COMPETITIVENESS AND RHIZOSPHERE COLONIZATION OF *BRADYRHIZOBIUM* SP STRAINS ON CHICKPEA[1]

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ABSTRACT - Factors affecting competition among strains of *Bradyrhizobium* sp for nodulation of chickpea (*Cicer arietinum* L.) were investigated in the greenhouse. Nodule occupancy by two strains varied when the strains were co-inoculated either in Leonard jars on in soil pots, or when inoculum ratios of the strains were changed. In another experiment, early rizosphere dominance in root tips or total rizosphere did not reflect nodule occupancy, as all the nodules were formed by the soil-born strain, which represented less than 1% of the early rizosphere population. In a time-course inoculation experiment with 2-day-old chickpea seedlings, a delay in adding the secondary inoculum by 4 h increased the nodule occupancy of the primary strain from 16 to 94%. The results indicate that actual rates of strains are important only when the strains are inoculated together under the same conditions, but when different inoculation methods were used, rhizosphere numbers did not define nodule occupancy by the strains.

Index terms: Leonard jars, soil pots, nodules, inoculation.

COMPETITIVIDADE E COLONIZAÇÃO DA RIZOSFERA DE ESTIRPES DE *BRADYRHIZOBIUM* SP EM GRÃO-DE-BICO

RESUMO - Foram estudados em casa de vegetação alguns dos fatores que afetam a competitividade de estirpes de *Bradyrhizobium* sp em grão-de-bico (*Cicer arietinum* L). A ocorrência das estirpes nos nódulos variou quando as mesmas foram co-inoculadas no solo ou em vasos Leonard, ou quando se alterou as proporções das duas estirpes inoculadas. Em outro experimento, a predominância de uma estirpe na rizosfera ou nos pontos apicais das raízes, não se refletiu em sua ocorrência nos nódulos, nos quais predominou a ocorrência da estirpe inoculada no solo, apesar desta representar menos de 1% da população de *Bradyrhizobium* na rizosfera. Em outro experimento, o retardamento na inoculação com o inóculo secundário em 4 h elevou a ocorrência da estirpe inoculada no inóculo primário de 16 para 94%. Esses dados sugerem que a proporção relativa das estirpes é importante apenas quando as estirpes são inoculadas juntas sob as mesmas condições. Contudo, quando diferentes métodos de inoculação são utilizados, o número total de células na rhizosfera não define a dominância das estirpes nos nódulos.

Termos para indexação: vasos de Leonard, potes com solo, nódulos, inoculação.

INTRODUCTION

The primary objective of a *Bradyrhizobium* strain selection program is to obtain strains effective in nodulation and N_2 fixation with their hosts. These goals are relatively easy to achieve under laboratory conditions or in field soil void of homologous strains. However, when the soil has a bradyrhizobial population capable of nodulating the legume being planted, the inoculated strain usually fail to form a significant proportion of the root nodules, as reported in previous papers (Boonkerd et al. 1978, Ellis et al. 1984).

The mechanism involved in the competitiveness among *Rhizobium* strains or the processes that confer a competitive advantage to soil strains over seed-inoculated ones are poorly understood. Some of the factors affect-

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ing competitiveness are: host selection of one strain over another (Robinson 1969, Masterson & Sherwood 1974, Russel & Jones 1975); selective inhibition by actinomycetes (Damirgi & Johnson 1966); bacteriocin production (Schwinghamer 1971, Barta & Tripett 1985), abiotic soil factors (Damirgi et al. 1967; Bezdicek 1972); the relative proportion of the inoculated strains (Demezas & Bottomley 1986) or the ratio of inoculated to resident strain (Bohlool & Schmidt 1973, Weaver & Frederick 1974, Kamicker & Brill 1987).

The objective of this study was to evaluate factors that affect the nodulating competitiveness of *Bradyrhizobium* sp. (Cicer), including methods, levels, and time course inoculation of two strains as related to nodule occupancy in chickpea.

