International Journal of New Technology and Research (IJNTR)
ISSN:2454-4116, Volume-2, Issue-12, December 2016 Pages 27-37

Characterisation and Biodiversity of a Fast-Growing Rhizobacterial Population Nodulating Lupine in Morocco

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***Abstract*— First investigation was made to assess the phenotypic and the genetic diversity of nodule occupants harvested from different lupine species in Morocco. The determination of the phenotypic characteristics of strains revealed a wide range of tolerance to pH, to high salt (NaCl) concentrations and to extreme temperatures. Strains were also highly resistant to many heavy metals and antibiotics. Particularly, strains exhibited a fast growth rate and cultural features typical to Rhizobium. Besides, symbiosis with Lupinus luteus showed to be more effective with strains isolated from wild species. Numerical analysis allowed the recovery of interesting strains for use as potential inoculants. PCR-RFLP patterns of 16S rRNA gene indicate a big heterogeneity between the strains with a clear distinction from the reference strains used. However, two strains were closely related to Rhizobium and another strain was grouped with the Mesorhizobium. Further analysis to precise the taxonomic position of these strains is claimed.**

***Index Terms*—ARDRA – Biodiversity – Lupinus – Phenotypic characterization – Rhizobacteria– Symbiosis.**

I. Introduction

Lupinus is one of the most diverse and widespread plant genera (Cowling et al., 1997). In Morocco, the genus is represented by six species: L. luteus, L. albus, L. angustifolius, L. pilosus, L. cosentinii, and L. atlanticus (Buirchell, 1992). The latest being an endemic species of Morocco. During the last century, lupine was given much attention for its economic and ecological values as soil improver, source of forage and green manure (Gladstones, 1998). Its ability to thrive in very diverse environments and in a wide range of soil types is due mainly to its symbiosis with N2-fixing bacteria. The first classification of lupine symbiotic bacteria was based upon the cross inoculation patterns (Jensen, 1967). The concept of the growth rate was then amended and consequently, the slow-growing strains of Rhizobium japonicum and Rhizobium lupini were transferred to a new genus Bradyrhizobium (Jordan, 1982). Classifications were, since then, subject to revision as many plant genera or species were studied and as additional screening techniques were proposed. Actually, Bradyrhizobia consists of various symbionts isolated from many legumes like soybean (Jordan, 1982, Kuykendall et al., 1992; Xu et al., 1995), Acacia albida (Dupuy et al., 1994), Aeschynomene (Ladha and So, 1994), Arachis hypogea (van Rossum et al., 1995; Urtz and Elkan, 1996), Lupinus (Bottomley et al., 1994, Barrera et al., 1997), and others.

Lupine bradyrhizobia were reported to be closely related to Br. japonicum. Hollis et al. (1981) founded 99% of DNA homology between the American lupine type strain ATCC 10319 and Br. japonicum. Scholla et al. (1990) found that the high percentage was due to the contamination of the lupine type strain with Bradyrhizobium japonicum and evaluated the DNA homology value to 35%.

Diversity within lupine strains assessed by several approaches including nitrogen-fixing ability (Legocki et al., 1997; Kozhemyakov et al., 2001), fatty acid-methyl ester analysis (Graham et al., 1995), multilocus enzyme electrophoresis with pyrolysis mass spectrometry (Barrera et al., 1997) and 16S rDNA analysis (Laguerre et al., 1994, 1997) showed the same strain relatedness to Bradyrhizobium japonicum. However, Lupinus spp. was reported to be nodulated by Bradyrhizobium as well as by Rhizobium strains (Jensen, 1967; Jordan, 1982; Schlinkert-Miller and Pepper, 1988). Wayne et al. (1987) and Graham et al. (1991) reported that a proper description of species should combine PCR-based methods, DNA/DNA hybridisation as well as physiological traits. A polyphasic approach has permitted so far to characterize different strains from different lupine species. Trujillo et al (2005) have isolated some endosymbionts which belong to the genus Ochrobactrum from Lupinus honoratus. The same authors have proposed two new species, Kribella lupini and Micromonospora lupini isolated both from Lupinus angustifolius (Andam and Parker, 2007; Trujillo et al., 2006; 2007 & 2010).

Regarding to the large number of lupine species, approximately 200 species (Cowling et al., 1997) and to the different molecular based analyses which permit to characterize bacterial strains and to determine their taxonomic and/or phyllogenetic positions, a more detailed investigations should be conducted to determine the accurate status of lupine symbionts. To achieve partly this objective, our study was based on the evaluation of the genetic and the phenotypic diversity of some indigenous strains nodulating Lupinus spp. in Morocco so to present a comparative analysis of these strains and to assess their diversity.

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II. MATERIAL AND METHODS

A. Isolation and selection of bacterial strains

Strains were isolated from root nodules harvested from six Lupinus species: L. luteus, L. albus, L. angustifolius, L. cosentinii, L. pilosus, and L. atlanticus. Species were collected in twenty-two sites from different geographical regions (figure 1). Soil samples were also collected from some of these regions for soil trapping with L. luteus and L. albus which are the cultivated species in the country. The harvested nodules were surface sterilized with mercuric chloride (0.1% HgCl2 in 0.06N HCl) for 3 to 5 min and rinsed several times with sterile distilled water. They were then cut aseptically and the red material inside each nodule was streaked on Yeast-Extract Mannitol (YEM) (Vincent, 1970) agar plates supplemented with 25µgml-1 congo red for the isolation of single colonies. Only well-formed round colonies were considered. Isolates obtained were purified by repeated streaking, gram-stained and then stored as a whole collection in 50% glycerol YEM stocks at –80°C.

