Effect of glyphosate on the tripartite symbiosis formed by Glomus intraradices, Bradyrhizobium japonicum, and genetically modified soybean

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Most soybeans grown in North America are genetically modified (GM) to tolerate applications of the broad-spectrum herbicide glyphosate; as a result, glyphosate is now extensively used in soybean cropping systems. Soybean roots form both arbuscular mycorrhizal (AM) and rhizobial symbioses. In addition to individually improving host plant fitness, these symbioses also interact to influence the functioning of each symbiosis, thereby establishing a tripartite symbiosis. The objectives of this study were to (1) estimate the effects of glyphosate on the establishment and functioning of AM and rhizobial symbioses with GM soybean, and (2) to estimate the interdependence of the symbioses in determining the response of each symbiosis to glyphosate. These objectives were addressed in two experiments; the first investigated the importance of the timing of glyphosate application in determining the response of each symbiosis to glyphosate. These objectives were addressed in two experiments; the first investigated the importance of the timing of glyphosate application in determining the responses of the symbionts and the second varied the rate of glyphosate application. Glyphosate applied at recommended field rates had no effect on Glomus intraradices or Bradyrhizobium japonicum colonization of soybean roots, or on soybean foliar tissue [P]. N₂ fixation was greater for glyphosate-treated soybean plants than for untreated-plants in both experiments, but only when glyphosate was applied at the first trifoliate soybean growth stage. These data deviate from previous studies estimating the effect of glyphosate on the rhizobial symbiosis, some of which observed negative effects on rhizobial colonization and/or N₂ fixation. We did observe evidence of the response of one symbiont (stimulation of N₂ fixation following glyphosate) being dependent on co-inoculation with the other; however, this interactive response appeared to be contextually dependent as it was not consistent between experiments. Future research needs to consider the role of environmental factors and other biota when evaluating rhizobial responses to herbicide applications.

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1. Introduction

The use of genetically modified (GM) crops is widespread in North America and market share around the globe is growing swiftly. The global area of approved GM crops in 2005 was 90 million hectares in 21 countries, up from 81 million hectares in 17 countries in 2004, with herbicide-tolerant (HT) varieties making up a large proportion of this area (James, 2006). For instance, U.S. growers seeded 36%, 65%, and 89% of all corn, cotton, and soybean acres, respectively, with GMHT varieties in 2006 (NASS, 2006). Most GMHT varieties have been modified for tolerance to glyphosate, a broad-spectrum herbicide (Duke, 2005). As a result, glyphosate is now extensively used in many cropping systems [e.g., over 80% of U.S. soybean acres were treated with glyphosate in 2002, up from 10% in 1994 (Baumoc and Mauricio, 2004)].

Glyphosate targets the shikimate pathway in plants, inhibiting the enzymatic activity of 5-enolpyruvoyl-shikimate-3-phosphate synthase (EPSPS), thereby preventing the synthesis of amino acids (Herrmann and Weaver, 1999; Duke, 2005). Glyphosate-sensitive forms of EPSPS are also present in many microorganisms and protozoans, some of which play key roles in soil nutrient cycling (Roberts et al., 1998; Zabloutowicz and Reddy, 2004; Feng et al., 2005). Impacts on these non-target organisms are thought to be insignificant because glyphosate is quickly adsorbed to soil particles where its activity is limited, while free glyphosate is rapidly degraded by microorganisms (Glass, 1987; Anderson et al., 1993). Nevertheless, glyphosate can accumulate and remain active in plant roots or be exuded into the rhizosphere, where it may affect colonization of roots by symbiotic microorganisms (Feng et al., 2005; Kremer et al., 2005).

