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Comprehensive Final Report

Alternative Carrier Materials for *Rhizobium phaseoli* Inoculants

Grants 701-15-64 and 801-15-97
AID-SEA/CR Symbiotic Nitrogen Fixation Program

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BRIEF HISTORY OF PROJECT

Grant 701-15-64, "Alternative Carrier Materials for *Rhizobium phaseoli* Inoculants," was awarded April 18, 1977; the original termination date was April 17, 1980. Because of promising results with coal-based inoculants, permission was granted on July 25, 1978 to largely restrict further research to coal--provided that peat controls were included in more critical experiments. Grant 801-15-97, "Alternative Carrier Materials for *Rhizobium* phaseoli Inoculants," was awarded September 26, 1978 to allow for the study of the application of coal-based inoculants to seed. The termination date for grant 801-15-97 was September 30, 1981; the same termination date was also established for grant 701-15-64.

INTRODUCTION

In order to obtain the full benefits of biological dinitrogen fixation in developing countries, high-quality inoculants must be used. It is important, therefore, to adapt inoculant technology to the conditions in different geographical regions. Inoculants are usually prepared by mixing broth cultures of appropriate rhizobia with a carrier material. Although peat is recognized as the most desirable of the relatively few carriers examined, peat suitable for use in inoculants is not universally available. One objective of this research was to identify carriers which may be substituted for peat in *R. phaseoli* inoculants.

Although the results of several investigations have suggested that coal has potential as a carrier material, no comprehensive study had been performed up to the time of the present research project. Because our results with eight different coals and three strains of *R. phaseoli*

were promising, the scope of the project was expanded to include methods for the application of coal-based inoculants to seed and a study of the survival of the rhizobia on seed.

MATERIALS AND METHODS

Bacteria

R. phaseoli strains 127K17, 127K26, and 127K35 were provided by Dr. J. C. Burton, The Nitragin Company, Milwaukee, Wisc. Cultures were maintained on mannitol-yeast extract (MYE) agar, pH 5.5-5.8; MYE agar contains: mannitol, 10.0 g; yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 1.0 g; $KH_2PO_4 \cdot 3H_2O$, 0.66 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; agar (Difco Laboratories, Detroit, Mich.), 15.0 g; and distilled H_2O, 1000 ml. Stock cultures were grown on MYE agar slants, in 20 X 150 mm screw-capped tubes, for 72 h at 30°C, and then stored at 4°C. Fresh stock cultures were prepared every 3 months.

Preparation of Coal-Based Inoculants

One anthracitic, three bituminous, two subbituminous, and two lignite coals (Table 1) were supplied by the United States 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 of each coal was determined by shaking a mixture of 10 g of coal dust and 50 ml of distilled H_2O for 20 min, and then electrometrically measuring the pH of the mixture. The moisture-holding capacity (MHC) of each coal was determined by measuring the amount of H_2O required to saturate a 25.0-g sample.

Inoculants were prepared by mixing samples of each coal powder with MYE-broth cultures, grown for 30 h at 30°C, of *R. phaseoli* 127K17, 127K26, or 127K35 in sterile metal canisters (one coal and one strain per inoculant). Portions (50-65 g) of the rhizobia-coal mixtures were packaged in 6-oz polyethylene (3 mil) Whirl-Pak bags (Nasco, Fort Atkinson, Wisc.). These packages were incubated at 30°C for 2 weeks, and then stored.

Table 1. Coals used in inoculants

Type	Source	pH	% Moisture	Moisture-holding capacity (ml/100 g)	Particle size (%)	
					100-Mesh	200-Mesh
Anthracite	PA	7.5	1.6	48	86	39
Bituminous	IL	5.0	10.0	80	100	82
Bituminous	PA	6.5	0[a]	60	100	74
Bituminous	UT	7.5	3.4	56	99	69
Lignite	ND	6.4	26.3	92	99	60
Lignite	TX	4.7	19.4	80	97	38
Subbituminous	NM	6.8	9.0	64	98	51
Subbituminous	WY	7.3	20.6	92	99	69

[a]This coal was oily.

Plate-Count Procedure

The number of viable rhizobia in each inoculant was determined by plate counts. One gram of inoculant was aseptically transferred to 9.0 ml of sterile saline (0.5% NaCl). After mixing, the suspension was serially diluted in saline and plated, in duplicate, on MYE agar supplemented with congo red (25 mg/liter; Harleco, Philadelphia, Pa.); colonies were counted after the plates were incubated for 72 h at 30°C.

Plate-Inoculation Procedures

Pinto beans (UI 111; provided by Dr. A. Schneiter, Department of Agronomy, North Dakota State University) were treated by rinsing in 95% ethanol, soaking for 5 min in 0.2% $HgCl_2$ (acidified with 5 ml of concentrated HCl per liter), and rinsing with distilled H_2O. The seeds were incubated on water agar for 48 h at 30°C to achieve germination. Seedlings were planted in 60-ml serum vials or 150-ml wide-mouth flasks containing sterile, chopped vermiculite saturated with a nitrogen-free plant nutrient solution; the nutrient solution (pH 6.5) contained: KCl, 0.5 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 1.0 mg; $ZnSO_4 \cdot 7H_2O$, 1.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; $MnCl_2 \cdot 4H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg; Fe-EDTA, 1.0 mg; and distilled H_2O, 1000 ml. After the seedlings were planted, the containers were wrapped with foil, and seedlings were covered with a sterile plastic bag. The plants were grown at 22°C with 18-h photoperiods provided by 40-watt plant lights (F40-PL, General Electric) which provided approximately 1200 lumens. As the seedlings grow, the plastic bags were removed and cotton was packed around the stems. Plants were maintained with nutrient solution as needed (about 30 ml per plant per week).

To characterize the infectivity (ability to induce nodule formation) and effectiveness (ability to fix N_2) of a given *R. phaseoli* isolate, two week-old plants were inoculated with cells grown in 10 ml of MYE broth for 40 h at 30°C. The cells were pelleted by centrifugation and resuspended in 2 ml of nutrient solution, and 1 ml of this suspension was used for inoculation. Approximately 18 days after inoculation, effectiveness was assayed by the acetylene-reduction technique. The stems of plants growing in 60-ml serum vials were cut, and the vials were tightly stoppered and partially evacuated. Then, 5 ml of acetylene was injected into each vial. After incubation of the vials for 2 h at 22°C, 0.5 ml of the gas phase was removed from each vial and analyzed for ethylene with a Beckman GC-45 flame ionization chromatograph, equipped with a 1.8 X 2 mm glass column packed with Porapak R (Applied Science Laboratories, Inc., State College, Pa.). Operating conditions were:

Inlet temperature	70°C
Column temperature.	35°C
Detector line temperature	90°C
Detector temperature.	120°C
Carrier gas flow (N_2)	50 ml/min
H_2 gas flow	45 ml/min
Air flow.	250 ml/min
Column flow	20 ml/min
Carrier make-up	70 ml/min

Following the acetylene-reduction assay, roots from each vial were examined for the presence and appearance of nodules; roots of plants grown in wide-mouth flasks were inspected 3 weeks after inoculation.

Seed-Inoculation Procedure

When survival of *R. phaseoli* on seed was monitored, spontaneous streptomycin-resistant mutants of strains 127K17, 127K26, and 127K35 were used. Pinto beans were randomly separated into 100-bean aliquots and stored in 18-oz Whirl-Pak bags. Approximately 2 g of inoculant containing a streptomycin-resistant *R. phaseoli* strain and 10 ml of 30% gum arabic (flakes; Mallinkrodt, Inc., St. Louis, Mo.) were added to each bag. The bags were shaken for 10 seconds to facilitate even coating of seeds, and the seeds were transferred to sterile 10-mesh sieves. These were shaken for 10 seconds to remove excess inoculant, and the seeds were removed and stored at 20-22°C in 18-oz Whirl-Pak bags. Each inoculated sample was held for 1 month, with subsamples taken weekly. Ten seeds were aseptically transferred to 25 X 150 mm screw-capped tubes containing 10.0 ml of sterile saline and 10 glass beads (3 mm). After 10 seconds of vortexing, the resulting suspension was serially diluted in saline and plated in duplicate on MYE agar containing congo red, 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.

RESULTS

In initial experiments, North Dakota lignite obtained from the power plant at North Dakota State University was used. After the lignite was ground and sifted, 87% of the particles were smaller than 100-mesh, and 55% were smaller than 200-mesh. The pH of the coal was 7.6, and the moisture content was 20% by weight. When the dust was dried at 160°C for 4 h, contaminating microorganisms were virtually eliminated. The dried dust had an MHC of 88 ml/100 g.

To determine if the lignite contained any products which would be toxic to rhizobia, water extracts of the lignite dust were prepared by soaking 60 g of dust in 300 ml of distilled H_2O for 24 h. The coal suspension was then filtered through muslin and filter paper, and the filtrate was used in place of distilled H_2O to prepare MYE broth. R. phaseoli strains 127K17, 127K26, and 127K35 grew in the coal extract-MYE broth as well as they did in normal MYE broth in a 48-h period at 30°C; therefore, the lignite did not appear to be toxic to the rhizobia.

Because saline may adversely affect some strains of Rhizobium, saline and MYE broth were compared as diluents in the plate-count procedure. Counts obtained with the two diluents were comparable. Rhizobia could be held in saline at least 45 min without detrimental effects.

Inoculants having final moisture contents of 30, 40, 50, 60, and 70% of MHC were prepared by adding appropriate amounts of a broth culture of R. phaseoli 127K17, grown for 30 h at 30°C, to 100-g samples of lignite dust. After packaging, the inoculant preparations were incubated at 30°C for 2 weeks and then stored at 22°C. The results shown in Table 2 indicated that rhizobial survival was best in inoculants prepared at 60-70 of MHC. Additional experiments which examined rhizobial survival in inoculants prepared at 60, 70 and 80% of MHC also showed that 60-70% was optimal;

Table 2. Survival of R. phaseoli 127K17, at 22°C, in North Dakota lignite at different moisture levels

% of moisture-holding capacity	Log_{10} colony-forming units per g after:											
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo
30	6.11	<5.0	--	--	--	--	--	--	--	--	--	--
40	7.51	<5.0	--	--	--	--	--	--	--	--	--	--
50	8.20	6.72	8.91	8.77	9.20	8.48	8.23	7.60	6.95	6.76	6.34	5.78
60	8.80	8.76	9.71	9.00	9.26	9.18	9.11	9.91	8.53	8.66	7.34	7.00
70	9.51	6.73	7.75	8.64	9.15	9.32	9.04	8.90	8.74	8.43	8.11	7.88

at a higher moisture level the liquid and solid components of the inoculant separated, and rhizobial survival was not as good. Therefore, all further inoculants were prepared by adding broth culture to carrier to achieve 60-70% of MHC. Also, inoculants were packaged in 50- to 65-g portions because smaller quantities desiccated more rapidly.

