



Symbiotic performance of some modified *Rhizobium etli* strains in assays with *Phaseolus vulgaris* beans that have a high capacity to fix N₂

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Abstract

Rhizobium etli and *R. tropici* form nitrogen-fixing nodules on *Phaseolus vulgaris* (common bean). In the hope that *R. etli* strains with additional citrate synthase genes have better carbon economies, merodiploid strains were constructed. Previously, one such construct was shown to have an increased nodulation capacity in the standard bean cultivar Negro Xamapa. In the present work, derivatives from different *R. etli* strains carrying the *R. tropici* plasmid-borne or chromosomal citrate synthase gene were constructed and tested for nodulation in bean cultivars selected for their high capacity to fix nitrogen. Nodule numbers were dependent on the strain and the cultivar used. Differences in nodule number were not reflected in plant biomass.

Introduction

Phaseolus vulgaris (common bean) is the main source of protein in the nutrition of the low-income population in Latin America and in other developing countries. Bean was domesticated both in Mesoamerica and in the Andean region in South America (Gepts, 1990). Beans, as well as many other leguminous plants, are capable of establishing a symbiosis with nitrogen-fixing bacteria of the genus *Rhizobium*. Nevertheless, bean is one of the crops with the lowest levels of nitrogen fixation (Hardarson et al., 1993; Peña-Cabriales and Castellanos, 1993; Peña-Cabriales et al., 1993).

Numerous field assays have shown that the inoculation with *Rhizobium* has not been successful in increasing nitrogen fixation of bean and the failure may be attributed to the presence of native rhizobia with high competitive ability which form the majority of the nodules (Singleton and Tavares, 1986; Thies et al., 1991). We analyzed the native *Rhizobium* populations from bean nodules in agricultural fields and found that the bean rhizobia were genetically very diverse

(Martínez et al., 1985; Piñero et al., 1988), but that two distinct groups could be distinguished (Martínez et al., 1988). Further characterization led us to propose that these two groups be classified as novel *Rhizobium* species: *R. etli* (Segovia et al., 1993), and *R. tropici* (Martínez-Romero et al., 1991) with two sub-types (Geniaux et al., 1995; Martínez-Romero, 1994). *R. etli* was the *Rhizobium* most commonly isolated from bean nodules in Mesoamerica, while *R. tropici* was obtained from South American acid-soil regions. *R. tropici* strains were found to be very tolerant of high temperatures and more stable in their symbiotic properties than *R. etli* strains. Since high temperatures in the field limit nitrogen fixation in many tropical areas, *R. tropici* strains seem promising bean inoculants.

R. tropici and *R. etli* have been compared for their symbiotic properties, for their competitiveness for nodule formation in standard bean cultivars (Martínez-Romero and Rosenblueth, 1990), and for the production of Nod factors (Poupot et al., 1993, 1995a, b). Nod factors that trigger the nodulation process in plants (Relic et al., 1994; Truchet et al., 1991) are lipo-oligosaccharides with different chemical substituents related to nodulation specificity (Dénarié et al., 1992; Price et al., 1992; Spaink et al., 1991).

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R. etli and *R. tropici* produce different types of Nod factors which induce nodules in Negro Xamapa beans (Martínez-Romero et al., 1995).

In several cases research on bean-rhizobia has shown that genetically modified strains acquired increased nodulation or increased nitrogen-fixing abilities. For example, *R. etli* strain CFN42 with additional citrate synthase genes (Pardo et al., 1994) or *R. tropici* strain CFN299 with additional *nod* genes (*nodP* and *nodQ*) formed more nodules on the Negro Xamapa bean cultivar (Martínez et al., 1993).

