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Analysis of the legume–rhizobia symbiosis in shrubs from central western Spain

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ABSTRACT

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Aims: This work analyses the diversity of rhizobia associated with some of the predominant shrubby legumes in central-western Spain. Symbiotic promiscuity and effectiveness were studied using cross-inoculation experiments with shrubby species.

Material and Results: Six new bradyrhizobia strains were isolated from nodules collected from wild plants of six leguminous species, *Cytisus balansae*, *C. multiflorus*, *C. scoparius*, *C. striatus*, *Genista hystrix* and *Retama sphaerocarpa*. These isolates were genetically characterized by 16S rDNA partial sequencing and random amplification of polymorphic DNA–PCR fingerprinting. The phylogenetic analysis revealed that these isolates could represent three new *Bradyrhizobium* species. Shrubby legumes and bradyrhizobia displayed a high symbiotic promiscuity both for infectivity and effectiveness. Symbioses were effective in more than 70% of the associations established by four of the six plant species.

Conclusions: Native woody legumes in western Spain are nodulated by *Bradyrhizobium* strains. The high degree of symbiotic promiscuity and effectiveness highlights the complex dynamics of these communities in wild ecosystems under a Mediterranean-type climate. Furthermore, the results from this study suggest a potential importance of inoculation for these legume species in soil-restoration projects.

Significance and Impact of the Study: This is the first study, to our knowledge, that combines both molecular analysis and pot trials to study the rhizobia–legume symbiosis for wild legumes.

Keywords: *Bradyrhizobium*, nitrogen fixation, random amplification of polymorphic DNA–PCR, revegetation, woody legumes.

INTRODUCTION

The biological nitrogen fixation that takes place in the symbiosis between Fabaceae plants and Rhizobiaceae bacteria is the main natural input of nitrogen in the biosphere. In spite of the importance of this process for ecosystems functioning, research in this area has been mostly focussed on herbaceous species of agricultural interest. Nevertheless, in the last two decades many new rhizobial strains have been isolated and identified from legumes in natural systems around the world. These studies have been focussed on woody leguminous species from tropical (Trinick 1980; Faria *et al.* 1994; Saur *et al.* 1998; Vinuesa *et al.* 1998), temperate (Burdon *et al.* 1999; Ulrich and Zaspel 2000; Lafay and Burdon 2001) or arid and semi-arid environments (Jiti and Galiana 1996; Khbaya *et al.* 1998; Marsudi *et al.* 1999), showing that woody legume species can be nodulated by bacteria from the *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* genera.

Shrubby legumes are widely distributed in regions with a Mediterranean-type climate, as is the case for the Iberian

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Peninsula where there is a remarkable diversity of such species. However, little is known about the diversity of rhizobia associated with leguminous shrubs and about their importance in legume establishment and growth. In this study we have analysed the identity and promiscuity of bacterial strains that nodulate six leguminous shrubby species from central-west Spain, *Cytisus balansae* (Boiss.) Ball, *C. multiflorus* (L'Hér.) Sweet, *C. scoparius* (L.) Link, *C. striatus* (Hill) Rothm, *Genista hystrix* Lange and *Retama sphaerocarpa* (L.) Boiss. The distribution of these plant species is largely restricted by soil pH, nutrient availability and temperature (Pérez-Fernández *et al.* 2000). Their establishment and growth is also likely to be facilitated by their ability to enter into effective symbioses with multiple rhizobial species. Indeed, legumes that occupy large geographical areas usually display high symbiotic promiscuity and effectiveness (Ulrich and Zaspel 2000). Symbiotic promiscuity is a common trait in the relationship between legumes and rhizobia, with the exception of the tribes Trifolieae, Viceae and Cicereae (Perret *et al.* 2000). However, although most leguminous species are nodulated by several rhizobial strains, the infections do not always result in effective symbioses [i.e. an increase either in nitrogen content or biomass production (Turk and Keyser 1992)].

