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Meiyu Mao for the degree of Master of Science in Crop Science presented on March 14, 1989.

Title: Evaluation of a Rhizobium meliloti Transconjugant for Increased Nodulation and Biological Nitrogen Fixation in Alfalfa

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Dr. David B. Hannaway

Agrobacterium rhizogenes has been shown to cause root proliferation and increased water use efficiency in certain woody dicots. Root proliferation may be desirable in herbaceous legumes as a means of increasing potential sites for infection by Rhizobium species and subsequent nodulation and nitrogen fixation. Thus, A. rhizogenes was used to inoculate a series of forage legumes to evaluate its ability to stimulate root activity. Growth chamber experiments were conducted in which plants were grown in plant tubes with nutrient agar. Plants were evaluated for root development six weeks after inoculation. The preliminary experiment showed that alfalfa treated with A. rhizogenes 232 produced a significantly (159%) greater number of roots, twice the root volume, 183% greater root

fresh weight, and 214% more root dry weight than the uninoculated treatment. In white clover and field pea, however, A. rhizogenes 232 did not produce significantly increased root number or root mass. This experiment indicated that A. rhizogenes was effective in stimulating increased root initiating activity in alfalfa.

The positive effects of A. rhizogenes 232 on the initial growth of alfalfa roots led to experiments to evaluate A. rhizogenes's ability to stimulate root activity and the effectiveness of a R. meliloti transconjugant (pRi) in increasing nodulation and biological nitrogen fixation.

The R. meliloti (pRi) transconjugant was formed by introducing the Ri plasmid from A. rhizogenes into R. meliloti. This R. meliloti transconjugant BL105-9 was used to inoculate two alfalfa (Medicago sativa) cultivars (Ladak 65 and Vernema) to evaluate its effectiveness for increased nodulation and biological nitrogen fixation. Strain effectiveness was determined following six weeks in plant tubes containing nutrient agar in a growth chamber experiment. Although the R. meliloti transconjugant (BL105-9) did not produce significantly more root or shoot mass than the R. meliloti parent (BL116), it did produce significantly (72.8%) more nodules and 99.2% more nodule dry weight. No significant difference in either total plant nitrogen level or acetylene reduction rate was

observed between the R. meliloti transconjugant and the parent strain. Thus, this experiment indicates that introduction of the hairy root plasmid into R. meliloti resulted in increased nodulation of alfalfa, but this increased nodulation was not translated into increased total plant nitrogen or increased dry matter production.

Evaluation of a *Rhizobium meliloti* Transconjugant for Increased Nodulation and Biological Nitrogen Fixation in Alfalfa

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Head of Crop Science Department

Redacted for Privacy

Dean of Graduate School

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EVALUATION OF A *RHIZOBIUM MELILOTI* TRANSCONJUGANT FOR INCREASED NODULATION AND BIOLOGICAL NITROGEN FIXATION IN ALFALFA

I. INTRODUCTION

In 1650, the world population was 545 million. At the beginning of the twentieth century it stood at approximately 1.6 billion. Today the world population is 4.4 billion (Dudal, 1982). It is anticipated that it may increase by the end of this century to more than 7 billion and later stabilize at 10.5 billion (Heath and Kaiser, 1985). One of the principle needs of this population is food, yet on a world basis, food production is not keeping pace with population growth.

According to Hardy and Havelka (1975), in both the more and less developed areas of the world, the application of nitrogen (N) fertilizer remains the single most important agronomic input for increasing food production particularly for cereal cultivation. The greatest population growth, however, is in developing countries where dependence on imported industrially-fixed N fertilizer limits optimum agricultural production.

Nitrogen is the major limiting nutrient for corn, small grains, and grasslands. Nitrogen is also the most costly of the major crop nutrients on a land area basis

(Heichel, 1985).

Forages play a vital role in the production of milk, meat, and wool in the world. The common objective of forage workers is to establish a stable and profitable forage livestock sector in the agricultural economy through intensified forage production and utilization of each given land resource at the optimal economical level.

Legumes have long been valued for their capacity to be at least partly nitrogen self-sufficient. It is known that this property is associated with the symbiotic legume-Rhizobium association which converts atmospheric N_2 into ammonia (NH_3) (Evans, 1969).

Although this symbiotic relationship is utilized every time legumes are planted, commonly as much as 50% of the legume plant nitrogen is provided by soil and/or fertilizer nitrogen (Rennie and Dubetz, 1986). The scarcity of appropriate sources of energy, high costs for the manufacture of N fertilizers, and desire for more sustainable systems of agricultural production have stimulated a renewed worldwide interest in biological nitrogen fixation (BNF) (Emerich and Evans, 1980; Francis et al., 1987; Hardy and Havelka, 1975; Quispel, 1974).

Effectively nodulated leguminous crops may fix up to three hundred kilograms of nitrogen per hectare per year (Vincent, 1974). Fixation on a global scale has been

calculated at about $2*10^8$ kilograms of nitrogen per year. In the late 1970's, world production of fixed nitrogen from dinitrogen for chemical fertilizer was about $6*10^7$ kilograms. Amounts of atmospheric oxides of nitrogen washed into soil suggest that this source may provide about $5*10^6$ kilograms of fixed nitrogen per year (Burns and Hardy, 1975). This information reduced the estimate of global biological N_2 fixation from $175*10^6$ metric tons (Burns and Hardy, 1975) to about $122*10^6$ kilograms (Burris, 1980). Biological processes thus account for about 60% of the earth's newly-fixed nitrogen (Postgate, 1982).

There are two aspects of the benefits that can accrue from the use of nodulated legumes as crop or pasture plants. The first is the plant's independence of soil N. The second is the potentially improved N status of the soil following legume use (Vincent, 1974). In legume-nonlegume rotations, the amount of fertilizer N replaced by legume N depends upon the quantity of residue returned to the soil, the proportion of symbiotically fixed N in the residue, and the rate at which that N becomes available to the succeeding non-legume crop (Heichel, 1985). Therefore, leguminous plants, growing in symbiosis with the appropriate Rhizobium species, are of great economic importance in the conversion of atmospheric N_2 to a form

that can be used efficiently for the nutrition of plants and animals.

The degree to which forage legumes are N self-sufficient may be improved by various agronomic practices. Inoculation with Rhizobium spp. is now a common practice used to assure high numbers of highly effective nodule forming bacteria on legume roots. Considerable work has demonstrated that inoculating legumes such as soybeans, clover, alfalfa, peas, or beans with an appropriate strain of rhizobia improves the rate of nodulation (Abel and Erdam, 1964; Brockwell and Katznelson, 1976; Hunt et al., 1981; Weaver and Frederick, 1974), capacity to fix nitrogen (Dunigam et al., 1984; Eaglesham et al., 1983; Gibson and Pagan, 1977), and yield (Ham et al., 1971; Spparrow and Ham, 1983) when compared to depending on indigenous rhizobia.

The number of nodules formed, however, is often dependent on the formation of infection sites. The morphology of the legume root system, its nutrition, its growth and proliferation of root hairs also have been shown to influence the number of nodules formed (Andrew and Robin, 1969a, 1969b; Smith et al., 1981).

Agrobacterium rhizogenes is a soil inhabiting microorganism that occasionally enters plant roots via wounds or natural openings. This may result in a proliferation

of secondary roots leading to "hairy root syndrome" (Hildebrand, 1934; Riker, 1930). The factor responsible for the genetic transformation is a bacterial plasmid (Ri) which is positively correlated with the infectivity of A. rhizogenes (Chilton et al., 1982; Moore et al., 1979; White and Nester, 1980). This is in contrast to the effect of Agrobacterium tumefaciens, which causes gall or tumor formation on its hosts. Although A. rhizogenes can cause unsightly roots in some nursery stock, it has never been reported as an important pathogen on fleshy rooted species such as carrot, beet, radish, or sweet potato. A. rhizogenes has a wide host range, but it seems to be strictly confined to dicotyledonous plants (Strobel and Nachmias, 1985).

Moore et al. (1979) reported that apple trees possessing a form of non-infectious "hairy root" had greater root growth and demonstrated better drought tolerance than those not treated with A. rhizogenes. White et al. (1980) also showed that the infection of numerous species of vegetables by A. rhizogenes induced a large proliferation of their root system.

The rationale for treating the roots of such plants with A. rhizogenes is for the earlier development of numerous secondary roots, allowing better water uptake, and subsequently resulting in better stand establishment

and total top growth (Strobel and Nachmias, 1985). Plants that require especially rapid root growth and development are those that are developing nodules in preparation for biological nitrogen fixation.

The co-existence of A. rhizogenes bacteria in many different soils with Rhizobium has been demonstrated by Hooykass et al. (1977). Strobel et al. (1985) reported increased nodulation in the host after introducing the hairy root plasmid into Rhizobium meliloti. Although they reported that the R. meliloti (pRi) transconjugants were unable to cause root proliferation in any of the dicotyledonous plants tested, they were capable of nodulating alfalfa seedlings to a 87-142% greater extent than non-transconjugant forms of R. meliloti.

N_2 fixation by the Rhizobium-legume symbiosis is influenced by the genotype of both the bacterium and the plant (Phillips and Teuber, 1985). Many workers have considered the question of whether R. meliloti or alfalfa has the greater genetic variation for traits influencing N_2 fixation (Burton and Wilson, 1939; Erdman and Means, 1953; Gibson, 1962; Mytton et al., 1984; Tan, 1981). Both plant cultivars and bacterial strains have significant effects on N_2 fixation. All the studies showed that Rhizobium-strain X alfalfa-cultivar interactions were responsible for a large part of the variation. 'Nitro' is

the first alfalfa cultivar selected for specialized N accumulation attributes (Barnes et al., 1988).

Alfalfa has been called "Queen of the Forage Crops" because of its remarkable ability to produce high yields of rich, palatable, nutritious forage under a wide range of soil and climatic conditions. On a global basis it is the oldest and most widely used forage. Alfalfa is grown on nearly 15 million hectares in North America and 33 million hectares on a world scale, and it has been a major crop in the United states for more than 100 years (Walton, 1983). Annual rates of N_2-fixation in alfalfa have been reported to vary from 150 to 600 kg/ha (Hoffman and Melton, 1981).

Because of the importance and wide spread use of alfalfa, improvement of N_2 fixation in alfalfa has world-wide importance and is of interest to alfalfa producers, cropping system managers, plant and soil scientists, and microbiologists. The experiments described here sought to improve N_2 in alfalfa through the use of microbiological and genetic engineering methods.

Objectives

The objectives of this study were: 1) to test *Agrobacterium rhizogenes*' ability to cause root proliferation in forage legumes, and 2) to evaluate the effectiveness of a *Rhizobium meliloti* transconjugant for increased nodulation and biological nitrogen fixation in alfalfa.

II. REVIEW OF LITERATURE

Biological Nitrogen Fixation

Biological N_2 fixation is a unique property possessed by only a few genera of prokaryotic organisms that contain the genetic information to synthesize the enzyme nitrogenase. Nitrogenase catalyzes the conversion of N_2 to NH_3 under ambient temperature and atmospheric pressure. This is contrasted to the commercial Haber-Bosch process that requires high temperatures and pressures (Havelka et al., 1982). Nitrogen-fixing organisms use light energy, directly or indirectly, to produce ammonia and, since manufacture occurs on site, distribution costs do not arise (Sprent, 1979).

The microorganisms that possess the ability to fix N_2 include bacteria and blue-green algae (cyanobacteria) that may be free living or symbiotic or that may form associative relationships with higher plants. Some may function in either mode. They range from strict anaerobes to microaerophiles to obligate aerobes; a few are photosynthetic (Havelka et al., 1982). According to Genetic Engineering News (Anonymous, 1989), an organism discovered by scientists at Boyce Tompson Institute and named Photorhizobium thompsonum is the first known symbiotic

bacterium that is both photosynthetic and nitrogen fixing.

The most well-known and most agriculturally important relationship is the root nodule-forming symbiosis between Rhizobium species and some legumes. The legume/rhizobial symbiosis was first discovered about 100 years ago (Havelka et al., 1982).

Symbiotic nitrogen fixation is a mutually beneficial metabolic process involving two partners. Legume/Rhizobium symbiosis provides bacteria of the genus Rhizobium with carbon, or energy, in the form of simple sugars which are translocated from their production site in the green parts of the plant through the phloem to the bacteria-containing nodules. Nitrogenous compounds from fixation in the nodules are made available to the plant after translocation through the xylem of the host plant. Nitrogenous compounds vary according to legume species, but include glutamine, asparagine, allantoin, ureides, and a variety of amino acids (Sprent, 1979). These compounds are directly or indirectly used for protein synthesis and the resultant growth and maintenance of the legume.

