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# DESICCATION TOLERANCE OF FOUR STRAINS OF *RHIZOBIUM JAPONICUM*

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**Summary**—The survival of different strains of *Rhizobium japonicum* was evaluated in three soils with two matric- and three osmotic-induced moisture potentials. Both drying and added NaCl significantly decreased populations. Strains CC 709 and USDA 110 were less affected by the matric- or osmotic-induced desiccation than strains CB 1809 and USDA 123. The survival of CC 709 and USDA 110 at 2 weeks with 0.7% added NaCl was 33 and 46% of initial counts in soils undergoing drying, and 70 and 67% in soils maintained at the 30 kPa (0.3 bar) potential. Comparable survivals of CB 1809 and USDA 123 were 15 and 18% and 56 and 59%, respectively. The soil with the greatest clay and organic C contents maintained the highest populations during desiccation. Turbidity measurements indicated similar rates of growth of the four strains at a water activity ($A_w$) of 0.999 in yeast mannitol broth (YMB). When the YMB was adjusted with glycerol to lower water activities, strains CC 709 and USDA 110 consistently showed greater growth than did strains CB 1809 and USDA 123. No growth of CB 1809 and USDA 123 was observed at an $A_w$ of 0.975. Water adsorption isotherms of freeze-dried cells showed that the more desiccation-susceptible strains (CC 709 and USDA 110) retained greater amounts of water at a given relative vapor pressure than did the two more tolerant strains.

## INTRODUCTION

Many biotic and abiotic factors affect the survival and growth of root-nodule bacteria after addition to soil. One factor that has been studied is the decrease in viability due to desiccation. Danso and Alexander (1974) reported a decrease in living cells of *R. meliloti* from $10^8$ g$^{-1}$ to less than $10^1$ g$^{-1}$ after 4 weeks when dried in sand. They found that survivals at 8 weeks were approximately $10^6$ g$^{-1}$ when similar populations were dried in a silt loam or a loamy fine sand.

The rate of drying during desiccation also affects survival. Marshall (1964) and Bushby and Marshall (1977a) reported that the fast-growing clover, pea and lotus rhizobia were more susceptible to desiccation than the slow-growing species under conditions of relatively rapid drying. Bushby and Marshall related death of desiccated cells to changes in membrane permeabilities (1977b) and to quantities of water retained at a known relative vapor pressure (1977c). Jansen van Rensburg and Strijdom (1980) found that slow drying over $Ca(NO_3)_2$ allowed greater survival of fast-growing rhizobia than of slow-growing rhizobia. They further observed with forced-air drying, similar to Bushby and Marshall's procedure (1977a), that the pattern of survival changed and strains of slow-growing *Rhizobium* withstood desiccation better than those of fast-growing species. Slow drying, as evaluated by Jansen van Rensberg and Strijdom (1980), would more nearly mimic the natural drying in the field. Pena-Cabriales and Alexander (1979) studied the population decreases of nine streptomycin-resistant mutants of *Rhizobium* with slow drying; they failed to show any differences in susceptibility to desiccation between members of the fast- and slow-growing groups. But they did find statistical differences in resistance to desiccation among individual test organisms. Because they used only a single strain of each species, it was unclear from their study whether the differences in resistance were characteristic of species or characteristic of individual strains within a single species.

We evaluated differences in susceptibility to desiccation among four strains of a single species, *Rhizobium japonicum*. Desiccation tolerances among the strains were evaluated in three soils with two matric-induced and three osmotic-induced potentials. Water activities ($A_w$) of the soils were measured to compare the desiccation stress due to the various treatments. Tolerances to desiccation also were evaluated in sand with uniform slow drying and in yeast mannitol broth (YMB) adjusted with glycerol to varying water activities. Additionally, water adsorption isotherms of the test strains were determined to relate to the work of Bushby and Marshall (1977c), who found that greater quantities of water were retained at lower relative vapor pressures by desiccation-sensitive species (fast growers) than by the more resistant slow growers.

## MATERIALS AND METHODS

### *Strains*

Two of the four strains used, USDA 110 and USDA 123, were obtained from Dr D. F. Weber (Cell Culture and Nitrogen Fixation Laboratory, SEA-AR, Beltsville, MD). The remaining two strains, CC 709 and CB 1809, were obtained from Dr J. M. Vincent (102 Northwood Road, Northwood, NSW 2066, Australia).