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with the roots of a wide variety of plant species, including many crop species (e.g., corn, cotton, soybean). In exchange for photosynthates, AM fungi can provide their host plants with increased access to nutrients and water and enhanced protection against pathogens (Smith and Read, 1997). AM fungi are obligate symbionts, fully reliant on their plant hosts for carbon (Smith and Read, 1997). While the contribution of the AM symbiosis to agricultural productivity is not fully understood, several field studies suggest that it can promote crop yields in low-nutrient soils (Ryan and Graham, 2002). Due to the obligate nature of the AM symbiosis to the fungal partner (Smith and Read, 1997), it is difficult to estimate any direct effects that glyphosate may have on AM fungi. For example, negative effects of glyphosate on AM fungal colonization of carrot roots were observed in vitro, but glyphosate also reduced the growth of the roots themselves (Wan et al., 1998). In another study, glyphosate added to culture medium reduced AM fungal spore germination and germ tube growth in the absence of a host plant, but only at concentrations greater than those recommended for field use (Malty et al., 2006).

In addition, many leguminous plants have the ability to fix atmospheric nitrogen via associations with gram-negative soil bacteria belonging to the family Rhizobiaceae, universally known as rhizobia (Killham, 1994). In exchange for photosynthates, rhizobia stimulate the formation of nodules, plant organs where the bacteria have a suitable environment to convert N₂ into NH₃ (Long, 2001). Symbiotic N₂-fixation by legumes can account for as much as 97% of the total plant N (Peoples and Craswell, 1992). In the case of soybean, the average yearly amount of N₂ fixed by Bradyrhizobium japonicum and present in the above ground tissues is 80 kg N ha⁻¹ in central Ontario (Ravuri and Hume, 1992), but, on a global scale, that amount can be as high as 450 kg N ha⁻¹ (Peoples and Craswell, 1992). The effects of glyphosate on rhizobia have been studied to a greater extent. Glyphosate reduced growth rates of B. japonicum in glyphosate-amended media (Malty et al., 2006) and had negative effects on nodulation and N₂-fixation in greenhouse and field experiments (Reddy et al., 2000; King et al., 2001; Reddy and Zabloutowicz, 2003; Zabloutowicz and Reddy, 2004). However, rhizobial responses are often inconsistent, varying unpredictably with the rate and timing of glyphosate application. For example, Zabloutowicz and Reddy (2007) observed reduced shoot [N] at low and high application rates but not at medium rates in a field experiment, and, in one case, they observed increased acetylene reduction activity at only the medium rate.

In addition, a significant drawback of previous studies investigating the effects of glyphosate on root symbioses is that the symbioses are generally viewed in isolation. Many leguminous plants, including soybean, host AM fungi and rhizobia simultaneously and form a tripartite symbiosis that produces synergistic effects on the establishment and functioning of each symbiosis (Xie et al., 1995; Chalk et al., 2006). Therefore, it is plausible to hypothesize that the effect of glyphosate on one symbiosis could either indirectly affect or be mediated by the other symbiosis. Rhizobial and AM fungal colonization may buffer the negative effects of glyphosate on host physiology, which can occur in even glyphosate-tolerant varieties (De Maria et al., 2006), and its interaction with other symbionts; therefore, we would predict that reductions in the colonization and/or functioning of a symbiont would be lessened in the presence of the other symbiont. Alternatively, the frequently observed increase in nodulation and N₂-fixation in legumes simultaneously colonized by AM fungi is often attributed to the high demand for P during N₂-fixation (Barea et al., 1987); increased P availability, following glyphosate-degradation via microbial C-P lyase activity (Liu et al., 1991), may allow for greater AM fungal P uptake, thus supporting greater amounts of N₂-fixation.

The objectives of this study were (1) to estimate the effects of glyphosate on the establishment and functioning of co-occurring AM and rhizobial symbioses with GM soybean, and (2) to estimate the interdependence of the symbioses in determining the response of each symbiosis to glyphosate. Both of these objectives were addressed in two experiments; the first investigated the importance of the timing of glyphosate application in determining the responses of the symbionts and the second varied the rate of glyphosate application. We hypothesized that glyphosate would negatively affect the AM and/or rhizobial symbioses in the absence of the other microbial symbiont, but that the presence of the other microbial symbiont would negate or reverse the glyphosate effect.
2. **Materials and methods**