The first prerequistite for nitrogen fixation is the presence of genes to form the essential enzymes, while the final realization of the genetic potentialities depends on regulation phenomena, either with regard to induction or repression of enzyme formation, or to the activation and

regulation of enzyme activity (Quispel, 1974). Nitrogenases have been extracted from many diverse diazotrophs, representing the various physiological types (aerobes, anaerobes, microaerophilic bacteria, photosynthetic bacteria, blue-green algae, and nodule bacteria), and all are similar (Chen et al., 1973; Eady and Postgate, 1974). Symbiotic N_2-fixation is enhanced in legumes when effective and highly competitive strains of Rhizobium successfully nodulate host plants. Legume seed inoculation can be beneficial in soils in which the specific rhizobia are absent, or sparse, or where indigenous rhizobia are ineffective or submaximal in their N_2-fixing capacity (Vincent, 1974).

Legume/Rhizobium Symbiosis

Rhizobia are gram-negative rods, metabolically chemoorganotrophs that grow best at 25 to 30 C on complex media. They often contain poly-beta-hydroxybutrate storage granules. A general division of Rhizobium based on growth patterns into slow growers and fast growers has been recognized by Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). Fast growers lower the pH of a yeast mannitol agar and have a mean generation time of 2 to 4 hours. This is shorter than

slow growers (6 to 8 hours), which produce an alkaline endpoint on the same medium (Vincent, 1977). Fast growers include Rhizobium leguminosarum, Rhizobium trifolii, Rhizobium phaseoli, and Rhizobium meliloti. The slow growers include Rhizobium lupini and Rhizobium japonicum.

Another important classification of symbiotic diazotrophs is based on the host range and specificity. Approximately 19,700 species in the Leguminosae, the second largest family of seed plants, are arranged in about 750 genera with three subfamilies, the Mimosoideae, Caesalpinioideae, and Papilionoideae. Only 3100 of the species have been surveyed for the presence or absence of nodules (Allen and Allen 1981). Nodules form on about 90% of the surveyed species of Mimosoideae, 95% of which are woody species, and nearly all are of tropical origin. Only 30% of the surveyed species of Caesalpinioideae form nodules. Of these, 97% are woody species and almost exclusively of tropical origin. Nodules form on about 93% of the surveyed species of the subfamily Papilionoideae, which contains the major species of agronomic importance.

On the basis of normal host/diazotroph relationships, legumes have been commonly divided into seven infective or cross-inoculation groups. Groups of legumes that can be infected by a single Rhizobium species are classified in the same inoculation group. There are six crossinocula-

tion groups of single species (soybean-R. japonicum; lupin-R. lupini; clover-R. trifolii; peas and vetch-R. leguminosarum; bean-R. phaseoli; alfalfa-R. meliloti) and an additional seventh group, the cowpea miscellany (Havelka et al., 1982).

Rhizobium bacteria occur as free-living heterotrophs in soil, but are unable to independently fix atmospheric nitrogen. After they have infected the root hair, entered the root cortex, and been transformed into bacteroids, they are able to fix atmospheric nitrogen and exhibit true symbiosis. By definition, both the bacteria and the host legume benefit from this symbiotic relationship. Under most circumstances, neither the plant nor the rhizobia fixes N_2 individually. Rhizobium bacteroids are able to protect their nitrogen-fixing enzyme system from O_2 damage and create an O_2-free environment within the nodule where fixation is free to occur (Heichel, 1985). Nodulation does not normally occur in the field until the first leaves are unfolding and nitrogen fixation does not normally commence until the plant can safely divert a proportion of its photosynthate to this process (Sprent, 1979).

Rhizobia are generally present in soils. Nevertheless, inoculation usually is recommended to insure nodulation, and to provide large numbers of an effective N_2-fixing strain (Brill, 1977). Infection of the plant root

and production of a nodule does not guarantee vigorous N_2-fixation. A delicate balance governs an effective symbiosis between plant and bacteria. This is reflected in the phenomena of strain variation and host plant specificity (Burris, 1976). Plants can be nodulated, but the bacterial plant relationship can support only poor fixation in some instances or good fixation in others. These differences in effectiveness are poorly understood (Burris, 1976), but they are known to be dependent on the intrinsic properties of the partners, their mutual genetically controlled compatibility (Vincent, 1980), and the influence exercised by the environment on each separately and on their interaction (Vincent, 1980; Sprent, 1979).

Infection and Nodule Development

Prior to infection of a root hair, rhizobia must be present in the rhizosphere (Atlas and Bartha, 1987; Sprent, 1979). Rhizobial colonization of the root surface will generally be a necessary first step for both an indigenous population and an applied inoculum (Vincent, 1980). When a susceptible, leguminous plant and a compatible strain of rhizobia are brought together under conditions favorable for growth and infection, a nodule will form (Heichel, 1985).

The association between rhizobia and plant roots is very specific, and it has been the subject of a great deal of research. The initial communication between the bacterium and the plant appears to be the induction of Rhizobium nod gene expression by compound(s) secreted from the roots of legumes (Downie and Johnston, 1986; Zaat et al., 1988). A number of nod gene inducers have recently been isolated. These molecules, which can induce nod gene expression at very low concentrations (10 μM), include substituted flavone or flavonone molecules such as luteolin from alfalfa, 7,4'-dihydroxyl flavone from clover, and naringenin from peas and Vicia spp. (Downie and Johnston, 1986). In response to nod gene induction, the bacteria appear to synthesize an outer covering consisting of, for example, exopolysaccharide or lipopoly-saccharide to induce root hair curling. This altered bacterial surface may be recognized by a plant lectin. The mucigel at the top of the growing root provides a favorable site for rhizobial attachment (Atlas and Bartha, 1981; Bal et al., 1978; Brill, 1977; Dazzo et al., 1978; Postgate, 1982; Sprent, 1979; Vance, 1983, Vincent, 1982).

Upon recognizing the specific legume host, rhizobia enter the legume, in most cases, through root hairs or during the emergence of lateral roots, cause root hairs to become twisted, curled, or otherwise deformed (Newcomb,

1981), and grow within modified parts of the plant roots called nodules (Vincent, 1982). Infection may occur as early as 4 to 12 days after seed germination. The original infection rapidly develops into visible nodules 3 to 5 weeks after plants emerge, depending on the plant species and its growth rate.

Vincent (1974) summarized the steps in establishing the symbiosis, as follows: 1) colonization of the rhizosphere by rhizobia; 2) entrance of rhizobia via root hairs resulting in the formation of infection threads; 3) commencement of a persistent nodule meristem; 4) release of rhizobia from the infection thread; 5) multiplication of rhizobia within membrane envelopes of the nodule host cell; 6) conversion of rhizobia (motile rods) to nodule bacteroids (a non-motile form); 7) deposition of leghemoglobin (an oxygen binding, red pigment protein, which maintains the optimum oxygen concentration necessary for N_2 fixation) synthesized by the host in the membrane envelope; 8) establishment and continuance of a shared metabolism between plant and bacterium.

The type of nodulation which results in nitrogen fixation is called "effective" nodulation. Mature effective alfalfa nodules are large, elongated, often clustered on the primary roots, and have pink to red centers. The red color is attributed to leghemoglobin.

Hemoglobin is confined to those nodule cells that contain rhizobia and are fixing N_2. The change from hemoglobin (red) to choleglobin (green) indicates cessation of N_2 fixation and the onset of deterioration. Ineffective nodules are small, usually numerous, and are scattered over the entire root system. They have a white or pale green center (Burton, 1972).

Mechanism of Nitrogen Fixation

Industrial reduction of atmospheric N_2 to produce ammonia for fertilizer requires the transfer of hydrogen from natural gas to the N_2 molecule under high temperature (300-600 C) and pressures (20-80 M Pa). Plants achieve the reduction of N_2 at ambient temperatures and pressures with enzymes and energy from the oxidative phosphorylation of sugars produced by photosynthesis (Heichel, 1985).

The enzyme which catalyses N_2 fixation is nitrogenase. Nitrogenase consists of two components. The first contains molybdenum (Mo), iron (Fe), and sulfur (S) and is designated the Mo-Fe protein, Component I, or Protein 1. The second contains Fe and S, and is designated the Fe-protein, Component II, or Protein 2 (Brill, 1977; Goodwin and Mercer, 1983). A special characteristic of all nitrogenase systems is that both

protein components of the enzyme are denatured by contact with free molecular oxygen (O_2). In Rhizobium-legume symbioses, oxygen is trapped before it can reach the bacteria by an O_2-binding protein called leghemoglobin. As a result, Rhizobium can use an efficient aerobic metabolism while still protecting nitrogenase from O_2 (Brill, 1977).

The gaseous N_2 molecule combines with the Mo of the large component, while the small enzyme component gathers the electrons necessary for the reduction of N_2 to ammonia. Metabolic energy in the form of adenosine triphosphate (ATP) splits the triple bond of the N_2 molecule:

$$N{\equiv}N + 16ATP + 8e^- + 10H^+ \text{---> } 2NH_4^+ + 16ADP + 16P_i + H_2$$

Sixteen molecules of ATP are needed to split each N_2 molecule into two ions, and concurrently three electrons (e^-) are transferred to each of the two N ions formed. Two ammonium (NH_4^+) molecules are formed when the unpaired electrons on each N ion are balanced by hydrogen ions (H^+) from the interior of the bacteroid (Heichel, 1985).

Nitrogenase simultaneously reduces two H ions to H_2 (gas) during N_2 reduction in many legume-bacteria symbioses (Heichel, 1985; Postgate, 1982). While this concomitant reaction has been viewed by many to be wasteful, experimental data has shown some rhizobia capable of chemolithotrophic growth through the coupling

of the oxidation of this H_2 to ATP synthesis (Evans et al., 1987).

The ATP required to split the N_2 molecule and the electrons required for reduction of N_2 to ammonium are both produced during the respiration of photosynthate (sugars) in the mitochondria of the bacteroid. Fixation of 100 kg of N_2 as NH_3 requires the metabolic energy needed for production of 1500 kg of plant dry matter (LaRue and Patterson, 1981). While the energy cost of N_2 fixation is considerable, there is no direct evidence that N_2 fixation restricts legume productivity because of diversion of energy to the nodules. Non-N_2-fixing legumes adequately nourished with fertilizer N yield similarly to their N_2-fixing counterparts (Heichel, 1985).

Factors Affecting Nodulation and Nitrogen Fixation

There are many factors affecting nodulation and optimum N_2 fixation. These include: soil conditions, soil nutrient concentrations, other soil organisms, temperature effects, and light quality and quantity (Streeter and Hannaway, 1988). Since N_2 fixation is an energy-intensive process, it is apparent that factors controlling rates of photosynthesis or the distribution of photosynthate in the

plant will significantly affect N_2 fixation (Havelka et al., 1982).

Photosynthesis

A number of investigations have shown that nodules can indeed act as sinks for newly formed photosynthate from the leaves. Small and Leonard (1969), for example, have shown that functional nodules are active sinks for newly formed photosynthate from the tops of pea and subterranean clover plants.

In soybeans, Lawn and Brun (1974) reported that acetylene reduction activity increased in both 'Chippewa 64' and 'Clay' during the flowering period, reached a maximum near the end of flowering, and declined markedly during the early podfilling stage. The activity decline was due to a decline in the specific activity of the nodules, and occurred immediately prior to the time when the growth rate of the pods (including seed) exceeded that of the total plant tops. Treatments designed to enhance the photosynthetic source/sink ratio (supplemental light and depodding) maintained nodule activity well above the control in both varieties. Conversely, treatments designed to reduce the source/sink ratio (shading and defoliation) decreased nodule activity below the level of

the control. Treatment effects on total plant protein recovered at maturity closely reflected the treatment effects on total nodule activity. The results of their study were interpreted as evidence that symbiotic nitrogen fixation in these varieties declined during podfilling as the result of inadequate assimilate supply to the nodules.

Schweitzer and Harper (1985) also reported that root nodule fresh weight and acetylene reduction activity declined slightly more rapidly for fertile lines than for male-sterile lines in both years, with differences significant on the last two to three sampling dates as leaf loss occurred in the control plants.