MATERIALS AND METHODS

Experimental conditions

The experiments were carried out in the greenhouse in pots with soil or in Leonard jars with vermiculite, sand and nutrient solution (Sloger 1969). The soil was a Latah silt loam (fine, mixed, mesic, xeric, Argialbolls) collected at the Spillman Farm (Pullman, WA) from the surface 20 cm. The soil was dried, passed through a 2 mm-dia sieve and homogenized. Preliminary tests in pots showed that the soil was void of *Bradyrhizobium* sp capable of nodulating chickpea.

Inoculation

Strains of *Bradyrhizobium* (Cicer) used in this experiment were 27 a 8, 27 a 15 and 27 a 16 (hereafter called A8, A15 and A16, respectively) obtained from the culture collection of The Nitragin Co., Milwaukee, WI. All strains were maintained on yeast mannitol agar (YMA) slants and grown for inoculum in broth medium of the same composition. For the studies using Leonard jars, late log phase cultures (0.1 mL/seedling) were added directly over the pregerminated chickpea seed at variable cell concentrations. In the experiments with soil, peat inoculant was used for seed inoculation or broth culture was mixed with dry soil and wetted to -0.03 MPa.

Surface sterilization of the seeds was accomplished by soaking the seeds in 50% ethanol for 30 sec and in 20% chlorox for 1.5 min. followed by several rinses with sterile water. For the experiments in Leonard jars, seeds were pregerminated in petri dishes with filter papers soaked with sterile water.

FA counts in rhizosphere

A variable number of roots were added to dilution bottles containing 95 mL of extractant solution (Kingsley & Bohlool 1981), one drop of Antifoam B emulsion (Sigma Co.) and 5 drops of Tween 80. The flasks were shaken for 30 min. on a wrist action shaker and centrifuged for 10 min. by mild centrifugation (200 x g). An aliquot was taken from the extracting solution before centrifugation and dried for 3 days at 105°C to determine the dry weight of soil in the rhizosphere. A volume (1 mL) amount of supernatant was passed through a 0.45 um-pore size polycarbonate membrane filter (Nuclepore Corp., Pleasanton, CA.) stained with Irgalan Black (Ellis et al. 1984). Fluorescent antibodies to specific chickpea bradyrhizobia were prepared as described by Schmidt et al. (1968) and applied after treatment with rhodamine-gelatin conjugate to eliminate non-specific absorption of antibody to soil particles (Bohlool & Schmidt 1968). Forty to seventy microscopic fields were counted on each filter and the counts were converted to cells g^{-1} of oven-dry soil. Cells were visualized with a Zeiss microscope equipped for epifluorescence using a HBO Osram mercury light source and a 100K Neofluar objective.

Nodule serotyping by FA

Nodule-bearing roots were kept frozen at -20°C until use. Eighteen nodules were selected per pot, cleaned by shaking for 30 min. in 100 mL water containing 5 drops of Tween 80 and then rinsed in tapwater for 2-3 min. The nodules were individually crushed in 1-2 mL of saline and smears were placed on a glass slide (18 smears/slide). Smears were fixed and stained according to the procedure described by Robert & Schmidt (1983).

Rhizosphere establishment studies

Strain A15 was inoculated at 1.3 x 10^6 cells g^{-1} in soil that was packed into wooden boxes 10 cm deep and kept at about -0.03 MPa moisture tension. Sur-

face sterilized chickpea seeds were inoculated with a peat carrier and 20% Acacia powder adhesive at 9.2×10^5 cells/seed and planted into flats two days after soil inoculation. Seed inoculum level was determined by FA after the inoculum mixture was dried at the seed surface. At different intervals (3, 6, 9 and 12 days after planting), seedlings were removed from the flats, shaken gently to remove loosely adhering soil and enumerated for *Bradyrhizobium* sp (Cicer) by FA. At early samplings (3 days), the whole radicle without the seed was excised for FA enumeration, but at later stages, the root tips from taproots and lateral roots (about 20% of total root length) were separated from the rest of the root and enumerated separately. On the same dates seedlings removed from the flats were planted into Leonard jars (4 plants per jar) after removing loosely adhering soil. These seedlings containing the rhizosphere soil were grown in the greenhouse until early flowering for nodule serotyping.

