

INTERSTRAIN COMPETITION AND HOST CONTROL OF NODULATION

IN THE Phaseolus vulgaris-Rhizobium leguminosarum

bv. phaseoli SYMBIOSIS

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ABSTRACT

The legume-Rhizobium symbiosis is a plant-microbe interaction that results in the formation of nitrogen-fixing root nodules. Competition for nodulation among rhizobial strains is a significant, but poorly understood, ecological and agronomic problem. This study addressed the role of the host as a variable that may influence the outcome of interstrain competition. The Phaseolus vulgaris-Rhizobium leguminosarum bv. phaseoli symbiosis was studied to characterize the competitiveness of rhizobial strains and to correlate competitiveness with the ability to induce suppression, a host-mediated regulatory response that restricts the extent of modulation.

The results of this study showed that Rhizobium leguminosarum bv. phaseoli strains have differential intrinsic competitive abilities. There was a correspondence between competitiveness and effectiveness, but there was no correlation between competitiveness and nodulation characteristics. Two highly competitive and highly effective strains, TAL 1472 and TAL 182, were identified as good model strains for genetic manipulation. Nodulation in common bean was subject to modulation suppression. When a primary inoculation was done on one side of a split-root, followed by a secondary inoculation on the opposite side with increasing delay, secondary modulation was suppressed in a time-dependent manner, becoming more pronounced as the delay period was increased. Strains with varying competitive abilities induced a suppression response that partially corresponded with their competitive abilities. Suppressiveness was not related to the speed of nodule formation or to the nodulating potential of a strain. Suppression was correlated with rhizobial presence inside the nodules, but was not related to the ability to induce visible nodule primordia, or to the ability to fix nitrogen. Homologous complementation restored the nodulation abilities of the nonnodulating Class I and the nodule primordial-forming Class II mutants.

However, the complemented Class I mutants were ineffective and did not regain full ability to induce nodulation suppression. In contrast, the complemented Class II mutants regained the wild-type nodulation, fixation, and suppression characteristics. Restriction analysis of the cosmids complementing Class I mutants showed a 7.2-kb region: in common. Two complementation groups corrected the Class II mutants (MLC35 and MLC640), showing an 8.0-kb and a 13.4-kb region in common, respectively.

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

The technology aimed at exploiting the legume-Rhizobium symbiosis for agricultural production involves the selection of highly effective nitrogen-fixing rhizobial strains and their introduction into the soil. However, when indigenous rhizobia are present in the soil, the introduced strains usually do not nodulate the host plant. Even at high inoculum rates, the proportion of nodules formed by the introduced strain is generally less than 20 (Ham et al., 1971; Kuykendall and Weber, 1978). Thus, rhizobial inoculation does not often result in increased productivity.

Despite its acknowledged importance, not much is known of interstrain competition at the mechanistic level. It is possible that competition among strains may occur from the time the rhizobia are together in the same soil environment until they are inside the nodule, entailing competitive interactions at several different levels. The introduced rhizobia may initially compete with strains of the natural soil population to survive in the bulk soil and in the rhizosphere (Bushby, 1984; Chatel and Parker, 1973; Franco and Vincent, 1976). The rhizobia that persist may then compete for infection sites on the roots of the plant host (Brockwell and Dudman, 1968; Caldwell, 1969; Johnson et al., 1965). During nodule development, the host may exert a strain-specific selective pressure, favoring one of a number of strains to form nodules (Bromfield, 1984; Caldwell and Vest, 1968; Hardarson et al., 1982; Jones and Hardarson, 1979; Vincent and Waters, 1953).

Competitiveness is defined in terms of the ability of a strain to occupy the nodules of a particular host plant in the presence of another nodulating strain. Collectively, studies have shown that the outcome of competition varies with environmental factors and the genetic make-up of the

symbiotic partners. The intrinsic competitive ability of rhizobia has been examined as a variable in interstrain competition in studies that emphasized the autecology of strains. However, there is yet no single feature of the microsymbiont that is consistently correlated with competitiveness. Thus, it is difficult to predict the outcome of competition for nodulation from information on the rhizosphere behavior of Rhizobium strains (Moawad et al., 1984; Robert and Schmidt, 1983).

Within the framework of rhizobia-controlled functions are processes under the regulation of the plant host. The plant host influences the extent of nodulation by the process of autoregulation, as seen in the restriction of nodulation in developmentally younger regions of the root (Bhuvaneswari et al., 1981; Munns et al., 1968; Paau et al., 1985; Takats, 1986). The systemic nature of autoregulation is seen in split-root systems, where developing nodules on one side suppress subsequent nodulation on the opposite side. Split-root studies in alfalfa (Caetano-Anolles and Bauer, 1988), clover (Sargent et al., 1987), siratro (Djordjevic et al., 1988), and soybean (Kosslak and Bohlool, 1984) have shown that autoregulation is a common phenomenon.

There is considerable evidence that the plant host plays a significant role in the selection of the nodulating strain. The influence of the host on the nodulating success of a strain was observed in various legume-Rhizobium associations. Competition trials in soybean (Caldwell and Vest, 1968; Diatloff and Brockwell, 1976; Materon and Vincent, 1980), clover (Jones and Hardarson, 1979; Russel and Jones, 1975; Vincent and Waters, 1953), alfalfa (Hardarson et al., 1982), and lentils (May and Bohlool, 1983) showed that in addition to the differences in the competitive ability of strains, there was also significant effect due to host cultivar. The nature of host selection of the nodulating strain is still not clear. One hypothesis on host control is that the plant expresses, a systemic signal in response to developing

nodule meristems, thereby allowing the plant host to regulate nodulation (Rolfe and Gresshoff, 1988).

The goal of this study was to examine the interaction between the host and the microsymbiont, particularly how host control of nodulation affects strain competitiveness.

The specific objectives were:

1. To characterize the nodulation competitiveness of several effective R. leguminosarum bv. phaseoli strains;
2. To correlate the competitiveness of strains with the ability to induce nodulation suppression in the plant host;
3. To define the relationship between nodulation and nodulation suppression using Tn5 mutants of a model strain; and,
4. To clone the genes that complement defects in nodulation and nodulation suppression.

CHAPTER 2

LITERATURE REVIEW

A critical problem that hampers the enhancement of nitrogen fixation in the legume-Rhizobium symbiosis in the cultivation of legumes is the inability of inoculant strains to compete successfully with indigenous soil rhizobia. The indigenous rhizobia which are often less efficient in nitrogen fixation, are highly competitive under field conditions. Thus, the introduction of highly effective strains usually fails if a naturalized population is already well established in the soil (for review, see Ham, 1980). The competition problem is well documented in crops such as soybean (Ham et al., 1971; Johnson et al., 1965) and common bean (Graham, 1981).

Studies have shown that the displacement of indigenous strains by inoculant strains is extremely difficult. Indigenous rhizobia form over 90% of soybean nodules when seeds are inoculated with choice strains (Caldwell and Vest, 1968; Johnson et al., 1965). Weaver and Frederick (1974) reported that in order to obtain 50% occupancy of soybean nodules, the rate of inoculum application would have to be at least 1000 times the level of the indigenous rhizobial population.

Interstrain competition is a poorly understood phenomenon. In general, studies of various legume-Rhizobium symbioses have shown that the outcome of interstrain competition depends on environmental variables and the genetic factors that determine the basic compatibility between the plant host and the microsymbiont (for review, see Dowling and Broughton, 1986).

ENVIRONMENTAL FACTORS THAT AFFECT COMPETITION

The soil is a very complex environment and any soil factor that adversely affects one partner in the symbiosis will also profoundly affect the interaction. If competition is indeed a preinfection event,

environmental factors will certainly influence its outcome by directly affecting the growth and survival of the competing strains.

Abiotic factors

Temperature. Strains differ in their ability to survive and grow over a range of temperatures. Under extreme temperature conditions, heat-tolerant strains may have a competitive advantage over less tolerant rhizobia.

In a pot study containing soil with indigenous soybean rhizobia, Weber and Miller (1972) found different B. japonicum serogroups predominating in the nodules of soybean plants incubated at different temperatures. Roughley et al. (1980) also observed that temperature affected the pattern of competition between B. japonicum strains and indigenous rhizobia isolated from Nigerian soils. The B. japonicum strains were more competitive at 24° and 33°C while the Nigerian isolates were more competitive at 36°C. High temperature was also shown to alter the competitive ability of strains of R. leguminosarum bv. trifolii (Hardarson and Jones, 1979) and R. meliloti (Rice and Olsen, 1988).

pH. Acidity restricts the occurrence of rhizobia in soil. The adverse effects of low pH are also combined with Al and Mn toxicities. Studies have demonstrated that altering soil pH can change the outcome of competition between rhizobial strains. In B. japonicum, Damirgi et al. (1967) reported that serogroup 123 formed the majority of nodules at low pH (5.9), whereas another serogroup dominated at higher pH (8.3). Similar changes in competitiveness due to changes in soil pH were noted in R. meliloti (van Rensburg and Strijdom, 1982) and R. leguminosarum bv. trifolii (Dughri and Bottomley, 1983).

Combined nitrogen. The inhibitory effect of combined nitrogen has been examined more in relation to its effects on the plant rather than in relation to its possible effects on rhizobia. Nitrate and its reduction product (nitrite) are known to inhibit root hair curling, infection thread formation,

infection thread development, and nitrogen fixation (for review, see Streeter, 1988).

McNeil (1982) reported that B. japonicum strains varied in their ability to nodulate soybean in the presence of nitrate, and suggested that combined nitrogen had a selective effect on rhizobial competition. In his study, a decline in nodule occupancy by USDA 110 in relation to that of CB1809 was observed with increasing levels of nitrate in a sand-culture system. However, a study by Kosslak and Bohlool (1985) showed that, in soil, the presence of nitrate did not alter the outcome of competition between USDA 110 and a strain of B. japonicum serogroup 123. Abaidoo et al. (1990) also reported that the competition patterns of inoculum strains on field-grown soybean and common bean were independent of nitrogen treatment.

Soil type. The variety of physical and chemical properties of soils compound the complexity of the soil environment. The differential competitiveness of B. japonicum strains in various soil types has been reported (Damirgi et al., 1967; Johnson and Means, 1963; May and Bohlool, 1983). In the study by May and Bohlool (1983), the pattern of competition between three strains of R. leguminosarum for nodulation of four cultivars of Lens esculenta was examined under growth chamber and field conditions. In the growth chamber, a commercial inoculant strain consistently formed the majority of nodules. In the field, another strain was equally competitive in an Inceptisol and superior in an Oxisol.

However, other studies reported that in soils devoid of native B. japonicum (Abaidoo et al., 1990; George et al., 1987; Kosslak and Bohlool, 1985) or of R. leguminosarum bv. phaseoli (Abaidoo et al. 1990), the pattern of competition between introduced strains remained the same regardless of soil type. In addition, the competition patterns did not change with cultivar (George et al., 1987) or with site elevation (Abaidoo et al., 1990; George et al., 1987).

Biotic factors

The survival and competitive abilities of rhizobial strains in the soil may also depend upon how well a population can respond to various aspects of the biotic environment. There are reports that suggest the selective influence of soil microflora on rhizobial nodulation (Evans et al., 1979; Fuhrmann and Wollum, 1989; Handelsman and Brill, 1985).

Antagonistic interactions between the soil microflora and rhizobia often occur in a strain-specific manner. In one study, the presence of Erwinia herbicola on alfalfa seeds reduced the ability of one R. meliloti strain to compete for nodule sites against another (Handelsman and Brill, 1985). Fuhrmann and Wollum (1989) reported that rhizosphere bacteria, particularly fluorescent Pseudomonas spp., caused significant changes in the nodule occupancies of B. japonicum strains under conditions of low iron availability.

In addition to adverse effects from a variety of soil microorganisms, the competitive environment may consist of other rhizobia themselves. Winarno and Lie (1979) reported the occurrence of a native non-nodulating R. leguminosarum bv. viciae strain which completely suppressed nodulation by another strain. In R. leguminosarum bv. trifolii, Schwinghamer and Brockwell (1978) demonstrated that bacteriocinogenic strains could be used to suppress the growth of sensitive strains in broth and peat cultures. Increased competitiveness of an inoculant strain was achieved using a mixture of a bacteriocin-producing strain and a highly effective, bacteriocin-resistant strain (Hodgson et al., 1985). The increase in the proportion of nodules formed by the bacteriocin-resistant strain was attributed to bacteriocin-mediated inhibition of the competing indigenous soil rhizobia. Evidence of the relationship between bacteriocin production and strain competitiveness was provided by Triplett and Barta (1987). When a highly competitive, trifolitoxin-producing R. leguminosarum bv. trifolii strain was mutagenized

with Tn5, the trifolitoxin-minus mutants showed decreased competitiveness. The trifolitoxin genes were also cloned and, when transferred to other Rhizobium strains, increased the competitiveness of the recipient strains (Triplett, 1988).

It is important to note, however, that most studies on biotic interactions were done under artificial conditions and extrapolation to the complex soil environment with its normal microflora may not be warranted.

THE RHISOBIAL SYMBIONT

The intrinsic genetic diversity of rhizobia is a factor contributing to the differential competitiveness of strains.

Rhizobial attributes that may influence competition

Rhizobial attributes thought to be involved in the competitive ability of a strain have been the subject of investigations. Among these are motility, growth rate in the rhizosphere, colonization of the rhizosphere, speed to infection, and effectiveness (for review, see Triplett, 1990). The involvement of indigenous plasmids in strain competitiveness was also reported (Brewin et al., 1983).

Motility. Results of competition experiments between motile and nonmotile strains in artificial systems have been interpreted as evidence that motility confers a selective advantage. Ames and Bergman (1982) reported that an ethylmethane sulfonate-induced nonmotile mutant of R. meliloti was less competitive than the wild-type strain for nodulation of alfalfa. In B. japonicum serogroup 110, a nonmotile mutant formed only 20% of the nodules on plants inoculated with an equal mixture of nonmotile and motile strains (Hunter and Fehring, 1980).

In nonsterile soil, Liu et al. (1989) evaluated motility as a factor in competition among rhizobia. At normal soil moisture tension and in the presence of normal rhizosphere populations, motility was found to be of limited importance in the competitiveness of a strain.

Growth rate. Competition studies between fast- and slow-growing soybean rhizobia (McLoughlin et al., 1985) and cowpea rhizobia (Trinick et al., 1983) showed no evidence that a faster growth rate is necessarily correlated with better competitive ability. In soybean, the slow-growers formed greater than 60% of the nodules on cv. Peking even when the fast-growers were added at a 10:1 ratio in their favor (McLoughlin et al., 1985).

Dowdle and Bohlool (1987) studied the competition patterns between B. japonicum and R. fredii isolates in three different soils. Although the slow-growing B. japonicum were highly competitive in a soil devoid of soybean rhizobia or in a soil containing indigenous B. japonicum, R. fredii were highly competitive in a soil where they are indigenous.

Rhizosphere colonization. High proliferation of rhizobia in the host rhizosphere is considered a prelude to infection. However, observations made in the rhizosphere of soybean (Abaidoo et al., 1990; Bushby, 1984; Moawad et al., 1984), common bean (Abaidoo et al., 1990; Robert and Schmidt, 1983), and Leucaena leucocephala (Moawad and Bohlool, 1984) did not correlate strain competitiveness with numbers in the rhizosphere. In the study by Moawad et al. (1984), competition among three serogroups of B. japonicum was examined in rhizosphere and nonrhizosphere soils. There was no significant difference in the abundance of the three serogroups. Serogroup 123, which formed 60-100% of the nodules, showed no dominance in the rhizosphere. Abaidoo et al. (1990) found that inoculum strains of both soybean and common bean colonized their respective host rhizospheres in equal numbers, but differed significantly in their competitive ability in forming nodules.

Speed of nodule initiation. The delayed inoculation of a competing strain has been shown to influence the outcome of competition. Kossalak et al. (1983) observed that when a less competitive B. japonicum strain was inoculated on soybean roots before a more competitive strain, the nodule occupancy of the less competitive strain was increased. In their study,

even a 2-h preexposure of the roots to the less competitive USDA 110 resulted in a significant increase in its nodule occupancy.

Some studies have shown a correlation between speed of nodule formation with strain competitiveness (Bhagwat and Thomas, 1982; de Oliveira and Graham, 1990; Stephens and Cooper, 1988). In contrast, Pinto et al. (1974) reported that competitive superiority was not related to the speed of nodule formation or the number of nodules produced by a strain.

Strain effectiveness. There are varying reports on whether the ability to fix nitrogen plays a role in interstrain competition. Some studies suggest that the host plant selects effective strains (Labandera and Vincent, 1975; Pinto et al., 1974; Robinson, 1969), while others report that ineffective strains are more competitive than effective strains (Franco and Vincent, 1976; Nicol and Thornton, 1941; Vincent and Waters, 1953).

Genetics of nodulation

The development of the legume-Rhizobium symbiosis is an interactive process that is regulated by both partners. During this process, the two partners interact at the level of gene expression by reciprocal transmission of signals. A number of genes code for different steps of the nodulation process and the genes are activated only at a given stage of development, requiring precise timing of gene expression in the rhizobial symbiont and the plant host (for review, see Verma and Long, 1983).

In the fast-growing rhizobia that have been studied, the genes that control symbiotic functions such as host-range specificity (hsn), nodulation (nod), and nitrogen fixation (nif and fix) are located on indigenous plasmids called sym plasmids. In the slow-growing rhizobia, these genes are located on the chromosome (for review, see Prakash and Atherly, 1986).

The general features of genetic regulation of the nodulation process appear to be similar in many different legume-Rhizobium symbioses.

Nodulation genes regulate general functions that determine the basic

proportion of nodulation and specific functions that determine host specificity. In addition, other genes such as those involved in the synthesis of surface components also affect nodule development (for review, see Appelbaum, 1990).

Common nodulation genes. The common nod region comprises a group of nodulation genes that are structurally and functionally conserved in many rhizobial species (Appelbaum, 1990; Djordjevic et al., 1985a). The common nod genes (nodDABC) which are found in all rhizobial species, are essential for the nodulation of legumes. The nodABC and nodD genes are organized into two transcriptional units, with the constitutively expressed nodD being transcribed separately in the reverse direction (Egelhoff et al. 1985; Rossen et al., 1985). Two additional genes, nodI and nodJ are not required for symbiosis but appear to control the efficiency of nodulation (Evans and Downie, 1986).

Mutations in the nodD gene completely block nodulation in strains which contain a single copy of the gene, such as R. leguminosarum bv. viciae and R. leguminosarum bv. trifolii, and cause reduced or delayed nodulation in strains which contain multiple copies of the gene, such as R. fredii and B. japonicum which contain two copies, and R. meliloti which contain three copies (Appelbaum, 1990).

Mutations in the nodABC genes completely block nodulation, affecting steps such as root hair curling, formation of the infection thread, induction of cortical cell division, and induction of early nodulin gene expression (Appelbaum, 1990). In contrast, mutations in nodIJ only cause reduced or delayed nodulation (Evans and Downie, 1986).

Host-specificity genes. The other essential nodulation cluster comprises the hsn genes which impose on the symbiosis a degree of host-rhizobial specificity. These genes do not show homology with genes from other Rhizobium species and mutations in these genes cannot be complemented

by DNA from other species. The hsnD gene product is thought to interact with the nodABC products and makes them host-specific for root hair curling and nodule initiation while the hsnABC may determine the host-specificity of infection thread formation within the root hairs.

In many cases, transfer of hsn genes between species widens the host range of the recipient rhizobia, indicating that these genes are positively acting. In some cases, mutations in the hsn genes extend the host range of the strain (Djordjevic et al., 1985b; Horvath et al., 1986), suggesting that some genes may be negatively acting. Djordjevic et al. (1985b) reported host-range alterations as a result of mutations in the ha region of R. leguminosarum bv. trifolii. One mutant acquired the ability to nodulate a new host (peas) and retained the ability to nodulate white and subterranean clovers, while some mutants lost the ability to nodulate white clover but retained the ability to nodulate subterranean clover and peas. Horvath et al. (1986) reported that in R. meliloti, Tn5 insertions in hsnD blocked nodulation of alfalfa but not of Melilotus albus, and extended the host range to Vicia sativa and Vicia villosa.

Nod box. A 47-bp conserved promoter sequence called the nod box, is found in front of each of the nodABC, hsnABC and hsnD transcription units (Rostas et al., 1986). Mutations in the nod box result in the inactivation of downstream genes and the blockage of the nodulation process. The nodD gene product has been shown to bind specifically to the nod box and activate the transcription of the nodABC operon.

Induction of nodulation genes. The nod and hsn genes are coordinately regulated and are specifically turned on during the symbiotic process. Regulation of these nodulation genes requires a host-produced factor (Innes et al., 1985; Peters, et al., 1986; Redmond et al., 1986; Kosslak et al., 1987), a functional nodD gene (Innes et al., 1985; Mulligan and Long, 1985; Rossen et al., 1985), and a conserved nod box sequence (Rostas et al., 1985).

The nod and hsn genes are inducible by low-molecular weight phenolic compounds produced by leguminous plants. In the fast-growing rhizobia, the inducers are substituted flavones and flavanones, such as luteolin (3', 4', 5, 7-tetrahydroxyflavone) and apigenin (4', 5, 7-trihydroxyflavone) for R. meliloti (Peters et al., 1986), naringenin (4', 5, 7-trihydroxyflavone) for R. leguminosarum bv. viciae (Redmond et al., 1986), and 4', 7-dihydroxyflavone for R. leguminosarum bv. trifolii (Innes et al., 1985). In the slow-growing rhizobia, the natural inducers are substituted isoflavones. The most potent inducers for B. japonicum are daidzein, genistein, 5, 7-dihydroxyisoflavone, and 7-hydroxyisoflavone (Rosslak et al., 1987). The nod genes of the fast-growing R. fredii, whose host range overlaps with the slow-growing R. japonicum, are inducible by both flavones and isoflavones (Appelbaum, 1990).

Legumes also secrete compounds that repress nod gene transcription (Firmin et al., 1986; Djordjevic et al., 1987). The isoflavones daidzein and genistein, compounds that function as inducers in the soybean rhizobia, R. fredii and R. japonicum, both antagonize the induction of nod genes in R. leguminosarum bv. viciae (Firmin et al., 1986).

Host specificity reflects the net activation of the nodD gene product by the set of signal compounds produced by each specific legume host, whether stimulatory or inhibitory. Different nodD genes have different inducer specificities and thus, influence host specificity of nodulation. In a study by Horvath et al. (1987), a chimeric nodD gene containing the amino-terminal region of nodD from R. meliloti and the carboxy-terminal of the nodD gene isolated from a strain that nodulates siratro, enabled R. meliloti isolates carrying this construct to nodulate siratro.

Thus, the specificity of the symbiosis operates on two levels of control: the hsn genes, and the combined effect of nodD and flavonoid induction. The current hypothesis is that the nodD gene product is

autoregulatory and requires plant-secreted compounds to convert it to an active form (Inner et al., 1985; Mulligan and Long, 1985; Rossen et al., 1985). The nodD product probably functions as an environmental sensor that is sensitive to the concentrations of stimulatory and inhibitory compounds released in the root exudates of a specific legume. In the presence of these compounds, the nodD protein induces the expression of all other nodulation genes, possibly as a positive transcriptional activator. The coordinate activation of nodulation genes may be mediated by binding of the nodD product to the nod box, resulting in the binding of RNA polymerase to the nod or hsn promoters and the initiation of transcription.

THE PLANT HOST

Host genetic factors regulate infection and nodule development. Evidence that the host plant may play a role in the selection of the more competitive strain was initially seen in clover (Vincent and Waters, 1953). Russel and Jones (1975) showed that such selection could vary not only between species of clover but also between and within varieties. Although the mechanism of selection is still unclear, a host influence on the nodulating success of a strain is widely reported in various legume-Rhizobium associations (Caldwell and Vest, 1968; Diatloff and Brockwell, 1976; Hardarson et al., 1982; Jones and Hardarson, 1979; Materon and Vincent, 1980; May and Bohlool, 1983).

Mechanisms of host selection

Several hypotheses have been proposed to explain the selective role of the plant host during the process of infection and nodulation. Among these are the production of root exudates that selectively stimulate a particular strain in the rhizosphere, and the genetic make-up of the legume host that restricts its compatibility with the rhizobial symbionts.

Role of root exudates. Substances found in legume root exudates have been shown to influence early events in the symbiosis. Several studies indicate that root exudates affect the chemotaxis of a particular strain to the host plant (Currier and Strobel, 1976), elicit faster nodulation from rhizobia (Bhagwat and Thomas, 1982) and induce symbiosis-associated rhizobial genes (Innes et al., 1985; Mulligan and Long, 1985; Peters et al., 1986; Redmond et al., 1986).

The initial signaling from the host plant involves the exudation of compounds that may selectively stimulate some rhizobial strains. Ayanaba et al. (1986) reported that root exudate treatment increased the nodule occupancy of some, but not all B. japonicum strains in their study. Homoserine, a compound excreted from the roots of pea seedlings, stimulated the growth of R. leguminosarum bv. viciae, but had little effect on rhizobia belonging to other cross inoculation groups (van Egeraat, 1978).

Host restriction of nodulation. Some soybean lines restrict the nodulation of B. japonicum strains. The soybean recessive gene rj₁ when present in the homozygous condition, results in the failure to nodulate with most strains of B. japonicum in soil (Williams and Lynch, 1954). The dominant Rj₂ gene in the cultivars Hardee and CNS, conditions an ineffective response with B. japonicum strains in serogroups c1 and 122 (Caldwell, 1966) while the Rj₄ gene in the cultivars Hill, Dunfield, and Dare, conditions an ineffective response with B. japonicum strain 61 (Vest et al., 1972). Roots of Rj₂ and Rj₄ plants develop either cortical proliferations or rudimentary nodules when inoculated with these strains (Caldwell, 1966; Vest et al., 1972).

Cregan et al. (1989) reported the presence of a very specific soybean genotype-serocluster 123 strain interaction that affect both the nodulation ability and competitiveness of strains belonging to this serocluster. In their study, the competitiveness of strains from this serocluster was

severely reduced when a host genotype restricted their nodulation. Apparently, host control of competitiveness is quite specific and the soybean plant can effectively discriminate between serologically related B. japonicum strains.

