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Enhancement of Soybean Nodulation by *Bacillus cereus* UW85 in the Field and in a Growth Chamber

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Seed treatments with *Bacillus cereus* UW85 increased nodulation of soybeans in three field seasons and in three different sterilized soils in the growth chamber. In the field, 28 and 35 days after planting, UW85-treated plants had 31 to 133% more nodules than untreated plants. From 49 days after planting until seed harvest, there were no significant differences between nodulation of UW85-treated plants and untreated control plants. In the growth chamber, in sterilized soil-vermiculite mixtures, at 28 days after planting, UW85 seed treatments enhanced nodulation by 34 to 61%, indicating that the increase in nodulation was not dependent on the soil flora.

We previously described *Bacillus cereus* UW85, a bacterium that suppresses *Phytophthora* damping-off of alfalfa seedlings in the laboratory and in the field (7, 8). While conducting field experiments to examine the ability of UW85 to colonize the rhizosphere of soybeans, we observed that UW85 seed treatments increased soybean nodulation. Here we report that UW85 increases nodulation of soybeans in the field and in sterilized soil in the growth chamber. Our results suggest that the ability of UW85 to suppress disease is not the only mechanism by which it can improve plant health.

Bacterial strains used in this study included the wild-type *B. cereus* UW85 (ATCC 53522); UW85n1 and UW85s1, which are spontaneous neomycin- and streptomycin-resistant mutants of UW85, respectively (6); *B. cereus* 569; and *Bradyrhizobium japonicum* USDA 110. *B. cereus* 569, which was a gift of C. B. Thorne, Department of Microbiology, University of Massachusetts, Amherst, is a wild-type strain that does not have biocontrol activity in the alfalfa bioassay (6). *B. japonicum* USDA 110 was a gift of G. Stacey, Department of Microbiology, University of Tennessee, Knoxville. Soybean (*Glycine max* L. Merr) cultivar AP200 was provided by Agripro Seeds, Ames, Iowa.

For the 1986 experiment, the seed treatments involved mixing seeds with either a 1:1 volume of a UW85 spore paste and 50% methylcellulose or 50% methylcellulose alone. In the 1987 and 1989 field experiments, seed treatments involved coating the seeds with a paste of UW85 spores. To produce a spore paste, Trypticase soy agar (BBL, Cockeysville, Md.) plates were inoculated with 1.0 ml of a mid-log-phase culture of *B. cereus* grown in half-strength Trypticase soy broth. After 4 days of incubation at 28°C, the lawn of spores was scraped off the plates and the resulting paste of spores was either mixed with 50% methylcellulose or applied directly to 25 soybean seeds per plate of bacteria in a 50-ml conical centrifuge tube. Seeds coated with methylcellulose or spores of UW85 were then spread in a single layer and dried in a laminar flow hood. We determined populations of *B. cereus* on coated seeds by sonicating individual seeds in sterile distilled water for 15 s at 20% output (25 W) with a 250-W Vibra-cell sonicator (Sonics and Materials, Danbury, Conn.) and then serially diluting the suspension in sterile distilled water and plating the dilutions on half-strength Trypticase soy agar. UW85-methylcellulose seed treatments resulted in 2.5×10^8 to 3.7×10^8 CFU seed^{-1}, and the spore paste treatments resulted in 1×10^8 to 6.3×10^8 CFU seed^{-1}.

We conducted the field experiments at the University of Wisconsin Experimental Farms in Arlington, Wis. The Arlington site is on Joy silt-loam soil (fine-silty, mixed, mesic aquic Hapludolls, pH 6.8). Prior to planting at Arlington in 1989, we applied 11 mm of water to the plot by overhead irrigation. Seeds for field experiments were planted 24 June 1986, 19 May 1987, and 23 May 1989.

The design of the 1986 field experiment was a randomized complete block with two seed treatments and five blocks. The seed treatments were UW85-methylcellulose and methylcellulose alone as a control. Plots consisted of 24 seeds m^{-1} planted at a depth of 2.5 to 4 cm in rows that were 3 m long and spaced 76 cm apart. For each treatment, 10 plants were removed from each block 55 days after planting. All analyses were based on the block average number of nodules per plant per treatment.

