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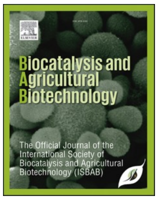

Diversity of *Bradyrhizobium* strains isolated from root nodules of the shrubby legume *Cytisus villosus* growing in the Moroccan Rif

Rajaa Chahboune[a,*], Fatima Ezzakkioui[b], Said Barrijal[b], Eulogio J. Bedmar[c]

[a] *Life Sciences and Health Laboratory, Faculty of Medicine and Pharmacy, Abdelmalek Essaadi University, Tangier, Morocco*
[b] *Department of Biology, Faculty of Sciences and Techniques, Abdelmalek Essaadi University, Tangier, Morocco*
[c] *Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Granada, Spain*

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ABSTRACT

The genotypic and phenotypic diversity of nineteen bradyrhizobial strains isolated from the root nodules of wild-grown *Cytisus villosus* in the central-western region of the Moroccan Rif were carried out. PCR-based RFLP (restriction fragment length polymorphism) of the 16S rDNA (ARDRA) of 19 strains using the restriction enzymes *Alu*I, *Cfo*I, *Dde*I, *Hinf*I, *Msp*I and *Rsa*I showed that they grouped in 3 clusters. The cluster I includes the type strain *Bradyrhizobium canariense* BTA-1^T, the cluster II the type strain *Bradyrhizobium japonicum* USDA6^T and the cluster III the type strains *Bradyrhizobium cytisi* CTAW11^T and *Bradyrhizobium rifense* CTAW71^T. The results from 63 physiological and biochemical tests confirmed the existence of the *B. canariense* and the *B. japonicum* groups but also allowed the separation of the *B. cytisi* and *B. rifense* strains in two groups. The phenotypic characteristics analysed permitted description of a wide physiological and biochemical diversity among the strains, and showed that the heavy metals resistance test was the most discriminating characteristic.

1. Introduction

Within the Genisteae tribe of the Fabaceae, the genus *Cytisus* includes about 60 species distributed from Northern to Southern and Western Africa and central Europe, reaching Germany and Poland to the North, and Western Russia, the Black Sea and Turkey to the East. The highest species diversity, however, is observed around the Mediterranean Sea (Cristofolini and Troia, 2006).

Broom (*C. villosus* Pourr., syn. *Cytisus triflorus* L'Hérit) is a perennial shrub native to northern Africa and widely distributed in the central-western region of the Moroccan Rif as natural stands. In these locations, a high anthropogenic pressure exists on native plants due to the growing area required for cultivation of *Cannabis sativa* and the increase in the frequency of forest fires which, in turns, leads to extinction of autochthonous flora and associated soil microorganisms. During the period between 1984 and 1990, the forest in the Ketama region of the Moroccan Rif was cleared on almost all of its surface ranging from 8000 ha in 1984 to only 500 ha in 1990; today, a few groves of around 100 ha remains in the region (Benabid, 2000).

Together with the actinorhizal plants, legumes are unique among the higher plants because they have the ability to establish symbiotic associations with soil bacteria, collectively known as rhizobia, that fix atmospheric dinitrogen (N_2) within the nodules formed in the roots, and occasionally on the stems, of the plants. The species *C. villosus* is a sylvo-pastoral legume of great economical interest because their seeds have a high protein content (Bourbouze, 1980; Le Houerou, 1980); brooms have a deep, wide root system which allows the plants to thrive in soils holding little water and nutrients, which together with their N_2-fixing capacity make the plants to be used for cover crops and pasture improvement, green manure, soil stabilization and recovery of degraded soils. In addition, *C. villosus* greatly contributed to the total 1.5×10^9 fodder units (Fikri-Benbrahim et al., 2004) required for feeding the national cattle. The Mediterranean climate is often characterized by irregular rainfall distribution and hot dry summers. In this region, most soils (about 25 million ha) are eroded and shallow, with low organic matter content, in some cases lower than the desertification index (<17 g organic matter kg^{-1}) (Holland, 2004; Van-Camp et al., 2004).

