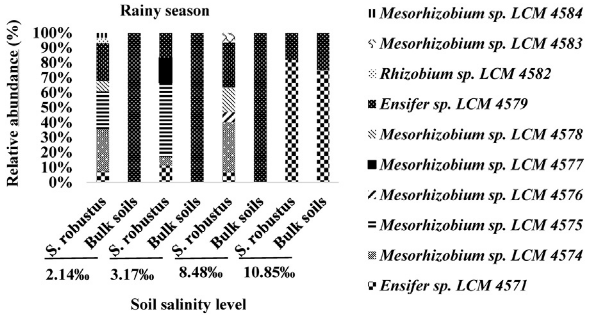

Fig. 2. Diagram of the relative abundance of 10 representative rhizobium genotype isolates in soil collected from the rhizosphere of *Sporobolus robustus* and the bulk soils at 4 salinity levels in the rainy season. Abundance is presented as a percentage of the total rhizobium sequences in a sample.

(Supplemental Table S2). The highest Shannon index value (1.45) was found for 2.10‰. Pielou's evenness index (J) was similar at 2.10 and 30.66‰.

During the rainy season, the results showed that, out of the 10 rhizobium genotypes found in the rhizosphere of *S. robustus* and in bulk soil, *Ensifer* sp. LCM 4579 was observed in all the soils sampled along the saline gradient (Fig. 2). For instance, LCM 4571 and LCM 4579 (*Ensifer*) were the only isolates occurring in soils sampled at the highest saline level (10.85‰). All the bulk soil samples were only composed of members of the *Ensifer* genus. At a low saline level (2.14‰), the isolates of the rhizosphere of *S. robustus* were the most diverse with seven rhizobium genotypes. Two genotypes, *Rhizobium* sp. LCM 4582 and *Mesorhizobium* sp. LCM 4584 were rare and found only in soil sampled around *S. robustus*. Two genospecies, *Mesorhizobium* sp. LCM 4576 and *Mesorhizobium* sp.

LCM 4583, were specific to soil covered by *S. robustus* at 8.48‰. *Mesorhizobium* sp. LCM 4578 occurred at a 2.14 and 8.48‰ soil salt content.

The Shannon diversity index (H') and richness index in soil sampled at 2.14‰ salinity were the highest (Supplemental Table S2). The lowest Shannon (0.50) and richness (02) index values were found in soil sampled at 10.85‰ salinity. The value of Pielou's evenness index varied between 0.72 and 0.83 in soil sampled at 10.85‰ and 2.14‰ salinity, respectively.

Sequence analyses of 16S-23S rRNA gene ITS

A phylogenetic tree based on the ITS gene was built with the 12 representative ITS genotypes from the study, together with *Mesorhizobium*, *Ensifer* and *Rhizobium* types, and reference strains (Fig. 3). Phylogenetic analyses showed that the strain genotypes from the rhizosphere soils of *S. robustus* were clustered into three main groups. Group I, representing eight genospecies belonging to the genus *Mesorhizobium*, was closely related to the type strain of *M. plurifarium* LMG 11892^T isolated from *Senegalia senegal*. The *Mesorhizobium* group was subdivided into three subgroups according to the blast result. The first subgroup was composed of genotypes LCM 4578, LCM 4583 and LCM 4580, which were closely related (99% similarity) to *Mesorhizobium* sp. ORS 3656, *Mesorhizobium* sp., ORS 3657 and *Mesorhizobium* sp. ORS 3359, respectively. A second subgroup contained genotypes LCM 4576 and LCM 4584, which were close to *Mesorhizobium* sp. CIRADF 154 and *Mesorhizobium* sp. LCM 3745, respectively, with 96% similarity. Genotypes LCM 4577, LCM 4575 and LCM 4574 formed a third subgroup with *Mesorhizobium* sp. LCM 3688, *Mesorhizobium* sp. LCM 3696 and *Mesorhizobium* sp. LCM 3714, with 96% similarity. Group II, belonging to the genus *Ensifer*, contained the representative isolates LCM 4571 and LCM 4579, which were clustered with the type strain *Ensifer fredii* LMG 6217^T, with 92% similarity. Group III comprised genotypes LCM 4573 and LCM 4582, which clustered with the type strain *Rhizobium pisi* DSM 30132^T with 85% similarity. The obtained sequences were deposited in the GenBank database under accession numbers MF993125 to MF993138. The accession numbers of study isolates are indicated on the tree (Fig. 3).

