Influence of glyphosate on Rhizoctonia and Fusarium root rot in sugar beet

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Abstract: This study tests the effect of glyphosate application on disease severity in glyphosate-resistant sugar beet, and examines whether the increase in disease is fungal or plant mediated. In greenhouse studies of glyphosate-resistant sugar beet, increased disease severity was observed following glyphosate application and inoculation with certain isolates of *Rhizoctonia solani* Kuhn and *Fusarium oxysporum* Schlecht. f. sp. *betae* Snyd. & Hans. Significant increases in disease severity were noted for *R. solani* AG-2-2 isolate R-9 and moderately virulent *F. oxysporum* isolate FOB13 on both cultivars tested, regardless of the duration between glyphosate application and pathogen challenge, but not with highly virulent *F. oxysporum* isolate F-19 or an isolate of *R. solani* AG-4. The increase in disease does not appear to be fungal mediated, since *in vitro* studies showed no positive impact of glyphosate on fungal growth or overwintering structure production or germination for either pathogen. Studies of glyphosate impact on sugar beet physiology showed that shikimic acid accumulation is tissue specific and the rate of accumulation is greatly reduced in resistant cultivars when compared with a susceptible cultivar. The results indicate that precautions need to be taken when certain soil-borne diseases are present if weed management for sugar beet is to include post-emergence glyphosate treatments.

Keywords: *Beta vulgaris*; *Rhizoctonia solani*; *Fusarium oxysporum*; glyphosate; shikimic acid

1 INTRODUCTION

Weed control is a costly and necessary part of sugar beet production, relying heavily on several post-emergence herbicide treatments with or without a pre-emergence herbicide applied at planting. Herbicide treatment cost ranges from 171 to 319 \$US ha^{-1},[1] with additional costs for cultivation and hand labor required to remove weeds left behind after herbicide applications. Recent developments in weed control include the use of transgenic plants resistant to herbicides such as glyphosate.[2] Glyphosate-resistant (GR) sugar beet was approved for production in the United States in 1998.[3] Use of glyphosate could provide sugar beet producers with broad-spectrum weed control at a fraction of the current cost for efficacious weed control in this crop.[1]

Glyphosate targets enolpyruvylshikimate-3-phosphate synthase (EPSPS), the enzyme responsible for converting shikimate to chorismate.[4] The inhibition of EPSPS blocks the shikimic acid (aka shikimate) pathway which produces precursors necessary for the biosynthesis of aromatic compounds,[5] including phenylalanine, tyrosine and tryptophan.[6] The shikimic acid pathway also gives rise to salicylic acid[7] and phytoalexins,[8] both of which are important in plant defense processes. GR sugar beet was developed through introduction of an *Agrobacterium* sp. strain CP4 EPSPS that is resistant to glyphosate.[9]

Repeated glyphosate application impacts soil microbial population dynamics,[10] and microorganisms have a variable response to glyphosate. Certain pseudomonads can convert glyphosate into essential amino acids,[11] and some fungi are able to utilize glyphosate as a nitrogen[12] or phosphorus[13] source. Alternatively, glyphosate can have negative effects on certain fungi, and it inhibits growth of yeast by preventing melanization.[14]

The effect of glyphosate use in GR crops on disease severity has been examined with variable results. GR soybean was more susceptible, following glyphosate application, to certain isolates of *Fusarium* in greenhouse[15] and field[16] tests. Use of glyphosate on GR soybean also increased disease severity caused by *Sclerotinia sclerotiorum* (Lib) de Bary in some soybean cultivars but not in others,[17] but had no impact on disease severity in a single GR cultivar in another study.[18] In yet another study, GR soybean was more susceptible to cyst nematode infection than the same glyphosate-susceptible (GS) cultivar following glyphosate application.[19] Alternatively, use of glyphosate on GR cotton reduced general seedling disease severity.[20] To date, the potential interactions

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of GR plants, glyphosate and microorganisms have not been examined extensively.

GR sugar beet is not currently in commercial production but is expected to be in the near future. Therefore, it is important to understand the potential impact of glyphosate on sugar beet–pathogen interactions before commercial production begins. The present study examines the interaction of glyphosate treatment with two major soil-borne fungal diseases in sugar beet: Fusarium yellows, caused by *Fusarium oxysporum* Schlecht. f. sp. *betae* Snyd. & Hans, and Rhizoctonia root rot, caused by *Rhizoctonia solani* Kühn. Both fungal pathogens are widespread threats to sugar beet production in the United States and abroad, causing reduced yield and, in the case of Fusarium yellows, increased impurity in the extracted syrup.[21] Additional studies sought to determine the basis for the increase in disease by examining the impact of glyphosate on (1) fungal growth and overwintering structure production and viability and (2) the efficacy of EPSPS in GR and GS sugar beet cultivars.

2 EXPERIMENTAL METHODS

2.1 Plant culture

Sugar beet (*Beta vulgaris altissima* Doell) varieties B4RR (GR; Betaseed Inc., Kimberly, ID), H16 (GR; Hilleshog, Longmont, CO) and B4 (GS; Betaseed Inc., Kimberly, ID) were seeded into 20 cm diameter pots containing pasteurized (3 h at 72 °C and 0.4 bar) Metro-Mix 200 (The Scotts Company, Marysville, OH). For disease severity analysis, at 1 week post-planting, seedlings were individually transplanted into 12 cm pots or 6.7 cm diameter ×25 cm planting cones for Rhizoctonia root rot or Fusarium yellows tests respectively. For shikimic acid analysis, at 1 week post-planting, seedlings were transplanted to 3.8 cm diameter ×21 cm deep planting cones (Steuwe & Sons, Inc., Corvallis, OR) for plants 2, 3 and 4 weeks of age and to 6.7 cm diameter ×25 cm planting cones when allowed to mature to 6 weeks of age. Plants were maintained in a glasshouse at 22 ± 5 °C, were watered daily and were kept under 16 h of daylight to maintain vigorous growth. B4RR has resistance to *Rhizoctonia solani* AG-2-2 (Stander JR, private communication). H16 is not reported to have resistance to *R. solani*, and neither cultivar has resistance to Fusarium yellows.

