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ORIGINAL PAPER

The effects of pesticides on bacterial nitrogen fixers in peanut-growing area

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Abstract In the peanut production, the applications of herbicides and fungicides are a common practice. In this work, studies done under field conditions demonstrated that pesticides affected negatively the number and nitrogenase activity of diazotrophic populations of soil. Agrochemical effects were not transient, since these parameters were not recovered to pre-treatment levels even 1 year after pesticides application. Results obtained from greenhouse experiments revealed that the addition of herbicide or fungicides diminished the free-living diazotrophs number reaching levels found in soil amended with the pesticides and that the number of symbiotic diazotrophs was not affected by the insecticide assayed. The soil nitrogenase activity was not affected by fungicides and glyphosate. The effect of pesticides on the nitrogen-fixing bacteria diversity was evaluated both in field and greenhouse experiments. Analysis of clone libraries generated from the amplification of soil *nifH* gene showed a diminution in the genetic diversity of this bacterial community.

Keywords Peanut · Pesticides · Soil diazotrophs · *nifH*

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Introduction

Many kinds of pesticides, including insecticides, fungicides, herbicides and other pesticides are of fundamental importance in agricultural production. Continual widespread use and release of such synthetic chemicals has become an everyday occurrence, resulting in environmental pollution. It has been estimated that only 0.1 % of applied pesticides reaches the target pests and the remaining 99.9 % accumulates in soils and affects directly or indirectly the microbial density and enzyme activities (Pimentel 1995; Das and Debnath 2006; Singh and Singh 2005; Pal et al. 2006). Microorganisms are fundamental components of soil ecosystem as they degrade soil organic matter and sustain biogeochemical transformations of elements (Lin et al. 2007). As they respond rapidly to any changes in soil composition, soil microbial communities are regarded as bioindicators and used for the assessment of soil quality and for the prediction of soil degradation (Groffman and Bohlen 1999). Moreover, it has been recently recognized that they play an evolutionary role in maintaining a gene pool from which new genotypes probably emerge in response to selective pressures resulting from changes in soil management (Fulthorpe et al. 1995). Therefore, if an evolution toward sustainable agriculture is desirable, it is needed to consider the potential harmful side effects of pesticides on the soil microflora. In this context, many researchers have carried out "in vitro" or at field studies to analyze the influence of pesticides on the viability and activity of soil microorganisms (Martinez-Toledo et al. 1998; Das and Debnath 2006; Ampofo et al. 2009). Results from assays done under field conditions have often been controversial, probably related with variations that could be influencing the effects of pesticides such as the type of soil or the structure of soil microbial community.

Products to control weeds, plant diseases and insect pests are typically applied as mixtures only once or sequentially during the growing season. A common practice in crop production is the application of one or more herbicides at planting, followed by sequential foliar post-emergence fungicide applications. This approach is used almost universally for peanut (*Arachis hypogaea* L.) by farmers in Argentina.

Nitrogen is often a limiting factor for crop productivity, affecting plant communities and ecosystems in all scales (Tan et al. 2003). In order to increase the yield of agronomical crops, nitrogen fertilization is widely used. However, the use of commercial nitrogen fertilizers is usually an expensive resource to supplement nitrogen for plant growth. Furthermore, improper or excessive use of nitrogen fertilizer may lead to nitrate pollution of grounds or water surfaces.

Many of the nitrogen-fixing bacteria that have been identified are free living diazotrophs such as *Azospirillum brasilense*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Erwinia herbicola* (Pedersen et al. 1978; James et al. 1994; Hirano and Upper 2000; Silva et al. 2003), while others are symbiotic nitrogen fixers. Among these, rhizobia are able to induce nodule formation on leguminous plant such as peanut.

A molecular dialogue between the two partners (plants and bacteria) is required to coordinate the events leading to the symbiosis (Fabra et al. 2010). Plant roots continuously release flavonoids that accumulate in the rhizosphere and constitute the first signals. These molecules activate the bacterial transcriptional regulator protein NodD, which in turn induces the translation of other rhizobial nodulation genes (*nod*, *nol* and *noe* genes) involved in the synthesis and secretion of the main bacterial nodulation signals called Nod factors or lipo-chito oligosaccharides (Spaink 2000). Nod factors cause morphological changes in legume root hairs, leading to infection thread formation, nodule development and symbiotic nitrogen fixation.

Rhizobial genes called *nodA*,*B*,*C* are common for almost all the rhizobia strains studied. Specifically, NodC protein is a progressive β-glycosyl transferase that forms chitin oligomers from UDP-GlcNAc. Since the NodC protein determines the Nod factor chain length, it could be involved in the Nod factor host-specificity (Perret et al. 2000).

