

AN ABSTRACT OF THE THESIS OF

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Title: Nitrogen Nutrition of Seedling Alfalfa (Medicago
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Nitrogen fertilization of seedling legumes is a controversial practice intended to provide N to the plant during periods when seed N, soil N, and N₂ fixation may not meet plant requirements; eg. prenodulation, postharvest, or in the event of an inoculation failure. Field experiments were conducted in 3 consecutive years (1980, 1981, and 1982) on a Woodburn silt loam soil (Aquultic Argixeroll) containing low soil N to evaluate the effect of 9 rates of NH₄NO₃-N (0-224 kg N ha⁻¹) on nodulation, acetylene reduction (AR), percentage herbage N, percentage nitrate N, and dry matter yield of seedling alfalfa (Medicago sativa L. cv. 'Apollo'). The R. meliloti at the field site were found to be ineffective at N₂ fixation, thus an inoculation treatment was added in 1982 by the use of a split-block design. The response of the variables to applied N was evaluated at 10 weeks, and over a postharvest time course. At ten weeks after planting, only the uninoculated, ineffectively nodulated plants exhibited increased herbage yield and %N in response to the applied N. All rates of N fertilization decreased nodulation and AR in a curvilinear response, regardless of rhizobial effectiveness. The applied NH₄NO₃ continued to decrease AR and nodulation of seedlings through the third week after the initial harvest. Acetylene reduction activity of control plants dropped to 68% of

preharvest levels at 2 to 4 days postharvest, then recovered during the next 4 weeks. Herbage nitrate N was increased in one year out of three. Dry matter yield and total herbage N of uninoculated plants were increased during the initial growing season, and unaffected the following spring. Since the inoculated plants showed little response to the N rates, except for decreased N_2 fixation, it was concluded that during establishment, managerial emphasis should be placed on inoculation rather than N fertilization to obtain a consistent source of N for herbage protein. An ancillary investigation was conducted to characterize native Rhizobium taken from uninoculated, ineffectively nodulated, M. sativa nodules. Results suggested that Phaseolus vulgaris L. may be an alternate host for the native Rhizobium. Agricultural implications of this finding were discussed.

Nitrogen Nutrition of Seedling Alfalfa
(Medicago sativa L.)

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NITROGEN NUTRITION OF SEEDLING ALFALFA (Medicago sativa L.)

INTRODUCTION

Alfalfa (Medicago sativa L.) is a widely adapted forage legume which is unsurpassed in herbage dry matter yield and forage quality. The following study examines several management practices which are associated with the nitrogen nutrition of seedling alfalfa. The primary focus of the study was to examine the controversial practice of nitrogen fertilization of seedling alfalfa (Medicago sativa L.). It is widely known that when alfalfa is grown in the presence of the soil bacteria, Rhizobium meliloti Dang., the plant forms a symbiotic relationship with the bacteria, which enables the plant to obtain all its N requirements from atmospheric N₂. In an effort to maximize seedling development, alfalfa growers frequently apply fertilizer N to ameliorate supposed N deficiencies which occur during early seedling growth prior to nodulation and development of N₂ fixation activity. Previous greenhouse research has indicated that N deficiencies may also occur immediately postharvest. When the study was initiated, the primary goal was to investigate the practice of N fertilization of alfalfa at establishment and during regrowth. Preliminary results provided valuable insight into a possible explanation for the widespread interest in the use of alfalfa N fertilization in this particular region of the U. S. The results suggested that the moderately acid soils in this region may contain only ineffective R. meliloti which are unable to provide adequate fixed nitrogen for plant development. Thus, alfalfa growers may

apply fertilizer N at seeding as insurance against an inoculation failure. The sensitivity of R. meliloti to acid soil conditions is a major limiting factor to the widespread adaptation of alfalfa worldwide. Rhizobium species vary in their tolerance to acid soil conditions, and the explanation for the high sensitivity of R. meliloti is obscure. Thus, the objectives of the experiment were expanded to include a characterization of the ineffective native population of R. meliloti at the field site.

