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Occurrence of *Azospirillum brasilense* in soils amended with swine manure

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Abstract - Restriction analysis of amplified 16S rDNA from bacterial strains isolated from soil was used to evaluate the effects of swine manure and urea fertilisation on indigenous populations of the plant growth promoting bacterium, *Azospirillum brasilense*, in corn- cultivated experimental plots. Several operational taxonomic units (OTUs) were found among the isolates. One of them, identified as *A. brasilense*, was found only in the control and in the organically amended plots, while treatment with urea reduced the presence of this species to under the detection threshold. Neither the organic nor the chemical treatment reduced the general biodiversity of the sampled soils, which was defined by the number of different OTUs within each plot. The spreading of pig slurry seems to be a good management practice in corn-cultivated agrosystems, as it does not affect the presence of *A. brasilense*.

Key words: swine manure fertilisation, *Azospirillum brasilense*, ARDRA.

INTRODUCTION

A current theme in applied soil microbial ecology is to understand the effects of agricultural practices on the composition of soil microflora, as it is generally recognised that the composition of the microbial community could represent a good indicator of soil fertility (Benedetti *et al*., 2000). To establish if a particular fertilisation treatment constitutes a stress condition for microorganisms, it is necessary to know the microbial diversity and composition occurring in that particular soil, with and without treatment. Consequently, the availability of suitable tools for measuring this effect is the first step toward the potential use of microorganisms as bioindicators in soils affected by human treatments. Bacteria involved

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in the nitrogen cycle are particularly affected by organic fertilisation, thus they could be exploited as appropriate bioindicators in agricultural soils (Ceccherini *et al.*, 1998; Miclaus *et al.*, 1994). Bacteria of the genus *Azospirillum*, microaerophilic nitrogen-fixing Gram-negative rods belonging to the α-subclass of Proteobacteria (Krieg and Döbereiner, 1984), are often associated with the roots of many agriculturally important crops. They promote root growth (Okon and Yutrhak, 1987) by inducing beneficial morphological and physiological changes through the release of phytohormones, enhancing both water and mineral uptake (Fallik and Okon, 1995) and providing fixed nitrogen. A large number of *Azospirillum* strains have been isolated from soils of temperate regions showing high survival capability outside the rhizosphere (Bashan *et al.*, 1995). Five species have been described: *A. lipoferum, A. brasilense, A. amazonense, A. halopraeferens* and *A. irakense* (Fani *et al.*, 1995).

Since many *Azospirillum* strains are used as agricultural inocula, evaluation of the effects of agricultural practices on this genus and, in particular, on the more commonly studied *A. brasilense* has become necessary in order to exploit the presence of this species as a bioindicator of disturbed soils (Vannini *et al.*, 1990).

Amendment of agricultural soil with manure is of general use, but its effects on indigenous microbial communities and on the dynamic equilibrium of the nitrogen cycle in intensive agricultural systems have been only partially evaluated (Miclaus *et al.*, 1994; Ceccherini *et al.*, 1998).

The development of molecular techniques made possible the rapid and precise detection of bacterial taxa from discrete habitats (Moyer et al., 1996). A variety of molecular methods have been developed during the last decade for the detection of *Azospirillum* isolates in the environment (Giovannetti *et al.*, 1990; Fani *et al.*, 1993; Fancelli *et al.*, 1998; Kabir *et al.*, 1995; Assmuss *et al.*, 1995). Among these techniques, the restriction fragment length polymorphism of 16S rRNA amplified genes (ARDRA-Amplified Ribosomal DNA Restriction Analysis) (Vaneechoutte *et al.*, 1992) has already been utilised for *A. brasilense* identification (Grifoni *et al.*, 1995). It is considered a powerful tool in the study of bacterial communities since rRNA genes can be easily amplified and analysed by polymerase chain reaction (PCR) (Moyer *et al.*, 1996).

In the present work, we used the restriction analysis of amplified 16S rDNA from soil isolated bacterial strains to evaluate the effects of swine manure fertilisation on indigenous populations of *Azospirillum* to find out whether this amendment could affect, by either increasing or decreasing the occurrence of isolated *A. brasilense*s.