Host control of nodulation

The development of nodules is carefully regulated and optimized by the plant host. This internal regulation was seen in a study by Singleton and Stockringer (1983). When the soybean plant was inoculated with varying ratios of ineffective and effective strains, the host compensated for the low number of effective nodules by increasing their size. Thus, the plant can also regulate the maintenance of nodules to optimize nitrogen fixation.

Autoregulation. The extent of nodulation is restricted by a process called autoregulation, in which the formation of nodules on one part of the root systemically inhibits subsequent nodule formation in other parts. Autoregulation occurs when developing infection centers in the roots trigger a general plant response that inhibits cell division activity in the roots, thereby preventing excessive nodulation (for review, see Rolfe and Gresshoff, 1988).

Bhuvaneswari et al. (1980) reported the developmental restriction of infection and nodulation on the roots of soybean in the area between the root tip and the smallest emergent root hair. The transient nature of infectibility in this zone was also observed in alfalfa, white clover, cowpea, and peanut (Bhuvaneswari et al., 1981). Pierce and Bauer (1983) proposed that a rapid host-mediated response to infecting rhizobia controls the nodulation process. This was based on their observation that spot inoculation of soybean roots with B. japonicum prior to a second inoculation substantially reduced subsequent nodulation. Furthermore, nodulation suppression appears to be specific, occurring only when the first inoculation involved live, homologous rhizobia.

A study by Calvert et al. (1984) indicated that the regulatory phenomenon occurs at a stage after meristem formation, and is mediated by the suppression of nodule emergence rather than by inhibition of root hair infection. It is thought that the high multiplicity of infections might be a host adaptation to ensure optimal modulation, permitting a positive control of nodule number through selective abortion of infections.

Systemic nature of autoregulation. Split-root studies demonstrate the systemic nature of autoregulation, where prior inoculation of one side results in the suppression of modulation of the other side. Split-root studies in alfalfa (Caetano-Anolles and Bauer, 1988), clover (Sargent et al., 1987), siratro (Djordjevic et al., 1988), and soybean (Rosslak and Bohlool, 1984; Olsson et al. 1989) indicate the general occurrence of autoregulation in various legumes.

The concept of infection-stimulating and infection-inhibiting substances controlling the nodulation process was proposed by Nutman (1952). In his study, the development of emergent nodules in clover seedlings inhibited the rate of subsequent infection initiation, with only a few of the infections developing into functional nodules. Furthermore, nodulation was increased by the excision of previously formed nodules, suggesting that nodules produce inhibitory substances that reduce further nodulation.

Rolfe and Gresshoff (1988) proposed the involvement of a shoot-derived systemic inhibitor elicited by early infections that is exerted on late infections, thus preventing the development of the latter into nodules. Using grafts between supernodulating mutants and wild-type soybeans, Delves et al. (1986) demonstrated that regulation of nodule formation is controlled by the plant shoot. The mechanism of autoregulation is yet to be elucidated. The eliciting signals are still not known, nor are the molecular events involved in the process defined.

CHAPTER 3

VARIATION IN COMPETITIVENESS AND EFFECTIVENESS AMONG

Rhizobium leguminosarum bv. phaseoli STRAINS

Abstract

Competitiveness and effectiveness are two rhizobial attributes required of a good inoculant strain. Six Rhizobium leguminosarum bv. phaseoli strains were characterized for effectiveness and competitiveness on common bean (Phaseolus vulgaris L.), as well as for their cellular attributes. The strains could be distinguished on the basis of their serology, antibiotic resistance patterns, plasmid profiles, melanin production and Calcofluor reactions. Competitive abilities were compared in pairwise inoculations using all possible strain combinations and nodule occupancy was determined with strain-specific fluorescent antibodies. The strains were divided into three groups according to their overall competitive abilities on pole bean cv. Kentucky Wonder and bush bean cv. Bountiful. Strains TAL 1472 and TAL 182 were highly competitive (greater than 70% nodule occupancy); strains KIM-5, Viking 1, and CIAT 899 were moderately competitive (approximately 50% nodule occupancy); and strain CIAT 632 was poorly competitive (less than 5% nodule occupancy). There was no evidence of strain preference by the plant host. The proportion of competing strains in the inoculum influenced the nodule occupancy of the highly competitive and moderately competitive strains. Changes in the inoculum ratio caused a shift in nodule occupancy, becoming higher if inoculated 9:1 in favor of a strain and lower if inoculated 1:9 in favor of the competing strain. Strain CIAT 632 was a consistently poor competitor, occupying less than 10% of the nodules even when it outnumbered the competing strain in the inoculum. Strain effectiveness was evaluated on two cultivars each of pole bean (cv. Kentucky

Wonder and cv. Manoa Wonder) and bush bean (cv. Blue Lake and cv. Brazil). The six strains showed different levels of effectiveness. There was a correspondence between the competitiveness and effectiveness groupings of the strains. The two highly competitive strains had the highest effectiveness indices while the poorly competitive strain had the lowest effectiveness index. The three moderately competitive strains showed intermediate effectiveness. No correlation between competitiveness and nodulation characteristics was found.

Introduction

A yield response to inoculation depends on the use of highly effective rhizobia that are able to compete for nodulation of their legume hosts. Successful nodulation by an introduced strain in soil containing an indigenous population of rhizobia has been attributed to various factors. Among these are environmental variables such as soil type, pH, temperature, combined nitrogen, and biotic factors (for review, see Dowling and Broughton, 1986) as well as the genetic compatibility of the symbiotic partners (Caldwell and Vest, 1968; Hardarson et al., 1982; Jones and Hardarson, 1979; Russel and Jones, 1975; Vincent and Waters, 1953).

Numerous competition studies have been done to isolate the factors that influence competitiveness (Dowling and Broughton, 1986) and to define a predictive component in strain performance in the field (Amarger and Lobreau, 1982; Beattie et al., 1989; Weaver and Frederick, 1974). However, the results of laboratory and greenhouse evaluations of various strains to predict competitive ability in the field have been variable (Amarger, 1981; Johnson and Means, 1964; Johnson et al., 1965; van Rensburg and Strijdom, 1982). Although field studies permit the interplay of all factors that impact on the symbiosis, the complexity of the natural system does not permit the easy comparison of the intrinsic competitive abilities of various strains.

The objective of this study was to examine the competitive ability of six effective Rhizobium leguminosarum bv. phaseoli strains for nodulation of common bean vulgaris L.). The experiments were designed to assess strain competitiveness in the absence of biotic and abiotic soil variables, thus allowing the evaluation of the intrinsic competitive ability of the strains.

This study will allow the identification of a highly competitive and effective strain that can serve as a model strain for in situ competition studies as well as for genetic manipulation, and the assessment of a possible

relationship between nodulation competitiveness and other features of the symbiosis.

Materials and Methods

Bacterial strains and media. The R. leguminosarum bv. phaseoli strains used in this study were selected on the basis of their reputation for effectiveness or competitiveness (Table 3.1). All strains were maintained on yeast extract-mannitol (YEM) agar slants (Bohloul and Schmidt, 1970) containing 0.05% CaCO₃, and routinely streaked on YEM agar plates containing 0.025 mg ml⁻¹ bromthymol blue to check for purity.

Serology of strains. Two-day-old YEN broth cultures were centrifuged at 10,000 X g for 10 min and resuspended in phosphate buffered saline (PBS) (NaH₂PO₄·H₂O, 4.47 g; Na₂HPO₄, 11.55 g; NaCl, 9 g; H₂O, 1 L; pH 7.2) to produce a slight turbidity. Culture smears were air dried, heat fixed, treated with gelatin-rhodamine isothiocyanate (Bohloul and Schmidt, 1968), and stained with the appropriate fluorescent antibody (FA) (Schmidt et al., 1968) obtained from the NifTAL Project, University of Hawaii. The slides were rinsed twice with distilled water, air dried, and observed by epifluorescence microscopy. Reactions of the stained smears were scored by assessment of the degree of fluorescence on a scale of 0 to 4+.

Adsorption of crossreacting FAs was carried out with the heterologous cells. Ten ml of a 2-day old YEM broth culture (approximately 10¹⁰ cells) of the cross-reacting strain were centrifuged at 10,000 X g and washed once in PBS. The pellet was mixed with 10 ml of FA and adsorbed at room temperature for 30 min. The FA was collected after centrifugation and filtration through a 0.2-µm pore-size membrane. One cycle of adsorption was sufficient to eliminate cross reactions, as verified by staining the heterologous strain with the previously adsorbed FA.

Intrinsic antibiotic resistance (IAR) of strains. IAR patterns were determined using the procedure of Josey et al. (1979). Filter-sterilized

TABLE 3.1. Source of R. leguminosarum bv. phaseoli strains used in this study.

Strains	Source	References
CIAT 632 (TAL 1383)	NiftAL	Abaidoo et al., 1990
CIAT 899 (TAL 1797)	NiftAL	Graham et al., 1982
KIM-5 (TAL 943)	D. Bezdicek	Josephson and Pepper, 1984
TAL 182	NiftAL	Abaidoo et al., 1990
TAL 1472	NiftAL	M. Kingsley, Ph.D. thesis, 1987
Viking 1 (TAL 1865)	F. Robert	Robert and Schmidt, 1983

antibiotic solutions were added to YEM agar, to give the following final concentrations ($\mu\text{g ml}^{-1}$, Sigma Chemical Co.): chloramphenicol 12, 25; kanamycin sulfate 10; nalidixic acid 10; neomycin sulfate 2.5; polymyxin B sulfate 20; rifampicin 1, 6; streptomycin 2.5, 10; tetracycline hydrochloride 1; and vancomycin hydrochloride 1.5, 5. The antibiotic stock solutions were prepared at a concentration of 10 mg ml^{-1} in distilled water except chloramphenicol (10 mg ml^{-1} in 95% ethanol), nalidixic acid (10 mg ml^{-1} in 1 N NaOH), and rifampicin (10 mg ml^{-1} in methanol). YEN plates without antibiotics served as controls.

Small volumes (200 μl) of two-day old YEN broth cultures were transferred to the wells of a sterile 96-well tissue culture plate and then inoculated into duplicate plates with a sterile multiple inoculator. Isolates showing growth similar to that of the controls after three days were scored as positive for antibiotic resistance.

Plasmid profiles of strains. An in-well lysis procedure (Eckhardt, 1978; Rosenberg et al., 1982) was used to isolate plasmid DNA in a 3-mm thick vertical 0.7% agarose gel. Cells were grown for two days in tryptone-yeast extract (TY) (Beringer, 1974) broth and 200- μl volumes of the cultures were centrifuged to harvest the cells. The cells were washed with a dilute solution of TEN buffer (0.05 M Tris-base, 0.02 M EDTA, 0.02 M NaCl, pH 8.0) containing 0.1% Sarkosyl (Sigma Chemical Co., St. Louis, MO). The pellet was resuspended in the same solution, and 40 μl of spheroplasting solution was added. This solution contained $7,500 \mu\text{g ml}^{-1}$ lysozyme (Sigma), 30 U ml^{-1} RNase I (Worthington Diagnostics, Freehold, NJ), 20% Ficoll 400 (Sigma), and 0.05% bromophenol blue in TEB buffer (89 mM Tris-base, 8.9 mM EDTA, 89 mM boric acid, pH 8.3). The cell suspension was placed in a well beneath 200 μl TEB, incubated for 15 min, overlaid with a solution of 0.2% (w/v) sodium dodecyl sulfate (SDS) and 10% Ficoll 400 in TEB, and followed by a second solution 0.2% SDS and 5% Ficoll 400 in TEB. The wells were sealed with molten

agarose, and electrophoresis was done at constant current (8 mA for 1 h and 40 mA for 3 h). The gel was stained with ethidium bromide ($5\text{ }\mu\text{g ml}^{-1}$) to visualize the plasmid DNA and photographs were taken using Polaroid 107C film. The approximate molecular weights of the plasmids were estimated from their relative mobilities using the 130-Mdal pJB5JI and other plasmids of R. leguminosarum bv. viciae 6015 (Hirsch et al., 1980) as standards.

Other characteristics of strains. The strains were assayed for the production of melanin on TY agar plates supplemented with $30\text{ }\mu\text{g ml}^{-1}$ L-tyrosine and $10\text{ }\mu\text{g ml}^{-1}$ CuSO_4 (Johnston et al., 1982). After three days of growth, the cells were lysed with a drop of 10% SDS. Colonies that turned dark after 30 min were scored melanin-positive.

Calcofluor, a fluorescent brightener, was used to screen the strains for acidic EPS production (Leigh et al., 1987). The strains were streaked on YEM agar plates containing 0.02% Calcofluor White M2R (Sigma). After three days, the colonies were examined for fluorescence with a hand-held long-wave UV lamp.

Motility was assayed in YEM medium containing 0.005% triphenyltetrazolium chloride and 0.4% agar. Stabs were made in culture tubes and the extent of reddening of the medium was observed after three days.

Plant inoculation and growth conditions. Plant tests for competitiveness and effectiveness of the strains were all done in five replicates in jars (Leonard, 1943) which were filled with a 3:1 (v/v) mixture of vermiculite-perlite. The nitrogen-free plant nutrient solution was that of Hoagland and Arnon (1938) modified to provide the plants with quarter-strength salts and full-strength trace elements. All growth units and solutions were sterilized by autoclaving for 20 min (121°C , 15 psi).

Seeds of common bean (P. vulgaris L.) were selected for uniformity, surface-sterilized in 30% H_2O_2 for 20 min, rinsed five times with sterile

distilled water, and germinated on moist sterile filter paper for two days. The seedlings were transferred aseptically to each jar (one seedling/jar) and inoculated with R. leguminosarum bv. phaseoli.

For all inoculations, each strain was grown in 10 ml of YEM broth for two days at 28°C, centrifuged at 10,000 X g for 10 min and resuspended in plant nutrient solution. The cells were counted in a Petroff-Hausser chamber, diluted to 10⁶ cells ml⁻¹, mixed in different proportions (9:1; 1:1; 1:9) in the case of the competition experiments, and one ml was inoculated unto each plant. Viable cell counts were done on YEN plates for verification of cell numbers. The jars were topped with waterproofed sand (5 kg sand:100 ml Water Seal, E. A. Thompson Co., Memphis, TN) to prevent cross-contamination. The jars were completely randomized in a growth chamber (25°C/18°C day/night temperature). The daylength was 16 h and light intensity was 500 microEinsteins m⁻² s⁻¹ at plant level.

Assessment of strain competitiveness. The strains were evaluated for nodulation competitiveness on pole bean cv. Kentucky Wonder (Burpee Seed Co., Clinton, IA) and bush bean cv. Bountiful (NifTAL Project, University of Hawaii). The competitive abilities of the six strains were compared in pairwise inoculations of all possible combinations. To determine if the competition pattern of the strains was altered by the inoculum input ratio, cv. Kentucky Wonder was inoculated with three proportions of competing strains in the various strain combinations. The experimental controls included uninoculated plants as well as plants inoculated singly with each of the competing strains.

Nodules were collected four weeks after inoculation and frozen at -20°C until use. Nodule occupancy by each strain was determined using strain-specific fluorescent antibodies. In the case of TAL 182 and TAL 1472, the Fas were adsorbed with the crossreacting strain prior to use. Ten nodules per plant were selected at random for a total of 50 nodules per

strain combination. The nodules were crushed singly in H₂O and smears were made on duplicate slides. Two additional control smears of nodules containing individual strains were included as positive and negative controls. The two slides were stained separately with FA specific for each of the competing strains.

Competitiveness groups were derived by averaging the percent nodule occupancy of each strain against those of all the other competing strains in the various treatment combinations.

Assessment of strain effectiveness. The strains were evaluated for effectiveness on two pole beans, cv. Manoa Wonder (Department of Horticulture, University of Hawaii) and cv. Kentucky Wonder, as well as two bush beans, cv. Brazil (NifTAL Project, University of Hawaii) and cv. Blue Lake (Burpee Seed Co., Clinton, IA) by single-strain inoculations. Five replicates of uninoculated plants per cultivar served as controls. Plants were harvested five weeks after inoculation and total number of nodules, nodule dry weight, and shoot dry weight were measured. Shoot and nodule dry weights were determined after drying at 75°C to constant weight. Effectiveness was expressed as the proportionate increase in shoot dry weight of the inoculated plant over the uninoculated nitrogen-free control. Specific nodule dry weight was determined by dividing the total dry weight of nodules by the total number of nodules. The effectiveness index and nodulation parameters were subjected to analysis of variance using PC-SAS (Statistical Analysis System for Personal Computers, SAS Institute, Inc., Cary, NC).

Results

Strain characterization. The uniqueness of the R. leguminosarum bv. phaseoli strains used in this study was established by serology, IAR patterns, plasmid profiles, melanin production and Calcofluor reactions. The strains were serologically distinct (Table 3.2), with the exception of TAL

182 and TAL 1472 which had crossreactive antibodies. However, crossreaction between the two strains was eliminated after adsorption with the heterologous cells. All six strains had characteristic IAR patterns (Table 3.3). Some strains were easily distinguishable, such as CIAT 632 and KIM-5, which were clearly different with their unique resistance to kanamycin and tetracycline, respectively. In contrast, the differences between the IAR patterns of TAL 1472 and TAL 182, or of TAL 1472 and Viking 1 were very slight. The additional tests confirmed the uniqueness of each strain (Table 3.4). Each of the strains had distinctive plasmid profiles. The plasmids, ranging in number from two to five in a strain, were 100 to 300 Mdal in size. Of the six strains, two (CIAT 899 and CIAT 632) were melanin minus while one (CIAT 899) was Calcofluor positive. All the strains were motile.

Strain competitiveness. Table 3.5 shows the nodule occupancy of the six strains when two competing strains are mixed together in equal proportions using all possible pairwise strain combinations. The overall competitive ability of each strain was derived by averaging the percent nodule occupancies of the individual strains from the different strain combinations. The six strains can be grouped according to their competitive abilities on two cultivars (Figure 3.1). Two strains (TAL 1472 and TAL 182) were highly competitive, occupying greater than 70% of the nodules. Three strains (CIAT 899, Viking 1, and KIM-5) were moderately competitive, occupying approximately 50% of the nodules. One strain (CIAT 632) was poorly competitive, occupying less than 5% of the nodules. There was no evidence of strain host preference. The competitiveness of the strains was similar on pole bean cv. Kentucky Wonder and bush bean cv. Bountiful.

Effect of inoculum ratio on nodule occupancy. Figure 3.2 shows the effect of varying the proportion of the two competing strains in each of the strain combinations. The overall relationship between the inoculum ratio and nodule occupancy was derived by averaging the nodule occupancies of each

TABLE 3.2. Fluorescent antibody reactions of *R. leguminosarum* bv. *phaseoli* strains.

Strain	FA reaction ^a					
	CIAT 632	CIAT 899	KIM-5	TAL 182	TAL 1472	Viking 1
CIAT 632	4+	0	0	0	0	0
CIAT 899	0	4+	0	0	0	0
KIM-5	0	0	4+	0	0	0
TAL 182	1+	0	0	3+ ^b	0	1+
TAL 1472	0	0	0	0	3+ ^b	0
Viking 1	1+	0	0	0	1+	4+

^aIntensity of fluorescence (0 to 4+).

^bFA reaction after reciprocal adsorption of TAL 182 and TAL 1472 Fas with heterologous cells.

TABLE 3.3. Intrinsic antibiotic resistance patterns of *R. leguminosarum* bv. *phaseoli* strains.

Strain	Antibiotic resistance ^a												
	<u>Cm</u> ^b		<u>Km</u>	<u>Nl</u>	<u>Nm</u>	<u>Pm</u>	<u>Rf</u>		<u>Sm</u>		<u>Tc</u>	<u>Vc</u>	
	12 ^c	25	10	10	2.5	20	1.0	6.0	2.5	10	1.0	1.0	5.0
CIAT 632	-	-	+	+	+	+	+	-	-	-	-	+	+
CIAT 899	+	-	-	+	+	+	+	-	-	-	-	+	+
KIM-5	+	+	-	+	+	+	+	+	+	+	+	+	+
TAL 182	+	+	-	+	+	+	+	-	-	-	-	+	-
TAL 1472	+	+	-	+	+	+	+	-	+	-	-	+	-
Viking 1	+	-	-	+	+	+	+	-	+	-	-	+	-

^a+, growth similar to control indicating resistance; -, no growth indicating susceptibility.

^bCm, chloramphenicol; Km, kanamycin sulfate; Nl, nalidixic acid; Nm, neomycin sulfate; Pm, polymyxin B sulfate; Rf, rifampicin; Sm, streptomycin; Tc, tetracycline hydrochloride; Vc, vancomycin hydrochloride.

^cAntibiotic concentrations were in $\mu\text{g ml}^{-1}$.

TABLE 3.4. Other characteristics of *R. leguminosarum* bv. *phaseoli* strains.

Strain	Indigenous plasmids (Mdal)	Melanin production	Calcofluor reaction	Motility
CIAT 632	300, 275, 150, 130, 100	-	-	+
CIAT 899	220, 130	-	+	+
KIM-5	290, 185, 100	+	-	+
TAL 182	180, 150, 100	+	-	+
TAL 1472	195, 150	+	-	+
Viking 1	285, 180	+	-	+

^aDetermined on YEM agar plate containing 30 $\mu\text{g ml}^{-1}$ L-tyrosine and 10 $\mu\text{g ml}^{-1}$ CuSO_4 .

^bDetermined on YEM agar plate containing 0.02% Calcofluor.

^cDetermined on YEM semisolid agar tube containing 0.005% triphenyltetrazolium chloride.

TABLE 3.5. Nodule occupancy^a by *R. leguminosarum* bv. *phaseoli* strains on two *P. vulgaris* (L.) cultivars after double-strain inoculation in 1:1 ratio.

Strain Combination	Cultivar		Significance ^b	
	K. Wonder	Bountiful	K. Wonder	Bountiful
	-----%-----			
Viking 1:TAL 182	26:68 (6) ^c	36:64	***	NS
Viking 1:CIAT 899	26:66 (8)	44:48 (8)	***	NS
Viking 1:CIAT 632	94:4 (2)	90:10	***	***
Viking 1:TAL 1472	26:68 (6)	6:94	***	***
Viking 1:KIM-5	46:48 (6)	50:50	NS	NS
TAL 182:CIAT 899	74:24 (2)	84:16	***	***
TAL 182:CIAT 632	100:0	100:0	***	***
TAL 182:TAL 1472	36:58 (6)	20:66 (14)	NS	***
TAL 182:KIM-5	82:16 (2)	82:18	***	***
CIAT 899:CIAT 632	98:2	84:16	***	***
CIAT 899:TAL 1472	12:80 (8)	4:94 (2)	***	***
CIAT 899:KIM-5	60:34 (6)	24:74 (2)	NS	***
CIAT 632:TAL 1472	0:100	0:100	***	***
CIAT 632:KIM-5	0:100	2:98	***	***
TAL 1472:KIM-5	76:18 (6)	84:14 (2)	***	***

^aNodule occupancy was determined from five plants per strain combination for a total of 50 nodules.

^bA Chi-square analysis was used to test the deviation of the observed nodule occupancy of the singly occupied nodules from the expected ratio (df = 1), NS, not significant; ***, $P \geq 0.005$.

^cParenthetical values represent the percentage of double occupancy.

FIGURE 3.1. Overall competitiveness of R. leguminosarum bv. phaseoli strains on P. vulgaris (L.) cv. Kentucky Wonder and cv. Bountiful. The values represent the nodule occupancy of each strain averaged from every combination where the strain competed with the other strains in 1:1 proportion.

