Influence of PRE-Emergence Herbicides on Soybean Development, Root Nodulation and Symbiotic Nitrogen Fixation

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ABSTRACT

Widespread occurrence of herbicide-resistant weeds has further limited effective POST-emergence herbicide options in soybean (*Glycine max* (L.) Merr.) leading to an increased adoption of PRE-emergence herbicides. The objective of this study was to investigate the influence of 11 commonly used PRE-emergence herbicides on soybean development, root nodulation, and symbiotic N fixation. Soybean plants were grown under greenhouse conditions in pots (10 L; 4 plants per pot) filled with silt loam soil and treated one day after planting with a labeled field rate of imazethapyr, chlorimuron-ethyl, cloransulam-methyl, metribuzin, sulfentrazone, flumioxazin, saflufenacil, acetochlor, *S*-metolachlor, dimethenamid-P, pyroxasulfone and no herbicide (untreated control). Sulfentrazone reduced soybean canopy at the VC growth stage but no canopy reduction was observed at the V2 growth stage from any of the herbicide treatments. At the R2 growth stage, herbicides had no effect on soybean development (root and shoot biomass), root nodulation (# nodule per plant, nodule diameter, and nodule biomass) and symbiotic N fixation (acetylene reduction assay and ^{15}N natural abundance). According to our findings, although PRE-emergence herbicides may slightly affect early-season soybean development, the impacts on plant growth, root nodulation, and symbiotic N fixation were negligible. Thus, when sprayed according to the label, the benefits of PRE-emergence herbicides for weed control likely outweigh any potential concern regarding soybean development, root nodulation, and N fixation.

Keywords: PRE-emergence herbicides; *Bradyrhizobium japonicum*; root nodulation; soybean; nitrogen fixation

Abbreviations: ALS, acetolactate synthase; ARA, acetylene reduction assay; DAT, days after treatment; GR, glyphosate-resistant; N, nitrogen; Ndfa, nitrogen derived from the atmosphere; OM, organic matter; POST, POST-emergence; PPO, protoporphyrinogen oxidase; PRE, PRE-emergence; PSII, photosystem II; SOA, sites of action; US, United States; VLCFA, very-long-chain fatty acid; 1×, label rate; 5×, five times label rate

1. Introduction

PRE-emergence (PRE) herbicides are recommended in soybean (*Glycine max* (L.) Merr.) production systems for management of weed species with extended emergence window. Additionally, the use of PRE herbicides is considered a crucial component for management of glyphosate-resistant (GR) weeds (Norsworthy et al., 2012). PRE herbicides were commonly used in soybean production; however, herbicide use trends changed drastically due to the rapid and widespread adoption of GR soybean cultivars in the United States (US) in the late 1990s, leading to increased reliance on glyphosate alone for POST-emergence (POST) weed control (Young, 2006; Benbrook, 2016; Kniss, 2017). Overreliance on glyphosate has resulted in rapid evolution of GR weeds (Johnson et al., 2009), consequently between 1990 and 2020, 17 different weed species evolved resistance to glyphosate in the US alone (Heap, 2020).

Due to the widespread prevalence of GR weeds and limited effective POST herbicide options in soybean, the use of PRE herbicides has become a standard recommendation for weed management in the US (Norsworthy et al., 2012). As a result, total soybean planted area treated with herbicides applied PRE in the US has increased from 2006 to 2017 where the area treated with metribuzin [Photosystem II (PSII)-inhibitor],

62 sulfentrazone [Protoporphyrinogen oxidase (PPO)-inhibitor] and *S*-metolachlor [Very-long-
63 chain fatty acid (VLCFA)-inhibitor] increased from 2 to 18%, 1 to 22% and 1 to 16%,
64 respectively (USDA, 2020).

