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Co-introduction of exotic rhizobia to the rhizosphere of the invasive legume *Acacia saligna*, an intercontinental study

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ABSTRACT

Invasive woody legumes have profound impacts in the nitrogen content and cycling of invaded ecosystems due to the ability to enter into symbiosis with nitrogen-fixing bacteria. In spite of the relevance of this symbiosis, the identity and origin of the symbionts involved in invasion are not well understood. We conducted a study to assess the diversity of symbiotic root-nodulating bacteria associated with the invasive *Acacia saligna*, in newly colonized areas in Portugal and Australia. BOX-PCR was used to discriminate the isolated bacteria and 16S rRNA and *nifD* genes were sequenced to identify the different isolates and their geographic origin. *Bradyrhizobium* and *Mesorhizobium* nodulated *A. saligna* in Australia while only *Bradyrhizobium* spp. were found in Portugal. The dominant strains nodulating *A. saligna* were related to *Bradyrhizobium liaoningense* and *Bradyrhizobium canariense*. Co-occurring *Acacia longifolia* and *A. saligna* in Australia harbor different rhizobial communities. As an example, we found *Mesorhizobium sp.* and *Phyllobacterium trifolii* in *A. saligna* and *A. longifolia* respectively, being this the first report for this association. The analysis of the phylogeographic marker *nifD* clustered most of the sequences obtained in this study with sequences of Australian origin, indicating that exotic bradyrhizobia might have been co-introduced with *A. saligna* in Portugal. This result highlights the risks of introducing exotic inoculants that might facilitate the invasion of new areas and alter native soil bacterial communities, hindering the recovery of ecosystems.

1. Introduction

One of the major threats to biodiversity worldwide is the invasion by exotic species (Hulme, 2007), a process that can change relatively diverse communities into near monocultures. The invasion by plants and microbes is a major threat to the composition and functioning of ecosystems but the basic mechanisms governing invasion by these exotic organisms are not well understood (van der Putten et al., 2007).

About twenty Australian *Acacia* species are aggressive invaders worldwide having major impacts in nutrient cycling in the invaded areas (Richardson et al., personal communication). Most of these *Acacia* species were introduced for sand and slope stabilization, wind protection, forestry and ornamental purposes. Among these, four species – *Acacia longifolia*, *Acacia saligna*, *Acacia dealbata* and *Acacia melanoxylon* – can be highlighted as the most aggressive invaders of European Mediterranean and temperate ecosystems (Richardson et al., 2011). These species form mutualistic associations with nitrogen fixing bacteria, a trait that contributes to an increase of nitrogen content and changes in N-cycling regimes in invaded areas (Stock et al., 1995; Yelenik et al., 2004). This change in the available nitrogen can cause an increase or decrease of the growth rates of other species, which may lead to changes in the structure and function of plant communities (Yelenik et al., 2004). Furthermore, some *Acacia* species can also change the morphological structure of the soil in places where they are the dominant species (Musil, 1993), reduce bulk soil density and increase its organic carbon content (May, 2003), and reduce plant diversity (Costello, 2000; Holmes and Cowling, 1997; Richardson et al., 1992).

The legume-rhizobia symbiosis is a key factor of legume ecology but the symbiotic interactions during the host range expansion are still poorly understood (Parker et al., 2006). When legumes are entering a new habitat, the symbiosis can be a limiting factor for plant establishment because they may have a reduced performance in the lack of compatible symbionts (Parker, 2001; Parker et al., 2006). In spite of that, many introduced woody legumes nodulate abundantly in the introduced range, either due to a high symbiotic promiscuity or because there are cosmopolitan rhizobial symbionts with a wide host range (Richardson et al., 2000). Alternatively, compatible root nodulating bacteria might have been accidentally or deliberately introduced into new areas with exotic legumes

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(Pérez-Ramírez et al., 1998; Rodríguez-Echeverría, 2010; Stepkowski et al., 2005), making it possible for invasive legumes to establish in the new range without having to enter into new symbioses with local rhizobia (Chen et al., 2005; Weir et al., 2004). Legumes can also be responsible for shifts in the soil bacterial community composition (Diallo et al., 2004; Junier et al., 2009; Lorenzo et al., 2010), including changes in the communities of symbiotic nitrogen-fixers (Rodríguez-Echeverría, 2010). In spite of the increased research in this area in the last five years, little information is available on the diversity of rhizobia associated with invasive legumes. Recent research on *A. longifolia* has shown that nodulation is a key trait on the invasion process for this species (Rodríguez-Echeverría et al., 2009), which supports different rhizobial diversity in areas with different times of invasion (Rodríguez-Echeverría et al., 2007). The work presented here aims at increasing the present knowledge on the rhizobial diversity associated with exotic invasive acacias by focusing on *A. saligna*, native to Western Australia and an invader of coastal sand dune ecosystems in Europe and Africa. This acacia is considered one of the best woody species for stabilizing shifting sand (Kutiel et al., 2004), the main reason why it has been introduced in Southern Europe and Africa. *Acacia saligna* has also been introduced in other places in Australia where it is currently naturalized.