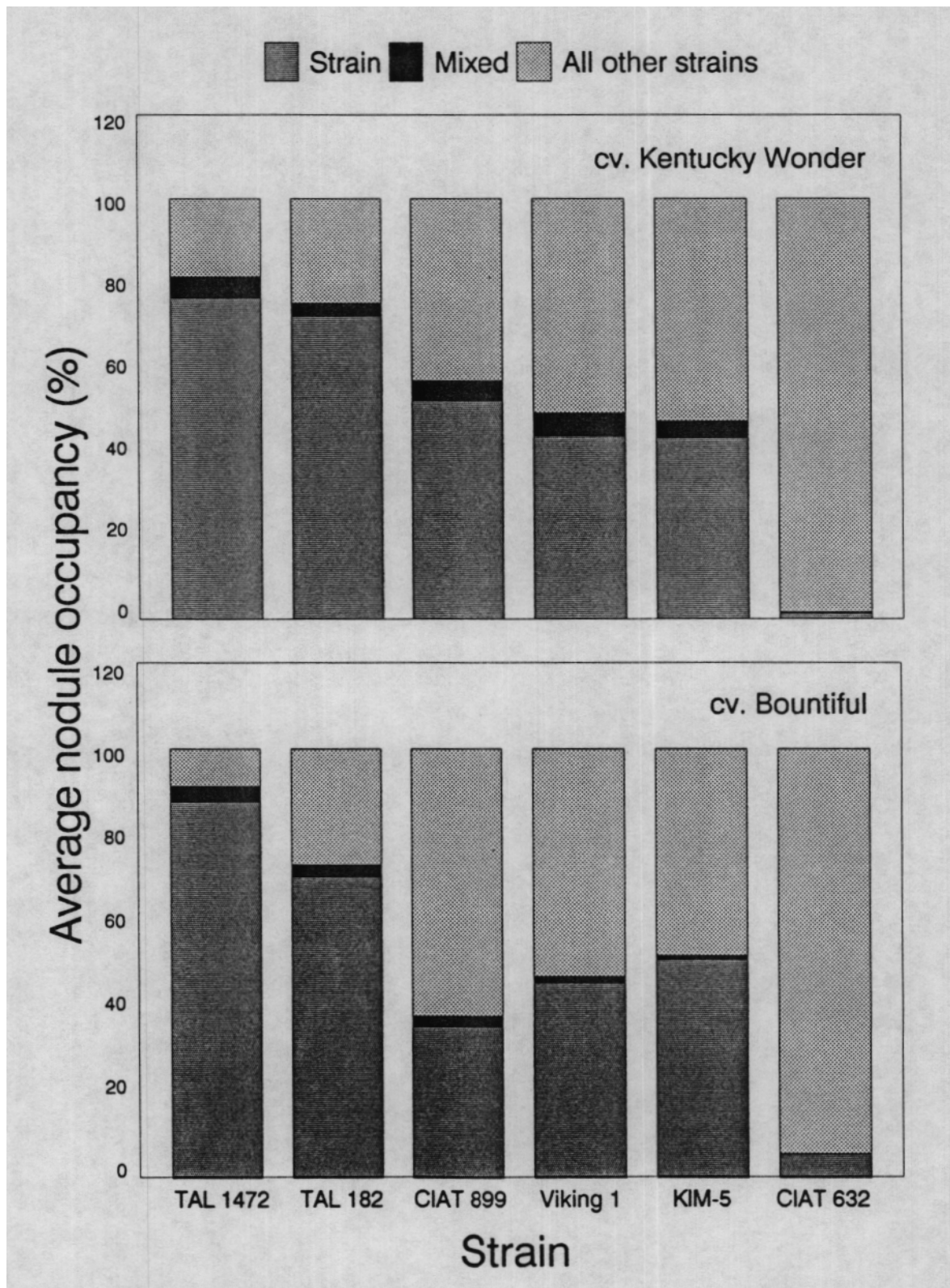
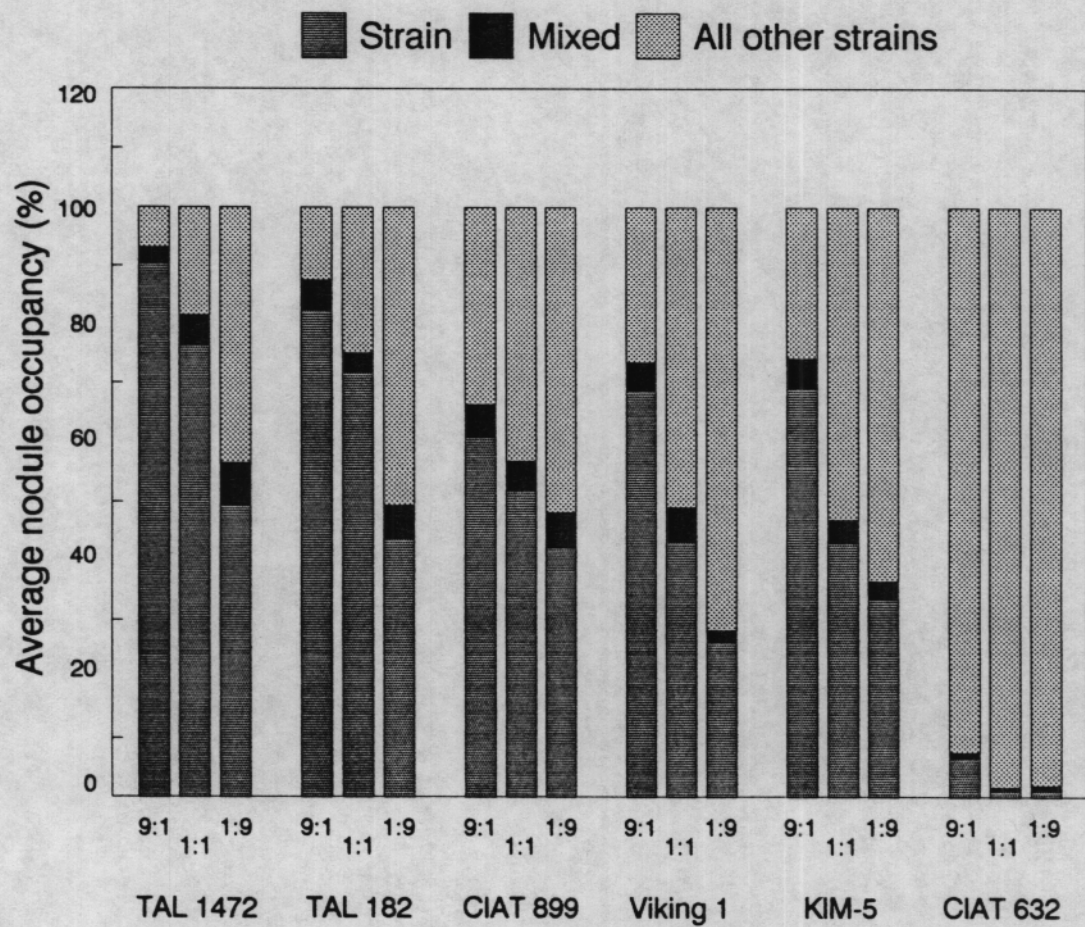


FIGURE 3.2. Effect of inoculum proportions on the competitiveness of R. leguminosarum bv. phaseoli strains on P. vulgaris (L.) cv. Kentucky Wonder. The values represent the nodule occupancy of each strain averaged from every combination where the strain competed with the other strains in specific proportions.



strain from all the strain combinations within one inoculum ratio. The effects varied with the individual strain combinations, but on average, the nodule occupancy of a strain depended on the proportion of competing strains in the inoculum. A change in the inoculum ratio of the competing strains were associated with a shift in nodule occupancy, becoming higher if inoculated 9:1 in favor of a strain and lower if inoculated 1:9 in favor of the competing strain. However, this shift was notable only in the highly competitive and moderately competitive strains. The magnitude of the shift is such that nodule occupancy of the moderately competitive strains when inoculated in 9:1 ratio in their favor, became comparable to that of the highly competitive strains when inoculated in equal proportions. Strain CIAT 632 was consistently a poor competitor, showing minimal change in nodule occupancy even when it outnumbered its competitor in the inoculum.

Strain effectiveness. Strain effectiveness on P. vulgaris L., which was evaluated on two cultivars each of pole bean and bush bean, was standardized by calculating an effectiveness index (Table 3.6). The index was expressed as the proportionate increase in shoot dry weight of the inoculated plant over the uninoculated nitrogen-free control. There were no significant cultivar by strain interactions, indicating that no specific combination was superior over the range of treatments. The effectiveness index was significantly influenced by strain ($P<.05$) and cultivar ($P<0.001$). The six strains showed varying levels of effectiveness: high (TAL 1472 and TAL 182), intermediate (CIAT 899, Viking 1, and KIM-5), and low (CIAT 632). The pole bean cv. Manoa Wonder showed the highest effectiveness index among the four bean cultivars.

Nodulation characteristics of strains. There were significant differences among strains in their nodulation characteristics. On cv. Kentucky Wonder (Figure 3.3), the strains significantly influenced nodule number ($P<0.05$), nodule dry weight ($P<0.01$), and specific nodule dry weight

TABLE 3.6. Effectiveness index^a of *R. leguminosarum* bv. *phaseoli* strains on four *P. vulgaris* (L.) cultivars five weeks after inoculation.

Strain	Cultivar ^b				Mean
	<u>Bush beans</u>		<u>Pole beans</u>		
	BL	BR	KW	MW	
TAL 1472	3.8	2.8	4.0	6.8	4.4a ^c
TAL 182	3.2	3.8	3.1	6.8	4.2a
KIM-5	3.0	3.4	2.6	6.4	3.8ab
Viking 1	2.3	3.9	2.0	6.1	3.6ab
CIAT 899	2.6	3.7	4.2	5.2	3.9ab
CIAT 632	2.6	2.9	2.4	4.8	3.2b
Mean	2.9B ^c	3.4B	3.0B	6.0A	

^aEffectiveness index was calculated by dividing the shoot dry weight of the inoculated plant by the uninoculated nitrogen-free control.

^bCultivar BL-Blue Lake, BR-Brazil, KW-Kentucky Wonder, MW-Manoa Wonder

^cStrain by cultivar interaction was not significant. Values followed by the same lower case letter in a column or the same upper case letter in a row are not significantly different by Duncan's multiple range test ($P < 0.05$).

($P < 0.01$). CIAT 632 had significantly higher nodule number than four other strains (TAL 182, CIAT 899, Viking 1, and KIM-5) while CIAT 632 and KIM-5 had significantly higher nodule dry weights than two other strains (CIAT 899 and Viking 1). KIM-5 had the highest specific nodule dry weight.

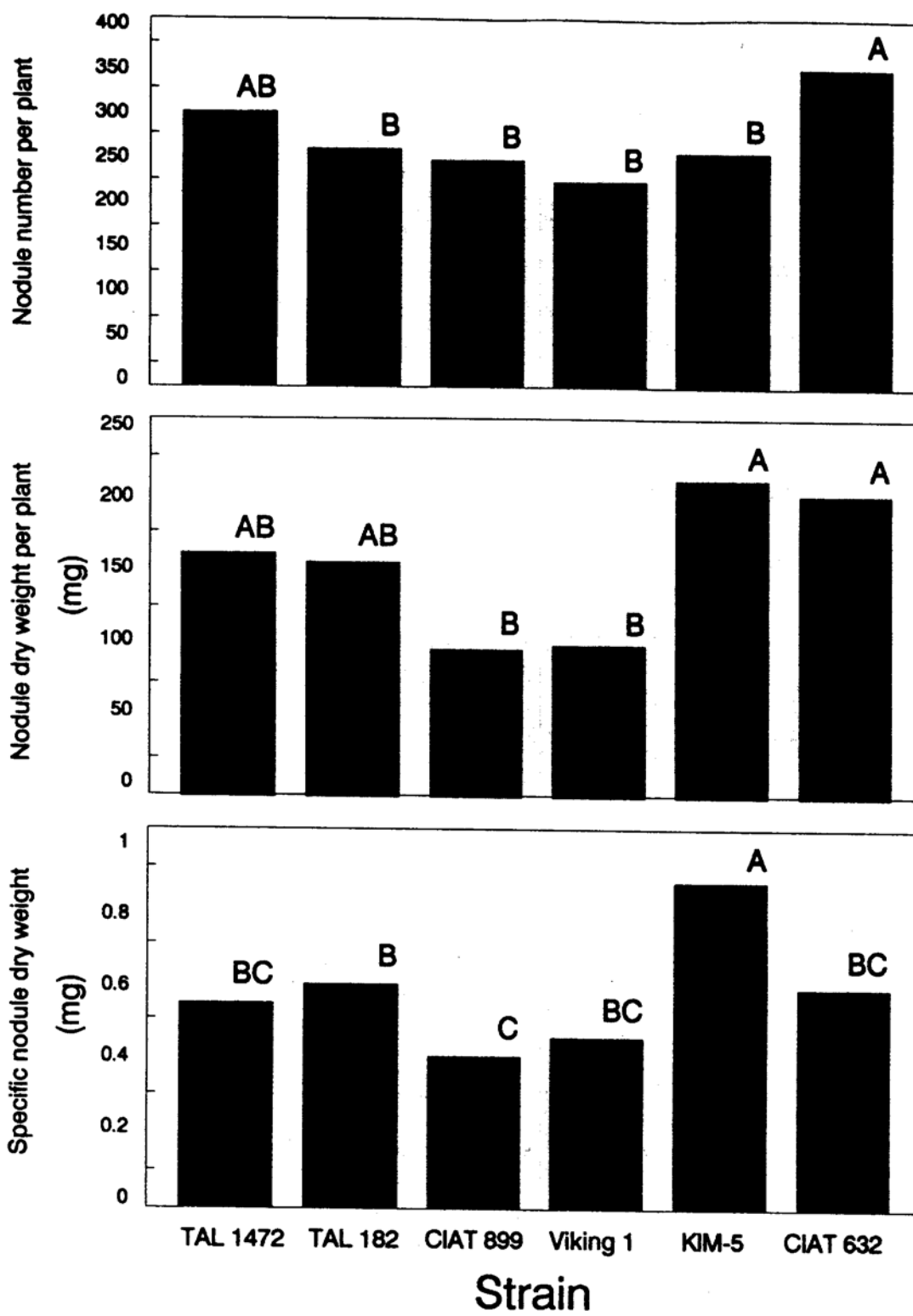
Discussion

In this study, the intrinsic competitive ability of a strain was evaluated based on its nodule occupancy when it competes with another in a wide spectrum of strain combinations. An index of strain competitiveness was derived by averaging the nodule occupancy of a particular strain when paired with another in various combinations. In this way, the separate effects of large numbers of combinations were pooled into a meaningful grouping. This provides an estimate of the overall competitive ability of a strain. In the absence of biotic and abiotic soil variables, R. leguminosarum bv. phaseoli strains showed distinctly different competitive abilities (Figure 3.1).

In field studies, the differential competitive abilities of R. leguminosarum bv. phaseoli strains were also observed. Introduced strains showed nodule occupancies of 10-15% (Beynon and Josey, 1980), 3-68% (Beattie et al., 1989), and 100% (Robert and Schmidt, 1983) in the presence of indigenous populations. One of the highly competitive strains identified in this study, TAL 182, was also found to dominate in the nodules when inoculated in the field (Abaidoo and van Kessel, 1989; Abaidoo et al., 1990). However, KIM-5 (Josephson and Pepper, 1984; Beattie et al., 1989) and Viking 1 (Robert and Schmidt, 1983), which are considered highly competitive in the field, were only moderately competitive when challenged by the strains used in our study.

The capacity to outcompete a competitor appears to be a strain characteristic. The competitiveness grouping of the six strains did not change with the host cultivars (Figure 3.1), indicating that the plant did not discriminate between the six strains.

FIGURE 3.3. Nodulation parameters of R. leguminosarum bv. phaseoli strains on P. vulgaris (L.)_cv. Kentucky Wonder. Specific nodule dry weight was determined by dividing the total dry weight of nodules by the total number of nodules. Bars with the same letters are not significantly different by Duncan's Multiple Range Test ($P < .05$).



There was a general tendency for nodule occupancy to change with the relative proportion of the competing strains in the inoculum (Figure 3.2). The overall effect was such that moderately competitive strains can achieve dominance comparable to that of highly competitive strains when inoculated in high enough numbers relative to the competing strains. However, this effect was not seen in the poorly competitive strain. Strain CIAT 632 still occupied an insignificant proportion of the nodules even when it outnumbered the competing strain in the inoculum. Thus, competitiveness appears to be a flexible character only among those strains that possess an intrinsic ability to compete. However, this plasticity may be true only under controlled conditions. In the field, there is no evidence that strain competitiveness is a function of cell numbers (Abaidoo et al., 1990; Moawad et al., 1984; Robert and Schmidt, 1983).

The frequency of mixed infections ranging from 0 to 16% (not all data shown), was within the range (up to 40%) reported in the literature on plants grown under bacteriologically controlled conditions (Beatie et al., 1989; Bromfield and Jones, 1980; Franco and Vincent, 1976; Labandera and Vincent, 1975; Lindemann et al., 1974). While a higher incidence of double occupancy was seen when highly competitive and moderately competitive strains were paired with others, this was not the case in the poorly competitive strain. Thus, the occurrence of mixed infections seems related to the competitiveness of a strain. Such relationship was also noted in other studies (May and Bohlool, 1983; Josephson and Pepper, 1984).

There was a correspondence between the competitiveness and the effectiveness groupings of the strains. On the four cultivars used in the study, the two highly competitive strains (TAL 1472 and TAL 182) had the highest effectiveness indices while the poorly competitive strain (CIAT 632) had the lowest effectiveness index (Table 3.5). The results of other studies correlating competitiveness and effectiveness have been variable. Among

equally effective strains, differences in competitiveness were found (May and Bohlool, 1983; Means et al., 1961; Roughley et al., 1976). In some studies contrasting the competitive abilities of effective and ineffective strains, the effective strain appeared to have an advantage over an ineffective competitor (Labandera and Vincent, 1975; Pinto et al., 1974; Robinson, 1969). In others, ineffective strains were described as being more competitive in nodule formation than effective strains (Franco and Vincent, 1976; Nicol and Thornton, 1942; Vincent and Waters, 1953).

The results of this study showed no correlation between competitiveness and nodulation characteristics (Figure 3.3). CIAT 632, a poorly competitive strain, formed significantly more nodules than a highly competitive strain (TAL 182) and the moderately competitive strains (CIAT 899, Viking 1, and KIM-5) while strains KIM-5 and CIAT 632 showed significantly higher nodule dry weights than two moderately competitive strains (CIAT 899 and Viking 1). KIM-5 also had the highest specific nodule dry weight. The highly competitive strains, on the other hand, were generally intermediate in their nodulation characteristics. This lack of correlation was also observed in other studies (George et al., 1987; Pinto et al., 1974).

Of the six R. leguminosarum bv. phaseoli strains, TAL 1472 and TAL 182 make good candidates for genetic manipulation to study the molecular basis for competitiveness. The superior competitive ability of TAL 182 was consistently demonstrated under different soil types and environmental conditions (Abaidoo and van Kessel, 1989; Abaidoo et al., 1990; Somasegaran and Bohlool, 1990) and its high effectiveness was also shown on several cultivars of common bean (Pacovsky et al., 1981; Somasegaran and Bohlool, 1990). Furthermore, the contrast provided by the highly competitive-poorly competitive strain combination of TAL 1472 or TAL 182 versus CIAT 632 represents an ideal model for autecological studies.

CHAPTER 4

HOST NODULATION SUPPRESSION AS A FACTOR IN COMPETITION

AMONG Rhizobium leguminosarum bv. phaseoli STRAINS

Abstract

The plant host influences the extent of nodulation by autoregulation. It is postulated that the plant expresses a systemic signal in response to infecting rhizobia, resulting in the suppression of subsequent nodulation. In this study, host nodulation suppression was examined as a factor in interstrain competition for nodulation of common bean (Phaseolus vulgaris L.) cv. Bountiful using split-root systems. The pattern of suppression induced by R. leguminosarum bv. phaseoli TAL 182 was characterized in a time-course experiment. A primary inoculation was done on one side of a split-root at day zero, followed by a secondary inoculation on the opposite side with increasing delay (one to 14 days). The extent of suppression of secondary nodulation was time-dependent, becoming more pronounced as the delay period was increased. A 14-day delay in secondary inoculation resulted in 94% suppression of secondary nodulation. When six R. leguminosarum bv. phaseoli strains with varying competitive abilities were used as primary inoculants to compare their ability to induce suppression, all strains induced nodulation suppression but varied in their degree of suppressiveness. A highly competitive strain (TAL 1472) was also highly suppressive whereas a poorly competitive strain (CIAT 632) was also less suppressive. Suppression ability was not related to the speed of nodule formation or to the nodulating potential of a strain. On cv. Bountiful, most of the strains formed nodules five days after inoculation and the number of newly emerged nodules peaked one day later. RIM-5, a moderately competitive strain, formed nodules and reached its maximum number of new nodules one day

earlier than the rest of the strains, but was less suppressive than some strains. Similar nodule numbers were formed by the strains but there were differences in nodule weights. The strain with the highest nodule weight (KIM-5) was less suppressive than other strains.

Introduction

The symbiotic process is initiated once rhizobia encounter their host plant. However, successful nodulation by the introduced strains is not assured if indigenous rhizobia are already present in the soil. The ability of a strain to dominate the nodules is dependent on environmental factors as well as the genetic compatibility between the plant host and the rhizobia.

Host selection of the competing strain may constitute an important factor that affects the ability of rhizobia to compete and nodulate successfully. The influence of the host on the nodulating success of a strain is well documented in various legume-Rhizobium associations (Bromfield, 1984; Caldwell and Vest, 1968; Hardarson et al., 1982; Jones and Hardarson, 1979; Materon and Vincent, 1980; May and Bohlool, 1983; Vincent and Waters, 1953). However, only a few studies have addressed the role of the host as a variable that can significantly influence the outcome of competition (Sargent et al., 1987).

While the mechanism of host selection is still not known, it is clear that the development of nodules is under the regulation of the plant host. Autoregulation is thought to occur when the plant expresses a signal in response to invading rhizobia, allowing the plant to optimize nodulation and prevent excessive modulation (for review, see Rolfe and Gresshoff, 1988). The systemic nature of autoregulation is seen across a split-root system where a suppression of nodulation is seen on one half of the root system when the other half has been previously nodulated. Such a host response was reported in alfalfa, clover, siratro, and soybean (Caetano-Anolles and Bauer, 1988; Djordjevic et al., 1988; Kosslak and Bohlool, 1984; Olsson et al., 1990; Sargent et al., 1987).

Root infectibility was found to be a transient property (Bhuvaneswari et al., 1981) due to a rapid host suppression of nodulation (Pierce and Bauer, 1983). If an inhibitor of nodulation is induced after primary

infection and nodule initiation, the early period of the symbiosis may assume a critical importance in the competition process. Several studies provide evidence that early events do affect the outcome of competition (Kosslak et al., 1983; Kosslak and Bohlool, 1985; de Oliveira et al., 1990; Skrdleta, 1970; Stephens and Cooper, 1988). In the study of Kosslak et al. (1983), preexposure of soybean roots to a less competitive strain of B. japonicum resulted in a significant increase in the proportion of nodules occupied by that strain under competitive conditions. Thus, the speed of a strain to initiate nodulation may become a determining factor in nodule occupancy.

In this study, host modulation suppression was studied as a factor in competition among strains of Rhizobium leguminosarum bv. phaseoli for nodulation of common bean (Phaseolus vulgaris L. cv. Bountiful). The objectives were 1) to characterize the process of nodulation suppression in common bean, 2) to determine whether the ability to induce suppression is a factor in the nodulation competitiveness of rhizobial strains, and 3) to correlate a strain's speed to initiate nodule formation and its nodulating potential to suppressiveness and competitiveness.

Materials and Methods

Bacterial strains and media. The six R. leguminosarum bv. phaseoli strains used in this study, their maintenance, and growth conditions were as described in Chapter 3. The inherent competitive abilities of the strains were previously characterized as highly competitive (TAL 1472 and TAL 182), moderately competitive (CIAT 899, Viking 1, and KIM-5), and poorly competitive (CIAT 632).

Plant inoculations and growth conditions. Plant tests for all nodulation and nodulation suppression experiments were done using plastic growth pouches (Northrup King Seed Co., Minneapolis, MN) that contained nitrogen-free nutrient solution (Hoagland and Arnon, 1938). The nutrient solution was modified to one eighth-strength salts and one half-strength

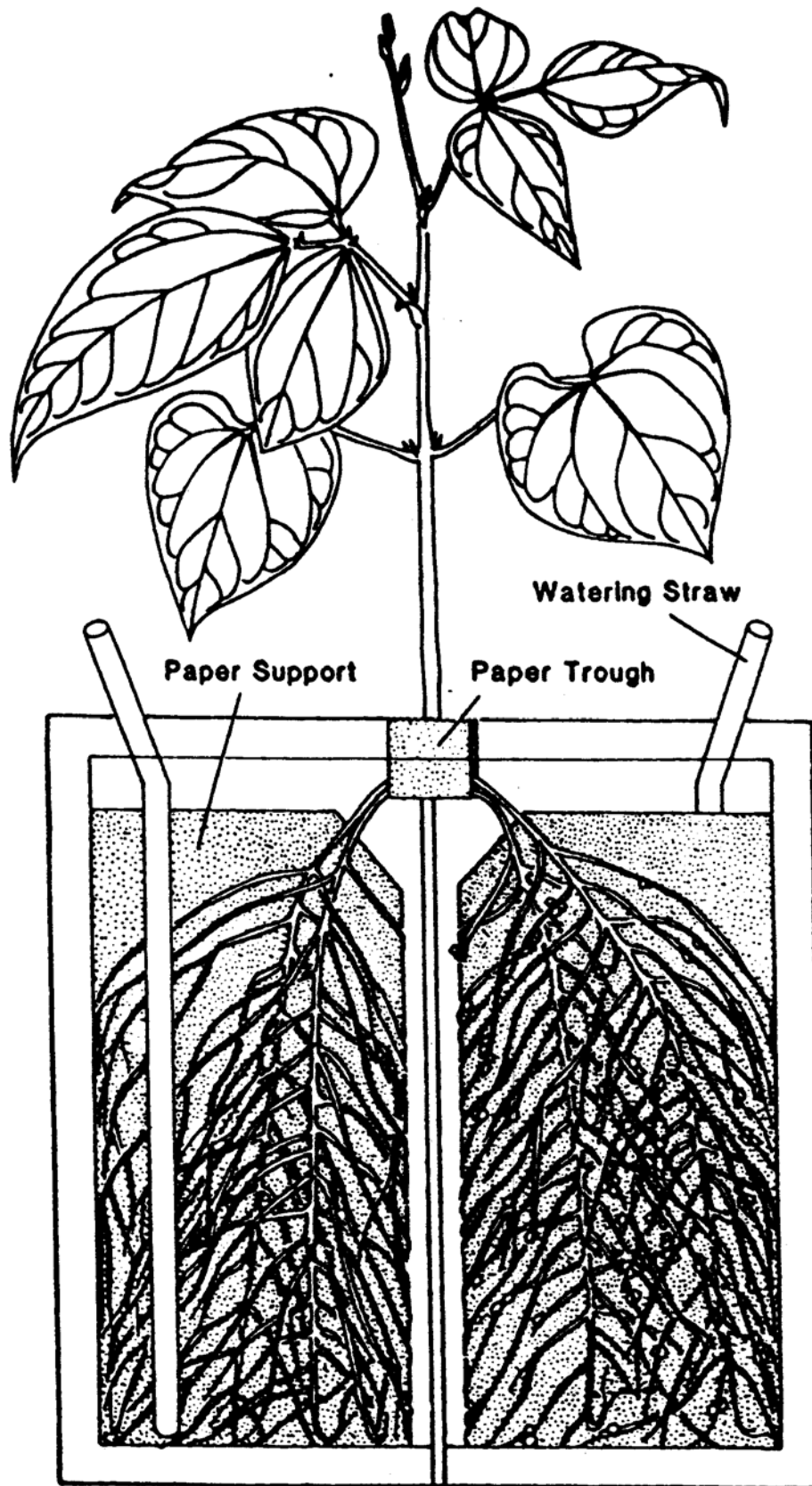
trace elements. The nutrient solution was sterilized by autoclaving for 20 min (121°C).

The split-root plastic growth pouch used in the nodulation suppression experiments was prepared by cutting the paper wick into two and dividing the plastic pouch by heat sealing (Figure 4.1). A paper trough in the middle supported the plant and plastic straws on both sides of the pouch were used to deliver the nutrient solution. The pouches were tested for interchamber leaks by pouring 15 ml of plant nutrient solution first on one side, waiting for 10 min to check that the other side remained dry, then pouring another 15 ml on the other side. This volume of nutrient solution was maintained on each side of the growth pouch by periodic watering.

