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Rhizobial diversity, symbiotic effectiveness and structure of nodules of *Vachellia macracantha*

Irene Cordero [a, b], Beatriz Ruiz-Díez [a], Teodoro Coba de la Peña [a], Luis Balaguer [b, †], M. Mercedes Lucas [a, 1], Ana Rincón [a, 1], José J. Pueyo [a, *, 1]

[a] *Instituto de Ciencias Agrarias, ICA-CSIC, C/Serrano 115 bis, 28006 Madrid, Spain*
[b] *Departamento de Biología Vegetal I, Universidad Complutense de Madrid, C/Jose Antonio Nováis 12, 28040 Madrid, Spain*

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ABSTRACT

The diversity and symbiotic effectiveness of rhizobia isolated from root nodules of native American acacia *Vachellia macracantha* (formerly *Acacia macracantha*) grown in soils from different locations in Peru was investigated. Thirteen bacterial isolates were characterized by RFLP analysis of the IGS region, and sequencing and phylogenetic analyses of the 16S rRNA and the symbiotic *nodC* and *nifH* genes. Isolates were ascribed to the genera *Ensifer* or *Rhizobium*, and phylogenetic analyses of the symbiotic genes allowed relating them to rhizobial strains of American origin, probably comprising a new symbiovar. Most strains formed highly effective nitrogen-fixing indeterminate nodules with *V. macracantha*. Nodules presented distinctive structural characteristics, including tannin deposits in the inner cortex and a cap of cells with dense vacuole content over an area of intermingled meristematic and freshly infected cells in the apical central zone. Typical zones I, II and interzone II/III of indeterminate nodules were not clearly defined. Unusually complex mitochondrial structures were observed in mature and senescent infected cells of nitrogen-fixing nodules. The effect of inoculation on seedling growth and physiology was evaluated in both *V. macracantha* and the often co-existing non-nodulating tree legume *Caesalpinia spinosa*. Inoculation had an overall positive effect on the growth of *V. macracantha* seedlings, which correlated with the symbiotic effectiveness of isolates, and some strains significantly improved the photochemical efficiency of *C. spinosa*. To our knowledge, this is the first report on *V. macracantha* nodulating rhizobia and nodule structure and ultrastructure. This work provides the basis for the formulation of effective inoculants to be used in restoration programmes in the Andean region.

1. Introduction

Acacia s.l. has been recently divided into five genera, among which, the genus *Vachellia* contains approximately 160 species with a pan-tropical distribution (LPWG, 2013). In general, *Acacia s.l.* species are highly tolerant to drought and show large root systems enabling them to access deep layers of water, a highly relevant trait in arid regions. This ability induces an increase in the water potential of superficial soil layers, a hydraulic lift effect from which the neighbouring plants also benefit, highlighting the facilitative potential of acacias (Ludwig et al., 2003). *Acacia s.l.* species are often pioneers in extreme environments aided, to a great extent, by their nitrogen fixing capacity in symbiosis with rhizobial bacteria (Franco and de Faria, 1997), and by the establishment of other symbioses (e.g. endo and/or ectomycorrhiza) (Duponnois et al., 2007), playing a key role in preserving the fertility and structure of poor and eroded soils (Munzbergova and Ward, 2002). Besides their ability to fix nitrogen, many acacias are fast-growing species, a valuable criterion in reforestation programmes under harsh conditions (Duponnois et al., 2007; Bakhoum et al., 2015).

Vachellia macracantha (Humb. & Bonpl. ex Willd.) Seigler & Ebinger is a legume tree distributed across neotropical regions, spreading from the Southern United States to Northern Argentina, the Caribbean region included (Aronson, 1991). It thrives in a variety of environments, around permanent or intermittent water courses, from relatively dry to humid areas, from sea level up to 2500 m, in altered or well preserved zones (Aronson, 1991), and it is used for wood and fodder, inks and gum extraction and traditional

* Corresponding author. Tel.: +34 917452500.
E-mail address: jj.pueyo@csic.es (J.J. Pueyo).
† Deceased.
1 M.M. Lucas, A. Rincón and J.J. Pueyo contributed equally to this work.

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medicine (Sanchez et al., 2006; Balaguer et al., 2011). In Peru, Ecuador, Chile and Bolivia, *V. macracantha* grows naturally in association with the non-nodulating legume tree tara, *Caesalpinia spinosa* (Mol.) Kuntze (Aronson, 1991; Balaguer et al., 2011; Larrea, 2011), a highly ecological and economical valuable legume tree growing in natural forests, and also cultivated, making this acacia species a good facilitative candidate for co-reforestation/afforestation in the area. Synergistic benefits have been reported when acacias are interplanted with other tree species (Brockwell et al., 2011), and restoration strategies should consider, whenever possible, co-occurring species. Restoration projects with *C. spinosa* have already been implemented in some areas of Peru like the fog forest of Atiquipa (Balaguer et al., 2011), but co-reforestation strategies with *V. macracantha* have not been implemented yet.