Different *P. vulgaris* bean cultivars possess distinct nitrogen-fixing abilities (Hardarson et al., 1993; Graham, 1981; Graham and Rosas, 1977; Wolyn et al., 1989). By screening plant cultivars, J Z Castellanos (unpublished data) identified some *Phaseolus vulgaris* lineages with superior nitrogen-fixing abilities. Genetically modified *Rhizobium* strains have not been tested with these cultivars. The aim of this work was to test if the modified *Rhizobium* strains increase nodulation in beans with high capacity to fix nitrogen.

Materials and methods

Bacterial strains and culture conditions

R. etli strains CFN42, Viking1 and F8 and *R. tropici* strain CFN299 were grown in PY medium (1 g CaCl₂·2H₂O, 3 g yeast extract, and 5 g peptone L⁻¹) and *E. coli* in Luria Broth (LB). As pRK7813 is stably maintained in *Rhizobium*, modified strains were constructed by introducing this vector with the cloned plasmid-borne citrate synthase (pMP7) or the chromosomal citrate synthase (*ccsA*) from *R. tropici*. Transconjugants were obtained by mating with *E. coli* HB101 containing pMP7 or pRK7813 *ccsA* (Hernández-Lucas et al., 1995a) and selecting *R. etli* transconjugants on nalidixic acid (10 mg L⁻¹) and Tc (5 mg l⁻¹). Two independent colonies of each strain were tested for nodulation. Transconjugants were tested by hybridization to the *R. tropici* citrate synthase genes as described (Hernández-Lucas et al. 1995a).

Nodulation assays

Negro Xamapa, Bat-477 and N-8-116 were surface sterilized as described (Martínez and Rosenblueth, 1990) and seeds germinated on plates with agar. Seedlings with roots approximately 1 cm in length

were transferred to 250 mL Erlenmeyer flasks containing agar-Fahraeus or vermiculite saturated with Fahraeus N-free solution (Fahraeus, 1957). Plants were maintained in a growth chamber at 28–30 °C and watered twice a week with Fahraeus medium diluted 1:1 with water. Nodules were counted 15 and 35 days after inoculation. Aerial plant parts were dried at 65 °C for 1 week. Five different plants per transconjugant were counted for nodule numbers, and ten plants per strain were considered for dry weight.

Results

Nodulation assays with wild-type strains

R. etli strains CFN42 and Viking1 formed more nodules than did the *R. tropici* strains CFN299, BR835 and BR859 with the selected cultivar N-8-116 when tested in flasks with agar (Figure 1). When *R. etli* strain CFN42 was co-inoculated with *R. tropici* strain CFN299 (at a 1:1 ratio) in N-8-116 cultivar, CFN299 blocked nodulation of CFN42. Nodule kinetics (not shown) was similar to that of CFN299 alone. CFN42 formed about twice as many nodules as did CFN299 in *P. vulgaris* cultivar BAT-477 at 15 days after inoculation. In many previous experiments, both *R. tropici* and *R. etli* strains formed similar numbers of nodules on Negro Xamapa beans.

Nodulation assays with modified R. etli strains

A cultivar- and strain-dependent nodulation response was observed. Slight increases in nodule numbers were obtained when CFN42 contained additional citrate synthase genes from *R. tropici* (Figure 2A: a, b, c). Larger increases were obtained with transconjugants derived from Viking1 (Figure 2A: d, e, f), but Viking1 had lower nodulation levels and its derivatives only reached the levels obtained with CFN42 transconjugants. In Bat-477, the effect was reversed, with decreased nodulation obtained with CFN42 strains carrying additional citrate synthase genes (Figure 2B: a, b, c). The observed differences were not statistically significant. The response with Viking1 was similar in Bat-477 (Figure 2B: d, e, f) and in N-8-116. Best nodulation performances were obtained with CFN42 in Bat-477 under our conditions. *R. etli* strain F8 was not improved for nodulation in Bat-477 by the addition of citrate synthase genes (not shown).

