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

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The long-term survival of *Rhizobium phaseoli* strains 127K17, 127K26, and 127K35 in legume inoculants prepared with eight different coals (one strain and one coal per inoculant) was studied. The coals used were Pennsylvania anthracite, bituminous coals from Illinois, Pennsylvania, and Utah, lignite from North Dakota and Texas, and subbituminous coals from New Mexico and Wyoming; they ranged in pH from 4.7 to 7.5. All coals, with the exceptions of Illinois bituminous coal and Texas lignite (pH's of 5.0 and 4.7, respectively), supported the growth and survival of all *R. phaseoli* strains. All coal-based inoculants in which rhizobial viability was maintained had more than 10^6 rhizobia per g for at least 7 months, and most contained more than 10^7 rhizobia per g after 12 months. It appears that most coals, regardless of grade or source, may be acceptable carriers for *R. phaseoli* inoculants.

The legume inoculant industry provides rhizobia for leguminous seeds (1). Presently, inoculants consist of rhizobial cultures mixed with carrier materials. The most widely used carrier is dried, milled peat (8). Although peat is the best carrier identified to date, it has disadvantages: its suitability varies depending on the source (9), and it is not readily available in all areas of the world (11).

Coal has recently received attention as an alternative carrier material for inoculants. Kandasamy and Prasad (6) showed that a peanut *Rhizobium* sp. multiplied well in Indian lignite, but their experiment lasted only 21 days. Dube et al. (2, 3) obtained satisfactory survival of *R. japonicum* in inoculants prepared with Indian lignite and Indian inferior-grade coal. Carriers consisting of 40% coal, 40% bentonite, and 20% lucerne meal (CBL carriers) have been studied in South Africa by Strijdom and Deschodt (11). The CBL carriers, prepared with anthracite from two different sources or a low-grade coal, were inoculated with cultures of *R. meliloti* or cowpea *Rhizobium*. After a 140-day incubation period, rhizobial survival in the CBL carriers was comparable to that in peat.

This paper examines the long-term survival of *R. phaseoli* in coal-based inoculants. Coals from diverse sources, and of various grades, were used so that a determination of the general suitability of coal as a carrier material could be made.

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MATERIALS AND METHODS

Bacteria. *R. phaseoli* strains 127K17, 127K26, and 127K35 were provided by J. C. Burton, The Nitragin Company, Milwaukee, Wis. Cultures were maintained on mannitol-yeast extract (MYE) agar, pH 5.8, prepared essentially as described by Vincent (13); MYE agar contained 1.0% mannitol, 0.1% yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 0.066% $KH_2PO_4 \cdot 3H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, and 1.5% agar (Difco Laboratories, Detroit, Mich.).

Coals. Samples of anthracitic, bituminous, lignitic, and subbituminous coals were obtained with the cooperation of the Grand Forks Energy Research Center, U.S. Department of Energy. All coals were ground in a hammer mill, sifted to pass an 80-mesh sieve, and dried for 4 h at 160°C. The pH and moisture-holding capacity of each coal were determined as described by Roughley and Vincent (9).

Preparation of inoculants. Inoculants were prepared by mixing samples of each coal powder with MYE broth cultures (grown on a shaker for 30 h at 30°C) of *R. phaseoli* 127K17, 127K26, or 127K35 in sterile metal canisters (one coal and one strain per inoculant). Sufficient broth culture was added to each coal to achieve 60 to 70% of its moisture-holding capacity. Portions (50 to 65 g) of the rhizobia-coal mixtures were packaged in 3-mil polyethylene bags (6-oz Whirl-Pak; Nasco, Fort Atkinson, Wis.). These packages were incubated at 30°C for 2 weeks and then stored at room temperature (20 to 22°C).

Enumeration of rhizobia. The number of viable rhizobia in each inoculant was determined by plate counts. A 1-g amount of inoculant was aseptically transferred to 9.0 ml of saline (0.5% NaCl). After being mixed, the suspension was serially diluted in saline and plated, in duplicate, on MYE agar supplemented with congo red (25 mg/liter; Harleco, Philadelphia,

Pa.) (13); colonies were counted after the plates had been incubated for 72 h at 30°C.

Plant inoculation procedure. Pinto beans (University of Idaho 111) were surface-sterilized by being rinsed in 95% ethanol, soaked for 5 min in 0.2% $HgCl_2$ (acidified with 5 ml of concentrated HCl per liter), and rinsed with distilled water (13). The seeds were incubated on water agar for 48 h at 30°C to achieve germination. Seedlings were planted in 150-ml wide-mouth flasks containing sterile, chopped vermiculite saturated with a nitrogen-free plant nutrient solution (10). After the seedlings had been planted, the flasks were wrapped with foil, and each seedling was covered with a sterile polyethylene bag. The plants were grown at 22°C with 18-h photoperiods provided by 40-W plant lights (F40-PL, General Electric) which yielded approximately 1,200 lumens. As the seedlings grew, the bags were removed and sterile cotton was packed around the stems. Plants were maintained with nutrient solution as needed (about 30 ml per plant per week).

