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Differences Among Cowpea Rhizobia in Tolerance to High Temperature and Desiccation in Soil

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Strains of cowpea rhizobia grew in mannitol-amended, nonsterile soil at 29 to 35°C but not at 40°C. Little decline in numbers of these bacteria occurred in dry, nonsterile soil incubated at 42°C for 7 days. Strains of cowpea rhizobia differed widely in their tolerances to drying at 30°C in nonsterile and sterile soil, and from less than 1 to 50% of the bacteria were still viable after 11 days. No relation was evident between tolerance to desiccation and the degree of aridity of the site from which the bacteria were isolated or their growth rates in culture, but strains not producing extracellular polysaccharide were often more tolerant than those producing extracellular polysaccharide. It is suggested that desiccation-tolerant rhizobia be used for the production of legume inoculants.

Cowpeas (*Vigna unguiculata* L.) are an important source of food for people and an important crop in farming systems of the humid tropics. Good growth of this legume in regions where fertilizers are unavailable or expensive requires that the plant be nodulated by an effective strain of *Rhizobium*, but the high soil temperatures and frequent drought characteristic of much of the tropics may have detrimental effects on the needed rhizobia. For a cowpea inoculation program for the tropics to be successful, *Rhizobium* strains are needed that are, besides being effective and competitive, tolerant of these adverse environmental conditions.

Some attention has been given to the effects of high temperatures on the root-nodule bacteria. Thus, it has been reported that rhizobia which nodulate tropical legumes show a wide range of maximum growth temperature in agar media (1, 10). Munevar and Wollum (13) observed significant differences among *Rhizobium japonicum* strains in their tolerance to high temperature in culture, and Weber and Miller (18) noted that serogroups of *R. japonicum* that were infrequently recovered at 10 to 15°C became dominant at 30°C, whereas serogroups forming the majority of nodules at 10 or 15°C formed fewer nodules at 30°C.

The survival of *Rhizobium* in soil is adversely affected by moist heat (1, 19). In dry soils, these bacteria are quite resistant to high temperatures, although significant differences in tolerance to high temperatures exist among the rhizobia (12, 19). It has been suggested that a major source of the differences among *Rhizobium* species in tolerance to high temperature in dry soils is the geographical origin of the species (19). Desiccation has a markedly detrimental influence on the rhizobia even at moderate temperatures both in soil (12, 15) and on seeds (17). Although differences among species of *Rhizobium* in sensitivity to drought have been reported (2, 12), the extent of variation within individual species has not been established.

The objectives of this study were to evaluate the effect of high soil temperature and desiccation on the persistence of cowpea *Rhizobium* and to determine the degree of variation among the cowpea rhizobia in their sensitivities to these stresses.

MATERIALS AND METHODS

Soils. The soils used were a sandy soil (pH 6.1; organic C, 0.7%) from the experimental field of the International Institute for Tropical Agriculture at Maradi, Niger, and Collamer silt loam (pH 5.7; organic C, 1.3%) from Ithaca, N.Y. The samples were from the surface 23 cm of soil and were passed through a 2-mm sieve and allowed to dry in air before use. When sterile soil was used, 10-g portions in milk-dilution bottles were autoclaved for 1 h on each of 3 consecutive days, and sterility of the soil was ascertained by noting the absence of growth when dilutions were plated on nutrient agar.

Cultures. The cowpea rhizobia were from the International Institute of Tropical Agriculture (strain designations beginning with I [except IRj, which refers to *R. japonicum*], III, M, or T) or from Boyce Thompson Institute (strain designations beginning with CP). Cultures 179, 197, 401, and 403 nodulated cowpeas, and 176, 297, 383, 389, 390, and 417 were *R. japonicum*; both groups were from this laboratory. Isolates resistant to 2.0 mg of streptomycin sulfate per ml of medium were obtained from some of these cultures by the method of Danso et al. (5). For strains from which antibiotic-resistant isolates could not be obtained by this method, 2.0 mg of streptomycin per ml was added to cultures growing in liquid media when distinct

turbidity was evident, and after 7 days of further incubation on a rotary shaker, antibiotic-resistant isolates were obtained by plating portions of the liquid on agar medium supplemented with the antibiotic at the same concentration. All cultures were grown in a modified yeast extract-mannitol medium (14).

Rhizobia in nonsterile soil were enumerated by the plate-count method on the yeast extract-mannitol agar supplemented with 2.0 mg of streptomycin sulfate and 200 μg of actidione per ml. The plates were incubated at 30°C for 9 days before counts were made.