REVIEW OF LITERATURE

Importance of Nitrogen Fixation in Alfalfa

Of all field crops grown in Oregon, alfalfa hay ranks second only to wheat in terms of dollar value and acres harvested (Miles, 1984). It should be noted that this estimate does not consider the thousands of acres on which alfalfa is grown for grazing and the value of alfalfa hay grown and used on the farm. Reasons for its desirability as a forage include its adaptability to a wide range of climatic and soil conditions, its high mineral and vitamin content (Hanson and Barnes, 1973), and its unsurpassed feeding value as a hay crop (Barnes and Gordon, 1972). Alfalfa produces more protein per hectare and as much energy per hectare as any of the crops commonly used to feed livestock (Niedermeier et al., 1972).

The protein content of alfalfa is dependent upon the amount of combined N available to the plant. In the absence of fertilizer N, the alfalfa plant has three potential sources of combined N; seed N, soil N, and N obtained from symbiotic N_2 fixation. In a 4 year field study of the N requirements of alfalfa, symbiotic N_2 fixation was found to be responsible for two-thirds of the total N content of the plant (Heichel et al. 1984).

Overview of the Nitrogen Fixation Process

The sequence of events in nodule formation has been described in detail elsewhere (Dart, 1974; Sprent, 1979), and thus only a brief description will be given here. The development of symbiotic N_2 fixation in alfalfa begins within a few days after germination when the plant roots are grown in media containing R. meliloti (Munns, 1968). The earliest conclusive evidence of the role of these bacteria was provided by the German scientists Hellriegel and Wilfarth in 1888. During the initial stage of the infection process, it is thought that the bacteria are antigenically recognized and attached to the legume root hairs via plant-produced glycoproteins called lectins (Dazzo and Brill, 1978). The Rhizobium bacteria then invade the roots of the young alfalfa seedling through the root hairs, where they are immediately encased in a plant-derived tube, or infection thread. Inside the infection thread, the bacteria progress to the cortical cells of the root via coordinated bacterial cell division and cell wall deposition. Meristematic activity occurs in the plant cells in advance of the ramifying infection thread, and eventually a vascular system is formed as the dividing cortical cells differentiate and grow outward from the root. As the infection thread progresses intercellularly during nodule formation, Rhizobium are released from the thread wall, and surrounded by host plasmalemma. The bacterial cells then differentiate into enlarged pleomorphic bacteroids, which are capable of N_2 fixation.

The enzyme inside the bacteroids which is responsible for N_2

fixation is called nitrogenase. Several reviews on the biochemistry of this enzyme have been published (Eady and Postgate, 1974; Zumft and Mortensen, 1975). Nitrogenase is oxygen labile, and contains 2 component proteins; component I, which contains 2 molybdenum atoms and 32 nonheme iron atoms, is the site of substrate binding and substrate reduction; component II, which contains 4 nonheme iron atoms, reduces component I in the active enzyme. The process of N_2 fixation requires Mg^{++} , ATP, a source of reducing power, N_2 , and an anaerobic environment. In legume nodules, the O_2 labile nitrogenase is protected by the O_2 binding protein leghemoglobin (Bergersen and Turner, 1975). Other triple bonded compounds such as acetylene can also serve as the reducible substrate for nitrogenase (Dilworth, 1966; Schollhorn and Burris, 1966). The product of N_2 fixation is ammonium, and in alfalfa nodules this product is exported from the bacteroid into the cytoplasm of the surrounding nodule plant cell (O'Gara and Shanmugam, 1976). Subsequently, it is converted to glutamine via glutamine synthetase (Groat and Vance, 1981). The amide of glutamine is then transferred to either aspartate by asparagine synthetase with the input of ATP to yield asparagine and AMP, or it is transferred to 2-oxoglutarate by glutamate synthase with the input of NADH to yield glutamate and NAD^+ . The major portion of fixed N is transported from alfalfa nodules to the rest of the plant as asparagine.

The Influence of Combined N on Nitrogen Fixation

The influence of combined N on N_2 fixation activity has been one of the most studied aspects of N_2 fixation. Many reviews have been written on this subject (Fred et al., 1932; Roberts and Brill, 1981), and thus the discussion here will be limited to the salient features of the influence of combined N on symbiotic N_2 fixation in the Rhizobium-legume symbiosis. Unlike the free-living N_2 fixing bacteria, the synthesis of nitrogenase by Rhizobium in nodules is apparently not repressed relative to other bacterial proteins by the addition of combined N to nodules (Bishop et al., 1976; Bisseling et al., 1978; Noel et al., 1982). Once nitrogenase is formed in the nodules however, its activity is decreased by application of combined N (Bishop et al., 1976; Feenstra et al., 1982; Noel et al., 1982). This effect has been shown to be reversible over a short time period with detached nodules (Houwaard, 1979).