To determine if heat of wetting would have any detrimental effect on rhizobial viability, inoculants were prepared with lignite and a broth inoculum of R. phaseoli 127K17. When inoculants were prepared with ingredients equilibrated to 5 or 22°C, the temperatures rose to 35 and 48°C, respectively, within 5 min. These inoculants were incubated at 30°C for 2 weeks and then stored at 22°C. The rhizobial numbers were comparable in both inoculants. Apparently, the heat produced by mixing the carrier and broth inoculum quickly dissipated and had no deleterious effect on rhizobial survival.

Mixtures of lignite and various materials were prepared to determine if amended carriers were superior to coal alone in promoting rhizobial survival. The mixtures included: 80% coal: 20% vermiculite, 80% coal: 20% corn meal, coal plus 1% sucrose, coal plus 1% yeast extract, and coal plus 1% catalase (Sigma Chemical Co., St. Louis, Mo.) The results in Table 3 show that the additives examined did not improve rhizobial survival as compared to that in inoculants without supplementation. 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.

Twenty-four inoculants were prepared using eight different coals (Table 1) and three strains of R. phaseoli (one coal and one strain per

Table 3. 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	7 mo	8 mo	9 mo
Coal	7.95	7.63	8.26	8.54	8.30	9.00	9.08	8.30	8.60	8.08	7.90
80% Coal: 20% vermiculite	9.13	7.77	7.94	8.13	8.08	8.56	8.42	8.25	8.26	8.03	7.79
80% Coal: 20% corn meal	8.46	8.84	9.91	9.67	10.11	9.75	8.51	8.63	8.28	8.24	7.81
Coal + 1% sucrose	7.84	7.48	8.80	9.04	8.61	9.04	8.67	8.48	7.70	7.49	7.26
Coal + 1% yeast extract	8.52	8.83	9.84	9.61	9.73	9.04	8.62	7.90	7.51	7.12	6.85
Coal + 1% catalase	8.86	--	9.38	9.23	9.48	8.80	8.83	8.72	8.59	--	--

inoculant). These inoculants were incubated at 30°C for 2 weeks and then stored at 22°C. Tables 4, 5, and 6 show that all coals, except bituminous coal from Illinois and Texas lignite, supported the growth and survival of all strains of R. phaseoli. The numbers of rhizobia in Illinois bituminou coal and Texas lignite decreased to less than 1 X 10^5/g in the first 2 wee the low pH's of these coals (5.0 and 4.7, respectively) may have adversely affected the rhizobial viability. The numbers of rhizobia in the other si coals were at least 1 X 10^7/g after 6 months. Two-way analyses of varianc (α = 0.05) performed on the survival data at 4 and 8 months indicated that there were significant differences in rhizobial survival among the six coa however, these differences could not be correlated with the grade of coal. Two-way analyses of variance also indicated no significant differences amo strains as to survival; that is, survival was not strain-dependent.

The influence of storage temperature on rhizobial viability was studi by comparisons of rhizobial survival in inoculants stored at 4, 22, and 30 The results in Table 7 show comparable rhizobial survival (>1 X 10^7 rhizob per g) in six coals stored at 4 and 22°C after 10 months. A two-way analy of variance (α = 0.05) indicated no significant difference in survival at two temperatures. However, the data in Table 8 indicate that rhizobial su vival is significantly better at 22°C than at 30°C after only 5 months of storage (two-way analysis of variance, α = 0.05). Also, when one set of inoculants was held at 37°C, rhizobial death was rapid.

Inoculants were prepared in which the initial rhizobial populations varied from 1 X 10^7 to 1 X 10^9 colony-forming units per g. After 2 months the rhizobial counts were comparable in all inoculants (Table 9); therefor within limits, the initial number of rhizobia in coal-based inoculants doe not seem critical to the quality of the final product.

Table 4. Survival of R. phaseoli 127K17 in coal

Coal	Log_{10} colony-forming units per g after:													
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo	11 mo	12 mo
AP[a]	8.76	8.96	8.60	8.83	8.57	8.34	8.59	8.15	8.68	8.36	8.63	7.38	7.89	7.89
BI	6.32	<5.0	--	--	--	--	--	--	--	--	--	--	--	--
BP	8.41	8.86	8.67	8.86	8.34	8.32	8.08	8.04	8.11	7.58	7.76	7.46	7.45	6.78
BU	8.40	9.89	8.89	8.91	8.93	8.90	8.46	8.64	8.45	9.32	8.64	8.76	8.72	8.20
LN	8.78	9.38	9.15	9.04	9.15	8.92	8.98	9.41	8.74	8.48	8.63	8.67	8.65	8.51
LT	7.08	<5.0	--	--	--	--	--	--	--	--	--	--	--	--
SN	8.45	9.58	9.57	9.51	9.32	9.45	9.11	9.20	8.63	8.64	8.60	8.57	8.49	7.81
SW	8.58	8.54	8.69	8.43	9.08	8.48	8.54	8.26	8.15	8.41	8.04	8.57	8.04	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 5. Survival of R. phaseoli 127K26 in coal

Coal	Log_{10} colony-forming units per g after:													
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo	11 mo	12 mo
AP[a]	8.69	9.11	9.32	8.87	8.85	8.81	8.08	7.75	7.65	7.00	7.11	7.99	7.41	7.79
BI	5.30	<5.0	--	--	--	--	--	--	--	--	--	--	--	--
BP	8.54	9.04	9.76	8.49	8.65	8.26	8.36	7.93	7.28	7.56	7.58	6.85	4.78	<3.0
BU	8.79	9.64	9.56	9.69	9.11	9.04	8.99	8.80	8.56	8.18	8.68	8.00	8.11	8.08
LN	8.96	9.58	9.51	9.20	9.18	8.85	8.73	8.40	8.26	8.11	7.88	8.08	8.04	8.00
LT	8.[illegible]	[illegible].0	--	--	--	--	--	--	--	--	--	--	--	--
SN	8.87	[illegible]3	9.49	9.48	9.11	9.18	9.04	8.89	8.66	8.56	8.30	8.52	8.52	8.48
SW	[illegible]	7.95	8.96	8.87	8.96	8.91	8.79	8.58	8.08	8.08	7.99	7.97	7.95	7.79

[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 6. Survival of R. phaseoli 127K35 in coal

Coal	Log_{10} colony-forming units per g after:													
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo	11 mo	12 mo
AP[a]	8.81	9.18	9.88	9.46	8.54	8.64	8.45	7.63	7.51	8.18	7.20	7.20	7.04	7.00
BI	5.30	<5.0	--	--	--	--	--	--	--	--	--	--	--	--
BP	8.72	8.34	8.59	8.53	8.18	8.04	8.04	7.28	6.81	6.60	<4.0	<3.0	--	--
BU	8.75	9.11	8.89	8.99	8.84	8.64	8.62	8.34	8.36	8.66	8.30	8.53	8.11	8.04
LN	8.79	8.71	8.45	8.36	8.08	8.28	8.36	7.61	7.63	7.72	7.74	7.49	7.52	7.54
LT	8.60	<5.0	--	--	--	--	--	--	--	--	--	--	--	--
SN	8.83	9.66	9.59	9.63	9.45	9.34	9.20	8.86	9.08	8.95	8.85	8.79	8.70	8.66
SW	8.72	6.72	5.30	<5.0	6.20	7.64	8.30	8.54	8.51	8.52	8.77	8.83	8.45	8.62

[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 7. Survival of *R. phaseoli* 127K17 in coal at 4 and 22°C after 10 months

Coal	Log_{10} colony-forming units per g at:	
	4°C	22°C
Anthracite, PA	7.64	7.38
Bituminous, PA	7.58	7.46
Bituminous, UT	8.64	8.76
Lignite, ND	8.72	8.67
Subbituminous, NM	7.84	8.57
Subbituminous, WY	7.92	8.57

Table 8. Survival of *R. phaseoli* 127K17 in coal at 22 and 30°C after 5 months

Coal	Log_{10} colony-forming units per g at:	
	22°C	30°C
Anthracite, PA	8.59	7.58
Bituminous, PA	8.08	4.00
Bituminous, UT	8.46	8.97
Lignite, ND	8.98	5.45
Subbituminous, NM	9.11	7.15
Subbituminous, WY	8.54	7.20

To confirm the results of the long-term survival studies using the eight coals and three strains of *R. phaseoli*, 24 inoculants were again prepared. However, the eight coals (which had been stored 8 months by this time) had lowered pH's due, most probably, to spontaneous oxidation of aliphatic groups. Table 10 shows the pH's of the coals after storage

Table 9. Effect of inoculum level on the resultant number of rhizobia in the inoculant

Coal	Log_{10} colony-forming units per g after:				
	0 h	2 h	2 wk	1 mo	2 mo
	9.08	8.16	8.79	8.81	8.87
Bituminous, UT	8.08	6.92	8.93	10.30	8.76
	7.08	6.04	8.87	8.95	8.92
	9.11	8.72	9.46	9.18	9.11
Subbituminous, NM	8.11	7.62	9.38	8.96	9.20
	7.11	6.52	9.04	9.04	8.69

Table 10. Coal pH after 8 and 13 months of storage

Coal	pH		
	Initial	After 8 mo	After 13 mo
Anthracite, PA	7.5	6.7	6.7
Bituminous, IL	5.0	--	4.5
Bituminous, PA	6.5	5.8	5.8
Bituminous, UT	7.5	6.6	6.6
Lignite, ND	6.4	5.8	5.8
Lignite, TX	4.7	--	3.4
Subbituminous, NM	6.8	6.3	6.3
Subbituminous, WY	7.3	6.6	6.6

for 8 and 13 months. Virtually all oxidation seems to have occurred within the first 8 months. Coal pH does not appear to be affected by storage with R. phaseoli (Table 11); the inoculant pH's were comparable to the pH's of the coals alone after 13 months.

Table 11. Inoculant pH after 13 months

Coal	pH		
	127K17	127K26	127K35
Anthracite, PA	6.7	6.9	6.9
Bituminous, PA	4.8	4.3	4.2
Bituminous, UT	6.7	6.5	6.6
Lignite, ND	5.8	5.7	5.5
Subbituminous, NM	5.9	5.7	5.9
Subbituminous, WY	6.8	6.5	6.7

Tables 12, 13, and 14 indicate that rhizobial survival in the second trial was similar to that in the first, except that some of the coals having more acidic pH's after 8 months of storage (Pennsylvania bituminous coal and North Dakota lignite) did not maintain rhizobial viability as well as they had previously. Two-way analyses of variance ($\alpha = 0.05$) performed on the second trial results at 4 and 8 months indicated significant differences in rhizobial survival among the six coals that allowed long-term viability. Survivals of the three strains within each of the six coals were again comparable; two-way analyses of variance ($\alpha = 0.05$) at 4 and 8 months indicated that survival was not strain-dependent. Therefore, the results of the second trial strongly supported those of the first.

