

## ORIGINAL ARTICLE

# Glyphosate affects micro-organisms in rhizospheres of glyphosate-resistant soybeans

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*Fusarium* spp., glyphosate, Glyphosate-resistant soybean (*Glycine max* L.), indoleacetic acid, Mn, plant biomass, rhizobacteria.

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**Abstract**

**Aims:** Glyphosate-resistant (GR) soybean production increases each year because of the efficacy of glyphosate for weed management. A new or 'second' generation of GR soybean (GR2) is now commercially available for farmers that is being promoted as higher yielding relative to the previous, 'first generation' (GR1) cultivars. Recent reports show that glyphosate affects the biology and ecology of rhizosphere micro-organisms in GR soybean that affect yield. The objective of this research was to evaluate the microbiological interactions in the rhizospheres of GR2 and GR1 soybean and the performance of the cultivars with different rates of glyphosate applied at different growth stages.

**Methods and Results:** A greenhouse study was conducted using GR1 and GR2 soybean cultivars grown in a silt loam soil. Glyphosate was applied at V2, V4 and V6 growth stages at three rates. Plants harvested at R1 growth stage had high root colonization by *Fusarium* spp.; reduced rhizosphere fluorescent pseudomonads, Mn-reducing bacteria, and indoleacetic acid-producing rhizobacteria; and reduced shoot and root biomass.

**Conclusions:** Glyphosate applied to GR soybean, regardless of cultivar, negatively impacts the complex interactions of microbial groups, biochemical activity and root growth that can have subsequent detrimental effects on plant growth and productivity.

**Significance and Impact of the Study:** The information presented here will be crucial in developing strategies to overcome the potential detrimental effects of glyphosate in GR cropping systems.

**Introduction**

Glyphosate-resistant (GR) soybeans (*Glycine max*) genetically modified to produce glyphosate-insensitive 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) (Reddy *et al.* 2001) were commercialized in 1996 in US (Duke 2005). The 'first generation' transgenic cultivars (GR1) were developed by insertion of the cp4 EPSPS coding sequence derived from the common soil bacterium *Agrobacterium* spp. strain CP4 (Franz *et al.* 1997). Improved trait selection combined with transgenic modification led to 'second generation' (GR2) cultivars that were commercially available in 2008 and promoted as

higher yielding relative to GR1 cultivars. Recent reports on plant injury in some GR soybean cultivars after glyphosate application (Zablotowicz and Reddy 2007) stimulated greenhouse and field research showing that photosynthesis, plant nutrient content and biomass were significantly decreased in some GR1 and GR2 soybean cultivars (Zobiolo *et al.* 2010b,c) by glyphosate applied at different rates and at different soybean growth stages.

In addition to affecting plant physiological functions, glyphosate may enhance higher incidence of many diseases as a result of the reduced nutritional status of the plant and may detrimentally impact many beneficial soil micro-organisms (Kremer *et al.* 2005; Johal and Huber

2009). Gressel (2002) suggests that the transgenic EPSPS in GR1 soybean is considerably less efficient than the wild-type enzyme and produces insufficient amounts of phytoalexins to prevent fungal infection. Key inducible defence components associated with the shikimic acid pathway include antimicrobial phytoalexins that accumulate rapidly at the site of pathogen infection (Johal and Huber 2009). Lignification of cell walls at and around the infection site also depends on a shikimate-derived component to fortify cells and ensure isolation of the pathogen at the infection site. Decreased lignin content may also be because of the reduced photosynthesis in soybean caused by glyphosate (Zobiolo *et al.* 2010a). Decreased lignification and phytoalexin production allow increased root colonization by *Fusarium* in plants damaged by glyphosate (Johal and Rahe 1988). Thus, considerable concern exists regarding potential detrimental effects of rhizosphere micro-organisms on soybean productivity resulting from either direct effects of glyphosate or indirect effects on plant physiological functions.

Long-term studies on field-grown soybean during 1997–2007 demonstrated consistently higher root colonization by *Fusarium* spp. on GR cultivars with glyphosate (Kremer and Means 2009). Soybean roots from plants receiving no or conventional postemergence herbicides exhibited low *Fusarium* colonization, and nontransgenic (non-GR) cultivars always had the lowest root colonization. Subsequent studies showed that label rates of glyphosate applied to GR soybean resulted in exudation of glyphosate from roots with simultaneous exudation of high concentrations of soluble carbohydrates and amino acids that favoured fungal infection (Kremer *et al.* 2005). Other reports indicate that although glyphosate is considered inactivated by rapid soil adsorption, glyphosate may serve as a substrate to some micro-organisms (Araújo *et al.* 2003; Kuklinsky-Sobral *et al.* 2005). Thus, rhizosphere-inhabiting *Fusarium* may metabolize glyphosate in root exudates as a source of P, C and energy (Castro *et al.* 2007).

Rhizosphere-inhabiting *Pseudomonas* spp. are important multifunctional bacteria that are capable of producing numerous secondary metabolites including siderophores, hydrogen cyanide, extracellular enzymes and various antibiotics that enhance nutrient availability and suppress competing microbial groups (Schroth *et al.* 2006). Many fluorescent pseudomonads in the rhizosphere antagonize fungal pathogens (Schroth and Hancock 1982) and mediate Mn transformations, primarily Mn reduction (Rengel 1997), and thus have a major impact on plant nutrient availability and metabolic processes (Thompson and Huber 2007). Manganese is a critical cofactor in enzymes necessary to drive many essential pathways in plants including photosynthesis and amino acid synthesis; thus, disruption of Mn availability in the soil environment

through microbial processes can negatively affect plant productivity. Manganese is easily oxidized and reduced in soils and biological systems and can exist in either  $Mn^{+2}$ ,  $Mn^{+3}$  and  $Mn^{+4}$  oxidation states; however, the soluble  $Mn^{+2}$  ion is the predominant form taken up from the soil by plants (Clarkson 1988; Marschner 1988, 1995).

