Research Article

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Impact of glyphosate-resistant corn, glyphosate applications and tillage on soil nutrient ratios, exoenzyme activities and nutrient acquisition ratios

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Abstract

BACKGROUND: We report results of the last two years of a 7 year field experiment designed to test the null hypothesis: applications of glyphosate on glyphosate-resistant (GR) and non-resistant (non-GR) corn (*Zea mays* L.) under conventional tillage and no-till would have no effect on soil exoenzymes and microbial activity.

RESULTS: Bulk soil (BS) and rhizosphere soil (RS) macronutrient ratios were not affected by either GR or non-GR corn, or glyphosate applications. Differences observed between exoenzyme activities were associated with tillage rather than glyphosate applications. In 2013, nutrient acquisition ratios for bulk and rhizosphere soils indicated P limitations, but sufficient assimilable N. In 2014, P limitations were observed for bulk and rhizosphere soils, in contrast to balanced C and N acquisition ratios in rhizosphere soils. Stoichiometric relationships indicated few differences between glyphosate and non-glyphosate treatments. Negative correlations between C:P and N:P nutrient ratios and nutrient acquisition ratios underscored the inverse relation between soil nutrient status and microbial community exoenzyme activities.

CONCLUSIONS: Inconsistent relationships between microbial community metabolic activity and exoenzyme activity indicated an ephemeral effect of glyphosate on BS exoenzyme activity. Except for ephemeral effects, glyphosate applications appeared not to affect the function of the BS and RS exoenzymes under conventional tillage or no-till.

Keywords: corn; exoenzymes; glyphosate; rhizosphere; soil; tillage

1 INTRODUCTION

In contrast to conventional tillage, conservation tillage, such as reduced- and no-till systems, conserve soil, water and energy resources and target retention of at least 30% of the previous crop residues on the soil surface after planting.[1] With conservation tillage practices, crops are planted into the previous year's plant residue, and integral to this practice is the use of herbicides for weed control.[2–4] The implementation of glyphosate-resistant crops along with strategic applications of the non-selective herbicide glyphosate [N-(phosphomonomethyl)glycine] for controlling weeds has facilitated the adoption of conservation tillage practices[5] and has become a general means of weed management across the United States.[6] By 2013, 90% of all corn that was planted across the United States was genetically modified to be resistant to the herbicide glyphosate (www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx, accessed 1 February 2016).

Because glyphosate applications have become a significant part of weed management, its use has expanded parallel to the increased acreage of glyphosate-resistant crops. Although glyphosate appears to adsorb readily to soil, its range of K_f values between 0.6 and 215 contradicts this assertion.[5] The half-life of glyphosate in soil is variable, ranging from 1.7 to 142 days. Thus, glyphosate is detected, as well as its metabolite, aminomethylphosphonic acid (AMPA), in surface waters in agricultural regions where glyphosate applications regularly occur.[7] Results of a study on the fate of glyphosate in soil[8] indicated that, under controlled conditions, phosphate may compete with glyphosate for soil binding sites, thus keeping glyphosate in solution. As Coupland and Caseley[9] demonstrated, foliar applications of glyphosate move through plants, and glyphosate is released into the rhizosphere where it may diffuse or be hydrologically transported into bulk soil. Thus, the detection of glyphosate and AMPA in surface waters implies that, because of its presence in soil water, it interacts with rhizosphere and bulk soil microbial

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communities and their extracellular enzymes that are involved in the initial steps of crop residue degradation.

Few studies have assessed the effects of glyphosate resistance on soil ecology by directly comparing cropping systems with and without the glyphosate resistance trait.[5] A few reports from both laboratory and field studies have indicated no or insignificant effects of either glyphosate or glyphosate-resistant crops on bulk and rhizosphere soil microbial biomass carbon, microbial functional diversity[10–13] and exoenzyme β-glucosidase activity.[13] In contrast, Zobiole *et al.*,[14] in a greenhouse study, observed that glyphosate applications to a glyphosate-resistant soybean (*Glycine max* L. Merr.) cultivar had a negative impact on components of the rhizosphere microbial community. Sannino and Gianfreda[15] reported results of laboratory experiments on several Italian soils that demonstrated inhibitory effects of glyphosate on phosphatase activity.

The initial steps in the mineralization of crop residues and soil organic matter in water, sediment and soil are undertaken by exoenzymes, catalysts synthesized and excreted by components of the microbial community, which underlie global nutrient cycles and plant productivity.[16] In this study we have focused on four exoenzymes that are linked to terminal reactions of biogeochemical processes involved in the C, N and P nutrient cycles – β-1,4-glucosadase (BG) involved in cellulose degradation, leucine aminopeptidase (LAP) involved in proteolysis, β-1,4-*N*-acetylglucosaminidase (NAG) involved in chitin and peptidoglycan degradation and acid (alkaline) phosphatase (AP) involved in the hydrolysis of phosphate from phosphosaccharides and phospholipids – and the exoenzymatic ratios BG:AP, BG:(LAP + NAG) and (LAP + NAG):AP which represent metrics for exoenzymatic C:P, C:N and N:P nutrient acquisition activities respectively.[16] Our objective for the last 2 years of a 7 year field study was to measure the effects of routine applications of glyphosate to genetically modified glyphosate-resistant corn and its non-glyphosate resistant isogenic cultivar and appropriate controls, and to test the null hypothesis that agronomic application rates of glyphosate would have no observable effect on soil nutrient cycling as observed in bulk and rhizosphere soil C:N, C:P and N:P nutrient ratios, exoenzyme activities, their nutrient acquisition activities as defined by Sinsabaugh *et al.*[16] and total microbial activity as measured with fluorescein diacetate (FDA) activity.

