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Jodie M. Scheele
University of Arkansas, Fayetteville

C. Andy King
University of Arkansas, Fayetteville

Marilynn K. Davies
University of Arkansas, Fayetteville

Larry C. Purcell
University of Arkansas, Fayetteville

Bradyrhizobium japonicum and soybean symbiotic response to glyphosate in glyphosate-tolerant soybean

Jodie M. Scheele[*], *C. Andy King*[§], *Marilynn K. Davies*[†], *and Larry C. Purcell*[∞]

ABSTRACT

Soybean (*Glycine max*) grain contains approximately 40% protein and 6.5% nitrogen (N) on an elemental basis. Therefore, the plant requires an abundant N supply throughout its life cycle, and symbiotic N fixation of soybean with *Bradyrhizobium japonicum* provides 40 to 85% of the soybean N. Although soybean cultivars have been genetically engineered to withstand the herbicide glyphosate, *B. japonicum* grown in culture is sensitive to glyphosate. We hypothesized that glyphosate applied to glyphosate-tolerant soybean would inhibit nodulation by *B. japonicum* unless *B. japonicum* could also be selected for glyphosate tolerance. Cultures of *B. japonicum* were challenged with sublethal doses of glyphosate, and individual colonies were selected for growth in the presence of glyphosate. Of the 40 isolates that were originally selected for glyphosate tolerance, all isolates in subsequent experiments had similar sensitivity to glyphosate as wild-type *B. japonicum*. To determine if glyphosate affected *B. japonicum* in plants, soybean seeds were imbibed with differing levels of glyphosate and water and then planted and inoculated with *B. japonicum*. After several weeks of growth the plants were harvested and nodules were scanned and analyzed by digital imagery. Glyphosate application to glyphosate-tolerant soybean did not affect the ability of *B. japonicum* to form nodules and fix nitrogen. These data do not agree with previous responses of small soybean plants sprayed with glyphosate, which showed delayed nodulation and decreased nodule size. It may be that the dosage applied to plants and the timing of the application affect the response of glyphosate on symbiotic effectiveness.

[*] Jodie M. Scheele is a senior majoring in crop management in the Department of Crop, Soil, and Environmental Sciences.

[§] C. Andy King is the project manager for the Soybean Physiology Program in the Department of Crop, Soil, and Environmental Sciences.

[†] Marilynn K. Davies is a research specialist III in the Department of Crop, Soil, and Environmental Sciences.

[∞] Larry C. Purcell, faculty sponsor, is an associate professor in the Department of Crop, Soil, and Environmental Sciences.

Jodie Scheele

MEET THE STUDENT-AUTHOR

I am a senior from Bristol, Wisconsin, majoring in crop management in the Department of Crop, Soil, and Environmental Sciences and will graduate in May 2002. I received a Chancellor's Scholarship to attend the University of Arkansas, and throughout my college career I have received various awards and honors including the Presidential Scholar Award and the First Ranked Senior Scholar Award. I have been involved in many activities at the University of Arkansas including the Crop, Soil, and Environmental Science Club and Alpha Zeta Fraternity.

I decided to do this project upon the encouragement of my advisor, Dr. Larry Purcell. I learned many things about soybeans, herbicides, and the factors that affect plant growth. After graduation I plan to attend law school at the University of Wisconsin-Madison. This experience has given me the chance to learn more about research techniques and write a paper for publication. All in all, I consider this a valuable experience that will assist me in the future.

INTRODUCTION

In the legume family, plants form a symbiotic relationship with bacteria in the soil. In this relationship the bacteria infect the roots of the plant and chemically reduce (fix) nitrogen (N_2) gas from the atmosphere into a form that the plant can use. In return, the plant provides the bacteria with a source of carbon and an appropriate environment for bacterial growth. The morphological structure that results from the bacterial infection of the plant root is called a nodule, and this is where N fixation occurs. Nitrogen fixation does not occur until about three weeks after the bacteria infect the plant root when large, irregular shaped nodules with a red interior indicate that N fixation is occurring (Graham, 1998).

Soybean is a member of the legume family and forms this symbiotic relationship with *Bradyrhizobium japonicum* (Harper, 1987). Nitrogen fixation by this bacterium is very important to the production of soybean and allows farmers to produce soybean in the absence of costly N fertilizer.

