Genetic variability in Bradyrhizobium japonicum *strains nodulating soybean [*Glycine max *(L.) Merrill]*

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SHORT COMMUNICATION

Genetic variability in *Bradyrhizobium japonicum* strains nodulating soybean [*Glycine max* (L.) Merrill]

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Abstract Brazil has succeeded in sustaining production of soybean [*Glycine max* (L.) Merrill] by relying mainly on symbiotic N_2 fixation, thanks to the selection and use in inoculants of very effective strains of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*. It is desirable that rhizobial strains used in inoculants have stable genetic and physiological traits, but experience confirms that rhizobial strains nodulating soybean often lose competitiveness in the field. In this study, soybean cultivar BR 16 was single-inoculated with four *B. japonicum* strains (CIAT 88, CIAT 89, CIAT 104 and CIAT 105) under aseptic conditions. Forty colonies were isolated from nodules produced by each strain. The progenitor strains, the isolates and four other commercially recommended strains were applied separately to the same cultivar under controlled greenhouse conditions. We observed significant variability in nodulation, shoot dry weight, shoot total N, nodule efficiency (total N mass over nodule mass) and BOX-PCR fingerprinting profiles between variant and progenitor strains. Some variant strains resulted in significantly larger responses in terms of shoot total N, dry weight and nodule efficiency, when compared to their progenitor strain. These results highlight the need for intermittent evaluation of stock bacterial cultures to guarantee effective symbiosis after inoculation. Most importantly, it indicates that it is possible to improve symbiotic effectiveness by screening rhizobial strains for higher N_2 fixation capacity within the natural variability that can be found within each progenitor strain.

Keywords *Bradyrhizobium* · N_2 fixation · BOX-PCR fingerprinting · Inoculant

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Introduction

Brazil has succeeded in maintaining soybean [*Glycine max* (L.) Merrill] production by relying largely on symbiotic N_2 fixation thanks to the selection of very effective strains *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*. Soybean was introduced to Brazil in the late 1800s, and large-scale commercial crop production began in the early 1960s. As Brazilian soils were originally void of soybean-nodulating bacteria, rhizobial inoculants were introduced mainly from the United States and Australia and massive application of inoculants containing selected rhizobial strains have continued ever since (Ferreira and Hungria 2002). Most Brazilian soils cropped with soybean now contain populations of 10^3–10^6 soybean bradyrhizobial cells per gram of dry soil, but field-growing soybeans positively respond to inoculation with elite strains (Hungria et al. 2006). Part of this phenomenon is explained by the fact that naturalized strains undergo intense environmental stress conditions in the field, mainly high temperatures and drought (Hungria et al. 1996, 1998; Santos et al.

1999; Batista et al. 2007), and reinoculation increases the likelihood of obtaining nodules with physiologically healthy, effective strains (Hungria et al. 2006; Kaschuk et al. 2010).

Repeated renewal of rhizobial slants can result in spontaneous genetic variability of colonies obtained from a single progenitor strain (Peres et al. 1984; Hungria et al. 1996; Kober et al. 2004). If genetic instability is strong in laboratory conditions, rhizobial strains may randomly lose their ability to nodulate or, perhaps, may become more effective in fix N_2. Indeed, elite soybean bradyrhizobial strains have been identified during studies of the natural variability detected in some groups of strains (Peres et al. 1993; Hungria and Vargas 2000).

In this study, we evaluated natural variability of pure strains of *B. japonicum* for N_2-fixation traits. Our objective was to confirm that it is possible to isolate rhizobial variant strains with superior symbiotic capacity and to quantify the probability of identifying elite genotypes by using this selection approach.

Materials and methods

Seeds of soybean cultivar BR 16 were surface-sterilized (Vincent 1970) and inoculated with rhizobial bacteria grown in yeast-mannitol (YM) broth at 28°C for 7 days. Two groups were chosen: (1) progenitor strains, *Bradyrhizobium japonicum* CIAT 88, CIAT 89, CIAT 104 and CIAT 105, received from the International Center for Tropical Agriculture (CIAT), Cali, Colombia, and (2) reference strains, *B. japonicum* SEMIA 5079 and SEMIA 5080, *B. elkanii* SEMIA 587 and SEMIA 5019. Details on reference strains are given by Hungria et al. (1998). Plants were grown in Leonard jars containing sand and vermiculite (2:1) and sterile N-free nutrient solution under temperatures of 28/23°C (day/night ± 2.5°C) and 12 h photoperiod. In the first step of the study, soybean was individually inoculated with progenitor strains, and, from their individual nodules, 40 variant colonies were isolated on YM agar-plates (Vincent 1970). In the second step, four completely randomized experiments were performed; each one containing three replicates of a progenitor, its 40 variant strains and the references. When plants were flowering, shoot and root were harvested, washed with distilled water and held at 60–70°C until dry mass was constant. Nodules were detached, counted and weighed. Shoot was weighed, ground and digested using the Kjeldahl method. The nitrogen content was determined by the indo-phenol blue colorimetric method. Statistical analysis checked for data normality and homogeneity. Differences between variants, progenitor and reference strains were analyzed by the two-tailed Duncan test at $P \leq 0.05$. Eight variant colonies of each *B. japonicum* strain (CIAT 88, CIAT 89, CIAT 104 and CIAT 105) were randomly chosen for genetic analysis. The PCR amplification of repetitive regions of the DNA (*rep*-PCR) was performed with BOX-A1R primer (Versalovic et al. 1994) following methodology as described by Kaschuk et al. (2006).

