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EFFECTIVENESS STABILITY OF COWPEA RHIZOBIA
AS AFFECTED BY SOIL TEMPERATURE AND MOISTURE

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ABSTRACT

Inoculation of a soil with rhizobia puts them under various environmental stresses that leads to selection of subpopulations that may not be as effective as the original population. The hypothesis was tested by incubating different strains of rhizobia in a sandy pH 5.7 soil obtained from Thailand. Three strains of cowpea rhizobia were utilized; two nodulated peanuts, Tha-201 and T-1; and one nodulated cowpea, TAL-309. The rhizobia were incubated under dry and moist (-0.33 bar) soil conditions at 40°C in sterilized soil and at 45°C in non-sterilized soil. Eighty single colony isolates of each strain were obtained from nodule isolations of cowpea and peanut which were inoculated with soil samples immediately following soil inoculation and 15 days later for non-sterilized soil. Single colony isolates were also obtained from pour plates of soil dilutions of sterilized soil immediately following inoculation and again after 45 days of incubation. Results indicated that there were changes in effectiveness of some strains due to treatments. Relatively ineffective isolates (<60% of plant dry matter produced as compared to parent culture) were found in relatively high frequency (30% of isolates) in the population of nodule isolates of strain TAL-309. The variation was not as great when strain TAL-309 isolates were obtained directly from soil. Strains Tha-201 and T-1 were comparatively stable in effectiveness. Selection of strains that have stable effectiveness characteristics is important for inoculant production.

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INTRODUCTION

Spontaneous loss of the symbiotic characteristics infectivity and effectiveness of rhizobia have been reported (Roughley, 1976, Schwinghamer, 1964, and Labandera and Vincent, 1975). Frequency of mutation in a single gene, in bacteria is about 10^{-7} and 10^{-8} per cell per generation (Stent and Calendar, 1978). There are a number of genes involved in effectiveness in nitrogen fixation in Rhizobium, including 17 for nitrogenase (Brill, 1980), a hydrogen uptake system (Schubert and Evans, 1976), and symbiosis between legume and rhizobia (Caldwell and Vest, 1977, and Vincent, 1980). Chance for loss of effectiveness by spontaneous mutation is considerable since many genes are involved.

There is also evidence that genes required for nodulation and nitrogen fixation are plasmid-borne in rhizobia (Nutti et al., 1979, Brewin et al., 1980, Buchanan-Wollaston et al., 1980, and Casadesus et al., 1980). Therefore, loss of plasmids from effective rhizobia may also result in ineffective or non-nodulating mutants as was demonstrated by Casadesus et al., (1980).

Variation in symbiotic effectiveness of cowpea rhizobia has been reported by Herridge and Roughley (1975). They found that 17 sub-strains of CB756 varied in symbiotic effectiveness; 9 were fully effective, 5 were moderately effective, 2 were relatively ineffective and 1 was completely ineffective.

The majority of rhizobial isolates obtained from nodules on peanuts in peanut growing areas were either only moderately effective or relatively ineffective (Weaver, 1974). Similar results were obtained with isolates from nodules of clovers (Bergersen, 1970, Bergersen et al., 1971, and Brockwell et al., 1968). Holding and King (1963) and Nutman (1946) reported that soil factors have an influence on variation in effectiveness of rhizobia.

From an economic standpoint ineffective variants in a field environment compete with effective rhizobia for formation of nodules on legumes and decrease the yield potential. The frequency of effective rhizobial strains mutating to become ineffective under stress in the

soil environment has not been adequately examined. The purpose of the experiment reported here was to determine the relative symbiotic stability of various strains of rhizobia to soil temperature and moisture.

MATERIALS AND METHODS

Symbiotic stability of cowpea rhizobia in sterilized and non-sterilized soil conditions was investigated. The soil was a Korat sandy loam (Ustoxic Dystopepts) from Thailand having a pH of 5.7. Three strains of cowpea rhizobia were selected. Two strains were isolated from peanut, Tha-201 (Thailand) and T-1 (Texas A&M University), and were effective on cowpea and peanut. The third strain, TAL-309 was obtained from the NIFTAL culture collection, Paia, Hawaii but the original culture designation is CB756 and belonged to the Sydney University collection, New South Wales, Australia and is effective on cowpea. Two moisture regimes, air dry and moist (-0.33 bar) and two temperatures, 40°C for sterilized soil and 45°C for non-sterilized soil, were used for incubations. All treatments were replicated twice. Soil was sterilized by autoclaving 10 g samples in test tubes for 1 hour on each of 2 successive days.

