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Rhizobacterial Populations of Glyphosate-Resistant Soybean (*Glycine Max*) as Affected by Glyphosate and Foliar Amendment

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ABSTRACT: Increased application of glyphosate (Gly) in glyphosate-resistant (GR) soybean cropping systems may affect rhizospheric microorganisms including IAA-producing rhizobacteria (IPR) and their effect on the growth of soybean. This field experiment was conducted to assess IPR populations in the rhizosphere of GR soybean ('Roundup-Ready' DeKalb DKB38-52) treated with glyphosate and foliar amendment treatments such as PT21® (urea solution with N 21%) and Grozyme® (Biostimulant: mixtures of micronutrients and enzymes). Effects of herbicide, sampling date, and their interaction on total bacterial numbers were significant ($P < 0.001$, 0.001, 0.013, respectively). Total bacteria (TB) numbers were increased with glyphosate treatment at 20 d after application and highest TB populations were associated with Grozyme® application, possibly due to the additional substrate from this product. The IPR of the soybean rhizosphere was significantly affected by herbicide, sampling date, and the herbicide*foliar amendment interaction. The ratios of numbers of IPR to TB ranged from 0.79 to 0.99 across the sampling dates irrespective of treatments. IPR numbers were slightly hindered by glyphosate application regardless of foliar amendment.

Key Words: Rhizobacteria, Glyphosate-resistant Soybean, Glyphosate, Foliar Amendments, IAA-producing Bacteria

INTRODUCTION

Application of glyphosate herbicide in crop production systems has increased since the introduction of the first glyphosate-resistant (GR) crops in 1997. Glyphosate (N-[phosphonomethyl]glycine) is a broad spectrum, non-selective herbicide of grasses and broadleaf weeds and a single post-emergence application is usually sufficient to control weeds in crops planted in narrow rows[1-3]. The mode of action of glyphosate appears to be inhibition of 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase, an enzyme in the shikimic acid pathway of aromatic amino acid biosynthesis, in which the final products are the amino acids: phenylalanine (Phe), tyrosine (Tyr), and trytophan (Try), preceded by the production of chorismate, the important branch point intermediate[4-6]. Significant concentrations (< 0.3 mM) of glyphosate were translocated within root tissues of susceptible plants at 55 to 70% of the applied glyphosate after 24 h[7]. Recently, application of glyphosate to GR soybean significantly increased glyphosate concentrations in GR soybean seeds[8]; frequent application (more than two times) increased glyphosate in GR soybean leaves, stems, and grains[9]. Furthermore, increased applications of glyphosate to GR soybean can cause injury, which includes decreased chlorophyll contents under certain environmental conditions such as high or low temperature, water availability, or nutritional status, suggesting a potential to decrease grain yield[10-12].

Continued cultivation of GR crops and frequent use of glyphosate could lead to modifications in the soil ecology including altered soil microbial populations and activity. Soil microorganisms continually interact with plants, especially in the rhizosphere, where microenvironments exist in which microorganisms readily metabolize organic compounds released from plant roots[13]. It is well demonstrated that plant-growth

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regulators (PGRs) are among the numerous organic compounds released into the rhizosphere[14]. The PGRs such as auxins, gibberellins, and cytokinins, originate partly from plants and some may be produced by a variety of microorganisms. Indole-3-acetic acid (IAA) is a representative auxin and the final compound in the auxin metabolic pathway. Biosynthesis of IAA is mediated by up to 80% of bacteria in rhizospheres depending on soil conditions and plant species. Therefore, herbicide application may positively or negatively affect microorganisms that synthesize auxin-like compounds in the rhizosphere due to the direct effects of the herbicide on microbial activity or indirectly by promoting those microorganisms able to metabolize the herbicide[15].

Various products are commercially available to improve crop growth and productivity and may offset possible adverse side-effects of herbicides or other plant-protecting agents applied to the crop. Soil biostimulants are biological preparations developed to improve plant productivity directly or indirectly via widely varying mechanisms including: inoculation of soil with beneficial microorganisms, activation of soil microbial activity, promotion or augmentation of the activities of critical soil enzymes, addition of plant growth hormones, and supplementation with micro-nutrients.