2.2 Fungal culture

Two isolates each of *Fusarium oxysporum* and *Rhizoctonia solani* were used in this study. *Rhizoctonia solani* isolates R-9 (AG-2-2) and R-1411 (AG-4) are highly and moderately virulent respectively, as determined by pathogenicity tests.[22] *Fusarium oxysporum* f. sp. *betae* isolates were F-19 (highly virulent) and FOB13 (virulent) (Hanson L and Hill A, unpublished data). Stock cultures of the isolates were maintained on potato dextrose agar (PDA) (Becton, Dickinson and Co., Sparks, MD) at 25 ± 2 °C with 8 h of supplemental light per day. For long-term storage, *Fusarium* isolates were stored dried on sterile filter paper at −20 °C as described by Peever and Milgroom,[23] except that isolates were grown on water agar (WA) (Becton, Dickinson and Co., Sparks, MD), and glass microfiber filter paper was used. For long-term storage, *R. solani* isolates were stored on colonized autoclaved barley grains at −20 °C.[24] *Rhizoctonia* inoculum was prepared by growing each *R. solani* isolate on moist autoclaved barley grains. Infested barley was air dried and ground according to the methods of Pierson and Gaskill.[25] *Fusarium* inoculum was prepared by transferring a 4 mm plug of fungal hyphae from the actively growing edge of a fungal colony on PDA to half-strength V8 agar.[26] Plates were incubated under 8:16 h light:dark photoperiod at 22–25 °C for 2 weeks. Sterile distilled water (7.5 mL per plate) was used to scrape hyphal material and spores from the plates with a sterile bent glass rod. The contents of 24 plates were strained through sterile cheesecloth, and the spore concentration was determined with a hemacytometer and adjusted to approximately 1×10^5 conidia mL^{-1}.

2.3 Plant treatments

Glyphosate-potassium 540 g AE L^{-1} SL (Roundup WeatherMax; Monsanto Co., St Louis, MO) was applied to sugar beets at a rate of 0.84 kg AE ha^{-1} in an overall spray volume of 180 L ha^{-1} using a Research Track Sprayer (DeVries Manufacturing, Hollandale, MN) with a TeeJet 8002 nozzle (Spraying Systems Co., Wheaton, IL). Ammonium sulfate (120 mg L^{-1}) was added to the spray liquid according to the manufacturer's recommendation to give increased glyphosate efficacy. The surfactant control treatment consisted of 10 μL L^{-1} Tween 20 containing 120 mg L^{-1} ammonium sulfate. Tween was selected for use as a surfactant control as it is non-toxic to fungi at this level and the carrier from Monsanto was not available. Ammonium sulfate was added to the control to remain consistent with the glyphosate treatment.

2.4 Disease severity tests

For Rhizoctonia root rot tests, ground inoculum was applied to the crowns of six-week-old sugar beets [approximately 0.6 mL (20–40 cfu) per plant, 10 plants per treatment]. Plants were arranged in a completely randomized design, watered daily and maintained at 26–28 °C. At 30 days post-inoculation, roots were harvested and individually rated using a scale of 0 (no visible damage) to 7 (plant dead and root completely rotted).[27] For Fusarium yellows tests, six-week-old plants were removed from soil and rinsed under running tap water. Roots were soaked in a *Fusarium* spore suspension or sterile water (control) for 8 min with intermittent agitation. Following inoculation, beets were replanted into their original planting cones. Plants were arranged in a completely randomized design, watered daily and maintained at 26–28 °C. Plant symptoms were rated

weekly for 6 weeks using a modified rating scale[28] of 0 to 5 (0 = no visible disease; 1 = leaves may be wilted, small chlorotic areas on lower leaves, but most of leaves still green; 2 = leaves showing interveinal chlorosis, with entire leaves chlorotic; 3 = leaves with necrotic spots or becoming necrotic and dying, but less than half of the leaves affected; 4 = half or more of the leaves dead, plants stunted, most living leaves showing some symptoms; 5 = death of the entire plant). After 6 weeks, plants were harvested and roots examined for vascular discoloration. The presence of the appropriate *Fusarium* isolate was confirmed through morphological comparison of isolates recovered from infected plants (at least two roots per treatment), with original isolates used for inoculation. The area under the disease progress curve (AUDPC) was determined for the 6 week period, allowing for examination of disease progress over time when analyzed with non-destructive sampling. The experiments were repeated 3 times.

2.5 Glyphosate effect on *Fusarium oxysporum* f. sp. *betae in vitro*

The methods of Sanogo *et al.*[15] were adapted for determination of fungal growth, sporulation and spore germination in the presence of glyphosate. Briefly, 7 mm agar plugs (three per isolate per glyphosate concentration) from the margin of five-day-old stock cultures of F-19 and FOB13 isolates were transferred to half-strength PDA (30 mL per 9 cm diameter petri plate) amended with filter-sterilized (0.2 μm) glyphosate (pure technical grade; Supelco, Bellefonta, PA) at 0, 1.0, 4.0, 8.0 or 40 $\mu g\,mL^{-1}$, representing negative control, environmental breakdown, standard application rate, accidental duplicate spray and spillage respectively, as determined by concentration of active ingredient per unit area. Plates were incubated ($25 \pm 2\,^{\circ}C$; 8 h light) and measurements were taken daily until mycelia reached the edges of the plates (approximately 7 days after plug transfer). Plates were incubated until 21 days after plug transfer, at which time spores were harvested in 15 mL distilled water and concentrations were determined using a hemaecytometer. The viability of spores in the presence of glyphosate was determined as follows: 7 mm plugs of F-19 and FOB13 were transferred to half-strength PDA (30 mL per 9 cm diameter petri dish) and incubated ($25 \pm 2\,^{\circ}C$; 8 h light) until 14 days after plug transfer, at which time spores were harvested in 15 mL sterile distilled water. For each fungal isolate, 10 μL of spore suspension (three per isolate per glyphosate concentration) was added to one 90 μL glyphosate dilution (1.0, 4.0, 8.0 or 40 $\mu g\,mL^{-1}$) or sterile distilled water (negative control). Spores were incubated for 6 h in the dark at room temperature. The total number of germinating and non-germinating conidia was determined using a hemaecytometer. Percentage germination was calculated by dividing the number of germinating conidia by the total number of conidia in the suspension. The experiments were replicated 3 times.