Most rhizobacteria synthesize plant growth regulators such as auxins (Khalid *et al.* 2004) that are comprised primarily of indole-3-acetic acid (IAA), which plays an important role in growth promotion by stimulating cell elongation (Taiz and Zeiger, 1998). IAA biosynthesis in bacteria (i.e., *Agrobacterium*, *Pseudomonas*, *Bradyrhizobium*) occurs via several pathways with many identifiable intermediates (Patten and Glick 1996); however, very little is known of the impact of repeated glyphosate use on rhizosphere micro-organisms associated with GR soybean. If the repeated application of glyphosate affects IAA-producing rhizobacteria (IPR), the growth and production of GR soybean could be negatively affected (Kim 2006).

There is considerable current interest regarding glyphosate effects on GR soybeans; however, little information is available on the performance of GR2 soybean beyond commercial and farmer testimonial statements. Based on previous controlled and field studies with GR1 soybean, we hypothesize that glyphosate released from roots may affect microbial populations and/or activity in the rhizosphere, regardless of transgenic soybean generation. Confounding the accurate assessment of glyphosate impacts on plant-microbial interactions are the multiple rates of glyphosate employed during the growing season that are often different depending on the global production region and the soybean growth stage when the herbicide is applied. Therefore, the objective of this study was to evaluate selected rhizosphere microbial communities in GR1 and GR2 soybeans receiving variable rates of glyphosate applied at different growth stages.

## Material and methods

### Soil, cultivation practices and seeds sources

Two experiments were conducted in greenhouse conditions at the University of Missouri, Columbia, MO, USA, between July and October, 2009. Temperatures were maintained at 26–30 : 22–26°C (day/night) with a 12-h photoperiod of full sunlight (midday irradiance of 400–700 nm) and a photosynthetic photon flux density of 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at the top of the leaf canopy. Plants were grown in 5-dm<sup>-3</sup> clay pots filled with sieved (5 mm) air dry soil from the A horizon of a Mexico silt loam soil (fine, smectitic, mesic Aeric Vertic Epiaqualfs). Soil in all pots was maintained at water content of 80% water-holding capacity (0.33 g/g) with N-free water throughout the

experiment. The soil physicochemical properties were ( $C_{\text{org}}$ : 25.2 g kg<sup>-1</sup>; P: 15.87; K: 45.86; Ca: 1782.64; Mg: 123.89; Fe: 80.70; Mn: 43.23; B: 14.06; Cu: 1.78; Zn: 9.82; Mo: 1.61 mg kg<sup>-1</sup>;  $\text{pH}_{\text{CaCl}_2}$ : 6.77).

Seeds of 'BRS 242' (GR1) and 'AG3539' (GR2) soybean cultivars were sterilized for 2 min in 2% NaClO and then inoculated with a mixed culture of *Bradyrhizobium japonicum*, strains SEMIA587 and SEMIA 5019 (100 ml 50 kg<sup>-1</sup> seed) at a concentration of  $5 \times 10^9$  rhizobia per gram. This inoculation rate resulted in effective root nodules clustered on the upper tap roots of soybean by 14 days after emergence, indicating efficient N fixation by the *B. japonicum* inoculant strains. Six seeds were sown per pot at 3 cm depth and thinned to one plant per pot at the one-leaf (V1) growth stage.

### Glyphosate application

Three glyphosate rates (800, 1200 and 2400 g a.e. ha<sup>-1</sup>) were sprayed at different growth stages (V2, V4 and V6). There was a slight difference for each cultivar among the days after sowing (DAS), with GR1 and GR2 being 12 and 10 DAS at V2, 25 and 22 DAS at V4 and 32 and 35 DAS at V6, respectively. The treatments were sprayed at 7:00 am using the commercially formulated potassium salt of glyphosate 540 g a.e. l<sup>-1</sup> (Roundup Weather Max<sup>®</sup>; Monsanto, St Louis, MO) as a single application. Plants were sprayed with a moving track sprayer using an even flat-fan (Teejet; Spraying Systems Co., Wheaton, IL, USA) nozzle tip delivering 187 l ha<sup>-1</sup> at 150 kPa. The sprayed solution did not cause run-off from leaves, and plants were irrigated using a sprinkler on the following day to ensure leaf absorption of the herbicide.

### Microbiological assays

At the R1 growth stage (42 and 38 DAS for GR1 and GR2, respectively), shoots were clipped close to the ground, roots were carefully removed from soil and soil tightly adhering to the root surface (rhizoplane) was rigorously removed using a camel-hair brush and retained as 'rhizosphere soil.' A subsample of the cleaned roots was washed under running water and gently blotted on paper towels in preparation for microbiological assays. Roots were surface sterilized in 1.25% sodium hypochlorite for 2 min followed by rinsing three times in sterile water and blotted dry on sterile paper towels. *Fusarium* colonization of the surface-sterilized soybean root segments (8, 2-cm segments per agar plate) was assessed by the root-planting procedure of Lévesque *et al.* (1993) on *Fusarium*-selective agar medium (Nash and Snyder 1962). After 5-day incubation at 25°C, the number of fungal colonies developing on root segments was recorded.

