Seasonal variations of *Bacillus* isolated from the rhizosphere of *Elaeagnus angustifolia* L.

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Abstract

The rhizosphere of *Elaeagnus angustifolia* L. was sampled monthly during one year and the genera of the isolated bacteria were determined. *Bacillus* predominated in all seasons. Eight groups of *Bacillus* were identified according to biochemical tests described in the Bergey's Manual, from which, group VII, with *B. mycoides* and *B. laterosporus* as the most likely species to define it, predominated (total year average of 60.5%). In order to determine intrageneric variation, additional tests as resistance to antibiotics and use of different carbon sources were assayed. According to both tests, the *Bacillus* strains isolated from the rhizosphere of *E. angustifolia* differed significantly throughout the year. These results suggest the adaptation of rhizospheric microbial communities to environmental conditions, so variable in Mediterranean climates, and to the physiological status of the plant.

Key words: antibiotics, *Bacillus*, carbon sources, *Elaeagnus angustifolia*, rhizosphere.

Resumen. *Variaciones estacionales de* Bacillus *aislados de la rizosfera de* E. angustifolia

Se muestreó la rizosfera de *E. angustifolia* L. durante un año y se determinaron los géneros a los que pertenecían las bacterias aisladas. *Bacillus* predominó durante todo el año. Se identificaron ocho grupos de *Bacillus* según las pruebas bioquímicas del Manual Bergey's entre los cuales predominaba el grupo VII (media anual 60.5%). Con objeto de determinar la variación intragenérica, se realizaron pruebas de resistencia a antibióticos y metabolización de fuentes de carbono. En ambos casos se encontraron diferencias significativas anuales. Estos resultados sugieren la gran capacidad de adaptación de las comunidades microbianas rizosféricas tanto a las condiciones ambientales, tan variables en climas mediterráneos, como al estado fisiológico de la planta.

Palabras clave: antibióticos, *Bacillus*, fuentes de carbono, *Elaeagnus angustifolia*, rizosfera.

Introduction

The rhizosphere is a region of intense microbial and microfaunal activity, where root exudates allow the development of many rhizosphere communities (Curl and Truelove, 1986). In this environment, molecular and trophic interactions form a complex web, in which there is an intense selection of bacterial types. Conse-

quently, bacteria isolated from the rhizosphere are often resistant to a wide selection of antibiotics, while non-rhizospheric strains are not (Gilbert et al., 1993); the same applies to many other metabolic attributes. Rhizospheric bacteria sometimes exhibit a certain degree of specificity (Chanway et al., 1988). This specificity may be either adaptative, as a result of natural coexistence of plants and bacteria (Sumner, 1990) or a simple metabolic compatibility that is independent of recent coexistence (Holl & Chanway, 1992). This system is also affected by environmental physicochemical characteristics (Lynch, 1990). For example, seasonal changes have been reported in the bacterial genera in the rhizosphere of diazotrophic plants such as alder, particularly in those that participate in the nitrogen cycle (Probanza et al., 1996).

Despite of the above, little is known about the ecology of the rhizosphere, being this lack of knowledge the limiting factor to introduce bacteria in soils in order to improve plant physiology (Schippers et al., 1995). Our main concern in this sense was to get a better understanding of the steady rhizosphere community as well as the adaptative processes they have undergone, in order to evaluate chances of survival, success in colonization and possible alterations arising from the inoculation (Tiedje et al., 1989).

Studies on other diazotrophic species show that *Bacillus* is the predominant genera in their rhizosphere (Probanza et al., 1996), while *Pseudomonas* predominates in the rhizosphere of non diazotrophic species (Atlas & Bartha, 1993). The aim of this study was to characterize the structure, bacterial composition and some adaptative trends of the microbial community in the rhizosphere of *E. angustifolia* L., considering that it is a diazotrophic plant and that this type of plants have a rhizospheric environment specially rich in nitrogen and other nutrients (Acero et al., 1993). This affects the dynamics of the biogochemical cycles in the rhizosphere (Pozuelo et al., 1995), and provides a specific environment for microbial communities adapted to this area, defined by all the relationships that take place simultaneously.