There are several possible explanations for this decrease in nitrogenase activity, and the most common is termed the photosynthate deprivation hypothesis (Small and Leonard, 1969). This hypothesis suggests that the reduction of nitrate in the plant utilizes reducing power which would otherwise be used in the N_2 fixation process (Fig. 1). Supporting evidence for this hypothesis was reported by Noel et al., (1982) in work with detached nitrate-treated soybean nodules. When provided with an optimal carbon source, the nitrogenase specific activity of these nodules was equal to that of nodules from plants which had received no nitrate. In contrast, other studies using alfalfa nodules have indicated that

PHOTOSYNTHATE DEPRIVATION HYPOTHESIS

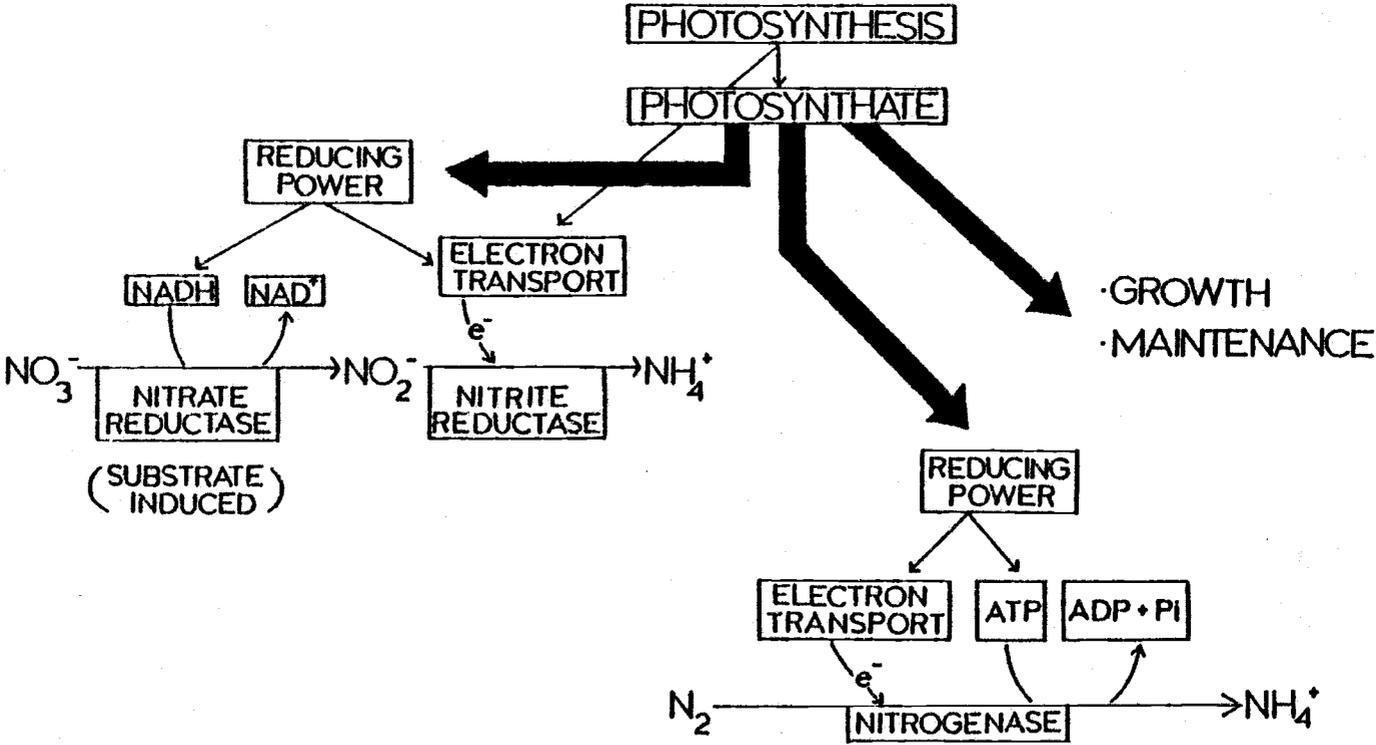


Figure 1. Diagram of the photosynthate deprivation hypothesis.