2.1. **Soil and growing conditions**

Soybean plants were grown in 4-L pots containing a 60:40 mixture of peat moss (Premier® Pro Moss; Premier Tech, Rivière-du-Loup, QC, Canada) and sand (Hillview®, Nu-Gro IP Inc., Brantford, ON, Canada). The growth medium was sterilized (autoclaved twice at 121°C (1.5 h), with 1 week between autoclave cycles) and stored for 3 weeks prior to use. Selected pots were inoculated with approximately 10 g of Daucus carota roots colonized by Glomus intraradices (Premier Tech, Rivière-du-Loup, QC, Canada); the nonmycorrhizal treatment received approximately 10 g of uncolonized D. carota roots. To account for the contribution of soil microorganisms other than AM fungi, all pots, including non-inoculated pots, received 20 mL of a microbial wash, filtered through a 20 μm-mesh (Ames et al., 1987), derived from field soil (Elora Research Station, ON, Canada in experiment #1; Guelph, ON, Canada in experiment #2) with no history of soybeans for at least 60 years. Selected pots were inoculated with 0.5 g of a peat-based B. japonicum 532C inoculum (Hi-Stick®; MicroBio, Saskatoon, SK, Canada), buried 5 cm below the soil surface. Washes from different soils were used in the two experiments to try (unsuccessfully) to control for nodulation observed in the nonrhizobial controls in the first experiment. The use of washes compromised the ability to establish nonrhizobial controls but were necessary to account for indirect ‘mycorrhizosphere’ effects associated with AM fungal inoculation (Linderman, 1988).

Thirty milliliters of a solution of $^{15}$NH$_4$,$^{15}$NO$_3$, 27.2 mM at 10.6% $^{15}$N, which corresponds to a rate of approximately 30 kg N ha$^{-1}$, were added to each pot prior to planting. According to Goss et al. (2002), such a fertilizer application rate is not likely to affect nodulation. To facilitate the incorporation and distribution of $^{15}$N, 100 ml of water were then added to each pot.

A registered glyphosate-tolerant soybean variety, OAC Rockwood (from Istvan Rajcan, Department of Plant Agriculture, University of Guelph), was grown. Soybean seeds were surface-sterilized with 0.5% NaOCl for 5 min, germinated in autoclaved moist vermiculite and transplanted to the pots. Experiments were conducted under a 16h light:8h dark photoperiod and a minimum greenhouse temperature of 20°C. Pots were irrigated with approximately 200 mL of water every 2–3 days.

2.2. **Experiment 1**

The experimental design consisted of three factors: symbiotic microbial inoculation (three levels: rhizobium, AM fungi, rhizobium and AM fungi), glyphosate (three levels: negative control (water), glyphosate applied 10 days following transplant (“early”); first trifoliate growth stage), or glyphosate applied 17 days following transplant (“delayed”)), and harvest date (four levels: 1 week following the early glyphosate application (control and early glyphosate treatments only), 1 week following the delayed glyphosate application (control and delayed glyphosate treatments only), and 4 and 8 weeks following the early glyphosate application (all glyphosate treatments)). Glyphosate was applied in formulation (Roundup WeatherMAX®) at a product rate equivalent to 0.9 kg acid equivalent (ae)/ha. The formulation was sprayed on foliar and soil surfaces from above, mimicking an application under field conditions. Experimental units were arranged on the greenhouse bench in a completely randomized design. Pots lacking rhizobial inoculation were placed behind a plastic barrier at the end of the bench to minimize contamination. This experiment was conducted in the summer months under a maximum photosynthetic photon flux density (PPFD) of ~2000 μmol/(m² s). A complete factorial design was not used; instead, the experiment was designed to address the following questions:

1. Do glyphosate application and the timing of application affect the formation and functioning of symbiotic relationships? The experimental design consisted of a full factorial with all levels of the glyphosate factor and three levels of harvest date factor (1 week post-glyphosate (i.e., 17 and 24 days after transplant in the early and delayed glyphosate treatments, respectively), and the 4 and 8 weeks treatments described above (i.e., 38 and 66 days after transplant for all glyphosate treatments)). All experimental units were inoculated with both B. japonicum and G. intraradices. Each factor combination was replicated five (control treatment) or seven (glyphosate treatments) times.