The design for the 1987 field experiment was a randomized complete block with three seed treatments and three blocks. The seed treatments were UW85n1 and UW85s1. One group of seeds was left untreated as a control. Plots consisted of two rows of seeds (60 seeds per row), with seeds spaced 15 cm apart and rows spaced 76 cm apart. The seeds were planted at a depth of 2.5 to 4 cm. For each treatment, three plants from each block were removed and the average number of nodules per plant was calculated for each block. We measured nodulation 21, 28, 35, and 49 days after planting.

The design for the 1989 field experiment was a randomized complete block with 10 blocks. There were two treatment factors: sampling time and seed treatment. The sampling times were 21, 28, 35, and 42 days after planting. The seed treatments were UW85n1 and UW85. One section was left untreated as a control. Each combination of seed treatment and sampling time was assigned to a different plot within each block. Each plot consisted of four seeds, which were spaced 15 cm apart in rows spaced 76 cm apart. The seeds were planted at a depth of 2.5 to 4 cm. For each treatment,

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we removed one plant from each block. None of the field plots were inoculated with *B. japonicum*.

In the laboratory, plants were grown in an autoclaved soil and vermiculite mixture (1:1 by volume) in glass tubes (25 by 200 mm). We used three different soils in these experiments: a Joy silt-loam soil from Arlington, an acid-washed sand, and a Plainfield sand (mixed, mesic typic Udipsamments, pH 6.2) from Hancock, Wis. Plants were grown in a growth chamber at 24°C with a 12-h photoperiod at a light intensity of 244 microeinsteins m^{-2} s^{-1} and watered with a nitrogen-free plant nutrient solution (25). An inoculum of *B. japonicum* USDA 110 for the growth chamber experiments was prepared by growing the bacterium in a yeast extract-mannitol broth (25) at 28°C to mid-log phase on a rotary shaker (170 rpm). USDA 110 cultures were diluted in sterile distilled water to a concentration of between 5.2×10^5 and 3.7×10^6 CFU ml^{-1} as determined by dilution plating on yeast extract-mannitol agar. Seeds were planted at a depth of 2 to 3 cm. Each seed was inoculated with 1.0 ml of the diluted USDA 110 culture at planting.

The design for the growth chamber experiments was a randomized complete block. There were two seed treatments, *B. cereus* UW85 and *B. cereus* 569, and one group of seeds was left untreated as a control. For each treatment there were two plants in each block, and there were 10 blocks in each experiment. For each treatment the average number of nodules per plant was calculated for each block. Nodule numbers, nodule dry weights, and acetylene reduction activity (ARA) were measured 28 days after planting. In both the growth chamber and the field experiments, the total number of nodules was defined as the number of nodules on the primary root and on the lateral roots.

We measured nodule dry weight and ARA in six separate growth chamber experiments. For acetylene reduction assays, the tops of plants were removed, the glass tubes in which plants had been grown were sealed with rubber stoppers, 3 ml of acetylene was injected into each tube, and the tubes were incubated at room temperature for 1 h. The air space was at least 10% of the tube volume. Air samples (100 μl) were withdrawn from each tube and injected into a gas chromatograph. Acetylene reduction is expressed as nanomoles of C_2H_2 reduced per plant per h. To determine nodule dry weight, nodules from each plant were removed, placed in a paper coin envelope, and dried overnight in a 75°C drying oven. Nodule dry weight is expressed as milligrams of dry weight per plant.