From an applied perspective, the leguminous species selected for this study are suitable for revegetation projects in the Iberian Peninsula. Desertification is one of the most important ecological problems in Mediterranean areas (Warren *et al.* 1996) and legumes are good candidates for soil-restoration projects. Woody legumes can prevent erosion, increase soil fertility and facilitate the establishment and growth of other plant species (Gutiérrez *et al.* 1993; Moro *et al.* 1997; Cross and Schlesinger 1999). In addition, the inoculation of seeds and seedlings with appropriate native rhizobia would guarantee root nodulation, enhance plant performance, and reintroduce these micro-organisms in the soil (Requena *et al.* 2001).

MATERIALS AND METHODS

Nodule collection and isolation of bacterial strains

Plants of six leguminous shrubby species, native to the Iberian Peninsula, were excavated at various sites in central-western Spain (Table 1). Fragments of roots with attached nodules were excised and transported in distilled water in plastic vials to the laboratory. Nodules were excised from roots using a scalpel blade sterilized in flaming ethanol. Individual nodules were surface-sterilized by sequential washing with 96% ethanol for 1 min, 3% sodium hypochlorite for 3 min and three rinses in sterile distilled water. Nodules were crushed on sterile plates, homogenized and streaked onto yeast-extract mannitol agar (YMA) plates (Somasegaran and Hoben 1994). Isolates were grown at 28°C for 14 days. Single colonies were visible after 8 days. Bacteria from single colonies were picked and restreaked on fresh YMA plates. Strains were designated according to the plant species from which they were isolated, i.e. cba isolated from *C. balansae* (Table 1).

DNA extraction

Single colonies from YMA plates were suspended in 100 μl of 0·05 M NaOH and boiled for 4 min. Deionized H_2O (900 μl) was added to the solutions and stored at −20°C.

RAPD–PCR

Box A1R-PCR (Martin *et al.* 1992; Versalovic *et al.* 1994) was used to compare the six isolated strains and the reference species *B. japonicum* LMG 6138 T, *B. elkanii* LMG 6134 T and *B. liaoningense* LMG 18230 T. Box A1R-PCR was carried out using the primer Box A1R (CTA CGG CAA GGC GAC GCT GAC G) synthesized by MWG-Biotech AG and a PTC-100 Thermocycler (MJ Research, Waltham, MA, USA). The reactions were carried out in a 100 μl volume containing 2 μl of template DNA solution, 2 μM of the primer, 200 μM of each deoxynucleoside triphosphate (Life Technologies Inc., Gaithersburg, MD, USA) and 2 U of *Taq*DNA polymerase (Ecogen, Barcelona, Spain). The amplifications were performed using the following protocol: initial denaturation at 95°C for 7 min; 30 cycles of 1 min at 94°C, 1 min at 53°C and 8 min at 65°C, and final extension at 65°C for 16 min. After the reaction, aliquots (5 μl) of the PCR products were examined by electrophoresis in a 2% agarose gel.

Table 1 Plant species, origin sites with coordinates, soil pH and names given for the isolated strains

Host species	Coordinates	Province	Soil pH	Strain name
Cytisus balansae	40°27′N 6°40′W	Salamanca	4·55	cba
Cytisus multiflorus	39°21′N 8°51′W	Badajoz	4·46	cmu
Cytisus scoparius	38°21′N 7°08′W	Badajoz	5·80	csc
Cytisus striatus	39°21′N 8°51′W	Badajoz	4·08	cst
Genista hystrix	41°00′N 7°34′W	Salamanca	5·05	ghy
Retama sphaerocarpa	38°31′N 7°08′W	Badajoz	7·05	rsp