In order to confirm that colonies enumerated in plate counts of inoculants stored for prolonged periods were due to R. phaseoli, plant inoculation tests were performed on isolates from inoculants prepared with R. phaseoli strains 127K17, 127K26, and 127K35 after 8 months of storage; all isolates were infective. In addition, acetylene-reduction assays were performed on the R. phaseoli 127K17 and 127K26 isolates, and they were all effective.

Table 12. Survival of R. phaseoli 127K17 in coal (second trial)

Coal	Log_{10} colony-forming units per g after:									
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo
AP[a]	7.77	8.85	8.58	8.30	8.46	8.72	8.23	8.23	8.08	8.04
BI	6.58	<5.0	--	--	--	--	--	--	--	--
BP	8.04	8.94	8.74	8.84	9.11	8.18	8.11	8.11	7.49	8.11
BU	7.56	8.96	10.18	8.85	9.32	9.15	8.80	9.23	8.59	8.67
LN	8.23	7.74	7.73	7.86	7.20	7.56	6.95	6.75	6.69	6.52
LT	6.15	<5.0	--	--	--	--	--	--	--	--
SN	8.18	10.15	9.63	9.54	9.20	9.18	9.15	8.91	9.04	8.70
SW	8.45	7.79	8.46	8.38	8.59	8.76	8.51	8.62	8.51	8.28

[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 13. Survival of R. phaseoli 127K26 in coal (second trial)

Coal	Log_{10} colony-forming units per g after:									
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo
AP[a]	8.18	7.54	7.76	7.53	8.74	8.18	8.18	7.82	7.73	8.04
BI	5.30	<5.0	--	--	--	--	--	--	--	--
BP	7.78	7.46	8.53	8.08	8.45	8.18	7.30	7.04	6.98	6.95
BU	7.76	8.76	8.76	8.74	8.81	8.94	8.81	8.58	8.54	8.78
LN	8.58	<5.0	6.04	7.91	8.38	8.20	7.88	7.75	7.53	7.66
LT	7.23	<5.0	--	--	--	--	--	--	--	--
SN	8.36	9.08	9.48	9.11	9.28	9.41	9.04	8.80	8.95	9.04
SW	8.34	6.04	5.90	5.40	7.61	7.99	7.90	7.73	7.86	7.99

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 14. Survival of R. phaseoli 127K35 in coal (second trial)

Coal	Log_{10} colony-forming units per g after:									
	2 h	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo
AP[a]	8.41	6.78	5.00	7.36	7.61	6.88	6.99	6.85	6.92	6.04
BI	5.78	<5.0	--	--	--	--	--	--	--	--
BP	8.38	<6.0	6.67	7.45	7.92	8.38	7.74	7.49	6.58	6.59
BU	8.34	8.38	8.32	8.49	8.15	8.59	8.54	8.51	8.15	8.18
LN	8.40	7.70	<5.0	<4.0	5.32	5.98	5.76	6.49	6.38	5.91
LT	8.15	<5.0	--	--	--	--	--	--	--	--
SN	8.71	9.45	9.11	9.08	9.23	9.04	8.95	9.04	9.08	8.72
SW	8.41	<6.0	7.65	8.54	8.50	8.51	8.43	8.32	8.28	8.15

[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.

Because survival of *R. phaseoli* in Illinois bituminous coal and Texas lignite was poor, probably due to the low pH's of these coals, several compounds were tested for their ability to raise coal pH. Of these, "precipitated" $CaCO_3$ was best. The results in Tables 15 and 16 show that the addition of 10% $CaCO_3$ to Illinois bituminous coal and Texas lignite substantially increased their pH's. Long-term rhizobial survival experiments were performed using bituminous coals from Illinois and Pennsylvania, and lignites from North Dakota and Texas containing 10% $CaCO_3$. Peat, also containing 10% $CaCO_3$, was used as a control. The survival data are given in Table 17. In all cases, greater than 1×10^6 viable *R. phaseoli* cells per g were present after 6 months. Illinois bituminous coal had greater than 1×10^6 viable cells per g after 10 months, and Texas lignite had greater than 1×10^6 viable cells per g after 12 months. Rhizobial survival in Texas lignite for 12 months was not significantly different from that in any of the other coals tested. Statistical analysis indicated that survival in $CaCO_3$-amended coals was not strain-dependent ($\alpha = 0.05$).

Table 15. Relationship between $CaCO_3$ concentration and pH of Texas lignite

Amount of coal (g)	Amount of $CaCO_3$ (g)	% $CaCO_3$	pH
10.0	0.0	0	4.0
9.5	0.5	5	6.2
9.0	1.0	10	6.5
8.5	1.5	15	6.7
8.0	2.0	20	6.9
7.0	3.0	30	7.1
6.0	4.0	40	7.1
5.0	5.0	50	7.2

Table 16. Relationship between $CaCO_3$ concentration and pH of Illinois bituminous coal

Amount of coal (g)	Amount of $CaCO_3$ (g)	% $CaCO_3$	pH
10.0	0.0	0	5.1-5.9
9.5	0.5	5	6.8
9.0	1.0	10	7.1
8.5	1.5	15	6.7
8.0	2.0	20	6.6
7.0	3.0	30	7.2
6.0	4.0	40	7.4
5.0	5.0	50	7.5

The pH's of the 15 inoculants were determined electrometrically after 13 months of storage, and the samples were also checked for moisture levels. Each value in Table 18 was the result of triplicate determinations, and one-way analysis of variance indicated no significant differences among bacterial strains ($\alpha = 0.05$). Changes in pH, known to be detrimental to rhizobial survival, did not appear to be a factor in rhizobial death in coal-based inoculants amended with 10% $CaCO_3$; all inoculant samples had pH's greater than 7.2 after 13 months.

After 13 months of storage, the moisture in all inoculants had decreased to approximately 30% of the original content (Table 18). Rhizobia are highly susceptible to desiccation, especially in the case of the fast-growing strains (*R. phaseoli*). No relationship between the degree of moisture loss and rhizobial death was noted. Illinois bituminous coal, which had the most marked decrease in rhizobial viability after 13 months, also had the highest remaining relative moisture. North Dakota

Table 17. Survival of *R. phaseoli* in coals and peat containing 10% $CaCO_3$

Carrier	Strain	Log$_{10}$ colony-forming units per g after:									
		0 h	2 wk	1 mo	2 mo	3 mo	4 mo	6 mo	8 mo	10 mo	12 mo
BI[a]	127K17	7.35	8.29	8.03	8.74	7.74	9.13	6.98	8.20	7.29	<6.0
	127K26	8.50	8.13	7.25	8.47	7.74	7.30	6.98	7.25	7.29	<6.0
	127K35	7.87	7.71	8.04	7.87	8.18	8.45	8.15	<6.0	7.71	<6.0
BP	127K17	8.48	8.90	8.95	7.90	9.50	9.85	9.41	9.62	8.50	8.38
	127K26	8.56	8.73	10.81	10.04	>11.0	10.65	10.56	7.65	7.37	8.71
	127K35	8.75	9.07	9.42	9.68	9.61	8.82	6.60	8.75	9.54	8.38
LN	127K17	7.85	8.62	8.41	9.10	8.38	8.78	7.65	9.87	7.38	8.93
	127K26	8.20	9.01	9.42	10.15	9.68	8.83	9.18	8.34	9.13	8.65
	127K35	8.90	10.01	9.77	9.89	9.16	10.01	9.45	8.18	8.40	8.73
LT	127K17	7.86	8.10	8.45	9.48	8.80	8.67	7.94	<6.0	7.62	8.15
	127K26	7.87	8.89	11.48	10.79	>11.0	10.70	9.69	8.05	8.48	7.87
	127K35	8.89	10.51	9.69	9.87	9.81	10.17	9.67	<6.0	7.99	8.09
Peat	127K17	8.29	7.62	8.64	8.71	8.48	8.92	8.10	6.65	8.45	8.11
	127K26	8.73	6.87	8.72	9.13	9.14	8.83	8.70	8.75	9.11	7.57
	127K35	8.71	8.90	9.97	9.57	9.33	10.30	9.74	8.54	9.85	6.87

[a]Abbreviations used: BI, bituminous coal from Illinois; BP, bituminous coal from Pennsylvania; LN, lignite from North Dakota; LT, lignite from Texas.

Table 18. Changes in coal-based inoculants after 13 months of storage

Carrier	pH after 13 mo	60% of MHC (ml/100 g)	Moisture content after 13 mo (ml/100 g)	Moisture content after 13 mo / 60% of MHC
Bituminous, Illinois	7.5	48	17	35.4
Bituminous, Pennsylvania	7.7	36	11	30.5
Lignite, North Dakota	7.3	55	15	27.2
Lignite, Texas	7.3	48	15	31.2
Peat	7.4	78	25	32.0

lignite, on the other hand, had adequate survival of R. phaseoli after 13 months, along with the lowest remaining moisture relative to MHC.

The problems encountered with low pH coals can apparently be circumvented with the addition of 10% $CaCO_3$ as is presently done with peat. However, rhizobial survival in coal during storage in apparently dependent on the coal itself. Statistical analyses indicated that rhizobial survival in coal-based inoculants was not strain-dependent.

Various methods of applying inoculant to seed employing dry inoculant, water plus inoculant, and an inoculant slurry were examined; due to a lack of adequate adhesion to seed, these methods were discarded. Application of coal-based inoculants using 30% gum arabic allowed for good numbers of bacteria per seed (Table 19). One-way analysis of variance indicated no difference in adhesion among coals ($\alpha = 0.01$), or between coals and peat ($\alpha = 0.01$). Data developed from Table 19 indicated that approximately 1 mg of inoculant adheres to each seed.

