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Competition Among *Rhizobium leguminosarum* Strains for Nodulation of Lentils *(Lens esculenta)*

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Thirty-one cultures of *Rhizobium leguminosarum* were screened for effectiveness (C_2H_2 reduction) on lentils *(Lens esculenta)*. Fluorescent antibodies prepared against three of the most effective strains (Hawaii 5-0, Nitragin 92A3, and Nitragin 128A12) exhibited a high degree of strain specificity; the antibodies reacted strongly with their homologous rhizobia in culture and with bacteroids in nodules. They did not cross-react with one another, and only weakly with 5 of the 47 other *R. leguminosarum* cultures tested. In competition studies in the growth chamber, whenever strain Nitragin 92A3 was included in the inoculum mixture, it consistently (but not always significantly, P = 0.05) occupied the majority of nodules on all four cultivars used. However, some degree of strain X cultivar interaction was apparent: Hawaii 5-0 was of equal competitiveness (P = 0.05) with Nitragin 92A3 on three of the varieties (Commercial, Tekoa, and Benewah), but inferior (P = 0.01) on the Chilean variety; Nitragin 92A3 completely dominated (P = 0.01) Nitragin 128A12 on all cultivars; and Hawaii 5-0 was of equal competitiveness (P = 0.05) to Nitragin 128A12 on the Chilean variety and more competitive (P = 0.01) on the commercial variety and less so on the other two varieties. In field experiments, Hawaii 5-0 proved of equal competitiveness (P = 0.01) with Nitragin 92A3 in one soil (an Inceptisol) and superior (P _< 0.05) to it in another (an Oxisol). Incidence of double-strain occupancy of nodules varied from 0 to 36% in vermiculite, depending on the strains in the mixture and the host variety, and from 0 to 38% in the field, depending on the strains in the mixture and the soil type. The results suggest a close relationship between the competitiveness of a strain and its occurrence in doubly infected nodules.

An important objective in legume inoculation research is to select highly effective strains of rhizobia for a particular host plant. Such inoculant strains must also be able to establish themselves in the rhizosphere and compete successfully for nodule sites against the indigenous soil rhizobia, which often include ineffective strains. There have been many reports of differential competition between effective and ineffective *Rhizobium* strains (17, 27-29, 32, 33) as well as among effective strains.

The mechanisms that confer competitive advantage to a strain are not fully understood. Some of the factors that have been suggested are: faster growth in the rhizosphere of the host (27); preferential selection by the host (32, 40); tolerance of climate, pH, and other soil factors (28, 31); antagonism between competing strains (37); and the relative ratio of inoculant to resident strains (4).

The present study analyzes the competitive ability of effective strains of *Rhizobium leguminosarum* for nodulation of the roots of lentils *(Lens esculenta)*. Experiments were designed to assess competitiveness in the absence of biological and abiological soil factors in the growth chamber and under field conditions in two soils with different properties. The fluorescent antibody (FA) technique was used to identify the strains in the nodules.

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MATERIALS AND METHODS

The strains of *R. leguminosarum* used in the competition experiments, Hawaii 5-0, Nitragin 92A3 (previously NZP5400), and Nitragin 128A12, were selected on the basis of growth chamber screening, which showed all three to be highly effective on lentils. The origin of these strains has been described previously (1). The sources of other cultures used in this study are listed in Table 1. Cultures were grown and maintained in modified yeast extract-mannitol medium (3) and routinely transferred every 6 months.

The above cultures were screened for effectiveness on lentils. Seedlings, grown in sterilized vermiculite in test tubes (25 by 200 mm) and moistened with nitrogen-free nutrient solution (11), were inoculated in duplicate with 3-day-old cultures of each of the strains (see Table 1). Eight units were left uninoculated.

Plants were grown in an EGC model M-31 growth chamber with a 14-h day, and a day/night temperature of 29°C/24°C. After 4 weeks, each tube was stoppered directly and injected with 10% acetylene. Nitrogenase activity was measured according to the method of Hardy et al. (18).

Preparation of FAs and immunofluorescent (IF) staining of cultures and nodules are described elsewhere (36). Smears from cultures and nodules were treated with gelatin-rhodamine isothiocyanate conjugate (2) to control nonspecific staining and autofluorescence. Nodules from all experiments were stored dry at 55°C until use.

Stained nodule and culture smears were examined with a Zeiss universal microscope equipped for epi-fluorescence and transmitted dark field. Incident illumination was from an HBO-200 (OSRAM) light source with a fluoroscein isothiocyanate filterpack. Transmitted dark-field illumination was from a 12-V quartzhalogen lamp, using a Zeiss Ultracondenser.