Material and methods

Sampling area and procedures

The rhizosphere samples were collected monthly for a year in an *E. angustifolia* copse in Valdemoro, 30 Km southwest of Madrid, central Spain (coordinates N40°11'30" W3°40'30"). The copse was divided into three sampling zones of equivalent areas (A, B and C). Three trees were randomly selected from each zone, and four samples (of approximately 100 g each) of the soil adhering to roots (ranging between 3 to 4 cm diameter) were collected from each tree at a depth of 10-20 cm. All samples of the three trees in the same zone were mixed in aseptic conditions and constituted replicate, giving a total of three replicates, one for each zone. Samples were kept in sterile bags at 4°C. For each of these samples, microbiological and chemical analysis were performed within the next 24 hours after sampling.

Chemical analysis of soils

Soil moisture content was determined gravimetrically after drying it at 105°C during 24 h. After the soils had been sieved through a 2 mm sieve and dispersed in a 5% calgon solution, soil texture was determined by densitometry (Day, 1965). For chemical analysis, soils were dried at 55°C and sieved through a 2 mm screen before routine laboratory analysis.

Organic-C was determined by the Walkley-Black method (Allison, 1965), while total-N was determined by the modified Kjeldhal method (Bremmer, 1965). Soil samples were suspended in distilled water 1:1, and pH was measured with a Crison glass-electrode-pH-meter. For NO_3^- determination, 4 g soil were vigorously shaken during 20 min in a solution containing: 16.66 g $Al_2(SO_4)_3.18H_2O$; 1.24 g H_3BO_3; 4.67 g Ag_2SO_4, 2.43 g $NH_2SO_3H_2$ and 1000 mL distilled water. After the soil had settled, nitrates were measured using a mv/PH meter (Crison digital 501), provided with a specific nitrate electrode (Orion 93-07-00) and a reference electrode (Orion 90-02-OO) (Milham et al., 1970). The NH_4^+–N concentration of 5 g soil samples suspended in 45 mL distilled water and 5 mL of 10 M NaOH was measured with a specific electrode (Orion 95-10-00).

Soil dilutions and plating

Serial 10-fold dilutions were prepared from 10 g of rhizospheric soil from each zone in 100 mL of sterile distilled water up to a dilution of 10^{-9}. After vigorous shaking for 10 minutes, 1 mL of each dilution was plated on a medium containing 23.5 g standard methods agar (DIFCO), 10 mL soil extract (Pochon & Tardieux, 1962), 25 mL of double concentrated Winogradsky saline solution (Pochon & Tardieux, 1962) and 1 mL oligoelements solution (Pochon & Tardieux, 1962) per litre. Incubation was at 28°C during 72 h.

Culture identification

At monthly intervals, 20 cfu (colony forming units) were randomly isolated in plates from each of the three sampling zones (60 cfu per month, 180 cfu per season) and pure cultures were obtained from the isolates, on the same culture medium used before. To ensure the objectiveness of the selection, all cfu on plates containing approximately 50 cfu were numbered, and 20 of them were selected with a computer random number program. A total of 720 cfu were obtained and these were identified to the genus level as described in Acero et al. (1993).

Biochemical characterization

Following the same method as described above, 72 colonies (6 from each month) were selected at random from the predominant genus of bacteria isolated during the year (*Bacillus*), and the following tests were done: oxidation/fermentation of rafinose (ra), glucose (gl), maltose (mt) and arabinose (ar); resistance to ampicillin (am), 10 µg, rifampicin (rf) 30 µg, tetracycline (te) 30 µg and neomycin (neo) 30 µg; production of H_2S (*HS2*), indole (ind) and nitrous oxide (nt); ß-galactosi-

dase (on), lysine-decarboxylase (LDC), ornithine decarboxylase (ODC), urease (URE), tryptophane-deaminase (TDA), catalase (ca) and cytochrome oxidase (ox) activities and use of citrate (ct).

Additionally, the 72 *Bacillus* strains were categorized into eight groups following the scheme in the Bergey's Manual (Claus & Berkley, 1989), based on the catalase activity, and the use of glucose, arabinose and citrate.

Statistics

Unidirectional analysis of variance (ANOVA) was used to contrast results from chemical analysis of soils among seasons (3 months each). When these differences were significant at 95 or 99% level, means were compared by least significant difference (LSD) test (Sokal & Rohlf, 1979).

