Microbial dynamics in soils under long-term glyphosate tolerant cropping systems

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Abstract
Glyphosate is a non-selective, broad-spectrum herbicide now widely used. Early research on short-term effects on soil microbiology was inconclusive, but now long-term applications of glyphosate to tolerant cropping (GTC) systems may be shifting microbial communities and causing plant nutrition deficiencies and diseases. A field survey of 10 pairs of fields (K sufficient and deficient) under >10 yrs GTC showed significant negative correlations between microbial biomass K and K crop uptake suggesting glyphosate applications caused microbial immobilization of K. A 28 day experiment with a single glyphosate application and another experiment with repeated glyphosate application was done on soils from >10 yrs GTC or soils that had never received glyphosate The soils that had been under GTC showed no shift in fatty acid profiling of microbial communities (suggesting they had adapted) compared to the non-GTC soils where unique fatty acids were stimulated by the addition of glyphosate. For example, gram positive bacteria responded to glyphosate in non-GTC soils but not GTC soils. This suggested that adding glyphosate does cause a shift in a sub population of the soil. DNA-DGGE analysis of GTC fields identified *Fusarium* species that are known to be pathogenic. The results suggest there is sub-population shift that is occurring with long-term GTC.

Introduction

Non Target Effects of Glyphosate

There is a growing body of anecdotal information and preliminary research that suggests long term and repeated use of glyphosate in both glyphosate tolerant cropping systems and in other agricultural applications is changing the soil microbial community, worldwide. This is manifesting various undesirable effects on crops that have included stimulation of diseases and nutrient deficiencies (International Conference Symposium: *Mineral nutrition and disease problems in modern agriculture: Threats to sustainability*, Informações Agronômicas 119, 2007). There is evidence that glyphosate causes nutrient deficiency on Fe, Ca, Mg (Carmak 2007), Ni (Wood 2007), and Mn (Huber 2007). Glyphosate kills plants by making it susceptible to fungal diseases caused by *Fusarium* and *Pythium* (Johal and Rahe 1990). A four year study in Canada showed that glyphosate-treated wheat had higher levels of *Fusarium* head blight (a toxic fungal disease) than wheat fields where no glyphosate had been applied (Fernandez *et al.* 2007). Furthermore, this study was the first to report the possibility of residual effects from RR wheat causing disease in barley. Although relatively few trials have been conducted in the US, the majority typically has shown yield decrease of 5 to 10% by RR soybeans compared to non-RR varieties (consistently in the northern US corn belt) (Gaska and Boerboom, UW, Madison, person comm. 2009). Exceptions to this were a study in Iowa that showed no significant yield differences between RR and non-RR soybeans and a study in Illinois RR varieties yielded 1 bu/ac more than conventional non-RR varieties. (Gaska and Boerboom, UW, Madison, person comm. 2009)

In very controlled studies under hydroponic conditions Kremer *et al.* (2005) showed that treated RR soybeans transported glyphosate to the roots where it was released and stimulated *Fusarium* in the plant roots, to such a degree that he considers the elevation of *Fusarium* levels to be glyphosate's "secondary mode of action." Although they did not investigate the pathogenesis of *Fusarium* colonies, this outcome raises the question of whether *Fusarium* is stimulated in soils of RR systems that could cause disease epidemics. In Australia recent concerns have been expressed that 90 percent of Australia's cotton belt could be inundated by the soil borne pathogen *Fusarium* wilt within the next decade because of the widespread use of RR cotton. *Fusarium* can be a serious disease such as Fusarium Head Blight (FHB) which for wheat and barley in Saskatchewan has been responsible for serious crop losses (Fernandez *et al.* 2007). About a fifth of the wheat crop in Europe every year is lost to FHB and in Michigan during 2002 there were 30 to 40% yield losses. There is a growing incidence of potassium deficiency on corn across the Midwest, which appears to be related to the widespread adoption of RR soybean systems that are in rotation with corn (Norton *et al.*

2005). This K deficiency is exacerbated by no-tillage, where it causes yield reduction over conventional tillage (Norton *et al.* 2005; Vyn *et al.* 2002). Fast growing, high yielding corn hybrids are more susceptible to K deficiency, but K deficiency has occurred with other cultivars as well. It is largely unknown why glyphosate based cropping systems might affect K availability. Preliminary research (Kremer *et al.* 2005), limited evidence from the literature, and our understanding of K cycling suggest that glyphosate causes an interaction of soil biology that may be inducing K deficiency in plants. Glyphosate has been shown to stimulate fungi (Arauj *et al.* 2003), and the roots of glyphosate tolerant crops leak glyphosate and elevated levels of C exudates into soils (Kremer *et al.* 2005). Weed *et al.* (1969) showed that fungi can rapidly take up K and there is evidence that microbial biomass K increases after K fertilization (Perrott *et al.* 1990) or fluctuates seasonally (Roberts 1968). We hypothesize that glyphosate systems cause a microbial shift towards fungal dominance or specific fungal genera such as Fusarium (Kremer *et al.* 2005), which rapidly take up K and transfer it to non-exchangeable/plant, unavailable forms.