2.6 Glyphosate effect on *Rhizoctonia solani in vitro*

The methods of Harikrishnan and Yang[29] were used to examine the effect of glyphosate on *R. solani in vitro*. Briefly, 7 mm mycelial plugs (three per isolate per glyphosate concentration) from the margin of two-day-old stock cultures of R-9 and R-1411 were transferred to the edge of PDA plates amended with filter-sterilized (0.2 μm) glyphosate (0, 1.0, 4.0, 8.0 or 40 $\mu g\,mL^{-1}$). Based on the rapidity of growth, R-1411 plugs were transferred to 15 cm petri plates (60 mL PDA) and R-9 plugs were plated on 9 cm plates (30 mL PDA). Plates were incubated ($25 \pm 2\,^{\circ}C$; 8 h light) and measurements of radial growth were recorded daily until mycelia reached the edges of the plates (approximately 7 days after plug transfer). R-9 plates were incubated for an additional 3 weeks to allow for sclerotia production. R-1411 plates were also incubated for 3 weeks, but no sclerotia were produced by R-1411. At 4 weeks after plug transfer, all sclerotia were manually harvested from plates using forceps (three plates per glyphosate concentration). Sclerotia were dried overnight in a laminar flow hood and the total weight was then determined. The following day, sclerotia were counted and 50 individual sclerotia were transferred to PDA (60 mL per 15 cm petri plate). Plates were incubated overnight ($25 \pm 2\,^{\circ}C$) and the number of germinating sclerotia was recorded. Percentage germination was calculated by dividing the number of germinating sclerotia by 50 (total number of sclerotia plated). The experiments were repeated 3 times.

2.7 Shikimic acid analysis

For sugar beet analyses in experiments testing age, all leaves were harvested and used in extractions. For tissue-specific response measurements, cotyledons, treated leaves (leaves present during glyphosate application), newly emergent untreated leaves (leaves that emerged after glyphosate was applied) and roots were harvested independently and used in extractions. For *Fusarium* studies, hyphae were harvested from either a non-glyphosate-supplemented PDA plate or a plate containing 40 $\mu g\,mL^{-1}$ glyphosate. Shikimic acid extractions from sugar beet and fungal material were carried out according to Singh and Shaner.[30] Briefly, tissue was collected (three plants per treatment per cultivar; three mycelia collections per treatment per isolate), weighed and immediately ground in liquid nitrogen. Following grinding, hydrochloric acid (0.25 M; 3 mL g^{-1} fresh weight) was added to each sample. Samples were centrifuged for 30 min at 14 000 × *g*. Supernatant was used immediately in the shikimic acid concentration assay. Extractions were repeated on three independent occasions for sugar beet analyses and two separate occasions for fungal analyses. A spectrophotometric approach for determining shikimic acid concentrations was employed.[30] Briefly, 25 μL of supernatant collected from the extraction was transferred to a microfuge tube containing periodic

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acid (100 mg L^{-1}; 500 μL). Samples were allowed to oxidize at room temperature for 3 h. Following the incubation, sodium hydroxide (1 M; 500 μL) and glycine (0.1 M; 300 μL) were added to each sample. The sample was vortexed briefly and the OD at 380 nm was determined immediately. A standard curve was established using 0.5–60 μmol shikimic acid (Sigma, St Louis, MO) added to periodic acid incubated under similar conditions.

2.8 Statistical analyses

All statistical analyses for disease severity tests were performed using SAS (SAS Institute, Cary, NC). Data from experiments were combined, and a linear model was fit to these data using PROC MIXED. Experiment was treated as a random effect. Because variance in disease indices differed among the isolates, the group option in the repeated statement was used to fit models that allowed heterogeneity of variance among factor combinations. Since the overall model fit indicated that there were significant isolate effects and significant interactions between isolates and other treatment factors, analyses were run on isolates separately. Mean separations for disease indices or AUDPC for each isolate were conducted using the macro PDMIX800.SAS[31] with Bonferroni adjustments to control the type I error rate. All experiments testing direct effects of glyphosate on *F. oxysporum* and *R. solani* were analyzed with a one-way ANOVA. Means separations were conducted with a Tukey–Kramer *post hoc* test ($\alpha = 0.05$) using PDMIX800.SAS. For shikimic acid experiments, data were analyzed using analysis of variance in the general linear models procedure of SAS. Since no difference between experiments was detected for shikimic acid analyses, experimental data were combined. Mean separations between treatments by tissue type for four-week-old sugar beet were conducted by Fisher's least significant difference (LSD) test ($\alpha = 0.05$) using SAS.

