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Comparison of Hup Trait and Intrinsic Antibiotic Resistance for Assessing Rhizobial Competitiveness Axenically and in Soil

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The competitiveness of dual-strain inocula of cowpea rhizobia for nodulation of Vigna unguiculata (L.) Walp. was studied axenically between one slow-growing strain (P132, HP147, 401, or 22A1) and one fast-growing strain (176A26 or 176A28) at logarithmic inoculum ratios ranging from 10^2 to 10^{-2}. Nodule infectivity was determined by multiple intrinsic antibiotic resistance, since both fast-growing strains were sensitive. Different hydrogen uptake (Hup) efficiencies of dual-strain inocula allowed for the comparison of an indirect rapid method. Infectivity data based on antibiotic resistance and Hup efficiency were fit to linearized Ivanoff plots of log-normal distributions to determine C_{50} (percent infectivity at a 1:1 inoculum density) or I_{50} (inoculum ratio at 50% infectivity). The slow growers were always better competitors and had I_{50} values which ranged from 7 to 160,000 and C_{50} values which ranged from 62 to 97%. P132 was the best competitor of all those tested. Antibiotic resistance and Hup efficiency methods were in agreement with 401 (Hup^+) and 176A26 (Hup^+), but the Hup efficiency method overestimated the I_{50} index with 22A1 (Hup^-) and 176A28 (Hup^+). The competition of each of the four slow-growing strains with indigenous rhizobia was examined in *Cajanus cajan* from three tropical soils. Nodule infectivity for all strains ranged from 42 to 94%, and P132 was the best competitor in all the soils. Hup efficiency overestimated infectivity by about 2-fold when Hup^+ inocula (P132 and HP147) were used but underestimated infectivity by more than 100-fold when Hup^- inocula (401 and 22A1) were used. Although the Hup trait has limited quantitative usage axenically, it is only qualitative in soil competition studies and can only be used with Hup^+ inocula.

The methods most commonly used for the identification of inoculum strains from nodules are immunofluorescence (3), immunodiffusion (4, 22), and antibiotic resistance (1, 9, 10, 16). These methods are all very time-consuming. A more rapid and less tedious procedure for assessing nodule infectivity between competing strains would be to measure specific differences in nodule metabolism. One such metabolic function that is directly pertinent to nitrogenase activity is the hydrogen uptake (Hup) trait (17). La Favre and Focht (11) showed that differences in Hup activity between inoculum strains and indigenous soil rhizobia were consistent with infectivity determinations from nodule typing by antibiotic resistance. Yet, to our knowledge, no attempt has been made to determine if the Hup trait can be used to assess nodule infectivity.

There has been considerable interest in fast-growing cowpea rhizobia because they are easy to work with and are suitable for genetic manipulation and large-scale production of inoculants. Consequently, we chose to evaluate the competitiveness between fast and slow growers as well as between Hup^+ and Hup^- rhizobia. Nicol and Thornton (15) stated that a fast-growing strain would be the better competitor for nodulation, but Franco and Vincent (5) found just the opposite. Trinick et al. (19) found that the fast-growing rhizobium strain NGR-234 was a better competitor against slow-growing rhizobia at 23 or 27°C but found that the opposite was true at the more realistic tropical temperature of 26 to 30°C.

We therefore undertook this study to determine if the Hup trait can be used as a marker for the identification of competing strains in both axenic and nonaxenic studies and to examine the competitive nodulation abilities of fast- and slow-growing rhizobia.

MATERIALS AND METHODS

Rhizobium strains. Strain 22A1, a Hup^- slow grower (doubling time [t_d], 13 h) was obtained from J. C. Burton of Nitragin Co., Milwaukee, Wis., and was originally isolated from *Vigna unguiculata* (L.) Walp. The sources and physiological characteristics of strains 176A26 (t_d, 1.7 h), 176A28 (t_d, 4.6 h), 401 (t_d, 11 h), P132 (t_d, 16 h), and HP147 (t_d, 30 h) have been described previously (3).