The objective of this research was to evaluate the effect of pesticides applied to soil at recommended rates on the number of bacteria and diversity of nitrogen-fixing bacteria in soil from peanut-growing area in Argentina.

Materials and methods

Field experiment

Assays were conducted at the experimental field of the University of Río Cuarto, located at 33°07′ south latitude, 64°14′ longitude W of G (central region of Argentina), at an altitude of 421 m above the sea level, on a sandy loam Typic Hapludoll soil. The field experiments were conducted in 10 m^2 microplots with three replications following randomized block design. One of the microplots was not treated with pesticides and served as control.

Seeds of peanut cultivar Oleico (obtained by Criadero "El Carmen," Córdoba, Argentina) were hand planted in November 2009. The first agrochemical application was done 2 days after planting seeds; this application was herbicides and insecticide. The pesticides applied to soil at recommended rates were shown in Table 1. The second agrochemical application was done 45 days after planting seed. At this time only, the commercial formulation containing the fungicides azoxystrobin + cyproconazole (1 l ha^{-1}) was applied. In the control microplots, weeds were removed manually every 10 days.

Five soil samples (each of them constituted by 25 subsamples) were taken at different times: (a) 2 days before each agrochemical application, (b) 2 days after each agrochemical application and (c) 12 months after the first agrochemical application. Soil sub-samples (cores of 2 cm diameter) were randomly taken at 10–15 cm deep from bulk soil, pooled, sieved and stored in bags at 4 °C. Before used for all the determinations done in this work, 10 g of soil samples was dried at 37 °C for a week in order to indicate the number of bacteria in dried weight.

Greenhouse experiments

Thirty-six undisturbed soil samples from field (cores of 30 cm diameter and 25 cm deep) without agrochemical treatment were taken from the experimental field. Each sample was put inside a plastic bag, and one surface-sterilized peanut seed was sown at 5 cm deep. After 2 days, one different agrochemical was added to each bag, at the same concentration used at field, by spraying the solution on the soil surface. Treatments were arranged in a randomized complete block with four replications. Plants were grown in a greenhouse under controlled environmental conditions (light intensity of 200 μE $m^{-2}S^{-1}$, 16-h day/8-h night cycle, at a constant temperature of 28 °C). Soil was watered as required to maintain 60 % field capacity.

Determination of the number of bacteria of soil nitrogen-fixing bacteria

Ten grams of each soil sample obtained as described above were transferred to Erlenmeyer flasks containing 45 mL of 0.1 % sterile sodium pyrophosphate (NaPP). The flasks were shaken for 30 min at 180 rpm and 0.1 mL aliquots of serial dilutions (up to 10^{-8}) were streaked onto Petri dishes containing nitrogen-free semisolid malate medium (NFb)

Table 1 Pesticides used in the experiment

Pesticide	Chemical	Recommended rates
Pre-emergence herbicides		
S-metolachlor	2-chloro-6′-ethyl-N-(2-methoxy-1-methylethyl) acet-o-toluidide	1 l ha^{-1}
Diclosulam	N-(2,6-dichlorophenyl)-5-ethoxy-7-fluoro [1, 2, 4] triazolo [1,5-c] pyrimidine-2-sulfonamide	20 g ha^{-1}
Pre- and post-emergence herbicide		
Glyphosate	*N*-(phosphonomethyl) glycine	3.5 l ha^{-1}
Imazethapyr	{2-[4,5-dihydro-4-methyl-4- (1-methylethyl)-5- oxo-1H -imidazole-2-yl]-5-ethyl-3-pyridine carboxylic acid}	1 l ha^{-1}
Imazapic	2-[4,5- dihydro-4- methyl-4- (1-methylethyl)-5-oxo- 1H-imidazol- 2-yl]-5-methyl-3-pyridinecarboxylic acid	30 ml ha^{-1}
Insecticide a commercial formulation containing gamma + cyhalothrin and Lambda-cyhalothrin	{(S-α-cyano-3-phenoxybenzyl(1R,3R)-3-[(Z)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate)}	
	{(S)-α-cyano-3-phenoxybenzyl (Z)-(1R,3R)-3-(2-chloro-3,3,3-trifluoro-prop-1-enyl)-2,2-dimethylcyclopropanecarboxylate and (R)-α-cyano-3-phenoxybenzyl (Z)-(1S,3S)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate}	25 ml ha^{-1}
Fungicides azoxystrobin + cyproconazole	(E) -2-{2-[6-(2-cyano-phenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxy-acrylate + 3-chloro-4-[(2RS, 4RS, 2RS, 4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl) -1,3-dioxolan-2-yl] phenyl -4-chlorophenyl ether	1 l ha^{-1}