Legume-nodulating Rhizobium nodulation and symbiotic host response

All 12 rhizobium isolates selected as being representative of ITS rRNA genotypes were able to re-nodulate *V. seyal* seedlings *in vitro*. However, only eight bacterial isolates belonging to the *Ensifer* (LCM 4571 and 4579) and *Mesorhizobium* genera (LCM 4574, 4575, 4577, 4578, 4580 and 4584) formed nodules on roots of *P. juliflora* (Table 3). In *V. seyal*, our results showed a significant effect of *Ensifer* sp. LCM 4571, *Ensifer* sp. LCM 4579, *Mesorhizobium* sp. LCM 4577 and *Mesorhizobium* sp. LCM 4580 inoculation ($P < 0.05$) on nodule dry weight, in comparison to the control plants. Only one strain, LCM 4579, belonging to the *Ensifer* genus, showed a significant effect ($P < 0.05$) on plant total dry weight (shoot + root) and had the highest SARA value. Of the 12 strains tested, the *Ensifer* LCM 4579, and the *Mesorhizobium* LCM 4580, had the highest acetylene reduction activity. In *P. juliflora*, only the strains belonging to the *Ensifer* genus (LCM 4571 and LCM 4579) had a significant effect ($P < 0.05$) on nodule dry weight and plant total dry weight, as compared to the control plants. One *Mesorhizobium* isolate (LCM 4574) had the highest SARA value, while acetylene reduction activity did not vary significantly depending on the inoculation treatment.

Discussion

To our knowledge, this is the first report on legume-nodulating rhizobia (LNR) in soil samples from the rhizosphere of *S. robustus* in saline areas. In this study, we found that *S. robustus* hosts higher LNR population densities in its rhizosphere in comparison with bulk soil. The decline in population numbers and diversity along the salinity gradient suggested that salinity exerts significant stress on soil LNR populations. Our findings are consistent with those of other studies showing that salinity stress decreases the numbers of soil LNR [5]. Salt stress may inhibit the initial steps of symbiosis (nodule initiation, nodule infection, and development), but it also has a depressive effect on nitrogen fixation (Nogales et al. [32]). The estimates of LNR populations were significantly higher in soils sampled in the rainy season compared to soils sampled in the dry season, as demonstrated by Hungria and Vargas [24] and Lazali [28]. This indicated that rhizobia need moisture to multiply. Sahelian legume species develop extensive root systems near the soil surface and usually have a large number of spontaneous nodules during the rainy period. This could naturally facilitate LNR multiplication through rhizosphere effects [26,11].

Based on the sequencing of the 16S-23S ITS region, the 232 rhizobium isolates obtained from roots of cultured *V. seyal* and *P. juliflora* were grouped into 12 genotypes belonging to the genera *Mesorhizobium*, *Ensifer* and *Rhizobium*. This demonstrated that *Sporobolus* hosts several rhizobia in its rhizosphere, which could enter into symbiosis with *V. seyal* and *P. juliflora*. Our results corroborate those of Roesch et al. [37], who showed that the rhizosphere soil of grass harbors the greatest diversity of diazotrophic bacteria. The author detected the presence of *Mesorhizobium*, *Rhizobium* and *Ensifer* associated with maize rhizospheres. In addition, Lovell et al. [30] highlighted the occurrence of the *Rhizobium* genus in the rhizosphere of *Spartina alterniflora*. Furthermore, several studies have shown that African vachellias are predominantly nodulated by the *Mesorhizobium*, *Rhizobium*, or *Ensifer* genera [40,14,42]. Together with our data, all these findings support the view that considerable genetic diversity exists within LNR populations in saline areas [12,14].

The identification of 128 isolates (amounting to 55%) as being *Mesorhizobium* and 102 isolates as being *Ensifer* (amounting to 44%) suggested that the rhizosphere soil of *S. robustus* was dominated by *Mesorhizobium* and *Ensifer* microsymbionts, partly in accordance with previous observations in saline areas of Senegal [14,13]. Additionally, the results showed that *V. seyal* and *P. juliflora* were mainly nodulated by both *Mesorhizobium* and *Ensifer*. The preference for symbiotic bacteria is also true for other legumes, such as *S. senegal*, predominantly associating with *Mesorhizobium* [1] and *Vachellia jacquemontii* selecting *Ensifer* [39] as their effective nitrogen fixation partners. As shown in Figs. 1 and 2, the *Ensifer* genus was found to be distributed on all saline levels. More interestingly, the results of our study revealed the relative abundance of *Ensifer* genospecies in the severe saline levels of the four saline grades. The universal distribution of the *Ensifer* genus in all the sampling plots demonstrated its broad ability to adapt to saline conditions. Similar results were found by Li et al. [29] on *Sesbania cannabina* plants in Chinese saline soils, which were essentially nodulated by *Ensifer*. Sankhla et al. [39] reported that saline soil conditions favor the presence of *Ensifer* species in root nodules of *V. jacquemontii*.

