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Effects of the glyphosate-resistance gene and of herbicides applied to the soybean crop on soil microbial biomass and enzymes

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ABSTRACT

Glyphosate, a broad-spectrum herbicide used for the non-selective control of weeds, inhibits 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme in the synthesis of aromatic amino acids in the shikimic acid pathway in plants, fungi and bacteria, thus impairing the synthesis of proteins required for various life processes. Soybean genetically engineered to be glyphosate resistant (GR or Roundup Ready, RR) represents the most cultivated transgenic crop globally, including Brazil. There are concerns about the effects of RR transgenic soybean and of glyphosate on soil microbial communities and their functioning. Our study was designed to detect changes in soil microbial biomass-carbon (MB-C) and -nitrogen (MB-N) and in enzyme activities [beta-glucosidase (GLU) and acid phosphatase (PHO)] in a large set of field trials performed at six sites in Brazil for two cropping seasons. We evaluated the effects of the RR transgene, glyphosate and weed management (RR soybean + glyphosate vs. conventional soybean + conventional herbicides), with three pairs of nearly isogenic soybean cultivars evaluated per site. Soils were sampled from the 0–10 cm layer, between cropped lines, during the cropping seasons 2004/2005 and 2005/2006, at the R2 stage of soybean growth. Univariate and contrast analyses were performed in addition to multivariate analyses including all four microbial variables, and denominated as soil microbial variables (SMV). In general, microbial parameters and SMV were not affected by the transgene, type of herbicide or weed management. Differences were, rather, related to site, cropping season and cultivar.

1. Introduction

Glyphosate-resistant (GR or Roundup Ready®, RR) soybean [*Glycine max* (L.) Merr.] represents the most-cultivated transgenic crop. In Brazil, cultivation of RR genotypes continues to increase, mainly due to broad adoption of glyphosate for weed control (Pereira et al., 2008; Zobiole et al., 2010a,b,c); in the 2010/2011 growing season, RR genotypes occupied 86% of the area cultivated to soybean globally (ISAAA, 2012). In response to the widespread use of RR soybean, the quantity and frequency of glyphosate applications have escalated worldwide in recent years (Gomez et al., 2009), raising concerns regarding potential environmental impacts (Weaver et al., 2007).

Glyphosate [N-(phosphomethyl) glycine; Roundup®, Monsanto, St. Louis, MO] is a broad-spectrum herbicide used for the non-selective control of weeds. It inhibits 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme in the synthesis of aromatic amino acids in the shikimic acid pathway in plants, fungi and bacteria (Gomez et al., 2009). Without these amino acids, organisms are unable to synthesize certain proteins essential for metabolism (Ware, 2000). Transgenic soybean produces a glyphosate-insensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Reddy et al., 2001).

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Glyphosate is often described as a simple molecule, easily degraded, exhibiting little or no activity in soil due to its rapid adsorption on inorganic and organic particles, with a half-life ranging from days to months (Forlani et al., 1999; Jonge and Jonge, 1999; Duke and Powles, 2008). Many soil microorganisms can metabolize pesticides and use xenobiotics as sources of carbon, energy and nutrients (Durkin, 2003). The rate at which glyphosate is mineralized is related to biomass and activity of microorganisms, determining its persistence in the soil (Wiren-Lehr et al., 1997).

Even though the herbicide is not applied directly to the soil, a significant amount may reach its surface during broadcast preplanting or early-season applications, with harmful effects on soil microorganisms. The quantity of herbicide available to the soil microorganisms depends on factors such as soil nutrient and pH status, temperature, and moisture content (Weber et al., 1993).

Glyphosate consumption is increasing globally, due both to the expanded area planted to RR crops, and to higher amounts applied per unit area. The increased application of glyphosate has raised concerns about changes in soil-microbial communities, demanding monitoring of microbial populations and activities (Kremer et al., 2005; Johal and Huber, 2009). Microbial biomass is correlated with essential functions in the soil, such as the decomposition of organic matter, cycling of mineral nutrients, plant-growth promotion, biological control of diseases and insects and degradation of xenobiotics. It has been proposed as a sensitive parameter to measure disturbances caused to the soil environment, particularly in relation to those resulting from agricultural management (e.g. Sylvia et al., 2005; Nogueira et al., 2006; Kaschuk et al., 2010, 2011; Lopes et al., 2013), including the monitoring of effects related to transgenic crops (Souza et al., 2008a,b, 2013). Similarly, microbial enzymatic activities are considered to be responsive to changes in the environment caused by natural or human-induced factors (Puglisi et al., 2006; Kaschuk et al., 2010; Mendes et al., 2012). The β-glucosidase enzyme plays a critical role in soils, releasing low molecular weight sugars that represent important energy sources for soil microorganisms (Bandick and Dick, 1999). The acid phosphatase (predominant in acidic soils) can provide useful information on soil biochemical activity in the tropical Brazilian soils characterized by low pH values (Balota et al., 2013). Urease is also an important enzyme; however, the experimental areas of our study have not received N-fertilizers and the soils are very poor on N and thus the activity of this enzyme was not evaluated.

Contrasting results have been observed by various researchers evaluating the impact of glyphosate on soil microbiota. Studies by Haney et al. (2000, 2002) indicated that glyphosate application can increase soil-microbial biomass, respiration and C and N mineralization. Evaluations on Brazilian soils indicated that glyphosate was rapidly metabolized to aminophosphonic acid, and increased respiration and fluorescein diacetate (FDA) hydrolytic activity were observed (Araujo et al., 2003). Investigation conducted under controlled conditions in the Midwestern USA found limited or no effect of glyphosate or of cropping RR soybean on soil-microbial biomass (Liphadzi et al., 2005). Similar results were observed in Brazil by Zilli et al. (2008), who found that no significant changes occurred in microbial-biomass content, soil basal respiration or metabolic quotient (qCO_2). Pereira et al. (2008), in a field experiment, also observed that glyphosate application had no impact on soil CO_2 production or on microbial biomass. The study of Gomez et al. (2009) showed that glyphosate application promoted an initial inhibitory effect that affected microbial biomass, microbial respiration rate, qCO_2 and dehydrogenase activity; however, the effect was transitory. In contrast, Bohm et al. (2007) showed that the application of glyphosate resulted in lower C incorporation by microbial biomass, as well as increased respiration.

