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SHORT COMMUNICATION

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Influence of biosolids compost on the bradyrhizobial genotypes recovered from cowpea and soybean nodules

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Abstract The interaction of biosolids compost application to soil and bradyrhizobial genotypes recovered from nodules was examined. Among 170 isolates, seven genotypes were recovered from soils receiving either no biosolids application or rates of 73 or 146 Mg/ha for three successive years. With the exception of one genotype, the distribution of the bacterial genotypes recovered from nodules was interrelated with the level of biosolids. Two of the genotypes nodulated both soybean and cowpea. Because soybean-nodulating bradyrhizobia were not recovered from control plots, it is possible that they had been introduced together with the biosolids compost application.

Keywords Biosolids compost · Cowpea · Soybean · Nodulation · Nitrogen fixation · *Bradyrhizobium* · Genotype · Symbiosis · Plant-microbe interaction

Introduction

Biosolids is a semi-solid organic material produced in wastewater treatment facilities. In addition to organic nutrients, biosolids contains soluble salts and trace amounts of heavy metals such as cadminum, copper, lead, nickel, iron, mercury and zinc (Hornick, et al. 1984). In the District of Columbia, large quantities of biosolids are produced with little or no land area available for waste disposal. The application of biosolids to farmland offers a means for disposal and recycling of urban waste (Guisquiani, et al. 1988; Hornick et al. 1984). The U.S. Environmental Protection Agency (EPA) has recommended the use of biosolids compost as a means for disposal and waste recycling (US EPA 1989, 1993).

It has been suggested that the rhizobia of clover in soils amended with biosolids eventually become ineffective for symbiotic nitrogen fixation (Angle et al. 1993; Rothe et al. 1983). A possible explanation offered for this observation is that the applied biosolids induce a change in the population of *Rhizobium leguminosarum* (Hirsch et al. 1993; Obbard et al. 1993) from one that is effective to one that is ineffective. However, Robson et al. (1989) indicated that nitrogen fixation in white clover need not necessarily be influenced by biolsolids amendments since effective rhizobia in other locations may well be more resistant.

The influence of biosolids application to soil on nitrogen fixation and yield of soybeans also has been investigated. There were no adverse effects on yield of soybean grown in soil that had been treated with biosolids for 15 years prior to planting (Heckman et al. 1987). However, comparison of these results with those of clover probably is not justified since the bacterial symbionts forming the nitrogen-fixing symbioses with each crop species are different. Soybeans growing in the USA form nitrogen-fixing symbioses with *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, while clover is nodulated by *Rhizobium leguminosarum* bv. *trifolii*. It is possible for cowpea to nodulate with *B. japonicum* and *B. elkanii* (Keyser et al. 1983), but it is more common for this crop to form symbioses with *Bradyrhizobium* spp. collectively referred to as “the cowpea miscellany group”. The “cowpea-miscellany” bradyrhizobia are not able to nodulate the roots of the soybean cultivar Williams (Keyser et al. 1983; Nautiyal et al. 1988).

Since the microbial symbionts of cowpea may be different from those of soybean and clover, there is no information available about the effects of biosolids treatment of soil on the symbioses of cowpea. Therefore, the objective of our study was to determine the effects of biosolids

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compost amendment of soil on the genetic diversity of *Bradyrhizobium* recovered from nodules of cowpea.

Materials and methods

Isolation of bradyrhizobia

Soils were obtained from plots at the Muirkirk Research Farm in Beltsville, Md., USA, which are silty loam with a pH of 5.2. This experimental site has no history of crop production and the land was covered with grass before it was used as an experimental site. The plots were arranged in a completely randomized block design replicated three times. Replicated plots received three annual applications of biosolids compost, in 1995, 1996, and 1997, at the rates of 73 and 146 Mg/ha that elevated the pH from 5.2 to 7.5. The biosolids compost was tilled into the top 15-cm layer of the soil. The amendment led to elevated levels of Pb (64.2–95.3 μg/g), Cd (1.3–2.2 μg/g), Zn (1.0–1.2 μg/g) and Cu (78.3–99.2 μg/g). Soils from replicated control plots (containing 48.3, 0.8, 0.5, and 45.1 μg/g of Pb, Cd, Zn, and Cu, respectively) not receiving biosolids compost also were collected. These soil samples were used to isolate bacterial symbionts of cowpea and soybean under N-limiting conditions for plant growth. Sterilized Leonard jar assemblies (Leonard 1943), containing sand:vermiculite 1:1 (w/w) mixtures moistened with N-free plant nutrient solution (Norris 1964), were used together with soil samples of approximately 500 mg for each seed to trap the bacteria. Seeds of *Vigna unguiculata* cultivar California black eye 5 were surface-sterilized with 1% Clorox for 5 min and then were rinsed six times with sterile distilled water. The surface-sterilized seeds were germinated on moist sterile filter paper in petri dishes at 30 °C for 24 h. Three germinated seeds were planted in each jar in furrows made in the sand:vermiculite mixture. Plants were grown for 30 days in a greenhouse receiving supplemental lighting set at light/dark cycles of 14/10 h and temperatures were controlled to 24/20 °C. Plants were harvested and all nodules were used to isolate the bacteria. Nodules were surface-sterilized with 1% Clorox for 5 min, subsequently treated with 70% ethanol for 30 s and then rinsed six times with sterile distilled water in preparation to isolate the bradyrhizobia according to the method described by Vincent (1970) using modified arabinose-gluconate (MAG) agar media (van Berkum 1990). Each isolate was successively purified on MAG agar plates from single colonies. Bradyrhizobia were isolated from nodules of *Glycine max* cultivar Williams using the same approach as described for cowpea. Each isolate was tested for nodulation of the original host plant using 10 ml of 10^9 cells/ml MAG broth cultures as inoculum.