The common bean (P. vulgaris L.) cv. Bountiful, provided by the NifTAL Project, University of Hawaii, was the host plant used in all assays. The seeds were selected for uniformity, surface-sterilized in 30% H₂O₂ for 20 min, rinsed five times with sterile distilled water, and germinated on a sterile bed of a 3:1 (v/v) mixture of vermiculite-perlite. The split-root systems were generated by cutting off the root meristem of 2-day-old seedlings. Each seedling was placed on top of the paper trough with moist paper strips connecting the trough and the paper support in the two chambers, allowing equally vigorous lateral roots to develop in each chamber of the plastic pouch. After three days, extraneous lateral roots were severed, leaving two secondary roots of equal length on each side. The connecting paper strips were then removed to create a discontinuity between the two chambers, preventing cross-contamination. The cotyledons were excised at this time to minimize plant variability due to nitrogen reserves.

For all inoculations, each strain was grown in 10 ml of YEM broth for two days and diluted in nutrient solution to approximately 10⁶ cells ml⁻¹. Five days after germination, a primary inoculation consisting of one ml of diluted culture, was done on one side of the split-root (E, denoting early

Figure 4.1. Diagram of a split-root plastic growth pouch
used in the study.



inoculation). A secondary inoculation was done on the opposite side of the split-root after a given delay period (D, denoting delayed inoculation). All experiments included uninoculated split-root plants as controls. The plants were grown in a temperature-controlled (25°C) room providing a 16-h daylength and a light intensity of 500 microEinsteins m⁻² sec⁻² at plant level.

In all experiments, the plants were harvested four weeks after the primary inoculation. Nodules on each side of the split-root were counted and collected, and shoot and nodule weights were determined after drying at 75°C for two days.

Nodulation suppression by TAL 182. Strain TAL 182, a highly effective and highly competitive strain (Chapter 3), was the standard strain used in this study. The pattern of nodulation suppression induced by TAL 182 on common bean was characterized in a time-course experiment using five replicate split-root systems per treatment. One side of a split-root system was inoculated at zero time, and the second side with increasing delay (E/D treatment). At zero time, the controls included an early inoculation on one side only (E/0 control) and early inoculation on both sides of the split-root (E/E control). The delay period consisted of 1-day increments for ten days after the primary inoculation and a 14-day delay. For each delay period, there were controls consisting of delayed inoculation only on one side (0/D control) as well as delayed inoculation on both sides of the split-root (D/D control). In this experiment, TAL 182 was both the primary (E) and secondary (D) inoculant.

The delay period of secondary inoculation showing maximal suppression of nodulation was determined and served as the standard delay period in subsequent nodulation suppression experiments.

Nodulation suppression patterns of different strains. Each of the six R. leguminosarum bv. phaseoli strains was used as the primary inoculant on a split-root system. Strain TAL 182 was used as the standard secondary

inoculant on the delayed side two weeks after the primary inoculation. At zero time, the controls consisted of E/0 and E/E inoculations with each strain. At the delayed time, the controls consisted of D/D and 0/D inoculation with the standard strain. All treatments and controls consisted of five replicate split-root systems.

Statistical analysis was performed using PC-SAS (Statistical Analysis System for Personal Computers, SAS Institute, Inc., Cary, NC).

Assessment of nodulation profiles. To compare the speed of nodule formation by the individual strains, bean seeds were surface sterilized and germinated as previously described. After two days, the seedlings were transferred to unmodified plastic growth pouches (1 plant/pouch). After another three days, each of the six strains was inoculated individually on five replicate plants. The location of newly emerged nodules was marked on the plastic pouch and their number was counted daily for 10 days after inoculation.

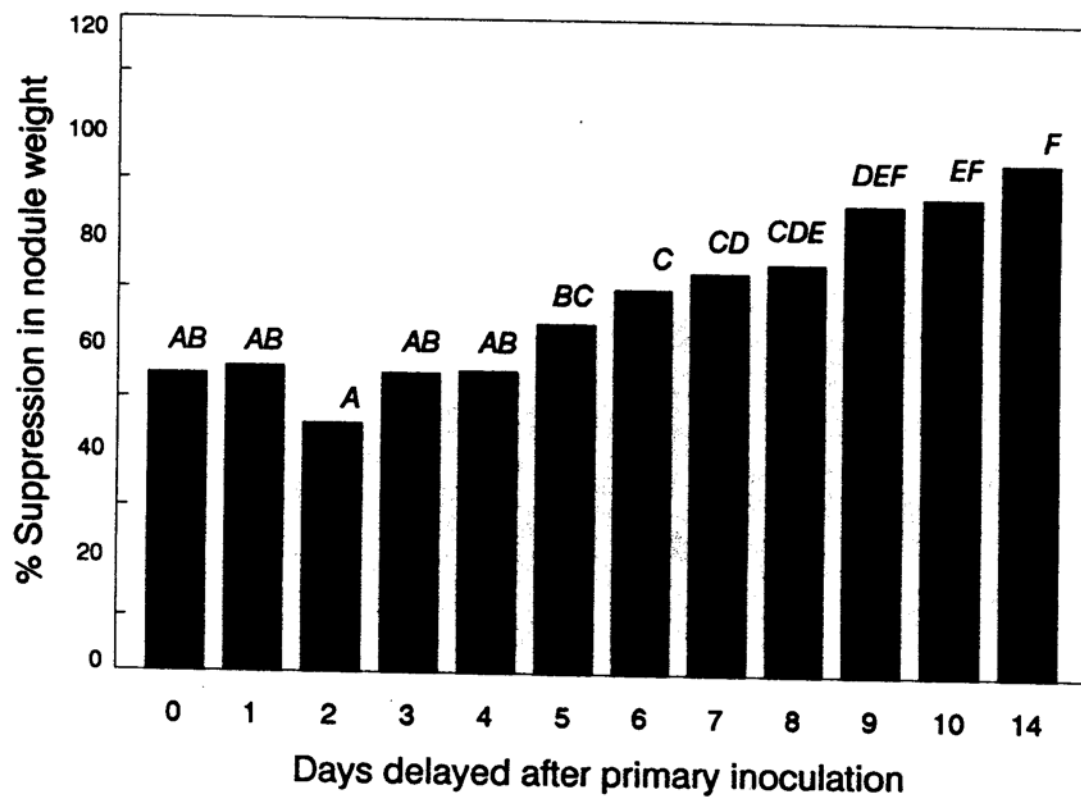
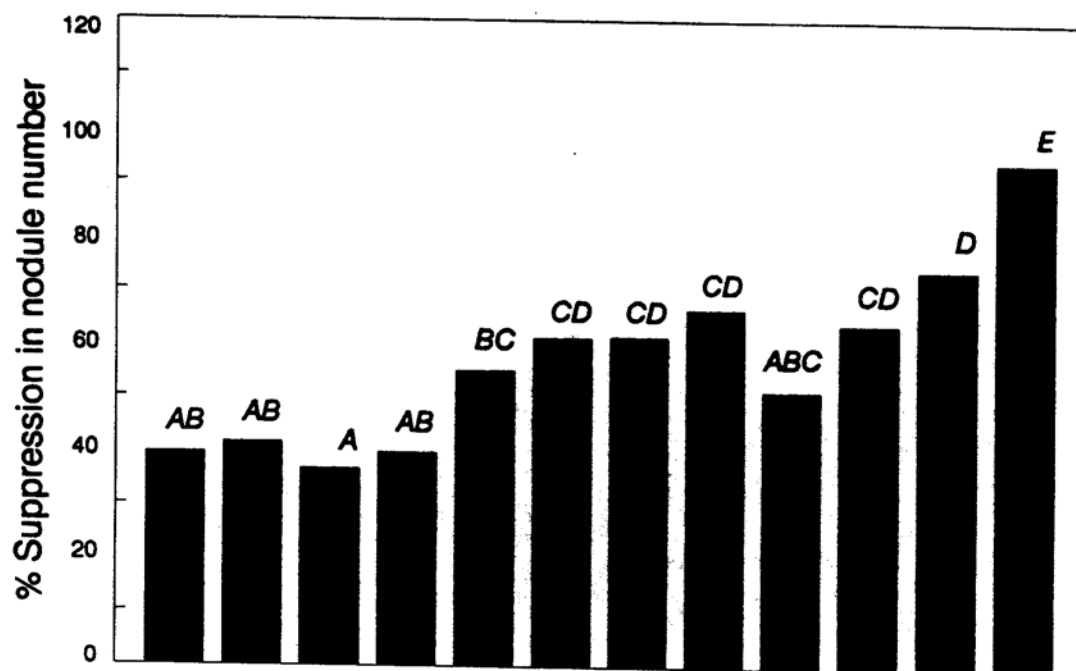
Results

Time-course of nodulation suppression by TAL 182. Figure 4.2 shows the pattern of nodulation suppression induced by R. leguminosarum bv. phaseoli TAL 182 on common bean as secondary inoculation was delayed for increasing periods of time. The data used to generate this figure are in the Appendix. Inhibition of secondary nodulation was expressed as percent suppression, using the formula:

$$\% \text{ Suppression} = 100 - \% \text{ Nodulation}$$

The percent nodulation was derived by expressing the extent of secondary nodulation (D side of E/D treatment) as a percentage of the nodulating potential of that side of the split-root at the time of the delayed inoculation (D side of 0/D control) at each delay period. For example, on day 1:

FIGURE 4.2. Time-course of nodulation suppression induced by R. leguminosarum bv. phaseoli TAL 182 on common bean (P. vulgaris L.) cv. Bountiful. The plants were harvested four weeks after the primary inoculation. Inhibition of secondary nodulation was expressed as percent suppression, using the formula:
$$\% \text{ Suppression} = 100 - \% \text{ Nodulation}$$
Percent nodulation is the nodule number and nodule weight from the secondary inoculation (D side of E/D, Early/Delayed treatment), expressed as a percentage of the nodulating potential of that side of the split-root at the time of the delayed inoculation (D side of 0/D, Uninoculated/Delayed control) at each delay period. Bars with the same letters are not significantly different by LSD ($P \leq 0.05$).



$$\% \text{ Nodulation} = \frac{\text{D1 nodule number (of E/D1)}}{\text{D1 nodule number (of 0/D1)}} \times 100$$

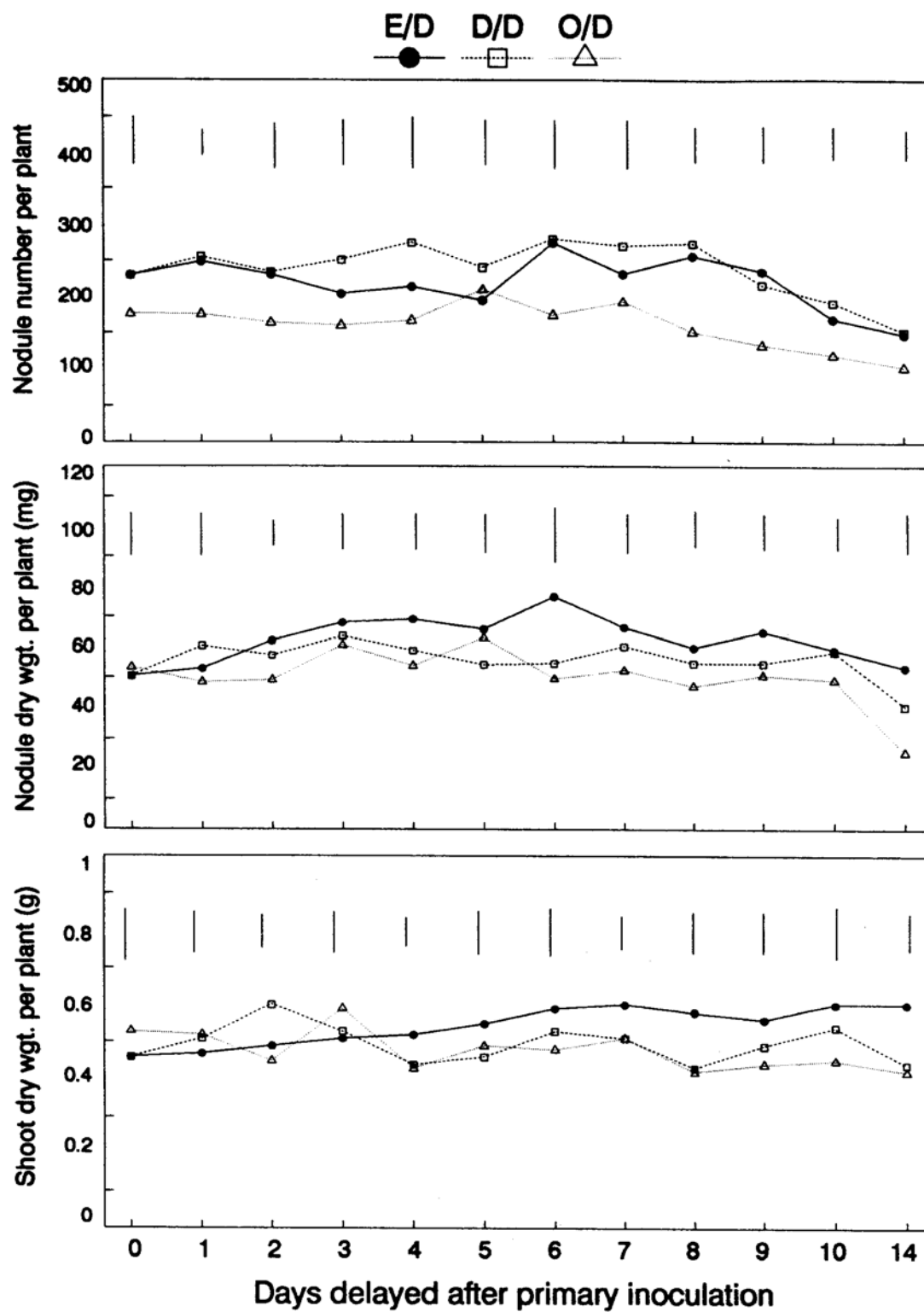
By quantifying suppression this way, the analysis was standardized with the comparison of nodules at the same developmental stage.

When a primary inoculation was done on one side of a split-root at day zero and was followed by a secondary inoculation on the opposite side with increasing delay, the suppression of nodulation on the side that received the delayed inoculation was time-dependent. As the delay period was increased, the extent of suppression became more pronounced. When the secondary inoculation was placed 14 days after the primary inoculation, there was 94% suppression of secondary nodulation in nodule number and nodule weight. The split-root systems in which only one side was inoculated (0/E or 0/D controls) confirmed the absence of cross-contamination between the two sides of the growth pouch.

The effect of different inoculation treatments over time on plant parameters are shown in Figure 4.3. At harvest, nodule weight and shoot weight were similar in most cases, regardless of time of inoculation of the two sides or the number of sides inoculated (E/D, D/D, or 0/D), indicating that plant growth was limited by the growth system. In general, there was a significant reduction in nodule number when only one side of the split-root was inoculated (0/D) as compared to when two sides were inoculated (E/D or D/D), possibly due to the decreased size of the root system exposed to inoculation.

Nodulation suppression by R. leguminosarum bv. phaseoli strains. When six R. leguminosarum bv. phaseoli strains with varying competitive abilities were used as primary inoculants, all strains induced nodulation suppression (Figure 4.4). The 0/D control, representing the nodulation potential of the secondary inoculant in the absence of suppression, showed an average nodule number of 151 and nodule weight of 25 mg, as compared to a range of 11-40

FIGURE 4.3. Nodule and shoot parameters of common bean (*P. vulgaris*) cv. Bountiful as influenced by different inoculation treatments with *R. leguminosarum* bv. *phaseoli* TAL 182. The plants were harvested at day 28. Bars indicate the least significant difference (LSD) between E/D (Early/Delayed), D/D (Delayed/Delayed), and O/D (Uninoculated/Delayed) treatments.



and 0.8-4.8 mg, respectively, resulting from the occurrence of suppression induced by the six strains. Although the suppression patterns of the strains looked similar, there were significant differences ($P < 0.001$) among the strains in their degree of suppressiveness. Overall, a comparison of the nodule number and nodule weight resulting from the secondary inoculation on the delayed side of the E/D treatment showed that TAL 1472 and CIAT 899 were more suppressive than KIM-5 and CIAT 632.

Strain speed of nodulation. Figure 4.5 shows the number of nodules formed by each strain as they became visible on the roots everyday for ten days after inoculation. Four of the strains formed nodules five days after inoculation and the number of newly emerged nodules peaked one day later. The nodulation patterns of three strains (TAL 1472, TAL 182, and CIAT 632) were very similar. KIM-5 formed nodules and reached its maximum number of new nodules one day earlier than the rest of the strains. Viking 1 formed nodules at the same time as KIM-5, but reached its peak at the same time as the other strains.

Symbiotic characteristics of individual strains. These data in Figure 4.6 were derived from the E/E controls for each strain, measured four weeks after inoculation. On cv. Bountiful, although the six strains formed similar nodule numbers, some differed significantly ($P < 0.001$) in nodule weights. KIM-5 showed the highest nodule weight. No significant differences were found in the shoot weight of plants inoculated with each of the six strains, perhaps due to the limiting growth conditions in the growth pouches.

Discussion

The mechanisms that determine the ability of a strain to dominate the nodules of its host in the presence of another nodulating strain are far from understood. In this study, a split-root system was used to characterize nodulation suppression in common bean and to examine whether differences in the inherent competitiveness of R. leguminosarum bv. phaseoli strains could

FIGURE 4.4. Nodulation suppression induced by six R. leguminosarum bv. phaseoli strains on common bean (P. vulgaris L.) cv. Bountiful. The early side of the E/D (Early/Delayed) treatment was inoculated with each of the primary strains at day zero while the delayed side was inoculated with a standard strain (TAL 182) at day 14. The O/D (Uninoculated/Delayed) control represents the nodulation potential of the plant at the delayed time in the absence of suppression. Bars with the same upper case or lower case letters are not significantly different by Duncan's multiple range test.

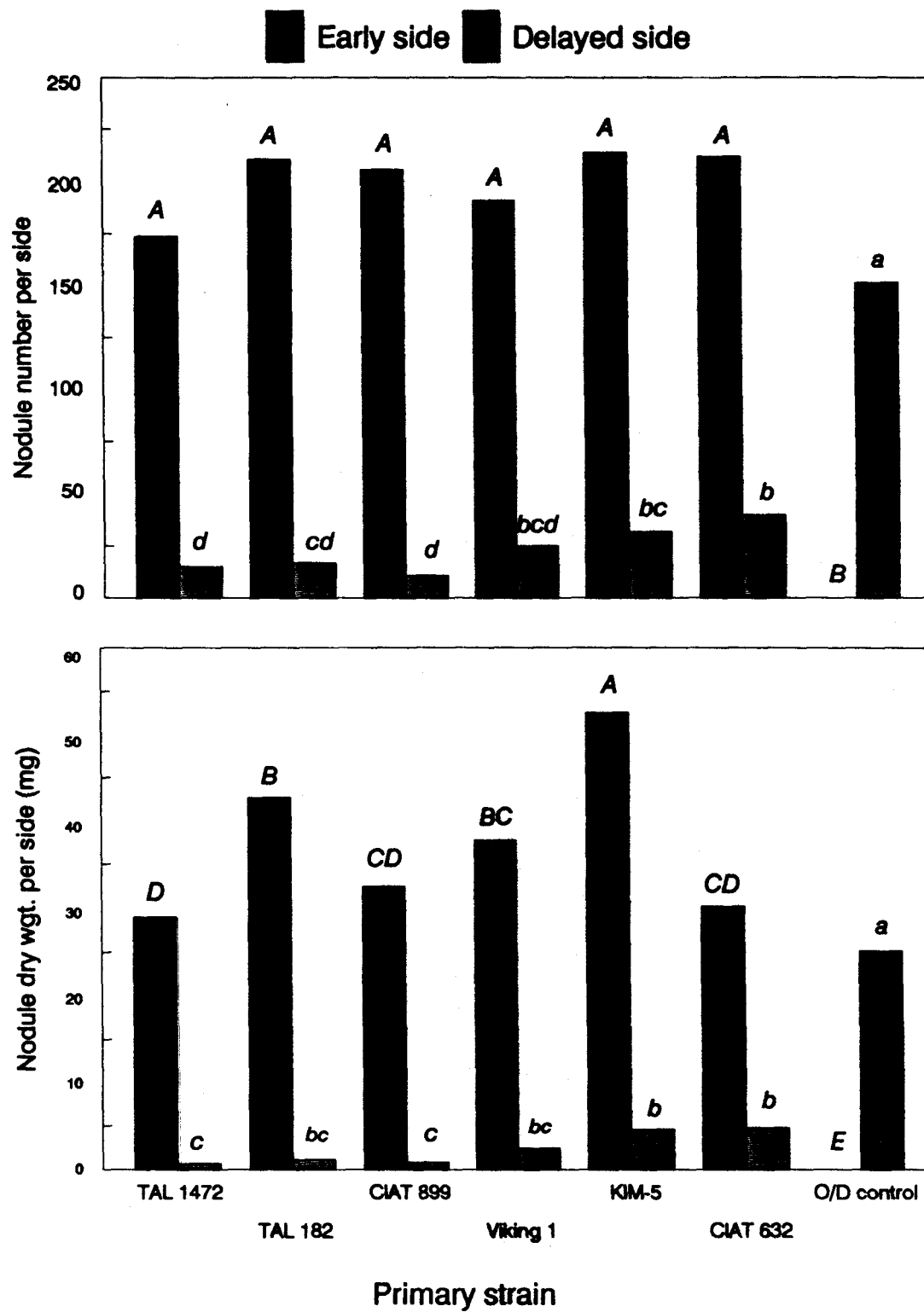


FIGURE 4.5. Speed of nodule formation of six R. leguminosarum bv. phaseoli strains on common bean (P. vulgaris L.)_cv. Bountiful. The location of newly emerged nodules was marked on the growth pouch as they became visible, and their number was counted daily for ten days after inoculation.

