APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 4260–4262 Vol. 62, No. 11
0099-2240/96/$04.00+0

Inoculation with *Sinorhizobium meliloti* RMBPC-2 Increases Alfalfa Yield Compared with Inoculation with a Nonengineered Wild-Type Strain

ALEXANDRA J. SCUPHAM,[1] ANDREW H. BOSWORTH,[1] WILLIAM R. ELLIS,[2] THOMAS J. WACEK,[2] KENNETH A. ALBRECHT,[1] AND ERIC W. TRIPLETT[1]*

Department of Agronomy and the Center for the Study of Nitrogen Fixation, University of Wisconsin-Madison, Madison, Wisconsin 53706-1597[1], and Research Seeds, Inc., St. Joseph, Missouri 64502[2]

Received 27 June 1996/Accepted 12 August 1996

Inoculation of alfalfa seeds with any of three recombinant strains of *Sinorhizobium meliloti* significantly increased overall plant biomass compared with inoculation with the wild-type strains over a 3-year period at three locations. A high proportion of nodules were occupied by the inoculum strains throughout the 3-year period.

A number of approaches to the improvement of leguminous plant production have been attempted by altering the ability of root nodule bacteria to fix nitrogen. An approach to constructing bacterial strains with enhanced nitrogen fixation capabilities was described recently by Bosworth et al. (1) and Ronson et al. (3). Bosworth et al. described the construction of eight recombinant strains of *Sinorhizobium meliloti* by Biotechnica Corp. and presented the seeding year (1992) biomass yield results from alfalfa inoculated with those strains. These yield trials were planted at Arlington, Hancock, Lancaster, and Marshfield, Wis. Bosworth et al. (1) found that the addition of *nifA* or *dctABD* enhanced alfalfa yield response, in the seeding year, under nitrogen-limiting conditions. Characteristics of the strains field tested in this work are outlined in Table 1.

Because alfalfa is a perennial crop it was necessary to determine the long-term effects of the recombinant strains on dry matter yield, forage nitrogen content, and nodule occupancy, and field studies were continued at the Lancaster and Marshfield sites through 1994 and at Hancock through 1995 (Tables 2 to 4). The Arlington site was dropped after the seeding year as a result of extensive winter kill. Most yield effects seen in this experiment can be related to the inoculum strains. However, the Hancock yield in 1993 was depressed by leaf disease pressure following heavy rains during that summer. The yield at Marshfield was much lower than at other locations because a low proportion of nodules were occupied by the inoculum strains at that site.

Three engineered strains caused an alfalfa yield increase over plants inoculated with wild-type strains for all sites and all years. These strains were RMBPC-2, and to a lesser extent, strains RMB7240 and RMB7201. The effects of RMB7240 and RMB7201 are surprising, and analysis of the results suggests that the combined effects of the cassettes and the sites of cassette introduction were more important symbiotically than the cassettes alone.

The Ω cassette provides streptomycin and spectinomycin resistance to all engineered strains except RMB7240. When placed into the inositol locus in strain RMB7201, this cassette caused an increase in alfalfa yield over wild-type levels. When the Ω cassette was combined with the nitrogen-fixing gene *nifA* in strains RMB7103 and RMB7203, no yield effect was apparent regardless of the insertion site. The Ω/*nifA*/*dctABD* cassette inserted into the inositol locus in strain RMBPC-2 caused the greatest observed yield increase. In contrast, when the Ω cassette was combined with the *dct* genes in the P3 site, the cassette decreased yield. Because of the different effects of the

TABLE 1. Relevant characteristics of the *S. meliloti* strains used in alfalfa field trials[a]

Inoculant strain	Parent strain	Genotype of added genes	Integration site	Yield phenotype in relation to the parent
PC		None	None	Wild type
RMB7201	PC	Ω	Inositol	Increase
RMB7203	PC	*nifA*/Ω	Inositol	No change
RMB7240	PC	*nifA*/Km^r	P3	Increase
RMBPC-2	PC	*dctABD*/Ω/*nifA*	Inositol	Increase
RCR2011		None	None	Wild type
RMB7103	RCR2011	*nifA*/Ω	Inositol	No change except decrease at Marshfield
RMB138Ω 710A	RCR2011	*dctABD*/Ω	P3	Decrease at Marshfield and Hancock
RMB139Ω 710B	RCR2011	2 (*dctA*/Ω)	P3	Decrease at Hancock

[a] The construction of the strains was as described in Bosworth et al. (1994).