One concern regarding the use of transgenic plants and their effects on soil microorganisms is the so-called "unexpected effect". Donegan et al. (1995) suggested that unexpected changes in plant traits resulting from genetic modification may impact soil microbial communities. Novel proteins have been shown to be released from transgenic plants into the soil ecosystem, and their presence can influence microbial communities by selectively stimulating the growth of specific organisms able to use these molecules (Dunfield and Germida, 2004). However, as pointed out by Dunfield and Germida (2004), genetically engineered plants differ by only one or two genes, begging the question of whether the difference is enough to influence rhizospheric microorganisms.

The great majority of the studies assessing the effects of transgenic plants and specific herbicides application is limited to greenhouse conditions, using only the transgenic treatments without comparison against the parental non-transgenic plants. When the studies are carried out under field condition, few sites are evaluated and in general for only one cropping season. The present work is part of a large set of experiments performed in six field sites of Brazil, covering several edaphoclimatic conditions, for two cropping seasons, aiming to investigate the effects of transgene resistance to glyphosate, of herbicides and of weed management strategies on soybean crop. The RR gene was introduced in the parental genotypes, in an agreement between Embrapa and Monsanto®; therefore, the comparison between transgenic and nearly isogenic parental genotypes was possible, what is rare in studies with transgenic soybean, once the material usually belongs to the private sector. The results from our study were designed to detect changes in soil microbial biomass and enzyme activities, evaluated both individually and pooled as a multivariate response.

2. Materials and methods

2.1. Geographic location, general description of the field sites, treatments and experiment logistics

The experiments were set up during the cropping seasons of 2003/2004, 2004/2005 and 2005/2006, under no-tillage management, at six sites in Brazil (States between parentheses): Passo Fundo (Rio Grande do Sul); Ponta Grossa (Paraná) (except in 2003/2004); Londrina (Paraná); Uberaba (Minas Gerais); Planaltina (Federal District); and Luiz Eduardo Magalhães (Bahia). Details about the location, climate and soil classification at each site were given elsewhere (Hungria et al., 2014), and the locations of the sites are shown in Fig. 1. Soil chemical and physical properties were described before (Hungria et al., 2014), but are also supplied in Supplementary Table S1. Evaluation of microbial parameters started from the second year after the establishment of the field experiments.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2014.03.010.

Each trial was conducted in a completely randomized block design, with 5 treatments × 3 cultivars, with 6 replicates, in a total of 90 plots. The five treatments consisted of: (T1) RR soybean + glyphosate (2 L ha^{-1}) [Roundup Transorb®, Monsanto, applied 20–30 days after emergence]; (T2) RR soybean + conventional herbicides [0.5 L ha^{-1} of Select 240 (Clethodim, Milenia), mixed with Assist mineral oil at 0.5% of the volume (narrow-leaf weeds), and Classic (Chlorimurum-ethyl, DuPont) at 80 g ha^{-1} (broad-leaf weeds)]; (T3) conventional soybean + conventional herbicides; (T4) RR soybean + hand-weed control; (T5) conventional soybean + hand-weed control.

Three pairs of cultivars, each of which included a parental soybean genotype and its respective nearly isogenic RR genotype, were cropped at each site. Soybean cultivars tested in Ponta Grossa and Londrina were Conquista/ValiosaRR (Cultivar 1 = C1); BRS133/BRS245RR (Cultivar 2 = C2); and Embrapa 59/BRS244RR

