INTERACTIONS BETWEEN THE SOYBEAN HOST (*Glycine max*) AND ITS MICROSYMBIONT (*Rhizobium japonicum*) WHICH AFFECT COMPETITION BETWEEN STRAINS

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ABSTRACT

An important, but poorly understood aspect of the Rhizobium/legume association is the differential competitiveness between strains of the microsymbiont for nodulation of their legume host. The competitive ability of strains of R. japonicum for nodulation of soybean, Glycine max L. Merrill, was examined under different environmental conditions to determine whether: 1) the presence of soil microorganisms differentially antagonistic to strains of R. japonicum would affect the outcome of competition between those strains; 2) changes in available soil nitrogen would affect nodule occupancy by different strains; 3) relative nodule occupancy by strains of R. japonicum differs with plant age; 4) preexposure of the soybean host to poorly competitive strains could alter the pattern of competition between strains; and, 5) what is the role of the soybean host during the process of nodulation.

The results from this investigation indicate: 1) the pattern of competition between strains of R. japonicum was not affected by establishment of antagonistic microorganisms on soybean roots; 2) changes in soil available nitrogen did not affect the percent of nodules occupied by strains R. japonicum in three different soils; 3) relative nodule occupancy by a strain changed with plant age. Relative nodule occupancy was different during vegetative growth from that observed during pod-fill; 4) preexposure of young seedlings to poorly competitive effective, or ineffective strains, significantly altered the outcome of competition with a highly competitive strain.

This occurred in vermiculite and in soil; 5) use of a split-root system to separate strain/strain interactions from those of the host during the process of nodulation, indicated that the number of infection sites which developed into functional nodules was under host control. Host control of nodulation was expressed through the suppression of nodulation on half-root systems which were inoculated four or more days after inoculation of the opposite side. Suppression of nodulation was not associated with the time of appearance of nodules, or nitrogenase activity on half-sides inoculated at zero-time. The total number of nodules and mass supported by the soybean host, however, appeared to be linked to the amount of light available to the host for photosynthesis; and 6) the competitive ability of strains of R. japonicum for nodulation of the soybean host was influenced by interactions which occurred between a strain and its host during the early stages of the infection process.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS..... 3

ABSTRACT..... 4

LIST OF TABLES..... 7

LIST OF FIGURES..... 10

CHAPTER I. INTRODUCTION AND OBJECTIVES..... 11

CHAPTER II. LITERATURE SURVEY..... 13

CHAPTER III. INFLUENCE OF ENVIRONMENTAL FACTORS ON COMPETITION BETWEEN TWO STRAINS OF Rhizobium japonicum..... 36

CHAPTER IV. COMPETITION OF Rhizobium japonicum STRAINS ON THE EARLY STAGES OF SOYBEAN MODULATION..... 60

CHAPTER V. USE OF A SPLIT-ROOT GROWTH SYSTEM FOR PARTITIONING OF STRAIN/STRAIN AND STRAIN/HOST INTERACTIONS IN THE MODULATION OF SOYBEANS BY Rhizobium japonicum..... 88

CHAPTER VI. GENERAL SUMMARY..... 116

APPENDIX..... 120

LITERATURE CITED..... 122

LIST OF TABLES

TABLE		
III-1	Effect of abiotic/biotic components on pattern of competition between *Rhizobium japonicum* strains USDA 110 and 123	45
III-2	Pattern of antibiotic resistance of USDA 110 and 123 in culture	48
III-3	Effect of rhizosphere microorganisms on the growth of *R. japonicum* strains USDA 110 and 123 in culture	49
III-4	Effect of actinomycetes on the pattern of competition between *R. japonicum* strain USDA 110 and USDA 123	50
III-5	Effect of combined nitrogen on competition pattern of *R. japonicum* strains USDA 110 and 123 in three soils	51
III-6	Effect of combined nitrogen on competition pattern of *R. japonicum* strains USDA 110 and USDA 123 in three soils: Nitrate levels	53
III-7	Competition pattern of *R. japonicum* strains in a Hawaiian inceptisol: Field study	54
III-8	Percent nodule occupancy of *R. japonicum* strains in a Hawaiian inceptisol: greenhouse trial	56
III-9	Effect of pretreatment of *R. japonicum* strain USDA 110 on its nodule occupancy in competition with four other strains: field experiment	57

LIST OF TABLES (CONTINUED)

IV-1 Effect of preexposure of two-week-old roots to a poorly competitive strain (USDA 138) on nodule occupancy of a highly competitive strain (USDA 110) of *R. japonicum*..... 72

IV-2 Effect of preexposure of roots to an ineffective poorly competitive strain on nodule occupancy by an effective highly competitive strain of *R. japonicum*.. 73

IV-3 Effect of binding of gamma-irradiated cells of one strain on nodule occupancy of another strain of *R. japonicum*..... 75

IV-4 Effect of preexposure of soybean roots on rhizosphere colonization by a poorly competitive and a highly competitive strain of *R. japonicum*..... 76

IV-5 Effect of preexposure to USDA 110 on competition pattern of *R. japonicum* strains in a soil containing indigenous *R. japonicum* (Waseca)..... 80

IV-6 Effect of preexposure to *R. japonicum* strains on competition pattern in a *Rhizobium*-free soil: Harvest I..... 81

IV-7 Effect of preexposure to *R. japonicum* strains on competition pattern in a *Rhizobium*-free soil: Harvest II..... 82

IV-8 Effect of preexposure to *R. japonicum* strains on nodule and plant parameters in a *Rhizobium*-free soil: Harvests I and II..... 83

LIST OF TABLES (CONTINUED)

V-1 Suppression of nodulation of half-root systems caused by delayed inoculation (short-day season)..... 96

V-2 Suppression of nodulation of half-root systems as a result of delayed inoculation (long-day season): nodule parameters..... 98

V-3 Suppression of nodulation of half-root systems as a result of delayed inoculation (long-day season): plant parameters..... 105

V-4 Effect of light intensity on suppression of nodulation of half-root systems as a result of delayed inoculation (long-day season): nodule parameters..... 107

V-5 Effect of light intensity on suppression of nodulation of half-root systems as a result of delayed inoculation (long-day season): plant parameters..... 109

V-6 Pattern of competition between a highly competitive strain and a poorly competitive strain of R. japonicum on half-root systems..... 110

V-7 Pattern of competition on half-root systems between effective and ineffective R. japonicum strains..... 111

APPENDIX TABLES

Appendix-1 Suppression of nodulation of half-root systems caused by delayed inoculation: partitioning of soluble carbohydrates..... 121

LIST OF FIGURES

Figure		
IV-1	Effect of preexposure of roots to an effective strain of *R. japonicum* on nodule occupancy by another effective strain	70
IV-2	Fluorescent antibody stain of USDA 138 colonizing the surface of soybean roots	78
V-1	Diagram of split-root assembly	93
V-2a	Pattern of nodulation in split-root system: inoculated/uninoculated	100
V-2b	Pattern of nodulation in split-root system: inoculated/inoculated	102
V-2c	Pattern of nodulation in split-root system: inoculated/inoculation delayed	104

CHAPTER I

INTRODUCTION AND OBJECTIVES

An example of an economically important symbiosis in nature is the nitrogen fixing association of leguminous plants with soil bacteria of the genus Rhizobium. This association leads to the formation of nitrogen fixing root nodules. Legumes are distributed worldwide and include trees, shrubs, woody vines, as well as major grain, oil, fodder and forage crops (for review see 117). Cropping systems using nodulated legumes benefit from the independence of the plant on soil nitrogen and the improvement of the nitrogen status of the soil (52). Estimates of atmospheric nitrogen fixed by the Rhizobium/legume association range from 20 to 500 kg/ha depending on environmental conditions, the effectiveness of the Rhizobium/legume association, and method of determination (for reviews see 39,52,117). Some strain/host combinations are more efficient than others in the conversion of atmospheric nitrogen to biologically useable forms.

One of the goals of recent investigations has been the selection of strains of rhizobia which are highly effective nitrogen fixers. However, the introduction of highly effective inoculum strains into the soil does not always increase crop yields because nodules continue to be formed by indigenous rhizobia (for review see 39). Thus, an important consideration in legume cultivation is the inability of selected inoculant strains to compete successfully with indigenous rhizobia.

Competitive ability as a characteristic of a particular strain has been documented for several Rhizobium/legume systems (12,34,36, 53,67). The results from these studies have demonstrated that the competitive ability of strains may vary with environmental conditions and host genotypes. There does not seem to be any relationship between a strain's ability to fix nitrogen efficiently and its ability to compete (82,86). The mechanisms that confer competitiveness to a strain rain poorly understood.

The objectives of this research were:

I. To determine the effect of selected environmental factors on the outcome of competition between strains of Rhizobium japonicum.

II. To determine which stages in the infection process are important in determining the outcome of competition between strains.

III. To determine the role of the soybean host in regulating the number of sites available for infection by different strains of Rhizobium japonicum.

CHAPTER II

LITERATURE SURVEY

Members of the genus Rhizobium are gram-negative, aerobic, chemoheterotrophic bacteria that are capable of forming symbiotic associations with leguminous plants (for review see 56). This association leads to the formation of nitrogen fixing root nodules. Within the nodule, the rhizobia convert atmospheric nitrogen to ammonia using the enzyme complex nitrogenase. The ammonia thus formed is excreted into the nodule cytoplasm where it is incorporated into nitrogenous compounds and translocated by the xylem from the roots. In several legume species, ammonia is incorporated into asparagine, while in soybeans and cowpeas, ureides (allantoin and allantoic acid) are the major carriers of ammonia in the xylem (for reveiw see 52). The efficiency of conversion of atmospheric nitrogen into ammonia varies with host and bacterial genotypes, as well as with various environmental stresses (for review see 50,115).

Carbon and nitrogen balances of nodulated and non-nodulated legumes indicate that the cost to the legume host in terms of energy and photosynthate for nodule maintenance, nitrogenase functioning and the provision of energy and carbon necessary for bacteroid growth and reproduction can be substantial. It has been estimated that approximately 27 moles of ATP are consumed for each two moles of ammonia formed from dinitrogen (52). This represents ATP used directly by the nitrogenase complex for the formation of ammonia, as well as the loss of ATP through hydrogen evolution. If the

microsymbiont possesses a hydrogen uptake system, some of the protons lost as hydrogen gas, can be recycled back into the nitrogenase complex (83).

Comparisons of carbon:nitrogen balances between nodulated and nitrate-fed legumes have shown that the respiratory efficiency of nodulated roots with respect to nitrogen fixation is similar to that of uninoculated roots assimilating nitrate (70). However, in terms of dry matter yield, nitrate-fed plants converted 69% of assimilated carbon into dry matter, whereas, only 57% of the assimilated carbon was converted into dry matter in nodulated plants (79). The 12% difference in carbon assimilation between the nitrate-fed legume and the nodulated legume was assumed to be the cost to the host plant for the maintenance of the symbiotic association. In a similar study, a comparison of shoot and root respiration rates on the utilization of gross photosynthate in legumes (soybean, cowpea, and white clover) grown with or without a source of combined nitrogen indicated that the source of nitrogen had a significant effect on root respiration (91). The source of nitrogen had no effect on gross photosynthesis or shoot respiration. Respiration by nodulated roots accounted for 22 to 34% of the total carbon, whereas in nitrate-grown plants, root respiration utilized 11 to 21% of the total carbon assimilated (91). As in the previous study, the higher respiratory rates in nodulated plants were attributed to the cost of maintaining the symbiotic state and were also reflected in slower growth rates and lower dry matter yields in nodulated hosts.

To maintain an effective symbiotic association, the legume host has to divert a greater proportion of its assimilated carbon to its underground organs. This occurs at the expense of its vegetative organs and results in slower growth rates, lower dry matter and seed yields (90). The majority of the carbon utilized by the underground organs of nodulated legumes has been attributed to the nodules (70). Carbon and energy acquired through photosynthesis are used for nodule growth and maintenance, bacteroid growth and maintenance, nitrogenase functioning and provision of carbon skeletons for the export of fixed nitrogen (77,78,79). A significant portion of the carbon utilized by the nodules is lost as CO_2 as the result of hydrogen evolution during nitrogen fixation and rhizobial metabolism (78, 79,83). Thus, the costs of growing legumes dependent on nitrogen fixation must be weighed against those using sources of combined nitrogen in terms of dry matter yield and seed production.

A. Overview of the Infection Process

A brief overview of the infection process is presented to clarify the stages in nodule formation which may be important in determining the outcome of competition between strains of rhizobia. The symbiotic association formed between members of the genus Rhizobium and their leguminous hosts, involves a series of interdependent steps which begin exterior to the root and culminate in the establishment of nitrogen fixing root nodules. These steps can be separated into three main stages. These are: 1) preinfection; 2) infection and nodule formation; and, 3) nodule function (118). Whether or not this association results in an effective symbiosis depends on the genetic

make-up of both partners, their mutual compatibility, and the influence of the environment on them in both the free-living and symbiotic state.

Steps classified as preinfection events include, root colonization, attachment, branching, and marked curling of root hairs (118). Rhizobia in the free-living state are found in the rhizosphere of both legumes and non-legumes, as well as in the soil. In the presence of a host legume, rhizobia must be able to successfully compete against a variety of other soil microbes and colonize the root surface. Several studies have indicated that the numbers of rhizobia in the rhizosphere of legumes increases, in comparison to the numbers found in the rhizosphere of non-legumes (74,85,89,109). Whether this stimulation is specific for a particular species of Rhizobium remains questionable (for review see 94).

Initiation of infection in legumes has been extensively reviewed by Dart (25), Bauer (6), Grahman (37), and Schmidt and Bohlool (94). In general, for a successful infection to occur, Rhizobium must attach to root hair cells. However, there are several species of tropical legumes in which infection does not occur through root hairs (21). Only a very small percentage of rhizobia attached to root hair cells are able to initiate successful infections. This has led to the idea of specific recognition sites located in certain zones of the root. Recognition by a legume host for its specific microsymbiont is thought to involve specific carbohydrate-binding proteins, lectins, which serve in the binding and/or bridging of Rhizobium to the host root. One of the first visible indications that an interaction has occurred

is the elongation and deformation of root hairs, manifested as curling of the root hair tips (Shepherd's Crook) and marked branching. Root hair branching is a moderately specific response, while marked curling is more host specific (118).

The second stage in the infection process, infection and nodule formation, includes the following events: formation of infection threads, nodule development and differentiation, "intracellular" release of rhizobia and development of the bacteroid forms (for reviews see 6,25,118). Infections are usually restricted to areas on the root with deformed hairs, and occur in highly curled regions. Infections may also occur in straight root hairs or at a point of contact between two root hairs. Examination of attachment of rhizobia to root hair cells and epidermal cells has shown that only a small percentage of the attached organisms are able to initiate infection. Even fewer of these develop into functional nodules.

The first visible sign of infection is the presence of a localized swelling or refractile spot (for review see 25,30,74). This area is characterized by rapid cytoplasmic streaming and the presence of the root hair nucleus. Two major hypotheses have been proposed to explain the entry of rhizobia into the root hairs. These are: 1, invagination of the root hair cell wall, and, 2, direct penetration, which could involve either a mechanical rupture or enzymatic activity.

In the process of invagination, rhizobia become trapped within the highly curled region of the root hair. This stimulates the synthesis of new plant cell wall material and leads to the formation of a tubular infection thread, containing the entrapped rhizobia. The

localized production of enzymes or hormones is thought to be necessary for the process to occur. A study of the ultrastructure of infection thread formation supports this theory (72). However, this study failed to explain satisfactorily how invagination of the root hair cell occurs against the hydrostatic pressure of the cell cytoplasm (30).

A more recent study by Callaham and Torrey (20) on the mode of infection thread formation in Trifolium repens indicated that direct penetration of the root hair cell is involved. Electron micrographs showed that infection threads did not develop as an invagination of the root hair tip, but involved degradation of the root hair wall in the area in which the rhizobia were entrapped. The formation of the thread wall appeared to be the result of the apposition of a new, fibrillar layer of wall material around the site containing the rhizobia. The authors suggested the involvement of rhizobial enzymes in the degradative penetration of the root hair wall.

The involvement of hormones and enzymes, either of plant or rhizobial origin, in the infection process has been proposed by several investigators been proposed by several investigators (5,20,51,57,64,106,121,122). However, the involvement of hormones and enzymes remains an area of controversy, since efforts to validate the work of other investigators has often resulted in negative results. Although it has been possible to demonstrate the production of indole3-acetic acid (IAA), cytokinins, and gibberellin-like substances by rhizobia, the role of these hormones in the infection process has not been demonstrated (5,106,121,122,126). Recent work by

Badenoch-Jones et al (5) and Wang et al (121) indicated that IAA levels differed between effective and ineffective nodules. Thus, it was proposed that Rhizobium derived IAA may be a factor involved in nodule initiation and morphogenesis during the early stages of the infection process.

Following the initiation of infection, the infection thread grows through the root hair until it reaches the cells of the outer root cortex (for review see 25). Prior to the advance of the infection thread, meristematic activity occurs in the cortical cells, along with cell division. As the infection thread continues to grow into the root, it branches and ramifies through the cells in the inner cortex. Rhizobia are either released through vesicle formation, or through a break in the thread wall. Released rhizobia are enclosed by the cell membrane, the peribacteroid membrane, and remain separate from the host cell contents.

Detailed descriptions of legume nodule development and morphology may be found in reviews by Dart (25) and Sutton et al (105). The majority of nodule tissue differs from other plant cell tissue in being polyploid. It is generally accepted that polyploidy in nodules is a result of the infection process and is not due to the presence of preformed tetraploid cells. Vascular connections are found within the nodule cortex and connect with tissue in the central stele of the root.

After release from the infection thread, rhizobia undergo a period of multiplication (for review see 105). This is followed by development of the mature bacteroid state, in which both morphological

and physiological changes are evident. The most striking morphological change is in cell shape, since bacteroids are usually longer and often times, swollen and branched when compared to cells in the vegetative state. Nodule tissue containing mature bacteroids is characterized by high levels of nitrogenase activity, and a high leghemogloblin content.

The final stage, nodule function is concerned with the efficiency of conversion of nitrogen to ammonia, complementary biochemical functions required for an effective symbiosis, and persistence of nodule function. The genetic make-up of both partners affects the functioning of nitrogenase, as well as complementary biochemical functions. Nodule persistence is affected by the onset of reproduction, and the growth habit of the host (for review see 118). Some other factors affecting the efficiency of the symbiosis are: the presence of a hydrogenase uptake system (83), the activity of nodule phosphoenolpyruvate carboxylase (62,113), nature and exchange of nitrogen compounds between the shoot and root, and plant-provided enzymes (for review see 50).