Despite the limited information available, efforts can be made to recover eroded areas in the Moroccan Rif. In this sense, crop yield during revegetation studies using leguminous plants is limited not only by the nitrogen-fixing potential of the rhizobial symbionts, but also by the harsh edaphoclimatic conditions of the region. This highlights the interest of identifying effective native rhizobial species well adapted to the local environmental conditions prevailing in the area.

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* Corresponding author. Life Sciences and Health Laboratory, Faculty of Medicine and Pharmacy, University Abdelmalek Essaadi, Tangier, Morocco.
E-mail address: chahboune82rajaa@yahoo.fr (R. Chahboune).

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From a number of bacterial strains isolated from root nodules of *C. villosus* grown in soils of the central-western region of the Moroccan Rif, 11 strains were characterized as *Bradyrhizobium canariense* and other 3 were *Bradyrhizobium japonicum* (Chahboune et al., 2011a). In addition two new lineages were identified as the novel species *Bradyrhizobium cytisi* (Chahboune et al., 2011b) and *Bradyrhizobium rifense* (Chahboune et al., 2012) that were included in the symbiovar genistearum. More recently, endosymbiotic bacteria from *C. villosus* growing in Northeastern Algeria have been described, which belong to the species *B. japonicum* and *B canariense* (Ahnia et al., 2014).

The study of rhizobia nodulating wild-grown legume shrubs is attracting great interest because of their high potential for environmental applications (Cardinale et al., 2008; Mahdhi et al., 2008; Boulila et al., 2009; Ruiz-Diez et al., 2009). In this sense, because of the potential value of brooms in reforestation programs, the aim of this study was to analyze the phenotypic and genotypic variability of the native bradyrhizobial strains isolated from wild-grown plants of *C. villosus* in the central-western region of the Moroccan Rif.

2. Material and methods

2.1. Bacterial strains and soil characteristics

The bacteria used in this study were 19 *Bradyrhizobium* strains isolated from nodules of the shrubby legume *C. villosus* grown wild near the villages of Fifi (34°57′N, 5°14′W) and Aoudal (35°08′N, 5°20′W) in the central-western region of the Moroccan Rif (Fig. 1). Eleven strains were previously identified as *B. canariense*, 3 belonged to *B. japonicum*, 3 grouped with *B. cytisi* and the remaining 2 strains belonged to *B. rifense* (Chahboune et al., 2012, 2011a; 2011b). Cells were routinely grown in yeast extract-mannitol (YEM) medium (Vincent, 1970) at 28 °C.

2.2. DNA extraction and PCR amplifications

For DNA extraction and PCR amplifications, genomic DNA was isolated from the bradyrhizobial cells using the RealPure Genomic DNA Extraction kit (Durviz, Spain), according to the manufacturer's instructions. The quantity of DNA was determined by using a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, USA). PCR amplifications of 16S rRNA gene fragments were carried out using the primers fD1 and rD1 (Weisburg et al., 1991) and the procedures described earlier (Herrera-Cervera et al., 1999). The 19 strains were analysed for similarity using restriction fragment length polymorphism (RFLP) of the 16S rRNA gene (amplified ribosomal DNA restriction analysis, ARDRA). The enzymes *Alu*I, *Cfo*I, *Dde*I, *Hin*fI, *Msp*I and *Rsa*I (Roche Molecular Biochemicals) were used for digestion of the PCR products. After restriction, the DNA fragments were separated by electrophoresis in 3% agarose gels.

Fingerprint profiles were analysed using GelCompar II V.2 (Applied Maths BVBA, Belgium) with gel images normalized using the molecular weight marker XIV (Sigma-Aldrich). Similarities between profiles were calculated from bands in the range of 100–1500 base pair molecular size. Dice coefficient, and an average linkage (UPGMA) dendrogram derived from the combined profiles.

