Systematic and Applied Microbiology 42 (2019) 448–456

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

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

Genetic diversity of rhizobia associated with root nodules of white lupin (*Lupinus albus* L.) in Tunisian calcareous soils

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ARTICLE INFO

Article history:
Received 24 December 2018
Received in revised form 26 March 2019
Accepted 4 April 2019

Keywords:
Lupinus albus
Calcareous
soils
Agrobacterium
MLSA
Rhizobium
Neorhizobium

ABSTRACT

With a view to introducing white lupin (*Lupinus albus* L.) for cultivation in Tunisian calcareous soils, compatible indigenous rhizobia for nitrogen-fixing symbiosis were investigated and characterized. Two *L. albus* varieties, Mekna and Lumen, were used to trap rhizobia in soil samples collected from 56 sites with high active lime contents (0–49%). Nodulation occurred in only 15 soils. The local variety, Mekna, developed significantly more root nodules and had a trapping capacity in more soils than the imported variety Lumen. A phylogenetic analysis based on the partial 16S-23S ribosomal RNA internal transcribed spacer region (ITS) and multi-locus sequence analysis (MLSA) of three chromosomal housekeeping genes, *recA*, *atpD* and *dnaK*, showed that strains were affiliated to *Agrobacterium*, *Rhizobium*, and *Neorhizobium*, with large internal diversity, including separate lineages. Infectivity tests highlighted some nodulation specificity at the plant variety level, since the strains originating from Mekna could only nodulate this variety, while strains trapped in Lumen could nodulate both varieties. When inoculated, almost all strains resulted in a significant increase in plant shoot dry weight on *L. albus*. Although *Agrobacterium* sp. strains isolated from *L. albus* could nodulate and had a plant growth promoting effect, no *nodA* and *nodC* genes could be amplified. This is discussed together with the absence of bradyrhizobia and the general infrequency of *L. albus*–nodulating rhizobia in Tunisian soils. The adapted and efficient rhizobial strains reported here were promising candidates for inoculant development and represent a contribution towards successful cultivation of *L. albus* in Tunisia, especially the most promising Mekna variety.

Introduction

The genus *Lupinus* (tribe *Genisteae*) groups more than 280 species of annual herbs, as well as perennial herbaceous woody shrubs distributed mainly across the American continent, the Mediterranean region and Africa [9]. However, only 12 of the 280 *Lupinus* species originate from the Mediterranean region and North Equatorial Africa. Only four species have been domesticated and have agricultural importance, including the white lupin *Lupinus albus* L., which has been known since the Late Neolithic period and was probably already cultivated during the Bronze Age with other lupin species in Greece, Cyprus and Egypt. Five lupin species exist in Tunisia (*Lupinus albus* L., *upinus angustifolius* L., *upinus luteus* L., *upinus micranthus* Guss and *upinus cosentinii* Guss), but they are scarce and restricted to a limited area in the north of the country [16], and are not cultivated.

To date, livestock feeding in Tunisia has been dependent on expensive imported soybean [49] and there is a need for alternative local resources, especially legumes, and *Lupinus* species are good candidates because of their agronomic, food and feed values. Recently, attention has focused on *L. albus* due to the high quality proteins (33%–47%) and fat in its seeds [11], and this legume has been considered a good alternative to soybean oilcakes [25]. Apart from its potential as a forage crop, *L. albus* can also be used as green manure [23]. When intercropped with wheat, *L. albus* has been reported to improve wheat shoot growth and shoot phosphorus (P) uptake by 33% and 45%, respectively, without significantly affecting its own development and nutrition [54]. This may be due to its abil-

Abbreviations: ITS, 16S-23S rRNA internal transcribed spacer; MLSA, multi-locus sequence analysis; PCR, polymerase chain reaction; PGP, plant growth promoting.

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https://doi.org/10.1016/j.syapm.2019.04.002
0723-2020/

ity to form cluster roots in response to phosphorus starvation [4]. The meta-analysis recently conducted by Cernay et al. [3] showed that preceding cultivation of *L. albus* improved the following wheat yield by 36%. Furthermore, this grain legume contributes to a significant increase in nitrogen content in wheat grain and to lower crop disease incidence [45] through its ability to cover its nitrogen (N) requirements mainly from the atmosphere by symbiotic interaction with rhizobia soil bacteria.

Lupinus spp. are generally reported to be nodulated by slow-growing rhizobia affiliated to *Bradyrhizobium* [51,58], including *Bradyrhizobium canariense* and *Bradyrhizobium japonicum* for European lupin species, and *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* for most American species [9]. Specifically, strains nodulating *L. albus* in Spain, the Canary Islands and Chile group into four different *Bradyrhizobium* lineages [58].

However, several reports have mentioned fast-growing rhizobia nodulating *Lupinus* spp. in Morocco and Poland, and 70% of *Lupinus*-nodulating bacteria were found to be affiliated to *Allorhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Rhizobium* [10,44]. The *Phyllobacterium trifolii* [56] and *Ochrobactrum* strains [54] were also reported to nodulate *L. albus* L. in Spain, albeit not efficiently. Recently, Msaddak et al. [38,39] affiliated rhizobial strains nodulating *L. luteus* and *L. micranthus* growing naturally in North Tunisia to *Bradyrhizobium* and *Microvirga*; in addition, two strains nodulating *L. micranthus* were found to be related to *Phyllobacterium.*

Similarly to soybean-nodulating rhizobial populations, whose composition and distribution are related to environmental conditions such as soil temperature [52], soil pH and climatic features [61,1], the large rhizobial diversity associated with *Lupinus* spp. may be explained by the soil and eco-climatic conditions prevailing in each region and country.