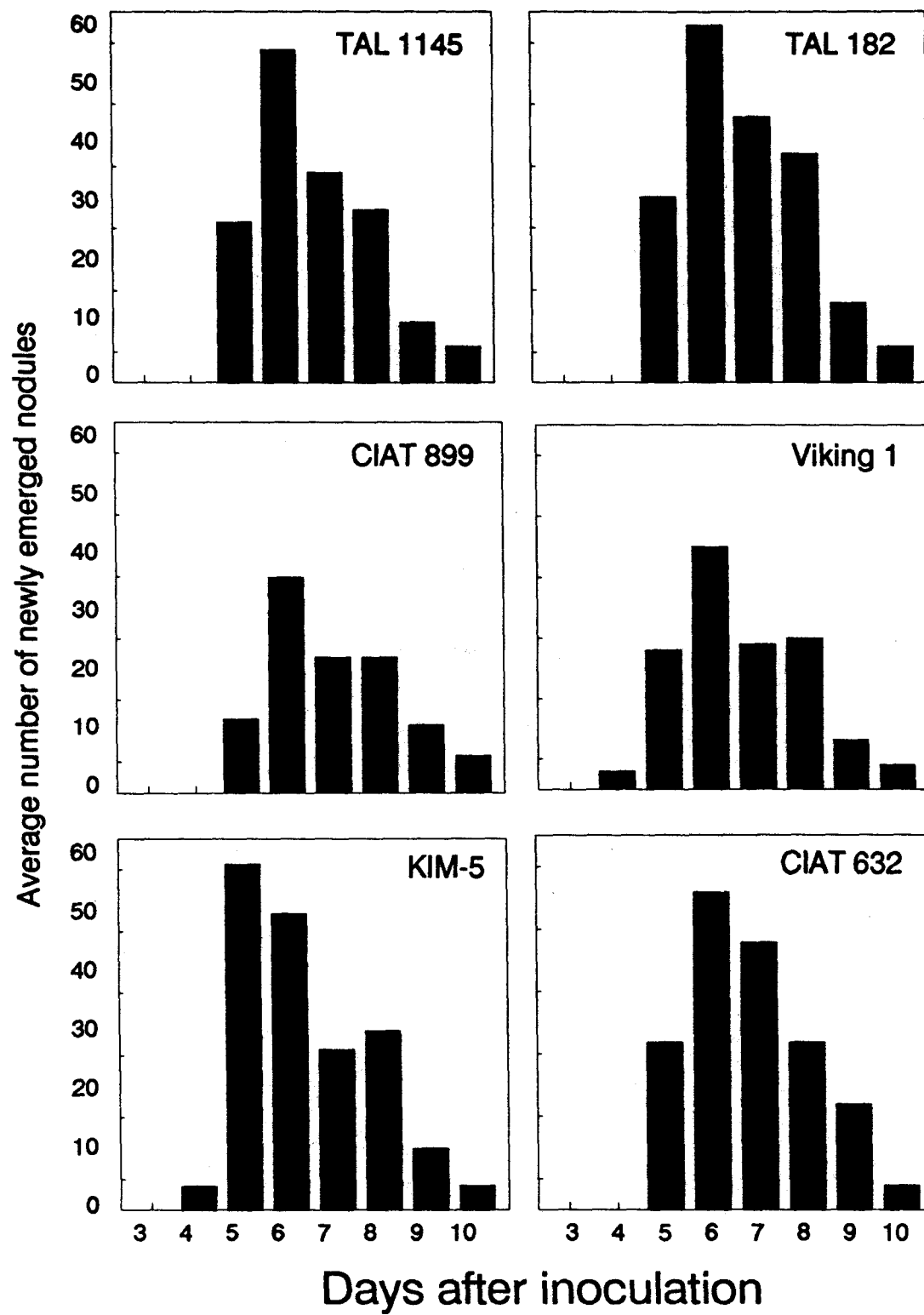
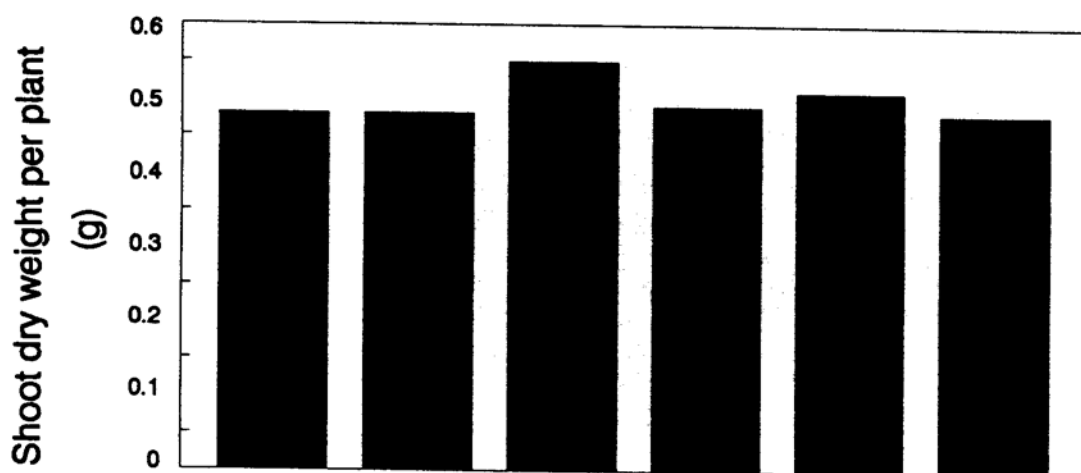
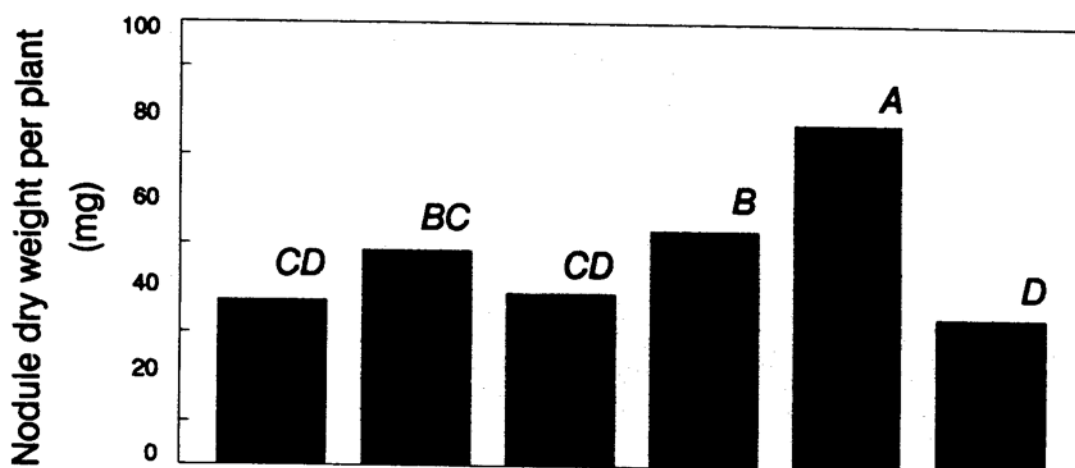
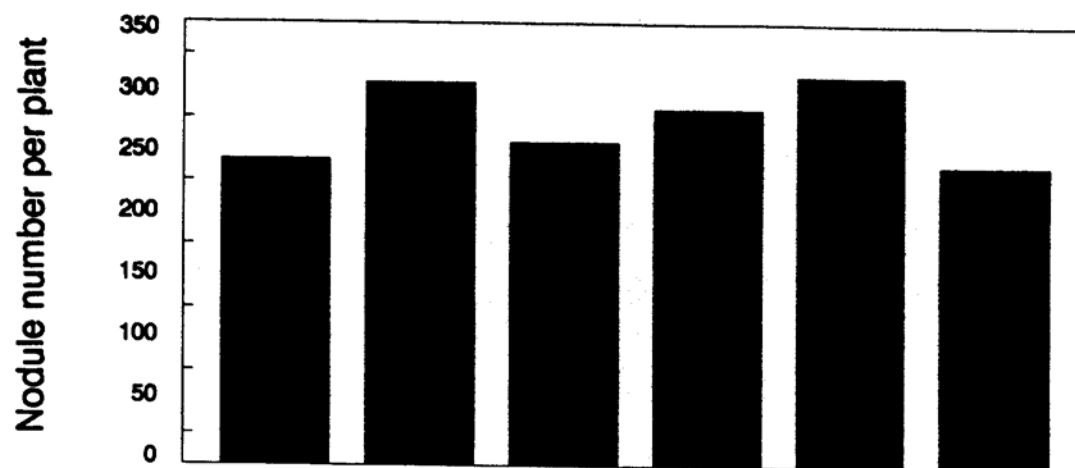


FIGURE 4.6. Symbiotic characteristics of common bean (*P. vulgaris* L.) cv. Bountiful inoculated with six *R. leguminosarum* bv. *phaseoli* strains. Data were derived from the E/E (Early/Early) controls for each strain, measured four weeks after primary inoculation. Letters represent the separation of means by Duncan's multiple range test when differences are significant ($P < 0.05$).



TAL 1472 TAL 182 CIAT 899 Viking 1 KIM-5 CIAT 632

Strain

be explained by the their abilities to induce an autoregulatory response from the plant host.

The results show that nodulation in common bean is subject to suppression (Figure 4.2). Despite the limiting effect of the pouch system on plant growth in this study, measurable suppression was evident. The inherent limitation of this growth system is also apparent in the similar shoot weights of plants inoculated by six strains (Figure 4.6) despite significant differences in effectiveness (Chapter 3) shown by the strains on other cultivars in a less restrictive growth system (Leonard jars). Nevertheless, some of the strains showed differences in nodule weights that were not reflected in the shoot weights (Figure 4.6), but might have been reflected in shoot nitrogen content not measured in this experiment.

Suppression was apparent regardless of the constancy of plant parameters at harvest (Figure 4.3). The fact that differences between treatments in time, from inoculation to harvest, did not translate into differences in total nodule number, total nodule weight, or shoot weight (Figure 4.3), indicate that these parameters have reached their maximum in this growth system. Thus, the observed suppression is likely to have resulted from differential partitioning of a constant photosynthate supply to the two sides.

In this study, suppression was measured through time independent of the developmental stage of the nodules resulting from the secondary inoculation. Since the plants from all treatments were harvested at the same given time from primary inoculation, nodules on the side that received the secondary inoculations with increasing delay were of decreasing age. However, the confounding effect of nodule age on the measurement of suppression was eliminated by standardizing secondary nodulation in relation to the nodulating potential of the plant without primary inoculation at that particular delayed time (Figure 4.2).

All six strains with differential competitive abilities induced nodulation suppression (Figure 4.4). Differences in the degree of suppressiveness of the strains were not striking. A statistical analysis of the results did not show a clear correspondence between the suppressiveness and the competitiveness of the strains. However, it appeared that TAL 1472, a highly competitive strain, was more suppressive than CIAT 632, a poorly competitive strain.

A possible relationship between systemic suppression and strain competitiveness was previously suggested by Sargent et al. (1987), who used Tn5 mutants slightly impaired in their ability to nodulate to show an associated impairment in suppression in clover split-roots. In this study, nodulation-competent wild-type strains whose inherent competitive abilities were well-characterized (Chapter 3), were used, thereby eliminating the complication of a nodulation defect in the competition process.

A comparison of the nodulation profiles of the six strains showed that strain suppressiveness or competitiveness could not be explained by a higher potential for nodule initiation or nodule development. KIM-5, a moderately competitive strain, was the best nodulator in terms of speed (Figure 4.5) and weight (Figure 4.6) but was less suppressive than some strains (Figure 4.4). The poorly competitive and less suppressive strain (CIAT 632) initiated nodules at the same rate as a highly competitive and more suppressive strain (TAL 1472). Furthermore, the strain (KIM-5) with the highest nodule weight (Figure 4.6), was less suppressive than some strains (Figure 4.4).

Variable results have been described relating the speed of nodule formation to the outcome of competition. In some studies, strains of R. leguminosarum bv. trifolii (Stephens and Cooper, 1988) and R. leguminosarum bv. phaseoli (de Oliveira and Graham, 1990) which nodulated their plant hosts rapidly also showed a competitive edge over slower nodulating strains.

In other studies, competitiveness did not correlate with the relative speed with which strains produced nodules or with the number of nodules that they produced (Pinto et al., 1974; Smith and Wollum, 1989).

The data on the suppression patterns of differentially competitive strains are not conclusive. Clearer differences in the suppression levels of the strains might have been detected with a shorter delay period of the secondary inoculation (between 11-13 days) or with a larger number of plants to decrease experimental variability.

However, the results of this study seem to suggest the involvement of a postinfection event in interstrain competition and highlight a more prominent role of the host in the process. One may speculate that all nodulation-competent competing strains are able to infect the host, but when a strain fails to induce a strong suppression response, nodule primordia formed by the competing strain may continue to develop and eventually form the majority of the nodules.

CHAPTER 5

AUTOREGULATORY RESPONSE OF Phaseolus vulgaris L. TO SYMBIOTIC MUTANTS OF Rhizobium leguminosarum bv. phaseoli

Abstract

The plant host controls and optimizes the nodulation process by autoregulation. In this study, Tn5 mutants of R. leguminosarum bv. phaseoli which are impaired at various stages of symbiotic development, were used to examine autoregulation in common bean. TAL 182, a highly competitive and effective strain, was randomly mutagenized with Tn5 and the transconjugants were screened for symbiotic defects. Three classes of symbiotic mutants were identified. Class I mutants were nonnodulating, Class II mutants induced small swellings on the roots, and a Class III mutant formed pink, bacteroid-containing, but ineffective nodules. In addition, metabolic mutants with differential nodulation abilities, were isolated. A purine mutant (Ade⁻) was nonnodulating while a pyrimidine mutant (Ura⁻) formed small swellings on the roots. Amino acid mutants (Leu⁻, Phe⁻, Cys⁻) formed white, empty nodules. Southern hybridization analysis indicated that each mutant had a single Tn5 insertion. Each of the mutants was used as a primary inoculant in split-root systems to assess its ability to induce nodulation suppression in common bean. Suppression was correlated with the presence of the microsymbiont inside the nodules, but not with the ability to initiate nodule formation or with the ability to fix nitrogen. All mutants with defects in nodulation ability, regardless of the particular stage of blockage, failed to induce a suppression response from the host. Only the nodulation-competent, bacteroid-containing, but ineffective Class III mutant induced a suppression response similar to that induced by the wild-type. Thus, alteration in the

nodulation competence of the mutants apparently also altered the autoregulatory response of the plant host.

Introduction

The development of the legume-Rhizobium symbiosis requires a precise interaction between host and microbe. Reciprocal control by the two partners allows the symbiotic process to occur in a regulated manner, leading to a mutually beneficial association.

The plant exerts control by autoregulation, which involves the shoot-controlled inhibition of further nodulation in the presence of developing nodules (Rolfe and Gresshoff, 1988). The systemic nature of autoregulation has been demonstrated in split-root systems of alfalfa (Caetano-Anolles and Bauer, 1988), clover (Sargent et al., 1987), siratro (Djordjevic et al., 1988), and soybean (Kosslak and Bohlool, 1984; Olsson et al., 1989). In common bean, the nodulation process is also subject to autoregulation (Chapter 4).

There is evidence that nodule meristems are the target of suppression by developing nodules. In soybeans, microscopic studies by Calvert et al. (1984) showed that prior nodulation did not suppress the initiation of infection in younger regions of the root but instead suppressed the development of nodule meristems into emergent nodules.

Suppression appears to be a response to some, as yet unidentified, aspect of nodule development that is exerted during the early stages of the process. Soybean split-root studies showed that suppression of nodulation on the side that received a delayed inoculation was not related to the onset of nodulation or to the onset of nitrogenase activity on the side inoculated at an earlier time (Kosslak and Bohlool, 1984), but that suppression was triggered much earlier in the symbiotic process. Exactly at what stage of symbiotic development the autoregulatory response is initiated still remains unclear.

In this study, Tn5-induced mutants of R. leguminosarum bv. phaseoli TAL 182 capable of developing only to specific stages of symbiosis, were used to

define the stage at which autoregulatory occurs in common bean. The mutants were used as primary inoculants in split-root systems to correlate a particular symbiotic stage with the suppression response of the plant host.

Materials and Methods

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 5.1. R. leguminosarum bv. phaseoli TAL 182, a highly competitive and effective strain, served as the model strain.

Rhizobium strains were grown and maintained as previously described (Chapter 3).

Media. TAL 182 Tn5 mutants were grown on TY medium (Beringer, 1974). E. coli strains were grown on LB medium (Miller, 1972). When selective conditions were needed, antibiotics (Sigma) were used at the following concentrations: kanamycin ($50 \mu\text{g ml}^{-1}$), nalidixic acid ($20 \mu\text{g ml}^{-1}$), and rifampicin ($20 \mu\text{g ml}^{-1}$).

The minimal (MM) medium contained sodium glutamate (1.8 g l^{-1}) as a combined carbon and nitrogen source, the following salts (mg l^{-1}): KNO_3 (600), K_2HPO_4 (32), KH_2PO_4 (16), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (250), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (67), KCl (112), Fe-EDTA (10), and trace elements. The trace element solution was based on that of M. Kingsley (Ph.D. thesis, 1987).

Mutagenesis procedure. Four Tn5-carrying suicide vector plasmids were evaluated for their transposition frequency and efficiency on TAL 182. Approximately 10^8 cells each of late-log phase donor and recipient cells were washed in sterile saline (0.9% NaCl, w/v) and collected by filtering successively onto a $0.45\text{-}\mu\text{m}$ pore-size filter (Millipore Corp., Bedford, MA). The filters were incubated on TY plates at 28°C overnight. The cells were resuspended in two ml of sterile saline, and plated on selective medium. The frequency of transposition in each cross was expressed as the number of transconjugants (from TY plates supplemented with kanamycin and nalidixic acid or with kanamycin and rifampicin) per total number of recipients (from

TABLE 5.1. Bacterial strains and plasmids.

Strains	Relevant characteristic	Reference
<u>R. leguminosarum</u> bv. <u>phaseoli</u>		
TAL 182	Nl', Rf', Km', wild-type	This study (Chapter 3)
TAL 1472	highly competitive	This study (Chapter 3)
CIAT 632	poorly competitive	This study (Chapter 3)
<u>E. coli</u>		
S17.1	Nl', pSUP1021 donor	Simon et al., 1986
HB101	Rf', pGS9 and pBLK-2 donor	Boyer and Roulland-Dussoix, 1969
1830	Rf', pJB4JI donor	Berlinger et al., 1978
Plasmids		
pJB4JI	Tn ₅ suicide vehicle	Berlinger et al., 1978
pGS9	Tn ₅ suicide vehicle	Selvaraj and Iyer, 1983
pSUP1021	Tn ₅ suicide vehicle	Simon et al., 1986
pBLK1-2	Tn ₅ suicide vehicle	Kim et al., 1988
pSKS101	Tn ₅ probe	Shapira et al., 1983

TY plates supplemented with nalidixic acid or with rifampicin). The efficiency of transposition in each cross was assessed by replica-plating 1,200 transconjugants on MM medium containing kanamycin, and expressed as the number of auxotrophs per total number of transconjugants. The most efficient Tn5 delivery system was chosen for large-scale mutagenesis of TAL 182.

Tn5 was introduced into TAL 182 from pSUP1021, using E. coli S17.1 as the mobilizing strain (Simon et al., 1986). Spot mating was done on TY plates, and the transconjugants were selected by replica-plating on TY medium containing kanamycin and nalidixic acid. To ensure that independent mutations were isolated, only two random colonies were purified from each spot mating.

Isolation of auxotrophic mutants. All transconjugants were replica-plated on MM medium containing kanamycin to isolate auxotrophs. The transconjugants which failed to grow on MM were screened using the auxonography scheme by Holliday (1956), to identify their specific auxotrophic requirements.

To examine the stability of the Tn5 insertion, the reversion frequencies of the auxotrophs were determined by growing cells to saturation in 10 ml of TY (approximately 10^9 cells ml⁻¹), washing with sterile saline, concentrating 10-fold, and then plating on MM. Prototrophic colonies were subsequently streaked on MM medium containing kanamycin to determine their loss of resistance.

Isolation of symbiotic mutants. A total of 1,050 prototrophic mutants were screened on plants for symbiotic defects. All plant tests were done in plastic growth pouches (Northrup Ring Seed Co., Minneapolis, MN) using P. vulgaris L. cv. Bountiful (provided by NifTAL Project, University of Hawaii) grown for four weeks. Seed preparation and plant growth conditions were as previously described (Chapter 4).

Genomic DNA preparation. Total genomic DNA from TAL 182 wild-type and mutants was isolated using the following procedure. Cells from a 50-ml stationary-phase culture of TY or TY containing kanamycin, were collected and washed successively in 25 ml of 1.0 M NaCl and in 25 ml of cold TES buffer (10 mM Tris, pH 8.0; 25 mM EDTA; 150 mM NaCl). The pellet was resuspended in 5 ml of cold TE₂₅ buffer (10 mM Tris, pH 8.0; 25 mM EDTA) and 0.5 ml of lysozyme solution (2 mg ml⁻¹ lysozyme in TE₂₅ buffer) was added to the cells. After a 15-min incubation at 37°C, 0.6 ml of a predigested Sarkosyl-Pronase solution (10% Sarkosyl and 5 mg ml⁻¹ Pronase in TE₂₅ buffer) was added and the mixture was incubated for another h. The lysed cells were extracted at least twice with an equal volume of Tris-saturated phenol (pH 7.8) and once with an equal volume of chloroform. The concentration of NH₄Ac was diluted to 0.3 M, and the DNA was precipitated in 0.6 volume of isopropanol at room temperature. The DNA was spooled with a glass rod, washed in 1 ml of 70% ethanol, and then dissolved in 300 µl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA).

Restriction enzyme digestions. The restriction enzymes EcoRI, HindIII, and BamHI were used according to the supplier's specifications (New England Biolabs, Beverly, MA). DNA fragments were separated in 0.8% agarose gels prepared in TAE buffer (40 mM Tris, 10 mM NH₄Ac, 1 mM EDTA, pH 7.8).

Southern hybridization analysis. To verify the location and number of Tn5 insertions in the mutants, genomic DNA was digested to completion with EcoRI and HindIII for Southern blot analysis. Blotting and hybridization to GeneScreen Plus (New England Nuclear, Boston, MA) were done according to the manufacturer's instructions. The filters were prehybridized for 30 min at 65°C in a hybridizing solution containing 10% dextran sulfate, 1 M NaCl, 1% SDS, and 100 µg ml⁻¹ denatured salmon sperm DNA. Hybridization with the denatured probe was done overnight at 65°C in the same solution. After hybridization, the filters were washed once in 2 x SSC (30 mM NaCl; 30 mM Na

citrate, pH 7.0) at room temperature for 5 min, twice in 2 x SSC containing 1% SDS at 55°C for 15 min, and once in the same solution at 65°C for 30 min. The filters were dried and exposed to Kodak type RP5 X-ray film at -70°C overnight with an intensifying screen.

The probe was a 2-kb internal region of Tn₅ cloned in pSKS101 (Shapira et al., 1983) which was cut out as a BamHI fragment. Plasmid pSKS101 was digested with BamHI, and the fragments were separated by electrophoresis. The 2-kb fragment was recovered from the agarose gel using a DEAE membrane. The DNA was eluted with high salt NET buffer (10 mM Tris, pH 7.6; 1 mM EDTA; 60 mM NaCl) and purified by extraction with phenol/chloroform and chloroform. The DNA was precipitated in cold 95% ethanol, washed in 70% ethanol, and resuspended in 20 µl TE buffer. The DNA fragment was checked for purity on an agarose gel. About 200 ng of the DNA fragment was used for ³²P-labelling by random priming using a kit (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's instructions.

Phenotypic characterisation of mutants. The symbiotic phenotype of each mutant was determined by inoculating five plants grown in plastic pouches. Three-day old seedlings of uniform size were inoculated with 10⁶ rhizobia.

The ability to induce root hair deformations was examined by light microscopy. At the time of inoculation, the position of the root segment most susceptible to infection (from the zone of the smallest emergent root hairs to the root tip) was marked on the plastic pouch. Three days after inoculation, wet mounts of these root segments were stained with 0.05% (w/v) toluidine blue for 10 min, destained in water for 30 min, and observed for root hair curling, bulging, or branching.

Nodule initiation, as indicated by the presence or absence of nodules or pseudonodules, was continually appraised by visual inspection until four weeks after inoculation. The nodulation profile of each mutant was also

determined by examining the roots of five plants for newly emerged nodule primordia every day for 14 days after inoculation.

The presence of rhizobia in the nodules was judged by their recovery on selective medium. Four-week old nodules or pseudonodules (30 nodules for each mutant) were surface-sterilized by a 5-min immersion in 0.1% HgCl_2 (w/v), followed by one rinse in sterile water, a 3-min immersion in 95% ethanol, and three rinses in sterile water. The nodules were individually crushed with a sterile wooden stick, and plated on TY medium containing kanamycin. In addition, the presence of rhizobia in the nodules was verified by staining a smear from the crushed nodule with fluorescent antibody specific for TAL 182, and examined by epifluorescence microscopy.

Nitrogenase activity was evaluated by the acetylene reduction assay (Hardy et al., 1968), determined at 25°C by incubating excised roots in serum bottles with 10% (v/v) acetylene in air for 30 min. Gas samples were analyzed for ethylene with a Varian Aerograph 2700 gas chromatograph equipped with Porapak-T column (80-100 mesh).

Competitiveness for nodulation of common bean was evaluated by double-strain inoculations. In the case of mutant MLC191, the inoculum consisted of equal proportions of the mutant and a highly competitive strain (TAL 1472) or of the mutant and a poorly competitive strain (CIAT 632). Nodule occupancy was determined with strain-specific fluorescent antibodies after four weeks as previously described (Chapter 3). In the case of the auxotrophs, the inoculum consisted of equal proportions of the mutant and the wild-type. Nodule occupancy was determined by plating on selective medium. Nodules were surface-sterilized, individually crushed, and replica-plated on TY medium containing kanamycin to identify the mutant and on MM medium to identify the wild-type.

Induction of host nodulation suppression. The ability of the mutants to induce autoregulation in common bean was evaluated in split-root systems.

Each of the mutants was used as the primary inoculant on one side at zero time (E, early inoculation), and the wild-type was used as the secondary inoculant on the opposite side after a two-week delay (D, delayed inoculation). At zero time, the controls included inoculation with each mutant of both sides of a split-root at the same time (E/E control) and of one side only (E/0 control). At the delayed time, the controls included inoculation of the wild-type of two sides of a split-root at the same time (D/D control) and of one side only (0/D control). The split-root systems were generated and maintained as previously described (Chapter 4). The plants were harvested four weeks after the primary inoculation. Analysis of variance was performed using PC-SAS (Statistical Analysis System for Personal Computers, SAS Institute, Inc., Cary, NC).

Results

Tn5 mutagenesis of TAL 182. Table 5.2 shows the evaluation of four suicide vectors for transposon mutagenesis of TAL 182. E. coli S17.1 (pSUP1021) was the best Tn5 delivery system in terms of transposition frequency and efficiency.

There was no evidence of hotspots for insertion of Tn5 in the TAL 182 genome. Transposition of Tn5 appeared random based on the diversity of auxotrophies that were identified. Mutants that were auxotrophic for a purine (adenine), a pyrimidine (uracil), and amino acids (leucine, phenylalanine, and cysteine) were isolated (Table 5.3). No prototrophic revertants were observed on MM medium in 10^{10} cells of each of the auxotrophs, indicating that the Tn5 insertions were stable. However, in the case of the Cys⁻ auxotroph, Km⁻ prototrophic revertants were recovered from effective nodules, indicating an instability of the Tn5 insertion in this mutant after plant passage. The reversion of the Cys⁻ auxotroph to prototrophy and kanamycin sensitivity, and the accompanying restoration of symbiotic competence, provide evidence that the nodulation defect in the

TABLE 5.2. Assessment of various vector systems for Tn5 mutagenesis of *R. leguminosarum* bv. *phaseoli* TAL 182.

Plasmid	<i>E. coli</i> Donor	Transposition frequency ^a	Transposition efficiency ^b
pJB4JI	1830	$< 10^{-7}$	ND ^c
pGS9	HB101	$< 10^{-7}$	ND
pSUP1021	S17.1	4.6×10^{-6}	0.5%
pBLK1-2	HB101	1.1×10^{-6}	0.02%

^aCalculated by dividing the number of transconjugants by the number of recipients, average of two conjugation experiments.

^bIndicated by the frequency of auxotrophic mutants in 1,200 Tn5 mutants screened on MM medium.

^cND, not determined

TABLE 5.3. Phenotypic characterization of *R. leguminosarum* bv. *phaseoli* TAL 182 Tn5 mutants.

Mutant group	Strain	Metabolic defect	Phenotype				
			Had ^a	Noi ^b	Bad ^c	Fix ^d	Cmp ^e
Class I	MLC357	none	+	-	-	-	ND ^f
	MLC648	none	+	-	-	-	ND
	MLC778	none	+	-	-	-	ND
Class II	MLC35	none	+	+	-	-	ND
	MLC640	none	+	+	-	-	ND
Class III	MLC191	none	+	+	+	-	+
Auxotrophs	182A	Ade ⁻	+	-	-	-	-
	182U ^f	Ura ⁻	+	+	-	-	-
	182L	Leu ⁻	+	+	+/-	-	-
	182P	Phe ⁻	+	+	+/-	-	-

^aRoot hair distortion, indicated by curling, branching, or bulging.

^bNodule initiation, indicated by presence of nodules or pseudonodules.

^cBacteroid development, indicated by presence of bacteria inside the nodules or pseudonodules by recovery on selective plates or reaction with TAL 182 fluorescent antibodies, +/- means that bacteria were recovered in selective plates in less than 30% of the nodules.

^dNitrogen fixation, indicated by shoot color or nitrogenase activity.

^eCompetitiveness, + means nodule occupancy similar to the wild-type (50% when competed against TAL 1472 and 100% when competed against CIAT 632), - means 0% nodule occupancy when competed against the wild-type.

^fTwo mutants were distinct genotypically but could not be differentiated phenotypically.

^gND, not determined

mutant was due to the Tn5 insertion, and that reversion was probably due to a precise excision of the transposon. Nevertheless, this mutant was not used further.