2 METHODS

2.1 Experimental design

The study was established on the USDA-ARS Crop Production Systems Research Unit Farm, Stoneville, Mississippii, in the fall of 2007. A split-split plot experimental design was established with four replications: tillage (conventional and reduced tillage) was the main treatment, isogenic cultivar (glyphosate-resistant, GR, and non-glyphosate-resistant, non-GR) was the split treatment and glyphosate (with and without glyphosate application) was the split-split treatment. Treatments were randomly assigned within each level. Field preparation consisted of disking, subsoiling, disking and bedding in the fall of 2007. The two tillage treatments were conventional and reduced tillage. Conventional tillage plots were disked and seedbeds prepared each fall after corn harvest. Reduced-tillage plots did not receive any further tillage operations after establishment in the fall of 2007. A furrow sweep was used to remove excess corn residue from the previous year to enable irrigation during the crop year. Plots were kept weed free either by planned herbicide applications or by hand-hoeing. An established herbicide application regime was continued throughout the 7 years of the experiment: burndown of all plots with 2,4-D (1.1 kg AI ha^{-1}) in February and with paraquat (2.2 kg AI ha^{-1}) before planting, and pre-emergence herbicides for all plots with atrazine (1.7 kg AI ha^{-1}) and metolachlor (1.7 kg AI ha^{-1}) immediately after corn planting. Glyphosate, as potassium salt of glyphosate, was applied in a formulation of Roundup Weathermax (Monsanto Agricultural Co., St Louis, MO), and applications were (1) glyphosate at 2.2 kg ae ha^{-1} applied twice at early (first) and late (second) crop season, and (2) no glyphosate control. In GR corn plots that received glyphosate treatments, the first glyphosate application was applied over the top, and the second application was applied as post directed to base of the corn. In non-GR corn plots that received glyphosate treatments, both early and late post-emergence applications were made using a hooded sprayer only between corn rows to ensure no damage to non-GR corn. The purpose was to expose the soil to glyphosate under non-GR corn. Halosufuron (0.07 kg ha^{-1}) was applied in the third week of May on all plots to manage yellow nutsedge. All plots received 225 kg N ha^{-1} in the form of a mixture of liquid urea and ammonium nitrate. Each plot consisted of eight rows spaced 102 cm apart and 32 m long. Non-GR soybean [*Glycine max* (L.) Merr.] was planted in 2007 to establish a baseline with no transgenic influence, as the experimental area was under transgenic soybean in 2005 and transgenic cotton (*Gossypium hirsutum* L.) in 2006. During the first five years (2008–2012) of the study, GR and non-GR commercial corn hybrids were planted in respective plots. During the last 2 years, GR DeKalb DKC65-17 (RR2) and non-GR DeKalb DKC65-18 (conventional) hybrids were planted on 20 March 2013 and 20 March 2014. DKC65-17 and DKC65-18 are isogenic cultivars and are commercially available. In 2013 and 2014, a comprehensive evaluation was conducted to assess long-term (after 6 and 7 years) effects of transgene or glyphosate application within conservation no-tillage or conventional tillage systems on soil ecology and soil chemical and physical parameters (N, P, SOC). This paper presents results of exoenzyme activities associated with bulk and rhizosphere soils sampled in 2013 and 2014.

2.2 Soil sampling and processing

2.2.1 *Bulk soil*

At the corn's R2 growth stage, 27 days and 42 days after the last glyphosate application in 2013 and 2014 respectively, five 10 cm depth soil samples from each plot were taken randomly using a flame-sterilized probe, composited in a sterile plastic bag and mixed. Soil in the plastic bag was stored at 4 °C until analysis (enzymes, soil moisture, extractable P, TC, TN).

2.2.2 *Rhizosphere soil*

Concurrent with bulk soil sampling, seven root balls were removed from each field plot with a flame-sterilized shovel; loose soil was shaken off by knocking root balls against the shovel. Root balls were then placed in a paper bag for transit on ice to the laboratory for processing. In the laboratory, seven root balls per field plot were placed in a sterile (autoclaved) bucket with 4 L of sterile deionized water and washed one after the other by gentle agitation to remove rhizosphere soil. The rhizosphere soil slurry was poured through a large sterilized stainless steel funnel and through a sterilized 2 mm sieve into a sterilized pickle jar. Subsamples of soil slurry from the pickle jar were poured through a small surface

sterilized funnel into six sterile 250 mL centrifuge bottles. The samples were centrifuged at 10 000 × *g* for 15 min. The pellet was resuspended in a minimal volume of supernatant, which was then aseptically transferred to sterile Falcon tubes. The soil slurry was stored at 4 °C before exoenzyme activity determinations and chemical analyses. An aliquot of slurry was set aside for later air drying and dry weight determination.

2.3 Exoenzyme determinations

The enzyme activities of phosphatase (AP), *β*-1,4-glucosidase (BG), *β*-1,4-*N*-acetylglucoaminidase (NAG) and leucine aminopeptidase (LAP) were estimated using *p*-nitrophenyl (pNP)-linked substrates (pNP – phosphate, pNP – *β*-glucopyranoside, pNP – *β*-*N*-acetylglucosaminide and leucine *p*-nitroanilide respectively (Sigma Aldrich, St Louis, MO), as previously described.[17] An estimate of overall microbial activity was made using fluorescein diacetate (FDA) hydrolysis, as previously described.[18] Assays were performed on soil slurries. Field-moist bulk soil or rhizosphere soil slurry was weighed into a 50 mL sterile conical bottom tube, and UV-irradiated MilliQ water was added to achieve an approximate soil concentration of 0.5 g mL^{-1} in the slurry. Actual slurry concentrations were determined by removing an aliquot and drying at 105 °C. For the pNP-linked substrates, six replicates of 0.15 mL of slurry were pipetted into six wells of a 96-deep-well assay block using a 1 mL pipet tip clipped to a 0.5 mm opening. The soil slurry was homogenized by vortexing before pipetting. A volume of 0.15 mL of the requisite pNP-linked substrate at concentrations of 5 mM for AP and BG and 2 mM for NAG and LAP in 100 mM buffer were added to four wells of sample, and 0.15 mL of buffer only to the remaining two wells as sample control. Acetate buffer at pH 5 was used for the AP, BG and NAG assays, and TRIS buffer at pH 8 was used for the LAP assay. Six wells containing MilliQ water only were treated in the same fashion as controls (substrate and buffer controls). Each substrate was assayed on a separate set of four replicates, as were two replicates of non-sample (blank) controls. The FDA hydrolysis assay was approached in the same manner, with 0.1 mL of slurry and 0.2 mL of substrate dissolved in buffer or buffer only. The buffer was 50 mM phosphate at pH 7.6, and the substrate concentration was 0.06 mM. All samples in an assay block, as well as the MilliQ water controls, were pipetted, and then the substrates in buffer and buffer only were added using an eight-channel pipetter. The time was noted, and the block was sealed, inverted several times to mix and then placed in an incubator/shaker at 28 °C and 100 oscillations min^{-1}. Incubation times for the assays were 1 h for AP, BG and FDA and 4 h for NAG and LAP. For the pNP-linked substrates, the assay block was removed from the incubator/shaker and centrifuged for 10 min at 2000 × *g*. Using an eight-channel pipetter, 100 µL of supernatant was removed from each well and transferred to the appropriate wells of a polystyrene reader plate prepped with 190 µL of MilliQ water and 10 µL of 1 M NaOH per well. Absorbance was read at 405 nm using a Biotek ELx808 microplate reader (Bio Tek U.S., Winooski, VT). The FDA blocks were removed from the incubator shaker, and a 'short' spin to 1000 × *g* was used to clean the soil suspension from the underside of the sealing mat. The mat was removed, 200 µL of distilled acetone was added to each well and the mat was replaced. The sample was resuspended and mixed by inverting the block several times, and the block was centrifuged for 10 min at 2000 × *g*. Using an eight-channel pipet, 200 µL of supernatant was removed from each well and pipetted into the appropriate wells of a polypropylene reader plate that had previously been read with no contents for absorbance at 490 nm. The plate was read again at 490 nm. Absorbance was read with the Biotek ELx808 microplate reader. Final absorbance values for the pNP assays were calculated as follows:

$$\text{Final Abs} = (\text{Sample Abs} - \text{Sample control Abs}) - (\text{Substrate control Abs} + \text{Buffer control Abs})$$

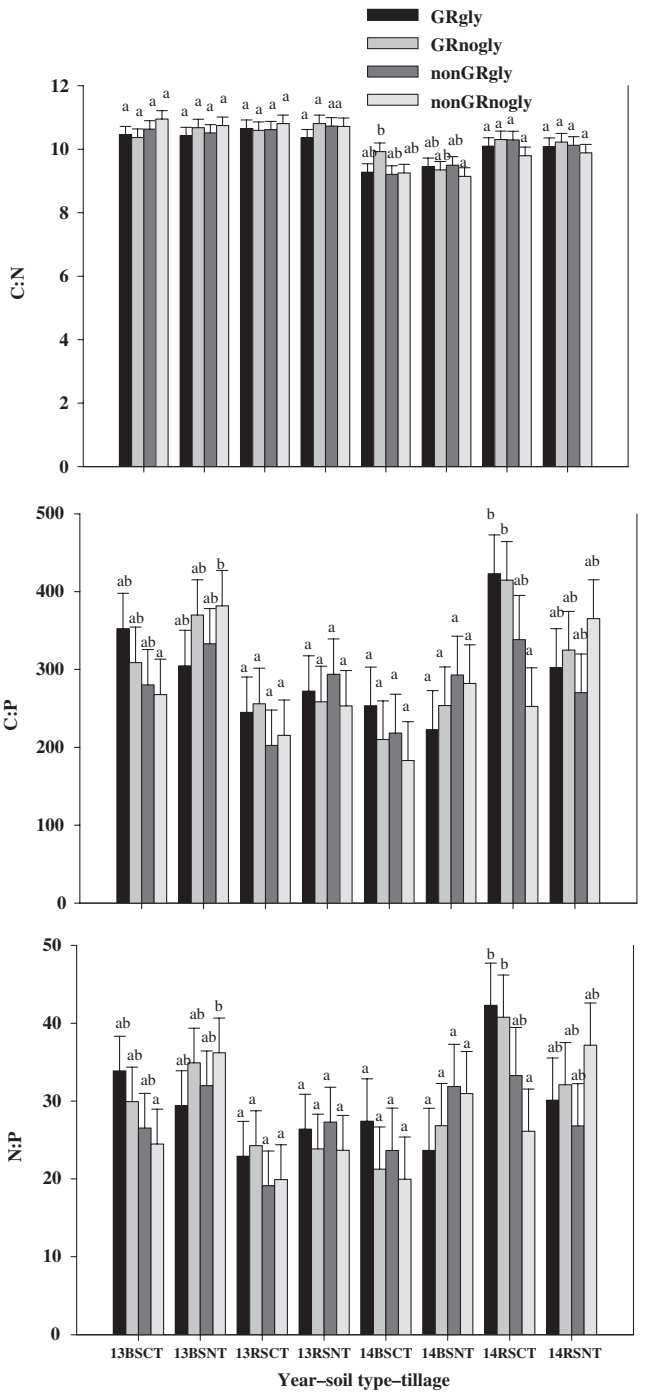

Figure 1. Mean C:N, C:P and N:P ratios of bulk soil (BS) and rhizosphere soil (RS) associated with conventional tillage (CT) and no-till (NT) and the following treatments: GR corn with and without glyphosate (gly, nogly) applications (GRgly, GRnogly), and non-GR isogenic cultivar with and without glyphosate applications (nonGRgly, nonGRnogly) in 2013 and 2014. Different letters above error bars associated with treatments per sampling year[13,14] and soil type by tillage indicate differences at $P < 0.05$.

Table 1. Correlation determinations between bulk and rhizosphere soil C and N, C and P and N and P concentrations, as affected by glyphosate applications on GR corn and its non-resistant isogenic cultivar (non-GR). Significant R^2 values at $P < 0.05$ are indicated in bold

		C versus N		C versus P		N versus P	
				Bulk	Rhizosphere		
Year	GR, non-GR	Bulk	Rhizosphere	Slope, R^2		Bulk	Rhizosphere
2013	GR	**1.03, 0.818**	**1.12, 0.905**	0.07, 0.155	0.13, 0.231	0.028, 0.028	0.126, 0.318
	Non-GR	**1.28, 0.590**	**1.25, 0.965**	0.07, 0.064	0.27, 0.301	−0.01, 0.004	0.224, 0.336
2014	GR	**1.08, 0.667**	**0.94, 0.919**	0.17, 0.135	0.06, 0.033	−0.03, 0.005	0.091, 0.041
	Non-GR	**1.00, 0.836**	**1.00, 0.923**	0.01, 0.002	0.12, 0.064	−0.03, 0.014	0.05, 0.010

The absorbance values of the FDA reads were first corrected for plate background using the dry plate read, and final absorbance values were calculated as above. Enzyme activities were calculated as follows:

$$\text{Activity } \left(\text{nM h}^{-1} \text{ sample}^{-1}\right) = \text{Final Abs}/C/\text{incubation time}/\text{sample}$$

where C is the appropriate standard curve conversion factor for the assay in question, 'incubation time' is the incubation time (in hours) and 'sample' is the sample amount expressed as desired (i.e. g soil, g OM or g OC). The standard curves for the assays were constructed from a dilution series of either *p*-nitrophenol or fluorescein disodium salt dissolved in the appropriate type and concentration of buffer. The absorbance of the known concentrations was corrected for background matrix, and a linear regression was performed to generate a value for C.