To our knowledge, *L. albus* has never been cultivated or consumed by humans (seeds) in Tunisia and there is no scientific report describing rhizobial populations nodulating the plant in this country. Cultivation of this valuable legume in Tunisian calcareous soils requires information on potential local rhizobial strains adapted to it in calcareous soils, as lupins are reported to be calcifugous plants adapted to poor acid soils [13].

In this context, this study explored the diversity and distribution of local rhizobial populations able to nodulate *L. albus* in different geographical areas of Tunisia, where calcareous soils prevail. After characterizing a strain collection by the 16S-23S ITS region, as well as *recA*, *atpD* and *dnaK* housekeeping gene sequencing, their PGP efficiency was tested on two *L. albus* varieties, one local and one imported.

Materials and methods

Soil sampling and physico-chemical analysis

As *L. albus* has never been cultivated in Tunisia, it was not possible to collect nodules from fields. Soil samples were therefore collected from the root zone (0–25 cm in depth) at 56 distant sites scattered across northern Tunisia with no record of any rhizobial inoculation, in order to trap potential symbionts of white lupin. The corresponding GPS coordinates, including altitude, latitude and longitude, together with the province name, were also recorded. The physico-chemical properties of the soil samples in which nodulation was observed were recorded using standard procedures (Table 1).

Rhizobial trapping and isolation

There is no commercial source for white lupin seeds in Tunisia. Therefore, seeds were collected from a local variety of white lupin,

Table 1
Geographical coordinates and soil characteristics at sampling sites where rhizobial isolates were obtained.

	Latitude	Longitude	Altitude (m)	Clay (%)	Silt (%)	Sand (%)	pH	EC (µS/cm)	CaCo3 (%)	Active CaCo3 (%)	Organic matter (%)	Total N (%)	Available P (ppm)
Mateur	37°01'N	9°62'E	13	49	22	29	7.81	152.40	18.52	17.25	2.97	0.11	66.00
Ouedbeja	36°74'N	9°23'E	151	46	34	20	6.77	140.00	25.8	15.00	0.34	0.28	10
Sidithabet	36°91'N	10°04'E	6	28	15	57	8.05	160.00	21.33	12.00	2.00	0.13	40
Borjmassaoudi	36°30'N	9°08'E	418	26	36	38	8.02	120.00	22.52	7.50	3.73	0.18	65
Krib 1	36°37'N	9°19'E	363	15	26	59	7.32	12.00	2.58	2.50	2.21	0.12	67
krib 2	36°30'N	9°18'E	435	20	28	52	7.36	71.50	1.25	0.95	1.66	0.18	62
Azmour	36°93'N	11°01'E	49	15	28	57	8.20	80.00	8.53	0.25	1.24	0.14	110
Mraissa	36°75'N	10°55'E	11	21	30	49	7.18	36.00	1.70	0.00	0.83	0.03	90
Sejnen	37°06'N	9°24'E	133	20	28	52	4.98	23.00	0.00	0.00	1.38	0.03	88
Tejerouine	35°93'N	8°55'E	596	28	40	32	7.70	135.10	49.73	28.75	0.90	0.15	43
Madian	36°30'N	9°03'E	352	14	39	47	7.98	140.30	32.21	26.25	2.62	0.14	73
Zaghouan	36°74'N	10°03'E	55	18	30	52	6.68	143.00	34.58	15.00	2.55	0.12	48
Benouria	36°30'N	9°08'E	418	28	35	37	7.56	139.80	56.36	31.25	1.17	0.15	68
jrissa	35°85'N	8°58'E	616	28	35	37	7.81	214.00	44.05	21.25	1.38	0.21	61
Teboursouk	36°38'N	9°23'E	436	19	50	31	7.80	120.90	28.89	24.25	1.73	0.14	22

Table 2
Primers and PCR reaction conditions used for amplification of the ITS region, and the *recA*, *atpD* and *dnaK* genes.

Target	Primer	Sequence (5'-3')	PCR conditions	References
ITS	Br5ITS 23S-38	CTTGTAGCTCAGTTGGTTAG CCGGGTTTCCCCATTCGG	3 min 96 °C, 30 × (30 s 95 °C, 30 s 55 °C, 40 s 72 °C), 3 min 72 °C	[60] [43]
recA	recA(63)f recA(504)r	ATCGAGCGGTCGTTCGGCAAGGG TTGCGCAGCGCCTGGCTCAT	5 min 96 °C, 35 × (30 s 96 °C, 30 s 57 °C, 30 s 72 °C), 7 min 72 °C	[14]
atpD	atpD352f atpD871r	GGCCGCATCATSAACGTCATC AGAGCCGACACTTCMGARCC	5 min 96 °C, 35 × (30 s 96 °C, 30 s 57 °C, 2 min 72 °C), 7 min 72 °C	[31]
dnaK	TSdnaK3 TSdnaK2	AAGGAGCAGCAGATCCGCATCCA GTACATGGCCTCGCCGAGCTTCA	1 min 94 °C, 35 × (1 min 94 °C, 1 min 62 °C, 40 s 72 °C), 3 min 72 °C	[51]

known as Mekna, growing in soils of Sejnen located in North Tunisia. The commercial variety Lumen was imported from France.

In order to trap soil rhizobia, the soil samples were used as a substratum in sterilized 500-mL plastic pots. Lupin seeds were surface sterilized by treatment with 95% ethanol for 1 min, 25% sodium hypochlorite for 3 min, and finally rinsed with sterile distilled water ten times, then soaked in sterile water for 1 h. The surface-sterilized seeds were immediately sown in the different collected soils.

