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Inoculant Maturity Influences Survival of Rhizobia on Seed

L. A. MATERON AND R. W. WEAVER*
Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas 77843

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Survival of *Rhizobium trifolii* on seeds of arrowleaf clover (*Trifolium versiculosum* Savi) and subclover (*Trifolium subterraneum* L.) was affected by the maturity of peat-, vermiculite-, and charcoal-based inoculants. Ten times more rhizobia survived on seed 4 days after inoculation when inoculants were stored (cured) before being utilized as compared with uncured inoculants. Increasing the curing time of inoculants beyond 4 weeks had little effect on increasing survival of seed-applied rhizobia.

Solid-base inoculants such as peat, charcoal, and vermiculite materials have been used as carriers for rhizobia in legume inoculants (1, 10). Such carriers have a high water-holding capacity, provide a nutritive medium for growth of rhizobia, and favor rhizobial survival during inoculant distribution to farmers and after inoculation onto seed (2–5). Peat-based inoculants are generally kept for a storage (curing) period of 4 to 5 weeks at 20 to 27°C after inoculation to allow populations to increase (3, 4). Curing also increases the ability of the rhizobia to survive on seed (3). Burton (3) reported that survival of *Rhizobium meliloti* inoculated onto alfalfa seed was enhanced when the peat-based inoculant was cured. He surmised that the rhizobia became adapted to the carrier environment and were better conditioned to withstand drying on the seed; he stated that an optimum curing time was 4 weeks, but did not present supporting data.

While conducting inoculation trials on clovers, we recognized that rhizobia in freshly prepared peat inoculants did not survive as well as did rhizobia in stored inoculant (unpublished data). Therefore, we initiated experiments to compare the survival of *Rhizobium trifolii* on clover seeds with three solid-base carrier materials to determine the relationship between the curing time of inoculants and the survival of rhizobia on seed.

Strains of *R. trifolii* 162Y10 (Nitragin Co., Milwaukee, Wis.) and C6 (6) were maintained on yeast extract-mannitol agar (11). Yeast extract-mannitol broth was used for liquid cultures. The numbers of rhizobia in the inoculants and on seed were determined by plate counts. For inoculants, 1 g was aseptically transferred into dilution bottles containing 100 ml of peptone water (1 g of peptone per liter); the mixture was vigorously shaken by hand, serially diluted, and plated in duplicate on yeast extract-mannitol agar supplemented with actidione (100 mg/liter) and congo red (100 mg/liter). The number of rhizobia on seed was determined at each sampling time by transferring duplicate lots of 100 seeds into 100-ml dilution blanks containing the peptone water for serial dilutions.

To prepare the inoculants, dried and milled peat (Nitragin Co., Milwaukee, Wis.), charcoal (Darco, G-60; Fisher Scientific Co., Pittsburgh, Pa.), and vermiculite (Grace Horticultural, Cambridge, Mass.) were ground to pass a 200-mesh screen. The acidic peat was adjusted to a final pH of 7.15 (9). The pH values of charcoal and vermiculite were 7.43 and 7.68, respectively. To reduce seed coat toxicity, charcoal was incorporated into the peat carrier at the rate of 10 g into 90 g of dried peat (8). The carrier materials were sterilized by autoclaving, and sterility was confirmed by plating on yeast extract-mannitol agar. Sterile carriers were aseptically packaged, in 50 g quantities, into polyethylene bags (11.4 by 21.6 cm, Whirl-Pak; Nasco West Inc., Modesto, Calif.) having a wall thickness of 76 μm. Each package was inoculated with 25 ml of broth culture containing rhizobia in the log phase of growth. Inoculants were incubated at 27°C and were mixed twice each day by kneading the packages for the first 3 days after inoculation.

Seeds of Yuchi arrowleaf clover (*Trifolium vesiculosum* Savi) and Mt. Barker subclover (*Trifolium subterraneum* L.) were prepared for inoculation by submersing them for 30 s in 95% ethanol to disinfect seed surfaces without removing toxic factors in the seed coats (8). After disinfection, seeds were dried at 24°C under a laminar clean air hood equipped with bacteriological filters. For each experiment, 10 g of seed was inoculated with approximately 10,000 rhizobia per seed. The quantity of inoculant used was different for the two clover types because of seed size; arrowleaf had 800 seeds per g, and subclover had 150 seeds per g. Arrowleaf and subclover, respectively, received 200 and 40 mg of inoculant per 10 g of seed. The inoculant was adhered to the seed by a solution of gum arabic (250 mg per ml of water), which was used at a rate of approximately 0.5 and 0.3 ml, respectively, for 10 g of arrowleaf and subclover seed. Inoculated seed was divided into lots of 100, placed in petri plates, and incubated for the appropriate time period in a moisture-tight chamber. Excess seed was discarded. Humid-