Viable cells per seed were determined by plate counts. Other researchers have shown a close correlation between plate-count data and nodulation, thus supporting the reliability of this method. Seeds were stored at room temperature in polyethylene bags to allow for gas exchange with a minimum of moisture loss. Samples were taken weekly and plated on agar containing congo red, dihydrostreptomycin sulfate, and cycloheximide. Congo red, due to its slow uptake by R. phaseoli, assisted in the recognition of rhizobial colonies. While the sterility of the applied inoculant can be controlled, seed-sterilization methods are tedious and can affect the rhizobial survival in the applied inoculant. Dihydrostreptomycin, which did not inhibit the resistant R. phaseoli mutants, eliminated most contaminating microorganisms and thereby allowed accurate counting. Cycloheximide prevented fungal contamination. When

Table 19. Average numbers of rhizobia applied to seed using coal-based inoculants and 30% gum arabic as an adhesive

Carrier	Strain	Log_{10} colony forming units: Per g of inoculant	Per seed
Anthracite, Pennsylvania	127K17	9.91	6.39
	127K26	9.48	7.03
	127K35	8.41	6.68
Bituminous, Illinois	127K17	7.32	5.59
	127K26	7.41	5.74
	127K35	9.79	5.70
Bituminous, Pennsylvania	127K17	9.23	7.11
	127K26	9.45	7.15
	127K35	8.78	7.09
Bituminous, Utah	127K17	9.96	7.15
	127K26	8.53	6.88
	127K35	9.08	7.45
Lignite, North Dakota	127K17	8.00	6.23
	127K26	9.34	7.24
	127K35	8.13	6.73
Lignite, Texas	127K17	8.70	5.77
	127K26	8.06	6.04
	127K35	7.87	5.50
Subbituminous, New Mexico	127K17	8.62	6.66
	127K26	8.60	7.21
	127K35	8.60	7.08
Subbituminous, Wyoming	127K17	8.68	7.25
	127K26	7.85	7.30
	127K35	8.50	7.30
Peat, Nitragin Company	127K17	10.30	8.00
	127K26	9.70	8.01
	127K35	11.02	8.20

the R. phaseoli strains were plated on both MYE agar and MYE agar containing dihydrostreptomycin sulfate and cycloheximide, there were only negligible differences in counts.

Survival experiments were continued for 4 weeks, and the data were converted to logarithms for statistical analysis (Tables 20-27). Each

Table 20. Survival of R. phaseoli in Pennsylvania anthracite on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	6.2	5.4	5.1	4.8	4.1
	6.5	-	4.8	4.8	4.4
	6.1	5.7	4.8	4.5	4.1
127K26	6.2	6.0	5.8	5.8	5.0
	6.3	-	5.2	5.5	4.5
	6.2	6.2	5.3	5.3	4.5
127K35	6.4	5.9	5.2	5.5	5.0
	6.3	-	5.6	-	5.1
	6.4	5.9	5.5	5.1	5.0

Table 21. Survival of R. phaseoli in Illinois bituminous coal on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	4.6	3.1	3.9	3.4	3.8
	5.7	3.6	3.0	-	2.9
	4.3	4.5	4.1	-	3.1
127K26	4.7	3.8	3.4	3.1	3.9
	5.8	3.2	3.3	-	2.6
	4.5	3.4	-	-	-
127K35	4.7	4.5	4.0	4.5	4.1
	5.9	4.6	5.4	-	3.8
	3.6	5.4	-	-	-

Table 22. Survival of *R. phaseoli* in Pennsylvania bituminous coal on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	5.7	5.5	4.7	4.0	3.5
	6.2	5.4	5.0	-	4.6
	6.0	4.2	-	3.9	4.4
127K26	5.9	4.7	3.7	3.2	3.1
	6.1	5.6	5.2	-	5.0
	6.0	5.5	5.2	4.4	4.7
127K35	5.8	-	4.4	4.6	3.8
	6.0	5.4	5.0	-	4.3
	5.8	4.4	4.9	3.7	3.7

Table 23. Survival of *R. phaseoli* in Utah bituminous coal on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	6.1	4.5	3.0	4.5	3.7
	6.2	5.5	5.4	5.0	4.1
	6.0	5.4	4.6	5.2	4.5
127K26	6.0	4.7	5.5	4.7	4.0
	6.1	5.4	4.7	4.6	4.3
	6.2	5.6	5.1	5.2	4.9
127K35	5.8	4.6	5.5	4.9	4.2
	5.9	4.9	-	4.3	3.6
	5.8	4.8	4.3	4.3	3.5

Table 24. Survival of R. phaseoli in North Dakota lignite on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	5.0	3.9	3.8	5.2	3.3
	5.1	4.2	-	4.2	4.9
	5.7	5.2	4.7	4.6	3.9
127K26	-	5.8	5.6	5.0	5.1
	6.4	5.4	6.2	5.8	4.6
	6.3	6.2	5.5	5.7	5.1
127K35	6.3	5.4	5.5	5.2	5.2
	6.3	5.9	6.0	5.9	4.9
	-	5.9	5.6	5.7	5.5

Table 25. Survival of R. phaseoli in Texas lignite on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	5.8	4.4	4.3	3.5	-
	5.7	4.5	4.3	4.1	-
	5.6	5.3	4.3	4.4	4.2
	5.6	5.6	4.4	4.3	4.0
127K26	6.0	5.8	5.3	6.2	4.7
	6.1	6.0	5.8	5.1	5.1
	6.2	6.2	5.6	4.6	5.0
	6.1	5.5	5.0	5.4	5.3
127K35	5.5	4.9	5.6	4.5	4.5
	5.2	5.0	4.7	4.5	4.6
	5.9	6.0	5.3	4.8	4.8
	5.6	5.1	4.6	4.1	4.7

Table 26. Survival of *R. phaseoli* in New Mexico subbituminous coal on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	5.8	5.0	4.5	3.9	4.5
	6.3	-	5.8	4.8	4.4
	-	6.1	5.7	5.7	5.2
127K26	-	6.0	5.8	5.6	5.2
	6.4	-	6.1	5.5	4.5
	-	6.1	5.9	6.0	5.1
127K35	6.2	5.5	5.8	5.0	5.1
	5.8	-	5.6	4.0	4.1
	-	5.2	4.9	4.9	4.2

Table 27. Survival of *R. phaseoli* in Wyoming subbituminous coal on seed

Strain	Log_{10} colony-forming units per seed after:				
	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	3.7	2.8	3.2	2.7	2.4
	5.1	-	4.3	4.0	4.4
	6.0	5.9	5.8	5.6	4.6
127K26	5.4	3.5	4.8	3.2	3.0
	5.5	3.5	3.6	4.5	3.6
	5.1	3.0	-	-	-
127K35	5.3	3.9	4.6	3.7	3.9
	4.7	3.5	3.1	2.6	2.8
	5.4	4.9	3.4	3.1	3.0

experiment was usually performed in triplicate to increase statistical validity. Linear regression analysis of rhizobial survival in each coal yielded regression lines shown in Figs. 1-8. Two-way analyses of variance of the slopes indicated no significant differences among bacterial strains ($\alpha = 0.05$), but significant differences among coals. All of the coals tested, except for the subbituminous coal from Wyoming, had greater than 600 viable cells per seed for at least 4 weeks; this includes both of the coals (Texas lignite and Illinois bituminous coal) that previously had not supported rhizobial survival without the addition of 10% $CaCO_3$. The peat-based inoculant, used as a control, gave significantly better survival on seed than did any of the coal-based inoculants tested (Table 28 and Fig. 9). The average logarithmic decrease in the rhizobial

Table 28. Survival of R. phaseoli in peat on seed

	Log_{10} colony-forming units per seed after:				
Strain	0 wk	1 wk	2 wk	3 wk	4 wk
127K17	-	5.8	5.9	5.8	6.0
	-	5.5	6.3	5.8	5.0
127K26	6.0	6.1	5.8	5.7	5.4
	-	6.1	5.8	5.9	5.8
127K35	6.0	5.5	5.1	5.4	5.2
	6.3	5.9	5.6	5.4	5.6

population in peat over 4 weeks was approximately 0.5 units. Most of the coal-based inoculants applied to seed had between a 1- and 2-unit decrease in viable cells per seed in 4 weeks.

To demonstrate the need for an inoculant carrier, a 30-h broth culture of R. phaseoli was applied to seed using 30% gum arabic as an adhesive. The seeds were treated exactly as described above, but

Fig. 1. Regression analysis of *R. phaseoli* survival when inoculants containing Pennsylvania anthracite were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.

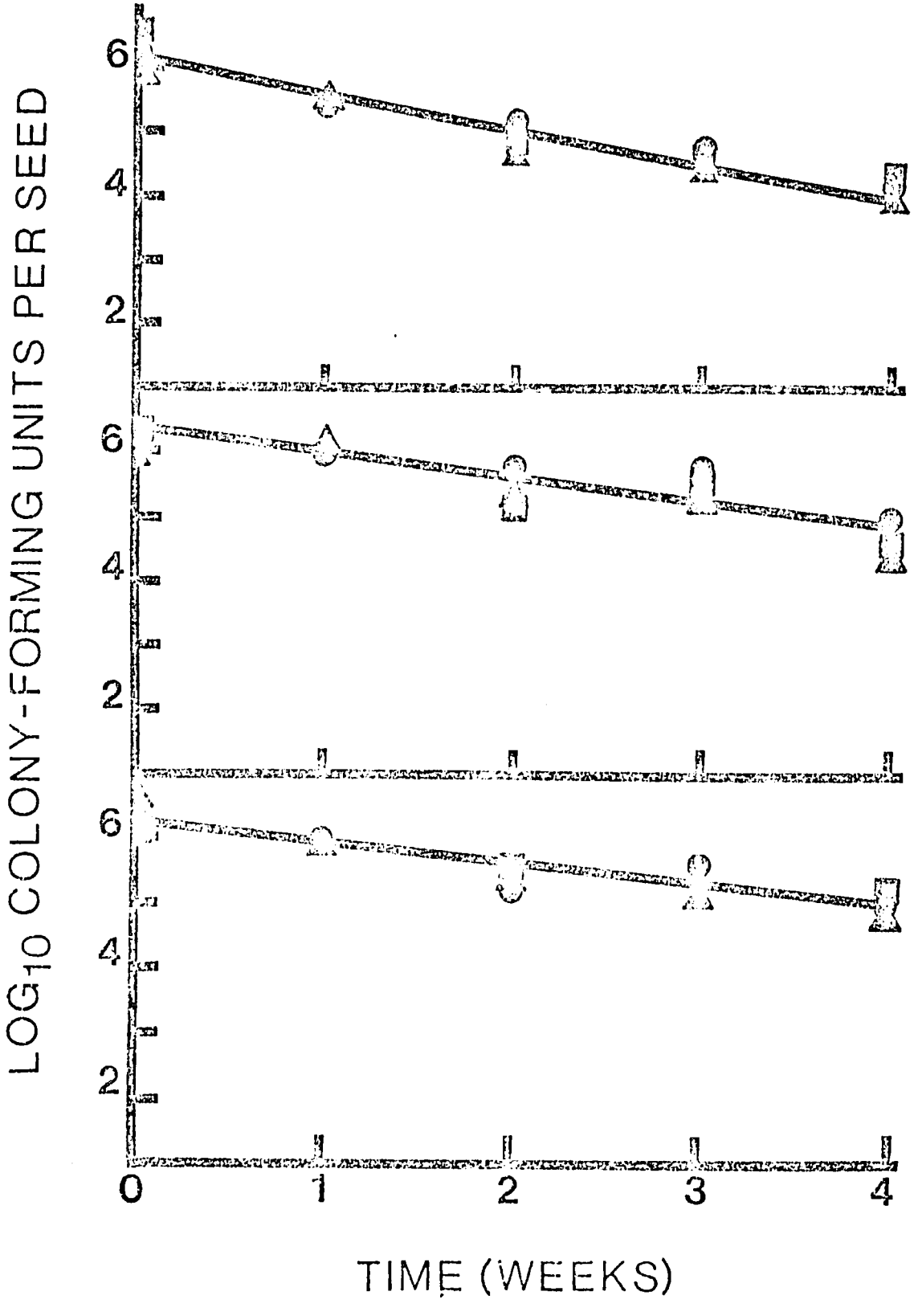

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Fig. 2. Regression analysis of *R. phaseoli* survival when inoculants containing Illinois bituminous coal were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different sumbols indicate different survival experiments.

