Determination of competitive abilities of *Bradyrhizobium japonicum* strains in soils from soybean production regions in South Africa

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Abstract *Bradyrhizobium japonicum* strain CB 1809 was recently chosen to replace strain WB 1 in commercial soybean (*Glycine max* (L.) Merr.) inoculants in South Africa, the selection criterion being N₂-fixing effectiveness. Nodulation competitiveness is an additional characteristic required of inoculants and was determined for CB 1809 and WB 1 as well as two other strains, USDA 110 and a Brazilian strain 965, using the *gusA* marker gene to identify strains. Initial experiments with plants grown in sterile sand showed that the competitive index of strain WB 1 was less than that of the other strains. Further comparisons used plants grown in five soils containing established populations of *B. japonicum*. When strains were applied in peat inoculum to seed at a rate of 1,000 cells per seed in a soil containing 300 rhizobia g⁻¹, significant differences in nodule occupancy were detected and strains ranked in the order 965 > CB 1809 > USDA 110 > WB 1. The remaining four soils each contained about 10⁶ rhizobia g⁻¹ and 5 × 10⁶ cells were applied per seed. Nodule occupancy by inoculant strains ranged from 22% to 81% between soils. In this experiment, WB 1 was consistently the poorest performer and its competitiveness was significantly less than CB 1809. The competition results supported the recent decision to replace WB 1 with CB 1809 in commercial inoculants. Although WB 1 had been used in inoculants over a period of 19 years, this strain was detected in only one soil, where it comprised 8% of isolates. In contrast, a substantial proportion (32–78%) of isolates from the soils corresponded serologically to a former inoculant strain WB 66, which had been discontinued in 1966. This illustrates the difficulty of replacing a resident population with an introduced strain. The effect of naturalized populations on the establishment of CB 1809 in South African soils will need monitoring.

Keywords *Bradyrhizobium japonicum* · *Glycine max* · Rhizobial competitiveness · Soil rhizobia · β-Glucuronidase marker

Introduction

Maximum benefit of N₂ fixation by soybean (*Glycine max* (L.) Merr.) often requires the inclusion of selected strains of *Bradyrhizobium* in seed inoculants. The inoculant strain must be effective in its ability to fix N₂ with the cultivar concerned and possess the ability to compete for nodulation of the plant with other strains of rhizobia that might be present in the soil. Strain competitiveness is influenced by the genetic diversity of both symbiotic partners (Triplett and Sadowsky 1992) as well as the soil environment in which nodulation occurs (Streeter 1994). Efforts to enhance competitiveness have included the isolation of bacteriocin-producing strains of rhizobia (Triplett 1990), use of selective genetic techniques such as random DNA amplification (Mavingui et al. 1997) and the development of soybean lines that are nodulated only by specific strains of rhizobia (Cregan and Keyser 1986). These have either achieved limited success or are still in the developmental stage. Selection for competitiveness remains an empirical process based on the ability of strains to nodulate roots in the presence of other competing rhizobia.

Although South African soils do not contain indigenous rhizobia that effectively nodulate soybean (Jansen van Rensburg et al. 1976), naturalized populations of *B. japonicum* have become established in soybean production regions as a result of previous inoculation (Jansen van Rensburg and Strijdom 1985). This is an important factor as the success of an inoculant strain is often lim-
ited by the presence of soil rhizobia that compete for nodulation. Brockwell et al. (1995) consider that inoculation is invariably futile in soils with populations greater than 1,000 rhizobia g⁻¹ whereas in soils with smaller, less effective populations a response would depend on the ability of the inoculant to compete with rhizobia in the soil. There is evidence, however, that a significant response to inoculation is possible in soils containing large numbers of established rhizobia when strains with both superior N₂-fixing efficiency and nodulation competitiveness are inoculated (Sylvester-Bradley et al. 1991; Hungria et al. 1998).

Determination of strain competitiveness was previously seldom undertaken in inoculant selection programmes because of technical difficulties. Earlier techniques relied mainly on antibiotic-resistant mutants or immunological methods to identify the inoculant strain (eg. Strijdom et al. 1987; Lochner et al. 1989). Mutation for antibiotic resistance often resulted in lowered effectiveness and competitiveness of rhizobia containing these markers (Lochner et al. 1989, 1991; Swelim et al. 1996) while immunological methods were often limited by the presence of cross-reactive antibodies. Both methods were arduous and expensive and identifications could only be made from small, statistically inadequate, samples of the large numbers of nodules formed (Wilson 1995a).