65 Benefits of incorporating PRE herbicides into weed management programs include
66 reduced early season weed competition and delayed critical time for weed removal, thus
67 optimizing weed control strategies and minimizing potential crop yield loss (Oliveira et al.,
68 2017a; Knezevic et al., 2019). PRE herbicides can delay the first POST application by 2 to
69 5 weeks reducing the need for repeated POST herbicide applications (Knezevic et al.,
70 2019). Oliveira et al. (2017b) reported effective use of PRE herbicides for control of several
71 annual broadleaf and grass species in Nebraska. Additionally, the use of PRE herbicides is
72 considered a foundation for management of troublesome weeds such as kochia (*Bassia*
73 *scoparia* (L.) A.J. Scott) and *Amaranthus* spp. (Whitaker et al., 2011; Kumar & Jha, 2015).

74 On the other hand, early-season soybean injury due to PRE herbicide applications is
75 a common concern amongst growers (Mahoney et al., 2014a, 2014b). For instance, soybean
76 injury by metribuzin, sulfentrazone, flumioxazin, saflufenacil and *S*-metolachlor
77 applications have been documented in previous research (Miller et al., 2012; Mahoney et
78 al., 2014b; Belfry et al., 2015). The level of soybean injury can be related to both cultivar
79 tolerance and environmental conditions. Cool and wet environmental conditions increase
80 the likelihood of soybean injury as these herbicides are readily available in the soil for plant
81 uptake and cool temperatures decrease the crop's ability to metabolize the herbicides
82 (Hulting et al., 2001; Poston et al., 2008; Miller et al., 2012).

83 The inoculation of soybean seeds with the Rhizobia bacteria *Bradyrhizobium*
84 *japonicum* (Kirchner 1896) Jordan 1982, is a common practice in soybean production as

these bacteria symbiotically colonize soybean roots and fix atmospheric nitrogen (N), providing a renewable source of N for soybean plants (Mohammadi et al., 2012; Zimmer et al., 2016). For instance, Salvagiotti et al. (2008) documented that soybean symbiotic N fixation ranged from 0 to 337 kg N ha^{-1} and 50-60% of soybean N demand came from the atmospheric N_2 fixing process. Comparatively, Mastrodomenico and Purcell (2012) observed a higher contribution, where approximately 90% of seed N content and 97% of total plant N uptake came from symbiotic N fixation. There has been limited research investigating the impact of PRE herbicides on this symbiotic relationship (Chikoye et al., 2014; Aliverdi & Ahmadvand, 2018). If PRE herbicides negatively impact soybean development and root nodulation, symbiotic N fixation may be decreased and could negatively affect soybean grain yield and soil N availability for subsequent crops. As PRE herbicides continue to be integral to weed control in soybean production systems, research evaluating their impact on soybean development, root nodulation, and symbiotic N fixation is necessary as such information is not readily available in the literature. Thus, the objective of this study was to investigate the influence of 11 commonly used PRE herbicides on soybean development, root nodulation, and symbiotic N fixation. Our hypothesis was that PRE herbicides, applied following label recommendations, would not impact soybean development, root nodulation, and symbiotic N fixation. This research consisted of a comprehensive list of PRE herbicides from four sites of action (SOA) commonly used in soybean production throughout the US and beyond.

2. Materials and Methods

107 2.1 Experiment Background

108 A greenhouse experiment was conducted in 2019 to investigate the influence of 11
109 PRE herbicides from four different SOA on soybean development, root nodulation, and
110 symbiotic N fixation. The experiment was conducted at the Walnut Street Greenhouse
111 (43°04'33" N, 89°25'27" W), University of Wisconsin-Madison, Madison, WI, US. The
112 soil used in this experiment [silt loam (16% sand, 61% silt and 23% clay), pH of 6.9 (H_2O)
113 and 6.4% organic matter (OM)] was collected from a certified organic field (no history of
114 synthetic herbicide use) at Arlington Agricultural Research Station (43.301890º N,
115 89.344900º W). The experimental unit consisted of a 10 L pot (29 and 28 cm in diameter
116 and height, respectively) filled with the field soil. The soil was not fertilized during the
117 greenhouse experiment. Soybean seeds, cultivar AG24X7 (Bayer Crop Science, St. Louis,
118 MO), were inoculated with *B. japonicum* (Cell-Tech Liquid, Bayer Crop Science, St. Louis,
119 MO) at the rate of 1.4 mL inoculant per 500 g seeds. Six seeds were sown per experimental
120 unit (at 5 cm depth) following inoculation with *B. japonicum*. To standardize comparisons
121 amongst treatments, experimental units were thinned to a final density of 4 plants per
122 experimental unit, 7 days after planting (thinned plants were randomly selected). The
123 treatments consisted of 11 PRE herbicides plus an untreated control (Table 1).