From the stocks of each strain six working cultures were prepared on yeast extract mannitol agar slants (Vincent, 1970). Slants were stored in a refrigerator at 4°C after approximately 7 days of incubation at room temperature. The working cultures were used as standards over a period of 1 year without additional transfers.

Inoculum for soil was prepared by transferring a loopful of culture from a working culture to 20 ml yeast extract mannitol broth (Vincent, 1970). The broth cultures were cultured in an incubator shaker at 29°C for 7 days at which time the cultures were in the late log phase of growth. The cell densities of strain Tha-201 and TAL-309 used for inoculating non-sterilized soil were respectively, 5.6×10^8 and 7.0×10^8 cells ml⁻¹. The cell densities of strains Tha-201, TAL-309, and T-1 used for inoculating sterilized soil were respectively, 5.7×10^8 , 5.5×10^8 and 6.7×10^8 cells ml⁻¹. Soil was inoculated by adding 0.5 ml of broth culture to 10 g samples of soil contained in 15 x 100 mm test tubes with plastic caps. The appropriate quantity of sterile dis-

tilled water was added to adjust the soil to -0.33 bar moisture. For the dry treatment, caps were removed from the tubes and the soil was air dried in a laminar flow hood at room temperature for 2 hours before replacing caps and placing the tubes in an incubator. Tubes containing moist soil were kept in a moist chamber to reduce moisture loss. Non-sterilized soil was incubated at 45°C for 15 days but sterilized soil was incubated at 40°C for 45 days.

Two replicates of non-sterilized soil were used for isolation of rhizobia immediately after inoculation and again 15 days after inoculation. The entire 10 g samples were added to 90 ml of sterile distilled water contained in screw-cap bottles and mechanically shaken for 5 minutes. Peanut (Arachis hypogaea L.) seeds were surfaced sterilized with a 10% solution of commercial household bleach for 5 minutes and rinsed several times with tap water (60°C) to remove all traces of the bleach. Four sterilized seeds were sown in modified Leonard jars containing vermiculite. The seeds were inoculated with a soil suspension of Tha-201 by dribbling the entire sample onto the seeds. After inoculation seeds were covered with vermiculite. Seedlings were thinned to two plants per pot. For TAL-309, the same procedure was employed except that cowpea (Vigna unguiculata (L.) Walp) was the host plant. Uninoculated soil was used for inoculation of peanut and cowpea in some pots to provide a check for indigenous rhizobia.

Plants used for isolating rhizobia were supplied with 1/4 strength plant nutrient solution (Munns, 1968) except for Mg which was used at 1/2 strength and were placed in a light room providing a photon flux density of 650 $\mu\text{E m}^{-2}\text{s}^{-1}$ at the top of the plants. The temperature of the room was maintained at 29°C during a 14 hour light period and at 21°C during 10 hours of dark each day. At 5 weeks of plant growth all nodules were collected and more than 40 nodules from plants in each container were used for isolation of rhizobia. Nodules were surface sterilized by first rinsing in 95% ethanol and then soaking in 10% bleach for 3 to 4 minutes. The nodules were rinsed repeatedly in sterile water to remove residual bleach. Rhizobia were isolated by piercing a nodule with a flamed, 24 gauge, needle and streaking on YMA (yeast extract mannitol agar) containing congo red. After 7 to 10 days colonies characteristic of rhizobia were transferred to agar slants.

A total of 40 isolates were obtained from each of the two replicates. After culturing on slants for approximately 1 week the cultures were stored in a refrigerator at 4°C.

Immediately following inoculation of sterilized soil and 45 days after inoculation YMA spread plates were utilized to obtain single colony isolates. After incubation of plates for 10 days, 30 colonies of each strain were transferred to slants. Slants were stored in a refrigerator at 4°C after culturing. Isolates obtained from nodules and sterilized soil were confirmed as the isolates of the inoculum strains by using the fluorescent antibody technique (Schmidt *et al.*, 1968).

For effectiveness assays a loopful of rhizobia was aseptically transferred from YMA slants to 6 ml of YMB (yeast extract mannitol broth) in 15 x 100 mm culture tubes having plastic closures. Tubes were placed in test tube racks and were shaken on an incubator shaker at 29°C for 5 days. The populations of rhizobia in the broth after 5 days ranged between approximately 1×10^7 to 10^8 cells ml⁻¹. Six siratro (*Macroptilium atropurpureum* (DC.) Urp) seedlings in separate compartments of plastic pouches were inoculated singly with each isolate at a rate of 1 ml broth per plant. The plants were grown in the same light room as those used for nodule isolations. Because of labor and space restrictions all treatments could not be tested at the same time. To compensate for this procedure 12 to 24 plants inoculated with a working culture were included in each test so that plant growth conditions over time could be compared.