2.3. Phenotypic characterization

Tests were performed in liquid or solid media inoculated with an exponentially growing liquid culture (~10^8 cells ml^{-1}) at 150 rpm. Bacterial growth was checked 3–10 d after inoculation. All experiments were carried out in triplicate. Strains were considered tolerant or effective when growth was at least 50% of that exhibited by the corresponding control strain. The ability of the strains to grow at different salt concentrations was tested by streaking them on solid YEM medium

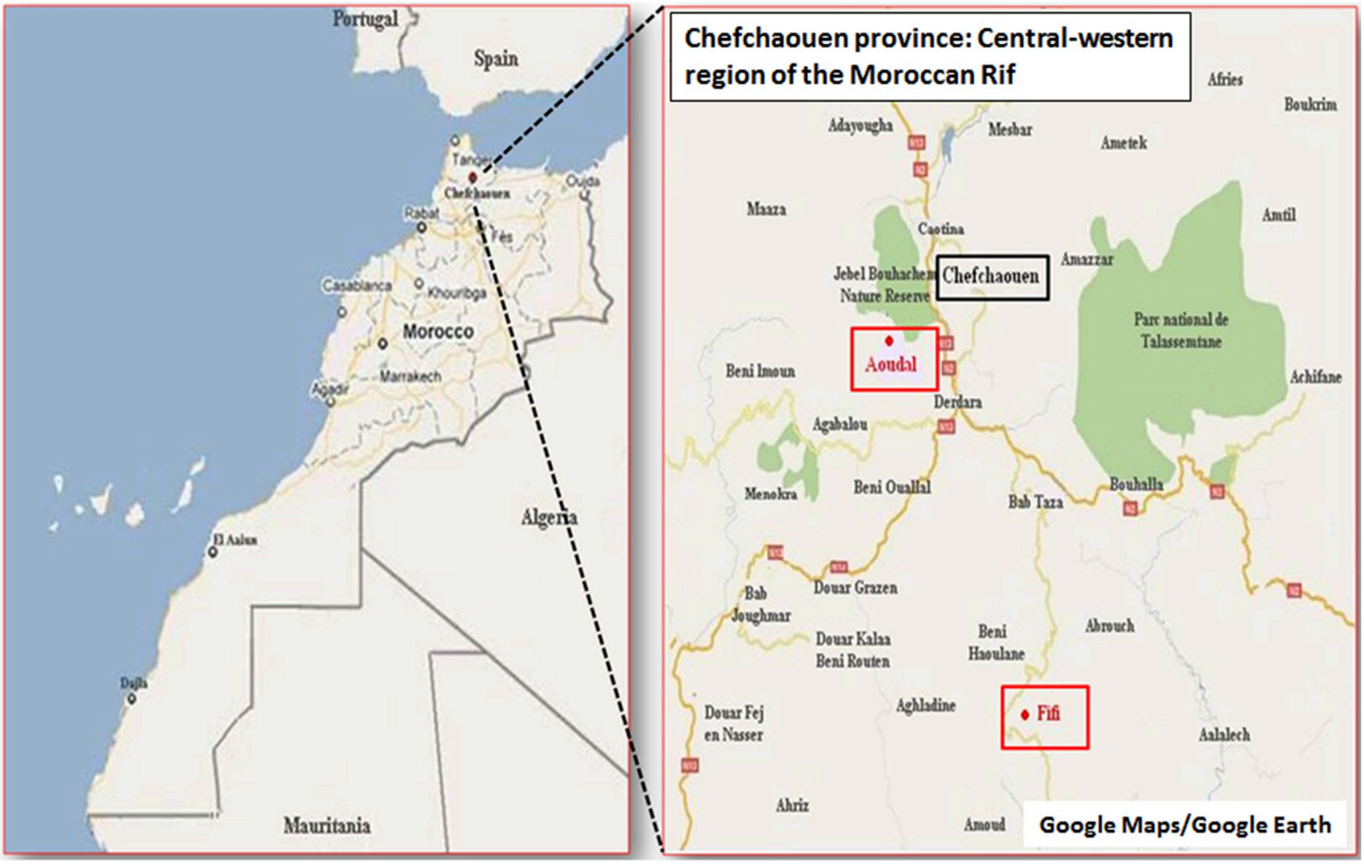

Fig. 1. Geographical situation of the sampling sites located near the villages of Fifi and Aoudal in the central-western region of the Moroccan Rif.

containing 0.5, 1 and 2% (w/v) NaCl. The ability of the strains to grow in acidic or basic pH was tested in liquid YEM medium whose pH had been adjusted and buffered to 4.0, 6.8 and 9.0. The tolerance of the bradyrhizobial strains to high temperatures was tested on solid YEM medium at 4, 28 and 37 °C. Carbohydrate assimilation was performed by using a modified solid YEM medium containing 0.05 g l^{-1} yeast extract and 0.01% NH_4NO_3 as a nitrogen source. Mannitol (1%, w/v) was replaced by the same concentration of each of the following carbohydrates: glucose, fructose, arabinose, inositol, lactose, sucrose, maltose, raffinose and starch. YEM with no carbon source or containing mannitol were used as negative and positive controls respectively. The intrinsic antibiotic resistance was tested on YEM agar plates containing the following filter-sterilized antibiotics (μg ml^{-1}): ampicillin (5, 25, 100), kanamycin (10, 50, 100), streptomycin (5, 20, 100), tetracycline (10, 50, 100), chloramphenicol (10, 50, 200), gentamycin (5, 25, 100), spectinomycin (5, 25, 100) and erythromycin (5, 25, 100). The intrinsic resistance of the strain to heavy metals was