For growth chamber experiments, commercial lentil seeds (Spokane Seed Co.) were surface sterilized and germinated aseptically in petri dishes containing 1% water agar. Seedlings were planted in modified Leonard jars (23) (three per jar) containing sterile vermiculite and a nitrogen-free nutrient solution (11). Three day-old shake cultures of Hawaii 5-0, Nitragin 92A3, and Nitragin 128A12 were used to prepare inocula. For single-strain inocula, cultures were diluted in sterile water to 10^6 rhizobia per ml. Equal volumes of these were mixed to prepare the appropriate multistrain mixtures. Each seedling received 1 ml of a particular inoculum. A randomized complete-block design was used with three replicates. Plants were grown as described above. After 4 weeks, the plants were harvested. The roots were washed in distilled water containing 0.1% Tween 80 and rinsed four times, and a random sample of at least 50% of the nodules (but not fewer than 20) from each plant was removed for FA staining. To determine strain X cultivar interactions, lentil cultivars (Benewah, Chilean, and Tekoa [courtesy of D. Bezdicek, Washington State University, Pullman, Wash.]) were grown as above, and their nodules were tested for strain occupancy after 4 weeks.

For the field experiments, the inoculum strains were grown separately in gamma-irradiated peat (31). Mixtures of different peat cultures were made to contain equal numbers of the desired strains. The peat preparations were coated onto commercial lentil seeds, using the procedure outlined by Vincent (39). Coated seeds were pelleted with calcium carbonate (8). Each seed harbored approximately 5 x 10^4 cells of the desired rhizobial mixture, as determined by viable count on yeast extract-mannitol agar and the IF quantitative membrane-filter technique (5, 35).

Pelleted seeds were planted at two sites: Mauka Field Station, the University of Hawaii, with an Andic Ustic Humitropept soil (Inceptisol), pH 6.1, containing a low number of indigenous ineffective rhizobia (less than 100/g, as determined by the most-probable number method [14]); and Poamoho Experiment Station, Hawaii Institute of Tropical Agriculture and Human Resources, with a tropeptic Eustrostox soil (Oxisol), pH 5.8, characterized by low phosphorus in soil solution (0.003 ppm P) and high phosphorus-sorption properties (16) and lack of R. *leguminosarum* (no nodules developed when the soil was used as an inoculum [1 g per seedling] on lentils).

In all field experiments, randomized complete-block designs with three replicates were used. Plants were harvested after 10 days, 5 weeks, and 8 weeks; there were 6 to 15 plants per replicate per treatment. Tops were removed, and the roots were tested for nitrogenase activity by the acetylene reduction assay (18). The roots were then washed in 0.1% Tween 80, and at least 50 nodules per replicate per treatment were picked at random for FA identification. To determine the persistence of the inoculum strains in the soil, uninoculated lentil seeds were planted at the same plots in the Mauka Field Station 1 year after the original competition experiment. After 10 weeks, ten plants per replicate per treatment were harvested, and their nodules were identified as described above.

TABLE 1. Effectiveness of *R. leguminosarum* strains on lentils (*L. esculenta*)

Source of strains or isolates[a]	Effectiveness groups[b]		
	Ineffective	Effective	Highly effective
Hawaii (5)	I9	I2 I7 I13	Hawaii 5-0
New Zealand (2)	NZP 5225	NZP 5262	
Australia (1)	TA 101		
Indonesia (2)	I20 I22		
Morocco (16)	F4 F8 F9 F10D F20D F24	I40 I41 I42 I43 I44 I45 I46 I47 F30	F15
Nitragin Co. (5)		175P1	92A3 128A12 128C53

[a] The strains from Hawaii and Indonesia were isolated (40) from nodules of lentils growing in soils from different sites in these areas; the New Zealand and Australian strains were courtesy of R. M. Greenwood, Division of Scientific and Industrial Research, New Zealand; those from Morocco were isolated on *Vicia faba*, courtesy of E. L. Schmidt, University of Minnesota, Minneapolis; and the Nitragin strains were from J. C. Burton, Nitragin Co. Number in parentheses indicates the number of cultures tested.

[b] Effectiveness groups: ineffective, no ethylene produced in 1 h; effective, 100 to 300 nmol of ethylene produced per plant per h; highly effective, >300 nmol of ethylene produced per plant per h.

