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Rates of Drying and Survival of *Rhizobium meliloti* Strains During Storage at Different Relative Humidities

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An investigation was made of the survival of six strains of *Rhizobium meliloti* filtered on membrane filters and held in atmospheres of controlled relative humidities (RH) of from 0 to 100% at 30°C in the presence of air. The rate of water loss in the desiccator was determined by the humidity-controlling solution used. Drying was accelerated by a mild evacuation of the desiccator during the drying step. Survival rates of *R. meliloti* strains were much higher after slow drying to 0% RH than immediately after rapid drying. Fast drying (drying period less than 3.4 h) was shown to adversely affect the tolerance to storage at all RH values tested (no survival after 2 to 5 days of storage). When survival during storage was measurable (after slow drying), the optimum RH values for storage were 43% for strains A145 and Wu498, 22 to 43% for strains RCR2011, Wu499, and Ar16, and 83% for strain RCR2004. The most favorable drying periods were 8, 9.2, 14.2, and 50.1 h for the subsequent storage of strain RCR2011 at RH values of 0, 22, 43, and 83%, respectively. The damaging effects of rapid drying on the tolerance of strain RCR2011 to storage at different RH values could be prevented either by rehydration and subsequent slow redrying or incomplete rapid drying followed by slow drying. It is suggested that *R. meliloti* strains are susceptible to desiccation stresses. However, the quantitative differences among strains appear to be large enough to permit selection with regard to tolerance to desiccation and storage in dried states.

Although rhizobia are soil microorganisms, they are not found in large numbers in fields where no legumes are growing. The difficulties of establishment and persistence of these bacteria in soils in the absence of their specific leguminous hosts have emphasized the need for legume inoculation and prompted studies of their survival in soil under adverse conditions.

Many biotic and abiotic factors affect the growth and survival of rhizobia in soil. Most *Rhizobium* strains, which nodulate important crops, are sensitive to desiccation in soils (10, 19), on seeds (11, 24), and in peat cultures (23). Thus, tolerance to desiccation may be an important part of saprophytic competence and competitiveness in rhizobia.

Although the data showing the different sensitivity of fast- and slow-growing rhizobia are not conclusive, they demonstrate the importance of the rate of drying during desiccation. Bushby and Marshall (6) reported that the fast growers are more susceptible than the slow growers to rapid and severe desiccation in a forced drought oven (12-h drying period). The opposite result was obtained when the bacteria were subjected to slow drying with $Ca(NO_3)_2$ (31% relative humidity [RH]) (15). Pena-Cabriales and Alexander (20) failed to find a clear difference in tolerance to desiccation between species of rhizobia subjected to slow drying in air. Moreover, the survival of *Rhizobium japonicum* in soil was not consistently different immediately after rapid (24 h) or slow (11 days) drying. However, recent studies have shown that counts of different *Rhizobium* species immediately after fast drying in soil were 2 or 4 orders of magnitude lower than those observed after slow drying (8, 14).

The effect of drying rates on the survival of several genera of microorganisms during storage at 31% RH is well documented (2). However, the effects of the drying rate on the subsequent survival of *Rhizobium meliloti* strains during storage in different dried states have not been investigated. The objectives of this paper are to evaluate the effects of rate of drying, RH values during storage, and their interaction on survival of *R. meliloti* strains.

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MATERIALS AND METHODS

Strains. *R. meliloti* RCR2004 and RCR2011 (Rothamsted Collection of *Rhizobium*, Harpenden, Hertfordshire, United Kingdom), Wu498 and Wu499 (The University of Western Australia, Nedlands, Australia), A145 (Rijksdienst voor de ijsselmeerpolders, Kampen, The Netherlands), and Ar16 (Station de Recherches de Microbiologie du Sol, Dijon, France) were used in this study.

Medium and buffer. All strains were grown in *Rhizobium* complex medium (12) of the following composition (in grams per liter of distilled water): K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract, 1.0; mannitol, 10. The pH was adjusted to 7.2. A buffer containing 1 g of K_2HPO_4 and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water was employed to wash the cells and prepare the 10-fold dilution series.