A new method for identifying inoculant rhizobia was recently developed, based on transfer of the gusA marker gene coding for the enzyme β-glucoronidase (GUS) into the desired strain (Wilson et al. 1995). This has helped in the isolation and selection of marked strains equally competitive with the wild type (Streit et al.1995; Sessitch et al. 1997). As nodules containing the marked strain are identified by colour formation after reaction with a suitable substrate, accurate typing of large numbers of nodules and accurate determination of competitive ability is possible (Wilson et al. 1995; Sessitch et al. 1997).

In an endeavour to improve the protein content of soybean [Glycine max (L.) Merr.] cultivated in South Africa, 20 strains of B. japonicum were recently evaluated for nitrogen-fixing ability on 30 local soybean cultivars (Bloom and Staphorst 1998). This resulted in selection of the Australian inoculant strain CB 1809 to replace strain WB 1. The former is an aggressive colonizer of the host plant rhizosphere and is persistent in the soil (Brockwell et al. 1995). In the present study, the gusA marker gene system was used to compare the competitive abilities of the two strains as well as that of USDA 110, ranked second by Bloom and Staphorst (1998). A Brazilian inoculant strain 965 of variable efficiency was also included in strain comparisons (Boddey and Hungria 1997; Bloom and Staphorst 1998; Hungria et al. 1998). The relative abilities of the individual strains to compete for nodulation of soybean were compared using plants grown in sterile sand as well as in soils containing established populations of B. japonicum.

Materials and methods

Bacterial strains and their origin are described in Table 1. Soybean cultivars (A5308, Highveld top, Mukwa, PAN 854) were provided by the ARC-Grain Crops Institute. The transposon mTnSSgusA31 was introduced into B. japonicum using a modification of the conjugation procedure described by Wilson et al. (1995). Wild-type strains of B. japonicum were grown in yeast-mannitol (YM) broth (Vincent 1970) at 28°C for 5 days (Avos=0.3–0.6). Escherichia coli strain S17–1(A-pr) harbouring pCAM131 was grown overnight at 37°C in LB broth (Miller 1972) containing 100 µg ampicillin ml⁻¹, then diluted 1:100 in LB medium and incubated a further 2.5 h (Avos = 0.3). Five millilitres of E. coli suspension was mixed with 10 ml B. japonicum and the cells were centrifuged and washed by re-suspension in yeast salts (YS) broth (YM medium minus mannanitol). Finally, cells were re-suspended in 200 µl YS broth and spread either on YM agar containing an increased yeast extract concentration of 0.5%, or on YSA agar (YS salts containing 0.1% yeast extract and 0.1% arabinose). After overnight incubation at 28°C, cells were scraped off the agar surface and suspended in 2 ml water containing 0.01% Tween 20. Transconjugant strains of B. japonicum were selected by plating on minimal medium (Law and Strijdom 1984b) modified to contain 10% glycerol (m/v) as carbon source. The medium contained streptomycin at a concentration of 200 µg ml⁻¹ for strains CB 1809 and USDA 110, or 400 µg ml⁻¹ for strains 965 and WB 1. After incubation, individual colonies were streaked on minimal medium agar containing spectinomycin (500 µg ml⁻¹) and observed for growth. Colonies that were resistant to spectinomycin and streptomycin were selected for further use. All B. japonicum cultures were subsequently maintained on YM agar slants. Wild-type strains and gusA-marked derivatives used to prepare inoculants for plant tests were stored in water at 22°C (Crist et al. 1984).

Serological comparisons

Rabbit antiserum was prepared against each B. japonicum strain and the IgG antibody fraction purified (Law and Strijdom 1984a) for use in gel immunodiffusion tests (Dudman 1964). Preparation of fluorescein isothiocyanate (FITC)-labelled strain WB 1 antibody, immunofluorescence and whole cell agglutination reactions were essentially as described by Somasegaran and Hoben (1994). Whole antiserum against strain WB 66 was from a previous study (Jansen van Rensburg et al. 1976) and FITC-labelled antibodies against B. japonicum strains CB 1809 (TAL379), USDA 110 (TAL 102) and USDA 138 (TAL 377) were from NiFTAL, University of Hawaii, Hawaii.

DNA analysis

Total genomic DNA was isolated from wild-type and putative transposon-containing strains and subjected to dot blot hybridization analysis using a non-radioactive kit (Boehringer Mannheim, Germany) and digoxigenin-labelled probes prepared from Nor1 transposon fragments containing gusA. DNA from selected strains containing mTnSSgusA31 insertions was digested separately with EcoRV and Nor1 and subjected to electrophoresis on 0.8% agarose before Southern hybridization using the gusA probe.

Greenhouse experiments

Experiments with gusA-marked rhizobia were carried out under contained greenhouse conditions with approval from the South African Committee for Genetic Experimentation. Initial strain