3 RESULTS

3.1 Disease severity

The GR plants showed no visible damage from the glyphosate treatments. There were no significant differences in foliar yellowing or necrosis between glyphosate-treated and control plants in the absence of pathogens. There was no significant effect of experimental replication on disease severity, and therefore data from the three experiments were pooled and analyzed together. For Rhizoctonia root rot tests, analysis of variance showed two main effects (cultivar and isolate) that were highly significant and one main effect that was borderline significant (spray, $P = 0.0519$). Of the two-way interaction effects, cultivar × spray, spray × isolate and cultivar × isolate were all highly significant. Additionally, the three-way interaction effect of cultivar × spray × isolate was highly significant. All other interaction effects were not statistically significant (Table 1). Therefore, no significant effect of the amount of time between glyphosate application and pathogen inoculation was detected in this test. In the absence of glyphosate, sugar beet cultivar B4RR (Rhizoctonia root rot resistant) had significantly lower disease severity ratings than the other sugar beet cultivar (H16) when the AG-2-2 isolate, R-9, was used, but B4RR was not significantly different from H16 in disease severity caused by the AG-4 isolate, R-1411 (Fig. 1(A)). However, following treatment with glyphosate, both sugar beet cultivars showed statistically indistinguishable disease levels for each of the two isolates ($P \leq 0.05$), and the rating for cultivar B4RR was significantly higher than the surfactant treatment when challenged with isolate R-9 regardless of the duration between glyphosate treatment and pathogen challenge.

In Fusarium yellows tests, minimum evidence of disease was observed in non-inoculated controls, but both pathogen isolates caused detectable disease (Fig. 1(B) and (C)). The presence of the appropriate *Fusarium* isolates was confirmed and the control roots were free from *Fusarium*. As no statistically significant effect of experiment was observed, data for all experiments were analyzed together. All main effects (spray, cultivar, isolate and day) were significant. The two-way interaction effects of spray × isolate, cultivar × isolate, day × cultivar and day × isolate were all significant. The two-way interaction of day × spray was borderline significant ($P = 0.0674$). The only three-way interaction with significance was day × cultivar × isolate (Table 1). Isolate F-19 was more virulent than FOB13. F-19 killed 40% or more of the plants within the 6 week rating period. FOB13 did not cause any plant death during the time period examined with any of the treatments. Disease severity caused by isolate FOB13 was significantly higher following glyphosate treatment than on surfactant-treated plants for both cultivars regardless of the duration between treatment application and pathogen inoculation (Fig. 1(B) and (C)). There were no statistically significant differences between plants treated with isolate F-19 for either spray treatment at either time, although in the plants treated at 1 day after spraying the two cultivars differed significantly in disease severity caused by F-19 (Fig. 1(B) and (C)). Cultivar H16 showed a significantly higher AUDPC than cultivar B4RR for both treatments with F-19 when inoculated 1 day after spraying, but there was no significant difference between the two cultivars in average AUDPC when they were inoculated at 9 days after spraying.

3.2 Fungal growth

To determine the effect of glyphosate on fungal growth, the two isolates of *F. oxysporum* (FOB13 and F-19) and *R. solani* (R-1411 and R-9) were examined *in vitro*. All isolates showed a similar rate of growth at glyphosate concentrations between 1

Table 1. Analysis of variance for main and interaction effects of glyphosate on disease severity caused by *Rhizoctonia solani* and *Fusarium oxysporum* in two glyphosate-resistant sugar beet cultivars

Pathogen	Effect	*F* value	*P* > *F*
Rhizoctonia solani[a]	Spray[b]	3.79	0.0519
	Cultivar[c]	9.57	0.0021
	Cultivar × spray	8.65	0.0034
	Isolate[d]	1290.57	<0.0001
	Spray × isolate	10.05	<0.0001
	Cultivar × isolate	17.90	<0.0001
	Cultivar × spray × isolate	7.95	0.0004
	Day[e]	1.59	0.2080
	Day × spray	0.04	0.8486
	Day × cultivar	1.79	0.1818
	Day × cultivar × spray	0.07	0.7893
	Day × isolate	2.09	0.1246
	Day × spray × isolate	0.63	0.5324
	Day × cultivar × isolate	0.31	0.7370
	Day × cultivar × spray × isolate	1.84	0.1591
Fusarium oxysporum[f]	Spray	27.16	<0.0001
	Cultivar	14.20	0.0002
	Cultivar × spray	0.23	0.6340
	Isolate[g]	907.60	<0.0001
	Spray × isolate	36.93	<0.0001
	Cultivar × isolate	7.06	0.0010
	Cultivar × spray × isolate	0.07	0.9302
	Day	5.01	0.0257
	Day × spray	3.36	0.0674
	Day × cultivar	3.91	0.0485
	Day × cultivar × spray	0.94	0.3339
	Day × isolate	4.38	0.0131
	Day × spray × isolate	1.58	0.2061
	Day × cultivar × isolate	4.63	0.0102
	Day × cultivar × spray × isolate	0.63	0.5334

[a] Results are from ratings taken at 4 weeks post-inoculation, using a rating scale of 0 to 7.
[b] Two spray treatments were used, either a standard field application rate of glyphosate (0.84 kg AE ha^{-1}) or a surfactant control.
[c] Two sugar beet cultivars were used: B4RR and H16. Cultivar B4RR has resistance to *Rhizoctonia solani* AG-2-2. Both are susceptible to *Fusarium oxysporum*.
[d] Two isolates of *R. solani* were used: R-1411 (AG-4) and R-9 (AG-2-2). Control was sterile ground barley.
[e] Plants were sprayed either 1 day or 9 days before inoculation with fungal isolates.
[f] Results are from average area under the disease progress curve (AUDPC), representing ratings taken weekly for 6 weeks post-inoculation using a rating scale of 0 to 5.
[g] Two isolates of *F. oxysporum* were used: FOB13, moderately virulent, and F-19, highly virulent. Control was sterile water.

and 40 $\mu g\,mL^{-1}$ when compared with the negative control ($P \leq 0.05$). At a glyphosate concentration of 400 $\mu g\,mL^{-1}$ there was a significant inhibition of fungal growth for all isolates tested; however, the inhibition was incomplete (17 and 11% inhibition for *F. oxysporum* isolates FOB13 and F-19 respectively, and 30.7% and 25% inhibition for *R. solani* isolates R-1411 and R-9 respectively).