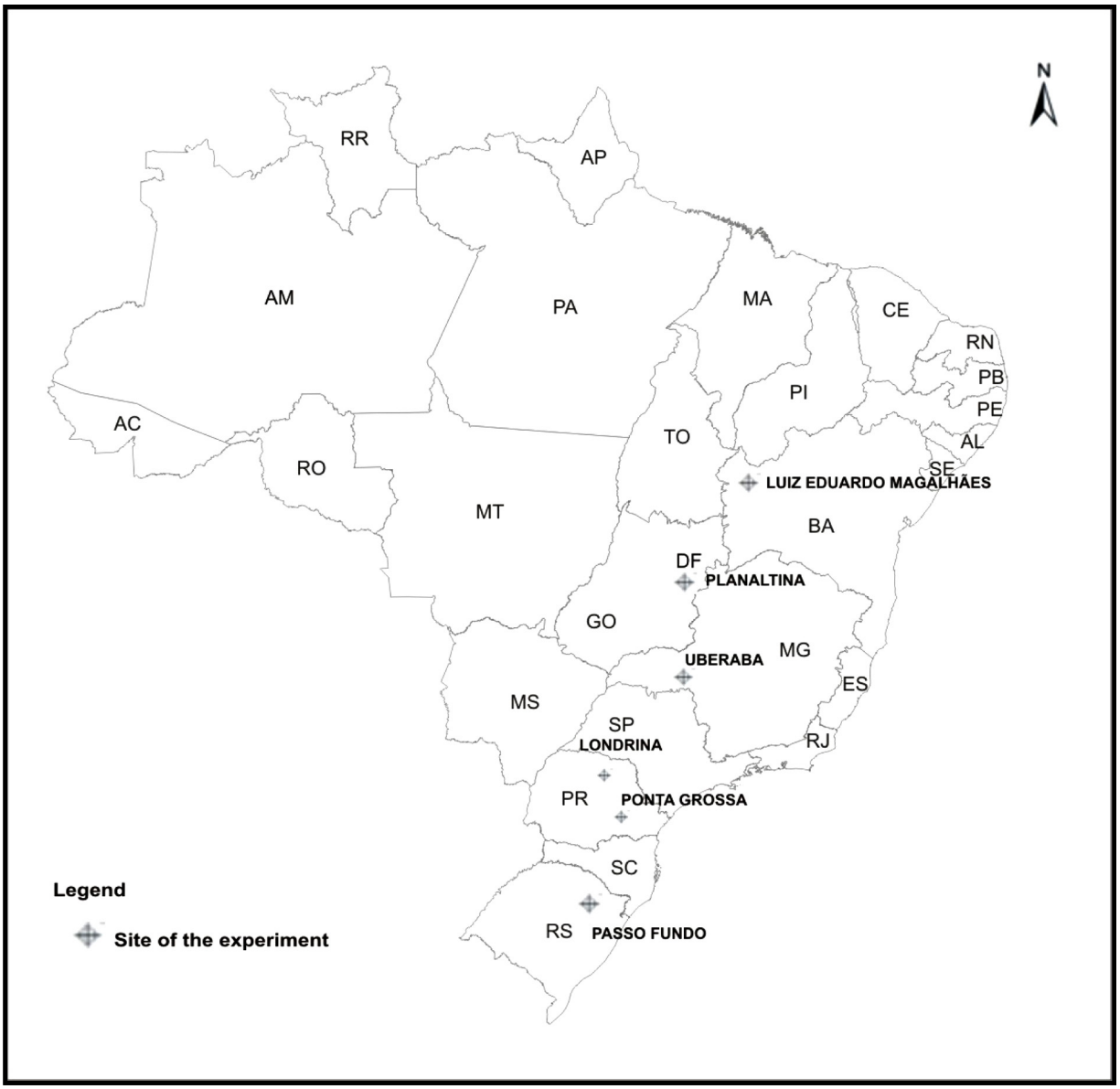

Fig. 1. Map indicating the nine sites where the field experiments were performed.

(Cultivar 3 = C3). These same cultivars were tested in Passo Fundo, except in 2004/2005 and 2005/2006, when Conquista/ValiosaRR were replaced by Embrapa58/BRS242RR. In 2003/2004, the cultivars tested in the Central region areas (Uberaba, Planaltina, Luiz Eduardo Magalhães) were Conquista/ValiosaRR (Cultivar 1 = C1); BRS133/BRS245RR (Cultivar 2 = C2); and Jataí/SilvâniaRR (Cultivar 3 = C3). In 2004/2005 and 2005/2006, the same cultivars were tested, except for BRS133/BRS245RR, which were replaced by Celeste/BalizaRR. Genealogy and maturity groups of parental conventional types were as follows: Conquista MG/BR 46 (Lo76-4484 × Numbaíra, G.8.1); BRS 133 (FT Abyara × BR 83-146, G.7.3); Embrapa 59 (FT Abyara × BR83-147, G.7.3); Embrapa 58 (Paraná × BR83-143, G.7.4); BRS/GO Jataí [Embrapa 313 (Anhanguera) × BR92-31910 (Cristalina CARDF-30*3 × FT Estrela), G.8.9]; BRS Celeste (Bossier × BR 1 T, G.8.1); they all have determinate type of growth. Replacements were made when the cultivars used failed to show good performance, being replaced by better-adapted, newly released cultivars.

All areas were managed under no-tillage system and cropped to soybean in the summer. In the winter, the areas were sown with black oat (*Avena strigosa* L.) or wheat (*Triticum aestivum* L.) in the South Region (Passo Fundo, Ponta Grossa and Londrina), and pearl-millet (*Pennisetum americanum*) in the Central Region (Uberaba, Planaltina and Luiz Eduardo Magalhães).

The soybean seeds were inoculated with a peat-based inoculant produced at Embrapa Soja and containing a 1:1 mixture of *B. elkanii* strain SEMIA 587 and *B. diazoefficiens* strain CPAC 7 (=SEMIA 5080) (10^9 cells g^{-1} of peat). Inoculation involved application of a slurry prepared with 200 g of inoculant in 300 mL of a 10% sucrose sticker solution per 50 kg of seeds, and the inoculant was applied to result in a concentration of 1.2 million cells $seed^{-1}$. Sowing was done manually, with 25–30 viable seeds per meter of row. The experimental plots were 5.0 m × 6.0 m in size, and spaced by 1.0 m. Each plot consisted of 10 rows, sown 50 cm apart.

2.2. Sampling and laboratory analyses

For evaluation of microbial parameters, soil samples were collected from the top layer (0–10 cm) between cropped lines. Microbial biomass-carbon (MB-C) and -nitrogen (MB-N) were

analyzed in 2004/2005 and 2005/2006 for cultivars C1, C2 and C3, whereas beta-glucosidase (GLU) and acid phosphatase (PHO) data were obtained only in season 2005/2006 for cultivar C1. At the R2 stage of soybean growth (Fehr et al., 1971) ten soil samples from 0- to 10-cm depth between the cropped lines were randomly taken in each plot (replicate) and mixed to obtain a composite sample. At the laboratory, samples were homogenized and sieved (4 mm, 5 mesh), and their moisture content determined by drying a 10-g subsample for 12 h at 105 °C. On the following day, the moisture content of the samples was adjusted to 40% (dry basis) of the water-holding capacity (WHC) (Vance et al., 1987) by adding distilled water.

MB-C and MB-N were determined by the fumigation-extraction method of Vance et al. (1987), slightly modified as described before (Hungria et al., 2009). Eight subsamples (20 g) of each composite soil sample were weighted and stored in glass jars (300 mL), four of which were submitted to fumigation, and the other four were left non-fumigated. Fumigated and non-fumigated samples were incubated in the dark at 25 ± 2 °C for 16 h. After the incubation, the C was extracted from the samples by adding 50 mL of extractor solution (0.5 mol L^{-1} K_2SO_4), shaking (175 rpm, 1 h), centrifuging (3000 rpm, 10 min) and filtering as described by Franchini et al. (2007). The C contents of the extracts were determined by oxidation with Mn^{3+} and evaluation on a spectrophotometer (Bartlett and Ross, 1988).

