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ORIGINAL PAPER

Impact of the *ahas* transgene and of herbicides associated with the soybean crop on soil microbial communities

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Abstract Although Brazil has recently reached the position as the second largest producer of genetically modified soybean [*Glycine max* (L.) Merr.], there are few reports on the effects of transgenic crops and the associated use of specific herbicides on soil microbial communities, both under the edaphoclimatic conditions in Brazil, and in other producer regions in the southern hemisphere. The aim of this study was to evaluate the effects of transgenic soybean containing the *ahas* gene conferring resistance to herbicides of the imidazolinone group, and of the herbicides associated with transgenic soybeans on the soil microbial community. Twenty field experiments were carried out during three growing seasons (summer of 2006/2007, short-season of 2007 and summer of 2007/2008), in nine municipalities located in six Brazilian states and in the Federal District. The experiments were conducted using a completely randomized block design with four replicates and three treatments: (1) conventional (non-transgenic) soybean cultivar Conquista with conventional herbicides (bentazone + acifluorfen-sodium and other herbicides, depending on the level of infestation in each region); (2) near-isogenic transgenic Cultivance (CV127) containing the *ahas* gene, with conventional herbicides; (3) transgenic Cultivance with specific herbicide of the imidazolinone group (imazapyr). As the objective of the study was to verify impacts of the transgene and herbicides on the soil microbial community of the whole area and not only a punctual rhizospheric effects, samples were

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taken at the 0–10 cm layer prior to cropping and at R2 soybean growth stage, between plant rows. Quantitative (microbial biomass C and N, MB-C and MB-N) and qualitative (DGGE of the 16S rDNA region) parameters of soil microbial community were evaluated. No qualitative or quantitative differences were found that could be attributed to the transgene *ahas*. A comparison of Cultivance soybean with conventional and imidazolinone-group herbicides applications also failed to reveal differences that could be attributed to the specific use of imazapyr, even after three consecutive croppings at the same site. Finally, no differences were detected between conventional (Conquista and conventional herbicides) and transgenic soybean managements (Cultivance and imazapyr). However, marked differences were observed in MB-C and MB-N between the different sites and times of year and, for the 16S rDNA-DGGE profiles, between different sites. In conclusion, microbial community evaluations were found to be sensitive and viable for monitoring different technologies and agricultural management methods, but no differences could be attributed to the *ahas* transgene for three consecutive cropping seasons.

Keywords Soil microbial biomass · DGGE · Soil microbial diversity · *Glycine max* · Imidazolinones · Environmental monitoring · Transgenic soybean

Introduction

Genetically modified (GM) soybean (*Glycine max* (L.) Merr.) from Monsanto (Roundup Ready or RR soybean) was released for commercialization for the first time in the United States in 1996, with the cultivated area increasing from 2 to 81 % by 2003. In 1997, a transgenic soybean containing the *ahas* gene resistant to herbicides of the imidazolinone group was also developed, and genetic work on soybeans resistant to other herbicides is under way. In global terms, with approximately 81.9 million hectares, soybean is the most widely cultivated GM crop in the world, and of the 29 countries with transgenic crops, Brazil ranked second in 2010, with 17.8 million hectares (James 2010).

The first transgenic soybean event in Brazil (RR) was approved in 1998 (CTNBIO 1998), but released for commercialization only in 2005, when the new Brazilian Biosecurity Law was passed. Since then, the intensity of use of specific herbicides on the crop has become significant, with the possibility of application at post-emergence (Petter et al. 2007). The most widely-used products for controlling weeds in the crop are glyphosate and imidazolinone-group herbicides. The imidazolinone group includes imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz and imazaquin herbicides, whose common molecular structure is imidazol (Kraemer et al. 2009). These molecules are absorbed by the roots and leaves and translocated to the phloem and xylem, accumulating at growth points and can control a broad spectrum of weeds. Imidazolinones control a wide range of weeds, by acting in the growing points after absorption by roots and leaves, and translocation to phloem and xylem.This group of herbicides inhibits the synthesis of the enzyme acetohydroxyacid synthase (EC 4.1.3.18, AHS or AHAS, also known as acetolactate synthase and ALS), responsible for the biosynthesis of the amino acids valine, leucine and isoleucine. Inhibition interrupts protein synthesis, which in turn interferes with DNA synthesis and cellular growth (Brighenti et al. 2002; Shaner and Singh 1993; Tan 2006). With the *ahas* gene isolated from *Arabidopsis thaliana* and containing a mutation at position 653 bp, combined with the use of imazapyr and a multiple shooting induction protocol, Aragão et al. (2000) developped a protocol to select transgenic meristematic cells. The approach proved to be successful with soybean, common bean (*Phaseolus vulgaris* L.) and cotton (*Gossypium hirsutum* L.) (Rech et al. 2008).

Assessing the environmental impact of transgenic technologies, including transgenic plants and management methods calling for specific herbicides, is both a government requirement and a social obligation. Regarding their impact on soil quality, it is known that soil microbial biomass is responsible for essential functions, including the decomposition of organic matter, nutrient cycling, biological control of diseases and degradation of pesticides. Changes in soil quality are associated with changes in soil biological properties, highly sensitive to disturbances resulting from management methods, and especially from the use of pesticides (Kaschuk et al. 2010, 2011). Furthermore, it is known that plants are capable of exudating substances that have been applied to the aerial part (Tuffi Santos et al. 2005). Moreover, as root

exudates can deeply affect soil microbial community (Koranda et al. 2011), differences may arise by the use of herbicides, or due to the exudation of proteins expressed by transgene. In general, the impact of herbicides on soil microbial biomass is lower than that of other pesticides (Kinney et al. 2005; Li et al. 2004; Santos et al. 2005), but the exclusive application of specific herbicides associated with transgenics still needs further investigation. In relation to the transgenes, studies searching for effects on soil microorganisms have been reported, e.g., for the Bt toxin of cotton (Donegan et al. 1995), maize (*Zea mays* L.) (Devare et al. 2004), rice (*Orya sativa* L.) (Wei et al. 2008), among others. Effects of the transgenes on bacterial and fungal populations have been reported (Devare et al. 2004; Donegan et al. 1995; Flores et al. 2005), but in general most studies report absence of effects (Wei et al. 2008). Several other transgenic plants have been released, but in general there is absence of long-term and broad-range studies on the impact of the transgenes on soil microbial community.