3.3 Production of overwintering structures

Spore production and germination were determined for the two isolates of *F. oxysporum* in the presence of glyphosate. FOB13 showed a significant decrease in spore production and germination at 8 $\mu g\,mL^{-1}$ when compared with the control (Table 2). However, spore production was similar to the control from the higher glyphosate concentrations (12 $\mu g\,mL^{-1}$, 20 $\mu g\,mL^{-1}$ and 40 $\mu g\,mL^{-1}$). Spore production remained comparable with the control levels at all glyphosate concentrations for F-19, but spore germination was significantly reduced at a glyphosate concentration of 40 $\mu g\,mL^{-1}$. Spore production remained similar to the control for FOB13 and F-19 at a glyphosate concentration of 400 $\mu g\,mL^{-1}$ (data not shown). Spore germination showed a statistically significant reduction at 1 mg mL^{-1} glyphosate for both FOB13 and F-19–40.6% and 13.0% respectively. Sclerotia production, weight and viability were analyzed in the presence of glyphosate for *R. solani* R-9. Between 0 and 40 $\mu g\,mL^{-1}$ of glyphosate there was no significant ($\alpha = 0.05$) effect of glyphosate on sclerotia production, with sclerotia ranging from 96 to 104 per plate. However, at 400 $\mu g\,mL^{-1}$ of glyphosate there was a significant ($\alpha = 0.05$) 60% reduction in sclerotia production compared with the non-amended media control. There was no significant ($\alpha = 0.05$) difference in sclerotia weight or sclerotia viability at any of the glyphosate concentrations tested. In tests examining shikimic acid levels in the presence of glyphosate, no difference was noted between *F. oxysporum* isolates FOB13 and F-19 when grown in 40 $\mu g\,mL^{-1}$ glyphosate-supplemented media or an unamended control (data not shown).

Table 2. Effect of glyphosate on spore production and germination of two isolates of *Fusarium oxysporum*

Glyphosate concentration	Spore production[a,b]		Spore germination (%)[b,c]	
($\mu g\,mL^{-1}$)	FOB 13[d]	F-19[d]	FOB 13	F-19
0	1.98 ab	2.57 a	51.9 ab	31.0 a
1	2.01 ab	2.27 a	57.7 a	31.9 a
4	2.56 a	1.93 a	51.6 b	35.9 a
8	0.81 c	2.03 a	38.0 c	24.5 b
12	1.27 bc	n.d.[e]	n.d.	n.d.
20	1.31 bc	n.d.	n.d.	n.d.
40	1.67 abc	2.63 a	25.9 d	16.9 c

[a] Total spore count 1×10^6 at 4 weeks after mycelial agar plug transfer.
[b] Numbers followed by the same letter are not statistically different as determined by Tukey–Kramer *post hoc* test ($P < 0.05$). Experiments were repeated 3 times.
[c] Percentage of total spore count following 6 h of exposure to glyphosate.
[d] FOB13 = *Fusarium oxysporum* f. sp. *betae* isolate FOB13; F-19 = *Fusarium oxysporum* f. sp. *betae* isolate F-19.
[e] n.d. = no data.

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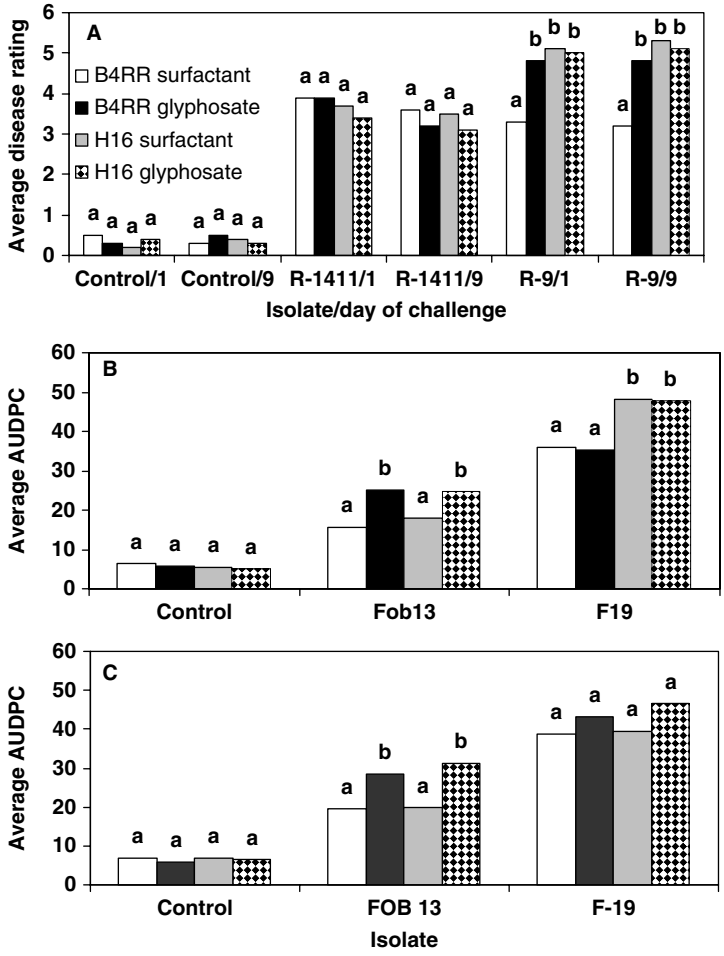