B. Importance of Inter-strain Competition in Legume Inoculation

An important concern in legume cultivation is the inability of a selected inoculant strain to compete successfully with indigenous rhizobia (for reviews see 39,92,117). Inoculant strains are selected on the basis of their ability to fix dinitrogen effectively. The ability to fix nitrogen effectively varies significantly between strains. Some strains are highly effective nitrogen fixers, while others may be totally ineffective. In many instances, the indigenous

soil rhizobia are either partially effective or ineffective, but are highly competitive under field conditions. Thus, efforts to introduce highly effective strains as inoculum usually fail if a naturalized soil population of competitive rhizobia is already well established (1,18,40,53,124).

Competitive ability as a characteristic of a particular strain has been studied with several Rhizobium/legume symbioses. Results from these studies showed that the competitive ability of a strain varied with environmental conditions, inherent characteristics of a strain, and host genotypes. In spite of the many studies which have been done over the past few decades, no single factor is correlated with the competitive ability of a strain. Thus, the mechanisms that confer competitiveness to a strain remain poorly understood.

C. Influence of Environmental Factors on Competition between Strains of Rhizobium

In recent years, the effect of a variety of environmental factors on the outcome of competition between strains of Rhizobium for nodulation of a host legume has been examined. Some of these factors are: temperature (19,42,65,88,125), pH (31,41,113), soil composition (3 7,15,67), varying levels of mineral nitrogen (32,44,68,99), and the presence of other microorganisms in the rhizosphere (4,23,33,45,53,96,97,107). These studies have shown that the pattern of competition between some strains may be affected by one or more of the above factors. However, other influences, such as the host genotype, are also involved in determining the outcome of competition between strains of Rhizobium (29,50).

The influence of temperature on various aspects of the Rhizobium/legume association has recently been reviewed by Vincent (115). Growth and survival of Rhizobium strains, as well as nodule formation and function are influenced by temperature. A survey of the distribution of Rhizobium japonicum serogroups recovered from nodules of field grown soybeans showed that a shift in nodule occupancy occurred between early planting dates as compared to later planting dates (19). Similar results were obtained in a greenhouse study involving the same serogroups (125). In general, serogroups which predominated during the early planting dates, occupied a higher percentage of the nodules at lower temperatures. Similarly, serogroups recovered at later planting dates formed a greater proportion of the nodules at the higher temperatures (125). A similar survey on the distribution of R. leguminosarum serogroups in eastern Washington also showed that shifts in nodule occupancy of the two predominant serogroups were correlated with temperature and moisture (65). Root temperature affected the pattern of competition between strains of R. japonicum and indigenous Rhizobium spp isolated from Nigerian soils (88). At temperatures of 30°C or less, R. japonicum strains formed the majority of nodules on Glycine max cv. Malayan. However, at temperatures greater than 30°C, the majority of the Nigerian isolates were more competitive. Root temperature also affected the outcome of competition between two strains of R. trifolii(42). In this instance, however, significant soil/strain and strain/host interactions also affected the competitiveness of the two strains.

The major effect of pH is on the survival and growth of rhizobia. Soil acidity is an important factor in restricting the occurrence of rhizobia in soils, although there is considerable species variability in the degree to which acid conditions are tolerated (for review see 115). In a survey of R. japonicum serogroups, serogroup 123 occupied the majority of nodules in soils below pH 7.5. In contrast, isolates belonging to serogroup 135 predominated in soil where the pH was greater than 7.8 (24,41). Distribution of the other serogroups was not related to soil pH. An evaluation of the competitive abilities of strains of R. meliloti, indicated that the competitiveness of some of the strains was affected by soil acidity. Soil type and host cultivar also influenced the pattern of competition between the strains (112). The nodulating ability of two serogroups of R. trifolii, obtained from an indigenous soil population was also affected by pH. One of the groups formed the majority of nodules at a low pH, while the other group dominated at a high pH. An analysis of cellular proteins within the two groups, revealed four to six gel patterns. Isolates which were represented by one or two gel types, formed the majority of the nodules attributed to each serogroup (31). Thus, differences within a serogroup were also an important factor in determining the competitive ability of strains of R. trifolii.

A variety of physical and chemical characteristics are used to classify soils. Many of these properties can affect the soil microbial populations. Soil properties, such as pH, texture, and chemical composition may play a role in determining which rhizobial strains will persist and which will become the dominant population in

nodules of a particular host legume. However, it is difficult to identify any single property of a given soil to be the deciding factor in determining the competitive ability of a given strain.

An analysis of the percent of nodules formed by three inoculum strains of R. leguminosarum, in competition with naturalized populations of R. leguminosarum, indicated that there were significant effects due to strain, number and soil type (3). Competitive ability of the soil strains varied considerably from one soil to another, although numbers were similar. In another study, the pattern of competition between three strains of R. leguminosarum for nodulation of Lens esculenta was examined under growth chamber and field conditions (67). A commercial inoculant strain, Nitragin 92A3, consistently formed the majority of nodules on four cultivars under growth chamber conditions. However, in the field another strain, Hawaii 5-0, was equally competitive in an Inceptisol and superior to Nitragin 92A3 in an Oxisol. The distributions of two serogroups of R. japonicum, 110 and 123, were examined in a silt and a sandy loam (7). In the silt loam, the presence of serogroup 110 was correlated with organic matter and mineral nitrogen. However, this was not true for the sandy loam. The presence of serogroup 123 in the silt loam was positively correlated with soil pH, magnesium, and negatively correlated with organic nitrogen. In the sandy loam, the only consistent parameter for 123 was a negative correlation with organic nitrogen. In contrast to the above studies, the competitive abilities of inoculum strains of R. trifolii were not affected by soil types or plant age (15). In the first year, the inoculum strains occupied the

majority of the nodules. The occurrence of the strains in the nodules the following year was significantly less. The persistence of the commercial strains was related to host cultivar and levels of the indigenous R. trifolii population.

The influence of combined nitrogen on the process of nodulation and nitrogen fixation has been documented for several systems (for review see 115). Nitrate has been shown to delay the appearance of nodules, depress nodule number and mass, reduce the number of infection threads, and degree of infection thread curling. Nitrate also inhibits nitrogen fixation. Thus, in the presence of mineral nitrogen, it would be desirable to maintain an effective symbiosis in order to reduce the depletion of soil nitrogen.

The effect of added nitrogen on serogroup representation in soybean nodules was examined in soils in which soybeans had not been cultivated and in soils where soybeans had been grown for various lengths of time (99). Results from this study indicated that the addition of nitrogen had only a slight effect on serogroup distribution. Variations between sites were due to environmental factors or host cultivar. Low levels of nitrogen did not significantly affect nodulation of either alfalfa or soybeans (32,44). However, high levels of nitrogen significantly affected both nodulation and nitrogenase activity. In both studies, significant strain/nitrogen interactions with respect to nodulating ability were found. The pattern of competition between R. japonicum strains, USDA 110 and CB1809, was examined in the presence of varying levels of nitrate (68). Decreases in nodule mass and activity were observed

with higher levels of nitrate. Only the highest level of nitrate (10 mM) affected the outcome of competition between the two strains. R. japonicum strain CB1809 occupied a greater percentage of the nodules, than USDA 110 at this level of nitrate.

The introduction of inoculant strains into soils containing naturalized population of rhizobia is often unsuccessful, due to the failure of these strains to nodulate the host legume and persist (53, 124). Increasing the rate of inoculation has sometimes resulted in a greater percentage of the nodules being formed by the inoculum strains (123,124), but this is not always true for other strains (53). Poor nodulation by inoculum strains has been encountered in soils where naturalized populations of rhizobia are low or absent (45,80). The failure to establish inoculant strains may be the result of microbial antagonism in the legume rhizosphere. The addition of soil extracts to the rhizosphere of legumes grown under controlled conditions delayed the appearance of nodules and depressed nodule numbers (22,45, 80). Exudates from grasses inhibited rhizobia multiplication and decreased nodulation (80). Microorganisms isolated from soils and from the rhizosphere of legumes had an inhibitory effect on some Rhizobium strains growing on laboratory medium (4,23,107). Only a small minority of the microorganisms which inhibited rhizobia in laboratory medium, had an inhibitory effect on nodulation when present in the rhizosphere of the host legume (4,23,107). Bacteriocin producing strains of Rhizobium had a competitive advantage over sensitive strains in mixed culture in the laboratory (33,95,97). The significance of these findings to conditions in the soil is not known.

D. Attributes of the Microsymbiont Which May Play a Role in Competitiveness

The differential competitiveness of strains of Rhizobium for nodulation of their legume host has been recognized for many years (73). However, the cellular attributes which make one strain a better competitor than another remain largely unknown. The ability of a particular strain to survive in the soil in the absence of its host, to respond rapidly to the appearance of a suitable host and to initiate successful infections on the host are all part of the competitive ability of a strain.

A recent review of advances in the area of genetic engineering by Denarie et al (27) showed that some of the genes required for the symbiotic state are located on plasmids. In the fast-growing rhizobia, some of the genes controlling host range specificity, early steps in the infection process, and some of the metabolic functions of mature bacteroids (e.g. nitrogen fixation) have been located on plasmids. The transfer of symbiotic plasmids from R. leguminosarum into recipient strains indicated that both the plasmid and the background genotype of the recipient strain influence competition for nodulation (14). Survival and growth in the rhizosphere of the host depended entirely on the background genotype of the Rhizobium strain, and was not due to the introduction of the plasmid. Another example of the influence of the genetic make-up of Rhizobium on its symbiotic characteristics comes from the work of Gibson et al (35). An examination of isolates belonging to R. japonicum serogroup 123, indicated that these isolates represented a heterogenous population,

with at least one common antigen. It was also possible to differentiate the majority of these isolates based on symbiotic effectiveness, and nodule-forming ability (35). Thus, even within a serogroup, inherent characteristics of a given organism vary. Variation in symbiotic effectiveness between laboratory cultures and field isolates has also been observed within a single strain of R. trifolii (36).

It is much more difficult to correlate symbiotic properties with plasmid-borne genes in the slow-growing species of Rhizobium because of difficulties in consistently demonstrating the presence of plasmids in this group (for review see 27). R. japonicum mutants defective in nitrogen fixation and nodulating ability have been obtained, but the location of genes involved was not known. In another study however, R. japonicum isolates obtained from alkaline soils were placed into four groups based on the presence or absence of plasmids (38). The different plasmid groups could not be associated with any specific phenotypic characters required for symbiosis.

Some of the other factors thought to be involved in the competitive ability of a strain are: growth rate in the rhizosphere (73, 129), colonization and attachment to the root surface (34,82,84,111), and time to infection thread initiation (8,34,82). The dominance of a soybean and a pea strain in nodules was the result of the early rapid growth of both strains in the rhizosphere of their respective hosts (73). However, when fast- and slow-growing strains of cowpea rhizobia were compared, no relation between the growth rate of the strains in in vitro and the ability of these strains to compete

for nodule sites was found (129). In a related study, the population density of a highly competitive indigenous strain of R. japonicum was followed (84). Colonization of soybean roots by this strain was sparse and essentially the same as on non-host plants. The addition of another R. japonicum strain had no effect on the colonization ability of the indigenous strain. Thus, the competitiveness of this indigenous strain was not due to its ability to colonize the surface of its host legume.

The role of specific versus non-specific attachment as related to competition between strains has recently been reviewed by Schmidt (92). Differences in the selective adsorption of an infective strain over that of an non-infective strain of R. trifolii have been observed (26). A significant correlation was found between the ratio of cells firmly attached and those loosely attached, with respect to representation in the nodules on Medicago sativa (111). However, the use of attachment as an indication of nodulating success for a particular strain in the field did not hold, since the strain which was the best competitor in soil, was one of the worst under laboratory conditions.

Attempts to correlate the speed of nodule initiation with competitiveness have also been inconclusive. In one study (8), the more competitive strain nodulated more rapidly, while in other studies (34,82), this relationship was not found.

In general, attempts to correlate competitiveness with effectiveness or persistence have shown that these characteristics are unrelated. The outcome of competition between effective and

ineffective strains of R. trifolii favored the effective strains, regardless of differences in inoculum densities (86). Competition between effective and ineffective strains of R. japonicum also favored the effective strains when competed against an ineffective strain (29). However, there were marked differences in competitive ability between the effective strains. Significant cultivar/strain interactions with respect to nodulating success were observed(29). In contrast, results from other studies have shown that some ineffective strains were more competitive than effective strains in various Rhizobium/legume systems (34,73,82). A recent study using ineffective mutants and their effective parent strains demonstrated that competitive ability was not related to effectiveness (2). In most cases, the ineffective mutants retained the competitive ability associated with the wild-type parent and were equally competitive in nodulation trials. The outcome of competition between equally effective strains was host related.

E. Effect of the Legume Host on Competition between Strains

There is considerable evidence that the host plant plays a significant role in the selection of the "more competitive" strain. However, the mechanisms through which the host exerts its influence remain poorly understood. Several hypotheses have been proposed to explain the role of the host during the process of infection and nodulation (for reviews see 50,74,92,118). Some of these are: selective stimulation of one strain in the rhizosphere; presence of specific lectin binding sites on the root which are involved in the recognition of a particular strain; and the genetic background of the

host, which governs which strains are capable of forming an association and the effectiveness of the association.

Specific stimulation of the appropriate Rhizobium in the rhizosphere of the legume host as a prelude to infection is an appealing hypothesis, although little experimental evidence supports it (for review see 92). Evidence that the legume rhizosphere exerts a stimulatory effect on rhizobia has been obtained, but the secretion of a particular compound stimulatory for one Rhizobium species has not been substantiated. The only indirect evidence for specific stimulation of rhizobia comes from the work of van Egeraat (110). Under aseptic conditions, he found that a compound excreted from the roots of pea seedlings, homoserine, stimulated the growth of R. leguminosarum, but had little effect on rhizobia belonging to other cross-inoculation groups. Studies by Vincent and Waters (119), and Reyes and Schmidt (84) indicated that there was no specific stimulation of one Rhizobium strain over another in the rhizosphere of the host legume growing in either agar (119) or soil (84).

The concept of specific recognition sites on the surface of legume roots which would recognize the appropriate Rhizobium species was proposed by Bohlool and Schmidt (13). They observed that the majority of R. japonicum strains tested bound soybean seed lectin, but rhizobia belonging to other plant groups were not bound by lectin. Since then, other studies have either confirmed these findings or have not found any correlation between lectin binding and host specificity (for reviews see 6,94). One of the difficulties in establishing the significance of binding as a prelude to infection has been associated

with the isolation of lectins from roots. Root lectins are present in much lower quantities than seed lectins, and this has made it difficult to determine the distribution of lectins on the root surface. It is also possible that another step is required after binding, which involves either activation of a host or bacterial component, and that this step, has not yet been elucidated. Thus, the exact role of root lectins in the infection process remains unclear.

Observations of a host influence on the nodulating success of a strain, has come from several studies using different Rhizobium legume associations (29,43,55,87,1114,119). Competition trials with R. trifolii strains showed that although there were marked differences between the competitive ability of the strains used, there was also a significant effect due to host cultivar (55,87,114,119). In alfalfa, the outcome of competition between strains of R. meliloti was significantly influenced by the host. In particular, the competitive ability of one of the strains was enhanced when combined with one of the alfalfa cultivars (43). The competitive success of three R. japonicum strains, CB1809, CC709, CB1795, was also influenced by the host. On Glycine max cv. Hampton, strain CB1809 was the most competitive, whereas, it was the least competitive on the cv. Hardee (29).

Observations of host incompatibility with particular strains of Rhizobium, led to the discovery of some of the genes responsible (for review see Nutman 75). Soybean lines carrying the rj_1 gene in the homozygous condition fail to nodulate with most commercial strains of R. japonicum (28). The presence of the Rj_2 gene, a dominant gene,

causes an ineffective nodulation response with R. japonicum strains in serogroups c1 and 122. The association of soybean lines carrying the Rj_4 gene with R. japonicum strain 61 results in the formation of cortical proliferations instead of nodules (28). Examination of root colonization and nodule representation using Rj_2 compatible and incompatible strains on the Rj_2 soybean cv. Hardee, indicated that one of the incompatible strains was able to colonize the roots, but representation on the roots by the other incompatible strain was sparse (66). Nodules were formed primarily by the compatible strains. All of the strains were found to be equally competitive on a soybean line in which the Rj_2 gene was not dominant (66). Kvien et al (61) used several soybean lines in an attempt to enhance the recovery of inoculum strains under field conditions. Two of the lines recovered one of the introduced strains at enhanced levels. In clover, some of the genes controlling nodulation, time until nodules appear, nodule number and effectiveness have been found (75). Thus, for some Rhizobium/legume associations, specific host genes have been identified that play a major role in determining whether or not the association can occur, and the relative effectiveness of the association.

Other indications concerning the role of the host during the process of infection and nodulation come from the work of Nutman (74), Lim (63), Munns (71), Bhuvaneswari et al (9), Pierce and Bauer (81), and Singleton and Stockinger (101). Nutman (74) followed the rate and number of infection threads initiated in clover seedlings and found that the distribution of infectible hairs was not random, but occurred

in discrete zones. However, only a few of the infectible hairs developed into functional nodules. The number of hairs which developed into nodules depended on the host. Nutman (74) also observed that the presence of primary nodules suppressed further nodulation. Based on these results, he proposed that the host produces both "infection stimulating" and inhibitory substances which control the process of nodulation.

Lim's (63) results showed that two phases were involved in the infection process in clover. The first phase occurred prior to the appearance of nodules, while the second phase occurred after the initiation of primary nodules. After nodulation had been initiated, the number of rhizobia required to double the number of infections had to be significantly greater than that needed during the first phase. These results indicated that the total number of infectible sites was under host control. Work with the R. meliloti/alfalfa system supports the results obtained with the clover system (71). In alfalfa, the appearance of the first group of nodules inhibited or suppressed subsequent nodule formation. This suppression did not appear to be related to the supply of nitrogen from the nodules since the addition of nitrate did not prevent prior nodules from inhibiting further nodulation.

Bhuvaneswari et al (9) found that infectibility of soybean root hairs occurred in specific zones and was a transient property. Further work by Pierce and Bauer (81), showed that the number of nodules which developed on the developmentally younger sections of soybean roots were suppressed by older regions of the root which were

inoculated 15 hours earlier. Suppression of later developing nodules did not occur when heterologous rhizobia or UV-killed homologous rhizobia were used in place of living bacteria. Thus, suppression of further nodulation appears to be host-mediated and is specific with respect to the Rhizobium/legume symbiosis.

Singleton and Stockinger (101), working with the R. japonicum soybean system found that the soybean host compensated for low numbers of effective nodules by increasing the size of these nodules. When the soybean host was inoculated with varying ratios of ineffective and effective strains of R. japonicum the average size of an effective nodule was at least twice the size of an ineffective nodule. As the proportion of total effective nodules decreased, the size of these nodules increased. Thus, the host plant maintained the amount of effective nodule tissue even when the total number of effective nodules decreased.