RESULTS

The effectiveness (acetylene reduction) pattern of 31 strains and isolates of R. *legumino*

sarum on lentils (commercial variety) is shown in Table 1. Eleven of the cultures proved ineffective, five highly effective, and the rest intermediate. Three of the cultures from the highly effective group, Hawaii 5-0, Nitragin 92A3, and Nitragin 128A12, were chosen for further field testing and competition studies.

The FAs prepared against the somatic antigens of Hawaii 5-0, Nitragin 92A3, and Nitragin 128A12 exhibited a high degree of strain specificity, as shown in Table 2. The FAs reacted strongly with the homologous bacteria in culture and with the bacteroids in nodules. They did not cross-react with one another and cross-reacted only weakly with 5 of the 41 other *R. leguminosarum* cultures tested.

The competition pattern of strains Hawaii 5-0, Nitragin 92A3, and Nitragin 128A12 on four varieties of lentils grown in sterilized vermiculite is given in Table 3. Nitragin 92A3, whenever included in the inoculum, occupied the majority of nodules. This was significant ($P < 0.05$) in two-strain competitions against Nitragin 128A12 on all varieties and against Hawaii 5-0 on the Chilean variety. Hawaii 5-0 was of equal competitiveness ($P = 0.01$) with Nitragin 128A12 on the Chilean variety, more competitive ($P = 0.01$) on the commercial variety, and less competitive ($P \leq 0.05$) on the other two varieties. Table 3 also shows that incidence of double-strain occupancy (the same nodules containing two strains) varied from 0 to 36% of the total number of nodules, depending on the strain combinations and the host varieties used.

TABLE 2. Specificity of FAs prepared against three *R. leguminosarum* strains

R. leguminosarum strains and isolates	Amt of fluorescence[a]		
	Hawaii 5-0	Nitragin 92A3	Nitragin 128A12
Cultures			
Hawaii 5-0	4+	−	−
Nitragin 92A3	−	4+	−
Nitragin 128A12	−	−	4+
Nitragin 128C53	−	−	2+
WSU serogroup 2 (3)[b]	−	−	2+
O. N. Allen strains 301, 311, 312	−	−	1+
Other (34)[c]	−	−	−
Nodule bacteroids			
Hawaii 5-0	4+	−	−
Nitragin 92A3	−	4+	−
Nitragin 128A12	−	−	4+
From Mauka experimental plots	−	−	−

[a] Intensity of fluoresence: 4+, very bright fluorescence; 2+ and 1+, definite fluorescence, but very subdued; −, no fluorescence.

[b] WSU, Washington State University; courtesy of D. Bezdicek.

[c] All other cultures listed in Table 1 in addition to seven strains from WSU serogroups 1 and 3; five other strains from the O. N. Allen collection (at NifTAL Project), and TAL 218.

The inoculum strains completely dominated the indigenous rhizobia in the experiments at the Mauka Field Station (Table 4). The results show that strains Hawaii 5-0 and Nitragin 92A3 were of equal competitiveness ($P = 0.01$) on lentils in this field, with 38% of the nodules containing both strains. Single and mixed infections in nodules of lentils grown in the field are shown in Fig. 1. Smears from two nodules from the same plant were stained with FA against Hawaii 5-0. In a single-strain nodule, (Fig. 1A), all of the bacteroid cells stain, whereas in a double-strain nodule (Fig. 1B), only a proportion of the cells stain. The unstained cells (arrows) can be visualized by using a double-light system with a darkfield condenser. They were shown to be Nitragin 128A12 by staining a similar smear with anti-Nitragin 128A12 FA. The results in Table 4 (numbers in parentheses) also show that the inoculum strains persisted well in this soil, for they still formed the majority of nodules on uninoculated plants 1 year after they were first introduced into the soil. However, Hawaii 5-0 was dominant in the single-strain nodules ($P = 0.05$).

Hawaii 5-0 occupied the majority of nodules at all treatments at the Poamoho field sites, except at high P levels after 8 weeks, when its nodule occupancy was not significantly different from that of the other strains (Table 5). The results in Table 5 also show that the incidence of doublestrain occupancy varied depending on the strains used. In the majority of the cases, there were higher proportions of Hawaii 5-0-Nitragin 92A3 and Hawaii 5-0-Nitragin 128A12 in double-strain nodules than other combinations.

The uninoculated plants remained free of nodules in samplings 1 and 2 at Poamoho. However, by the harvest at 8 weeks, approximately 50% of the uninoculated control plants harbored nodules. Immunofluorescence typing of these nodules revealed that all of these nodules were formed by one or more of the three inoculum strains used in this experiment.