2.4 C, N and P analyses

For analyses of bulk and rhizosphere soil C and N contents, duplicate 1.0 mL aliquots of slurries from the enzyme analyses were analyzed on a vario Max CNS analyzer (Elementar Americas, Inc., Mt Laurel, NJ). The Mehlich 3 extraction procedure was undertaken for determining soil P concentrations.[19,20]

2.5 Data analysis

Differences in mean C:N, C:P and N:P ratios, enzyme activities and C:P, C:N and N:P acquisition ratios (as defined by the four exoenzymes and their ratios of activity: BG:AP, BG:(LAP + NAG) and (LAP + NAG):AP respectively[16]) were determined with Proc Mixed of SAS[21] with replicate ratios as random variables and tillage and treatments as fixed variables. Regression analyses on natural-log-transformed ratios (at $P < 0.05$) were determined with the linear regression application of SAS.[22]

3 RESULTS

3.1 Macronutrient ratios

Treatment comparisons were confined to soil types BS and RS. No differences in C:N ratios within treatments in 2013 and 2014 were observed between soil types and soil by tillage interactions (Fig. 1). This apparent homogeneity of C:N ratios appears to be supported by the significant regression slopes, which ranged between 0.94 and 1.28 for bulk and rhizosphere soil total C regressed against total N (Table 1). In contrast to C:N ratios, tillage and treatment differences were observed for C:P ratios (Fig. 1). In 2013, tillage differences occurred between bulk soil by conventional tillage (BSCT) and bulk soil by no-till (BSNT) for the nonGRnogly treatment with NT (381.8), which was greater than the CT treatment (267.8). A treatment difference occurred in 2014 for the rhizosphere soil under conventional tillage (RSCT) with C:P ratio: treatment GRgly (423.1) was greater than the nonGRnogly treatment (252.6). In 2013, tillage differences were observed for N:P ratios (Fig. 1) between BSCT (24.5) and BSNT (36.2) nonGRnogly treatment. In 2014, the N:P ratio for GRgly (42.3) treatment was greater than the ratio of the nonGRnogly (26.1) treatment for the RSCT interaction. In contrast to the significant regression slopes for C against N, regression slopes for BS and RS C against P and N against P indicated no correlations (Table 1). In contrast to BS, no treatment differences were observed for RS C:P and N:P ratios in 2013; in 2014 no treatment differences were observed for BS C:P and N:P ratios (Fig. 1).

3.2 Exoenzyme and FDA activities

3.2.1 Tillage and treatment by tillage differences

In 2013, a tillage difference was observed for BG activity: BSNT nonGRgly treatment (38.9) was greater than the analogous BSCT treatment (34.3) (Fig. 2). In 2014, a tillage by treatment difference in BG activity was observed: BSNT nonGRnogly treatment (40.5) was greater than the BSCT nonGRgly treatment (34.3). No tillage or treatment differences were observed for the rhizosphere soils in either sampling year. In 2013, treatment by tillage differences in AP activity were observed between nonGRgly (95.4) and nonGRnogly (95.0) within BSCT and GRgly (130.3) within BSNT. Tillage differences were observed in 2014 for both BS and RS. AP activities were greater for BSNT treatments GRnogly (92.7), nonGRgly (113.7) and nonGRnogly (116.0) than for corresponding BSCT treatments (33.4, 34.3 and 35.4 respectively); AP activity of RSCT treatments GRgly (306.4) and GRnogly (374.0) were greater than corresponding RSNT treatments (263.1 and 239.7 respectively). With the exception of treatment differences between GRnogly (29.8) and nonGRgly (24.3) of BSNT soil tillage interaction for 2013 LAP + NAG activities, no other differences were observed for either sampling year.

In general, the overall mean of AP and LAP + NAG activities was greater for rhizosphere soil ($P < 0.0001$) than for bulk soil for both sampling years. In 2013, the overall mean of BS BG activity of 38.8 ± 2.8 was less than the overall mean of RS BG activity of 50.4 ± 2.8; the overall mean of BS AP activity of 109.2 ± 7.7 was less than the overall mean of RS AP activity of 171.3 ± 7.7; the overall mean of BS LAP + NAG activity of 26.2 ± 1.1 was less than the overall mean of RS LAP + NAG activity of 35.3 ± 1.1. In 2014, the overall mean of BS BG activity of 35.2 ± 8.4 was less than the overall mean of RS BG activity of 70.2 ± 8.4; the overall mean of BS AP activity of 94.3 ± 14.9 was less than the overall mean of RS AP