determined on solid YEM medium supplemented with the following heavy metals (μg ml^{-1}): $NiCl_2$ $6H_2O$ (50, 100, 200), $BaCl_2$ $2H_2O$ (500, 600, 700), $MnCl_2$ $4H_2O$ (500, 1000), $CuCl_2$ $2H_2O$ (50, 100, 200), $CoCl_2$ $6H_2O$ (50, 100, 200), $HgCl_2$ $2H_2O$ (10, 50) and $CdCl_2$ $2H_2O$ (5, 10, 50). The growth rate was studied by growing the strains in liquid YEM (pH 6.8) on a rotary shaker at 180 rpm. For each strain, the generation time was calculated at the exponential growth phase.

2.4. Numerical analysis

A computer cluster analysis of 63 phenotypic traits using Statistica 6 Program (Statsoft www.statsoft.com) was carried out for the 19 bacterial isolates. The resemblance between pairs of isolates was calculated using the Pearson correlation coefficient and plotted as a dendrogram with the unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973).

3. Results

3.1. Soil characteristics

Location of the sites and main physicochemical properties of the soils are shown in Table 1. The soils have a franc-clay texture and are best characterised by pH as low as 4.8 in the soil from Aoudal and 5.3 in the soil from Fifi, respectively.

3.2. ARDRA

PCR amplification of the 16S rRNA gene yielded DNA bands of approximately 1500 base pair and the DNA restriction analysis (ARDRA) of the 16S rDNA distributed the strains in 3 separated clusters (Fig. 2). The cluster I contained *B. canariense* strains CTAW25, CTAW31, CTAW33, CTAW36, CTAW43, CTFI26, CTFI28, CTFI47, CTFI52, CTFI54 and CTFI410 together with the type strain of *B. canariense* BTA-1^T. The cluster II was composed of *B. japonicum* strains CTAW56, CTFI12, CTFI32 and the type strain *B. japonicum* USDA 6^T. The cluster III was formed by the *B. cytisi* strains CTAW22 and CTAW38 and the *B. rifense* strain CTAW69 that grouped together with *B. cytisi* $CTAW11^T$ and *B. rifense* $CTAW71^T$.