Growth rates. The antibiotic-resistant strains were used to determine rhizobial growth rates in nonsterile soil. An aqueous rhizobium suspension was inoculated into soil samples to give about 10^3 to 10^4 cells per g. The soil was also amended with mannitol to a final concentration of 1% (wt/wt), and the moisture content was adjusted to and maintained at 25% (wt/wt). Duplicate samples were removed to determine rhizobium numbers after 0, 2, 4, 6, and 8 days. A plot of cell numbers against time showed that growth in soil was logarithmic for most of the bacteria between days 2 and 6.

To determine growth rates of the antibiotic-resistant strains in pure culture, 100-ml portions of yeast extract-mannitol broth in 250-ml Erlenmeyer flasks were inoculated with 1.0 ml of a rhizobial suspension containing 10^2 to 10^3 cells per ml. The flasks were incubated at 30°C on a rotary shaker operating at 120 rpm. Cell numbers were determined at 8-h intervals for 5 days. The specific growth rate (μ) was determined as the slope of the linear regression of the logarithm of the counts during the exponential phase of growth.

Drying procedure. A slow- and a fast-drying procedure were used. The slow-drying process involved inoculating 10 g of an air-dry soil sample contained in a milk dilution bottle with 1.0 ml of an aqueous rhizobium suspension. The soil was thoroughly mixed, and the bottle was capped with a cotton plug. The bottles were incubated vertically at 30°C for 11 days, by which time the soil had dried, as shown by tests indicating that no further loss of weight occurred with longer incubation.

In the fast-drying process, 10 g of an air-dry soil sample was placed in a plastic dish (diameter, 55 mm; height, 10 mm). After adding 1.0 ml of a rhizobium suspension to the sample, the soil was thoroughly mixed with a spatula and then incubated at 30°C for 24 h, by which time it had dried.

Determination of crude EPS. The bacteria in 20 ml of 7-day-old cultures were removed by centrifugation at 10,000 × *g* at 4°C for 30 min. The insoluble polysaccharide that formed a loose, easily detachable layer on top of the cell pellet was washed three times in sterile distilled water to remove the cells, the extracellular polysaccharide (EPS) being collected each time by centrifugation at 40,000 × *g* for 30 min.

To obtain soluble EPS, the supernatant fluid, after centrifuging the suspension at 10,000 × *g* for 30 min, was centrifuged at 40,000 × *g* for 30 min at 4°C. The resulting supernatant fluid was dialyzed against deionized water at 4°C for 48 h, and absolute ethanol was then added to a final concentration of 75%. The liquid was allowed to stand at 4°C for 24 h, and the resulting EPS precipitate was collected by centrifugation at 8,000 × *g* for 10 min. The data presented are for the total insoluble and soluble EPS.

TABLE 1. Growth response of rhizobia in mannitol-amended Collamer silt loam at four temperatures

Strain	Generation time (h) at following temp (°C):			
	29	31	33	35
CP IV	33.0	14.5	10.1	19.8
179	13.7	12.4	11.8	19.3
CP XIII	12.2	10.5	9.6	28.9
176	16.1	13.6	16.1	50.0
389	9.4	9.3	8.7	11.6
IRj 114	18.2	17.3	12.0	11.0
401	18.7	14.4	12.8	22.4
390	13.6	11.4	10.8	11.4
CP III	10.2	8.9	8.2	10.3
197	18.7	16.9	16.5	17.8
417	12.0	11.2	12.0	12.4
IRj 101	9.2	8.5	6.2	11.4
297	9.0	9.3	9.3	13.3
IRj 128	12.4	12.2	12.0	15.4
IRj 111	10.0	9.4	6.6	12.1
M/I63A	43.3	8.0	5.4	6.6
M/I63C	30.1	9.1	5.7	7.2
M/IV55A	49.5	53.3	46.2	30.1
M/IV55D	17.3	12.0	10.7	10.3
M/III19D	23.1	9.9	5.6	7.3

TABLE 2. Survival of antibiotic-resistant cowpea rhizobia in dry nonsterile Collamer silt loam at 42°C (±2°)

Strain	No. of rhizobia ($\times 10^8$/g of dry soil)	
	Before exposure	After exposure
CP IV	15.0	13.1
179	36.9	23.0
CP XIII	52.1	34.5
176	4.0	4.3
389	6.3	6.5
IRj 114	5.4	5.2
401	6.1	4.0
390	9.8	10.4
CP III	3.7	3.9
197	20.8	24.1
417	11.1	11.5
IRj 101	6.7	5.7
297	10.4	11.7
CP IX	1.3	1.6
CP II	11.5	11.3
CP VI	6.2	6.2
CP VIII	14.7	16.8
CP X	4.4	5.1

RESULTS

Effect of temperature on growth and survival. Antibiotic-resistant *Rhizobium* strains were used to determine the effect of temperature on growth in nonsterile soil amended with 1% mannitol. No strain grew at 40°C, but all grew at 29, 31, 33, and 35°C. Most strains had an optimum at 33°C (Table 1).