A *Fusarium* colony that developed on a root segment was counted as a single colony-forming unit (CFU) of rhizoplane *Fusarium*. Total numbers of *Fusarium* colonies per plate were converted to *Fusarium* CFU per 100 cm of root. *Fusarium* colonies were randomly selected, subcultured on potato dextrose agar and tentatively identified using descriptions of cultural and microscopic morphologies (Nelson *et al.* 1983). Identification of isolates was confirmed by the USDA-ARS Microbial Genomics Unit, Peoria, Illinois, by molecular analysis using partial translation elongation factor sequences (Skovgaard *et al.* 2001).

A 1-g portion of rhizosphere soil from each sample was suspended in 0.01 mol l<sup>-1</sup> MgSO<sub>4</sub> buffer, and appropriate 10-fold dilutions were plated on S1 agar medium selective for fluorescent pseudomonads (Gould *et al.* 1985) and on Gerretsen's agar medium for detecting Mn-oxidizing and Mn-reducing micro-organisms (Huber and Graham 1992). After incubation at 25°C (48 h for pseudomonads; 7 day for Mn-transforming bacteria), colonies developing on agar medium were recorded as colony-forming units in the rhizosphere. Mn-oxidizing colonies are brown to black on Gerretsen's medium, and Mn-reducing bacteria are white to opaque within a cleared halo. Mn-transforming bacterial components were further expressed as ratios of Mn reducers to Mn oxidizers to detect potential effects of microbial activity on plant-available Mn (Rengel 1997). Rhizosphere soils were screened for indoleacetic acid-producing (IAA) bacteria by plating on S1 agar medium supplemented with 5 mmol l<sup>-1</sup> L-tryptophan. A nitrocellulose membrane (90 mm diameter) was placed directly on the agar surface after plating prior to incubation (Bric *et al.* 1991). After 48 h, the nitrocellulose membrane was removed from each plate and placed on filter paper (Whatman 42) saturated with Salkowski reagent (2% 0.5 mol l<sup>-1</sup> FeCl<sub>3</sub> in 35% HClO<sub>4</sub>) in a clean petri plate for up to 4 h (Gordon and Weber 1951). Colonies that developed a pink colour were scored positive for IAA production and were considered 'IAA-producing rhizobacteria.' Representative bacterial colonies from the pseudomonad, Mn transformer and IAA-producing assays were selected and subcultured on S1 and tryptic soy agars to obtain pure, single-colony isolates. Isolates were characterized for colony morphology and fluorescent pigment production; gram stain and oxidase reactions; and classified taxonomically by cellular fatty acid profiles using gas chromatography-fatty acid methyl ester analysis (Kennedy 1994).

### Shoot and root biomass

Shoots and roots were packed in paper bags to dry in a forced-air oven at 65–70°C until a constant weight was achieved. Biomass was determined by weighing plant parts.

### Statistic and experimental design

For both two experiments, treatments were distributed in a randomized block design in a factorial scheme ( $3 \times 3 \times 2$ ) + 1 with four replicates. The first factor was the glyphosate rates (800, 1200 and 2400 g a.e. ha<sup>-1</sup>), the second was the growth stage (V2, V4 and V6) and the third factor was cultivar representing the 'first and second generation' cultivars (cv. BRS 242, GR1 and cv. AG3539, GR2, respectively). The additional treatment was a nonglyphosate treated.

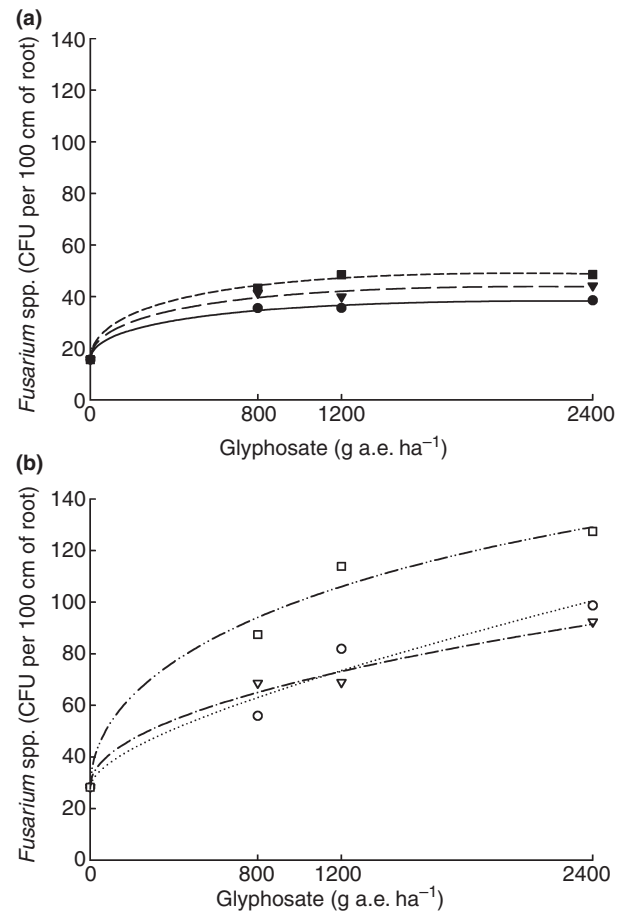
The data errors passed the normality test of Shapiro and Wilk (1965), and because there was homogeneity of error variances, the data for the two repeated experiments were combined. No transformations were necessary. Data were subjected to analysis of variance, and when *F* values were significant ( $P < 0.01$ ), to regression analysis. Data were analysed using PROC MIXED by SAS statistical program (SAS Institute, Cary, NC), and equations were adjusted using the polynomial model  $\hat{y} = a + bx + cx^{0.5}$  by SIGMAPLOT 10.0 statistical package (Systat Software, SPSS, Chicago, IL).