Media and inoculants. The maintenance medium for all the strains was yeast extract-mannitol agar. It was used as a solid medium either on petri plates or in tubes as slants to maintain the strains. Glutamate-yeast extract was used as a solution medium to grow cells for inocula in axenic studies, and yeast extract-mannitol was used for the preparation of inoculants for soil studies. Both media have the following mineral salts composition per liter of water: 1.0 g of KH_2PO_4, 1.0 g of K_2HPO_4, 0.2 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of yeast extract, 0.5 mg of $FeSO_4$, and 1.0 mg of $CaCl_2$. In addition, glutamate-yeast extract contains 3.6 g of glutamate (monosodium salt), and yeast extract-mannitol agar contains 1.8 g of KNO_3, 10.0 g of mannitol, and 15.0 g of agar. The pH was adjusted to 7.0 with NaOH before autoclaving at 121°C and 15 lb/in² for 15 min.

Inocula were prepared by growing rhizobia in shaken flasks (250 ml containing 25 ml of glutamate-yeast extract or yeast extract-mannitol medium. Flasks were placed on rotary shakers (100 rpm) and incubated at 28°C until an optical density (at 525 nm) of 0.70 was obtained.

Axenic plant cultures. Cowpeas (*V. unguiculata* (L.)

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Walp., var. California no. 5 blackeyed) were grown in culture tubes (38 by 200 mm). The tubes were filled to 2 cm with gravel and then fine vermiculite (washed twice with deionized water), and a watering tube (8 mm in diameter) was inserted into the culture tube on top of the gravel to facilitate the addition of N-free nutrient solution and water (21). The tubes were saturated with N-free nutrient solution, covered with aluminum foil, and autoclaved for 30 min at 121°C. Autoclaved gravel (1 cm) was added to the top of the tube to minimize contamination and reduce evaporation.

Cowpea seeds were surface sterilized in 0.1% $HgCl_2$ for 4 min and then rinsed in 10 changes of sterile distilled water before they were planted aseptically in the culture tubes, one seed per tube. Extra tubes were planted to ensure that a sufficient number of healthy plants would be available prior to the start of the experiment. The aluminum cover was returned on top of the tube until plant emergence. At that time, each plant was inoculated with a 2-ml mixture of glutamate-yeast extract broth cultures containing 1 ml of each of two competing strains in the following inoculum ratios: 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, and 10^{-3}. Gravel (~1 cm) added after inoculation, and sterilized nutrient solution and water were added alternately to the culture tubes through the watering tube as needed. Four replicate tubes for each inoculum strain and four uninoculated controls were used. The tubes were glazed in a randomized block design on a rack which was covered on all sides with wood boards to protect the roots from light. The temperature in the greenhouse was maintained at between 21 and 27°C. A mercury lamp was installed in the greenhouse to add supplemental lighting, since the experiment was performed during the winter months. Plants were harvested 6 weeks after planting for the determination of acetylene reduction rates, H_2 evolution rates, dry mass, and nodule occupancy.

Soil plant cultures. Pigeon pea (*Cajanus cajan* (L.) Millsp., var. 64–2d) seeds were inoculated separately with P132 (Hup$^+$), HP147 (Hup$^-$), 401 (Hup$^-$) and 22A1 (Hup$^-$) and placed in pots containing 3.5 kg of soil. The method of inoculation and the Bayano soil used in this study have been described previously (7a). The other two tropical soils used in this study were a fine, mixed, hyperthermic Typic Hapludult (Tocumen) of pH 5.4 and a fine, mixed, hyperthermic Oxtic Haplorthox (Los Lotes) of pH 4.2. All pots, including the uninoculated control, were replicated four times and treated with PO_4^{-3}-P (75 kg · ha^{-1}), Ca^{+2} (989 kg · ha^{-1}), and MoO_4^{-2} (3.2 kg · ha^{-1}) to alleviate mineral deficiencies and acidity. Plants were grown in greenhouses at the University of Panama for 60 days, after which determinations of dry mass, H_2 production, acetylene reduction activity, and nodule occupancy were made.