Results and discussion

Although most of the variant strains maintained the same response pattern as their progenitors in relation to total N and nodule efficiency, bradyrhizobial strains expanded their genetic variability, such that, some resulted in different responses than their progenitors and the reference strains. Figure 1 depicts the normal distribution of shoot total N (highly correlated with shoot dry weight, data not shown) and nodule efficiency in the population of variant strains of CIAT 88, CIAT 89, CIAT 104 and CIAT 105. The mean values of progenitors CIAT 88 and CIAT 89 were located in the central part of the distribution, while the mean values of progenitors CIAT 104 and CIAT 105 were located towards the left side due to lower values, especially of shoot total N. In relation to shoot total N, variant 104.07, followed by variant 104.06, and variant 105.21 followed by variant 105.25 showed superior performances than their progenitor and reference strains. In relation to nodule efficiency, variants 104.26 and 104.34, and variants 105.07 and 105.21 resulted in the best results (Fig. 2). Following the same trend, albeit less pronounced, variants 88.24, 88.36 and 88.37 showed superior performances in comparison with both their progenitor and the commercial strains, but not with reference strain SEMIA 5080, in terms of shoot N and nodule efficiency. Finally, variants 89.01 and 89.03 showed higher performance when compared to progenitor and commercial strains in relation to shoot total N, whereas variants 89.09 and 89.36 were superior for nodule efficiency (Fig. 2).

Until recently, it was evident that expanding bradyrhizobia diversity resulted from association of strains in a harsh soil environment (Hungria and Vargas 2000; Batista et al. 2007). However, this study shows that it is likely that physiological and genetic diversity of a given bradyrhizobial strain can expand without contacting other different individuals. Since we worked with controlled environment and pure-culture inoculants, divergence between progenitor and variant strains is explained by intra-population genetic recombination (Peres et al. 1984; Hungria et al. 1996; Kober et al. 2004).