Partial sequencing and phylogenetic analysis

The 16S rDNA genes were amplified by PCR using 63f and 1387r primers based on the *E. coli* numbering system (Marchesi *et al.* 1998) in a 100 μl volume containing 0·2 μl of template DNA extract, 20 pmol of each primer, 250 μM of deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 2 U of PlatinumTaq Polimerase (Life Technologies/Invitrogen, Carlsbad, CA, USA). The reaction was carried out with a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) using the following protocol: initial denaturation at 94°C for 1 min; 30 cycles of 60 s at 94°C, 60 s at 55°C and 90 s at 72°C; final extension at 72°C for 7 min. The PCR products were purified from an agarose gel using the Concert^{TM} Rapid Gel Extraction System kit (Life Technologies Inc.) and, afterwards, the QIAquick Spin Gel Extraction kit (Qiagen, Valencia, CA, USA). A fragment of around 850 bp was sequenced with an ABI 3100 Genetic Analyser using the 63f primer and Big Dye Terminator Chemistry. The 16S rDNA sequences were submitted to the GenBank database to search for significant alignments. The partial sequences were compared with those of the following organisms *B. japonicum* (D11432), *B. japonicum* (AY050540), *Rhizobium tropici* (U89832), *Sinorhizobium meliloti* (X67222) and *Brucella melitensis* bv. *ovis* (L26168). All the sequences were manually aligned, and a phylogenetic tree was inferred using the neighbour-joining algorithm (Saitou and Nei 1987) derived from a Jukes–Cantor distance matrix (Jukes and Cantor 1969) with the programme TREECON (Van de Peer and De Watcher 1994).

Plant inoculation test

Seeds from the six leguminous shrubby species were collected from several field sites in central-west Spain and stored in paper envelopes at 18°C under dry conditions. Seeds were mechanically scarified and surface sterilized by immersion in 96% ethanol for 1 min, 4% sodium hypochlorite for 2 min and rinsed with sterile distilled water. Seeds were germinated on 1·5% water agar plates and then transferred to sterile sand in 15-cm diameter pots. Seedlings were inoculated with 2 ml of the appropriate bacterial inoculum (heavy suspension of the log-phase culture on YMA in 20 ml of yeast-mannitol broth). The pots were covered with polyurethane beads to prevent evaporation and contamination. Treatments included the inoculation with the six bacterial isolates and a control without inoculum. Twelve plants of each species were used for these treatments. Plants were maintained in a greenhouse for 14 weeks, watered with sterile distilled water every 2 days and, once a week, with a sterile N-free nutrient solution (1 mM $MgSO_4{\cdot}7H_2O$; 1 mM KH_2PO_4; 2·5 mM K_2SO_4; 1 mM $CaSO_4{\cdot}2H_2O$; 0·2 mM Fe–EDTA; 1·5 μM H_3BO_3; 0·02 μM Na_2MoO_4; 0·06 μM $ZnSO_4$; 0·05 μM $MnSO_4$; 0·2 μM $CoSO_4$; 0·15 μM $CuSO_4$). Plants were harvested to check for the presence of nodules on the roots. Biomass production was estimated as dry weight of plants oven-dried at 75°C for 48 h. Data were analysed by one-way ANOVA and Tukey test using the statistical programme SPSS v10·0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Six slow-growing bacterial isolates were recovered from nodules of the six selected leguminous species. The genetic analysis of these isolates and the reference *Bradyrhizobium* strains by Box-PCR showed nine different fingerprintings (Fig. 1). The comparisons of the six 16S rDNA partial sequences revealed that they were highly homologous (94–98%). The highest homology (99%) was found for the strains cba and csc. The strain cmu had a similarity of 94–95% when compared with the other five strains whereas homology in the rest of comparisons was of 98%.