Table 1
Physicochemical properties of the soils.

Characteristics	Site	
	Aoudal	Fifi
Coordinates	35°08′ N, 5°20′ W Franc-clayey	34°57′N, 5°14′W Franc-clayey
Texture	22.35% sand 34.55% silt 43.10% clay	27.05% sand 33.40% silt 39.55% clay
pH_{water}	4.8	5.3
Organic matter (%)	5.29	10.42
Carbon (%)	3.08	6.05
Nitrogen (%)	0.28	0.39
Electrical conductivity (μS/cm)	93.0	110.0

3.3. Phenotypic characterization

Differential characteristics among the four species of *Bradyrhizobium* isolated from root nodules of *C. villosus* are recorded in Table 2. All 19 strains had mean generation times varying from 8 to 12 h and, therefore, can be considered as slow growing rhizobia. Colonies are less than 1 mm in diameter, pearl white on solid YEM medium after incubation for 7 days at 28 °C. Only *B. japonicum* and *B. rifense* strains grew well at 0.5% NaCl and none of the 19 strains tolerated higher levels of NaCl. None of the strains survived at 37 °C and a slight growth of the 3 *B. japonicum* strains was observed at 4 °C. Only the two *B. rifense* strains and 5 out of the 11 *B. canariense* strains tolerated pH 4.0, and none of the bradyrhizobial strains grew at pH 9.0.

All bradyrhizobial strains used arabinose, inositol and mannitol as the only C sources for growth. Except for *B. japonicum* strains, the remaining 16 strains representing the species *B. canariense*, *B. cytisi* and *B. rifense* used glucose and fructose for growth and did not metabolized maltose and raffinose; these latter compounds, however, were used by the *B. japonicum* strains. *B. japonicum*, *B. cytisi* and *B. rifense* grew well with starch and *B. canariense* did not. None of the bradyrhizobial strains grew using lactose or sucrose as the sole C source.

Growth responses of the *Bradyrhizobium* strains to heavy metals were variable, especially in the presence of $CuCl_2$, $NiCl_2$ and $CdCl_2$. This variability is noted even between strains of the same species.

All the strains grew vigorously in the presence of $BaCl_2$, $MnCl_2$ and $CoCl_2$, but they did not tolerate relatively low $HgCl_2$ concentrations. All the strains grew well with ampicillin and were tolerant to 10 μg ml^{-1} of chloramphenicol. Higher chloramphenicol concentrations inhibited bacterial growth. Kanamycin was the most potent antibiotic as it prevented the growth of all the bradyrhizobial strains. *B. japonicum* strains were more tolerant to spectinomycin, gentamycin, erythromycin and streptomycin than the others species. Only *B. japonicum* CTFI32 and CTAW56 were tolerant to 200 μg ml^{-1} of chloramphenicol.

Based on results of 63 cultural, physiological and biochemical tests, a dendrogram (Fig. 3) was constructed from similarity matrix using the UPGMA algorithm. The dendrogram shows a relatively high diversity among strains isolated from root nodules of *C. villosus*, which were grouped at a similarity level of 60% into 4 main clusters. The use of various heavy metals at various concentrations proved to be the most discriminating test. The cluster I includes the species *B. japonicum* whose three strains showed very similar response to the different physiological, albeit *B. japonicum* CTFI12 was unable to tolerate spectinomycin μg/ml 100 and 200 μg/ml of chloramphenicol. In addition, the strains in cluster I tolerated streptomycin and NaCl (1%). The large cluster 2 contains the 11 strains of *B. canariense* whose common characteristics are the tolerance to NaCl and assimilation of carbohydrates and antibiotics. The cluster 3 includes the species *B. cytisi* represented by the strains *B. cytisi* $CTAW11^T$, CTAW22 and CTAW38 which differ in their ability to tolerate some heavy metals. Finally, cluster 4 grouped the *B. rifense* strains CTAW69 and $CTAW71^T$ which differs from each other in their ability to use fructose and tolerate $CuCl_2$ and $CdCl_2$.