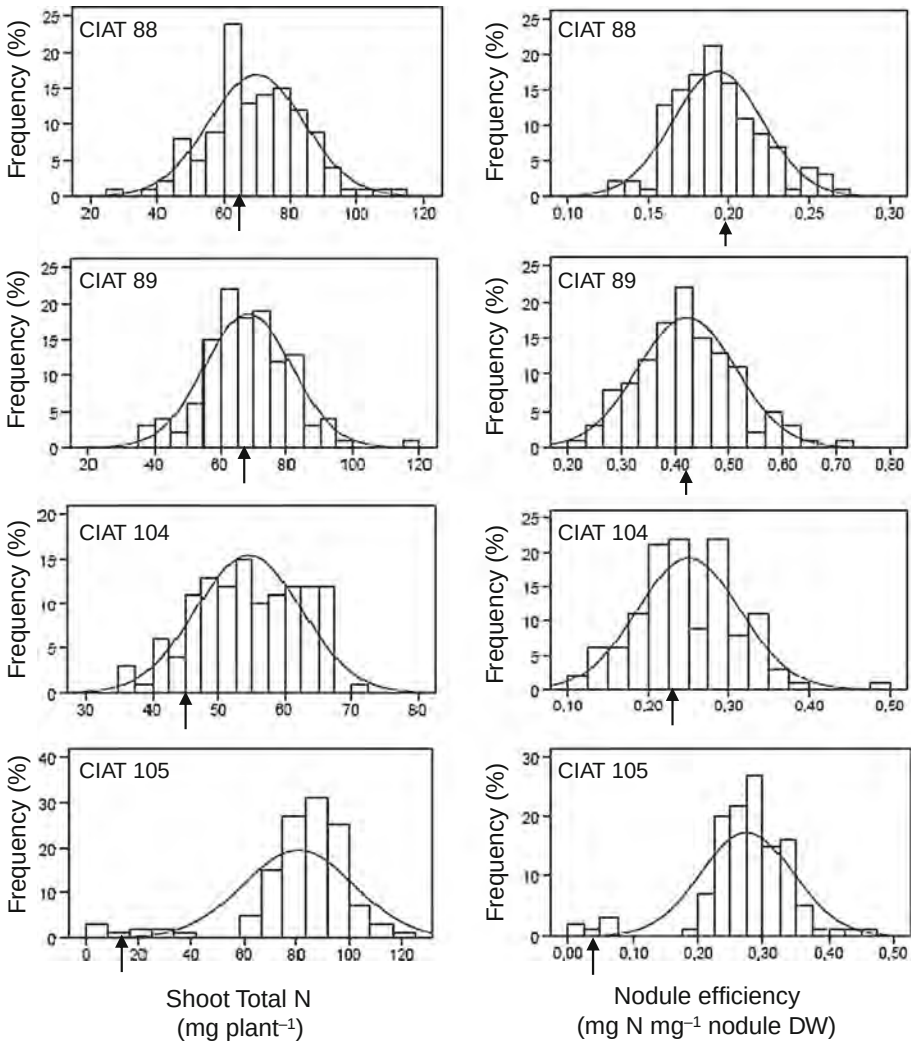

Fig. 1 Histograms of shoot total N and nodule efficiency of soybean c.v BR 16 nodulated by 40 variant and progenitor strains of *B. japonicum* CIAT 88, CIAT 89, CIAT 104 and CIAT 105. *Arrows* indicate the values referring to the progenitor strain

In this study, modifications in the DNA profile were clearly detected, and probably resulted from genomic rearrangement. *rep*-PCR is based on repetitive intergenic non-coding regions, and our knowledge about the variability and role of those regions is still very poor. Some variant strains of CIAT 104 and CIAT 105 (among others, 104.06, 104.07, 105.24, 105.34) were characterized by a low level of genetic similarity in comparison to the progenitor strains (Fig. 3). Presumably, expanded genetic variability is explained by the fact that rhizobial genomes are inserted with discrete segments of DNA, transposable between sites, both in bacterial plasmids (absent in *B. japonicum* and *B. elkanii*) and chromosomes, increasing their copy numbers at each bacterial generation (Minamisawa et al. 1998). Furthermore, it is possible that these transposons rearrange symbiotic genes by carrying them when located close to each other in the genome (Kaluza et al. 1985; Hahn and Hennecke 1987a, b). As a matter of fact, several strains showing dissimilarity in relation to the progenitor genotypes (Fig. 3, i.e. 104.06, 104.07, 105.24 and 105.34 and CIAT 104 and CIAT 105), accumulated more shoot total N and increased nodule efficiency in comparison to their parents (data not shown).

The expansion of bradyrhizobial diversity in soybean fields of Brazil is indeed very intriguing. It is often believed that Brazilian soybean varieties are usually less promiscuous than Chinese and Japanese types, and, therefore, inoculation is generally more successful. However, in addition to the studies on field-grown soybeans, we detected that genetic diversity occurred under aseptic conditions, showing that the legume-bradyrhizobia symbiosis tends to promiscuity. An interesting point is that, despite all variability observed between variant and progenitor strains, all plants nodulated satisfactorily, and there was a positive correlation between nodulation and shoot total N, demonstrating that, despite variability, the symbiosis per se is very stable. More recently, it has been