Time-course inoculation studies

Two-day-old seedlings (radicles of 1 to 2 cm) obtained from surface-sterilized seeds were placed on the sand surface in Leonard jars (4 plants per jar), and covered to prevent water loss. The experiment was divided in two groups: one group was inoculated with strain A8 and the other group with A15 at approximately the same inoculum level (0.90×10^7 and 2.0×10^7 cells/seed for strains A15 and A8, respectively). At different times (zero, 2, 4, 9 and 26 h), each group was challanged by the other strain as a secondary inoculum. Following application of the challanged strain, seedlings were covered with sand. At early flowering (34 days after addition of the secondary inoculum), the plants were harvested and the nodules were separated and classified as to early (taproot) and late (lateral roots) and serotyped using FA as described previously.

RESULTS AND DISCUSSION

Competition studies in soil and sterile jars

Nodule occupancy was quite different when seeds were inoculated in sterile jars as compared to soil-inoculation (Fig. 1). Although in both cases strains A8 and A16 were inoculated at about the same rate, the recovery in nodules of strain A16 was 90% from seed-inoculation and 40-50% from strains inoculated directly into soil. These results suggest application methods x strain interactions, that permitted strain A8 to be equally competitive in soil as compared to strain A16 which dominated the nodules in sand culture.

Differences in nodule occupancy have been reported for plants growing in soil as compared to plants growing in mineral salts solution (Demezas & Bottomley 1987) or in growth pouches (Moawad et al. 1984).

These observations point out the importance of the soil environment in determining nodule occupancy by a single strain. However, competitiveness as measured in Leonard jars or in growth pouches should be interpreted with caution, since it does not take into consideration the many possible interactions in soil.

The effect of increasing rates of strain A16 in the soil on nodule occupancy is shown in Fig. 2. When the level of strain A8 was kept constant at 2.5×10^3 cells/g soil the nodule occupancy of strain A16 increased from 11 to 95% when population of strain A16 varied in the inoculum from 2.1×10^2 to 4.1×10^4 cells g^{-1}. By inoculating both strains together in a soil void of *Bradyrhizobium* sp (Cicer) we avoided the confonding effect of one strain being adapted to the soil as has been the case with some previous studies (Bohlool & Schmidt 1973), Weaver & Frederick 1974). The pattern of recovery of A16 was similar when challanged with strain A8 at 2.5×10^4 and 2.5×10^5 cells/g soil (lines two and three, respectively).

These results confirm that the relative proportion of both strains rather than their total population in the soil is the important parameter in determining nodule occupancy. Similar results were reported with *B. japonicum* by Bohlool & Schmidt (1973) and Weaver & Frederick (1974), although in these studies the soil was already colonized by one or more strains of *B. japonicum* and very high levels of the inoculated strain had to be added to overcome the native rhizobial population.