2. Is the response of a symbiosis to glyphosate influenced by the presence of another symbiont? The experimental design consisted of a full factorial with all levels of the symbiont inoculation factor, two levels of the glyphosate factor (control, applied 10 days following transplant), and three levels of the harvest date factor (1, 4, and 8 weeks following glyphosate application). Effects of glyphosate on the tripartite symbiosis were determined by comparing individual symbiotic responses in the presence versus absence of the other microbial symbiont. Each treatment combination was replicated three times, except for the experimental units including both symbionts, which were replicated five (control treatment) or seven (glyphosate treatment) times.

2.3. **Experiment 2**

A full-factorial design was used, consisting of two factors: symbiotic microbial inoculation (three levels: rhizobium, AM fungi, and glyphosate), and glyphosate (three levels: negative control (water), “low” product rate (equivalent to 0.9 kg ae/ha), and “high” product rate (1.8 kg ae/ha)). Both the low and high doses represent recommended application rates in Ontario (OMAFRA, 2006). Experimental units were arranged on the greenhouse bench in a completely randomized design. Pots lacking rhizobial inoculation were placed behind a plastic barrier at the end of the bench to minimize contamination. Glyphosate was applied, in formulation (Roundup WeatherMAX®), 12 days following soybean transplant (first trifoliate growth stage) and the experiment was harvested 28 days following the herbicide application. This experiment was conducted during the winter months under a maximum PPFD of ~600 μmol/(m² s).
2.4. Measurement of response variables

Shoots were clipped at the soil surface and soil was gently washed away from roots. Nodules were counted, dried to constant mass (60 °C) and weighed. Five small subsamples of roots, selected systematically from different locations in the root system, were pooled, fixed in 70% ethanol, stained with chlorazol black E (Brundrett et al., 1984), and examined with a compound microscope at 200× magnification using a gridline-intersection method, which estimates the percentage of root length colonized by AM fungal hyphal structures (McConigle et al., 1990). AM hyphal colonization measures the prevalence of all AM fungal structures in roots, including arbuscules and vesicles, indicating overall hyphal growth. Arbuscular colonization specifically measures the prevalence of arbuscules, which are the AM fungal structures presumably associated with nutrient uptake by the host plant (Smith and Read, 1997), potentially indicating the physiological status of the mycorrhizal association.

Nutrient analyses were performed on shoot material harvested 4 and 8 weeks after soybean transplant in experiment #1 and all shoot material in experiment #2. Shoot material was oven-dried, to constant mass, at 60 °C for at least 48 h, then finely ground in a model 3 Wiley mill (Thomas Scientific, Swedesboro, NJ). Subsamples were pulverized in a MM300 Brinkmann Retsch sphere mill (Retsch Inc., Newtown, PA) then analyzed for total N content and the ratio of 15N:14N by mass spectrometry (Tracermass, ANA-MS, Europa Scientific Ltd., Crewe, UK) (Mulvaney, 1993). N₂-fixation was estimated by the isotopic dilution method and assessed based on the percentage of N derived from the atmosphere, which was calculated according to the equations reported by Fried and Middelboe (1977), with the non-inoculated soybean plants as the non-fixing control. We estimated total N derived from the atmosphere in the inoculated soybean plants as described by Fried and Middelboe (1977), as well as N derived from the atmosphere standardized by shoot biomass. No difference in 15N uptake was observed between AM and nonmycorrhizal soybean plants, whether or not they were rhizobial, in previous experiments (Antunes, unpublished data); therefore, the nonrhizobial, AM soybean plants were used as controls to calculate N derived from the atmosphere for both AM and nonmycorrhizal, rhizobial soybean plants. In experiment #1, additional subsamples were digested by dry ashing (Greweling, 1976) and analyzed for total P content using colorimetric methods (Thomas et al., 1967).