A preliminary test of nodulation was conducted with all the isolates to determine their ability to infect the two cultivated species. Only those that gave positive result were retained.

REP-PCR analysis (data not included; El Hilali, 2006) was conducted for the selection of representative strains to be used for the following tests.

B. Generation time

The generation time of each strain was assayed in duplicate in YEM broth medium incubated at 28°C in a generator shaker at 200rpm and was evaluated by the increase of the culture optical density at 600nm and at subsequent intervals of 30min. from the logarithmic phase of growth. Isolates were also examined for acid or alkali production after growth.

C. Physiological characterization

Strain synthesis of acid or alkali production was evaluated after 24h on YEM plates amended with Bromophenol blue (BTB) at 0.0025% (w/v). For all the following tests, plates were inoculated with 10µl of fresh YEM broth culture at the concentration of 108 cells ml-1. Twenty isolates were spotted onto each plate and three replication plates were used for each treatment. Controls of each test were grown in YEM plates under standard conditions: 0.1% (w/v) NaCl, pH 7.0 at 28°C. Strains were scored sensitive (no growth) or resistant relatively to control plates after 7 days of growth. Strains were tested for their tolerance to pH by adjusting the pH of the media to different values with the following buffers: Homopipes (25mM) for 3, 3.5, 4, 4.5 & 5 units, MES (20mM) for 5 , 6, & 7 units and with phosphate buffer supplemented with NaOH (5N) for 7, 8, 8.5, 9, & 9.5 units. Tolerance to salinity was tested with NaCl at concentrations varying from 170mM to 1700mM. The ability to grow at different temperatures was determined at variable degrees: 4°C, 10°C, 15°C, 20°C, 30°C, 35°C, 38°C, & 42°C. Intrinsic antibiotic resistance was tested by the capacity of strains to grow on YEM plates amended with 8 solutions of antibiotics (Sigma chemical Co.) at the final concentrations in µg/ml of spectinomycin (10, 15, 50, 100), tetracycline, nalidixic acid and streptomycin (10, 25, 50 100), chloramphenicol (10, 25, 50 100, 150), kanamycin (10, 25, 100), erythromycin (25, 100, 200), and ampicillin (15, 100, 200). Similarly, resistance to heavy metals was evaluated at variable concentrations in µg/ml for AlCl3.6H2O (200, 300, 400), ZnCl2 (200, 300, 400), MnCl2.4H2O (200, 300, 400), CoCl2.6H2O (100), CdCl2.2H2O (5, 20, 30, 50), and HgCl2 (5, 20, 40).

D. Auxanographical tests

The assimilation of 49 carbohydrates as a sole carbon source was tested by using API galleries (API 50CH, BioMérieux, France). Cells of a well-formed colony, obtained after 24h of incubation on YEM agar plates at 28°C, were dispersed in 1ml sterile distilled water, then resuspended in the following media in gl-1 of K2HPO4: 0.46; KH2PO4: 0.13; MgSO4: 0.2; NaCl: 0.1; Yeast nitrogen base (Difco): 2; (NH4)2SO4: 2; and agar (Sigma): 7.5. The galleries were inoculated and incubated at 28°C. Results were scored every day for 7 days as described by the manufacturer.

E. Symbiotic effectiveness

Effectiveness of strains was tested with L. luteus as a host plant. Seeds of the host were surface sterilized by 0.1% (w/v) HgCl2, scarified with concentrated H2SO4 (95%), rinsed thoroughly with sterile distilled water, and germinated on water agar 0.7% (w/v) for 3 days. Well-germinated seeds were transferred into pots filled with sterile nitrogen-free sand. Three pots, each containing three plants were inoculated with 1ml of the bacterial suspension (about 108 cells). Pots were placed in a plant growth chamber with a 16/8 light/dark photoperiod, and 25/18°C day/night temperature. Plants were watered as needed with N-free nutrient solution (Broughton and dillworth, 1971); The pH was adjusted to 6.6 units. Non-inoculated plants were used as negative controls while nitrogen fertilized control plants were watered with the nutrient solution amended with KNO3 at 0.5% (w/v). After seven week period, plants were harvested, then nodules were counted and shoots were oven-dried at 70°C for 48h and used to estimate the relative efficiency.

F. Hydrolysis of urea

10µl of YEM broth culture of each strain were inoculated onto YEM agar containing 2% urea and 0.012% phenol red (Jarvis et al., 1977). Results were scored after 24h incubation at 28°C.

G. Nitrate reduction

Strains were cultivated for 3 to 5 days in YEM broth modified medium free of phosphorus compounds, buffered with MES at pH 7.0 and containing Na2SO4 at 0.003% and KNO3 at 5mM. Each strain was assayed in four replication tests. The reduction of nitrate to nitrite was assessed as described by Lindström and Lehtomäki (1988).

H. DNA extraction

Stains were streaked onto ½ TY agar plates (Beringer, 1974) and incubated for two days at 28°C. A well- formed colony of each isolate was picked and cells were suspended into 10µl sterile distilled water. DNA of the suspension was extracted via alkaline lysis according to Maniatis et al. (1989).

DNA of gummy strains was obtained by phenol-chloroform method (Wilson, 1989).