Seventy-seven days after sowing, the root nodules of the trap plants were carefully collected, washed in water, then surface sterilized by immersion in 95% ethanol for 30 s, 15 min in 15% (v/v) H_2O_2, and finally rinsed ten times in sterile distilled water. The nodules were aseptically crushed and streaked on yeast extract mannitol agar (YMA) containing 0.025 g kg^{-1} Congo red, then incubated at 28 °C for 2–20 days for colony development [50]. Single colonies were picked and repeatedly streaked on the same medium for purification. The pure isolates were maintained on YMA-Congo red slants, kept at 4 °C, and at −80 °C for long-term storage in yeast mannitol liquid medium adjusted with 20% (v/v) glycerol.

Plant nodulation and symbiotic efficiency

All isolates were tested for their nodulating capacity on the two *L. albus* varieties, Mekna and Lumen. Lupin seeds were surface-sterilized by treatment with 95% ethanol for 1 min, 25% sodium hypochlorite for 3 min, and finally rinsed with sterile distilled water ten times, then soaked in sterile water for 1 h. The surface-sterilized seeds were sown in sterile 500-mL plastic pots containing sterilized sand.

After germination, lupin seedlings were inoculated with 1 mL of culture (approx. 10^9 bacterial cells). Uninoculated unfertilized plants were used as negative controls (T0), while uninoculated nitrogen-fertilized plants (equivalent to 90 units of nitrogen applied per ha) served as positive controls (TN). All treatments were carried out in triplicate. A sterile nitrogen-free nutrient solution containing 1.65 mM $CaCl_2$, 1 mM $MgSO_4$, 0.36 mM KH_2PO_4, 0.7 mM K_2SO_4, 4 μM H_3BO_3, 6.6 μM $MnSO_4$, 1.56 μM $CuSO_4$, 1.55 μM $ZnSO_4$, 0.12 μM Na_2MoO_4, 0.12 μM $CoSo_4$, and 1.26 mg L^{-1} iron (Sequestrene) was supplied to the plants twice a week [55]. Nodulation was examined 40 days after inoculation and the nodule number per plant was recorded. In the case of infective strains, nitrogen fixing efficiency was estimated from the shoot dry weight, and nitrogen content was determined in 100 mg of ground-dried shoots using the Kjeldahl method [24] and compared to the positive and negative controls. Symbiotic effectiveness (%) was expressed as the test-plant ratio of the nitrogen content (mg) per plant to that of the nitrogen-fertilized control plants (TN). Data were analysed for variance using R statistical software version 3.5.1.

DNA extraction

Total genomic DNA was extracted from the isolates by lysis of single colonies picked from yeast extract mannitol agar (YEMA) plates. Colonies were suspended in 50 μL sterile ultrapure water, then 10 μL proteinase K solution (1 mg mL^{-1}) and 50 μL Tris EDTA (10 mM) were added. The mixture was maintained at 52 °C for 2.5 h for cell lysis and boiled at 95 °C for 10 min to inactivate the proteinase K. The resultant lysate was stored at −20 °C pending PCR amplification.

PCR amplification, sequencing and phylogenetic analysis of the ITS region, and the recA, atpD *and* dnaK *genes*

PCR amplification was carried out in a 25 μL final reaction volume containing 2 μL bacterial cell suspension, 1X reaction buffer (final 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.8 μM of each primer, and 0.62 U of GoTaq® DNA polymerase (Promega Corporation, Madison, Wi). The primers and thermal cycle conditions used for each gene are listed in Table 2.

Sequencing was carried out by the Genoscreen Company (France). Sequences were manually corrected using FinichTV software (version 1.4.0) and blasted for phylogenetic relatedness with reference sequences in the NCBI database (www.ncbi.nlm.nih.gov). Sequences were aligned using Seaview software (version 4.6.2) [17]. All the new sequences were deposited in the DDBJ (DNA Data Bank of Japan) database with the following accession numbers: LC436109 to LC436171 for ITS, LC436172 to LC436230 for *recA*, LC436298 to LC436343 for *atpD*, and LC436231 to LC436297 for *dnaK*.

ITS-based phylogenetic reconstruction was carried out using the maximum likelihood phylogeny method with 100 bootstrap replications using Seaview software (version 4.6.2). MLSA was performed with individual and concatenated sequences of three housekeeping genes (*recA*, *atpD* and *dnaK*). Due to the lack of *dnaK* sequences for several reference strains in the NCBI database, a concatenated phylogeny tree based on the *atpD* and *recA* housekeeping genes was also constructed. The percentage sequence similarity was calculated using BioEdit software version 7.2.5 [19].

Results

Isolation of root nodule bacteria

Soils were sampled from 56 different sites mainly located in northern Tunisia and they were examined for their nodulation potential on two varieties of *L. albus*, Mekna and Lumen. Lupins had never been grown at these sites, but other legumes, such as *Vicia faba*, *Pisum sativum*, *Cicer arietinum* and *Medicago sativa*, are traditionally cultivated at some of them, although there is no record of rhizobial inoculation. No nodules could be detected on the roots of either the Mekna or Lumen varieties grown in soil samples from 41 of the sites. However, nodulation occurred in 15 soil samples for Mekna but only in 8 soils for Lumen (Table 3). Nodulation records were heterogeneous for nodule numbers and fresh weights, depending on the site and the lupin variety.

