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Effect of Salinity on *Rhizobium* Growth and Survival†

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This study examines the effect of salinity on the growth and survival of *Rhizobium* spp. in culture media and soil. Eleven isolates from saline and nonsaline environments were compared. The growth (mean doubling time) of all strains and species tested decreased when the electrical conductivity of the culture medium (yeast extract-mannitol) was raised from 1.2 mS cm^{-1} to 6.7 mS cm^{-1} (15% seawater equivalent) or to 13.1 mS cm^{-1} (28% seawater equivalent). Three of eleven strains failed to grow at 13.1 mS cm^{-1}. Although growth was affected by salinity, four strains selected from the growth rate study could survive in extremely high concentrations of salt. Two strains with growth rates sensitive to salt and two strains with growth rates relatively unaffected by salt were inoculated into solutions with electrical conductivities of up to 43.0 mS cm^{-1} (92% seawater equivalent). Not only did all four strains survive the initial osmotic shock (at 5 h after inoculation), but it was not until 27 days after inoculation that the sensitive strains exhibited a significant reduction in viable numbers. The salt-tolerant strains survived for more than 65 days with no reduction in viable counts. The interaction between soil moisture tension and soil salinity in relation to *Rhizobium* survival in gamma-irradiated soil was also examined. Six treatment combinations were used, ranging from −0.1 bars and 0.2 mS cm^{-1} to −15 bars and 12 mS cm^{-1}. Sensitive strains declined from 10^7 to 10^5 organisms per g of soil after 84 days of incubation at −15 bars and 12 mS cm^{-1}. Tolerant strains survived for the same period with no loss in viable numbers. The results of these experiments indicate that many strains of *Rhizobium* can grow and survive at salt concentrations which are inhibitory to most agricultural legumes. The emphasis of research concerning the effects of salinity on symbiotic nitrogen fixation should, therefore, be directed to aspects of the symbiosis other than the survival of the *Rhizobium* spp.

Excessive soluble salts affect more than 4×10^6 km^2 of the potentially arable lands of the world (7). The agricultural potential of these lands is generally not limited by a lack of solar radiation or temperature, and if managed properly, these lands can become productive. Measurements of soil salinity are commonly made by determining the electrical conductivity (EC) in millisiemens per centimeter or the equivalent osmotic pressure in bars of the soil solution at saturation (15). Seawater at 20°C has an EC of 46.6 mS cm^{-1} (14). The actual concentrations of soluble salts that are present in soil moisture films fluctuate with changes in soil water content.

Little work has been done concerning the effect of salinity on the legume-*Rhizobium* symbiosis (11). It has been well documented that nitrogen accumulation by the symbiotic systems of soybean, alfalfa, and *Glycine javanica* is reduced by salinity (2, 18).

Saline conditions may limit the symbiosis by (i) affecting survival and proliferation of *Rhizobium* spp. in the soil and rhizosphere, (ii) inhibiting the infection process, (iii) directly affecting root nodule function, or (iv) reducing plant growth, photosynthesis, and demand for nitrogen. Since soil salinity may directly affect either symbiont or affect their interaction (11), it is essential to identify the processes most sensitive to salinity. Efforts then may be directed toward improving the tolerance of the most sensitive symbiont or process of the symbiosis.

The reported effects of salts and soil moisture tension are mixed. Enhancement of growth of *Rhizobium* spp. in media with 1% NaCl (approximately 19 mS cm^{-1}) has been reported by Pillai and Sen (12). Steinborne and Roughley (13) have reported a reduction in growth rates of *Rhizobium trifolii* and *Rhizobium meliloti* in the presence of salt. Bhardwaj (3) claimed that isolates