In addition to traditional methods to quantify microbial biomass, DNA-based methods have brought significant advances in the study of microbial ecology. Among these methods is the evaluation of microbial community by the denaturing gradient gel electrophoresis (DGGE) profiling of amplified ribosomal genes based on the soil total DNA. The 16S rDNA-DGGE has proved to be an efficient way of diagnosing alterations in the rhizosphere and can be applied in studies monitoring environmental risks associated with transgenics (Fang et al. 2005; Lottmann et al. 2010; OECD 2010; Souza et al. 2008a, b; Zilli et al. 2008).

In Brazil and other countries in the southern hemisphere with significant levels of transgenic soybean production, there are still very few studies evaluating the impacts of transgenic crops and of the use of specific herbicides on soil microbial community, especially under field conditions. Another critical point is that, in global terms, the availability of comparative studies involving parental soybean and the respective near-isogenic transgenic lines with resistance to herbicides is limited, since the parents remain within the domain of private corporations. However, in Brazil a public–private partnership for developing transgenic cultivars has made this type of material accessible.

The aim of this study was to conduct a large-scale evaluation, involving twenty field experiments over three growing seasons, to assess the impact of transgenic soybeans containing the *ahas* gene and of the herbicides used in conventional and transgenic management on soil microbial community. In our study, the main objective was to evaluate possible effects of the transgenic soybean by using a long-term and broad-range analysis. Therefore, we did not focus on rhizospheric soil; instead, we took samples from the whole areas.

Materials and methods

Sites

The experiments were conducted over three growing seasons (summer of 2006/2007, short-season of 2007 and summer of 2007/2008), at nine sites, with a total of 20 experiments. Details of the sites are given in Table 1 and locations indicated on the map in Fig. 1. It should be pointed out that the sites were selected as representative of soybean farming regions in Brazil and are located in edaphoclimatic conditions suitable for developing of the Conquista cultivar (both GM and conventional). The main characteristics of the soil at each experimental site are also given in Table 1. All experimental stations had Brazilian biosafety certification for performing transgenic experiments.

Treatments and experimental design

The experiments consisted of three treatments: (1) Cultivance soybean (GM soybean, near isogenic event CV127), containing the *ahas* gene, and imidazolinone-group herbicides as the sole agents for weed control; (2) Cultivance and conventional herbicides for weed control; (3) the parental non-transgenic cultivar Conquista (conventional soybean) and conventional herbicides for weed control.

The imidazolinone herbicide used was imazapyr at a rate of 70 g a.i. (active ingredient) ha^{-1} and the conventional herbicide consisted of a mixture of bentazon (400 g L^{-1}) + acifluorfen (170 g L^{-1}) (Volt) (570 g a.i. ha^{-1}), in addition to other herbicides such as tepraloxydim, according to the level of weed infestation in each area (ranging from 80 to 240 g a.i. ha^{-1}).

The experiments were conducted with four replicates in a completely randomized block design. The plots consisted of four rows of 5 m long, spaced at 0.5 m between lines, with eight lines per plot.

Table 1 Information about the field sites where the experiments were conducted

Sites and acronyms	Crop seasons	Latitude (S)	Longitude (W)	Climate Koeppen's classification	Great groups of the American System	Previous crops
Santo Antonio da Posse, SP (EEA)	2006/2007; 2007/2008	22°37′	46°54′	Cwa	Ultisol	Corn and soybean (summer)/potato (winter)
Ponta Grossa, PR (SNT)	2006/2007	25°05′	50°09′	Cfb	Oxisol	Soybean (summer)/ oats (winter)
Londrina, PR (CNPSo)	2006/2007; 2007/2008	23°18′	51°09′	Cfa	Oxisol	Soybean (summer)/ oats (winter)
Uberaba, MG (CTTP)	2006/2007; 2007-short-season; 2007/2008	19°45′	47°55′	Aw	Oxisol	Soybean (summer)/ fallow (winter)
Sete Lagoas, MG (CNPMS)	2006/2007; 2007-short-season; 2007/2008	19°27′	44°14′	Cwa	Ultisol	Fallow (summer)/ fallow (winter)
Santo Antonio de Goiás, GO (CNPAF)	2006/2007; 2007-short-season; 2007/2008	16°29′	49°18′	Aw	Ultisol	Fallow (summer)/ fallow (winter)
Brasília, DF (CNPH)	2006/2007; 2007-short-season; 2007/2008	15°46′	47°55′	Cfb	Oxisol	Fallow (summer)/ fallow (winter)
Teresina, PI (EMN)	2007-short-season	5°05′	42°48′	Aw	Ultisol	Fallow (winter)/ fallow (summer)
Vilhena, RO (ER)	2007-short-season; 2007/2008	12°44′	60°08′	Aw	Oxisol	Fallow (winter)/ soybean (summer)

Experiments conduction

Information on rainfall and average temperature for each experiment is given in Table 1S. All farming practices (fertilizing, irrigation, pest control, etc.) were carried out uniformly in the experimental areas, following the soybean cropping recommendations for each region. Seeds were sown manually, mainly in the month of November and the beginning of December (summer growing seasons of 2006/2007 and 2007/2008) and in March (short-season of 2007).

