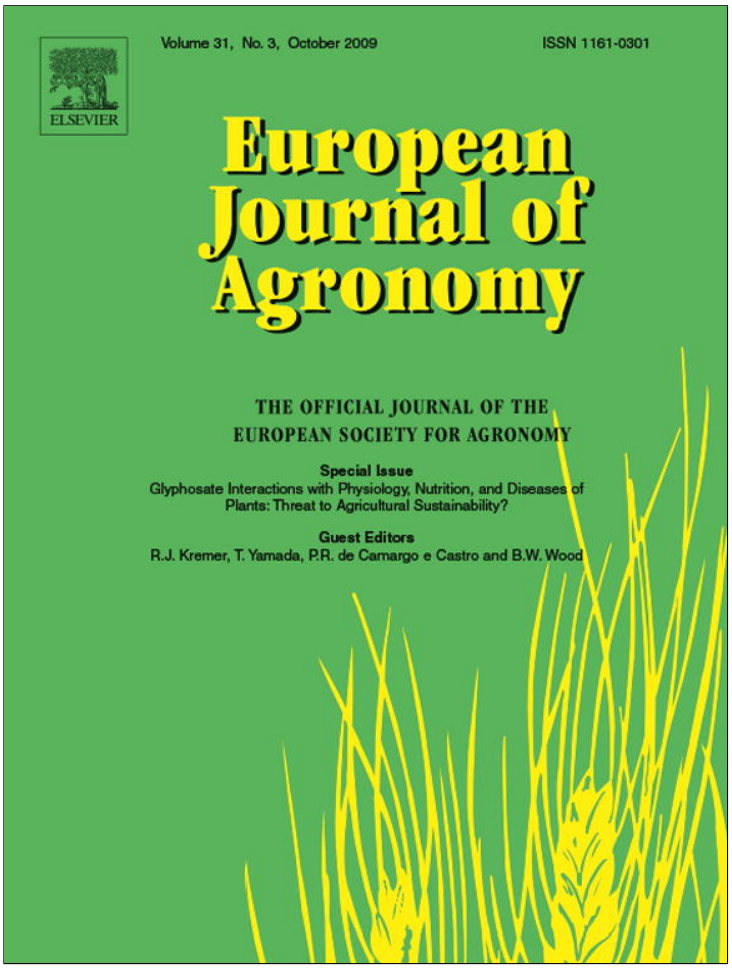

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Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms

Robert J. Kremer[a], Nathan E. Means[b,*]

[a] USDA-ARS Cropping Systems & Water Quality Research Unit, 302 Natural Resources Bldg., University of Missouri, Columbia, MO 65211, USA
[b] Science Department, Columbia College, 1001 Rogers St., Columbia, MO 65216, USA

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ABSTRACT

Current crop production relies heavily on transgenic, glyphosate-resistant (GR) cultivars. Widespread cultivation of transgenic crops has received considerable attention. Impacts of glyphosate on rhizosphere microorganisms and activities are reviewed based on published and new data from long-term field projects documenting effects of glyphosate applied to GR soybean and maize. Field studies conducted in Missouri, U.S.A. during 1997–2007 assessed effects of glyphosate applied to GR soybean and maize on root colonization and soil populations of *Fusarium* and selected rhizosphere bacteria. Frequency of root-colonizing *Fusarium* increased significantly after glyphosate application during growing seasons in each year at all sites. Roots of GR soybean and maize treated with glyphosate were heavily colonized by *Fusarium* compared to non-GR or GR cultivars not treated with glyphosate. Microbial groups and functions affected by glyphosate included Mn transformation and plant availability; phytopathogen–antagonistic bacterial interactions; and reduction in nodulation. Root-exuded glyphosate may serve as a nutrient source for fungi and stimulate propagule germination. The specific microbial indicator groups and processes were sensitive to impacts of GR crops and are part of an evolving framework in developing polyphasic microbial analyses for complete assessment of GR technology that is more reliable than single techniques or general microbial assays.

1. Introduction

Glyphosate [*N*-(phosphomonomethyl)glycine] is a water-soluble, non-selective herbicide applied to foliage resulting in death of most herbaceous plants. The mode of action of glyphosate is inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphatase synthase (EPSPS) in the shikimic acid pathway blocking the synthesis of essential aromatic amino acids and precursors of other critical aromatic compounds including plant growth regulators and phytoalexins (Duke et al., 2003a). Glyphosate is an extremely effective herbicide because the compound remains intact in the plant with little degradation and is systemically transported to metabolically active sites throughout the plant before inducing symptoms (Cerdeira and Duke, 2006). Glyphosate is often described as exhibiting little or no activity in soil due to potential rapid adsorption on soil inorganic and organic particles (Duke and Powles, 2008).

1.1. Glyphosate and soil microorganisms

In contrast to generalizations that glyphosate is tightly bound and inactivated in soil, numerous studies show that glyphosate is available to soil and rhizosphere microbial communities as a substrate for direct metabolism leading to increased microbial biomass and activity (Haney et al., 2000; Wardle and Parkinson, 1990). Indeed, Simonsen et al. (2008) recently demonstrated that agricultural soils amended with phosphorus fertilizers are high in unbound glyphosate because soil sorption sites are occupied by competing phosphate ions; thus, glyphosate remaining in the soil solution is vulnerable to potential uptake by plant roots, microbial metabolism, or leaching into groundwater. The main degradation product, aminomethylphosphonic acid (AMPA), is frequently detected in soils subjected to frequent glyphosate applications (Fomsgaard et al., 2003). Reports on impacts of glyphosate and its degradation products on specific microbial species inhabiting non-rhizosphere soil are limited.

Response to glyphosate appears to vary among soil bacteria based on sensitivity of intracellular EPSPS to the herbicide. Screening assays with glyphosate in minimal medium identified five pseudomonad species (*P. maltophilia*, *P. putida*, *P. aeruginosa* [two strains], *Pseudomonas* sp.) that were not growth inhibited due to a 'glyphosate-resistant EPSPS' (Schulz et al., 1985). Interestingly,

* Corresponding author.
E-mail address: nemeans@ccis.edu (N.E. Means).