Glyphosate Effects on Soil Microbial Communities

Glyphosate application on agricultural fields has been shown to penetrate the upper 2 mm of the soil (Haney *et al.* 2000) at recommended field application rates. Single application or short term studies in the field and lab have shown that microbial biomass is unaffected or stimulated (Hart and Brookes 1996; Liphardzi *et al.* 2005; Haney *et al.* 2000). In similar, short term applications, glyphosate even at rates significantly above recommended rates generally show minimal or transitory effects on soil biology (Savin *et al.* 2007; Zaboly *et al.* 2008; Ratcliff *et al.* 2008). However, detailed studies in the soil rhizosphere have shown that glyphosate leaks out of the roots within hours after application (Carmak 2007; Kremer *et al.* 2005). Root exudation of glyphosate tolerant soybeans under hydroponic growth releases greater amounts of glyphosate than a non-tolerant cultivar after foliar application of glyphosate (Kremer *et al.* 2005). The glyphosate tolerant soybean exudates, besides glyphosate, had high levels of carbohydrate and amino acid content. Exposing this exudate to isolated fungi and bacteria stimulated several fungi (Kremer *et al.* 2005). Other studies that directly exposed microbes to glyphosate have found: 1) stimulation of mycorrhizal fungi (Laatikainen and Hinonen-Tanski 2002); 2) that fungi are the main glyphosate degraders (Krzysko-Lupicka *et al.* 1997); and 3) that bacteria are the main incorporators of glyphosate (Charney *et al.* 2004). Thus, short term or single applications may not have permanent changes of the soil community that are measurable in the bulk soil, but there still may be microsites in the rhizospheres of crops that could negatively affect crop production (Norton *et al.* 2005). One of the few studies on long-term glyphosate applications (6 yrs) did cause a shift in microbial communities (Araujo *et al.* 2003). Indeed, the reports on RR cotton, RR wheat, and anecdotal information indicates it takes a number of years of RR cropping before detrimental impacts are measurable.

Procedures

A field survey on GTC fields and lab incubation were done to investigate how long-term GTC may be impacting soil microbial communities. The objective of this study was to determine the effect of long-term GTC on: (1) soil K dynamics; (2) soil microbial structure (ester linked fatty acid methyl ester, EL-FAME profiling); (3) biomass K (Kmic); and (4) *Fusarium* diversity.

Objective 1: Determine microbial community and microbial biomass K shifts in soils from fields deficient or sufficient in plant available K under long-term glyphosate tolerant cropping.

Based on information from farmers experiencing unexplained K deficiency under GTC and recent reports we hypothesized that long-term GTC is stimulating K immobilization and possibly *Fusarium* species. Hence the latter could further contribute to reduced K uptake in crops by damaging roots or otherwise impeding crop growth. The use of glyphosate could stimulate a specific fungal genus or cause a general shift in the microbial community towards fungal dominance.

Procedure 1

Ten paired sites (deficient vs. sufficient in plant available K) across the mid-west corn belt of the US sites under no-tillage were selected that had been under GTC for > 10 years. In April, June and July 2007 as well as in March, June and August 2008 sites were sampled. In the summers of 2007 and 2008, bulk soil samples were taken in the vicinity of the corn and soybean plants and another set of soil samples was strictly taken in the rhizosphere. All soils were passed through a 2 mm sieve and stored at 4ºC until further analysis. Soils for DNA extraction were stored at -80ºC. In July 2007 and August 2008 plant leaf tissue was collected. At each sampling spot, three plants were selected and leaf tissue from the V5 to V7 growth stage was collected. Microbial fatty acids were extracted using the EL-FAME protocol described in Schutter and Dick (2000).

Methyl nonadecanoate served as an internal standard, which allowed calculation of FAME concentrations (Zelles 1996). Soil microbial biomass potassium (K_{mic}) was extracted after chloroform fumigation extraction (Lorenz *et al.* 2009). Soil DNA was extracted using a MoBIO® Power soil DNA extraction kit. Subsequently, extracted DNA was stored at -80ºC until further processing. For PCR, an aliquot of the ultra-frozen DNA was diluted (1:10) and stored at -20ºC. Subsequently, PCR was performed using a PTC-200 Thermal Cycler (MJ Research®). Fusarium-specific PCR was performed targeting a partial region of the translation elongation factor-1 alpha gene (Yergeau *et al.* 2005, Wakelin *et al.* 2008). PCR products were verified on an Agarose gel and cleaned with a QIAquick PCR product cleaning kit (QIAGEN®). Subsequently, PCR products were applied on a denaturing gradient gel and separated electrophoretically at 90V overnight using a Dcode system (Bio-Rad®). Denaturing gradient gels were stained using SYBR green immediately after electrophoresis and pictures were taken using a Digital Gel Logic 4 camera (Kodak®). Cloning was done with a TOPO TA cloning kit with DH5α-T1 chemically competent E.coli (Invitrogen®). Plasmids were isolated from E.coli clones with a PureLink quick plasmid miniprep kit (Invitrogen®) and a QIAprep spin miniprep kit (QIAGEN®). Sequencing of the Plasmids was performed by the Plant-Microbe Genomics Facility at the Ohio State University, Biological Sciences Department, Columbus, Ohio.