The phylogenetic tree based on ITS sequence analyses showed that most of the *Mesorhizobium* isolates clustered with the type strain *M. plurifarium* LMG 11892^T. The blast analysis showed also that they were closely related to other strains unassigned to any of the recognized *Mesorhizobium* species (named *Mesorhizobium* sp.), which were mostly isolated in Senegal. These results suggest a widespread distribution of *Mesorhizobium* in Senegalese soils, as demonstrated by Bakhoum et al. [1] and Diouf et al. [12]. *Mesorhizo-*

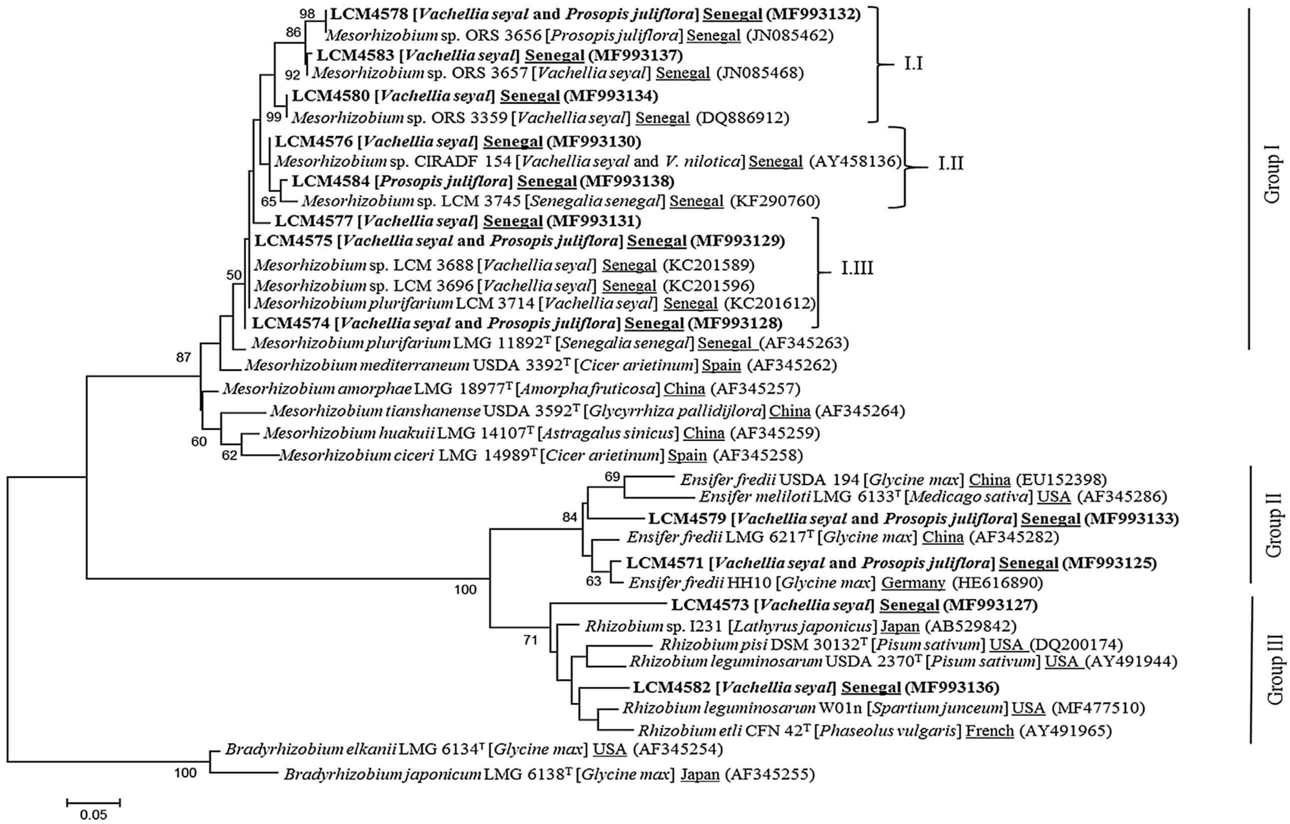

Fig. 3. Phylogenetic tree based on the sequence of the 16S-23S rRNA gene ITS, showing the relationships between the legume-nodulating rhizobium isolates from the rhizosphere of *S. robustus* and in bulk soil (shown in bold) and some related sequences for recognized species of the genera *Mesorhizobium*, *Ensifer* and *Rhizobium*. The accession numbers for the sequences are indicated in brackets. The isolate hosts are indicated in square brackets. The geographical locations of the rhizobia are underlined. The tree was constructed by the Maximum Likelihood method with the Tamura Nei model using MEGA version 6. Bootstrap values over 50% (using 1000 replications) are shown at each node. The horizontal branches are drawn proportionally to the number of nucleotide substitutions per site. Type strains are indicated as (T). *Bradyrhizobium japonicum* and *Bradyrhizobium elkani* were used as outgroup References.

Table 3
Effect of inoculation with the 12 different representative rhizobium isolates on the percentage of nodulation (PN), nodule dry weight (NDW), total plant dry weight (TDW), acetylene reduction activity (ARA), and specific acetylene reduction activity (SARA) of *Vachellia seyal* and *Prosopis juliflora* plants grown for 60 days under *in vitro* conditions.