Comparison of these partial sequences with sequences in GenBank indicated a high degree of similarity (95–98%) with other *Bradyrhizobium* strains. A phylogenetic tree including other bacterial species was constructed to determine the phylogenetic position of these six new isolates (Fig. 2). The new species were assigned to the *Bradyrhizobium* genus and submitted to the GenBank database (accession numbers from AF461191 to AF161196). The strains csc, cba and rsp were clustered in a separate group

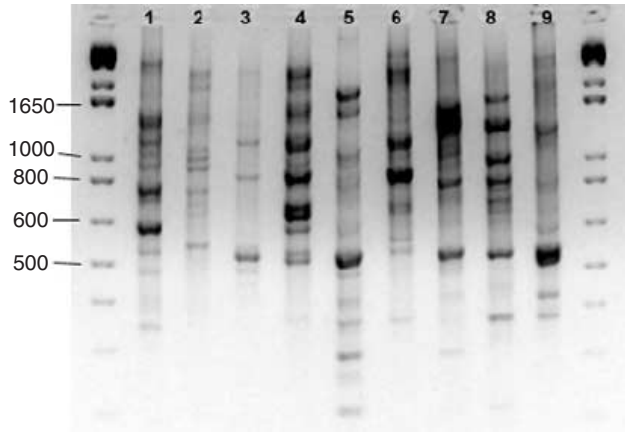

Fig. 1 Genetic fingerprinting obtained by Box A1R-PCR. 1, *Bradyrhizobium elkanii* LMG 6134 T; 2, *B. japonicum* LMG 6138 T; 3, *B. liaoningensis* LMG 18230 T; 4, cba (strain isolated from *Cytisus balansae*); 5, cmu (isolated from *C. multiflorus*); 6, csc (isolated from *C. scoparius*); 7, cst (isolated from *C. striatus*); 8, ghy (isolated from *Genista hystrix*); 9, rsp (isolated from *Retama sphaerocarpa*). Outside lanes: DNA size markers (1 kb Plus DNA Ladder, Invitrogen)

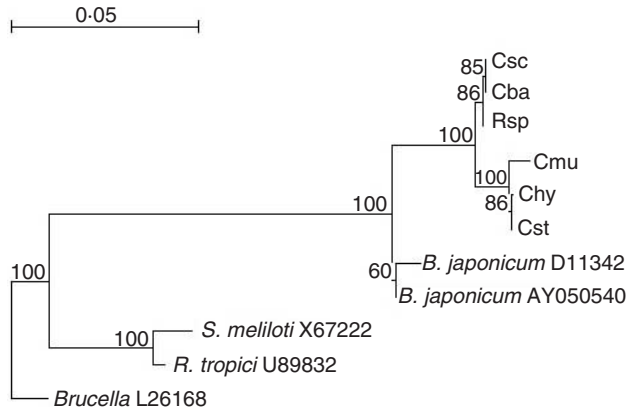

Fig. 2 Phylogenetic tree showing the relationships between the 16S rDNA sequences of the new six isolates and those from other *Rhizobium* and *Bradyrhizobium* species (GenBank accession numbers given). The tree is based on a 822 bp alignment and was constructed using the neighbour-joining method (Saitou and Nei 1987). *Brucella* L26168 was used as outgroup. The numbers correspond to the bootstrap support for internal branches based on 100 replications. The scale bar represents 0·05 substitutions per site

from ghy, cst and cmu. Within these two different groups, the strongest similarity was between csc and cba in one case and between cst and ghy in the other.

Retama sphaerocarpa was infected by three different isolates whereas *C. scoparius* was nodulated by all six strains (Fig. 3). The remaining leguminous species produced nodules in four of the six inoculation treatments. No nodules were found in the control treatment without inoculation. The number of nodules was statistically different ($P < 0{\cdot}001$) from the zero value found in the control treatment in all the symbiotic associations for *G. hystrix* and *R. sphaerocarpa*, in 75% of the cases for *C. balansae*, *C. multiflorus* and *C. striatus*, and in 33% for *C. scoparius* (Fig. 3). The highest number of nodules was observed for *C. striatus* inoculated with the strains cmu and rsp (20 and 13 nodules, respectively) and for *R. sphaerocarpa* inoculated with cba and rsp (10 and 15 nodules, respectively). The lowest number of nodules per plant was observed for *C. balansae* with less than three nodules.