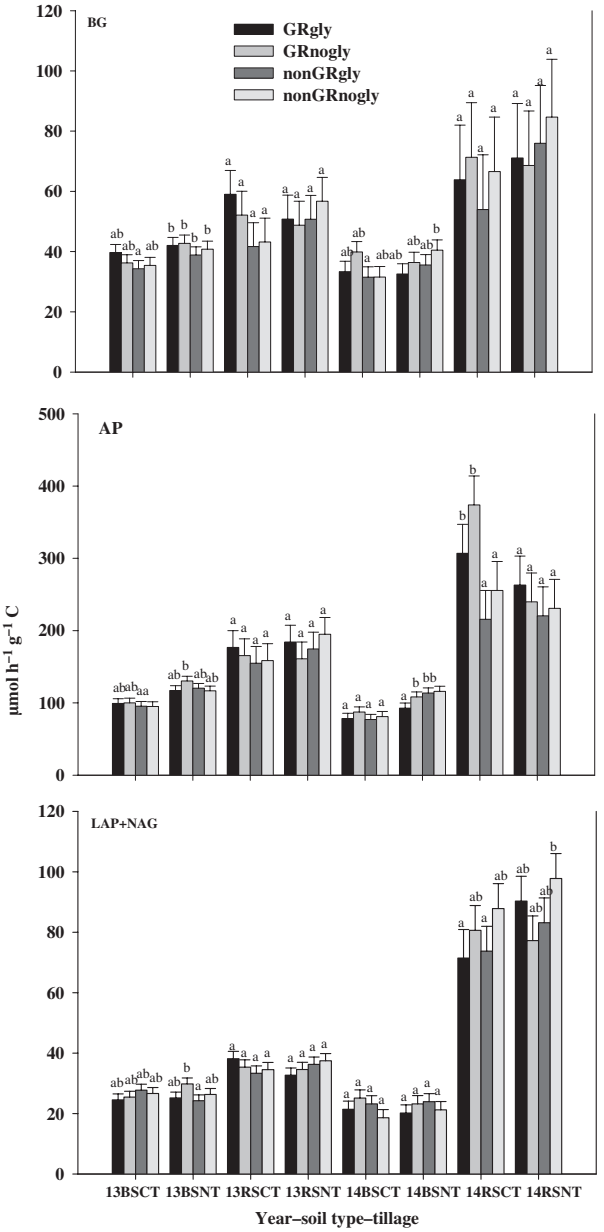

Figure 2. Mean BG, AP and LAP + NAG activities of bulk soil (BS) and rhizosphere soil (RS) associated with conventional tillage (CT) and no-till (NT) and the following treatments: GR corn with and without glyphosate (gly, nogly) applications (GRgly, GRnogly), and non-GR isogenic cultivar with and without glyphosate applications (nonGRgly, nonGRnogly) in 2013 and 2014. Different letters above error bars associated with treatments per sampling year[13,14] and soil type by tillage indicate differences at $P < 0.05$.

activity of 263.2 ± 14.9; the overall mean of BS LAP + NAG activity of 22.1 ± 2.2 was less than the overall mean of RS LAP + NAG activity of 83.0 ± 2.2.

In contrast to the 2013 exoenzyme activities, FDA activity (Fig. 3), an indicator of overall microbial activity, was less in RS than in BS (Fig. 3). Unlike 2013 FDA activities, no differences in FDA activity between BS and RS were observed in 2014.

3.3 Nutrient acquisition ratios

The P nutrient acquisition ratio, BG:AP, for all treatments in both 2013 and 2014 ranged between 0.239 and 0.474 (Fig. 4). The mean BG:AP ratio for BS treatments was greater than the mean

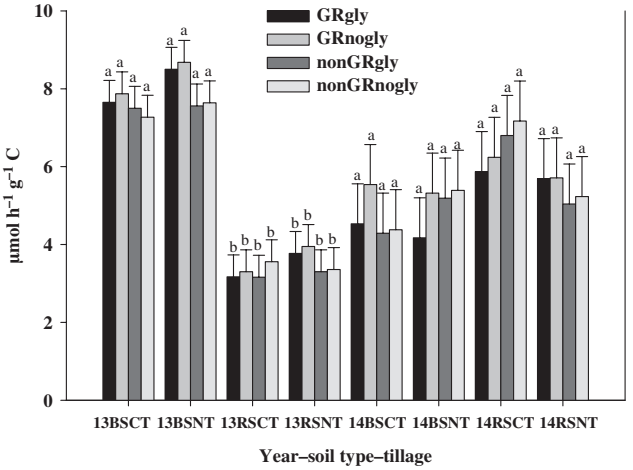

Figure 3. Mean FDA activities of bulk soil (BS) and rhizosphere soil (RS) associated with conventional tillage (CT) and no-till (NT) and the following treatments: GR corn with and without glyphosate (gly, nogly) applications (GRgly, GRnogly), and non-GR isogenic cultivar with and without glyphosate applications (nonGRgly, nonGRnogly) in 2013 and 2014. Different letters above error bars associated with treatments per sampling year (2013, 2014) and soil type by tillage indicate differences at $P < 0.05$.

RS treatment ratio ($P < 0.0001$) in 2013, indicating an overall soil type difference. In 2014, with the exception of nonGRnogly treatment, the other treatments for BSCT BG:AP acquisition ratio for the soil by tillage interactions were greater than those associated with RSCT, indicating also a soil type difference. The N acquisition ratio, BG:(LAP + NAG), for all treatments in 2013 and 2014 ranged between 0.725 and 1.827. In 2013, the BSNT nonGRgly treatment (1.605) was greater than the same BSCT treatment (1.263) and thus indicated a particular treatment by tillage difference; otherwise, no further differences between treatments or soil type by tillage interactions were observed. In 2014, a soil type difference was observed, as the mean BG:(LAP + NAG) ratios of the bulk soil (BSCT and BSNT) treatments (1.636 ± 0.108) were greater than the mean rhizosphere soil (RSCT and RSNT) treatments (0.863 ± 0.110), indicating a soil type difference. The (LAP + NAG):AP nutrient acquisition ratios for all treatments in both 2013 and 2014 ranged between 0.181 and 0.437. The differences observed within this range of ratios were for the BSCT nonGRgly treatment (0.292), which was greater than the analogous BSNT treatment (0.206) in 2013, indicating a treatment by tillage difference, and in 2014 the mean (LAP + NAG):AP ratio of RSNT treatments (0.346 ± 0.026) was greater than ($P < 0.0001$) the mean of the BSNT treatments (0.243 ± 0.026), indicating a soil type difference.

3.4 Effects of glyphosate on bulk and rhizosphere soil activities

As the difference between the two isogenic corn cultivars was the insertion of a glyphosate-resistant genetic determinant, and no differences in exoenzyme activities or nutrient acquisition ratios were observed between the two corn cultivars, we have focused on the impact of glyphosate applications as the determining effect on soil macronutrient ratios and nutrient acquisition ratios. Thus, regression analyses on stoichiometric relationships between BS and RS macronutrient ratios and nutrient acquisition ratios, with a focus on effects of gly and nogly treatments, were undertaken to discern statistically significant ($P < 0.05$) connections between them (Table 2). In 2013, significant relationships were observed