The SAS statistical computer program was used for statistical analyses (22). For the 1986 field experiment, an analysis of variance was performed on the number of nodules per plant, and a least significant difference (LSD) was calculated by the method of Snedecor and Cochran (24). For the 1987 field experiment, a repeated-measures analysis of variance was performed on a square-root transformation of the number of nodules per plant, and an LSD was calculated by the method of Milliken and Johnson (19). The repeated-measures analysis consisted of comparing in a single analysis the number of nodules per plant 21, 28, 35, and 49 days after planting. For the 1989 experiment, the number of nodules per plant was analyzed as a split-plot experiment. In this analysis, seed treatments were the whole-plot treatment factors and sampling times were the subplot treatment factors. Analyses were performed on the square-root transformation of the number of nodules per plant to reduce the effect of greater variability on the analysis of variance at later sampling times. To compare differences among seed treatments, an LSD was calculated by the method of Milliken and Johnson (19). For growth chamber experiments, an analysis of variance was performed on the number of nodules, ARA, and nodule dry weight per plant. An LSD of $P = 0.05$ was used for all field and growth chamber experiments, unless indicated otherwise. No significant treatment-time interactions were observed in either the 1987 or the 1989 experiment.

TABLE 1. Effect of UW85 seed treatments on nodulation of soybeans in 1987 and 1989 field experiments

Year of expt and treatment strain	No. of nodules (mean ± SE)[a] on the following day after planting:			
	21	28	35	42 or 49[b]
1987				
None	6.4 ± 1.1 A	9.0 ± 1.8 A	16.7 ± 0.6 A	42.0 ± 3.4 A
UW85n1	9.0 ± 2.1 B	17.8 ± 1.5 B	21.9 ± 3.4 B	39.2 ± 8.7 A
UW85s1	8.8 ± 1.1 B	21.1 ± 2.3 B	23.4 ± 2.2 B	45.7 ± 3.7 A
1989				
None	6.2 ± 2.0 A	12.5 ± 2.8 A	11.8 ± 2.7 A	22.9 ± 3.3 A
UW85n1	4.7 ± 0.8 A	23.4 ± 2.3 B	24.8 ± 2.9 B	29.2 ± 4.0 A
UW85	7.6 ± 1.7 A	23.2 ± 2.5 B	17.6 ± 2.5 B	26.5 ± 1.8 A

[a] Means followed by the same letter in a column do not differ significantly at $P = 0.05$.

[b] Estimates of nodulation were made 49 and 42 days after planting in the 1987 and 1989 field experiments, respectively.

UW85 seed treatments increased the nodulation of soybeans by indigenous *B. japonicum* in three field experiments. In the 1986 Arlington experiment, at 55 days after planting, UW85-methylcellulose-treated soybean plants had an average of 19.5 nodules $plant^{-1}$, whereas the methylcellulose controls had an average of 14.3 nodules $plant^{-1}$. The values differed significantly at $P = 0.05$ (LSD = 3.4 nodules). In the 1987 and 1989 field experiments, the enhancement of nodulation associated with UW85, UW85n1, and UW85s1 seed treatments was evident at 21 days after planting and was greatest at 28 and 35 days after planting (Table 1). Nodulation of UW85-treated plants 28 days after planting was 87 to 134% greater than nodulation of untreated plants, and at 35 days after planting, UW85-treated plants had 31 to 49% more nodules than untreated plants (Table 1). In the 1987 and 1989 field experiments, UW85 treatments significantly increased nodulation of both the primary root and the lateral roots compared with that of the untreated control at $P = 0.05$ (data not shown). Increased nodulation of UW85-treated plants appears to be transient, since no significant differences ($P > 0.1$) in nodulation among the treatments were detected 42 and 49 days after planting (Table 1). In the 1987 experiment, from 49 days after planting until seed harvest, no significant differences in nodulation among treatments were observed (data not shown).

To determine whether UW85 would enhance the nodulation of soybeans grown in the laboratory, we applied it to soybeans inoculated with 5.5×10^5 to 1.4×10^6 cells of *B. japonicum* USDA 110 and grown in sterilized soil-vermiculite mixtures. The ability of UW85 seed treatments to increase nodulation does not appear, from our data, to be dependent on the indigenous flora or on soil type, since we observed increases in nodulation with all sterilized soil-vermiculite mixtures used in the laboratory (Table 2). Overall, UW85 seed treatments increased the number of nodules by 34 to 61% compared with those of untreated plants (Table 2). In the silt-loam soil, UW85 significantly ($P = 0.05$) increased nodule mass by 33% (6; data not shown), whereas

TABLE 2. Effect of *B. cereus* strains on nodulation of soybeans inoculated with *B. japonicum* in sterilized soil-vermiculite mixtures