I. *ARDRA analysis*

Thirty-two reference strains were included in this analysis (table. 1). PCR amplifications were carried out in a 70µl volume using the primers 1488r (5'-CGGTTACCTTGTTACGACTTCACC-3') and 41f (5'-GCTCAAGATTGAACGCTGGCG-3') as described by Herrera-Cervera and coll. (1999). A reaction mixture containing pure water instead of template DNA was used as a negative control. PCR amplifications were performed with the following temperature profile: an initial denaturation at 94°C for 3min. followed by 35 cycles of denaturation: 1min. at 94°C, annealing: 1min. at 55°C, extension: 1min. at 72°C, and a final extension at 72°C for 7min., then PCR products were kept at 4°C. 10µl of each amplified sample were digested with four restriction enzymes HinfI and MspI (Promega), TaqI and HhaI (Biolabs). Restriction products were mixed with the bromophenol blue dye (0.25% bromophenol blue, 50% glycerol, 50% TE) then loaded on 1.8% (w/v) agarose gel (Promega). Electrophoresis was run in TBE buffer (89mM Tris- borate, 89mM boric acid, 2mM EDTA, pH. 8.0) at 80V for 4h. The 100pb (Promega) was used as molecular weight marker. Gels were stained with ethidium bromide and scanned under UV illumination (Perfect Image, V6.1). Sizes of the different generated fragments were evaluated by comparison with those of the molecular weight marker used.

J. *Data analysis*

Results of the genetic analysis were converted into two-bidimentional binary matrix using a simple matching of band presence (1) or absence (0) at a specific molecular size. Positive or negative result of each of the other tests was similarly scored. The resulting data were analysed by UPGMA (unweighted pair group method using averages) linkage analysis (Sneath and Sokal, 1973) performed with STATISTICA/ w (V.6).

III. RESULTS AND DISCUSSION

A. *Bacterial strains*

One hundred and fifty-nine isolates were obtained from the collected root nodules of lupine species. Nodules were present on the main and the secondary roots at variable number and at dimensions varing from 3mm to 25mm. Isolates were obtained mainly by direct isolation from fresh root nodules and only two strains were obtained by soil trapping (MSMC 5155 and MSMC 5156). Isolates formed circular, gummy, and translucent or creamy visible colonies of 1 to 3mm within 2- 3 days of incubation on YEM agar at 28°C.

All the isolates were able to infect the cultivated species L. luteus and L. albus. The initial characterization performed by REP-PCR analysis to assess the intraspecific diversity between the isolates revealed a high level of genetic diversity (El Hilali et al., 2003; El Hilali, 2006); and so we selected fifty two representative isolates of each the formed groups from the clustering analysis to be used in this research.

B. *Growth rate*

Strains exhibited in general a fast growth (Fig. 2) and produced either acid or neutral reactions in the growth medium. 10% of strains were very fast growing and more acid producing with a growth rate less than 1h. Most strains, with a threshold of 73% have a growth rate between 1h and 2h and were either neutral or acid producing. MSMC556 and INRA L29 were the only strains recorded to have a doubling time above 3h. According to their cultural characteristics, stains were more related to the fast growing rhizobia. This result corroborate with the description given by Jordan (1982) for fast-growing rhizobia.These rhizobia were reported to be acid-producing; However, the variation of pH at the end of growth should not be taken as an indicative feature of the growth rate since many reports showed the presence of alkali producing rhizobia (Hernandez and Focht, 1984) as well as acid producing bradyrhizobia (Moreira et al., 1993).

C. *Physiological characterization*

Results showed that 66% of strains were able to give positive result with the BTB dye test (they turned the dye to yellow). The remaining strains, which made no noticeable color change or slightly turned the dye to blue, were scored to give negative results. Fast growing strains were reported to be acid producing (Jordan, 1984) and therefore would give a positive result with the BTB test. In our case, most strains were fast growing. However, both reactions with the dye were registered as it was also noticed with the variation of pH at the end of growth.

Strains were highly tolerant to alkalinity (Fig. 3). 100% of tolerance was obtained at pH 9 and 79% of strains grew at pH 9.5. Generally rhizobial growth was reported to be less affected at high pH. Jordan (1984) reported that rhizobial cells can grow well at pH 9 but show variable responses under acidic conditions. For acid pH, 80% of tolerance was obtained at pH 4.0. 34% could even grow at pH 3.0. This result is in a perfect agreement with that of Cordero and Blair (1978) who found that the lowest limit of pH tolerance for lupine symbionts is about 3.2 to 4.2.

Many reports have associated the acid pH tolerance to the slow growth of strains (Jordan, 1984; van Rossum et al., 1994). However, acid-tolerant strains from fast-growing species have been recovered as well (Cooper, 1982; Cooper et al., 1985; Graham et al., 1994).

Concerning salinity (Fig. 4), 80% of strains were tolerant to 850mM NaCl, 40% tolerated 1360mM NaCl, and 8% could even grow at 1700mM NaCl. Zahran et al. (1994) have reported the same result with one Lupine strain from Egypt that tolerates 10% NaCl (1700mM NaCl).

The optimum temperature of growth for all the strains ranged between 10°C and 35°C (Figure 5). This result corroborates with the temperature growth range of most rhizobia (Graham, 1992). Furthermore, more than 95% of strains could grow both at 38°C and at 4°C.

68% of the strains tested were tolerant to 42°C. Similarly to our result, Moawad and Beck (1991) have identified some lens rhizobia in the Nile valley that tolerate 35°C and 40°C.

Strains showed variable responses to the different antibiotics tested. In fact, antibiotic resistance may vary from

a rhizobium species to another and between strains of the same species (Shishido and Pepper, 1990; Zhang et al., 1991).