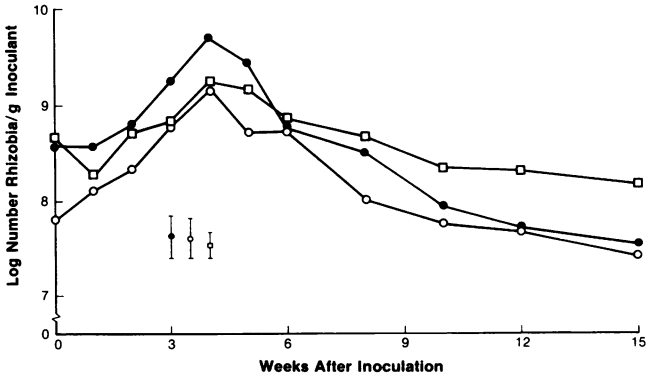

FIG. 1. Population of *R. trifolii* in three inoculant carriers incubated at 27°C. Symbols: ●, peat; ○, charcoal; □, vermiculite. Vertical bars denote the standard error of each value.

* Corresponding author.

TABLE 1. pH and moisture levels at various times after preparation and incubation of three inoculant carriers

Carrier	Inoculant maturity (wk)	pH (± SD)	% Moisture (± SD)
Peat	0	7.15 ± 0.07	50.7 ± 0.6
	6	6.41 ± 0.06	46.0 ± 2.5
	12	6.13 ± 0.06	41.8 ± 1.8
	15	6.04 ± 0.01	37.9 ± 1.1
Charcoal	0	7.43 ± 0.00	50.8 ± 0.8
	6	7.21 ± 0.03	46.8 ± 2.5
	12	7.01 ± 0.11	39.6 ± 1.3
	15	7.00 ± 0.04	35.3 ± 1.1
Vermiculite	0	7.68 ± 0.00	50.3 ± 0.9
	6	7.16 ± 0.06	47.2 ± 2.0
	12	7.11 ± 0.03	41.1 ± 0.7
	15	7.08 ± 0.08	38.7 ± 1.1

ity was kept high in the chamber by having a free water surface and wet paper towels to increase the air-water interface.

Three experiments were conducted. Each experiment consisted of using a different inoculation carrier in a completely randomized design with two replications. Treatments were subclover and arrowleaf clover seeds each inoculated with two strains of rhizobia in inoculants cured for 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 15 weeks. The parameter measured was the population of rhizobia surviving on seed immediately after inoculation and 1, 2, 3, and 4 days after inoculation. Populations were transformed to a logarithmic basis before statistical analysis. The data were analyzed using the general linear model procedure of the statistical analysis system (7). Linear regression lines were fitted to the data for each treatment to determine the mortality rate of rhizobia. The rates were the slopes of the regression lines. For comparison of treatment main effects (i.e., strain of rhizobia, seed type, and curing time of the carrier), the slopes of the regression lines were utilized in analysis of variance. Significant differences for main effects and the presence of interactions between main effects were determined by the *F*-test.

Figure 1 illustrates that the populations of *R. trifolii* in solid-based carriers reached maximum densities by the fourth week of incubation. At this time, the population exceeded 10^9 organisms per g of inoculant for all carriers. Thereafter, the rhizobial populations declined, but still remained reasonably high by the end of the 15-week incubation. A gradual decline in the pH and moisture content of the carriers occurred during the incubation period (Table 1).

The rhizobial populations on seed declined linearly with time regardless of carrier material (Fig. 2). The correlation coefficients indicated that approximately 96% of the decreases in populations were due to elapsed time after inoculation of seed. Thus, the rate of rhizobial population decline (slope of the regression lines) served as a good indicator of the treatment response. Analysis of variance on the slopes of the regression lines obtained for treatments with each inoc-