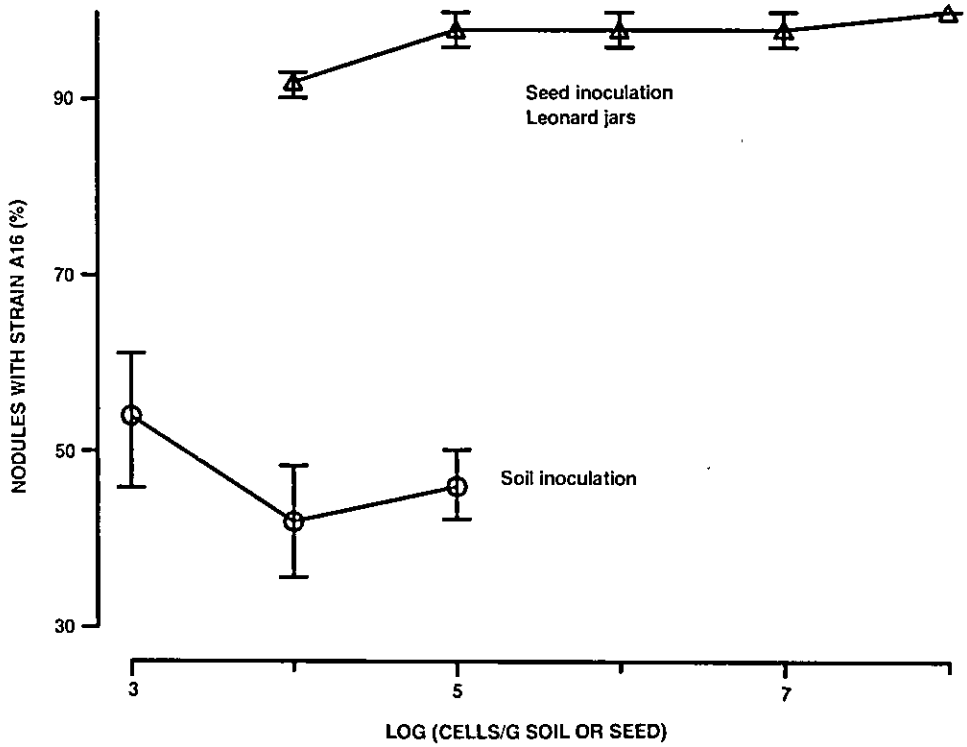

FIG. 1. Effect of inoculum population on nodule occupancy in chickpea grown in pots (soil inoculation) or Leonard jars (seed inoculation). Strains A16 and A8 were inoculated at approximately the same rates. Each point is the mean of four replicates ± standard error.

Bohlool & Schmidt (1973) described a "competitive curve" similar to the ones in Fig. 2, but the proportion of inoculated strain to native strain had to be as high as 30:1 in order for the inoculated strain to form about 30% of the nodules.

Rhizosphere establishment studies

From seed inoculation of A15 and soil inoculation of strain A8, seedlings were removed periodically and enumerated for strains A8 and A15 in the rhizosphere after removing loosely adhering soil particles from the roots (Fig. 3). On the same date, seedlings submitted to the same treatment were planted in Leonard jars and allowed to grow until flowering stage for nodule serotyping. Strain A8 populations were considerably higher than strain A15 in the early chickpea rhizosphere in the early samplings, although differences were less evident at later sampling dates. However, seed inoculation always resulted in a significantly greater number of strain A8. The erarly rhizosphere dominance of strain A8 was not reflected in nodule occupancy, as the soil inoculated strain A15 formed 100% of the nodules in all the plots. Reyes & Schmidt (1979), Moawad et al. (1984) and Robert & Schmidt (1985) observed that rhizosphere numbers of *R. japonicum* strains did not relate to nodule occupancy in soybean roots. However, since in their studies the rhizobial populations were evaluated on the basis of the total rhizosphere,