The N contents of the extracts were determined by adding to the extract 1.5 mL of concentrated H_2SO_4 and 50 mg of catalyst (K_2SO_4 + $CuSO_4$, 10:1); after digestion, the product was diluted with distilled water and N quantified by the spectrophotometric determination of NH_4 using the indophenol-blue method (Feije and Anger, 1972).

MB-C and MB-N values were estimated from the differences between the fumigated and non-fumigated samples, employing a K_{CE} value of 0.38 and a K_{NE} value of 0.54 (Brookes et al., 1985).

Beta-glucosidase (GLU) and acid phosphatase (PHO) activities were determined according to Tabatabai (1996), based on the hydrolysis of *p*-nitrophenyl β-glucoside and *p*-nitrophenyl phosphate substrates and the colorimetric determination of the resulting *p*-nitrophenol. Yield parameters obtained at harvest [soybean grain yield (SGY, kg ha^{-1}), adjusted to 13% moisture; total grain N content (TGN, kg N ha^{-1}); percentage grain N (%GN, g kg^{-1})] are presented in our accompanying study (Hungria et al., 2014), and were used in this study for the correlation analyses with individual microbial variables and with the pooled variables, denominated as soil microbial variables (SMV).

2.3. Statistical analyses

Prior to the analysis of variance (ANOVA), data were tested for normality of variables and uniformity of variance (SAS Institute, 1999). After that, data from season 2005/2006 were submitted to means contrast analysis, to compare the effects of the transgenic trait (Contrast 1), type of herbicide in transgenic cultivars (Contrast 2), and weed-management strategies (Contrast 3) on soil microbiological variables (SMV), as follows: Contrast 1, transgenic vs. non-transgenic (only conventional herbicide and hand-weed control treatments) [(μT2 + μT4)/2] – [(μT3 + μT5)/2]; Contrast 2, transgenic with glyphosate vs. transgenic with conventional herbicide [μT1] – [μT2]; and Contrast 3, transgenic with glyphosate vs. non-transgenic with conventional herbicide [μT1] – [μT3], where T1, T2, T3, T4, T5 represent the treatments and μ represents the means. Contrast significance was assessed by the Student's *t* test ($p \leq 0.05$). The same contrasts were used to analyze MB-C and MB-N data, averaged over the 2004/2005 and 2005/2006 seasons.

In addition, we used a multivariate analysis to evaluate the effects of the transgenic event and herbicides on soil SMV, described by the analysis of four pooled variables (MB-C, MB-N, GLU, PHO). A two-dimensional ordination of samples in the space of microbial variables, representing differences in SMV among treatments, was attained by non-metric multidimensional scaling (NMS, Sokal, 1979), with Sorensen distances. Prior to analysis, data were standardized by totals within each variable (option "relativization by column totals" in PC-ORD v.6.0) to eliminate the differences in the expression units between variables. The ordination was run in the autopilot mode, using the "slow and thorough" option in the PC-ORD v.6.0 software (McCune and Mefford, 2011). The number of dimensions to be interpreted was selected considering the criteria of stress and stability of the graphical solutions.

Variations in the SMV among the different sites were characterized by Pearson correlation between the sample scores on the NMS Axes 1 and 2 and the values of microbiological variables. Correlation analyses were also performed to describe the relationship between productivity components (SGY, %NG and TNG) and SMV NMS scores.

A blocked multiresponse permutation procedure (blocked-MRPP, Mielke and Berry, 2000) was employed to test the hypotheses of no effect of transgenic trait, herbicide type, and weed-management strategy on SMV, using the same three contrasts described for the univariate analysis as "grouping variables" and sites as "blocking variables." Because PC-ORD v.6.0 permits only two levels of explanatory variables (grouping and blocking variables; in our case, contrasts and regions, respectively) for blocked-MRPP the mean values among replicates (blocks) from each treatment were used. This analysis approach allowed us to test the hypotheses after considering the differences in SMV between regions. In all cases, Sorensen distance measure was used. Values of *p* associated with test statistics (*T*) were determined by numerical integration of the Pearson type III distribution. All multivariate tests were performed using the statistical program PC-ORD v. 6.0 (McCune and Mefford, 2011).

For each tested contrast, MB-C and MB-N were modeled using sums of squares multivariate regression tree (SS-MRT) models (De'Ath, 2002), with site, season, cultivar, and contrast treated as categorical explanatory variables. This analysis allowed us to evaluate the effect size of each contrast on MB-C and MB-N relative to those of site, season and cultivar. A series of 20- to 10-fold cross-validations (Breiman et al., 1984) was run to choose the most frequently occurring (modal) tree size with a minimum error rate (De'Ath and Fabricius, 2000). The final tree size was chosen using the 1-SE (standard error) rule (Breiman et al., 1984), which results in a tree smaller than that suggested by the minimum cross-validated-error rate (at most 1-SE). A library of S-Plus functions for tree routines (RPART: Recursive partitioning), developed by T. Therneau (unpublished data), was used for all SS-MRT models. S-Plus (version 4.0) statistical software (Mathsoft, 1999) was used for these analyses.

Multivariate techniques (NMS and MRPP) used to evaluate the response of the four pooled variables (SMV) to the factors tested were carried out only with data from season 2005/2006 and cultivar C1.