124

Table 1. PRE-emergence herbicide active ingredients, site of action, herbicide family, and rate used in the greenhouse experiment.

Treatment	Site of Action†	Herbicide Family	Rate (g ai ha^{-1})
untreated control	-	-	-
imazethapyr	ALS	Imidazolinone	70
chlorimuron-ethyl	ALS	Sulfonylurea	53

cloransulam-methyl	ALS	Triazolopyrimidine	35
metribuzin	PSII	Triazinone	563
sulfentrazone	PPO	Aryl triazinone	280
flumioxazin	PPO	N-phenylphthalimide	107
saflufenacil	PPO	Pyrimidinedione	25
acetochlor	VLCFA	Chloroacetamide	1260
S-metolachlor	VLCFA	Chloroacetamide	1787
dimethenamid-P	VLCFA	Chloroacetamide	945
pyroxasulfone	VLCFA	Pyrazole	179

† Acetolactate synthase (ALS)-, photosystem II (PSII)-, protoporphyrinogen oxidase (PPO)-, and very-long-chain fatty acid (VLCFA)-inhibiting herbicides.

Herbicides were applied one day after planting the soybean seeds using a research track sprayer (DeVries Manufacturing, Generation 3, Hollandale, MN) equipped with a TP8002E (Teejet, Springfield, IL) nozzle calibrated to deliver 140 L ha^{-1}. Experimental units were watered to field capacity immediately following herbicide application and repeated daily for the remainder of the experiment. The experiment was conducted in a randomized complete block design with six replications and replicated twice over time (14 days apart). Greenhouse conditions (21 ºC minimum, 26 ºC average, 32 ºC maximum with 45% average relative humidity) were monitored with a WatchDog A150 Temp/RH logger (Spectrum Technologies, Aurora, IL). Artificial lighting was provided using metal halide lamps (600 µmol m^{-2} s^{-1}) to ensure 15 h photoperiod.

2.2 Soybean Development and Root Nodulation

To investigate potential herbicide injury on early-season soybean development, soybean canopy was assessed at the VC (vegetative stage cotyledon) and V2 (two open trifoliates) growth stages, 10 and 20 days after treatment (DAT), respectively, through photos taken of each experimental unit approximately 30 cm above plant canopy using an Apple iPhone 8 plus camera (Apple Inc., Cupertino, CA) in the square mode. The photos were processed using the Canopeo Software (Canopeo App, Oklahoma State University, Stillwater, Oklahoma) which was developed in Matlab programming language (Mathworks, Inc., Natick, MA) to evaluate fractional green canopy cover. In the Canopeo Software, green canopy coverage is estimated on a 0 (no green canopy cover) to 1 (100% green canopy cover) range (Patrignani & Ochsner, 2015).

To evaluate soybean root and shoot biomass and root nodulation, plants were sampled at the R2 growth stage (45 DAT). Entire plants were carefully collected from each experimental unit, shoots and roots were separated, roots were gently washed in a bucket with water to remove excess soil, and the nodules were manually removed from the roots. Nodules were enumerated and the diameter of 20 randomly selected nodules from each experimental unit were measured using a digital caliper (IP54, EAGems, Palmdale, CA). Nodule activity was assessed from these same 20 nodules by slicing in half and considering those with internal pink coloration as fixing and those not pink as non-fixing nodules (Somasegaran & Hoben, 1985). Soybean shoots, roots and nodules were force air-dried (70 ºC) to constant weight and their respective biomass recorded.