Sample preparation for desiccation experiments. *Rhizobium* strains were harvested from *Rhizobium* complex medium after 1 day of growth at 30°C (end of logarithmic phase), washed twice by centrifugation, and suspended in buffer. About 2×10^9 rhizobium cells were placed on a membrane filter (Sartorius SM 11307; 0.2-μm pore size; 37-mm diameter) by rapid suction of 1 ml of the cell suspension. This amount of bacteria was considered to cover the filter in a thin layer. The filters were then placed on dry absorbent pads to remove the excess water. The water content of the filter sample with bacteria was about 70 ± 2 mg.

Drying experiments. The filter samples were dried and stored in small desiccators (125 ml) in which the RH of air was controlled by silica gel (0% RH) or by saturated $KC_2H_3O_2$, K_2CO_3, NaCl, and KCl solutions to give RH values of 22, 43, 75, and 83%, respectively. Desiccators containing distilled water were included for comparison

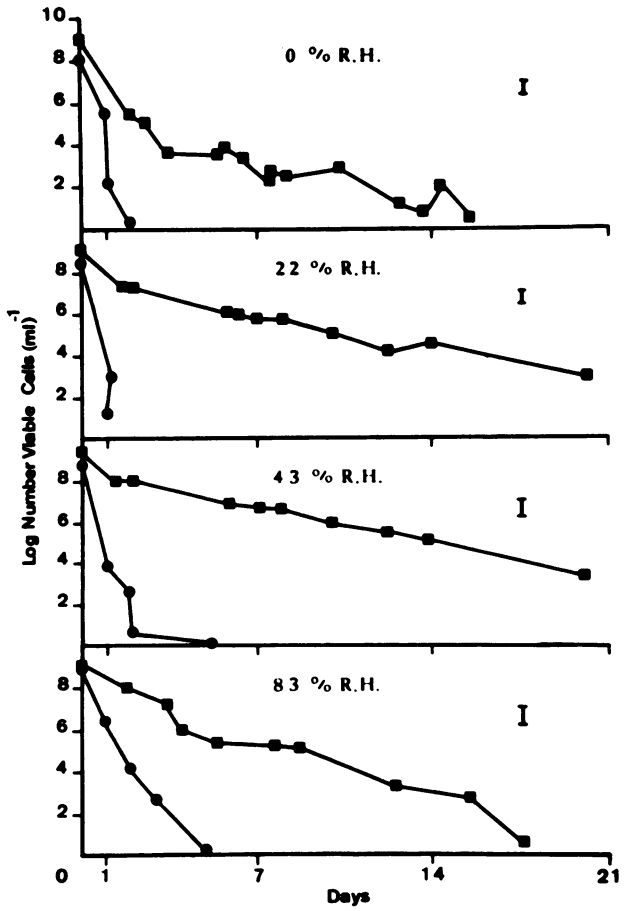

FIG. 1. Effect of rapid and slow drying on the viability of *R. meliloti* RCR2011 after storage at different RH values. Viability of rapidly (●) and slowly (■) dried cells is shown. Vertical bars indicate least significant difference (0.05) between treatment means.

(RH, 100%). Samples in desiccators of known RH were kept in the dark at 30°C for various periods. Slow drying occurred in desiccators at atmospheric pressure. Rapidly dried cells were obtained by mild evacuation (ca. 280 torr [37,240 Pa]) of the desiccator to accelerate moisture equilibration between sample and environmental RH. The water loss of sample in the desiccator at particular RH was determined by weighing the filter sample with bacteria.

The following additional experiments were carried out with strain RCR2011. Cells were dried rapidly or slowly in desiccators with an RH of 75 or 43%, respectively. When equilibria were obtained in these desiccators, samples were transferred rapidly to storage enclosures with lower RH values kept at atmospheric pressure, and the bacteria were further dried at the rate characteristic of the storage desiccator. In another experiment, rapidly dried cells were remoistened (to 70 mg of water content) and slowly redried.

Enumeration of rhizobium survival. After the drying step and at regular intervals during storage, filter samples were resuspended in 2 ml of buffer. The release of dried cells was facilitated by mechanical shaking with a Vortex mixer. Viable cell counts were identical for periods of mixing from 5 to 30 s and were the same as counts of the same cell suspensions carried out before filtration. A 30-s mixing step was used throughout this study. Optical density readings of suspended dried cells revealed no significant decrease with increasing length of storage in the dried state. Any decrease in the plate count with duration of storage was therefore attributed to a decrease in viability and not to an increase in adherence of the cells to the membrane filters. At each sampling time, counts were made on duplicate samples under specified treatment, and triplicate plates were prepared at each dilution. Viable cells were counted by spreading 10-fold dilutions on *Rhizobium* complex agar. To ascertain whether sterility, due to desiccation, had been achieved, nil dilution and drained membrane filter were included for enumeration of surviving cells. Statistical analysis of results was performed by the calculation of the least significant difference.