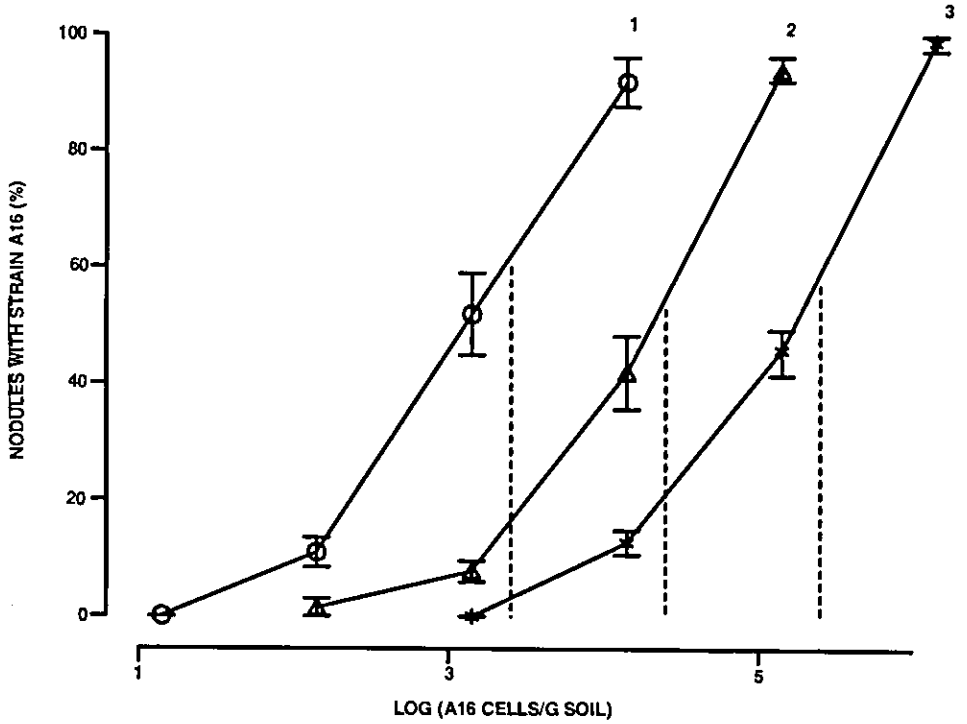

FIG. 2. Effect of increasing rates of strain A16 in the soil on nodule occupancy. Each point is the mean of four replicates ± standard error. The levels of strain A8 (log N cells/g soil) were 3.4, 4.4 and 5.4 for lines 1, 2 and 3, respectively. Dashed vertical lines indicate points of equal additions for the strains.

it may be more reasanable to assume that rhizosphere colonization at discrete sites on the roots rather than total numbers in the rhizosphere is responsible for nodule dominance by one strain. Bhuvaneswari et al. (1980), Pueppke (1986) and Sargent et al. (1987) demonstrate that root regions most susceptible to nodulation are located near the root tips; Rai & Patil (1978) reported chemotaxis of *Rhizobium* sp (Cicer) towards chickpea root exudates, and Gulash et al. (1984) showed that rhizobial cells are attracted to localized regions closer to the root tips and are found in high numbers, forming "clouds" at these points, three to five hours after inoculation. However, the hypothesis that nodule occupancy by one strain is due to root colonization at these possible infection sites was not borne out. Populations of the seed-inoculated strain A8 were much higher than the soil-inoculated strain A15 in early rhizosphere at the root tips, theoretically the sities for nodular infection (Fig. 4). However, all the nodules from plants grown in Leonard jars derived from seedlings transplanted with rhizosphere soil were formed by soil strain A15 which represented less than 1% of the rhizobial population at the rhizosphere root tips at the time of transplanting. Even when 3-day-old seedlings with very small radicles (1-2 cm) which carried very little rhizosphere soil with whem when transplanted to Leonard jars, no nodules were formed by the seed-inoculated strain A8.

Kosslak et al. (1983) indicated that critical interactions among competing strains occurred at very early stages of soybean growth and a few hours of difference in the presence or ab-