Because soybean seed contains a large amount of protein, the plant must be supplied with an abundant N supply throughout its life cycle, and bacterial N fixation provides 40 to 85% of the soybean N requirement (Graham, 1998). Past research has shown that *B. japonicum* strains differ in their ability to form nodules and in how well they fix N. Selection for superior N-fixing *B. japonicum* has been accomplished, and in controlled environments, these strains demonstrated increased N fixation (Vasilas and Fuhrman, 1993). However, in field environments these bacteria have not been effective in increasing N fixation and yield. This is due to existing *B. japonicum* populations in the soil. These indigenous strains out-compete the superior strains and form over 90% of the nodules (Johnson et al., 1965). The indigenous *B. japonicum* strains are often inefficient at N fixation compared to the superior strains, and full yield potential may not be realized.

Recent advances in biotechnology have led to the engineering of soybean cultivars that are tolerant to the chemical glyphosate. Glyphosate is the active ingredient in the non-selective herbicide Roundup™ (Duke, 1988). These glyphosate-tolerant (GT) cultivars allow for better post-emergence weed control in soybean fields. Glyphosate works by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the plant that leads to the synthesis of aromatic amino acids. Glyphosate-tolerant soybean expresses a gene for EPSPS that is tolerant to the herbicide. Extensive research under field conditions has shown that yields of glyphosate-tolerant soybean sprayed with glyphosate are comparable to glyphosate-sensitive cultivars in conventional herbicide systems (Delannay et al., 1995).

Although engineered soybean cultivars are tolerant to glyphosate, the N-fixing bacteria that form the symbiotic relationship with the soybean are not tolerant to glyphosate (Jaworski, 1972). In culture, *B. japonicum* growth is inhibited by glyphosate, depending upon concentration of glyphosate in the culture and the sensitivity of the bacterial strain. Glyphosate-tolerant soybean plants do not readily degrade glyphosate, and it concentrates in metabolic sinks such as roots and nodules (Duke, 1988). Since the bacteria live in these areas, the concentration can have a negative effect on their growth, and research has shown that early application of glyphosate to plants delays N fixation and nodulation (King et al., 2001). The observation that glyphosate delays nodulation by *B. japonicum* may provide a clue as to how to increase the competitiveness of superior N-fixing *B. japonicum* over poor N-fixing indigenous strains.

We hypothesized that glyphosate applied to GT soybean would inhibit nodulation by *B. japonicum* unless *B. japonicum* could also be selected for glyphosate tolerance. A practical corollary of this hypothesis is that *B. japonicum* selected for glyphosate tolerance would be more competitive for nodulating GT soybean seedlings treated with glyphosate, which could increase the competitive advantage of superior N-fixing *B. japonicum* strains over inferior indigenous strains. In this experiment our objectives were to determine the effect of glyphosate applied as a seed-imbibition solution, to evaluate the effect on nodulation with wild type *B. japonicum*, and to select for GT *B. japonicum* by challenging existing strains with glyphosate.

MATERIALS AND METHODS

Greenhouse Experiment One

Glyphosate solutions of 50, 25, 12.5, 6.25, and 3.125 mM were prepared using serial dilution of a 50 mM solution. Six petri dishes were filled with a single layer of GT soybean cultivar DK5961RR seed, and seeds were weighed. Each petri dish then received 20 mL of one of the glyphosate treatments or water. Several hours after imbibition, 15 mL of each solution was added to ensure there was free solution in the bottom of each petri dish. After the overnight soaking, the seeds were blotted with paper towels and weighed.

B. japonicum (strain USDA 110) was cultured in a defined medium that lacked amino acids and included arabinose as the carbon source (Karr and Emerich, 1989). During mid log-phase, a culture was diluted with deionized water to an optical density at 600 nm (OD600) of 0.0654 (approximately 3.08 x 106 cells/mL, Mahler and Wollum, 1981), which served as inoculum.