The quantity of dry matter produced in plants top after 5 weeks of growth was used to group plants into 12 categories. Categories were in 10 mg increments beginning with 20-30 mg. Plant dry weights of parent cultures and uninoculated controls were not included in the analysis of variance. The variances of subpopulations within each date of sampling were analyzed using the SAS (Statistical Analysis System) computer program. Homogeneity of variance between treatments was tested by the F-max ratio method which is the ratio of the largest to the smallest treatment variances (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Stability of effectiveness of cowpea rhizobia in non-sterilized soil was investigated using plant dry weight as the effectiveness characteristic. Since N free plant nutrient solution was utilized the quantity of N fixed would be expected to be highly correlated with plant dry weight (Erdman and Means, 1952, Wynne *et al.*, 1980, and Haydock *et al.*, 1980). Plants used for effectiveness assays of rhizobial isolates from different treatments were not grown at the same time but in the same controlled environment. Inoculation of some plants, in each assay, with parent stock cultures provided controls for comparing growth conditions between treatments. Growth conditions for treatments using non-sterilized soil were not significantly different (Table 1).

Incubation of TAL-309 in non-sterilized soil for 15 days greatly increased the variability of effectiveness in the surviving population (Fig. 1). Square root or logarithmic transformations of data did not result in homogeneity of variance. The mean effectiveness of the populations following incubation were less effective than the parent culture and the mean effectiveness of the 0 day population (Fig. 1). Approximately 37% of the isolates from samples incubated for 15 days produced plants with dry weights less than 60% of the parent cultures whereas none of the isolates from the 0 day treatment were so ineffective. Of the isolates from the moist soil treatment 8% were classified as ineffective (Fig. 1) since the plants were yellowish in color and dry weights were similar to uninoculated plants.

Variances for treatments of strain Tha-201 in non-sterilized soil were not significantly different (Fig. 1). Incubation of this strain in non-sterilized soil did not increase the variability of effectiveness in the population (Fig. 1).

The populations of strains TAL-309 and Tha-201 incubated in dry and moist soil for 15 days at 45°C were decreased by approximately 1000 times indicating pressure against survival was applied. The increased variation in effectiveness of the TAL-309 population indicates that selective pressures were also acting on the effectiveness trait.

Confounding, in the first experiment, due to the possibility of plant selectivity during nodulation occurred since isolates were obtained from nodulated roots and not directly from soil. In a second experiment sterilized soil was used so that rhizobia could be isolated directly from soil by plating. Because of reduced labor requirements an additional strain T-1 was included. The incubation time was increased to 45 days because reduced stress from removal of biotic factors and lowering incubation temperature from 45°C to 40°C increased the time needed for selective pressures on survival (Boonkerd and Weaver, 1982). Populations of strains in moist soil did not decrease in 45 days but in the dry soil the populations decreased by a factor of 10, 1000 and 100 for strains Tha-201, T-1 and TAL-309 respectively (Boonkerd and Weaver, 1982).

Plant growth conditions were not consistently the same during the effectiveness assays of isolates obtained from the soil (Table 1) and must be considered when making treatment comparisons. The mean effectiveness of the populations immediately following inoculation and after incubation for 45 days in moist soil were similar to their respective controls (Fig. 2). The variances of populations for these two treatments were only different for TAL-309 in which case the variance was reduced.

Exposure to dry soil conditions for the 45 day period resulted in treatment effects on all strains (Fig. 2). Variance for strain Tha-201 was increased but the population mean was similar to the parent culture mean. Variances for the other two strains were similar to the respective preincubation treatments. The mean effectiveness of the populations was probably significantly lower than the respective parent cultures considering the magnitude of the differences and the low standard errors of the sample means (± 2) and the standard errors of the parent culture means (Table 1). For TAL-309 the dry treatment resulted in a statistically significant (.05 level) reduced mean effectiveness as compared to the 0 day treatment.

Inspection of the data in Figures 1 and 2 of day 0 treatments of both experiments reveals that the shape of the frequency distributions for effectiveness of respective strains were very similar. Apparently

there was no detectable difference due to sampling of the soil population for effectiveness by inoculating plants and isolating rhizobia from the nodules or by isolating rhizobia directly from soil by plating.