Wheeler (1971) found a distinct diurnal variation in nitrogen fixation by nodulated Alnus glutinosa. The time of the mid-day peak in N_2 fixation corresponded well with the time of the maximum influx of new photosynthate into the nodules.

Ching et al. (1975) showed that acetylene reduction by 'Chippewa' soybean roots was reduced 50 percent after one day of exposure to darkness. They attributed this decrease to a lack of photosynthate. The treatment caused nodule sucrose to decrease by 60 percent, nodule ATP content by 70 percent, and the ATP/ADP ratio by 44 percent.

Minchin and Pate (1973) have developed carbon and nitrogen budgets of the shoot, root, and nodules of garden

pea plants over a nine-day interval just prior to flowering. Almost half of the incoming carbon is respired into the soil and the remainder is partitioned between shoot, root, and nodule growth in the ratio of 41:7:5.

When soybean pods were removed in another kind of source-sink manipulation experiment, the decline in nodule activity was delayed but total plant and protein yield did not change significantly (Lawn and Brun, 1974).

Crafts-Brandener et al. (1984), conducted field studies in 1981 and 1982 to ascertain the effect of pod removal on senescence of nodulating and non-nodulating isolines of soybean plants. Their test hypothesis was that nodules act as a nitrogen source and a carbohydrate sink which would prevent or delay senescence in the absence of pods. The results indicated that senescence patterns of soybean plants were the same for nodulated and non-nodulated plants, and that pods did not control the initiation of senescence, but rather altered only the partitioning of plant constituents and the visual manifestations of senescence.

A previous study on dry matter and nitrogen accumulation in male-sterile and male-fertile soybeans done by Burton and his co-workers in 1979, also concluded that the retention of green leaves and the resultant increased carbohydrate supply to the roots may not increase N_2 fixa-

tion in the absence of a strong sink in pods.

In addition to sink competition between root nodules and developing seeds, decreases in the photosynthetic capacity of the leaf canopy also contribute to the late season loss of root nodule AR activity as leaves complete monocarpic senescence (Schweitzer and Harper, 1984). The loss of foliar protein during late podfill places restrictions on photosynthetic potential since RuBP carboxylase constitutes 40% (Wittenbach, 1982) to 50% (Ogren, 1976) of total foliar protein. During seed development, demands for nitrogen must also be met. Huber and Israel (1982) reported that nodulation of some soybean cultivars increased photosynthetic sucrose formation at the expense of starch accumulation. In 1983, Huber et al. studied the effect of nodulation on photosynthesis and carbon partitioning in leaves. They also found that nodulated plants exhibited increased sucrose formation as compared to nitrate-dependent plants.

These experiments support the conclusion that current photosynthate is the most limiting factor in rates of biological nitrogen fixation.

Temperature

Temperature effects on N_2 fixation may be manifested

through either root or shoot effects (Havelka et al., 1982). Low root temperature of both temperate and tropical legumes causes a greater degree of nodule development. This could compensate for the lower specific N_2-fixing activity per unit of tissue at low temperatures. With Trifolium subterraneum, there was a proportionately higher increase in total dry weight and total nitrogen in the nodules when plants were growing at 8 C relative to those at 15 and 22 C.

Low soil temperatures retard root hair infection and nodule development and dinitrogen fixation. The threshold temperature for nodulation varies with species and bacterial strain, but with temperate legumes generally falls in the range of 7-10 C. With soybeans and many tropical species, this minimum temperature is 15-18 C (Gibson, 1976a, 1976b).

Rhizobium species typically grow and fix nitrogen best at 25 to 30 C (Vincent, 1970), although there is considerable variation among species. Alfalfa strains have an optimum temperature between 15 and 30 C, and a sharp drop in activity below 12 C and above 35 C (Burton, 1972). Limited survival of Rhizobium is observed at extremely high temperature (above 38 C) (Eaglesham and Ayanaba, 1984).

Soil Conditions

Soil pH

Soil pH is one of the most important external factors affecting the nitrogen fixing symbiosis. Low soil pH is inhibitory to root hair infection and early nodulation in peas and lucerne. Low soil pH may also reduce the supply of nutrients required for nodulation (Havelka et al., 1982). Poor survival of rhizobia has been reported under acid conditions with lupin bacteria being the most acid-tolerant and those of alfalfa the most acid-sensitive (Burton, 1972).

Soil moisture

Rhizobium achieve optimal N_2 fixation at soil moisture levels that are most beneficial to the host plant, usually at or near field capacity (Graham, 1984). The soil must be moist enough to meet the needs of both the plant and the rhizobia, yet not so moist as to inhibit nodule respiration. Since rhizobia are aerobic organisms, the anoxic condition of flooded soils has a detrimental affect on their survival, although there is variation in tolerance among species (Eaglesham and Ayanaba, 1984). It has been demonstrated (Graham, 1984) that flooding not only reduces fixation, but in plant species intolerant of

flooded conditions, nodules may be shed after only 1 to 2 days of inundation.

Nodule activity is affected by solutes in a variety of ways. Primarily, if the concentration is high enough, water can be withdrawn osmotically from nodules.

Nutritional Factors and Soil Nitrate

Nutritional factors also affect N_2 fixation through their affect on either the host or the rhizobia. The fact that nitrate and nitrite ions inhibit nodule formation is well documented (Atlas and Bartha, 1987; Burns and Hardy, 1975; Lang and Collins, 1981).

Molybdenum (Mo) deficiency is commonly the most important trace element deficiency. Work by Payne (1973) and Notton (1983) showed Mo to be a component of a cytochrome necessary for the respiration of certain bacteria under anaerobic conditions which enable the organism to use nitrate as a terminal oxidant in place of O_2. Mo is well known as a component of the Mo-Fe protein complex of nitrogenase and is essential for its activity (Mengel and Kirby, 1982).

Cobalt deficiency resulted in lowered bacteroid densities within the nodules and a 50 to 80% decrease in acetylene reduction activity (Dilworth et al., 1979).

Klueds et al. (1983) also found that trace amounts of nickel are necessary for hydrogen-dependent growth of R. japonicum and for urease activity expression in soybean leaves.

Calcium (Ca) deficiency and excess aluminum (Al) and manganese (Mn) tend to occur together in soils and to interact in their affect on nodulation and plant growth (Munns, 1977). Calcium is required in greater amounts for nodule function than for plant metabolism (Robson, 1978). Calcium also moderates toxic effects of manganous ions in leguminous plants (Robson and Loneragan, 1970).

Specific roles in the nodule are amply established for phosphorus (P), as a constituent of nucleotides, and its impact on nodulation, nitrogen fixation, and growth; for sulfur (S) as a constituent of the Fe-S proteins; and for potassium (K) for its osmotic regulation and enzyme activation (Evans and Russell, 1971; Evans and Sorger, 1969; Epstein, 1972). Potassium may greatly stimulate nodule activity, possibly by improving carbohydrate supplies (Sprent, 1979; Collins and Duke, 1981).

Munns (1977) reported that nodule formation is prevented by boron (B) deficiency, but it is affected little and inconsistently by deficiencies of the other micronutrients unless the deficiency is severe enough to injure several other phases of the symbiosis.

Copper (Cu) is required in greater amounts for nodule function than for plant metabolism (Robson, 1978). Iron (Fe) is a constituent of leghemoglobin, which is important for nodule function. In the Fe-S form, Fe is intimately involved as a constituent of both components of nitrogenase and of a bacterial ferredoxin which may function as a reductant of nitrogenase (Bergersen, 1971). Sulfur deficiency is known to retard protein synthesis, and as a consequence adversely affects both nodulation and N_2-fixation of legumes (Adams and Sheard, 1966; Smith, 1982; Zaroug and Munns, 1979).

Other Soil Organisms

Agrobacteria share with some rhizobia the capacity for fast growth, utilization of a wide range of carbon sources, and acid production (Vincent, 1974). Agrobacterium rhizogenes populations have been found which are capable of producing organic chemicals which stimulate the growth of root hairs and other plant tissues (Atlas and Bartha, 1987). This increase in root hairs could increase the number of potential infection sites for rhizobia.

Another indirect and beneficial relationship exists between Rhizobium and mycorrhizal root fungi which form mutualistic relationships with plants. Mycorrhizal

associations occur with many plant species, including most agricultural crops. In this association, the mycelium of the fungi forms a loose network in the soil and functions as an extension of the root hairs, resulting in increased uptake of phosphate and other ions (Atlas and Bartha, 1987). The improved phosphorus nutrition results in the enhancement of nitrogen nutrition in mycorrhizal legumes. Symbiotic N fixation by rhizobia in legume nodules has a high P requirement (Mosse, 1981).

In addition to beneficial relationships, there are antagonistic relationships among soil organisms. For example, Bdellovibrio species are parasitic on Rhizobium and some bacteriophages (bacterial viruses) have been isolated and found to be specific for Rhizobium (Atlas and Bartha, 1981).

Techniques for Estimating Nitrogen Fixation

Indirect Methods

Acetylene Reduction Assay

The acetylene reduction assay arose from the independent observations of Dilworth (1966) and Schollhorn and Burris (1966) that the nitrogen-fixing enzyme, nitrogen-

ase, reduced acetylene to ethylene. The theoretical relationship between acetylene reduction and N_2 fixation dictates that 3 moles of C_2H_2 are reduced for each mole of N_2 fixed. Since that time, the reliability of acetylene reduction as an indicator of nitrogenase activity has been established for a wide range of biological systems and the technique has assumed a vital role in N_2 fixation studies (Hardy et al., 1968, 1973). The development of the acetylene reduction (AR) assay as an index of dinitrogen fixation (N_2-fixation) has permitted substantial progress in laboratory and controlled environmental investigations, providing a means of estimating the contribution of fixed N_2 by many systems (Emerich and Evans, 1980; Evans and Barber, 1977; Hardy et al., 1975; Heichel et al., 1981; Westermann and Kolar, 1978). Alfalfa in particular has been the subject of many investigations utilizing the acetylene reduction assay (Collins and Duke, 1981; Duke and Doehlert, 1981; Duke et al., 1980; Eardly et al., 1985; Hardarson et al., 1981; Hoffman and Melton, 1981; Tan, 1981).

For the acetylene reduction assay, nodules, soil containing nodules or whole plant-soil systems are enclosed in gas-tight containers and exposed to an atmosphere containing acetylene. The atmosphere is sampled repeatedly after a suitable incubation period (0.5 to 24 h depend-

ing on the system) and analysed for ethylene using gas-liquid chromatography with a flame ionization detector (Vincent, 1982).

Acetylene reduction is a short term, kinetic measurement. The existence of diurnal and seasonal variations in N_2 fixation and plant cultivar-rhizobial strain specificity in expression of H_2 evolution makes the extrapolation to total N_2 fixed over a growing season questionable (Rennie, 1985). This technique should be used for ranking of treatments rather than quantitative estimates of fixation.

Direct Methods

Nitrogen Balance Sheets

A plant may have two or three sources of N (atmosphere, soil, and perhaps fertilizer). The nitrogen balance sheet method, used extensively to estimate N_2 fixation (LaRue and Patterson, 1981), is based on the assumption that the fixing system (fs) and non-fixing system (nfs) assimilate identical amounts of soil and fertilizer N. Thus,

%Ndfa (% nitrogen (plant) derived from the atmosphere)

$$= \frac{\text{N yield (fs)} - \text{N yield (nfs)}}{\text{N yield (fs)}} * 100$$

If the N yield of the fs exceeds that of the nfs, the difference is attributed to N_2 fixation.

This technique depends upon the ability of the resercher to match growth rates, and to ensure insects, diseases, and other factors known to affect plant growth are similar in both crops (Vincent, 1982).

^{15}N Methods

^{15}N is a stable isotope of nitrogen occurring naturally in atmospheric N at 0.366%. Being stable, it poses none of the health hazards associated with radioactive isotopes and is suitable for long-term studies. It has the disadvantage that methods for detection and determination of ^{15}N are complicated and time-consuming and require expensive instruments which are difficult to operate and maintain. The isotope is expensive but, except in field situations, only small quantities are needed, and the cost compared with the cost of the analytical instruments is small (Vincent, 1982).