2.5. Statistical analyses

AM fungal colonization responses were arcsine [square root (proportion root segments colonized)]-transformed and rhizobial colonization and shoot biomass responses were square root-transformed to realize normality of error distributions (Shapiro-Wilk’s test). Nutrient responses did not require transformation. All analyses were performed in R v.1.16 (R Development Core Team, 2006). For the first experiment, we analyzed the responses with linear mixed effects models using the “nlme” package (Pinheiro et al., 2006). Harvest date was treated as a random effect. Because of the unbalanced design, fixed effects (glyphosate, inoculum) and their interactions with other fixed or random effects were evaluated by testing the likelihood ratio observed when comparing the null and alternative models against a random distribution of 999 simulated likelihood ratios (Faraway, 2006). We also compared models with different variance assumptions using likelihood ratio tests (Pinheiro and Bates, 2000). Contrast coefficients in the model summary, specified a priori, were used to separate treatment means. For the second experiment, which employed a balanced design with all factors fixed, we analyzed the responses with conditional F-tests in ANOVA and, where appropriate, performed multiple comparisons with Tukey’s honest significant differences test. We confirmed homogeneity of error distributions using Levene’s test.

P-values of significance (<0.05) and marginal significance (<0.10) are reported for interpretive purposes. P-Values greater than 10% are reported as such.

3. Results and discussion

3.1. Establishment and functioning of the symbioses

AM fungal colonization of soybean roots was observed for all AM fungal-inoculated pots in both experiments. Functional consequences were observed in the symbiosis in the first experiment: both shoot P (P < 0.001) and whole-shoot P (P < 0.001) were greater for AM fungal inoculated soybean plants. However, shoot dry mass (P = 0.053) and root dry mass (P = 0.045) were significantly/marginally significantly reduced for AM fungal-inoculated soybean plants, indicating a negative interaction between the symbionts. This result is not entirely unexpected as Klironomos (2003) observed symbiotic outcomes on plant biomass ranging from increases to decreases when combining different plant and fungal species. Others (Johnson, 1993; Johnson et al., 1997) have observed that symbiotic outcomes are dependent on environmental factors (e.g., nutrient availability). We observed no effects of AM fungal inoculation on shoot or root dry mass in the second experiment (P > 0.10); tissue P was not estimated.

Rhizobial colonization was observed for all B. japonicum-inoculated pots in both experiments, and also for some non-inoculated pots. The latter was likely a result of using microbial washes (see Section 2.1) to establish a microflora with which AM fungi could potentially interact. Whole-plant nodulation was greater on B. japonicum-inoculated relative to uninoculated root systems (P < 0.001 in both experiments). Whole-plant nodule mass, on the other hand, was similar for inoculated and uninoculated root systems (P > 0.10 for both experiments), due to a few large nodules on uninoculated root systems. Rhizobial inoculation reduced shoot dry mass (P = 0.004) and root dry mass (P < 0.001), suggesting a negative association in the first experiment; no effect of rhizobial inoculation was observed for either estimate in the second experiment (P > 0.10). Functionally, however, N uptake from soil was reduced [in experiment one (P = 0.004); marginally significant in experiment two (P = 0.08)], and whole-plant N unaffected (P > 0.10 for both experiments) when soybean plants were inoculated with B. japonicum, suggesting N-fixation was enhanced for these plants.
The lack of a proper negative rhizobial control hindered interpretation of the potential modifying effects of rhizobial colonization on AM fungal responses to glyphosate. However, it was still possible to make comparisons between soybean plants with low and high levels of rhizobial colonization and functioning.

Harvest date explained a large proportion of variation for all responses in the first experiment. For the most part, symbiont responses were consistent across all harvest dates (i.e., \( P > 0.10 \) for interactions between the fixed factors and harvest date). Therefore, harvest date is only discussed for responses where a significant interaction was detected.