4. Discussion

In spite of their importance, the shruby wild legume *C. villosus* is understudied and few data are published about their symbiotic status. Brooms were found to be infected by *B. canariense*, *B. japonicum*, *B. cytisi* and *B. rifense* (Ahnia et al., 2014; Chahboune et al., 2012, 2011a, 2011b), which indicates that, like most Genisteae so far studied,

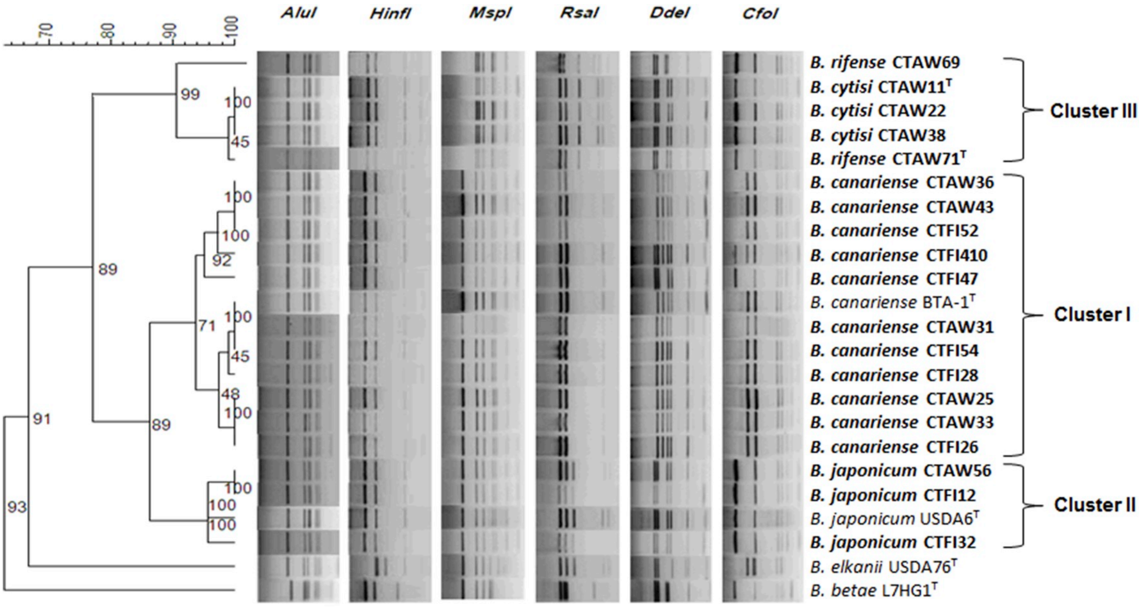

Fig. 2. UPGMA dendrogram showing similarity between the PCR-RFLP (ARDRA) profiles of the 16S rDNA of 19 bradyrhizobial strains isolated from root nodules of *C. villosus*, *B. betae* L7HG1^T and *B. elkanii* USDA76^T restricted with endonucleases *Alu*I, *Cfo*I, *Dde*I, *Hin*fI, *Msp*I or *Rsa*I.

Table 2
Differential characteristics among the four species of *Bradyrhizobium* isolated from *C. villosus*. + : positive; –: negative; W: weak.