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the ubiquitous environmental bacterium, *Pseudomonas fluorescens*, exhibited a 'glyphosate-sensitive EPSPS' and was inhibited by glyphosate. Using enrichment culture, several bacterial strains isolated from both untreated and glyphosate-treated soils metabolized glyphosate using an initial cleavage of the carbon–phosphorus bond to yield sarcosine (Dick and Quinn, 1995). A majority of the isolates was classified as *Pseudomonas* and *Arthrobacter* species. Liu et al. (1991) also demonstrated that several members of *Rhizobiaceae* including *Rhizobium* spp. and *Agrobacterium* spp. grew on glyphosate and the glyphosate metabolite, AMPA, as the sole source of P under P-limiting conditions in liquid culture medium. Mineralization of glyphosate to CO_2 in five agricultural soils in a microcosm study was highly correlated with *Pseudomonas* spp. population in the soils, suggesting that the mineralizing activity of specific pseudomonads was a major factor controlling the fate of glyphosate in soil (Gimsing et al., 2004).

Several studies document effects of glyphosate on soil fungal community response and function. Wardle and Parkinson (1990) found that glyphosate applied to soil amended with wheat (*Triticum aestivum* L.) straw and incubated with fungi significantly enhanced straw colonization by *Trichoderma harzianum*, *Mortierella alpina*, and *Arthrinium sphaerospermum*. Subsequent research revealed that antagonistic interactions between the fungal species were eliminated by glyphosate suggesting that the herbicide might influence overall soil fungal community structure (Wardle and Parkinson, 1992). Glyphosate added to sandy clay with a history of repeated glyphosate treatment appeared to select for specific fungal species that were able use the herbicide as a nutrient source (Krzysko-Lupicka and Orlik, 1997). A follow-up study verified that long-term exposure of soil microorganisms to glyphosate led to a fungal community dominated by *Fusarium* spp. (Krzysko-Lupicka and Sudol, 2008). Similarly, Means (2004) detected a significant increase in soil *Fusarium* within 2 weeks after glyphosate was applied at recommended rates in the field. In culture-based studies, five strains of *Fusarium* spp. isolated from soil were able to metabolize glyphosate and use it as a phosphorus source (Castro et al., 2007). Population growth and sporulation of soil *Fusarium* spp. increased after glyphosate application to soils containing maize (*Zea mays* L.) or peanut (*Arachis hypogeae* L.) crop residues compared with lower *Fusarium* populations in similar fallowed soils lacking crop residues (Meriles et al., 2006).

1.2. Glyphosate and rhizosphere microorganisms

Because the rhizosphere is rich in organic substances and high microbial activity with fluctuating soil moisture (Kennedy, 2005), the fate of glyphosate entering this environment is difficult to predict although microbial metabolism, movement through soil pores or root channels, absorption by living plant roots, and soil sorption are all possible. Early research showed that glyphosate absorbed through plant foliage after application was transported systemically toward roots and was eventually released into the rhizosphere (Coupland and Casely, 1979). Indeed, Neumann et al. (2006) demonstrated that glyphosate released through the roots of dying plants was transferred to living plants (not treated with glyphosate) via root absorption, and suggested that glyphosate applied to vegetation in orchard alleys may be similarly transferred to trees causing disease and yield losses. Microbial activity may increase in rhizospheres of glyphosate-treated plants through translocation and release from roots, where it is immediately metabolized resulting in stimulation of activity and changes in functional diversity of the heterotrophic microbial community (Mijangos et al., 2009).

Glyphosate applied to susceptible plants resulted in heavy colonization of roots by soilborne fungi, primarily *Fusarium* and *Phytophthora* (Johal and Rahe, 1984; Lévesque and Rahe, 1992). Lévesque et al. (1987) observed that glyphosate not only increased root colonization of various susceptible weeds by *F. oxysporum* but also increased propagule density of the fungus in soil. Severity of root and crown rot diseases was highest on cereal crops planted immediately after glyphosate treatment of weeds and volunteer cereal plants in which facultative (opportunistic) pathogens rapidly built up to provide inocula for subsequent colonization of seedling roots of the cereal crop (Smiley et al., 1992). Glyphosate treatment of bean (*Phaseolus vulgaris* L.) plants caused heavy root colonization by *Pythium* spp., significantly building up the soil populations of *Pythium* spp. as the dying roots provided substrate increasing inocula levels of the phytopathogen (Descalzo et al., 1998). The severity of damping-off disease of sunflower (*Helianthus annuus* L.) seedlings growing in the colonized soil significantly correlated with *Pythium* population density. Bacterial endophytic communities in soybean [*Glycine max* (L.) Merr.] can also be altered by pre-plant applications of glyphosate (Kuklinshy-Sobral et al., 2005).

These studies suggest that glyphosate exhibits non-herbicidal effects manifested by enhancement or suppression of activity of latent pathogenic and/or plant growth-promoting bacteria and fungi, which may subsequently impact growth of non-target plants. Because glyphosate blocks the shikimate pathway and subsequent synthesis of aromatic compounds including phytoalexins, pathogen defense mechanisms are suppressed, as shown in early studies in which glyphosate contributed to infection of soybean by *Phytophthera megasperma* f. sp. *glycines* (Keen et al., 1982) and of tomato (*Solanum lycopersicon* L.) by crown and root rot *Fusarium* spp. (Brammall and Higgins, 1988). In addition to suppression of phytoalexin synthesis, glyphosate is implicated in immobilization of micronutrients including Mn and Fe, essential in many metabolic pathways (Eker et al., 2006; Jolley et al., 2004; Neumann et al., 2006; Ozturk et al., 2007), and increased excretion of substrates from roots that may be selectively metabolized by pathogens (Lévesque and Rahe, 1992). Regarding the latter, Liu et al. (1997) showed that roots of bean seedlings were vulnerable to infection by *Pythium* spp. 2 days after glyphosate application to the foliage. Increased amino acid contents as well as suspected translocated glyphosate released in root exudates apparently enhanced germination and growth of *Pythium* propagules which, combined with suppression of plant cell lignification defense responses to pathogen attack, rapidly infected and colonized roots (Liu et al., 1997). Glyphosate applied to the winter annual weeds henbit (*Lamium amplexicaule* L.) and downy brome (*Bromus tectorum* L.) significantly increased rhizosphere populations of *Fusarium solani* f. sp. *pisi* and *Pythium ultimum* (Kawate et al., 1997). Proliferation of these phytopathogens on winter annual weeds may boost soil populations suggesting that weed control with a burndown application of glyphosate could lead to disease incidence in subsequent leguminous crops planted into residues of the killed vegetation.