(Döebereiner 1995). Colonies obtained after 3 days of incubation at 28 °C were transferred to tubes containing soft NFb medium, and their nitrogen-fixing capability was evaluated by the formation of a growth halo under the surface. For the isolation of symbiotic N_2-fixing rhizobial bacteria, yeast-extract mannitol agar medium (YEMA) (Vincent 1970) was used. It was confirmed that colonies growing on YEMA were rhizobia by PCR amplification of *nifH* and *nod*C genes. The *nodC* gene was amplified using primers NodCrev1160 (5-CGY GAC ARC CAR TCG CTR TTG-3) and NodCfor540 (5-TG ATY GAY ATG GAR TAY TGG CT-3) (Sarita et al. 2005). The *nifH* gene was amplified using primers PolF (5-TGCGAYCCSAARGCBGACTC-3) and PolR (5-ATSGCCATCATYTCRCCGGA-3) (Poly et al. 2001).

DNA isolation

DNA was extracted from field and greenhouse soil samples (0.25 g) using Mo BIO Kit (MOBIO laboratories, California, USA) and purified using polyvinyl-poly pyrrolidone (PVPP) columns (Petric et al. 2011). DNA were quantified using a PicoGreen-based method (Quant-iT™ dsDNA quantification, Invitrogen) against standard curve of λ-phage DNA calibrated using a spectrophotometric method.

nifH PCR amplification and clone library construction

The *nifH* gene fragments (360 bp) were amplified by PCR with the primers PolF and PolR following the method described by Poly et al. (2001). The 50 μL reaction mixture contained 1 μL of template DNA (50–100 ng), 10 mM Tris–HCl, pH 8.3, 10 mM KCl, 0.5 mM of each primer, 200 mM of each dNTPs, 2.5 mM $MgCl_2$ and 2 U of Taq polymerase (Promega). PCR comprised 30 cycles (denaturation, at 94 °C for 1 min, annealing 55 °C for 1 min and primer extension at 72 °C for 2 min) with a final extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 1.4 % agarose gel and purified using the QIAquick PCR Purification kit (Qiagen) and cloned into the pGEM-T easy vector (Promega), following suppliers instructions. After transformation of *Escherichia coli* DH5α, 220 clones were picked and the presence of the expected size inserts was evaluated by PCR using M13f (5′-GTAAAACGACGGCCAG-3′) and M13r (5′-CAGGAAACAGCTATGAC-3′) primers.

Restriction fragment length polymorphism (RFLP) from *nifH*: DNA extracted from soil of the experiment carried out in greenhouse

Ten microliters of each PCR product were directly used for restriction enzyme cleavage by *Msp*I or *Hinf*I (Promega). These endonucleases provide fragments in the range of 20–360 bp. Digestions were performed overnight to ensure that complete fragmentation was achieved. Digested DNA fragments were analyzed in 11 % polyacrylamide gel (acrylamide/bis-acrylamide 19:1) using manufacture recommendations, staining with ethidium bromide and washed for 10 min in deionized water prior to UV transillumination.

The band patterns of *nifH* RFLP fingerprinting were converted into a binary matrix through a binary scoring system

(one for the presence of band and zero for the absence). Computer-assisted analysis of the fingerprints was carried out using Cross Checker system software 2.91 (Buntjer 1999). With the assistance of the FAMD (Fingerprint Analysis with Missing Data) software package (Schlüter and Harris 2006), a dendrogram was constructed from the distance matrix by the means of Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

Phylogenetic analysis

Twenty-one clones were randomly selected to evaluate their phylogenetic relationship. Their partial *nifH* sequence analyses were performed by using the algorithm BLASTN (Altschul et al. 1997) to identify similarities. Phylogenetic analyses were conducted using BioEdit using CLUSTAL X (Thompson et al. 1994), MEGA version 5 (Tamura et al. 2011) and PhyML (Guindon and Gascuel 2003; Posada 2008) softwares. The best-fit nucleotide substitution model was selected according to jModeltest 0.1.1 software (Posada 2008).

Clone library evaluation

Analyses of the clone libraries were followed by calculation of coverage (C), where C is expressed by 1-n1/N, in which n1 is the number of clones that appeared only once, and (N) the total number of clones (Chelius and Triplett 2001). Shannon–Wiener index, $H' = -\sum_{i=1}^{S} pi \ln pi$ (Margelef 1958) and Evenness, $E = H'/\ln S$ (Pielou 1969) were also determined.

Soil nitrogenase activity determination

It was measured using the acetylene reduction assay (ARA) (Hardy et al. 1968). Three control or treated 10-g soil samples were incubated individually with 10 % acetylene during 21 days. Ethylene production was quantified by using a Konik KNK 3,000 series gas chromatograph equipped with a hydrogen flame ionization detector and a microbore column. Nitrogenase activity was determined using linear regression analysis from a standard curve generated with diluted ethylene gas. The amount of reduced nitrogen was estimated by using a conversion factor of 3.8 acetylene molecules reduced per one dinitrogen molecules (Jensen and Cox 1983). A negative control (soil incubated without acetylene) was also included.