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Table 1. Soils used to study the survival of *R. japonicum* strains

| Soil | pH (1:2 $H_2O$) | Total C (%) | Total N (%) | Sand (%) | Clay (%) |
|---|---|---|---|---|---|
| Hayden | 6.9 | 3.18* | 0.219* | 54* | 10* |
| Nicollet | 6.3 | 3.02 | 0.248 | 43 | 23 |
| Okoboji | 6.4 | 6.11 | 0.562 | 18 | 41 |

* Total C was determined by the Leco method (Tabatabai and Bremner, 1970); total N by the Kjeldahl method (Bremner, 1960); and texture by the pipette method (Kilmer and Alexander, 1949).

*Matric osmotic potentials of soils*

The three soils used, chosen to represent a range in textures, were collected from the surface 15 cm of the Hayden, Nicollet, and Okoboji series (Table 1). Matric potentials were controlled during the 6-week investigation by maintaining one set of samples at the 30 kPa (0.3 bar) tension and allowing the second set of samples, initially at the 30 kPa tension, to dry uniformly in desiccators over $CaCl_2$. Osmotic potentials were varied by treating each soil with 0.0, 0.3, or 0.7% (w/w) NaCl. The relative humidities of soils at different moisture and salt contents were measured with a hygrometer (American Instrument Co., Division of Travenol Laboratories Inc., Silver Springs, MD), as suggested by Bouyoucos and Cook (1967). One hundred grams of soil was placed in a 300-ml glass container; the lid had a hole sufficiently large to accommodate the sensor adaptor. Readings were taken at 8 h after equilibrium was reached.

Strains were prepared by centrifugation of 4-day-old cultures grown in YMB. Cells were washed three times with 0.1 M phosphate buffer (pH 7.1) and adjusted to equal turbidity. One milliliter of inoculum was added to 30 g γ-irradiated soil (4.2 Mrad during 9 h), and soils were incubated overnight at 28 C before initial counts were taken. Viable populations were determined weekly by plate counts on yeast extract mannitol (YEM) agar.

*Drying sand*

Desiccation tolerance of the four strains was determined in sterilized sand. One milliliter of washed cells at a uniform turbidity was added to 6.0 g dry quartz sand, providing an initial moisture content of 17%. Initial populations of each strain were determined by serial dilution, and the dishes containing the sand were placed in desiccators over $CaCl_2$; this was a similar procedure to that used by Danso and Alexander (1974). Triplicate plates were periodically prepared for cell counts on YEM to determine survival.

*Broth media with controlled $A_w$*

Lower water activities ($A_w$) were obtained by adding glycerol to YMB (glycerol was used instead of salts to eliminate the specific ion effect). The amount of glycerol required to attain the desired $A_w$ was calculated by using Raoult's law of mole fractions (Bone, 1973). After the glycerol was dissolved in water, the mixtures were sterilized by autoclaving, and sterile water was added to restore volume. One milliliter of each cell culture was added to 50 ml YMB in nephelo-culture flasks, and the flasks were shaken on a rotary shaker (140 rev min$^{-1}$) at 28 C. Changes in turbidity at 525 nm were recorded daily.

*Water-adsorption isotherms*

Water adsorbed at 40 C by freeze-dried cells of the four *R. japonicum* strains was measured in a vacuum apparatus similar to the one described by Bateman *et al.* (1962). Cell preparation was similar to that used by Bushby and Marshall (1977c). To obtain salt-free suspensions of rhizobia, 1 ml of Triton X-100 was added to 100 ml of broth culture and the cells were dialyzed in flowing water at 2 C for 4 days. The rhizobial suspensions then were frozen in dry ice and acetone, and dried overnight in a lyophilizer. Water adsorption isotherms of solid egg albumin were obtained to test the performance of the apparatus and values were in good agreement with those obtained by Bull (1944). Changes in the weight of samples after equilibrium were measured by observing with a traversing microscope the extension of a precalibrated silica spring.

## RESULTS AND DISCUSSION

*Survival in soil*

The biologically available water in this study was determined by measuring $A_w$, which is defined as the relative humidity above the sample after equilibrium.