These reports show that glyphosate imposes diverse effects on the biology and ecology of rhizosphere microorganisms and on their interactions with plant roots when released into the rhizosphere. Although the interactions suggest a "secondary mode of action" of glyphosate by pre-disposing susceptible plants to microbial infection (Johal and Rahe, 1984), the potential for developing critical pathogen inocula levels in soils that affect crop health, altering rhizosphere microbial communities involved in nutrient transformations, and shifting the balance of beneficial and detrimental plant-associated microorganisms are legitimate concerns regarding the impact of glyphosate on crop productivity and environmental sustainability. This is especially significant with consideration to the current widespread use of glyphosate in glyphosate-resistant (GR) cropping systems.

1.3. Rhizosphere microorganisms and glyphosate-resistant crops

One of the most significant advancements in intensive agriculture was the introduction of GR crops in the mid-1990s. By 2007 the global area planted to all genetically modified crops exceeded 113 million ha on which GR soybean occupied 58.6 million ha (52% of the global area planted to biotech crops), followed by GR maize (35.2 million ha at 31% of global area), and GR cotton (13.4 million ha at 12% of global area) (James, 2007). Indeed, based on average application rate and frequency and area treated, the amount of glyphosate applied to GR soybean in the U.S. increased by 52 million kg from 1997 to 2004 (Benbrook, 2004). The GR cropping system provided a more cost-effective option for farmers, allowing them flexibility in weed management to spray a broad spectrum of weeds with glyphosate on an "as needed" basis and reducing the need for pre- and post-emergence herbicides. Production of transgenic crops, resistant to glyphosate, represents a drastically new approach in weed management that allows broad-spectrum weed control without crop injury, may reduce preemergence herbicide use, and better conserve soils by increasing the use of no-tillage (Zablotowicz and Reddy, 2004; Locke et al., 2008). Because current soybean production relies heavily on use of transgenic, GR cultivars (Roundup Ready™), considerable interest has developed concerning the impacts of widespread cultivation of genetically modified crops and use of one herbicide class on agroecosystems, especially on the potential effects on biological processes including phytopathogenic activity and disease incidence. Glyphosate-resistant soybean was developed by insertion of a transgene (*cp4*) from an *Agrobacterium* species that codes for an insensitive version of EPSPS (Franz et al., 1997). The limited research conducted to date suggests that, in GR soybean, very little glyphosate is degraded within the plant (Arregui et al., 2003) with most of the herbicide translocated to active metabolic sinks including seeds (Duke et al., 2003b), nodules (Reddy and Zablotowicz, 2003), and roots (Duke, 1996). Nodulation and nitrogen fixation are reduced in some early-season GR soybean cultivars receiving glyphosate applications at field rates (King et al., 2001).

Since the introduction of GR crops in the mid- to late-1990s many consequences associated with production of GR crops were soon reported based on field observations of apparent increased disease and nutritional deficiencies relative to conventional or non-transgenic cultivars. A limited number of studies attempted to quantify or repeat anecdotal observations of glyphosate influences on crop-microorganism interactions, yielding variable results. In a controlled field study, increased susceptibility to sudden death syndrome (SDS) in soybean caused by *F. solani* pv. *glycines* [reclassified as *F. virguliforme* (Aoki et al., 2003)] was reported for GR soybean treated with glyphosate compared with no herbicide treatment (Sanogo et al., 2000). However, subsequent research (Njiti et al., 2003) failed to reproduce SDS in other GR soybean cultivars. *Corynespora cassiicola* may cause severe root rot on GR soybean in proximity to giant ragweed (*Ambrosia trifida* L.) treated with glyphosate (Huber et al., 2005). Altered root exudates and/or glyphosate released from roots of dying ragweed plants appeared to modify the rhizosphere environment and predispose adjacent GR soybean roots to severe *Corynespora* root rot. Research with *Sclerotinia sclerotiorum* (Sclerotinia root rot) was inconsistent in describing a definite relationship between susceptibility and glyphosate use on GR soybean, leading to the conclusion that effects of the GR trait or glyphosate use on disease susceptibility may be cultivar specific (Lee et al., 2002). Disease severity caused by *F. oxysporum* and *Rhizoctonia solani* in GR sugarbeet [*Beta vulgaris* (*saccharifera*) L.) increased with glyphosate application (Larson et al., 2006).

Studies on glyphosate interactions with microbial communities in GR cropping systems reveal that the most pronounced effects are detected with specific genera or species of microorganisms (i.e., *Fusarium*, *Pythium*) rather than with broader measurements of soil microbial diversity and functions. In a review on impacts of disturbance on soil microbial communities, Wardle (1995) suggests that measurement of response by functional groupings may be problematic because individual taxonomic species are likely to be more sensitive to disturbance rather than an entire functional group comprised of multiple taxonomic groups. If herbicide application is considered an external ecosystem disturbance, it is not surprising that glyphosate is often reported to have no effect on soil microbial biomass and diversity and broad functional activities such as soil respiration and soil enzymatic activity in GR cropping systems (Liphadzi et al., 2005; Lupwayi et al., 2007; Means et al., 2007; Hart et al., 2009). Based on numerous studies of *Fusarium* spp., Powell and Swanton (2008) proposed possible mechanisms involved in promoting the proliferation of an individual taxonomic group by glyphosate in GR crops including direct stimulation of fungal growth, indirect stimulation due to alteration of root exudate components, increase in host susceptibility to pathogens, and decrease in effectiveness of pathogen antagonists.