The evaluations were carried out at two soybean cropping stages, at pre-sowing and R2 (50 % of plants in full bloom, scale of Fehr and Caviness 1977) stages.

Soil sampling

At pre-sowing and R2, we obtained four subsamples at 0–10 cm of soil depth in each plot, as described before (Andrade and Hamakawa 1994), to make up composite samples. Around 200 g soil per replicate were packed in plastic bags and sent to the laboratory. Samples were homogenized and sieved (4 mm sieve, 5 mesh), immediately processed for microbial biomass and stored in plastic bags and maintained in a ultra-low freezer at −80 °C for the analysis of microbial diversity.

Quantitative evaluation of microbial biomass carbon (MB-C)

Soil MB-C was determined using the fumigation-extraction method (Vance et al. 1987), a variation of the traditional fumigation-incubation method proposed by Jenkinson and Powlson (1976). The method involves eliminating microorganisms from the soil by fumigation with chloroform ($CHCl_3$) (Jenkinson and Powlson 1976), and then determining the C released from the dead microorganisms by chemical extraction (Vance et al. 1987).

A 10 g subsample was taken from each soil replicate to determine soil moisture content. Based on the moisture content, the soil samples were standardized to a moisture content of 75 % of field capacity. Next, 20 g quantities were weighed out in

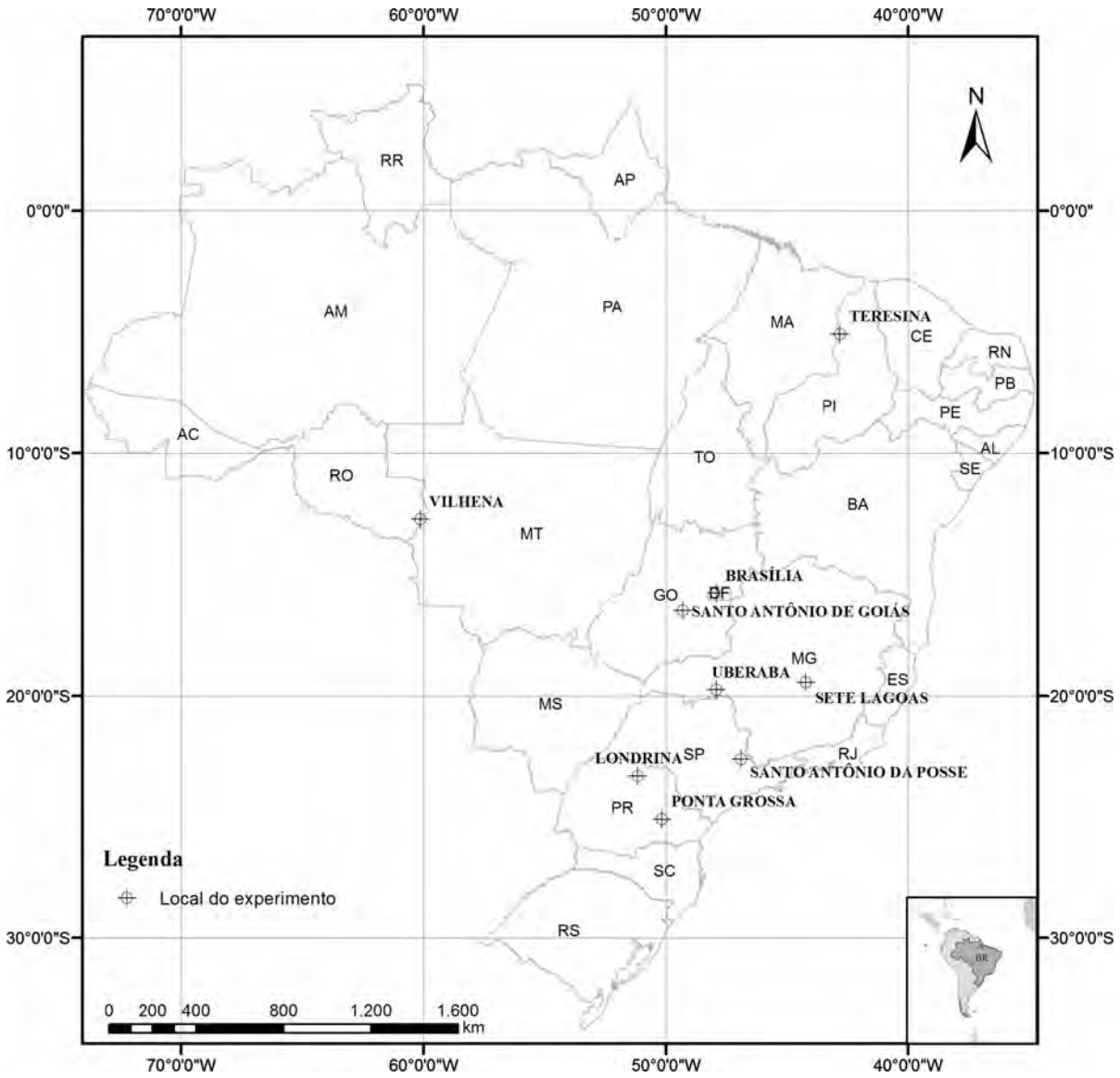

Fig. 1 Map indicating the nine sites where the field experiments were performed in the summer 2006/2007, short-season 2007 and summer 2007/2008 crop seasons

snap-cap tubes for two repetitions (two fumigated and two non-fumigated) and the procedures for incubation and extraction were conducted as described above (Franchini et al. 2007; Hungria et al. 2009).