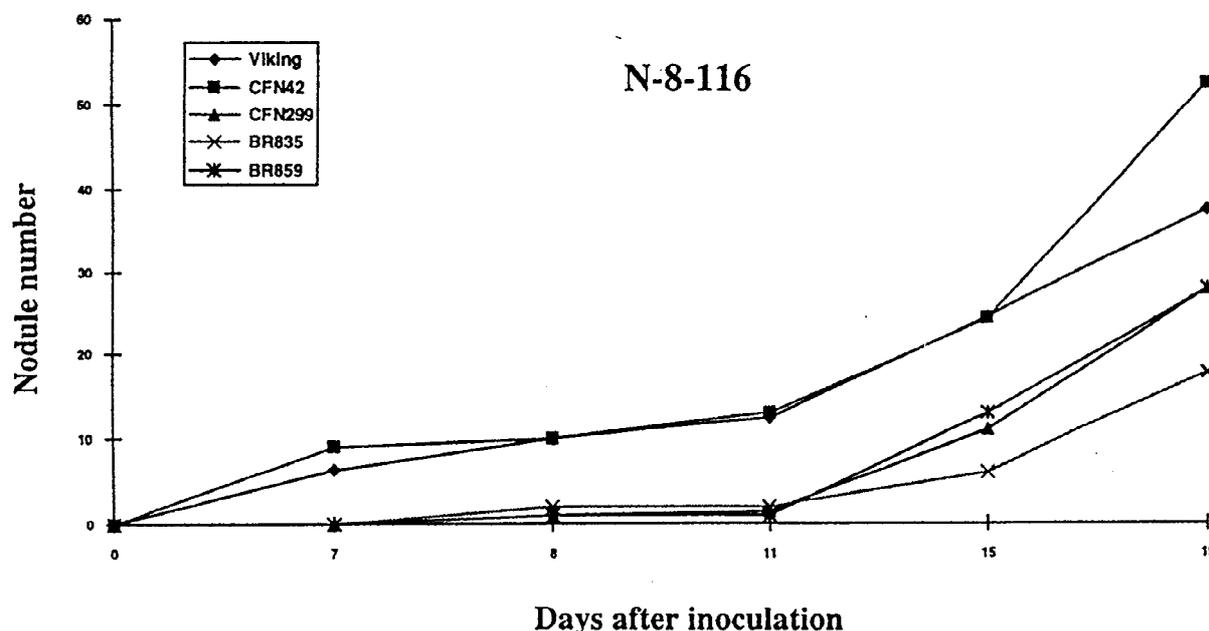


Figure 1. Nodulation kinetics of *Rhizobium* strains on the selected cultivar N-8-116 in agar flasks with Fahraeus medium. Average nodule per plant, 10 plants considered.

Discussion

Following the identification of superior bean cultivars for nitrogen fixation, it was imperative to test if modified *Rhizobium* strains were capable of increasing even further the symbiotic performance of these beans.

R. etli strain CFN42 containing the plasmid-borne citrate synthase gene from *R. tropici* strain CFN 299 has an increased nodulation capacity in the standard bean cultivar Negro Xamapa (Pardo et al., 1994). The biological reason for the increase in nodule formation is not known. The citrate produced by *R. tropici* CFN299 might be excreted to the medium and act as an iron chelator as in *B. japonicum* and other bacteria (Guerinot et al., 1990). We tested if citrate was present in CFN299 culture medium, but were unable to detect it by HPLC with serial Shodex Ion Pak columns (data not shown). Alternatively, the effect of having extra copies of the citrate synthase gene may be to provide additional substrates for energy production in the symbiotic process.