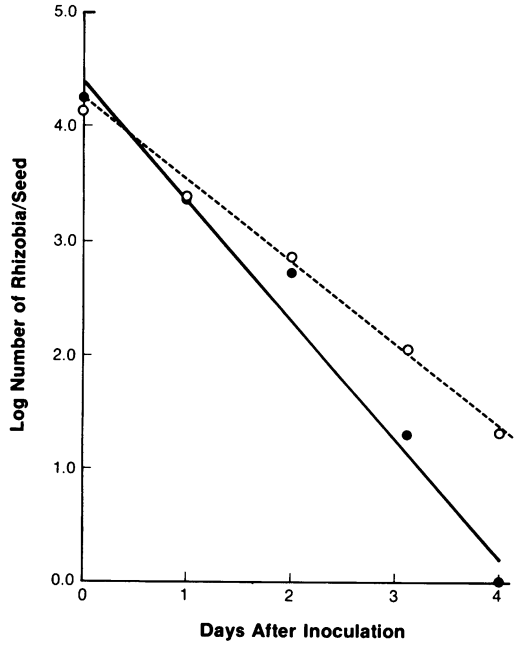

FIG. 2. Survival of *R. trifolii* on clover seeds as affected by maturity of peat-base inoculants. Symbols: ●, age 0 week ($y = 4.392 - 0.044x$; $r^2 = 0.96$); ○, age 6 weeks ($y = 4.124 - 0.029x$; $r^2 = 0.96$).

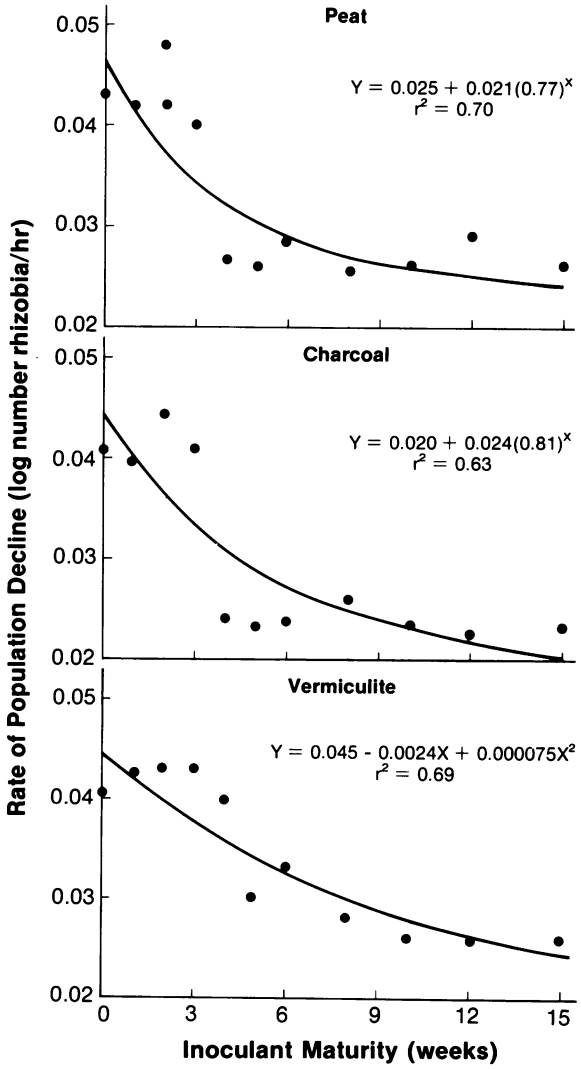

FIG. 3. Curvilinear relationships between the rate of rhizobial population decline on clover seed with the maturity of solid-based inoculants.

ulant carrier indicated that the rate of population decline was not significantly (0.05 level of P) affected by seed type or strain of rhizobia. But the time of curing the inoculant before seed inoculation significantly (0.01 level of P) influenced the survival of rhizobia on seed. Interactions between various factors were not significant, which facilitated evaluating the effect of curing time on rhizobial survival.

Increasing the time of curing reduced the rate of rhizobial population decline on seed in a curvilinear manner (Fig. 3). The data in Fig. 3 indicates that for peat and charcoal carriers increasing the curing time beyond 4 weeks had little effect on survival of rhizobia on seed. However for the vermiculite carrier increasing the curing time an additional 4 weeks tended to increase rhizobial survival. The net result of the curing process on survival of rhizobia is best demonstrated by inspection of Fig. 2. The slope (−0.044) of the equation for uncured inoculant and that of the cured inoculant (−0.029) resulted in a 10-fold difference in the sizes of populations remaining 4 days after inoculation.

A limitation of our research is that the carriers were sterilized and aseptically inoculated with rhizobia. Commercial products in the United States utilize carriers that have not been sterilized. Determining the population of rhizobia under such circumstances is very difficult. We have no reason to expect that the effect of curing time on rhizobia would be different for nonsterile carriers.

Most solid-based inoculants prepared for commercial consumption are usually exposed to a curing period for maximum rhizobial numbers before they reach the farmer. It is apparent from these results that the practice is also important for increasing survival of rhizobia on seed and should be continued. It is particularly important that those conducting research on survival of rhizobia on seed be aware that the curing of inoculants influences the survival of rhizobia. Often it is tempting to prepare and use inoculants on the same day, but if the research results are to be extrapolated to those obtained with commercial products a curing period should be provided before the inoculants are utilized.

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