After incubation, 50 mL of 0.5 M potassium sulfate (K_2SO_4) extractor were added to each sample, stirred for 1 h at 175 rpm and centrifuged for 10 min at 2,500 rpm. The extract obtained was filtered through quantitative filter paper. C-content for MB-C was obtained by oxidizing organic C using potassium permanganate ($KMnO_4$), according to Bartlett and Ross (1998).

MB-C was determined using the formula: $MB\text{-}C = (C_F - C_{NF})/K_{EC}$, where C_F and C_{NF} represent the C extracted from the fumigated and non-fumigated soils and K_{EC} is a constant for all samples. A K_{EC} factor of 0.33 was used, as suggested by Vance et al. (1987).

Quantitative evaluation of microbial biomass nitrogen (MB-N)

To evaluate MB-N, 20 mL of the filtered extract described in the MB-C procedure were transferred to

digestion tubes and a catalyst (K_2SO_4 + $CuSO_4$, 10:1) and 1.5 mL concentrated H_2SO_4 were added (Bremner 1965). After sulfuric digestion, the resulting N (NH_4^+) was determined using the indophenol blue method (Feigl and Anger 1972).

MB-N was determined using the formula: MB-N = $(N_F - N_{NF})/K_{EN}$, where N_F and N_{NF} represent the N extracted from the fumigated and non-fumigated soils; a $K_{EN} = 0.45$ was applied to all samples (Brookes et al. 1985a; b).

Qualitative evaluation of microbial community by denaturing gradient gel electrophoresis (DGGE)

DNA was extracted from the soil samples using the UltraClean™ soil DNA isolation kit (Mobio Inc., Solana, CA, USA) and following the manufacturer's instructions. Total DNA extracted from the soil was verified on 2 % agarose gel, after running at 80 V for 45 min. The DNA Mass Ladder (Life Sciences) was used as a mass marker for size and quantity. Gels were stained with ethidium bromide and visualized under UV light.

Two successive amplifications were carried out for each DNA sample. First, soil DNA was amplified with universal primers fD1(5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1(5′-AAGGAGGTGATCCAGCC-3′), which amplify nearly the entire region of the DNA coding for 16S rDNA (~1,500 bp), as described by Weisburg et al. (1991). The PCR reaction consisted of: 3.0 μL deoxynucleotides (dNTPs) 1.5 mM; 1.5 μL $MgCl_2$ 50.0 mM; 5.0 μL buffer 10X [20 mM Tris–HCl (pH 8.4)]; 1.5 μL of each primer (fD1 and rD1) 10 pmols; 0.2 μL 5 U of Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA); 1 μL of soil DNA (30 ng); sterile Milli-Q water to complete a final volume of 50 μL. The PCR program consisted of: an initial desnaturation at 95 °C for 2 min; 15 cycles of denaturation at 94 °C for 15 s; 93 °C for 45 s; primer annealing at 55 °C for 45 s, and extension at 72 °C for 2 min; the reaction was finalized by holding at 4 °C.

The second amplification was performed using 1 μL (~20 ng) of the products of the first reaction as a template. This second amplification is needed to reduce the size of the fragment, as the resolution of the method is of a maximum of 800 bp and mainly to introduce the GC clamp, needed to separate the bands. We chose one of the most variable regions of the 16S rDNA genes, amplified with the primers F-968 (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′), with a GC-clamp (underlined) and R-1401 (5′-GCGTGTGTACAAGACCC-3′) (Nübel et al. 1996). The region amplified has approximately 430 bp, corresponding to the V3 hypervariable region. PCR mixtures were prepared as: 5.0 μL dNTPs 1.5 mM; 1.3 μL $MgCl_2$ 50.0 mM; 2.5 μL buffer 10X [20 mM Tris–HCl (pH 8.4)]; 1.0 μL of each primer (F-968 and R-1401) 10 pmol; 0.2 μL 5 U of Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA); 1 μL of the PCR product of the first reaction with fD1 and rD1 primers (~10 ng); sterile Milli-Q water to complete a final volume of 25 μL. The following amplification cycles were used: one initial denaturation cycle at 94 °C for 2 min; 2 cycles at 94 °C for 1 min, at 60 °C for 2 min, and at 72 °C for 2 min; 2 cycles at 94 °C for 1 min, at 59 °C for 2 min, and at 72 °C for 2 min, 94 °C for 1 min, 58 °C for 2 min, 72 °C for 2 min (2 cycles); 94 °C for 1 min, 57 °C for 2 min; 72 °C for 2 min (2 cycles); 94 °C for 1 min, 56 °C for 2 min, 72 °C for 2 min (2 cycles); 94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min; and for 10 min at 72 °C; the reaction was finalized by holding at 4 °C. Amplification was confirmed by running 2 μL of PCR product on a 1 % (w/v) agarose gel in 1X TBE, staining with ethidium bromide (0.3 μg mL^{-1}) and visualizing under UV light.

The products of the second PCR were then subjected to electrophoresis in a 6 % acrylamide gel, with a urea denaturing gradient ranging from 20 to 75 %, in a Bio-Rad DGGE apparatus (Bio-Rad DCode), as described before (Hungria et al. 2003; Souza et al. 2008a). The gels were run for 16 h and stained with ethidium bromide, visualized under UV light and photographed.

Statistical analysis

The data were tested for normality of variables and uniformity of variance (ANOVA) (SAS 1999). After verifying normality and uniformity, the means were compared using the Tukey test at $p \leq 0.05$.