### Results

The response of all microbial groups and plant measurements to increasing concentrations of glyphosate was consistent for both GR soybean varieties. Root colonization by *Fusarium* spp. increased in response to glyphosate applications and was further enhanced as soybean growth progressed. In contrast, the pseudomonad, Mn-reducing and IAA-producing microbial groups decreased in response to glyphosate regardless of GR soybean variety. Consistent with results for microbial groups, soybean root and shoot biomass also decreased with increasing glyphosate, indicating an influence of glyphosate on plant growth through its effect on rhizosphere microbial components.

#### *Fusarium* spp. root colonization

Both the glyphosate rates and the soybean growth stage when glyphosate was applied affected *Fusarium* spp. root colonization on GR1 and GR2 plants (Fig. 1). *Fusarium* spp. root colonization in GR2 was highest when glyphosate was applied at V6 growth stage relative to colonization at stages V4 and V2. Similar results were noticed for GR1; however, root colonization when glyphosate was applied at stages V2 and V4 was similar (Fig. 1). Glyphosate enhanced *Fusarium* spp. root colonization, proportionally with increasing glyphosate rates with both cultivars. Interestingly, roots of untreated GR2 soybean (without glyphosate) were colonized by *Fusarium* spp. at lower levels than on GR1 roots; however, glyphosate sig-



**Figure 1** Total *Fusarium* spp. root colonization, determined at the R1 growth stage, on (a) second generation (GR2) and (b) first generation (GR1) soybean with increasing rates of a single glyphosate application at different growth stages ( $n = 8$ ,  $P < 0.01$ ). (a) (●) V2 – GR2; (▼) V4 – GR2 and (■) V6 – GR2. (b) (○) V2 – GR1; (▽) V4 – GR1 and (□) V6 – GR1.

nificantly increased *Fusarium* spp. infection of both GR cultivars at all rates of application (Fig. 1, Table 1).

#### Fluorescent pseudomonads

In general, *Pseudomonas* spp. populations in the rhizosphere of GR1 and GR2 soybeans were reduced as the glyphosate rate increased but this effect was influenced by the growth stage when the glyphosate was applied (Fig. 2). Glyphosate applied early (at V2 growth stage) had a greater effect in suppressing *Pseudomonas* spp. than when it was applied at V4 and V6 growth stages. The untreated GR2 had a higher population of *Pseudomonas* spp. than the GR1 cultivar, and both cultivars had the highest population of *Pseudomonas* spp. in their rhizosphere when glyphosate was not applied to them (Fig. 2, Table 1).

**Table 1** Regression statistics and correlations for the variables analysed in different glyphosate-resistant soybeans treated with different rates of glyphosate applied as a single treatment

Growth stage	Estimation of model parameters adjusted			$R^2$
	a	b	c	
<i>Fusarium</i> spp.				
Fig. 1a				
GR2				
V2	15.64	-0.0101	0.9560	0.99*
V4	15.71	-0.0131	1.2175	0.99*
V6	15.49	-0.0164	1.4864	0.99*
Fig. 1b				
GR1				
V2	27.69	0.0115	0.9252	0.98*
V4	28.52	-0.0003	1.2976	0.99*
V6	27.73	-0.0133	1.2976	0.99*
<i>Pseudomonas</i> spp.				
Fig. 2a				
GR2				
V2	45.35	0.0143	-1.3151	0.99*
V4	45.58	0.0097	-0.8928	0.96*
V6	45.44	0.0139	-1.0262	0.99*
Fig. 2b				
GR1				
V2	38.39	-0.0002	-0.2302	0.99*
V4	38.45	0.0010	-0.1357	0.97*
V6	38.42	0.0019	-0.1705	0.99*
Ratio of Mn reducers/Mn oxidizers				
Fig. 3a				
GR2				
V2	0.74	0.0002	-0.0221	0.98*
V4	0.75	0.0002	-0.0166	0.99*
V6	0.75	0.0002	-0.0163	0.99*
Fig. 3b				
GR1				
V2	0.67	6.54E5	-0.0094	0.99*
V4	0.67	-5.33E5	-0.0020	0.98*
V6	0.67	-8.49E5	-9.24E5	0.99*
Proportion of indoleacetic acid-producing bacteria				
Fig. 4a				
GR2				
V2	0.85	5.28E5	-0.0124	0.97*
V4	0.85	0.0001	-0.0154	0.96*
V6	0.85	0.0002	-0.0155	0.99*
Fig. 4b				
GR1				
V2	0.75	1.09E5	-0.0096	0.99*
V4	0.76	0.0001	-0.0148	0.93*
V6	0.76	-4.05E5	-0.0068	0.94*
Root dry weight				
Fig. 5a				
GR2				
V2	1.64	0.0004	-0.0387	0.99*
V4	1.65	0.0004	-0.0356	0.99*
V6	1.64	0.0004	-0.0300	0.99*