Table 2. Water activity equivalencies of soils with different moisture and NaCl additions*

| Soil | NaCl (%, w/w) | Electrical conductivity (mmhos cm$^{-1}$) | Water content ($P_w$) at the 30 kPa tension (%, w/w) | Water Activity ($A_w$) 30 kPa | Air dry |
|---|---|---|---|---|---|
| Hayden | 0.0 | 0.23 | 16.7 | 0.991 | 0.505 |
| | 0.3 | 6.30 | ND | 0.982 | 0.465 |
| | 0.7 | 15.20 | ND | 0.975 | 0.440 |
| Nicollet | 0.0 | 0.30 | 30.4 | 0.990 | 0.495 |
| | 0.3 | 5.80 | ND | 0.980 | 0.465 |
| | 0.7 | 12.80 | ND | 0.975 | 0.425 |
| Okoboji | 0.0 | 0.35 | 50.4 | 0.990 | 0.500 |
| | 0.3 | 5.15 | ND | 0.984 | 0.470 |
| | 0.7 | 11.50 | ND | 0.975 | 0.430 |

* Average of duplicate determinations. Electrical conductivity was measured in a saturated soil extract, tensions by a pressure-gradient plate and water activity by a hygrometer.
ND = Not determined.

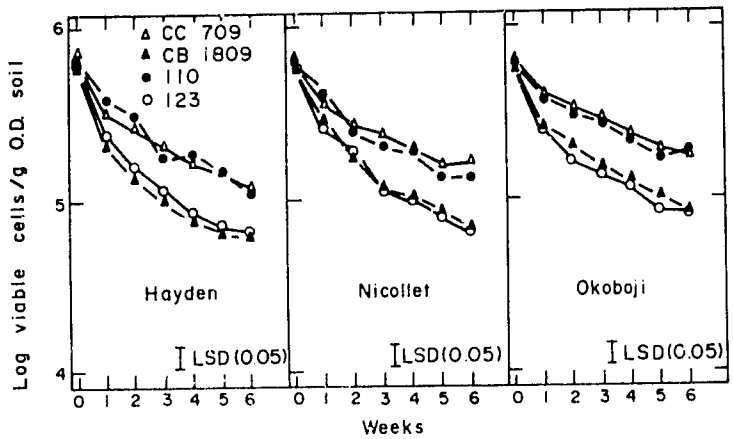


Fig. 1. Viable counts $g^{-1}$ oven-dry (O.D.) soil of four *R. japonicum* strains during slow drying over $CaCl_2$.

The quantitative relationship between matric- and osmotic-induced desiccation was not determined but, rather, differences were evaluated in the biological responses of *R. japonicum* strains to water stress from both sources. Adebayo and Harris (1971) and Cook *et al.* (1972) observed that matric potentials and osmotic potentials were not necessarily cumulative in their effect on biological systems.

The percentage of water by weight ($P_w$) of the three soils varied widely at the 30 kPa tension (Table 2). The measured $A_w$ values, however, at this potential were similar: 0.991, 0.990 and 0.990 for Hayden, Nicollet and Okoboji soils, respectively. Under air-dry conditions, similar water activities again were obtained at widely different moisture contents. The addition of NaCl reduced the measured water activities more in the air-dried soils than in moist soils. Drying salt-free soils over $CaCl_2$ for 6 weeks gave $P_w$ values similar to those of air drying, the lowest moisture contents likely to be attained under field conditions.

Similar patterns of survival among the strains in all three soils were found with slow drying over $CaCl_2$ (Fig. 1). Strains CC 709 and 110 persisted at higher populations than did strains CB 1809 and 123. Final counts were significantly higher in Okoboji, the soil having greater clay and organic C contents, than Nicollet or Hayden soils. Marshall (1964) and Bushby and Marshall (1977a) have shown that clay is beneficial in increasing survival during desiccation.

The effect of osmotic potential on the viable cell recovery at 0, 2, and 4 weeks when expressed as a percentage of initial counts without added NaCl shows that as salt concentrations increased from 0 to 0.7%, the counts of viable rhizobia decreased (Table 3). At the 30 kPa tension, populations increased in the three soils with no salt, but decreased with the addition of 0.3% NaCl. At all salt concentrations, there was a more rapid decrease in populations during drying than when soils were maintained at the 30 kPa tension. Strains CC 709 and 110 consistently had higher populations at a given salt concentration, both in air-dried soils and in soils at the 30 kPa potential, at 2 and 4 weeks than did strains CB 1809 and 123. Thus, increased osmotic potential resulted in a greater mortality of the latter two strains than of the former strains.

*Survival in sand during slow drying*

Susceptibility to desiccation of the four strains also was evaluated in sterilized sand desiccated over $CaCl_2$. After 36 days, both strains CC 709 and 110 had more than 10 times as many surviving cells as did strains 123 and CB 1809 (Fig. 2).