Objective 2: Evaluate the effect of repeated applications of glyphosate on soil microbial communities in a long term soil incubation study

Roundup Ready (RR) systems, with up to 2 or 3 applications per year of glyphosate on RR soybeans in rotation with corn, are widespread in the Midwest. With the increasing use of RR corn, soils will be receiving double the glyphosate of the past 10 years. Analyzing the soils today from such fields does not reveal the historical shift in microbial communities that may have occurred. The goal of this experiment is to replicate 5 years of RR applications in a microcosm study. The approach was be to manipulate soils that had no or varying degrees of long-term glyphosate applications in a short-term incubation with repeated applications on a 21 day basis (equivalent to the cumulative amount of a.i. of glyphosate equal to that of 5 years of RR systems in the field). Then we will monitor the shift in microbial communities over months rather than the years required for a field study.

Procedure 2

Two experiments were done. The first was a 28 day incubation of soil that was sampled at 2, 7 14 and 28 d after applying glyphosate to either GTC (>10 yrs) or non-GTC soils. The second experiment was a 3 x 2 factorial design with: three soils from farmers' fields (organic farm that has never had glyphosate applied; non-GMO soybean-corn farm that has had occasional applications of glyphosate in pre-growing season burn downs; or a farm with >10 years of repeated applications of glyphosate using RR soybeans) and two levels of repeated glyphosate applications (control or recommended a.i. glyphosate/application). The sites were chosen because the texture and total C (soil organic matter) are virtually identical and all are of the same classification. The glyphosate treatment was applied every 21 days for 8 months (simulating 5 yrs of field applications). This application interval is based on a preliminary study where we found that 21 days after the glyphosate application, respiration returned to base line levels - suggesting the community had reached an equilibrium and exhausted the available glyphosate. At 0.5, 1, 2, 4, 6, and 8 months soil samples were collected and analyzed for ester linked fatty acid methyl esters (EL-FAME) as described above.

Results and Discussion

Experiment 1

All the deficient and sufficient K fields had > 100 ppm exchangeable K, that by classic interpretations would indicate there was no K deficiency. However, results showed that 5 out of the 10 sites where farmers had experienced K deficiency in the past, there was deficiency based on tissue analysis of K in corn plants. For these fields we found a negative correlation between Kmic and K uptake – suggesting there was microbial immobilization of K. Analysis of FAME profiles showed that the long-term effect of glyphosate on microbial communities was not a 'broad band' change in microbial community diversity or structure but rather a shift in certain subgroups. DGGE profiling followed by sequencing revealed certain pathogenic species were present in GTC fields and K deficient soils.

Experiment 2

In the 28 day incubation where soils from long-term glyphosate or no glyphosate were amended with glyphosate, EL-FAME profiling showed certain groups of soil bacteria responded to the glyphosate addition only when the soil had never received glyphosate before (e.g. Gr+ were suppressed over Gr- bacteria with 19

unique FAs changing with the addition of glyphosate). Conversely, the long-term GTC soils showed no shift in the microbial community due to glyphosate and had elevated levels of respiration, suggesting that the community had adapted to glyphosate applications. In the second experiment with repeated applications after 60 days unique fatty acids were stimulated only in the organically managed soils. This is shown in Table 1.

Additional Studies

Greenhouse manipulative studies will be presented which evaluates responses to GRC soybeans and corn treated with glyphosate growing in soil that have had long-term GTC or non-GTC (organic and non-GMO fields) management. We will report on *Fusarium* species' responses and the results of a novel stable carbon isotope technique (^{13}C) which is allowed us to track glyphosate-^{13}C into rhizosphere microbial groups to determine which microbes are feeding on glyphosate leaked by glyphosate tolerant roots.

Table 1. Unique fatty acid biomarkers that were stimulated in organically managed soils after glyphosate application that were unaffected in the long-term glyphosate treated soil.

FAME Biomarker		Organism
13.0 ISO	**	
16.1 ω9c	**	
16.1 ω5c	**	Arbuscular Mycorrhizal Fungi
16.0 10 ME	***	Actinomycetes
19.0 Cyclo w8c	**	Gram- Bacteria
20:4 ω6,9,12,15c	ns	Protozoa