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TABLE 1. Source of cultures

Culture	Host	Soil environment	Location	Source
17E[a]	*Leucaena leucocephala*	Beach sand	Kualoa, Oahu, Hawaii	This study
Hawaii 5-0	*Lens esulenta*	Acid tropical soil	Molokai, Hawaii	S. N. May (M.S. thesis, University of Hawaii, Honolulu, 1979)
7B[a]	*Indigofera suffruticosa*	Beach sand	Kualoa, Oahu, Hawaii	This study
Web 48	*Glycine max*	Soybean field	Midwestern United States	B. Bohlool, (M.S. thesis, University of Minnesota, Minneapolis, 1979)
TAL 425	*Vigna radiata*	Acid tropical soil	Thailand	Culture collection, NifTAL Project, Paia, Hawaii
23B[a]	*Macroptilum lathyroides*	Flood plain	Kahuku, Oahu, Hawaii	This study
8[a]	*Mimosa pudica*	Beach sand	Kualoa, Oahu, Hawaii	This study
14E[a]	*Croatalaria mucronata*	Irrigated cane field	Ewa, Oahu, Hawaii	This study
TAL 426	*Vigna unguiculata*	Acid tropical soil	Thailand	Culture collection, NifTAL Project, Paia, Hawaii
21A[a]	*Canavalia* spp.	Beach sand	Laie, Oahu, Hawaii	This study
USDA 110	*Glycine max*	Soybean field	Florida	Culture collection, U.S. Department of Agriculture, Beltsville, Md.

[a] Isolate from saline soil. EC of saturation extract, ≥5.0 mS cm^{-1}.

from nonsaline soils could not proliferate or survive in salt-affected soils. Carr and Ballard (5) found that a strain of *R. trifolii* was able to withstand a short exposure to fertilizer solutions with ECs in excess of 60 mS cm^{-1}.

This paper compares rhizobia isolated from salt-affected soils with random inoculum strains from nonsaline areas for their ability to grow and survive in salt solutions and in saline soil with different moisture tensions. Salinity treatments were, for the most part, selected to encompass a range of tolerance found in agricultural legumes.

Although salinity slowed the growth of all strains tested in these experiments, there was considerable variation within the species tested for tolerance to salt. The salt tolerances of the strains were not related to their ecological origins or to their growth rates in normal media. Rhizobia were able to withstand large changes in osmotic concentrations with little reduction in viable numbers. Sensitive strains were only affected after 5 days of exposure to salt solutions which approached the concentration of seawater. There appears to be no interaction between soil moisture tension and salinity in relation to rhizobial survival in clay soil. A reduction in total water potential, whether due to osmoticum or to high moisture tension, is the factor that affects rhizobial survival. Both "tolerant" and "sensitive" strains are much more tolerant of soil salinity and dry soil conditions than are their leguminous hosts.

MATERIALS AND METHODS

Source of cultures. Isolates of rhizobia were made from legumes growing on beach sands and salt-affected irrigated fields on the island of Oahu, Hawaii. Many of the isolates from beach sands were made close to standing seawater. *Rhizobium* isolation and plant infection tests were carried out as described by Vincent (16). All isolates were confirmed as rhizobia on *Macroptilum lathyroides*, except 17E which was confirmed on its host, *Leucaena leucocephala*. Sources of standard cultures from nonsaline soils are given in Table 1. All cultures were maintained on yeast extract-mannitol (YEM) slants (16).

Enumeration. Viable counts were performed by using standard serial dilutions and plated by the drop plate count method (16). The entire content of incubated soil vials was diluted; solution cultures were subsampled. All serial dilutions of salt treatments were made with isotonic diluents.

Effect of salt on growth. Inocula of 11 strains were diluted, and 1 ml of each diluted culture was added to tubes containing 9 ml of YEM broth amended with either no NaCl (1.2 mS cm^{-1}), 50 mM NaCl (6.7 mS cm^{-1}), or 100 mM NaCl (13.1 mS cm^{-1}). The initial cell density was 10^3 viable cells ml^{-1}. Mean doubling times (MDTs) were determined by viable counts made three times during the log phase of growth of each strain. The relative tolerance of different strains was expressed as the ratio of MDT at 13.1 mS cm^{-1} to MDT in normal YEM broth (1.2 mS cm^{-1}).