Statistical analysis

Data analysis was carried out using the Infostat software and treatments were analyzed by ANOVA and differences among treatments were detected by LSD test ($p < 0.05$).

Results

Effects of pesticides on the number of bacterial free-living diazotrophs

The addition of pesticides to agricultural soil negatively affected the population of aerobic nitrogen-fixing bacteria. The number of diazotrophs in soil samples amended with pesticides was significantly reduced, particularly after the first application, but also after the second one. Twelve months after the first application of pesticides, the size of free-living diazotroph population was still not recovered (Fig. 1a).

In an attempt to elucidate if this negative effect could be attributed to a specific agrochemical, greenhouse experiments were done. The addition of herbicides, fungicides or insecticides separately also affected negatively the size of nitrogen-fixing bacterial population (Fig. 2a). However, only in soils treated with the herbicide imazapic or with the fungicide composed by azoxystrobin + cyproconazole, the diazotroph number diminution was as low as in soil treated with the agrochemical mixture.

Effects of pesticides on the number of bacterial symbiotic diazotrophs

Thirty-eight bacteria isolated from the soil samples assayed were able to grow on YEMA plates, and presence of the *nodC* gene was analyzed in all of them. A fragment of the expected size (640 bp) was obtained from the 43 % of the analyzed colonies (data not show). The number of bacteria of this symbiotic diazotrophic population decreased by 70 % after the first application of pesticides and remains without changes even after the second application. Twelve months after the first pesticide application, the number of symbiotic diazotrophs increased but do not reached the control one (Fig. 1b).

When the effect of each agrochemical was evaluated, it was found that only the application of the commercial mixture of insecticides do not affect the number of symbiotic diazotrophs. The diminution in the number of bacteria produced by all the other treatments did not differ from that obtained in soils treated with the agrochemical mixture (Fig. 2b).

Effects of pesticides on soil nitrogenase activity

Nitrogenase activity was determined in soil samples. This enzyme activity decreased after the first treatment in field experiment, since there was a diminution of 34 % in the amount of fixed nitrogen in the soil, remaining without changes after the second treatment. Twelve months after the first application of pesticides, soil nitrogenase activity did not reach the level found in untreated soil (Fig. 1c).

