Changes in the bacterial communities structure of the rhizosphere of four wild *Lupinus* species from flowering to fruiting

J.A. Lucas García, A. Probanza, B. Ramos,
M.L. Barrientos, F.J. Gutiérrez Mañero
Universidad San Pablo CEU. Departamento de Biología
P.O. Box 67. 28668 Boadilla del Monte. Madrid (Spain)
Fax 913 510 496
lgutma@ceu.es

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Abstract

Microbial rhizosphere communities of four different *Lupinus* species were studied at two plant growth stages in order to determine the dominance of r/K strategists and the pattern of taxa and diversity (Shannon index). Five hundred and seventy-six strains were isolated, determined and classified into eleven different bacterial genera and the Enterobacteriaceae family. *Aureobacterium* dominated on *L. hispanicus* and *L. angustifolius* at both sampling times (GS1: Growth Stage 1 (flowering); GS2: Growth Stage 2 (fruiting)), whereas it only dominated in the rhizosphere of *L. albus* at GS1 (58.45%). *Cellulomonas* dominated at GS2 (42.93%). In *L. luteus, Bacillus* dominated at GS1 (41.21%) and *Aureobacterium* at GS2 (39.74%). Principal Component Analysis (PCAs) showed that the microbial composition of the rhizosphere of *L. albus* is the only one that changed considerably from GS1 to GS2. These changes were due to a proportional increase of r strategists at GS2. Sample size (18 to 54 colonies, depending on lupin species) was adequate according to the Shannon index. Despite the fact that diversity between GS1 and GS2 hardly varied in *L. albus*, *L. luteus,* and *L. hispanicus*, the PCAs revealed the largest differences in the microbial structure associated with *L. albus*. A tentative successional model for the lupin microbial population of the rhizosphere related to its biological cycle, is proposed.

Key words*:* bacterial communities, biological diversity, *Lupinus*, rhizosphere, r/K strategies.

Resumen. *Cambios en la comunidad microbiana rizosférica de cuatro especies salvajes de* Lupinus *desde la floración hasta la fructificación*

Se estudia la comunidad microbiana rizosférica de cuatro especies de *Lupinus* en dos momentos de su ciclo biológico, con el objeto de determinar la dominancia de r y K estrategas, el patrón de taxones y la diversidad biológica (índice de Shannon). Se aislaron 576 cepas, y se encontraron representantes de once géneros bacterianos y de la familia Enterobacteriaceae. *Aureobacterium* fue mayoritario en *L. hispanicus* y *L. angustifolius* en ambos momentos de muestreo (GS1: floración; GS2: fructificación), mientras que sólo dominó en *L. albus* en GS1 (58.45%). *Cellulomonas* dominó en GS2 (42.93%). En *L. luteus* fue *Bacillus* el mayoritario en GS1 (41.21%) y *Aureobacterium* en GS2 (39.73%).

Los Análisis de Componentes Principales (ACPs) indican que sólo *L. albus* tiene cambios considerables en sus poblaciones microbianas rizosféricas de GS1 a GS2. Estos cambios se deben al incremento de estrategas de la r en GS2. El tamaño de la muestra (de 18 a 54 colonias, dependiendo de la especie de lupino) fue la adecuada, según indica el índice de Shannon. A pesar de que la diversidad apenas varía de GS1 a GS2 en *L. albus, L. luteus* y *L. hispanicus*, los ACPs reflejan cambios importantes en la estructura microbiana rizosférica en *L. albus*. Se propone un modelo sucesional tentativo de las poblaciones microbianas de la rizosfera del lupino, relacionándolo con su ciclo biológico.

Palabras clave: comunidades bacterianas, diversidad biológica, estrategias de crecimiento r/K, *Lupinus*, rizosfera.

Introduction

Interactions plant-soil-microorganism have been known for a long time, however, little research has been done to understand microbial diversity, soil functioning, and ecosystem sustainability (Kennedy & Smith, 1995). The knowledge of microbial community structure, successional phenomena and growth strategies within the rhizosphere are essential for understanding of the Ecological Theory in this environment. This lack of knowledge of Ecological Theory (Margalef, 1968, 1973) is probably due to inconsistencies between evolutionary ecology (Kauffman, 1993, 1995), ecosystem ecology (Schulze & Mooney, 1993), and information theory (Rasmussen, 1988; Yockey, 1990, 1995). Previous cited inconsistences to be in other natural systems. The concept of r/K strategy (Luckinbill, 1978) is derived from evolutionary ecology and posits that there are genetic differences between organisms in their ability to exploit and survive in different kinds of environments (Luckinbill, 1978; Pianka, 1970).

Knowledge of these interactions is also essential when considering the use of biological agents to improve crop yields. First, because the successful inoculation of biological agents depends on the bacterial ability of microorganisms to colonise the plant root (Kloepper et al., 1980; Michiels et al., 1989), and secondly, because the incidence and consequences of introducing foreign microorganisms in the rhizosphere must be known.

Among the factors that determine the structure of microbial communities in the rhizosphere, the plant plays a major role (Marilley et al., 1998) by releasing organic compounds to soil, a process called rhizodeposition or exudation (Whipps & Lynch, 1985). The effect of specific compounds released by the plant root on the selection of the microbiota of the rhizosphere was first demonstrated by Gunner et al. (1966). The exudation pattern is affected by genetic factors of the plant (Bolton et al., 1993, Hedges & Messens, 1990), plant age, physiological status of the plant and environmental factors (Grayston et al., 1996). Chanway & Nelson (1991) suggested that rhizobacteria play a significant role in rhizodeposition, assuming that bacteria may modify the plant physiology. This hypothesis is supported by Wiehe & Höflicht (1995) and others, who demonstrated that certain bacteria had different colonization patterns depending on the plant species. In view

of this, rhizobacterial composition must be defined by an adaptive process based on the natural coexistence (Sumner, 1990) and metabolic compatibility (Chanway et al., 1989) between the plant and the bacteria. As proposed by Lynch (1990), soil also influences rhizobacterial composition to some extent.