Acetylene reduction and hydrogenase assays. Hup efficiency in axenic and soil systems was determined with the formula of Schubert et al. (18), whereby percent relative efficiency = [1 − (H_2 evolved/C_2H_2 reduced)] × 100. Plant shoots were cut off and roots were removed from tubes. Vermiculite or soil was gently washed from the roots, and the entire root system of each plant was placed in a widemouthed Erlenmyer flask (250 ml) containing a moistened filter paper. Each flask was closed with a rubber stopper containing a glass tube and screw cap for gas sampling purposes. Each flask was incubated for 1 h and subsequently sampled for H_2 by withdrawal of a 10-ml sample, which was then stored in a VACUTAINER (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) bottle for not more than 3 days prior to analysis by gas chromatography. Several preliminary experiments with known amounts of H_2 indicated no loss when the samples were stored under these conditions. A 25-ml quantity of air was removed from each flask and replaced with a 25-ml injection of acetylene. Each flask was incubated for 25 min, whereupon a sample was removed and analyzed by gas chromatography for ethylene. Gas chromatographic analyses for H_2 and ethylene have been described elsewhere (7a, 11).

The fractional efficiency of each of the competing strains was determined with the following equation: $f_B = (C - A)/(B - A)$, where f_B = the fraction of activity due to strain B, B = the efficiency of strain B, A = the efficiency of strain A, and C = the observed efficiency of the mixture.

Nodule typing from axenic plant cultures. Roots were washed free of vermiculite, and 15 nodules were randomly selected from each replicate tube and pooled together to yield 60 nodules per determination. The nodules were surface sterilized by immersion in 95% ethanol for 30 s and then treated with 0.2% $HgCl_2$ (acidified with a 5-ml · liter^{-1} HCl solution) for 4 min. The nodules were then rinsed in five changes of sterile distilled water. Further handling of the nodules was done under aseptic conditions. Each nodule was cut in half with a razor blade that was sterilized prior to each cutting by immersion in ethanol followed by flaming. Each half was pressed onto an agar plate (yeast extract-mannitol agar) containing the following concentrations of antibiotics to which the four slow-growing strains were resistant: rifampin (60 μg · ml^{-1}) and streptomycin (200 U · ml^{-1}); HP147: kanamycin (50 μg · ml^{-1}) and tetracycline (60 μg · ml^{-1}); P132: and penicillin (200 U · ml^{-1}) and streptomycin (200 U · min^{-1}); 401 and 22A1. The plates were incubated at 28°C, and results were recorded after 5 to 7 days of incubation. All media contained 20 μg of cycloheximide^{-1} (Calbiochem-Behring, La Jolla, Calif.) to inhibit the growth of fungal contaminants.

Nodule typing from soil plant cultures. Preliminary studies were conducted to determine the concentrations of three antibiotics which permitted the growth of the four inoculum strains used for assessing competitiveness against indigenous rhizobia. P132, HP147, 401, and 22A1 were resistant to the following combined concentrations in yeast extract-mannitol agar: nalidixic acid (800 μg · ml^{-1}), erythromycin (50 μg · ml^{-1}), and rifampin (100 μg · ml^{-1}). 176A26 and 176A28 were not used in this study because they were sensitive to the levels of antibiotics needed to suppress the growth of indigenous rhizobia. The preparation and methodology for nodule typing were identical to those described above except that 10 nodules were randomly selected from each replicate pot and pooled together to yield 40 nodules per determination.