RESULTS

Estimation of the time required to achieve equilibrium between sample water content and environmental RH. In the experiments in which only one specimen was exposed per desiccator, the total amount of water to be taken up by the humidity-controlling solution or silica gel was minimized. Under these conditions, it was very difficult to determine the water content of cells in equilibrium with the environmental RH because of the small amount of cells on a membrane filter. Nevertheless, for a large part of the drying period (from 70 to 1 mg of residual water content), the water loss was linearly related to time. Residual errors from the fitted regression lines were not significant. The equation of the water loss kinetics of the sample at a specified RH allowed an estimation of the time required to reach equilibrium between the sample water content and the environmental RH value. Equilibria in desiccators at 0, 22, 43, 75, and 83% RH values were attained in 1.8, 0.6, 1, 2.1, and 3.4 h, respectively, with the rapid-drying method, whereas 8, 9.2, 14.2, 36, and 50.1 h, respectively, were necessary for the slow drying.

Survival of *R. meliloti* strains during storage in air at different RH values after slow- or rapid-drying periods. Results for *R. meliloti* RCR2011 dried rapidly or slowly and subsequently stored at different degrees of desiccation are illustrated in Fig. 1. Significantly fewer viable cells were observed immediately after rapid drying to 0% RH compared with counts obtained immediately after slow drying. When the cells were rapidly dried, viable numbers of rhizobia during storage declined sharply until sterility was approached in 2 days at 0, 22, and 43% RH and 5 days at 83% RH. After slow drying and subsequent storage at all RH values tested, appreciable survival rates were still observed after 14 days, and strain RCR2011 was shown to withstand 22 and 43% RH better than 0 and 83% RH. After slow drying, viable numbers of rhizobia decreased markedly throughout the first stage of storage at 0, 22, and 43% RH. Thereafter, numbers of cells declined at a slower rate. During the first 3.5 days of storage at 0% RH, the rate of death was 1.5 log units per day. This rate was approximately 1 log unit per day within the first 2 days of storage at 22 and 43% RH. Beyond these steps, population fell by 0.23 log unit per day. During storage at 83% RH, population declined at a constant rate of 0.5 log unit per day.

A maximum decrease in cell numbers of 1 log was observed during a 10-day storage period at 100% RH (data not shown). Survival data for five other strains of *R. meliloti* examined in the follow-up study are presented in Tables 1 and 2. These five strains revealed similar survival trends with strain RCR2011. The harmful effects of rapid drying on survival of rhizobia during storage were confirmed (Table 1). Viable counts observed immediately after rapid drying to 0%

TABLE 1. Effect of rapid desiccation on the survival of *R. meliloti* strains during storage periods at four different RH values

Strains	Log initial no. of cells	Period of storage (days)	Log no. of cells after storage at % RH[a]			
			0	22	43	83
A145	9.34	0	8.71	8.77	9.23	9.27
		0.75	5.22	5.42	7.05	8.58
		1.25	4.15	5.50	5.98	7.62
		2	1.41	2.02	2.60	4.62
Wu498	9.01	0	8.21	8.71	8.91	9.05
		0.75	2.46	4.03	5.35	8.17
		1.25	1.88	2.52	2.05	4.98
		2	0.00	0.30	0.00	0.90
Wu499	9.39	0	8.46	8.91	9.08	9.28
		0.75	6.10	8.56	6.30	8.55
		1.25	2.60	5.75	5.75	4.43
		2	0.77	1.41	2.82	0.90
Ar16	9.04	0	8.40	8.42	8.40	8.99
		0.75	5.50	4.84	3.79	8.04
		1.25	4.54	2.32	3.03	6.17
		2	1.20	0.60	0.00	1.89
RCR2004	9.25	0	8.79	8.75	9.07	9.05
		0.75	5.68	4.75	7.21	8.65
		1.25	3.46	4.11	5.81	7.95
		2	2.10	0.00	2.50	6.50

[a] Least significant differences (0.05): Strain A145, 0.6; Wu498, 0.57; Wu499, 0.64; Ar16, 0.59; RCR2004, 0.48.