Strains were highly resistant to erythromycin at 200µg/ml with a threshold of 77%. At the same concentration, ampicillin showed some growth inhibition effect; the percentage of strain resistance recorded was 37%. Strains appeared to be moderately tolerant to chloramphenicol, nalidixic acid and spectinomycin. At the same concentration of 50µg/ml, we obtained 94%, 79% and 63% of resistance respectively. Tetracycline, streptomycin and kanamycin, inhibitors of the plasmic membrane and the protein synthesis, had the most drastic effect on the growth of the strains. Less than 50% of resistance was obtained with tetracycline and streptomycin tested at 50µg/ml. The effect was more detrimental with kanamycin; only 15% of strains could grow at the concentration of 25µg/ml.

The last three antibiotics were also revealed to be the most growth inhibitors for some fast-growing strains of lupine (Schlinkert and Pepper, 1988) and the same effect of kanamycin was reported for other rhizobial species (Mdrzak et al., 1995; Zeghari et al., 2000).

The evaluation of heavy metals effects indicated that 86% and 80% of strains were respectively resistant to manganese and aluminium at 400µg/ml. 67% of strains were also resistant to the zinc at 400µg/ml. These exhibited resistances, combined with the acidity tolerance already described, are of particular interest, especially under acid soil conditions where excess of aluminium and manganese are major problems for the establishment of the symbiosis (Graham, 1992).

The sensitivity of the strains under different concentrations of mercury and cadmium appeared to be similar. 68% and 64% of resistance were recorded respectively for the two metals at 40µg/ml. These metals caused the highest decrease in the extent of growth in comparison to control plates. Cadmium is considered as a current problem for the survival of rhizobial strains in soil, for plants and for the formation of effective N2-fixing nodules (Tiller et al., 1994; Purchase et al., 1997; Figueira et al., 2005).

Large differences of growth were observed for the cobalt and only one-quart of strains were responsive at the concentration tested. Cobalt was reported to play a negative effect on the survival of lupine symbionts and on the first steps of nodule initiation (Riley and Dillworth, 1985).

D. Auxanography

Strains presented a good growth on the following monosaccharides: L-arabinose, fructose, ribose and mannitol. Chakrabati and coll. (1981) reported that only lupine strains were able to assimilate the arabinose among all the bradyrhizobia they tested. All strains were able to use glycerol, gluconate, N-acetyl glucosamine, 77% were able to assimilate glucose, 73% used sucrose and 12% could use dulcitol. Most strains were able to assimilate disaccharides. 75% of strains were also able to use esculin, 8% used amidon, and 4% used glycogen. The same percentage was recorded for inuline. However, none of the strains was able to assimilate L-sorbose. These results corroborate with the carbon sources utilization scheme of fast-growing strains reported by Stowers (1985).

E. Infectivity and effectiveness

The test of infectivity showed that L. luteus was responsive to the inoculation by all the rhizobial strains tested and all the strains were recorded to establish an effective symbiosis. Nodules formed were placed evenly on the whole root system instead of the tap-root nodulation specific to L. luteus. The mean number of nodules formed per plant varied from 8 to 109. The most infective strains were MSMC 5142 and INRA L 212 with 108 and 80 nodules per plant. Strains MSMC5127 and MSMC 5114 were the less infective with 8 and 10 nodules formed per plant respectively. Symbiotic potential of the strains was evaluated by the increase of the shoot dry weight of inoculated plants compared with that of the controls (Figure 6). All the strains showed higher efficiencies when compared to the non-fertilised control that represented an average of 31% of relative efficiency. Effectiveness values varied between 87% and 35%. The highest shoot dry weights were obtained with the strains MSMC 518 and MSMC 549 for which the relative efficiency was estimated to 87% and 83% respectively. While the lowest effectiveness was 35% obtained with the strain MSMC 5115. In general, strains were highly effective: 70% of strains have efficiencies between 60% & 80%, and 19% of strains showed efficiencies between 50% & 60%. We have also noted that all the strains isolated from other species than the used host plant showed efficiencies more than 60%. While efficiencies of strains isolated from L. luteus varied considerably (from 35% to 87%). Besides, the variation of strains infectivity showed no correlation with the variation of their relative efficiency.

F. Biochemical tests

Urea was hydrolysed by 63% of strains. Nitrate reductase was scored to be active for 52% of strains. The ability to reduce nitrate is of a great ecologic importance. An excess of nitrate in soil causes inhibitory effect on the adsorption of rhizobia on the root surface (Sherwood et al., 1984) and a decrease of the infection and the nitrogen fixation capacity of strains (Davidson and Robson, 1986; Arreseigor et al., 1997).

G. Numerical analysis

The determination of the phenotypic characteristics is important for understanding the significance of the inter-specific differences found between the strains. The combined data analysis of 105 phenotypic characters was performed (figure. 7). At 75% of similarity level, the phenogram was divided into four heterogeneous clusters with two independent lineages formed by the strains INRA L 15 and MSMC 5419. The former strain presented some distinctness and many interesting features, it was able to grow in the presence on 1700mM NaCl, could assimilate esculin, inuline, and starch and it was efficient with a threshold of 74% of relative efficiency. Strain MSMC 5419 could tolerate 850mM NaCl, showed positive reaction with urea and nitrate tests and was also able to assimilate esculin, inuline, and starch. The strain presented a high relative efficiency of 83%.