CHAPTER III

INFLUENCE OF ENVIRONMENTAL FACTORS ON COMPETITION BETWEEN TWO STRAINS OF *Rhizobium japonicum*

Abstract

The effect of several biotic and abiotic factors on the pattern of competition between two strains of *Rhizobium japonicum,* USDA 110 and USDA123, was examined. In two soils from Minnesota, Waseca and Waukegan, USDA 123 occupied 69% and 24% of the nodules on *Glycine max* L. Merrill cv. Chippewa, grown without inoculation. Nodule occupancy for USDA 110 was 2% in the Waseca soil and 12% in Waukegan. Under a variety of other conditions - vermiculite, vermiculite amended with Waseca soil, and two Hawaiian soils devoid of naturalized *R. japonicum* strains -USDA 110 was more competitive than USDA 123. However, in another Hawaiian soil, containing a background population of soybean rhizobia, USDA 123 was more competitive and occupied 19% to 54% of the nodules on three soybean cultivars. Pretreatment of USDA 110 to soybean root exudate did not improve its competitiveness against other *Rhizobium japonicum* strains in this soil. The pattern of competition between the two strains was not affected by the addition of nitrate, or by inoculation of antibiotic-producing actinomycetes into the rhizosphere of soybeans.

Introduction

Environmental factors have been shown to influence the outcome of the symbiotic association formed between leguminous plants and members of the genus Rhizobium. Temperature (19,42,65,125), and various soil properties (7,24,68,99) have been found to affect the distribution of serogroups, and the nodulating ability of strains.

In an effort to determine which factors in the soil are important for the survival and nodulating success of the inoculum strain, several studies have focused on the problems of establishing selected inoculant strains in soils which contain a background population of indigenous rhizobia (1,15,24,41,123,124). In many soils of the north central United States, inoculation of soybeans with highly effective strains of R. japonicum, has failed to increase yields (for review see 39). This is partly due to the failure of the inoculant strains to displace highly competitive, yet less efficient, indigenous strains from the nodules. In particular, R. japonicum serogroup 123 is among the most successful of the native strains in these soils (24,41,84,124).

R. japonicum serogroup 123 formed the majority of nodules on soybeans in several Iowa soils, except for a few highly alkaline ones (24,41). Recovery of serogroup 123 from nodules also changed with planting dates (19). The percent of nodules on soybeans containing serogroup 123 decreased with the later planting dates. Attempts to correlate the distribution of USDA 123 with organic nitrogen levels, have been inconclusive. In one study, a negative correlation with

organic nitrogen was observed (7), while in another study, addition of combined nitrogen had no effect on nodule occupancy by USDA 123 (99).

Examination of root colonization by USDA 123, in field grown soybeans, showed that representation on the root surface by this and other indigenous strains was sparse (84). Its colonizing ability was not affected by nitrogen amendments or addition of another R. japonicum strain. Population densities of USDA 123 on the root surface of a nodulating and a non-nodulating soybean isoline, and on the root surface of a non-legume were also similar (84). In another study, susceptibility of USDA 123 to antimicrobial activity of actinomycetes isolated from soil was slight. Only two actinomycete isolates had an inhibitory effect on USDA 123 on laboratory medium (23).

In this study, the effect of some biotic and abiotic factors on the pattern of competition between two strains of R. japonicum, USDA 110 and USDA 123 was examined. R. japonicum strain, USDA 110 is a commercial inoculant strain, which increases fresh weight and soybean yields (16). However, inoculation with USDA 110 often does not result in yield increases. This is due to the failure of USDA 110 to form a significant proportion of nodules on field grown soybeans, when R. japonicum serogroup 123 is present in the soil (39, 84,124).

Materials and Methods

Bacterial Cultures. Culture of R. japonicum strains, USDA 110, USDA 123, USDA 138, and USDA 31, were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. R. japonicum strain, CB1809,

was obtained from the NiFTAL project, Paia, HI. Stock cultures were maintained on yeast-extract mannitol, YEM (11), slants at 28°C.

Soils. The two Minnesota soils, Waseca and Waukegan, were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. Both soils contain a background population of indigenous rhizobial strains, including R. japonicum serogroups, USDA 110 and USDA 123. Three Hawaiian soils were used. Two of the soils, Waimea and Kawaihae, were from the island of Hawaii, HI. The third soil was obtained from the Mauka Field Station, University of Hawaii, Oahu, HI. The Waimea soil was collected along the saddle road near the town of Waikii. Waimea soil is classified as an isothermic typic eutrandept (Inceptisol), pH 6.2, and is devoid of indigenous R. japonicum strains. The Kawaihae soil was collected along the northwestern coast of Hawaii, near the town of Kawaihae. The Kawaihae soil is classified as an medial isohyperthermic ustollic camborthid (Aridisol), pH 6.9, and also lacks indigenous R. japonicum strains. The soil from the Mauka Field Station is classified as an andic ustic humitropept soil (Inceptisol), pH 6.1, and contains a background population of indigenous R. japonicum strains.

Source of Soybean Cultivars. Seeds of Glycine max L. Merrill cv. Lee were obtained from H. H. Keyser, United States Department of Agriculure, Beltsville, MD. Soybean seeds of cv. Chippewa were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. Soybean seeds of cv. Davis, were obtained from the NiFTAL Project, Paia, HI. For all of the experiments, seeds were surface sterilized for 20 minutes in 4% calcium hypochlorite, and washed five times in

sterile water. The seeds were either pre-germinated on water agar plates, or planted directly into the soil.

Strain Identification. The percent of nodules occupied by each strain was determined by immunofluorescence. At least 25% of the nodules from each replicate was identified. Nodule smears were stained with strain-specific fluorescent antibodies (FA) according to the method of Schmidt et al (93). Gelatin-rhodamine isothiocyanate (Rh-ITC) was used to suppress non-specific adsorption (10). Colonization by actinomycete isolates in the soybean rhizosphere was determined by staining buried microscope slides with acridine orange. Microscopy was done as described previously (67).

Statistical Analyses. Analysis of variance and Duncan's multiple range tests were done using the GLM program from the SAS statistical package at the University of Hawaii, Honolulu, HI. For data given as percents, the values were converted to ranks before being analyzed.

Effect of Rhizosphere Microorganisms on Nodule Occupancy. To determine if the resistance of USDA 110 and USDA 123 to antibiotics was related to their competitiveness in Waseca soil, the sensitivity of the two strains to soil microorganisms associated with the roots of soybeans was examined. Nodule isolates of USDA 110 and USDA 123, were obtained as described by Vincent (116). Soil bacteria and actinomycetes associated with soybean roots were isolated following the methods of Waksman (120). Stock cultures were maintained on tryptone-yeast extract, TY (47), slants, containing 1.0 g of arginine per liter.

Background resistance of USDA 110 and USDA 123 to antibiotics of actinomycete origin was determined according to the methods of Pankhurst (76). Antibiotics used were: spectinomycin sulfate (Upjohn Company) at 1, 10, 50 and 100 micrograms per ml, chloramphenicol (Sigma) at 50, 100, 200, 250, 350 and 400 micrograms per ml, streptomycin sulfate (Sigma) at 10, 50, 100, 150 and 250 micrograms per ml, and, tetracycline HCl (Calbiochem-Behring Corp.) at 1, 50, 100, 150 and 200 micrograms per ml. The antibiotics were filter sterilized, and added to modified YEM, containing 1:4 strength mannitol. YEM plates were inoculated with 0.1 ml (1×10^8 cells/ml) of four-day-old YEM cultures using the spread plate method, and incubated at 28°C. Controls consisted of modified YEM, without antibiotics.

The formation of antibiotics by bacteria and actinomycetes isolated from the roots of soybeans, and the sensitivity of USDA 110 and USDA 123 to these products were examined on TY-arginine plates using the cross-streaking method (120). The root isolates were inoculated either one or two days before inoculation of USDA 110 and USDA 123. Plates were incubated at 28°C. Controls consisted of plates inoculated with sterile distilled water, in place of the root isolates.

The effect of four antibiotic-producing actinomycete isolates on the pattern of competition between USDA 110 and USDA 123 was examined in the presence of the soybean host. Uniform, two-day-old seedlings of cv. Chippewa were planted in sterile vermiculite moistened with 1:4 strength Hoagland nitrogen-free solution (48) in 250-ml Erlenmeyer flasks. The seedlings were inoculated with either 1.0 ml of a spore

suspension of the four isolates, singly, or with a mixture (1.0 ml) of the four isolates. Seven days later, 1.0 ml (1 x 10^9 cells /ml) of YEM broth, containing USDA 110 and USDA 123 was added to the flasks. After inoculation, the top of the vermiculite was covered with a 2-cm layer of sterile perlite and a 2-cm layer of paraffin coated sand. Plants were grown in a Sherer model CEL4-7 controlled environment growth chamber at 27^{o}C with a light intensity of 170 micro einsteins/cm^2/second and a photoperiod of 16 hours. Four weeks after the inoculation of USDA 110 and USDA 123, the plants were harvested and nodule occupancy determined. In a similar experiment, the pattern of competition between USDA 110 and USDA 123 was examined in vermiculite, either left unamended or amended with 1.0 g of Waseca soil.

Effect of Combined Nitrogen on Nodule Occupancy. To test the effect of available nitrogen on the pattern of competition between USDA 110 and USDA 123, three soils were selected. T hey were: Waimea, Kawaihae, and Waukegan. Available nitrogen in the soils was tied up by adding finely ground bagasse (sugar cane waste) to give a final concentration of 1.1% (wgt/wgt). Calcium nitrate was added at either 70 ppm (0.615g per 1500g soil) or at 300 ppm (2.636g per 1500g soil), to replenish available nitrogen in the three soils. The cadmium reduction method was used to determine nitrate levels in the soils (104). The percent moisture for the Waimea and Kawaihae soils was maintained at 0.1 bars water potential. The percent moisture for the Waukegan soil was maintained at 60$ of the water holding capacity of the soil.

At the time of planting, seeds cv. Chippewa) in the Waimea and Kawaihae soils were inoculated with 1.0 g (4×10^6 cells/seed) of peat, containing an equal mixture of USDA 110 and USDA 123. Soil surfaces were covered with a 2-cm layer of perlite. Plants were grown in a greenhouse, and harvested at pod-fill. Nodule number, dry weight, shoot dry weight, and nodule occupancy were determined.

Effect of Harvest Time on Nodule Occupancy. For both the field and greenhouse experiments, the inoculum strains were grown separately in modified YEM broth, containing 1:4 strength mannitol and yeast-extract. A peat inoculum, containing an equal number of the desired strains (1×10^8 cells/g) was used to inoculate the seeds. In all experiments, randomized complete block designs with three replicates were used.

Seeds of cultivars Chippewa, Lee and Davis, were planted at the Mauka Field Station, University of Hawaii. A peat inoculum, containing R. japonicum strains USDA 110, USDA 123, USDA 138, USDA 31 and CB1809, was used to inoculate the seeds. Plants were harvested after one, two and three months. For each time period, three to five plants per replicate per treatment were harvested and nodule occupancy determined.

In a similar experiment, soil from the Mauka Field site was collected and used in the greenhouse. In this experiment, however, only two harvest times were chosen. Plants were harvested after four and eight weeks. Nodule occupancy was determined for each harvest time.

Effect of Pretreatment of Inoculum Strain to Soybean Roots. R. japonicum strain USDA 110 was grown in a soybean root medium, to determine if there were any components present in the medium which would enhance the competitiveness of this strain. Two-day-old seedlings of cv. Lee were grown in a liquid culture system containing 1:4 strength Hoagland nitrogen-free solution (48). After three days, one set of the seedlings was inoculated with a washed cell suspension of USDA 110. Four days after the inoculation of USDA 110, the plants were harvested, and the roots plus the growth medium were homogenized in a blender. The resulting material was filter sterilized. Washed cells of USDA 110 were inoculated into either modified YEMS, soybean root medium, or soybean root medium preconditioned with USDA 110.

Three different peat mixtures were used to inoculate seeds of cv. Lee planted at the Mauka Field Station. For all mixtures, R. japonicum strains USDA 123, USDA 138, USDA 31, and CB1809 were grown separately in modified YEMS broth. The peat mixtures used were: 1, all five strains grown in modified YEMS, 2, USDA 110 grown in soybean root medium, and 3, USDA 110 grown in soybean root medium preconditioned with USDA 110. A randomized block design with three replicates per treatment was used. Plants were harvested at pod-fill and nodule occupancy determined.

Results

Effect of Rhizosphere Microorganisms on Nodule Occupancy. The pattern of competition between USDA 110 and USDA 123 in Waseca soil, and in vermiculite, left unamended or amended with Waseca soil is

Table III-1. Effect of abiotic/biotic components (Waseca soil) on pattern of competition between R. japonicum strains USDA 110 and 123

Treatment			% Total Nodules[a]			
Substratum	Amendment	Inoculum	110	123	110+123	Other
Waseca soil	None	None	2± 1	70± 1	2± 1	26± 1
Waseca soil	None	110	15± 1	60± 1	9± 1	16± 1
Vermiculite	None	110+123	53±16	31± 6	16±16	None
Vermiculite	1 g Waseca soil	None	34±12	24±12	5± 4	37± 4
Vermiculite	1 g Waseca soil	110+123	69± 9	10± 3	0	21± 6

[a]Pattern of competition between USDA 110 and 123 was not affected by the addition of gamma-irradiated or heat treated Waseca soil. Numbers are the Mean±S.E. of three replicates.

given in Table III-1. In the Waseca soil, USDA 123 was the more competitive strain, while in vermiculite, USDA 110 was more competitive than USDA 123. When 1.0 g of Waseca soil was used to inoculate the vermiculite, the percent of nodules occupied by the two strains was approximately equal. However, when the vermiculite was inoculated with both the soil and the two strains, the percent of nodules occupied by USDA 110 increased, while the percent of nodules occupied by USDA 123 decreased.

The pattern of antibiotic resistance of USDA 110 and USDA 123 to four antibiotics of actinomycete origin is given in Table III-2. USDA 110 was resistant to low concentrations of all four of the antibiotics tested. In contrast, three out of the four antibiotics tested, inhibited the growth of USDA 123.

Sensitivity of USDA 110 and USDA 123 to rhizosphere microorganisms isolated from the roots of soybeans is given in Table III-3. The growth of USDA 123 was inhibited by actinomycete isolates #2 and #3, while only actinomycete isolate #3 was found to inhibit the growth of USDA 110. None of the bacteria isolated from the roots of soybeans, inhibited the growth of either strain in culture.

Establishment of the four actinomycete isolates in the rhizosphere of soybeans did not alter the pattern of competition between USDA 110 and USDA 123 (Table III-4). USDA 110 was significantly more competitive than USDA 123 in either the presence or the absence of the four actinomycete isolates. Examination of buried microscope slides, indicated that the actinomycete isolates were able to colonize the soybean rhizosphere.

Effect of Combined Nitrogen on Nodule Occupancy. The addition of bagasse or calcium nitrate to the Waimea, Kawaihae, and Waukegan soils, did not change the percent of nodules occupied by USDA 110 and USDA 123 (Table III-5). In the Waimea and Kawaihae soils, USDA 110 was highly competitive and formed a significantly greater percent of the nodules than did USDA 123. In contrast, in the Waukegan soil, USDA 123 formed a greater percentage of the nodules than did USDA 110. However, the majority of the nodules in the Waukegan soil were occupied by other R. japonicum strains which did not react with the two FAs specific for USDA 110 and USDA 123.

Nodule number and dry weight were affected by the addition of nitrogen to the two Hawaiian soils (Table III-5). The addition of calcium nitrate, decreased nodule number and dry weight in these two soils. In the Waukegan soil, the addition of nitrate had no significant effect on nodule number or dry weight. The addition of bagasse or nitrate, had no observable effect on shoot dry weight in the three soils.

Nitrate levels for the three soils are given in Table III-6. Levels of nitrate in the unamended treatments and in the treatments amended with bagasse remained relatively constant over the duration of the experiment. Nitrate levels in the treatments which were amended with calcium nitrate increased on the fifth day of sampling. By harvest time, however, the level of nitrate in these treatments had decreased to the levels detected on the first day of sampling.

Table III-2. Pattern of antibiotic resistance of USDA 110 and 123 in culture.

Antibiotics	110	123
I. Chloramphenicol		
5 µg/ml	+	+
10 µg/ml	+	-
II. Spectinomycin		
10 µg/ml	+	-
50 µg/ml	-	-
III. Streptomycin		
10 µg/ml	+	-
50 µg/ml	-	-
IV. Tetracycline		
15 µg/ml	+	-
25 µg/ml	+	-

[a] + = growth
- = no growth

Table III-3. Effect of rhizosphere microorganisms on the growth of *R. japonicum* strains USDA 110 and 123 in culture.[a]

Rhizosphere Isolates	110	123
None	4+	4+
Actinomycete 1	2+	2+
Actinomycete 2	2+	0
Actinomycete 3	0	0
Actinomycete 4	2+	2+
Bacteria (4)	4+	4+

[a] 4+ = growth
2+ = partial growth
0 = no growth

Table III-4. Effect of actinomycetes on the pattern of competition between *R. japonicum* strains USDA 110 and USDA 123

Treatment[a]	% Nodule Occupancy[b] 110	123	Both
110+123	58±12	22±14	20± 3
110+123+Actinomycete #1	50± 0	20± 7	30± 7
110+123+Actinomycete #2	53±18	25±14	22± 4
110+123+Actinomycete #3	50± 4	20± 4	30± 7
110+123+Actinomycete #4	53± 4	35±14	12±11
110+123+Actinomycete #1-4	77±15	8±10	15± 9
	c	d	d

[a] Two to three replicates per treatment.

[b] Mean±S.E. Columns with the same letter (c-d) do not differ significantly (p=0.05). No significant differences (p=0.05) between treatments were found.

Table III-5. Effect of combined nitrogen [$Ca(NO_3)_2$] on competition pattern of *R. japonicum* strains USDA 110 and 123 in three soils

Soil	Amendment	Shoot[a] Dry Wgt (g) per plant	Nodule[a] Dry Wgt (mg) per plant	Nodule[a] Number per plant	Percent Nodule Occupancy[b] 110	123	110+123	Negative[c]
Waimea	None	4.0±0.2^{e}	502±104^{d}	115±16^{d}	66	24	10	None
(Inceptisol)	Bagasse	3.0±0.4^{e}	370± 29^{d}	56± 6^{e}	52	35	13	"
C/N = 9	+70ppm	2.2±0.2^{f}	282± 16^{e}	60±15^{e}	64	23	13	"
%N = 1.2	+300ppm	5.1±1.0^{d}	321± 84^{e}	120±11^{d}	53	38	9	"
					d	e	f	
Kawaihae	None	1.3±0.2^{g}	78± 18^{f}	60± 8^{f}	86	8	6	"
(Aridisol)	+70ppm	1.3±0.2^{g}	27± 8^{g}	20± 6^{g}	80	10	10	"
C/N = 9					g	h	h	
%N = 0.09								
Waukegan	None	3.1±0.7^{h}	195± 23^{h}	46± 6^{h}	12	24	13	51
	Bagasse	3.6±0.5^{h}	248± 83^{h}	65± 8^{h}	13	23	12	52
	+300ppm	3.3±1.2^{h}	246± 83^{h}	55±19^{h}	22	18	7	53
					ij	i	j	k

[a]Numbers are the Mean±S.E. of three replicates. Treatments followed by the same letter (d-h) do not differ significantly (p=0.05) within a given column.