Characteristics	*B. japonicum*	*B. canariense*	*B. cytisi*	*B. rifense*
Growth at:				
pH 4.0	–	w	–	+
4 °C	+	–	–	–
Carbon sources used:				
Glucose/fructose	–	+	+	+
Maltose/raffinose	+	–	–	–
Amidon	+	–	+	+
Resistant to:				
Spectinomycin (100 µg ml^{-1})/	+	–	–	–
Streptomycin (100 µg ml^{-1})/	+	–	–	–
Erythromycin (25 µg ml^{-1})/	+	–	–	–
Gentamycin (5 µg ml^{-1})	+	–	–	–
Chloramphenicol (50 µg ml^{-1})	+	+	–	–
Ni (200 µg ml^{-1})/Cd (50 µg ml^{-1})	+	–	–	–
Mn (1000 µg ml^{-1})	–	+	+	–

C. villosus is nodulated by a broad range of *Bradyrhizobium* lineages. In this study, the phenotypic and genotypic diversity of bradyrhizobial strains isolated from root nodules of *C. villosus* is reported. These studies are necessary for the characterization and selection of isolates adapted to marginal edapho-climatic conditions and to provide information about their diversity.

After restriction with the enzymes *Alu*I, *Cfo*I, *Dde*I, *Hin*fI, *Msp*I *and Rsa*I, the RFLP-PCR (ARDRA) of the 16S DNA sequences of the 19 strains used in this study showed that they clustered in 3 groups, each containing 11 strains of *B. canariense*, 3 strains of *B. japonicum* and 3 strains of *B. cytisi* (CTAW11, CTAW22 and CTAW38) together with 2 strains of *B. rifense* (CTAW69 and CTAW71) (Fig. 2). These results show that the RFLP-PCR fingerprinting technique used here did not distinguish between *B. cytisi* and *B. rifense*. This is not surprising as *Bradyrhizobium* species have highly conserved 16S rRNA gene sequences and multilocus sequence analyses (MLSA) are required to elucidate the taxonomic affiliations among them (Vinuesa et al., 2008). In fact, only DNA-DNA hybridization experiments confirmed that strains CTAW71^T and CTAW69 hybridized with *B. cytisi* and *B. canariense* showing values lower than 50% in all cases, which is below the 70% threshold value of DNA-DNA similarity for definition of bacterial species (Wayne et al., 1987), indicating that strains CTAW71^T and CTAW69 belong to the new species *B. rifense*. The presence of *Bradyrhizobium* species among the rhizobia nodulating Genisteae spp. could be due to different factors, including their symbiotic potential that is responsible for their nodulation of a wide spectrum of Fabaceae spp. and their adaptability to various edaphic and climatic conditions, resulting in this genus having a broad host range (Parker, 2015; Sprent et al., 2017). On the other hand, based on results of 63 cultural, physiological and biochemical tests, the dendrogram in Fig. 3 shows that the 19 strains grouped into 4 main clusters. In contrast to the ARDRA-based genotypic affiliation, the phenotypic characterization followed in this study allowed differentiation between the *B. rifense* and *B. cytisi* strains, which formed two clearly different clusters. Our results show that all the 19 strains grew well at 0.5% NaCl, but only the *B. japonicum* and *B. rifense* strains tolerated 1.0% NaCl. Salinity tolerance may vary due to the growth habit of rhizobial strains and fast-growing rhizobial species are usually more salt tolerant, whereas *Bradyrhizobium* species are comparatively less salt tolerant due to slow growth rate (Elsheikh and Wood, 1995). Most soils in the central-western region of the Moroccan Rif are characterized by acidic pH, more concretely 4.6 in Aoudal and 5.3 in Fifi; in acidic soils nutrient availability is reduced and aluminium and proton concentrations can reach toxic levels for plants and rhizobia (Graham et al., 1994). However, all the 19 strains grew at pH 6.8, but only 5 strains of *B. canariense* and the 2 *B. rifense* strains grew at pH 4.0. Because slight variation in the pH of the medium might have significant effects on bacterial growth (Singh et al., 2008), it is possible that the pH 4.0 used in this study prevented growth of the *B. cytisi*, *B. japonicum* and of 6 *B. canariense* strains. All the 19 strains were very sensitive to alkaline pHs and none of them grew at pH 9.0. In this sense, both the plant and many of the bradyrhizobial strains are adapted to those soil conditions, which guarantees their success on revegetation strategies.