Other authors have reported that modifications in the respiratory chain in *R. etli* led to earlier nodulation and to increased nitrogen fixation in Negro Xamapa bean (Soberón et al., 1989). The effects were more pronounced when *R. etli* strain Viking1 was used instead of CFN42 (M. Soberón, personal com-

munication). Mutants derived from *R. etli* CFN42 that are incapable of synthesizing poly- β -hydroxybutyrate (PHB⁻) have increased nitrogen-fixation ability (Cevallos et al., 1996). We have obtained *Agrobacterium tumefaciens* transconjugants containing the symbiotic plasmid from *R. tropici* CFN299 that form nodules in *P. vulgaris* but with a low level of nitrogen fixation. A *tumefaciens* transconjugants that also contain a smaller plasmid (bp) from the same *R. tropici* strain formed more nodules and fixed more nitrogen (Martínez et al., 1987). This effect was not observed if the plants were grown at higher light intensity. *R. etli* transconjugants with the same plasmid from *R. tropici* had a higher nodulation capacity and increased competitiveness for nodule formation in Negro Xamapa (Martínez and Rosenblueth, 1990).

Bacteria may compensate for some deficiencies in cultivars that are not efficient for symbiosis or nitrogen fixation (such as Negro Xamapa). In this case, nodulation and nitrogen fixing are improved with bacteria modified in their metabolism. By contrast, there would be no advantage in utilizing *Rhizobium* strains with optimized metabolism in bean cultivars selected for their high nitrogen fixing capacity.