The first cluster was formed by strains tolerating 850mM NaCl or more. They all have a positive urease activity. For nitrate reduction, only strains isolated from L. luteus showed a positive result. Strains of this species were all resistant to mercuric chloride at 40µg/ml. However, they were less

efficient (39% ≤ RE ≤ 57%). While the other strains did not tolerate concentrations above 5µg/ml HgCl2 but they were highly efficient (62% ≤ RE ≤ 78%).

Cluster 2 was formed by strains belonging to L. luteus, and L. cosentinii. These strains were distinguished especially by their high salt tolerance to 1190mM and 1700mM NaCl. Strains of L. luteus showed efficiencies lower than 60%, while the other strains were more efficient (62% ≤ RE ≤ 74%).

Cluster 3 appeared to be the most heterogeneous. At the branch point corresponding to 77% of similarity level, the cluster was divided into two sub-clusters: I and II. Strains of the sub-cluster I were not able to hydrolyse urea or to reduce nitrate. However, they exhibited a high tolerance to salinity (1190mM and 1700mM NaCl) and they were also highly efficient (66% ≤ RE ≤ 77%). Strains of the sub-cluster II could tolerate from 680 to 1190mM NaCl. They presented efficiencies comprised between 55% and 70%. They all had positive urease and nitrate reductase activity, and they were particularly resistant to spectinomycine (100µgml-1) and to tetracycline (50µgml-1).

Cluster 4 is formed by strains belonging to L. luteus and L. angustifolius. These strains were tolerant to NaCl concentrations comprised between 680mM and 1190mM (except for MSMC 532, and MSMC 5328). They had mostly positive responses with biochemical tests, were all not able to assimilate sucrose and showed efficiencies comprised between 55% and 78%.

H. ARDRA analysis

16S rDNA band of 1500pb was obtained for all the strains tested. 16S rDNA gene restriction analysis has been applied to characterize rhirobial strains and to give insights into the classification of these strains with the defined species of Rhizobia (Weisburg et al., 1991; Vanechoutte et al., 1992; Laguerre et al., 1994).

RFLP fingerprints generated with each of the four restriction enzyme used were very distinct. More than thirty restriction combinations were obtained (data not shown).

Pair-wise comparisons of restriction profiles obtained for both lupine and reference strains were used to generate a phylogenetic tree (figure 8). The clustering analysis revealed a clear distinction between strains. One independent lineage corresponding to Allorhizobium undicola and seven clusters were delimited at the genetic similarity level of 70%. The first cluster is represented exclusively by strains isolated from L. luteus species. Cluster 2 is formed by Bradyrhizobium strains. Clusters 3, 4 and 5 are formed by lupine strains. Cluster 6 combine Mesorhizobium and Sinorhizobium and Cluster 7 combine on the first branch some lupine strains and on the second branch Agrobacterium with Rhizobium species.

Phylogenetic clustering of the reference strains is in a perfect agreement with other well established results based either on ARDRA analysis (Laguerre et al., 1994, 1997) or on the complete sequence of the 16S rDNA (Terefework et al., 2001).

Concerning lupine strains, three strains showed to be more closely related to the reference strains. While all the other strains were distributed in distinct clusters. The three strains were the INRA L 61 which form a unique lineage with Rhizobium leguminosarum bv. vicae and Rhizobium leguminosarum bv. trifolii strains, INRA L 23 which shares with Rhizobium giardinii 88% of genetic similarity and INRA L 29 which is classified into the Mesorhizobium genus but shows however 25% of divergence. At higher level of genetic similarity, the four clusters of lupine were divided into many branches reflecting an extensive genetic heterogeneity between strains. However, no obvious relation with the host plant was noted except for cluster 1.

IV. GENERAL DISCUSSION

Rhizobium – lupine symbiosis can yield high quality of forage and seeds and can be efficiently used for the rehabilitation of infertile soils. In Morocco, no information concerning nodules occupants of lupine species has been given so far. This study was then conducted for two reasons, the selection of interesting strains as potential inoculants under specific environments and the evaluation of their taxonomic position.

Strains presented cultural aspects related to the fast-growing rhizobia. Indeed, they were exhibiting a fast growth rate. Although symbionts of lupine were assigned to belong to bradyrhizobia (Jordan, 1982; Barrera et al., 1997; Jarabo-Lorenzo et al., 2003; Stepkowski et al., 2007; Velázquez et al., 2010), there were different lupine fast-growing symbionts identified until now belonging either to the α- or β-proteobacteria. Jordan (1984) has isolated some fast growing strains from Lupinus densiflorus. Pudelko and Mdrzak (1996) reported the presence of both rhizobial and bradyrhizobial distinct populations nodulating Lupinus in Poland. Trujillo et al (2005; 2006; 2007 & 2010) have also identified some other fast-growing strains than rhizobia nodulating Lupinus honoratus and Lupinus angustifolius.

In our case, strains were isolated from different species and in different geographic sites, so it can be inferred that fast-growing strains of lupine are dominant in Moroccan soils and this is of a great practical use in inoculation strategies.

Strains tested presented some distinction features through their aptitude to thrive in very diverse environments. They presented a broad range of tolerance to pH, salinity, and extreme temperatures. Many strains can be also used as potential inoculants in arid regions where high salt concentration and alkalinity are the most inhibiting factors of an effective symbiosis.