Most of the strains in this study possessed optimum growth at 28 °C; only the *B. japonicum* strains were able to grow at 4 °C and those of *B. canariense* did at 37 °C. This is surprising because most rhizobia show optimal temperature for their growth in the range from 25 to 31 °C and cannot grow at 37 °C (Graham, 1992; Harwani, 2006).

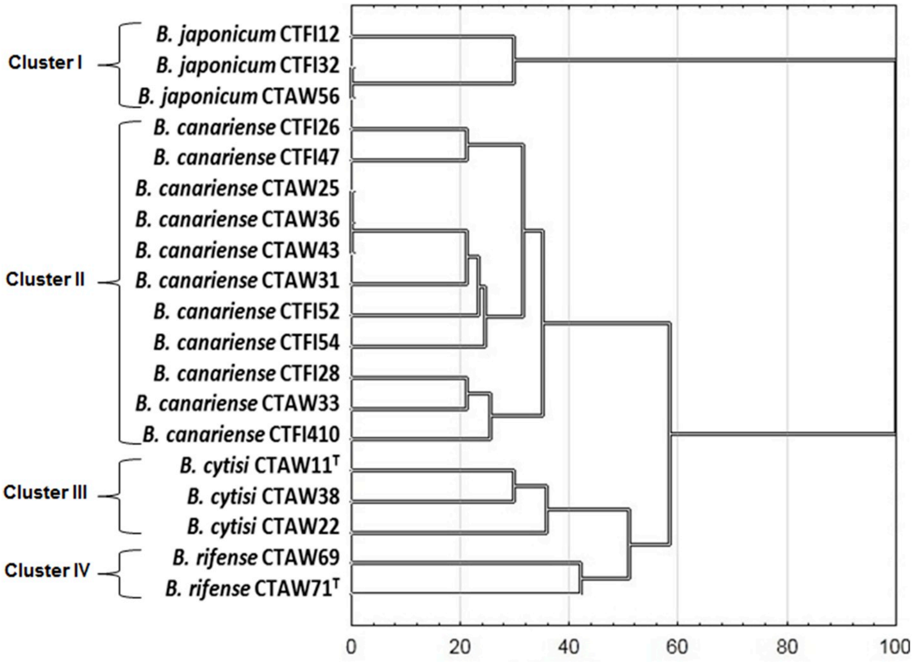

Fig. 3. Dendrogram showing the phenotypic relationships among *Bradyrhizobium* strains investigated based on UPGMA program analysis of 63 characteristics.

Utilization of different compounds by the rhizobial strains as the sole C source is a useful trait for their differentiation and identification (Hungria et al., 2001). Bradyrhizobial strains associated with *C. villosus* were able to utilize different carbohydrate sources, but none of them can be used for distinction among strains. None of the *B. canariense* strains utilized starch for growth, which suggests those strains lack amylases enzymes. Determination of the intrinsic antibiotic resistance has been shown to be useful for strain differentiation (Chanway and Holl, 1986). Our results show that the strains of *B. japonicum* were the most tolerant to antibiotics as compared to the remaining strains and that *B. canariense* strains were tolerant to tetracycline and chloramphenicol. In addition, *B. cytisi* and *B. rifense* showed similar response to the antibiotics used in this study to which they were highly sensitive.

Taken together, our result show that the phenotypic diversity of the isolates that nodulate *C. villosus* can be envisaged as a trait of ecological importance, as it permits the establishment of a functional symbiosis even under changing, severe soil conditions. The strains adapted to these variations will continue to grow in the plant rhizosphere, and only the individuals with efficient potentials will compete and survive (Zahran, 2010, 1999).

Declaration of interest statement

Declarations of interest

None.

Acknowledgments

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