Three techniques that employ ^{15}N are commonly used for assaying N fixation. The first and most popular

involves exposure of the N-fixing system under study to $^{15}N_2$ in a gas-tight container. This is a relatively simple procedure when studying free-living soil organisms but is a complex problem when fixation by whole plants is to be measured. This is because the atmosphere within the sealed container must be controlled within close tolerance limits. These systems tend to be expensive since they must be gas-tight, and must be capable of precise monitoring and control of O_2, CO_2, and N_2 levels in the atmosphere. Adequate temperature control is also important (Vincent, 1982).

The second technique, isotope dilution, involves labeling the soil N with ^{15}N. By comparing the depletion of ^{15}N in the legume with that in a non-fixing control plant (non-nodulating legume or non-legume), an estimate of the amount of N fixed can be made (Heichel et al., 1981; Vincent, 1982).

The third isotope technique measures the variation in natural abundance caused by discrimination during N fixation in favor of the lighter (^{14}N) isotope. The very small deviations in abundance can be detected on sensitive mass spectrometers (Kohl et al., 1980). This technique is not recommended for routine use (Vincent, 1982).

III. EVALUATION OF *AGROBACTERIUM RHIZOGENES* FOR ROOT PROLIFERATION OF SEVERAL FORAGE LEGUMES

Abstract

Agrobacterium rhizogenes has been shown to cause root proliferation and increased water use efficiency in certain woody dicots. Root proliferation may be desirable in herbaceous legumes as a means of increasing potential sites for infection by *Rhizobium* species and subsequent nodulation and nitrogen fixation. Thus, *A. rhizogenes* was used to inoculate a series of forage legumes to evaluate its ability to stimulate root activity. Growth tubes and nutrient solution were used in growth chamber experiments. Plants were evaluated for root development six weeks after inoculation. Alfalfa treated with *A. rhizogenes* 232 produced significantly (159%) greater root number, twice the root volume, 183% greater root fresh weight, and 214% more root dry weight than the uninoculated treatment. In white clover and field pea, *A.* rhizogenes 232 did not produce significantly increased root number or root mass. This experiment indicated that *A. rhizogenes* was effective in stimulating increased root initiating activity in alfalfa.

Introduction

Agrobacterium rhizogenes is a soil inhabiting microorganism that occasionally enters plant roots via wounds or natural openings. This may result in a proliferation of secondary roots leading to "hairy root syndrome" (Hildebrand, 1934; Riker, 1930). This is in contrast to Agrobacterium tumefaciens, which causes gall or tumor formation on its hosts. The factor responsible for the genetic transformation is a bacterial plasmid (Ri) which is positively correlated with the infectivity of A. rhizogenes (Chilton et al., 1982; Moore et al., 1979; White and Nester, 1980). Although Agrobacterium rhizogenes has a wide host range, it seems to be strictly confined to dicotyledonous plants (Strobel and Nachmias, 1985).

Moore et al. (1979) reported that apple trees possessing a form of non-infectious "hairy root" had greater root growth and seemed to possess better drought tolerance than those not treated with Agrobacterium rhizogenes. The proliferation of roots may be favorable to the establishment, survival, and reproduction of some plant species, especially those growing in areas of limited rainfall (Moore et al., 1979; Strobel and Mathre, 1982). Plants that require especially rapid root growth

and development are those that are developing nodules in preparation for biological nitrogen fixation (BNF). Strobel et al. (1985) reported increased nodulation in the host after introducing the hairy root plasmid into Rhizobium meliloti.

Legumes have long been valued for their capacity to be at least partly nitrogen self-sufficient. The degree to which forage legumes are N self-sufficient may be improved by various agronomic practices. Inoculation with Rhizobium spp. is now a common practice used to assure high numbers of highly effective nodule forming bacteria on legume roots. The number of nodules formed, however, is often dependent on the formation of infection sites.

Objective

The objective of this study was to evaluate the ability of Agrobacterium rhizogenes to stimulate greater root growth and increased infection sites leading to potentially greater nodulation and enhanced BNF in forage legumes.

Materials and Methods

Bacterial Strains and Culture Conditions

The strains of Agrobacterium rhizogenes 232 and A. radiobacter NT1 were provided by G.A. Strobel of Montana State University. Strains were received on agar slants and grown in yeast extract mannitol broth/agar (Vincent, 1970).

Species of Legumes Selected for Study

The three species of legumes evaluated were alfalfa (Medicago sativa), white clover (Trifolium repens), and field pea (Pisum sativa subsp. arvense). Seeds were provided by the Seed Lab, Crop Science Department, Oregon State University.

Plant Growth and Analyses

Seeds of legumes were surface sterilized with 95% ethanol and 1% sodium hypochlorite (Chlorox) and rinsed in sterile distilled H_2O (Vincent, 1970). Seeds were then planted directly into the water agar in petri dishes. Petri dishes were inverted to provide seedlings with

uniform straight roots. Germinated seedlings were transferred to nutrient agar slants under laminar flow hood conditions. After growing bacteria in yeast extract mannitol to a concentration of approximmately 10^7 cells per ml, bacterial inoculum solution (1 ml) was added with a pipette. Plastic caps were placed over tubes to allow for elongation of the hypocotyl under dark conditions. When cotyledons were above the lip of the tube, sterile 1/5 strength Jensen's solution (containing 0.5 g/l KNO_3) was added to within 12 mm of the lip. A needle was used to make a small hole in foil caps (big enough for the cotyledons to fit through). After removing the plastic cap from the tube, the foil cap was carefully pulled down over the seedling and onto the tube. A small amount of cotton was placed around the seedling to prevent contamination. Each tube was covered with a plastic bag to prevent desiccation. Roots were protected from the light by wrapping each tube with foil. The tubes were placed in open racks in the growth chamber. The racks and the chamber had been thoroughly scrubbed with Chlorox followed by 75% ethanol. Plants received a 14/10 hour light/dark period and a 20/16 C temperature regime. Light intensity was 330 μmol m^{-2} s^{-1}. Root number, root volume, and root fresh and dry weight were measured 6 weeks after seedling transfer. Each species of legume received three treat-

ments (inoculated with A. rhizogenes 232, inoculated with A. radiobacter NT1, and uninoculated), and each treatment was replicated 4 times. Analysis of variance procedures were used to evaluate treatment effects.

Results

Alfalfa

Root number

In alfalfa, root number was significantly greater with the 232 treatment while the uninoculated treatment had the fewest roots (Figure III.1). Strains 232 and NT1 produced 159.1% and 36.4% greater root number than the uninoculated treatment, respectively. Root number mean values are shown in Table III.1.

Root volume

Root volume was also significantly higher with the 232 treatment, producing three times the root volume of the uninoculated treatment (Figure III.2). Root volume with the NT1 treatment was 150% greater than the uninoculated treatment. Root volume mean values are shown in Table III.1.

Root fresh weight and dry weight

The 232 treatment also resulted in significantly higher root fresh weight and dry weight (Figure III.3 and III.4). The fresh weight of the 232 and NT1 treatments were 182.7% and 80.6% more than the uninoculated

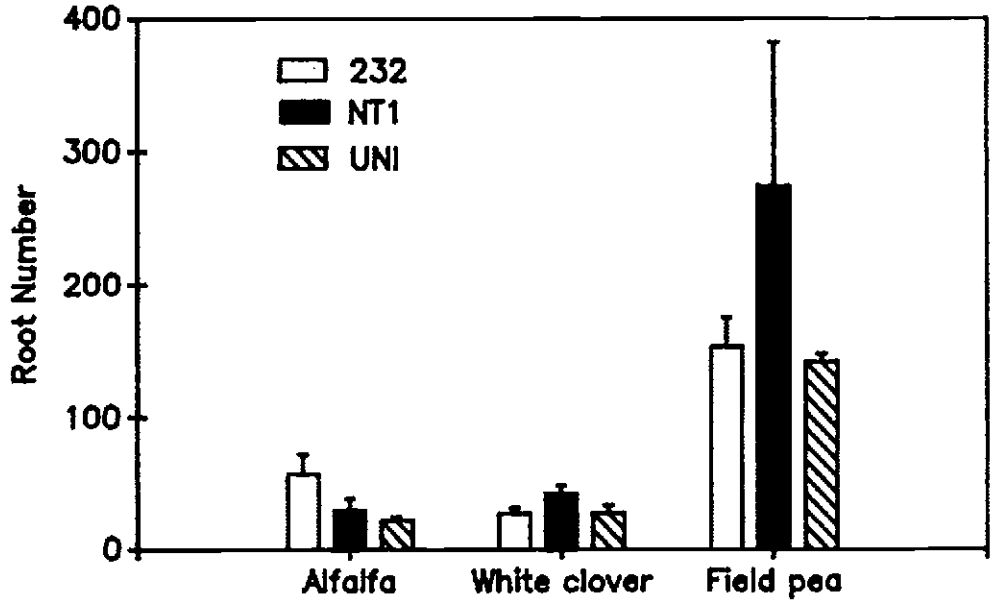

Figure III.1. Mean root number and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

Table III.1. Mean root number, volume (ml), fresh weight and dry weight (mg) per plant of 42 day-old alfalfa plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

Treatment	Number	Volume(ml)	Fresh Weight (mg)	Dry Weight (mg)
232	57a	0.18a	134.3a	11.0a
NT1	30ab	0.15ab	85.8ab	9.3a
UNI	22b	0.06b	47.5b	3.5a
LSD	33	0.11	82.95	8.39

Means followed by the same letter are not significantly different at the .05 probability level.

treatment; the dry weight of the 232 and NT1 treatments were 214.3% and 165.7% greater than the uninoculated treatment. Root fresh weight and dry weight mean values are shown in Table III.1.

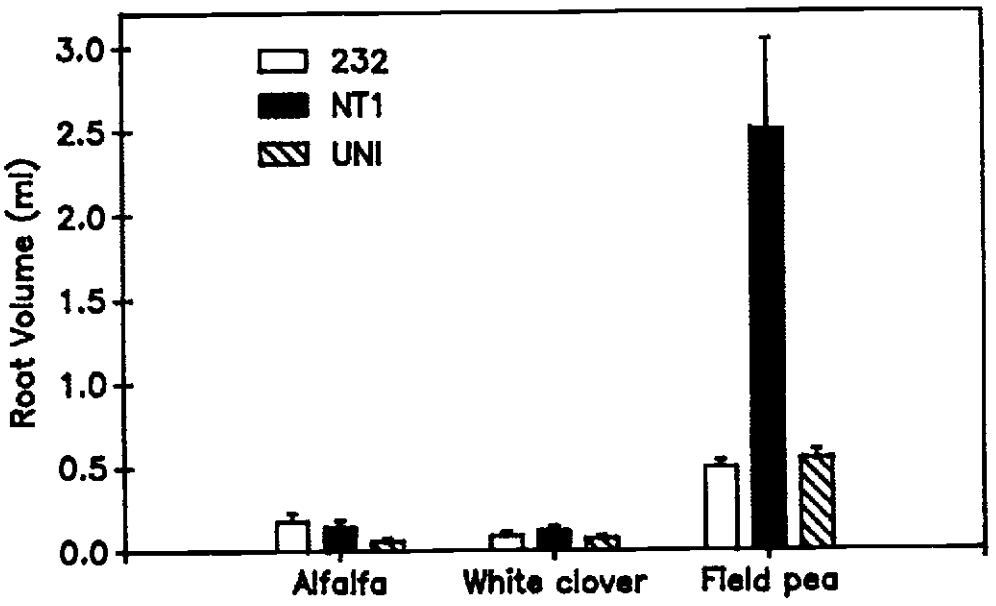

Figure III.2. Mean root volume and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

Figure III.3. Mean root fresh weight and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

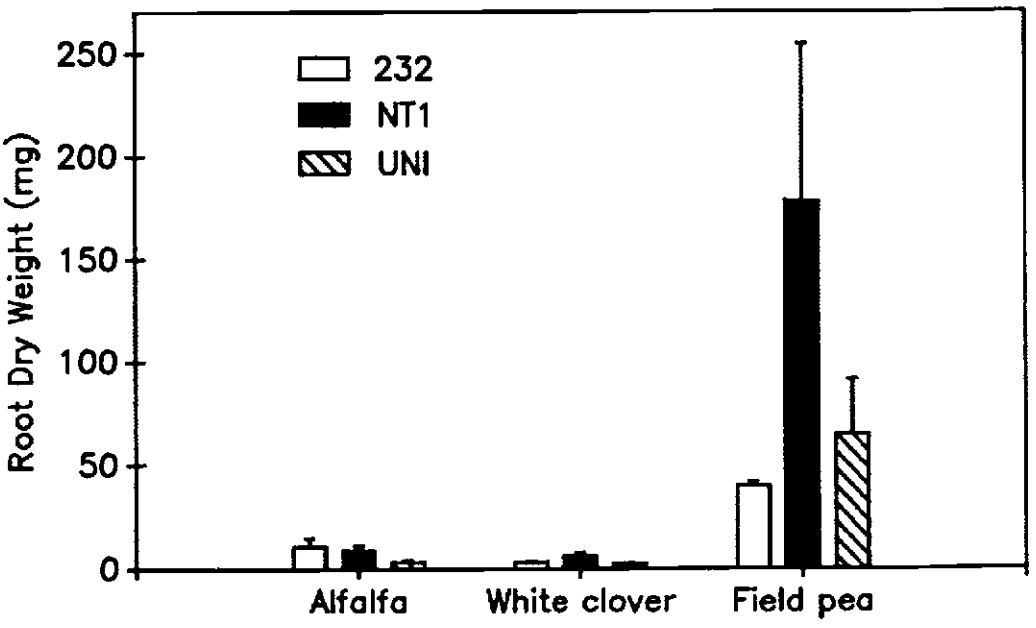

Figure III.4. Mean root dry weight and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

White Clover

Root number

Root number in white clover was highest with the NT1 treatment, producing 53.6% more roots than the uninoculated treatment (Figure III.1); however, the difference was not significant. There was no significant difference in root number between strain 232 and the uninoculated treatments. Root number mean values are shown in Table III.2.

