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Survival of *Rhizobium phaseoli* in Coal-Based Legume Inoculants Applied to Seeds†

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Eight coals used as carriers in legume inoculants promoted the survival of *Rhizobium phaseoli* on pinto bean seeds. Although peat was more protective, most coal-based inoculants provided $>10^4$ viable rhizobia per seed after 4 weeks.

Finely ground peat is the carrier of choice in legume inoculants. However, peat is not always available in areas most in need of symbiotic nitrogen fixation (8). Recent research has shown that coal may be a workable alternative to peat as an inoculant carrier (4–8). In the most comprehensive study to date, Paczkowski and Berryhill (7) examined the long-term survival of *Rhizobium phaseoli* in inoculants prepared with eight coals of various grades and found that most coals tested promoted acceptable rhizobial survival. Our study concerned the survival of *R. phaseoli* in coal-based inoculants applied to seeds. One of the desirable properties of peat is its ability to protect rhizobia on seeds (1, 9), and we wished to determine whether coal is also protective.

We used anthracitic coal from Pennsylvania, bituminous coals from Illinois, Pennsylvania, and Utah, lignitic coals from North Dakota and Texas, and subbituminous coals from New Mexico and Wyoming (7). Inoculants were prepared as described previously (7), except that *R. phaseoli* 127K17, 127K26, and 127K35 mutants spontaneously resistant to streptomycin (>100 μg/ml) were used, and "precipitated" $CaCO_3$ was routinely added to the carrier (10 g per 90 g of coal powder) so that the pH was at least 6.5. The addition of $CaCO_3$ improved rhizobial survival, especially in inoculants containing the two coals with the lowest pH values (Illinois bituminous coal and Texas lignitic coal). An inoculant for each *R. phaseoli* strain was also prepared with peat provided by the Nitragin Co., Milwaukee, Wis. All inoculants were at least 1 month old and contained 10^7 to 10^{10} viable rhizobia per g when used to inoculate seeds.

To test survival on seeds, 2 g of inoculant and 10 ml of 50% gum arabic (Mallinckrodt, Inc., St. Louis, Mo.) were added to an 18-oz (532-ml) Whirl-Pak bag (Nasco, Fort Atkinson, Wis.) containing 100 randomly chosen pinto bean seeds (University of Idaho 111). The bags were shaken for 10 s to facilitate an even coating of seeds, and then the seeds were vigorously shaken in a sterile 10-mesh sieve to remove excess inoculant before they were stored at 20 to 22°C in 18-oz Whirl-Pak bags. Gum arabic allowed uniform adhesion of inoculants to seeds. About 1 mg of inoculant adhered to each seed; there were no significant differences in adhesion among coal-based inoculants ($\alpha = 0.01$) or between coal-based and peat-based inoculants ($\alpha = 0.01$).

The numbers of viable rhizobia on seeds were determined weekly by plate counts. Ten seeds were aseptically transferred to a screw-capped tube (25 by 150 mm) containing 10.0 ml of sterile saline (0.5% NaCl) and 10 glass beads (3 mm). The tube contents were vortexed for 1-s intervals 10 times. The resulting suspension was serially diluted in saline and plated, in duplicate, on mannitol-yeast extract agar (7) containing Congo red (25 μg/ml; Harleco, Philadelphia, Pa.), dihydrostreptomycin sulfate (50 μg/ml; ICN Pharmaceuticals, Inc., Cleveland, Ohio), and cycloheximide (50 μg/ml; Sigma Chemical Co., St. Louis, Mo.). The plates were incubated for 72 h at 30°C before the colonies were counted. Because seed sterilization procedures were avoided, it was critical to include streptomycin and cycloheximide to control bacterial and fungal contamination, respectively. Pure cultures of the *R. phaseoli* strains grew equally well on media with and without these substances.