DISCUSSION

The three strains of *R. leguminosarum* used in these studies were among the most efficient (C_2H_2 reduction) of the 31 cultures screened (Table 1). They were also shown to be distinct by immunofluorescence staining (Table 2). This distinction was previously demonstrated by the enzyme-linked immunosorbent assay for the same organisms (1). It was thus possible to use

TABLE 3. Percentage of nodules formed on four lentil varieties by inoculum strains of *R. leguminosarum*

Lentil variety (cultivar)	Strains in inoculum mixture[a]	Strains recovered in nodules (percent of total)							Significance[b]	
		A	B	C	A + B	A + C	B + C	A + B + C	1:1	1:1:1
Commercial (Spokane Seed Co.)	A + B	29	41		30				NS	NS
	A + C	59		23		18			**	**
	B + C		94	3			3		**	**
	A + B + C	28	38	2	12	18	2	0		
Tekoa	A + B	30	39		31				NS	NS
	A + C	31		54		15			*	**
	B + C		79	16			5		**	**
	A + B + C	12	56	23	2	0	7	0		
Benewah	A + B	31	49		31				NS	NS
	A + C	27		73		0			**	**
	B + C		80	17			3		**	**
	A + B + C	17	36	28	0	8	11	0		
Chilean	A + B	18	46		36				**	**
	A + C	38		26		36			NS	NS
	B + C		82	12			6		**	**
	A + B + C	4	31	17	17	7	24	0		

[a] Mixture of strains contained equal numbers of rhizobia of two or all three strains (see text). A, Hawaii 5-0; B, Nitragin 92A3; C, Nitragin 128A12.

[b] Chi-square analysis was used to test the deviation of results from the expected ratio of 50:50 (1:1) for the single-strain:single-strain nodules (df = 1) and 33:33:33 (1:1:1) for single-strain:single-strain:double-strain nodules (df = 2); NS, not significant; *, $P = 0.05$; **, $P = 0.01$.

these strains in competition against one another and easily identify the one(s) present in the nodules.

Under bacteriologically defined conditions in the growth chamber, one of the strains (Nitragin 92A3) dominated the nodules in two-strain competition trials (Table 3). The competitive advantage of Nitragin 92A3 was consistent, but significant (P = 0.01) only against Nitragin 128A12 on all four varieties and against Hawaii 5-0 on the Chilean variety. Vincent and Waters (40) and Russell and Jones (32) have reported that some clover species preferentially select one strain over another. Our data indicate that the overall pattern of competition of the strains used in this study varies depending on the host variety.

In the field that contained low numbers of resident R. *leguminosarum* (Mauka Station), the inoculum strains outcompeted the resident rhizobia completely. This might have been due, in the first year, to the high numbers of the inoculum strains applied to the seeds. However, even after 1 year in the soil, when these strains no longer had the advantage of direct seed placements, they still formed the majority of nodules on uninoculated plants (Table 4).

The local isolate, Hawaii 5-0, performed better than the other inoculum strains in an Oxisol field (Table 5). Thornton (38) has also reported that local strains survive in the same soil better than commercial strains in two of three instances.

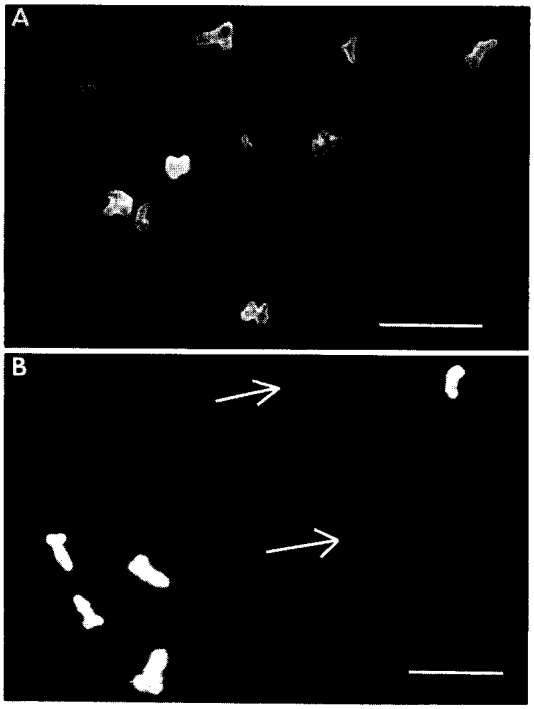

FIG. 1. IF stain of bacteroids in lentil nodules containing (A) a single strain, Hawaii 5-0, and (B) two strains, Hawaii 5-0 and Nitragin 128A12. Nodule smears in A and B were stained with the FA against Hawaii 5-0. The unstained cells in B (arrows) are visualized by the use of a double-light system, using a dark-field condenser (from reference 6). Bar, 10 μm.