Humans have used the legume *Lupinus* for nutritional purposes because of the high protein content (36-52%) in seeds (Petterson & Mackintosh, 1994). *Lupinus* tolerates low temperatures, drought and poor soils, three qualities that make this crop quite suitable for marginal soils, where other crops cannot be cultivated (Mohamed & Rayas-Duarte, 1995).

The aim of this study is to determine the influence of plant growth stage (flowering and fruiting) on i) pattern of bacterial taxa, ii) degree of diversity, and iii) community structure and natural succession of rhizosphere bacterial communities in the populations of four wild *Lupinus* species (*Lupinus albus* L.*, Lupinus luteus* L.*, Lupinus hispanicus* Boiss & Reuter, and *Lupinus angustifolius* L.). The two growth stages have been determined according to traditional agricultural cropping practices for *Lupinus*.

Materials and Methods

Field sites

The four populations of *Lupinus* were located in different areas within the Comunidad de Madrid (Spain). Table 1 shows the geographical location, vegetation, type of soil, annual mean rainfall, and annual mean temperature. *L. luteus* grew in a soil with pH 6.3, 0.698% nitrogen, and 0.474 mg g^{-1} carbon and *L. albus* in a soil with pH 6.02, 1.077% nitrogen, and 0.741 mg g^{-1} carbon. Although these two populations probably originated from nearby cropping areas, they have been established in the area for at least four years.

L. hispanicus was sampled in the Sierra de Ayllón, in a soil with a pH, carbon, and nitrogen content of 5.71, 0.416 mg g^{-1}, and 1.306%, respectively. *L. angustifolius* was sampled in the Valle del Lozoya, a soil with a pH, carbon, and nitrogen content of 6.03, 0.633 mg g^{-1}, and 1.378%, respectively. These two species are indigenous to the Madrid area.

Total nitrogen was determined colorimetrically, following the Smith method (1980), after a Kjeldhal digestion of 2 g of soil in a Prolabo Maxidigest-Mx350 microwave digestor. Organic C was determined as in Walkley & Black (1934).

Root Sampling

Three populations of each *Lupinus* species (50 x 50 m), separated by 500 to 1000 m, were marked on a map (1:10) and each population was divided into 1 x 1 m plots, numbered on the map. Ten plots were selected with a random number computer programme, sampling one plant from each of them. Roots from these 10 plants for each one population constituted a replicate (therefore, there were three replicates for each lupin species).

Samples were transported to the laboratory in sterile plastic bags at 4ºC. Roots from each replicate were cut into 2 cm pieces, mixed and 10 g were put into Erlenmeyer flasks filled with 100 ml of sterile distilled water. Roots were shaken at 2000 rpm with 4 mm-diameter glass beads for 10 minutes. Then, 10-fold dilutions were prepared in sterile distilled water and 1 mL of each was plated on a general solid media (23.5 g standard methods agar (Pronadisa), 10 mL soil extract, 50 mL Winogradsky saline solution, 1 mL oligoelement solution, 1000 mL distilled water) (Pochon & Tardieux, 1962) and incubated at 28ºC for 72 hours; cfus were isolated after 36 and 72h. Two sampling moments were determined at two Growth Stages (GS) of the plants, one at the beginning of flowering (GS**1**) and the other when fruiting was completed (GS**2**).

Identification of isolates

12 isolates were randomly taken after 36 h of incubation at 28ºC from plates with approximately 100 cfus and another 12 in the following 36 h, that is, after 72 hours. Therefore, 24 colonies were isolated for each replicate (three populations of each *Lupinus* species), giving a total of 72 colonies for each lupin species (4). Two different growing stages were sampled, giving a total of 576 isolates.

All isolates, except those that belonged to the Enterobacteriaceae family, were identified untill genus level according to the Acero et al. (1994) procedure modified by us. This methodological modification were done in order to a better identification of the Coryneforme genera (figure 1). Results are presented as frequencies (percentage) of total isolates.

Diversity Analysis

The Shannon index (Shannon & Weaver, 1949; $H(e) = -\Sigma (p_i \log_2 p_i)$, where p_i is the proportion of isolates belonging to each taxa) was applied to assess rhizobacterial diversity in each *Lupinus* species and growth stage (Bianchi & Bianchi, 1982).

Statistical Analysis

Three two-way ANOVAs with three replicates each were made (Sokal & Rohlf, 1969). The variation factors being the four *Lupinus* species and the frequencies of the isolated bacterial taxa in the two plant growth stages. ANOVA a was done with data after 72 h of incubation, ANOVA b with data after 36h of incubation, and ANOVA c with data from 36 to 72h of incubation.

Three Principal Component Analysis (PCAs) were undertaken to determine main trends in microbiological data, previously log(%+1) transformed (Hartmann, 1967). PCAs nomenclature were the same as the ANOVAs and both were carried out with the same data.

