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Influence of *Glycine max* nodulation on the persistence in soil of a genetically marked *Bradyrhizobium japonicum* strain

L.D. KUYKENDALL
USDA, Agricultural Research Service, Plant Sciences Institute, Nitrogen Fixation and Soybean Genetics Laboratory, Bldg. 011, HH-19, BARC-West Beltsville, MD 20705, USA

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Abstract

Since competition with indigenous strains limits nodule occupancy by bacteria applied to seeds, the ecology of Bradyrhizobium inoculum strains used for soybean is of concern. A genetically marked strain, *B. japonicum* I-110 ARS, was directly enumerated from soil on selective medium. A clear long-term positive influence of even limited *Glycine max* nodulation was shown by comparisons of population densities obtained with or without plant removal prior to nodule senescence in the first year and with an incompatible as well as a compatible soybean variety after 5 years.

Introduction

Bradyrhizobium japonicum strain I-110 ARS, with antibiotic resistance markers, was identifiable as the inoculum strain in nodules of field-grown soybeans (Kuykendall and Weber, 1978). This strain was used to indirectly demonstrate that nodulation of wild-type soybeans, in contrast to the $rj_1 rj_1$ 'non-nodulating' genotype, promotes the establishment of soybean bradyrhizobia in soils not previously containing *B. japonicum* (Kuykendall *et al.*, 1982). Genetically marked strains of legume microsymbionts have been shown to be useful for quantitative recovery from soil and rhizosphere (Bushby,1982; Cooper, 1979). The purpose of this study was to directly examine the influence of soybean nodulation on the long-term survival and establishment of *B. japonicum* in field soil. Strain I-110 ARS was found to be directly recoverable from soil on a selective medium containing rifampicin, streptomycin, and pimaricin. This technique was used to test the effect of competitively limited nodule occupancy on cell densities of *B. japonicum* in soil.

Materials and methods

Strain I-110 ARS is a symbiotically competent strain of *Bradyrhizobium japonicum*, derived from strain I-110 (Kuykendall and Elkan, 1976), and has resistance to azide ($10\,\mu g\,ml^{-1}$), rifampicin ($500\,\mu g\,ml^{-1}$), and streptomycin ($1\,mg\,ml^{-1}$). HM salts basal medium (Cole and Elkan, 1973) was used with the addition of $1\,g\,l^{-1}$ arabinose and $1\,g\,l^{-1}$ yeast extract (Difco). For field inoculation, cultures were grown from a 10% inoculum in 10 liter batches in glass carboys with aeration by sparging. For direct enumeration of strain I-110 ARS from soil, one-gram soil samples were suspended in HM salts + 0.01% Tween 20 by vigorous vortexing for 1 minute. One-tenth ml aliquots of diluted suspensions, containing between 0.5 and 2.2 mg of soil per ml, were routinely spread onto agar plates containing R + S + P medium ($500\,\mu g\,ml^{-1}$ rifampin SV, $1\,mg\,ml^{-1}$ streptomycin, and $100\,\mu g\,ml^{-1}$ pimaricin in AIE medium). At the above concentration, pimaricin (Sigma), a potent fungicide, did not interfere with growth of strain I-110 ARS. Antibiotics were filter-sterilized in 100-fold concentrations and

added to fresh sterile agar (1.5% purified agar) medium just prior to pouring the plates. Results of viable counts, expressed as colony-forming units (CFU) per gram of soil, were taken after 7 days incubation at 30°C.