* Corresponding author. Mailing address: Department of Agronomy and the Center for the Study of Nitrogen Fixation, University of Wisconsin-Madison, 1575 Linden Dr., Madison, WI 53706-1597. Phone: (608) 262-9824. Fax: (608) 262-5217. Electronic mail address: ewtriple@facstaff.wisc.edu.

TABLE 2. Yield of alfalfa inoculated with wild-type rhizobia strains and the genetically engineered derivatives at Hancock, Wis.[a]

Strain	Yield (Mg of dry forage/ha) during:				
	1992	1993	1994	1995	1992–95
RMBPC-2	10.08a	6.51a	11.61ab	10.01ab	38.21a
RMB7240	9.32b	6.20ab	12.03a	10.16a	37.71a
RMB7201	9.20b	6.11ab	11.58ab	10.07a	36.96abc
RMB7203	9.12b	5.98ab	11.41b	9.77ab	36.27bc
PC	8.93bc	6.08ab	11.23b	9.82ab	36.06c
RMB138Ω710A	8.93bc	5.92b	11.58ab	9.80ab	36.22c
RMB139Ω710B	8.98bc	6.44ab	11.20b	9.56b	36.19c
RMB7103	8.98bc	6.37ab	11.95a	10.16a	37.47ab
RCR2011	9.40b	6.43ab	11.70ab	10.02a	37.55ab
Uninoculated	8.55c	6.09ab	11.79ab	9.96ab	36.40bc
LSD[b]	0.52	0.53	0.53	0.45	1.28
c.v.[c]	6.81	10.30	5.51	5.45	4.16

[a] Yield results followed by the same letter within a year are not significantly different at the 10% level of confidence as determined by Tukey's least-significance test.
[b] LSD, least significant difference (10% level of confidence).
[c] c.v., coefficient of variance (%).

TABLE 4. Average annual yield of alfalfa from 1992 to 1994 inoculated with wild-type rhizobia strains and the genetically engineered derivatives at Hancock, Lancaster, and Marshfield, Wis.[a]

Strain	Yield (Mg of dry forage/ha) at:			
	Hancock	Lancaster	Marshfield	All sites
RMBPC-2	9.40b	9.73a	6.22abc	8.45a
RMB7240	9.18bc	9.68ab	6.31ab	8.39ab
RMB7201	8.97cd	9.70ab	6.41a	8.36ab
RMB7203	8.84cd	9.53ab	6.36a	8.24bc
PC	8.75de	9.44b	6.25abc	8.14c
RMB138Ω710A	8.81d	9.61ab	6.02bc	8.15c
RMB139Ω710B	8.88cd	9.30bc	6.34ab	8.17c
RMB7103	9.10c	9.55ab	6.08bc	8.24bc
RCR2011	9.18bc	9.34bc	6.36a	8.29abc
Uninoculated	8.81d	9.46ab	6.23abc	8.16c
LSD[b]	0.27	0.27	0.27	0.16
c.v.[c]	4.63	4.16	5.72	6.91

[a] Yield results followed by the same letter within a year are not significantly different at the 10% level of confidence as determined by Tukey's least-significance test.
[b] LSD, least significant difference (10% level of confidence).
[c] c.v., coefficient of variance (%).

various Ω cassettes in regard to their insertion point, it appears that interruption of the bacterial inositol site is beneficial to the host plants, while the Ω cassette itself may have no beneficial effect on yield.

It was expected that addition of the *nifA* gene to the engineered bacterial strains would increase the nitrogen-fixing potential of the microsymbionts and therefore increase plant biomass. To test this hypothesis, strains RMB7201 and RMB7203 were designed with the cassettes Ω and Ω/*nifA*, respectively, in the inositol locus. Strain RMB7240 was designed with the cassette *nifA*/Km^r in the P3 site. The yield comparison of plants inoculated with those strains showed that, when inserted into the inositol site, the *nifA* gene seemed to have very little effect on yield. The exception occurred with strain RMB7103 at the Marshfield site where a decrease in yield was noted. In contrast, insertion of *nifA*/Km^r into the P3 site increased yield overall.