[b]Differences between treatments, within a soil, were non-significant (p=0.05). Columns with the same letter (d-k), for each soil, do not differ significantly (p=0.05).

[c]Percent of nodules which did not react with two FA's.

Effect of Harvest Time on Nodule Occupancy. The percent of nodules occupied by the five R. japonicum strains on soybeans grown at the Mauka Field Station is given in Table III-7. USDA 123 occupied a significantly greater percent of the nodules on all three cultivars for the first two harvests. The percent of nodules occupied by USDA 138 on cultivars Lee and Davis, and by CB1809 on all three cultivars did not differ significantly from the percent of nodules containing USDA 123 for the final harvest. Under these conditions, USDA 110 was poorly competitive and was present in less than 10$ of the nodules on the three cultivars.

When soybeans were grown in the greenhouse, using soil collected from the field site, the pattern of competition between the strains was different (Table III-8). USDA 123, which was the most competitive strain in the field, was the least competitive in the greenhouse. The percent of nodules formed by the other four strains was similar on all three cultivars for both harvests. In contrast to the results obtained in the field, the percent of nodules occupied by unidentified soybean rhizobia was significantly greater than those occupied by the five inoculum strains.

Effect of Pretreatment of Inoculum Strain to Soybean Roots. Pre treatment of R. japonicum strain USDA 110 to soybean root media had no effect of the competitive ability of this strain in the field (Table III-9). As in the previous field experiment, USDA 123 was more competitive than the other four strains. The percent of nodules formed by unidentified soybean rhizobia varied from 1 to 15.

Table III-6. Effect of combined nitrogen [$Ca(NO_3)_2$] on competition pattern of *R. japonicum* strains USDA 110 and 123 in three soils: Nitrate Levels[a]

Soil	Amendment	g Nitrate per 1500 g Soil					
		Days after Inoculation: 0	2	5	8	10	37
Waimea	None	0.06	0.05	0.03	0.02	0.02	0.04
	Bagasse	0.02	0.02	0.03	0.01	0.02	0.05
	+ 70ppm	0.61	0.93	7.17	5.19	1.70	0.70
	+300ppm	1.74	9.39	9.81	39.30	42.30	1.50
Kawaihae	None	0.01	0.02	0.63	0.06	0.03	0.02
	+ 70ppm	0.98	1.32	8.90	3.32	3.48	0.80
Waukegan	None	0.04	ND	ND	ND	0.09	ND
	Bagasse	0.01	ND	ND	ND	0.04	ND
	+300ppm	1.15	ND	ND	ND	55.20	ND

[a]Values are the Means of three replicates. Plants were harvested at pod-fill (day 37). ND = not determined.

Table III-7. Competition pattern of R. japonicum strains in a Hawaiian inceptisol: field trial.[a]

Strains	Cultivar:	Lee			Chippewa			Davis		
	Harvest:	I	II	III	I	II	III	I	II	III
USDA 110		3^{de}	3^{j}	9^{mn}	1^{g}	2^{j}	3^{lm}	0^{f}	10^{hi}	11^{l}
USDA 123		36^{c}	29^{f}	19^{kl}	44^{c}	41^{h}	31^{k}	54^{c}	28^{g}	25^{k}
USDA 138		7^{d}	11^{ghi}	18^{lm}	8^{ef}	11^{i}	3^{lm}	6^{ef}	7^{i}	14^{kl}
USDA 31		6^{de}	10^{hif}	1^{o}	1^{g}	0^{j}	1^{m}	1^{f}	1^{j}	4^{m}
CB 1809		10^{d}	14^{gh}	11^{lm}	12^{e}	12^{i}	35^{k}	9^{ef}	20^{gh}	20^{kl}
Double Infection		31^{c}	15^{g}	22^{k}	24^{d}	16^{i}	21^{kl}	20^{d}	20^{gh}	13^{kl}
Triple Infection		4^{de}	4^{ij}	6^{n}	4^{f}	1^{j}	7^{lm}	0^{f}	1^{j}	2^{m}
Negative[b]		1^{e}	14^{gh}	14^{lm}	6^{ef}	17^{i}	0^{m}	10^{e}	13^{hi}	11^{l}

[a]For each cultivar, numbers within the same harvest, followed by the same letter (d-m) do not differ significantly (p=0.05).

[b]Percent of nodules which did not react with the five FA's.

Discussion

Under controlled growth conditions (e.g. growth chamber), the addition of Waseca soil to vermiculite did not change the pattern of competition between USDA 110 and USDA 123. The pattern of competition between the two strains was not altered by the presence of antibiotic producing actinomycete isolates in the rhizosphere of soybeans. Changes in available nitrogen in three different soils had no affect on the percent of nodules occupied by USDA 110 and USDA 123. However, the modulating ability of strains of R. japonicum was affected by host cultivar, soil type, and plant age.

The presence of antagonistic microflora has been postulated to affect the outcome of competition between strains of rhizobia (98). Studies by other investigators have shown that inhibition of Rhizobium by soil microorganisms may occur under laboratory conditions. However, the presence of inhibitory microorganisms in the legume rhizosphere has little effect on the nodulating ability of strains of rhizobia (23,107). In this study R. japonicum strains USDA 110 and USDA 123 were found to differ in their sensitivity to four antibiotics (Table III-2) and to actinomycete isolates associated with the roots of soybeans (Table III-3). Although, USDA 110 was more tolerant to antibiotics and to the four actinomycetes isolates, its competitive ability was not enhanced by the presence of the four isolates in the soybean rootzone (Table III-4).

The effect of combined nitrogen on the nodulating ability of strains is variable. In some studies, significant strain/nitrogen interactions have been found (44,68). In other studies, available

Table III-8. Percent nodule occupancy of R. japonicum strains in a Hawaiian inceptisol: greenhouse trial[a]

Strains	Cultivar:	Lee		Chippewa		Davis	
	Harvest:	I	II	I	II	I	II
USDA 110		12.5^{d}	12.0^{gh}	13.0^{l}	11.0^{o}	11.0^{rs}	15.0^{vw}
USDA 123		5.0^{e}	3.0^{jk}	3.0^{m}	5.0^{op}	1.0^{t}	1.0^{y}
USDA 138		15.0^{d}	7.0^{hij}	10.0^{l}	10.0^{o}	4.0^{st}	6.0^{x}
USDA 31		10.0^{d}	8.0^{ij}	15.0^{l}	7.0^{op}	20.0^{rs}	10.0^{wx}
CB 1809		11.0^{d}	9.0^{ghi}	11.0^{l}	4.0^{op}	10.0^{r}	8.0^{wx}
Double Infection		12.5^{d}	15.0^{g}	11.0^{l}	9.0^{o}	7.0^{st}	26.0^{u}
Triple Infection		1.0^{e}	1.0^{k}	1.0^{m}	1.0^{f}	1.0^{t}	0.0^{uv}
Negative[b]		33.0^{c}	45.0^{f}	36.0^{k}	53.0^{n}	46.0^{q}	34.0^{y}

[a]Values given are Means of three replicates. Numbers followed by the same letter (d-x) do not differ significantly (p=0.05) within a given column.

[b]Percent of nodules occupied by other R. japonicum strains, which did not react with the five FA's.

Table III-9. Effect of pretreatment of R. japonicum strain USDA 110 in its nodule occupancy in competition with four other strains: field experiment.

Pre-Treatment of USDA 110	Percent Nodule Occupancy[a]							
	110	123	138	CB1809	31	Double	Triple	Negative[b]
I. None	0	28	18	7	<1	29	3	15
II. Root Exudate	3	28	15	8	1	29	7	14
III. Root Exudate + USDA 110	2	23	16	10	1	30	3	15
	fg	c	d	e	g	c	f	d

[a]Differences between treatments, non-significant (p=0.05). Columns with the same letter (c-g) do not differ significantly (p=0.05).

[b]Percent of nodules which did not react with five FA's.

nitrogen had no effect on the nodulating ability of strains of rhizobia (32,99). Depletion of soil nitrogen by the addition of bagasse, or replenishment of soil nitrogen by the addition of calcium nitrate, had no effect on the percent of nodules occupied by USDA 110 and USDA 123 in the three soils (Table III-5). In the two Hawaiian soils, USDA 110 out-competed USDA 123, while in the Minnesota soil, USDA 123 was the more competitive strain.

Although, no significant strain/nitrate interactions were observed, significant strain/soil interactions were detected (Table III-5). Significant strain/soil interactions have been reported for the R. leguminosarum/Lens esculenta (67), R. leguminosarum/Vicia faba (3), R. japonicum/Glycine max (24) and the R. meliloti/Medicago sativa (112) associations. As observed by others (32,44,68, 71), the addition of nitrate reduced nodule number and dry weight in the two Hawaiian soils (Table III-5).

The results from the field and greenhouse studies (Tables III-7 and III-8), showed that host cultivar and plant age significantly affected the nodule occupancy of five R. japonicum strains. In the field, USDA 123 was highly competitive and occupied a greater percentage of the nodules than either of the four inoculum strains or the indigenous soybean rhizobia (Table III-7). In the greenhouse, however, the majority of the nodules were occupied by the indigenous soybean strains (Table III-8). Nodulating abilities of R. leguminosarum (67), R. meliloti (2,112) and R. japonicum (29,68) were also affected by the host cultivar. Changes in nodule occupancy of R. japonicum strains have also been reported (19).

Pretreatment of Rhizobium species with cowpea root exudate was found to improve the nodulating ability of one of the strains under laboratory conditions (8). However, similar results were not observed in this study. Pretreatment of USDA 110 to soybean root medium had no effect on the nodulating ability of this strain under field conditions (Table III-9).

The percent of nodules occupied by R. japonicum strains USDA 110 and USDA 123 was influenced by the conditions under which the soybean host was grown, the host cultivar, soil type and the time at which the plants were harvested. USDA 110 was more competitive than USDA 123 in vermiculite and in the Waimea and Kawaihae soils which lacked an indigenous soybean rhizobia population. USDA 123 was more competitive than USDA 110 in the Waseca, Waukegan and Mauka Field Station soils, all of which were characterized by the presence of an indigenous R. japonicum population. No single factor was found which explained the relative competitiveness of either strain under a given set of conditions.

CHAPTER IV

COMPETITION OF *RHIZOBIUM JAPONICUM* STRAINS IN THE EARLY STAGES OF SOYBEAN NODULATION

Abstract

Preexposure of soybean roots to poorly competitive strains of *Rhizobium japonicum* significantly enhances nodule occupancy by those strains. The effects of preexposure of soybean [*Glycine max* (L) Merr.] roots to strains of *Rhizobium japonicum* were investigated by using combinations of effective (USDA 110, USDA 138, and USDA 123) and effective/ineffective (USDA 110 and SM-5) strains. Strain USDA 110 was a better competitor than either USDA 138 or SM-5 on cultivars Lee and Peking. USDA 110 also out-competed USDA 123 on cultivar Chippewa, in a *Rhizobium japonicum*-free Hawaiian soil. However, when one of the three less competitive strains was inoculated onto 2-day old seedlings before USDA 110, the nodule occupancy of the less competitive strains increased significantly on all cultivars. With USDA 138 as the primary inoculum and USDA 110 delayed for 6, 48 and 168 hours, the incidence of USDA 138 in nodules increased on cultivar Peking from 6% (at zero-time) to 28%, 70% and 82% respectively, and on cultivar Lee from 17% (at zero-time) to 32%, 88% and 95% respectively, for the three time delays. Preexposure of 2-week-old roots of cultivar Lee to USDA 138 had essentially the same effect: the incidence of USDA 138 nodules increased from 23% at zero-time to 89% and 97% when USDA 110 was delayed for 24 and 72 hours, respectively. When the ineffective

strain SM-5 was used as the primary inoculum, followed by USDA 110 72 hours later, the percentage of nodules containing SM-5 increased from 7% to 76%. Preexposure of two-day-old seedlings of cultivars Lee or Chippewa to USDA 110, USDA 123 or SM-5 before transplantation into soil, was also found to alter the pattern of competition. In a Minnesota soil, preexposure of cultivar Chippewa to USDA 110, increased the percent of nodules occupied by USDA 110 from 2% to 55% after a 72 hour preexposure. Similarly, in a Hawaiian soil, nodule occupancy by USDA 123 increased from 7 to 33% after a 72 hour preexposure, while nodule occupancy by USDA 110 decreased from 62% to 39% for the two time periods. When seedlings were-preexposed to SM-5, similar results were obtained. These results indicate that the early events in the nodulation process of soybeans are perhaps the most critical for competition among strains of R. japonicum.

Introduction

Competition among strains of Rhizobium for nodulation of their legume host is an important aspect of the ecology of the root-nodule bacteria. This can be of agronomic concern when the desired inoculum strain fails to establish in nodules because of competition from indigenous, and often less efficient, rhizobia. This problem is highlighted in the soybean-growing regions of the United States, where inoculation with highly effective strains does not always result in yield increases. This is in part because of the failure of the inoculum strains to compete with resident rhizobia (40,61).

The mechanisms that confer competitive advantage to a strain are poorly understood. It is clear that many factors of both the microsymbiont and the host play important roles in determining which strain succeeds in occupying the majority of the nodules (39). Some of the characteristics of Rhizobium species that have been studied are effectiveness (29,54,82), numbers in inoculum (69,84,123,124), and bacteriocinogenicity (97). Other studies (17,29,67,74,119) have indicated that the host plant can play an important role in selecting the successful strain. None of these studies, however, have identified the essential factors or the critical stages in the infection process responsible for success or failure of a strain.

In this study delayed inoculation with specific strains was used to pinpoint the period of time in the nodulation process most critical to competition among strains.

Materials and Methods

Bacterial Cultures. Effective strains of R. japonicum, USDA 110, USDA 138, and USDA 123, were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. The ineffective strain, SM-5, was obtained from W. Brill, University of Wisconsin, Madison, WI. Rhizobium cultures were maintained on yeast-extract-mannitol, YEM (11) slants at 28°C.

Soils. The Minnesota soil, Waseca, containing a background population of indigenous rhizobial strains, was obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. The Hawaiian soil, Waimea, was from the island of Hawaii, HI. The Waimea soil was collected along the saddle road near the town of Waikii. Waimea is classified as an isothermic typic eutrandept (Inceptisol), pH 6.2, and is devoid of indigenous R. japonicum strains.

Source of Soybean Cultivars. Seeds of Glycine max L. Merrill cultivars Lee and Peking, were obtained from H. H. Keyser, United States Department of Agriculture, Beltsville, MD. Lee is a commercial cultivar, and Peking is an unimproved cultivar reported to exclude USDA 110 under field conditions if other R. japonicum strains are present (17,60). Soybean seeds of cultivar Chippewa, were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. For all of the experiments, seeds were surface sterlized for 20 minutes in 4% calcium hypochlorite, washed five times in sterile water and pregerminated in sterile vermiculite.

Preexposure of Soybean Roots: in Vermiculite. Two-day-old seedlings, with radicles of 2 to 3 cm, were selected for the

experiments. They were planted in sterile vermiculite moistened with 1:4 strength Hoagland's nitrogen-free solution (48), in 250 ml Erlenmeyer flasks. The seedlings were inoculated with 1.0 ml (1×10^9 cells/ml) of four day-old YEM broth cultures at the designated times. After primary inoculation with the desired strain(s), the top of the vermiculite was covered with a 2 cm layer of sterile perlite anda 2 cm layer of paraffin-coated sand. A 19-gauge needle, pushed through a rubber stopper into the flask, facilitated inoculation and watering.

In the time course experiment with USDA 110 and USDA 138, three different treatments were used for two-day-old seedlings of cultivars Lee and Peking. At zero-time, one group (six plants) received both strains, another group received only USDA 110, and the last group received USDA 138. At selected times (6, 48, and 168 hours) thereafter, three flasks each from groups 2 and 3 received the other strain as the secondary inoculum. Controls consisted of uninoculated seedlings and seedlings inoculated with only one strain at each time period. Plants were grown in a Sherer model CEL4-7 controlled-environment growth chamber at 27°C with a light intensity of 170 micro einsteins/cm^2 per second and a photoperiod of 16 hours. Four weeks after the secondary inoculation, the plants were harvested, and the nodules were separated into early (taproot) and late (lateral root) groups based on their location on the root and into large (>1 mm) and small (<1 mm) groups based on their size. Of these, approximately 32 to 72% were classified as early nodules. A similar experiment was done with two-week-old plants (cv. Lee), instead of seedlings. The design of the experiment with the ineffective strain,

SM-5, was essentially the same, except that the secondary inoculum was introduced 72 hours after the primary one.

Effect of Preexposure of Soybean Roots on Rhizosphere Colonization. An experiment, similar to the ones described above, was designed to determine if the introduction of one strain at zero time had an effect on colonization of the rootzone by the delayed strain. At zero time, two-day-old seedlings of cultivar Lee were inoculated as follows: one group (six plants) received both strains, another group received USDA 110, and the last group received USDA 138. Ninety-six hours later, groups 2 and 3 received the other strain as the secondary inoculum.

One week after the inoculation of the second strain, three plants from each of the groups were harvested. Roots were divided into three zones (measured from the crown): zone I, first 5 cm; zone II, second 5 cm; and zone III, the last 5 cm, and examined for the presence of the two strains. Four weeks after the inoculation of the second strain, the remaining plants were harvested. Nodules were separated into two groups based on their location on the roots. These were: zone I, first 5 cm; and zone II, the second 5 cm. A third zone, zone III, was also noted, but nodulation in this zone was rare.

Effect of Binding of Gamma-Irradiated Cells on Nodule Occupancy. To determine if binding of gamma-irradiated cells to soybean roots would block nodulation by another strain, irradiated cells of USDA 138 were used as the primary inoculum. Five mls of a four-day-old YEM culture of USDA 138 were inoculated on YEM flats and incubated at 28℃. Four days later, the cells were harvested and gamma-irradiated (1.5 x

10^6 Rads). After irradiation, the cells were checked for viability on YEM plates. The presence and organization of lectin binding material was observed before and after irradiation.

Seeds of soybean cultivar Lee were surface sterilized and pregerminated on water agar plates. Two-day-old seedlings were selected and divided into two groups. Group one was placed in flasks containing Hoagland nitrogen-free solution (48). Group two was placed in flasks containing Hoagland nitrogen-free solution and gamma-irradiated cells of USDA 138. At zero time, the first group received either USDA 138, or both strains. Seventy-two hours later, plants which received USDA 138 at zero time received USDA 110. The second group was either left uninoculated, or received USDA 138, USDA 110, or both strains at zero time. Seventy-two hours later, flasks from the uninoculated group, received the other strain as the secondary inoculum.