the principle effect of exogenous nitrate was a premature degradation of bacteroids released from the infection thread (Truchet and Dazzo, 1982). These results may be consistent with the photosynthate deprivation hypothesis in that a similar type of degradation is observed when alfalfa is harvested, which probably also results from a limitation of reducing power available to the nodules (Vance et al., 1980). This connection was not discussed in either study. In a more recent study, the combined effects of harvesting and nitrate application on N_2 fixation in alfalfa were evaluated (Vance et al., 1983). It was determined that PEP carboxylase activity, which is responsible for approximately 25% of the carbon required for assimilation of fixed N in alfalfa, was decreased 26% by the application of either 40 or 80 kg N ha⁻¹.

Ammonium has similar inhibitory effects on N_2 fixation and growth in active nodules, however explanations for its inhibitory effects on N_2 fixation are unclear. Accumulated ammonium in root nodules may influence combined N assimilatory enzymes which in turn may decrease nitrogenase activity (Duke and Ham, 1976). It has also been suggested that accumulated ammonium may inhibit electron transport to nitrogenase, thus decreasing the reducing power available for the reduction of N_2 (Laane et al., 1979; Haaker et al., 1982). Unfortunately, it is difficult to examine these processes in intact root systems.

Another possible N_2 fixation regulatory mechanism involves leghemoglobin. Decreased leghemoglobin activity resulting from the addition of combined N to nodules also may limit the respiratory

activity of the bacteroids, thus decreasing nitrogenase activity (Bisseling et al., 1978; Rigaud and Puppo, 1977). The main criticism of this hypothesis has been that leghemoglobin content does not always parallel nitrogenase activity. During the early development of the nodule, the appearance of leghemoglobin precedes nitrogenase activity (Verma et al., 1979), and after the addition of combined N to active nodules, leghemoglobin breakdown does not begin until after a detectable decrease in nitrogenase content (Chen and Phillips, 1977; Klucas, 1974).

Although N_2 fixation in developed nodules is diminished by exogenous combined N, the primary influence of combined N occurs during early nodule development. The presence of combined N in rooting media decreases the number of root hairs and thus decreases the number of potential infection sites (Thornton, 1936). The inhibitory effect of nitrate on nodulation is well known (Fred et al., 1932), and several steps of the nodulation process are affected. These include both the lectin-induced binding of Rhizobium to root hairs (Dazzo and Brill, 1978), as well as infection thread development (Munns, 1970). Early experiments in which roots of a single plant were split between two containers, one containing nitrate in nutrient solution and the other without, provided convincing evidence that the effect of nitrate was localized, in that only the root exposed to nitrate showed decreased nodulation (Wilson, 1917). Recent evidence obtained with a pea mutant having a high nodulation density in either the presence or absence of nitrate indicates that the susceptibility of the nodulation response of the

plant to nitrate is monogenic and recessive (Jacobsen and Feenstra, 1984). The mutants which were used in this study developed nodules in the presence of 15 mM KNO_3 , however the AR specific activity of these nodules exhibited the same decrease in activity as nonmutant control plants. Nevertheless, because of the abundance of nodules formed, the mutant plants had substantially higher total AR activity per plant. These results support the widely held view that the primary influence of combined N on symbiotic N_2 fixation is exerted during nodule development. These results also support the idea that the regulation of N_2 fixation is relatively independent of the overall nitrogen nutrition of the host plant, as was suggested by Wilson's split root experiment.

Nitrogen Deficiency in Seedling Alfalfa

The alfalfa seedling requires a constant supply of combined N for protein synthesis. The initial source of N for the seedling is from storage proteins in the seed, which contain sufficient N to sustain the seed through the first trifoliolate leaf stage of development. The seedling then requires an alternate source of combined N, which can be supplied either as mineral N, or N derived from N_2 fixation. If both sources are available, N_2 fixation is inhibited, and the plant will preferentially use the mineral N source. It is often suggested that low levels of mineral N supplied to the seedling prior to full nodule development may be beneficial to long-term development of symbiotic N_2 fixation in the plant (Allos and Bartholomew, 1959; Gibson, 1977). This suggestion is based on the assumption that the seedling enters a stage of nitrogen deficiency prior to nodule development, and that by supplying the seedling with 'starter' N, the deficiency will be alleviated and the plant will develop a larger root system with more potential nodulation sites.