Effect of concentrated saline solutions on survival of rhizobia. Four strains from the growth rate experiment were selected: 17E (fast growing, salt tolerant), Hawaii 5-0 (fast growing, salt sensitive), 21A (slow growing, salt tolerant) and USDA 110 (slow growing, salt

TABLE 2. Effect of NaCl on the MDT of 11 *Rhizobium* strains

Strain	DT (h) in the following NaCl concn:			Relative sensitivity[a]
	0 mM (1.2 mS cm^{-1})	50 mM (6.7 mS cm^{-1})	100 mM (13.1 mS cm^{-1})	
17E[b]	2.7	5.3	8.4	3.1
Hawaii 5-0	3.9	11.8	NG[c]	
7B[b]	6.0	7.7	69.7	11.6
Web 48	8.7	11.9	NG	
TAL 425	7.4	7.5	17.4	2.4
23B[b]	6.2	7.9	10.1	1.6
8[b]	5.9	7.1	NG	
14E[b]	7.4	7.8	69.0	9.3
TAL 426	8.4	7.5	26.4	3.1
21A[b]	10.3	12.6	17.6	1.7
USDA 110	5.5	6.3	27.2	5.0

[a] Ratio of MDT at 13.1 mS cm^{-1} to MDT at 1.2 mS cm^{-1}.
[b] Isolated from salt-affected soils.
[c] NG, No growth.

sensitive). Cultures were grown in YEM broth, diluted to 10^7 cells ml^{-1} in solutions containing only YEM broth salts, and then inoculated into tubes containing broth salts only (1.4 mS cm^{-1}), broth salts plus 233 meq of total salts NaCl plus $CaCl_2$ per liter (18.4 mS cm^{-1}), or broth salts plus 564 meq of total salts NaCl plus $CaCl_2$ per liter (43.0 mS cm^{-1}). The proportions of NaCl and $CaCl_2$ were adjusted to maintain a constant Na activity (SAR) as defined by the U.S. Salinity Laboratory (15). Viable counts were made at 5 h, 5 days, 27 days, and 65 days after inoculation.

Growth and survival of rhizobia in saline soil at two moisture tensions. Wahiawa subsoil (Tropeptic Eutrustox; clayey, kaolinitic, isothermic) was air dried and passed through a 2-mm sieve. A moisture release curve was developed by using hanging water columns and a membrane pressure plate apparatus (6). The soil was also calibrated for the EC when increasing amounts of $CaCl_2$ and NaCl were added at a constant Na activity (SAR = 10). Air-dried soil samples, each weighing 34.4 g (30 g of dry soil at 65°C), were added to 120-ml glass vials with airtight polyethylene caps. Vials and soil were gamma irradiated with 6×10^6 rads and inoculated with appropriate mixtures of salt solutions and dilutions of YEM suspension cultures of strains 17E, 21A, Hawaii 5-0, and USDA 110 to impose treatments consisting of all combinations of −0.1 and −15 bars of moisture tension and ECs of 0.2, 5.0, and 12.0 mS cm^{-1}. The initial inoculum densities ranged between 1.5×10^6 and 5.2×10^6 viable cells g^{-1} of oven-dry soil.

Viable counts were made at 3, 7, 24, and 82 days after inoculation for fast-growing strains (Hawaii 5-0 and 17E) and at 6, 29, 49, and 86 days after inoculation for slow-growing strains (USDA 110 and 21A).

RESULTS

The addition of NaCl to YEM broth increased the MDT for all but one strain tested (Table 2). Sensitive strains such as Hawaii 5-0, Web 48, and 8 failed to grow in the highest level of salt. Isolates from saline soils were not consistently more tolerant to salt stress than were other isolates.

All four strains selected from the growth rate experiment (17E and 21A for salt-tolerant growth and Hawaii 5-0 and USDA 110 for salt-sensitive growth) were able to survive the initial exposure (5 h) to solutions with an EC of 43 mS cm^{-1} (Fig. 1). Only after 5 days of exposure of strain USDA 110 to solutions at 43 mS cm^{-1}, and after 27 days of exposure of strain Hawaii 5-0, was any significant decline in viability detected. The viability of the tolerant strains, 17E and 21A, was maintained in all of the treatment solutions for the duration of the experiment. Viable numbers in YEM broth cultures (no salt) declined over time.

The survival of strains USDA 110 and 21A was affected in soil, but only at the most extreme treatment combination of 12 mS cm^{-1} and −15 bars of moisture tension, whereas strains 17E and Hawaii 5-0 remained unaffected (Fig. 2). All strains grew slightly when exposed to less extreme conditions.