The strategy adopted included: *i)* the isolation of bacterial clones in a nitrogen-free minimal medium for *A. brasilense, ii)* the lysis of the isolated colonies and the PCR amplification of 16S rDNA gene sequences with eubacterial primers, *iii)* the restriction analysis of amplified 16S rDNA (ARDRA), and *iv)* the screening of restriction patterns to group clones in operational taxonomic units (OTUs) (Moyer *et al.*, 1994).

MATERIALS AND METHODS

Soil samples. Experimental corn-cultivated plots at the San Prospero Agronomic Experimental Centre of Modena, Italy were fertilised for three years (May 1993, 1994, 1995) with stored pig slurry manure whose whose composition is shown in Table 1. The soil was alluvial and classified as Vertic-Calcic Gleyic Cambisol 3a (FAO 1998); its chemical and physical characteristics were: tot. N: 1.88‰; organic matter: 2.43%; C/N ratio: 7.5; P tot. (P as P_2O_5): 1.73‰; assimilable P (ppm P_2O_5): 24.6; exchangeable K (ppm K_2O): 389; pH (H_2O): 7.9 (Costantini and Spallacci, 1987). The quantities of total N applied as manure were 75 and , 225 kg ha^{-1} per plot L2 and L4, respectively. Plot L5 received 150 kg ha^{-1} of N as urea per year; the control plot L1 did not receive any fertilisation.

Soil samples were collected from the four plots considered, L1, L2, L4 and L5, during the third year of fertilisation, at on 0-15 cm from the surface layer. Five samples from each plot were randomly collected, mixed together and passed through 4mm and 2mm sieves. Samples were taken seven days after the fertilisation (May) and a few days after the corn crop the harvest ingin November. Plot L2 (75 kg N ha^{-1}) samples were not taken at this time.

Enrichment and isolation of *Azospirillum* strains. Sieved soil samples (10g) were homogenised in 90 ml sterile distilled water for 20 min; 1 ml of 10^{-3} and 10^{-4} dilutions was transferred into tubes containing 9 ml of liquid N-free MSP medium (Bani *et al*., 1980) and incubated 7 days at 35 °C; 100 µl of serial dilutions were plated on N-free MSP agar. After an incubation of for 2 days at 35 °C, 30 well-isolated colonies per plot were randomly chosen to be re—isolated ontoto N-free MSP agar plates ands used for DNA analysis.

Amplification and restriction analysis of 16S rDNA (ARDRA). Amplification of nearly the entire 16S rDNA was performed directly on a single bacterial colony with two universal primers 27f and 1495r, as described by Grifoni *et al*., (1995). An aliquot of each PCR reaction, containing about 400 ng of DNA, was treated

TABLE 1 – Composition of the pig slurry applied to the soil samples in the three years (Spallacci and Marchetti, 1995)

Determination (%)	1993 mean (n=9)	*CV (%)	1994 mean (n=5)	CV (%)	1995 mean (n=3)	CV (%)
Dry matter	0.73	6.7	5.75	3.6	4.90	3.8
pH	7.90	0.9	7.54	0.7	7.62	0.4
total N	0.135	2.0	0.263	2.4	0.286	2.8
$N\text{-}NH_4$	0.111	5.0	0.139	9.2	0.124	4.0
P	0.011	18.6	0.170	2.2	0.170	0.0
K	0.141	22.1	0.100	11.1	0.107	9.9

* CV = coefficient of variability.

with 10 U of the restriction enzyme *Alu*I (Roche, Germany) in a total volume of 30 µl, at 37 °C for 2 h.

The reaction products were analysed by agarose (2.5% wt/vol) gel electrophoresis. The electrophoretic patterns from of the isolated clones were compared with those of the following reference strains: *A. brasilense* Cdr (ATCC 29710), *A. brasilense* SPF94 (Fani *et al*., 1988), *A. lipoferum* (ATCC 29731), *A. amazonense* Y2 (ATCC 35120).