The nineteen variables (biochemical tests and resistance to antibiotics) and the eiht groups (frmed by the 72 *Bacillus* strains) were used as an input o a Principal Component Analysis (PCA) (Hartman, 1967). This method is a widely employed statiscal tool to detect successional structures in vegetation (Pineda et al., 1981) and in microorganisms (Acero et al., 1993).

Monthly frequencies of groups in each season were compared with a two way analysis of variance (ANOVA) with two factors (groups and seasons). Also, monthly results for each variable were considered by seasons (3 months each) and another two way analysis of variance (ANOVA) with two factors (carbon sources and antibiotics) was used to compare results (Sokal & Rohlf, 1979). When differences were significant at 95 or 99% level, means were compared by least significant diference (LSD) test (Sokal & Rohlf, 1979).

Results

Average soil texture was 62% sand, 26% clay and 11% silt. Physico-chemical characterization of soil was done monthly, although results are shown seasonally (Table 1). No significant differences were detected in total nitrogen and pH. Both $N\text{-}NH_3^+$ and $N\text{-}NO_3^-$ concentration in soil peaked in summer (119 µg/g and 137.3 µg/g soil respectively) although there was a time lag between lowest values: while $N\text{-}NH_3^+$ was minimum in winter (3.1 µg/g soil), NO_3^- was minimum in spring (84.6 µg/g soil); NO_3^- showed higher values along the year. Organic carbon levels fluctuated from 2.9% in autumn to 2.1% in winter.

E. angustifolia rhizobacteria belonged to the following genera (year average values): *Bacillus* (88.5%), *Pseudomonas* (0.4%), coryneformes (2.7%), *Streptomyces* (1.1%) and *Erwinia* (0.9%). *Bacillus* dominated seasonally and in all sampling areas. Seasonal results appear in Table 2.

Taxonomic groups of *Bacillus* and the species that most likely belong to them according to the Bergey's Manual are shown in Table 3. Frequencies of all groups along the year appear in table 4 and it is evident the predominance of Group VII throughout the year except in autumn, when group V was more abundant. Nevertheless, these differences were significant ($p<0.01$) according to the ANOVA.

Table 1. Chemical analysis of soils.

	Moisture %	**pH**	**Organic C %**	**NH_4^+ µg/g soil**	**NO_3^- µg/g soil**	**Total N mg/g soil**
Autumn	31.0a	7.6	2.9a	25.3a	113.2ab	0.6
Winter	36.0b	7.5	2.1b	3.1b	88.6a	0.4
Spring	33.8ab	7.8	2.2b	67.0c	84.6a	0.6
Summer	24.9c	7.8	2.4c	119.0d	137.3b	0.6

Values not sharing same letter(s) in the same column are statistically different by LSD test ($p<0.05$).

Table 2. Seasonal (a) frequencies of bacterial genera (%).

	Autumn	**Winter**	**Spring**	**Summer**
Bacillus	80	93	92	89
Pseudomonas	0.6	1.34	0	0
Coryneformes	3.6	1.34	1.8	1.3
Streptomyces	6.6	0	1.3	0.65
Acinetobacter	0.6	0	0	0
Erwinia	0	0	3.7	0
Flavobacterium	0	0.7	0	0
Rhizobium	0	1.34	0	0
Staphilococcus	0.6	0	0	0
Micrococcus	0	0	0	0.65
Unknown	7.1	2.2	1.2	7.7

Table 3. Biochemical characterization and annual frequencies of the groups of *Bacillus* and *Paenibacillus* and species that will most probably belong to each group (according to the Bergey's Manual).

Group	**ca**	**gl**	**ara**	**ct**	**% annual**	**– Species**
I	+	fer	fer	*	1.4	*B. insolitus, B. sphaericus*
II	–	ox	*	*	0	*B. larvae*
III	–	fer	*	*	2.8	*B. azotoformans*
IV	+	ox	fer	+	1.4	*B. subtilis, B. pumilus, B. licheniformis*
V	+	ox	fer	–	14.05	*B. lentus, Paenibacillus polymyxa*
VI	+	ox	ox	+	8.44	*B. cereus, B. megaterium, B. thuringiensis*
VII	+	ox	ox	–	60.52	*B. mycoides, B. laterosporus*
VIII	+	fer	ox	*	12.6	Not determined

(*) Data not available; (+) Positive; (–) Negative; (ox) oxidation; (fer) fermentation; (ct) citrate; (ca) catalase; (gl) glucose; (ara) arabinose.