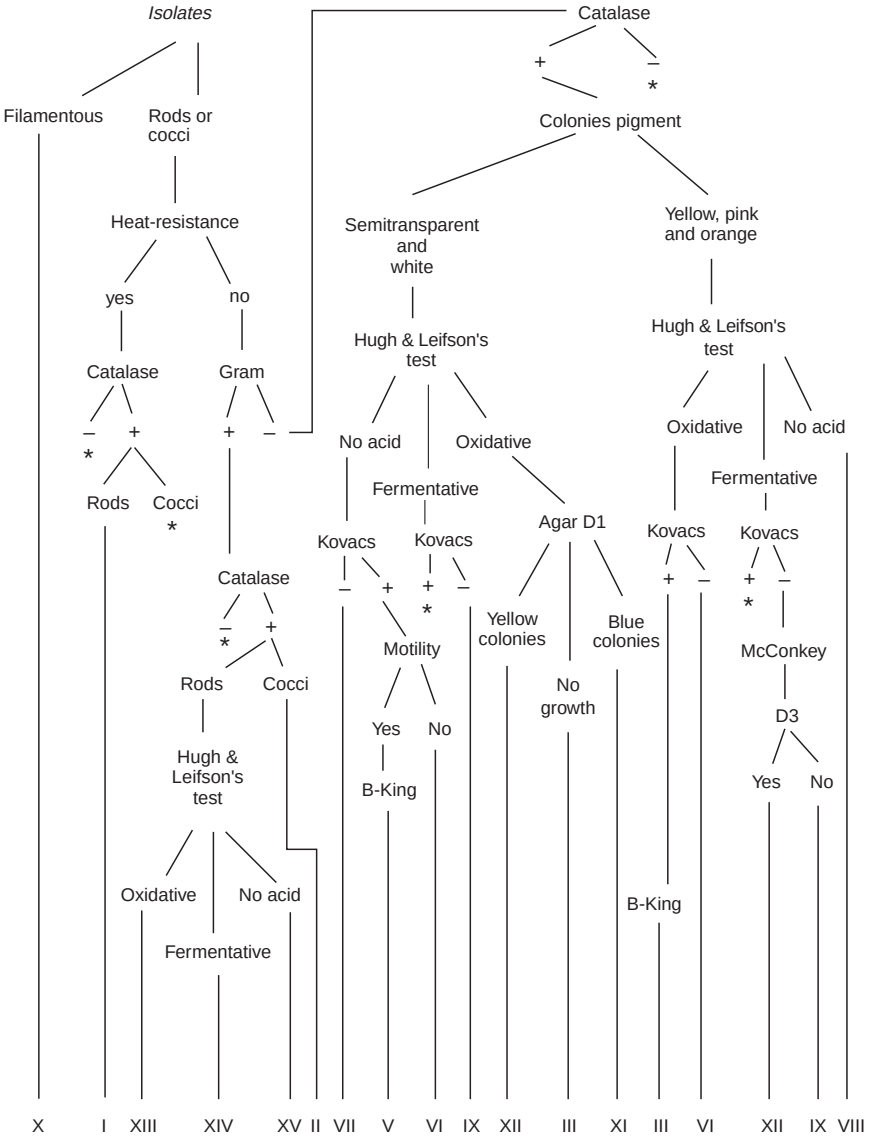


Figure 1. Modified Acero et al., (1994) protocol: I; *Bacillus*, II; *Micrococcus*, III; *Pseudomonas*, IV; *Pseudomonas-Alcaligenes*, V; *Moraxella*, VI; *Xanthomonas*, VII; *Acinetobacter*, VIII; *Flavobacterium*, IX; Enterobacteriaceae, X; *Streptomyces*, XI; *Agrobacterium*, XII; *Erwinia*, XIII; *Aureobacterium*, XIV; *Cellulomonas*, XV; *Arthrobacter*, asterisk; not found.

Table 1. Geographic coordinates, vegetation characteristics and type of soil, annual mean rainfall and annual mean temperature under each *Lupinus* species.

	Geographic coordinates	Vegetation and type of soil	Annual mean rainfall	Annual mean temperature
Lupinus albus	40º24´N; 3º50´W	Junipero-oxycedri Quercetum rotundifoliae. Anfisol soil	464-508 mm	14-14.25 ºC
Lupinus luteus	40º24´N; 3º50´W	Junipero-oxycedri Quercetum rotundifoliae. Anfisol soil	464-508 mm	14-14.25 ºC
Lupinus hispanicus	41º3´30´´N; 3º31´30´´W	Luzulo forsteri Quercetum pyrenaicae. Brown forest soil on igneous rocks	650-700 mm	10-10.3 ºC
Lupinus angustifolius	40º58´N; 3º43´W	Junipero-oxycedri Quercetum rotundifoliae. Brown forest soil on metamorphic rocks	487-608 mm	10.8-11.4 ºC

Table 2. Frequency of bacterial taxa isolated from the rhizosphere of *Lupinus* at the beginning of flowering (GS1) and fruiting (GS2) isolated at different times of incubation: 1a) after 72h, 1b) after 36h and 1c) from 36 to 72h. LA: *Lupinus albus*; LL:*L. luteus*; LH: *L. hispanicus*; LAN: L. angustifolius.

a) 0 ⇒ 72h	LAGS1	LAGS2	LLGS1	LLGS2	LHGS1	LHGS2	LANGS1	LANGS2	Total Mean
Bacillus	19.84	2.77	41.21	34.75	4.33	3.00	5.26	7.12	**14.78**
Aureobacterium	58.45	15.27	33.77	39.74	40.47	35.95	56.66	71.61	**43.99**
Cellulomonas	3.50	42.93	0	4.34	24.60	20.47	3.50	6.20	**13.19**
Pseudomonas	10.75	1.38	11.84	11.86	21.70	22.17	12.01	4.42	**12.01**
Arthrobacter	4.54	33.51	3.4	7.78	0	2.90	8.46	10.62	**8.90**
Micrococcus	0	0	6.73	0	0	0	0	0	**0.84**
Streptomyces	0	0	1.66	0	0	0	1.73	0	**0.42**
Erwinia	0	0	0	0	1.43	4.44	3.50	0	**1.17**
Acinetobacter	3.03	2.77	0	0	0	1.43	3.46	0	**1.33**
Flavobacterium	0	0	0	0	0	0	3.50	0	**0.43**
Enterobacteriaceae	1.51	1.56	0	1.43	2.86	0	1.73	0	**1.13**
Xanthomonas	0	0	0	0	1.43	5.66	0	0	**0.88**