The largest numbers of nodules were recorded for the local variety, Mekna, grown on soil samples from Mateur, Sejnen and Mraissa, with 15, 20 and 42 nodules per plant, respectively. How-

Table 3
Nodulation of white lupin and corresponding bacterial isolates.

Soil sample	Nodule number (nodules plant^{-1})		Nodule fresh weight (mg plant^{-1})		Number of isolates		Isolates
	L[a]	M[b]	L	M	Site	Plant variety	
Mateur	2.00d	15.0b	19.33ef	290.00a	4	4(M)	M:LAb4;LAb23;LAb24;LAb26
Oued Beja	12.00b	1.3c	81.20c	50.00b	7	4(L); 3(M)	M:LAa25;LAa26;LAa27, L:LAa30;LAa31;LAa33;LAa34
Sidithabet	1.66d	2.0c	29.30de	66.96b	3	1(L); 2(M)	M:LAa35;LAa37, L:LAa38
Borjmassaoudi	1.33d	3.0c	44.19de	79.46b	16	9(L); 7(M)	M:LAa7;LAa8;LAa9;LAa11;LAa12;LAa13;LAa14, L:LAa15;LAa16;LAa17;LAa19;LAa20; LAa21;LAa21;LAa22;LAa23;LAa24
Krib 1	1.66d	2.0c	ND	ND	0	0	
Krib 2	0.00d	3.00c	0.00f	14.76b	1	1(M)	M:LAb12
Azmour	6.66c	3.00c	51.15c	33.90b	2	2(L)	L:LAb21;LAb22
Mraissa	14.00b	42.00a	113.79b	307.62a	10	6(L); 4(M)	M:LAa39;LAa40;LAa41;LAa42, L:LAa43;LAa44;LAa45;LAa46;LAa47;LAa48
Sejnen	21.00a	20.00b	391.25a	385.26a	7	4(L); 3(M)	M:LAa50;LAa51;LAa57, L:LAa53;LAa54;LAa55;LAa56
Tejerouine	0.00d	4.33c	0.00f	112.18b	6	6(M)	M:LAb13;LAb19;LAb17;LAb15;LAb18;LAb14
Madian	0.00d	4.00c	0.00f	117.07b	3	3(M)	M:LAb8;LAb9;LAb33
Zaghouan	0.00d	3.33c	0.00f	9.40b	3	3(M)	M: LAa3;LAa5;LAa6
Benouria	0.00d	1.66c	0.00f	18.68b	2	2(M)	M:LAb29;LAb31
Jrissa	0.00d	1.66c	0.00f	3.51b	2	2(M)	M:LAb30;LAb32
Teboursouk	1.00d	2.33c	2.10f	1.10b	6	3(L); 3(M)	M:LAb34;LAb35;LAb6, L:LAb3;LAb2;LAb7

ND: not determined; Different letters indicate significant differences according to the LSD-test ($p \leq 0.05$).
[a] L: Lumen variety.
[b] M: Mekna variety.

ever, nodulation was rather poor and only a few nodules were observed on this variety grown in soil samples from the other 12 sites, with averages ranging from 1.3 to 4.33 nodules per plant.

Only a few nodules were detected on the imported variety Lumen in the majority of the soil samples (1.0–6.66 nodules per plant), except those from Oued Beja, Mraissa and Sejnen, for which 12, 14 and 21 nodules were recorded per plant, respectively.

The nodules collected from the two varieties were few in number, but were pink inside, indicating the presence of leghaemoglobin and suggesting nitrogen fixation (data not shown). It should be noted though that this efficient nodulation was observed despite the adverse soil conditions of a high lime content ranging from 0 to 31%. Further studies were conducted on a total of 72 strains, all fast-growing, obtained from 14 sampling sites, with 43 originating from Mekna and 29 from Lumen.

Plant nodulation and symbiotic efficiency

The symbiotic efficiency of the 72 isolates was tested on the two *L. albus* varieties and 60 of them induced nodulation on Mekna plants, of which only 25 nodulated the imported variety Lumen. The positive and negative control plants did not develop any nodules, confirming aseptic experimental conditions. Contrasting nodulation scores were recorded for the two varieties: their average root nodule numbers ranged from 1.7 to 16.3 and from 1 to 6.7 nodules per plant for Mekna and Lumen, respectively. The effectiveness of the strains was shown by the pink colour inside the nodules and the dark green colour of leaves compared to the negative controls, as well as the positive effect of inoculation on *L. albus* shoot dry weight and the nitrogen content under controlled conditions, as an indirect measurement of the nitrogen fixation benefit. Table 4 shows the results for the 21 most efficient strains, and all the records are detailed in Supplementary Table S1. Compared to the negative control, all the strains except La11 significantly increased the shoot dry weights (SDW) of Mekna plants. Specifically, the highest SDW values were recorded with strains LAb15, LAa46, LAa38 and LAa42, resulting in significant increases (+11 to 31%) compared to the positive controls. A significant increase in SDW (+2%) was observed on Lumen plants inoculated with strain LAa43 in comparison to the positive control. Based on the nitrogen content in shoots and the negative control plant (T0) comparisons, all the strains could be considered as efficient nitrogen fixing rhizobia, except LAa19, LAa11 and LAb18. Specifically, by considering symbiotic effectiveness, the negative control plants (T0) had 15% and 34% positive control symbiotic effectiveness values for Mekna and Lumen varieties, respectively.