TABLE 4. Competition and persistence of introduced *R. leguminosarum* strains in a soil containing resident *R. leguminosarum* (Mauka Field Station)

Treatment	Plant wt (g)[a,b]	Ethylene per plant/h (μmol)[a,b]	Nodules/ plant[a,b]	Strains recovered in nodules (percent of total)[b]			
				A	B	A + B	Unknown
Uninoculated	4.9a (3.8a)	0.26d (0.96e)	50h	6 (40)	12 (24)	0 (12)	82 (24)
Hawaii 5-0 (A)	5.2a (3.7a)	0.93c (0.91e)	137g	100 (84)	0 (3)	0 (11)	0 (2)
Nitragin 92A3 (B)	6.5a (3.5a)	0.90c (0.97e)	173g, f	0 (4)	100 (80)	0 (6)	0 (10)
A + B[c]	5.4a (3.0a)	1.38b (0.80e)	232f	30 (51)	32 (19)	38 (24)	0 (6)

[a] Values within columns followed by the same letter do not differ significantly ($P = 0.05$).
[b] Numbers in parentheses are data from uninoculated plants grown in the same plots 1 year after the first experiments.
[c] Chi-square analysis of percentage of nodule occupancy revealed that the year 1 results were not significantly different ($P = 0.01$) but that the year 2 results (values in parentheses) were ($P = 0.05$).

Read (28) has shown that strains of R. trifolh establish differently at different field sites. Our data (Tables 4 and 5) also show that at one field site, Mauka Field Station (Inceptisol), Hawaii 5-0 and Nitragin 92A3 were equally competitive (P = 0.01), whereas at another site, Poamoho (Oxisol), Hawaii 5-0 had a significant (P:5 0.05) advantage at all harvests and fertility treatments except after 8 weeks at the highest phosphorus treatment, when the strains were not significantly (P = 0.05) different. It is interesting to note that this strain (Hawaii 5-0) was originally isolated (1) from nodules of lentils growing in another Hawaiian Oxisol (Typic Torrox).

Many investigators (12, 21, 26, 28, 30, 40) have suggested the use of inocula containing several strains adapted to a wider range of soil and plant differences. Some workers (7, 12, 15) have even shown that plants inoculated with mixtures of effective strains give better yield and higher nitrogen content than those with a single-strain inoculum. Considering that our data (Table 4) do not show significant differences in plant weight between uninoculated and inoculated treatments, the value of multistrain inocula cannot be evaluated in this study.

Lindemann et al. (24) used immunofluorescence to provide evidence for double infection in nodules of soybeans in sterilized sand and reported a frequency of such occurrence as high as 32%. Other investigators have reported incidences of double-strain occupancy exceeding 20% in nodules on plants grown under bacteriologically controlled conditions (10, 17, 20, 22, 25), but only <1.0 to 3.5% in the field (9, 10).

In our studies, the incidence of double-strain occupancy in nodules varied from 0 to 36% in vermiculite (Table 3), depending on the strains in the mixture and the host variety, and from 0 to 38% in the field (Tables 4 and 5), depending on the strain mixture and the soil type. The data also suggest a close relationship between the competitiveness of a strain and its involvement in the formation of doubly infected nodules.

TABLE 5. Competition of inoculum strains of *R. leguminosarum* for nodulation of lentils grown in an Oxisol at three different phosphorus levels

Phosphorus level[a]	Sample period	Strains recovered in nodules (percentage of total)[b]							
		A	B	C	A + B	A + C	B + C	A + B + C	Significance[c]
Low		46	15	6	15	15	2	0	**
Medium	10 days	41	9	25	5	18	2	0	*
High		37	15	17	5	26	0	0	*
Low		54	16	5	14	3	8	0	**
Medium	5 wk	48	10	10	14	15	1	0	**
High		60	5	10	17	3	5	0	**
Low		44	4	18	10	18	4	0	**
Medium	8 wk	32	8	21	15	18	4	0	*
High		27	21	8	23	15	4	0	NS

[a] Amount of phosphorus in soil solution: low, 0.003 ppm P; medium, 0.05 ppm P; high, 0.8 ppm P (15).
[b] A, Hawaii 5-0; B, Nitragin 92A3; C, Nitragin 128A12.
[c] Chi-square analysis was used to test the deviation of the results of A:B:C from the expected ratio of 1:1:1 for single-strain nodules (df = 2); NS, not significant; *, $P = 0.05$; **, $P = 0.01$.

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