159 2.3 Symbiotic Nitrogen Fixation

160 2.3.1 Acetylene Reduction Assay

161 Symbiotic N fixation was estimated using the acetylene reduction assay (ARA)
162 which is a technique that measures the nitrogenase activity through the reduction of
163 acetylene (C_2H_2) to ethylene (C_2H_4) (Dilworth, 1966; Hardy & Knight, 1967; Stewart et al.,
164 1967). Specific ARA methodology used in this experiment was adapted from David et al.
165 (1980). The ARA was performed using 10 cm root samples with approximately 10 nodules
166 attached as well as 10 cm non-nodulated root samples (serving as negative control)
167 collected from each experimental unit at the R2 growth stage. The non-nodulated roots
168 were used to measure the ethylene produced naturally by the plant in response to the tissue
169 damage during sampling. The root samples were placed into a 10 mL airtight glass
170 container with 1 mL of sterile water and sealed with a rubber septum lid. One mL of air
171 was collected from each container and replaced with 1 mL of Atomic Absorption 2.6 Grade
172 Acetylene (Airgas #AC AA4). After 24 hours of incubation at room temperature (22 ºC), 1
173 mL gas sample was taken by a HS-10 Headspace Gas Chromatography Sampler
174 (Shimadzu, Columbia, MD) and injected in a GC-2010 gas chromatograph (Shimadzu,
175 Columbia, MD) equipped with Rt®-Alumina BOND/KCL 50m, 0.53mmID, 10µm
176 RESTEK (CAT#19760) column set at 100 ºC for analysis. Methane, acetylene, and
177 ethylene gases present in the samples were recognized through the LabSolutions software
178 (Shimadzu, version 5.82, Columbia, MD) at the retention peaks of 1.853, 2.082 and 3.015
179 minutes after sample injection, respectively. The ethylene peak area produced per sample
180 was converted using a standard curve of ethylene dilutions into 10% acetylene to estimate
181 the ethylene concentration in each sample (Hardy et al., 1968). The ethylene production

182 from the non-nodulated root samples (negative control; with average of 0.00013 and error
183 of $1.4x10^{-5}$ µmol of ethylene) was subtracted from each reading, and the nitrogenase
184 activity was estimated as µmol ethylene $nodule^{-1}$ $hour^{-1}$. The gas chromatograph was
185 configured with SPL1 at 200 ºC, 73.5kPa with 171mL min^{-1} of total flow, and 3mL min^{-1} of
186 purge flow. The detector (flame ionization) was set at 200 ºC, with 30mL min^{-1} of makeup
187 flow, 40mL min^{-1} of H_2 and 400mL min^{-1} of air flow (Ye et al., 2013). Since the nodulated
188 and non-nodulated root samples used to perform the ARA analysis were collected from the
189 specific plants used to estimate root and shoot growth and root nodulation, their final dry
190 biomass, nodule counts, and nodule weight were added to the corresponding measurements
191 before statistical analysis.
192
193 2.3.2 ^{15}N Natural Abundance
194 The ^{15}N natural abundance method relies on the ratio of stable N isotopes in the
195 plant tissue, which can come from either the atmosphere (atmospheric N_2) or soil (soil
196 mineral N) (Amarger et al., 1979; Mariotti, 1983; Unkovich et al., 2008). To conduct this
197 analysis, a tissue sample from the newest fully developed trifoliate leaf was collected from
198 a random soybean plant from each experimental unit at the R2 growth stage (Shearer &
199 Kohl, 1986). Soybean leaf samples were placed in 2 mL microcentrifuge tubes
200 (Fisherbrand, Pittsburgh, PA), dried at 70 ºC for 48 hours and ground into a fine powder
201 using a glass bead per tube and processed in a Mixer Mill MM 400 (Retsch, Haan,
202 Germany) for 2 minutes agitating 30 times per second. Approximately 2.0-2.2 mg of the
203 powder was weighed into tin capsules for analysis of N content and ^{15}N abundance. The
204 ^{15}N natural abundance was estimated as:

$$\delta 15N\ (‰) = \frac{atom\%15Nsample - atom\%15Natmosphere}{atom\%15Natmosphere}\ x\ 1000 \quad \text{Equation 1:}$$

where atom% ^{15}Nsample is the abundance of ^{15}N atoms expressed as a percentage of the total N present (^{15}N / (^{14}N + ^{15}N)) x 100; and atom% ^{15}Natmosphere is the ^{15}N abundance of atmospheric N_2 which for the standard is 0.3663 (Shearer & Kohl, 1986; Unkovich et al., 2008). Additionally, the ^{15}N natural abundance in the soil was assessed from 4 composite soil samples obtained from sample cores collected from 4 random experimental units in each replication of the study. The samples were placed in 50 mL Falcon tubes and stored in a freezer (-20 ºC). The soil samples were air dried, ground by mortar and pestle, and weighed (38 mg) into tin capsules. The soil and plant samples were analyzed for total N and δ^{15}N using a PDZ-Europa ANCA elemental analyzer linked to a PDZ-Europa 20-20 stable isotope mass spectrometer (Sercon, Ltd., Crewe, United Kingdom). The percentage of plant N derived from the atmosphere (%Ndfa) was estimated as:

$$\%\ Ndfa = \frac{\delta 15N\ of\ soil\ N - \delta 15N\ of\ N2\ fixing\ legume}{\delta 15N\ of\ soil\ N - \delta 15N\ of\ N2}\ x\ 100 \quad \text{Equation 2:}$$

where δ^{15}N of soil N reflects the ^{15}N abundance of the soil which was found to average 7.07 $^{0}/_{00}$; δ^{15}N of N_2 fixing legume is the natural ^{15}N abundance in the legume; and δ^{15}N of N_2 is the aboveground ^{15}N abundance which is 0 $^{0}/_{00}$ assuming the plant is using atmospheric N_2 as the only N source for growth (Shearer & Kohl, 1986; Unkovich et al., 2008).

2.4 Statistical Analyses

The statistical analyses were performed using R statistical software (version 3.5.1; R Core Team, 2018). The Shapiro-Wilk test was performed to test for normality and the

228 Levene's test assessed homogeneity of residual variance of the dataset. Root biomass per
229 plant, shoot biomass per plant, nodule biomass per plant, number of nodules per plant,
230 nodule diameter, ARA, $\delta^{15}N$ and %Ndfa were subjected to ANOVA using a mixed-effect
231 model. No statistical analysis was conducted for nodule activity as all nodules were
232 determined to be active. In the models, herbicide treatments were considered as fixed effect
233 and the replications nested within experimental runs were treated as random effect. The
234 ARA data were square root transformed to meet the ANOVA assumptions of normality and
235 homogeneity of residual variance before analysis; back-transformed data are presented
236 herein for ease of result interpretation. Soybean canopy data (0 to 100%) at the VC and V2
237 growth stage were subjected to ANOVA using the beta distribution (family logit); herbicide
238 treatments were considered as fixed effect and the replications nested within experimental
239 runs were treated as random effect. For all response variables evaluated herein, if ANOVA
240 indicated significant treatment effects ($P<0.05$), the means were separated using Fisher's
241 protected LSD test.
242
243 3. Results
244 3.1 Soybean Development and Root Nodulation
245 The PRE herbicides tested in this study had minimal to no influence on early season
246 soybean canopy development. Soybean canopy was only affected during the VC growth
247 stage assessment ($P<0.01$; Table 2), when the sulfentrazone treatment reduced soybean
248 canopy by 27% compared to the untreated control. Herbicide treatments had no impact on
249 soybean canopy development at the V2 growth stage assessment when compared to the
250 untreated control treatment ($P=0.096$; Table 2). Moreover, there was no impact of any PRE

herbicide tested in this study on soybean root (P=0.207) and shoot (P=0.454) biomass per plant at the R2 growth stage (Table 3). The PRE herbicides tested in this study also had no impact on root nodulation including number of nodules per plant (P=0.154), nodule diameter (P=0.362), nodule activity (all nodules evaluated were pink in color thus considered fixing nodules; data not shown), and nodule biomass per plant (P=0.203) at the R2 soybean growth stage (Table 3).