Because the GR soybean cropping system is in widespread use and little information is available on the impact of continuous use of glyphosate on the soil microbial community, a series of experiments in field environment were conducted to determine the response of specific bacterial populations and activities to glyphosate in GR soybean. The objective of this study was to describe changes in bacterial populations synthesizing IAA in the GR soybean rhizosphere.

MATERIALS AND METHODS

Soils

Field experiment was conducted with a split-plot randomized complete block design with four replications at Bradford Research and Extension Center (BREC) of the University of Missouri. The soil is classified as a Mexico silt loam (fine, smectitic, mesic, Aeric Vertic Epiaqualf). The field was disked with a disk harrow, fertilized and managed consistent with practices common to soybean production in Missouri[16]. Composite soil samples consisting of four sub-samples were collected with a probe (5-cm diameter by 16-cm deep) at each treatment area and analyzed by the Soil and Plant Testing Laboratory at the University of Missouri, Columbia for selected soil properties (Table 1).

GR-soybean treatments

Glyphosate-resistant soybean ('Roundup-Ready' soybean, DeKalb DKB38-52) was planted in 0.76 m rows. Each treatment replicate plot was 3.66 m wide by 3.05 m long with four rows. Glyphosate (Roundup Ultra Max®) and non-glyphosate treatments were applied to main plots; the foliar amendments, urea solution (21.0% nitrogen; PT-21®), biostimulant (containing a mixture of boric acid, cobalt sulfate, copper sulfate, ferric nitrate, manganese nitrate, sodium molybdate, zinc nitrate and enzyme systems; Grozyme®), or no foliar amendment were applied to the split-plots. PT-21® and Grozyme® were obtained from Ag Spectrum Company (DeWitt, Iowa, USA). Glyphosate was applied at 0.84 kg ae·ha^{-1} rate with a 130 L·ha^{-1} spray volume at pressure of 138 KPa using 11003 nozzles (Spraying Systems). At the time of application, soybeans were at the V4-V5 vegetative stage of growth[17]. At 10 days after glyphosate application, urea solution (9.2 kg·ha^{-1}) and biostimulant (33.5 ml·ha^{-1}) were applied to soybean

Table 1. Changes of the selected chemical properties of Mexico silt loam with treatments of glyphosate or foliar amendments

Treatments	pH	OM¶	Ava. P_2O_5	Exc. Ca	Exc. Mg	Exc. K	CEC
		g kg^{-1}	mg kg^{-1}	----------	cmol$_c$ kg^{-1}	----------	cmol$_c$ kg^{-1}
Control	6.6	33	55.2	9.69	1.64	0.65	21.5
Glyphosate	6.6	35	62.1	10.11	1.70	0.66	22.5
Biostimulant (Grozyme®)	6.6	33	55.2	9.13	1.52	0.69	20.3
Urea Solution (PT-21®)	6.6	37	56.0	9.78	1.68	0.59	21.8

¶OM : organic matter

foliage using a backpack sprayer.

Sampling of the rhizosphere soil

Soybean roots and associated soil were collected from the outer rows of each plot for the field study by carefully removing 3-4 plants with a shovel at 0, 10, 20, and 30 d after herbicide application. Samples of a single plant and the attached soil were collected from each plot of the field. Each plant sample with soil attached to roots was placed in a plastic bag. All bags with plant samples were transported in a cooler, to maintain viability of the plants, and then kept in a cold room at 4°C before processing within 24 to 48 h of collection.

Culture conditions for total rhizobacteria and IAA-producing rhizobacteria

Each root with rhizosphere soils was added to sterile phosphate buffered saline (PBS; 10mM K_2HPO_4 - KH_2PO_4, 0.14M NaCl; pH 7.2), shaken on a rotary shaker for 20 min at 200 rpm, removed and blotted on paper towel, and exact fresh root weight was determined. Tenfold dilutions of soil suspensions were made in PBS and plated on half-strength King's B medium (KBM). The numbers of total bacteria (TB) were enumerated after 5 days of incubation. Rhizobacteria were screened for IAA production using an *in situ* nitrocellulose membrane assay[18]. A nitrocellulose membrane (90-mm diameter) was placed directly on bacterial colonies immediately after enumeration of TB and incubated an additional 24 h to 48 h. The numbers of colonies grown after enumeration were added to the TB population. After the membrane was removed from the plate, it was placed on filter paper (Whatman 42) saturated with Salkowski reagent (2% 0.5M $FeCl_3$ in 35% $HClO_4$) for no more than 4 h[19]. Colonies which developed a pink color were scored positive for IAA production (Fig. 1). Intensity of color development was rated representing no IAA production, faint, readily detectable, and deep and dark color as 0, 1, 2, and 3, respectively.