The DGGE gels were analyzed using Bionumerics software (Applied Mathematics, Kortrijk, Belgium, v.4.6). Similarities between fingerprints were analyzed statistically using the unweighted pair-group method with arithmetic averages (UPGMA, Sneath

and Sokal 1973) and the Jaccard (J) coefficient (Jaccard 1912) and the standard parameters of the software to create a distance matrix.

Results

Quantitative evaluation of MB-C and MB-N

In the pre-sowing samples taken in the summer season of 2006/2007, there was no statistical difference between treatments for MB-C and MB-N at any of the seven sites evaluated (Table 2). The MB-C values found at all sites were close to 500 μg C g^{-1} dry soil, with little difference between the sites, except for Ponta Grossa (SNT), where MB-C levels were numerically lower. For MB-N, the highest values were also found in Ponta Grossa, characterized by a humid subtropical climate. Fairly low MB-N values were observed in Santo Antonio da Posse (EEA), what could be attributed to climatic conditions, favorable to rapid mineralization of crop residues (Table 2). In this first evaluation, no differences between treatments were expected because it was before the experiment establishment, but rather an indication of the area homogeneity.

In the evaluation carried out at R2 in the 2006/2007 season, no statistical differences were observed between the treatments at the seven sites, indicating no effects that could be attributed to the transgenic (comparison of treatments with Conquista and Cultivance, accompanied by conventional herbicides), or herbicides (comparison of treatments involving Cultivance accompanied by imazapyr or conventional herbicides), or weed and soybean management (Cultivance and imazapyr vs. Conquista and conventional herbicides) (Table 2). There was also no statistical difference in MB-N among treatments at the seven sites, and the highest values were observed in Sete Lagoas (CNPMS). Also in the 2006/2007 season, by comparison with the pre-sowing samples, there was generally a marked drop in MB-N, indicative of rapid N mineralization, except in Sete Lagoas (CNPMS) (Table 2).

In the short-season of 2007, the experiments were carried out for the first time at two sites, Teresina (EMN) and Vilhena (ER), in addition to four other sites previously cropped in the summer. In the pre-sowing collection, no statistical differences were detected for MB-C and MB-N between treatments at any of the sites evaluated (Table 2). MB-C values clearly varied from one site to another, with the highest values obtained at Vilhena (ER) and the lowest at Uberaba (CTTP) and Brasília (CNPH), probably due to the strong influence of a drought period before sowing. In addition, no statistical differences between treatments were detected at R2 and the lowest MB-C values were obtained once more at Brasília (CNPH), possibly reflecting a period of prolonged drought at this site (Table 1S). For MB-N, the lowest values for pre-sowing and at R2 were also obtained at Brasília (CNPH) (Table 2).

In the last growing season evaluated (summer of 2007/2008), all sites had already been cropped at least once with the three treatments. Consequently, any residual effect over and above the effect of the treatments could be detected. However, there was no statistical difference in MB-C or MB-N between treatments at the pre-sowing or R2 stages at any of the seven sites evaluated (Table 2). In general, the MB-C values obtained at pre-sowing were high, especially at Londrina (CNPSo), with low values detected only at Uberaba (CTTP), and related to water stress. For the evaluation at R2, the highest MB-C values were obtained at Vilhena (ER), but there was a marked drop in MB-C at all sites evaluated. Similar behavior was observed for MB-N (Table 2).

Qualitative evaluation of microbial community by 16S rDNA-DGGE

Considering all 40 DGGE analyses, representing 20 field experiments analyzed at pre-sowing and R2, the number of bands obtained ranged from 38 to 71. The results obtained at all sites during the 2006/2007 summer crop season in the pre-sowing evaluation confirmed the homogeneity of the areas, with similarity of 100 % among all plots (Table 3). At R2, the similarity among the treatments was also 100 %, this time indicating that there was no difference among the treatments at each of the seven sites at which the experiments were conducted (Table 3).

In the 2007 short-season experiments conducted in Teresina (EMN), Vilhena (ER), Sete Lagoas (CNPMS), Santo Antônio de Góias (CNPAF) and Brasília (CNPH), the profiles representing each treatment, in both pre-sowing and R2, still had 100 % of similarity (Table 3). In Teresina (EMN) and Vilhena (ER), the experiments were conducted for the first time in 2007 and indicated homogeneity of the area, whereas at the other sites the results indicated the absence of residual effects from the

Table 2 Microbial biomass carbon (MB-C) and nitrogen (MB-N) (μg C and N g^{-1} dry soil) at pre-sowing and R2 soybean stages in the field experiments performed in summer of 2006/2007, short-season of 2007 and summer of 2007/2008