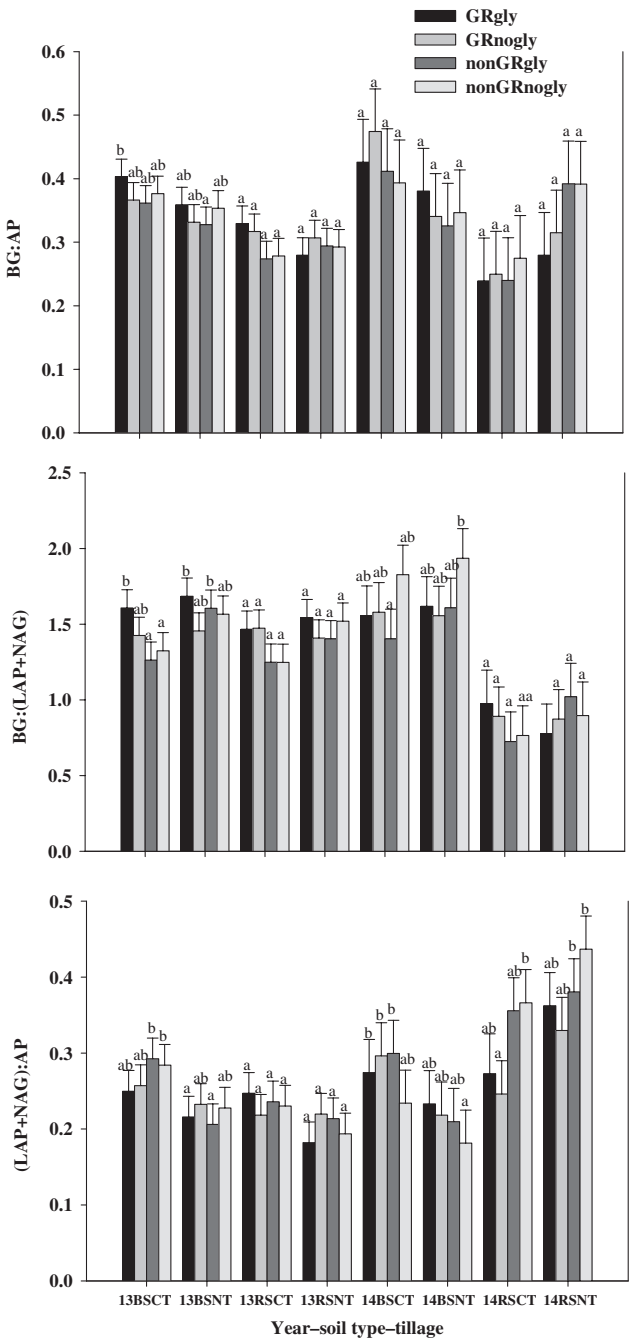

Figure 4. Mean nutrient acquisition ratios BG:AP, BG:(LAP + NAG) and (LAP + NAG):AP of bulk soil (BS) and rhizosphere soil (RS) associated with conventional tillage (CT) and no-till (NT) and the following treatments: GR corn with and without glyphosate (gly, nogly) applications (GRgly, GRnogly), and non-GR isogenic cultivar with and without glyphosate applications (nonGRgly, nonGRnogly) in 2013 and 2014. Different letters above error bars associated with treatments per sampling year[13,14] and soil type by tillage indicate differences at $P < 0.05$.

between C:N ratios of BS gly and nogly treatments and acquisition ratios BG:AP and (LAP + NAG):AP. These relationships were not observed for RS gly or nogly treatments in 2013 or for both BS and RS gly and nogly treatments in 2014. Significant regression slopes around 1.00 for both BS and RS nutrient ratios between C:P and N:P were observed for both gly and nogly treatments in both sampling years. In 2013, negative regression slopes between both C:P and N:P ratios of BS nogly and RS gly and nogly treatments and (LAP+NAG):AP acquisition ratios were observed. In 2014, analogous relationships between RS gly and nogly treatments were observed but not for the BS treatments. In 2013, the BS nogly treatments of both C:P and N:P ratios were also negatively correlated with BG:AP acquisition ratios, whereas RS gly and nogly C:P and N:P ratios were positively related to BG:(LAP + NAG) acquisition ratios. In 2013, the BG:(LAP + NAG) acquisition ratios for all BS and RS treatments were negatively correlated with (LAP + NAG):AP acquisition ratios. In contrast, in 2014 only the BS nogly treatments had an analogous negative correlation with (LAP + NAG):AP, whereas for both RS treatments BG:(LAP + NAG) was positively correlated with BG:AP acquisition ratios. In 2013 and 2014, BG:AP ratios were correlated with (LAP + NAG):AP for all treatments and interactions except for RS gly treatments in 2014.

In 2013, regression slopes with significant R^2 values between FDA activities and BG, AP and LAP + NAG exoenzyme activities were observed for bulk soil nogly treatments only (Table 3). In 2014, the only significant regression slopes observed were between FDA and BG activities for bulk soil gly and nogly treatments and for FDA and (LAP + NAG) activities for BS gly treatments. We observed no significant regression slopes or correlations between FDA and exoenzyme activities for rhizosphere soil in either 2013 or 2014.

4 DISCUSSION

4.1 Macronutrients

None of the treatments (tillage, GR and non-GR corn and glyphosate application) appeared to affect BS and RS C:N ratios. The homogeneity of C:N ratios between treatments of the two soil types appeared to be underscored by the correlation determinations oriented around 1.00, which indicates a stoichiometric balance between C and N.[23] In 2014, the C:N ratios of the BS treatments were on the whole significantly less than the C:N ratios of the RS treatments. This difference appeared to indicate a different nutrient dynamic between BS and RS ecology. The greater C:N ratio of RS appears to indicate competition for N between corn roots and the rhizosphere microbial community.[24]

The greater C:P and N:P ratios for BSNT nonGRnogly treatment compared with the corresponding BSCT treatment in 2013 appeared to indicate that CT may conserve more soil P than NT. A similar difference was observed in which P sorption capacity of subtropical soils under CT was greater than under NT.[25] This tillage difference, however, was not observed in 2014. Unlike the significant correlation determinations between BS and RS C and N (Table 1), no significant correlation determinations between BS and RS C and P and N and P were observed. This observation may indicate a decoupling of C and P and N and P dynamics. This apparent decoupling, however, appeared not to be connected to any of the experimental treatments.