RH were significantly fewer than counts carried out before the desiccation (Table 1), whereas viable counts after slow drying were statistically equivalent to the controls (Table 2). After rapid drying, higher survival rates during storage were observed at 83% RH (except for strain Wu499) compared with other RH values (Table 1). Under conditions of slow drying, the optimum RH values after 14 days of storage were 43% for strains A145 and Wu498, 22 to 43% for strains

TABLE 2. Effect of slow desiccation on the survival of *R. meliloti* strains during storage periods at four different RH values

Strains	Log initial no. of cells	Period of storage (days)	Log no. of cells after storage at % RH[a]			
			0	22	43	83
A145	9.22	0	9.06	8.88	9.20	9.17
		2	6.39	7.52	8.10	8.55
		7	2.92	4.49	5.50	6.20
		14	0.60	2.86	4.42	2.22
Wu498	9.19	0	8.96	8.91	9.14	9.15
		2	6.12	7.93	8.33	9.05
		7	3.63	4.79	7.68	7.74
		14	1.08	4.78	6.73	4.20
Wu499	9.23	0	9.02	8.95	9.19	9.05
		2	7.22	7.50	8.50	8.99
		7	4.56	7.87	7.95	7.66
		14	2.89	7.60	7.82	5.42
Ar16	9.09	0	8.92	8.90	8.99	9.05
		2	6.55	8.31	8.32	8.21
		7	4.20	6.31	7.78	6.89
		14	3.32	5.88	6.35	4.11
RCR2004	9.26	0	9.08	8.84	9.17	9.16
		2	5.61	6.57	7.85	8.97
		7	1.50	4.52	6.08	7.83
		14	0.78	1.00	4.74	5.83

[a] Least significant differences (0.05): Strain A145, 0.72; Wu498, 0.64; Wu499, 0.58; Ar16, 0.62; RCR2004, 0.55.

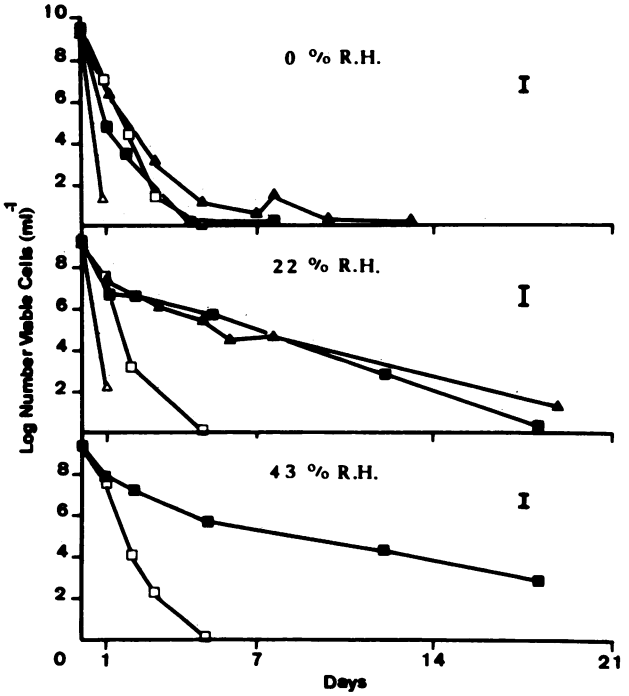

FIG. 2. Effect of rapid and slow drying to RH values of 75 or 43% and subsequent slow drying to lower RH levels on the survival of *R. meliloti* RCR2011 after storage at different RH values. Slow (■) and rapid (□) drying to 75% RH and subsequent slow drying to lower RH values and slow (▲) and rapid (△) drying to 43% RH and subsequent slow drying to lower RH values are shown. Vertical bars indicate least significant difference (0.05) between treatment means.

Wu499 and Ar16, and 83% for strain RCR2004. Strains Wu499 and Ar16 survived well (at all RH values tested) relatively to the other strains (Table 2). Survival during storage at 0, 22, and 43% RH values (Table 2) again occurred in two steps, but owing to the exploratory nature of these experiments it was difficult to determine the time at which the second step occurred in the decline of surviving cells.