Most of the symbiotic associations were effective with total plant dry-weight statistically different ($P < 0{\cdot}001$)

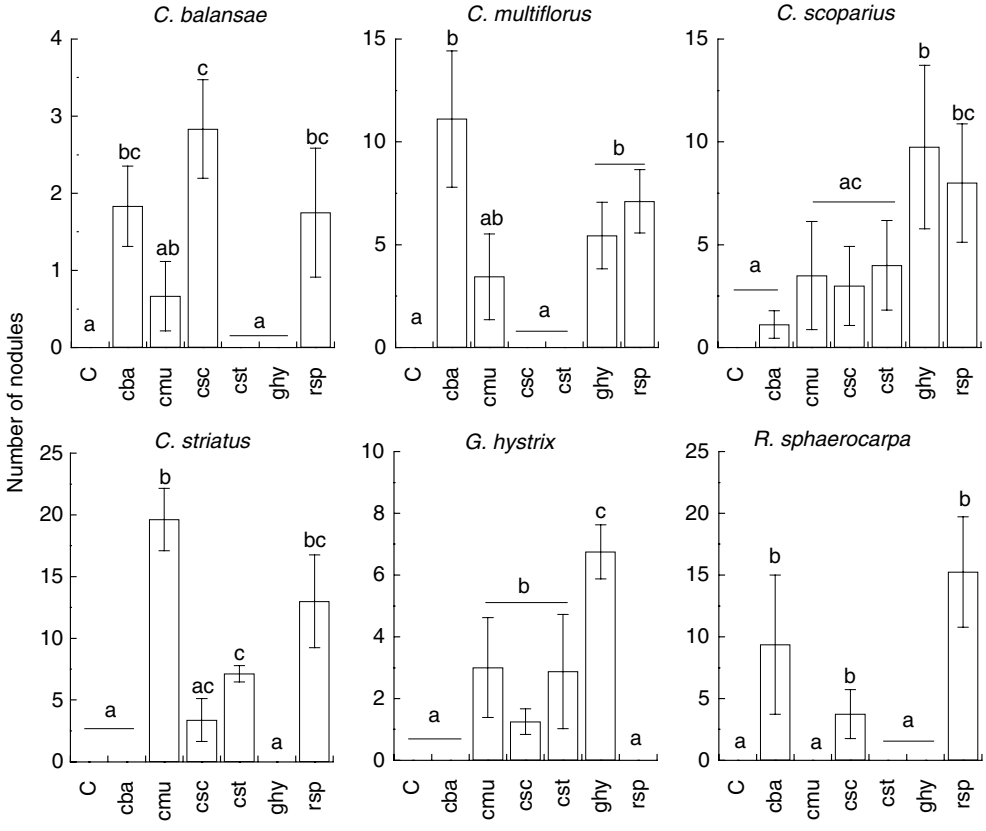

Fig. 3 Number of nodules produced by each plant species under each inoculation treatment. Control without inoculation (C). Different letters indicate significant differences ($P < 0{\cdot}001$) between the treatments after one-way ANOVA and Tukey test for multiple comparisons. Error bars represent the standard deviation of the mean (note different scale)