This hypothesis has been tested in many studies using forage legumes, and in general the results are inconclusive (Munns, 1977; Tesar and Jackobs, 1972). The reason for disagreement on this subject may be due to the methodology used to conduct these studies, and the influence of environmental conditions on the relative rate of development of symbiotic N_2 fixation in the plants involved. Experiments which are conducted under greenhouse or growth chamber conditions generally indicate that low levels of combined N may enhance symbiotic N_2 fixation and also have beneficial effects on

growth and N concentration of young alfalfa (Allos and Bartholomew, 1959; Fishbeck and Phillips, 1981). In contrast, field studies generally suggest no beneficial effects of combined N application on seedling alfalfa, and typically indicate decreased symbiotic N_2 fixation potential (Peters and Stritzke, 1970; Ward and Blaser, 1961). The methodology used in these various experiments may have determined the type of response observed. Controlled environment experiments have certain limitations which may result in restricted ability to predict field responses to combined N. In controlled environments, temperature, light, and hydroponic nutrient solutions are usually manipulated to obtain optimal growth rates; conditions which may not be entirely realistic in comparison to field conditions. If optimal conditions are not provided, however, other problems may develop. Gibson (1977) concluded that symbiotically grown plants are more sensitive to adverse environmental conditions (eg. high temperatures, low light intensities, flooding injury, and low O_2 concentration) than plants grown on combined N. Thus caution is required in assessing fertilizer N effects in controlled environments. The chemostatic maintenance of low levels of N in hydroponic nutrient solutions also presents a difficult problem. If the nutrient solution is recycled, and very low levels of N are not constantly replenished, a temporary stimulation in nodulation can result. Field studies can also be criticized for lack of environmental and chemostatic control. For example, seasonal mineralization or immobilization of available soil N may occur, thus resulting in variable levels of available N over time. For this reason field

experiments are also difficult to use for the prediction of discrete physiological responses. If all of these aspects are considered, then the apparent anomalies in the literature may be rationalized.

Nitrogen deficiency in seedling alfalfa may also arise due to a lack of effective R. meliloti in the soil. Ineffective R. meliloti, i.e. microsymbionts which do not provide adequate combined N to the plant via N_2 fixation, are ubiquitous in most areas where alfalfa is grown (Burton, 1972). Their importance, however, is usually insignificant due to the widespread occurrence of effective R. meliloti and the common practice of applying effective and competitive R. meliloti as peat inoculants during planting. Problems with ineffectively nodulated alfalfa are virtually nonexistent in soils of the northcentral U.S., and it has been suggested that this may be due to deposition of effective R. meliloti from areas where alfalfa had previously been grown (Barnes et al., 1979). In general, problems associated with ineffective R. meliloti are principally associated with acid soils, or soils derived from acid parent material (Burton, 1972). In North America, the problem of ineffectively nodulated alfalfa has received attention only in the northwestern U.S. (Barber, 1980; Bottomley and Jenkins, 1983; Eardly et al., 1985; Weber and Leggett, 1966) and western Canada (Rice et al., 1977). The common factor which arises in connection with the widespread occurrence of predominately ineffective R. meliloti is moderately acid soil conditions. Rhizobium meliloti is the most sensitive species of the genus to soil acidity (Graham and Parker, 1964). It has been suggested that this sensitivity is primarily due

to the inability of R. meliloti to proliferate under acid soil conditions (Lowendorf et al., 1981), however it has been suggested that R. meliloti may lose its effectiveness in acid soils (Burton, 1972). Alfalfa grown on combined N can tolerate soil acidity to pH 5.5, while symbiotically grown alfalfa requires a pH of 6.0 or greater for optimal plant development (Munns, 1965). Studies which have examined the possibility of developing acid-tolerant strains of R. meliloti suggest that acid-tolerance of a given strain is dependent on the presence of a plant host (Lowendorf and Alexander, 1983). These same studies indicate that growth in acidified broth culture is not a good indicator of acid-tolerance under soil conditions. There is little information in the literature concerning characteristics of acid-tolerant Rhizobium which enable them to tolerate acid soil conditions. In a survey of R. meliloti isolates obtained from diverse locations within the U.S., Ellis and Barnes (1983) concluded that acid-tolerance was related to geographic origin. They also suggested that acid-tolerant strains seemed to be associated with alfalfa cultivars of Chilean ancestry.