Effect of rate of drying on survival of *R. meliloti* RCR2011 during storage at different RH levels. Four additional periods of desiccation, imposed by desiccators at RH values of 75 or 43% (as described in Materials and Methods), were carried out on samples of *R. meliloti* RCR2011 stored at a final RH of 0, 22, and 43%. Though the RH was reduced from 75 to 43%, from 75 to 22%, or from 75 to 0% at the slow rate characteristic of the storage enclosure with RH values of 43, 22, or 0%, respectively, cell survival during storage at a particular RH was significantly reduced when the 75% RH step was reached by rapid drying (except with subsequent slow drying to 0% RH) (Fig. 2). Similar results were obtained when the rhizobia were rapidly or slowly dried to an RH of 43% and then slowly dried to RH values of 22 and 0% (Fig. 2). The highest cell survival levels during storage at 0, 22, and 43% RH values occurred when equilibria were attained in 8, 9.2, and 14.2 h, respectively (Fig. 1 and 2). Cells that were dried in less than 3.4 h subsequently died during a 5-day storage period. Moreover, the lowest water content per specimen to which *R. meliloti* RCR2011 could be rapidly desiccated without causing serious damage was determined by comparison with the work of Antheunisse and Arkesteijn-Dijksman (2). These authors reported that harmful effects of rapid drying on survival during storage at 31% RH occurred

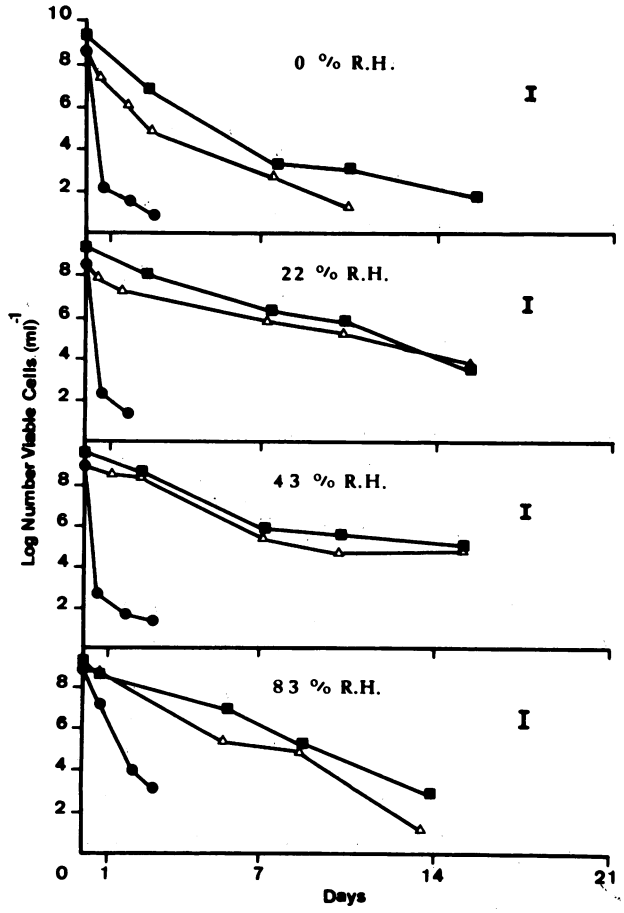

FIG. 3. Effect of rapid and slow drying on the survival of *R. meliloti* RCR2011 with and without remoistening of the rapidly dried cells during storage at different RH values. Viability of rapidly (●) and slowly (■) dried cells and cells which were rapidly dried, remoistened, and slowly redried (△) is shown. Vertical bars indicate least significant difference (0.05) between treatment means.

when the water content was less than 8 mg for 10^8 cells of *Escherichia coli*. We found that detrimental effects of rapid drying did not occur when a residual water content of 5 mg per specimen (with about 2×10^9 cells of *R. meliloti*) was reached by rapid drying followed by slow drying to lower RH values (data not shown).

Effects of rehydration and slow redrying steps on the survival during storage of the rapidly dried cells. Survival of cells which were rapidly dried, remoistened, and slowly redried was significantly higher during storage than cells which were rapidly dried (Fig. 3).