3. Results

3.1. Impact of transgenic soybean, glyphosate and weed-management strategy on soil-microbiological variables

First, results of season 2005/2006 are presented, where the soil microbiological condition was assessed from microbial biomass-carbon (MB-C) and -nitrogen (MB-N), beta-glucosidase (GLU) and acid phosphatase (PHO) activities, since the soil enzyme activities were not measured during the earlier seasons. Following, results from MB-C and MB-N in two cropping seasons (2004/2005 and

Table 1
Microbial biomass carbon (MB-C) and nitrogen (MB-N) and activity of beta-glucosidase (GLU) and acid phosphatase (PHO) as affected by glyphosate resistant trait (contrast 1) in soybean cultivars grown in six major producing sites in Brazil in the crop season of 2005/2006.

Site	Contrast	MB-C mg C kg^{-1} soil	MB-N mg N kg^{-1} soil	GLU μg p-nitrophenol g^{-1} soil h^{-1}	PHO
Passo Fundo	Transgenic	253.82	12.97	126.33	497.27
	Non-transgenic	268.03	15.13	135.66	507.92
	p	ns	ns	ns	ns
Ponta Grossa	Transgenic	153.68	17.94	115.35	453.94
	Non-transgenic	141.82	19.83	106.95	473.97
	p	ns	ns	ns	ns
Londrina	Transgenic	248.00	24.21	150.54	423.69
	Non-transgenic	253.37	25.48	155.46	441.63
	p	ns	ns	ns	ns
Uberaba	Transgenic	152.75	7.46	54.16	407.11
	Non-transgenic	121.92	8.16	48.83	450.20
	p	**	ns	ns	*
Planaltina	Transgenic	224.09	33.18	138.91	521.83
	Non-transgenic	239.80	33.25	152.30	533.62
	p	ns	ns	ns	ns
Luiz Eduardo Magalhães	Transgenic	179.04	15.05	45.79	281.95
	Non-transgenic	178.44	11.95	42.31	264.10
	p	ns	ns	ns	ns

* $p < 0.05$.
** $p < 0.01$.

2005/2006) are presented, allowing us to evaluate the effects of the transgene, crop season, site and cultivar on these two microbiological variables.

Except for a few cases, soil microbiological parameters evaluated in 2005/2006 were not affected by either the transgenic trait (Table 1), type of herbicide (Table 2), or weed-management method (Table 3). Yet, in these few cases, effects were not consistent in terms of specific treatments and were dependent on the type of variable and site. When compared to their nearly isogenic non-transgenic counterparts, transgenic cultivars increased MB-C (+25%) and decreased PHO values (−11%) at Uberaba (Table 1).

There were no differences between types of herbicide in terms of MB-N, regardless of the site. Compared with glyphosate, conventional herbicides resulted in higher MB-C (+24%) at Uberaba, GLU at Passo Fundo (+25%), and PHO at Planaltina (+14%), but in lower PHO at Londrina (−13%) (Table 2).

Compared with the weed-management strategy based on non-transgenic cultivars and conventional herbicides, the combined use of glyphosate and RR soybeans resulted in higher PHO (+12%) at Londrina and MB-C (+22%) at Uberaba. However, the opposite was true for GLU (−51%) and PHO (−25%) at Passo Fundo, PHO at Uberaba (−19%), and MB-N at L.E. Magalhães (−36%) (Table 3)

When the two seasons (2004/2005 and 2005/2006) were analyzed together, no effects on MB-C and MB-N were observed for any of the microbial contrasts evaluated (Table 4).

A two-dimensional NMS graph represented 97% of the variability of the soil microbial variables (SMV), described by the analysis of four pooled biological variables (MB-C, MB-N, GLU and PHO) (Fig. 2).

Table 2
Microbial biomass carbon (MB-C) and nitrogen (MB-N) and activity of beta-glucosidase (GLU) and acid phosphatase (PHO) as affected by the type of herbicide (glyphosate vs. conventional herbicides) used to control weeds (contrast 2) in RR soybean crops in six major producing sites in Brazil in the crop season of 2005/2006.

Site	Contrast	MB-C mg C kg^{-1} soil	MB-N mg N kg^{-1} soil	GLU μg p-nitrophenol g^{-1} soil h^{-1}	PHO
Passo Fundo	Transgenic with glyphosate	298.86	12.74	99.13	420.11
	Transgenic with conventional herbicide	258.10	13.34	123.67	478.03
	p	ns	ns	*	ns
Ponta Grossa	Transgenic with glyphosate	161.51	18.01	89.66	461.49
	Transgenic with conventional herbicide	148.41	17.24	112.81	512.64
	p	ns	ns	ns	ns
Londrina	Transgenic with glyphosate	274.57	27.31	144.09	484.03
	Transgenic with conventional herbicide	240.47	24.79	134.59	430.16
	p	ns	ns	ns	*
Uberaba	Transgenic with glyphosate	164.14	6.03	49.68	392.47
	Transgenic with conventional herbicide	203.69	7.55	56.22	431.85
	p	**	ns	ns	ns
Planaltina	Transgenic with glyphosate	252.86	33.12	155.76	530.03
	Transgenic with conventional herbicide	229.57	34.46	148.71	604.41
	p	ns	ns	ns	*
Luiz Eduardo Magalhães	Transgenic with glyphosate	235.53	15.95	48.17	273.80
	Transgenic with conventional herbicide	204.62	17.35	48.50	303.10
	p	ns	ns	ns	ns

* $p < 0.05$.
** $p < 0.01$.

Table 3
Microbial biomass carbon (MB-C) and nitrogen (MB-N) and activity of beta-glucosidase (GLU) and acid phosphatase (PHO) as affected by the strategy of weed management (transgenic soybean cultivars managed with glyphosate against non-transgenic cultivars managed with conventional herbicides) (contrast 3) in soybean crops in six major producing sites in Brazil in the crop season of 2005/2006.