Table 2. Soybean canopy (% green canopy cover plant^{-1}) assessed at the VC (10 DAT) and V2 (20 DAT) soybean growth stages in the greenhouse experiment.

Treatment	% canopy (LCI - UCI)†	
	VC (10 DAT) ††	V2 (20 DAT)
untreated control	3.34 (2.87-3.90) abc	6.45 (5.70-7.29)
imazethapyr	3.56 (3.07-4.13) ab	6.53 (5.77-7.37)
chlorimuron-ethyl	2.87 (2.43-3.37) cd	5.20 (4.54-5.95)
cloransulam-methyl	3.19 (2.73-3.73) abc	6.75 (5.98-7.60)
metribuzin	3.17 (2.71-3.70) bc	6.08 (5.36-6.89)
sulfentrazone	2.41 (2.02-2.87) d	6.23 (5.49-7.05)
flumioxazin	2.76 (2.34-3.25) cd	5.65 (4.96-6.44)
saflufenacil	3.34 (2.86-3.89) abc	6.16 (5.43-6.97)
acetochlor	3.86 (3.30-4.51) a	6.49 (5.73-7.33)
S-metolachlor	3.63 (3.13-4.21) ab	6.10 (5.38-6.92)
dimethenamid-P	2.86 (2.43-3.37) cd	5.59 (4.90-6.37)
pyroxasulfone	3.58 (3.09-4.16) ab	6.42 (5.68-7.26)
p-value	<0.001	0.096

†Means within a column followed by the same letter are not different at the 5% level according to Fisher's LSD test. Lower Confidence Interval (LCI) and Upper Confidence Interval (UCI) at 95%.

†† VC, unifoliate leaves; V2, two trifoliates; DAT, days after treatment

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Table 3. Soybean root, shoot, nodule biomass and number of nodules plant^{-1}, nodule diameter, ARA, δ^{15}N and %Ndfa assessed at the R2 growth stage (45 DAT) in the greenhouse experiment.