Southern hybridization analysis. The Tn5 (5.7 kb) insertions were localized by analyzing restriction digests of mutant DNA. Southern analysis of EcoRI-restricted DNA with a 2.0-kb internal region of Tn5 (Figure 5.1A) showed that each mutant had a single insertion. Since Tn5 does not contain an EcoRI site (Jorgensen_i et al., 1979), EcoRI-restricted DNA from a mutant carrying a single insertion was expected to show a single hybridizing fragment (Figure 5.1B and C). The insertions were verified by analysis of HindIII-restricted DNA, showing three hybridizing bands in each mutant (data not shown). Since Tn5 has two HindIII sites 3.3 kb apart in the inverted repeats (Figure 5.1A), a single insertion was indicated by three hybridizing bands representing the internal 3.3-kb fragment and two other fragments containing the 1.2-kb fragments of the inverted repeats and the flanking DNA. Analysis of the restricted DNA also showed that the Tn5 transposed into different fragments, indicating that each mutation was distinct.

Symbiotic phenotypes of mutants. All the mutants were capable of inducing root hair deformations on common bean (Table 5.3). The Class I and the Ade⁻ mutants were nonnodulating while the Class II and the Ura⁻ mutants were able to induce visible nodule primordia but lacked hypertrophy (Figure 5.2). The Class III and amino acid auxotrophic mutants formed normal-sized nodules. However, while the Class III mutant formed bacteroid-containing pink nodules, the amino acid auxotrophs formed empty white nodules.

The nodulation efficiency of the mutants capable of initiating nodulation (Noi⁺) were evaluated. The Class III mutant showed a nodulation profile similar to that of the wild-type (Figure 5.3) and eventually formed a significantly higher total number of nodules than the wild type and other mutants four weeks after inoculation (Figure 5.4). However, its specific

FIGURE 5.1. Southern hybridization analysis of R. leguminosarum bv. phaseoli TAL 182 mutants. (A) Map of Tn5 showing the position of the neomycin phosphotransferase (kan) gene, the two inverted repeats (thick lines), and the 2-kb internal region used as probe. (B) EcoRI-restricted genomic DNA separated by electrophoresis. (C) Autoradiogram of DNA blotted into nitrocellulose and hybridized with the labelled 2-kb region of Tn5. The size markers are HindIII-digested lambda DNA. Lanes A (182A); B,C (182L), D,E (182P), F,H (182U1); G (182U2); I (MLC35); J (MLC191); K (MLC357); L,M (MLC640); N (MLC648); O,P,Q (MLC778); R (wild-type).

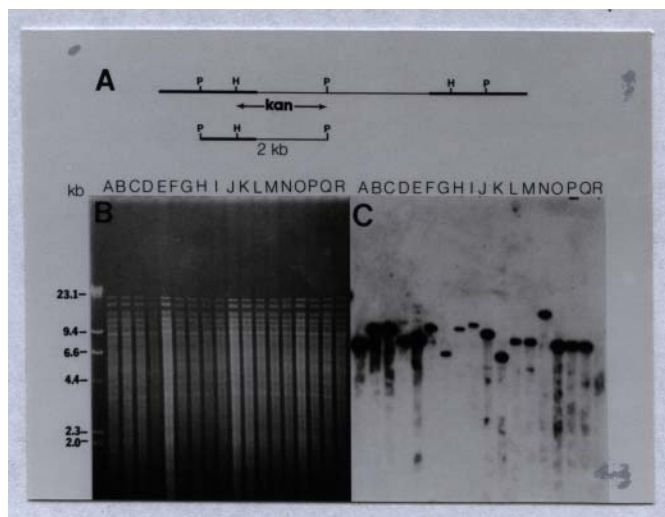


FIGURE 5.2. Nodulation phenotype of a Class II mutant (MLC35). Arrows point at small swellings formed on the roots.



nodule weight (average weight per nodule) was significantly lower than that of the wild-type (Figure 5.4). The rest of the mutants showed a marked impairment in their ability to form nodules. Compared to the wild-type, they developed nodule primordia at a slower rate, even though the onset of nodulation was not delayed (Figure 5.3 and 5.5). The total nodule number and nodule weights of plants inoculated with the mutants were also lower than those inoculated with the wild-type (Figures 5.4 and 5.6). The two amino acid auxotrophs showed similar nodule weights, although the Leu⁻ auxotroph formed only half as many nodules as the Phe⁻ auxotroph (Figure 5.6).

All plants inoculated with the mutants had yellow color (Table 5.3). In the case of the Class III mutant, the Fix phenotype was confirmed by the lack of nitrogenase activity. All plants inoculated with the mutants had significantly lower shoot weights than those inoculated with the wild-type (Figures 5.4 and 5.6).

The Nod⁺ Class III mutant was as competitive as the wild-type for nodulation of common bean (Table 5.3). Like the wild-type, it occupied 50% of the nodules when coinoculated with a highly competitive strain (TAL 1472) and 100% of the nodules when coinoculated with a poorly competitive strain (CIAT 632). In contrast, the nodulation-defective mutants were unable to compete with the wild-type for nodulation (Table 5.3). When each of the nodulation-defective auxotrophs was coinoculated with the wild-type on bean plants, the wild-type occupied 100% of the nodules.

Nodulation suppression by mutants. An inability of the nodulation-defective mutants to induce autoregulation in common bean was observed (Figures 5.7 and 5.8). When the wild-type was inoculated on one side of a split-root at zero time and on the opposite side two weeks later (E/D control), there was a significant inhibition of nodulation, both in nodule number and in nodule weight, on the side that received the delayed

FIGURE 5.3. Nodulation profiles R. leguminosarum bv. phaseoli TAL 182 symbiotic mutants on common bean (P. vulgaris L.) cv. Bountiful.

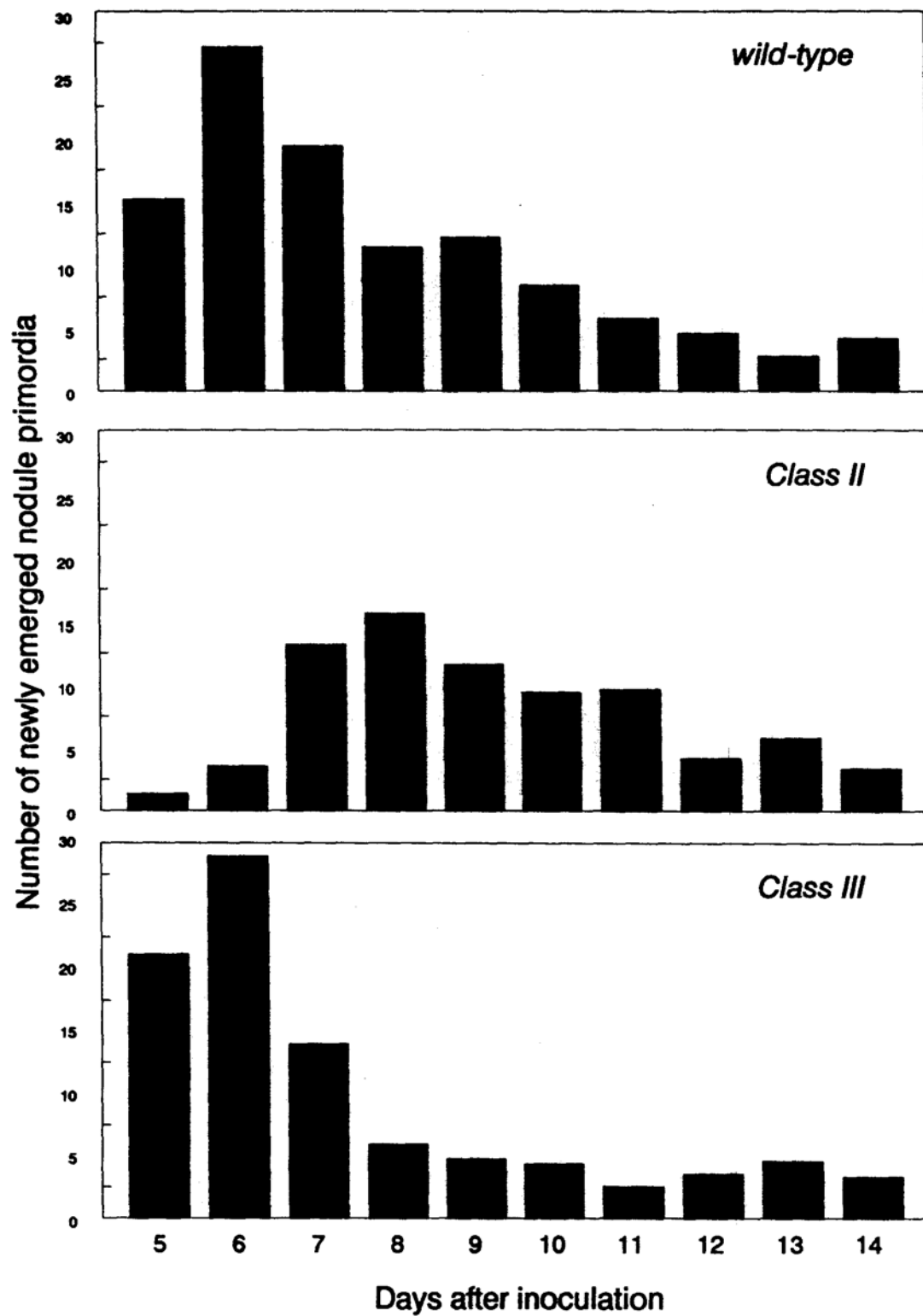


FIGURE 5.4. Symbiotic characteristics of common bean (P. vulgaris L.) cv. Bountiful inoculated with symbiotic mutants of R. leguminosarum bv. phaseoli TAL 182. Letters represent the separation of means by Duncan's multiple range test.

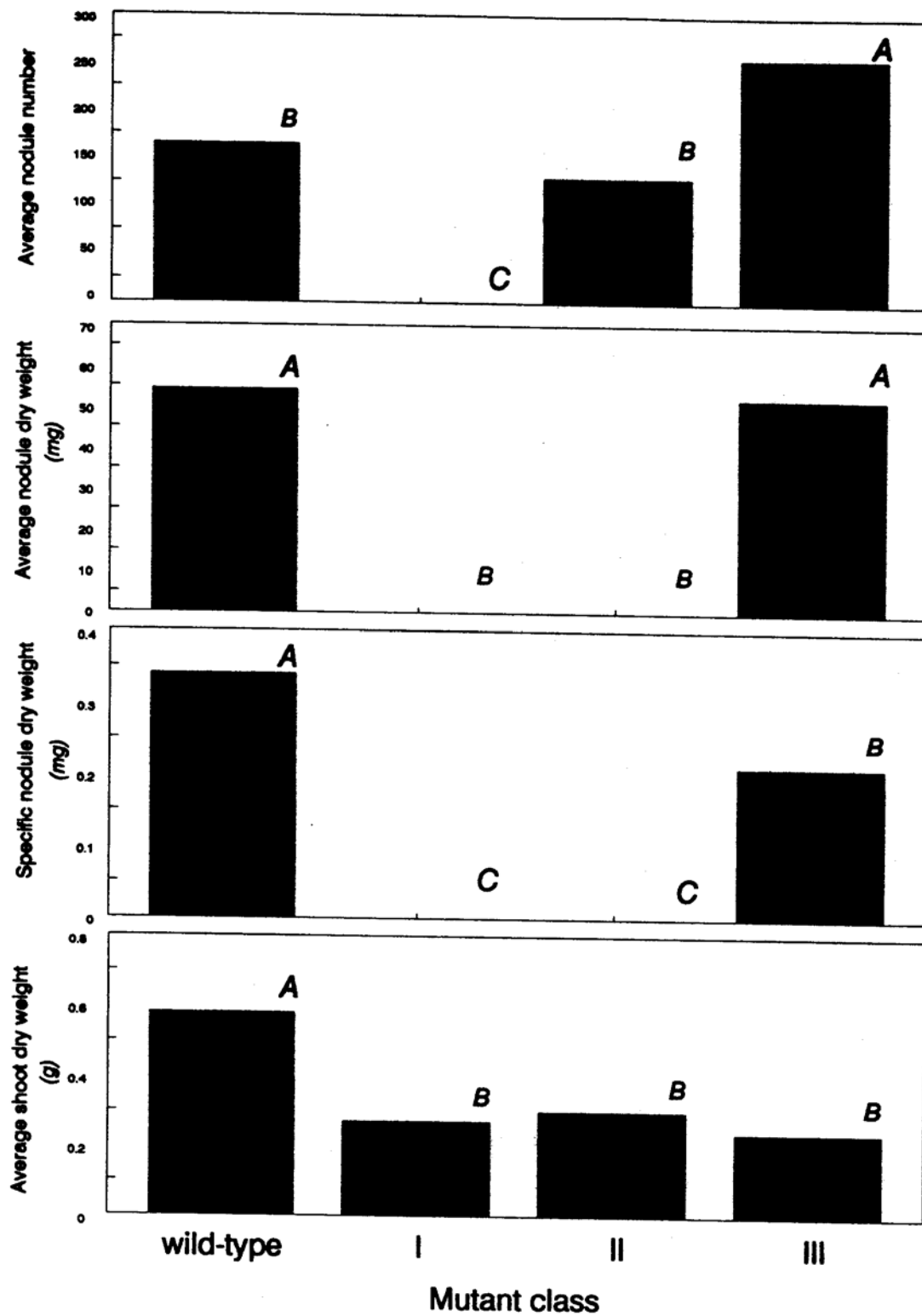


FIGURE 5.5. Nodulation profiles R. leguminosarum bv. phaseoli TAL 182 auxotrophic mutants on common bean (P. vulgaris L.) cv. Bountiful.

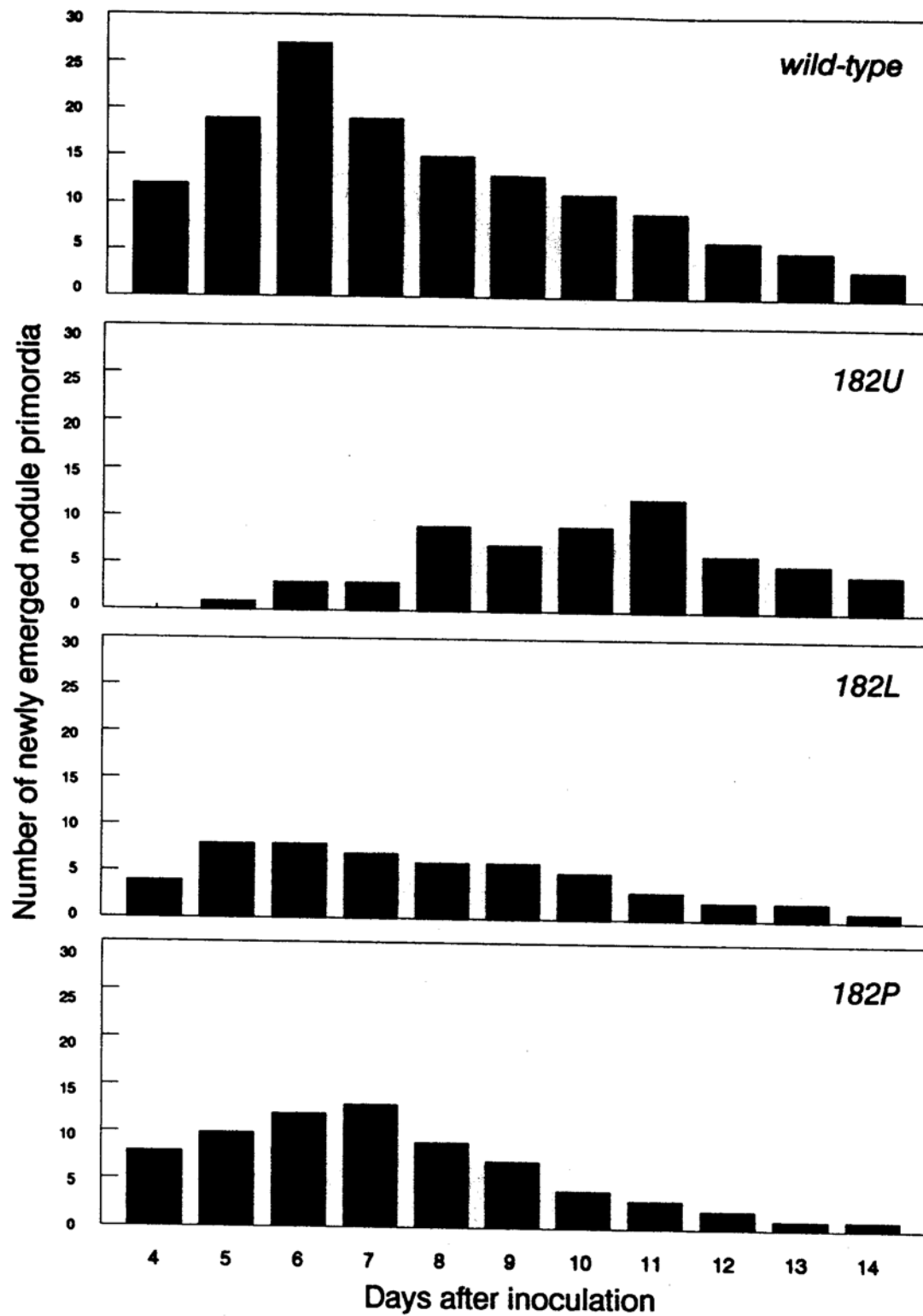


FIGURE 5.6. Symbiotic characteristics of common bean (P. vulgaris L.) cv. Bountiful inoculated with auxotrophic mutants of R. leguminosarum bv. phaseoli TAL 182. Letters represent the separation of means by Duncan's multiple range test.

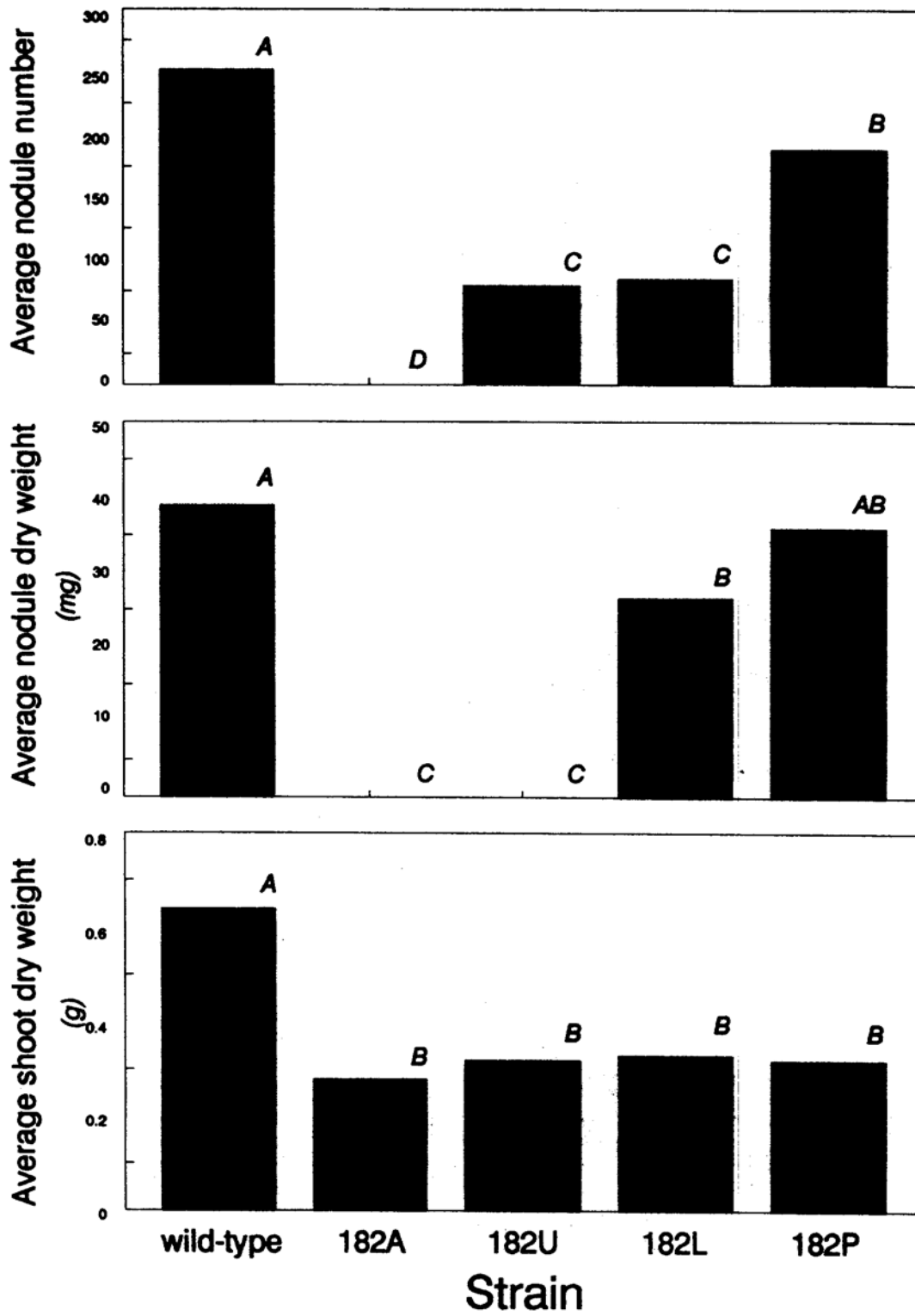


FIGURE 5.7. Nodulation suppression induced by symbiotic mutants of R. leguminosarum bv. phaseoli TAL 182 on common bean (P. vulgaris L.) cv. Bountiful. The E/D (Early/Delayed) control represents the suppression response induced by the wild-type while the 0/D (Uninoculated/Delayed) control represents the nodulation potential of the plant in the absence of suppression. Bars with the same upper case or lower case letters are not significantly different by Duncan's multiple range test.

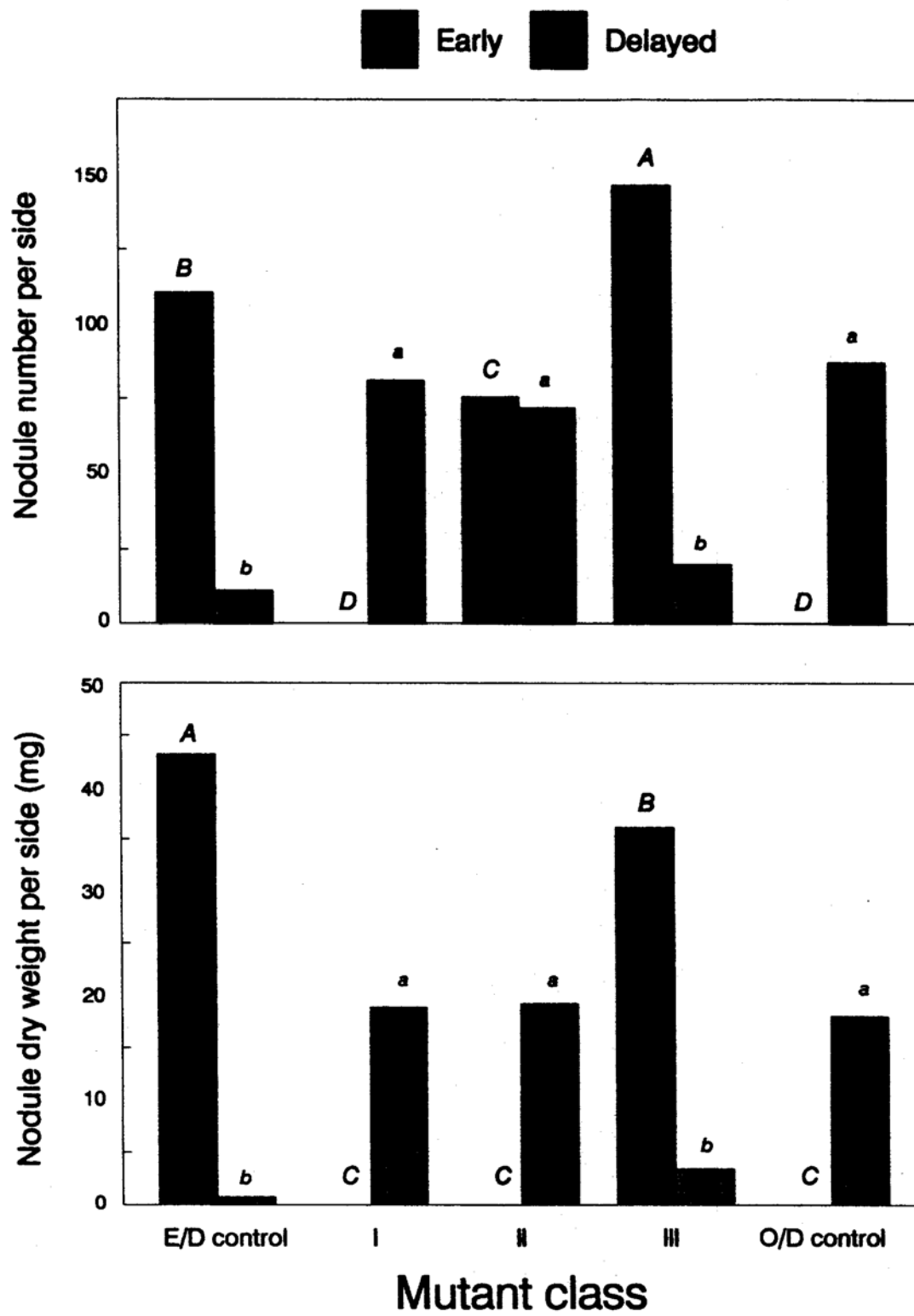
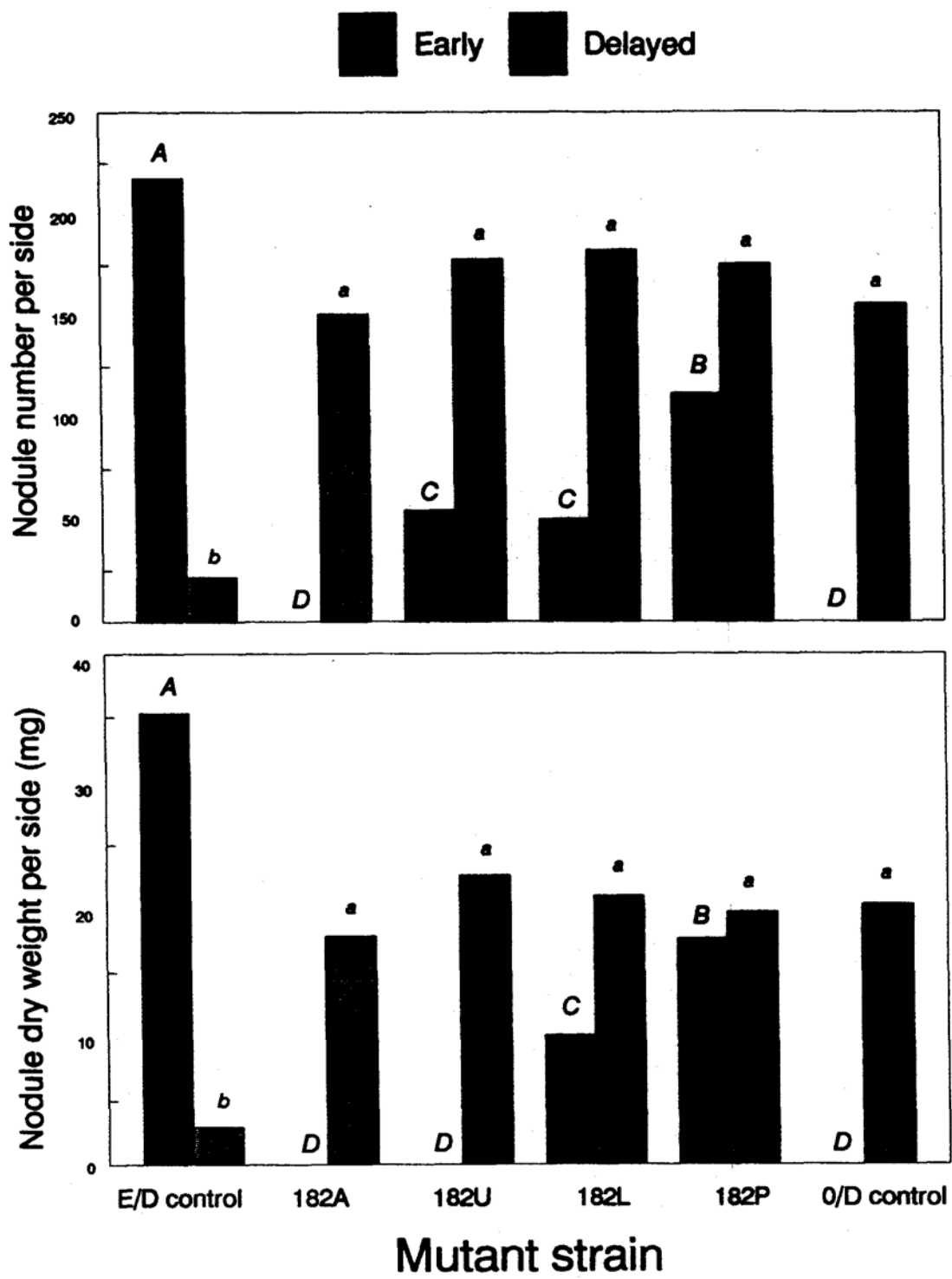