Site	Contrast	MB-C mg C kg^{-1} soil	MB-N mg N kg^{-1} soil	GLU μg p-nitrophenol g^{-1} soil h^{-1}	PHO
Passo Fundo	Transgenic with glyphosate	298.86	12.74	99.13	420.11
	Non-transgenic with conventional herbicide	267.47	16.13	149.26	526.88
	p	ns	ns	**	*
Ponta Grossa	Transgenic with glyphosate	161.51	18.01	89.66	461.49
	Non-transgenic with conventional herbicide	148.30	20.77	107.57	507.81
	p	ns	ns	ns	ns
Londrina	Transgenic with glyphosate	274.57	27.31	144.09	484.03
	Non-transgenic with conventional herbicide	290.37	26.84	153.08	433.32
	p	ns	ns	ns	*
Uberaba	Transgenic with glyphosate	164.14	6.03	49.67	392.47
	Non-transgenic with conventional herbicide	134.68	7.04	52.64	465.72
	p	*	ns	ns	*
Planaltina	Transgenic with glyphosate	252.86	33.12	155.76	530.03
	Non-transgenic with conventional herbicide	248.85	34.14	163.35	551.09
	p	ns	ns	ns	ns
Luiz Eduardo Magalhães	Transgenic with glyphosate	216.38	6.25	48.17	273.80
	Non-transgenic with conventional herbicide	248.39	9.72	44.14	251.33
	p	ns	*	ns	ns

* $p < 0.05$.
** $p < 0.01$.

Table 4
MB-C and MB-N as affected by RR trait in soybean cultivars (transgenic × non-transgenic), the type of herbicide (transgenic with glyphosate × transgenic with conventional herbicide) used to control weeds in areas with transgenic cultivars, and strategy of weed management (glyphosate + RR cultivars vs. conventional herbicide + non-RR cultivars) in soybean in the crop seasons of 2004/2005 and 2005/2006 in six main producing sites in Brazil.

Contrast	MB-C mg C kg^{-1} soil	MB-N mg N kg^{-1} soil
Transgenic × Non-transgenic		
Transgenic	314.82	32.09
Non-transgenic	308.40	32.09
P	ns	ns
Transgenic with glyphosate × Transgenic with conventional herbicide		
Transgenic with glyphosate	329.38	31.98
Transgenic with conventional herbicide	323.00	32.48
P	ns	ns
Transgenic with glyphosate × Non-transgenic with conventional herbicide		
Transgenic with glyphosate	329.38	31.98
Non-transgenic with conventional herbicide	317.05	31.37
P	ns	ns

Table 5
Correlation coefficients (*r*) of soil microbial variables (SMV) with non-metric multidimensional scaling (NMS) ordination axis.

	Axis 1	Axis 2
Microbial biomass carbon	0.291	0.984***
Microbial biomass nitrogen	0.835***	−0.016
Beta-glucosidase activity	0.926***	0.530
Acid phosphatase activity	0.781***	0.278

*** $p < 0.001$.

Axis 1 represented most of the total variability (72%), whereas only a minor portion (25%) was represented by Axis 2. GLU and PHO activities and MB-N were predominantly correlated with Axis 1, whereas MB-C was correlated with Axis 2 (Table 5). SMV clearly shifted between the six sites ($p < 0.001$), possibly related to the contrasting chemical properties of the soils (Table S1). The arrangement of soil samples from different sites along axis 1 of SMV was strongly and positively correlated with Al + H ($r = 0.89$, $p < 0.01$) and CEC ($r = 0.81$, $p < 0.05$). Therefore, shifts in SMV from L. E. Magalhães toward Planaltina were associated with increases in MB-N,

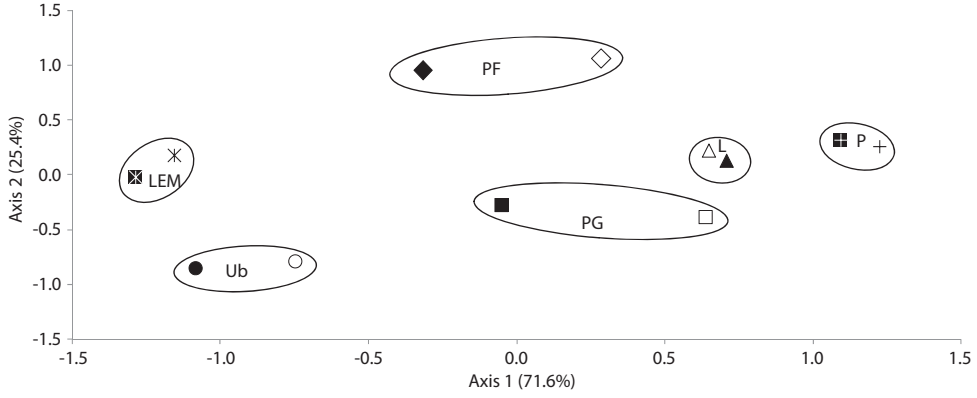

Fig. 2. Non-metric multidimensional scaling (NMS) ordination of the sample plots with respect to soil microbial variables (SMV), as affected by site and weed management strategy. Symbols represent the centroids of samples ($n = 4$) from plots with weed management based on glyphosate + RR cultivars (dark symbols) and on conventional herbicides + non-RR cultivars (light symbols), in the regions of Luis Eduardo Magalhães (LEM), Uberaba (Ub), Passo Fundo (PF), Ponta Grossa (PG), Londrina (L) and Planaltina (P). *p* value for comparisons of SMV between the two weed management strategies was 0.052, after considering differences due to sites.

Table 6
Percentage of the data variability explained by the tree model for microbial biomass-carbon (MB-C) and nitrogen (MB-N) in contrasts 1, 2 and 3, as a function of site, year of sampling, cultivar and the tested contrast. Crop seasons of 2004/2005 and 2005/2006.