Preexposure of Soybean Roots: in Soil. An experiment was designed to determine if preexposure of soybean seedlings to USDA 110 would enhance its competitiveness in the Minnesota soil, Waseca. Two-day-old seedlings of cultivar Chippewa, in vermiculite, were inoculated with a four-day-old YEM broth culture of USDA 110. Seedlings were removed after 2, 48, and 72 hours and transplanted into pots containing Waseca soil. Controls consisted of uninoculated seedlings and seeds which received a peat inoculum (2×10^8 cells/g) of USDA 110 at the time of planting. The percent moisture was maintained at 60% of the water holding capacity of the soil. Plants were grown in a greenhouse and harvested at pod-fill.

In a similar experiment, preexposure of soybean seedlings to USDA 110, USDA 123, or SM-5, was examined in the originally Rhizobium free soil, Waimea. The inoculum strains were grown separately for ten days in peat and then introduced into the soil. Mixtures of different peat cultures were made to contain equal numbers of the desired strains. The peat preparations were mixed in with the soil and allowed to equilibrate for 24 hours before planting of the seedlings.

Two-day-old seedlings of cultivar Chippewa were inoculated with USDA 110 only, USDA 123, or SM-5. After 2, 24, 48, and 72 hours, the seedlings were transplanted into the soil. The percent of moisture in the soil was maintained at 0.1 bars water potential. Plants were harvested at four weeks and at pod-fill. For both harvests, nodule number, dry weight, nitrogenase activity, shoot dry weight, and nodule occupancy were determined.

Determination of Nitrogenase Activity. Nitrogenase activity was determined by the acetylene reduction assay (108). Acetylene reduction was determined at 25°C by incubating roots in jars with 10% (v/v) acetylene for 15 and 30 minutes. Gas samples were analyzed for ethylene with a Bendix 2500 Gas Chromatograph equipped with a Porapak-T column (80-100 mesh).

Identification of Strains. The percent of nodules occupied by each strain was determined by immunofluorescence. At least 25% of the nodules from each replicate per treatment were identified. Nodule smears were stained with strain-specific fluorescent antibodies (FA) according to the method of Schmidt et al (93). Gelatin-rhodamine isothiocyanate (Rh-ITC) was used to suppress non-specific adsorption

(11). For the examination of lectin binding material, smears of USDA 138 were stained with fluorochrome-labeled lectin according to the method of Bohlool and Schmidt (13). For the examination of rhizosphere colonization, roots were stained for 30 minutes with strain-specific FA, followed by a 30 minute rinse in phosphate-buffered-saline (PBS), pH 7.0. Roots were counterstained with crystal violet (100 mg/1, dissolved in PBS) for five minutes and rinsed three times in PBS (49). Root segments were mounted in PBS, pH 9.0, for microscopic examination. Microscopy was done as described previously (67).

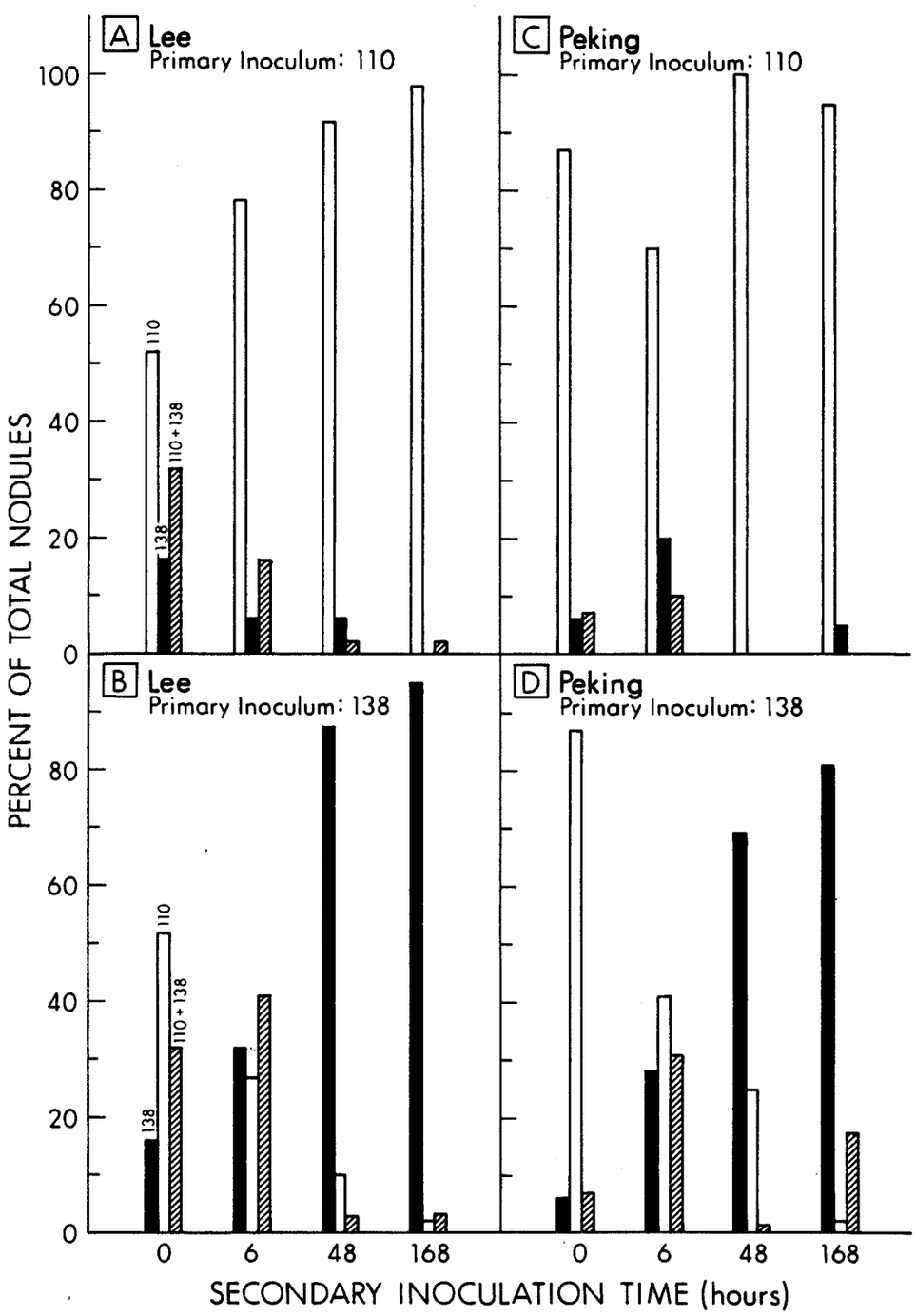
Statistical Analyses. Analysis of variance and Duncan's multiple range tests were done using the GLM program from the SAS statistical package at the University of Hawaii, Honolulu, HI. For data given in percentages, the values were converted to ranks before being analyzed.

Results

Effect of Preexposure of Soybean Roots: in Vermiculite. Nodule occupancy of strains, USDA 110 and USDA 138, did not differ for early (small or large) or late (small or large) nodules. In groups receiving both strains simultaneously, USDA 110 occupied the majority of the nodules regardless of size and location. Likewise, in the delayed inoculation experiments, the pattern of nodule occupancy of the strains was found to be the same. Therefore, the percentage of nodules occupied by the two strains were pooled (Figure IV-1).

When seedlings were inoculated with both strains at the same time, USDA 110 was more competitive and occupied the majority of the

Figure IV-1. Effect of preexposure of roots to an effective strain of R. japonicum on nodule occupancy by another effective strain. (A) and (C), Glycine max cultivars Lee and Peking, respectively, with USDA 110 as primary and USDA 138 as secondary inoculum; open bars, USDA 110; solid bars, USDA 138; hatched bars, USDA 110 and USDA 138. (B) and (D), cultivars Lee and Peking, respectively, primary inoculum USDA 138, secondary inoculum USDA 110; solid bars, USDA 138; open bars, USDA 110; hatched bars, USDA 110 and USDA 138.


A Lee
Primary Inoculum: 110
C Peking
Primary Inoculum: 110
B Lee
Primary Inoculum: 138
D Peking
Primary Inoculum: 138
PERCENT OF TOTAL NODULES
100
80
60
40
20
0
110
110+138
138
0
6
48
168
SECONDARY INOCULATION TIME (hours)

nodules on both cultivar Lee (52% ± 16.5, standard error) and cultivar Peking (87% ± 16.5). Approximately 30% of the nodules on cultivar Lee and 10% of the nodules on cultivar Peking contained both strains. When USDA 138 was allowed a headstart in the rootzone (Fig. IV-1, B and D), its ability to compete against USDA 110 was improved. The 48 hour delay in the introduction of USDA 110 enabled USDA 138 to become dominant and occupy 69% of the nodules on cultivar Peking and 87% of the nodules on cultivar Lee. Even with a 6 hour delay in the introduction of USDA 110, the nodule occupancy of USDA 138 increased substantially. When USDA 110 was used as the primary inoculum (Fig. 1V-1, A and C), it completely out-competed USDA 138 at every time point.

In the experiment with two-week-old plants (cv. Lee), when USDA 138 was introduced 24 hours before USDA 110, its nodule occupancy increased significantly (Table N-1). A 72 hour preexposure to USDA 138 resulted in virtual elimination of USDA 110 from the nodules.

The results from the experiment using the effective strain, USDA 110, and the ineffective strain, SM-5, are given in Table IV-2. When the two strains were introduced together, USDA 110 occupied the majority of the nodules. However, when SM-5 was introduced 72 hours before USDA 110, its nodule occupancy increased to 76%. The total number of nodules formed by SM-5 was greater than that formed by USDA 110, whereas the total nodule mass per plant was much less for SM-5 formed nodules than for USDA 110 formed nodules. Plants which were inoculated with SM-5 at zero-time, and then with USDA 110 72 hours later, rained chlorotie (yellow) for approximately one week or more

Table IV-1. Effect of preexposure of two-week-old roots to a poorly competitive strain (USDA 138) on nodule occupancy of a highly competitive strain (USDA 110) of R. japonicum.

Inoculum[a]		No. of nodules[b] per plant	Nodule Occupancy (% of total)[c]		
Primary (zero time)	Secondary (hours)		USDA 110	USDA 138	USDA 110 and USDA 138
USDA 110	None	ND	100[d]	0[e]	0[f]
USDA 138	None	ND	0[g]	100[h]	0[f]
USDA 110+USDA 138	None	104±24	47[i]	23[j]	30[h]
USDA 138	USDA 110 (24)	87±14	8[g]	89[h]	3[f]
USDA 138	USDA 110 (72)	121± 7	0[g]	97[h]	3[f]

[a] All uninoculated controls were devoid of nodules.

[b] Values are the means of three replicates ± S.E. ND, not determined.

[c] Values are the means of three replicates. Numbers followed by the same letter (d-f) within a given column do not differ significantly (p=0.05).

Table IV-2. Effect of preexposure of roots to an ineffective poorly competitive strain (SM-5) on nodule occupancy by an effective highly competitive strain (USDA 110) of R. japonicum.

Inoculum[a]		Total Nodule Mass (mg)[b]	No. of Nodules per plant[b]	Nodule Occupancy (% of total)[b]		
Primary (zero time)	Secondary (72 hours)			USDA 110	SM-5	USDA 110 and SM-5
USDA 110	None	53^{cd}	25^{e}	100^{f}	0^{g}	0^{h}
SM-5	None	32^{d}	57^{ij}	0^{k}	100^{i}	0^{h}
None	USDA 110	64^{c}	40^{ej}	100^{f}	0^{g}	0^{h}
None	SM-5	39^{d}	73^{i}	0^{k}	100^{l}	0^{h}
USDA 110+SM-5	None	49^{cd}	28^{e}	89^{m}	7^{n}	3^{h}
None	USDA 110+SM-5	53^{cd}	31^{e}	92^{mo}	9^{n}	0^{h}
USDA 110	SM-5	44^{cd}	18^{e}	96^{fo}	4^{gn}	0^{h}
SM-5	USDA 110	52^{cd}	41^{ej}	18^{p}	76^{q}	5^{h}

[a] All uninoculated controls devoid of nodules.

[b] Values are means of three replicates. Numbers followed by the same letter (c-q) within a given column do not differ significantly (p=0.05).

longer than those which were inoculated with both strains at zero-time, or which received USDA 110 as primary inoculum.

Effect of Binding of Gamma-Irradiated Cells on Nodule Occupancy. Saturation of soybean roots with gamma-irradiated cells of USDA 138 did not prevent nodulation by live cells of USDA 110 or USDA 138 (Table IV-3). The presence of gamma-irradiated cells also had no effect on nodule occupancy by either strain. When the two strains were introduced together, USDA 110 occupied the majority of the nodules. As was found previously, when USDA 138 was used as the primary inoculum, its nodule occupancy increased significantly. Examination of cells of USDA 138 after irradiation, showed that lectin binding material was still present, but unevenly distributed on the cell surface, as compared to the unirradiated cells, which were evenly surrounded by the lectin binding material.

Effect of Preexposure of Soybean Roots on Rhizosphere Colonization. When USDA 110 and USDA 138 were introduced together, the numbers of USDA 110 in the rhizosphere was slightly greater than that observed for USDA 138 (Table IV-4 and Figure IV-2). The introduction of USDA 138 96 hours before USDA 110, had little effect on rhizosphere colonization by USDA 110. When USDA 110 was used as the primary inoculum, the number of USDA 138 cells in the rhizosphere decreased, compared to that observed when both strains were introduced at zero time. Preexposure of soybean roots to either strain, however, significantly increased the percent of nodules occupied by that strain (Table IV-4).

Effect of Preexposure of Soybean Roots: in Soil. In the

Table IV-3. Effect of binding of gamma-irradiated cells of one strain (USDA 138) on nodule occupancy of another strain (USDA 110) of R. japonicum.

Pretreatment[a] (USDA 138)	Inoculum		Nodule Occupancy (% of total)[c]		
	Primary (zero time)	Secondary[b] (hours)	USDA 110	USDA 138	USDA 110 and USDA 138
None	None	USDA 110 + 138 (0)	51[d]	21[h]	28[k]
None	None	USDA 110 + 138 (72)	56[d]	25[h]	19[k]
None	USDA 138	USDA 110 (72)	7[e]	92[i]	1[l]
Gamma-irradiation	USDA 138	USDA 138 (0)	0[g]	100[i]	0[l]
"	USDA 138	USDA 110 (0)	100[f]	0[j]	0[l]
"	USDA 138	USDA 138 (72)	0[g]	100[i]	0[l]
"	USDA 138	USDA 110 (72)	100[f]	0[j]	0[l]
"	USDA 138	USDA 110 + 138 (0)	60[f]	17[h]	23[k]
"	USDA 138	USDA 110 + 138 (72)	53[d]	23[h]	24[k]

[a]Cells were killed by the irradiation.

[b]Secondary inoculation of live cells were at either zero time or 72 hours after primary inoculum.

[c]Values are means of two to three replicates. Numbers followed by the same letter (d-l) do not differ significantly (p=0.05) within a given column.

Table IV-4. Effect of preexposure of soybean roots on rhizosphere colonization by a poorly competitive (USDA 138) and a highly competitive (USDA 110) strain of R. japonicum.

Inoculum			Degree of Colonization by:[b]		No. of Nodules[c] per zone per plant	Nodule Occupancy (% of total)[d]		
Primary (zero time)	Secondary (96 hours)	Zone[a]	USDA 110	USDA 138		USDA 110	USDA 138	USDA 110 + 138
USDA 110	None	I	4+	±	16± 4			
		II	2-4+	±	7±12			
		III	±	±	0± 0	100[e]	0[i]	0[m]
USDA 138	None	I	±	4+	9± 1			
		II	±	2-4+	0± 0			
		III	±	±	0± 0	0[h]	100[j]	0[m]
USDA 110+138	None	I	4+	±-3+	15± 3			
		II	±-3+	±-3+	0± 0			
		III	±	±	0± 0	57[f]	30[l]	13[n]
USDA 138	USDA 110	I	2-3+	1-3+	22±13			
		II	±-1+	±-1+	1± 1			
		III	±-1+	±-1+	0± 0	12[g]	61[k]	27[n]
USDA 110	USDA 138	I	4+	±-2+	19± 6			
		II	1-3+	±-2+	8± 8			
		III	±-1+	±-1+	0± 0	100[e]	0[i]	0[m]

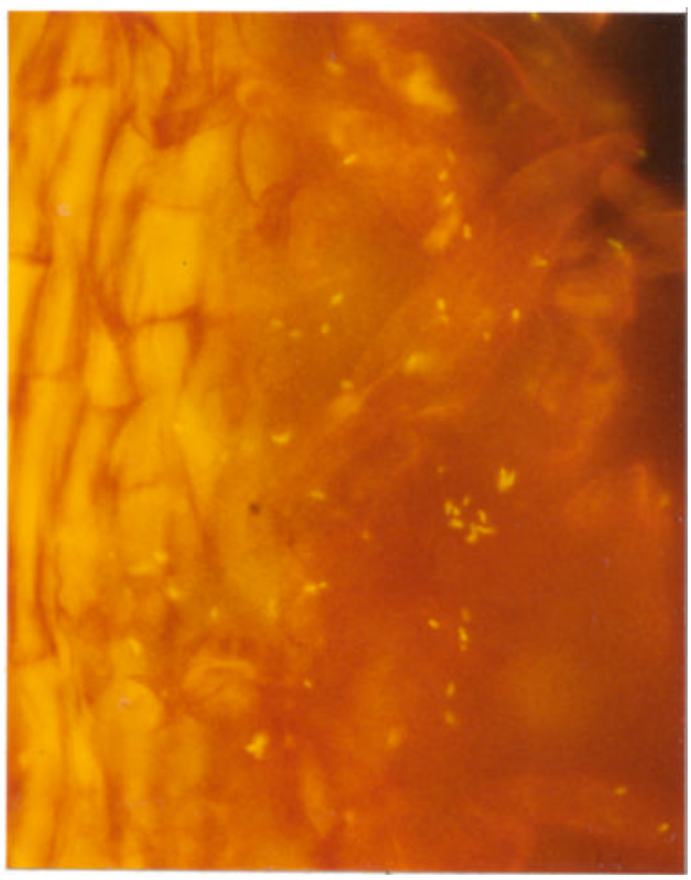
[a]Root zones defined as: I= first 5 cm; II=second 5 cm; III=last 5 cm.

[b]Colonization rating: 4+=>100 cells/field; 3+=25 to 100 cells/field; 2+=10 to 25 cells/field; 1+=<10 cells/field; ±=0 cells/field.

[c]Values are Mean±S.E. of three replicates.