RESULTS AND DISCUSSION

There were no significant differences in shoot dry matter between axenic and monoxenic inoculated cultures. The greatest differences in dry matter occurred in soils, in which the following yields (grams · plant^{-1}) and ranges were observed: Los Lotes (2.58 to 2.72), Tocumen (3.87 to 4.29), and Bayano (8.48 to 8.80). Only with the axenic treatments were the yields greater in inoculated (1.13 ± 0.15 g · plant^{-1}) than in uninoculated (0.73 ± 0.081 g · plant^{-1}) cultures.

If a 1:1 mixture of two different rhizobial strains produced equal infectivity, the ratio of population density to infectivity would be arithmetic and normally distributed. This is obviously not the case, as shown by others (1, 16, 19) who formulated equations which described competitiveness as a logarithmic function. Thus, populations which are log-normally distributed should be transformable to the fractile

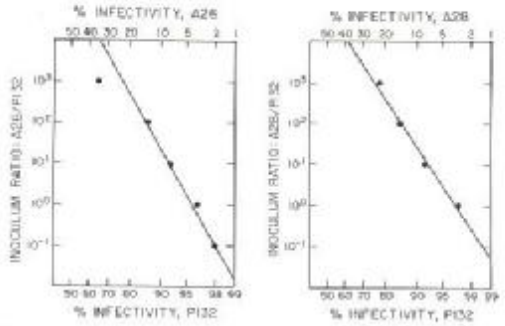

FIG. 1. Nodule infectivity as a function of dual-inoculum ratios of strain PI32 versus 176A26 and 176A28. Each point was derived from 60 samples.

plots shown in Fig. 1 to 4. The midpoint (50%) representing the ratio of the dual-inoculum density which produces equal infectivity of nodules is referred to as the I_{50} index. Others (1, 20) have used the C_{AB} index, which represents the frequency of infectivity of two different strains present in equal quantities. This index can be found from the fractile plots in Fig. 1 to 4 where the line intersects the 10^0 inoculum ratio. Further details regarding transformations of log-normally distributed populations to fractile plots can be found elsewhere (6).

Since the data generally conform to the linearized transformations shown in Fig. 1 to 4, the I_{50} index can be determined among highly competitive strains; such a determination would otherwise be empirically impossible. PI32 was clearly the best competitor against the two fast-growing strains. The I_{50} values of 1.6×10^5 and 6.3×10^4 against 176A26 and 176A28, respectively, were orders of magnitude greater than the I_{50} values attained by the other three slow-growing strains (HP147, 22A1, and 401) against the same fast-growing strains. In no instance were the fast growers the dominant competitors.

Although it has been inferred that growth rate might be an important criterion for competitiveness in nodulation (15), the results reported here do not support this concept. In all cases, the two fast-growing strains 176A26 and 176A28 required 5 and 1.6×10^5 more cells, respectively, per slow-growing competitor to effect an equal infectivity (I_{50}) of the plant. Thus, the observation by Trinick et al. (19) that the rate of growth on laboratory media did not always correlate with better competitive ability was consistent with our own.

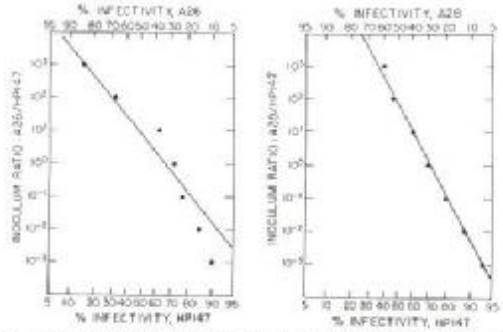

FIG. 2. Nodule infectivity as a function of dual-inoculum ratios of strain HP147 versus 176A26 and 176A28. Each point was derived from 60 samples.

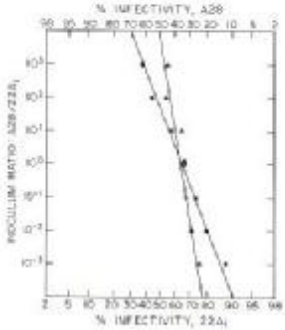

FIG. 3. Comparison of nodule infectivity with dual-inoculum ratios of strains 22A1 and 176A28 as determined by antibiotic resistance (●) and Hup efficiency (▲). Antibiotic resistance points were derived from 60 samples, and Hup efficiency points were derived from 3 samples.