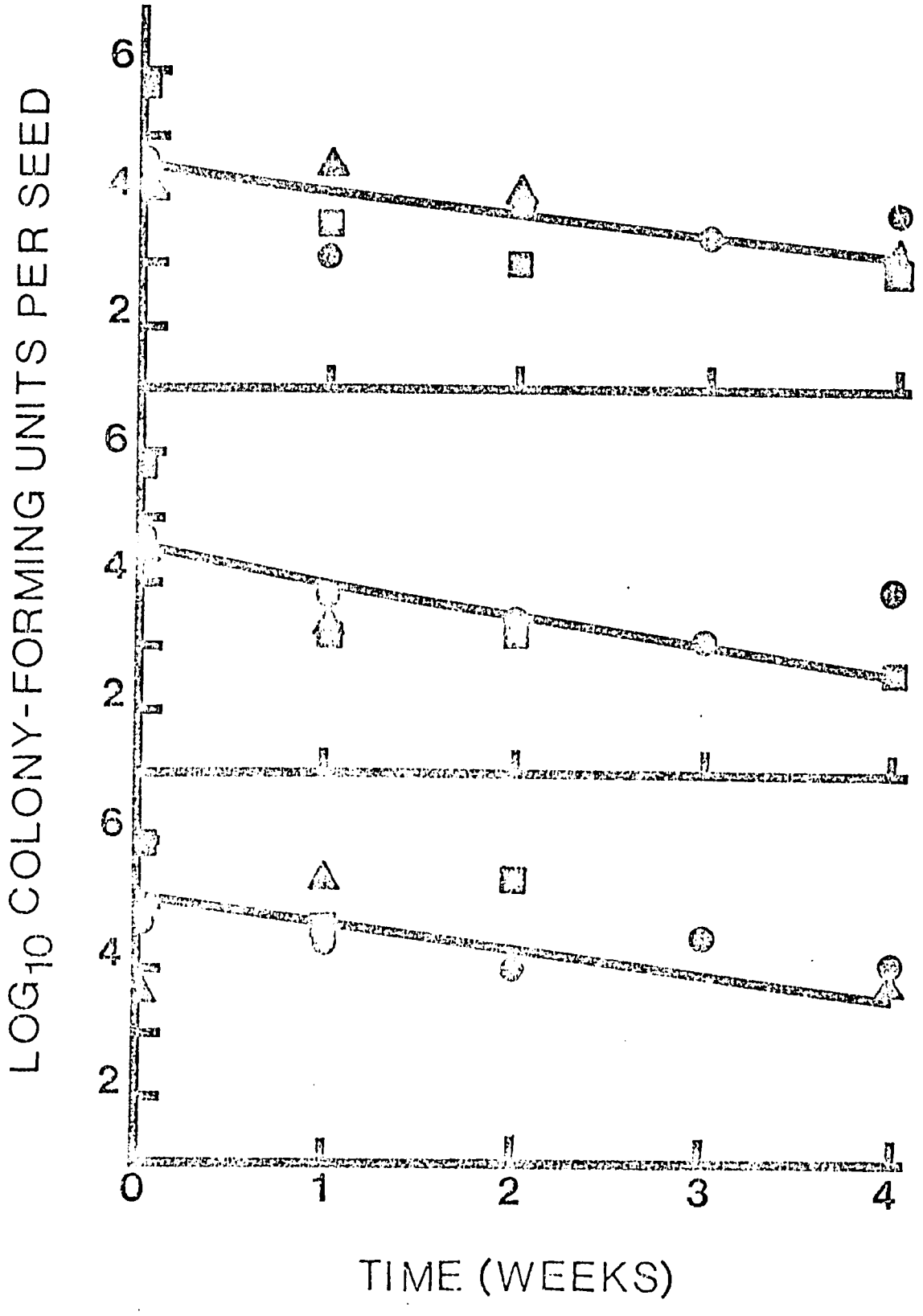

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Fig. 3. Regression analysis of *R. phaseoli* survival when inoculants containing Pennsylvania bituminous coal were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.

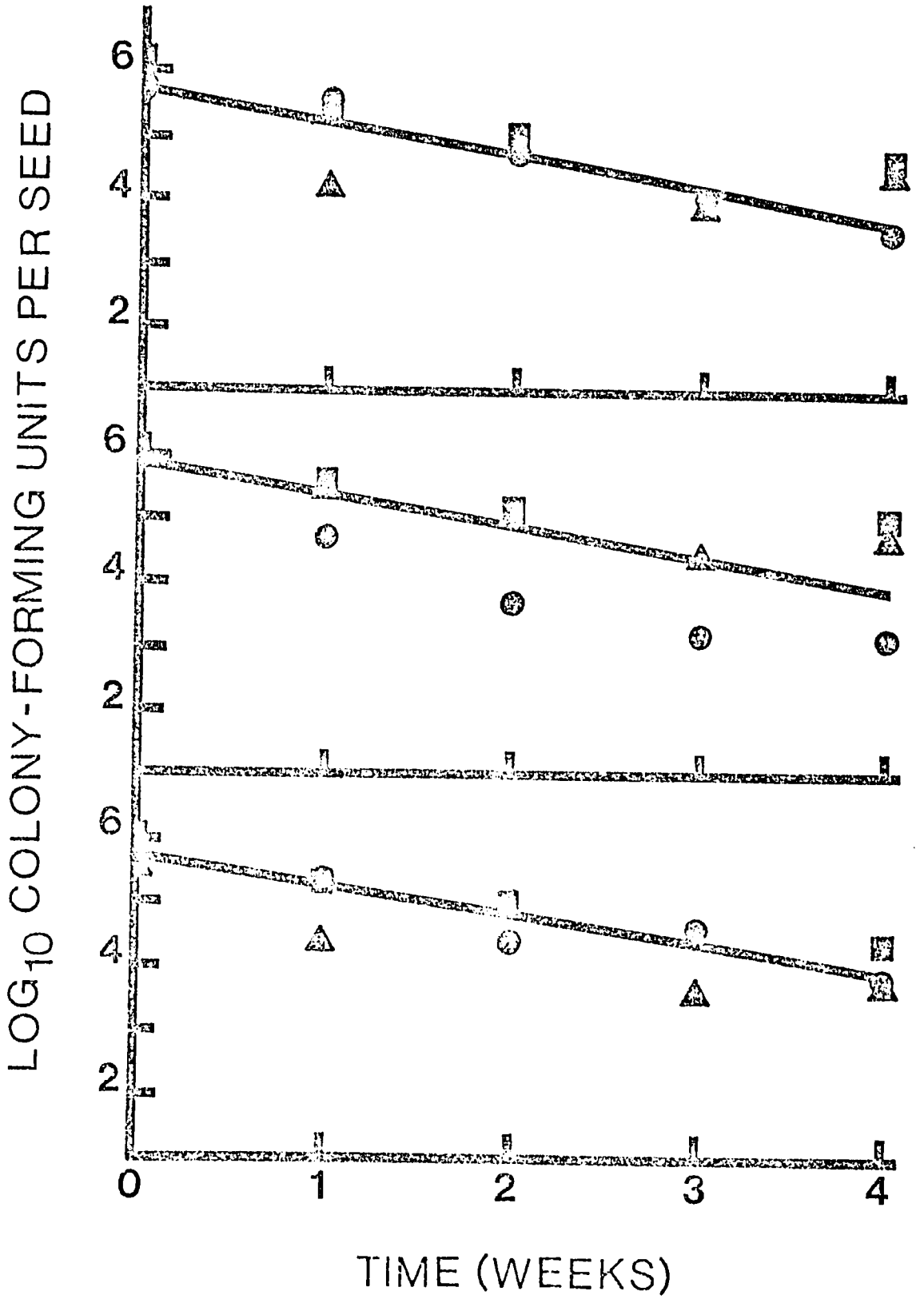

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Fig. 4. Regression analysis of *R. phaseoli* survival when inoculants containing Utah bituminous coal were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.