Table III.2. Mean root number, volume (ml), fresh weight and dry weight (mg) per plant of 42 day-old white clover plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

Treatment	Number	Volume(ml)	Fresh Weight (mg)	Dry Weight (mg)
232	27a	0.09a	40.75a	3.25a
NT1	43a	0.13a	72.75b	6.50b
UNI	28a	0.08a	32.50a	2.50a
LSD	18	0.07	30.26	2.43

Means followed by the same letter are not significantly different at the .05 probability level.

Root volume

Root volume was also greatest with the NT1 treatment, which yielded 62.5% greater volume than the uninoculated

treatment (Figure III.2). The 232 treatment resulted in 12.5% more root volume than the uninoculated treatment. Root volume mean values are shown in Table III.2.

Root fresh weight and dry weight

Significant increases in root fresh weight (123.8%) and dry weight (160%) were observed with the NT1 treatment relative to the uninoculated plants. No significant differences in root weight were observed between the 232 and the uninoculated treatments (Figure III.3 and III.4). Root fresh weight and dry weight mean values are shown in Table III.2.

Field Pea

Root number

Root number was greatest with the NT1 treatment, in which 93.7% more roots were observed compared to the uninoculated treatment (Figure III.1). High variability in the values, however, resulted in no significant differences. Strain 232 and uninoculated plants had similar root number values. Root number mean values are shown in Table III.3.

Table III.3. Mean root number, volume (ml), fresh weight and dry weight (mg) per plant of 42 day-old field pea plants as affected by different treatments. Treatments consisted of inoculation with A. rhizogenes 232, A. radiobacter NT1, and an uninoculated treatment (UNI).

Treatment	Number	Volume(ml)	Fresh Weight (mg)	Dry Weight (mg)
232	153a	0.50a	597.50a	39.75a
NT1	275a	2.50b	1464.00a	178.50a
UNI	142a	0.55a	757.25a	64.75a
LSD	203	0.99	986.11	148.67

Means followed by the same letter are not significantly different at the .05 probability level.

Root volume

Root volume was significantly higher with the NT1 treatment, producing 354.5% more than the uninoculated treatment. There was no significant difference, however, between the 232 and uninoculated treatments (Figure III.2). Root volume mean values are shown in Table III.3.

Root fresh weight and dry weight

Greater root fresh weight (93.3%) and dry weight (175.7%), were observed with the NT1 treatment. No significant differences were obtained, however, due to high variability in root weight values (Figure III.3 and III.4). Root fresh weight and dry weight mean values are shown in Table III.3.

Discussion

The effect of A. rhizogenes 232 on rooting of three forage legumes was tested in a short term (42 days) growth chamber experiment. In alfalfa, significantly increased root number and root mass were observed. This shows that root proliferation (root mass) may have increased due to the genetic transformation of the roots via the Ri plasmid carried by A. rhizogenes 232 (as previously shown by Moore et al. (1979), Chilton et al. (1982), Strobel and Nachmias (1985)). In contrast, white clover and field pea root characteristics were not significantly affected by inoculation with A. rhizogenes 232. These results suggest that roots of these species may need to be wounded for effective infection by A. rhizogenes 232 (Strobel et al., 1985). A. radiobacter NT1 increased root number and root mass in field pea. NT1 does not contain the Ri plasmid and these root effects were not anticipated.

Root proliferating organisms did not cause pathogenic response in any the legumes tested, information not previously reported. This indicates that A. rhizogenes 232 is not an important plant pathogen in forage legumes.

The positive effects of A. rhizogenes 232 on the initial growth of alfalfa roots suggests that future experiments should be conducted to further evaluate A.

rhizogenes' ability to stimulate root activity and the effectiveness of a Rhizobium meliloti transconjugant (pRi) in increasing nodulation and biological nitrogen fixation.

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IV. EVALUATION OF A *RHIZOBIUM MELILOTI* TRANSCONJUGANT FOR INCREASED NODULATION AND BIOLOGICAL NITROGEN FIXATION IN ALFALFA

Abstract

Rhizobium meliloti transconjugant BL105-9 was used to inoculate two alfalfa (*Medicago sativa*) cultivars (Ladak 65 and Vernema) to evaluate its effectiveness for increased nodulation and biological nitrogen fixation. Strain effectiveness was determined following six weeks in plant tubes containing nutrient agar in a growth chamber experiment. The results showed that the *R. meliloti* transconjugant (BL105-9) increased root number in both cultivars. *R. meliloti* transconjugant (BL105-9) did not, however, produce significantly more root and shoot mass than the *R. meliloti* parent (BL116). Treatment with the transconjugant produced significantly (72.8%) greater nodule numbers and 99.2% more nodule dry weight than the parent (BL116). No significant difference in either total plant nitrogen level or acetylene reduction rate was observed between the *R. meliloti* transconjugant and parent strains. Thus, this experiment indicates that introduction of the hairy root plasmid into *R. meliloti* resulted in increased nodulation of alfalfa. Under these

specific growing conditions, however, the increased nodulation was not translated into increased total plant nitrogen or increased dry matter production.

Introduction

Forages play a vital role in the production of milk, meat, and wool in agriculture worldwide. In oregon, cattle and calves consistently rank first in agricultural production, with forages contributing largely to the rations of these animals. Alfalfa hay represents the largest hay crop in the state, vying for leadership with wheat and potatoes for the highest crop value in the state.

The common objective of forage workers is to establish a stable and profitable forage livestock sector in the agricultural economy through intensified forage production and utilization at the maximum economic level for each given land resource. One of the ways this objective can be achieved is through utilizing the special relationship that forage legumes have with Rhizobium bacteria, the symbiotic relationship of N_2 fixation. By improving nitrogen fixation, fewer of the variable expenses needed to produce feed and fiber through forages will be spent on fertilizer nitrogen, thus improving the economic position of farmers.

Legumes have long been valued for their capacity to be at least partly nitrogen self-sufficient (Evans, 1969). The degree to which forage legumes are N self-sufficient

may be improved by various agronomic practices. Inoculation with Rhizobium spp. is now a common practice used to assure high numbers of highly effective nodule forming bacteria on legume roots. The number of nodules formed, however, is often dependent on the formation of infection sites (Andrew and Robin, 1969a, 1969b; Smith et al., 1981).

Perhaps one means to increase the number of infection sites is through the use of the plasmid pRi from Agrobacterium rhizogenes which is responsible for the induction of the hairy root syndrome in dicotyledonous (Chilton et al., 1982; Moor et al., 1979; White and Nester, 1980). The Rhizobium meliloti (pRi) transconjugant is formed by introducing the Ri plasmid from A. rhizogenes into R. meliloti to stimulate greater root activity, increased infection sites and nodulation, and enhanced biological nitrogen fixation (Strobel et al., 1985).

Objective

The objective of this study was to evaluate the potential of a Rhizobium meliloti (Ri) transconjugant (BL105-9) for improving nodulation and biological nitrogen fixation of alfalfa.

Material and Methods

Cultivars Selected for Study

Ladak 65 and Vernema were the cultivars selected for study. Ladak 65 is the cultivar previously used for evaluating transconjugants (Strobel, 1985). Vernema is a recently released cultivar (Peaden, 1983) with resistance to verticillium wilt and frequently used in new alfalfa plantings in the Pacific Northwest.

Seed Source

Ladak 65 was obtained from G.A. Strobel, Montana State University, Bozeman, MT. Vernema was obtained from R.N. Peaden, USDA-ARS, Prosser, WA.

Seed Sterilization

Using a laminar flow hood, seeds were rinsed with 95% ethanol (15 seconds) and immersed for 8-10 minutes in 1% sodium hypochlorite. Seeds were then washed thoroughly with eight changes of sterile water.

Bacterial Strains and Culture Conditions

Rhizobium meliloti parent (BL116) and R. meliloti (pRi) transconjugant (BL105-9) strains were provided by Dr. Strobel. They were grown in yeast extract/mannitol (YM) medium. All cultures of bacteria were started from single colonies.

Seed Transfer and Inoculation

The seeds receiving treatment were thoroughly coated with bacteria by placing them onto a colony of the appropriate organism on the surface of a YM agar plate. Each seed received about 10^6 bacterial cells (Strobel, 1985). Two seeds were planted on the surface of the growth medium in each tube (25 X 240 mm) containing 60 ml of Jensen's nutrient solution plus 0.8% agar. Prior to cooling of the agar solution, tubes were inclined to create a long growing surface. For N treatments, 0.5 g/l NH_4NO_3 was added to the Jensen's solution. To assure rapid bacterial growth, the growth media of Rhizobium treatment received an additional 4% yeast extract mannitol to serve as a nutrient source.

Seedlings were capped with Kaputs (Belco Glass Co.), and kept in the dark for 2 days to allow for stem etio-

lation. Following etiolation, caps were removed and foil caps (with a hole large enough to fit over the cotyledons) were placed on the tubes and secured with rubber bands. The area around the stem in the holes was carefully filled with loose cotton. Each tube was covered with a chemically sterilized plastic bag to prevent desiccation. Treatments and replications were labeled, randomly placed in tube racks, and transferred to growth chambers (Vincent, 1970).

Growth Chamber Conditions

Seedlings were grown in a growth chamber (Conviron, model E8VH) under a 16 hour day cycle with a light intensity of 300 μmol m^{-2} s^{-1} and 25/18 C day/night temperature regime (Lopez-Jurado and Hannaway, 1985).

Analytical Procedures and Measurements

Acetylene Reduction

Acetylene reduction was evaluated following a 6 week growing period. Nodulated plants had attained a size and robust nature indicative of successful nitrogen fixation. Roots of intact plants were subjected to a 10% acetylene

concentration with 5 cc samples withdrawn at 30, 60, and 90 minute intervals. During this procedure, the plants were left in the temperature and light conditions under which they have been growing (Streeter and Hannaway, 1988).

Chambers for Acetylene Reduction

Acetylene reduction analyses were performed in polyvinyl chloride (pvc) pipe chambers of 40 mm inside diameter which were cut to a length of 230 mm. Each chamber accommodated 250 cc of air (Monaco et al. 1981). A #9 (45 mm top, 37 mm bottom, 25 mm long) rubber stopper was placed in one end of the pipe. The stem of a plant, just above the root, was wrapped with kneaded rubber and placed in an 8 mm hole cut in the center of a second #9 stopper. This stopper, split from the center out with a razor blade, was placed in the other end of the pipe. A thin sheet of kneaded rubber was placed in the split to prevent gas leakage. Acetylene was injected into the chamber through a rubber septum secured in a hole drilled into the side of the pvc pipe.

Gas Chromatography

Samples drawn during incubation with acetylene were injected into a gas chromatograph (model 5830A, HP) for the quantification of ethylene produced. Linear regression was used to calculate the acetylene reduction activity (nmole ethylene evolved per mg nodule dry weight per hour).