In fact, by the salinity test, we noted that some strains originating from non-irrigated areas were inhibited by the low salt concentrations used (170mM and 340mM NaCl). While all the strains, originating either from the cultivated-irrigated areas or near the irrigated sites, were salt-tolerant. It is obviously known that one of the principal causes of salinization can be dragged by irrigation (Szabolcs, 1986). The wide range of salt tolerance expressed by the stains was then related to the rate of salinity in the original site of isolation.

Leguminous tree rhizobia were supposed to be more tolerant to NaCl. Strains isolated from Acacia, Mesquite and Leucaena proved to be tolerant to 500mM and 850mM NaCl (Zeghari et al., 2000, Lal and Khanna, 1995). However,

growth at saline concentration of 850mM NaCl or even more has been recorded for many strains isolated from annual cultivated or forage plants in Morocco. Maâtallah and coll. (2002) have identified high salt tolerant strains (850mM NaCl) nodulating chickpea. Some strains nodulating fenugreek were able to grow at salt concentration at high as 14% (Abdelmoumen et al., 1999). Lupine strains characterized in this study can tolerate up to 1700mM NaCl.

Extreme temperatures are listed among the main limiting factors of symbiotic nitrogen fixation in very humid and cold regions (Zhang and Smith, 1996) and have a great impact on strain persistence in arid environments (Zahran, 1999; Hungria and Franco, 1993; Kishinevsky et al., 1992).

Strains can persist under temperature stress. Besides, they can loose their symbiotic characteristics. This has been demonstrated for some Rhizobium leguminosarum bv. Phaseoli strains which can tolerate from 45°C to 47°C but were unable to induce nodulation (Karanja and Wood, 1988).

In this study, strains showed a large tolerance to extreme temperature but there was no correlation between the temperature range of tolerance for most of the strains and the extreme temperatures recorded for each of the sampling sites.

The number and the survival of rhizobial cells in contaminated soils can be severely affected (Obbard et al., 1994). It is obviously known that mineral toxicity inhibits the symbiosis (Biro et al., 1998; Pal et al., 1996). Besides, valuable rate of N2-fixation was recorded at variable extent with various legumes under high metal concentration. Actually, resistant Rhizobium-legume symbiotic systems are used as an efficient mean of bioremediation in contaminated soils (Abbes and Kamel, 2004; Vasquez et al. 2006).

On the basis of the heavy metal resistance profile, one interesting strain MSMC 541 was selected to be used in association with L. Luteus species for the remediation of metal contaminated soils. The experiment gave interesting results and a great potential usage of rhizo-phytostabilization of metals in soils (El Aafi et al., 2012)

Concerning carbohydrate assimilation, strains showed a large preference to mono- and disaccharides. Fast growing strains were reported to show in general a large preference towards hexose, pentose, disaccharides and organic acids (Stowers, 1985) while slow-growing strains show variable usage of disaccharides and a rare affinity to monosaccharide. Besides, variability of the carbohydrate nutrition among rhizobia (Graham, 1964; Stowers and Eaglesham, 1984) was also reported.

All the strains were able to infect L. luteus, which is the first cultivated species of lupine in Morocco, but they showed variable degrees in terms of efficiency. Strains isolated from wild species proved to be in general more efficient than those isolated from cultivated species. Thus, obtaining data about the N2-fixation capacity of strains from indigenous population is important in order to select useful strains adapted to various environments. The recovery of effective strains from wild lupine would be promiscuous for inoculating cultivated species and for improving their symbiosis under N2-limiting conditions.

The polymorphism obtained by ARDRA analysis allowed us to differentiate several and distinct ribotypes. Clustering of the reference strains was highly correlated with the classification based on 16S rDNA full sequence. A high level of genetic diversity was revealed between lupine strains and between these strains and the reference strains used. Particularly, the strains of cluster 1 were grouped apart from the other strains. In fact, these strains were all isolated from a single huge nodule located on the tap first root of the plant (El Hilali et al., 2007).

More interesting is the clustering of lupine strains distinctly of the Bradyrhizobium branch and from the other reference strains used in the analysis, except for three strains already mentioned.

On the basis of the partial sequence analysis of 16S rDNA gene, the strain MSMC541 showed 99% homology with some species belonging to the genus Serratia (El Aafi et al., 2012). Many new endosymbionts belonging to the alphaproteobacteria and associated with different species of lupine are also and continuously reported (Ardley et al., 2012; De Meyer et al., 2012; Carro Lorena et al., 2014; Flores-Félix et al., 2014).

V. CONCLUSION

The numerical and the ARDRA analysis of the different lupine strains studied which were extracted from different lupine species and from different geographical locations showed a high level of biodiversity. Some interested strains were selected and tested for their tolerance to extreme environments (Annicchiarico et Thami Alami, 2012) and others for their resistance to metals in soils (EL Aafi et al., 2012). Results were interesting; however, the precise taxonomic status and the phylogenetic classification of these strains are still to be determined.

VI. ACKNOWLEDGMENTS

This work was financed by the National Education Ministry: PROTARS research project N°P5T2/13 and AIRE development project 01-2-MAR- 28-1. Dr. J-C.Cleyet-Marrel is acknowledged for reference strains.

This work is dedicated to Mrs BRHADA Fatiha who died on February 2013 and who has supervised the enrollment of the survey presented in this article.