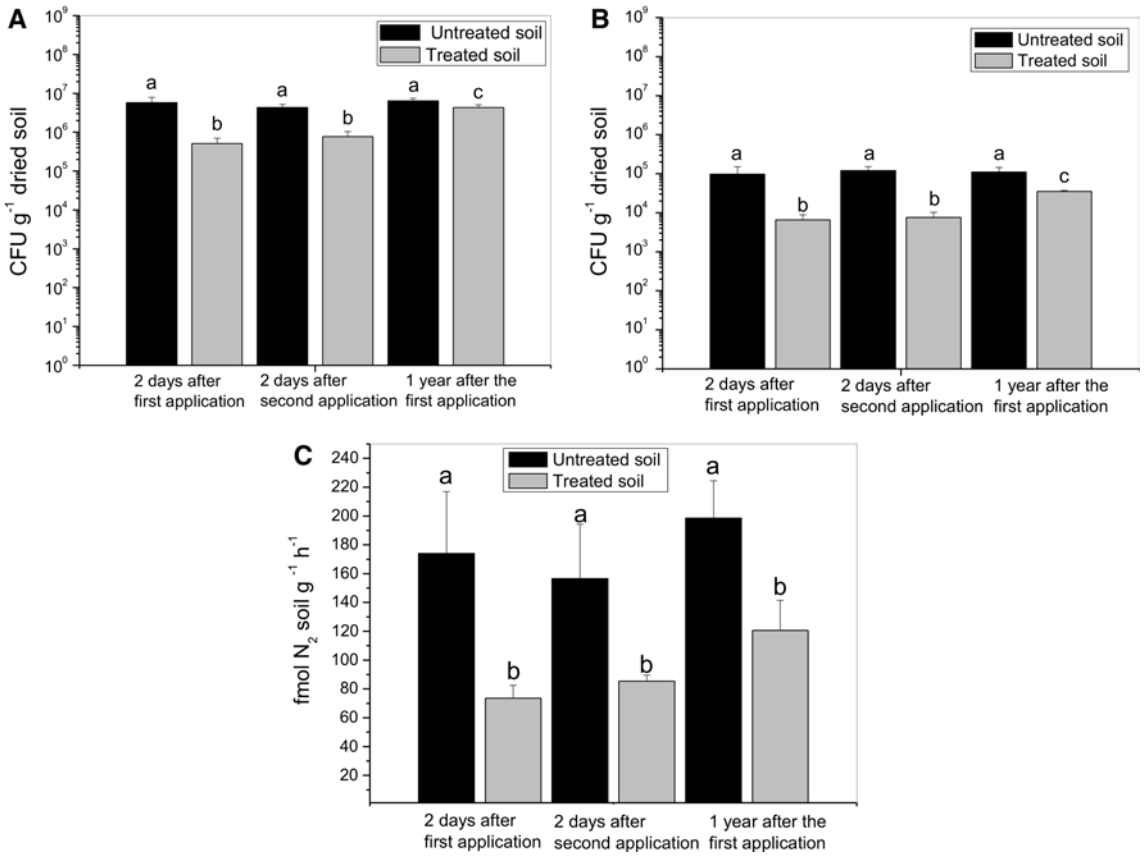

Fig. 1 Experiment on field effects of pesticides in soil. **a** Number of free-living diazotrophs in soil samples. **b** Number symbiotic diazotrophs in soil samples. **c** Nitrogenase activity Values are the mean ± SE of five replicates ($N = 5$). Different letters in each column indicate significant differences ($p < 0.05$) among control without pesticides according to LSD multiple range test. The figure a and b *y-axis* is CFUg^{-1} dried soil in log scale

In greenhouse experiments only the addition of fungicides and glyphosate did not affect the soil nitrogenase activity. Although among the treatments the differences were not remarkable, the lowest activity was determined in soils amended with the pre-emergence herbicide diclosulam (Fig. 2c).

Effects of pesticides on the diversity of nitrogen-fixing bacteria from DNA extracts from soil in experiment of field

From a total of 220 *nifH* clones (110 *nifH* clones from control and 110 *nifH* clones from treatment soil), 21 were randomly selected and sequenced (6 from untreated soil samples and 15 from treated soil samples). All clones analyzed were identified as *nif H* sequence, making them firmly meaningful for phylogenetic analyses. The phylogenetic distribution of clones based on *nifH* gene sequence in the samples was quite homogeneous and clustered in the taxonomic groups Alphaproteobacteria and Cyanobacteria (Fig. 3).

The phylogenetic dendrogram obtained from *nifH* sequences of nitrogen-fixing bacteria isolated from untreated control samples differed from that of treated ones. Values of the Shannon-Wiener index were 1.26 and 0.23 in the libraries obtained from control or treated soil samples, respectively. The highest evenness value was observed in untreated soil samples. In order to check whether the size of clone libraries was reflecting the real diversity, the coverage index was checked according to Chelius and Triplett (2001).

Since libraries obtained in this study covered 34 % (untreated soil samples) and 99 % (treated soil samples) of the total diversity based on the BLAST-N identification, results obtained could be indicating a change in the bacterial diversity in soil treated with pesticides.

RFLP analysis of *nifH* PCR products from DNA extracts from soil in experiment of greenhouse

Restriction fragment length polymorphism of *nifH* PCR products from soil DNA using *MspI* or *HinfI* enzymes