*Growth in media with adjusted water activities*

The basal YMB medium had an $A_w$ of 0.999. Water activities were adjusted with glycerol to 0.990, 0.980 and 0.975, and turbidity increases of the four strains were determined during growth. Figure 3 indicates growth at an $A_w$ of 0.980; this should be roughly equivalent to a matric potential of 3 MPa (30 bars) (Griffin, 1972). Beginning at 3 days, the rate of loss in transmittance was greater with strains 110 and CC 709 than with strains 123 and CB 1809. This resulted in large differences in turbidity by 8 days. Comparable 8-day losses of transmittance due to growth for strains 110 and CC 709 were 96 and 95% at an $A_w$ of 0.999 (basal medium without added salt) and 68 and 69% at an $A_w$ of 0.985 for the two strains, respectively. For strains 123 and CB 1809, comparable values were 95 and 95% at an $A_w$ of 0.999 and 57 and 54% at an $A_w$ of 0.985, respectively. Only slight growth of strains

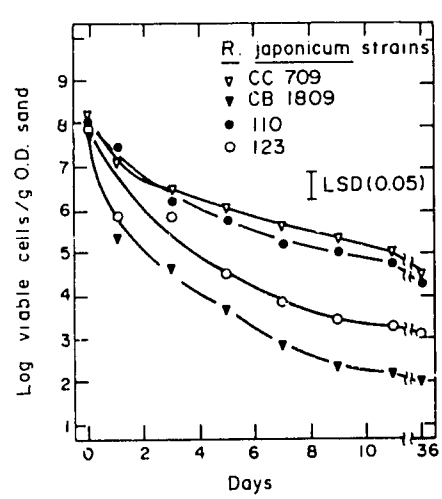


Fig. 2. Viable counts $g^{-1}$ oven-dry (O.D.) sand of four *R. japonicum* strains during slow drying over $CaCl_2$.

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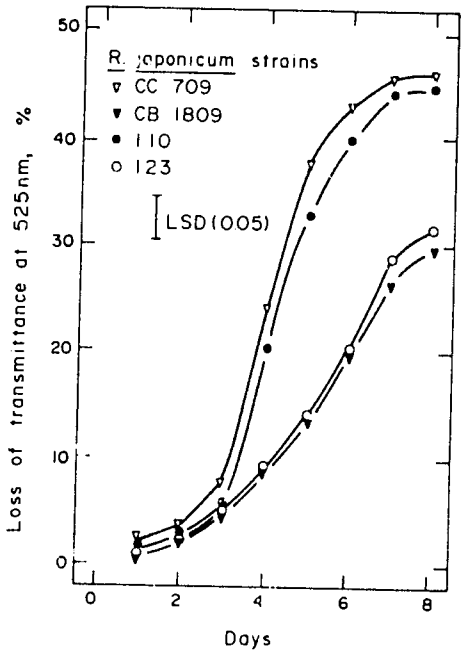


Fig. 3. Turbidities in yeast extract broth at an $A_w$ of 0.980 due to growth of four *R. japonicum* strains.

110 and CC 709 and no growth of strains 123 and CB 1809 was obtained with an $A_w$ of 0.975. Thus, as water activities were lowered below 0.999, strains 110 and CC 709 were better able to grow at the reduced osmotic potentials.

Attempts to improve the ability of these strains to withstand desiccation in sterilized sand by previous growth in YMB adjusted to lower $A_w$ values were not successful, except for small increases with strain CB 1809. Bushby and Marshall (1977a) also reported that the survival of rhizobia in a desiccated sandy soil was not influenced by previous growth in media of lower water activity.

### *Water adsorption isotherms*

Bushby and Marshall (1977c) reported that the susceptibility to desiccation of rhizobia was related to the amounts of water retained by the cells at a given relative vapor pressure. Strains 123 and CB 1809 (sensitive to desiccation) adsorbed more water than did strains 110 and CC 709 (less sensitive to desiccation) at all water activities (Fig. 4). These results were consistent with those of Bushby and Marshall (1977c), who reported greater amounts of water retained at lower relative vapor pressures by the fast-growing rhizobia (sensitive to desiccation) than by the slow-grow-