Table 4. Seasonal frequencies (%) of groups.

Group	**Autumn**	**Winter**	**Spring**	**Summer**	**Total**
I	0	1,4	0	0	1.4
II	0	0	0	0	0
III	0	0	1.4	1.4	2.8
IV	1.4	0	0	0	1.4
V	9.85	4.2	0	0	14.05
VI	5.63	0	2.81	0	8.44
VII	5.63	16.9	14.08	22.5	60.52
VIII	0	5.6	5.6	1.4	12.6

Anova: Differences between groups D.F. = 22; LSD (0.05) = 0.929, LSD (0.01) = 1.268
Differences between groups and seasons D.F. = 4; LSD (0.05) = 1.785, LSD (0.01) = 2.9.12

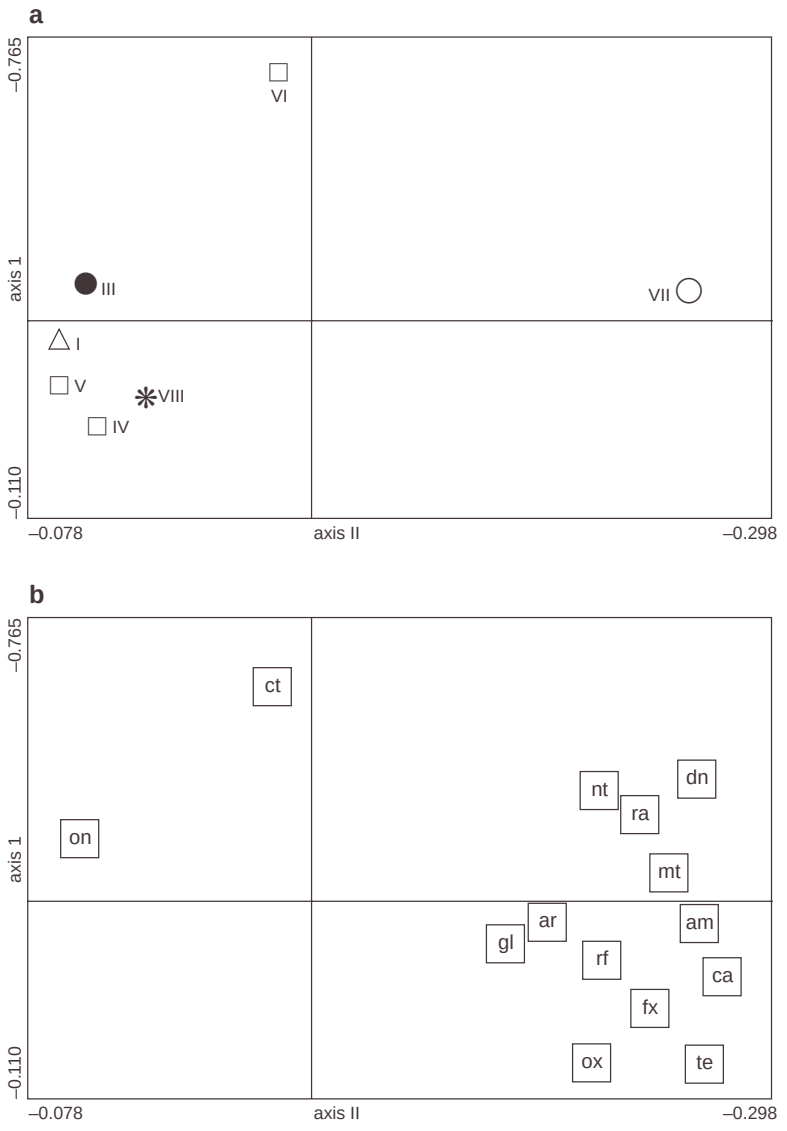

Figure 1. a) Distribution of the groups of *Bacillus* according to the PCA; symbols indicate groups that predominate autumn (□), winter (△), winter, spring and summer (○), winter and spring (✳), and spring and summer (●), b) Representation of weights according to PCA: oxidation/fermentation of rafinose (ra), glucose (gl) maltose (mt), arabinose (ar); resistance to ampicillin (am), rifampicin (rf), tetracyclin (te); production of nitrous oxides (nt), ß-galactosidase (on), catalase (ca) and cytochrome oxidase (ox) activities, use of citrate (ct), nitrogen fixation (fx), denitrification (dn). The first PCA axis absorbs 79.23% of the variance, and the second, 12.98%.