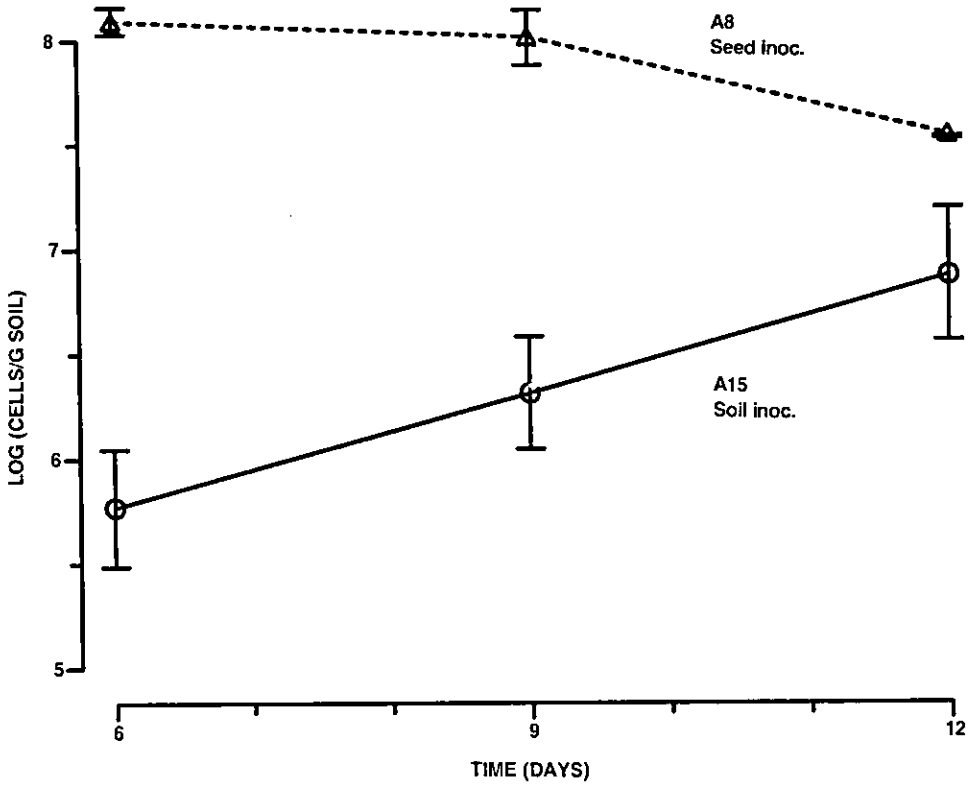

FIG. 3. Total populations of strains A8 and A15 in the chickpea rhizosphere after removal of the seed. Rates of inoculation were 9.2 x 10^5 cells/seed and 1.3 x 10^6 cells/g^{-1} soil for strains A8 and A15, respectively. At each sampling time, seedlings with rhizosphere soil were planted in Leonard jars and grown for nodule serotyping. Each point is the mean of four replicates ± standard error.

sence of one strain is enough to define nodule occupancy. In the present study, when both strains were inoculated at the same time in the soil, the relative proportion of the two strains was the determining factor for nodule occupancy (Fig. 2) whereas rhizosphere numbers were meaningless when different inoculation methods were used. Therefore, from our results, we may speculate that although the seed inoculated strain was able to colonize and dominate the rhizosphere, the soil-inoculated strain had the advantage of being present at the infection site during some critical time of root development, and before the actual colonization of the root tips by the seed-inoculated strain. In that case, the time course would be more important than the actual number of the cells at the infection sites.

Time-course inoculation studies

Nodule occupancy by two strains submitted to a time-course inoculation of a secondary strain is shown in Fig. 5. In all the treatments, nodule occupancy did not differ for early (taproot) or late (lateral root) nodules within each treatment and therefore the data were pooled for analysis. When the two strains were inoculated together (zero time), strain A8 occupied only 16% of the nodules, which confirms our earlier results about the low com-