4.2 Exoenzyme and FDA activity

In both sampling years, tillage and tillage by treatment differences were observed for BS, but not for RS. All of the exoenzyme assays displayed significantly greater activities overall in RS than in BS. In particular, the increased AP and LAP + NAG activities of RS either appeared to be associated with P and N limitations[26] or was an indication that the rhizosphere microbial community was in competition with the corn roots for assimilable N and P.[24] In

Table 2. Regression slopes and coefficients of determination (R^2) for stoichiometric relationships between nutrient ratios and nutrient acquisition ratios observed for bulk soil (BS) and rhizosphere soil (RS) and contrasting glyphosate (gly) and non-glyphosate (nogly) treatments for 2013 and 2014. Significant slopes and R^2 values at $P < 0.05$ are in bold

2013						
		Slope, R^2				
Treatment		Soil C:P	Soil N:P	BG:(LAP + NAG)	BG:AP	(LAP + NAG):AP
BS gly	Soil C:N	−0.020, 0.027	0.010, 0.005	−0.060, 0.065	**0.144, 0.247**	**0.128, 0.354**
	Soil C:P	**1.01, 0.991**	−0.194, 0.007	−0.660, 0.056	−0.204, 0.010	
	Soil N:P		−0.133, 0.003	−0.801, 0.078	−0.330, 0.024	
			BG:(LAP + NAG)	0.031, 0.068	**−0.625, 0.490**	
				BG:AP	**0.375, 0.287**	
BS nogly	Soil C:N	−0.040, 0.053	−0.070, 0.153	−0.161, 0.061	**0.235, 0.323**	**0.190, 0.333**
	Soil C:P	**0.932, 0.972**	0.997, 0.079	**−1.19, 0.274**	**−1.01, 0.312**	
	Soil N:P		1.158, 0.095	**−1.42, 0.352**	**−1.20, 0.395**	
			BG:(LAP + NAG)	−0.087, 0.019	**−0.312, 0.379**	
				BG:AP	**0.688, 0.748**	
RS gly	Soil C:N	0.016, 0.027	0.007, 0.005	0.015, 0.010	−0.043, 0.045	−0.025, 0.044
	Soil C:P	**1.01, 0.990**	**0.893, 0.349**	−0.787, 0.134	**−0.905, 0.507**	
	Soil N:P		**0.879, 0.345**	−0.744, 0.123	**−0.88, 0.490**	
			BG:(LAP + NAG)	0.077, 0.003	**−0.677, 0.651**	
				BG:AP	**0.323, 0.297**	
RS nogly	Soil C:N	0.013, 0.007	−0.012, 0.006	0.002, 0.001	0.013, 0.002	0.006, 0.001
	Soil C:P	**0.987, 0.975**	**1.00, 0.367**	−0.410, 0.050	**−0.873, 0.426**	
	Soil N:P		**1.00, 0.366**	−0.424, 0.053	**−0.555, 0.406**	
			BG:(LAP + NAG)	0.174, 0.024	**−0.561, 0.482**	
				BG:AP	**0.439, 0.363**	
2014						
		Slope, R^2				
Treatment		Soil C:P	Soil N:P	BG:(LAP + NAG)	BG:AP	(LAP + NAG):AP
BS gly	Soil C:N	0.002, 0.0001	−0.058, 0.056	0.221, 0.168	0.084, 0.070	0.006, 0.0004
	Soil C:P	**0.941, 0.939**	−0.808, 0.145	−0.213, 0.029	0.061, 0.003	
	Soil N:P		−1.03, 0.222	−0.298, 0.053	0.055, 0.002	
			BG:(LAP + NAG)	0.118, 0.040	−0.207, 0.137	
				BG:AP	**0.793, 0.699**	
BS nogly	Soil C:N	−0.024, 0.033	−0.039, 0.093	0.049, 0.027	0.104, 0.206	0.051, 0.073
	Soil C:P	**0.961, 0.984**	0.205, 0.008	−0.588, 0.116	−0.473, 0.112	
	Soil N:P		0.156, 0.004	−0.692, 0.151	−0.524, 0.129	
			BG:(LAP + NAG)	0.042, 0.003	**−0.358, 0.332**	
				BG:AP	**0.642, 0.615**	
RS gly	Soil C:N	−0.037 0.149	−0.042, 0.214	0.003, 0.002	0.018, 0.073	0.021, 0.018
	Soil C:P	**0.957, 0.993**	0.338, 0.163	−0.130, 0.029	**−0.925, 0.371**	
	Soil N:P		0.335, 0.150	−0.148, 0.035	**−0.945, 0.362**	
			BG:(LAP + NAG)	**0.807, 0.735**	−0.292, 0.026	
				BG:AP	0.708, 0.136	
RS nogly	Soil C:N	−0.057, 0.179	−0.067, 0.276	0.002, 0.0004	0.005, 0.007	0.010, 0.007
	Soil C:P	**0.933, 0.987**	−0.121, 0.028	−0.213, 0.208	**−0.549, 0.373**	
	Soil N:P		−0.122, 0.026	−0.218, 0.197	**−0.560, 0.341**	
			BG:(LAP + NAG)	**0.565, 0.772**	0.533, 0.195	
				BG:AP	**1.533, 0.667**	

contrast to the greater exoenzyme activities associated with RS in 2013, the corresponding FDA activities of RS were significantly less than for BS. On the other hand, this inverse relation between RS and BS exoenzyme and FDA activities may indicate that the rhizosphere microbial community in 2013 had access to less labile carbon and energy sources and thus displayed a decrease in metabolic activity.

4.3 Nutrient acquisition ratios

The BG:AP ratios for both BS and RS were lower than the synoptic BG:AP ratio of 0.62 that Sinsabaugh *et al.*[16] reported for soils and may reflect the effects of agricultural perturbations associated with management practices. These BG:AP nutrient acquisition ratios much lower than 1.00 may be indicative of a general limitation of available P for both BS and RS microbial communities.[27] The

Table 3. Coefficients of determination (R^2) of metabolic relationships between natural-log-transformed fluorescein diacetate (FDA) activities and activities of exoenzymes β-glucosidase (BG) and acid (alkaline) phosphatase (AP), and the sum of the activities of leucine aminopeptidase (LAP) and β-*N*-acetylglucosaminidase (NAG) associated with bulk and rhizosphere soil exposed to glyphosate (gly) or no glyphosate (nogly) in 2013 and 2014. Significant slopes and R^2 values at $P < 0.05$ are indicated in bold