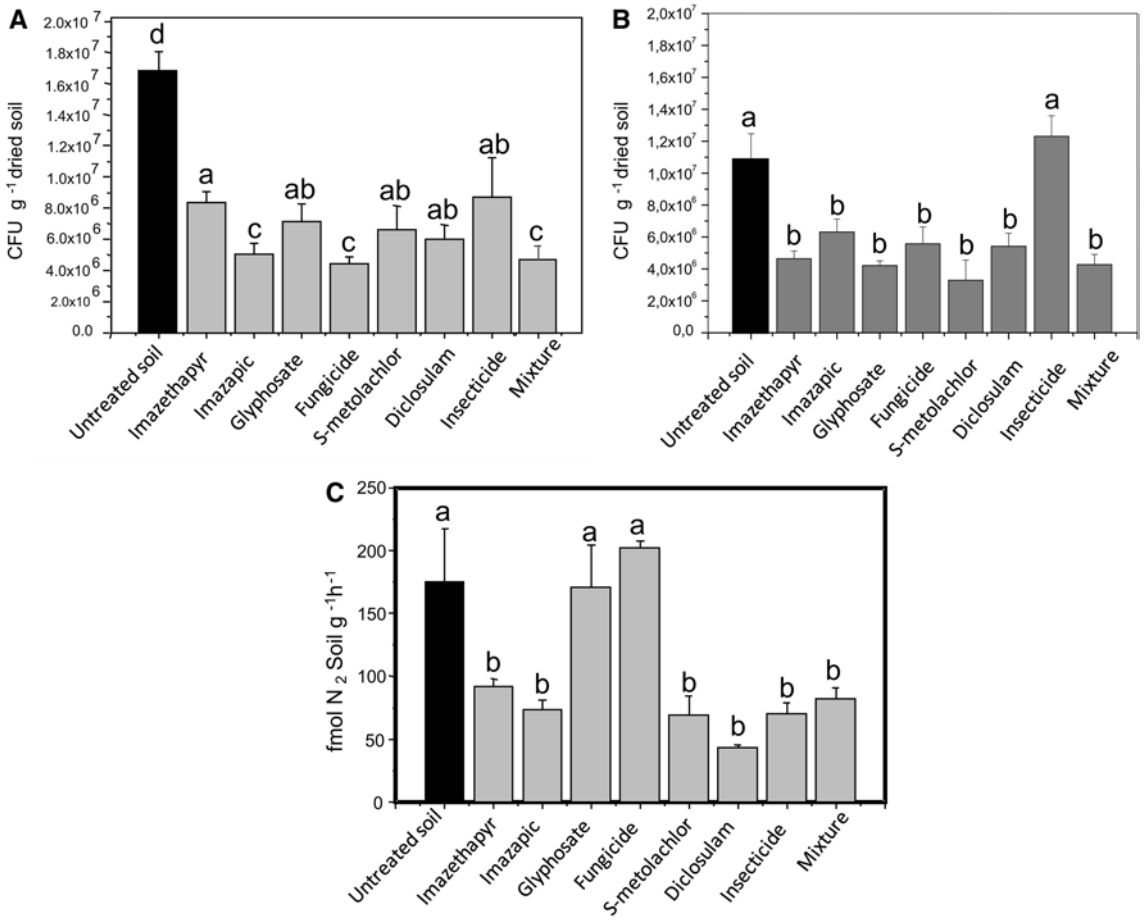

Fig. 2 Experiment on greenhouse effects of pesticides in soil. **a** Number of free-living diazotrophs in soil samples. **b** Number symbiotic diazotrophs in soil samples. **c** Nitrogenase activity Values are the mean ± SE of five replicates ($N = 5$). Different letters in each column indicate significant differences ($p < 0.05$) among control without pesticides according to LSD multiple range test

showed good restriction pattern (data not shown). Each enzyme produced a specific profile for each DNA sample. The dendogram shows that, at 90 % of similarity coefficient, different soil samples grouped into four different clusters (Fig. 4). Group IV clustered RFLP derived from *nifH* genes from soil treated with herbicides imazapic, glyphosate and with the pesticides mixture. Group III included those derived from herbicide S-metolachlor and untreated soils, while those from insecticides and herbicide diclosulam clustered in Group I and II, respectively.

Discussion

Agricultural soils are heterogeneous environments and different conditions may affect microbial diversity and activity. Soil nitrogen-fixing bacteria are important contributors to primary productivity and their population may be greatly affected by the agrochemical application. The practical importance of these microorganisms is evident because they convert yearly about 200 million tons of nitrogen to ammonia, being the major quantity of nitrogen biologically fixed due to the activity of symbiotic nitrogen fixers (Glazer and Nikaido 2007). A better understanding of factors affecting the biological nitrogen fixation will help to maximize the effectiveness of this process, improving agricultural sustainability. Therefore, it is important to determine how addition of synthetic chemicals to the soil may affect the composition of this bacterial community, considering that these changes can be regarded as early and sensitive indicators of ecosystem disturbance. In this study, long-term fluctuation in the number, diversity and nitrogen fixation ability of diazotrophic community in soil treated with pesticides were investigated.

From the results obtained, it is evident that the number of bacteria of symbiotic and non-symbiotic diazotrophs decreased significantly after the first application of pesticides. These effects were not transient, since this parameter was not recovered to pre-treatment levels, even 1 year