Treat-ment	Microbial Parameters[a]															
	MBC	MBN	MBC	MBN	MBC	MBN	MBC	MBN	MBC	MBN	MBC	MBN	MBC	MBN	MBC	MBN
Summer-season 2006/2007																
	EEA(SP)		SNT(PR)		CNPSo(PR)		CTTP(MG)		CNPMS(MG)		CNPAF(GO)		CNPH(DF)			
Pre-sowing[ns]																
T1	602	9.7	339	62	560	36	596	37	497	29	655	24	586	23		
T2	599	16	263	48	503	21	578	35	508	16	503	16	485	10		
T3	505	14	320	62	505	26	505	32	548	17	560	19	690	16		
CV(%)	46	48	37	57	25	42	46	32	31	46	36	58	30	68		
R2[ns]																
T1	1,005	15	715	5.1	351	11	446	15	702	43	387	18	213	7.1		
T2	1,007	27	520	8.8	324	7.7	378	12	696	29	455	12	255	6.9		
T3	701	7.6	571	11	384	5.6	539	30	581	36	526	16	405	7.3		
CV(%)	32	71	32	78	26	54	32	60	47	31	39	51	52	30		
Short-season 2007																
	EMN(PI)		ER(RO)				CTTP(MG)		CNPMS(MG)		CNPAF(GO)		CNPH(DF)			
Pre-sowing[ns]																
T1	669	14	1,219	23			297	20	794	24	421	20	303	9.0		
T2	807	14	1,082	24			378	14	630	15	590	12	356	8.0		
T3	704	24	1,201	21			220	15	713	18	673	17	264	11		
CV(%)	15	97	18	35			60	28	16	33	40	59	24	66		
R2[ns]																
T1	468	25	636	28			509	48	597	16	547	22	170	9.0		
T2	541	17	790	26			544	53	706	24	601	23	149	7.a		
T3	468	24	748	30			552	39	720	28	526	28	169	13		
CV(%)	24	36	25	18			19	23	19	30	22	48	37	77		
Summer-season 2007/2008																
	ER(RO)		EEA(SP)		CNPSo(PR)		CTTP(MG)		CNPMS(MG)		CNPAF(GO)		CNPH(DF)			
Pre-sowing[ns]																
T1	696	26	583	13	942	32	356	25	749	22	859	21	713	22		

Table 2 continued

Summer-season 2007/2008																
	ER(RO)		EEA(SP)			CNPSo(PR)		CTTP(MG)		CNPMS(MG)		CNPAF(GO)		CNPH(DF)		
T2	760	24	770	13		1,106	41	218	25	864	29	886	18	670	32	
T3	703	31	695	8.4		1,143	44	302	28	925	22	830	17	511	35	
CV(%)	25	35	45	81		21	43	31	26	44	41	39	43	43	44	
R2 [ns]																
T1	635	17	399	11		246	14	259	8.8	272	17	237	34	530	44	
T2	530	24	439	10		232	12	298	14	265	21	281	32	310	60	
T3	591	21	496	10		276	14	190	10	255	22	266	36	508	56	
CV(%)	12	48	34	4		60	38	45	39	48	63	26	63	39	55	

T1, Cultivance soybean (GM) and imazapyr herbicide; T2, Cultivance (GM) with conventional herbicides; T3, Conquista (parental conventional soybean), with conventional herbicides

[a] Data represent the means of four replicates, and "ns" denotes absence of statistical differences between the treatments of each site and each sampling (Tukey. $p < 0.05$)

previous cropping. Only in Uberaba (CTPP) there was a detectable difference among the treatments, both at pre-sowing and R2. However, it is possible that these differences are related to the effect of high water stress, observed in the Uberaba region during most of the crop season, and it should be remembered that the lowest MB-C levels were also detected at this site (Table 2), a further indication of microbial stress. At R2, the absence of differences among the treatments at each of these sites (except Uberaba) indicates that none of the treatments had a qualitative effect on the soil bacterial community, evaluated by the 16S rDNA-DGGE methodology (Table 3).

In the experiments conducted during the third season (summer of 2007/2008) in Santo Antônio da Posse (EEA), Vilhena (ER), Londrina (CNPSo), Uberaba (CTTP), Santo Antônio de Goiás (CNPAF) and Brasília (CNPH), again no qualitative differences were found in the soil microbiota that could be attributed to the cultivars, or to the herbicides, or to cultivar x weed management, confirming that the treatments exhibited a level of genetic similarity of 100 % (Table 3). This shows that there were no detectable effects of treatments in this growing season, nor any residual effects from previous cropping, since in some of these areas the experiments were being conducted for the third time. In Sete Lagoas (CNPMS), various differences in the levels of genetic similarity were observed among the pre-sowing treatments, and to a lesser degree, in the R2 sampling. Before sowing, there was a period of heavy drought in Sete Lagoas (data not shown), which could have placed the bacterial community under high stress levels.

Fig. 1S gives an example of the similarity of the DGGE profiles in each area. This figure also shows that, although the DGGE profiles are similar in each area, they do differ from one area to another. Furthermore, in practically all cases, the pre-sowing profiles obtained were identical or fairly similar to the R2 profiles. Fig. 1S shows an example from Uberaba, taken in the summer season of 2007/2008.

Discussion

Effect of herbicides on soil microbial community

The importance of soil microbial community relies mainly on its role as a transformation agent for organic

Table 3 Final level of genetic similarity (%) in the cluster analysis of the DGGE-16SrDNA profiles at each site and crop season

Treatments	T1	T2	T3
Summer 2006/2007			
EEA (SP), SNT (PR), CNPSo (PR), CTTP (MG), CNPMS (MG), CNPAF (GO), CNPH (DF)			
Pre-sowing and R2			
T1	100		
T2	100	100	
T3	100	100	100
Short season 2007			
EMN (PI), ER (RO), CNPMS (MG), CNPAF (GO), CNPH (DF)			
Pre-sowing and R2			
T1	100		
T2	100	100	
T3	100	100	100
CTTP (MG)			
Pre-sowing			
T1	100		
T2	69.2	100	
T3	71.4	81.8	100
R2			
T1	100		
T2	88.9	100	
T3	80	90	100
Summer 2007/2008			
EEA (SP), ER (RO), CNPSo (PR), CTTP (MG), CNPAF (GO), CNPH (DF)			
Pre-sowing and R2			
T1	100		
T2	100	100	
T3	100	100	100
CNPMS (MG)			
Pre-sowing			
T1	100		
T2	72.7	100	
T3	90.9	80	100
R2			
T1	100		
T2	81.8	100	
T3	90	90.9	100

T1, Soybean Cultivance (GM) and herbicide imazapyr; T2, Cultivance (GM) with conventional herbicides; T3, Conquista (parental conventional soybean) with conventional herbicides

matter and a labile reservoir of plant nutrients. However, this microbial biomass can undergo changes due to temperature, soil water content and aeration, availability of nutrients and organic substrates, among others (Balota et al. 1998; Franchini et al. 2007; Silva et al. 2010), causing alterations in nutrient cycling rates and availability (Wang et al. 2009). It has been shown that methods used to assess soil microbial biomass in general can promptly detect environmental changes and different agricultural practices, which is

the reason for a strong tendency to adopt parameters as MB-C and MB-N to evaluate soil quality and environmental monitoring (e.g., Balota et al. 1998, 2003, 2004; Bending et al. 2004; Franchini et al. 2007; Hungria et al. 2009; Kaschuk et al. 2010, 2011; Mendes et al. 2003; Souza et al. 2008a, b).