DISCUSSION

The salt tolerance of each symbiont of the legume-*Rhizobium* symbiosis may differ (11). It is practical, therefore, to examine the tolerance of one symbiont in relation to the tolerance of the other. Despite a large range in tolerance to salts among species of legumes, there are no agricultural legumes that can be considered to be highly salt tolerant (Table 3). Comparing the sensitivity of the microsymbiont, *Rhizobium*, with that of the legume host will then indicate the relative value of efforts to increase rhizobial tolerance to salts in strain selection programs.

The growth of all but one of the rhizobia tested was slowed by the presence of NaCl (Table 2). These results are contrary to those obtained by Pillai and Sen (12), who showed that the growth rate of *Rhizobium* spp. increased

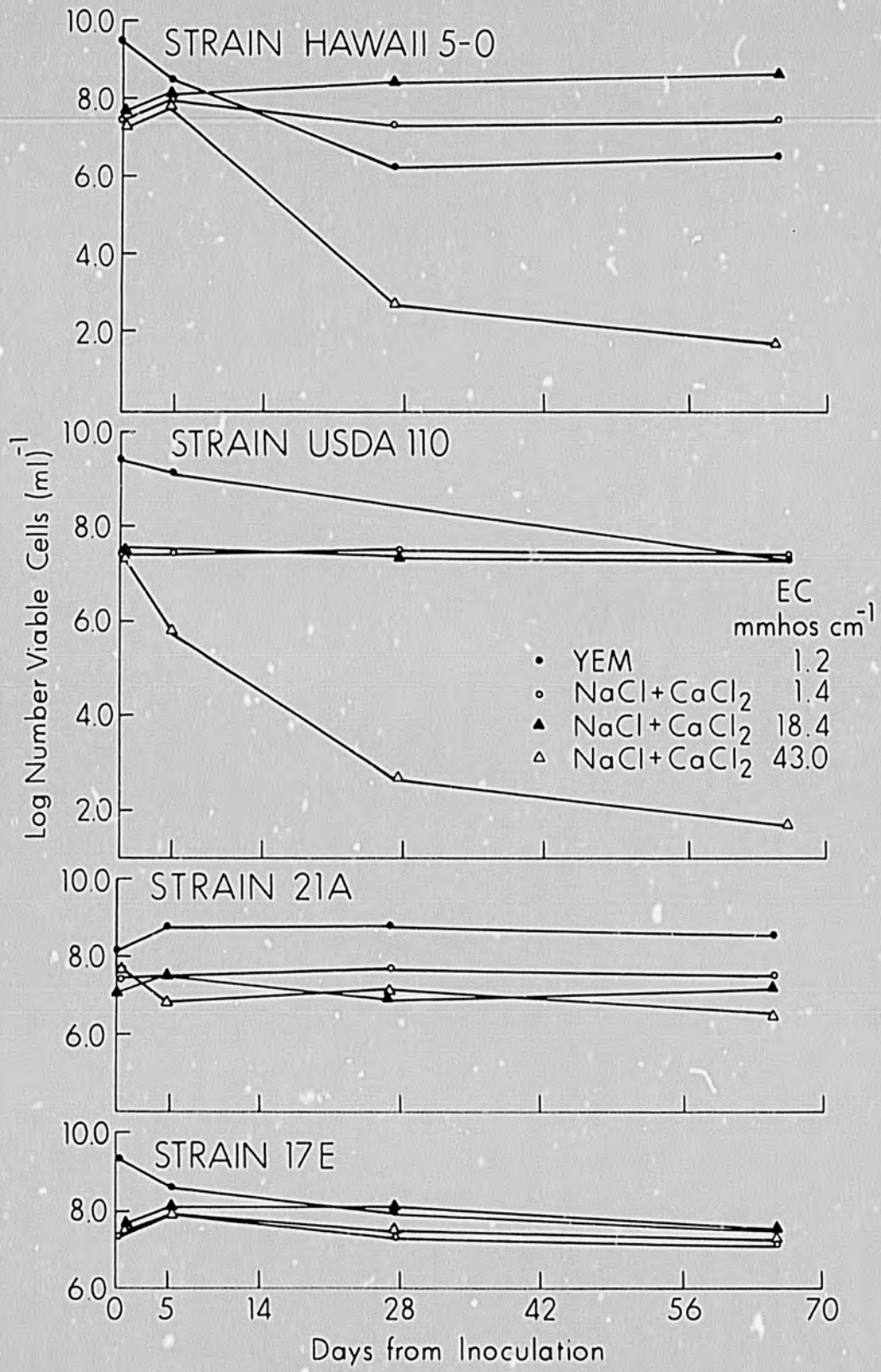

FIG. 1. Survival of four *Rhizobium* strains in YEM and in salt solutions. One mmohs = one mS.