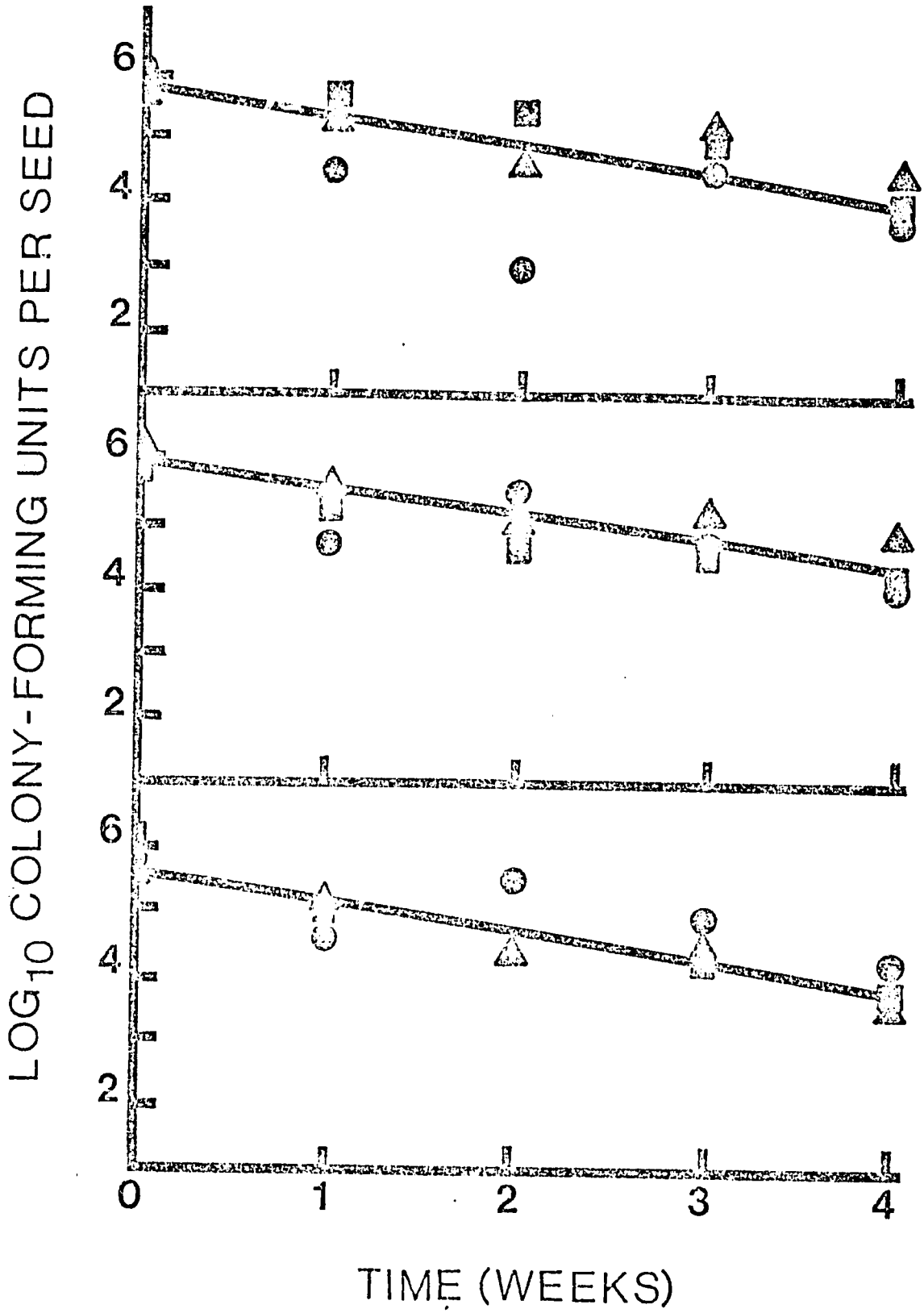

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Fig. 5. Regression analysis of *R. phaseoli* survival when inoculants containing North Dakota lignite were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.

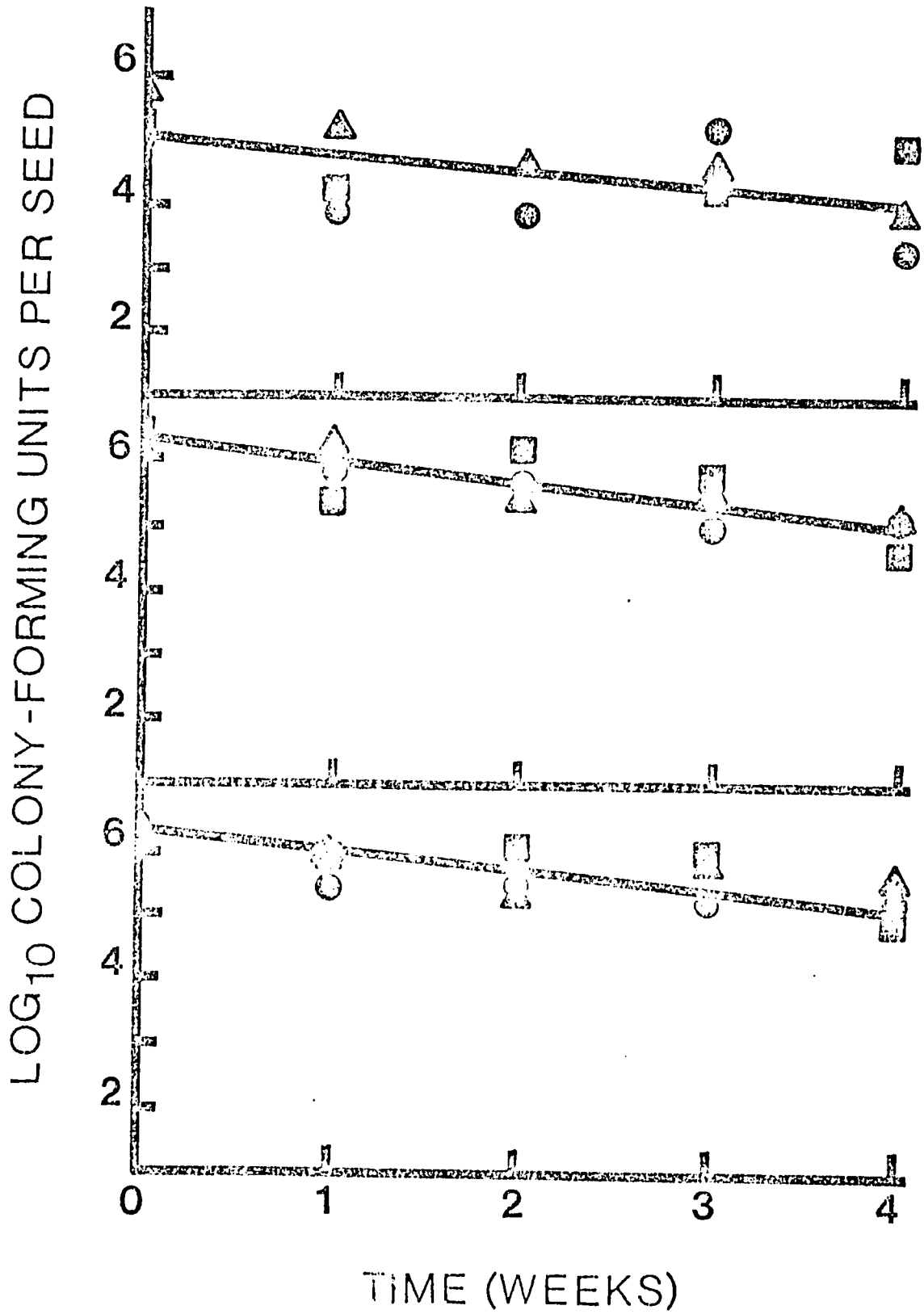

LOG10 COLONY-FORMING UNITS PER SEED
6
4
2
6
4
2
6
4
2
0
1
2
3
4
TIME (WEEKS)

Fig. 6. Regression analysis of *R. phaseoli* survival when inoculants containing Texas lignite were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.

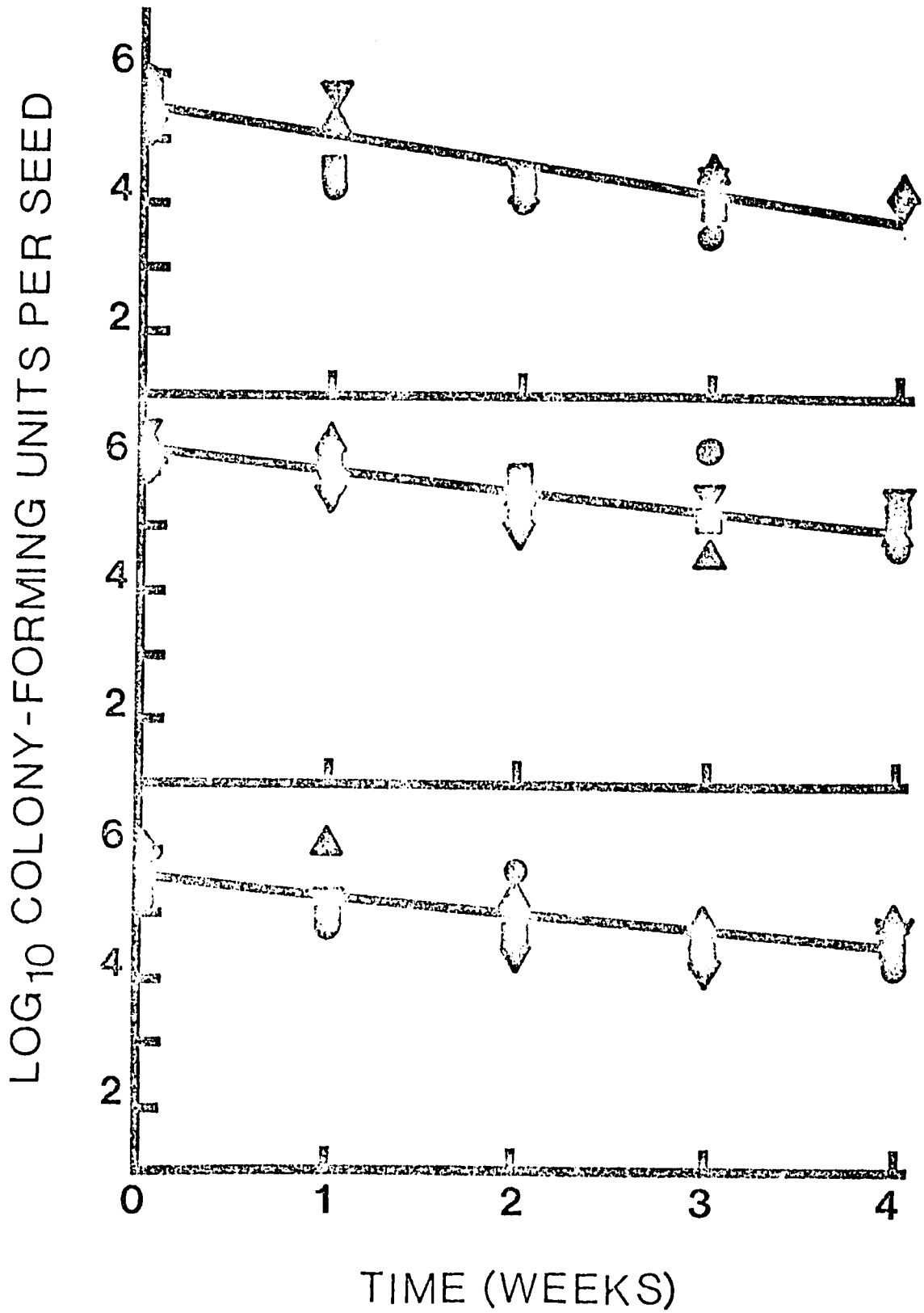

LOG10 COLONY-FORMING UNITS PER SEED
6
4
2
6
4
2
6
4
2
0
1
2
3
4
TIME (WEEKS)

Fig. 7. Regression analysis of *R. phaseoli* survival when inoculants containing New Mexico subbituminous coal were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different sumbols indicate different survival experiments.