Treatment	Biomass (g plant^{-1})†			Nodule†		Nitrogen fixation†		
	Root	Shoot	Nodule	# Plant^{-1}	Diameter (mm)	ARA (µmol ethylene nodule h^{-1})	δ^{15}N ($^{0}/_{00}$)	%Ndfa
	------ mean (LCI – UCI)†† ------							
untreated control	0.83 (0.65-1.00)	5.0 (4.3-5.6)	0.14 (0.10-0.18)	51 (38-62)	2.9 (2.7-2.9)	0.04 (0.01-0.07)	5.21 (4.16-6.26)	26.3 (11.4-41.2)
imazethapyr	0.94 (0.77-1.11)	4.8 (4.1-5.4)	0.14 (0.10-0.18)	49 (37-60)	2.8 (2.6-2.8)	0.05 (0.01-0.09)	4.33 (3.27-5.38)	38.8 (23.9-53.7)
chlorimuron-ethyl	0.89 (0.71-1.06)	4.2 (3.6-4.8)	0.08 (0.03-0.11)	35 (23-46)	2.7 (2.6-2.8)	0.01 (0.01-0.04)	5.85 (4.80-6.91)	17.2 (2.3-32.1)
cloransulam-methyl	0.96 (0.78-1.13)	4.8 (4.1-5.3)	0.14 (0.10-0.18)	48 (36-59)	2.8 (2.7-2.9)	0.04 (0.01-0.08)	4.43 (3.38-5.48)	37.4 (22.4-52.3)
metribuzin	0.73 (0.55-0.90)	4.5 (3.9-5.1)	0.10 (0.05-0.13)	55 (43-66)	2.7 (2.6-2.8)	0.02 (0.01-0.05)	4.82 (3.77-5.88)	31.8 (16.9-46.7)
sulfentrazone	0.90 (0.72-1.07)	4.7 (4.1-5.3)	0.11 (0.07-0.15)	47 (35-58)	2.7 (2.6-2.8)	0.05 (0.01-0.09)	4.55 (3.49-5.60)	35.7 (20.7-50.6)
flumioxazin	0.98 (0.80-1.15)	5.2 (4.5-5.8)	0.12 (0.08-0.16)	50 (38-61)	2.7 (2.5-2.8)	0.04 (0.01-0.07)	4.38 (3.32-5.43)	38.1 (23.2-53.0)
saflufenacil	0.80 (0.62-0.97)	5.0 (4.3-5.6)	0.11 (0.07-0.15)	47 (35-58)	2.7 (2.6-2.8)	0.04 (0.01-0.07)	4.51 (3.46-5.56)	36.2 (21.3-51.1)
acetochlor	0.81 (0.63-0.98)	4.7 (4.0-5.3)	0.12 (0.07-0.15)	44 (32-55)	2.8 (2.7-2.9)	0.03 (0.01-0.05)	4.96 (3.91-6.01)	29.8 (14.9-44.7)
S-metolachlor	0.85 (0.67-1.02)	4.5 (3.8-5.1)	0.10 (0.05-0.13)	41 (28-52)	2.8 (2.6-2.8)	0.05 (0.01-0.08)	4.08 (3.03-5.13)	42.3 (27.4-57.2)
dimethenamid-P	0.78 (0.60-0.95)	4.6 (3.9-5.2)	0.11 (0.06-0.14)	44 (32-55)	2.7 (2.6-2.8)	0.06 (0.02-0.1)	5.80 (4.70-6.89)	18.0 (2.53-33.5)
pyroxasulfone	0.85 (0.67-1.02)	4.6 (3.9-5.2)	0.12 (0.08-0.16)	50 (38-61)	2.8 (2.6-2.9)	0.02 (0.01-0.04)	4.39 (3.25-5.53)	37.9 (21.7-54.1)
p-value	0.207	0.454	0.203	0.154	0.362	0.254	0.215	0.215

† # plant^{-1}, number of nodules plant^{-1}; ARA, acetylene reduction assay; δ^{15}N, natural abundance relative to atmospheric N_2; %Ndfa, percentage of plant N derived from the atmosphere.

†† Lower Confidence Interval (LCI) and Upper Confidence Interval (UCI) at 95%.

260

261 3.2 Acetylene Reduction Assay and ^{15}N Natural Abundance

262 Corroborating the root nodulation findings, the PRE herbicides also did not
263 influence soybean N fixation according to the ARA (P=0.254), $\delta^{15}N$ (P=0.215) and %Ndfa
264 (P=0.215) assessments (Table 3). The ^{15}N natural abundance method depends on the ^{15}N
265 content difference between a legume plant and a sample reference source, a non-nitrogen
266 fixing neighbor plant or soil. The average of $\delta^{15}N$ natural abundance in the field soil used in
267 this experiment was 7.07 $^{0}/_{00}$, higher than in the sampled soybean leaves, 4.79 $^{0}/_{00}$, which
268 reflects the relative contribution from fixed atmospheric N_2 (Table 3) (Unkovich et al.,
269 2008). According to our results, an average of 32.4% (%Ndfa; Table 3) of the N was
270 derived from the atmosphere across treatments.

271

272 4. Discussion

273 The 11 PRE herbicides applied at their respective label rate to a silt loam soil did
274 not influence soybean growth, root nodulation, and N fixation at the R2 growth stage in this
275 greenhouse experiment. The only impact observed was a slight reduction on soybean
276 canopy by sulfentrazone at the VC growth stage. Early season soybean injury is a common
277 concern amongst producers who adopt PRE herbicides (Walsh et al., 2015). Early season
278 sulfentrazone injury in soybeans has been documented by Arsenijevic et al. (2020);
279 however, soybeans overcame injury and no yield loss was observed in their field study.
280 Taylor-Lovell et al. (2001) also reported that 15 soybean varieties were sensitive to
281 sulfentrazone applied at three rates (112, 224 and 446 g ai ha^{-1}) and injury increased as rate
282 increased. Contrasting our results, metribuzin (450 g ai ha^{-1}) and trifluralin (120 g ai ha^{-1})
283 were observed to negatively affected soybean shoot and root biomass in a Eutric Cambisol