b) 0 ⇒ 36h	LAGS1	LAGS2	LLGS1	LLGS2	LHGS1	LHGS2	LANGS1	LANGS2	Total Mean
Bacillus	37.11	0	58.33	49.99	11.44	6.11	12.50	13.88	**23.67**
Aureobacterium	47.13	16.66	22.22	25.15	19.69	26.11	61.43	36.11	**31.43**
Cellulomonas	7.40	47.22	0	6.06	32.31	38.33	6.66	11.61	**18.69**
Pseudomonas	0	2.77	11.10	15.75	16.66	14.99	3.03	2.77	**8.38**
Arthrobacter	2.77	27.77	0	0	0	2.77	6.36	0	**4.95**
Micrococcus	0	0	8.33	0	0	0	0	0	**1.04**
Streptomyces	0	0	0	0	0	0	0	0	**0**
Erwinia	0	0	0	0	2.77	6.11	3.33	0	**1.52**
Acinetobacter	2.77	2.77	0	0	0	0	0	0	**0.69**
Flavobacterium	0	0	0	0	0	0	3.33	0	**0.41**
Enterobacteriaceae	2.77	0	0	3.03	5.55	0	3.33	0	**1.83**
Xanthomonas	0	0	0	0	0	5.55	0	0	**0.69**

c) 36h ⇒ 72h	LAGS1	LAGS2	LLGS1	LLGS2	LHGS1	LHGS2	LANGS1	LANGS2	Total Mean
Bacillus	3.33	2.77	8.33	15.58	2.77	5.80	0	0	**4.82**
Aureobacterium	68.18	13.88	52.38	58.40	53.02	48.73	51.51	71.62	**52.21**
Cellulomonas	0	38.38	0	2.77	17.17	3.03	0	0	**7.66**
Pseudomonas	18.48	0	21.42	8.33	23.98	28.02	20.87	9.09	**16.27**
Arthrobacter	6.66	39.14	8.94	14.89	0	3.03	10.43	22.30	**13.17**
Micrococcus	0	0	4.76	0	0	0	0	0	**0.59**
Streptomyces	0	0	4.16	0	0	0	3.03	0	**0.89**
Erwinia	0	0	0	0	0	2.77	3.70	0	**0.81**
Acinetobacter	3.33	2.77	0	0	0	3.03	6.73	0	**1.98**
Flavobacterium	0	0	0	0	0	0	3.70	0	**0.46**
Enterobacteriaceae	0	3.03	0	0	0	0	0	0	**0.38**
Xanthomonas	0	0	0	0	3.03	5.55	0	0	**1.07**

ANOVA a: Differ. between genera D.F.=46; LSD (0.05)=5.432; LSD (0.01)=7.420, differ. Interaction between genera and sampling moments D.F.=4; LSD (0.05)=21.101; LSD (0.01)= 34.973
ANOVA b: Differ. between genera D.F.=46; LSD (0.05)=7.427; LSD (0.01)=10.147, differ. Interaction between genera and sampling moments D.F.=4; LSD (0.05)=28.856; LSD (0.01)= 47.827
ANOVA c: Differ. between genera D.F.=46; LSD (0.05)=5.97; LSD (0.01)=7.97, differ. Interaction between genera and sampling moments D.F.=4; LSD (0.05)=23.196; LSD (0.01)= 38.445

Results

Identification and frequency of isolates.

The 576 isolates belonged to 11 different bacterial genera and to the Enterobacteriaceae family. Table 2 shows frequencies of each bacterial genera for each *Lupinus* species at the two growth stages determined (table 2a shows data of colonies grown from 0 to 72h of incubation; table 2b: data of colonies grown from 0 to 36h; table 2c: data from colonies that grew between 36 and 72h) .

Considering total averages after 72 h (table 2a), *Aureobacterium* was the dominant genus (43.99%) followed by *Bacillus* (14.78%), and the least abundant were *Streptomyces* (0.42%) and *Flavobacterium* (0.43%). *Aureobacterium* showed the highest frequency (71.61%) when associated with *L. angustifolius* at GS2 and the lowest (15.27%) associated with *L. albus* at GS2. The corresponding ANOVA (ANOVA **a**, table 2a) shows significant differences between genera ($p<0.01$) and between genera and growth stages ($p<0.01$). The dominant genus was different depending on the plant species and growth stages. *Aureobacterium* dominated in *L. hispanicus* and *L. angustifolius* in both growth stages but it only dominated in *L. albus* in GS1 (58.45%), while *Cellulomonas* did in GS2 (42.93%). In *L. luteus, Bacillus,* and *Aureobacterium* dominated in GS1.

Data from isolates that grew from 0 to 36h (table 2b) and that grew between 36 and 72h (table 2c) of incubation are treated separately in ANOVAs **b** and **c**, respectively. These ANOVAs show significant differences between genera and between genera and growth stages ($p<0.01$ in all cases).

Our results after 36h of incubation (table 2b) showed that *Aureobacterium* was the dominant genus (31.43%), followed by *Bacillus* and *Cellulomonas* (23.67% and 18.69%, respectively). Considering the dominant genera under each *Lupinus* species, *Aureobacterium* dominated only under *L. angustifolius* at GS1 and GS2, and under *L. albus* at GS. From 36 to 72h (table 2c), the frequency of *Aureobacterium* increased from 31.43% to 52.21%, while *Bacillus* and *Cellulomonas* frequencies decreased significantly (4.82 and 7.66%, respectively). In this case, *Aureobacterium* dominated in all the *Lupinus* species at both sampling moments, except for *L. albus* at GS2.