The results obtained in our study confirm the viability of using microbial biomass parameters, promptly altered with edaphoclimatic conditions and support the use of these parameters in environmental monitoring studies, or as soil quality bioindicators. Other studies conducted in Brazil reported that, using microbial biomass parameters, it was possible to detect changes caused by soil management and crops at an earlier stage in comparison with chemical and physical parameters (Balota et al. 1998, 2003; Franchini et al. 2007; Hungria et al. 2009; Kaschuk et al. 2010, 2011; Silva et al. 2010).

Some studies reported that cropping practices, such as the use of pesticides, may interfere directly in soil microbial community, with both negative and positive effects (Araújo et al. 2003; Busse et al. 2001; Haney et al. 2002; Kennedy 1999; Zilli et al. 2008). However, various results in the literature indicate that, when microbial biomass is analyzed over a longer period of time, the effects of herbicides are generally less extensive and of lower intensity than those of other pesticides, such as fungicides (Topp et al. 1997; Wardle 1992, 1995).

Considering the effects of herbicides, Zilli et al. (2008) reported that glyphosate did not cause any significant changes in MB-C contents. Similarly, under field conditions, Liphadzi et al. (2005) did not detect changes in MB-C on comparing the use of glyphosate at a rate of 1,120 g a.i. ha^{-1} with other herbicides recommended for maize (*Zea mays* L.) and soybean. However, in a study conducted in Georgia and Texas (USA), Haney et al. (2002) reported a significant increase in MB-C when glyphosate was applied at a rate of 234 mg a.i. kg^{-1} on nine types of soil, with different pH, organic-C and clay contents. According to the authors, soils with higher levels of organic matter tend to mineralize glyphosate faster, possibly due to the higher microbial biomass. In addition, Kremer et al. (2005) reported that applying glyphosate to soybean plants increased the quantity of exudates and altered their composition, what could increase the soil microbial biomass, possibly due to the high levels of soluble carbohydrates and amino acids in soybean plants treated with glyphosate. Contrarily, examples of results that show a reduction in microbial activity include the study of Pereira et al. (2008), who observed that the soil microbial biomass under RR transgenic soybean dropped in treatments involving the application of endosulfan alone or in a mixture with glyphosate, in comparison with the control and when only the herbicide glyphosate was applied. There are some indications that the soil biota are capable of metabolizing glyphosate, whereas endosulfan in contact with the soil can form endosulfan diol and endosulfan sulfate, both highly toxic to the microbial community in the soil. Other authors have reported a drop in microbial biomass with the application of herbicides such as prosulfuron (Kinney et al. 2005; Santos et al. 2005). Overall, as reported by Wallis et al. (2010), depending on the soil, region, cultivation conditions (field, greenhouse, in pots or in vitro), rates of application and the time at which the herbicide is applied, glyphosate may or may not affect the soil biota.

A significant proportion of herbicides of the imidazolinone group, when applied to crops, reach the soil and can be absorbed by roots, adsorbed in the soil colloids, or dissolved, and can undergo photolysis, hydrolysis, microbial degradation or leaching. When environmental conditions are favorable to microorganism development, degradation of imidazolinones increases, since the main mechanism for its dissipation in the soil is microbial degradation (Flint and Witt 1997). However, under field conditions, Li et al. (2004) reported a drop in soil microbial biomass and in the mineralization of organic compounds with the application of imazapyr.

In our study, taking all twenty experiments conducted at the nine sites over three cropping seasons and during two different periods in each season, we found no differences in MB-C and MB-N when comparing the transgenic cultivar (Cultivance) treated with imazapyr or with a conventional mixture of conventional herbicides. The lack of differences was confirmed under various edaphoclimatic conditions, covering the main Brazilian biomes, thus we can safely confirm that there was no difference between the effects of imazapyr and conventional herbicides on the soil microbiota under field conditions. It is also possible that other factors, with an emphasis on environmental conditions, soil physical and chemical properties, among others prevailed for determining

soil MB-C and MB-N in comparison with the transgene *ahas* and the herbicides.

In relation to the microbial diversity, a study conducted by Zilli et al. (2007) comparing the effects of glyphosate-, imazaquin- and trifluralin-based herbicides on bacterial community associated with the soybean rhizoplane verified that the imazaquin altered the bacterial DGGE profile more intensely than glyphosate. In another study, Zilli et al. (2008) observed that both glyphosate- and imazaquin-based herbicides altered the bacterial community (DGGE) associated with the soybean rhizoplane in all samplings (14, 30 and 62 days after herbicide application). In our study, we did not have an absolute control without herbicide, because this is not a feasible practice in soybean cropping, and we evaluated microbial parameters in the soil between lines and not in the rhizoplane, where we detected no differences between the use of imazapyr and conventional herbicides in the 16S rDNA-DGGE profiles. However, large differences were related to different edaphoclimatic conditions.