In this work we studied the rhizobial diversity associated with *A. saligna* populations in Portugal and New South Wales (Eastern Australia), locations where this species has been introduced during the last century. The rhizobial diversity associated with co-occurring native *A. longifolia* in New South Wales was also investigated to try to ascertain whether they share the same symbionts. Rhizobial isolates were analyzed by using BOX-PCR fingerprinting and 16S rDNA and *nifD* sequences. Sequencing of the 16S rRNA gene has been widely used to study the rhizobial diversity associated with leguminous species (Cardinale et al., 2008; Kaschuk et al., 2006; Khbaya et al., 1998; Rodríguez-Echeverría et al., 2007; Ulrich and Zaspel, 2000) and specifically with *A. saligna* (Amrani et al., 2010; Zerhari et al., 2000). It is a highly conserved gene, very useful for identifying nitrogen-fixing bacteria because there is a large collection of available sequences on GenBank that can be used for comparison (Benson et al., 2005) and, specifically for this work, because there are many studies of rhizobia associated with Australian legumes in Australia based on 16S rDNA sequences (Lafay and Burdon, 2006, 2001, 1998). *Bradyrhizobium* species have only one copy of the rRNA gene (van Berkum et al., 1998; Klappenbach et al., 2000), making possible the study of relationships between rhizobial species (Parker et al., 2002). The *nifD* gene was selected because it is a useful marker for phylogeographic studies of bradyrhizobia. Although *nifD* gene sequences are highly polymorphic they are more conserved within a certain geographical area than between different geographic sites (Parker et al., 2002; Qian et al., 2003) and thus can reveal geographical affinities between isolates (Parker, 2003; Parker et al., 2002; Qian and Parker, 2002). *nifD* genes can spread in bacterial populations through lateral transfer, independently of vertical transferred genes such as 16S rRNA, and thus are more homogeneous within a certain geographical area than genes that are exclusively vertically transferred (Parker et al., 2002; Qian et al., 2003). Consequently, 16S and *nifD* sequences can give very different results in terms of phylogenetic relationships (Andam and Parker, 2008; Parker et al., 2002; Haukka et al., 1998). *nod* genes have also been described as useful markers to detect the origin of *Bradyrhizobium* species (Stepkowski et al., 2005). In fact, previous studies have shown that phylogenies using *nif* and *nod* genes are very similar not only for *Bradyrhizobium* but also for other rhizobial genera such as *Mesorhizobium* or *Sinorhizobium* (Haukka et al., 1998; Laguerre et al., 2001; Risal et al., 2010; Rodríguez-Echeverría, 2010). As for *nif* sequences, *nod* phylogenies are usually different from those obtained using 16S rRNA or other core genes (Moulin et al., 2004; Stepkowski et al., 2011). We therefore assumed that the information provided by *nif* and *nod* genes would be very similar. With these approaches we expected to reveal both the phylogenetic diversity and the origin of the rhizobia associated with invasive populations of *A. saligna* both in Portugal and Australia.

2. Material and methods

2.1. Bacteria isolation

Root nodules were collected from *A. saligna* plants sampled at five sites in Portugal, with the following coordinates in latitude and longitude: Alvor – N 37°07′20″ W 8°37′01″; Caparica – N 38°35′57″ W 9°12′24″; Ilha do Pessegueiro – N 37°50′00″ W 8°47′14″; Santo André – N 38°02′19″ W 8°48′47″; Tocha – N 40°19′49″ W 8°49′31″. In Eastern Australia, we collected nodules from *A. saligna* and *A. longifolia* sampled at three sites with the following coordinates in latitude and longitude: Shellharbour – S 34°29′55″ E 150°53′45″; Thomas Dalton Park – S 34°23′31″ E 150°54′24″; Wollongong – S 34°25′30″ E 150°54′14″. In each site, depending on population size, we sampled root nodules from two up to twelve plants that were separated at least 10 m. Roots with attached nodules were excised and transported to the laboratory in plastic tubes for further bacterial isolation. Four nodules per plant were surface-disinfected by immersion in ethanol (96%, v/v) for 15 s, commercial bleach (4%, v/v) for 2 min and then washed six times with sterile distilled water.

Individual nodules were crushed with a sterile glass rod in a Petri dish and a loopful of each suspension was streaked on a plate containing yeast mannitol agar (YMA) (Somesegaran and Hoben, 1994). Plates were incubated at 28 °C during 15 days and pure cultures were obtained with one or more further subculturing steps. Pure cultures were then stored at 4 °C in YMA plates and at −80 °C in glycerol (40%, v/v).

2.2. DNA extraction

For each isolate, a loopful of one bacterial colony was suspended in 100 μl of NaOH at 0.05 M, boiled at 95 °C for 8 min and then 900 μl of autoclaved bi-distilled water was added. Cell lysates were stored at −20 °C.

2.3. BOX-A1R PCR

In order to discriminate between genetically different isolates, a genetic fingerprinting was performed for each isolate using the primer BOX-A1R (Versalovic et al., 1994) manufactured by Sigma-Genosys (Cambridge, UK). The final volume of the PCR reactions was 25 μl containing 1 μl DNA template solution, 2 μM of primer, 200 μM of each dNTP (Bioron GmbH, Germany), 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (Bioron GmbH, Germany) in Taq DNA polymerase reaction buffer (Bioron GmbH, Germany). The PCR program was the following: initial denaturation at 95 °C for 7 min; 30 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 8 min; final extension at 72 °C for 16 min. All the PCR reactions were carried out in a Gene Amp 9700 (Applied Biosystems, Perkin Elmer, CA, USA). Aliquots of 9 μl were examined by electrophoresis in a 1.8% agarose gel stained with GelRed™ (Biotium, Hayward, CA), which was ran at 90 V for 180 min. Digital images of each gel were obtained and further processed and analyzed with GelCompar II (Applied Maths, Belgium). A cluster analysis for each sampling site was carried out using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm and the Pearson product-moment correlation coefficient. Those isolates with a similarity lower than 90%