Treatment strain	No. of nodules (mean ± SE)[a] for plants grown in:				
	Joy silt-loam	Acid-washed sand		Plainfield sand	
		Expt 1	Expt 2	Expt 3	Expt 4
None	6.1 ± 0.4 A	5.4 ± 0.7 A	5.1 ± 0.7 A	7.4 ± 1.1 A	5.2 ± 1.4 A
B. cereus 569	5.7 ± 0.7 A	6.0 ± 0.7 A	5.5 ± 0.6 A	ND[b]	ND
B. cereus UW85	8.2 ± 0.8 B	7.7 ± 0.7 B	8.1 ± 0.8 B	9.7 ± 0.9 B	8.4 ± 1.2 B
LSD[c]	0.9	1.1	1.6	2.0	2.2

[a] Values represent the mean number of nodules ± standard error, at 28 days after planting, for five independent experiments for Joy silt-loam and for each individual experiment for acid-washed and Plainfield sands. Means followed by the same letter in a column do not differ significantly at $P = 0.05$.
[b] ND, not determined.
[c] Number of nodules.

in the Plainfield sand, UW85 increased nodule mass by 13 to 70%, but this increase was only significant at $P = 0.1$ (data not shown). The data also show that nodulation enhancement is not simply a physical effect of treating the seeds with a spore paste, since *B. cereus* 569 seed treatments did not enhance soybean nodulation (Table 2).

UW85 seed treatments increased the ARA of soybeans in all three sterilized soil-vermiculite mixtures tested (data not shown). Overall, UW85 seed treatments increased the ARA of soybeans by 25 to 73% compared with that of the untreated control. Since plants that were not inoculated with *B. japonicum* did not show detectable ARA, we conclude that the ARA of *B. japonicum*-treated plants is not due to endogenous ethylene and that the higher ARA of plants treated with UW85 is likely due to the greater number of nodules on these plants. In Joy silt-loam soil-vermiculite mixtures and in one of the two experiments with a Plainfield sand-vermiculite mixture (experiment 3), the ARA of UW85-treated plants was significantly greater than that of the untreated control plants at $P = 0.06$, while the rest of the experiments showed significant differences in ARA at $P = 0.05$ (6; data not shown).

In conclusion, we have shown that UW85 seed treatments increased the nodulation of soybeans in three field seasons and in laboratory experiments and that increased ARA was usually associated with increased nodulation in the laboratory. The 1986 and 1989 field experiments that showed the ability of UW85 to increase nodulation under field conditions in which no *Phytophthora* or *Pythium* disease was detectable (6) and our growth chamber experiments with sterilized soil (Table 2) show conclusively that nodulation enhancement does not depend on the suppression of *Phytophthora* or *Pythium* disease. Enhanced nodulation between 21 and 35 days after planting could increase the amount of nitrogen available to the plant and thereby improve plant growth.

The ability of UW85 to enhance nodulation in a sterilized soil suggests that UW85 has a direct effect on either the bradyrhizobia or the plant. In contrast to previous reports of microbes that enhance nodulation (1–3, 5, 9, 11, 14, 21, 23), enhancement of nodulation of plants in the field by UW85 did not require coinoculation with *B. japonicum*. UW85 treatments increased nodulation within 21 to 28 days after planting. Since soybean nodules appear 10 to 12 days after the initiation of nodulation, UW85 likely affects the nodulation process soon after planting by stimulating bradyrhizobial infections or by suppressing the abortion of infections. Other microbes produce similar effects by deforming root hairs (15), producing toxins (14), or enhancing phosphorous nutrition (5, 20).

Bacterial inoculants are used to improve crop performance by stimulating plant growth (2, 12, 13, 16), by increasing nutrient availability for the plants (4, 20), or by suppressing plant diseases (7, 10, 17, 18). Our results suggest that UW85 as a seed inoculant may have the potential to enhance the nitrogen status of soybeans. Further work will address the mechanism by which UW85 transiently enhances the nodulation of soybeans and the consequences of increased nodulation on plant productivity.

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