Franco and Vincent (5), who used an agar test tube method, also found that slow growers were better competitors than fast growers.

The dominance of P132 as a competitor was also demonstrated in all three soils tested (Table 1). It occupied 68, 87, and 96% of all the nodules typed from the Bayano, Los Lotes, and Tocumen soils, respectively. The recoveries were numerically higher than those found with any of the other three slow-growing strains; in only two comparisons

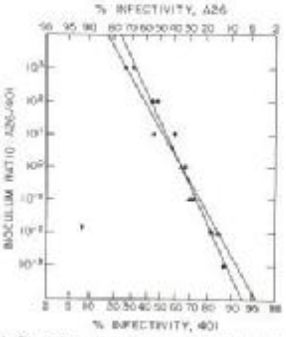

FIG. 4. Comparison of nodule infectivity with dual-inoculum ratios of strains 401 and 176A26 as determined by antibiotic resistance (●) and Hup efficiency (▲). Antibiotic resistance points were derived from 60 samples, and Hup efficiency points were derived from 3 samples.

TABLE 1. Frequency of inoculum strain infectivity in nodules of *C. vulgaris* as determined by selective multiple antibiotic resistance

Soil	% of nodules infected by inoculum strain[a]				
	None	P132	HP147	401	22A1
Bayano	0.02a	0.68d	0.42b	0.40b	0.54bc
Tocumen	0.02a	0.98f	0.89cf	0.73de	0.79de
Los Lotes	0.00a	0.87ef	0.66cdf	0.78de	0.72cd

[a] Numbers followed by the same letter are not significantly different at $\alpha = 0.05$ among columns and rows by multiple paired comparisons of normalized binomial distributions (14).

were these values statistically the same (Table 1, Tocumen HP147 and Los Lotes 401).

It is unclear whether inoculum density has any effect upon competition between strains. Since the number of viable cells will drastically be reduced as a function of time (24h.e., from inoculation to infection), the I_{50} will also be dependent upon the survival of inoculum strains. Bohlool and Schmidt (3) showed that the percentage of *R. japonicum* 110 nodules declined from 85 to 55 to 41% in 0 to 10 to 50 days, respectively, indicating that the initial population density was reduced by the time infection occurred. Thus, depending on differences in survival rates between two competing populations, the true I_{50} value at the time of infection may be higher or lower than the indices reported here. Ireland and Vincent (8) reported that both relative numbers and differences in competitive ability among strains are important in determining which rhizobium strain will form nodules on the legume host. Moreover, Weaver and Frederick (20) indicated that a high inoculum density may shorten the time of nodulation if the soil population is below a certain level, even though the total nodule weight formed on the plant later may not increase. On the other hand, Means et al. (13) reported that competition was often found to be independent of relative numbers of rhizobium strains, since strain 76, which induced chlorosis, was responsible for the formation of 85% of the nodules formed on soybeans even though it constituted as little as 1% of the population of the seed-applied inoculum.

The Hup trait was not as accurate as antibiotic resistance in determining nodule infectivity. Although there was good agreement between the axenic mixture of 401 and 176A26 (Table 2 and Fig. 4), the agreement between the axenic mixture of 22A1 and 176A28 was poor (Table 2 and Fig. 3). Coincidentally, the C_{A0} values (68 and 35%, respectively) were the same for both mixtures and both methods. Since the Hup trait is theoretically applicable only when the indigenous rhizobia have Hup efficiencies significantly different from those of the inoculum strains, only limited comparisons could be made between the two methods. With the Hup$^-$ strains in the Bayano soil, infectivities as deter-

TABLE 2. Infectivity index (I_{50}) in axenic plant culture tubes as determined from nodule typing by intrinsic antibiotic resistance

Inoculum strains	Infectivity index[a]
P132 and 176A26	160,000
P132 and 176A28	63,000
HP147 and 176A28	20
HP147 and 176A26	3
22A1 and 176A28	50 (7,000)
401 and 176A26	25 (16)

[a] Numbers in parentheses were determined by the Hup efficiency method.