Glyphosate and root exudates released into the rhizosphere from GR soybean influence microbial populations and/or activity in the rhizosphere. Previous findings that glyphosate and high concentrations of soluble carbohydrates and amino acids exude from roots of glyphosate-treated GR soybean (Kremer et al., 2005) suggested that impacts on root microbial interactions and micronutrient uptake might mirror those described for glyphosate interactions in non-transgenic cropping systems.

Based on the previous research on glyphosate-induced microbial root infection of susceptible weeds and crops and limited reports for GR crops, we hypothesized GR soybean and maize might be more susceptible to root infection by fungi than conventional, non-transgenic cultivars. Our research was justified because of the numerous reports by producers of seemingly more frequent incidence of diseases appearing in GR soybean than that observed previously with conventional soybean (Sanogo et al., 2000; Njiti et al., 2003). A primary focus of our long-term field studies is documenting effects of glyphosate applied to GR soybean and maize cultivars on root colonization and soil populations of *Fusarium* spp. *Fusarium* spp. were selected as indicators of the microbial ecology of the soybean rhizosphere because they exist in soil saprophytically and are prevalent in rhizospheres of many plants where they may dominate microbial communities and become pathogenic, often in response to root exudation (Nelson, 1990). Additional potential consequences of GR cropping systems are alterations in the microbial ecology and biological processes carried out in the crop rhizosphere environment (Lupwayi et al., 2009). Several functions are affected including nutrient cycling and plant availability; potential phytopathogen and antagonist interactions; and activities and composition of beneficial microorganisms including plant-growth promoting rhizobacteria (Johal and Huber, this issue). Due to the complexity of soil and microbial communities, such plant-associated components are often neglected in evaluations of crop productivity or risk assessments of GM crops (Azevedo and Araujo, 2003). Our objective is to present a research summary to illustrate approaches for understanding the impacts of GR crops on microbiological interactions in the rhizosphere.

2. The glyphosate-resistant crop production and rhizosphere microbial ecology project

Field trials with soybean and maize were conducted at the University of Missouri Bradford Research and Extension Center (Boone County; 38°53′N, 92°12′W), the Delta Center (Pemiscot County; 36°23′N, 89°36′W), and six mid-Missouri on-farm sites

from 1997 through 2007. Soils are representative of the Central (U.S.) Claypan Region, developed in loess overlain on glacial till, and contain a claypan that limits water percolation and promotes surface runoff. The soils are generally classified as aeric, vertic Epiaqualfs, and are located on landscapes of 1–2% slopes. The experimental design consisted of crop variety + herbicide combination treatments, each replicated four times: GR variety + glyphosate, GR variety + non-glyphosate herbicide, GR variety + no herbicide, non-GR variety + non-glyphosate herbicide, and non-GR variety + no herbicide. Plot size area varied from 3.7×6.1 m to 3.7×24.4 m depending on availability of field space each year. All treatment-combinations were not established every year; however, GR variety + glyphosate and GR variety + no herbicide treatments were evaluated every year. Field plots were prepared using minimum tillage consisting of one or two passes of a disk-harrow as necessary to incorporate crop residues before planting. Soybean and maize were planted in 76-cm rows from mid-May to mid-June depending on the annual weather conditions. Supplemental fertilization was consistent with recommended management practices. Glyphosate at 0.84 kg a.e. ha^{-1} was applied to GR soybean and GR maize at the V4–V5 and V5–V6 growth stages, respectively. Non-glyphosate herbicides included post-emergence application of clethodim (0.42 kg a.i. ha^{-1}) + fomesafen (0.175 kg a.i. ha^{-1}) on GR and conventional soybean and pre-plant incorporation of atrazine at 2.24 kg a.i. ha^{-1} on GR maize. All herbicides were applied to the appropriate crop variety-herbicide combination plots at a 130 L ha^{-1} spray volume at a pressure of 138 kPa using 11,003 spray nozzles. Plots receiving no herbicides were hand-weeded periodically during the season.

Intact soybean and maize plants were randomly sampled immediately prior to and periodically (up to 42 days) after herbicide application. All nodules present on the roots were removed for fresh and dry mass measurements; a subsample of roots were cleaned of soil and fresh and dry biomass. *Fusarium* colonization was assessed on surface-sterilized root segments following the root plating procedures of Lévesque et al. (1993) and using *Fusarium*-selective agar medium (Nash and Snyder, 1962). After a 7-day incubation, numbers of fungal colonies developing on root segments were recorded. *Fusarium* colonies were randomly selected from colonized roots, subcultured on potato dextrose agar, and tentatively identified using descriptions of cultural and microscopic morphologies (Nelson et al., 1983). Identification was confirmed by molecular analysis using partial translation elongation factor sequences (Skovgaard et al., 2001).

Soil tightly adhered to roots was rigorously removed using a sterile camel-hair brush, suspended in buffer and appropriate dilutions plated on S1 agar medium selective for fluorescent pseudomonads (Gould et al., 1985) and on Gerretsen's agar medium for detecting Mn-oxidizing and -reducing microorganisms (Huber and Graham, 1992). After incubation, colonies developing on agar were recorded and represented an estimate of colony-forming units in the rhizosphere. Mn-transforming bacterial components were further expressed as ratios of Mn reducers to Mn oxidizers to provide a means of detecting potential effects of microbial activity on plant available Mn (Rengel, 1997). Representative bacterial colonies from the pseudomonad and Mn-transformer assays were selected and subcultured on S1 and tryptic soy agars to obtain pure, single-colony isolates. These isolates were further assayed for in vitro antagonism of *Fusarium* (Kremer et al., 1990), extracellular protease production (Harley and Prescott, 1999), and extracellular polysaccharide (EPS) production (Kelman, 1954). Isolates were characterized for colony morphology and fluorescent pigment production; gram stain and oxidase reactions; and classified taxonomically by determining cellular fatty acid profiles using gas chromatography-fatty acid methyl ester analysis (Kennedy, 1994). Rhizobial-induced nodules on soybean root samples were removed and weighed for estimation of relative symbiotic nitrogen fixation.