Legume species	Representative isolate	PN (%)	NDW (mg)	TDW (mg)	ARA (μmol C2H4/h/plant)	SARA (μmol C2H4/h/mg nodule)
V. seyal	Control	0	00.00^{b}	154.25^{b}	0.000^{b}	0.000^{b}
	LCM 4571	100	12.75^{a}	190.25^{b}	0.123^{b}	0.010^{ab}
	LCM 4573	50	02.75^{b}	132.25^{b}	0.015^{b}	0.002^{b}
	LCM 4574	50	00.32^{b}	082.00^{b}	0.000^{b}	0.000^{b}
	LCM 4575	50	00.62^{b}	096.75^{b}	0.000^{b}	0.000^{b}
	LCM 4576	100	05.65^{ab}	116.75^{b}	0.028^{b}	0.000^{b}
	LCM 4577	100	12.00^{a}	178.75^{b}	0.059^{b}	0.005^{ab}
	LCM 4578	100	03.90^{b}	097.75^{b}	0.043^{b}	0.011^{ab}
	LCM 4579	100	11.75^{a}	407.25^{a}	0.241^{a}	0.020^{a}
	LCM 4580	100	12.25^{a}	203.50^{b}	0.229^{a}	0.018^{ab}
	LCM 4582	25	00.02^{b}	115.50^{b}	0.000^{b}	0.000^{b}
	LCM 4583	100	07.50^{ab}	161.50^{b}	0.059^{b}	0.010^{ab}
	LCM 4584	75	00.08^{b}	092.25^{b}	0.000^{b}	0.000^{b}
P. juliflora	Control	0	00.00^{b}	061.75^{b}	0.000^{a}	0.000^{b}
	LCM 4571	100	05.37^{a}	120.50^{a}	0.013^{a}	0.002^{b}
	LCM 4573	0	00.00^{b}	103.25^{ab}	0.000^{a}	0.000^{b}
	LCM 4574	100	01.20^{b}	080.75^{ab}	0.048^{a}	35.948^{a}
	LCM 4575	25	00.75^{b}	091.75^{ab}	0.031^{a}	3.759^{b}
	LCM 4576	0	00.00^{b}	060.75^{b}	0.000^{a}	0.000^{b}
	LCM 4577	25	00.75^{b}	101.25^{ab}	0.000^{a}	0.000^{b}
	LCM 4578	25	00.50^{b}	070.50^{ab}	0.014^{a}	7.952^{b}
	LCM 4579	100	05.25^{a}	112.25^{a}	0.041^{a}	0.007^{b}
	LCM 4580	25	00.23^{b}	105.75^{ab}	0.000^{a}	0.000^{b}
	LCM 4582	0	00.00^{b}	078.00^{ab}	0.000^{a}	0.000^{b}
	LCM 4583	0	00.00^{b}	108.00^{ab}	0.000^{a}	0.000^{b}
	LCM 4584	50	01.25^{b}	099.75^{ab}	0.023^{a}	9.757^{b}