Genetic characterization of isolates using Box PCR

Single colonies of each isolate, taken from the surface of MAG plates, were suspended in 200 μl of 0.1% (v/v) Tween 20. The cell suspensions were lysed at 95 °C for 10 min. Subsamples (1.5 μl) of the lysed cells were used in 25-μl PCR reactions according to the method described by Martin et al. (1992) using a Taq DNA polymerase buffer combination (Promega, Madison, Wis.) with Extender (Stratagene, La Jolla, Calif.). The PCR products in 10 μl of each reaction mixture were separated by horizontal gel electrophoresis with 1% (w/v) agarose gels amended with 1% (w/v) Synergel (Diversified Biotech, Boston, Mass.) and 0.5 μg ethidium bromide/ml using 0.5×TBE buffer (Sambrook et al. 1989).

Nodulation studies with selected bradyrhizobial genotypes

Seeds of *Glycine max* cultivar Williams, and *Vigna unguiculata* cultivar California black eye 5 were surface-sterilized, pre-germinated and planted in sterilized 120-cm diameter pots containing sterile sand:vermiculite 1:1 mixtures as described before. Broth cultures of isolates 0–13, 0–25, 0–40, 73–3, 73–28, 146–11 and 146–31 were inoculated at sowing as described before. The inoculated and the uninoculated plants were grown in a greenhouse and harvested 6 weeks after planting. Plants were cut at the cotyledonary node, the tops were dried at 60 °C for 48 h to measure dry weights, the nodules were collected from the roots and dry weights were determined.

Results

Cowpea was effectively nodulated when grown in Leonard jars in the presence of small quantities of all the soils collected from Muirkirk Research Farm. A combined total of 152 nodules were formed on all the plants and isolates were recovered from each nodule. These cowpea isolates were grouped into seven distinct genotypes according to the Box PCR analysis (Fig. 1).

More than half of the isolates belonged to genotype I, which was detected in control soils and soils containing both levels of biosolids compost (Table 1). Genotypes II and III were only detected in control soils, genotypes IV and V were isolated only from soils with 73 Mg biolsolids/ha, and genotypes VI and VII were recovered only from soils with 146 Mg biosolids/ha (Table 1).

The fingerprint patterns obtained with these isolates and those of reference cowpea-miscellany strains of *Bradyrhizobium* differed (data not shown).

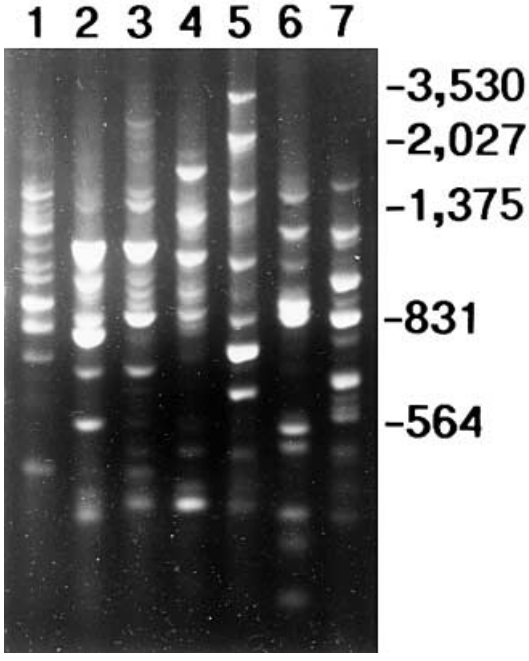

Fig. 1 Box PCR fingerprint patterns generated with the seven representative bradyrhizobial isolates from cowpea nodules. The representative isolates chosen for each genotype were 0–13, 0–25, 0–40, 73–3, 73–28, 146–11, and 146–31, where the first number indicates the control soil (0) or level of biolsolids compost (73 or 146 Mg/ha) and the second number represents the purified colony from each nodule. *Lane 1* Isolate 0–13, *lane 2* isolate 0–25, *lane 3* isolate 0–40, *lane 4* isolate 73–3, *lane 5* isolate 73–28, *lane 6* isolate 146–11, lane 7 isolate 146–31. Molecular size markers in kb are indicated on the *right margin*

Table 1 Isolates recovered from the nodules of cowpea nodulated by bradyrhizobia present in control soils and soils amended with 73 and 146 Mg/ha biosolids compost