TABLE 3. Average yield of alfalfa inoculated with wild-type rhizobia strains and the genetically engineered derivatives over three sites (Hancock, Lancaster, and Marshfield) and 3 years[a]

Strain	Yield (Mg of dry forage/ha) during:			
	1992	1993	1994	1992–94
RMBPC-2	6.46a	8.06a	10.83b	25.35a
RMB7240	6.15b	7.84abc	11.19a	25.18ab
RMB7201	6.14bc	8.08a	10.85b	25.07ab
RMB7203	6.05bc	8.01abc	10.66b	24.72bc
PC	5.98bc	7.74bc	10.72b	24.44c
RMB138Ω710A	6.02bc	7.77bc	10.66b	24.45c
RMB139Ω710B	5.84d	8.06a	10.62b	24.52c
RMB7103	5.87c	7.97abc	10.89b	22.73bc
RCR2011	6.11bc	8.04ab	10.72b	24.87abc
Uninoculated	5.89bc	7.81abc	10.79b	24.49c
LSD[b]	0.27	0.27	0.27	0.48
c.v.[c]	9.54	7.24	5.19	4.73

[a] Yield results followed by the same letter within a year are not significantly different at the 10% level of confidence as determined by Tukey's least-significance test.
[b] LSD, least significant difference (10% level of confidence).
[c] c.v., coefficient of variance (%).

The *dctABD* genes include the regulation and structural genes necessary for bacteroid uptake of C4-dicarboxylic acids from the plant. These genes, inserted into the inositol locus, caused the largest observed increase in plant biomass. However, when inserted into the P3 site, *dctABD* and 2 (*dctA*) caused yield decreases. These results represent the strongest evidence that the inositol site may not be symbiotically silent. Unfortunately, these results do not allow us to determine whether addition of the *dctABD* genes are beneficial to yield. The amount of nitrogen per plot increased where yield increased, but in no case did we see an increase of nitrogen concentration in the harvested plant tissue.

Another significant and somewhat surprising result of this work was the observation that newly formed alfalfa nodules were occupied by the original inoculum strains up to 4 years after inoculation. Very little work has been done on nodule occupancy of perennial legumes over two or more seasons. Jenkins and Bottomly (2) showed that identical isolates of *S. meliloti* occupied alfalfa root nodules in 1980 and 1982. Our data show that at Hancock, nodule occupancies by the recombinant derivatives of PC were very high for 3 of 4 years, while the RCR2011 derivatives were found to occupy nodules only in low numbers after 2 years (Fig. 1A). Similarly, at Lancaster where nodule occupancy was examined for 3 years, the PC derivatives were capable of occupying a large number of nodules for all 3 years, while RCR2011 and its derivatives were uncommon occupants of nodules in the third year (Fig. 1B). At Marshfield where the indigenous rhizobial population was large (1), the nodule occupancy was very low even during the seeding year (Fig. 1C). This experiment demonstrates that inoculant strains are capable of nodulating a perennial plant for up to 4 years in the absence of a large indigenous population.

The yield increases observed in this work from RMBPC-2 (3.8%), RMB7240 (3.1%), and RMB7201 (2.7%) over the three sites and the 3- and 4-year periods were small but statistically significant. Whether these increases are sufficient to encourage the use of these strains in agriculture depends on many economic factors. At current Wisconsin hay prices, the yield difference at Hancock between alfalfa inoculated with PC-2 and PC over the 4 years of this experiment would have