Although no strain grew at 40°C, the possibili-

TABLE 3. Frequency distribution of tolerance to desiccation in soil among slow-growing rhizobia

Range in % survival	Frequency
<1.0	5
1.0–2.5	4
2.5–5.0	5
5.0–7.5	4
7.5–10.0	4
10.0–12.5	3
12.5–15.0	3
15.0–17.5	2
17.5–20.0	1
20.0–22.5	1
>22.5	1

ty of their survival at a high temperature was tested by measuring the tolerance of 20 isolates in dry soil, in which extremes of temperature may occur in the tropics. After inoculation, the soil samples were dried by the slow-drying process and incubated at 42°C (±2°C) for 7 days. The results show that rhizobia are remarkably resistant to high temperature under these conditions (Table 2). The extent of the decline varied from 0 to 38%. Thus, the density of all of the strains was maintained in dry soil, although some isolates exhibited slightly greater degrees of tolerance to the stress.

Tolerance to desiccation. To ascertain the differences among cowpea rhizobia in tolerance to drying, 33 strains were tested in duplicate in sterilized Collamer silt loam. For this purpose, the slow-drying process was used. The initial cell count for all isolates was about 10^9 cells per g of dry soil. The data show that the strains differed widely in their resistances to desiccation (Table 3). Because the percent survival of all but two strains was less than 20%, these bacteria appear to be susceptible to drying.

The susceptibilities to drying of 20 randomly selected strains were tested (four replicates) in nonsterile soil. The initial counts were about 10^9/g of soil. The results (Table 4) confirm the wide range of sensitivity to desiccation that was observed in the first screening tests. It is noteworthy that the percent survival in the nonsterile soil was generally higher than that previously noted in sterile soil.

To determine whether the differences among strains in tolerance to desiccation were related to the soil from which they were obtained, rhizobia of known geographical origin were tested, using the fast-drying process and a sandy soil from Maradi. The isolates were from soils of Onne in the tropical rain forest of Nigeria, Ibadan in a derived savanna of Nigeria, and Maradi in a Sahel savanna of Niger. These are sites of increasing aridity. No close relationship existed between tolerance to desiccation and geographical origin of these strains (Table 5).

Members of the slow-growing group of *Rhizobium* are reported to be more resistant to desiccation than are the fast-growing rhizobia (2, 12). If resistance to desiccation of species is related to growth rate, it is conceivable that differences in tolerance to desiccation among strains of a species would also be related to growth rates. Hence, the specific growth rates of the cowpea rhizobia were measured in liquid culture. No marked relationship between these rates and survival was evident (Fig. 1). The few strains with growth rates below 0.11 were more resistant than most of the isolates with high specific growth rates, but some of the latter were also tolerant to drying. However, among the rhizobia with growth rates greater than 0.12, those not producing EPS were more tolerant to drying than strains synthesizing EPS.

The relationship between the amount of EPS formed and susceptibility to desiccation is shown in Table 6. Among the first five strains, which were chosen because they represented isolates previously noted to be markedly different in susceptibility to drying, it is clear that strains producing abundant EPS were most susceptible. Among the other isolates, a similar trend was evident; i.e., rhizobia producing 12 μg of EPS per ml or more exhibited, with one exception, less than 0.17% survival, and the two

TABLE 4. Survival of antibiotic-resistant cowpea rhizobia in nonsterile Collamer silt loam subjected to slow drying

Strain	% Survival		
	Mean	95% Confidence limits	
		Lower	Upper
179	49.8	42.1	57.5
CP IV	38.5	30.5	46.5
389	37.1	26.1	48.1
CP II	34.9	17.6	52.2
197	31.6	23.5	39.7
CP VI	26.8	23.5	30.1
CP VIII	24.8	23.0	26.6
417	24.7	21.2	28.2
297	15.7	14.5	16.9
IRj 101	13.5	9.4	17.6
390	13.0	11.1	14.9
CP XIII	12.6	10.4	14.7
CP IX	12.1	7.6	16.6
CP III	11.1	9.9	12.3
401	8.1	7.0	9.2
176	6.8	5.5	8.1
IRj 114	2.5	1.8	3.2
CP X	1.2	0.91	1.5
403	0.60	0.40	0.80
383	0.13	0.07	0.21

TABLE 5. Survival of cowpea rhizobia during fast drying in a sandy soil in relation to their geographical origins

Origin of bacterium and strain	% Survival
Maradi	
M/II-10A	0.54
M/I-50C	0.16
M/IV-17A	0.12
M/IV-55B	0.054
M/I-63D	0.071
M/IV-55D	0.020
M/II-27D	0.042
Ibadan	
T/II-61B	0.052
T/I-100D	0.29
T/I-177D	0.091
T/I-95C	0.051
CP XIII	0.22
Onne	
IRj 2007	0.10
IRj 2029	0.022
IRj 2001	0.073
IRj 2012	0.034
I-62B	0.033
I-62C	0.061

strains excreting less EPS survived drying much better.