Table 3. Percentage survival of four *R. japonicum* strains as influenced by matric and osmotic potentials

| Soil | NaCl (% w/w) | Strain | Percentage survival at week 0: 709 | 110 | 1809 | 123 | 2: 709 | 110 | 1809 | 123 | 4: 709 | 110 | 1809 | 123 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Air dry | | | | | | | |
| Hayden | 0.0 | | 100* | 100 | 100 | 100 | 35 | 49 | 23 | 27 | 22 | 30 | 13 | 16 |
| | 0.3 | | 91 | 100 | 107 | 100 | 30 | 37 | 17 | 19 | 13 | 14 | 7 | 8 |
| | 0.7 | | 81 | 89 | 91 | 89 | 28 | 36 | 13 | 16 | 10 | 11 | 2 | 0.8 |
| Nicollet | 0.0 | | 100 | 100 | 100 | 100 | 39 | 43 | 31 | 30 | 28 | 33 | 19 | 16 |
| | 0.3 | | 93 | 100 | 95 | 93 | 32 | 37 | 32 | 28 | 25 | 27 | 14 | 11 |
| | 0.7 | | 89 | 100 | 93 | 79 | 34 | 42 | 15 | 17 | 19 | 22 | 6 | 6 |
| Okoboji | 0.0 | | 100 | 100 | 100 | 100 | 50 | 54 | 35 | 29 | 35 | 38 | 21 | 21 |
| | 0.3 | | 93 | 93 | 91 | 98 | 40 | 41 | 28 | 30 | 28 | 30 | 13 | 15 |
| | 0.7 | | 91 | 93 | 85 | 91 | 37 | 60 | 16 | 21 | 22 | 26 | 9 | 11 |
| | | | | | | | 30 kPa | | | | | | | |
| Hayden | 0.0 | | 100 | 100 | 100 | 100 | 166 | 166 | 166 | 141 | 170 | 170 | 191 | 166 |
| | 0.3 | | 95 | 100 | 102 | 102 | 78 | 78 | 68 | 56 | 87 | 79 | 58 | 37 |
| | 0.7 | | 87 | 96 | 93 | 89 | 52 | 60 | 39 | 53 | 42 | 36 | 13 | 14 |
| Nicollet | 0.0 | | 100 | 100 | 100 | 100 | 138 | 162 | 151 | 148 | 151 | 155 | 151 | 138 |
| | 0.3 | | 98 | 98 | 105 | 102 | 115 | 91 | 71 | 76 | 91 | 93 | 58 | 72 |
| | 0.7 | | 89 | 102 | 105 | 110 | 81 | 65 | 72 | 59 | 63 | 49 | 55 | 42 |
| Okoboji | 0.0 | | 100 | 100 | 100 | 100 | 120 | 123 | 112 | 126 | 120 | 132 | 112 | 120 |
| | 0.3 | | 76 | 93 | 83 | 102 | 78 | 85 | 72 | 76 | 76 | 81 | 60 | 74 |
| | 0.7 | | 89 | 91 | 95 | 95 | 78 | 83 | 56 | 65 | 69 | 81 | 19 | 21 |

* Numbers are expressed as the percentage of survival of initial populations without NaCl. Data also were collected at 1, 3, 5 and 6 weeks; because trends were similar, values are not included in this table. Each value is an average of three replicates. CV = 6.4%.

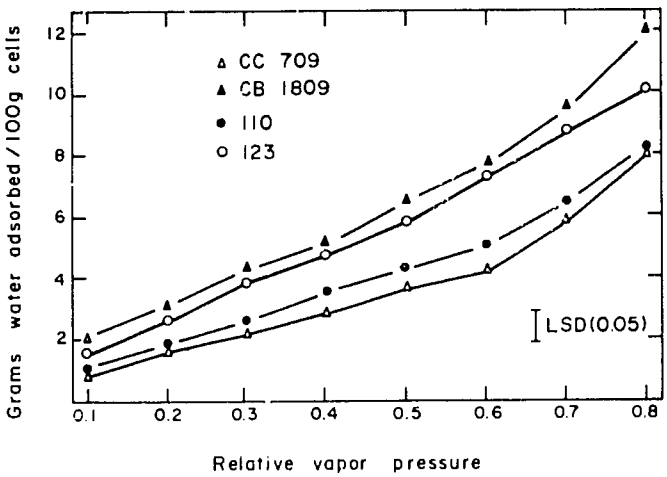


Fig. 4. Water adsorption isotherms at 40 C for four *R. japonicum* strains.

ing rhizobia (less sensitive to desiccation). The reason that cells sensitive to desiccation retained more water than tolerant cells at low relative vapor pressures is unclear. Bushby and Marshall (1977c) concluded that isotherm characteristics may be determined by differences in the number of water-accessible polar sites, and that this may affect internal enzymatic activities.

Because differences do exist between strains of the same species in tolerance to desiccation, our study suggests that strains should be screened for their survival during desiccation before being added to inoculants intended for use in arid or semi-arid regions or for use in saline soils.

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