**Table 1** (Continued)

Growth stage	Estimation of model parameters adjusted			$R^2$
	a	b	c	
Fig. 5b				
GR1				
V2	2.05	0.0003	-0.0429	0.99*
V4	2.05	0.0003	-0.0432	0.99*
V6	2.05	0.0003	-0.0390	0.97*
Shoot dry weight				
Fig. 6a				
GR2				
V2	6.16	0.0018	-0.1541	0.99*
V4	6.15	0.0020	-0.1664	0.99*
V6	6.15	0.0020	-0.1832	0.99*
Fig. 6b				
GR1				
V2	7.54	0.0014	-0.1403	0.99*
V4	7.51	0.0018	-0.1698	0.99*
V6	7.52	0.0021	-0.2064	0.99*

\* $n = 8$ ,  $P < 0.01$ .

#### Ratio of potential Mn reducers/Mn oxidizers

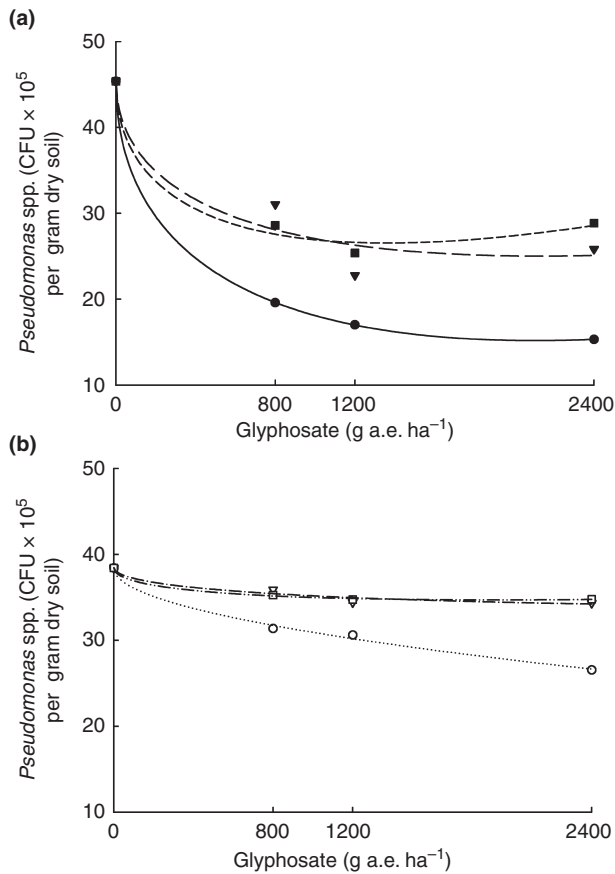
A high ratio of potential Mn reducers/Mn oxidizers was associated with GR cultivars without glyphosate application (Fig. 3), with the highest ratio exhibited by GR2 (Table 1). Although glyphosate reduced the ratio in both cultivars, the influence of increasing glyphosate rates on Mn reducers/Mn oxidizers ratio was clearly demonstrated in GR1 compared with GR2, with significant decreases with increasing glyphosate rates (Fig. 3). The greatest reduction in the ratio of potential Mn reducers/Mn oxidizers occurred when glyphosate was applied at early (V2) compared with later growth stages (V4 and V6) (Fig. 3).

#### IAA-producing rhizobacteria

Rhizobacteria producing IAA were composed mainly of fluorescent pseudomonads (>90% of total pseudomonad population). Increasing glyphosate rates decreased IAA-producing rhizobacteria in both GR cultivars (Fig. 4). These rhizobacteria were influenced less by the plant growth stage when glyphosate was applied than by glyphosate rate. The GR2 soybean had a higher population of IAA-producing rhizobacteria than GR1 in the absence of glyphosate (Fig. 4, Table 1), but both populations declined greatly by the highest rate of glyphosate.

#### Root and shoot dry weight

Glyphosate rate and growth stage at application influenced root and shoot dry weight of both GR cultivars



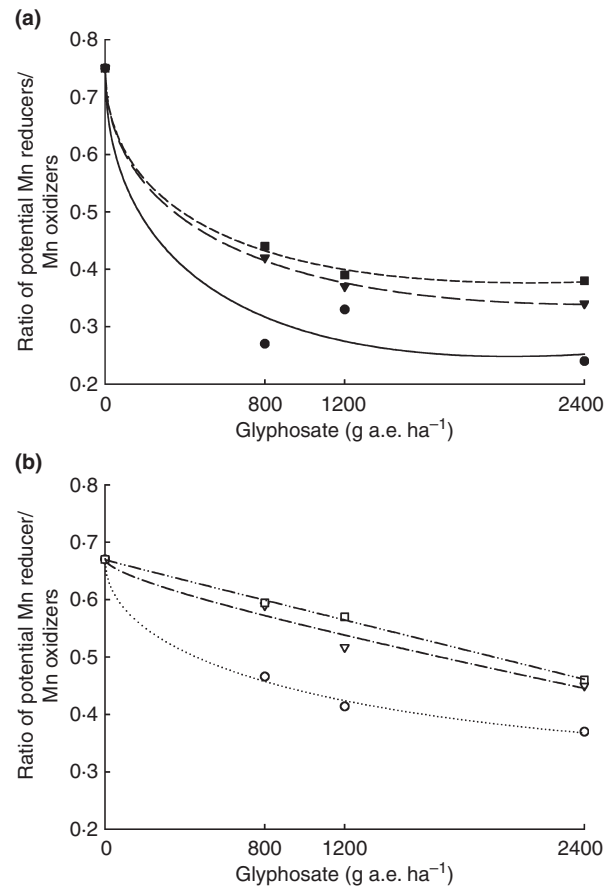
**Figure 2** Population of *Pseudomonas* spp., determined at the R1 growth stage, on (a) second generation (GR2) and (b) first generation (GR1) soybean with increasing rates of a single glyphosate application at different growth stages ( $n = 8$ ,  $P < 0.01$ ). (a) (●) V2 – GR2; (▼) V4 – GR2 and (■) V6 – GR2. (b) (○) V2 – GR1; (▽) V4 – GR1 and (□) V6 – GR1.