[d]Values are means of three replicates combined with respect to zones. Numbers followed by the same letter (e-n) do not differ significantly (p=0.05) within a given column.

Figure IV-2. Fluorescent antibody stain of USDA 138 colonizing the surface of roots, zone I. Taken from the treatment in which both strains were inoculated at zero-time.



midwestern soil, Waseca, preexposure of soybean seedlings (cv. Chippewa) to USDA 110, significantly increased nodule occupancy by this strain (Table IV-5). Even a two hour preexposure to USDA 110 resulted in a significant increase in nodule occupancy by USDA 110. When seedlings were preexposed to USDA 110 for 48 hours or longer, USDA 110 became the dominant strain in the nodules. The majority of nodules on plants which were not preexposed to USDA 110, or which received USDA 110 as a peat inoculum, were occupied by R. japonicum strain USDA 123.

The patterns of competition between USDA 110, USDA 123, and SM-5 in the Hawaiian soil Waimea, are given in Tables IV-6 and IV-7. USDA 110 was the most competitive of the three strains, and formed 62% of the nodules on plants harvested after four weeks (Table IV-6) and 73% of the nodules on plants harvested at pod-fill (Table IV-7). The percent of nodules occupied by USDA 110, was significantly increased when seedlings were preexposed to USDA 110 for two or more hours. Similarly, preexposure of young seedlings to USDA 123 caused a significant increase in the percent of nodules occupied by USDA 123 for both harvest I (Table IV-6) and harvest II (Table IV-7). Preexposure of young seedlings to the ineffective strain, SM-5, also resulted in an increase in nodule occupancy by this strain for the first harvest (Table IV-6).

Nodule number, dry weight, nitrogenase activity and shoot dry weight for harvest I and II are given in Table IV-8. Differences between the three strains in nodule number, dry weight and nitrogenase activity were found. Nodule number, dry weight and nitrogenase

Table IV-5. Effect of preexposure to USDA 110 on competition pattern of *R. japonicum* strains in a soil containing indigenous *R. japonicum* (Waseca).

Inoculum[a]	Preexposure Time	Nod. No.[b] per plant	Nodule Occupancy (% of Total)[b]			
			USDA 110	USDA 123	USDA 110 + 123	Other[c]
None	None	127[d]	2[g]	70[j]	2[l]	26[m]
110 Peat Inoculated	None	127[d]	15[h]	60[j]	9[l]	16[m]
USDA 110	2 hours	163[f]	26[h]	46[j]	4[l]	24[m]
USDA 110	48 hours	120[de]	51[i]	26[k]	6[l]	17[m]
USDA 110	72 hours	75[e]	55[i]	27[k]	7[l]	11[m]

[a]Values are means of four replicates.

[b]Numbers followed by the same letter (d-m) within a given column to not differ significantly (p=0.05).

[c]Percent of nodules which did not react with two FAs specific for USDA 110 and USDA 123.

Table IV-6. Effect of preexposure to R. japonicum strains on competition pattern in a Rhizobium-free soil (Waimea): Harvest I.

Soil Inoculated with	Preexposure Treatment: Strain	Time (hours)	Nodule Occupancy (% of total)[a] USDA 110	USDA 123	SM-5	USDA 110 + 123	USDA 110 + SM-5	USDA 123 + SM-5
USDA 110	None	0	100^{b}	0^{e}	0^{i}	0^{m}	0^{r}	0^{t}
USDA 123	None	0	0^{d}	100^{f}	0^{i}	0^{m}	0^{r}	0^{t}
SM-5	None	0	0^{d}	0^{e}	100^{j}	0^{m}	0^{r}	0^{t}
110/123/SM-5	None	0	62^{b}	7^{e}	13^{kl}	7^{np}	8^{s}	3^{t}
110/123/SM-5	123	2	39^{b}	33^{f}	11^{kl}	0^{m}	13^{rs}	4^{t}
"	123	24	40^{b}	25^{f}	17^{kl}	8^{oq}	7^{rs}	3^{t}
"	123	48	31^{b}	31^{f}	13^{kl}	17^{no}	7^{rs}	1^{t}
"	123	72	22^{c}	35^{f}	11^{kl}	22^{n}	6^{rs}	4^{t}
"	SM-5	2	52^{b}	6^{g}	27^{jk}	1^{moq}	13^{s}	1^{t}
"	SM-5	24	55^{b}	0^{e}	33^{jk}	1^{moq}	10^{s}	1^{t}
"	SM-5	48	44^{b}	4^{gh}	32^{jk}	0^{m}	18^{s}	2^{t}
"	SM-5	72	37^{b}	1^{eh}	43^{jk}	0^{m}	18^{s}	1^{t}

[a]Values are means of three replicates. Numbers followed by the same letter (b-t) do not differ significantly (p=0.05) within a given column. Duncan's Multiple Range test used to distinguish between treatments within a strain.

Table IV-7. Effect of preexposure to R. japonicum strains on competition pattern in a Rhizobium-free soil (Waimea): Harvest II

Soil Inoculated with	Preexposure Treatment: Strain	Time (hours)	Nodule Occupancy (% of total)[a] USDA 110	USDA 123	SM-5	USDA 110 + 123	USDA 110 + SM-5	USDA 123 + SM-5
USDA 110	None	0	100^{b}	0^{j}	0^{o}	0^{t}	0^{u}	0^{v}
USDA 123	None	0	0^{i}	100^{k}	0^{o}	0^{t}	0^{u}	0^{v}
SM-5	None	0	0^{i}	0^{j}	100^{p}	0^{t}	0^{u}	0^{v}
110/123/SM-5	None	0	73^{cde}	7^{m}	7^{qr}	5^{uv}	6^{vwxy}	2^{vw}
110/123/SM-5	123	2	68^{def}	17^{l}	3^{ors}	8^{uv}	3^{uwxy}	1^{vw}
"	123	24	62^{def}	15^{l}	10^{q}	7^{uv}	4^{uvwxy}	2^{vw}
"	123	48	47^{fgh}	22^{l}	19^{q}	7^{uv}	2^{uyx}	3^{vw}
"	123	72	55^{efg}	18^{l}	14^{q}	5^{uvw}	4^{uvwxy}	4^{w}
"	SM-5	2	71^{efg}	4^{mn}	6^{qrs}	3^{uvw}	15^{vw}	1^{vw}
"	SM-5	24	70^{efg}	4^{mn}	11^{q}	4^{uvw}	10^{vwx}	1^{vw}
"	SM-5	48	56^{gh}	2^{jn}	15^{q}	8^{u}	16^{v}	3^{vw}
"	SM-5	72	62^{h}	5^{mn}	17^{q}	3^{uvw}	13^{vw}	0^{v}
"	110	2	94^{b}	0^{j}	2^{os}	2^{vw}	2^{uy}	0^{v}
"	110	24	85^{bcd}	5^{mn}	4^{os}	4^{uvw}	0^{u}	2^{vw}
"	110	48	89^{bcd}	0^{j}	2^{os}	2^{vw}	7^{uvwxy}	0^{v}
"	110	72	90^{bc}	2^{jn}	0^{o}	0^{t}	8^{vwxy}	0^{v}

[a]Values are means of three replicates. Numbers followed by the same letter (b-y) do not differ significantly (p=0.05) within a given column. Duncan's Multiple Range test used to distinguish between treatments within a given strain.

Table IV-8. Effect of preexposure to R. japonicum strains on nodule and plant parameters in a Rhizobium-free soil (Waimea): Harvests I and II.

Soil Inoculated with	Preexposure Treatment: Strain	Time (hours)	Nod. No[a] per plant		Nod. Dry Wgt[a] (mg) per plant		N_2-ase activity[b] µmoles/g/hour		Shoot Dry[a] wgt (g) per plant	
			I	II	I	II	I	II	I	II
USDA 110	None	0	38^{c}	94^{i}	102.0^{c}	363.9^{e}	91.57	72.75	0.90^{dc}	6.41^{fgh}
USDA 123	None	0	63^{cd}	198^{jk}	129.0^{cd}	427.9^{e}	38.63	46.69	0.92^{d}	6.30^{fgh}
SM-5	None	0	132^{gh}	215^{j}	129.0^{cd}	183.9^{fg}	00.00	00.00	1.00^{de}	1.38^{i}
110/123/SM-5	None	0	73^{cde}	141^{iklmn}	139.8^{cd}	359.2^{e}	127.44	74.22	1.14^{e}	6.70^{fgh}
110/123/SM-5	123	2	69^{cde}	97^{ino}	137.7^{cd}	326.3^{efg}	96.22	28.19	1.00^{de}	5.10^{fgh}
"	123	24	82^{cdef}	157^{jklm}	148.3^{cd}	391.0^{e}	89.30	28.47	0.93^{de}	6.66^{fgh}
"	123	48	95^{defg}	118^{jkl}	144.0^{cd}	282.0^{efg}	79.12	29.91	1.00^{de}	6.15^{fgh}
"	123	72	107^{defgh}	141^{iklmn}	134.0^{cd}	371.7^{e}	61.29	41.34	0.90^{dc}	6.45^{fgh}
"	SM-5	2	85^{def}	144^{iklm}	125.7^{cd}	352.0^{e}	102.20	22.92	0.97^{de}	7.81^{g}
"	SM-5	24	122^{cdef}	176^{jkl}	152.0^{d}	423.7^{e}	122.02	49.88	1.00^{de}	7.97^{g}
"	SM-5	48	142^{h}	160^{jkl}	166.3^{d}	271.7^{efg}	46.19	19.75	0.70^{c}	4.87^{fh}
"	SM-5	72	113^{efgh}	165^{jkl}	144.3^{cd}	300.3^{efg}	100.95	8.99	0.79^{dc}	4.13^{f}
"	110	2	ND	83^{o}	ND	331.0^{eg}	ND	23.16	ND	5.58^{fgh}
"	110	24	ND	100^{imno}	ND	343.3^{eg}	ND	32.43	ND	7.41^{gh}
"	110	48	ND	76^{o}	ND	280.3^{efg}	ND	15.10	ND	7.29^{gh}
"	110	72	ND	79^{o}	ND	170.7^{f}	ND	33.99	ND	5.80^{fgh}

[a]Values are means of three replicates. Numbers followed by the same letter (or n) do not differ significantly (p=0.05) within a given column. Duncan's Multiple Range test used to distinguish between treatments. Each harvest analyzed separately. ND = not determined.

[b]For each treatment, replicates combined for acetylene reduction assay.

activity on SM-5-infected plants, differed significantly from plants infected by either of the two effective strains. No significant differences between nodule number, dry weight, nitrogenase activity or shoot dry weight were observed on plants which were infected by all three strains.

Discussion

The competitive superiority of USDA 110 over USDA 138 and SM-5 was demonstrated in all of the experiments in which the strains were introduced simultaneously. USDA 110 out-competed USDA 138 on both cultivars Lee and Peking. This is particularly surprising with cultivar Peking, as this cultivar previously excluded USDA 110 in soils containing other strains of R. japonicum (17,60). USDA 110 was less competitive than USDA 123 in the Waseca soil, but was more competitive than USDA 123 in the Waimea soil.

In vermiculite, delaying the introduction of USDA 110 into the root zone resulted in an enhancement of nodule occupancy by either USDA 138 or SM-5 (Figure IV-1, Tables IV-1 and IV-2). When either of the two less competitive strains was introduced 48 hours or longer before USDA 110, they formed the majority of the nodules. The effect of delayed inoculation on nodule occupancy of strains of Rhizobium has been shown by others (102,127). Skrdleta (102) found that the delayed introduction of one strain of R. japonicum favored the strain applied at sowing. However, the shortest period for delayed inoculation chosen by Skrdleta (102) was three days. The results from the present study show, that even a short preexposure (2 hours) is adequate to

significantly alter the competition pattern. Skrdleta (102) also found that all of the taproot nodules were formed by the primary strain, whereas in this study, the majority of both taproot and lateral root nodules were occupied by the primary strain. Winarno and Lie (127) showed that nodulation of pea cultivar Afghanistan by a nodulating strain of R. leguminosarum, TOM, was suppressed by a non-nodulating strain, PF2. They found that when TOM was introduced 24 hours before PF2, normal nodulation occurred.

Saturation of soybean roots with gamma-irradiated cells of USDA 138 did not prevent nodulation by live cells of either USDA 110 or USDA 138 (Table IV-3). The addition of gamma-irradiated cells of USDA 138 72 hours before USDA 110, had no affect on the percent of nodules occupied by USDA 110. Changes in the pattern of competition between the two strains occurred only when live cells were used as the primary inoculum.

Rhizosphere colonization by USDA 138 or USDA 110, was not affected by preexposure of soybean roots to either strain (Table IV-4). When USDA 138 was introduced 72 hours before USDA 110, the number of cells of USDA 110 on the surface of soybean roots was similar to that observed when both strains were introduced together. Similarly, the introduction of USDA 110 72 hours before USDA 138, had little effect on rhizosphere colonization by USDA 138. Thus, the increases in nodule occupancy by the strain to which the soybean host was preexposed, was not related to the ability of that strain to colonize the soybean rhizosphere.

In soils from the north central region of the United States, the use of highly effective strains as inoculum has not resulted in increases in soybean yields when other indigenous strains of R. japonicum are present. In particular, strains belonging to the USDA 123 serogroup form the majority of the nodules on soybeans grown in this area (39,84,124). In the Minnesota soil, Waseca, USDA 123 out-competed USDA 110 and formed the majority of nodules on cultivar Chippewa (Table IV-5). However, when young seedlings were preexposed to USDA 110 and transplanted into pots containing the Waseca soil, as little as a two hour preexposure to USDA 110 significantly increased the percent of nodules formed by this strain. Longer periods (48 hours or more) of preexposure resulted in the majority of nodules containing USDA 110.

In the Hawaiian soil, Waimea, USDA 110 was highly competitive and out-competed USDA 123 and the ineffective strain SM-5 (Tables IV-6 and IV-7). Preexposure of seedlings to either of the two less competitive strains increased the percent of nodules occupied by these two strains significantly. However, preexposure to soybean roots did not result in either USDA 123 or SM-5 becoming the dominant strain in the nodules. In contrast, when seedlings were preexposed to USDA 110, 85% to 94% of the nodules contained USDA 110. The percent of nodules occupied by the strain to which the host was preexposed appeared to be related to the effectiveness of that strain. Nodule occupancy by the ineffective strain SM-5 decreased on plants harvested at pod-fill, while the percent of nodules containing the two effective strains, USDA 110 or USDA 123, remained significantly greater (Table IV-7).

The results from these studies indicate that interactions which occur during the early period of infection between the soybean host and its microsymbiont are perhaps the most critical for competition among strains of R. japonicum. The role of the host in determining the outcome of competition between strains was highlighted by the observation that young seedlings with only a small (1-2 cm) radicle present at the time of primary exposure to one strain became predisposed to that strain for further nodulation. The results from this study also indicate that the soybean host is able to provide new sites for nodulation in response to infection by an ineffective strain. When seedlings were preexposed to the ineffective strain, SM-5, the percent of nodules on the mature host occupied by SM-5 decreased, while the percent of nodules containing the highly effective strain, USDA 110 increased.

CHAPTER V

USE OF A SPLIT-ROOT GROWTH SYSTEM FOR PARTITIONING OF STRAIN/STRAIN AND STRAIN/HOST INTERACTIONS IN THE NODULATION OF SOYBEANS BY *Rhizobium japonicum*

Abstract

In a split-root system of soybeans inoculation of one half-side suppressed subsequent development of nodules on the opposite side. At zero-time, the first side of the split-root system of soybeans (*Glycine max* (L) Merr) received *R. japonicum* strain USDA 138 as the primary inoculum. At selected time intervals the second side was inoculated with the secondary inoculum, a mixture of *R. japonicum* strain USDA 138 and strain USDA 110. In a short-day season, nodulation by the secondary inoculum was inhibited 100 when inoculation was delayed 10 days. Nodulation on the second side was significantly suppressed even when the secondary inoculum was delayed for only 96 hours. In a long-day season, nodule suppression on the second side was highly significant, but not always 100%. Nodule suppression on the second side was not related to the appearance of nodules, or nitrogenase activity on the side of split-roots which were inoculated at zero-time. When the experiments were done under different light intensities, nodule suppression was significantly more pronounced in the shaded treatments. When the splitroot system was used to separate a highly competitive strain, USDA 110, from a poorly

competitive strain, USDA 38, nodule number and dry weight on the two sides of the split-root system did not differ significantly. However, when USDA 110 and USDA 38 were introduced together USDA 110 occupied 90% to 93% of the nodules. Similar results were obtained using USDA 110 and the ineffective, poorly competitive strain SM-5.

Introduction

The nitrogen-fixing association formed between leguminous plants and soil bacteria of the genus Rhizobium has recently become an area of intense scientific research because of the economic and agricultural benefits derived from cropping systems using modulated legumes. One of the specific goals of recent investigations has been to understand the mechanisms involved in the formation of the legume/Rhizobium association. Although both the host plant and the bacteria contribute to the specificity of the association, the mechanisms by which each partner exerts its influence remain poorly understood (for reviews see; 6,37,50,94). Kosslak et al (59) have previously shown that the early events in the process of infection of soybeans play a critical role in determining nodule occupancy by two strains of R. japonicum competing for nodulation of the host legume. They found that when either a less competitive, or poorly competitive, ineffective strain was introduced into the soybean rhizosphere six or more hours before the introduction of a highly competitive strain, the percent of nodules occupied by either of the less competitive strains increased significantly. In this study, a split-root system (100) was used to separate strain/strain interactions from those involving the host during the process of nodulation. This system permits partitioning of these interactions in space and in time.