DISCUSSION

In our experiments, for a large part of the drying period (rapid or slow), water loss was a linear function of time, in agreement with the results of Vincent et al. (24). These authors showed that the viability of *Rhizobium trifolii* rapidly decreased under nondrying conditions (100% RH) when spread on glass beads; our data and that published by Jansen van Rensburg and Strijdom (15) did not support this finding.

The results reported here indicate that the rate of drying was of prime importance for survival of rhizobia during storage. Rapid drying to 0% RH caused significant decreases in cell numbers immediately after the desiccation process. These results confirm previous reports on the behavior of *Rhizobium* strains immediately after slow and fast drying (8, 14). Storage survival of cells in the dried or semi-dried states was significantly reduced by rapid drying (no survival after 2 to 5 days of storage). After slow drying, appreciable survival levels were still evident at 14 days. These data are in agreement with results of Antheunisse and Arkesteijn-Dijksman (2) and enlarge their findings to the genus *Rhizobium* desiccated and stored within the range of 0 to 83% RH.

After slow drying, the optimum RH level for storage in the presence of air was generally 43%. Nevertheless, strains RCR2011, Wu499, and Ar16 showed significantly equivalent survival rates at 43 and 22% RH. Strain RCR2004 maintained relatively high population under 83% RH but a significantly higher survival rate was observed at 43% RH compared with 0 and 22% RH conditions. It is well known that the lethal effects of oxygen on microorganisms increase when the RH falls below 40% RH (22, 25). In the same way, the frequency of single and double strand breaks in the DNA increases as the RH is reduced from 53 to 0% RH (3). Death of rhizobium cells below 43% RH could be a result of these effects. Further experiments are needed to investigate these hypotheses. The reason for the poor survival at an RH of 83% is not obvious. Fraser (13) reported that increasing the RH from 44 to 75% adversely affected the number of *R. meliloti* survivors. Jansen van Rensburg and Strijdom (15) found similar survival levels at RH values of 31 and 92% for three of the six strains of cowpea rhizobia studied. They speculated that it would be deleterious to the survival of rhizobia to expose them to RH too low to allow vital enzymes to function properly but not low enough to reduce or inhibit their activities. Dysfunction of intracellular enzymes may be responsible for cell death at 83% RH. Experiments on behavior of rhizobium cells stored at slightly higher RH values are necessary to support these hypotheses. The mechanism of the two-step decrease in survival which occurred after slow drying and subsequent storage at low RH values is unknown. Similar results were obtained by other drying processes (13, 15, 18). In some reports, the rhizobium population fell markedly as most of the water was lost. This rapid decline in survival was followed by a period with a slower death rate (11, 17, 20, 24).

Survival rates obtained at different RH levels in the present investigation were in agreement with previous reports (1, 6, 9, 24). *Rhizobium* spp., like other gram-negative bacteria, are particularly susceptible to desiccation. Moreover, increasing the temperature from 15 to 37°C adversely affects rhizobium survival in dried states (4, 8, 11, 13).

Very little is known about the causes of death of *Rhizobium* spp. after desiccation and rehydration. It has been attributed, by various authors, to changes in membrane permeability (7), quantities of water retained at a known relative vapor pressure (5), or the dysfunction of intracellular enzymes (15). Recently, it has been reported that the combination of drying and rehydration caused the rupture of the cell envelope when the internal pressure overcame its weakened resistance (21). The experiments, carried out with *R. meliloti* RCR2011, suggest that the rapid removal of water from the cell surfaces (until reaching 83% RH) damages the bacterial cells so that they become rapidly nonviable during storage. Such a phenomenon could be prevented by a remoistening step, as demonstrated by the similar behavior of cells which were rapidly dried, remoistened, and slowly redried and cells which were only slowly dried. These results are in good agreement with the previous report of

Antheunisse and Arkesteijn-Dijksman (2), who suggest that the cells are torn by rapid drying.

Though it may not be possible to directly extrapolate the results of desiccation experiments to the in vivo situations in soils (16), in peat cultures, or on inoculated seeds, the behavior of *Rhizobium* strains at different RH levels by the slow-drying method may be a convenient and rapid tool for screening for drought-tolerant rhizobia. Quantitative differences between strains are large enough to permit this selection. Considerable attention should be given to strains that are able to withstand large variations of RH. Experiments at higher RH values (e.g., >90%) should be of particular interest in regard to survival of rhizobia in soil undergoing drying.

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