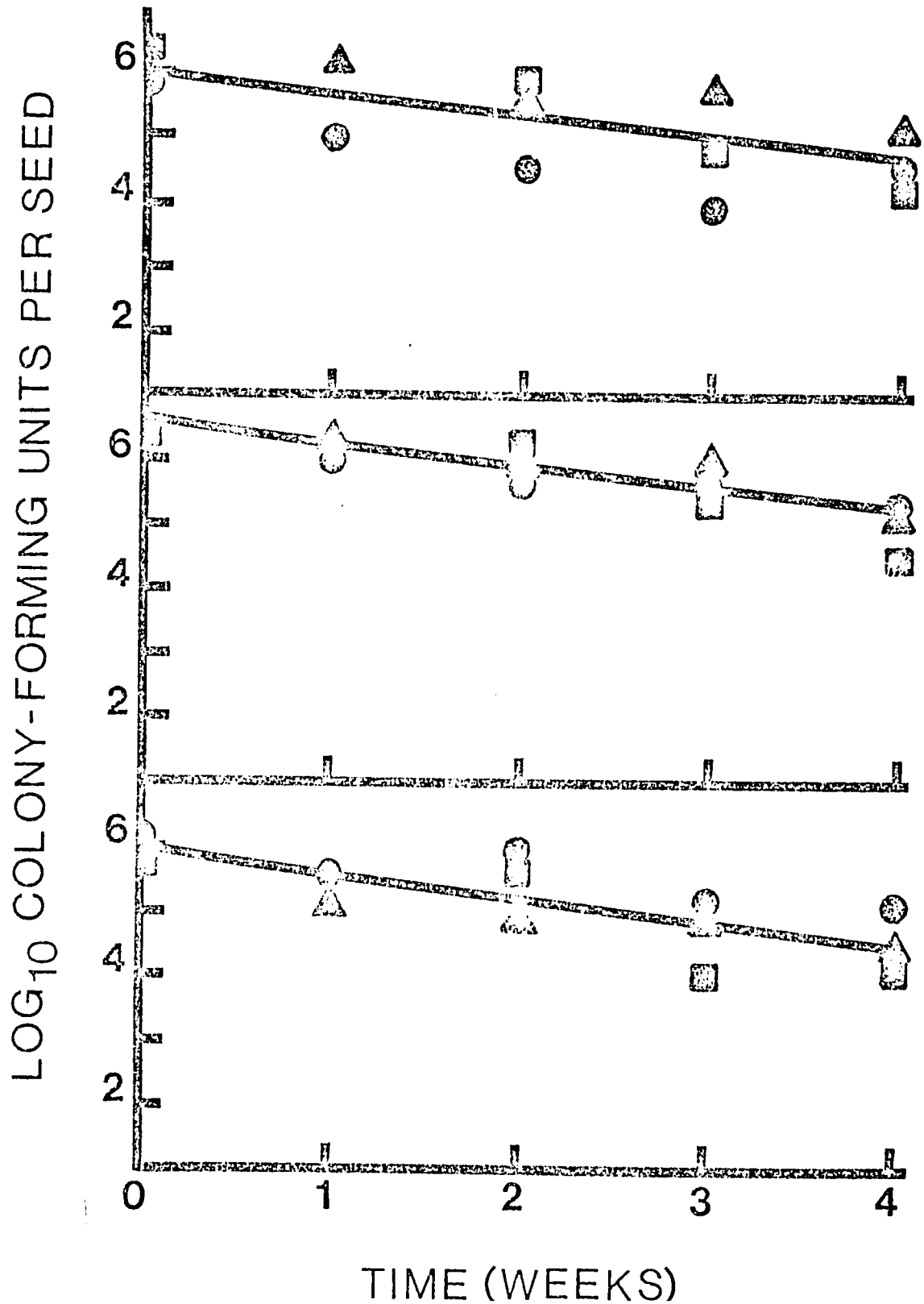

LOG10 COLONY-FORMING UNITS PER SEED
6
4
2
6
4
2
6
4
2
0
1
2
3
4
TIME (WEEKS)

Fig. 8. Regression analysis of *R. phaseoli* survival when inoculants containing Wyoming subbituminous coal were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.

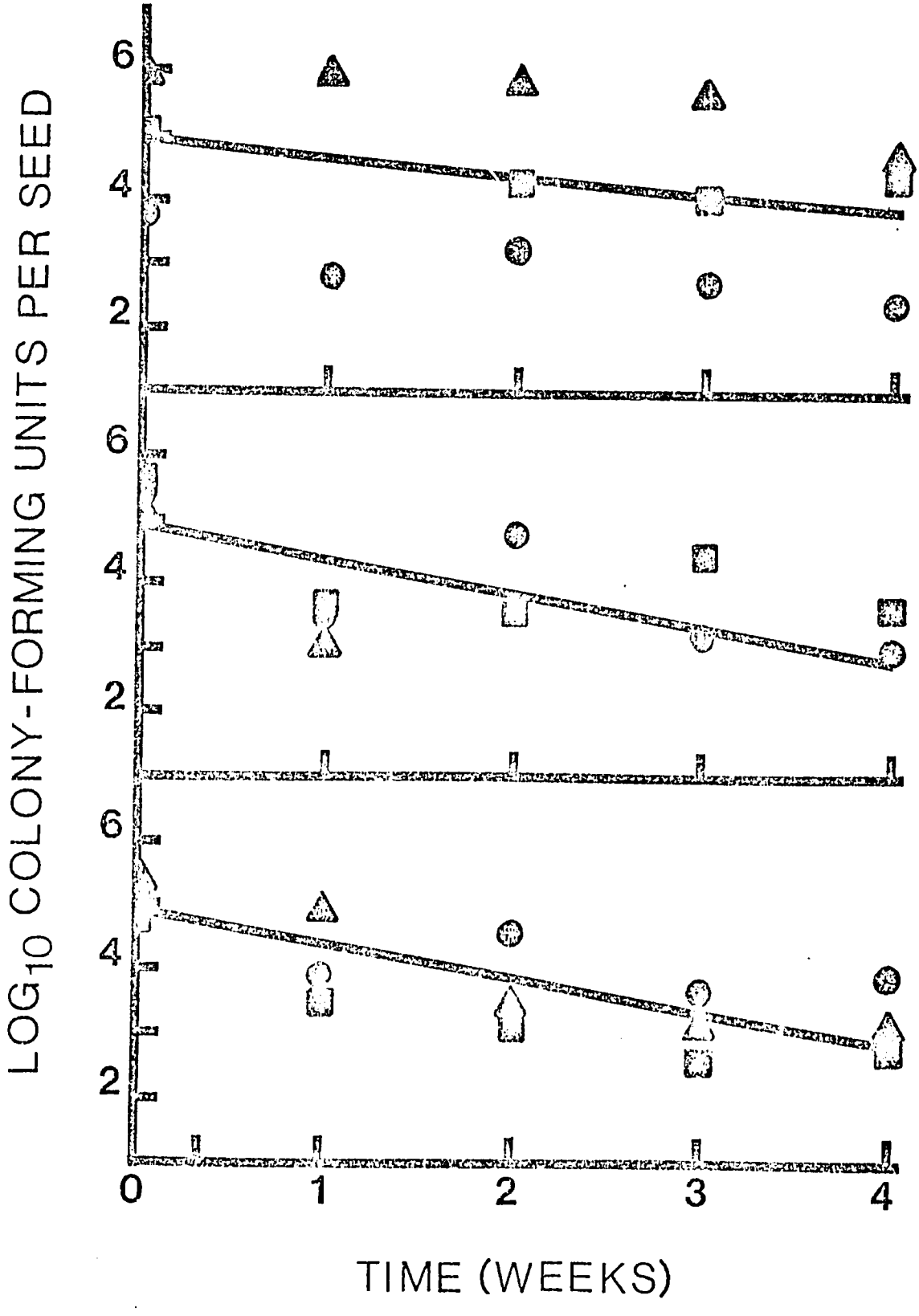
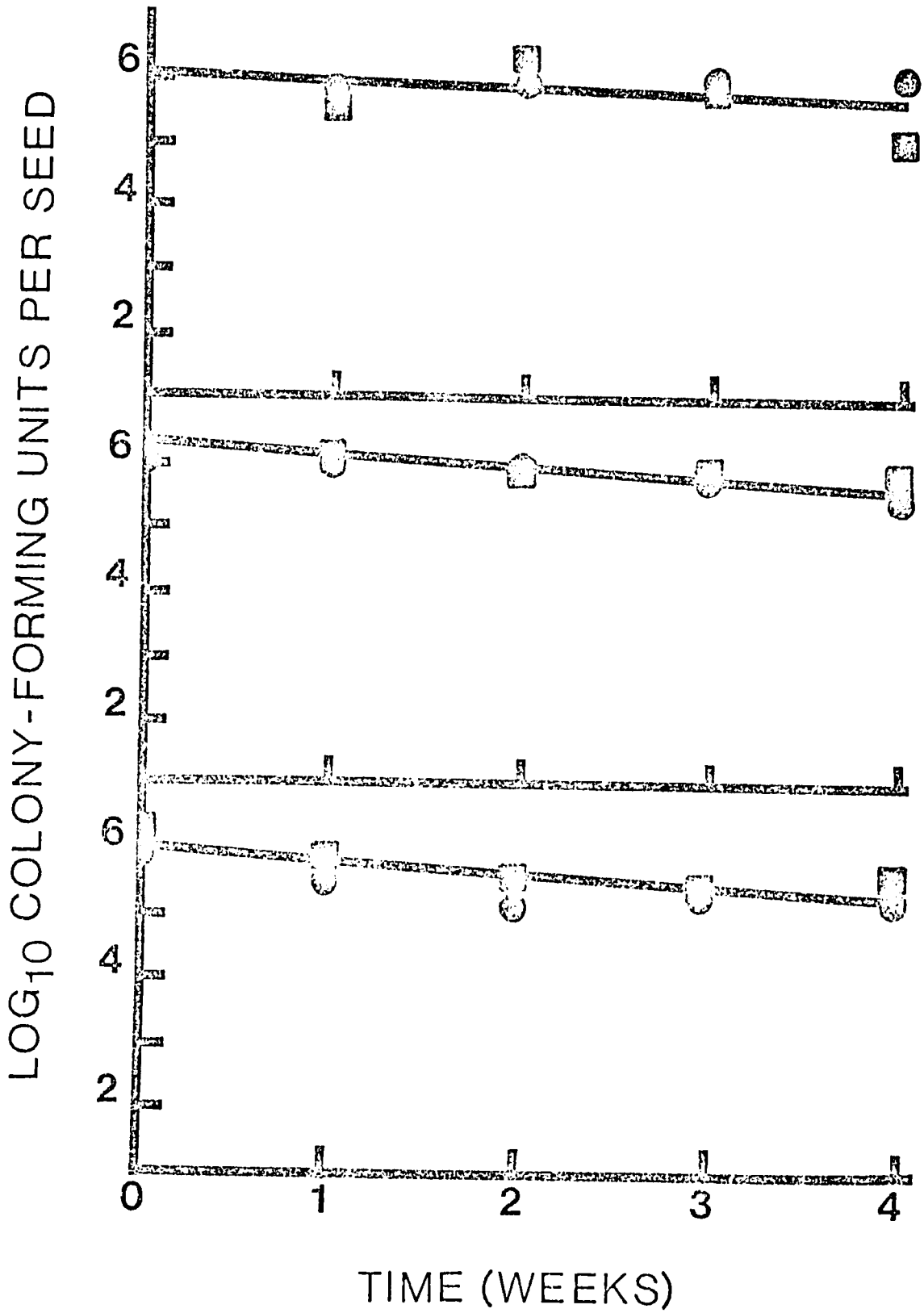

LOG10 COLONY-FORMING UNITS PER SEED
6
4
2
6
4
2
6
4
2
0
1
2
3
4
TIME (WEEKS)

Fig. 9. Regression analysis of *R. phaseoli* survival when inoculants containing peat were applied to seed. *R. phaseoli* strains 127K17 (top), 127K26 (middle), and 127K35 (bottom) were studied. Different symbols indicate different survival experiments.