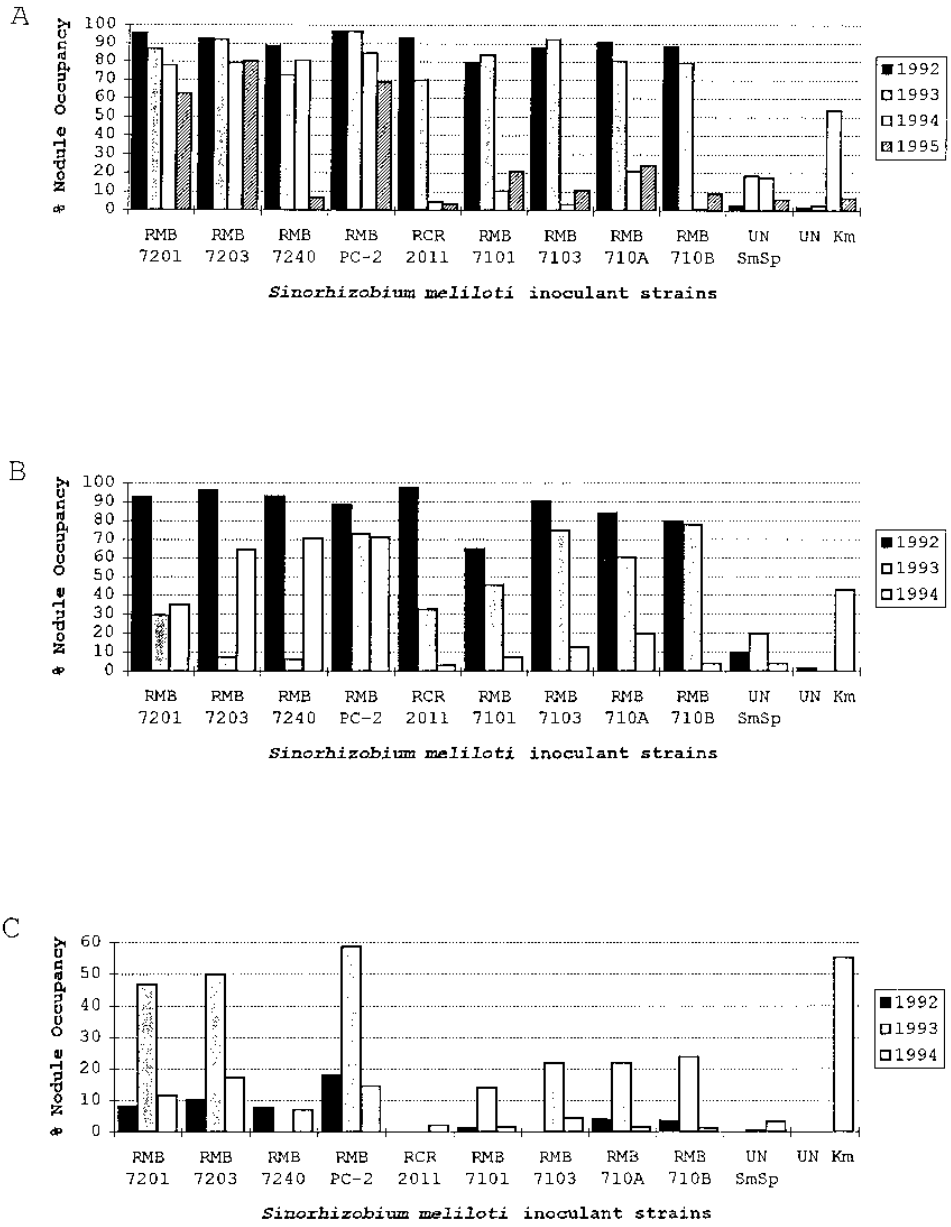

FIG. 1. Percent nodule occupancies at Hancock (A), Lancaster (B), and Marshfield (C) over 4- or 3-year periods for all inoculated strains except wild-type PC. Occupancy for strain RMB7240 was tested on kanamycin (150 μg/g). All other strains were tested on streptomycin (100 μg/g) and spectinomycin (100 μg/g). Uninoculated controls are shown in the last two column sets.

been worth about $237 per hectare. The yield data presented here are interesting for their implication that inositol utilization by the microsymbiont may be detrimental to alfalfa yield, but they should also be taken as a warning that insertion of genetic material into poorly characterized chromosomal loci may have unexpected consequences.

This work was supported by funds from Research Seeds, Inc., St. Joseph, Mo., and Hatch project no. 5201 and 3270 from the University of Wisconsin-Madison College of Agricultural and Life Sciences.

We also gratefully acknowledge the assistance of the staff of the University of Wisconsin Agricultural Research Stations for maintaining these field plots.

REFERENCES

1. **Bosworth, A. H., M. K. Williams, K. A. Albrecht, T. R. Hankinson, R. Kwiatkowski, J. Beynon, C. W. Ronson, F. Cannon, T. J. Wacek, and E. W. Triplett.** 1994. Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* carrying an extra copy of *dct* and/or modified *nifA* expression. Appl. Environ. Microbiol. **60:**3815–3832.
2. **Jenkins, M. B., and P. J. Bottomly.** 1985. Evidence for a strain of *Rhizobium meliloti* dominating the nodules of alfalfa. Soil Sci. Soc. Am. J. **49:** 326–328.
3. **Ronson, C. W., A. Bosworth, M. Genova, S. Gudbrandsen, T. Hankinson, R. Kwiatkowski, H. Ratcliffe, C. Robie, P. Sweeney, W. Szeto, M. Williams, and R. Zablotowicz.** 1990. Field release of genetically engineered *Rhizobium meliloti* and *Bradyrhizobium japonicum* strains, p. 397–403. *In* P. M. Gresshoff, L. E. Roth, and W. E. Newton (ed.), Nitrogen fixation: achievements and objectives. Chapman and Hall, New York.