The results of this research clearly indicate the importance of developing further understanding on the ecology of rhizobia in soil to include stability of symbiotic character for nitrogen fixation. An incubation temperature increase from 40°C to 45°C resulted in a change of 5% to 26% of the isolates of TAL-309 becoming only 60% as effective as the parent culture. Other strains were more stable but some isolates of low effectiveness (less than 60% as effective as the parent culture) were obtained for all strains. Additional research should be directed towards determining the relative abilities of the ineffective isolates to survive in soil. Also it would be beneficial to determine if the relative instability of TAL-309 is due to plasmids that control effectiveness. Certainly it is important for inoculant producers to utilize strains of rhizobia known to have symbiotic characteristics that will be stable in the environments where the inoculum will be utilized.

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Table 1. Effectiveness of the parent cultures used as controls for each treatment for the purpose of determining similarity of growth conditions between assays.

Treatment	Strain			
	TAL-309	Tha-201	T-1	
-----dry weight mg plant ⁻¹ -----				
Soil				
nonsterile	control	110 (+3.4) [†]	114 (+6.2)	
	dry	111 (+4.3)	100 (+6.3)	
	moist	118 (+5.1)	112 (+7.2)	
		N.S.	N.S.	
Soil				
sterilized	control	114 (+11.6) a [*]	118 (+6.9) a	98 (+3.3) b
	dry	116 (+ 5.1) a	110 (+5.1) a	113 (+4.8) a
	moist	92 (+ 4.7) b	82 (+3.2) b	86 (+3.8) b

[†]Number in parenthesis is the standard error of the mean. For nonsterile soil treatments averages are of 21 to 24 plants and for sterilized soil treatments averages are of 11 plants.

^{*}Averages within strains for nonsterile soil were not significantly different at 0.05 probability level by the F. test. For sterilized soil treatments averages having a letter in common are not significantly different by Duncan's new multiple range test.

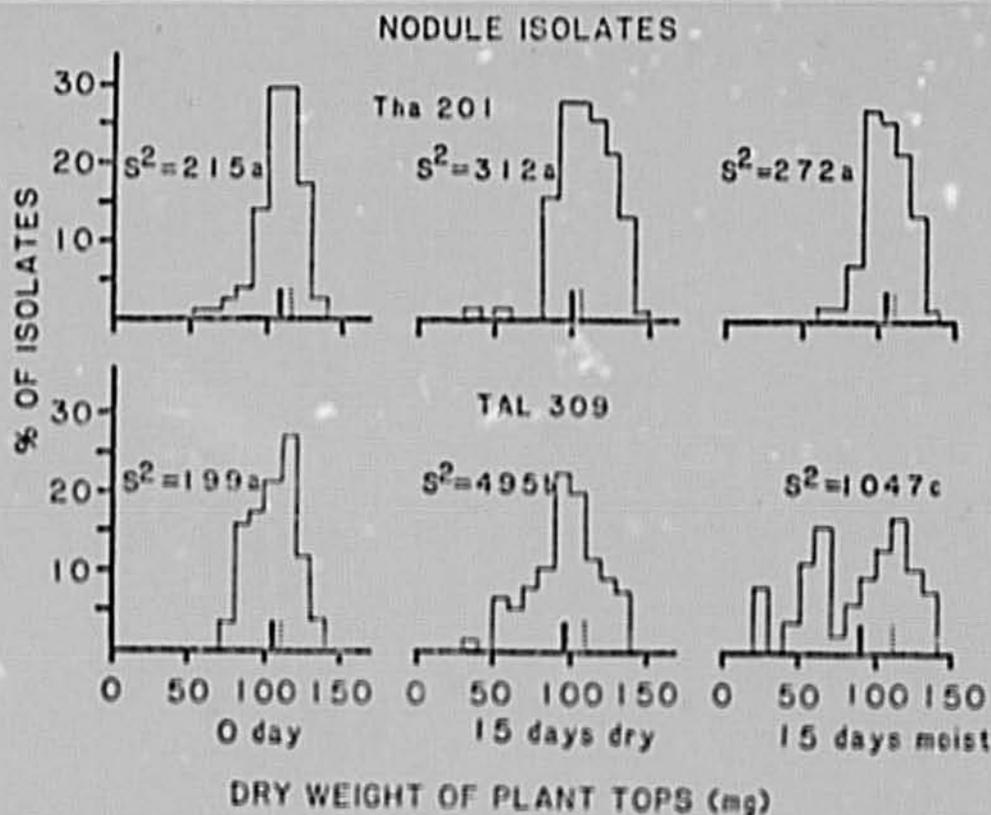


Figure 1. Effectiveness of rhizobial isolates obtained from nodules of plants inoculated with nonsterile soil immediately following inoculation (0 day) with two strains of rhizobia and after 15 days incubation under dry or moist conditions. Each figure is based on 80 nodule isolates. The variance (s^2) of each population is indicated. The variances for treatments of individual strains are not significantly different ($\alpha = 0.05$) if they have a letter in common. The bars on the abscissa indicate the relative effectiveness of the parent culture (broken) and the mean of the 60 isolates (solid).