ITS-based phylogeny

Sixty-three strains were successfully amplified and sequenced for their partial 16S-23S ribosomal RNA internal transcribed spacer region (ITS). All the strains were able to renodulate white lupin, except LAa14, LAa16, LAa54, LAb2, LAb6, LAb9, LAb12, LAb29, LAb32, and LAb34. In the phylogenetic reconstruction (Supplementary Fig. S1), and at a sequence similarity cut-off of 95% [53], the new strains clustered into 34 different lineages inside three main clades corresponding to *Agrobacterium* (49%), *Rhizobium* (49%) and *Neorhizobium* (8%). In each clade, the majority of isolates did not align with known reference strains and may represent new lineages.

*Multi-locus sequence analysis (MLSA) (*dnaK, atpD *and* recA*)*

The forty-five strains for which sequences were obtained for the three housekeeping genes *recA*, *atpD* and *dnaK*, were further compared by phylogenetic analyses based on either their individual *recA*, *atpD* and *dnaK* gene sequences, including reference sequences (Supplementary Figs. S2–S4), or on their concatenated sequences (*recA*, *atpD* and *dnaK*) (Fig. 1). For greater detail and resolution of the phylogeny of the new strains, a concatenated phylogenetic tree was constructed based on two gene sequences, *recA* and *atpD*, which allowed the inclusion of more reference sequences (Supplementary Fig. S5). All representations were congruent. White lupin-nodulating rhizobia grouped into three main clades corresponding to *Agrobacterium*, *Rhizobium*, and *Neorhizobium*. Consistent with ITS phylogeny, 53% of the studied strains grouped in the *Agrobacterium* clade, but the majority of them separated from the reference strains at a 95% identity cut-off. Only strains LAb6, LAb7, LAb12, LAb17, LAb29, LAa26 and LAa47 formed

Table 4
Results of nodulation and efficiency plant tests on *L. albus* inoculated with the new most efficient rhizobial strain collection.

Strains	Number of nodules (nodules plant^{-1})		Shoot dry weight (g plant^{-1})		Shoot nitrogen content (mg plant^{-1})		Symbiotic effectiveness (%TN)	
	Mekna	Lumen	Mekna	Lumen	Mekna	Lumen	Mekna	Lumen
Rhizobium								
LAb33	12	0	1.63	ND	42.82	ND	99.48	ND
LAa34	9.33	3	1.71	0.65	44.16	17.71	102.62	89.06
LAa43	7.33	7	1.76	0.73	45.46	22.48	105.66	113.02
LAb15	10.33	0	1.77	ND	47.70	ND	110.87	ND
LAa48	12.33	6	1.72	0.74	45.28	20.35	105.26	102.29
LAa57	9	0	1.65	ND	43.72	ND	101.62	ND
LAb8	9.66	0	1.6	ND	42.60	ND	99.03	ND
Neorhizobium								
LAa30	5	0.66	0.65	0.65	12.22	16.12	28.40	81.03
LAa46	12	1	1.86	0.44	49.90	9.24	115.99	46.44
Agrobacterium								
LAa15	14	2	1.59	0.67	34.66	17.06	80.56	85.78
LAa6	11	0	1.64	ND	37.39	ND	86.91	ND
LAa21	7	5.33	1.66	0.56	35.73	11.87	83.06	59.67
LAa26	8	0	1.61	ND	37.67	ND	87.56	ND
LAa38	14.33	5	1.96	0.66	54.19	16.05	125.96	80.71
LAa42	16.33	0	2.1	ND	59.49	ND	138.27	ND
LAa44	12.66	0.66	1.65	0.39	44.35	6.16	103.08	30.97
LAa56	10.33	3	1.6	0.54	36.48	12.09	84.79	60.80
LAb7	8.33	1	1.75	0.45	48.42	9.05	112.54	45.53
LAb17	14.33	0	1.6	ND	41.80	ND	97.17	ND
LAb21	14	1	1.52	0.44	39.71	8.65	92.31	43.48
LAb31	8.33	0	1.72	ND	45.51	ND	105.78	ND
TN	0	0	1.6	0.72	43.024	19.8936	100.000	100.000
T0	0	0	0.52	0.42	6.4116	6.72	14.902	33.780
LSD at 0.05 level	2.331	2.004	0.2	1.336	4.210	1.882		

T0: uninoculated unfertilized negative control; TN: positive control (fertilization equivalent to 90 units of nitrogen applied per ha); ND: not determined.

a group with 98.9–99.9% internal sequence similarity and shared only 85.1%–86.4% similarities with the most related strain *Agrobacterium radiobacter* LMG140^T. In addition, strains LAa5, LAa11, LAa6 and LAa9 formed two groups and were related, albeit distantly, to *A. rubi* LMG17935^T (79.6–80.7% sequence similarity).

A total of 40% of the studied strains could be affiliated to *Rhizobium*, and the single strain LAb4 may be affiliated to *Rhizobium gallicum* R602^T (95.2% sequence similarity). LAa57 and LAa41 formed two lineages sharing 92.6% similarity and they were distantly related to *Rhizobium etli* CFN42^T (76.5–77.3% sequence similarity) and *Rhizobium mesosinicum* CCBAU25010^T (77.6–78% sequence similarity). Three groups were distantly related to the most related strain *Rhizobium leguminosarum* LMG14904^T. The first group clustered the three strains LAb13, LAa22 and LAb19 sharing 98.2–99.3% similarity and they were distantly related to *R. leguminosarum* LMG14904^T (79.9–80.3% sequence similarity).