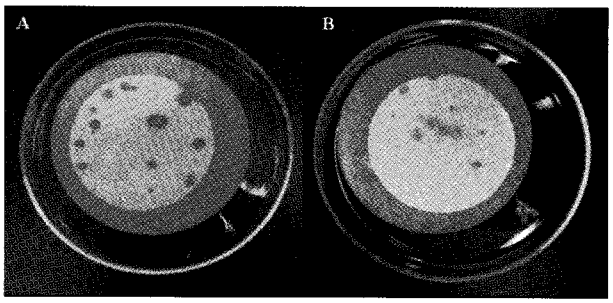

Fig. 1. IAA production using nitrocellulose membrane assay. (A) red color-developed colonies cultured of rhizospheres without glyphosate treatment; (B) colonies cultured of rhizospheres with glyphosate treatment.

Statistical analysis

Effects of glyphosate and two foliar amendments on IAA-producing bacteria in the rhizosphere of glyphosate-resistant soybean were analyzed using SAS PROC GLM[20]. The multiple comparison test used was Fisher's protected LSD at $P \leq 0.05$. Data were normally distributed and had equal variance using the test for homogeneity of variance.

RESULTS AND DISCUSSION

Determination of rhizobacteria populations

Chemical properties of the soil were not significantly altered by treatments of glyphosate or foliar amendments such as biostimulant (Grozyme®) and urea solution (PT-21®) (Table 1), possibly due to the inherent properties of the chemicals applied. Charge properties of glyphosate and micronutrients amended might be tied on the surface soil but glyphosate and foliar amendments might affect the rhizospherial populations and activities through the soil-root-microorganism interactions[13-15].

The effects of herbicide, sampling date, and herbicide *date on TB numbers were significant at $P < 0.001$, 0.001, 0.013, respectively but the foliar amendment effect was not significant (Table 2 and 3). For all treatments, TB decreased at 10-d followed by an increase at 20-d, after which numbers remained somewhat higher at 20 d, with the greatest increase (7 times higher than glyphosate alone) shown when the biostimulant (Grozyme®) was applied after glyphosate treatment (Table 3).

Microbial activity can be modified by many chemical and biological factors affected by application of herbicide and foliar amendments[21-24]. Rhizobacteria of GR soybean may be affected directly by herbicide and foliar amendments released through roots and/or indirectly by altered root exudation due to genetic modification of the plant[25,26]. Glyphosate caused slight

Table 2. Analysis of variance (ANOVA) of total bacteria population and IAA-producing bacteria on roots of glyphosate-resistant (GR) soybean for main and interactive effects of glyphosate (Gly), foliar amendment treatments (FA), and sampling date (date) of 20 to 30d after glyphosate application

Source of variations	P>F	
	Total rhizobacteria population	IAA-producing bacteria
Glyphosate (Gly)	0.001	0.008
Foliar amendment (FA)	0.486	0.886
Gly*FA	0.133	0.016
Date	0.001	<0.001
Gly*Date	0.013	0.092
FA*Date	0.826	0.298
Gly*FA*Date	0.952	0.533

Table 3. Average total bacteria population on roots of glyphosate-resistant (GR) soybean at selected days after glyphosate, Grozyme® or PT-21® applications

Treatment	Average Total Bacteria Populations at Days After Application			
	0	10	20	30
	Log CFU g^{-1} fresh root			
Control	8.40	7.74	9.39	9.20
Glyphosate	8.40	7.56	9.82	9.18
Grozyme®	8.61	7.74	9.17	9.14
Glyphosate + Grozyme®	8.61	7.56	10.08	9.49
PT-21®	8.81	7.74	9.31	9.09
Glyphosate + PT21®	8.81	7.56	9.80	9.15
LSD (0.05)	NS	0.13	0.18	0.41

decreases in TB numbers compared with the control within 5 to 10-d after glyphosate application (Table 3). Araújo et al. (2003) demonstrated that soil exposed to glyphosate for 6 years not only had significantly higher numbers of bacteria but also retained very low amounts of glyphosate after a 32-day incubation[27]. Highest TB populations were associated with Grozyme®, which suggested that additional substrate from Grozyme® enhanced bacterial numbers and possibly glyphosate metabolism.