Figure 1. Response of two glyphosate-tolerant sugar beet lines to different fungal isolates in the presence or absence of the herbicide glyphosate. All results are the average ratings of 30 plants. (A) Plants inoculated 1 day or 9 days after glyphosate or surfactant control application with two different *Rhizoctonia solani* isolates, R-9 (AG-2-2) and R-1411 (AG-4), in ground barley. The control plants were treated with sterile ground barley. Each root was rated for disease on a 0 (no disease) to 7 (completely rotted) rating scale. (B) Plants inoculated with *Fusarium oxysporum* isolates F-19 or FOB13 at 1 day after glyphosate or surfactant treatment. (C) Plants inoculated with *Fusarium oxysporum* isolates F-19 or FOB13 at 9 days after glyphosate or surfactant treatment. Values are average area under the disease progress curve (AUDPC) of six weekly ratings (0–5 rating scale). Control treatment for *Fusarium* experiments was inoculation with sterile water. Since analysis of variance showed a statistically significant effect of time of glyphosate application on disease severity for *F. oxysporum*, the data were analyzed separately. Bars on each graph for a given isolate with the same letter are not significantly different by mean separation with Bonferroni adjustments ($P = 0.05$).

3.4 Effects of glyphosate treatment on shikimic acid levels in glyphosate-resistant and susceptible sugar beet cultivars

The impact of glyphosate application on sugar beet physiology in GS and GR sugar beet cultivars was monitored by measuring the accumulation of shikimic acid following glyphosate application. The analysis of variance showed that all main effects (cultivar, treatment, age and day), two of the two-way interactions (cultivar × treatment and treatment × day) and the three-way interaction (cultivar × treatment × day) were highly significant (Table 3). However, the two-way interaction of plant cultivar × age was not significant. Analysis of the main effects by cultivar showed that treatment, age and day were all highly significant regardless of glyphosate tolerance (Table 4). There was a significant increase in shikimic acid following both surfactant and glyphosate treatment ($P < 0.0001$) for all cultivars, but the rate of accumulation was greatest for the glyphosate-treated GS variety, B4 (data not shown). The difference between shikimic acid levels in both GR varieties when treated with glyphosate versus surfactant control was only significant in three- and four-week-old beet samples (data not shown). The growth rate of the GS variety was negatively affected with glyphosate

Table 3. Analysis of variance across cultivars for main and interaction effects of glyphosate on shikimic acid levels in glyphosate-resistant and glyphosate-susceptible sugar beet cultivars

	F	*P* > *F*
Cultivar	80.47	<0.0001
Treatment	218.41	<0.0001
Age	6.29	0.0003
Day	15.97	<0.0001
Cultivar × treatment	92.6	<0.0001
Treatment × age	0.87	0.4586
Treatment × day	4.49	<0.0001
Cultivar × treatment × day	2.41	0.0015

Table 4. Analysis of variance for main effects by cultivar to examine accumulation of shikimic acid following glyphosate application on glyphosate-susceptible and glyphosate -resistant sugar beet cultivars

	Sugar beet cultivar					
	B4[a]		B4RR[a]		H16[a]	
	F	*P* > *F*	*F*	*P* > *F*	*F*	*P* > *F*
Treatment[b]	416.58	<0.0001	49.85	<0.0001	41.79	<0.0001
Age[c]	6.85	0.0001	18.17	<0.0001	9.98	<0.0001
Day[d]	17.33	<0.0001	15.55	<0.0001	10.88	<0.0001

[a] B4 is a GS sugar beet cultivar and B4RR and H16 are both GR sugar beet cultivars.
[b] Each cultivar was treated with a standard field application rate of glyphosate (0.84 kg AE ha^{-1}) or a surfactant control.
[c] Shikimic acid accumulation was monitored in all plant cultivars at 2, 3, 4 and 6 weeks of age.
[d] Leaves were sampled at 0, 1, 2, 3, 4, 7, 9, 11, and 14 days post-application of glyphosate or the surfactant control. The rate of shikimic acid accumulation (slope of line over 14 days of sampling) between treatments was 0.119–0.631 (B4), 0.146–0.220 (B4RR) and 0.144–0.160 (H16) for surfactant control and glyphosate application respectively.

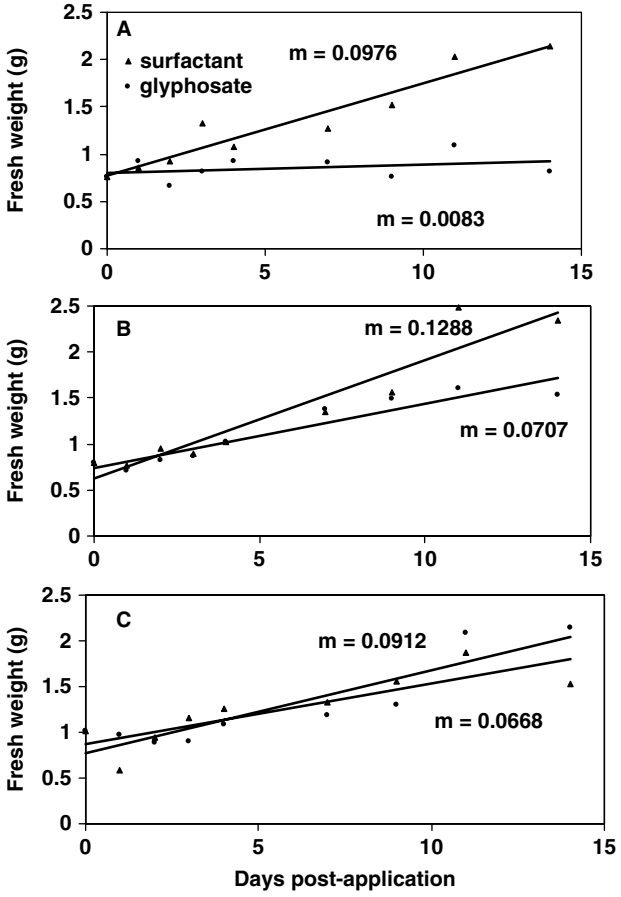

Figure 2. Leaf tissue accumulation in glyphosate- or surfactant-treated GS, B4 (A), and two GR cultivars of sugar beet, B4RR (B) and H16 (C). Data are the mean of three independent replications, containing three samples per replicate. The slope of the line, representing tissue accumulation, is noted as *m*. Differences between slopes for GS cultivar are significant ($P < 0.0001$), whereas the slopes for GR cultivars are not significantly different.

treatment, and the affect on the GR varieties was negligible (Fig. 2).