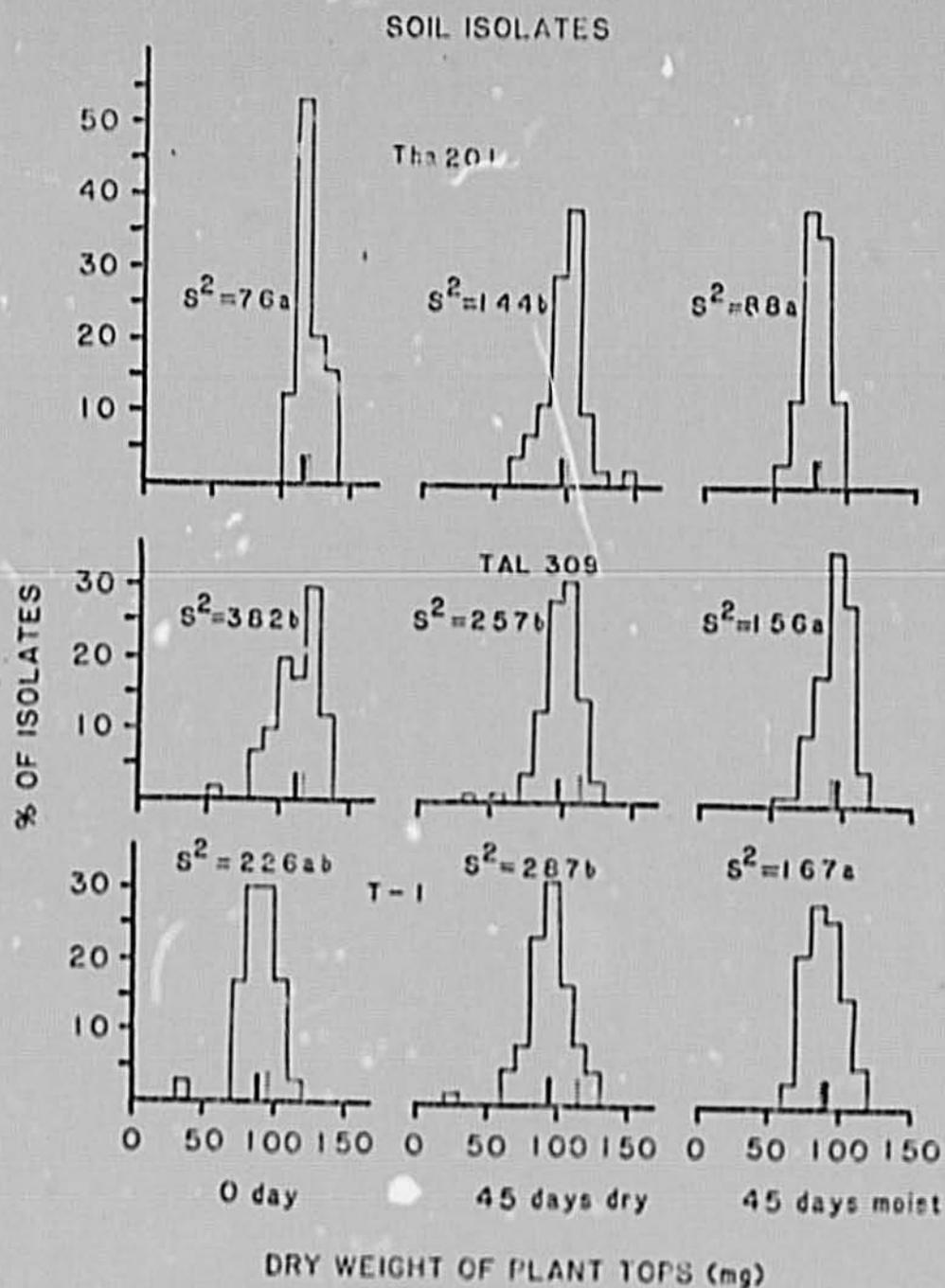


Figure 2. Effectiveness of rhizobial isolates obtained from sterilized soil immediately following inoculation (0 day) with three strains of rhizobia and after 45 days incubation under dry or moist conditions. Each figure is based on 60 isolates obtained by plating soil on yeast extract mannitol agar. See caption to Figure 1 for additional information.

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