Statistical analyses were performed with the general linear model procedure; analysis of variance and, where F values were significant ($\alpha = 0.05$), mean separations were conducted using Fisher's protected least significance difference (LSD) test (SAS Institute, Cary, NC).

3. Results and discussion

3.1. *Fusarium* root colonization of glyphosate-resistant crops

Fusarium colonization was higher on GR soybean treated with glyphosate throughout the growing season, generally two to five times higher compared with soybean receiving no herbicide or a conventional (non-glyphosate) herbicide. The frequency of root-colonizing *Fusarium* increased significantly within 1 week after glyphosate application throughout the growing season in each year at all sites (Fig. 1A). *Fusarium*-like colonies developed on surface-sterilized soybean root segments indicating colonization or infection of root tissue by the fungi. Populations of *Fusarium* in rhizosphere soil from soybean receiving glyphosate were also significantly increased during each growing season at all locations (Means, 2004). Several studies report rhizosphere and soil *Fusarium* spp. increase in response to glyphosate addition (Lévesque et al., 1987; Powell and Swanton, 2008) including infection and disease severity by *F. solani* f. sp. *glycines*, which increased with GR soybean treated with glyphosate compared to no herbicide treatment (Sanogo et al., 2000). *Fusarium* colonization of roots of GR maize receiving glyphosate was 3–10 times higher colonization levels than for colonization levels for the atrazine treatment, similar to colonization patterns documented for soybean with

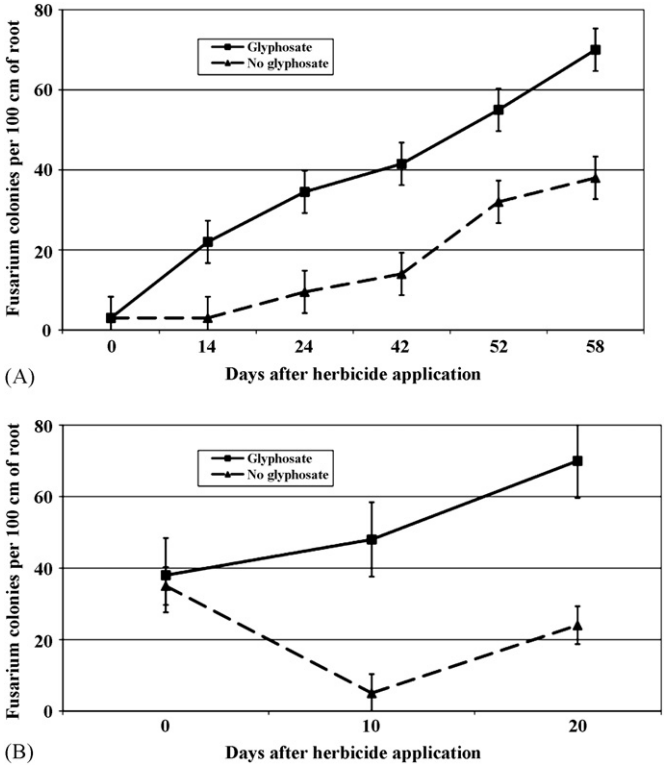

Fig. 1. Relationship of glyphosate application with root colonization of (A) glyphosate-resistant soybean ('Pioneer 94B01') and (B) glyphosate-resistant maize ('DeKalb DKC60') by *Fusarium* spp. Data in (A) based on Kremer (2003); data in (B) based on Means (2004). In both graphs, significant differences (*$P < 0.05$) between glyphosate and no glyphosate treatments within dates are indicated by the vertical bars representing Fisher's protected LSD.

glyphosate treatment (Fig. 1B; Means, 2004). These results suggest that glyphosate induces fungal colonization of both soybean and maize rhizospheres via similar mechanisms. Taxonomic diagnosis of representative cultures collected from annual field studies has been distributed among the following groupings: 70% *Fusarium oxysporum* complex, 18% *Fusarium solani* complex, and 10% *Fusarium equiseti*.

A summary of root colonization results of field-grown soybean roots during the period 1997–2007 demonstrates consistently higher colonization of GR cultivars by *Fusarium* spp., especially when glyphosate is applied (Fig. 2). Soybean roots from plants receiving no or conventional post-emergence herbicides exhibited low *Fusarium* colonization; non-transgenic (non-GR) cultivars always showed lowest root colonization. Rhizosphere-inhabiting *Fusarium* may readily metabolize glyphosate in root exudates as a sole source of P and also as a C and energy source (Castro et al., 2007). Also, glyphosate may stimulate propagule germination and early growth by *Fusarium* (Krzysko-Lupicka and Orlik, 1997). When root exudation is excessive, as for glyphosate-treated soybean, root infection by soilborne pathogens is enhanced (Nelson, 1990). Also, Griffiths et al. (1999) indicate that as concentrations of soluble carbohydrates and amino acids increased in root exudates, the proportion of fungi in the rhizosphere community also increased compared with that of bacteria. Therefore, the structure of the microbial community is not solely governed by composition of exudates. Optimal growth of different microorganisms is also related to quantity of available substrates. Thus glyphosate released into the rhizosphere of GR soybean combined with release of high concentrations of carbohydrates and/or amino acids favor increased growth of *Fusarium* spp., illustrated by the results from our repeated field studies with GR soybean (Kremer, 2003; Means, 2004). These findings summarized in Fig. 2 demonstrate that monitoring for effects of glyphosate on environmental processes is prudent especially in light of its current widespread use in agricultural, horticultural, and timber production systems.