Position on the plane defined by the two PCA axis of the *Bacillus* groups is shown in Figure 1a. The first PCA axis accounts for 89.23% of the variance and the second axis for 12.98%. Groups VI and VII are markedly separated from the others. Figure 1b shows that the citrate test separates group VI towards the positive end of component II; the ß-galactosidase (on) test separates most groups and all the other tests define group VII on the positive end of component I.

Approximately 80% of the bacteria tested used monosaccharides (glucose and arabinose) and almost 20% used disaccharides (rafinose and maltose). Differences between the use of monosaccharides and disaccharides were significant ($p<0.01$). Differences between monosaccharides as well as those between dissaccharides were also significant ($p<0.01$). As far as seasonal variations are concerned, the increase between spring and summer (70%) in the number of cfu's able to use arabinose was significant ($p<0.05$). The use of the other carbon sources assayed decreased simultaneously, although not significantly.

Regarding resistance to antibiotics, the ANOVA revealed seasonal differences for ampicillin. The isolates obtained in summer were significantly ($p<0.05$) more resistant to ampicillin than those isolated in other seasons; jdifferences between autumn and winter were highly significant ($p<0.01$), and only significant ($p<0.05$) between autumn and spring. Resistance to tetracycline, rifampicin and neomycin was maximum throughout the year.

Discussion

The analysis of the seasonal variation in the rhizosphere in this study is based on physiological attributes in order to avoid a strictly taxonomic classification, which would give little information on the important ecological role played by these microorganisms within this environment (Gilbert et al., 1993). Therefore, isolates were classified under taxonomic and physiological criteria, being their ability to use carbon sources with a different degree of complexity, and their resistance to antibiotics of variable spectra, the evaluated parameters for that purpose. The carbon sources and antibiotics considered in this study are used as simple markers that only tag groups of bacteria, but do not determine the presence or absence of a certain bacterium in this habitat.

The actual composition of the microbial community in the root zone is dependent on root type, plant species, plant age and soil type (Campbell, 1985) as well as other selection pressures. Typically, the rhizosphere is colonized by a predominantly Gram-negative microbial community (Atlas & Bartha, 1923). Many authors cite *Pseudomonas* as the dominant genera in this rhizosphere, probably since under favorable environmental conditions, its growth rate is higher than that of *Bacillus* (Bowen & Foster, 1978). However, previous studies in diazotrophic plants show *Bacillus* as the dominant genera in the rhizosphere of *Alnus glutinosa* (Probanza et al., 1996).

In this study, we think that the key to explain the predominance of *Bacillus* is its ability to use the nutrients provided by the plant through exudates. As other authors have pointed out, other diazotrophic plants release high quantities of easily

mineralizable nitrogen compounds by root exudation (Gutiérrez Mañero et al., 1994); therefore, the availability of these compounds may contribute to the fact that all (100%) of the *Bacillus* isolated in this study were ammonifiers. Consistent with this, a significant increase on organic carbon, ammonium and nitrate is detected between spring and summer (table 1), remarking that ammonium and nitrate concentrations peak in summer. These changes result from the activity of rhizospheric microorganisms on available organic substrates, and therefore, values should change noticeably between both periods, when a significant increase on the frequency of group VII (table 4) is detected, considering that this group has the strongest and widest physiological potential. In addition, *Bacillus* has the ability to inhibit the growth of other strains. Many strains of *Bacillus* have been reported to produce substances that act as growth inhibitors for other microorganisms (Llinares et al., 1994). Also, these bacteria can develop resistance to antibiotics, an advantage in the complex web of interactions that occurs in the rhizosphere.