Effect	Contrast 1		Contrast 2		Contrast 3	
	MB-C	MB-N	MB-C	MB-N	MB-C	MB-N
Site	29.43	27.53	31.86	38.63	30.90	36.10
Crop season	26.92	30.03	22.32	25.79	20.97	24.20
Cultivar	13.97	16.49	17.13	12.96	20.32	14.51
Contrast	0.18	0.56	0.88	0.62	1.71	4.89
Explained %	70.50	74.60	72.20	78.00	73.90	79.70

Contrast 1: transgenic vs. non-transgenic; contrast 2: transgenic with glyphosate vs. transgenic with conventional herbicide; contrast 3: transgenic with glyphosate vs. non-transgenic with conventional herbicide.

GLU and PHO, which in turn, were correlated with increments in Al + H and CEC. Also, variations in SMV between regions along axis 2 were correlated with Ca + Mg ($r = 0.85$, $p < 0.05$) and SB ($r = 0.81$, $p < 0.05$).).

However, according to blocked-MRPP, SMV was not affected by transgenic traits ($P_{\text{Contrast 1}} = 0.376$); type of herbicide in transgenic cropping ($P_{\text{Contrast 2}} = 0.249$); or weed-management strategy ($P_{\text{Contrast 3}} = 0.052$), after considering differences associated with the six experimental sites. Although the effects of weed-management strategies were marginally non-significant ($p = 0.052$), it is noteworthy that the pattern of change in SMV was, except for Londrina, the same for all sites, with samples from conventional herbicides with non-RR cultivars placed at the right side of samples with glyphosate and RR-cultivar-based management (Fig. 2). These shifts are in agreement with the significant increases observed in response to the use of conventional herbicides with non-RR cultivars in MB-N at Luiz Eduardo Magalhães, in PHO in Uberaba, PHO and GLU in Passo Fundo, and with trends of increments in MB-N, PHO and GLU in the majority of the evaluated sites (Table 3), especially in Ponta Grossa, Passo Fundo, Uberaba, and Planaltina.

The results of the tree model selected to measure the contribution of different factors (site, cropping season, cultivar and contrast) on the data variability of MB-C and MB-N in 2004/2005 and 2005/2006 for each contrast tested are shown in Table 6. It was verified that variability in the MB-C and MB-N data can be explained mainly by the factor "site" (from 28% to 36%), followed by "crop season" (from 21% to 30%) and "cultivar" (from 13% to 20%). On the other hand, the tested contrasts are responsible for only a small fraction of MB-C and MB-N variability (from 0.2% to 4.9%). Therefore, site, cropping season and cultivar were far more influential on soil MB-C and MB-N than the use of a transgenic crop or glyphosate.

Table 7
Correlation coefficients between the soil microbial variables (SMV) and soybean yield from five sites in Brazil. Crop season of 2005/2006.

	SGY	%GN
MB-C	−0.09	0.16
MB-N	0.34***	−0.40***
GLU	0.31***	−0.55***
PHO	0.58***	−0.67***

MB-C, carbon microbial biomass; MB-N, nitrogen microbial biomass; GLU, beta-glucosidase; PHO, acid phosphatase; SGY, soybean grain yield; %GN, % of grain nitrogen. Soybean grain yield data from Passo Fundo were lost due a drought event.
*** $p < 0.001$.

3.2. Correlation between the soil microbial variables (SMV) and soybean grain yield

Highly significant correlations ($p < 0.001$) were observed between soybean yield parameters SGY and %GN with Axes 1 of NMS ordination describing SMV (Fig. 3A and B). These correlations were positive and significant between SGY and MB-N, GLU, and PHO. Conversely, the correlations were negative and significant between %NG and these three microbiological variables, and non-significant correlations were found with MB-C (Table 7).

4. Discussion

Despite the benefits associated with transgenic soybean, such as increased grain yield and reduction of agrochemical use, concerns about the implications of the transgenes on human health and environmental safety are often raised (Cerdeira et al., 2007). In relation to the soil environment, it is well known that soil microorganisms play key roles that affect soil quality, justifying the need for continuous studies to monitor the effects of disturbances, with an emphasis on those associated with new agricultural technologies and pesticides (e.g. Kaschuk et al., 2010, 2011). The quantification of soil microbial biomass—C and N—is justified in the present study because they are sensitive variables to evaluate environmental impacts. In fact, Souza et al. (2008a,b) in an extensive study performed in Brazil, found soil microbial biomass to be part of a minimum dataset to assess the impacts of new technologies in agriculture. Similarly, soil enzymes play an important role in the nutrient cycling and are thus useful tools to evaluate soil microbial activity, since most soil enzymes are released by microorganisms (Stott et al., 2010; Lopes et al., 2013).

In addition, soil microbial parameters may be useful indicators of sustainability and yield; indeed, direct relations between crop yield and microbial biomass (e.g. Hungria et al., 2009; Silva et al., 2010) and soil enzymes (Zhang et al., 2012; Lopes et al., 2013) have been reported by other authors and were confirmed in our

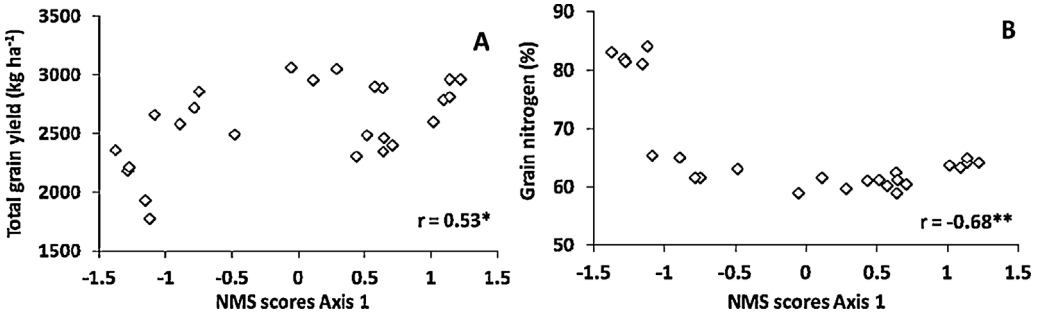

Fig. 3. Correlation between soybean grain yield (A) and soybean grain nitrogen (B) and NMS scores of Axis 1 (71.6%) of soil microbial variables (SMV). $n = 25$ for soybean grain yield and grain nitrogen content. Data from Passo Fundo were lost due to a drought event. *$p < 0.005$, **$p < 0.0005$. Crop season of 2005/2006.

study, with positive, significant correlations between three microbial parameters (MB-N, GLU and PHO) and soybean yield.