Annual variations in weather conditions affected *Fusarium* colonization of soybean roots, accounting for the variable infection levels shown in Fig. 2. For example, low colonization during 2003 was suspected to be related to low rainfall during the growing season, which was about 30% of normal. A greenhouse study in which soil water contents were controlled verified that glyphosate-treated soybean under extreme moisture stress exhibited about 75% reduction in *Fusarium* colonization of roots (Means and Kremer, 2007). Moisture stress reduces activity of other plant-microbial associations, including nitrogen fixation in glyphosate-treated GR soybean (King et al., 2001), considerably more than in untreated plants. These findings partly explain reported problems with soybean productivity and yields experienced by many farmers using the GR cropping system in the Midwestern U.S.

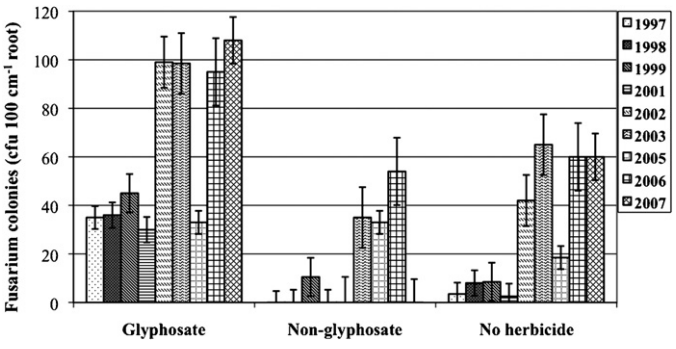

Fig. 2. Average seasonal colonization of glyphosate-resistant soybean roots by *Fusarium* spp. across all sites during 1997–2007, influenced by glyphosate, non-glyphosate, or no herbicide applications. Within year, significant differences ($^*P < 0.05$) between colonization of glyphosate-treated roots compared with the non-glyphosate and no herbicide treatments are indicated by the vertical bars representing Fisher's protected LSD. Partly based on data from Kremer (2003).

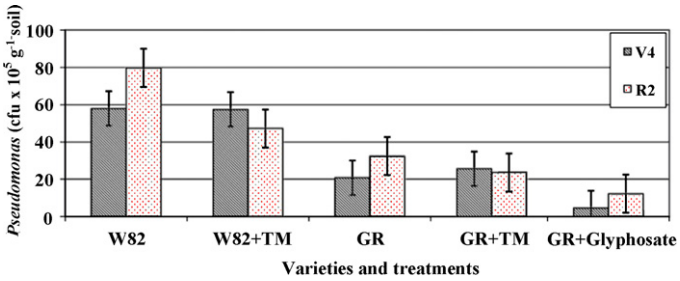

Fig. 3. Populations of rhizosphere *Pseudomonas* spp. detected in 2006 field study on conventional soybean (W82, 'Williams 82') and GR soybean (GR, 'DeKalb DK-838-52') at two growth stages (V4 and R2), influenced by herbicide treatment: TM, tank-mix of fomesafen + clethodim (non-glyphosate); glyphosate; or no designation indicates hand-weeding without herbicide. *Pseudomonas* spp. populations for W82 at both growth stages were significantly higher ($^*P < 0.05$) than all GR treatment combinations indicated by the vertical bars representing Fisher's protected LSD.

3.2. Glyphosate and GR soybean interactions with the rhizosphere bacterial community

3.2.1. Fluorescent pseudomonads

Rhizosphere-inhabiting *Pseudomonas* spp. are important multifunctional bacteria in the rhizosphere capable of producing numerous secondary metabolites including siderophores, hydrogen cyanide, extracellular enzymes, and various antibiotics that suppress competing microbial groups (Schroth et al., 2006). A majority of fluorescent pseudomonads is associated with antagonism of fungal pathogens (Schroth and Hancock, 1982), which contribute to Mn transformations, primarily Mn reduction (Rengel, 1997), in plant rhizospheres. Glyphosate and soybean cultivar significantly decreased rhizosphere fluorescent pseudomonads detected using selective culture (Fig. 3). Fluorescent pseudomonads, always higher in non-GR soybean rhizosphere, may be reduced in GR soybean due to their reported sensitivity to glyphosate (Schulz et al., 1985) that is exuded into the rhizosphere or because antagonistic activity is overcome by glyphosate (Wardle and Parkinson, 1992). Also, any negative effects of cultivar on rhizosphere pseudomonads might be further reduced by glyphosate. A negative relationship between population size of fluorescent pseudomonads and *Fusarium* root colonization further demonstrated that GR soybean and/or glyphosate was involved in altering the microbial composition in the rhizosphere (Fig. 4). Bioassays of single cultures of fluorescent pseudomonads confirmed that most (≈85%) were potentially antagonistic toward *Fusarium*; antagonism was also associated with strong extracellular protease activity by many of the isolates. Thus, as suggested by Powell and Swanton

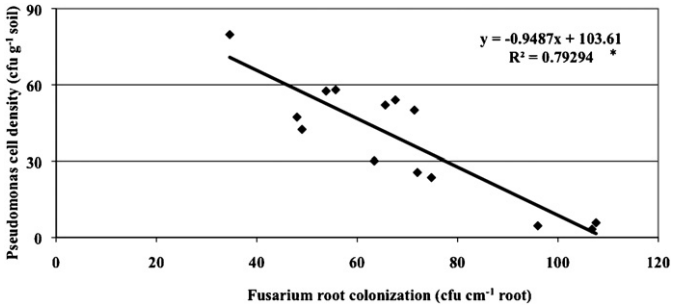

Fig. 4. Relationship between *Pseudomonas* spp. populations and *Fusarium* root colonization on soybean at R2 growth stage based on data collected in 2006 field study. Regression is significant at $^*P < 0.05$.

(2008), glyphosate and GR soybean may enhance fungal root colonization and potential disease through not only stimulating growth of the fungal pathogen but also by suppressing bacterial antagonists.