Shikimic acid accumulation was monitored across tissue types for both the GR and GS varieties (Fig. 3). Levels of shikimic acid were significantly different between glyphosate- and surfactant-treated plants for all tissue types in the GS cultivar ($P < 0.0001$) and for all tissue types except the roots ($P = 0.0727$ and 0.1170 for B4RR and H16 respectively) for the GR varieties. In all instances, the level of shikimic acid accumulation was higher for the GS variety than for the GR varieties following glyphosate application. In the GS variety, shikimic acid increased rapidly and reached a plateau over time. In comparison, the response to glyphosate treatment was delayed and transient in the GR cultivars. The greatest and most prolonged difference in response to glyphosate appeared in the newly emergent leaf tissue for the GR cultivars. The shikimic acid level was higher following glyphosate treatment at all sampling time points.

4 DISCUSSION AND CONCLUSIONS

There have been several reports of increased disease incidence following glyphosate application in GR crops.[15,16,19] In the present study, the Rhizoctonia root rot resistant GR cultivar (B4RR) had a significant increase in disease severity when inoculated with an AG-2-2 isolate of *R. solani*, but not with an AG-4 isolate, indicating a possible loss of resistance following glyphosate application. The cultivar B4RR has resistance to AG-2-2 but not to AG-4 which typically infects seedlings.[32] This increased disease severity is probably not a fungal-mediated response. There was no significant difference in growth rate in the presence of glyphosate for either isolate tested. Additionally, isolate R-9 produced equal numbers of equivalently sized sclerotia in the presence of glyphosate as compared with a water-amended control. This is in contrast to other studies where, in the presence of glyphosate, other *R. solani* isolates produced more, smaller sclerotia following exposure to glyphosate without affecting viability.[29] Alternatively, the increased disease severity caused by R-9 could reflect a possible cultivar- or isolate-specific response to glyphosate treatment. Other researchers have reported increased disease severity in some soybean cultivars with some pathogenic isolates and not in others.[17]

The increase in disease severity at both times tested and on both cultivars for one of the

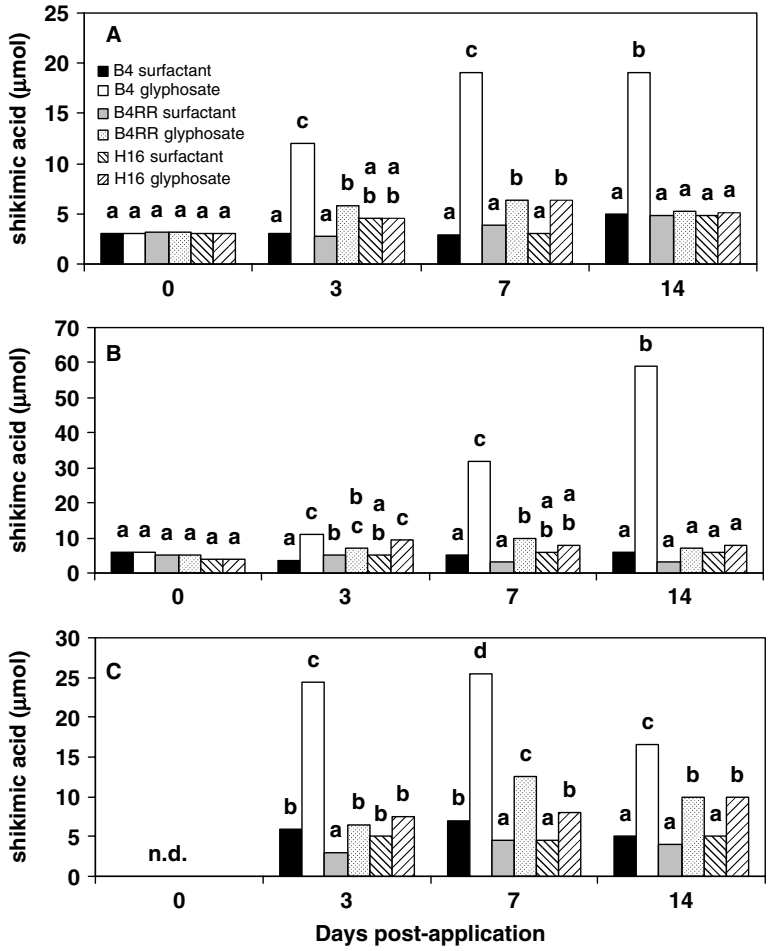

Figure 3. Shikimic acid accumulation in GR (B4RR and H16) and GS (B4) cultivars of sugar beet following glyphosate or surfactant application. Shikimic acid concentrations were monitored in (A) cotyledons, (B) treated leaves and (C) newly emergent, untreated leaves. Data are the mean of three independent replications containing three samples per time point per treatment. Analysis of variance for each cultivar across time showed statistically significant differences in shikimate levels between treatments in several tissue types ($P < 0.0001$ for all B4 tissue types; $P < 0.0001$ for cotyledons, treated leaves and newly emergent leaves for B4RR and $P = 0.0727$ for roots; $P < 0.0001$ for cotyledons and newly emergent leaves for H16 and $P < 0.05$ and 0.177 for H16-treated leaves and roots respectively). Columns with the same letter designation are not statistically different as determined using Fisher's least significant difference test ($P = 0.05$). n.d. = no data for those time points.