Survival experiments were continued for 4 weeks (2), and each inoculant was tested in at least three separate experiments. Tables 1, 2, and 3 show the logarithm averages of the viable counts for each of the strains and the mean death rates (logarithmic decline per week) calculated by linear regression analyses. In general, viable counts per seed decreased 1 to 2 logs during 4 weeks. Two-way analyses of variance of the regression slopes indicated no significant differ-

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TABLE 1. Survival of *R. phaseoli* 127K17 in inoculants applied to pinto bean seeds

Carrier[a]	Log_{10} CFU per seed after wk: 0	1	2	3	4	Mean death rate (log decline per wk)
AP	6.29	5.57	4.91	4.70	4.21	0.55
BI	4.88	3.74	3.60	3.40	3.27	0.35
BP	6.00	5.01	4.87	3.96	4.17	0.45
BU	6.07	5.13	4.35	4.88	4.10	0.42
LN	5.28	4.41	4.24	4.65	4.06	0.22
LT	5.61	4.95	4.34	4.10	4.10	0.42
SN	6.06	5.53	5.33	4.80	4.69	0.32
SW	4.90	4.32	4.44	4.07	3.80	0.26
Peat	5.97	5.67	6.11	5.80	5.50	0.08

[a] AP, Anthracitic coal from Pennsylvania; BI, bituminous coal from Illinois; BP, bituminous coal from Pennsylvania; BU, bituminous coal from Utah; LN, lignitic coal from North Dakota; LT, lignitic coal from Texas; SN, subbituminous coal from New Mexico; SW, subbituminous coal from Wyoming.

ences in survival among bacterial strains (α = 0.05), but significant differences among coals. Rhizobial survival in peat-based inoculants applied to seeds was superior to that in any coal-based inoculant under the same conditions.

Because gum arabic has been noted to improve rhizobial longevity on seeds (2, 10), the survival of *R. phaseoli* 127K17 applied to seeds with 30% gum arabic was determined. The seeds were treated as described above, except that 30-h mannitol-yeast extract broth (7) cultures were used instead of inoculants, and counts were made daily instead of weekly. In these experiments, strain 127K17 had a mean death rate of 3.53 logs per week. Therefore, although gum arabic may aid *R. phaseoli* viability on seeds, coal apparently promotes a substantially greater degree of rhizobial survival.

The ability to protect rhizobia on seeds, a valuable characteristic of peat, has seldom been examined in studies of alternative carriers (8).

TABLE 2. Survival of *R. phaseoli* 127K26 in inoculants applied to pinto bean seeds

Carrier[a]	Log_{10} CFU per seed after wk: 0	1	2	3	4	Mean death rate (log decline per wk)
AP	6.24	6.06	5.44	5.52	4.67	0.36
BI	5.01	3.48	3.32	3.10	3.25	0.51
BP	6.03	5.26	4.67	3.83	4.29	0.47
BU	6.09	5.25	5.08	4.84	4.42	0.38
LN	6.39	5.81	5.77	5.50	4.96	0.32
LT	6.11	5.89	5.43	5.31	4.99	0.28
SN	6.39	6.06	5.92	5.74	4.95	0.33
SW	5.35	3.34	4.23	3.85	3.36	0.50
Peat	6.01	6.09	5.82	5.84	5.59	0.16

[a] See Table 1, footnote *a*.

TABLE 3. Survival of *R. phaseoli* 127K35 in inoculants applied to pinto bean seeds

Carrier[a]	Log_{10} CFU per seed after wk: 0	1	2	3	4	Mean death rate (log decline per wk)
AP	6.33	5.92	5.45	5.38	5.04	0.32
BI	4.74	4.81	4.69	4.53	3.95	0.40
BP	5.85	4.92	4.77	4.12	3.94	0.46
BU	5.84	4.76	4.89	4.50	3.78	0.44
LN	6.29	5.72	5.96	5.63	5.21	0.27
LT	5.56	5.24	5.05	4.48	4.64	0.26
SN	5.99	5.32	5.43	4.64	4.46	0.38
SW	5.11	4.09	3.72	3.13	3.19	0.48
Peat	6.16	5.73	5.39	5.40	5.42	0.18

[a] See Table 1, footnote *a*.

Our experiments showed that the coals used as carriers for *R. phaseoli* inoculants benefited the rhizobia once the inoculants were applied to seeds. Although coal was not as effective as peat in maintaining rhizobia, most coal-based inoculants provided $>10^4$ rhizobia per seed for 4 weeks after inoculation, and all provided $>10^3$ rhizobia per seed for the same period. These numbers compare favorably with a minimum standard of 7×10^4 rhizobia per seed at inoculation (3). Although peat remains the best of all inoculant carriers tested, the ability of coal to promote the survival of *R. phaseoli* on seeds, as well as in inoculants (7), justifies its consideration as a carrier when an alternative to peat is needed.

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3

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