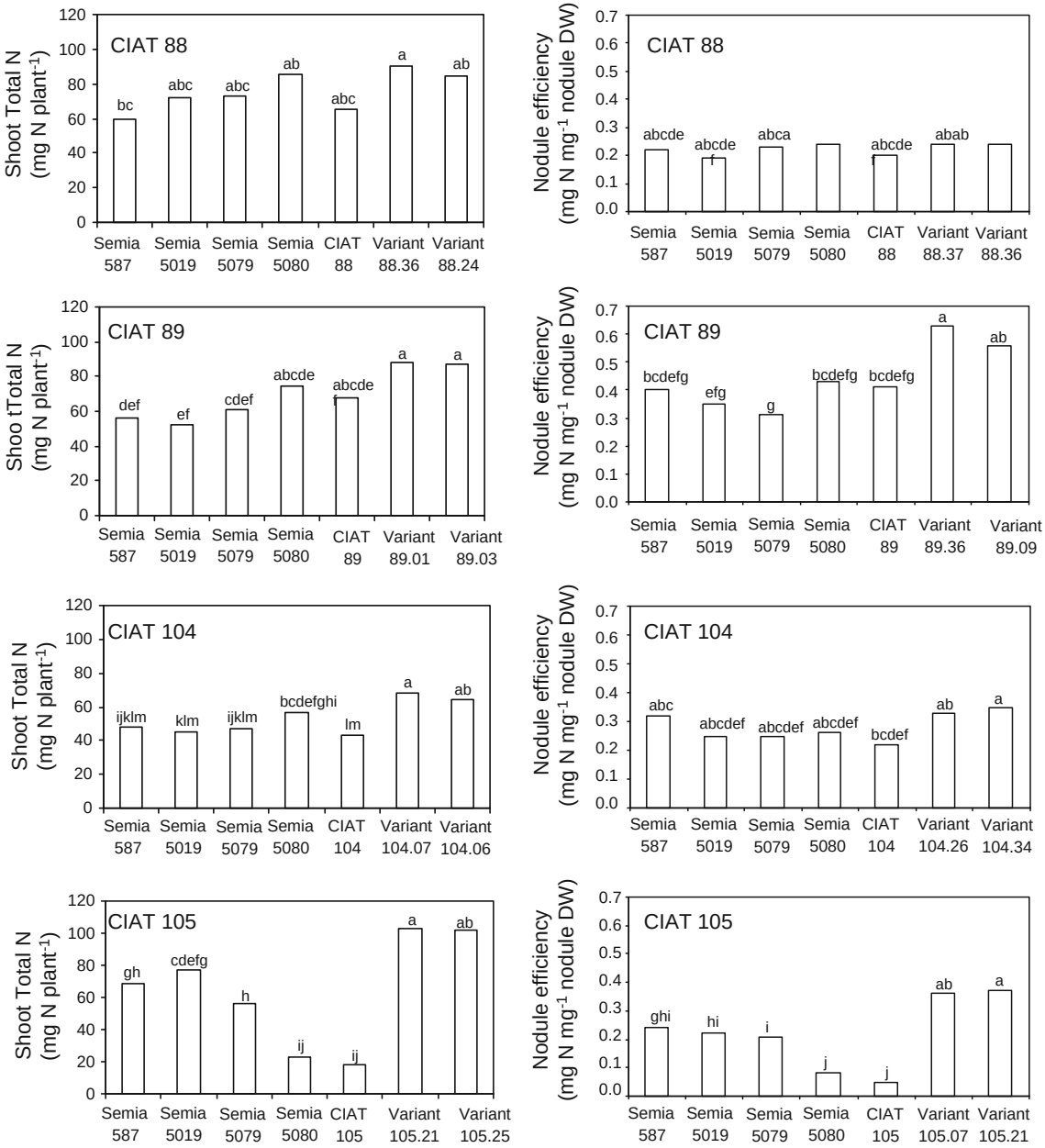

Fig. 2 Comparison of total N and nodule efficiency between variant strains of *B. japonicum* CIAT 88, CIAT 89, CIAT 104 and CIAT 105, their progenitors and four standard *B. japonicum* or *B. elkanii* strains. Different *letters on bars* indicate statistical differences in Duncan's test ($P \geq 0.05$). For clarity, the 40 variant strains of each progenitor were omitted from the graphics, but see Fig. 1 for normal distribution of that population

highlighted that rhizobia function at a population level, in which a quorum sensing of the population reacts to its environment (Sprent 2009). Since symbiotic N_2 fixation is rather a complex process, expanding diversity within similar serological groups would be very advantageous in evolutionary terms. Indeed, as we have discussed, many of the variant strains showed better symbiotic performance than their progenitors.

The expanding diversity of bradyrhizobia is a clear indication that the soybean symbiosis is not yet genetically settled. These results highlight the need for intermittent evaluation of stock bacterial cultures to guarantee effective symbiosis after inoculation. More importantly, it indicates that it is possible to improve symbiotic effectiveness of commercial inoculants by screening for higher N_2-fixation capacity within its own population variability.

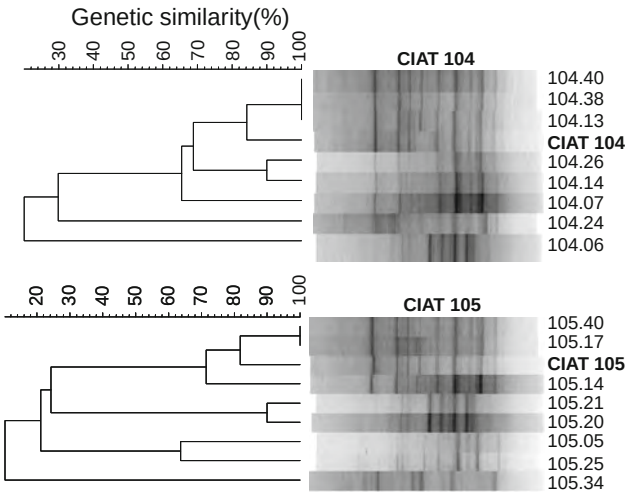

Fig. 3 Genetic distance of variants of *B. japonicum* CIAT 104 and CIAT 105 expressed by PCR-BOX A1R profiling

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