Amplification of the *nif* H gene. Total DNA from the most frequent OTUs was extracted as described by Ausbel *et al*. (1987) and then amplified. The reaction mixture was composed of 400µM of each *nif* H primers (Kloos *et al*., 1995), 400µM dNTP mix, 1x PCR buffer, 1.5 mM $MgCl_2$, and 1U Expand Taq polymerase (Roche) in a 50µl final volume. The amplification cycle was: 95 °C 3 min, two cycles of 94 °C 1 min, 60 °C 1 min, 72 °C 2 min; two cycles of 94 °C 1 min, 55 °C 1 min, 72 °C 2 min; two cycles of 94 °C 1 min, 50 °C 1 min, 72 °C 2 min; 25 cycles of 94 °C 1 min, 60 °C 1 min, 72 ° C 2 min. The PCR products were then run on 2% agarose gel in TAE 1x.

Hybridisation. Digoxigenin labelling, Southern blotting and hybridisation were performed as described in the Dig System User's Guide (Roche) under stringent hybridisation conditions. The probe was a 432bp *nif* H fragment from *A. brasilense*, as described by (Kloos *et al*.. (1995).

N_2 fixation assay. Nitrogen fixing activity was assayed by the acetylene reduction method described by Bani *et al*.. (1980).

Sequencing and analysis of partial 16S rDNA. The amplified 16S rDNA was purified with the a gel extraction kit (Gene clean – Bio 101) according to the manufacturer's instructions. The DNA was then sequenced by the method of Sanger *et al*. (1977), using the New England BioLabs "CircumVent" thermal cycle dideoxy-DNA sequencing kit and ^{35}S – dATP to label the DNA synthesised, according to the instruction manual. The sequencing reactions were performed using the primers 27f and 559r (Di Cello *et al*., 1997). The nucleotide sequence of the 16S rDNA fragments was ranked against the Ribosomal Database (Maidak *et al*., 2000) to find similar sequences.

RESULTS AND DISCUSSION

The 16S rDNA was amplified from thirty isolated strains for each soil plot, for a total of 210 strains. The amplicons were digested with the endonuclease *Alu*I, which generates species-specific restriction patterns in *Azospirillum* (Grifoni *et al*., 1995). Strains with identical profiles were grouped and 9 different OTUs were obtained. There was an average of 5 different OTUs per plot, with little variation among plots (Table 2).

ARDRA profiles from the four the more numerous OTUs, represented by more than five strains, are shown in Fig. 1. The *Alu*I restriction patterns indicated

TABLE 2 – Number of OTUs per plot

Sampling	Plot	Number of different OTUs
May	L1	5
May	L2	3
May	L4	5
May	L5	6
November	L1	5
November	L4	6
November	L5	5

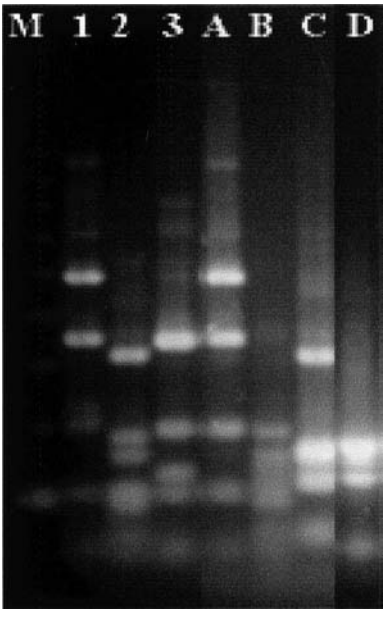

FIG. 1 – ARDRA profiles from *Azospirillum* and the representative OTUs (A, B, C, D) obtained with restriction endonuclease *Alu*I. Lane 1: *A. brasilense* SPF94; lane 2: *A. amazonense* Y2 (ATCC 35120); lane3: *A. lipoferum* (ATCC 29731); M: Ladder 123.

that OTU A corresponded to *A. brasilense*; other OTUs corresponded mostly to the *Pseudomonas* and *Agrobacterium* groups and were not investigated further on their ability to fix nitrogen (data not shown).