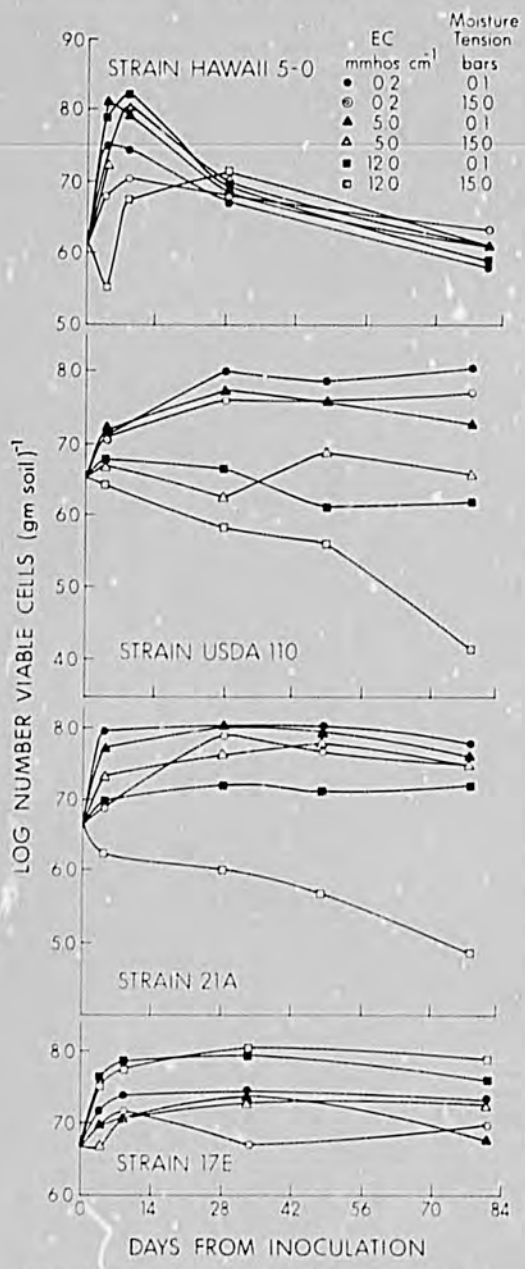

FIG. 2. Growth and survival of four *Rhizobium* strains in a Hawaiian Oxisol. One mmohs = one mS.

with 1% NaCl added to broth media (EC ≅ 18.0 mS cm^{-1}). Steinborne and Roughley (13), on the other hand, have shown that the growth of both *R. trifolii* and *R. meliloti* is slowed by the addition of salt.

The relative tolerances to salt are given in Table 2 to allow comparison of the effect of salt on the growth of strains with large differences in inherent MDTs. Large increases in MDT, or growth suppression, was not observed for any of the strains until the broth EC reached 13.1 mS cm^{-1} (28% seawater equivalent). This solution concentration is in excess of solution concentrations in which agricultural legumes can sustain an economic yield.

Slow-growing strains were not more tolerant to salt than were fast-growing strains. Isolates from saline environments were not consistently more tolerant to salt than were isolates from nonsaline soils. Isolates 7B, 8, and 14E from saline environments either did not grow or grew poorly in broth with an EC of 13.1 mS cm^{-1}. Strain TAL 425, an isolate from an acid tropical soil, had an MDT at 13.1 mS cm^{-1} that was only 2.4 times that in normal medium.

Subjecting two salt-tolerant isolates (17E and 21A) and two salt-sensitive strains (Hawaii 5-0 and USDA 110) to an extreme reduction in osmotic potential had no effect on survival over 5 h (Fig. 1). The solution with an EC of 43.0 mS cm^{-1} has a salt concentration equivalent to 92% of that of seawater (14). Since rhizobia can withstand large reduction in osmotic potential, they must be able to rapidly regulate and adjust their internal solute concentration. Vincent et al. (17) have shown that *R. trifolii* populations fall from log 4.35 to log ≤1.0 in a 160 mM NaCl (EC ≅ 16.0 mS cm^{-1}) solution after a 48-h exposure. The suspensions were, however, equilibrated with an atmosphere of 20% relative humidity which reduced the water content of the suspension to minimal amounts and left cells exposed to pure NaCl. Our results agree with those of Carr and Ballard (5), who found that a strain of *R. trifolii* could survive a short exposure to fertilizer solutions with ECs approaching 60 mS cm^{-1}.