Exudates released by roots play an important role in the processes of adaptation and selection (Klein et al., 1988). Among the pool of compounds found in exudates are organic acids such as citrate, and sugars, as dominant components (Lynch, 1990; Jones et al., 1994). Exudate composition varies with the plant physiological changes (Grayston and Campbell, 1996) that occur in response to different environmental conditions such as seasonal climatic variations (Whipps, 1984). Consequently, this variation would be reflected in the behaviour of bacteria towards the carbon sources provided by the plant. The increase in the number of isolates able to use monosaccharides from spring to summer should be understood, partially, as the response to seasonal changes in the composition of exudates. This variation in the amount and composition of exudates reflects in a greater availability of carbon sources, providing the rhizosphere with a number of relationships of competence, in which the production of antibiotic substances plays an important role (Gilespie-Sasse et al., 1991).

Our results are consistent with this hypothesis considering both aspects, resistance to antibiotics and use of carbon sources. The resistence to ampicillin (a wide spectra antibiotic) shown by the *Bacillus* strains fluctuated significantly along the year, peaking in summer when environmental conditions favour development of a great number of species; therefore, the dominant species are forced to increase their competing potential.

Connecting with this, the peak in the use of monosaccharides in summer could be explained as a general strategy to improve competing potential (Gilbert et al., 1933). As said before, the frequency of strains able to use monosaccharides (arabinose) in summer increases significantly and so does the frequency of strains of group VII (table 4). Group VII, defined by more biochemical activities than the other groups, would be able to perform more functions in the rhizosphere as shown by the principal component analysis (figure 1). However, despite the predominance of group VII along the year except in autumn, marked changes in the use of carbon sources as well as in the resistance to ampicillin were detected. This points out the internal variability of this group in which there are two possible species (*B. mycoides* and *B. laterosporus*), so these variations may be explained either by

the fluctuation in the abundances of the dominant species or by changes within their metabolic activities, assuming they maintain their relative abundances.

This study points out the relevance of two important topics: first, the adaptacion of microbial communities to rhizospheric environments and second, the variations of these communities presumed to be due to environmental conditions and to the phenologic status of the plant. In this sense, variations in the metabolic capacities of bacterial strains determined on certain key moments in the plant biological status could be used to mark the ecological role of these strains in the rhizosphere.

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References

Atlas, R.M.; Bartha, R. 1993. Microbial Ecology: fundamentals and applications. Menlo Park, CA: Benjamin/Cummings. p. 563.

Acero, N.; Probanza, A.; Blanco, B.; Gutiérrez Mañero, F.J. 1993. Seasonal changes in physiological groups of bacteria that participate in the nitrogen cycle in the rhizosphere of the Alder. Geomicrobiol. J.11: 133-140.

Allison, L.E. 1965. Organic carbon. In: Methods of soil analysis. C.A. Black (ed.). Madison. WI: American Society of Agronomy. p. 1367-1378.

Bremmer, J.M. 1965. Total nitrogen. In: Methods of soil analysis. C.A. Black (ed.). Madison. WI: American Society of Agronomy. p. 1149-1178.

Bowen, G.D.; Foster, R.C. 1978. Dynamics of microbial colonization of plant roots. In: Proc. Symp. Soil Microbiology and Plant Nutrition, Eds. W.J. Broughton and C.K. John. p. 98-101. Kuala Lumpur. University of Malaysia Press.

Campbell, R. 1985. Plant Microbiology. Baltimore: Edward Arnold. p. 191.

Claus, D.; Berkley, R.C.W. 1989. Genus *Bacillus.* In: «Bergey's Manual of Systematic Bacteriology». R.N. Krierg and J.G. Holt (eds.). 1: 1105-1139. Williams and Wilkins Company. Baltimore.

Chanway, C.P.; Holl, F.B.; Turkington, R. 1988. Genotypic coadaptation in growth promotion of forage especies by *Bacillus polymyxa.* Plant & Soil 106: 281-284.

Curl, E.A.; Truelove, B. 1986. The rhizosphere. Springer-Verlag. Bolin. Germany.

Day, P.R. 1965. Particle fractionation and particle-size analysis. In: Methods of soil analysis. C.A. Black (eds.). Madison. WI: American Society of Agronomy. p. 545-567.

Gilespie-Sasse, L.M.J.; Almassi, F.; Ghisalberti, E.L.; Sillasithampara, M. 1991. Use of a clean seedling assay to test plant growth promotion by exudates from a sterile red fungus. Soil. Biol. Biochem 23: 95-97.