Pots (15 cm diameter) were filled with N-free potting medium (LB2, Sungro Horticulture, Bellevue, Wash.) and inoculated with 1 mL of *B. japonicum* culture followed by 500 mL of –N nutrient solution (deSilva et al., 1996). Six seeds were planted in each pot, and there were six replications arranged in a randomized complete block design. Greenhouse lights were set for a 15-hour photoperiod from 6 am to 9 pm and provided a minimum of 300 umol PAR cm-2s-1 at plant height. Greenhouse temperatures were approximately 28 Å 3%C (day) and 22 Å 2%C (night). After 1 week, very poor germination was observed in all pots, indicating that this effect was not due to glyphosate. Cotyledons appeared small, yellow, and damaged, and plants were discarded. Due to the observance of poor germination in all treatments, a germination study was performed to find the best method of imbibition.

Germination/Imbibition Study

Two methods of seed imbibition were compared to planting dry seed. The first method involved placing a piece of filter paper in the bottom of a petri dish and then placing the seed on top of the filter paper. The filter paper was kept moist for the 3-hour imbibition. For the second method, seeds were placed in a petri dish, and the petri dish was kept half filled with water for the 3-hour period. Forty seeds were used per treatment, and both wet and dry weights were taken. After imbibition, four replications of 10 seeds from each imbibition treatment were planted and compared to a control treatment of planting dry seed. These data clearly indicated that imbibition on moist filter paper was superior to imbibition in a partially filled petri dish (Table 1). These results are discussed in more detail in the Results and Discussion section.

Greenhouse Experiment Two

After deciding to use the filter paper imbibition method, Greenhouse Experiment one was repeated.

Plants were harvested after 4 weeks of growth in the greenhouse. Shoots were harvested above the soil line and placed in a 65%C dryer. Roots were also harvested, and the nodules on the roots were separated into two groups, <2.36 mm and >2.36 mm, with a mesh sieve. Each group of nodules was scanned and then placed in the 65%C dryer. Dry weights were then taken of roots, shoots, and nodules. Nodule scans were made with a flatbed scanner and nodule number determined from the images using Sigmascan Pro (V. 5.0, SPSS Inc., Chicago, Ill.).

B. japonicum Selection for Roundup Resistance

A culture of USDA 110 was grown in defined media with arabinose as its carbon source and NH4+ as its N source (Karr and Emerich, 1989). Therefore, synthesis of proteins would require de novo amino acid production, including a functional EPSPS, which is the target enzyme inhibited by glyphosate. One hundred µL of the culture was plated out on defined media containing 10 mM glyphosate. One hundred individual colonies were selected from the agar plate and were grown in 5 mL of liquid culture (minus glyphosate). Liquid cultures were adjusted to an OD600 of approximately 0.16, and 100 µL was added to 5 mL of liquid media containing 5 mM glyphosate. Wild-type USDA 110 in the presence and absence of glyphosate was used as a control. The cultures were allowed to grow for 14 days, and then the OD600 of each culture was measured.

Greenhouse Experiment Three

The results from Greenhouse Experiment Two were used in designing Greenhouse Experiment Three with several modifications. In Greenhouse Experiment Three, seven different glyphosate-tolerant cultivars were used. The cultivars were: USG 540NRR, Progeny 5415RR, Delta Grow 5450RR, Asgrow AG5603, Asgrow AG5901, Delta King 5661RR, and Delta King 5961RR. Three different glyphosate treatments were used: 12.25, 3.06, and 0 mM glyphosate. Plants were harvested 23 days after planting.

RESULTS AND DISCUSSION

Germination/Imbibition Study

Germination was affected by imbibition treatments (Table 1). Germination was significantly decreased by imbibing seeds in a petri plate that was partially filled with water. Germination of seeds that were imbibed on wet filter paper was not significantly different from that of dry seeds. The amount of damaged seedlings did not differ significantly between any of the treatment groups. Due to these results, the filter-paper method was chosen for use in Greenhouse Experiment Two.

Table 1. Response of seedling emergence and seedling damage to imbibition treatments.

Imbibition treatment	Emergence %	Damaged seedlings %
3 hours partially submerged	35	35
3 hours filter paper	80	38
Dry seed	98	15
LSD[z]	20	N.S.

[z] LSD = least significant difference (P≤0.05); N.S. = nonsignificant.

Greenhouse Experiment Two

Glyphosate concentration did not affect nodulation or plant dry weight (Table 2). There were no significant differences among any of the treatments for average nodule weight, nodule number, or dry weights of other plant components.