FIGURE 5.8. Nodulation suppression induced by auxotrophic mutants of R. leguminosarum bv. phaseoli TAL 182 on common bean (P. vulgaris L.) cv. Bountiful. The E/D (Early/Delayed) control represents the suppression response induced by the wild-type while the 0/D (Uninoculated/Delayed) control represents the nodulation potential of the plant in the absence of suppression. Bars with the same upper case or lower case letters are not significantly different by Duncan's multiple range test.



inoculation. However, when a nodulation-defective mutant was inoculated on one side at zero time, and the wild-type on the opposite side two weeks later, nodulation of the wild-type on the side that received the delayed inoculation was comparable to its potential in the absence of prior nodulation (O/D control). This lack of suppression was true regardless of the particular stage of blockage in the nodulation process found in the mutants. All mutants that were defective in their ability to nodulate, whether as a direct result of the mutation itself (Figure 5.7) or an indirect result of a metabolic defect (Figure 5.8), failed to induce a suppression response from the host. Only the modulation-competent Class III mutant induced a suppression response similar to that induced by the wild-type (Figure 5.7).

Discussion

In legumes, the decreased frequency of nodulation in developmentally younger regions of the root is a common observation (Bhuvaneswari, et al., 1981; Munns, 1968; Paau et al., 1985; Sargent et al., 1987; Takats, 1986). The extent of nodulation by infecting rhizobia is restricted by a plant-mediated systemic response that prevents excessive nodule formation (Pierce and Bauer, 1983). It is postulated that autoregulation occurs as a shoot-controlled general plant reaction triggered by developing infection centers, resulting in the suppression of further cell division activity in root tissue (Rolfe and Gresshoff, 1988).

As in other legumes, the nodulation process in common bean is subject to autoregulation (Chapter 4). In this study, Tn5-induced mutants of R. leguminosarum bv. phaseoli that are blocked at specific stages of symbiotic development were used to examine autoregulation in common bean. With the wild-type strain TAL 182, prior inoculation of one side of a split-root induced a strong suppression of nodulation on the opposite side which received a delayed inoculation (Figures 5.7 and 5.8). The induction of

nodulation suppression was correlated with the ability of the microsymbiont to enter and persist inside the nodules, but was not related to the ability to initiate nodule formation or to the ability to fix nitrogen.

The nonnodulating Class I and Ade⁻ mutants (Hac⁺, Noi⁻) failed to induce a suppression response (Table 5.3, Figures 5.7 and 5.8), indicating that the induction of suppression does not occur at the root hair curling stage. Similarly, inoculation of one side of a clover split-root with a Sym plasmid-cured or a nonnodulating (nodD) mutant of R. trifolii did not inhibit nodulation of the wild-type inoculated 4 days later on the opposite side (Sargent et al., 1987). In alfalfa, suppression was also not induced by nonnodulating mutants of R. meliloti, but was induced by mutants that are capable of infection thread formation (Caetano-Anolles and Bauer, 1988).

The Class II and the Ura⁻, Leu⁻, and Phe⁻ mutants were capable of nodule initiation but formed empty nodules (Hac⁺, Noi⁺, Bad⁻) (Table 5.3). All failed to induce nodulation suppression (Figures 5.7 and 5.8), indicating that the ability to initiate cortical cell divisions is not sufficient to induce suppression. This was true regardless of differences in the degree of hypertrophic development induced by these mutants, ranging from small swellings (Class II and Ura⁻ mutants) to nodule-sized structures and Phe⁻ mutants). However, this observation differs from that seen in other studies. In siratro, an Ade⁻ mutant of the fast-growing, broad-host-range Rhizobium strain NGR234, that causes root hair curling and cortical cell division but not infection thread formation (Hac⁺, Noi⁺, Inf⁻), induced suppression when inoculated in a split-root 24-48 hours earlier than the wild-type (Djordjevic et al., 1988). Exopolysaccharide mutants of R. meliloti that were defective in their ability to invade and multiply inside the host, also induced suppression in an alfalfa split-root (Caetano-Anolles et al., 1990). It appears from these studies that the induction of suppression does not require rhizobial infection or proliferation inside the

host, implying that the signals for induction have to be transmitted into the root cortex without invasion.

A suppression response similar to that elicited by the wild-type was induced by the nodule-forming Class III mutant that formed pink, bacteroid-containing, but ineffective nodules (Hac^+ , Noi^+ , Bad^+ , Fix^-). This indicates that the presence of bacteria inside the nodules is required for the induction of suppression. A similar conclusion was drawn by Paa et al. (1985) in their study of a supernodulating mutant of R. meliloti. This mutant, which forms numerous infection threads but fails to develop into bacteroids, showed an impairment in its ability to elicit an autoregulatory response from alfalfa.

Suppression appears to be a response to an early stage of the symbiotic process prior to nitrogen fixation. As evidenced by the suppressiveness of an ineffective mutant, the ability to fix nitrogen is not required for the induction of nodulation suppression in common bean. This was also shown in soybean, where suppression of nodulation on one side of a split-root occurred even before the onset of nitrogen fixation on the other side that received an early inoculation (Kosslak and Bohlool, 1984).

In summary, our results point to the presence of rhizobia inside the nodules as a prerequisite for nodulation suppression. In alfalfa (Caetano-Anolles and Bauer, 1988) and in clover (Sargent et al., 1987), the suppression response was correlated with the presence of rhizobia in the infection threads. Studies in soybean (Morrison and Verma, 1987) and in alfalfa (Dunn et al., 1988) have shown that late nodulin proteins are not expressed in nodules lacking infection threads and intracellular bacteria. In a study of a B. japonicum mutant defective in early bacteroid development (Rossbach et al., 1989), the degree of nodulin (ENOD55) expression in soybean was strongly correlated with the number of bacteria present in the nodules.

Our results seem to indicate that nodulation suppression in P. vulgaris is a response to a collective signal from developing bacteroids.

CHAPTER 6

COMPLEMENTATION OF Rhizobium leguminosarum bv. phaseoli MUTANTS DEFECTIVE IN NODULATION AND NODULATION SUPPRESSION

Abstract

In this study, the genetics of modulation suppression in Phaseolus vulgaris L. (common bean) was investigated using Rhizobium leguminosarum bv. phaseoli TAL 182 mutants. Class I mutants are nonnodulating (Hac^+ , Noi^-) while Class II mutants induce small swellings on the roots (Hac^+ , Noi^+). Mutants with various auxotrophies (Ade^- , Ura^- , Leu^- , and Phe^-) are also differentially impaired in nodulation abilities. All these mutants fail to induce modulation suppression in split-root systems. Gene libraries from the wild-type and from Rhizobium loti TAL 1145, a strain which nodulates both common bean and Leucaena leucocephala, were conjugated en masse with the mutants, and cosmids that corrected the mutations were identified by in planta complementation. Homologous complementation restored the nodulation abilities of both Class I and Class II mutants. However, the complemented mutants of Class I were ineffective and did not regain full ability to induce nodulation suppression. When they were used as primary inoculants in a split-root, a secondary inoculation with the wild-type resulted in nodulation that was 32-70% of its potential, indicating that the primary inoculants did not induce a strong suppression response. In contrast, the complemented mutants of Class II regained nodulation, fixation, and suppression characteristics comparable to that of the wild-type. When they were used as primary inoculants, secondary inoculation with the wild-type resulted in nodulation that was 1-7% of its potential, a strong suppression response characteristic of the wild-type. Restriction analysis of the overlapping cosmids complementing Class I mutants showed a 7.2-kb region in common. As

predicted from a Southern hybridization analysis, the Tn₅ insertions in the Class I mutants were in 2.0-kb, 7.2-kb, and 3.0-kb EcoRI fragments that were located within a 21.6-kb region. Two complementation groups corrected the Class II mutations. Overlapping cosmids complementing MLC35 and MLC640 showed an 8.0-kb and a 13.4-kb region in common, respectively. Heterologous complementation with a gene library from R. loti TAL 1145, also yielded similar results. Restriction analysis of the overlapping cosmids complementing the Class I and Class II mutations showed a 6.7-kb region and a 10.1-kb region in common, respectively. All the auxotrophic mutations were complemented by heterologous DNA. Upon restoration to prototrophy, these mutants regained full symbiotic competence and induced strong suppression from the host.

Introduction

The development of nitrogen-fixing nodules is a complex process involving the expression of symbiotic genes in both the legume host and the microsymbiont. Nodule ontogeny has been studied in different legume-Rhizobium symbioses and a number of common features are observed (for review, see Rolfe and Gresshoff, 1988). One of these features is autoregulation, a host-mediated suppression mechanism that prevents excessive nodulation by invading rhizobia. The effect of autoregulation is seen in the decreased frequency of nodulation in younger regions of the root (Bhuvaneswari et al., 1981; Munns, 1968; Paau et al., 1985; Pierce and Bauer, 1983; Takats, 1986). It is also seen in split-root systems, where prior nodulation on one side suppresses subsequent nodulation on the other side (Caetano-Anolles and Bauer, 1988; Djordjevic et al., 1988; Rosslak and Bohlool, 1984; Sargent et al., 1987).

Such tight control of the nodulation process requires continuous signal exchange between the two partners. Exactly what or when signals trigger the autoregulatory response in the plant host is not yet clear. However, there is evidence that autoregulation is a shoot-controlled response to some early aspect of nodule development, that results in the inhibition of further nodule meristematic activity (Rolfe and Gresshoff, 1988).

Mutants defective in the early stages of the nodulation process are valuable biological tools for defining these early interactions between the host and invading rhizobia and for elucidating the molecular basis of autoregulation. In this study, we used genetic complementation of R. leguminosarum bv. phaseoli mutants to clone the genes that correct nodulation and suppression defects, and to examine the relationship between nodulation ability and the induction of suppression.

Materials and Methods

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 6.1. Rhizobium strains were grown in TY (Beringer, 1974), PA (Hirsch et al., 1980) or MM (Chapter 5) medium. E. coli strains were grown in LB (Maniatis et al., 1982) medium. The media were supplemented with kanamycin (50 $\mu\text{g ml}^{-1}$) and/or tetracycline (5 $\mu\text{g ml}^{-1}$) when selective conditions were required.

Construction of TAL 182 gene library. Total genomic DNA from R. leguminosarum bv. phaseoli TAL 182 was prepared from cells grown overnight in 1 liter of PA medium. The DNA isolation procedure described previously (Chapter 5) was scaled up and extraction was done three times with phenol, twice with phenol-chloroform, and twice with chloroform.

The wild-type DNA was partially digested with EcoRI to give fragments in the size range 20-30 kb. The DNA fragments were monitored for size in a 0.8% agarose gel prepared in TAE buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, pH 7.8), and isolated from the agarose gel by electroelution. Extraction was done on the eluted DNA, once each with phenol, phenol-chloroform, and chloroform. The aqueous phase was brought to 0.3 N sodium acetate, and the DNA was precipitated in one volume of isopropanol overnight at -20°C . The DNA was collected by spinning for 15 min, washed in 70% ethanol, dried, dissolved in 5 μl TE buffer, and checked for size on an agarose gel.

The gene library was constructed by insertion of the fragments into the unique EcoRI site of pLAFR1 (Friedman et al., 1982), a 21.6-kb, low-copy-number, broad-host-range mobilizable vector that confers tetracycline resistance (Tc^r). The vector DNA was prepared by digesting pLAFR1 with EcoRI and then dephosphorylating with calf intestinal alkaline phosphatase (CIAP). The insert (6 μg) and vector (0.5 μg) DNA were mixed together and ligated overnight at 16°C in a total volume of 20 μl using T4 DNA ligase. The ligated DNA was packaged in vitro using the Packagene Lambda DNA Packaging

TABLE 6.1. Bacterial strains and plasmids.

Strains	Relevant characteristics	Reference
<u>R. leguminosarum</u> bv. <u>phaseoli</u>		
TAL 182	wild-type	Chapter 3
MLC357	Hac ⁺ , Noi ⁻ , Nod ⁻ , Fix ⁻	Chapter 5
MLC648	Hac ⁺ , Noi ⁻ , Nod ⁻ , Fix ⁻	Chapter 5
MLC778	Hac ⁺ , Noi ⁻ , Nod ⁻ , Fix ⁻	Chapter 5
MLC35	Hac ⁺ , Noi ⁺ , Nod ⁻ , Fix ⁻	Chapter 5
MLC640	Hac ⁺ , Noi ⁺ , Nod ⁻ , Fix ⁻	Chapter 5
182A	Hac ⁺ , Noi ⁻ , Nod ⁻ , Fix ⁻ , Ade ⁻	Chapter 5
182U	Hac ⁺ , Noi ⁺ , Nod ⁻ , Fix ⁻ , Ura ⁻	Chapter 5
182L	Hac ⁺ , Noi ⁺ , Nod ⁻ , Fix ⁻ , Leu ⁻	Chapter 5
182P	Hac ⁺ , Noi ⁺ , Nod ⁻ , Fix ⁻ , Phe ⁻	Chapter 5
<u>E. coli</u>		
HB101		Boyer and Roulland-Dussoix, 1969
DH5 α mc ^r		BRL ^a
Plasmids		
pRK2013	Km ^r , RK2 transfer genes	Figurski and Helinski, 1979
pLAFR1	Tc ^r , cosmid vector	Friedman et al., 1982
pLAFR3	Tc ^r , contains <u>Sau</u> 3A-digested DNA	D. Borthakur
gene bank	of TAL 1145	

^aBethesda Research Laboratories, Inc., Gaithersburg, MD

System, and the packaged cosmids were used to infect E. Coli HB101.

EcoRI was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD) while CIAP, T4 DNA ligase, and Packagene Lambda DNA Packaging System were purchased from Promega Corp. (Madison, WI). They were all used according to the instructions of the supplier.

A gene bank from Rhizobium loti TAL 1145, a strain which nodulates both common bean and Leucaena leucocephala, (provided by D. Borthakur) was also used in this study.

Bacterial ratings. The TAL 182 gene library (pLkFR1 cosmids) and TAL 1145 gene library (pLAFR3 cosmids) were mass-conjugated with each of the Rhizobium mutants by the triparental mating system (Ditty et al., 1980), using pRK2073 (Figurski and Helinski, 1979) as the helper plasmid.

The donor (gene library), helper, and recipient cells were grown in selective media to late-log phase. One ml of each of the cultures was washed once in sterile saline (0.9% NaCl, w/v). The cells were resuspended in 250 µl of TY, mixed together, and placed on a 0.45-µm pore-size filter. The filters were incubated on TY plates overnight. The transconjugants were selected by plating on TY medium supplemented with kanamycin and tetracycline.

Isolation of complemented mutants. The transconjugants were inoculated directly onto common bean (Phaseolus vulgaris L. cv. Bountiful) for in planta selection of restored symbiotic competence. In crosses involving auxotrophic recipients, the transconjugants were plated onto MM medium supplemented with kanamycin and tetracycline for selection of restored prototrophy, prior to plant inoculation. The preparation of bean seeds was as previously described (Chapter 4).

Nodules which formed on the plants were collected after three weeks. The nodules were surface-sterilized as previously described (Chapter 5), and plated on TY containing kanamycin and tetracycline. The isolates were

purified for single colonies on the same medium, and then analyzed for the presence of complementing cosmids and tested for the restoration of their nodulation and nodulation suppression phenotypes.

Analysis of complementing cosmids. The complementing cosmids were isolated from the Rhizobium mutants, transformed into E. coli, and then isolated from E. coli for restriction analysis.

The complementing cosmids were isolated from the Rhizobium mutants for transformation using the alkaline lysis procedure. Each of the complemented mutants was grown for two days in 3 ml of TY medium containing kanamycin and tetracycline. The cells were collected, washed in 0.5 M NaCl solution, resuspended in 200 µl of GTE buffer (50 mM glucose; 25 mM Tris, pH 8.0; 10 mM EDTA), and then lysed by the addition of 400 µl of NaOH/SDS solution (0.2 M NaOH, 1% SDS). After 5 min, 300 µl of 3 M KAc (pH 8.0) was added. The suspension was mixed gently by inverting, left on ice for 5 min, and spun for 5 min to collect the supernatant. The DNA was precipitated in 0.6 volume of isopropanol at room temperature, washed in 70% ethanol, dried, and then dissolved in 30 µl of TE buffer.

The cosmids from the TAL 182 gene bank were transformed into E. coli HB101 while those from the TAL 1145 gene bank were transformed into E. coli DH5αmcr. Competent E. coli cells were prepared according to Maniatis et al. (1982) and were stored at -70°C. Transformation was carried out by mixing 10 µg of cosmid DNA with 100 µl of competent cells. The mixture was incubated on ice for 20 min and then subjected to heat shock for one min in a 42°C waterbath. The mixture was diluted with 1 ml of LB broth, incubated for 1 hr at 37°C with shaking, and then spun to collect the cells. The cells were plated on LB medium containing tetracycline and allowed to grow at 37°C. Tc^r transformants appeared after about 12 hours.

Small-scale preparation of cosmid DNA for restriction analysis was done by the alkaline lysis procedure for E. coli DH5 α mc^r and by the boiling method (Holmes and Quigley, 1981) for E. coli HB101.

The alkaline lysis procedure to prepare DNA for restriction analysis was modified to include additional extractions. After isopropanol precipitation, the DNA was dissolved in 100 μ l TE, and extraction was done once with phenol, and twice with chloroform. The aqueous phase was diluted to 0.3 M sodium acetate and the DNA was precipitated in 2.5 volumes of cold 95% ethanol, washed in 70% ethanol, dried, and dissolved in 20 μ l TE.

Cosmid DNA was prepared from E. coli HB 101 by the boiling method (Holmes and Quigley, 1981), from two ml of cells grown overnight in LB medium supplemented with tetracycline. The cells were spun down, washed in one ml of 0.5 M NaCl solution, and resuspended in 200 μ l of STET buffer (0.8% sucrose; 0.5% Triton-X-100; 50 mM EDTA; 50 mM Tris, pH 8.0). The cells were lysed by adding 60 μ l lysozyme solution (10 mg ml⁻¹ in H₂O) and left at room temperature for 5 min. The tube was placed in boiling water for 45 sec and spun for 10 min at room temperature. The supernatant was collected and the DNA was precipitated by adding an equal volume of isopropanol and spinning for five min. The DNA pellet was washed in 70% ethanol, dried, and dissolved in 30 μ l of TE buffer. About 10 μ l of this DNA was used for each digest with a restriction enzyme.

Restriction analysis was done with EcoRI and HindIII (Bethesda Research Laboratories, Inc., Gaithersburg, MD) which were used according to the recommendations of the supplier. DNA fragments were separated on 0.8% agarose gels prepared in TAE buffer.

Plant tests for nodulation and nodulation suppression. The complemented mutants were characterized for nodulation and nodulation suppression on common bean. Split-root systems of common bean were generated and maintained as described in Chapter 4, using three or five

replicate plants per treatment. The mutant or complemented mutant was used as the primary inoculant on one side of a split-root at zero time, and the wild-type was used as the secondary inoculant on the opposite side two weeks later. At this delayed time, the wild-type was also inoculated on one side of a split-root (unsuppressed control), representing the nodulation potential of the wild-type in the absence of prior inoculation. Suppression (Sup) was evaluated on the extent of nodulation of the wild-type when there was prior inoculation on the other side, as compared to when there was no inoculation on the other side. The phenotype of mutant or complemented mutant was considered Sup⁺ when nodulation by the secondary inoculant was reduced to less than 10% of its potential.

Results

Construction of TAL 182 gene library. A gene library of genomic DNA from R. leguminosarum bv. phaseoli TAL 182 was constructed in the mobilizable, broad-host-range cosmid vector pLAFR1 and maintained in E. coli HB101. The average length of the inserts in the clones was determined to be approximately 23 kb by EcoRI digestion of 9 randomly selected Tc^r colonies (data not shown), consistent with the size selection imposed by the lambda packaging system. The clone bank contained approximately 5,000 independent cosmids. Assuming that the Rhizobium genome has the same size as that of E. coli (4,200 kb), the probability of the clone bank containing a given 1-kb sequence of DNA was theoretically calculated, using the Clarke and Carbon formula (Collins, 1979), to be greater than 99%.

Isolation of cosmids that complement nodulation defects. Three groups of Tn5 mutants that are impaired at various stages of nodulation development were used for isolating genes that complement the phenotypic defects (Table 6.2). Class I mutants were nonnodulating while Class II mutants formed small swellings on the roots. Of the two Class II mutants, one (MLC35) showed autoagglutination in TY medium, a phenotype associated with an alteration in

lipopolysaccharide (LPS) production (Prifer, 1989). The third group consisted of auxotrophic (Ade⁻, Ura⁻, Leu⁻, and Phe⁻) mutants that were also differentially impaired in nodulation abilities. All of these mutants were unable to induce nodulation suppression in common bean (Chapter 5).

Cosmids that corrected the mutations were identified by in planta complementation with gene libraries from leguminosarum bv. phaseoli TAL 182 and from R. loti of TAL 1145. Variable-sized nodules ranging in number from 25-100, formed on bean plants inoculated with the Tc^r Km^r transconjugants. Three weeks after inoculation, rhizobia were isolated from the nodules, purified to single colonies, and the resident cosmid was transferred to E. coli HB101 by transformation.

The TAL 1145 gene library was also conjugated en masse with the TAL 182 auxotrophic mutants, and the Tc^r Km^r transconjugants were plated on minimal media for selection of restored prototrophy. Prototrophic transconjugants resulted from all four types of auxotrophic mutants. The transconjugants were purified to single colonies, and the resident cosmid was transformed into E. coli DH5 α mc^r.

Restriction analysis of complementing cosmids. Complementing cosmids were isolated from all mutants. Five overlapping cosmids from the TAL 182 gene library that complemented Class I mutants were isolated (Figure 6.1). The restriction map of the complementing region constructed from Figure 6.1 (lanes G, H, I, J, K) is shown in Figure 6.2. The region contained seven contiguous EcoRI fragments spanning approximately 32.1 kb with a 7.2-kb fragment common to the five cosmids. The Tn5 insertions in the three Class I mutants were located in different EcoRI fragments within a 21.6-kb region (Figure 6.2). As predicted from a Southern blot analysis of the mutants (Chapter 5), the Tn5 insertions in MLC357 and MLC648 were in 2.0-kb and 7.2-kb fragments, respectively, that were adjacent to each other, while that of MLC778 was in a 3.0-kb fragment located 9.4 kb away from that of MLC648.

TABLE 6.2. Symbiotic phenotypes of *R. leguminosarum* bv. *phaseoli* TAL 182 mutants and mutants complemented by TAL 182 and TAL 1145 gene banks.

Strain	Mutated Phenotype			Complemented Phenotype					
	Nod ^a	Fix ^b	Sup ^c	TAL 182			TAL 1145		
				Nod	Fix	Sup	Nod	Fix	Sup
Class I									
MLC357	-	-	-	+	-	+/-	+	-	+/-
MLC648	-	-	-	+	-	+/-	+	-	+/-
MLC778	-	-	-	+	-	+/-	+	-	+/-
Class II									
MLC35 ^d	-	-	-	+	+	+	+	+	+
MLC640	-	-	-	+	+	+	+	+	+
Auxotrophs									
182A	-	-	-	ND	ND	ND	+	+	+
182U	-	-	-	ND	ND	ND	+	+	+
182L	-/+	-	-	ND	ND	ND	+	+	+
182P	-/+	-	-	ND	ND	ND	+	+	+

^aNodulation, indicated by presence of normal-sized, bacteroid-containing nodules, +/- means that bacteroids were present in less than 30% of the nodules.