Root Number, Root Volume, Nodule Number, and Dry Weight of Plants and Nodules

Root numbers per plant were counted and root volumes were measured by water displacement after acetylene reduction. Roots, shoots, and nodules for each plant were dried and weighed. Nodules were counted when removed from roots and weighed separately following drying.

Plant Nitrogen

Roots and shoots for each tube were ground and analyzed for total plant nitrogen at the Plant Analysis Laboratory of Oregon State University.

Experimental Design and Statistical Analyses

Plants were arranged in a completely randomized design. Each cultivar was subjected to 4 treatments (*Rhizobium meliloti* parent, *Rhizobium meliloti* transconjugant, uninoculated + N, and uninoculated) with 8 replications. Analyses of variance were used to evaluate treatment and cultivar effects. Separation of treatment means was performed using Fisher's protected LSD value.

Results

Root number

Root number was highest for the +N treatment in both cultivars, being significantly greater than the transconjugant strain, which was greater than the parent strain (Figure IV.1). The uninoculated treatment had the fewest number of roots. For the BL116 treatment, Vernema had a significantly higher root number value than Ladak 65. Means of root number for the two cultivars at different treatment are shown in Table IV.1.

Root volume

The +N treatment produced significantly larger root volumes than other treatments (Figure IV.2). There were no significant differences between inoculum treatments or between cultivars. The R. meliloti transconjugant (BL105-9) and R. meliloti parent (BL116) exhibited a significantly larger volume than the uninoculated treatment. Mean root volume values for the different treatments are shown in Table IV.2.

Root dry weight

Treatment rankings showed the +N treatment highest, followed by the parent and transconjugant strains, with

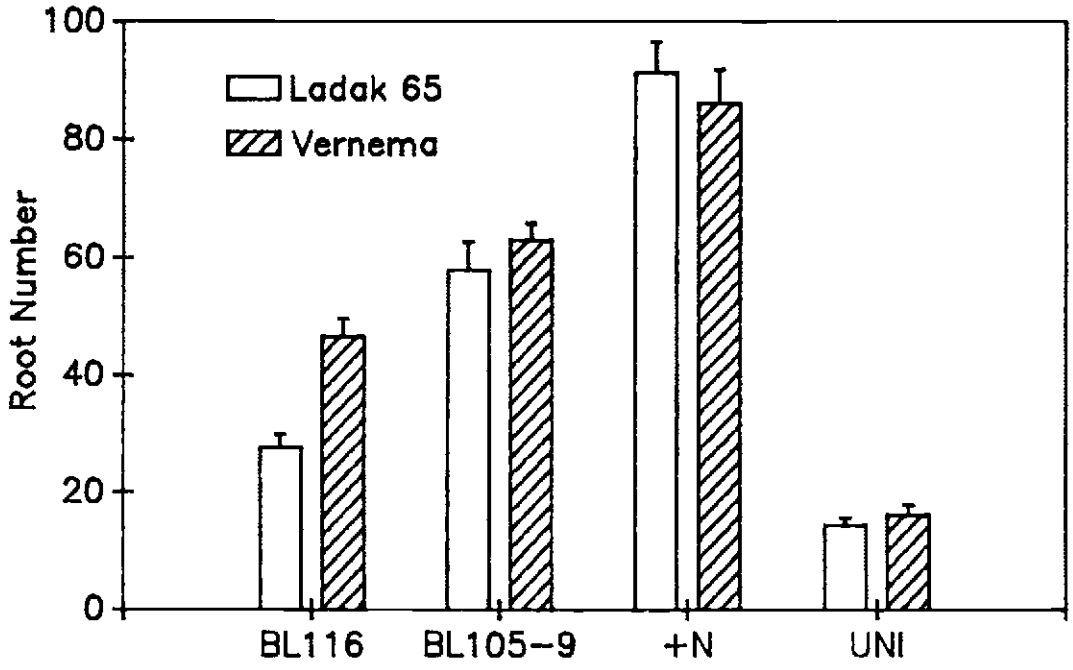

Figure IV.1. Mean root number per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

Table IV.1 Mean root number per plant of 42 day-old alfalfa plants as affected by different treatments and cultivars. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Cultivars consisted of Ladak 65 and Vernema.

Cultivar	Treatment	Root number
Ladak 65	BL105-9	58b
Ladak 65	BL116	28d
Ladak 65	+N	91a
Ladak 65	UNI	14e
Vernema	BL105-9	63b
Vernema	BL116	47c
Vernema	+N	86a
Vernema	UNI	16e
	LSD	11

Means followed by the same letter are not significantly different at the .05 probability level.

the unioculated treatment lowest in dry weight (Figure IV.3). Plants inoculated with the R. meliloti transconjugant (BL105-9) and the parent strain (BL116) exhibited 44% and 33% significantly less root dry weight than +N treatment, respectively. Vernema produced significantly heavier root dry weight values than cultivar Ladak 65 (21%), with less root dry weight for the transconjugant than the parent. Mean root dry weight values are shown in Tables IV.2 and IV.3 respectively.

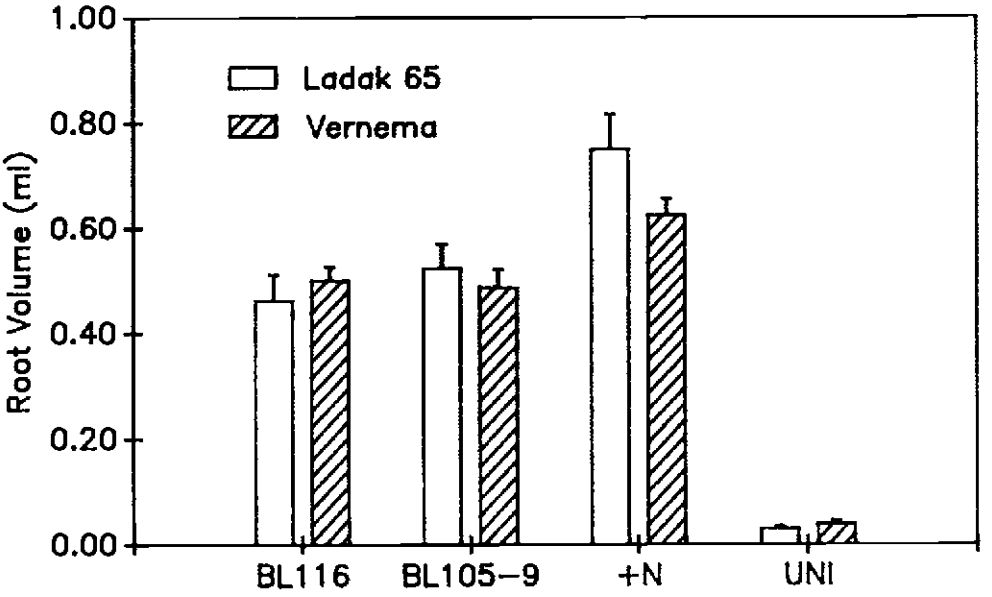

Figure IV.2. Mean root volume per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

Table IV.2 Mean root volume (ml) and root and shoot dry weight (mg) per plant of 42 day-old alfalfa plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

Treatment	Root volume(ml)	Dry weight (mg) root	shoot
BL105-9	0.51b	24.50b	55.75b
BL116	0.48b	28.88b	43.50b
+N	0.69a	43.38a	145.81a
UNI	0.03c	3.25c	3.87c
LSD	0.08	5.75	25.14

Means followed by the same letter are not significantly different at the .05 probability level.

Table IV.3 Mean root dry weight (mg) per plant of 42 day-old alfalfa plants as affected by different cultivars. Cultivars consisted of Ladak 65 and Vernema.

Cultivar	Root dry weight (mg)
Ladak 65	22.63a
Vernema	27.38b
LSD	4.07

Means followed by the same letter are not significantly different at the .05 probability level.

Shoot dry weight

Shoot dry weight of 42 day-old plants was significantly greater with the +N treatment. No significant differences were observed between inoculum treatments or between cultivars. The uninoculated treatment exhibited significantly lower shoot dry weight (Figure IV.4). Means of shoot dry weight for different treatments are shown in Table IV.2.

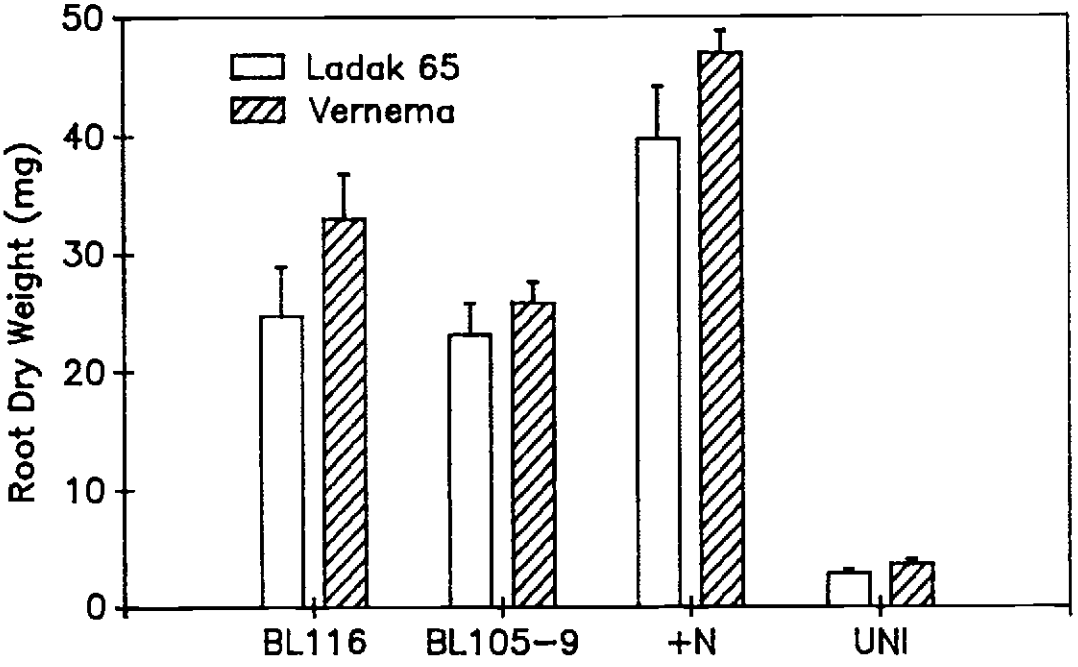

Figure IV.3. Mean root dry weight per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

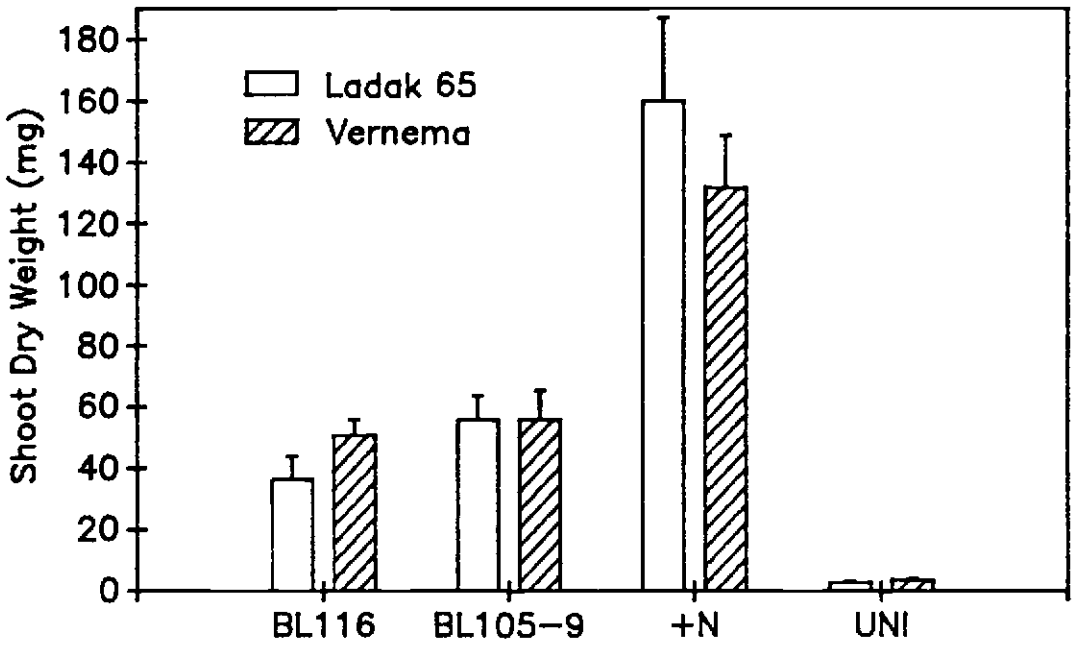

Figure IV.4. Mean shoot dry weight per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

Nodule number and nodule dry weight

Treatment with the R. meliloti transconjugant BL105-9 produced significantly (72.8%) more nodules and 99.2% higher nodule dry weight than the R. meliloti parent BL116 (Figures IV.5 and IV.6). No nodules were observed with the +N and uninoculated treatments. Means of nodule number and nodule dry weight for different treatments are shown in Table IV.4.