Rhizobia associated with the genus *Vachellia* have been studied for a reduced number of species, most of them from Africa, e.g. *Vachellia tortilis* (Forssk.) Galasso & Banfi (Ben Romdhane et al., 2006; Fterich et al., 2012) or *Vachellia seyal* (Del.) P.J.H. Hurter (Diouf et al., 2007; Bakhoum et al., 2015). However, there are few existing studies on *Vachellia* in America, e.g. *Vachellia caven* (Molina) Seigler & Ebinger (Frioni et al., 1998), *Vachellia cochliacantha* Humb. & Bonpl., *Vachellia farnesiana* (L.) Wight & Arn. or *Vachellia pennatula* (Schltdl. & Cham.) Seigler & Ebinger (Toledo et al., 2003) and to our knowledge, none on the rhizobia associated with *V. macracantha*. In general, there is a preferential nodulation of the different *Acacia s.l.* genera by specific rhizobial genera. The Australian genus *Acacia* is usually nodulated by *Bradyrhizobium*, even when it grows away from its original geographic range, whereas *Acaciella*, *Mariosousa*, *Senegalia* and *Vachellia* are mainly associated with *Ensifer*, *Mesorhizobium* and, to a lesser extent, with *Rhizobium* (Leary et al., 2006). American acacias have been essentially found to be nodulated by *Ensifer* (Toledo et al., 2003; Lloret et al., 2007), although nodulation by *Rhizobium* and *Mesorhizobium* has also been reported (Rincón-Rosales et al., 2009). The acacias investigated so far present indeterminate, frequently branched, and occasionally relatively big root nodules (Sprent and Parsons, 2000), although the structure and ultrastructure of nodules formed by acacias has not been extensively studied, particularly concerning the different species of *Vachellia* (Räsänen et al., 2001; Perrineau et al., 2012).

A multigene approach, e.g. 16S rRNA and symbiotic genes as *nod* (nodulation) and *nif* (nitrogen fixation) (Laguerre et al., 2001), is commonly used to approximate the systematics of rhizobia. Symbiotic genes can be differentially located in plasmids (*Ensifer*, *Rhizobium*), the chromosome (*Bradyrhizobium*), or in "symbiotic islands" within the chromosome (*Mesorhizobium*), depending on the rhizobial genus (Sprent, 2009). They are genes easily exchangeable among bacteria by lateral gene transfer (Wernegreen and Riley, 1999), and can define "symbiovars" within a rhizobial species according to the nodulating strain-legume species affinity (Rogel et al., 2011).

In ecological restoration projects using acacia, the physiological quality of seedlings and the presence of suitable symbiotic microorganisms in the soil can be important limiting factors. The use of selected inoculants could be crucial not only for producing high quality seedlings (Franco and de Faria, 1997), but also because specific rhizobia for a given species may be nonexistent or present in very low numbers within a given soil (Brockwell et al., 2011). Healthy plants with effective nitrogen fixing nodules are more likely to survive and grow faster in the field, enhancing their facilitative potential, particularly under harsh environmental conditions and in nitrogen deficient soils (Räsänen et al., 2001; Coba de la Peña and Pueyo, 2012).

In this study, we used a combined molecular and physiological approach to identify and characterize the rhizobia associated with *V. macracantha* growing in Peruvian soils, aiming to select potential inoculants for this valuable tree species. Due to its extensive distribution and pioneer character, we hypothesized that *V. macracantha* would be associated with taxonomically and functionally diverse rhizobia, allowing us to select the best performing inoculants. To test this we genotypically characterized rhizobia isolated from different soils collected in a latitudinal gradient in Peru, and subsequently determined their nodulation and nitrogen fixation potentials on *V. macracantha*. We also studied the structure and ultrastructure of nodules elicited by isolates that displayed contrasted nitrogen-fixing effectivity. Furthermore, we comparatively analyzed the response of nodulating (*V. macracantha*) and non-nodulating (*C. spinosa*) tree legumes to the selected rhizobial strains.

2. Material and methods

2.1. Bacterial isolation

Bacteria were isolated from nodules of *V. macracantha* trap plants growing in soils obtained from different locations along a latitudinal gradient in Peru (Fig. 1, Table 1). The characteristics of the soils are shown in Table 2. Nodules were surface-sterilized by immersion in 95% (v/v) ethanol for 30 s, then in 0.1% (v/v) $HgCl_2$ for 45 s, and finally 10-time washed in autoclaved distilled water. Nodules were cut in half and a loopful from the red interior part was plated on yeast extract mannitol (YM) agar medium (Vincent, 1970), under axenic conditions. Plates were incubated at 28 °C, single bacterial colonies were selected, and isolate purity was checked by streaking single colonies repeatedly on fresh medium. Bacteria liquid pure cultures were stocked in 20% glycerol at −80 °C.

2.2. Phenotypic characterization of isolates

Gram stain tests were performed and the morphology of the isolates was observed under the light microscope. A growth curve was individually determined for each isolate by adding 1 ml of pre-inoculum (A_{600} = 0.8) to 100 ml liquid YM medium, allowing bacteria to grow to the stationary phase (28 °C, 150 rpm). The

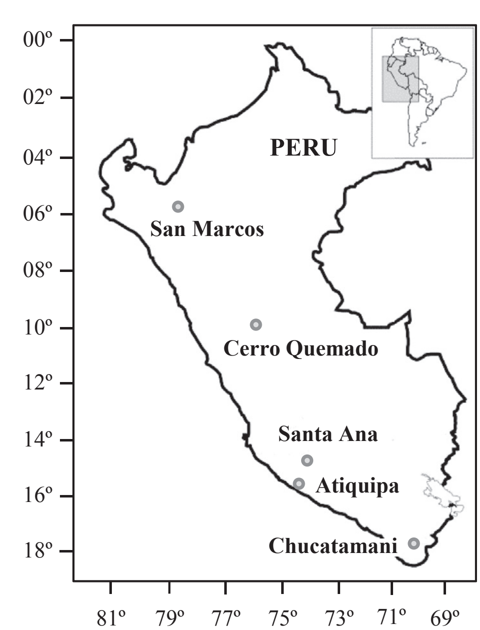

Fig. 1. Location of sites of soil sampling along a latitudinal gradient in Peru.