Patterns of Taxa from Plant Species

To establish differences between the *Lupinus* species at the two growth stages (GS) sampled, three PCAs were performed, based on the frequencies of bacterial genera. The loading factors of the variables (bacterial genera) determine the position of samples on to the corresponding diagram, defined by the two axes that account for the greatest variance percentages. PCA a was undertaken with data from 0 to 72h, PCA b with data from 0 to 36h, and PCA c with data from 36 to 72 h of incubation. Table 3 shows variance percentages for each PCA.

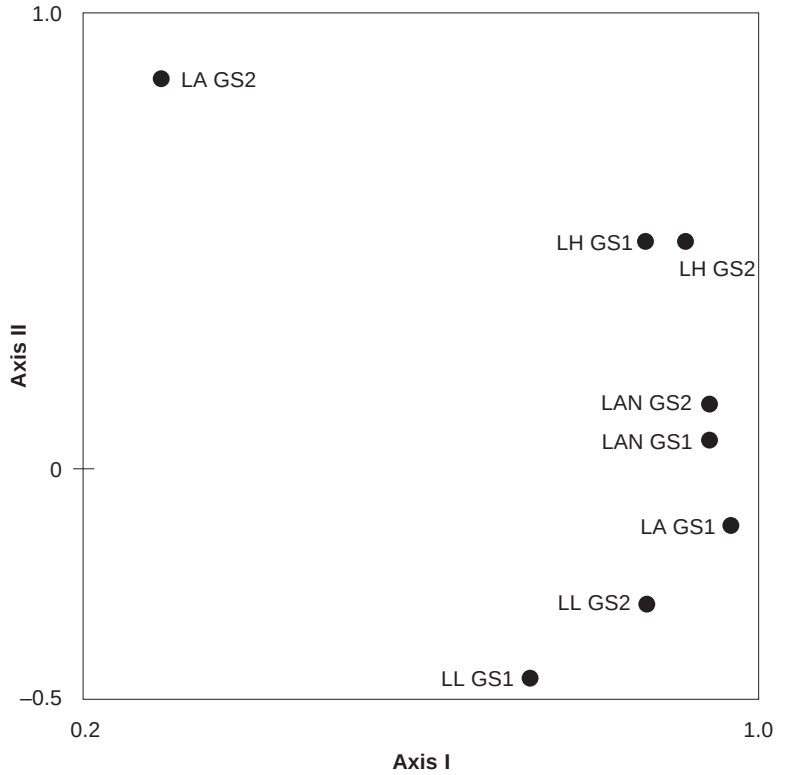


Figure. 2. PCA with bacterial genera isolated after 72h of incubation at two growth stages (GS1 and GS2) in the four species of *Lupinus: L.albus*, LA, *L.luteus*, LL, *L.hispanicus*, LH, and *L.angustifolius* LAN.

PCA a (figure 2) shows data from 0 to 72h. The first two axes account for 88.23% of the variation (table 3). The low frequencies of *Bacillus* (2.77%) and *Pseudomonas* (1.38%), and the high frequency of *Cellulomonas* (42.93%) separate *L. albus* GS2 from the other samples. *L. luteus* at GS1 and at GS2 are separated from the others because of the high percentage of *Bacillus* (41.21 and 34.75%,) and the absence of *Cellulomonas* in GS1. In PCA b (figure 3), all variables except for *Aureobacterium*, *Arthrobacter,* and the Enterobacteriaceae family have high load factors on axis I, accounting for 59.83% of the variation (table 3). *L. albus* at GS2 appears separated from all others due to the high *Cellulomonas* frequency. Once more this PCA evidences the differences between the two GS of *L. albus* because of the increase of *Cellulomonas* and the decrease of *Aureobacterium* at GS2.

Table 3. Loading factors and percentage of variance absorbed for each PCA.

	PCA I			PCA II			PCA III		
AXIS I	**% of Variance**	**Loading**	**Loading value**	**% of Variance**	**Loading**	**Loading value**	**% of Variance**	**Loading**	**Loading value**
	72.94	*Bacillus*	0.02	59.83	*Bacillus*	0.52	82.77	*Bacillus*	0.009
		Aureobacterium	–0.81		*Aureobacterium*	0.25		*Aureobacterium*	0.91
		Cellulomonas	0.21		*Cellulomonas*	–0.73		*Cellulomonas*	–0.91
		Pseudomonas	–0.40		*Pseudomonas*	–0.62		*Pseudomonas*	0.81
		Arthrobacter	0.36		*Arthrobacter*	0.11		*Arthrobacter*	–0.88
		Micrococcus	0.77		*Micrococcus*	0.56		*Micrococcus*	0.24
		Streptomyces	0.74		*Erwinia*	–0.73		*Streptomyces*	0.51
		Erwinia	0.44		*Acinetobacter*	0.77		*Erwinia*	0.67
		Acinetobacter	–0.54		*Flavobacterium*	0.71		*Acinetobacter*	0.24
		Flavobacterium	–0.040		*Enterobacteriac.*	0.36		*Flavobacterium*	0.47
		Enterobacteriac.	0.93		*Xanthomonas*	–0.62		*Enterobacteriac.*	–0.77
		Xanthomonas	0.89					*Xanthomonas*	0.35
AXIS II	**% of Variance**	**Loading**	**Loading value**	**% of Variance**	**Loading**	**Loading value**	**% of Variance**	**Loading**	**Loading value**
	15.29	*Bacillus*	–0.69	26.91	*Bacillus*	0.78	12.80	*Bacillus*	0.73
		Aureobacterium	0.23		*Aureobacterium*	–0.05		*Aureobacterium*	0.18
		Cellulomonas	0.69		*Cellulomonas*	–0.55		*Cellulomonas*	–0.09
		Pseudomonas	–0.04		*Pseudomonas*	0.65		*Pseudomonas*	0.14
		Arthrobacter	–0.28		*Arthrobacter*	–0.84		*Arthrobacter*	–0.25
		Micrococcus	0.38		*Micrococcus*	0.42		*Micrococcus*	0.59
		Streptomyces	0.09		*Erwinia*	0.07		*Streptomyces*	0.07
		Erwinia	–0.49		*Acinetobacter*	–0.48		*Erwinia*	–0.67
		Acinetobacter	0.69		*Flavobacterium*	–0.08		*Acinetobacter*	–0.91
		Flavobacterium	0.94		*Enterobacteriac.*	0.08		*Flavobacterium*	–0.71
		Enterobacteriac.	0.06		*Xanthomonas*	0.14		*Enterobacteriac.*	–0.22
		Xanthomonas	0.40					*Xanthomonas*	0.14