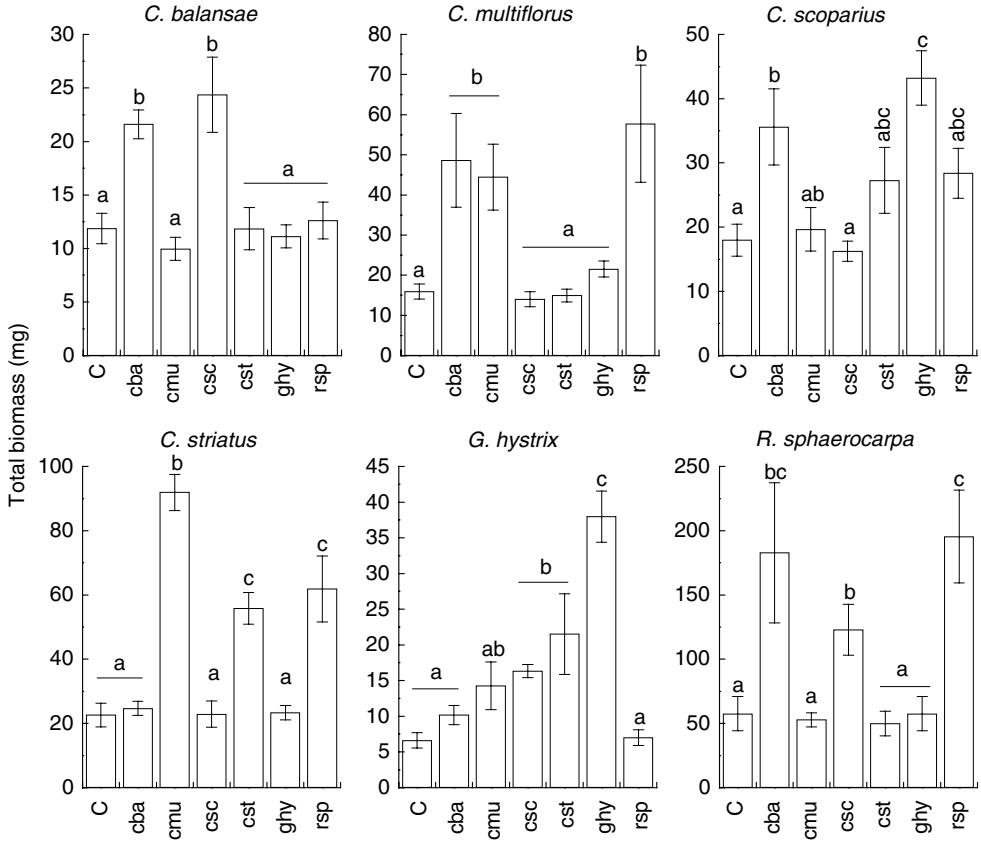

Fig. 4 Total plant biomass produced by each plant species under each inoculation treatment. Control without inoculation (C). Different letters indicate significant differences ($P < 0{\cdot}001$) between the treatments after one-way ANOVA and Tukey test for multiple comparisons. Error bars represent the standard deviation of the mean (note different scale)

from the control value (Fig. 4). Plants engaged in effective symbioses produced at least twice as much biomass as the control. The most promiscuous strain was cba, which established effective symbioses with *C. balansae*, *C. multiflorus*, *C. scoparius* and *R. sphaerocarpa*. The species *C. balansae* and *C. scoparius* entered into effective symbioses with two different isolates. The other four legumes established effective symbioses with three of the six *Bradyrhizobium* strains. The highest biomass increase was found for *C. striatus* nodulated by the strain cmu producing a total biomass four and half times higher than the control. The maximum plant biomass was observed for *R. sphaerocarpa* plants nodulated by the strains cba and rsp (180 and 190 mg, respectively).

DISCUSSION

Six new rhizobial strains were isolated from nodules obtained from six shrubby legumes native to the Iberian Peninsula. Their slow growth and the results obtained in the phylogenetic analysis placed them in the *Bradyrhizobium* genus. This genus could be the predominant group among the natural rhizobial populations in central-western Spain where soils are acidic or with near-neutral pH. Unlike other rhizobia genera, *Bradyrhizobium* species are not adversely affected by low soil pH (Graham *et al.* 1994). The actual distribution of the isolated bradyrhizobia is unknown. Presumably they could have the same large geographical distribution as the legumes from which they were isolated. Geographical partitioning has been shown for some rhizobial strains but not for others (Zhang *et al.* 1991; Novikova *et al.* 1994; Lafay and Burdon 1998). Therefore, further research is necessary to determine rhizobial diversity and distribution in western Iberian soils.