Our study has shown that glyphosate-resistance in soybean had no impact on soil microbial variables, assessed during one (2004/2005) or two consecutive cropping seasons (2004/2005 and 2005/2006), in agreement with other studies that showed little or no effect on soil microbial communities (Bruinsma et al., 2003; Motavalli et al., 2004; Liu et al., 2008; Böhm and Rombaldi, 2010; Weinert et al., 2010). Another transgenic soybean carrying the *ahas* gene—which confers resistance to herbicides of the imidazolinone group—also had no impact on soil microbial biomass compared to the non-transgenic parental cultivar treatment, in field trials carried out in six sites in Brazil for three cropping seasons (Souza et al., 2013).

We found that soil microbial biomass was more affected by site, cropping season and soybean cultivar than the transgenic trait. Although changes in soil microbial variables due to transgenic plants have been reported (Dunfield and Germida, 2001; Wei et al., 2006), the effects have usually been minor when compared to those related to natural variability and environmental factors, such as soil type, site, climate, time of year and tillage practice (Lottmann et al., 1999; Lukow et al., 2000; Dunfield and Germida, 2004; Griffiths et al., 2007). Even when changes related to a transgene were detected, they were short-lived and were absent from subsequent evaluations (Gyamfi et al., 2002; Dunfield and Germida, 2003; Mulder et al., 2006). According to Hart et al. (2009), EPSPS occurs naturally in agricultural soils, produced by the common soil bacterium *Agrobacterium tumefaciens*, therefore its effects—when produced by RR soybean—on soil microbial populations are likely to be minor in comparison with modifications that introduce novel or detrimental proteins into the soil.

In some of the findings of no effect of transgenic RR soybean on microbial communities, impacts of the herbicide glyphosate have been reported. Dallmann et al. (2010), in a field experiment performed in southern Brazil in the 2008/2009 crop season did not detect differences in fungal densities between soils planted with one transgenic (BRS 243RR) and one non-transgenic (BRS Cambona) soybean cultivar; however, glyphosate decreased fungal populations. The same authors reported that, under high application of glyphosate (1920 and 3840 g a.i. ha^{-1}), metabolic quotient (qCO_2) was higher, as a result of increased CO_2 production without change in MB-C. The effects of glyphosate on soil microorganisms depend on the dosage, soil texture and organic matter content (Giesy et al., 2000), as well as the site, cropping conditions and time at which it is applied (Wallis et al., 2010). Nevertheless, in our study, no such effects were detected, in agreement with the results of other studies reporting no impact of glyphosate on microbial biomass (Liphadzi et al., 2005; Pereira et al., 2008; Bohm et al., 2011). Zilli et al. (2008), in a greenhouse experiment, using a Brazilian Oxisol (Latossolo vermelho distroférrico, clayey texture), also found no effect of glyphosate on soil microbial biomass, and suggested that this may be explained by adsorption to colloids. Similarly, the herbicide would not be available to microorganisms as sources of C, N or P (Van Eerd et al., 2003); furthermore, degradation by hydrolysis and photolysis (Giesy et al., 2000) may contribute to reduced toxicity for soil microorganisms.

In relation to the weed management, we found no differences in the effect of transgenic soybean and glyphosate vs. non-transgenic soybean and conventional herbicides. Similarly, in the study with the transgenic *ahas* gene, differences were not detected between the treatments of transgenic soybean and imidazolinone vs. non-transgenic soybean with conventional herbicide (Souza et al., 2013). Regarding to glyphosate resistant cropping system, under field conditions in southern Brazil, in the cropping season of 2007/2008, Bohm et al. (2011) observed no changes on soil microbial biomass in the comparison between one transgenic soybean BRS 244RR with glyphosate, and one conventional soybean BRS 154 treated with the conventional herbicide imazethapyr.

It is important to highlight that the mentioned studies evaluating the effects of transgenic plants and/or glyphosate on soil microorganisms were conducted either under greenhouse conditions (Zilli et al., 2008), or under field condition in only one cropping season and one site (Dallmann et al., 2010; Bohm et al., 2011). In addition, the comparisons did not consider pairs of transgenic and the respective parental non-transgenic cultivar. In our study, treatments were evaluated at six field sites differing in edaphoclimatic conditions, during two consecutive cropping seasons (for MB-C and MB-N), and considering three pairs of transgenic vs. the nearly isogenic parental non-transgenic cultivar, giving higher credibility to the results that showed no effects of weed-management strategies on soil microbial variables.

Soil microbial biomass is composed by numerous microbial groups in constant interaction with the ecosystem, and in our study the introduction of transgenic plants and glyphosate application to the system did not change the microbial biomass as a whole. However, it is important to point out that our findings are valid for our specific case where few microbial attributes were evaluated in a maximum of two crop seasons. Further and long–term studies are needed using larger sets of microbial variables to confirm that the transgenic trait and the glyphosate application do not affect soil microorganisms.

5. Concluding remarks

Overall, soil microbial communities—evaluated in this study in terms of microbial biomass (MB-C and MB-N), and activities of acid phosphatase and beta-glucosidase—showed no differences in the comparisons of: RR transgenic × nearly isogenic parental non-transgenic cultivars; glyphosate × conventional herbicides; or RR soybean with glyphosate × conventional soybean and herbicide management. Moreover, where impacts on individual microbiological properties were observed, they were not consistently obtained with a specific treatment, but rather correlated with site, crop season and/or cultivar.

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