3.2. Effects of glyphosate on \( G. \) intraradices

No effect of glyphosate on AM fungal or arbuscular colonization of soybean roots was observed, regardless of the timing or rate of glyphosate applied or the amount of time elapsed since the application \( (P > 0.10) \). Responses were consistent over a wide range of colonization levels, both in the first experiment and between the two experiments (Table 1). This result is consistent with those of the only other study that has, to our knowledge, assessed direct effects of glyphosate on the AM fungal symbiosis through the use of GM host plants (Mujica et al., 1999). In addition, no effect of glyphosate on tissue \([P]\) or whole-plant \( P \) of soybean shoots was observed in the first experiment, regardless of application timing or time elapsed \( (P > 0.10) \). (Tissue \([P]\) was not estimated in the second experiment.) These results leave open the possibility that AM hyphal networks may be useful for crop plants accessing recently mobilized nutrients following weed management in GMHT cropping systems. Others have demonstrated that nutrient transfer from decomposing plants via AM fungal hyphal networks can enhance crop nutrient uptake and productivity (Johansen and Jensen, 1996) and that this interaction could be exploited during weed control (Bethlenfalvay et al., 1996a, 1996b; Mujica et al., 1999).

3.3. Effects of glyphosate on \( B. \) japonicum

Responses observed here differed from those in other studies investigating glyphosate effects on \( B. \) japonicum and, thus, did not agree with the proposed hypothesis. First, no effect on whole-plant rhizobial colonization (number of nodules or nodule mass) was observed in either experiment \( (P > 0.10) \). Also, no response of specific nodulation and specific nodule mass to glyphosate was observed in the first experiment \( (P > 0.10) \). Application rate affected specific nodule mass in the second experiment [low dose: 122 mg (g root dry weight) \(^{-1} \), high dose: 138 mg (g root dry weight) \(^{-1} \); \( P = 0.030 \)]; however, this was likely due to an indirect effect of a marginally significant reduction in root dry mass \( (low \) dose: 162 mg, high dose: 141 mg; \( P = 0.068 \)) associated with the high glyphosate rate; whole-plant nodulation was unaffected \( (P > 0.10) \). Retrospective power analyses \((1 - \beta = 0.9) \) indicated that sufficient replication was employed to estimate nodulation precisely enough to detect reasonable differences among treatments (e.g., over ranges of 10–20%), if they existed.

Second, glyphosate-treated soybean plants exhibited increased concentrations of atmosphere-derived \( N \), relative to untreated soybean plants (experiment #1: \( P = 0.041 \); experiment #2: \( P = 0.042 \); Fig. 1). Whole-plant, atmosphere-derived \( N \) was not observed to be significantly affected by glyphosate (experiment #1: \( P = 0.10 \); experiment #2: \( P = 0.12 \)), but this may have been an artifact due to the additive variability when multiplying the shoot mass and [atmosphere-derived \( N \)]

Table 1 – Rhizobial and arbuscular mycorrhizal parameter estimates that were unaffected by glyphosate. Except where indicated, estimates are averaged across the glyphosate and control treatments. All estimates were measured in the presence of both arbuscular mycorrhizal (AM) and rhizobial symbionts and were backtransformed where appropriate (see Section 2.5). Estimates of \( N_2 \)-fixation and \( N \) uptake from soil are presented in Figs. 1 and 2.