R. meliloti containing extra copies of citrate synthase genes does not have increased nodulation abili-

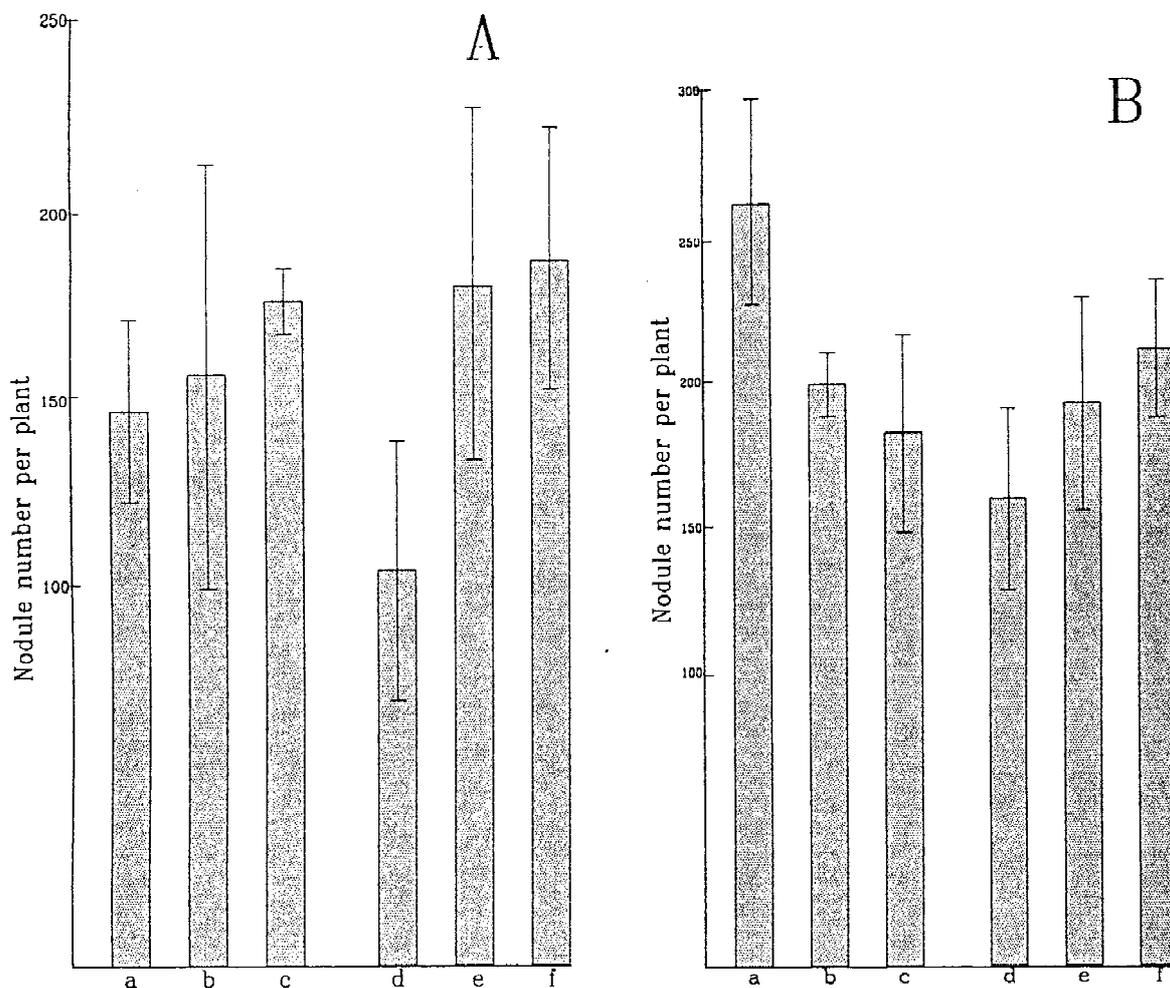


Figure 2. Effect of modified *Rhizobium etli* strains on nodulation of the *P. vulgaris* selected bean cultivars: (A) N-8-116 or (B) Bat-477. Plants were grown in flasks with vermiculite at 30 °C with a 12 h photoperiod in growth chambers. Nodule numbers per plant and plant dry weights were evaluated at 35 days after inoculation. Two independent experiments were performed. Average nodule number is presented and SDs are indicated (number of plants $n = 10$). *R. etli* strains CFN42 and Viking1 and their transconjugants (average of two independent colonies) were used (a) CFN42; (b) CFN42pcsA; (c) CFN42ccsA; (d) Viking1; (e) Viking1 pcsA; (f) Viking1 ccsA.

ties on *M. sativa* (M Kahn, personal communication). Similarly, we were unable to detect additive effects of *Rhizobium* modified strains and bean superior cultivars. Perhaps in these types of plants, symbiotic improvements may be obtained by different strategies; e.g. with higher nitrogenase activities. Recombinant *R. meliloti* strains with enhanced expression of the nitrogenase regulatory gene *nifA* and *dctABD* (for dicarboxylic supply) produced increased alfalfa yields under field conditions in some cases (Bosworth et al., 1994).

The plants from the selected cultivars were much greener and more developed than the Negro Xamapa

plants under the same conditions when nodulated with the unmodified wild type *R. etli* strains. We thus recommend the use of Bat-477 or N-8-116 as plant hosts for CFN42. With *R. tropici* strains, a very large number of nodules are formed in Jalo, a South American *Phaseolus vulgaris* cultivar (Nodari et al., 1993).

The different nodulation performances obtained with the various modified strains depicted in Figure 2 were not reflected in any differences in plant dry weight, neither in Bat-477 nor in N-8-116 cultivars at 35 days after inoculation (data not shown). Since the agronomic selection of beans rarely takes into account the nitrogen-fixing abilities, and as there are

many different cultivars used in agriculture (mainly based on local preferences), it is worth pursuing the improvement of *Rhizobium* strains. Improved constructs that consider more than one modification, e.g. additional citrate synthase genes with additional nitrogenase genes should be tested.

In addition, from our results with a very limited number of *R. tropici* and *R. etli* strains, it seems that the superior cultivars are better adapted to the *R. etli* strains than to *R. tropici* strains. *R. tropici* has been characterized as an opportunistic symbiont of bean due to its large host range (Hernández-Lucas et al., 1995b) and from the fact that *R. tropici* strains have been isolated from bean and *Leucaena* trees in regions where these plants are not native. It would be interesting to see if the selected Mesoamerican cultivars are more specific and better selectors of *R. etli* strains, which may be the symbionts that co-evolved with *P. vulgaris* (Segovia et al., 1993).

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