DISCUSSION

The effect of temperature on growth of rhizobia in nonsterile soil has not been studied heretofore, but the maximum temperatures for growth of the strains tested fell within the range of 30 to 42°C found for proliferation of rhizobia nodulating various tropical legumes (1, 10). The failure of the rhizobia in this study to proliferate in nonsterile soil at 40°C, together with similar observations on *Rhizobium lupini* and *Rhizobium trifolii* in sterilized soils (4), suggest that the maximum growth temperature of *Rhizobium* may be lower in soil than in laboratory media, where growth at higher temperatures has been observed. During dry and fallow periods, soil temperatures frequently exceed 40°C (6, 16), and the temperature at a depth of 5 cm may reach 42°C (11). The ability of a population to survive during these periods would ensure its presence in high numbers in the following season. The rhizobia investigated were all quite resistant to such high temperatures in dry soil; however, their growth rates at different temperatures varied markedly. The differences in temperature response among the strains point to the potential failure of *Rhizobium* strains used as inoculants in regions with high temperatures to which the bacteria are not adapted.

These data also indicate that susceptiblity to desiccation is common among the cowpea rhizobia. The quantitative differences among strains appear to be large enough to permit selection of drought-tolerant rhizobia. Because tolerance to drought was not related to the aridity of the site of origin of the isolates, evolution toward drought tolerance among rhizobia, as in many other microbial species (9), may be extremely slow or nonexistent. This observation, there-

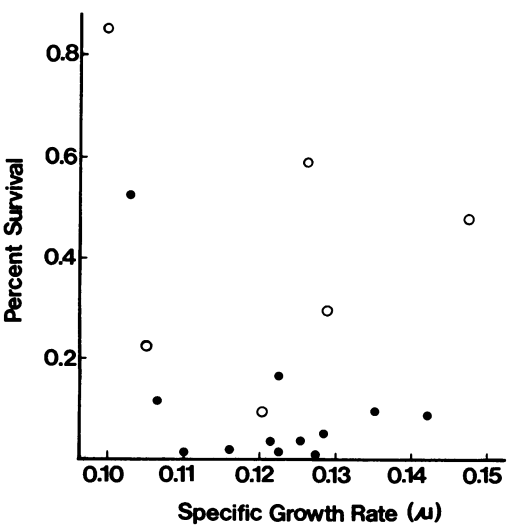

FIG. 1. Relationship between growth rate and survival in sandy soil subjected to fast drying of cowpea rhizobia producing (●) and not producing (○) EPS.

TABLE 6. Relationship between EPS production in culture and tolerance of rhizobia to desiccation in soil

Strain	EPS produced (μg/ml)	% Survival
383[a]	1,060	0.20
IRj 114[a]	400	3.1
CP XIII[a]	0.0	12.6
179[a]	0.0	42.2
389[a]	0.0	34.2
M/II-27A	260	0.042
IRj 2029	250	0.022
M/IV-55B	220	0.054
M/I-50C	210	0.16
IRj 2012	180	0.034
M/II-10A	75	0.54
M/IV-17A	37	0.12
I-62C	16	0.061
IRj 2001	12	0.073
I/II-81D	6	0.47
III-73B	5	0.33

[a] The slow-drying process and Collamer silt loam were used. All other cultures were tested by the fast-drying process in the sandy soil.

fore, emphasizes the potential advantage for using *Rhizobium* strains tolerant to drying as field inoculants.

The observation that slow-growing rhizobia may be more resistant to desiccation than fast-growing isolates (2, 12), a finding not confirmed in tests with other drying procedures (15), suggests a correlation between growth rate and drought tolerance, but the results obtained in the present study show that differences in growth rates within a species are not indicative of relative tolerances to drought. However, a relationship betwen EPS production and sensitivity to drying was found. The reason for this association is not clear. It is possible that EPS is produced at the expense of intracellular reserve material which could be used during periods of stress. In this regard, Calcott and MacLeod (3) reported a positive correlation between survival during freeze-drying and the carbohydrate content of *Escherichia coli* previously grown under nitrogen limitation, a condition that also favors EPS production (7, 8).

The results of the present investigation suggest that further study of the environmental factors affecting growth and survival of the rhizobia in soil and the differences in responses among strains to these factors should serve as the basis for obtaining inoculants that are able to give greater nitrogen fixation on crops of economic or agricultural importance.

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