284 soil (24% sand, 47% silt, 29% clay, 0.7% OM) under different pH levels (6.4, 7.8 and 8.0)
285 (Aliverdi & Ahmadvand, 2018). Alternatively, Mallik and Tesfai (1985) observed that
286 alachlor and trifluralin applied at 1.7 (1×) and 8.5 kg ha^{-1} (5×), and 0.56 (1×) and 2.8 kg ha^{-1}
287 (5×), respectively, had no impact on soybean growth in a sandy loam soil (79% sand, 14%
288 silt, 7% clay, pH=6.1 and 1.2% OM), except for trifluralin at the highest rate which reduced
289 soybean shoot biomass.

290 The presence of only N-fixing nodules at the R2 growth stage in this study indicated
291 no herbicide adversely affected soybean nodule activity. Bollich et al. (1985) demonstrated
292 that metribuzin (0.3 kg ha^{-1}) reduced nodule dry weight in a soil with coarse texture (57%
293 sand, 37% silt, and 6% clay) and low OM content (0.6%). However, no herbicide impact
294 was observed on nodule weight in the other soil types tested in their study, which were
295 finer in texture and had higher organic matter content (Bollich et al., 1985). Furthermore,
296 the number of nodules was not influenced by any PRE herbicide tested in their study
297 (Bollich et al., 1985). Conversely, Chikoye et al. (2014) reported that soybean grown in a
298 coarse soil (sand 56%, clay 10%, silt 34 %, pH=5.9 and 0.53% OM) treated with four doses
299 of pendimethalin (1.0, 2.0, 4.0 and 8.0 kg ai ha^{-1}) presented lower number of nodules and
300 nodule dry weight at the two higher doses. Similarly, Aliverdi and Ahmadvand (2018)
301 findings showed decreased number of nodules and nodule weight by metribuzin (450 g ai
302 ha^{-1}) and trifluralin (120 g ai ha^{-1}) in a Eutric Cambisol soil under different pH levels (6.4,
303 7.8 and 8.0). These previous findings indicate that under certain environmental conditions
304 some PRE herbicides can impact symbiotic N fixation.

305 The lack of PRE herbicide impact on soybean N fixation (estimated by ARA, $\delta^{15}N$
306 and %Ndfa methodologies) corroborates the lack of treatment effect on nodule

development and activity assessments in this study. Despite intensive labor requirements, nodule count, diameter, activity, and biomass measurements were shown as valuable response variables in this study to assess root nodulation and potential herbicide impact on N fixation. Analytical measurements of soybean N fixation through ARA, $\delta^{15}N$, and %Ndfa methodologies were important to validate results from the nodule assessments; however, these evaluations are equipment dependent and costly.

5. Conclusion

In this greenhouse experiment using a silt loam soil, the labeled rate of 11 PRE-emergence herbicides did not impact soybean development, root nodulation, and N fixation, other than reduced canopy early in the season (VC growth stage) with sulfentrazone. Thus, under similar soil and environmental conditions, and when sprayed according to their respective labels, the benefits of PRE-emergence herbicides in terms of residual weed control likely outweigh potential concerns regarding soybean development, root nodulation, and symbiotic N fixation. These results are relevant for soybean growers indicating that multiple PRE herbicide options with minimal to no impact on soybean development, root nodulation, and N fixation are available. However, future research is needed to validate these findings under field conditions at multiple environments. Additionally, investigating the influence of herbicide pre-mixes, which contain active ingredients from multiple SOA at different concentrations, would be beneficial as these herbicide pre-mixes have become more commonly recommended to and adopted by soybean growers across the US and beyond (Norsworthy et al., 2012).

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