Table 2. Plant dry weight, nodule number, and nodule size response to glyphosate seed treatments in Greenhouse Experiment Two. There were no significant effects (P≤0.05) for any treatments for any variables.

Glyphosate concentration	Nodule number		Nodule weight		Dry weight	
	Large	Small	Large	Small	Roots	Shoots
--(mM)--			--------(mg)-------		---------(g)----------	
0	23	23	15.6	80.2	0.32	1.32
3.13	26	23	15.5	97.3	0.33	1.33
6.25	31	31	10.6	90.1	0.40	1.42
12.5	29	25	13.3	84.6	0.36	1.26
25.00	25	24	16.1	74.0	0.39	1.23
50.00	29	32	13.0	77.6	0.35	1.15

B. japonicum Selection for Glyphosate Resistance

Glyphosate inhibited growth of all bacterial strains

Table 3. Growth of *B. japonicum* isolates in the culture containing 5 mM glyphosate.

Strain	Glyphosate	OD_{600}
USDA 110	-	1.33 a[z]
USDA 110	+	0.44 b
Selected[y]	+	0.36 b

[z] Means followed by the same letter within a column are not significantly different (P = 0.05).

[y] Forty individual cultures of USDA 110 were selected based upon their ability to form colonies on agar media containing glyphosate. There were no significant differences among selected strains, and an OD600 is presented that was averaged over all strains.

(Table 3). The cultures that were selected from the agar media containing glyphosate did not show growth that was significantly different from the wild type USDA 110 grown in the presence of glyphosate. The selected cultures also did not show differences among strains selected for glyphosate tolerance. The growth of the selected cultures in the presence of glyphosate was significantly less than that of the wild type USDA 110 grown in the absence of glyphosate. USDA 110 grown in the presence of glyphosate also showed growth that was significantly less than that from USDA 110 grown in the absence of glyphosate.

Greenhouse Experiment Three

There was no significant interaction of cultivar and glyphosate treatment in the study; alsp, there were no significant differences among any of any of the cultivars in response to glyphosate treatments for plant dry weight, average nodule weight, or average nodule number. There were significant differences among cultivars, however, for root and shoot weights, which were independent of glyphosate treatment (Table 4).

These experiments indicated that glyphosate had no effect on soybean nodulation when delivered to plants via seed imbibition. Previous research (King et al., 2001), in which glyphosate was applied foliarly to seedlings with 1 or 2 leaves, determined that glyphosate delayed nodulation and resulted in a decrease in nodule size. The difference between foliar delivery and seed imbibition of glyphosate may be due to the total amount of glyphosate delivered to roots during early stages of bacterial infection and nodulation. Seed imbibition for 3 hours generally resulted in a doubling of seed weight. Glyphosate content in the seed and young plant after 3 hours imbibition would expectantly range from approximately 60 to 500 µg as the concentration of glyphosate in the imbibition solution increased from 6 to 50 mM. In comparison, two sequential foliar applications of glyphosate at 1.12 kg ha-1 at the unifoliolate and first trifoliolate stages (King et al., 2001) would deliver approximately 730 µg per plant, half of which would be absorbed by the plant. An additional possibility for lack of response of glyphosate in an imbibing solution on nodulation is that the glyphosate would be primarily absorbed into the cotyledons, which may not transport glyphosate as readily to developing roots as would glyphosate delivered to leaves.

The rationale for imbibing seed in a glyphosate solution was that it would affect the infection and nodulation process from the initial stages of germination, beginning with radicle emergence from the seed. It was hypothesized that this treatment would affect nodulation similarly to or greater than a foliar application at the one- to two-leaf stage (King et al., 2001). Foliar application of glyphosate had a greater effect on nodulation than did seed imbibition, however, and foliar glyphosate application would have the added benefit of decreasing weed competition in a field environment.

Decreasing nodulation by foliar glyphosate applications in glyphosate-sensitive *B. japonicum* may be one important means of increasing the specificity with which *B. japonicum* strains infect soybean. Engineering glyphosate-tolerant *B. japonicum* that had N fixation capacity greater than indigenous strains could be one means of providing this specificity and greatly increasing the amount of N required for high yields in soybean.