(Figs 5 and 6). Earlier glyphosate applications caused greater decreases in root dry weight: V2 > V4 > V6. The lowest rate of glyphosate had the greatest proportional effect on root dry weight; however, root dry weight decreased further as glyphosate rates increased, especially when glyphosate was applied at growth stage V2 (Fig. 5).

Differential effects of glyphosate on root and shoot dry weight were observed depending on the growth stage when glyphosate was applied. Shoot dry weight of both varieties was most reduced when glyphosate was applied at the V6 growth stage and least at V4 and V2 stages, with the GR2 cultivar affected more than the GR1 cultivar (Fig. 6). In general, GR2 produced less biomass (shoot and root) than GR1 when glyphosate was not applied (Table 1).

### Discussion

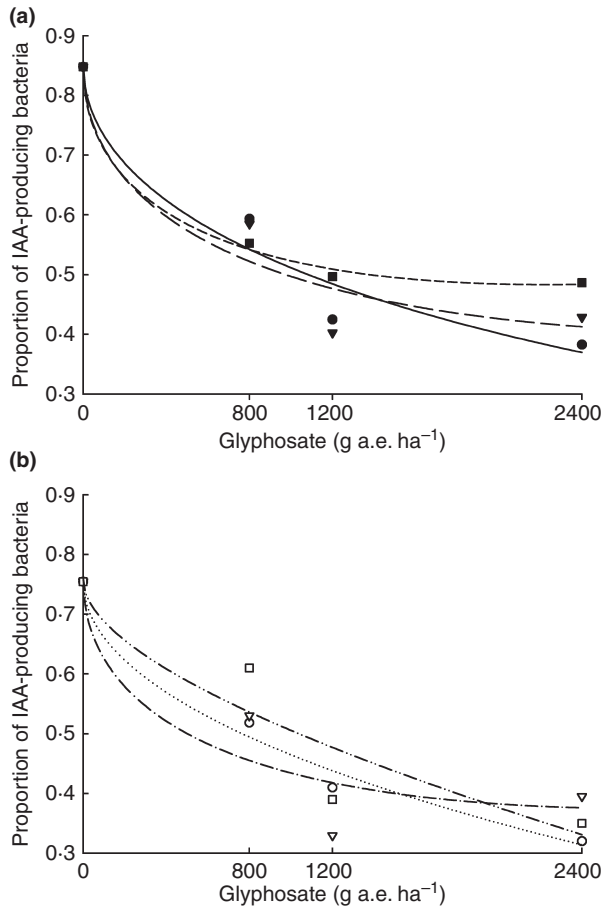
Previous studies reported that the roots of GR soybean and maize treated with glyphosate become heavily



**Figure 3** Ratio of potential Mn reducers/Mn oxidizers, determined at the R1 growth stage, on (a) second generation (GR2) and (b) first generation (GR1) soybean with increasing rates of a single glyphosate application at different growth stages ( $n = 8$ ,  $P < 0.01$ ). (a) (●) V2 – GR2; (▼) V4 – GR2 and (■) V6 – GR2. (b) (○) V2 – GR1; (▽) V4 – GR1 and (□) V6 – GR1.

colonized by *Fusarium* spp. relative to no glyphosate treatment (Kremer and Means 2009). GR1 soybeans treated with glyphosate released high concentrations of soluble carbohydrates and amino acids, which favour root colonization by the pathogen (Kremer *et al.* 2005). Similar findings were noticed in the present experiment, in which ‘first’ (GR1) and ‘second generation’ (GR2) soybean roots were densely colonized by *Fusarium* spp. after glyphosate application (Fig. 1). Glyphosate appears to compromise the ability of GR and non-GR plants to suppress against root-colonizing pathogens such as *Fusarium* (Johal and Rahe 1988; Johal and Huber 2009).