F. oxysporum isolates tested is consistent with reports that glyphosate treatment can increase disease by *Fusarium* in both GS[33] and GR[15] crops. Synergism between glyphosate and some soil-borne pathogens,[34] including *Fusarium*,[35] has been observed and can be expressed as increased disease severity when glyphosate treatment is combined with pathogen exposure.[36] From *in vitro* studies it was apparent that the two isolates of *F. oxysporum* were tolerant to glyphosate. Levels of glyphosate 100 times the recommended field application rate were necessary to achieve even slight growth inhibition. The slight decrease in spore production with FOB13 in the presence of $8\,\mu g\,mL^{-1}$ glyphosate was most likely an experimental artifact, since further examination at 12 and $20\,\mu g\,mL^{-1}$ showed no difference from that of the control (data not shown). Additionally, the two isolates appeared to have a similar physiological response to glyphosate, since shikimic acid levels in the presence of $40\,\mu g\,mL^{-1}$ glyphosate were similar for both isolates compared with a negative control (data not shown). Finding a statistically significant increase for only one of the two *F. oxysporum* isolates is consistent with reports for other pathogens that isolates can vary in their response to herbicide treatments.[15,17] Furthermore, it may be that, with the more virulent pathogen (F-19), the disease was so severe that no significant differences in disease could be detected with the methods used.

The lack of evidence for a fungal-mediated mode of increased disease severity led to the examination of sugar beet physiology following glyphosate application. Glyphosate inhibits the shikimic acid pathway which gives rise to essential aromatic amino acids and plant defense compounds.[6–8] Inhibition of the shikimic acid pathway could result in reduced defensive capabilities of sugar beet. This has been observed in GS tomato treated with a sublethal dose of glyphosate.[36] Application of the herbicide leads to an increase in shikimic acid,[37] which serves as an indicator of EPSPS efficacy and as a measure of the impact of glyphosate application on physiology. In preliminary studies using sugar beet seedlings, significant differences in shikimic acid levels between glyphosate

and surfactant negative controls were observed (data not shown). In all ages of sugar beet (two, three, four and six weeks old) tested, the rate of shikimic acid accumulation was greater following glyphosate application than following surfactant treatment in both GR cultivars. This suggests a random insertional event during transformation, or the background of the transformed material is not responsible for the sensitivity to glyphosate application and the impact of glyphosate is not tied to a particular development stage. As expected, shikimic acid levels following glyphosate application were significantly lower for the GR cultivars than for the GS cultivar.

Glyphosate had the greatest impact on actively growing tissue in sugar beet, which is expected since it is phloem mobile and is transported to metabolic sinks such as meristematic tissue.[38–40] In tissue type studies, newly emergent, actively growing tissues had the greatest, most prolonged shikimic acid accumulation. However, in every tissue type, accumulation was transient. Nevertheless, slight inhibition of EPSPS may inhibit plant defenses in the same manner as activation of plant defenses affects plant growth.[41] It is possible that the inhibition of EPSPS is substantial enough to limit substrate availability for conversion into salicylic acid and phytoalexins without negatively affecting growth, since growth rate was not affected by glyphosate in GR varieties. Future studies need to address the impact of shikimic acid accumulation on the production of key plant defense compounds, including phytoalexins. The induction of the phytoalexin betavulgarin has been reported in sugar beet roots inoculated with *R solani*,[42] but the role of phytoalexins in Fusarium yellows is not known. Alternatively, stress as a result of herbicide application has been associated with increases in disease.[39] Glyphosate is broken down into aminomethylphosphonic acid in GR soybean; this is a phytotoxic compound[43] that also may be a source of plant stress in sugar beet. Thus, examination of glyphosate movement, concentration and metabolic byproduct production in sugar beet would provide clues about whether phytotoxicity relates to disease severity. Additionally, although the evidence from *in vitro* studies suggests that the increase in disease is not fungal mediated, *in planta* observations of lack of effect of glyphosate on fungal pathogens could help confirm that increased disease severity is a result of a change in plant metabolism.

With the introduction of GR sugar beet into commercial production on the horizon, the response of GR sugar beet to glyphosate needs further investigation. The results of the present experiment suggest that the response to glyphosate differs by cultivar and pathogen isolate. The cultivars used in this study are not the cultivars that are now currently available for commercial production, and therefore these new events should be included in these broader-spectrum studies. Nevertheless, a cultivar by isolate disease severity study should be conducted for breeding lines being used or considered for GR sugar beet production. For *F. oxysporum* there was a statistically significant response to the amount of time between glyphosate application and pathogen inoculation (Table 1). While no significant direct effect on the interaction of spray treatment and disease severity was observed, this indicates that timing of application may be an important consideration in pathogen interactions. Other researchers have reported that the timing of glyphosate application can affect disease severity. For example, glyphosate reduced the severity of leaf rust in wheat if applied before infection had occurred, with efficacy lasting for 21 days after application but not more than 35 days after application.[44] A better understanding of the impact of the timing of glyphosate applications may allow the development of an optimized spray schedule for sugar beet to manage potential deleterious effects on disease severity.

A future direction includes understanding the impact of GR crops on rotation. Increases in Rhizoctonia root rot could increase the soil pathogen population and affect other susceptible crops in rotation with GR sugar beet, such as dry bean, soybean and corn.[45] Glyphosate application has been reported to increase propagules of *Fusarium*[33] in soil, changing the inoculum level in the agroecosystem. Furthermore, glyphosate application has made other crops susceptible to normally non-pathogenic isolates of *Fusarium*,[36] so raising concerns about new pathogenic threats to GR sugar beet, which serves as a symptomless host for certain *Fusarium* isolates.[46] Lastly, glyphosate can reduce the number of potential antagonists to pathogens in field soil[47] and alter the interaction among fungi on media and in soil.[48] Although this was not the underlying mechanism for loss of resistance in the greenhouse studies, this is an area that needs further investigation under field conditions.

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