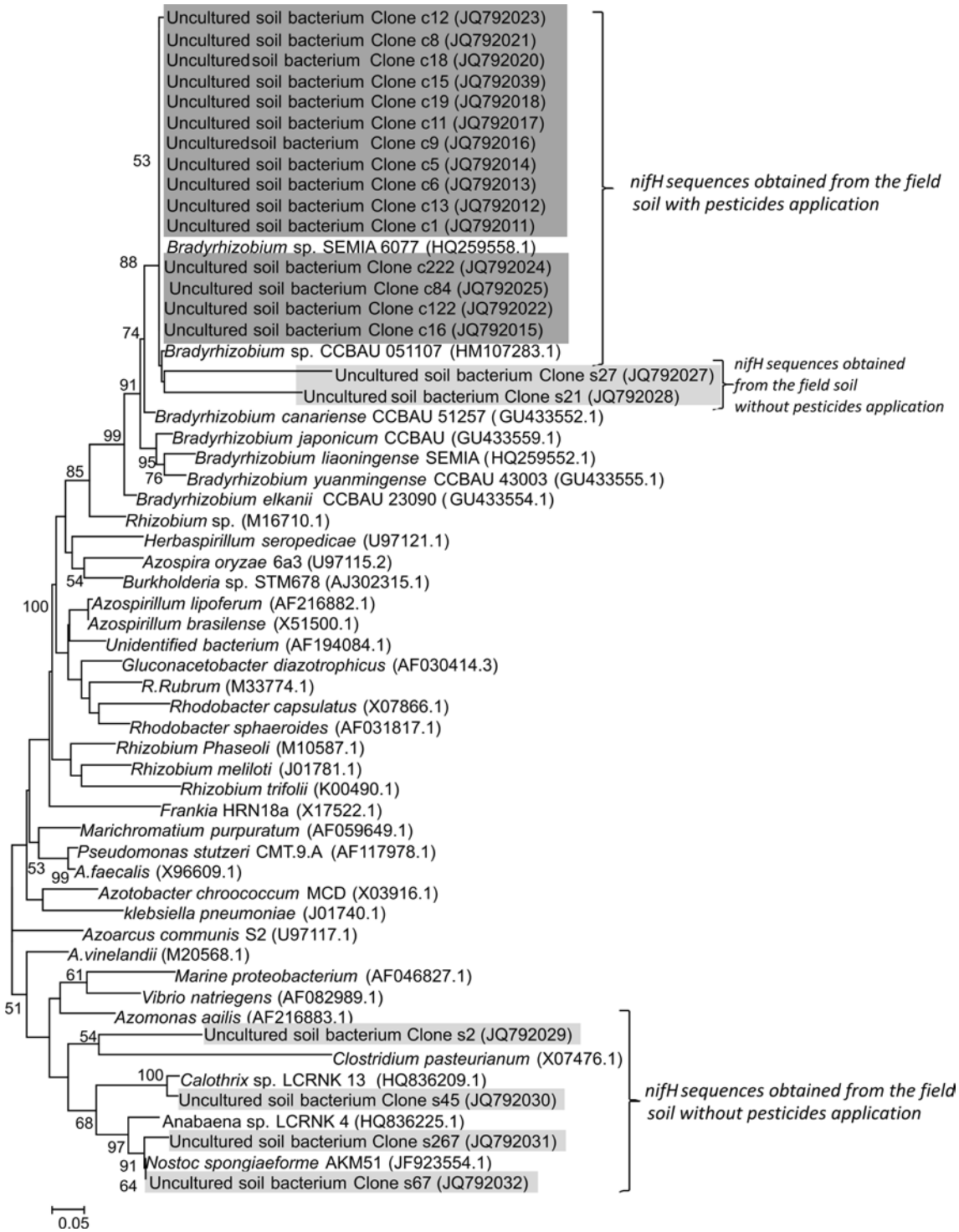

Fig. 3 Phylogenetic tree based on 360 bp of the *nifH* gene. The tree was inferred by the neighbor-joining clustering method under the Tamura–Nei + G substitution model. Bootstrap values (over 50 %) for 1,000 replicates are shown

after the treatment. Furthermore, the population of symbiotic nitrogen fixers seems to be somehow more sensitive, considering that 365 days after the first agrochemical application, only the 30 % of this population was recovered, compared with the 49 % from the free-living diazotrophic population (Figs. 1a, 2a).

The impact of a particular pesticide on soil microorganisms and biological functions are determined by a number

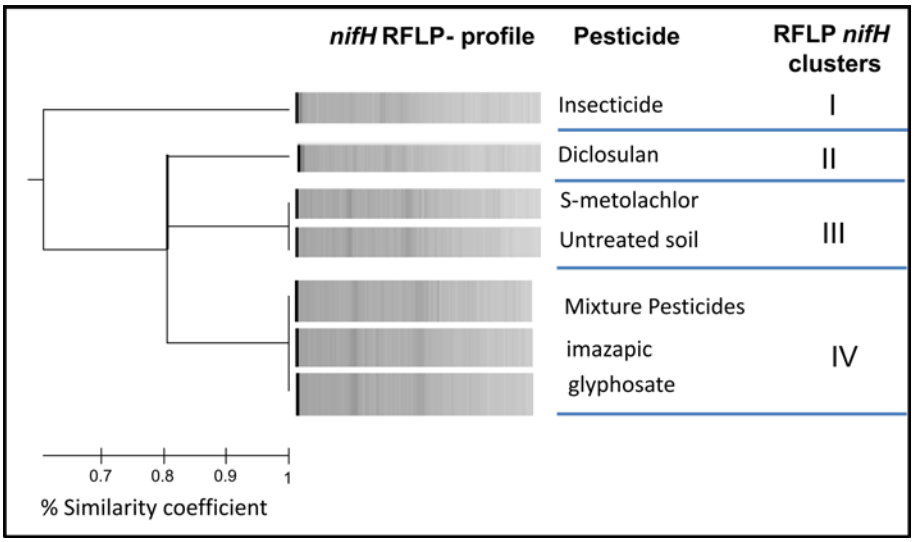

Fig. 4 UPGMA-dendrogram (Dice similarity coefficient) derived from RFLP analysis of nifH PCR products from soil in experiment in greenhouse. The RFLP profile, the pesticides and the clusters (I–IV) are also indicated