Determination of IAA-producing rhizobacteria populations

Production of IAA is widespread among rhizobacteria. Although it is not clear whether IAA synthesized by rhizobacteria is beneficial or pathogenic in bacteria-plant interactions, the presence of IAA, the naturally occurring auxin, is crucially important because it has broad physiological effects on the plant. The numbers of IAA-producing rhizobacteria (IPR) on roots of GR soybean are shown in Table 4.

The effects of herbicide, sampling date, and the herbicide*foliar amendment interaction on IPR of the soybean rhizosphere were significant (Table 2). IPR populations were enhanced due to glyphosate treatment at 20 d and were further increased at 10 days after the Biostimulant (Grozyme®) treatment (Table 4), and were sustained through 30 d. IPR populations were not affected by urea solution (PT-21®) treatment (Table 4). The ratios of numbers of IPR to TB ranged from 0.79 to 0.99 across the sampling dates irrespective of treatments (Table 3 and 4).

These proportions are considerably higher than a previous study reporting the percentage of IAA-producing rhizobacteria ranged from 16 to 34% depending on soybean cultivar[23]. However, up to 80% rhizobacteria can synthesize IAA depending on plant species and soil conditions[28,29]. The numbers of IPR were likely high in the present study because a novel application of the *in situ* membrane assay was demon-

Table 4. Average Indole 3-Acetic Acid (IAA) producing bacteria population on roots of glyphosate-resistant (GR) soybean at selected days after glyphosate, Grozyme® or PT-21® applications

Treatment	Average IAA-Producing Bacteria Populations at Days After Application			
	0	10	20	30
	Log CFU g^{-1} fresh root			
Control	7.96	7.37	9.26	8.48
Glyphosate	7.96	7.00	9.82	8.29
Grozyme®	7.13	7.37	9.02	8.05
Glyphosate + Grozyme®	7.13	7.00	10.00	8.93
PT-21®	7.02	7.37	9.17	8.70
Glyphosate + PT21®	7.02	7.00	9.37	8.46
LSD (0.05)	NS	0.11	0.26	0.47

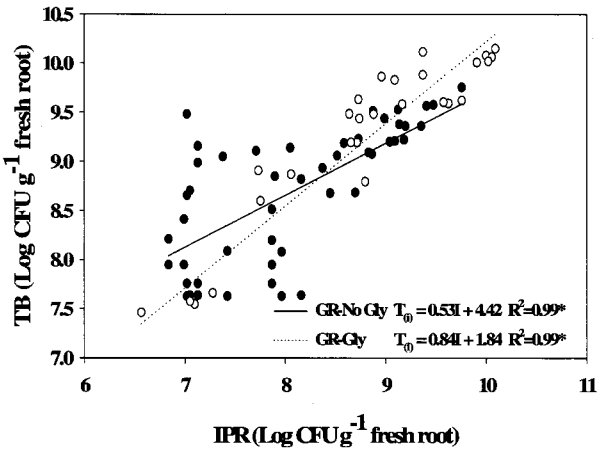

Fig. 2. Correlation models (Ti=a+b*I) between total bacteria (T: Log cfu g^{-1} fresh root) and IAA producing rhizobacteria (I: Log cfu g^{-1} fresh root) on roots of glyphosate-resistant (GR) soybean applied with glyphosate (Gly) and no glyphosate (No Gly). * represents significance at $P < 0.05$.

strated for the first time and the subjective determination of color reactions as positive for IAA production may have overestimated IPR[18]. In general, the numbers of IPR, as shown for TB, were affected by glyphosate and its interaction with foliar amendment applications. IPR numbers were slightly hindered by glyphosate application regardless of foliar amendment (Fig. 2), which support previous reports of the interference of glyphosate with the soil microbial population balance[23,24] and potential stimulation of soil microbial community by biostimulants[21,22].

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