<table>
<thead>
<tr>
<th>Units</th>
<th>1 week (^a)</th>
<th>4 weeks (^a)</th>
<th>8 weeks (^a)</th>
<th>4 weeks (^b)</th>
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<tr>
<td></td>
<td>Mean 95% CI</td>
<td>Mean 95% CI</td>
<td>Mean 95% CI</td>
<td>Mean 95% CI</td>
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<tr>
<td>AM colonization</td>
<td>% root length</td>
<td>6.3 2.9–10.8</td>
<td>52 37–67</td>
<td>86 73–95</td>
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<tr>
<td>Arbuscular colonization</td>
<td>% root length</td>
<td>4.4 1.7–8.2</td>
<td>39 26–53</td>
<td>53 41–65</td>
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<tr>
<td>Shoot P</td>
<td>g (kg plant (^{-1} ))</td>
<td>nd nd</td>
<td>2.3 2.1–2.6</td>
<td>1.3 1.2–1.4</td>
</tr>
<tr>
<td>Total shoot P</td>
<td>mg plant (^{-1} )</td>
<td>12 11–13</td>
<td>21 18–23</td>
<td>nd nd</td>
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<tr>
<td>Rhizobial parameters</td>
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</tr>
<tr>
<td>Nodules</td>
<td># plant (^{-1} )</td>
<td>22 14–30</td>
<td>99 83–117</td>
<td>178 156–201</td>
</tr>
<tr>
<td>Specific nodule</td>
<td># (g root (^{-1} ))</td>
<td>39 27–55</td>
<td>88 73–105</td>
<td>72 63–82</td>
</tr>
<tr>
<td>Nodule mass</td>
<td>mg plant (^{-1} )</td>
<td>nd nd</td>
<td>200 180–220</td>
<td>696 634–762</td>
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<tr>
<td>Specific nodule mass</td>
<td>mg (g root (^{-1} ))</td>
<td>178 156–202</td>
<td>284 265–303</td>
<td>differed (see text)</td>
</tr>
<tr>
<td>Shoot N</td>
<td>g (kg plant (^{-1} ))</td>
<td>nd nd</td>
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<td>25 22–28</td>
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<tr>
<td>Total shoot N</td>
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<td>424 389–458</td>
<td>15 13–17</td>
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<td>Root mass</td>
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<td>1.2 1.0–1.4</td>
<td>2.5 2.2–2.8</td>
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</table>

\( nd \): not determined.

\(^a\) Experiment 1.

\(^b\) Experiment 2.
estimates; no effect of glyphosate on shoot dry mass was observed when analyzed independently ($P > 0.10$ for both experiments). The effect was consistent across the two glyphosate application rates in the second experiment, but
was only observed for the early application treatment in the first experiment. No interaction was observed between glyphosate application and harvest date in the first experiment ($P > 0.10$).

Most other studies have observed negative effects of glyphosate on rhizobial colonization and/or N$_2$-fixation (Reddy et al., 2000; King et al., 2001; Reddy and Zablotowicz, 2003; Zablotowicz and Reddy, 2004). The reasons for the inconsistencies between these data and those of other studies are not clear. Variability among soybean varieties in glyphosate-tolerance or translocation of glyphosate to nodules may help explain the different reported outcomes (King et al., 2001). Similarly, variability exists among $B$. japonicum isolates in their tolerance to glyphosate or its metabolites (Moorman et al., 1992; dos Santos et al., 2005; Malty et al., 2006). Also, adjuvants and surfactants differ among glyphosate formulations and can have toxic effects on various biota, although these responses are usually more important (relative to glyphosate toxicity) for biota that do not synthesize the glyphosate-susceptible EPSPS (Atkinson, 1985).

One similarity between our results and those of others is the importance of application timing on the responses. In the first experiment, N$_2$-fixation by $B$. japonicum was only stimulated when glyphosate was applied within 2 weeks of transplanting seedlings. King et al. (2001) observed that significant effects on nodule biomass were only observed when glyphosate was applied within 12 days following emergence, and no effect was observed when applied after 18 days following emergence. Even though the responses were in opposite directions, they reveal a consistent pattern in the interaction between timing and the response of rhizobial parameters. This pattern may arise due to higher concentrations of translocated glyphosate in smaller root systems. The agronomical consequences of this result, however, are unclear as the soybean plants grown here compensated for increased rhizobial N$_2$-fixation by reducing N uptake from soil ($P = 0.013$ for experiment #1; $P < 0.001$ for experiment #2; Fig. 2). No overall glyphosate effect on tissue [N] was detected in the first experiment ($P > 0.10$), whereas a significant effect on tissue [N] was observed in the second experiment ($P = 0.042$; however, the multiple comparison test was unable to detect differences among the treatments, indicating marginal significance).