Comparisons were made of the population densities of the bacteria in soil with or without first year plant removal prior to senescence and with a compatible and an incompatible variety of soybeans after 5 years of soybean cultivation. Variety 'Peking' was selected as an incompatible soybean host. It does not accept nodulation by strain USDA 110 when grown in soils containing other strains although it does when inoculated with pure cultures and grown aseptically under greenhouse conditions (Caldwell and Vest, 1968; Kuykendall and Weber, 1978). The field plots were contained in open-ended 1-m diameter concrete cylinders, hereafter referred to as 'tiles', sunk into the ground vertically 1 m. The soil used in this study had a previsously-established rich population ($> 10^6 g^{-1}$) of diverse *B. japonicum* strains of various serogroups including 110 and 123. This pre-existing bacterial population was challenged by high density inoculation with strain I-110 ARS. In the initial inoculation, six tiles were densely planted in five mounds or 'hills' of 10 soybean seeds each of the variety 'Kent' and each hill received 300 ml of inoculum containing 3×10^9 CFU ml^{-1} of strain I-110 ARS. Sterile medium only was applied to three control tiles planted with Kent soybeans. In six additional tiles Peking soybean was sown as described above. The seeds in three tiles were inoculated with strain I-110 ARS and the seeds in the other three plots received only 300 ml of sterile medium. During the first year three replicates of the Kent plots were subjected to removal of all the plants including the nodulated root systems, at about 10 weeks after planting and inoculation. In subsequent years, the same cultivars were planted in each tile as in the first year, without further inoculation, and the plants were allowed to grow to maturity and senesce.

Nodule harvest, sterilization, and plating onto selective media were previously described (Kuykendall and Weber, 1978).

Results and discussion

The selective medium R + S + P was effective for direct enumeration of strain I-110 ARS from soil. The limit of detection was 50 viable cells per gram of soil. Serological tests confirmed that the reisolates from soil were *B. japonicum* serogroup 110. Soil from uninoculated plots did not contain bacteria able to grow on this selective medium. Nodule occupancy by strain I-110 ARS in the first year was about 7% for Kent and about 0.1% for Peking (Table 1). In subsequent years, Kent soybeans grown in the same plots without further inoculation had less than or equal to 1% nodule

Table 1. Direct counts from soil and nodule occupancy of *B. japonicum* strain I-110 ARS in field plots and in either "Kent" or "Peking" soybeans

Plot history/ 1st yr treatment	Nodule occupancy (%)		Direct soil estimation[a]
	1st yr	subsequent	(CFU g^{-1}), after 5 yr
Kent, 0 I-110 ARS	0.0	0.0	0
Kent, + I-110 ARS, Plants removed	7.0	$\leqslant 1.0$	5.0×10^3
Kent, + I-110 ARS, Allowed to Senesce[b]	7.0	$\leqslant 1.0$	2.5×10^4
Peking, 0 I-110 ARS	0.0	0.0	0
Peking, + I-110 ARS	0.1	0.0	4.0×10^3

[a] Differences in viable counts obtained between treatments within varieties were statistically significant ($P < 0.001$). Test for significant differences was Duncan's multiple range.

[b] A viable count of 1.3×10^5 CFU g^{-1} of soil was obtained six months after plant maturity in these plots. This was about 10-fold higher than in other treatments.

occupancy, whereas none of the nodules on Peking had strain I-110 ARS.

Soils in which soybeans had been previously cultivated may be expected to have about 1×10^4 rhizobia g^{-1} and an inoculation rate of about 1×10^3 times the soil population is required to produce 40–50% nodule occupancy by an inoculum strain (Weaver and Frederick, 1974). It has been hypothesized that release of viable *B. japonicum* from decaying nodules promotes the long-term survival of the bacteria in their soil environment. The results of this study provide clear evidence for the validity of this concept since, after 5 years, strain I-110 ARS was recovered at significantly higher levels that were five-fold greater in plots where nodulated soybean plants had remained in the soil the first year compared to plots where plants had been removed prior to nodule senescence (Table 1). Thus, host plant nodulation indeed plays an important positive role in the establishment of populations of *B. japonicum* in soil.

Strain I-110 ARS was nevertheless recovered from soil at levels about 80-fold higher than the limit of detection in plots where the incompatible variety 'Peking' soybeans had been grown for 5 years. Since there was no recovery of strain I-110 ARS in Peking nodules after the first year, this would suggest that the surviving bacteria were maintained as a rhizosphere population rather than by nodule passage. Rhizosphere effects are however apparently not a significant factor in competitiveness (Moawad *et al.*, 1984).

In conclusion, a genetically marked *B. japonicum* strain, permitting precise identification and enumeration from soil, was shown to have significantly higher persistence in soil due to even limited nodulation of a compatible host plant.

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