A second group was formed by seven strains, LAb33, LAb8, LAb18, LAb15, LAa24, LAa20 and LAb19, which showed 95.6–99.9% similarities with each other and 79.9–80.3% similarities with the reference strain *R. leguminosarum* LMG14904^T. The third group was formed by the two strains LAa37 and LAa23, which shared 95.7% and 78.6–78.9% similarities with reference strain *R. leguminosarum* LMG14904^T, whereas LAb14 stood alone, separated from all the new and reference strains, but was considered to be a *Rhizobium* sp.

Neorhizobium was represented by 7% of the studied strains. The two strains LAa46 and LAb34 shared 97.3% similarity and were distantly related to reference strain *Neorhizobium galegae* LMG6214^T. However, a single strain, LAa30, was separate and showed only 75.5% similarity with the most related reference strain *Neorhizobium huautlense* LMG18254^T.

Symbiotic genes

Several attempts were made to amplify the *nodA* and *nodC* genes in 14 representative strains chosen for their efficiency (3 strains for *Neorhizobium*, 4 strains for *Rhizobium* and 7 strains for *Agrobacterium*) using different primers and different amplification conditions (Supplementary Table S2). Amplification of *nodA* and *nodC* could only be obtained for strain LAb4 with different primer pairs. This strain harboured a *nodC* sequence similar to *R. gallicum* R602^T (Supplementary Fig. S6) and a *nodA* sequence similar to *R. giardinii* H152^T (Supplementary Fig. S7).

Discussion

Lupins have never been cultivated in Tunisia. Introducing *L. albus* as a forage crop and grain legume preceding cereal cultivation in Tunisia would: (1) reduce expensive soybean imports, (2) improve soil fertility, and (3) increase cereal yields. Therefore, in this study, local rhizobial strains adapted to calcareous soils in Tunisia were isolated and characterized that had the capacity to nodulate white lupin, display efficacy in promoting plant growth, and were likely to promote the adaptation of lupins to these stressful field conditions.

Soil samples collected from 56 sites representing various geographical and eco-climatic conditions were used to trap local symbiotic bacteria compatible with *L. albus* for efficient symbiosis. Nodulation did not occur in the majority of the soil samples (41), demonstrating a general lack of, or poor, lupin-nodulating rhizobial populations in Tunisia, which is easily explained by the historic absence of lupin plants in these soils [33]. However, although nodulation was poor it was efficient in 13 soil samples and even profuse on two lupin varieties, Mekna and Lumen, grown in the soil samples from two sites, Sejnen and Mraissa. The presence of rhizobia that were efficient on *L. albus* in these soils, which had adverse high lime contents (7–31%), can be seen as promising for introducing lupin growing in Tunisian calcareous soils.

Seventy-two trapped rhizobial strains were isolated from these *L. albus* root nodules. 16S rRNA gene sequencing has long been used for the molecular characterization of rhizobia, but many studies have shown its limitation in differentiating between closely related rhizobial species [15,20,48]. In recent years, MLSA analyses of housekeeping genes have proved useful for robust phyloge-