		Bulk soil			
		gly	nogly	Rhizosphere soil	
Year	Relationship	Slope, R^2		gly	nogly
2013	FDA versus BG	0.19, 0.036	**0.81, 0.446**	0.06, 0.009	−0.04, 0.001
	FDA versus AP	0.07, 0.004	**0.53, 0.261**	0.11, 0.030	0.08, 0.004
	FDA versus (LAP + NAG)	0.51, 0.199	**0.64, 0.290**	0.23, 0.029	0.55, 0.055
2014	FDA versus BG	**0.97, 0.344**	**0.95, 0.375**	−0.01, 0.0002	0.14, 0.038
	FDA versus AP	0.10, 0.006	0.42, 0.068	0.44, 0.088	−0.39, 0.109
	FDA versus (LAP + NAG)	**0.84, 0.531**	0.55, 0.208	0.38, 0.023	−0.08, 0.003

greater BG:AP ratios of BS are indicative of a lesser P demand from the BS microbial community than the P demand of the RS microbial community, and may be related to the differences between C:P and N:P nutrient ratios between CT and NT treatments in 2013. This difference between BS and RS microbial communities may also, as Schimel and Bennett[24] have noted, be attributable to the competition between the rhizosphere microbial community and the corn roots for available P. The differences observed between BS and RS P nutrient acquisition ratios indicated soil type differences that seem to override any individual treatment or treatment by tillage differences. Neither the application of glyphosate nor the GR corn cultivar appeared to have an effect on the P nutrient acquisition ratio.

Like the BG:AP P nutrient acquisition ratios, the (LAP + NAG):AP ratios were also well below 1.00 in 2013 and 2014. In contrast to the BG:AP ratios, the mean RS (LAP + NAG):AP ratio for 2014 was greater ($P < 0.0001$) than the mean ratio for BS. The mean ratios for both years and treatments were less than the synoptic value of 0.44 that Sinsabaugh *et al.*[16] reported and may be related to perturbations of the agricultural management practices employed. This soil type difference between RS and BS (LAP + NAG):AP ratios appears to indicate a differential demand for N and P between RS and BS microbial communities and is likely influenced by corn root uptake activities. The tillage difference between BSCT and BSNT nonGRgly treatment in 2013 may be considered an ephemeral example of a glyphosate tillage interaction.

In contrast to the P nutrient acquisition ratios (BG:AP), the N acquisition ratios of BG:(LAP + NAG) were near or greater than 1.00. With the exception of the treatment by tillage interaction difference between BSCT and BSNT nonGRgly treatment, overall mean tillage by soil type differences were not observed in 2013. In contrast, results of the 2014 mean BG:(LAP + NAG) acquisition ratios for the RS treatments were less than the ratios for the BS treatments, indicating a greater RS demand for N that was not observed in 2013. This apparent greater RS demand for N may parallel the observed RS demand for P, and thus may indicate competition between the rhizosphere microbial community and corn roots for available N. The BG:(LAP + NAG) N acquisition ratios that we have calculated, with the exception of the 2014 RS ratios, are around the mean of 1.41 that Sinsabaugh *et al.*[16] reported for bulk soils. The lack of a discernible pattern in treatment differences for BG:(LAP + NAG) ratios appears to indicate little or no effect of either glyphosate or the GR corn cultivar on N acquisition processes.

4.4 Effects of glyphosate on bulk and rhizosphere soil activities

Regression analyses of the stoichiometric relationships between BS and RS nutrient ratios and nutrient acquisition ratios as affected by glyphosate treatments indicated significant correlations for a limited number of comparisons. The significant correlations between 2013 and 2014 C:P and N:P ratios for both BS and RS gly and nogly treatments were one of two consistent relationships both years. With the exception of the 2014 RS gly set of treatments, the other consistent relationship was between BG:AP and (LAP + NAG):AP nutrient acquisition ratios in both sampling years and underscores the apparent connection between C:P and N:P ratios and the exoenzymes involved in P nutrition of the BS and RS microbial communities. These correlations between BS and RS C:P and N:P nutrient ratios and BG:AP and (LAP + NAG):AP nutrient acquisition ratios appear to indicate that neither the corn cultivar, glyphosate applications, tillage nor soil type affected their interactions. Whereas RS C:P and N:P ratios were significantly correlated with BG:(LAP+NAG) N acquisition ratios in 2013, analogous correlations were not determined for BS. This difference may indicate a soil type difference and corresponding differences between their associated microbial communities and their interactions with corn roots. As these relations were not observed in 2014, they may represent fluctuating ephemeral events. The results discussed above are in contrast to those that Bell *et al.*[28] reported regarding their investigation of the stoichiometric components of rhizosphere soil associated with non-agronomic plants growing in an arid grassland ecosystem, which indicated no correlation between C:P and N:P ratios. The apparent consistent negative correlations between C:P and BG:AP and N:P and (LAP + NAG):AP ratios underscore the inverse relation between soil nutrient status and nutrient acquisition demand of both BS and RS microbial communities. The contrasting 2013 negative relationship between BG:(LAP + NAG) and (LAP + NAG):AP and their respective 2014 positive correlations appear to underscore the dynamic character of the stoichiometric components that we measured, and appears to indicate that neither glyphosate applications, GR and non-GR corn nor tillage affected the functioning of BS and RS microbial communities' exoenzyme activities.

The relationships we observed between total microbial metabolic activity as measured with FDA and the exoenzymes suggest an ephemeral, if any real, effect of glyphosate on disrupting the BS microbial metabolic relationship to the stoichiometric

function of the exoenzymes. In contrast to BS, no relationships were observed between FDA and the exoenzymes for the RS. This lack of relationship between total microbial metabolic activity and RS exoenzyme activities appears not to be related to glyphosate applications, but is more likely related to competition between corn roots and the RS microbial community for mineralized macronutrients initiated by the exoenzymes.

The 7 years of agronomic applications of the herbicide glyphosate on GR and non-GR corn may have decoupled the stoichiometric relationship between C and P and N and P. This decoupling of soil P may, however, be more of an indication of a soil P limitation, as the BG:AP and (LAP+NAG):AP nutrient acquisition ratios, which were much less than 1.00, appeared to indicate. The lack of a relationship between bulk soil microbial metabolic activity and exoenzyme activities for glyphosate treatments as opposed to the significant relationships for no glyphosate treatments in 2013 may indicate that glyphosate has an ephemeral disrupting effect on the microbial community and its synthesis of exoenzymes. Our results appear to indicate that glyphosate applications on GR and its nonGR isogenic cultivar may have an ephemeral, but insignificant, effect on the stoichiometry of the initial steps in soil organic matter mineralization.

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