Comparing the occurrence of *A. brasilense* (OTU A) among the isolates of the four plots in the two samplings showed that this species was detected only in the control (L1) and in the organically amended plots (L2 and L4), both in May and November. Treatment with urea (plot L5) dramatically reduced the presence

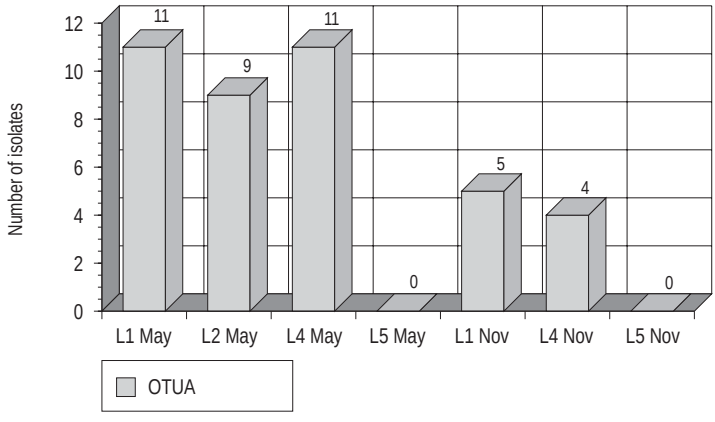

FIG. 2 – Presence and distribution of isolates belonging to Operational Taxonomic Unit A (OTU A) in the sampled plots. The considered plots, L1, L2 and L4, were amended, respectively, with: 0,75 and 225 kg ha^{-1} of N applied as swine manure per year; L5 received 150 kg ha^{-1} of N as urea per year.

of *A. brasilense* below the detection threshold (Fig. 2). One member of OTU A was tested for the presence of the *Azospirillum nif* H gene, both by PCR amplification and by hybridisation, and for nitrogen fixing activity. Finally, it was identified by 16S rDNA sequence analysis. As shown in Table 3, OTU A gave positive results for the presence of the *nif* H gene and nitrogenase activity, and the sequence analysis confirmed it was *A. brasilense*.

The occurrence of *A. brasilense* in agricultural soils could be an effective bioindicator of soil fertility conditions. We followed the presence of *A. brasilense* and its fluctuations according to soil treatment and season. In May, *A. brasilense* represented almost 1/3 of the isolates in the control and in the organically amended plots, while in November, after harvesting, it decreased to about 1/6 of the isolates in both the L1 and L4 plots. This decrease could be due to the end of the mutualistic relationship between plants and bacteria brought about by the start of winter, or, alternatively, to the growth of other competitive species. *A. brasilense* was never isolated from the soil treated with urea, indicating that this compound could reduce the presence of this species. According to Roper *et al*. (1994), nitrogenase activity by free-living bacteria in soil is depressed by urea and this should be evaluated when using this species as an inoculant. We can not exclude, however, that this reductive effect could be due to a urea-dependent induction of non-culturability for *A. brasilense*.

Though the enrichment and the isolation procedure used in this work could have limited the range of bacteria analysed, some conclusions can be drawn since a culturable population can also provide specific information about the effects induced by anthropogenic activities on biodiversity (Øvreås and Torsvik, 1998).

Neither the organic nor the chemical treatment reduced biodiversity (number of OTUs) during and after cultivation, although the species composition showed variations. This result indicates that, in the soil analysed, a dynamic equilibrium

TABLE 3 – Taxonomic affiliation of the OTU A

OTU	*nif* H amplification	*nif* H hybridisation	Nitrogenase activity	16S rDNA primers	Genebank accession number*	Similarity Rank Sab value	Taxonomic Affiliation**
A	+	+	+	27f	AF126448	0.614	*Azospirillum brasilense* 129/2
				559r	AF126449	0.763	*Azospirillum brasilense* str. Sp81

* The two stretches of nucleotide sequence obtained with the two primers were submitted to the Genebank and given the corresponding accession number.
** Strain with the most similar sequence (Ribosomal Database Project).
+ : positive result.

exists between bacterial populations and biodiversity is preserved in spite of organic or chemical fertilisation procedures. Pig slurry spreading seems to be an environmentally friendly procedure of fertilisation since even high doses did not affect the presence of *A. brasilense,* chosen in this work as a bioindicator of soil nitrogen regeneration capability.

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