



Genetic diversity of symbiotic *Bradyrhizobium elkanii* populations recovered from inoculated and non-inoculated *Acacia mangium* field trials in Brazil

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ABSTRACT

Acacia mangium is a legume tree native to Australasia. Since the eighties, it has been introduced into many tropical countries, especially in a context of industrial plantations. Many field trials have been set up to test the effects of controlled inoculation with selected symbiotic bacteria versus natural colonization with indigenous strains. In the introduction areas, *A. mangium* trees spontaneously nodulate with local and often ineffective bacteria. When inoculated, the persistence of inoculants and possible genetic recombination with local strains remain to be explored. The aim of this study was to describe the genetic diversity of bacteria spontaneously nodulating *A. mangium* in Brazil and to evaluate the persistence of selected strains used as inoculants. Three different sites, several hundred kilometers apart, were studied, with inoculated and non-inoculated plots in two of them. Seventy-nine strains were isolated from nodules and sequenced on three housekeeping genes (*glnII*, *dnaK* and *recA*) and one symbiotic gene (*nodA*). All but one of the strains belonged to the *Bradyrhizobium elkanii* species. A single case of housekeeping gene transfer was detected among the 79 strains, suggesting an extremely low rate of recombination within *B. elkanii*, whereas the nodulation gene *nodA* was found to be frequently transferred. The fate of the inoculant strains varied depending on the site, with a complete disappearance in one case, and persistence in another. We compared our results with the sister species *Bradyrhizobium japonicum*, both in terms of population genetics and inoculant strain destiny.

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1. Introduction

Acacia mangium is a leguminous tree native to northern Australia, Papua New Guinea and Indonesia. It is naturally found in tropical rainforests with a mean rainfall ranging from 1500 to 3000 mm per year. *A. mangium* has been planted widely in the tropics since the early 1980s, first in Indonesia and Malaysia for pulp production, but also for revegetation and rehabilitation purposes. Its main qualities are rapid early growth, good wood quality and its tolerance to different soil pH and composition. In 1998, Turnbull et al. [60] estimated the area planted to *A. mangium* in Asia at 600,000 ha. *A. mangium* belongs to the Mimosoideae tribe, and has the ability to form nitrogen fixing symbiosis with soil bacte-

ria collectively known as rhizobia. It has been found associated with various *Bradyrhizobium* strains in different countries and continents [23,50,55,66], and recently Le Roux et al. [22] suggested that *A. mangium* is preferentially nodulated by *Bradyrhizobium elkanii* species. It has also been found associated, albeit much less frequently, with other species, such as *Rhizobium* sp. [14,65], *Mesorhizobium* sp. [11], and *Ochrobactrum* sp. [39]. In South America, *A. mangium* has been introduced in several countries (Brazil, Colombia, Costa Rica, etc.), and two previous studies identified some isolates nodulating *A. mangium* in Brazil, such as *Bradyrhizobium* sp. [35,36].

Inoculating plants with efficient nitrogen-fixing bacteria is an environment-friendly way of improving the economic and natural growth of legumes, especially in poor or degraded soils. In *A. mangium*, regardless of the symbiotic rhizobial selected, inoculation improves survival in the field by 10% [28]. Galiana et al. [15] reviewed several inoculation field trials in different countries. They showed that inoculation had a positive effect on *A. mangium*

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Table 1
Sites sampled.

Geographical origin	Latitude	Longitude	Use of inoculant strain	Type of soil	Age of trees	Number of trees sampled	Number of isolates
Porto Trombetas	01°41'S	56°24'W	No	Subsoil	1–2 years	8	16
	01°41'S	56°25'W	Yes ^a	Tailings tank	1–2 years	10	18
Itatinga	23°02'S	48°38'W	No	Experimental field	11 years	6	10
	23°02'S	48°38'W	Yes ^a	Experimental field	6 years	10	22
Seropédica	22°45'S	43°40'W	No	Experimental field	15 years	9	13

^a Inoculated by *Bradyrhizobium* strains BR3609 and BR6009.

growth, whatever the soil type and the country, and improvement was still detectable after 2–3 years. In addition, the strains did not all display the same effect at the same stage of growth, from the nursery or greenhouse to the field [14]. One main question regarding legume inoculation, either directly in the field, or in the nursery prior to planting/sowing, is not only the survival and persistence of these inoculant strains, but also the possible genetic recombination between inoculant and indigenous strains. In the case of perennial species, the replacement of inoculant strains by local strains over the life span of the tree is also possible. In two *A. mangium* field trials in Ivory Coast in 1994, Galiana et al. [16] recovered an inoculated strain 42 months after field transplantation, whereas another one disappeared after 19 months. Martin-Laurent et al. [29] and Prin et al. [49] analysed nodules from *A. mangium* 4 and 6 months after transfer to the field in Singapore and Madagascar respectively. Molecular analyses showed the persistence and the overgrowth of one of the two inoculums to the detriment of the other. In 1990, McLoughlin et al. [30] showed that the success of inoculating soybean with *Bradyrhizobium japonicum* strains in the US was highly variable, and that successful inoculation the first year did not ensure that the inoculated strain would persist in forming nodules in subsequent years. More recently, Mendes et al. [31] showed that even 6 years after its inoculation on soybean in Brazil, the *B. japonicum* serogroup introduced was dominating nodules, occurring in more than 50% of the nodules from treatments where it had never been inoculated.