LOG10 COLONY-FORMING UNITS PER SEED
6
4
2
6
4
2
6
4
2
0
1
2
3
4
TIME (WEEKS)

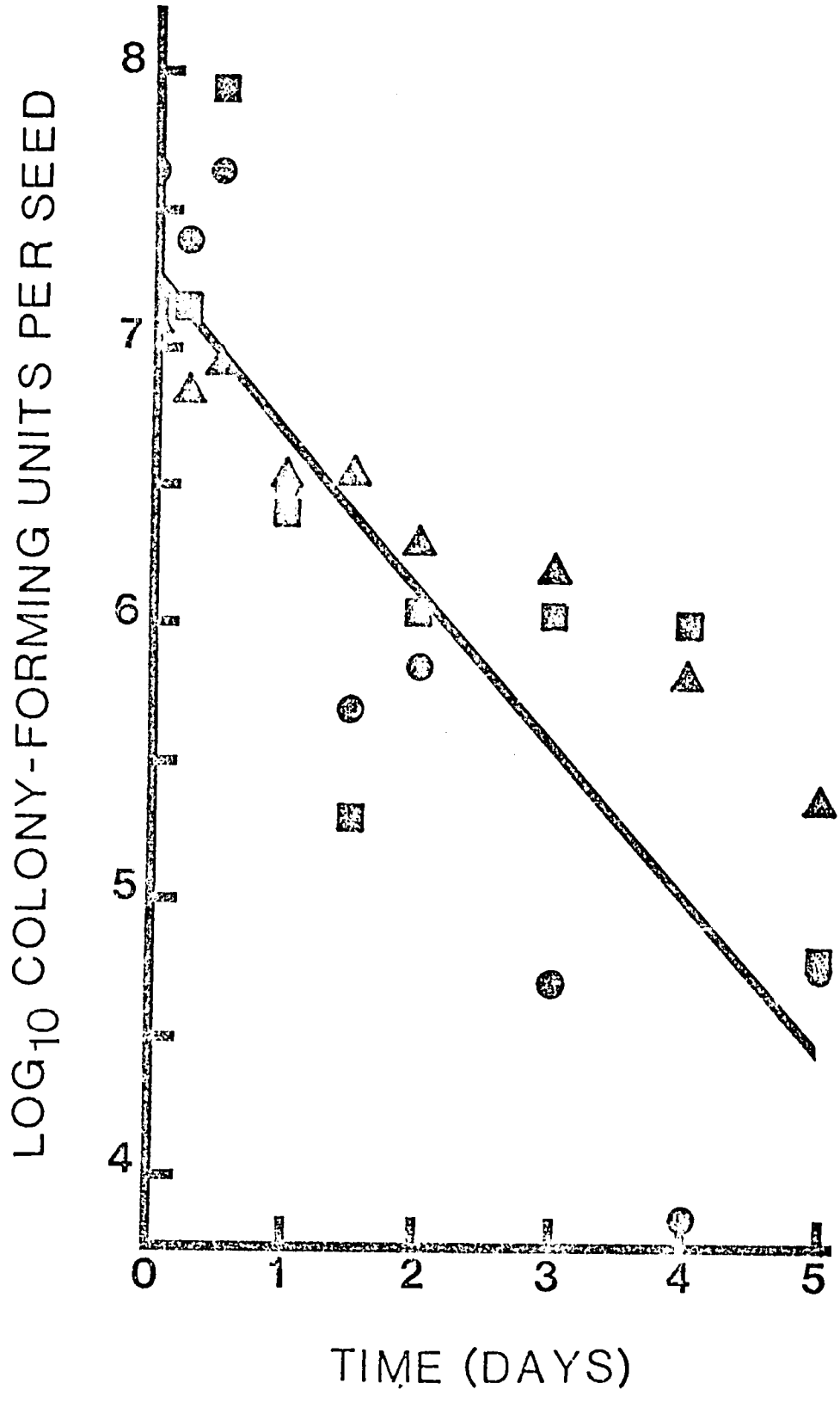
were sampled more frequently. The regression line in Fig. 10 shows that viable cells per seed decreased by greater than 3 logarithmic units in 5 days. These data were also collected in triplicate to increase statistical validity.

One of the coals tested, a subbituminous coal from Wyoming did not support greater than 600 cells per seed for 4 weeks. This inability was probably due to the low rhizobial count in the original inoculant. The regression line for this coal (Fig. 8) did not vary significantly from those of the other coals tested.

Some relationships between the MHC's of the coals and their abilities to support rhizobial survival on seed were noted. Those coals with the greatest MHC's had the greatest degree of rhizobial survival when applied to seed. Aside from the relationship between MHC and rhizobial survival, survival could not be correlated to the type of coal used. Peat, which has the highest MHC of any inoculant carrier tested, also gave the best rhizobial survival on seed. Many researchers have noted the detrimental effects of drying on rhizobial survival and these effects would be equally pronounced on seed.

To check nodulating ability, each inoculant was applied to seed and the seeds were allowed to germinate. All resulting plants were nodulated when examined after 4 weeks.

Fig. 10. Regression analysis of *R. phaseoli* 127K17 survival when a 30-h broth culture was applied to seed.


LOG10 COLONY-FORMING UNITS PER SEED
8
7
6
5
4
0
1
2
3
4
5
TIME (DAYS)

CONCLUSIONS

While peat remains the best of all inoculant carriers tested, a variety of organic powders show promise as alternatives when suitable peat is unavailable. The results of this research generally confirm and extend previous encouraging reports on the use of coal dust as a carrier. Not only does long-term survival of rhizobia in coal appear acceptable, but coal seems to protect rhizobia on seed as well.

The results of this study, which used eight coals of various grades and from divergent locations, demonstrate the general suitability of coal dust, or coal dust containing $CaCO_3$, as a carrier. Most coal-based inoculants stored at room temperature contained more than 1×10^8 rhizobia per g for at least 8 months. It is important that R. phaseoli survival was not strain-dependent, and that symbiotic properties did not appear affected. Additionally, no correlations were made between the grade of coal and rhizobial survival.

Although R. phaseoli survived best on pinto bean seeds when a peat-based inoculant was used, coal carriers afforded considerable protection to rhizobia on seed in view of the rapid death of R. phaseoli when no carrier was used. Using coal-based inoculants, virtually all seeds had more than 600 viable R. phaseoli cells after 4 weeks. It should be noted that the amounts of inoculant applied to seeds with 30% gum arabic were comparable for peat and coal.

RECOMMENDATIONS

Coal should be strongly considered as a carrier when peat suitable for inoculant manufacture cannot be obtained. The grade of the coal does not seem important, although preference should be given to coals having pH's near neutrality and relatively high MHC's. Should a coal be too acidic, adjustment of the pH with "precipitated" $CaCO_3$ is recommended. Other considerations when selecting a coal should include ease of milling, lack of toxicity, and lack of spontaneous clumping of the coal powder.

Coal should be processed for inoculant use in much the same manner as peat. It may be desirable to employ mineral oil to control dust. Heat sterilization of the coal carrier will probably be required in order to control contamination; heat has the additional advantage of reducing the moisture content of the coal and thereby allows more broth culture to be added. It is recommended that the coal powder be mixed with sufficient broth inoculum to obtain 60-70% of MHC. If the carrier requires neutralization with $CaCO_3$, the $CaCO_3$ can either be mixed in with the broth or added directly to the dry carrier. The latter was performed in the present study because of difficulties in dissolving sufficient $CaCO_3$ in broth. Various inoculant amendments such as sucrose and yeast extract should be avoided, for reasons of contamination, unless demonstrably superior rhizobial survival is obtained by their use. Procedures for curing and storing coal-based inoculants should be those used for peat-based inoculants.

The use of an adhesive for the application of coal-based inoculants to seed is recommended. Although coal-based inoculants adhere to wet seeds, distribution of the inoculant is not uniform. It should also be

remembered that adhesives such as gum arabic often have protective qualities which promote rhizobial survival.

IMPLICATION OF FINDINGS TO DEVELOPING COUNTRIES

It has been estimated that the world's population will nearly double by the year 2000, and much of this increase will occur in developing countries where food shortages already exist. All food production is ultimately dependent upon "fixed" nitrogen supplied either by industrially produced chemical fertilizers or biological nitrogen fixation. Because the industrial manufacture of fixed nitrogen requires immense capitalization, high energy consumption, and fossil fuels, biological nitrogen fixation offers the best prospects for providing fixed nitrogen in developing countries.

The *Rhizobium*-legume symbiosis is the most important of the biological nitrogen-fixing systems. A major constraint in exploiting the symbiosis is a lack of legume inoculants for supplying rhizobia appropriate for the crops of a given area. While it seems best to manufacture inoculants close to where they are used, the establishment of an inoculant industry may be impeded due to the lack of an acceptable inoculant carrier. Peat, the carrier of choice for *Rhizobium* inoculants, is not easily obtainable in all areas of the world. The present research has shown that coal, which is mined in numerous geographical areas, deserves consideration as an alternative to peat in *R. phaseoli* inoculants.

GRADUATE STUDENTS SUPPORTED

1. Mary Walker Paczkowski, M.S., 1979
2. Steven Lee Crawford, M.S., 1981

PUBLICATIONS

Journal article:

Paczkowski, Mary W., and D.L. Berryhill. 1979. Survival of *Rhizobium phaseoli* in coal-based legume inoculants. Appl. Environ. Microbiol. 38:612-615.

Abstracts:

Paczkowski, M., and D.L. Berryhill. 1979. Survival of *Rhizobium phaseoli* in coal-based inoculants. Seventh North American *Rhizobium* Conference, Proceedings, p. 39.

Crawford, S.L., and D.L. Berryhill. 1981. Survival of *Rhizobium phaseoli* in coal-based inoculants applied to seed. Eighth North American *Rhizobium* Conference, Proceedings, p. 113.

Theses:

Paczkowski, M.W. 1979. Survival of *Rhizobium phaseoli* in coal-based inoculants. M.S. thesis, North Dakota State University, Fargo.

Crawford, S.L. 1981. Survival of *Rhizobium phaseoli* in coal-based inoculants applied to seed. M.S. thesis, North Dakota State University, Fargo.