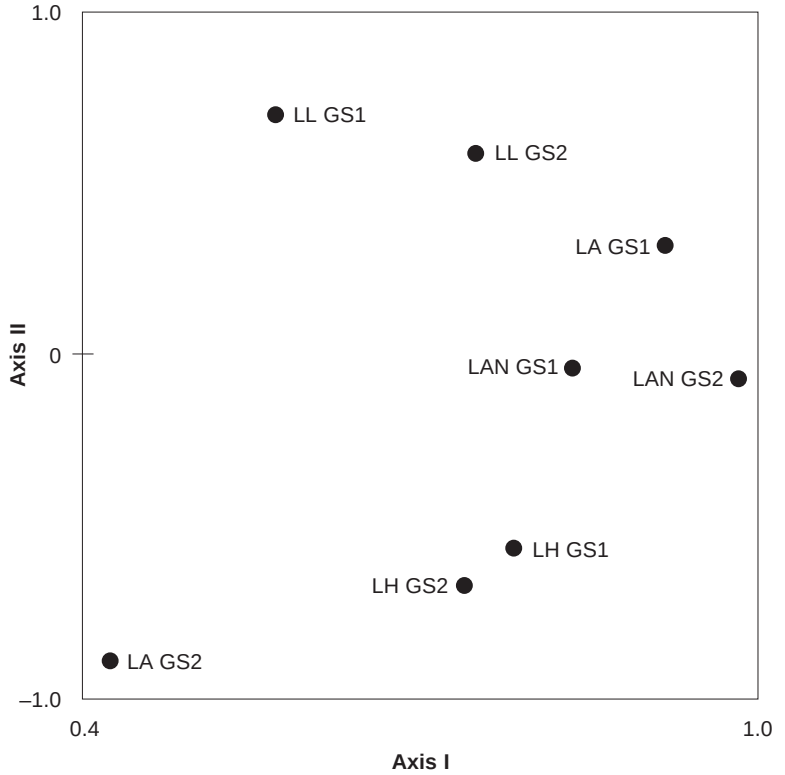


Figure 3. PCA with bacterial genera isolated after 36h of incubation at two growth stages (GS1 and GS2) in the four species of *Lupinus: L.albus*, LA, *L.luteus*, LL, *L. hispanicus*, LH, and *L. angustifolius* LAN.

In PCA c, axis I accounts for 82.77% of the variation and all samples have high load factors on axis I (table 3). Figure 4 shows all samples grouped towards the positive values of axis I, except for *L.albus* GS2, which separates towards the negative values of axis I and to the positive end of axis II; this is due, among other factors, to a low frequency of *Aureobacterium* (13.88%).

Diversity of Taxa

The Shannon diversity index for taxa sampled from the rhizosphere of all four *Lupinus* species was compared and assessed as to whether samples were large enough to calculate valid Shannon indexes, i. e, that their values do not increase substantially when larger samples are taken. From each sample, 12 random sub-

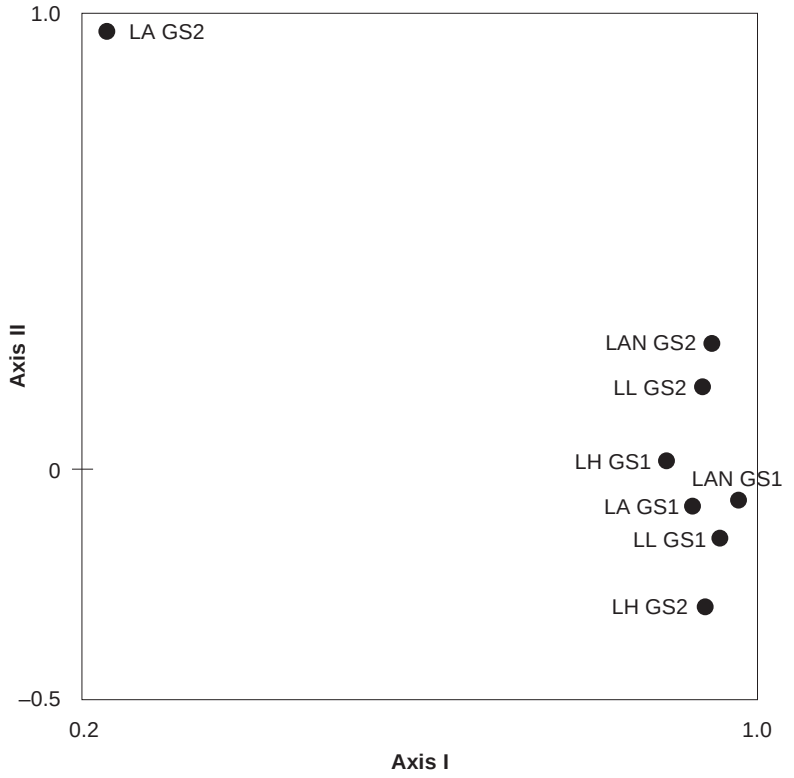
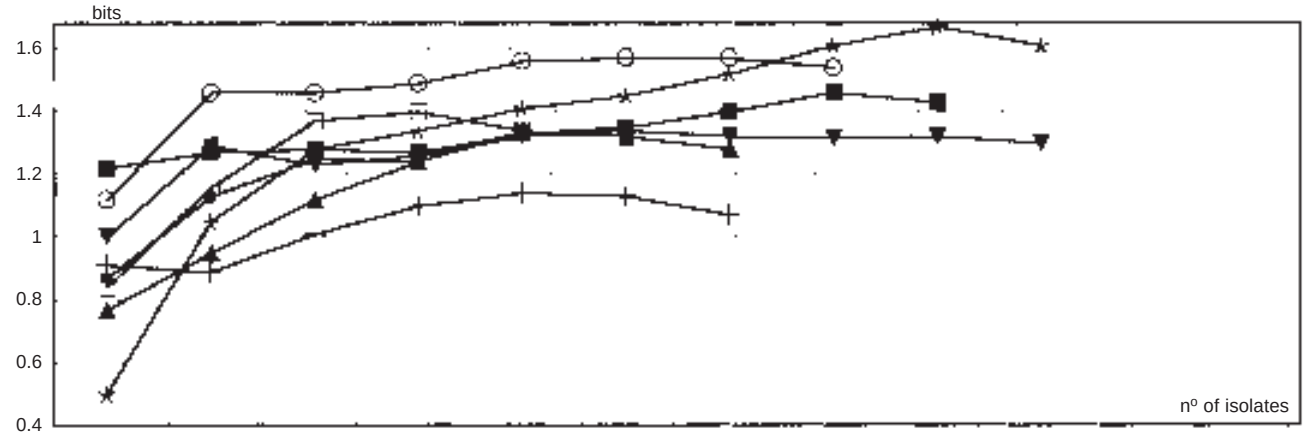