Normal YEM broth cultures with initial densities in excess of 10^9 cells ml^{-1} lost viability over time, declining until numbers were equal to or less than numbers in most salt treatments. Examinations of rhizobial tolerance to stress in liquid culture, therefore, should not begin with densities of 10^9 cells ml^{-1}, since these numbers cannot be maintained even in ideal conditions.

There is an inverse relationship between soil moisture tension and salinity in microenvironments. Reducing the soil moisture content necessarily concentrates salts in the soil solution. An inoculum placed into soil at planting encounters immediate fluctuations in soil water potential (the sum of matric and osmotic potentials). The treatment combinations selected to test the effects of both soil moisture tension and salinity on the survival of *Rhizobium* spp. ranged from soil conditions which may be considered optimal for plant growth (EC = 0.2 mS cm^{-1} and moisture tension = −0.1 bar) to extreme conditions which are unacceptable for supporting adequate growth of agricultural legumes (EC = 12 mS

TABLE 3. The salinity tolerance of some agricultural plants[a]

Crop	EC[b]
Barley	16
Birdsfoot trefoil	10
Sesbania	9
Soybean	9
Alfalfa	8
Clover	4
Kidney bean	3
Peas	2

[a] From Bernstein (1).
[b] EC (mS cm^{-1}) associated with a 50% decline in yield.

cm^{-1} and moisture tension = −15 bars). None of the four strains tested (17E, Hawaii 5-0, USDA 110, and 21A) lost viability rapidly with even the most extreme treatment (Fig. 2). *Rhizobium leguminosarum* strain Hawaii 5-0, which was sensitive to salt in both the growth rate study and exposure to salts in solution, grew slightly and survived well in this clay soil. Isolate 21A, which showed good tolerance to solutions with an EC of 43.0 mS cm^{-1}, lost viability over time with the combined effects of high salinity and dessication. The decline was not strictly due to the low moisture content, since strain 21A survived well at −15 bars and 5.0 mS cm^{-1}. *Rhizobium japonicum* strain USDA 110 lost viability as a function of increasing stress from both the osmotic and matric components of soil water potential. The fast-growing isolate 17E was completely resistant to all levels of stress in this experiment.

Marshall (10) and Bushby and Marshall (4) have concluded that slow-growing rhizobia tolerate dessicated sandy soil better than do fast-growing species. All species survived dessication better when sandy soil was amended with montmorillonite. Our study shows that the inherent growth rate of a strain does not determine its resistance to low soil moisture content or salinity. Both strain 17E and strain B73 survived the combination of high salinity and increased moisture tension in this clay soil better than did slow-growing strains USDA 110 and 21A.

Mahler and Wollum (8, 9) have shown that even clay soil at −15.0 bars of moisture tension is detrimental to the survival of many strains of *R. japonicum* and *R. leguminosarum*. Their soil was, however, autoclaved for 270 min, after which soil samples used for the incubation of rhizobia were contaminated with other bacteria. The growth of other bacteria in the incubation vessels decreased as a function of decreasing soil moisture content (8). We found that when sterile conditions were maintained in gamma-irradiated soil, none of the strains tested was affected by either the soil moisture tension levels or the salinity levels used in this study. There appears to be no differential effect of moisture tension and salinity on rhizobial survival. The reduced survival of strains USDA 110 and 21A seems to be caused by the additive effects of increasing salinity and moisture tension.

The results of the experiments described above emphasize that many strains of *Rhizobium* not only can withstand but may even grow at salt concentrations in excess of those tolerated by most agriculturally important legumes. This is consistent with the life cycle characteristics of the two symbionts. Whereas the host legume produces seed and enters dormancy at the onset of the dry season, its microsymbiont, *Rhizobium*, in order to survive, must be able to encounter much higher levels of salts in the soil solution as the soil dries.

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