3.2.2. Manganese-transforming rhizosphere bacteria

Mn transformations are primarily microbially mediated and thus have a major impact on plant nutrient availability and metabolic processes (Thompson and Huber, 2007). A low ratio of Mn reducers to Mn oxidizers determined for the GR soybean Dekalb 'DK-838-52' treated with glyphosate compared with 'Williams 82' suggested soil Mn was immobilized and not available for plant uptake (Fig. 5). This occurred despite standard chemical analysis showing the soils were Mn sufficient. Glyphosate as a major factor in enhancing Mn-oxidizing bacteria was further strengthened by results determined for both GR and non-GR soybean with or without non-glyphosate herbicides that were significantly different (higher ratio) from glyphosate treatments. Fluorescent pseudomonads are also primarily Mn-reducers involved in fungal suppression (Huber and McCay-Buis, 1993), thus low numbers on GR soybean (Fig. 3) likely further contribute to increased *Fusarium* root colonization due to reduction of this suppressive activity.

Interestingly, a majority of bacterial isolates with Mn-oxidizing ability detected on Gerritsen's medium produced copious amounts of exopolysaccharides (EPS), and was most frequently associated with GR soybean or GR soybean + glyphosate. Many isolates subcultured from this group were phenotypically characterized as *Agrobacterium* spp., which are resident saprophytic bacteria in the soybean rhizosphere (Kuklinsky-Sobral et al., 2004). The consistent detection of agrobacteria producing excessive amounts of EPS suggested that biofilm formation on the soybean root surface may be related Mn-oxidizing agrobacteria, enhanced in the presence of glyphosate. As members of the alpha–proteobacteria, comprised of many Mn-transforming bacteria, agrobacteria are known strong Mn oxidizers (Thompson and Huber, 2007; Johal and Huber, this issue). Agrobacteria also typically form biofilms composed of EPS matrices on the rhizoplane in which many biological functions are mediated (Matthysse, 2006), including biogenic Mn oxidation, reported for many biofilm-forming bacteria that produce precipitated Mn oxides, which are retained within the biofilm (Toner et al., 2005). Thus the frequency of rhizobacteria with both EPS-producing and Mn-oxidizing properties (primarily *Agrobacterium* spp.) in rhizospheres of GR soybean treated with glyphosate suggests that the herbicide alone or in combination with GR soybean enhances or selects this group of bacteria with potential detrimental effects on plant growth through Mn immobilization. Because GR crop cultivars contain the *cp4* gene, derived from an *Agrobacterium* sp., that encodes for the glyphosate-resistant EPSPS (Funke et al., 2006), it is possible that as glyphosate is released through roots of GR crops, genetically similar *Agrobacterium* spp. in soils may be preferentially selected for colonization of the rhizosphere and rhizoplane. The role of glyphosate in biofilm formation and associated Mn transformation by rhizosphere bacteria remains to be clarified.

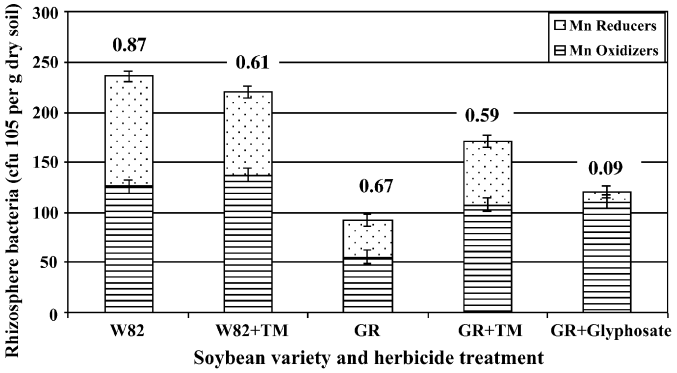

Fig. 5. Populations of rhizosphere Mn-transforming bacteria detected in 2006 field study on conventional soybean (W82, 'Williams 82') and GR soybean ('DeKalb DK-838-52') at growth stage R2 influenced by herbicide treatment (see Fig. 3 for explanation). Ratios of potential Mn-reducing to Mn-oxidizing bacteria are indicated at top of each bar. Significant differences ($^*P < 0.05$) among each bacterial group are indicated by the vertical bars representing Fisher's protected LSD.

3.2.3. Root nodulation

Symbiotic nitrogen fixation may contribute 40–70% of the nitrogen required by soybean during the growing season, thus sustaining this nitrogen input is critical for profitable grain yield and sustaining long-term soil productivity (Zablotowicz and Reddy, 2007). Legume nodules provide the necessary conditions for specialized plant and rhizobial structures to perform nitrogen fixation; thus nodule number and mass on roots qualitatively indicate the status of other parameters including nitrogenase activity, leghemoglobin content, and nitrogen accumulation. In our studies, nodulation was always lower on GR soybean with or without glyphosate compared with conventional varieties (i.e., Williams 82) with non-glyphosate or no herbicide (Fig. 6). These results confirm that glyphosate and perhaps the genetic modification in the GR plant may affect numerous processes associated with the nitrogen fixation symbiosis, including nitrogenase activity and leghemoglobin content (King et al., 2001; Reddy et al., 2000; Reddy and Zablotowicz, 2003; Zablotowicz and Reddy, 2007). A recent field study reporting that glyphosate significantly reduced nodule mass and nitrogen fixation in GR soybean yet did not affect grain yields suggests that an increased dependence of the crop on mineralized N from soil organic matter (Bohm et al., 2009) may contribute to a negative N balance and reduced sustainability of cropping systems based on GR soybean.

4. Recommendation

4.1. Polyphasic microbial analysis to assess impacts of GR crops

Results of our field studies emphasize the necessity for evaluation of the numerous and complex factors in the rhizosphere (root exudation, glyphosate release, microbial activities) to broaden our understanding of glyphosate interactions with root-associated microorganisms. Kowalchuk et al. (2003) recognized the limitations of general measurements such as soil microbial biomass and respiration for describing potential GM crop-induced effects on the microbial community and proposed that a polyphasic microbial analysis comprised of selected indicator groups and activities combined with general assays including microbial diversity analyses would yield a more comprehensive and informative assessment of GM crops.