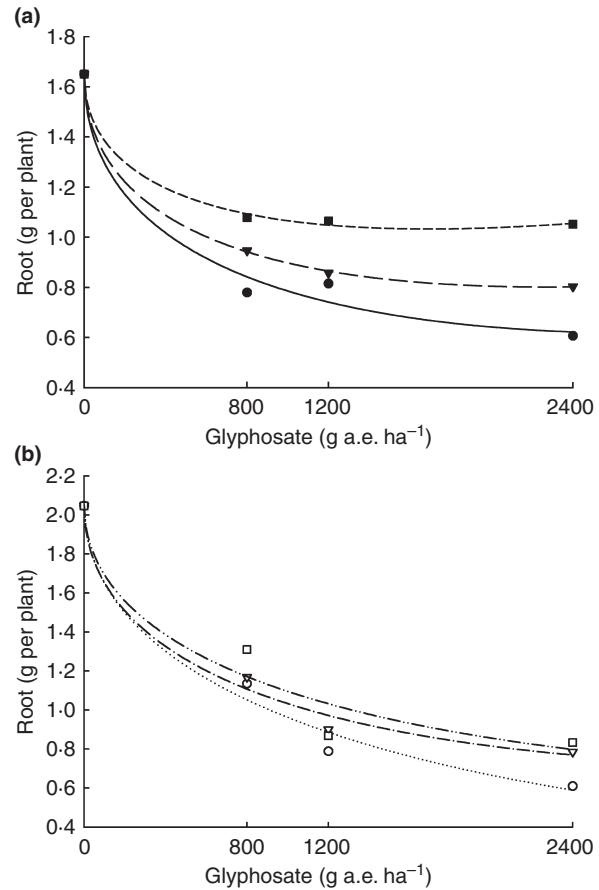
Harper (2007) reported that cotton growers in Australia and the Western United States observed a resurgence of *Fusarium* wilt since the introduction of Roundup Ready<sup>®</sup> cotton and that previously high levels of wilt resistance are less effective under glyphosate management



**Figure 4** Proportion of indoleacetic acid-producing bacteria, determined at the R1 growth stage, on (a) second generation (GR2) and (b) first generation (GR1) soybean with increasing rates of a single glyphosate application at different growth stages ( $n = 8$ ,  $P < 0.01$ ). (a) (●) V2 – GR2; (▼) V4 – GR2 and (■) V6 – GR2. (b) (○) V2 – GR1; (▽) V4 – GR1 and (□) V6 – GR1.

programmes. Reduced resistance to pathogen infection in response to glyphosate treatment would normally not be expected in GR plants resistant to glyphosate (Cerdeira and Duke 2006). Johal and Huber (2009) report that Roundup Ready® crops are vulnerable to glyphosate toxicity under certain conditions, occurring when the level of glyphosate exceeds the tolerance of the transgenic enzyme or if the transgene fails to match the transcriptional activity and profile of the native gene under biotic stress conditions. Both of these scenarios are possible and, if they develop, are very likely to increase the vulnerability of GR plants to fungal diseases following glyphosate application.

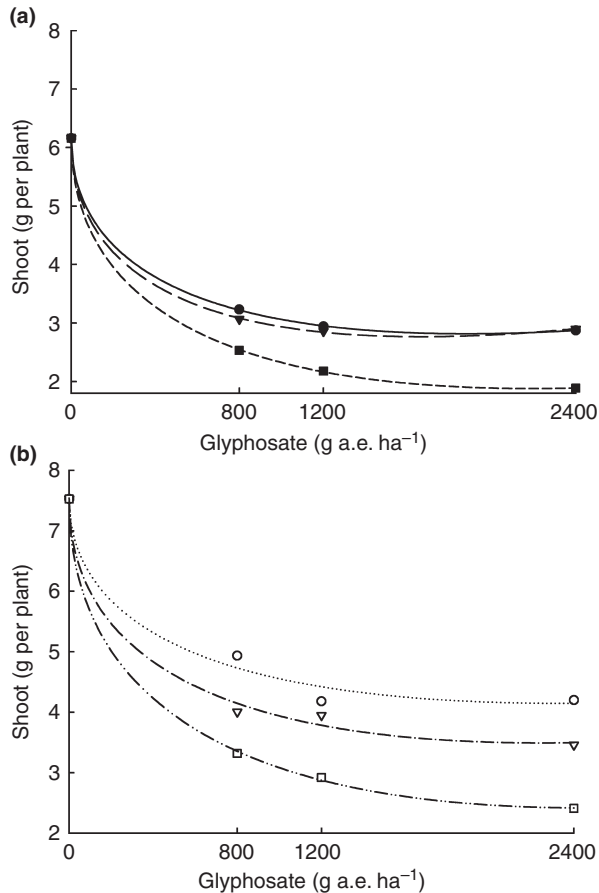
Based on the label rates used for single glyphosate application at V4 growth stage to GR soybeans (rates of 720–1200 g a.e. ha<sup>-1</sup>, Gazziero *et al.* 2008), the first scenario mentioned by Johal and Huber (2009) might occur



**Figure 5** Root dry weight, determined at the R1 growth stage, on (a) second generation (GR2) and (b) first generation (GR1) soybean with increasing rates of a single glyphosate application at different growth stages ( $n = 8$ ,  $P < 0.01$ ). (a) (●) V2 – GR2; (▼) V4 – GR2 and (■) V6 – GR2. (b) (○) V2 – GR1; (▽) V4 – GR1 and (□) V6 – GR1.

with rates higher than 1200 g a.e. ha<sup>-1</sup>; however, as shown in Fig. 1, applying the minimal glyphosate rate resulted in significantly increased *Fusarium* spp. root colonization. The high *Fusarium* spp. root colonization promoted by late glyphosate applications was probably because of higher root biomass (Fig. 5) that offered the nutritional stimulation from root exudates as well as numerous colonization sites for potential infection.