To confirm that colonies enumerated in plate counts were due to *R. phaseoli*, we used isolates to inoculate plants. Cells of each isolate were grown in 10 ml of MYE broth for 40 h at 30°C, collected by centrifugation, resuspended in 2 ml of nutrient solution, and injected into flasks containing 7-day-old plants (1 ml of suspension per plant). After 3 weeks, the roots of each plant were examined for the presence of nodules.

RESULTS

Table 1 lists the coals used and their pertinent characteristics. Each type of coal, except anthracite, was obtained from at least two different locations; in the United States, anthracite is mined only in northeastern Pennsylvania. The coals ranged in pH from 4.7 to 7.5 and varied considerably in both moisture content and moisture-holding capacity. Drying of the powdered coals at 160°C for 4 h virtually eliminated contaminating microorganisms.

Tables 2, 3, and 4 show the survival of the three strains of *R. phaseoli* in inoculants prepared with each of the eight coals. All coals, except bituminous coal from Illinois and lignite from Texas, supported the growth and survival of all strains of *R. phaseoli*. The numbers of rhizobia in Illinois bituminous coal and Texas lignite decreased to less than 10^5/g in the first 2 weeks; the low pH's of these coals (5.0 and 4.7, respectively) were probably responsible for the rapid death. The numbers of rhizobia in the other six coals exceeded 10^7/g for at least 6 months. Two-way analyses of variance ($\alpha = 0.05$) performed on the data at 4 and 8 months indicated no significant differences among strains as to survival; that is, survival was not strain-dependent.

TABLE 1. *Coals and their characteristics*

Type	Source	pH	Moisture (%)	Moisture-holding capacity[a] (ml/100 g)
Anthracite	Pennsylvania	7.5	1.6	48
Bituminous	Illinois	5.0	10.0	80
Bituminous	Pennsylvania	6.5	0[b]	60
Bituminous	Utah	7.5	3.4	56
Lignite	North Dakota	6.4	26.3	92
Lignite	Texas	4.7	19.4	80
Subbituminous	New Mexico	6.8	9.0	64
Subbituminous	Wyoming	7.3	20.6	92

[a] Milled coal was previously dried at 160°C for 4 h.
[b] This coal was oily.

TABLE 2. *Survival of R. phaseoli 127K17 in coal*

Coal[a]	Log_{10} colony-forming units per g after:					
	2 h	2 wk	1 mo	4 mo	8 mo	12 mo
AP	8.76	8.96	8.60	8.34	8.36	7.89
BI	6.32	<5.0	—	—	—	—
BP	8.41	8.86	8.67	8.32	7.58	6.78
BU	8.40	9.89	8.89	8.90	9.32	8.20
LN	8.78	9.38	9.15	8.92	8.48	8.51
LT	7.08	<5.0	—	—	—	—
SN	8.45	9.58	9.57	9.45	8.64	7.81
SW	8.58	8.54	8.69	8.48	8.41	7.89

[a] Abbreviations used: AP, anthracite from Pennsylvania; BI, bituminous coal from Illinois; BP, bituminous coal from Pennsylvania; BU, bituminous coal from Utah; LN, lignite from North Dakota; LT, lignite from Texas; SN, subbituminous coal from New Mexico; SW, subbituminous coal from Wyoming.

TABLE 3. *Survival of R. phaseoli 127K26 in coal*

Coal[a]	Log_{10} colony-forming units per g after:					
	2 h	2 wk	1 mo	4 mo	8 mo	12 mo
AP	8.69	9.11	9.32	8.81	7.00	7.79
BI	5.30	<5.0	—	—	—	—
BP	8.54	9.04	9.76	8.26	7.56	<3.0
BU	8.79	9.64	9.56	9.04	8.18	8.08
LN	8.96	9.58	9.51	8.85	8.11	8.00
LT	8.64	<5.0	—	—	—	—
SN	8.87	9.73	9.49	9.18	8.56	8.48
SW	8.74	7.95	8.96	8.91	8.08	7.79

[a] Abbreviations as in Table 2.

TABLE 4. *Survival of R. phaseoli 127K35 in coal*

Coal[a]	Log_{10} colony-forming units per g after:					
	2 h	2 wk	1 mo	4 mo	8 mo	12 mo
AP	8.81	9.18	9.88	8.64	8.18	7.00
BI	5.30	<5.0	—	—	—	—
BP	8.72	8.34	8.59	8.04	6.60	—
BU	8.75	9.11	8.89	8.64	8.66	8.04
LN	8.79	8.71	8.45	8.28	7.72	7.54
LT	8.60	<5.0	—	—	—	—
SN	8.83	9.66	9.59	9.34	8.95	8.66
SW	8.72	6.72	5.30	7.64	8.52	8.62

[a] Abbreviations as in Table 2.