	Number of isolates of genotypes						
Treatment	I	II	III	IV	V	VI	VII
Control	41	4	2	0	0	0	0
73 Mg/ha	17	0	0	18	27	0	0
146 Mg/ha	24	0	0	0	0	8	11

Table 2 Nodulation test of seven bradyrhizobial genotypes with cowpea or soybean as host plant. Each value is the mean of four determinations. Mean values within the same column flanked by different letters are significantly different ($P<0.01$) by Duncan's new multiple range test

	Cowpea Dry matter (mg/plant)		Soybean Dry matter (mg/plant)	
Isolate	Nodule	Plant top	Nodule	Plant top
0–13	65.0b	509.3c	0.0c	744.5abc
0–25	104.3a	538.8c	0.0c	709.8bc
0–40	114.3a	723.0a	0.0c	739.8abc
73–3	108.5a	859.5a	42.3b	877.5ab
73–28	110.3a	862.8a	57.3a	924.5a
146–11	116.8a	725.3ab	0.0c	667.0c
146–31	50.8b	615.0bc	0.0c	725.5bc
Control	0.0c	543.3c	0.0c	694.8bc

The seven isolates representing each genotype were used to evaluate nodulation and nitrogen fixation with cowpea and soybean. Isolates 0–13 and 0–25 (representing genotypes I and II, respectively) nodulated cowpea but were ineffective for nitrogen fixation since the top dry-weights of plants in these treatments and the controls were not significantly different (Table 2).

Isolates 0–40, 146–11, and 146–31 (representing genotypes III, VI, and VII, respectively) formed moderately effective nitrogen-fixing symbioses with cowpea, as determined from comparing plant dry matter accumulation with the uninoculated controls (Table 2). Plant dry matter accumulation in the treatments with isolates 73–3 and 73–28 (representing genotypes IV and V, respectively) was significantly higher than in the uninoculated controls. Only isolates 73–3 and 73–28 nodulated soybean and formed effective nitrogen-fixing symbioses (Table 2). In separate experiments, reference cowpea-miscellany bradyrhizobial strains (USDA 3384 and USDA 3456) did not nodulate Williams soybean, while reference soybean bradyrhizobial strains (USDA 6, USDA 76, and USDA 110) nodulated both cowpea and soybean.

A total of 18 nodules were formed on soybeans grown in the presence of soils collected at the Muirkirk Research Farm. Of the 18 isolates recovered, 11 and seven originated from plants grown in the presence of soils amended with 73 and 146 Mg biosolids/ha, respectively. In the presence of control soils soybean was not nodulated. All the soybean isolates had a Box PCR fingerprint pattern identical with isolate 73–28 and nodulated both cowpea and soybean.

Discussion

Many research articles have appeared in the literature that collectively indicate the existence of highly diverse native populations of rhizobia in soils (van Berkum and Eardly 1989). Diversity among populations of *Bradyrhizobium* also has been described (Barrera et al. 1997; Neves and Rumajanek 1997). Unlike most of these studies, we have found that the soils in our investigation harbor a very limited diversity of bradyrhizobia that nodulate cowpea and that this diversity is even more limited in the case of nodulation of soybean. We concluded this from the discovery that there were only three genotypes among 88 isolates recovered from cowpea nodules of plants grown with control soils as inoculum. Also, we observed that the majority of these isolates (82 isolates) belonged to only one of the genotypes (genotype I).

We are not aware of many studies that have attempted to interrelate rhizobial genotype with the level of biosolids treatment of soil. In our case, the distribution of the genotypes of *Bradyrhizobium* recovered from cowpea nodules appeared to be interrelated with the level of biosolids amendment of the soil, with the exception of genotype I. In the case of the rhizobia of clover, such an interrelationship was reported not to be apparent (Ibekwe et al. 1997). The different results between our study and that of Ibekwe et al. (1997) may be due to environmental change and/or the class of rhizobia investigated. The distribution of the bradyrhizobial genotypes may have been associated with the higher pH, changes in nitrogen availability and increased level of some of the metals.

From our data we conclude that adding biosolids to soils changed the population structure of the bradyrhizobia that were present in the soil of our experimental site. All the genotypes recovered from nodules of cowpea grown with control soils did not nodulate soybean. Furthermore, soybean grown in the presence of the control soil was not nodulated. Yet, genotypes IV and V recovered from biosolids-amended soils were capable of nodulating soybean, and soybean was nodulated when grown in the presence of biosolids-amended soils. Therefore, the amendment of soil with biosolids compost introduced bradyrhizobia that form symbioses with soybean to the soil of our experimental site.

Our determination for the presence of different bradyrhizobial genotypes was made using the host plant to extract the bacteria from the soils by sampling plant nodules. Because of this, we cannot completely exclude the possibility that other genotypes were present. Our results may have been affected by variation in competition for nodulation of cowpea among the genotypes. For example, genotype IV was only recovered from the 73 Mg biosolids/ha treatment with cowpea, even though we detected the presence of this genotype in the 146 Mg/ha treatment using soybean as trap host.

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