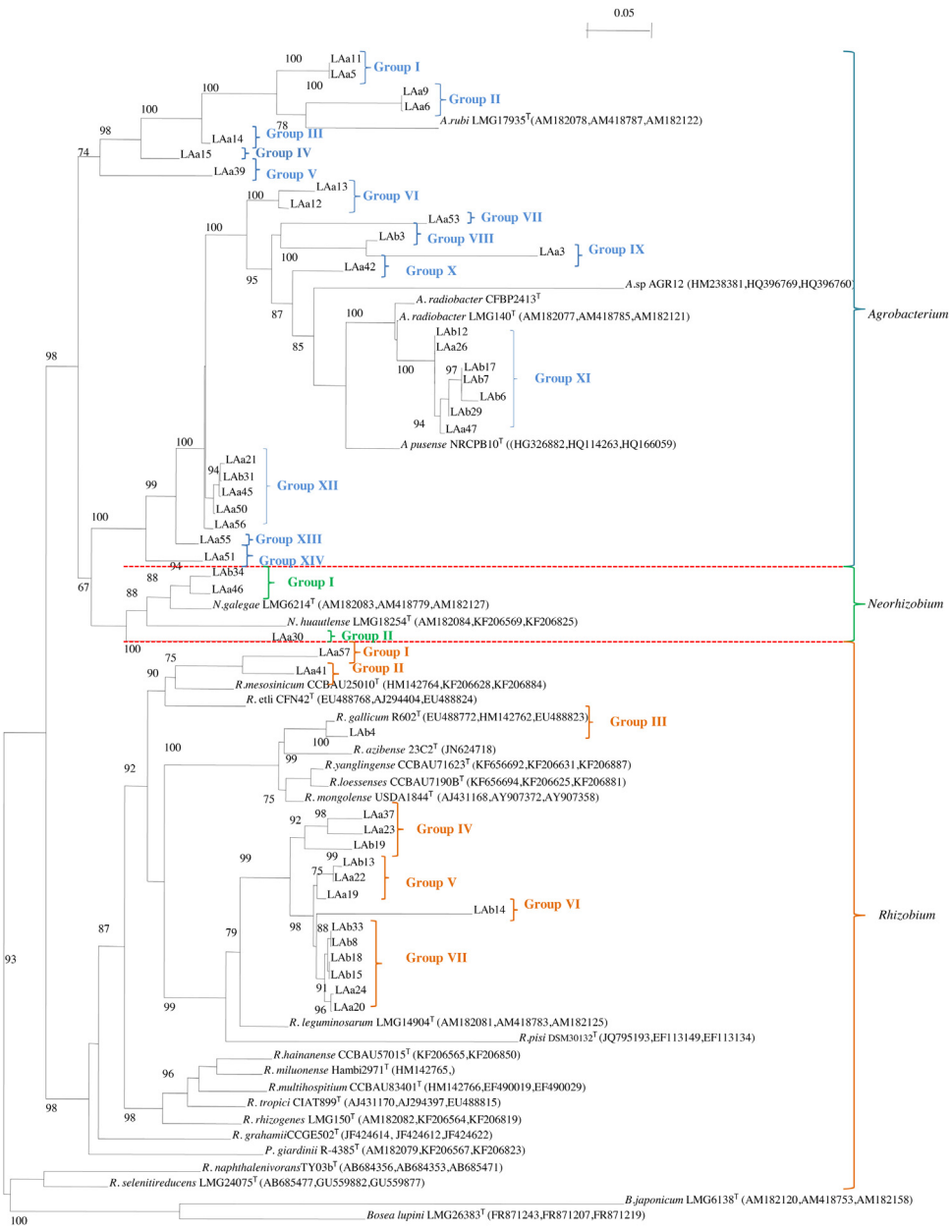

Fig. 1. Concatenated *dnaK*, *atpD* and *recA* gene sequence-based phylogenetic relationships of *Lupinus albus*-nodulating rhizobia and reference sequences. Bootstrap values were calculated for 100 replications, and those greater than 70% are indicated at the internodes. The scale bar represents the number of substitutions per nucleotide position. B = *Bradyrhizobium*, R = *Rhizobium*, M = *Mesorhizobium*, A = *Agrobacterium*, P = *Pararhizobium*, Allo = *Allorhizobium*, E = *Ensifer*, S = *Shinella*. (Orange = groups of *Rhizobium*, Blue = groups of *Agrobacterium*, Green = groups of *Neorhizobium*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

netic relationship studies in rhizobia [2,14,59,31]. In this current study, phylogenetic analyses were thus carried out based on ITS (for intraspecies diversity) and MLSA (for the taxonomic level) in order to characterize new rhizobial isolates from *L. albus* nodules. They were affiliated to *Rhizobium*, *Neorhizobium* and *Agrobacterium* but, surprisingly, none were affiliated to *Bradyrhizobium*, which is generally reported as the main efficient symbiont of *Lupinus* species. The absence (or low level) of *Bradyrhizobium* populations in Tunisia has been documented, especially for nodule isolates from species classically nodulated by this genus, such as *Retama raetam*, *Acacia tortilis*, *Astragalus corrugatus*, *Hippocrepis areolata* and lotus species, which are all affiliated to *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* [29,30,46,47], suggesting that soils in Tunisia are dominated by fast-growing rhizobia. This also occurs in Morocco, since six *Lupinus* species were reported to be associated with fast-growing strains, some of which were affiliated to *Mesorhizobium* and *Rhizobium*, and were able to infect the cultivated species *L. luteus* and *L. albus* [10]. The high lime content, ranging from 7% to 31%, in 90% of the soils sampled in the current study may help to explain the pre-eminence of fast-growing rhizobia known for their acid-producing capacity, possibly developed as their leguminous hosts became adapted to more alkaline soils [41], such as calcareous soils.

However, the results contrasted with those recently reported by Msaddak et al. [38,39] indicating that rhizobia isolated directly from nodules of spontaneous *L. micranthus* and *L. luteus* plants collected at 3–5 different sites in northern Tunisia were slow- or fast-growers depending on soil pH, since *Bradyrhizobium* were found in neutral to mildly alkaline soils (pH 6–8) and *Microvirga* were found in alkaline soils (pH 7.5–9). Only two strains affiliated to *Phyllobacterium* were detected in mildly alkaline soil, and they were associated with *L.*

micranthus. Most of the sample sites differed from those of Msaddak et al. [38,39], except Sejnen and Mraissa, although they had different GPS positions. It may seem unusual that, in this study, no *Bradyrhizobium* strains were found at these two sites, or at Krib2 and Azmour, where soils had acid pH and low lime contents. The different isolation methods used in the two studies cannot explain the discrepancies [32]. Strong genetic divergences may occur in populations at sites from a few kilometers to hundreds of kilometers apart [57]. Given that the plant is a stronger modulator of microbial richness than the soil type [6], that some rhizobial species are undetectable in the absence of their leguminous plant host [22], and that the root nodule forms a safe and favourable haven for soil rhizobia to multiply [27], it can be argued that non-cultivation of *L. albus* in the sampled soils mostly shaped their rhizobial populations. In addition, the huge stress levels in the soil throughout the dry season (our soil samples were collected in autumn after a very hot summer) may have contributed to the development of viable but, if present, undetectable bradyrhizobia [35]. Lastly, the hypothesis that slow-growing bradyrhizobial colonies may have been disregarded during the isolation process, to the benefit of fast-growing ones, can be ruled out, since: (1) the Petri dishes were inoculated with serial dilutions of the crushed nodule suspensions, in order not to miss any minor colony types, (2) dishes were kept and regularly examined for more than three weeks incubation, and (3) dishes were never invaded by convergent colonies. In the isolation dishes, the abundance of *Agrobacterium* sp. colonies ranged from 30% to 50% of the total.