Figure 4. PCA with bacterial genera isolated from 36 to 72h of incubation at two growth stages (GS1 and GS2) in the four species of *Lupinus: L.albus*, LA, *L.luteus*, LL, *L. hispanicus*, LH, and *L. angustifolius* LAN.

samples of 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 isolates were taken (figure 5).

Discussion

It is well know that a low percentage of soil bacterial community are able to grow on synthetic media (Colwell et al., 1985). Nevertheless, cultivable bacteria can be used to indicate trends in the whole community (Gilbert et al., 1993; Kennedy & Smith, 1995). Other studies on succession, either on bacterial community activity or structure of the rhizosphere, have used different methodologies, such as consideration of metabolic capacities through the BIOLOG system (Garland & Mills, 1991), or incorporation of radioactive precursors (Bååth, 1992). Models based on population growth, as the parameter λ (Bååth et al., 1988) or on the Shannon-



LL GS1	0.87	1.13	*1.25*	1.24						
LL GS2	0.84	1.16	1.37	*1.4*	1.34					
LA GS1	1.002	1.29	1.231	1.26	1.32	*1.34*	1.32	1.317	1.32	1.3
LA GS2	0.77	0.95	1.12	1.24	*1.337*	1.32	1.28			
LH GS1	1.22	1.27	1.28	1.27	1.33	1.35	1.4	*1.46*	1.43	
LH GS2	1.12	1.46	1.46	1.49	1.56	*1.57*	*1.57*	1.54		
LAN GS1	0.5	1.05	1.28	1.34	1.41	1.45	1.52	1.61	*1.67*	1.61
LAN GS2	0.91	0.89	1.01	1.1	*1.14*	1.13	1.07			

Figure 5. Shannon indexes calculated using random subsamples of bacteria from rhizosphere on the four *Lupinus* species (*L. albus* (LA), *L. luteus* (LL), *L. hispanicus* (LH), and *L. angustifolius* (LAN)) in two growth stages (GS) of the plant: at early flowering (GS1) and at fruiting (GS2). Highest values for each *Lupinus* species appear in bold.

Weaver equation, either isolating colonies after short incubation periods (De Leij et al., 1993), or in BIOLOG plates (Fuller et al., 1997), have also been used for this purpose. Other approaches to the study of the rhizosphere structure include identification of bacterial genera with fatty acid methyl esther (FAME) profiles (Lilley et al., 1996). In the present study, isolates were identified to the genus level by a classical biochemical test for taxonomy, similar to the procedures described by Degens & Harris (1997). Colony growth kinetics on plates (De Leij et al., 1993) has also been considered, since it yields information regarding the physiological state of cells in the environment, nutrient availability and adaptive strategies related to competitiveness.

The analysis of the Shannon index as the evaluation parameter of bacterial diversity of the rhizosphere, confirmed that the sample size was suitable. The minimum number of isolates necessary to ensure that the sampling size is appropriate is determined by the maximum value of the Shannon index or by its asymptotic value. The minimum number of isolates varied depending on the plant species and growth stage; this value ranged from 54 in *L. angustifolius* (GS1) to 18 in *L. luteus* (GS1). Other authors estimated a minimum sample size ranging from 40 to 80 isolates in *Beta vulgaris* (Lilley et al., 1996), and from 20 to 30 in beach sediment samples (Bianchi & Bianchi, 1982). In most of the species, a minimum of 30 to 54 isolates is necessary to detect the maximum Shannon index. *L. luteus* shows this maximum first in both GS. Interestingly, the most extreme values of diversity were detected at *L. angustifolius* in GS1 and GS2.