Initial studies in our GR crop-microbial ecology project focused on rhizosphere *Fusarium* spp. as a key indicator of potential impacts of GR soybean and maize on the rhizosphere ecosystem (Kremer, 2003; Means and Kremer, 2007) because this fungal group readily responds to alterations (i.e., introduction of glyphosate and altered root exudation) in the rhizosphere (Nelson, 1990). However, because of the complexity of the rhizosphere ecosystem, a more comprehensive examination of the structure and functions of the broader microbial community was implemented to provide a more complete assessment of potential effects induced by GR crops.

Our polyphasic microbial analysis entails a multiple assessment of sensitive indicators that provides a more reliable view of GR technology effects relative to any single technique (Kowalchuk et

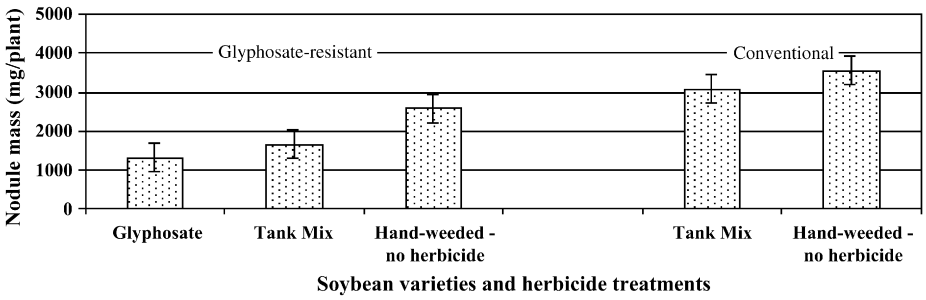

Fig. 6. Relationship of glyphosate application with root nodulation of glyphosate-resistant ('DeKalb DKB38-52') and conventional ('Williams 82') soybean based on 2005 field study. HW, hand-weeded check; TM, conventional herbicide tank-mix. Significant differences (*$P < 0.05$) between treatments are indicated by the vertical bars representing Fisher's protected LSD.

al., 2003; Powell, 2007). The framework for evaluating GR crop effects evolved during the course of our project based on progressive development of information in two areas: (1) non-herbicidal activity of glyphosate including systemic movement and release through GR plant roots; metal chelation within plant and in the rhizosphere; and growth stimulation or suppression of selected microbial groups; and (2) alteration of root exudate composition in GR plants with or without glyphosate treatment (Kremer et al., 2005). These are areas of concern because the structure of the rhizosphere microbial community is determined by the specific types and quantities of component chemicals and signaling compounds in root exudates (Grayston et al., 1998; El-Shatnawi and Makhadmeh, 2001; Broeckling et al., 2008); if the chemical composition of root exudates is drastically altered due to genetic transformation or treatment with glyphosate, it follows that physiological processes, pathogen defense mechanisms, and plant growth could be detrimentally affected. Therefore, our current specific analyses include fungal root colonization, Mn-transforming bacteria, symbiotic nitrogen fixation and nodulation, pseudomonad communities, and arbuscular mycorrhizal fungi (data not reported). We are also linking these key microbial relationships to community structure through analyses of DNA extracted from soybean rhizospheres to yield a molecular fingerprint. Preliminary results indicate that bacterial diversity based on molecular analyses was generally higher for conventional soybean compared with GR soybean treated with or without glyphosate, which agreed with the variations in composition and activities of the key indicators. The decreased microbial diversity in GR soybean rhizospheres is a concern because high diversity is essential in maintaining a stable ecosystem and plant productivity (Grayston et al., 1998).

5. Conclusions

The widespread adoption of transgenic GR crops is largely due to simplified crop management and greater flexibility in weed management offered by the GR cropping system. These crops have been released after assessments based on general soil biological analyses that often lack sensitivity to detect non-target effects (Kowalchuk et al., 2003). A more informative approach to assess the impact of GR crops on the environment is to target sensitive indicator microbial groups and/or processes in addition to the more general tests targeting microbial community diversity. Those microbial groups most neglected in assessments include specific soilborne and plant-associated (rhizosphere) microorganisms (Azevedo and Araujo, 2003; Kowalchuk et al., 2003).

In this overview we have attempted to present the current knowledge regarding impacts of glyphosate and GR crops on plant–microbial interaction. Our current view of the complex interactions in GR soybean and maize rhizospheres illustrates the limited information available and areas where research is desparately needed, such as impacts on mycorrhizal fungi. The report on our GR crop-microbial ecology project presented in this paper supports some of the literature reviewed, especially that dealing with glyphosate-induced *Fusarium* activity in the rhizosphere and negative impacts on soybean nodulation. Additionally, we document new information on changes in microbial components of GR soybean and maize rhizospheres: increases in the proportion of Mn-oxidizing bacteria; decreases in the pseudomonad component that antagonizes fungal pathogens; and increases in agrobacteria that may be involved in Mn oxidation and microbial community shifts due to apparent selection by glyphosate exudation. Analyses of GR soybean root exudates suggest that promotion of rhizosphere and root colonization of GR soybean by specific microbial groups may be a combination of stimulation by glyphosate released through root exudation and altered physiology leading to exudation into the rhizosphere of high levels of carbohydrates and amino acids (Kremer et al., 2005) possibly related to indirect, or pleiotropic, effects of genetic transformation for glyphosate resistance (Powell, 2007). This supports the ecological concept that plants actively modify their rhizospheres through production of specific root exudates that have a profound qualitative and quantitative effect in modifying the microbial communities (Grayston et al., 1998; El-Shatnawi and Makhadmeh, 2001; Broeckling et al., 2008). Overall, exposure of the rhizosphere microbial community to glyphosate and GR crops appeared to cause complex and varied responses; our project demonstrates the importance of using a multiphasic approach to yield a comprehensive analysis of rhizosphere microbial community structure and function in response to GR cropping systems.

It should be noted that the information generated from our project might also be used to improve productivity of GR crops by developing a combination of strategies including soil management for balanced biological activity, proper plant nutrition, and suppression of potential pathogens. Knowledge of the relationships between soil biology, plant nutrition, management factors, and disease potential provide a basis for addressing recurrent productivity problems associated with GR crops integrated in current agricultural production systems.

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