Gilbert, G.S.; Parke, J.L.; Clayton, M.; Handelsman, J. 1993. Effects of an introduced bacterium on bacterial communities in roots. Ecology 74 (3): 840-854.

Grayston, S.J.; Campbell, C.D. 1996. Functional biodiversity of microbial communities in the rhizosphere of hybrid larch (*Larix eurolepsis*) and stike spruce. Tree Physiol 16: 1031-1038.

Gutiérrez Mañero, F.J.; Probanza, A.; Pozuelo González, J.M.; Llinares, F. 1994. Effects of nodular extracts of *Alnus glutinosa* (L.) Gaertn on ammonification, nitrification and CO_2 production in different soils. Orsis 9: 3-12.

Hartmann, J.H. 1967. Modern factor analysis. 2nd ed. University Chicago Press. Chicago. 133 p.

Holl, F.B.; Chanway, C.P. 1992. Rhizosphere colonization and seedling growth promotion of lodgeopole pine by *Bacillus polymyxa.* Can. J. Micribiol. 38: 303-308.

Jones, D.L.; Edwards, A.C.; Donachie, K.; Darrah, P.R. 1994. Role of proteinaceous amino acids released in root exudates in nutrient acquisition from the rhizosphere microorganism effects on soluble aminoacids, sugars, and organic acids in the root zone of *Agropyron cristatum, A. smithii* and *Boutelona gracilis.* Plant & Soil 110: 19-25.

Klein, D.A.; Frederick, B.A.; Bioudini, M.; Trlica, M.Y. 1988. Rhizosphere microorganism effects on soluble aminoacids, sugars, and organic acids in the root zone or *Agropyron cristatum, A. smithii* and *Boutelona gracilis*. Plant & Soil 110: 19-25.

Lynch, J.M. 1990. The rhizosphere. J.M. Lynch (ed.). John Wiley and Sons. Chichester. 458 p.

Llinares, F.; Muñoz-Mingarro, D.; Pozuelo, J.M.; Ramos, B.; Bermúdez de Castro, F. 1994. Microbial inhibiton and nitrification potential in soils incubated with *Elaeagnus angustifolia* L. leaf litter. Geomicrobiol. J. 11: 149-156.

Milham, P.J.; Awad, A.S.; Paull, R.E.; Bull, J.E. 1970. Analysis of plant, soils and waters for nitrate by using and ion-selective electrode. Analyst. 95: 751-757.

Pineda, F.D.; Nicolás, J.P.; Pau, A.; Galiano, E.F. 1981. Ecological succession in oligotrophic pastures of central Spain. Vegetatio 44: 165-176.

Pochon, J.; Tardieux, P. 1962. Techniques d'analyse en microbiologie du sol. Editions de la tourelle. St. Mandé. 116 p.

Pozuelo, J.M.; Gutiérrez mañero, F.J.; Probanza, A.; Acero, N. 1995. Effect of Alder (*Alnus glutinosa,* L. Gaertn.) roots on distribution of proteolytic, ammonifying and nitrifying bacteria in soil. Geomicrobiol. J. 13: 129-138.

Probanza, A.; Lucas, J.A.; Acero, N.; Gutiérrez Mañero, F.J. 1996. The influence of native rhizobacteria on european alder [*Alnus glutinosa* (L.) Gaertn.] growth. I. Characterization of growth promoting and growth inhibiting bacterial strains. Plant & Soil 182: 57-62.

Schippers, G.; Scheffer, R.J.; Lugtenberg, B.J.J.; Weisbeek, P.J. 1995. Biocoating of seeds with plant growth-promoting rhizobacteria to improve plant establishment. Outlook on Agriculture 24 (3): 179-185.

Sokal, R.R.; Rohlf, F.J. 1979. Biometría. J. Blume (ed.). 832 p.

Sumner, M.E. 1990. Crop responses to *Azospirillum* inoculation. Adv. Soil Sci. 12: 52-123.

Tiedje, J.M.; Colwell, R.K.; Grossman, Y.L.; Hodson, R.E.; Lenski, R.E.; Mack, R.N.; Regal, P.J. 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology 70: 229-315.

Whipps, J.M. 1984. Environmental factors affecting the loss of carbon from the roots of wheat and barley seedlings. J. Exp. Bot. 35: 767-773.