Table 4. Plant dry weight, nodule number, and nodule size response to glyphosate seed treatments in Greenhouse Experiment Three. Values reported are averaged over glyphosate treatments (cultivar x glyphosate, interaction non-significant, P≤0.05).

		Nodule number		Nodule weight		Dry weight	
Cultivar		Large	Small	Large	Small	Roots	Shoots
				(mg)		(g)	
USG 540NRR		6	9	20.1	7.15	0.14	0.40
Progeny 5415RR		3	9	9.31	4.93	0.15	0.45
Delta Grow 5450RR		4	7	10.7	4.29	0.12	0.35
Asgrow AG5603		5	8	16.6	6.28	0.12	0.30
Asgrow AG5901		5	14	16.3	9.15	0.16	0.39
Delta King 5661RR		4	11	15.0	5.66	0.14	0.39
Delta King 5961RR		4	10	18.4	4.46	0.14	0.40
Source	DF			F-test P value			
Rep	3	0.20	0.18	0.34	0.29	0.82	0.26
Cult	6	0.72	0.20	0.59	0.27	0.01	0.01
Gly	2	0.98	0.23	0.96	0.43	0.53	0.30
Cult x Gly	12	0.43	0.62	0.31	0.51	0.35	0.14

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LITERATURE CITED

de Silva, M., L.C. Purcell, and C.A. King. 1996. Soybean petiole ureide response to water deficits and decreased transpiration. Crop Sci. 36: 611-616.

Delannay, X., T.T. Bauman, D.H. Beighley, M.J. Buettner, H.D. Coble, M.S. DeFelice, C.W. Derting, T.J. Diedrick, J.L. Griffin, E.S. Hagood, F.G. Hancock, S.E. Hart, B.J. LaVallee, M.M. Loux, W.E. Lueschen, K.W. Matson, C.K. Moots, E. Murdock, A.D. Nickell, M.D.K. Owen, E.H. Paschall II, L.M. Prochaska, P.J. Raymond, D.B. Reynolds, W.K. Rhodes, F.W. Roeth, P.L. Sprankle, L.J. Tarochione, C.N. Tinius, R.H. Walker, L.M. Wax, H.D. Weigelt, and S.R. Padgette. 1995. Yield evaluation of a glyphosate-tolerant soybean line after treatment with glyphosate. Crop Sci. 35:1461-1467.

Duke, S.O. 1988. Glyphosate. p. 1-70. In P.C. Kearney and D.D. Kaufman (eds.) Herbicides: Chemistry, degradation, and mode of action. Vol 3. Marcel Dekker, New York.

Graham, P.H. 1998. Biological dinitrogen fixation: Symbiotic p. 322-345. In D.M. Sylvia, J.J. Fuhrmann, P.G. Hartel, and D.A. Zuberer (eds.). Principles and applications of soil microbiology. Prentice Hall, Upper Saddle River, N.J.

Harper, J.E. 1987. Nitrogen Metabolism. pp. 497-533. In J.R. Wilcox (ed.). Soybeans: Improvement, production, and uses. Second edition. Amer. Soc. Agron., Madison, Wisc.

Jaworski, E.G. 1972. Mode of action of N-phosphonomethylglycine: Inhibition of aromatic amino acid biosynthesis. J. Agr. Food Chem. 20:1195-1198.

Johnson, H.W., U.M. Means, and L.R. Weber. 1965. Competition for nodule sites between strains of Rhizobium japonicum applied as inoculum and strains in the soil. Agron. J. 57:178-185.

Karr, D.B., and D.W. Emerich. 1989. Protein phophorylation in *Bradyrhizobium japonicum* bacteroids and cultures. J. Bacteriol. 171: 3420-3426.

King, C.A., L.C. Purcell, and E.D. Vories. 2001. Plant growth and nitrogenase activity of glyphosate-tolerant soybean in response to foliar glyphosate applications. Agron. J. 93:179-186.

Mahler, R.L., and A.G. Wollum, II. 1981. The influence of soil water potential and soil texture on the survival of Rhizobium japonicum and Rhizobium leguminosarum isolates in the soil. Soil Sci. Soc. Am. J. 45: 761-766.

Vasilas, B.L., and J.J. Fuhrmann. 1993. Field response of soybean to nodulation by a rhizobitoxine-producing strain of Bradyrhizobium. Agron. J. 85:302-305.