Mijangos *et al.* (2009) reported that microbial activity in the rhizosphere may change in glyphosate-treated plants from glyphosate accumulation within and/or released from root tissues that shift the composition of rhizosphere micro-organisms as the enzyme EPSPS, inhibited by glyphosate, is present in many bacteria and fungi (Padgett *et al.* 1995). Such an alteration in rhizosphere biology is illustrated in Figs 2 and 3 in which glyphosate applied to foliage of the plants decreased *Pseudomonas* spp. populations and the ratio of potential Mn



**Figure 6** Shoot dry weight, determined at the R1 growth stage, on (a) second generation (GR2) and (b) first generation (GR1) soybean with increasing rates of a single glyphosate application at different growth stages ( $n = 8$ ,  $P < 0.01$ ). (a) (●) V2 - GR2; (▼) V4 - GR2 and (■) V6 - GR2. (b) (○) V2 - GR1; (▽) V4 - GR1 and (□) V6 - GR1.

reducers/Mn oxidizers in the rhizosphere. This reduction may occur in GR soybean rhizospheres because of the reported sensitivity of *Pseudomonas* spp. to glyphosate (Schulz *et al.* 1985) exuded from roots or because antagonistic activity of this bacterial group to other species to overcome by glyphosate (Wardle and Parkinson 1992). The suppressive effect of glyphosate on pseudomonads was observed at early applications (growth stage V2) and became more significant with increased glyphosate rates, possibly because of alteration or decrease in specific carbon substrates released by the developing roots (Treseder *et al.* 2005). These findings are in agreement with previous reports in which glyphosate increased Mn oxidizers, decreased Mn reducers, and reduced the ratio of Mn-reducing/Mn-oxidizing bacteria (Johal and Huber 2009; Kremer and Means 2009).

Several rhizosphere microbial groups typically produce the plant growth hormone, auxin or IAA. In this study,

rhizobacteria producing IAA were mainly fluorescent pseudomonads and were similar to those described for the overall pseudomonad and Mn-reducing populations (Fig. 4) and in their susceptibility to increased glyphosate rates. Auxin compounds, as well as other aromatic compounds produced in plants and micro-organisms, are derived from the shikimic acid pathway that is blocked by the inhibition of EPSPS by glyphosate (Schulz *et al.* 1985). Thus, our results suggest that GR soybean can modify the composition of the rhizobacteria as well as IAA-producing potential. Increased cultivation of GR soybean with coincident use of glyphosate may potentially alter the rhizobacterial communities and activities crucial for soybean growth.

Zablotowicz and Reddy (2007) correlated the extent of injury in GR soybean with the levels of aminomethylphosphoric acid (AMPA) formed within the plant as evidenced by the reduction in shoot fresh weight (Reddy *et al.* 2004). In the present research, glyphosate decreased root and shoot dry weight proportional to the glyphosate rates applied (Figs 5 and 6). However, the greater reduction in root biomass suggested glyphosate presence in root tips where the Roundup Ready<sup>®</sup> gene is 'silent' resulting in root growth inhibition. These findings coincide with those of Zobiolo *et al.* (2010a), Reddy *et al.* (2001) and King *et al.* (2001). Bott *et al.* (2008) also noted that the glyphosate applied to a GR soybean significantly inhibited root biomass and root elongation.

In addition, a negative relationship between IAA-producing rhizobacteria and biomass production suggested that glyphosate suppression of IAA-producing populations also contributed to decreased biomass by reducing the plant growth promoting effect of IAA (Taiz and Zeiger, 1998). Furthermore, as glyphosate decreased growth of the root system at an early growth stage, subsequent decreases in root biomass may have been augmented by coincident enhancement of *Fusarium* spp. colonization (Means and Kremer 2007) and making the root conducive to succeeding infection by opportunistic pathogens. The effect of glyphosate on shoot dry weight exhibited a different trend from that observed for root dry weight, suggesting that plants treated early may have more time to recover from glyphosate effects. Huber *et al.* (2004) also found that late application of glyphosate generally resulted in lower yields because of increased weed competition and changes in rhizosphere microflora that predisposed roots to increased root rot disease.

Slightly more IAA-producing rhizobacteria were detected on the GR2 cultivar even though this cultivar showed lower root and shoot dry weights than the GR1 (Table 1). With the numerous GR cultivars currently available, it is not surprising to observe such differences because the physiological characteristics influencing root

exudation vary because of the genetics of the individual cultivar and the environmental conditions to which the cultivars are exposed (Reddy and Zablotowicz 2003).

## Conclusion

Glyphosate altered selected rhizosphere micro-organisms when applied to both 'first and second' generation GR soybeans at various growth stages and at variable rates. *Fusarium* spp. colonization of roots increased steadily as soybean growth progressed and as glyphosate rate increased suggesting that glyphosate affects the ability of plants to suppress potential pathogen colonization and root infection. Fluorescent pseudomonads and Mn-reducing rhizobacteria were suppressed by glyphosate, especially at the early growth stage which apparently lowers additional defense mechanisms in the rhizosphere that typically would be available to the plant to ward off pathogens. The reduction in IAA-producing rhizobacteria by glyphosate may suppress the formation of new roots or the rate of root growth leading to reduced nutrient uptake ability and added vulnerability of the plant to soil-borne pathogens. Overall, our study demonstrates that glyphosate applied to GR soybean, regardless of tolerance level for glyphosate, impacts the complex interactions of microbial groups, biochemical activity and root growth leading to subsequent effects on plant growth, health and productivity. This information is crucial in developing strategies to overcome the potential detrimental effects of glyphosate in GR cropping systems.

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