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Table 1: List of the reference strains used in ARDRA analysis

Species	Strain
Bradyrhizobium sp. (Arachis)	ISRA 616
Bradyrhizobium sp. (Arachis)	ISRA 601
Bradyrhizobium japonicum	USDA 110
Bradyrhizobium elkanii	ORS 2800
Bradyrhizobium canariense	BTA-1
Mezorhizobium plurifarium	ORS 1032
Mezorhizobium loti	NZP 2213
Mezorhizobium huakuii	ORS1752
Mezorhizobium tianshanense	ORS 2640
Mezorhizobium mediterraneum	ORS 2739
Mezorhizobium ciceri	ORS 2738
Rhizobium leguminosarum biovar	ORS 663
Rhizobium leguminosarum biovar	ORS 639
Rhizobium leguminosarum biovar	ORS 662
Rhizobium giadinii	STM 854
Rhizobium gallicum	STM 853
Rhizobium etli	ORS 645
Rhizobium galegae	STM 1834
Rhizobium mongolensis	STM 246
Rhizobium huautlense	STM 247
Sinorhizobium fredii	USDA 205
Sinorhizobium meliloti	ORS 665
Sinorhizobium arboris	ORS 1755
Sinorhizobium saheli	ORS 100
Sinorhizobium terangae	ORS 1007
Sinorhizobium medicae	ORS 504
Sinorhizobium kostiense	ORS 97
Agrobacterium tumefacience	ORS 1351
Agrobacterium rhizogene	ORS 1352
Agrobacterium rubi	ORS 1353
Agrobacterium vitis	ORS 2643
Allorhizobium undicola	ORS 992

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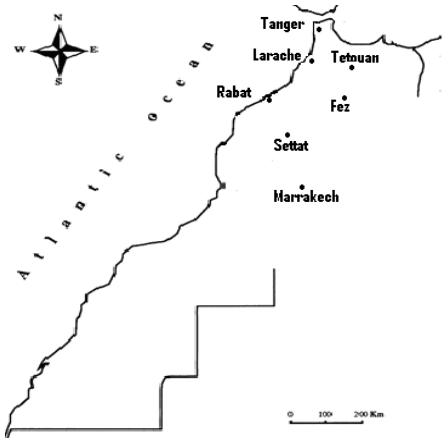

Fig 1: Principal geographic regions of plant and soil samples collection sites.

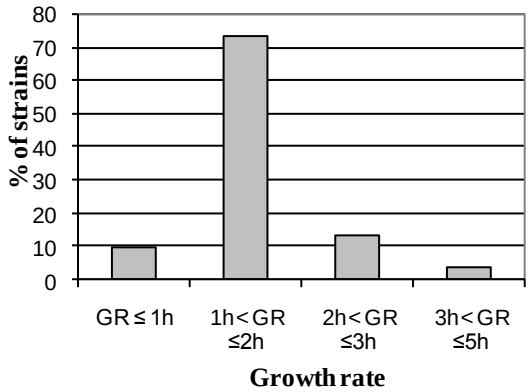

Fig 2: Generation time of the lupine strains studied.

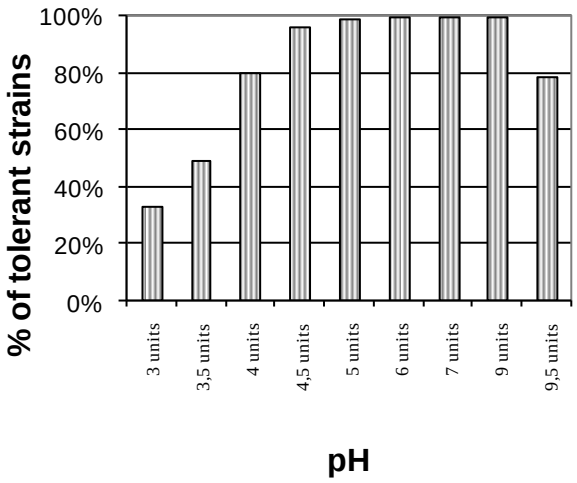

Fig 3: Effect of pH on the growth of the lupine strains studied.

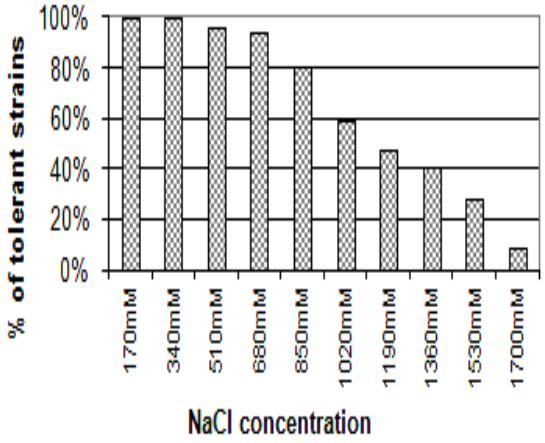

Fig 4: Tolerance of strains to different NaCl concentrations.

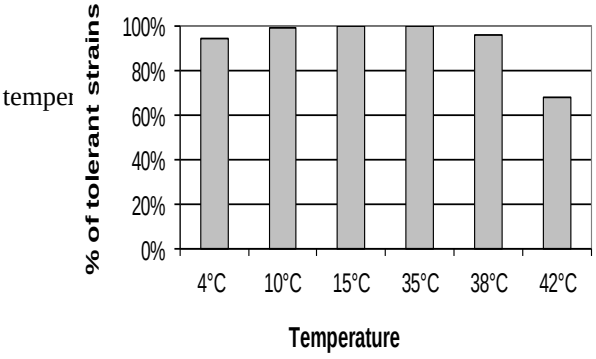

Fig 5: Tolerance of strains to variable temperatures.