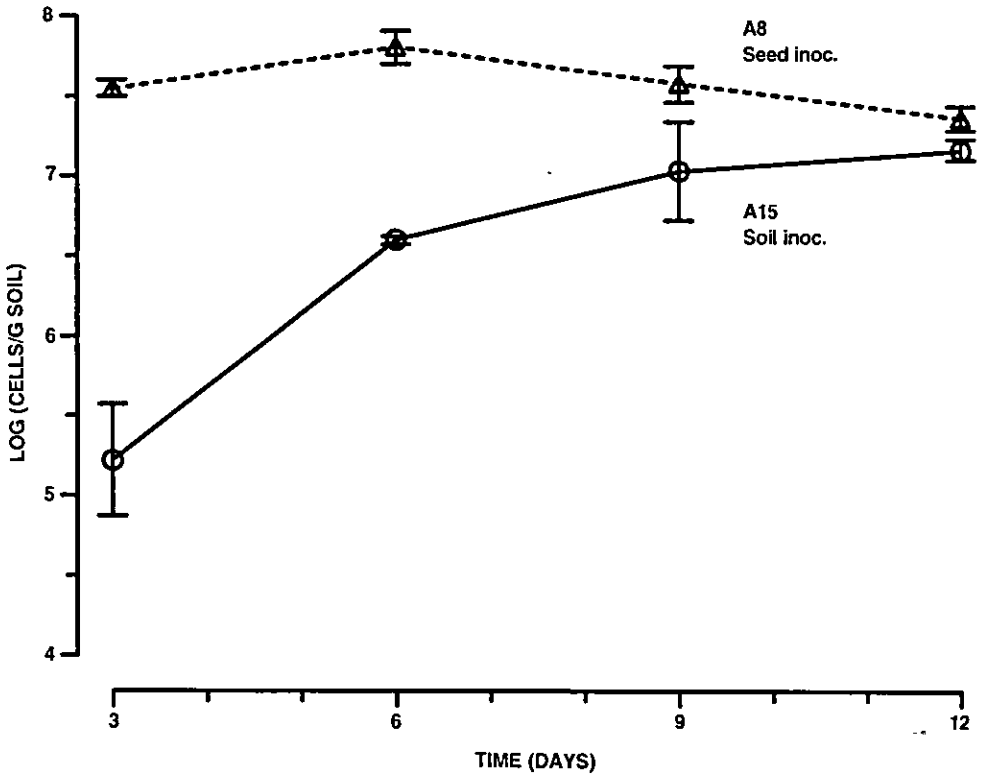

FIG. 4. Populations of strains A8 and A15 in the rhizosphere of chickpea root tips. See legend in Fig. 6 for further details. Each point is the average of four replicates ± standard error.

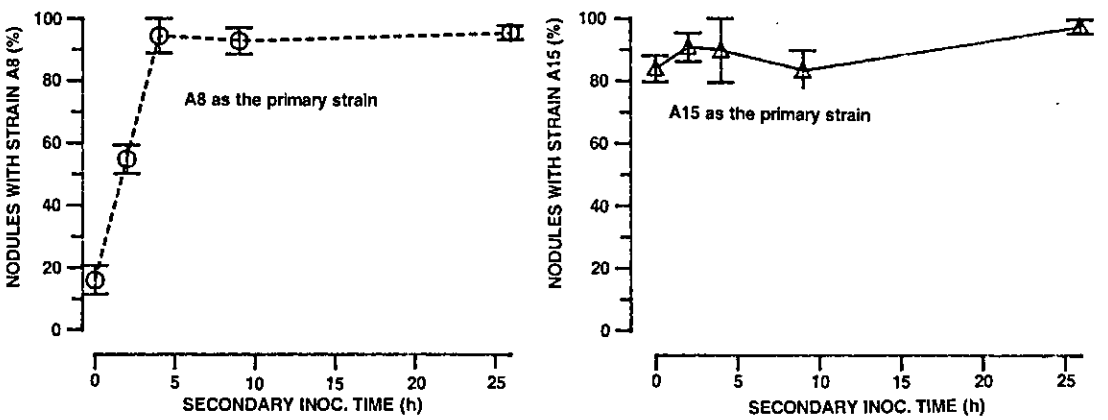

FIG. 5. Effect of preexposure of roots to a primary strain (A8 or A15) and to a delayed application of a secondary strain occupancy in Leonard jars. Each point is the mean of three replicates ± standard error.

petitiveness of this strain in Leonard jars. However, when inoculation was delayed for the secondary strain A15 by 4 h, strain A8 formed 94% of the chickpea nodules. Even a short delay of 2 h for the secondary inoculum (A15) was enough to greatly increase the competitive ability of strain A8 and allowing it to form 54% of the nodules. Strain A15 occupied 84% of the nodules when inoculated together with strain A8, and was little affected by delayed inoculation with strain A8 (Fig. 5).

Previous work (Skrdleta 1970, and Kosslak et al. 1983) reported that a delay in applying the secondary inoculum favored nodule dominance by the strain applied as primary inoculum. Kosslak & Bohlool (1984) and Sargent et al. (1987) working with a split-root system demonstrated that the contact of the legume root with a *Rhizobium* strain induces a systemic response that inhibits nodulation by a secondary strain inoculated on the other side of the split-root assembly. In this work, young seedlings with a very small radicle (1 to 2 cm), upon exposure to one strain became predisposed to that strain for further nodulation, as the majority of the nodules formed in the plants later in their cycle were affected by the preexposure of the seedlings to the primary strain.

CONCLUSIONS

1. The results of the present study indicate that nodule occupancy by one strain is determined by interactions of the host and inoculum strain at a very early stage of growth, possibly just after seed germination which determines the later nodule compostion of the legume. The effect of exposing seedlings with radicles smaller than 2 cm to one strain seems to last throughout the remaining cycle of the plant, as nodules formed later in the secondary root system were influenced by the exposure to the primary strain.

2. Bradyrhizobial enumeration in the host rhizosphere does not seem to be a useful parameter in competitiveness studies when different inoculation methods are used, whereas the relative proportion of two strains are critical in defining nodule occupancy when the same inoculation method is applied.

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