TABLE 3. Percent relative Hup efficiency as determined from root nodules of *C. rajan* previously inoculated with rhizobia

System	% Hup efficiency for inoculum strain[a]				
	None	PRH	HP147	401	22A1
Axenic		100a	98a	55c	56c
Bayano soil	82b	98a	98a	77b	84b
Tocumen soil	96a	93a	98a	95a	95a
Los Lotes soil	97a	98a	97a	98a	99a

[a] Numbers followed by the same letter are not significantly different ($P \leq 0.05$) among columns and rows by Duncan's multiple range test.

mined by antibiotic resistance and Hup efficiency (Tables 1 and 3) were, respectively, 68 versus 89% (PH52) and 42 versus 100% (HP147). There was even less agreement between the two methods when the Hup$^-$ strains were considered. The respective infectivities as determined by antibiotic resistance and Hup efficiency were as follows: strain 401, 48 versus 23% (Bayano soil), 77 versus 2% (Tocumen soil), and 78 versus 0% (Los Lotes soil); strain 22A1, 54 versus 0% (Bayano soil), 79 versus 2% (Tocumen soil), and 77 versus 0% (Los Lotes soil).

In soil, the Hup method overestimated the competitiveness of Hup$^+$ inoculum strains and underestimated the competitiveness of Hup$^-$ inoculum strains. Although it is possible that the more active Hup$^+$ nodules are a sink for exogenous H_2 produced by the Hup$^-$ nodules of the host system, we were never able to detect Hup in flasks incubated with Hup$^+$ nodules. D. J. Arp (personal communication) has observed that only crushed nodules take up H_2. Moreover, axenic mixtures of Hup$^-$ and Hup$^+$ nodules should give consistently higher infectivity frequencies when determined by Hup efficiency than when determined by antibiotic resistance. No such relationship was noted between 22A1 and 176A28 or between 401 and 176A26. Let us consider that a third member of the H_2 metabolic community may be present on the surfaces of nonaxenic nodules but not axenic nodules. Since it is not possible to remove the rhizoplane microflora from the entire root system, net measurements of H_2 consumption would always be greater than measurements of intranodule H_2 consumption alone. Therefore, the infectivity of Hup$^+$ inocula would be overestimated, whereas the infectivity of Hup$^-$ inocula would be underestimated. It has in fact been well established that H_2-oxidizing bacteria colonize the nodule in its rhizosphere in soil and that their numbers and activity are directly proportional to an H_2 gradient and inversely proportional to the Hup efficiency of the rhizobial endophyte (12). In light of these observations, it should come as no surprise that the agreement between the Hup efficiency and antibiotic resistance methods in determining nodule infectivity was poorer when inocula were Hup$^-$ than when they were Hup$^+$.

Nodule typing by antibiotic resistance is very tedious and time-consuming and requires 2 to 3 h for typing 50 to 60 nodules from a single treatment of four plants. By comparison, Hup efficiency requires only 40 min of analysis by gas chromatography (10 min per plant with four replicates) for a combined assay of ethylene and H_2. Thus, the ease and rapidity with which results are obtained with the Hup method make this a potentially useful screening tool for competition studies of rhizobia. However, the method can only be used for soil competition studies when Hup$^+$ inocula are used to challenge indigenous rhizobia, which have significantly lower Hup efficiencies. Until the contribution of H_2 metabolism from other bacteria in the rhizoplane can be determined or eliminated during the assay, the Hup method can only provide circumstantial evidence of competitive success.

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