Total plant nitrogen

In both cultivars tested, total plant nitrogen level was significantly highest for the +N treatment (Figure IV.7). For the +N treatment, Ladak 65 had significantly higher total N values. No significant difference was observed between transconjugant and parent strains, but in Ladak 65 and Vernema, transconjugant treatments fixed 41% and 10%, respectively, more N than parent treatments. The uninoculated treatment showed the lowest nitrogen level. Means of total nitrogen for different treatments and different cultivars are shown in Table IV.5.

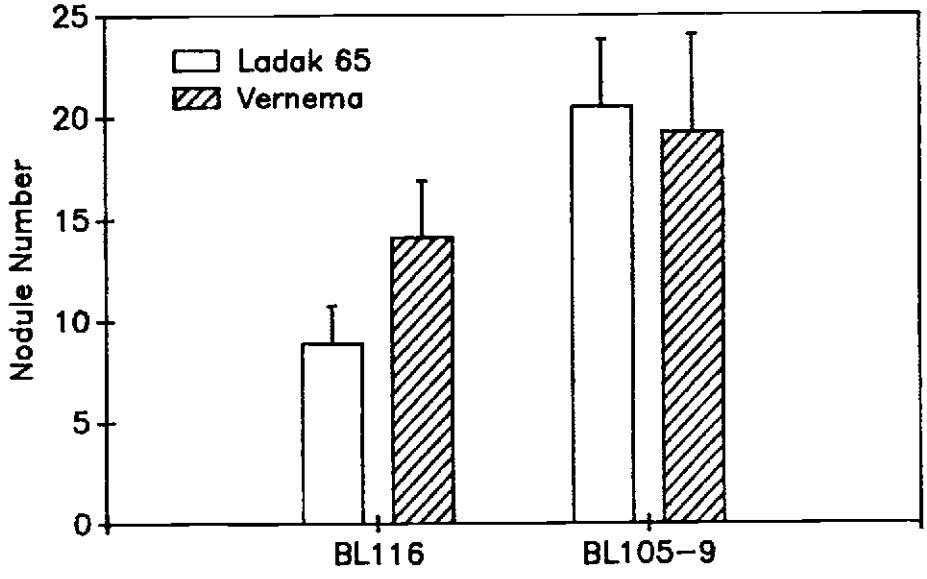

Figure IV.5. Mean nodule number per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9) and the R. meliloti parent (BL116).

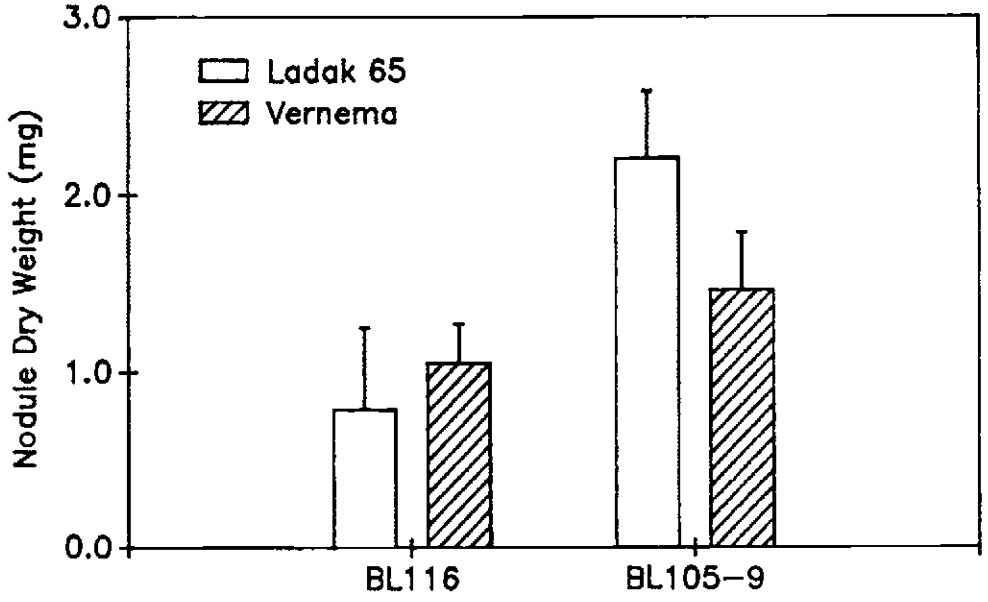

Figure IV.6. Mean nodule dry weight per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9) and the R. meliloti parent (BL116).

Table IV.4 Mean nodule number and nodule dry weight (mg) per plant of 42 day-old alfalfa plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

Treatment	Nodule number	Nodule dry weight (mg)
BL105-9	20a	1.83a
BL116	12b	0.92b
+N	0c	0.00c
UNI	0c	0.00c
LSD	5	0.51

Means followed by the same letter are not significantly different at the .05 probability level.

Acetylene reduction

of the four treatments evaluated, only the inoculated treatments showed significant acetylene reduction activity (Figure IV.8). Strain effectiveness, expressed as rate of Ethylene evolution is shown in Table IV.6. No significant difference in terms of rate of acetylene reduction was observed between the transconjugant and the parent strains.

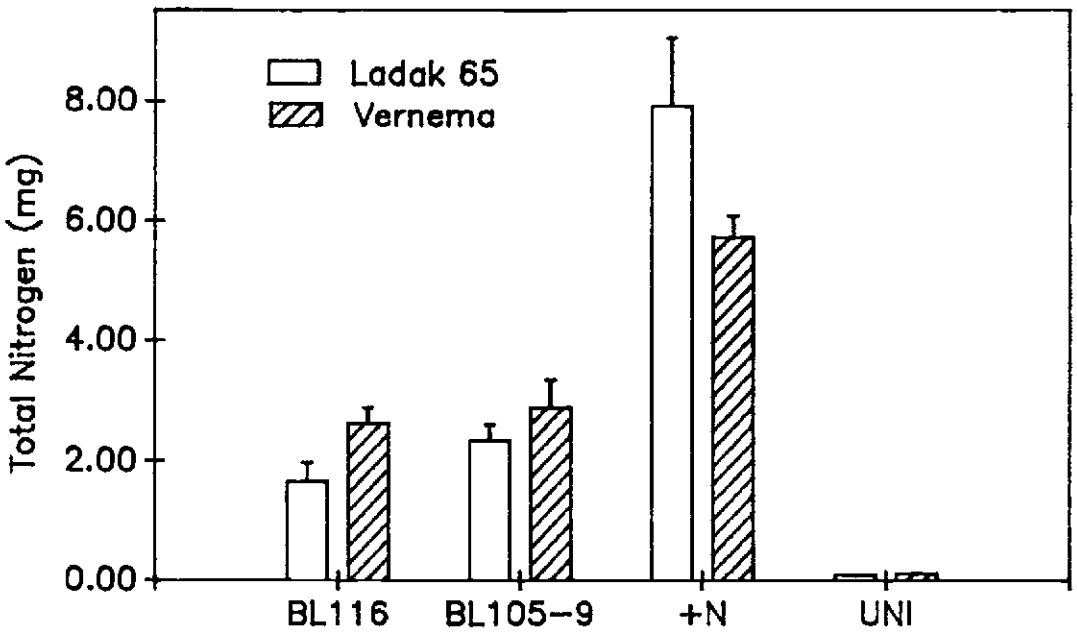

Figure IV.7. Mean total plant N per plant and standard error bars of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

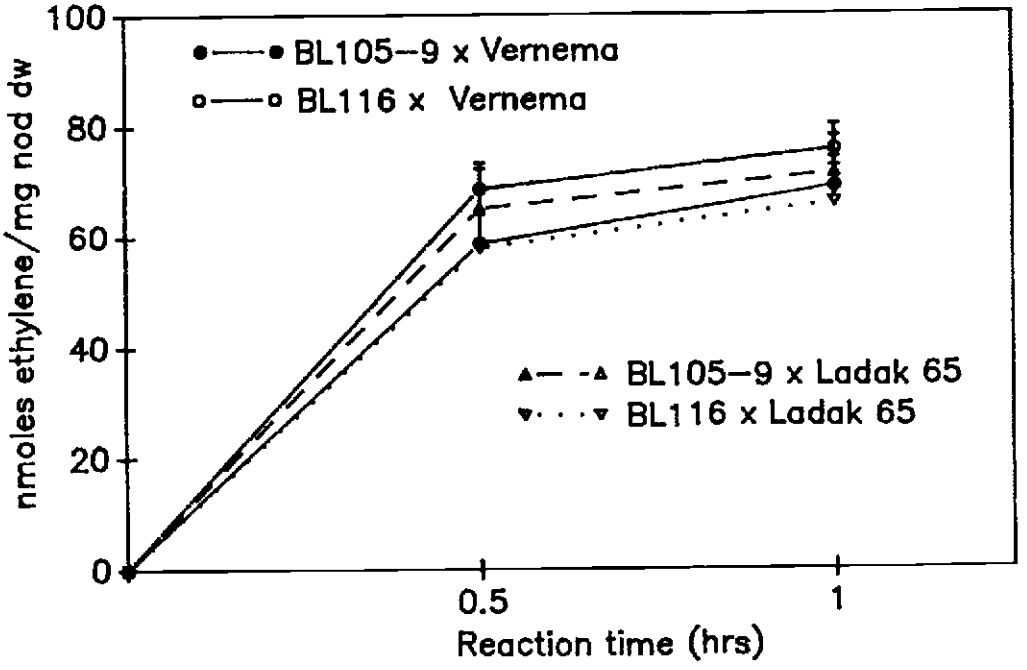

Figure IV.8. Mean acetylene reduction activity (nmole ethylene evolved.h^{-1}.mg^{-1} dw of nodules) per plant and standard error bars per plant of 42 day-old plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9) and the R. meliloti parent (BL116).

Table IV.5 Mean total plant N (mg) per plant of 42 day-old alfalfa plants as affected by different treatments and cultivars. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Cultivars consisted of Ladak 65 and Vernema.

Cultivar	Treatment	Total plant N (mg)
Ladak 65	BL105-9	2.33c
Ladak 65	BL116	1.65c
Ladak 65	+N	7.92a
Ladak 65	UNI	0.09d
Vernema	BL105-9	2.89c
Vernema	BL116	2.62c
Vernema	+N	5.72b
Vernema	UNI	0.11d
	LSD	1.35

Means followed by the same letter are not significantly different at the .05 probability level.

Table IV.6 Mean acetylene reduction activity (nmole ethylene evolved.h^{-1}.mg^{-1} dw of nodules) per plant of 42 day-old alfalfa plants as affected by different treatments. Treatments consisted of inoculation with a R. meliloti transconjugant (BL105-9), the R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI).

Treatment	Acetylene reduction
BL105-9	70.97a
BL116	70.37a
+N	0.00b
UNI	0.00b
LSD	8.15

Means followed by the same letter are not significantly different at the .05 probability level.

Discussion

Although root growth was highest with the +N treatment, root numbers were significantly increased with the Rhizobium meliloti transconjugant (BL105-9) over that of the R. meliloti parent (BL116). However, total root mass was not significantly different between the two treatments. This suggests that when R. meliloti acquires the Ri plasmid, it causes some root proliferation in alfalfa, but this proliferation does not change the entire root mass or partitioning of dry matter.

Significant enhancement of nodulation was observed in the transconjugant treatment. Although no significant differences in total plant N level and acetylene reduction rate were observed between transconjugant and parent strains, plants inoculated with the transconjugant fixed more N than that of the parent. This confirms the results of Strobel et al. (1985).

Conclusive evidence for the involvement of the Ri plasmid in enhancing nodulation by R. meliloti may come by the deliberate removal of the Ri plasmid from transconjugants (Strobel et al., 1985). The number and location of the genes that seem to be controlling the enhancement of nodulation of R. meliloti on the Ri plasmid are not known, nor have the biochemical factors increasing

the efficiency of nodulation been described.