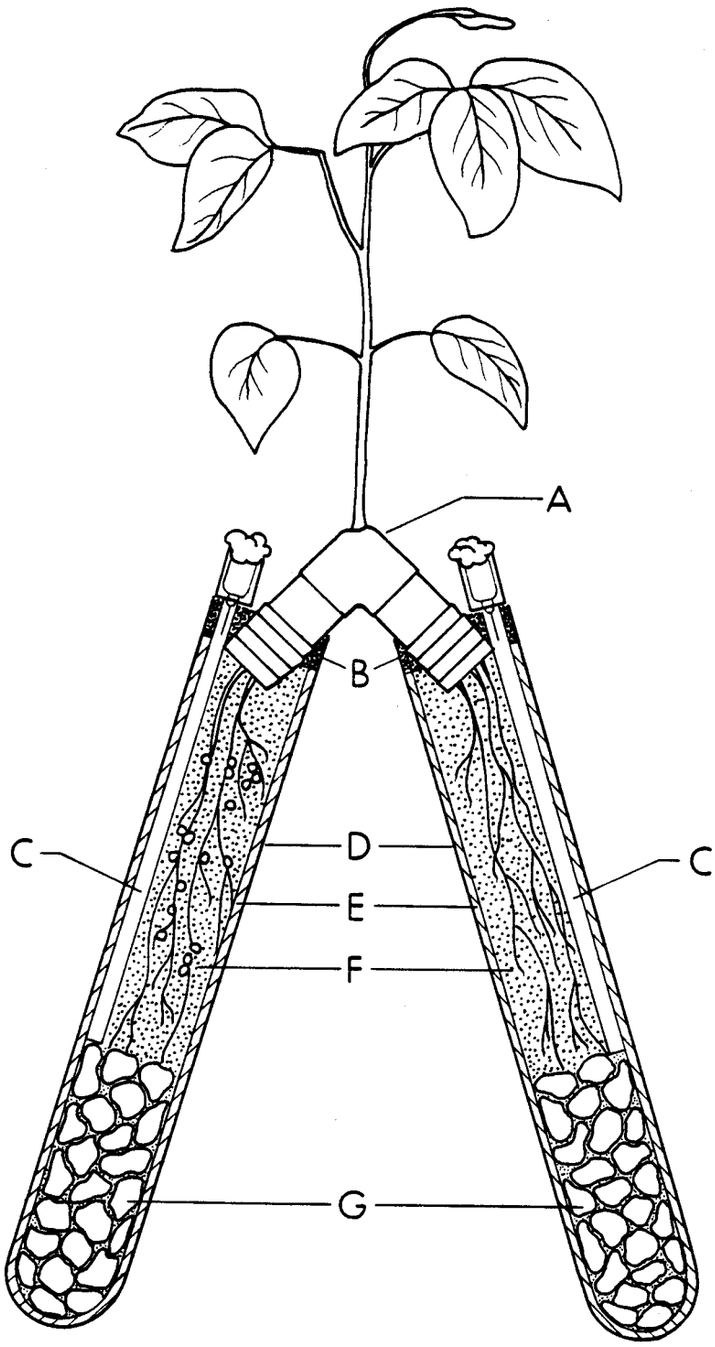
Materials and Methods

Rhizobium Culture. Cultures of R. japonicum strains, USDA 38, USDA 110, and USDA 138, were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. The ineffective strain, SM-5, was obtained from W. Brill, University of Wisconsin, Madison, WI. Stock cultures were grown on yeast-extract mannitol, YEM (11), slants at 28°C. Inoculation consisted of injecting 1.0 ml (1×10^9 cells) of four-day YEM broth cultures into the seedling vessel at the designated times.

Split-Root System. The split-root system used, was a modification of the split-root system described by Singleton (100). As seen in Figure V-1, glass tubes (Bellco 38mm x 300mm) were filled with 127 of coarse #3 gravel and overlaid with 170mm of moist vermiculite. Sixty-five ml of 1:4 strength Hoagland's nitrogen-free solution (48) were added to the gravel reservoir. A rope wick facilitated the movement of the solution into the vermiculite. Water was added with a 19 gauge needle inserted into a glass tube (8mm O.D., 185mm length) fitted with a serum stopper. The entire apparatus was sterilized for 20 minutes at 121°C. Polyvinylchloride (PVC) elbows (21.0mm O.D., 6.0cm sides), with a planting hole (10mm diameter) drilled into the joint of each elbow to receive seedling radicles, were packed with moist, vermiculite and sterilized by gamma-irradiation (1.5×10^6 Rads). After sterilization, the PVC elbows were placed into the tops of the growth vessels, covered with a 2 cm layer of paraffin-coated sand and sterile non-absorbent cotton.

Plant Growth Conditions. Soybean seeds of cultivar Lee were obtained from H. H. Keyser, United States Department of Agriculture,

Figure V-1. Diagram of Split-Root Assembly. (A): PVC elbow with planting hole; (B): Layer of paraffin-coated sand and cotton; (C): Glass tubing for watering with serum stopper and 19 gauge needle covered with cotton plugged tygon tubing; (D): Bellco glass tubes; (E): Rope wick; (F): vermiculite (G): Rock reservoir with Hoagland's solution.


A
B
C
D
C
E
F
G

Beltsville, Md. Seeds were surface-sterilized for 20 minutes in 4% calcium hypochlorite, washed five times with sterile water and soaked in water for two hours prior to germination in sterile moist vermiculite. Twenty-four-hour-old seedlings with radicles 1-2cm long were selected for all experiments. Before inserting the seedlings into the planting holes, the tips of the radicles (2-5mm) were removed to induce branching. The seedlings were watered with sterile water to ensure capillarity between the cut radicles and vermiculite. Twenty-four hours after planting, the seedlings were transferred to the greenhouse where they were grown without artificial lighting. Seedlings were inoculated one to two weeks after transfer to the greenhouse. At zero-time, the first side of the split-root systems received 1.0 ml(1×10^9 cells/ml) of the primary inoculum, USDA 138. At different times afterwards, the second side was inoculated with 1.0 ml (1×10^9 cells/ml) of the secondary inoculum, an equal mixture of strain USDA 110 and strain USDA 138. Plants were harvested three weeks after the inoculation of the second side.

In a similar set of experiments, the split-root system was used to separate strain/strain interactions from those of the host during the process of competition between strains. For these experiments, both sides of the split-root system were inoculated at zero time with either a single strain or an equal mixture of two strains. In the first experiment, two effective R. japonicum strains, USDA 110 and USDA 38, which differed in competitiveness were used, while in the second an effective (USDA 110) and an ineffective (SM-5) combination was chosen.

Determination of Nitrogenase Activity. Nitrogenase activity was determined by the acetylene reduction assay (108). Acetylene reduction was determined at 25°C by incubating roots in jars with 10% (v/v) acetylene for 15 and 30 minutes. Gas samples were analyzed for ethylene with a Bendix 2500 Gas Chromatograph equipped with a Porapak-T column (80-100 mesh).

Identification of Strains in Nodules. Immunofluorescenee was used to identify the strains within the nodules. Twenty-five percent of the nodules from each replicate per treatment was identified. In cases where the number of nodules was less than ten, all of the nodules were examined. Nodule smears were stained with strain-specific fluorescent antibodies (FA) according to the method of Schmidt et al. (93). Gelatin-rhodamine isothiocyanate (Rh-ITC) was used to suppress nonspecific adsorption (10). Microscopy was done as described previously (58).

Results

Suppression of Nodule Development. When soybeans were grown during a short-day season, delayed inoculation of one side of the split-root system resulted in suppression of nodulation on that side (Table V-1). When both the first and the second sides were inoculated at zero-time, nodule numbers and mass were not significantly different. A delay in inoculation of the second side for four days caused a significant suppression in nodule numbers and mass compared to the zero-time treatment. When inoculation was delayed for 10 days, no nodules were formed on the second side. However, total nodule

Table V-1. Suppression of nodulation of half-root systems caused by delayed inoculation (Short-day Season, February 1981)[a]

Treatment	First Side: 0-time Inoculation with USDA 138		Second Side: Delayed Inoculation						Total (1st + 2nd Sides)	
	Nodule		Inoculum	Delay (days)	Nodule		Occupancy[b]		Nodule/Plant	
	Mass(mg)	Number			Mass(mg)	Number	110	138	Mass(mg)	Number
1	58±18	61±15	None	0	0[c]	0[d]	0	0	58±18[e]	61±15[f]
2	39±14	48±14	110/138	0	32±5[g]	32±8[h]	57[NS]	54[NS]	71±11[e]	80±18[f]
3	49±15	52±21	"	4	15±6[i]	24±7[j]	70[NS]	54[NS]	61±16[e]	77±21[f]
4	71± 4	77±18	"	10	0[c]	0[d]	0	0	71± 4[e]	77±18[f]

[a]Four to five replicates used per treatment; all treatments harvested three weeks after inoculation of second side. Results are expressed as Mean ± S.E. Numbers ($^{c-j}$) in the same column flanked by the same letter are not significantly different ($p = 0.05$) as determined by the Mann-Whitney U-test (103). Day length, 10-11 hours; Mean temperature, 25°C. Light intensity for this time of the year, 1250 με per m^2 per sec.

[b]NS = not significant ($p = 0.05$) as determined by the t-test for paired comparisons (103).

number and mass did not differ significantly between treatments. R. japonicum strain USDA 110 was a slightly better competitor than USDA 138 for both the zero-time and four-day delayed treatments (Table V-1).

In the second experiment, done during a long-day season (July 1982), nodule suppression on the second side was significant but not always 100% (Table V-2). Nodule mass and number did not differ significantly when only one side was inoculated at zero-time (Table V-2 and Figure V-2a), or when both sides were inoculated at zero-time (Table V2 and Figure V-2b). However, when inoculation of the second side was delayed for 4, 6, 8, 10, or 14 days, nodule number and mass decreased significantly from the treatment in which both sides were inoculated at zero-time. Suppression of nodule mass was also reflected in decreased nitrogenase activity for the delayed treatments (Table V-2). A similar reduction in nodule numbers was also observed when inoculation of the second side was delayed for 4 days or longer (Table V-2 and Figure V2c). As in the previous experiment, USDA 110 was more competitive than USDA 138 and it occupied 90% to 96% of the nodules on the second side (data not shown). Total nodule numbers and mass per plant (Table V-2) did not differ significantly between treatments.

Shoot and root dry weights are summarized in Table V-3. Root dry weights for the first and second sides were approximately the same for the different treatments. An increase in total shoot and root dry weight was observed for the later harvest times.

Table V-2. Suppression of nodulation of half-root systems as a result of delayed inoculation (Long-Day Season, July 1982): Nodule parameters.[a]

First Side: 0-time Inoculation				Second Side: Delayed Inoculation					Total (1st & 2nd Sides)		
Inoculum	Nodule			Inoculum	Delay (days)	Nodule			Nodule/Plant		
	Mass (mg)	Number	N_2-ase Activity[b] (μmoles/side/hr)			Mass (mg)	Number	N_2-ase Activity[b] (μmoles/side/hr)	Mass	Number	N2-ase Activity[b] (μmoles/side/hr)
138	80±25	86±32	5.0±4.0	None	0	0[c]	0[e]	0	80±25[h]	86±32[i]	5.0±4.0
110	70±10	51±16	ND	None	0	0[c]	0[e]	0	70±10[h]	51±16[i]	ND
110/138	84± 8	75±13	5.0±5.0	None	0	0[c]	0[e]	0	84± 8[h]	75±13[i]	5.0±5.0
None	0	0	0	110/138	14	93±36[d]	94±36[f]	2.0±2.0	93±36[h]	94±36[i]	2.0±2.0
138	33±11	36±12	0.3±0.1	110/138	0	54±16[d]	40± 7[g]	1.0±0.1	87± 4[h]	76±14[i]	1.3±0.0
"	90± 3	59± 6	1.0±0.3	"	4	<1[c]	3± 2[e]	0.1±0.1	90± 3[h]	62± 8[i]	1.1±0.2
"	87±15	59±14	4.0±3.0	"	6	0[c]	0[e]	0	87±15[h]	59±14[i]	4.0±3.0
"	99±20	71± 7	1.0±0.1	"	8	<1[c]	2± 1[e]	0.1±0.1	99±20[h]	73± 7[i]	1.1±0.1
"	123±12	75±10	2.0±0.7	"	10	3± 2[c]	8± 7[e]	0.1±0.1	126±11[h]	83± 4[i]	2.1±0.8
"	100± 4	73±20	0.7±0.4	"	14	2± 2[c]	6± 5[e]	<0.1	102± 6[h]	79± 5[i]	0.7±0.4
110	120± 3	79±24	2.0±1.0	"	14	<1[c]	2± 1[e]	<0.1	120± 3[h]	81±24[i]	2.0±1.0

[a]Three to four replicates used per treatment; all treatments harvested three weeks after inoculation of second side. Results expressed as Mean ± S.E. Numbers (c-i) in the same column flanked by the same letter are not significantly different (p=0.10) as determined by the Mann-Whitney U-test (103). Day length, 13-14 hours; Mean temperature, 33.5°C.

[b]Two replicates used per treatment; ND = not determined.

Figure V-2a. An example of the type of nodulation observed when only one side of the split-root system was inoculated at zero-time.



Figure V-2b. An example of the type of nodulation observed when both sides of the split-root system were inoculated at zero-time.



Figure V-2c. Suppression of nodule development in a split-root system. Nodulated side was inoculated with R. japonicum strain USDA 138 at zero-time. The other side, received R. japonicum strains USDA 110 and USDA 138 six days later.



Table V-3. Suppression of nodulation of half-root systems as a result of delayed inoculation (Long-Day Season, July 1982): Plant parameters[a]

First Side: 0-time Inoculation		Second Side: Delayed Inoculation			Total (1st + 2nd Sides)	
Inoculum	Root Dry Wgt. (g/side)	Inoculum	Delay (days)	Root Dry Wgt. (g/side)	Root Dry Wgt. (g/plant)	Shoot Dry Wgt. (g/plant)
138	ND	None	0	ND	ND	0.46±.18
110	ND	None	0	ND	ND	0.39±.05
110/138	ND	None	0	ND	ND	0.45±.03
None	0.03±.00	110/138	14	0.08±.05	0.11±.05	0.77±.33
138	ND	110/138	0	ND	ND	0.49±.07
"	0.22±.17	110/138	4	0.09±.06	0.31±.11	0.87±.16
"	0.28±.20	"	6	0.28±.20	0.56±.30	1.20±.16
"	0.38±.10	"	8	0.19±.05	0.57±.13	1.40±.30
"	0.60±.06	"	10	0.31±.05	0.91±.10	1.80±.30
"	0.35±.21	"	14	0.16±.08	0.51±.13	1.80±.20
110	0.41±.15	"	14	0.22±.12	0.63±.20	1.80±.49

[a]Three to four replicates used per treatment; all treatments harvested three weeks after inoculation of second side. Results are expressed as Mean ± S.E. ND = not determined.

In addition to the experimental treatments, 15 split-root systems were used to determine whether suppression of nodulation on the sides where inoculation was delayed was related to the appearance of nodules or the onset of nitrogenase activity. From this group, three split-root systems were harvested 6, 8, 9, 10, and 12 days after the inoculation of USDA 138 (0-time) to the first side of the split-root systems. Split-root systems harvested after 6 days were devoid of nodules and no nitrogenase activity was detected. Nodules were visible on split-root systems harvested 8 days after inoculation, but nitrogenase activity was negligible. Significant nitrogenase activity was not detected until 12 days after inoculation.

Another experiment was done to determine whether suppression of modulation on one side of the split-root system was related to the quantity of light available to the soybean host. Split-root systems were grown during a long-day season (August 1982) at three different light intensities. One group of plants was grown without shading (full sun), another group under 47% shade cloth, and a final group under 73% shade cloth. Results in Table V-4 show that there was incomplete suppression of nodule development on the delayed sides in plants grown without shading. However, both nodule numbers and mass on the sides where inoculation had been delayed for 10 days differed significantly from the treatments inoculated at zero-time. In the shaded plants, no nodules developed on the delayed side of the split-roots. Microscopic examination of roots from the delayed sides showed that the process of modulation had been initiated. Nodule primordia were visible on the delayed side of split-root systems grown

Table V-4. Effect of light intensity on suppression of nodulation of half-root systems as a result of delayed inoculation (Long-Day Season, August 1982): Nodule Parameters[a]

Shading[b]	First Side: 0-time Inoculation				Second Side: Delayed Inoculation					Total (1st + 2nd Side)		
	Inoculum	Nodule			Inoculum	Delay (days)	Nodule			Nodule/Plant		
		Mass (mg)	Number	N_2-ase Activity[c] (μmoles/side/hr)			Mass (mg)	Number	N_2-ase Activity[c] (μmoles/side/hr)	Mass (mg)	Number	N_2-ase Activity[c] (μmoles/side/hr)
	None	0	0	0	110/138	0	45.0± 3[d]	32± 9[h]	0.51±.38	45.0± 3[k]	32± 9[o]	0.51±.38
None	None	0	0	0	110/138	10	44.3±13[d]	43±31[h]	0.61±.20	44.3±12[k]	43±31[o]	0.61±.20
(6600 με per	138	58.7±27	37±14	0.51±.10	None	0	0[g]	0[j]	0	58.7±27[k]	37±14[o]	0.51±.10
m^2 per sec)	"	21.7±19	19±18	0.30±.15	110/138	0	20.7±10[e]	22±15[h]	0.19±.13	42.4±11[k]	41± 7[o]	0.49±.02
	"	78.5±12	58± 6	0.39±.05	110/138	10	1.5± 1[g]	9± 4[i]	0.02±.03	80.0±13[l]	67± 9[o]	0.41±.08
	None	0	0	0	110/138	0	21.3± 9[e]	25± 9[h]	0.35±.20	21.3± 9[m]	25± 9[p]	0.35±.20
47%	None	0	0	0	110/138	10	23.5± 7[e]	46±20[h]	0.30±.20	23.5± 7[m]	46±20[o]	0.30±.20
(300 με per	138	11.8± 7	10± 8	0.13±.01	None	0	0[g]	0[j]	0	11.8± 7[m]	10± 8[p]	0.13±.01
m^2 per sec)	"	5.7± 5	6± 6	0.07±.02	110/138	0	10.0± 6[e]	7± 6[i]	0.12±.10	15.7± 6[m]	13±10[p]	0.19±.09
	"	36.8± 8	26± 2	0.15±.08	110/138	10	0[g]	0[j]	0	36.8± 8[m]	26± 2[p]	0.15±.08
	None	0	0	0	110/138	0	6.3± 4[f]	7± 4[i]	0.03±.00	6.3± 4[n]	7± 4[q]	0.03±.00
73%	None	0	0	0	110/138	10	10.8± 6[e]	12±12[i]	0.15±.10	10.8± 8[m]	12±12[p]	0.15±.10
(140 με per	138	5.0± 3	5± 4	0.02±.02	None	0	0[g]	0[j]	0	5.0± 3[n]	5± 4[q]	0.02±.02
m^2 per sec)	"	1.0± 1	2± 1	0.01±.00	110/138	0	5.1± 1[f]	6± 1[i]	0.02±.00	6.1± 2[n]	8± 1[q]	0.03±.00
	"	9.1±13	9± 8	0.06±.00	110/138	10	0[g]	0[j]	0	9.1±13[m]	9± 8[p]	0.06±.00

[a] Three to four replicates used per treatment; all treatments harvested three weeks after inoculation of second side. Results are expressed as Mean ± S.E. Numbers ($^{d-q}$) in the same column flanked by the same letter are not significantly different ($p = 0.10$) as determined by the Mann-Whitney U-test (103). Day length, 13-14 hours; Mean temperature, 31.5°C.

[b] Average maximum light intensity over the course of the experiment. Determined by Spectra illumination meter, Model #OP400.

[c] Two replicates used per treatment.

under 47% shade and only infection threads were observed on the split-roots of plants grown under 73% shade. Total nodule mass, nitrogenase activity and numbers (Table V-4) did not differ significantly within each light intensity group. However, there were significant differences in nodule numbers and mass between the three light treatments. These differences were also reflected in shoot and root dry weights (Table V-5). As was observed in the previous two experiments, USDA 110 was the more competitive strain and formed the majority of the nodules regardless of the light intensity under which the soybeans were grown (data not shown).