In columns, the means of values followed by the same letter do not differ significantly ($P < 0.05$) as determined by the Newman–Keuls test.

bium diversity has also been found in other regions and in various environments [9,49]. The strains LCM 4571 and LCM 4579 were found to be closely related to *Ensifer meliloti* LMG 6133^T. It is well known that many tree-nodulating rhizobia are more closely related to *Ensifer* species [39,38]. The strains LCM 4573 and LCM 4582, related to *Rhizobium* genospecies, were detected in cultured *V. seyal* and *P. juliflora* plants. Rhizobium diversity was also found by Jenkins et al. [25] and Diouf et al. [13] in native stands of *Prosopis*.

On the other hand, the inoculation experiments with *P. juliflora* and *V. seyal* showed that all the strains tested were able to nodulate *V. seyal*. In contrast, only 8 out of the 12 strains tested could nodulate *P. juliflora*, suggesting that this exotic legume species may have lower compatibility with the native LNR, when compared to *V. seyal*. In addition, several reports on inoculation tests indicated *V. seyal* as being a non-selective host for diverse rhizobium genotypes, including representatives of the genera *Ensifer*, *Mesorhizobium* and *Rhizobium* [13,17,34]. Acetylene reduction activity was found to be generally higher in *V. seyal* plants than in *P. juliflora* plants. Acetylene reduction activity did not vary significantly in *P. juliflora*. These results are contrary to those described by Diagne [10], who reported that experiments conducted in the nursery showed that *P. juliflora* responds positively to inoculation with *Rhizobium*, increasing its nitrogen-fixing ability. The results suggested that the potential of *P. juliflora* to fix significant proportions of nitrogen varied markedly depending on the rhizobium strain. Nitrogen-fixing efficiency must be a clear criterion for strain selection, with effective rhizobium strains stimulating legume growth. Of the 12 rhizobium isolates belonging to three genera (*Mesorhizobium*, *Ensifer* and *Rhizobium*), only those identified as representing the *Ensifer* genus had a significant effect on the nodule dry weight and growth of *P. juliflora* plants. Furthermore, the strain LCM 4579 (*Ensifer* sp.) was very effective in improving the ARA and SARA parameters, and growth for *V. seyal* plants. Similar results were found by Cordero et al. [8], who showed positive growth effects and higher nitrogenase activity for *Vachellia macracantha* due to rhizobium inoculation with *Ensifer* strains. The *Mesorhizobium* strain LCM 4580 improved both ARA and nodule dry weight when inoculated on *V. seyal*. However, strain LCM 4577 (*Mesorhizobium* sp.) only had a significant increasing effect on the nodule dry weight of *Vachellia* seedlings. The performance of *Mesorhizobium* inoculation strains in improving legume nodule production and nitrogen fixation potential have been reported for different nodulating tree species, including *V. seyal* and *P. juliflora* [13,2].

Conclusions

We conclude that the rhizosphere of *S. robustus* contains LNR and a major variation was found for the number of LNR depending on the soil saline level. For instance, the number of indigenous LNR decreased as soil salinity increased. A genetically diverse LNR population was supported by the phylogenetic analysis, which clustered the isolates into three genera: *Mesorhizobium*, *Ensifer* and *Rhizobium*. Our study also showed significant impacts of salinity on the LNR diversity indexes. The distribution of LNR was correlated to soil salinity and *Ensifer* spp. appeared to be more adapted to saline conditions. The tested *Ensifer* spp. strains that nodulated *V. seyal* and *P. juliflora* elicited greater total plant dry biomass, nodule dry biomass and ARA parameters than the rhizobium isolates belonging to the genera *Mesorhizobium* and *Rhizobium*, indicating that these *Ensifer* spp. strains had great potential as inoculums. Hence, the potential offered by *S. robustus* soils can be exploited in programs to reforest Senegalese saline soils with N_2 fixing trees.

Acknowledgments

We thank Joël Fardoux (LSTM, Montpellier), Oumar Sadio (IRD, Dakar) and Salif Gueye (UCAD, Dakar) for their valuable technical assistance. This work was funded by the Senegalese Ministry of Higher Education and Research (FIRST project). The authors wish to thank Peter Biggins for reviewing the English of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.10.006.

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