The temporal border to define growth strategists (r/K) is difficult to be established. Nevertheless, the really important is to define two growth population classes but not to define a limit between both growth strategists. De Leij et al. (1993), El-Beltagy & Hattori (1994), Gorlach et al. (1994), Ikeda et al. (1997) and Sarathchandra et al. (1997) define this temporal borders from 48 to 72 h. This approach allow us to distinguish between r and K strategists in the conditions used in this work. According to these criteria, *Aureobacterium* could be considered a K strategist, while *Cellulomonas* and *Bacillus* would belong to r strategists; *Pseudomonas* and *Arthrobacter* could be considered as K strategists, confirmed by PCA b and c results.

Considering all 576 isolates, PCA a (figure 2) showed that the microbial composition of the rhizosphere of *L. albus* is the only one that changed considerably from GS1 to GS2. These changes could also be observed in PCAs b and c (figure 3 and figure 4) and are caused by the increase of *Cellulomonas* (r strategists) parallel to the decrease of *Aureobacterium*, *Pseudomonas,* and *Arthrobacter* (K strategists) at GS2. De Leij et al. (1993) found that exudates of young roots are rich in simple sugars and aminoacids (Curl & Truelove, 1986), providing an uncrowded environment, rich in easily available nutrients, and therefore, favorable for r strategists. However, the environment provided by old roots is not as rich in nutrients, and carbon sources are less available for microorganisms, enhancing the development of K strategists. These results appear to be contradictory to ours, but it must be kept in mind that De Leij et al. (1993) worked on young wheat roots that is, very early growth stages, as compared to the flowering stage

(GS1 in our study). We agree with De Leij et al. (1993) that in early growth stages, r strategist development is enhanced, and these are progressively substituted by K strategists. However, during root necrosis that usually occurs in fruiting (GS2) in annual plants, a wide and abundant variety of organic compounds are released, favouring a new increase of the r strategists. In leguminous forbs, such as *Vicia villosa*, root necrosis is observed in fruiting with the subsequent increase in the biological activity of several groups of bacteria (Lucas García et al., 1999). Other studies also support the enhancement of r strategists when root exudation is increased (Andrews & Harris, 1986).

Differences in growth strategies and in the cenotic composition of the rhizosphere (see PCA) under each *Lupinus* species are revealed by frequencies of r/K strategists. In *L. albus*, the dominance of K strategists in GS1 switched to that of r strategists in GS2. In *L. luteus*, r and K strategists were even in the two GS, while in *L. hispanicus* and *L. angustifolius*, K strategists dominated in both GS. The literature reports that the dominance of r/K strategists reflects of the physiological status of the root; hence, it may be concluded that in *L. albus*, root necrosis would be more advanced than in other species, the plant would have lost control of exudation and organic compounds would be easily available via root necrosis. On the other hand, *L. hispanicus* and *L. angustifolius* still control root exudation, allowing the predominance of specialists (K strategists). The relation of r/K strategists in *L. luteus* would indicate an intermediate situation.

According to other authors, the rhizosphere is a very selective environment, where diversity is lower than in bulk soil (Marilley et al., 1998). However, the switch in the growth strategy detected from flowering to fruiting in the rhizosphere of *L. albus*, reflected in the dominance of K or r strategists respectively, would have to be associated with a decrease in biological diversity. On the contrary, the growth strategy remained the same in *L. hispanicus* and *L. angustifolius*, and it should not be associated with notable changes in bacterial diversity. This indicates that the bacterial diversity detected at a genus level is not representative of the real situation in the rhizosphere and suggests the need to work at a more specific level. All this is supported by the Ecological Theory, applied to different systems other than the rhizosphere: a low diversity indicates high connectance and development of competences and is related to the predominance of r strategists, not to K.

Figure 6 shows a tentative successional model for the rhizosphere of *Lupinus* during its biological cycle, relating the plant physiology with rhizospheric bacterial diversity and growth strategies (r/K). A low diversity is related to dominance of K strategists, feedback regulation by overcrowding and limitation of energy sources, while a high diversity would be represented by a dominance of r strategists. In this system, the plant exerts a strong control of the situation, given its role as a primary producer with the particularity that different carbon sources are being provided according to the physiological status of the moment.

Considering the current knowledge of the rhizosphere, many of the structural and functional aspects described for other natural systems will have to be discarded for this environment. As an example, succession in the rhizosphere is defined by an autotrophic succession, supported by plant exudates (early and late

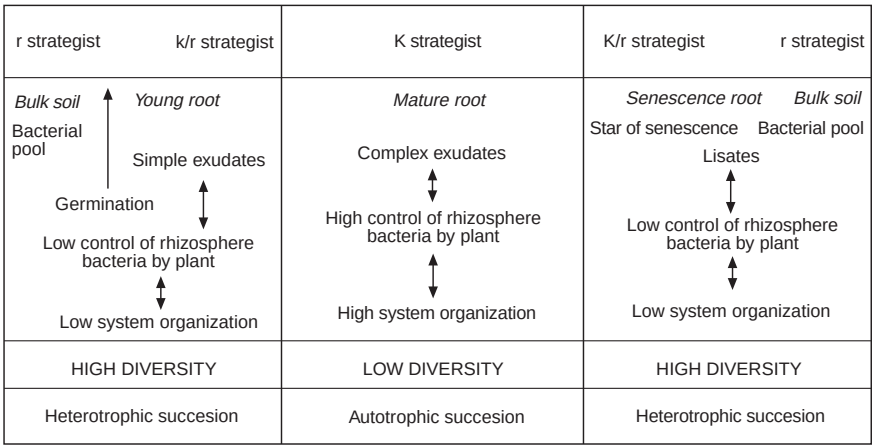


Figure 6. Successional model of the rhizosphere of *Lupinus* along the plant biological cycle that relates the plant's physiology with growth strategies (r or K), biological diversity and type of succession.

root growth), and linked to another heterotrophic succession (root necrosis with the corresponding rhizospheric soil turning to bulk soil).

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