Beyond the question of the persistence of a given inoculant strain in soil, its genomic stability and integrity, i.e. whether the strain introduced remains genetically isolated in soil, or whether gene exchanges occur with indigenous strains, is also relevant. One famous study [25,57,58] showed that a *Rhizobium loti* strain virtually disappeared 7 years after inoculation, but that its 500 kb chromosomal symbiosis island had been transferred to local non-symbiotic rhizobia. Nandasena et al. [37,38] also showed that endemic Australian *Mesorhizobium* sp. isolates received symbiotic genes carried on a mobile symbiosis island from inoculant strains through lateral transfer 5–6 years after inoculation with a *Mesorhizobium ciceri* strain. Barcellos et al. [2] demonstrated horizontal transfer of symbiotic genes from a *B. japonicum* parental strain to local *Ensifer fredii* and *B. elkanii*. The indigenous *B. elkanii* strain acquired at least one copy of the nodulation gene *nodC*, and the indigenous *E. fredii* strain received the whole symbiotic island and maintained an extra copy of its original *nifH* gene.

In the context of *A. mangium* inoculation successes and outcomes in Brazil, the first goal of this study was to analyse and compare the genetic diversity of bacteria spontaneously nodulating *A. mangium* at different sites in Brazil. Our second goal was to estimate the persistence and the genetic stability of introduced selected *Bradyrhizobium* strains in Brazilian soils, both by searching for the original inoculated strains (when applied), but also highlighting possible genetic recombination events between local and introduced strains. For this, we studied five bacterial populations nodulating *A. mangium*, distributed over three sites in Brazil: Porto Trombetas, Itatinga and Seropédica. At three sites, *A. mangium*

seedlings had not been inoculated prior to planting. At two, inoculation had been carried out in the greenhouse with two selected *Bradyrhizobium* strains, and seedlings were planted in nearby fields. The genetic diversity of all strains sampled from the fields (both inoculated and non-inoculated) was analysed for three housekeeping genes, *recA*, *dnkA* and *glnII*, selected for their phylogenetic congruence in the *Bradyrhizobium* genus [42] and one symbiotic nodulation gene, *nodA*, to test for possible horizontal transfer of the symbiotic island within and between local and inoculated bradyrhizobia.

2. Materials and methods

2.1. Site descriptions

Samples of *A. mangium* nodules were directly taken from 5 fields located at three sites in Brazil (Table 1). At Itatinga, we took samples from two experimental fields (2 km apart), each with a monoculture of *A. mangium* surrounded by *Eucalyptus* species. In one field, *A. mangium* seedlings had been planted 11 years earlier without any inoculation. In the other one, seedlings had been inoculated 6 years earlier in the nursery with the two rhizobia strains BR3609 (SEMIA6387) and BR6009 (SEMIA6404). These two strains were originally isolated in Porto Trombetas, from *Acacia auriculiformis* (Mimosoideae) and *Lonchocarpus costatus* (Papilionoideae) respectively [12], and were recommended by Embrapa Agrobiologia as efficient with *A. mangium*. These strains were subsequently described as belonging to the *B. elkanii* species [4,32].

The second site, Porto Trombetas, is exploited as a bauxite mine in the State of Pará. Two fields were sampled around the industrial site: the first was planted with *A. mangium* in a rehabilitated bauxite tailings tank (SP-1). This rehabilitation process was performed in a two-step procedure. In 1998, seeds of leguminous trees and shrubs, pre-inoculated with N-fixing bacteria and mycorrhizae (VAM fungi), were hydroseeded (i.e. seeds sown by distribution in a stream of water propelled through a hose) over the tailings with the addition of fertilizer [13]. In the second phase in 1999, after substrate consolidation, *A. mangium* seedlings were planted after inoculation in the nursery using the two selected strains BR3609 and BR6009. In the second field (1.8 km away from the first), spontaneous colonization of *A. mangium* occurred on subsoil, through seed migration from a neighbouring *A. mangium* reforested area. In both field trials, nodules were collected from 1 to 2-year-old plants. These trees were second generation trees in the inoculated plot.

The last site was at Seropédica, a 15 year-old experimental field trial planted with non-inoculated *A. mangium* trees.

We randomly sampled from 6 to 10 individual trees in each field, collecting as many nodules as possible from each (Table 1).

2.2. Isolation of bacterial isolates

Two nodules per sampled tree, chosen at random, were surface sterilised in 35% hydrogen peroxide for 4–6 min depending on the size of the nodules, rinsed and then crushed in sterile water. The

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