Competition between Strains. When the two effective strains were introduced together on one side of the split-root system, USDA 110 occupied between 90% to 93% of the nodules (Table V-6). The pattern of competition on the sides which received both strains, was not affected by the presence of either USDA 110 or USDA 38 on the other side of the split-root system. Nodule number and mass did not differ significantly in those treatments which received USDA 110, USDA 38 or USDA 110 plus USDA 38 on the two sides of the split-root system. For all treatments, total nodule number and mass per plant did not differ significantly.

When the effective strain, USDA 110 and the ineffective strain, SM-5 were present on the same side of the split-root system USDA 110 out-competed Sm-5 and occupied 63% to 75% of the nodules (Table V-7). Nodule numbers did not differ significantly in treatments which received USDA 110 on both sides, SM-5 on both sides or USDA 110 on one side and SM-5 on the other side. However, significant differences in

Table V-5. Effect of light intensity on suppression of nodulation of half-root systems as a result of delayed inoculation (Long-Day Season, August 1982): Plant Parameters[a]

Shade Level	First Side: 0-time Inoculation		Second Side: Delayed Inoculation			Total (1st + 2nd Side)	
	Inoculum	Root Dry Wgt. (g/side)	Inoculum	Delay (days)	Root Dry Wgt. (g/side)	Root Dry Wgt. (g/plant)	Shoot Dry Wgt. (g/plant)
None	None	0.13±.03	110/138	0	0.11±.06	0.24±.04	0.56±.04
"	None	0.13±.03	110/138	10	0.23±.06	0.36±.08	0.56±.22
"	138	0.18±.09	None	0	0.18±.07	0.36±.11	0.51±.08
"	"	0.22±.22	110/138	0	0.12±.02	0.34±.21	0.49±.01
"	"	0.49±.27	"	10	0.29±.14	0.78±.41	1.04±.05
47%	None	0.10±.10	110/138	0	0.07±.03	0.17±.04	0.38±.03
"	None	0.08±.02	110/138	10	0.12±.06	0.20±.07	0.52±.10
"	138	0.09±.07	None	0	0.09±.04	0.18±.06	0.30±.04
"	"	0.09±.03	110/138	0	0.09±.03	0.18±.05	0.36±.06
"	"	0.13±.06	"	10	0.09±.02	0.22±.06	0.61±.19
73%	None	0.08±.02	110/138	0	0.09±.04	0.17±.06	0.28±.03
"	None	0.07±.02	110/138	10	0.07±.04	0.14±.04	0.39±.03
"	138	0.09±.02	None	0	0.07±.02	0.17±.02	0.26±.04
"	"	0.07±.01	110/138	0	0.08±.01	0.15±.01	0.27±.00
"	"	0.05±.02	"	10	0.03±.00	0.08±.02	0.36±.06

[a]Three to four replicates used per treatment; all treatments harvested three weeks after inoculation of second side. Results are expressed as Mean ± S.E.

Table V-6. Pattern of competition between a highly competitive (USDA 110) strain and a poorly competitive (USDA 38) strain of R. japonicum on half-root systems.

First Side:			Second Side:						Total (1st + 2nd Sides)[b]	
Inoculum	Nodule[a]		Inoculum	Nodule[a]					Nodule/Plant	
	Mass(mg)	Number		Mass(mg)	Number	Occupancy (%)			Mass(mg)	Number
						110	38	Both		
110	42.4±10.5**	46±25**	None	0	0	0	0	0	42.4±10.5[c]	46±25[d]
38	38.1± 6.6**	49±11**	None	0	0	0	0	0	38.1± 6.6[c]	49±11[d]
110	20.8± 8.2[NS]	29±12[NS]	110	26.3± 9.8	35± 8	100	0	0	47.1± 4.4[c]	64± 8[d]
38	25.4±12.2[NS]	38±19[NS]	38	24.4±18.4	28±23	0	100	0	49.8±20.8[c]	66±18[d]
110	26.1±13.9[NS]	34±16[NS]	38	18.9±11.0	19±11	0	100	0	45.1±13.5[c]	54±17[d]
None	0**	0**	110/38	58.1±11.2	61±23	93± 6**	0±0	7±6	58.1±11.2[c]	61±23[d]
110	30.1±10.9[NS]	35±16[NS]	"	20.8± 9.3	23±11	90±12**	5±7	5±7	50.8± 6.9[c]	59±19[d]
38	23.3±14.5[NS]	33±17[NS]	"	27.8±12.0	30±14	91± 3**	4±4	5±3	51.1± 4.4[c]	63±14[d]

[a] Values are the Means ± S.E. of four to six replicates. For each treatment, differences between nodule mass and number for the first and second side determined by the paired T-test (103). Differences in percent occupancy determined by the T-test for differences between two means (103). NS = not significant; ** = significant (p = 0.05).

[b] Numbers in the same column followed by the same letter, are not significantly different (p = 0.05) as determined by the Mann-Whitney U-test (103).

Table V-7. Pattern of competition on half-root systems between effective (USDA 110) and ineffective (SM-5) R. japonicum strains.

First Side: Inoculum	First Side: Nodule[a] Mass(mg)	First Side: Nodule[a] Number	Second Side: Inoculum	Second Side: Nodule[a] Mass(mg)	Second Side: Nodule[a] Number	Second Side: Occupancy 110	Second Side: Occupancy SM-5	Second Side: Occupancy Both	Total (1st + 2nd Sides) Nodule/Plant[b] Mass(mg)	Total (1st + 2nd Sides) Nodule/Plant[b] Number
110	55.4± 9.4**	61±17**	None	0	0	0	0	0	55.4± 9.4[c]	61±17[e]
SM-5	39.5± 8.3**	106±12**	None	0	0	0	0	0	39.5± 8.3[d]	106±12[f]
110	30.9±11.6[NS]	47±24[NS]	110	31.0±16.3	42±16	100	0	0	61.9± 6.3[c]	89± 9[f]
SM-5	21.9±10.6[NS]	57±17[NS]	SM-5	15.1±11.6	59±26	0	100	0	36.9± 4.5[d]	99±15[f]
110	35.9± 9.5**	34±15[NS]	SM-5	10.4± 8.7	42±27	0	100	0	46.3± 8.1[c]	76±19[f]
None	0**	0**	110/SM-5	55.6±10.9	63± 6	63±13**	19±8	18±12	55.6±10.9[c]	63± 6[f]
110	30.2± 6.6[NS]	41±10[NS]	"	28.5±13.5	41±13	70±14**	8±3	22±11	58.7±11.2[c]	83± 7[f]
SM-5	10.2± 5.1**	38±13[NS]	"	41.6±10.0	40±15	75±10**	8±4	17± 8	51.8±10.6[c]	79±15[f]

[a]Values are Means ± S.E. of four to six replicates. For each treatment, differences between nodule mass and number for the first and second side determined by the paired T-test (103). Differences in percent occupancy determined by the T-test for differences between two means (103). NS = not significant; **= significant ($p = 0.05$).

[b]Numbers in the same column followed by the same letter, are not significantly different ($p = 0.05$) as determined by the Mann-Whitney U-test (103).

102 nodule mass were found in treatments which received SM-5 on one side and USDA 110 or USDA 110 plus SM-5 on the other side. Total nodule mass from plants which were inoculated with SM-5 differed significantly from plants inoculated with USDA 110. Total nodule number and mass per plantdid not differ significantly in treatments which received both strains.

Discussion

Delayed inoculation of one side of a split-root system for four or more days resulted in partial or complete suppression of nodulation on that side. The degree to which nodulation of the second side was suppressed depended on the conditions under which the soybean host was grown. There was a complete suppression of nodule development on the second side when soybeans were grown during a short-day season or under shade (Table V-1 and V-4). A significant reduction in nodule numbers and mass on the second side occurred when soybeans were grown during a long-day season (Table V-2). Suppression of nodulation on the second side was not related to the appearance of nodules or to nitrogenase activity on the sides inoculated at zero-time. Suppression of nodulation had already occurred in those treatments in which the introduction of the two strains was delayed for four days. This was four days prior to the appearance of nodules and eight days prior to the detection of significant nitrogenase activity on the sides which received the primary inoculum at zero-time.

Suppression of nodulation was also not found to be strain related, since a 14 day delay in inoculation of the second side

resulted in suppression of nodulation on that side with either R. japonicum strain USDA 138 or USDA 110 as the primary inoculum. In clover (63,74) and alfalfa (74) it has also been observed that the appearance of the first group of nodules has a suppressive effect on further infection and nodule development. This effect was also shown to be unrelated to the supply of nitrogen (46,71,74).

A preliminary analysis of total carbohydrates in root and nodule tissue between the two sides of the split-root system was done by the Anthrone method (128). Although no differences in total carbohydrates were detected in root tissue alone, when nodule carbohydrate data were included the results became significant. The data are presented in the Appendix. Layzell et al (62) have also shown that the nodules accounted for a significant percentage of the total plant photosynthate required by the roots of the host legume.

The total number of nodules and nodule mass supported by the host may be linked to the amount of light available to the host for photosynthesis, since significant differences in total nodule numbers and mass were observed between the three light intensity treatments (Table V-4). The degree to which the process of infection proceeded was also found to vary with light intensity. In plants grown under full sun, there was a significant suppression in nodule number and mass on the sides where inoculation was delayed. In contrast, when plants were grown under 47% shade, the process of infection was stopped at the nodule primordia stage and only infection threads were observed in plants grown under 73% shade. Host control of nodule numbers and mass has also been observed when soybeans were inoculated

with varying ratios of effective and ineffective strains of R. japonicum (101). Singleton and Stockinger (101) found that the average size of an effective nodule was twice that of an ineffective one, and that the average weight of an effective nodule increased as the proportion of effective nodule tissue decreased.

The number of nodules and mass formed by a highly competitive (USDA 110) and a poorly competitive (USDA 38) strain did not differ significantly when the two strains were present on different sides of the split-root system (Table V-6). Similar results were also obtained when the ineffective strain, SM-5, was used in place of USDA 38 (Table V-7). In contrast to the previous experiment, nodule mass differed significantly in those treatments which received SM-5 on one side and USDA 110 or USDA 110 plus SM-5 on the opposite side of the split-root system. The pattern of competition between the strains, was not affected by the presence of one of the strains on the other side of the split-root system. When either USDA 110 plus USDA 38 (Table V-6) or USDA 110 plus SM-5 (Table V-7) were introduced together, USDA 110 was highly competitive and occupied the majority of the nodules.

The results from this study indicate that the number of infection sites which develop into functional nodules are controlled by the host. Control by the soybean host over the number of sites leading to successful infections appears to be exerted during the early stages of the infection process and to the amount of light available to the host for photosynthesis. However, no direct role for the soybean host in the selection of the more competitive strain was found. Thus, the outcome of competition between strains of R. japonicum for nodulation

of the soybean host appears to involve both strain/strain and strain/host interactions.

CHAPTER VI

GENERAL SUMMARY

The introduction of highly effective strains of Rhizobium into soils is usually unsuccessful, because these strains are often unable to compete against resident rhizobia and occupy a significant proportion of the nodules on their legume host. The attributes which contribute to the competitiveness of a strain are poorly understood and often vary, depending on the conditions under which the investigation is conducted. The competitive ability of a strain is affected by environmental factors, its genetic composition and the physiological and genetic background of its host.

The results from this investigation indicate that interactions which occur between the soybean host and its microsymbiont are important in determining the outcome of competition between strains. The major findings of this research are:

I. Environmental factors such as the presence of antibioticproducing rhizosphere microorganisms and changes in available soil nitrogen did not affect the outcome of competition between strains of R. japonicum. It has been suggested that Rhizobium strains which are resistant to antibiotics produced by other rhizosphere microorganisms will be more competitive and form the majority of nodules on their legume host (98). However, this hypothesis is based on indirect results only (98,107). In this study,

resistance of two strains of R. japonicum to antibiotics was approached in two different ways. In the first experiment, soybeans were planted in sterile vermiculite and inoculated with 1.0 g of a Minnesota soil. Seven days later, R. japonicum strains USDA 110 and USDA 123 were added to the system. The percent of nodules occupied by the two strains did not change significantly in the presence or the absence of the soil inoculum. In the second experiment, the affect of four actinomycete isolates on the pattern of competition between USDA 110 and USDA 123 in vermiculite was examined. The four aetinomycete isolates were obtained from the roots of soybeans grown in soil and were found to inhibit the growth of either USDA 110 or USDA 123 in vitro. However, the establishment of the four actinomycete isolates in the rhizosphere of soybeans had no effect on the percent of nodules occupied by USDA 110 and USDA 123. It has also been suggested that competition between strains of Rhizobium is affected by different levels of soil nitrogen (68). In this study, depletion of soil nitrogen by the addition of bagasse or replenishment of soil nitrogen by the addition of calcium nitrate had no effect on the percent of nodules occupied by USDA 110 and USDA 123 in three soils. In two Hawaiian soils, USDA 110 out-competed USDA 123, while in a Minnesota soil, USDA 123 was the more competitive strain.

II. The early stages in the infection process were important in determining the outcome of competition between strains of R. japonicum. Preexposure of young seedlings (two-day-old) to poorly competitive strains increased the nodule occupancy by these strains significantly. This was particularly surprising in the Minnesota

soil, Waseca, where preexposure of young seedlings to USDA 110 for two or more hours enabled USDA 110 to displace the more successful indigenous strain USDA 123 from the nodules. Similar results were obtained in vermiculite and in the Hawaiian soil, Waimea, where preexposure of young seedlings to USDA 123, USDA 13% or the ineffective strain SM-5 enhanced their competitiveness against USDA 110. Although other studies (102,127) have used time delay experiments, none of the present studies were used to identify which stages in the infection process are critical to the outcome of competition between strains of Rhizobium.

III. The use of a split-root system to separate strain/strain interactions from those of the host during the process of nodulation indicated that the number of infection sites which developed into functional nodules were under host control. Delayed inoculation of one side of a split-root system for four or more days resulted in partial or complete suppression of nodulation on that side. The degree to which nodulation was suppressed depended on the conditions under which the soybean host was grown. Total nodule number and mass supported by the host appeared to be linked to the amount of light available to the host for photosynthesis. Host control of nodule numbers and mass has also been observed when soybeans were inoculated with varying ratios of effective and ineffective strains of R. japonicum (101). The soybean host compensated for low numbers of effective nodule tissue by increasing the weight of those nodules.

The outcome of competition between strains of R. japonicum for nodulation of their soybean host is dependent on a series of

interrelated steps which involve both the legume host and its microsymbiont. The interactions which occur between a strain and its host are affected by environmental factors and the physiological and genetic background of both partners. The results from this investigation emphasize that an understanding of both the ecology of these soil bacteria and of the mechanisms involved in the formation of the nitrogen fixing symbiotic association is required, before it will be possible to successfully introduce selected inoculum strains into the soil environment.

APPENDIX

Appendix Table 1. Suppression of nodulation of half-root systems caused by delayed inoculation: Partitioning of soluble carbohydrates.

Harvest (Days after 0-time inoculation)	First Side: 0-time inoculation[a]							Second Side: Delayed inoculation[a]						
	Inoculum	Nodule Number	Nodule Mass(mg)	Root Dry Wgt(mg)	µg Glucose per mg Dry Wgt: Nodules	Roots[b]	Total[b] (nodules+roots)	Inoculum	Delay (days)	Nodule Number	Nodule Mass(mg)	Root Dry Wgt(mg)	µg Glucose per mg Dry Wgt: Nodules	Roots[b]	Total[b] (nodules+roots)
4	None	0	0	35.2±17.4	0	18.7± 6.1^{NS}	18.7± 6.1^{NS}	None	0	0	0	22.3±17.1	0	22.5±20.8^{NS}	22.5±20.8^{NS}
	USDA 138	0	0	18.0±12.5	0	23.6± 6.3^{NS}	23.6± 6.3^{NS}	USDA 110	0	0	0	33.9±10.2	0	22.5± 2.4^{NS}	22.5± 2.4^{NS}
	USDA 138	0	0	37.7± 6.5	0	20.1±11.1^{NS}	20.1±11.1^{NS}	USDA 110	4	0	0	42.0±21.6	0	26.8± 6.4^{NS}	26.8± 6.4^{NS}
10	None	0	0	76.5±14.3	0	17.8± 3.5^{NS}	17.8± 3.5^{NS}	None	0	0	0	45.1±19.3	0	22.7± 5.1^{NS}	22.7± 5.1^{NS}
	USDA 138	0	0	65.4±34.2	0	26.7± 6.5^{NS}	26.7± 6.5^{NS}	USDA 110	0	0	0	72.1±10.3	0	24.9± 1.0^{NS}	24.9± 1.0^{NS}
	USDA 138	0	0	89.0± 8.9	0	21.2± 3.6^{NS}	21.2± 3.6^{NS}	USDA 110	10	0	0	48.9±17.3	0	18.6± 6.1^{NS}	18.6± 6.1^{NS}
25	None	0	0	80.5±52.4	0	26.0± 4.6^{NS}	26.0± 4.6^{NS}	None	0	0	0	106.5±35.7	0	30.0± 7.0^{NS}	30.0± 7.0^{NS}
	USDA 138	34±17	33.3±11.4	97.7±25.0	85.3±62.9	31.0± 2.6^{NS}	116.3±65.6^{NS}	USDA 110	0	26±11	36.3± 8.9	95.3±17.5	42.7± 2.5	33.3± 6.0^{NS}	76.0± 5.2^{NS}
	USDA 138	59±11	71.3±11.4	140.7± 3.7	117.3±44.7	32.3± 2.3^{NS}	149.6±44.8**	USDA 110	4	<1	<0.1	69.3±36.2	0	32.3± 4.0^{NS}	32.3± 4.0**
31	None	0	0	106.5±10.2	0	26.0± 7.2^{NS}	26.0± 7.2^{NS}	None	0	0	0	86.1±20.0	0	22.7± 2.3^{NS}	22.7± 2.3^{NS}
	USDA 138	23±22	22.0±21.5	89.0±25.6	40.5± 9.2	18.5± 1.0^{NS}	59.0± 8.5^{NS}	USDA 110	0	13±21	33.3±23.1	121.8±18.9	61.7±25.4	24.0± 1.7^{NS}	85.7±27.1^{NS}
	USDA 138	50± 9	79.2±12.7	107.7± 3.6	51.0± 2.0	26.0± 7.5^{NS}	77.0± 5.6*	USDA 110	10	1±1	0.1± 0.1	94.7±12.6	0	31.7±16.6^{NS}	31.7±16.6*

[a] Values are means ± S.E. of three replicates.

[b] For each treatment, differences between µg glucose per mg dry wgt for roots + nodules (total) determined by the paired T-test (103).
NS = not significant; * = significant at p=0.10; ** = significant at p=0.05.

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