of interacting factors, such as the physicochemical properties of the pesticide, and its behavior in the soil environment (its bioavailability, susceptibility to abiotic degradation, mobility and propensity to be adsorbed by soil particles; Kookana et al. 1998; Simpson et al. 2001). In this context, the herbicide imazapyr and the commercial fungicide used in this study have been described as highly persistent in the soil (Cheng 1990).

It has been informed that herbicides are less likely to impact adversely on soil microbial populations than fungicides or insecticides. This is the case for herbicides which act at the chloroplast levels. However, some herbicides such as glyphosate and the sulphonylurea group that inhibit the synthesis of certain amino acids are likely to also affect the growth of some microorganisms (Moorman 1989). On the other hand, herbicides may also affect soil microbial populations indirectly by changing the carbon substrate inputs into the soil following the death of targeted plants (Whitelaw-Weckert et al. 2004) or because they are used by microorganisms as a nutritional source. The herbicides S-metalochlor and diclosulam have been reported to be metabolized by soil microorganism (Martine et al. 2007; Rodrigues et al. 2010).

Fungicides and insecticides would have some impact on non-target microorganisms by perturbing the functioning of the soil food web (Magarey and Bull 2003; Pandey and Singh 2004). As found in the field assays, the evaluation in greenhouse experiments of the impact of a particular pesticide on soil microorganisms indicated differences between the sensitivity of symbiotic and free-living nitrogen-fixing populations. The symbiotic diazotrophs seem to be more affected since the range of bacterial number diminution in pesticide treated soil (39–70 %) was higher than that of the free-living diazotrophs (22–59 %) (Figs. 1b, 2b). Moreover, although all the evaluated pesticides affected the bacterial number, only the fungicides or the imazapic herbicide caused a reduction in the free-living diazotrophs number (54 and 59 %, respectively) which was equivalent to that found when the agrochemical mixture was applied (Fig. 1b). In contrast, in the symbiotic fixing population, all the pesticides evaluated reduced the size of this population to a similar level to that found with the mixture addition. A different sensitivity to insecticide between these populations was also observed, since it affected only the non-symbiotic nitrogen fixers. Then, the use of this insecticide in peanut crop may be a strategy to minimize adverse effects of pesticides on nodulation.

Soil enzyme activities are useful indicators of soil health and have been used widely to assess the effects of management practices on soil biological function (Dick 1997). Measurements of soil nitrogenase activity indicated that the negative effect of pesticide on the number of bacterial diazotrophs also impacted on this biological function since a diminution of soil ammonium content in treated soils was found. However, the percentage of recuperation of this activity 1 year after pesticide application (71 %; Fig. 3a) was greater than that determined for bacterial number, suggesting a high ability to fix nitrogen in the bacterial population that persists after treatment. Similar results were also obtained from greenhouse experiments (Fig. 2a–c).

Non-cultivated diazotrophs may be the dominant nitrogen-fixing organisms in soil systems (Widmer et al. 2001; Poly et al. 2001; Tan et al. 2003; Bürgmann et al. 2004) and could represent a new and unexplored group that can play an important role in the nitrogen cycle and nitrogen input in soil. *nifH* is one of the most important functional genes used to study structural and functional diversity in different environments (Mártensson et al. 2009). This gene has been used as a marker for nitrogen-fixing bacteria to indicate changes in the structure of symbiotic and non-symbiotic

nitrogen-fixing communities. Moreover, *nifH* gene is also used as soil fertility indicator in environmental samples (Ladha and Reddy 2003). In order to know how pesticides affect not only cultivable but also uncultivable soil diazotrophic communities we analyzed *nifH* bacterial sequences obtained from soil samples. The evaluation of *nifH* gene sequences from the two libraries obtained in this study suggested that pesticides diminished the diversity of the gene pool from nitrogen-fixing bacteria community.

Overall, our observations provide compelling evidence that, in soil from peanut-growing area, a one-time treatment with synthetic chemicals is enough to significantly disturb the composition of diazotrophic bacterial community and to reduce soil nitrogenase activity. As a result of this, these changes would also impact in the next culture since they persist even after 1 year post-application.

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