A high 16S rDNA genetic homology was found among those isolates. Nevertheless, the Box A1R-PCR showed that the six isolates were different. The analysis of 16S rDNA sequences is a powerful and reliable tool for phylogenetic classification, but fails in distinguishing between closely related strains (van Berkum and Eardly 1998). Even different bacterial species can have a 16S rDNA genetic similarity as high as 99·8% (Fox *et al.* 1992). The Box

A1R-PCR technique, however, is a useful and complementary method for rhizobia classification (Vinuesa *et al.* 1998; Bernal and Graham 2001). Our results validate this technique as a quick and successful approach to identify closely related rhizobia. The phylogenetic tree constructed using 16S rDNA partial sequences suggests that the new isolates could belong to three different species. The isolates named as cba, csc, rsp are very closely related and could be considered three different strains of the same species. The same conclusion can be applied to the strains cst and ghy. However, the isolate cmu could represent a different species. This hypothesis is supported by the lower degree of genetic similarity between this strain and the others.

In contrast with previous data from rhizobia associated with shrubby legumes (Turk and Keyser 1992; Valladares *et al.* 2002), the isolated *Bradyrhizobium* strains displayed a high infectivity and effectiveness. For instance, the strain cba entered into symbioses with four of the six species tested increasing plant biomass in all cases. A certain degree of promiscuity in species growing together can be expected because of the horizontal transfer of genes between bacteria in the soil. Most of the rhizobial genes responsible for nodulation and nitrogen fixation are located on plasmids and the transfer of these elements between soil bacteria is very common (Broughton *et al.* 1987; Schofield *et al.* 1987).

Cytisus scoparius was the most promiscuous plant species because it entered into symbiosis with all the rhizobial strains. Two of the six symbiotic associations resulted in statistically significant biomass increases and a further two symbioses clearly favoured plant growth. The high degree of promiscuity suggests the existence of complex and diverse relationships between bradyrhizobia and shrubby legumes in western Spain. The ability to develop effective symbiosis with different rhizobia could favour the colonization of new areas by these leguminous species. In fact, *C. scoparius* is an invader species in California, Australia and New Zealand (Williams 1981; Bossard 1991; Fogarty and Facelli 1999).

The high promiscuity and response to inoculation observed for these species could support their use in revegetation and soil rehabilitation projects. The use of woody legumes in these projects is valuable because they can be exploited as cattle fodder or fuel in some areas (Zahran 1999). In addition, legume litter is usually enriched in nitrogen and can increase soil fertility (Unkovich *et al.* 1997; Zahran 1999). Woody species are better than herbaceous species for revegetation projects because they can tolerate more disturbances (Piha *et al.* 1995) and reach deep underground water to survive during droughts (Jenkins *et al.* 1987). This is the case for most of the species analysed in this work (Pérez-Fernández 1996). The efficiency of the legume *Anthyllis cytisoides* L. in revegetation projects on the Iberian Peninsula has been widely verified in south-east Spain (Requena *et al.* 1997; Requena *et al.* 2001). However, the different nature of the soil in eastern and western Spain complicates the use of that shrub for the entire Iberian Peninsula. The species *C. balansae* (Boiss.) Ball, *C. multiflorus* (L'Hér.) Sweet, *C. scoparius* (L.) Link, *C. striatus* (Hill) Rothm, *G. hystrix* Lange and *R. sphaerocarpa* (L.) Boiss have not been previously considered in revegetation projects. The results presented in this work, and their large geographical distribution, indicate a remarkable ability to grow on different soils. We therefore, conclude that the use of these native species inoculated with appropriate native bradyrhizobia could be helpful for the revegetation of disturbed soils in the Iberian Peninsula.

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