^bNitrogen fixation, indicated by green color of plant shoot and red color of nodules.

^cSuppression, evaluated in a split-root by comparing nodulation resulting from a secondary inoculation with the wild-type when there is prior inoculation with a mutant or complemented mutant, and the nodulation potential of the wild-type in the absence of prior inoculation. +/- means reduced suppression.

^dMutant showed autoagglutination in TY medium and upon complementation, was restored to the wild-type phenotype.

FIGURE 6.1. LQRI restriction digest of cosmids from R. leguminosarum bv. phaseoli TAL 182 gene bank complementing the TAL 182 Class I and Class II mutations. MLC357 (2), MLC648 (4), and MLC778 (5) are Class I mutants. MLC35 (1) and MLC640 (3) are Class II mutants. The size markers are HindIII-digested lambda DNA. Lanes A,B,E,F (pUHR58); C,D (pUHR59); H,P (pUHR60); G,O,S,T,U,W,Y,Z (pUHR61); J,R (pUHR62); K,Q,V,X (pUHR63); I (pUHR64).

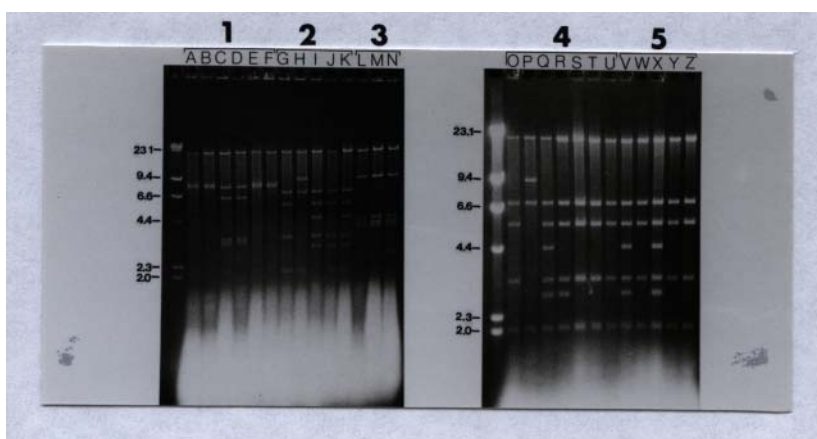
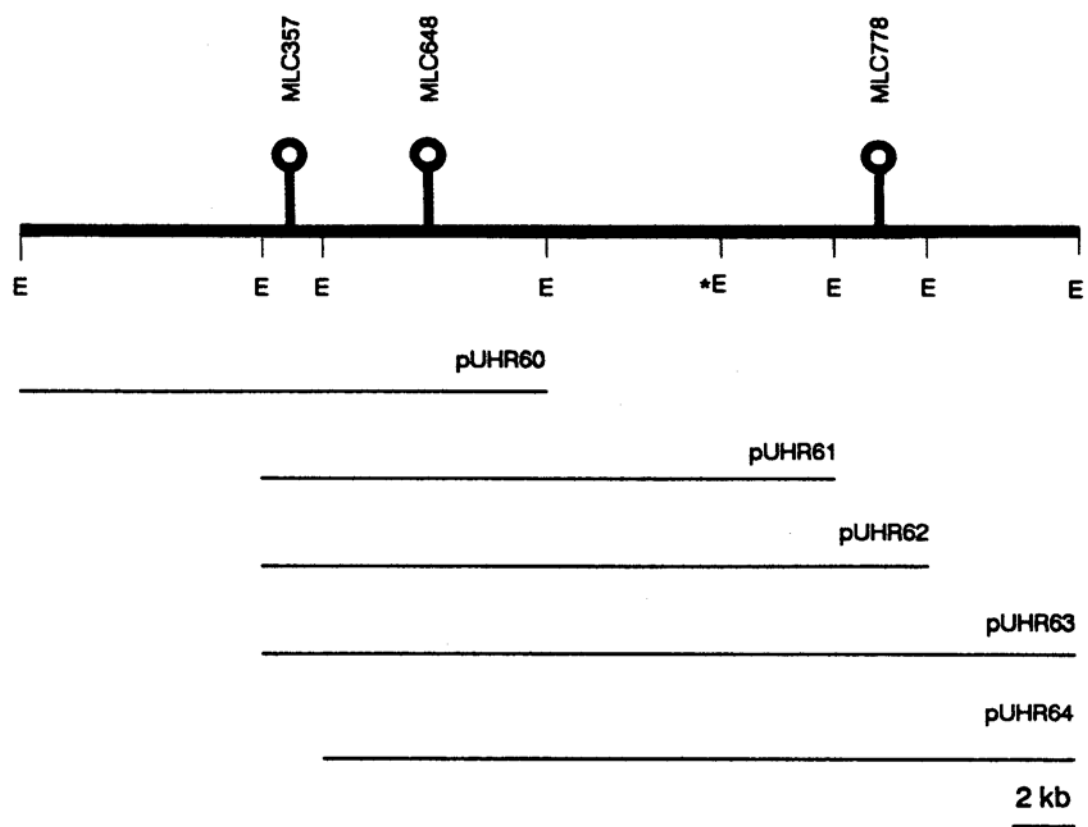


FIGURE 6.2. EcoRI (E) restriction map of the symbiotic region of R. leguminosarum bv. phaseoli TAL 182 complementing the TAL 182 Class I mutations. The map was constructed from the restriction digest shown in Figure 6.1 (Lanes G,H,I,J,K) and the approximate site of Tn5 insertions in the mutants were estimated from Chapter 5 (Figure 5.1). The exact location of the EcoRI site marked with an asterisk cannot be determined from present data, may be 5.8 or 3.6 kb from the adjacent sites.



The cosmids from the TAL 182 gene library that complemented the Class II mutants were separated into two groups (Figure 6.1). Two overlapping cosmids that complemented MLC35 contained five contiguous EcoRI fragments spanning 30.1 kb with an 8.0-kb common fragment. Two overlapping cosmids that complemented MLC640 contained six contiguous EcoRI fragments spanning 32.5 kb with three fragments (4.8 kb, 4.4 kb, 4.2 kb) in common. Southern blot analysis of the mutants (Chapter 5) localized the Tn₅ insertion in MLC35 and in MLC640 in the 8.0-kb and 4.2-kb fragments, respectively, that are common to each set of overlapping clones.

Restriction analysis of overlapping cosmids from the TAL 1145 gene library that complemented Class I and Class II mutants identified two complementation groups (Figure 6.3). Eight overlapping clones that complemented Class I mutants contained nine contiguous EcoRI fragments spanning 38.3 kb with two common fragments (4.5 kb and 2.2 kb). Three overlapping clones that complemented Class II mutants contained five contiguous EcoRI fragments spanning 32.7 kb with a 10.1-kb common fragment.

All the auxotrophic mutations were complemented by cosmids from the TAL 1145 gene library (Figure 6.4). The Ade⁻ auxotroph was complemented by three overlapping cosmids with three (3.5 kb, 3.1 kb, 2.5 kb) common EcoRI fragments. One cosmid each was isolated from the Ura⁻ and Leu⁻ auxotrophs. The Phe⁻ mutant was complemented by four overlapping cosmids with a 4.1-kb common EcoRI fragment.

Nodulation and nodulation suppression by complemented mutants.

Complementation with a cosmid library of wild-type DNA restored the nodulation abilities of both the Class I and Class II mutants (Table 6.2). However, only the Class II mutants regained full symbiotic competence, and in the case of MLC35, also the wild-type cell surface phenotype. Although the nodules formed in both complemented classes were all normal-sized, those formed by the Class I mutants were white and were scattered throughout the

FIGURE 6.3. Restriction digest of overlapping cosmids from Rhizobium loti (L. leucocephala) TAL 1145 gene bank complementing R. leguminosarum bv. phaseoli TAL 182 Class I (MLC648) and Class II (MLC640) mutations. The size markers are HindIII-digested lambda DNA. Lanes A (pUHR7); B,C (pUHR1); D,G (pUHR2); E (pUHR3); F (pUHR4) ; H,J,L (pUHR5) ; I (pUHR6) ; K (pUHR7) ; M,P (pUHR8); N (pUHR9); O (pUHR10); Q (pUHR11); R (pUHR12).

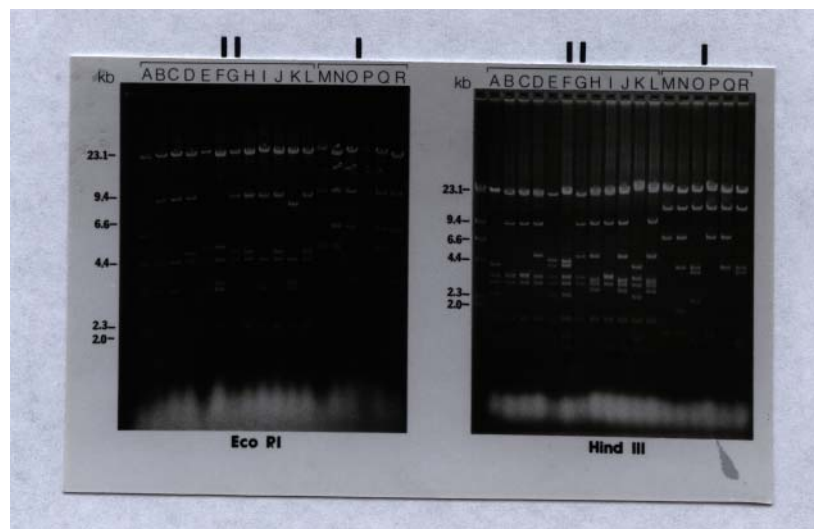
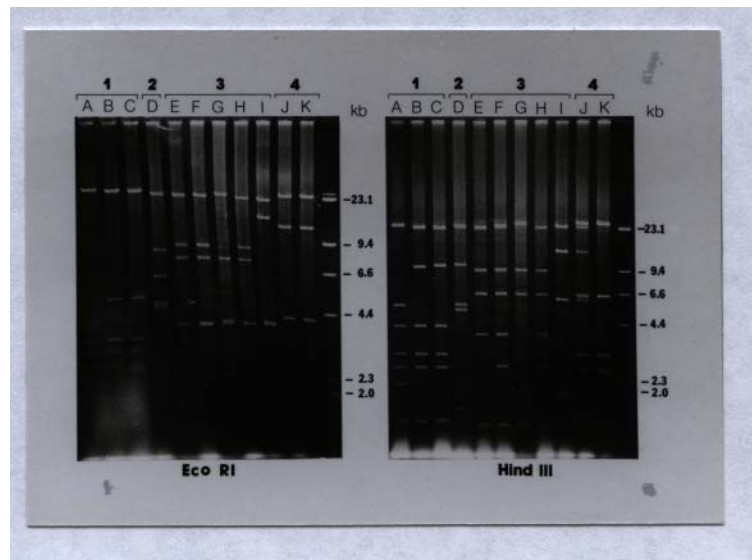


Figure 6.4 Restriction digest of overlapping cosmids from Rhizobium loti (L. leucocephala) TAL 1145 gene bank complementing R. leguminosarum bv. phaseoli TAL 182 auxotrophic mutations. The size markers are HindIII-digested lambda DNA. Group 1, cosmids complementing auxotrophy for adenine; 2, leucine; 3, phenylalanine; 4, uracil. Lanes A (pUHR49); B (pUHR50); C (pUHR51); D (pUHR52); E (pUHR53); F,H (pUHR54); G (pUHR55); I (pUHR56); J,K (pUHR57).



root system. Plants inoculated with these complemented Class I mutants were stunted and yellow, indicating nitrogen starvation.

The complemented Class I mutants did not regain their full ability to induce nodulation suppression (Table 6.2). When these were used as primary inoculants in a split-root, a secondary inoculation with the wild-type resulted in nodulation that is 32-70% of the nodulation potential of the secondary inoculant. In contrast, when the complemented Class II mutants were used as primary inoculants, nodulation resulting from a secondary inoculation was only 1-7% of the nodulation potential of the wild-type, a strong suppression response characteristic of the wild-type.

Heterologous complementation with a cosmid library from Rhizobium loti TAL 1145, also yielded similar results. All the complemented auxotrophs were restored to full symbiotic competence, and induced strong suppression from the host (Table 6.2).

Discussion

Homologous complementation restored the nodulation abilities of both Class I and Class II mutants (Table 6.2). However, the complemented Class I mutants were ineffective and did not induce nodulation suppression in split-roots. This impairment was also apparent in the scattered distribution of the nodules instead of a more clustered distribution (data not shown), indicating a lack of suppression in the younger regions of the root.

The complemented Class II mutants regained nodulation, nitrogen fixation, and suppression characteristics comparable to that of the wild-type (Table 6.2). The simultaneous restoration of the various wild-type characteristics indicate that the phenotypic defects were due to a single mutation. The same conclusion is drawn from the complemented phenotypes of the auxotrophs (Table 6.2). Upon restoration to prototrophy, these mutants regained full symbiotic competence and induced strong suppression from the host, indicating that the symbiotic defects may be an indirect consequence of

the mutated metabolic gene. The fact that the various auxotrophies were complemented by heterologous DNA also point to the functional generality of metabolic genes.

Along with the restoration of symbiotic competence, complementation of MLC35 also restored its cell surface phenotype. Single mutations leading to defects in both LPS synthesis and symbiotic ability were reported in other strains of *R. leguminosarum* bv. *phaseoli* (Cava et al., 1989; Noel et al., 1986) and in *R. leguminosarum* bv. *viciae* (de Maagd et al., 1989).

The Class I mutants (Hac^+ , Nod^-) which are blocked at an earlier stage of development, were not restored to full symbiotic competence, while the Class II mutants (Hac^+ , Nod^+), which are blocked at a later stage, were restored to full competence (Table 6.2). Heterologous complementation with a cosmid library from *R. loti* TAL 1145, also yielded similar results (Table 6.2). Hence, the possibility that the partial complementation in Class I may be due to partial homology can be ruled out. The contrast in the complemented phenotypes of the two classes of mutants may be due to a gene-dosage effect from genes elsewhere in the cosmid, leading to the lack of expression of some of the symbiotic genes. Such a mechanism was suggested by Scott et al. (1985). In their study, complementing cosmids restored nodulation but not nitrogen fixation to the Nod^- mutants. However, restoration of the Fix^+ phenotype was observed in a few nodules when plant growth was extended to five weeks, possibly after marker rescue has occurred. Since plants in this study were not grown this long, marker rescue was not observed. Subcloning of the common regions in this class that correspond to the region of Tn5 insertion and reintroduction of the subclones to the mutants, will demonstrate if in fact, the lack of full symbiotic expression is due to genetic interference.

The reduced ability of the Nod^+ , Fix^- complemented Class I mutants and the full ability of the Nod^+ , Fix^+ complemented mutants to induce nodulation

suppression does not necessarily indicate an effective phenotype as a requirement for suppressiveness, but does indicate that nodule development beyond the stage expressed in the complemented Class I mutants is required to attain and sustain a full suppression response. It is possible that while nodulation (Nod⁺) and fixation (Fix⁺) are the gross phenotypic characters that are most discernible, several symbiotic stages that are biochemically and ultrastructurally distinct may actually separate these phenotypes. Ultrastructural and biochemical comparison of the nodules of the nonsuppressive (uncomplemented mutant), partially suppressive (complemented Class I), and fully suppressive (wild-type or complemented Class II) mutants will help to define the specific phenotypic blockage associated with the extent of the host response.

The results of this study suggest that the autoregulatory response of the host may be effected by cumulative signals linked to the various stages of nodule development. While the initial trigger for suppression may occur much earlier in the process, within 24 h after primary inoculation in the case of soybean (Kosslak and Bohlool, 1984), the degree of suppression that is finally attained may depend on the stage of symbiotic development. Thus, for the suppression response to increase and be sustained would require the full development of the symbiosis.

SUMMARY

Inoculation with highly effective Rhizobium strains does not often result in increased productivity due to aggressive competition from less effective rhizobia that may be present in the soil. Despite its acknowledged importance, not much is known of interstrain competition at the mechanistic level.

There is evidence that the plant host may play an important role in the selection of the nodulating strain. It is postulated that the plant host expresses a systemic signal in response to invading rhizobia, thereby allowing the host to regulate nodulation. While the mechanism of host selection is still not known, it is clear that the development of nodules is under the regulation of the host. The plant host influences the extent of nodulation by the process of autoregulation, as seen in the restriction of nodulation in developmentally younger regions of the root. The systemic nature of autoregulation is seen in split-root systems, where developing nodules on one side suppress subsequent nodulation on the opposite side.

This study addressed the role of the host as a variable that may influence the outcome of competition. The major findings of this research are:

I. Six Rhizobium leguminosarum bv. phaseoli strains varied in their intrinsic competitive abilities. The strains were divided into three groups according to their overall competitive abilities on two bean cultivars. Strains TAL 1472 and TAL 182 were highly competitive (greater than 70% nodule occupancy); strains KIM-5, Viking 1, and CIAT 899 were moderately competitive (approximately 50% nodule occupancy); and strain CIAT 632 was poorly competitive (less than 5% nodule occupancy). The proportion of competing strains in the inoculum influenced the nodule occupancy of the highly competitive and moderately competitive strains, but not the poorly

competitive strain. Strain CIAT 632 occupied an insignificant proportion of the nodules even when it outnumbered the competing strain in the inoculum.

The capacity to outcompete a competitor appears to be a strain characteristic. The competitiveness grouping of the six strains did not change with the host cultivars, indicating that the plant did not discriminate between the six strains. There was a correspondence between the competitiveness and effectiveness groupings of the strains. The two highly competitive strains had the highest effectiveness indices while the poorly competitive strain had the lowest effectiveness index. However, no correlation between competitiveness and nodulation characteristics was found.

The highly competitive and highly effective strains, TAL 1472 and TAL 182, were identified as good candidates for genetic manipulation to study the molecular basis for competitiveness.

II. Nodulation in common bean was subject to autoregulation. When a primary inoculation was done on one side of a split-root, followed by a secondary inoculation on the opposite side with increasing delay, the extent of nodule inhibition on the side that received a delayed inoculation was time-dependent, and became more pronounced as the delay period was increased. A 14-day delay in secondary inoculation resulted in 94% suppression of secondary modulation.

When six R. leguminosarum bv. phaseoli strains with varying competitive abilities were used as primary inoculants to compare their ability to induce suppression, all strains induced nodulation suppression but varied in their degree of suppressiveness. Differences in their degree of suppressiveness partially corresponded with their competitive abilities. A highly competitive strain (TAL 1472) was also highly suppressive whereas a poorly competitive strain (CIAT 632) was also less suppressive. Suppression ability was not related to a strain's speed of nodule initiation or to its nodulating potential.

III. Three classes of symbiotic mutants were identified after Tn5 mutagenesis of TAL 182. Class I mutants were nonnodulating, Class II mutants induced small swellings on the roots, and a Class III mutant formed pink, bacteroid-containing, but ineffective nodules. In addition, metabolic mutants with differential nodulation abilities, were isolated. A purine mutant (Ade⁻) was nonnodulating while a pyrimidine mutant (Ura⁻) formed small swellings on the roots. Amino acid mutants (Leu⁻, Phe⁻, Cys⁻) formed white, empty nodules. Southern hybridization analysis indicated that each mutant had a single Tn5 insertion.

The mutants with differential symbiotic competence, were used as primary inoculants in split-roots to correlate a particular symbiotic stage with the suppression response of the plant host. Suppression was correlated with the ability of the microsymbiont to enter and persist inside the nodules, but was not related to the ability to induce the formation of visible nodule primordia, or to the ability to fix nitrogen. All mutants with defects in nodulation ability, regardless of the particular stage of blockage, failed to induce a suppression response from the host. Only the nodulation-competent, bacteroid-containing, but ineffective Class III mutant induced a suppression response similar to that induced by the wild-type. Thus, the results point to the presence of rhizobia inside the nodules as a prerequisite for nodulation suppression, indicating that suppression may be a response to signals from developing bacteroids.

IV. The genes that complement nodulation and suppression defects were cloned. Gene libraries from the wild-type TAL 182 and from R. loti TAL 1145, a strain which nodulates both common bean and Leucaena leucocephala, were conjugated en masse with the mutants, and cosmids that corrected the mutations were identified.

Homologous complementation restored the nodulation abilities of both Class I and Class II mutants. However, the complemented mutants of Class I

were ineffective and did not regain full ability to induce modulation suppression. In contrast, the complemented mutants of Class II regained nodulation, fixation, and suppression characteristics comparable to that of the wild-type.

Restriction analysis of the overlapping cosmids complementing Class I mutants showed a 7.2-kb region in common. As predicted from a Southern hybridization analysis, the Tn5 insertions in the Class I mutants were in 2.0-kb, 7.2-kb, and 3.0-kb EcoRI fragments that were located within a 21.6-kb region. Two complementation groups corrected the Class II mutations. Overlapping cosmids complementing MLC35 and MLC640 showed an 8.0-kb and a 13.4kb region in common, respectively.

Heterologous complementation with a gene library from R. loti TAL 1145, also yielded similar results. Restriction analysis of the overlapping cosmids complementing the Class I and Class II mutations showed a 6.7-kb region and a 10.1-kb region in common, respectively. All the auxotrophic mutations were complemented by heterologous DNA. Upon restoration to prototrophy, these mutants regained full symbiotic competence and induced strong suppression from the host.

The results of this study suggest that the autoregulatory response of the host may be effected by cumulative signals linked to the various stages of nodule development, and that the degree of suppression that is finally attained may depend on the stage of symbiotic development.

APPENDIX

Nodule number and nodule dry weight of common bean associated with the suppression response induced by *Rhizobium leguminosarum* bv. *phaseoli* TAL 182

Days Delayed	Early/Delayed			Delayed/Delayed			Uninoculated/Delayed		
	E side	D side	Total	D side	D side	Total	0 side	D side	Total
MODULE NUMBER									
0				123.6±13.0	106.4±12.0	230.0±19.8	0±0	175.8±27.0	248.6±14.2
1	145.6±12.6	103.0±5.4	248.6±14.2	123.0±11.4	132.4±11.7	255.4±14.2	0±0	175.6±7.1	175.6±7.1
2	126.6±11.3	104.2±8.1	230.8±12.6	112.2±11.3	121.8±19.3	234.0±30.3	0±0	163.8±4.6	163.8±4.6
3	106.0±10.0	97.4±4.5	204.0±11.3	109.4±10.3	141.8±19.7	251.2±28.1	0±0	160.0±16.6	160.0±16.6
4	138.6±13.5	75.4±10.0	214.0±22.0	139.0±18.8	136.4±19.1	275.4±33.0	0±0	167.2±12.4	167.2±12.4
5	113.6±11.6	81.8±6.0	195.4±16.2	120.8±13.0	120.0±9.2	240.8±19.1	0±0	210.0±24.1	210.0±24.1
6	207.4±17.2	67.8±16.7	275.2±22.6	130.8±9.7	149.8±5.6	280.6±9.7	0±0	174.8±27.4	174.8±27.4
7	166.4±11.2	64.8±11.3	231.2±15.5	139.2±11.8	131.4±18.1	270.6±27.4	0±0	192.8±20.4	192.8±20.4
8	182.8±18.2	73.6±8.1	256.4±23.2	147.6±13.2	125.8±18.3	273.4±16.0	0±0	151.4±7.8	151.4±7.8
9	186.6±12.9	48.2±16.6	234.8±25.5	117.6±13.4	98.6±7.1	216.2±8.8	0±0	133.2±9.3	133.2±9.3
10	137.8±15.2	31.2±3.9	169.0±17.4	122.2±15.1	79.2±15.6	191.4±17.3	0±0	119.6±8.1	119.6±8.1
14	142.0±14.2	6.2±2.8	148.2±16.1	98.8±21.5	52.6±10.2	151.4±12.8	0±0	103.0±11.8	103.0±11.8
MODULE DRY WEIGHT									
0				26.22±3.04	24.32±1.36	50.54±3.56	0±0	53.36±5.99	53.36±5.99
1	31.68±3.52	21.36±1.23	53.04±4.23	32.90±0.51	27.56±3.87	60.46±4.30	0±0	48.42±5.35	48.42±5.35
2	35.30±3.13	26.80±2.16	62.10±4.33	26.66±1.62	30.64±1.95	57.30±2.08	0±0	49.18±1.37	49.18±1.37
3	40.66±4.03	27.44±2.15	68.10±4.66	29.80±2.77	33.94±1.72	63.74±3.81	0±0	60.72±3.08	60.72±3.08
4	45.12±3.65	24.08±3.33	69.20±4.34	25.75±0.99	32.12±3.98	58.55±4.48	0±0	53.92±3.26	53.92±3.26
5	43.10±4.91	22.78±4.0	65.88±5.93	29.00±3.48	25.30±1.3	54.30±3.98	0±0	63.12±1.13	63.12±1.13
6	61.98±4.44	14.78±2.98	76.76±4.34	24.94±2.63	29.78±4.82	54.72±7.13	0±0	49.74±6.50	49.74±6.50
7	52.74±3.59	13.88±3.06	66.62±4.07	30.98±1.13	29.34±2.8	60.32±2.8	0±0	52.38±5.07	52.38±5.07
8	48.16±5.08	11.64±1.83	59.80±6.36	29.60±3.07	25.10±3.06	54.70±1.83	0±0	47.08±1.16	47.08±1.16
9	57.96±3.83	7.06±1.93	65.02±4.72	30.06±3.52	24.64±1.4	54.70±4.54	0±0	50.82±1.65	50.82±1.65
10	53.16±3.23	6.12±1.14	59.28±3.66	30.36±2.78	28.28±4.66	58.64±2.24	0±0	49.18±4.38	49.18±4.38
14	51.94±4.71	1.5±0.65	53.44±5.08	23.50±4.73	17.28±4.99	40.78±3.68	0±0	25.54±3.62	25.54±3.62

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