### 3.4. Effect of glyphosate on the tripartite symbiosis

There was partial support for the AM fungal symbiont enhancing rhizobial performance in the presence of glyphosate. In the first experiment, the stimulatory effect of glyphosate on N$_2$-fixation was dependent on simultaneous colonization by $G$. intraradices; N$_2$-fixation was not increased following glyphosate application in the absence of the AM fungus (Fig. 3). This glyphosate × AM fungal inoculation interaction was observed for both the concentration ($P = 0.014$) and whole-plant uptake ($P = 0.043$) of atmosphere-derived N. It was also strongest 4 weeks following the nonrhizobial treatment; these values underestimate absolute levels of N$_2$-fixation but are accurate for contrasting effects among treatments.

Fig. 3 – Interactive effects of glyphosate and inoculation with Glomus intraradices on N$_2$-fixation by Bradyrhizobium japonicum-colonized soybean plants, 4 weeks following herbicide application. The interaction between the glyphosate and G. intraradices factors was significant in experiment 1 ($P < 0.05$), but not experiment 2. For experiment 1, early refers to glyphosate (0.9 kg ae ha$^{-1}$) applied 10 days following seedling transplant. For experiment 2, low and high refer to glyphosate applied at 0.9 and 1.8 kg ae ha$^{-1}$, respectively, 12 days following seedling transplant. For both experiments, control refers to untreated soybean plants. NM and AM refer to nonmycorrhizal and arbuscular mycorrhizal treatments, respectively. Bars labeled with different letters, within each inoculation treatment for each experiment, are significantly different ($P < 0.05$); error bars indicate standard error estimates. Negative values for N$_2$-fixation are due to unintended nodulation of soybean plants in the
glyphosate application, disappearing by the 8-week point (harvest date interaction: \( P = 0.023 \)). Again, soybean plants compensated for increased N\(_2\)-fixation by reducing N uptake from soil ([soil-derived N]: \( P = 0.013 \), whole-plant soil-derived N: \( P = 0.084 \); [N] and whole-plant N: \( P > 0.10 \)). However, when this treatment combination was repeated in the second experiment, the stimulatory effect of glyphosate on N\(_2\)-fixation occurred regardless of AM fungal colonization by G. intraradices (Fig. 3).

Given that the experiments were conducted in the same soil medium and under identical nutrient conditions, perhaps other factors were responsible for mediating the response. For example, a fungus gnat (Diptera, Sciaridae) infestation may have influenced the outcome of the second experiment. Alternatively, the infestation may have been symptomatic of adequate or excessive levels of soil moisture. AM fungi can also enhance drought-tolerance of host plants (Khalvati et al., 2005); others have suggested that water stress, which was likely to have occurred more frequently in the first experiment when greenhouse temperature routinely exceeded 25 °C, can amplify the negative effects of glyphosate on nodulation (King et al., 2001). Additionally, light intensity varied between the two experiments, given the times of year that they were conducted; therefore, differences between the experiments in photosynthetic exchange in each symbiosis or manifestation of heat stress may have resulted in the different result. However, several physiological parameters that were not quantified are required to evaluate these hypotheses.

No effects of rhizobial inoculation on mycorrhizal responses to glyphosate were observed in either experiment (\( P > 0.10 \)), except for a marginally significant decline in shoot [P] (but not whole-shoot [P]) following glyphosate application in Br. japonicum-inoculated, but not uninoculated, soybean plants in the first experiment (\( P = 0.071 \)). It is possible that enhanced N\(_2\)-fixation following glyphosate application is responsible for the observed reduction in shoot [P].

4. Conclusions

These data support those of previous studies demonstrating a lack of effect of glyphosate on the AM fungal symbiosis. However, these data deviate from those of studies that observed negative effects of glyphosate on establishment and functioning of rhizobial symbioses. At least part of this confusion may be due to interactions between soybean symbioses before, during, and after glyphosate application; however, we currently know little about how the tripartite symbiosis responds to herbicide use or other types of environmental change, with the exception of tillage-related disturbance (Goss and De Varennes, 2002; Antunes et al., 2006; Chalk et al., 2006). Future research needs to consider the role of environmental factors and other biota when evaluating rhizobial responses to herbicide applications.

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