Effect of the transgenic gene *ahas* on soil microbial community

Genetically modified plants can express proteins or metabolic products that are released as root exudates and their effects can be evaluated by changes in soil or rhizosphere microbial community. In general, microbial community in these studies is evaluated at the rhizosphere level, where differences may not reflect long-term effects, as they can reflect other effects, e.g., differences associated to the release of different molecules in response to the application of different herbicides. Therefore, in our study, the main objective was to evaluate possible effects of the transgenic soybean by using a long-term and broad-range analysis, and for that soil was sampled in the whole area (pre-sowing) and between plant rows (R2) in experiments performed in the same site for three crop seasons.

By comparing the Conquista parental cultivar and the near-isogenic Cultivance containing the *ahas* gene, both treated with conventional herbicides, in twenty field experiments, it was possible to conclude that the expression of transgenes had no effect on microbial biomass parameters—MB-C and MB-N. It was also possible to confirm that there was no qualitative or quantitative difference in the soil microbial community by comparing transgenic soybean management (Cultivance with imazapyr) and conventional management (Conquista with conventional herbicides).

After reviewing studies on soil microbiota conducted under different edaphoclimatic conditions in soybean cropping ecosystems in Brazil, Bohm and Rombaldi (2010) concluded that the information on genetically modified soybean resistant to glyphosate could vary with the soil type and the crop management conditions, with no clear effect of the transgene. However, the effects were almost always associated with the herbicides. In general, the studies analyzed had a number of common characteristics, i.e., only a few variables in greenhouse experiments were evaluated, or the studies were conducted in vitro, and usually the authors concluded that genetic modification did not affect microorganisms in the soil.

Dunfield and Germida (2004) reported that the microbial community could undergo changes when associated with transgenic plants, by horizontal gene transfer to the soil indigenous microorganisms. However, the same authors have previously stated that they could not detect any horizontal gene transfer from the GM plant to environment soil microorganisms (Dunfield and Germida 2001). New proteins could also be released from transgenic plants into the soil and could influence the biodiversity by selectively stimulating the growth of specific microorganisms. However, these effects are minor in comparison to the effects of other factors such as the soil, site, climate and sampling time of year. Therefore, according to Ávila (2007), microorganism-plant interactions at different phenological stages and in different types of soil should be taken into account in evaluating the impact of transgenics. The author has shown that there were differences in the number of bacteria associated with the roots of Bt and non-Bt cotton only in specific development stages; the Bt rhizosphere contained higher densities of *Pseudomonas* spp. and of total bacteria during the budding and bud formation phases, respectively.

In another study on a GM variety of canola (*Brassica napus* L.) resistant to glyphosate and three varieties resistant to ammonium glyfosinate, compared with four non-transgenic varieties in four different regions of Canada, there was no significant difference in the total number of colony forming units in the rhizosphere (Dunfield and Germida 2001).

However, the authors reported that the microbial community associated with the rhizosphere of the transgenic variety resistant to glyphosate (Quest) was different from the others, whether transgenic or non-transgenic. However, the changes were short-lived and did not persist in subsequent evaluations (Dunfield and Germida 2003). Other studies have also reported an increase in the microbiota of soils cropped with transgenic plants in comparison with conventional plants (Donegan et al. 1995; Wei et al. 2006). Differences among cultivars can be explained by effects of the rhizosphere of the different cultivars, since the roots may exude different compounds that in turn change the composition of the microbial community in the soil (Gomes et al. 2001; Milling et al. 2004).

In our study, qualitative evaluation using 16S rDNA-DGGE failed to confirm any transgenic effect on soil bacterial community. Field studies using DGGE analyses in two separate areas over 3 years on transgenic potato (*Solanum tuberosum* L.) confirmed that parameters such as cultivar, soil type, seasonal climatic changes and plant age can have more significant effects on soil microbiota than transgenics (Heuer et al. 2002). In fact, in our study, the 16S rDNA-DGGE profiles were fairly different from one area to another, and also differed with environmental stresses, but we did not detect differences that could be attributed to the *ahas* gene.

According to Knupp et al. (2009), constant monitoring throughout the cropping cycle of genetically modified plants is necessary to verify whether the changes in the microbial community in the rhizosphere and in the soil persist throughout the plant's life cycle. Milling et al. (2004) observed variable effects in the profiles of the bacterial community from one plant development stage to another. In a further study conducted by Knupp et al. (2009) on GM common bean plants (*Phaseolus vulgaris* L.) resistant to Bean Golden Mosaic Vírus (BGMV) (Olathe M1-4) and conventional plants, the groupings obtained in the 16S rDNA DGGE profiles indicated changes in the bacterial community associated with the roots in development stages V4 and R6. However, in our experiments on soybean, no differences were detected between the pre-sowing and R2 soybean growth stages, and although we have not sampled rhizospheric soil, no effects were observed even after three consecutive crop seasons. Shen et al. (2006) also failed to observe any effects in microbial community activity evaluated in the vegetative, reproductive and senescent phases and after sampling in the Bt cotton rhizosphere, in comparison with non-Bt cotton.

After reviewing numerous studies, Bruinsma et al. (2003) concluded that the majority of transgenic plants do not significantly affect soil biota, and according to our study, the Cultivance soybean cultivar containing the *ahas* gene can be added to that list. We should point out that the results of our study are probably among the most extensive in terms of the number of evaluations carried out under field conditions, and indicate that *ahas* gene did not cause significant differences in soil bacterial community profiles evaluated by 16S rDNA-DGGE under a variety of edaphoclimatic conditions in Brazil. The results were included in the report to the National Commission on Biosafety and contributed to the approval of Cultivance soybean for commercial use in Brazil.

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