On the other hand, and unexpectedly, *Agrobacterium* sp. strains were observed as being dominant and they accounted for over 50% of the total nodule isolates. Most of them were related to *Agrobacterium rubi*, *Agrobacterium deltaense*, *Agrobacterium salinitolerans*, *Agrobacterium radiobacter* and *Agrobacterium nepotum*, and other *Agrobacterium* sp. isolates that were separated from any described taxa. The members of *Agrobacterium* are ubiquitous in soil and plant habitats [66], with different saprophytic, phytopathogenic or symbiotic forms. There are several reports on the isolation of non-pathogenic opportunistic *Agrobacterium* strains from surface-disinfected nodules, and their coexistence with rhizobial strains inside root nodules in several legumes around the world [2,28,29,62], but that lack the capacity to induce nodules on their hosts. Experimental nodulation with double inoculation (*Agrobacterium-Rhizobium*) may result in contrasting effects: no effect on nodulation and effectiveness [34]; an antagonistic effect [37]; enhanced competitivity of rhizobial strains; nodulation and growth promotion and enhanced seed quality [5].

Similar predominance of *Agrobacterium* strains in root nodules was observed on soybean in Egypt [65] and in northwestern China [67], as well as on faba bean in Egypt [66], at 55%, 30% and 57%, respectively. In this study, the nodules from which *Agrobacterium* spp. were isolated exhibited a normal nodule appearance, with no galls or proteoid roots, as mentioned for *L. albus* by Péret et al. [42], and no pathogenic effect was observed. Nodulation tests on their original host, *L. albus*, confirmed that these *Agrobacterium* sp. strains were not only non-pathogenic but, surprisingly, induced nodules of normal appearance according to Koch's postulate, suggesting their symbiotic characteristic. Such nodules were selected randomly for rhizobial strain isolation and sequencing to confirm their identity with the inoculated agrobacterial strains (data not shown). Different attempts with various primer sets failed to amplify *nodA* and *nodC* gene sequences and only unspecific amplificates were obtained (data not shown). Following Estrella et al. [12], it could be argued that consistent sequence divergence in the symbiotic genes of these isolates would explain their nodulation capacity. Positive effects on *L. albus* shoot dry weight and nitrogen content with the Mekna and Lumen varieties under controlled conditions indicated that plants benefited from symbiosis with these bacteria. The literature reports nodulating non-phytopathogenic *A. radiobacter* strains on several plants, such as *Phaseolus vulgaris* in Ethiopia [2], as well as *Glycine soja* [67] and *Sesbania cannabina* [7,28,63] in China. The nodulation gene (*nodA)* and/or nitrogen fixation gene *(nifH)* have been found in some of them [64,65,67].

In the current study, the new strains were not equally efficient and their efficiency varied with their genus affiliation and the plant variety. A total of 83% of the new strains increased plant growth of the local lupin variety (Mekna), while only 35% induced efficient nodules with the imported variety (Lumen). *Neorhizobium* sp. strains were more efficient on the local variety, Mekna, with symbiotic effectiveness (SE) ranging from 27% to 121%, followed by *Agrobacterium* sp. strains with SE varying from 11% to 147%, and 13% to 115% for *Rhizobium* sp. strains. Conversely, *Rhizobium* sp. strains were more efficient on the imported variety, Lumen, with SE varying from 35% to 119% versus 28% to 96% for *Agrobacterium* and 42%–87% for *Neorhizobium* sp. strains, which were equally efficient (Supplementary Table S3). To our knowledge, there has been no previous scientific report describing such symbiotic specificity at the variety level in white lupin, although cultivar specificity has been observed in other legumes. This plant variety-dependent efficacy of local rhizobia was also reported for cowpea [26]. It is also documented that domesticated crop species tend to have fewer compatible symbionts (higher specificity) than their wild counterparts [40] and that symbiotic efficiency varies according to the rhizobium × cultivar interaction match [21]. For instance, *P. sativum* cv. *afghanistanis* is only nodulated by specific strains of *Rhizobium leguminosarum* sv. viciae, occurring in soils in their native range in Afghanistan/Turkey [40]. In this case, nodulation is conferred by the plant *sym*2 gene and the specific *nodX* gene present in some *R. leguminosarum* sv. *viciae* strains [8]. Other examples of cultivar specificity are *Medicago rigiduloides* × *Ensifer meliloti* sv. *rigiduloides* and *Trifolium ambiguum* × *R. leguminosarum* sv. *trifolii* in their native region in the Mediterranean Basin and eastern Europe, respectively [18,36]. However, to date, the mechanisms underlying this high cultivar specificity have not been understood.

Some of the new strains, such as *Rhizobium* sp. LAa34, LAb15, LAb8, LAb33 and *Agrobacterium* sp. LAa38, LAa6, LAb17, LAb7, LAb31 originating from alkaline soils with a high lime content (12–31%), and displayed significant increases in shoot dry weight and nitrogen content of *L. albus* plants similar to the positive control (chemical nitrogen fertilization). This indicated their efficacy and designated them as candidates for further testing with a view to developing efficient field inoculants adapted to alkaline soil conditions and calcareous soils, in order to facilitate the introduction of white lupin cultivation in Tunisia.

Conclusion

This study provided information on the genetic diversity and phylogeny of local rhizobia present in Tunisian calcareous soils likely to nodulate *L. albus*. All the new strains were fast-growing rhizobia affiliated to *Rhizobium*, *Neorhizobium*, and *Agrobacterium*, with a majority of *Agrobacterium* sp. members. Some strains represented new lineages requiring further characterization in order to clarify their taxonomic positions. Symbiotic efficiency tests revealed the potential of some of these indigenous adapted rhizobial isolates as useful, efficient inoculants for promoting the introduction of lupin as a crop plant in order to make use of calcareous soils in Tunisia.

Acknowledgement

We are grateful to Peter Biggins for English editing of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.04.002.

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