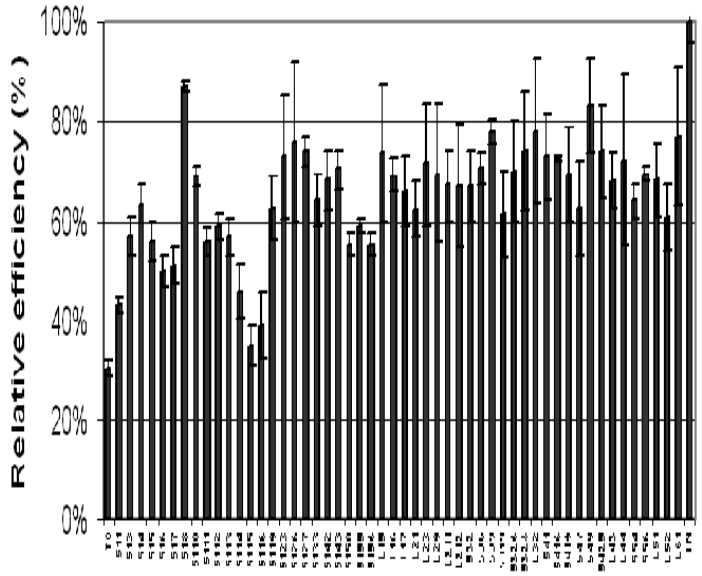

Fig 6: Relative efficiency of lupine strains evaluated after seven weeks of growth.
(T0= Negative control, TN= Nitrogen-fertilized control).

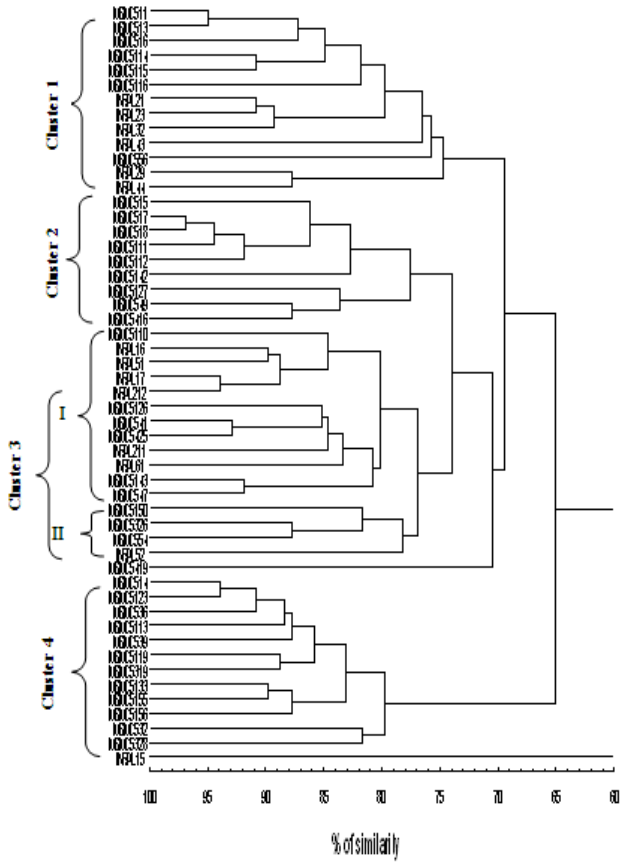

Fig 7: Phenogram based on the comparison of 105 phenotypic characteristics tested among the lupine strains.

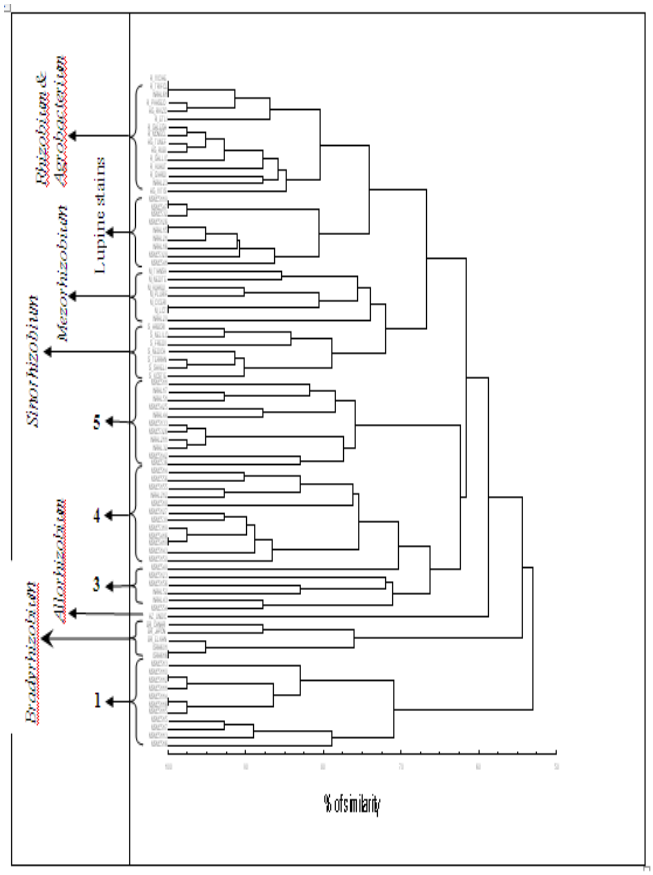

Fig 8: UPGMA dendrogram constructed from the restriction patterns of the 16S rDNA gene of the lupine strains tested and the reference strains used in the analysis.