At the 8-month sampling time, two representative colonies growing on plate-count plates for each of the inoculants were streaked for isolation and used to prepare pure cultures. All isolates caused the formation of mature, pink nodules on pinto bean plants.

To confirm the results of the first survival experiment, we prepared 18 inoculants (one strain and one coal per inoculant, using all three strains and the six best coals). The milled coals had been stored at room temperature for 8 months by this time, and all had lowered pH's (Table 5). The decreases in pH were due, most probably, to spontaneous oxidation of aliphatic groups (4). The results of the second survival experiment, performed for 8 months, strongly supported those of the first. However, inoculants prepared with Pennsylvania bituminous coal and North Dakota lignite did not maintain rhizobial viability as well as they had previously; both coals had a pH of 5.8.

The influence of storage temperature on the survival of *R. phaseoli* 127K17 was studied by comparisons of viable counts of inoculants (prepared with the six best coals) stored at 4, 22, and 30°C. Rhizobial survival was comparable in inoculants stored at 4 and 22°C after 10 months. A two-way analysis of variance ($\alpha = 0.05$) indicated no significant difference in survival at the two temperatures; however, rhizobial survival was significantly better at 22°C than at 30°C after only 5 months of storage.

Sterile mixtures of North Dakota lignite and powdered vermiculite, corn meal, sucrose, or yeast extract were prepared to determine whether amended carriers were superior to coal alone in promoting rhizobial survival. The results in Table 6 show that the additives did not improve the survival of *R. phaseoli* 127K17 as compared to that in unamended lignite. A two-way analysis of variance performed on the data at the 6-month sampling time indicated no significant difference in rhizobial survival in inoculants containing amended coal or coal alone ($\alpha = 0.01$). Also, the incidence of contamination was higher when corn meal, sucrose, or yeast extract was present in the inoculant.

TABLE 5. *Coal powder pH after 8 months of storage*

Coal	pH
Anthracite, Pennsylvania	6.7
Bituminous, Pennsylvania	5.8
Bituminous, Utah	6.6
Lignite, North Dakota	5.8
Subbituminous, New Mexico	6.3
Subbituminous, Wyoming	6.6

DISCUSSION

The unavailability of suitable peat in many areas of the world has prompted efforts to identify alternative carriers for legume inoculants. Previous investigations (2, 3, 6, 11) have shown that coal has potential as a carrier, but the value of these studies has been limited by the restricted scope of the experiments. Our results suggest that most coals, regardless of grade or source, can support the growth and survival of *R. phaseoli*. Strijdom and Deschodt (11) have also reported that the grade and source of coal used as a carrier had no appreciable effect on rhizobial growth and survival.

Those coal-based inoculants in which rhizobial viability was maintained exceeded the quality standard for peat-based inoculants ($>10^6$ rhizobia per g) (8, 13) for at least 7 months. In fact, most inoculants exceeded a 10-fold higher standard for at least 12 months, a period more than twice that reported in any previous study of coal-based inoculants.

Satisfactory survival of *R. phaseoli* was not obtained in coals having a pH of 5.8 or less. In all previous studies of coal-based inoculants, calcium carbonate was used to neutralize coal acidity. We are currently conducting experiments to determine whether Illinois bituminous coal and Texas lignite will support the long-term survival of *R. phaseoli* when neutralized with calcium carbonate. If so, virtually any coal may be usable as a carrier.

Among other things, low storage temperatures for inoculants have been reported to reduce desiccation and thus have a favorable effect on rhizobial survival (5, 7, 12). This beneficial effect of low storage temperatures was also observed

TABLE 6. *Survival of R. phaseoli 127K17 in North Dakota lignite and amended lignite*

Carrier	Log_{10} colony-forming units per g after:							
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo
Coal	7.95	7.63	8.26	8.54	8.30	9.00	9.08	8.30
Coal (80%)–vermiculite (20%)	9.13	7.77	7.94	8.13	8.08	8.56	8.42	8.25
Coal (80%)–corn meal (20%)	8.46	8.84	9.91	9.67	10.11	9.75	8.51	8.63
Coal + 1% sucrose	7.84	7.48	8.80	9.04	8.61	9.04	8.67	8.48
Coal + 1% yeast extract	8.52	8.83	9.84	9.61	9.73	9.04	8.62	7.90

for the inoculants prepared in this investigation. *R. phaseoli* survived better at 4 and 22°C than at 30°C; storage at 30°C accelerated drying and rhizobial death.

Amended North Dakota lignite was not significantly superior to lignite alone in supporting survival of *R. phaseoli*. Although lucerne meal and sucrose have been shown to be beneficial in other coal-based inoculants (11), the inclusion of corn meal, sucrose, or yeast extract in inoculants prepared with North Dakota lignite only resulted in an increased incidence of contamination. Because nutrients such as meals and sucrose may increase the risk of inoculant contamination, and may also stimulate the growth of competing microorganisms once an inoculant is applied to the soil, their use in inoculants should be avoided if they only marginally increase rhizobial survival.

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