In this experiment, acetylene reduction activity was expressed as nmoles of ethylene produced per hour per milligram of nodule dry weight. Due to the small nodule dry weight and sensitivities inherent in acetylene reduction measurement, the acetylene reduction analyses displayed high variability. Although 8 replications were utilized, this high variability prevented distinguishing treatment differences.

The most significant aspect of this experiment is that the introduction of the hairy root plasmid into R. meliloti resulted in increased nodulation in alfalfa. However, this increased nodulation was not translated into increased acetylene reduction rate or total plant nitrogen.

To maximize the potential for determining treatment differences in future experiments, further evaluation of the R. meliloti transconjugant for increased N fixation should utilize more plants per experimental unit, greater light intensity, and ^{15}N methods for estimating nitrogen fixation to reduce the variability associated with acetylene reduction methods. It is recommended that the future experiments also evaluate morphological and cytological changes in alfalfa nodules caused by the transconjugant.

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VI. APPENDIX

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Table 1. Root number data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	15	19	26
2	87	55	20
3	65	16	16
4	62	30	26
Means	57	30	22

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	6498.92			
Treatment	2	2732.17	1366.08	3.26	4.26
Error	9	3766.75	418.53		

Table 2. Root volume (ml) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	0.10	0.10	0.05
2	0.30	0.25	0.05
3	0.20	0.10	0.10
4	0.10	0.15	0.05
Means	0.18	0.15	0.06

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	0.072			
Treatment	2	0.028	0.014	2.83	4.26
Error	9	0.044	0.005		

Table 3. Root fresh weight (mg) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	43	52	40
2	202	118	32
3	94	49	64
4	198	124	54
Means	134.3	85.8	47.5

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	39325.67			
Treatment	2	15121.17	7560.58	2.81	4.26
Error	9	24204.50	2689.39		

Table 4. Root dry weight (mg) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	4	7	2
2	14	11	3
3	5	5	4
4	21	14	5
Means	11.0	9.3	3.5

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	370.92			
Treatment	2	123.17	61.58	2.24	4.26
Error	9	247.75	27.53		

Table 5. Root number data table and ANOVA of 42 day-old white clover plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	22	45	10
2	24	51	30
3	22	25	35
4	41	49	36
Means	27	43	28

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	1723.00			
Treatment	2	600.50	300.25	2.41	4.26
Error	9	1122.50	124.72		

Table 6. Root volume (ml) data table and ANOVA of 42 day-old white clover plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	0.05	0.10	0.05
2	0.05	0.10	0.10
3	0.10	0.10	0.10
4	0.15	0.20	0.05
Means	0.09	0.13	0.08

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	0.022			
Treatment	2	0.005	0.003	1.44	4.26
Error	9	0.017	0.002		

Table 7. Root fresh weight (mg) data table and ANOVA of 42 day-old white clover plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	25	41	23
2	42	107	32
3	53	56	40
4	43	87	35
Means	40.75	72.75	32.50

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	6838.67			
Treatment	2	3616.17	1808.08	5.05	4.26
Error	9	3222.50	358.06		

Table 8. Root dry weight (mg) data table and ANOVA of 42 day-old white clover plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	2	5	2
2	4	8	2
3	4	4	3
4	3	9	3
Means	3.25	6.50	2.50

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	56.92			
Treatment	2	36.17	18.08	7.84	4.26
Error	9	20.75	2.31		

Table 9. Root number data table and ANOVA of 42 day-old field pea plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	135	90	135
2	125	572	157
3	220	289	146
4	132	148	128
Means	153	275	142

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	188896.25			
Treatment	2	43614.50	21807.25	1.35	4.26
Error	9	145281.75	16142.42		

Table 10. Root volume (ml) data table and ANOVA of 42 day-old field pea plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	0.50	1.50	0.70
2	0.40	4.00	0.50
3	0.50	2.50	0.50
4	0.60	2.00	0.50
Means	0.50	2.50	0.55

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	13.96			
Treatment	2	10.41	5.20	13.19	4.26
Error	9	3.55	0.39		

Table 11. Root fresh weight (mg) data table and ANOVA of 42 day-old field pea plants as affected by treatment. Treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	450	325	479
2	660	2529	1490
3	650	1802	700
4	630	1200	360
Means	597.50	1464.00	757.25

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	5121508.92			
Treatment	2	1701117.12	850558.58	2.24	4.26
Error	9	3420391.75	380043.53		

Table 12. Root dry weight (mg) data table and ANOVA of 42 day-field pea plants as affected by treatment. treatments consisted of inoculation with A. rhizogenes (232), A. radiobacter (NT1), and an uninoculated treatment (UNI).

	TREATMENTS		
	232	NT1	UNI
Reps			
1	34	41	39
2	41	387	143
3	42	190	49
4	42	96	28
Means	39.75	178.50	64.75

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	11	121500.67			
Treatment	2	43754.17	21877.08	2.53	4.26
Error	9	77746.50	8638.50		

Table 13. Root number data table and ANOVA of 42 day-old alfalfa plants as affected by treatments. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	78	31	101	12	73	41	75	11
2	68	25	95	21	55	55	75	15
3	61	21	96	11	54	41	65	12
4	55	27	74	11	57	47	81	23
5	63	29	81	13	61	57	98	18
6	36	25	70	18	61	57	115	21
7	61	22	115	13	69	40	98	18
8	41	41	98	16	74	35	81	11
Means	59	28	91	14	63	47	86	16

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	55528.73			
Treatment	3	47578.30	15859.43	141.45	2.78
Cultivar	1	425.39	425.39	3.79	4.02
Treatment X Cultivar	3	1246.17	415.39	3.70	2.78
Error	56	6278.88	112.12		

Table 14. Root volume (ml) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	0.7	0.6	1.0	0.02	0.7	0.5	0.7	0.02
2	0.6	0.5	0.7	0.05	0.5	0.6	0.5	0.02
3	0.5	0.5	0.6	0.02	0.5	0.5	0.6	0.05
4	0.4	0.6	0.6	0.02	0.5	0.6	0.6	0.05
5	0.4	0.3	0.6	0.03	0.4	0.5	0.5	0.05
6	0.4	0.3	0.6	0.03	0.5	0.5	0.7	0.05
7	0.7	0.3	1.0	0.02	0.4	0.4	0.7	0.05
8	0.5	0.6	0.9	0.05	0.4	0.4	0.7	0.02
Means	0.53	0.46	0.75	0.03	0.49	0.50	0.6	0.04

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	4.4342			
Treatment	3	3.6998	1.2333	104.58	2.78
Cultivar	1	0.0135	0.0135	1.15	4.02
Treatment X Cultivar	3	0.0605	0.0202	1.71	2.78
Error	56	0.6604	0.0118		

Table 15. Root dry weight (mg) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	18	49	36	4	37	42	48	2
2	24	23	31	3	23	27	49	3
3	19	12	52	2	24	42	40	4
4	13	29	30	2	22	26	52	6
5	25	15	31	2	24	51	38	4
6	18	21	28	2	27	32	51	4
7	35	18	62	4	28	23	51	3
8	33	31	48	4	22	21	47	3
Means	23.13	24.75	39.75	2.88	25.88	33.00	47.00	3.63

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	17424.00			
Treatment	3	13215.50	4405.17	66.79	2.78
Cultivar	1	361.00	361.00	5.47	4.02
Treatment X Cultivar	3	154.00	51.33	0.78	2.78
Error	56	3693.50	65.96		

Table 16. Shoot dry weight (mg) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	93	81	223	2	98	34	98	2
2	74	36	77	7	59	84	74	2
3	69	22	245	5	41	44	62	3
4	39	32	71	5	60	46	173	4
5	43	43	78	4	37	57	150	2
6	25	22	144	4	26	44	137	5
7	59	12	201	4	91	51	168	4
8	44	43	241	6	34	45	191	3
Means	55.75	36.38	160.0	5.88	55.75	50.63	131.63	3.13

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	247159.48			
Treatment	3	172546.30	57515.43	45.64	2.78
Cultivar	1	260.02	260.02	0.21	4.02
Treatment X Cultivar	3	3788.80	1262.93	1.00	2.78
Error	56	70564.38	1260.08		

Table 17. Nodule number data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	21	17	0	0	22	8	0	0
2	41	4	0	0	32	12	0	0
3	11	4	0	0	19	9	0	0
4	15	13	0	0	39	19	0	0
5	20	10	0	0	9	31	0	0
6	12	5	0	0	3	15	0	0
7	23	4	0	0	28	7	0	0
8	21	14	0	0	2	12	0	0
Means	20.5	8.9	0	0	19.3	14.1	0	0

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	7168.44			
Treatment	3	4498.69	1499.56	32.89	2.78
Cultivar	1	16.00	16.00	0.35	4.02
Treatment X Cultivar	3	100.50	33.50	0.73	2.78
Error	56	2553.25	45.59		

Table 18. Nodule dry weight (mg) data table and ANOVA of 42 day-old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	2.3	4.0	0	0	2.8	0.6	0	0
2	4.5	0.3	0	0	2.2	1.0	0	0
3	1.5	0.2	0	0	1.5	1.0	0	0
4	1.2	0.6	0	0	1.7	2.0	0	0
5	1.5	0.2	0	0	0.7	0.7	0	0
6	1.5	0.2	0	0	0.4	0.8	0	0
7	2.8	0.2	0	0	2.1	0.3	0	0
8	2.3	0.6	0	0	0.3	2.0	0	0
Means	2.2	0.8	0	0	1.5	1.1	0	0

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	68.13			
Treatment	3	36.91	12.30	23.95	2.78
Cultivar	1	0.23	0.23	0.44	4.02
Treatment X Cultivar	3	2.22	0.74	1.44	2.78
Error	56	28.77	0.51		

Table 19. Total plant nitrogen (mg) data table and ANOVA of 42 day old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak 65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	3.48	3.56	7.90	0.09	5.50	2.25	5.87	0.07
2	2.90	1.54	4.76	0.10	3.10	3.98	5.52	0.10
3	2.90	0.93	11.76	0.07	2.01	1.93	3.55	0.10
4	1.55	1.39	4.05	0.07	2.83	2.37	5.99	0.15
5	2.01	1.44	4.86	0.07	1.84	2.98	5.47	0.14
6	1.34	1.18	7.72	0.07	1.89	1.96	6.15	0.16
7	2.66	0.81	10.68	0.12	3.89	2.06	6.15	0.11
8	1.82	2.34	11.62	0.12	2.04	3.43	7.04	0.07
Means	2.33	1.65	7.92	0.09	2.88	2.62	5.72	0.11

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	508.80			
Treatment	3	381.72	127.23	69.38	2.78
Cultivar	1	0.42	0.42	0.23	4.02
Treatment X Cultivar	3	23.97	7.99	4.36	2.78
Error	56	102.69	1.83		

Table 20. Acetylene reduction activity (nmole ethylene evolved.h^{-1}.mg^{-1} dw of nodules) data table and ANOVA of 42-day old alfalfa plants as affected by treatment. Treatments consisted of inoculation with R. meliloti transconjugant (BL105-9), R. meliloti parent (BL116), a +N treatment, and an uninoculated treatment (UNI). Alfalfa cultivars consisted of Ladak-65 and Vernema.

TREATMENTS

	LADAK 65				VERNEMA			
	BL105-9	BL116	+N	UNI	BL105-9	BL116	+N	UNI
Reps								
1	63.48	36.25	0	0	54.29	53.50	0	0
2	56.01	50.32	0	0	68.41	80.31	0	0
3	95.00	54.50	0	0	97.00	89.54	0	0
4	68.30	59.21	0	0	84.41	73.75	0	0
5	93.67	82.24	0	0	56.00	67.00	0	0
6	90.00	80.21	0	0	63.00	81.00	0	0
7	48.75	79.00	0	0	68.57	91.32	0	0
8	57.61	87.54	0	0	61.35	69.75	0	0
Means	71.60	66.16	0	0	69.13	75.77	0	0

ANOVA

Source	df	Sum of squares	Mean square	F	$F_{.05}$
Total	63	87707.31			
Treatment	3	79900.25	26633.42	201.20	2.78
Cultivar	1	50.96	50.96	0.38	4.02
Treatment X Cultivar	3	343.11	114.32	0.86	2.78
Error	56	7412.97	132.37		