OBJECTIVES

The central objectives of this study were to determine; 1) the rationale for the application of fertilizer N during legume establishment and; 2) the effects of NH_4NO_3 fertilization on symbiotic N_2 fixation, herbage N concentration and, herbage dry matter yield of field-grown seedling alfalfa. The approach was to select situations where previous research had indicated that N fertilization may be beneficial in supplementing the N nutrition of the alfalfa plant, and then apply rates of N fertilizer which could potentially sustain the plants through the deficiency periods. During the suggested deficiency periods, various aspects of the N nutrition of the plants were then evaluated to determine the effects of the N treatments. Measurements included herbage Kjeldahl N concentration, herbage dry matter yield, herbage nitrate concentration, nodule acetylene reduction activity, nodules per plant, and rhizobial effectiveness. Early results indicated that the native population of R. meliloti at the field site was ineffective in N_2 fixation and also possessed other anomalous cultural characteristics. Thus, an ancillary investigation was initiated to examine some symbiotic, nutritional and physiological properties of these organisms to explain their apparent persistence under acid soil conditions and their ineffectiveness in N_2 fixation.

CHAPTER I

Nitrogen Nutrition and Yield of Seedling Alfalfa as affected by
Ammonium Nitrate Fertilization

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ABSTRACT

Nitrogen fertilization of seedling legumes is a controversial management practice intended to provide N to the plant prior to the development of N_2 fixation activity. Field experiments were conducted in three consecutive years (1980, 1981, and 1982) to evaluate the effect of ammonium nitrate (NH_4NO_3) at establishment on nodulation, acetylene reduction (AR), percentage herbage N, and dry matter yield of 10 week old seedling alfalfa (Medicago sativa L. cv. 'Apollo'). The soil at the field site was a Woodburn silt loam, a member of the fine-silty, mixed, mesic Aquultic Argixerolls. The soil contained 8 to 12 mg kg^{-1} of available soil N. Six rates of NH_4NO_3 -N were applied preplant in 1980 and nine rates were applied preplant in 1981 and 1982 (0 to 224 kg N ha^{-1}) in a randomized complete block design. Data from the 1980 experiment suggested that the native population of Rhizobium meliloti Dang. was ineffective in N_2 fixation. This was confirmed by effectiveness tests of native strains of R. meliloti in 1981. In 1982 an inoculation variable was added in a factorial treatment combination to examine the effects of NH_4NO_3 fertilization on the N nutrition and yield of both effectively and ineffectively nodulated alfalfa. Results from the three field experiments indicated that only plants which were ineffectively nodulated exhibited substantial increases in herbage yield and Kjeldahl N concentration in response to the NH_4NO_3 applications. All rates of NH_4NO_3 fertilization decreased nodulation in a curvilinear response. For example, application of 45 kg N ha^{-1} reduced both nodulation and AR by 45% at 10 weeks (early bloom) regardless

of rhizobial effectiveness. Nitrogen fertilization also increased weed competition in 1981 and herbage nitrate levels in 1982.

Additional Index Words: Medicago sativa L.; Rhizobium meliloti Dang.; NH_4NO_3 ; Nodulation; Nitrogen fixation; Acetylene reduction; Nitrate.

INTRODUCTION

Fertilizer N is frequently used in the establishment of forage legumes (Hojjati et al., 1978). This application is intended to provide adequate combined N to the plant prior to the initiation of N_2 fixation in the nodules (Lie, 1974) and to insure high herbage N concentrations from initial harvests. It is unclear whether supplemental N provided during this period will result in a more vigorous plant capable of enhanced N_2 fixation at later stages of plant development. The negative influence of N fertilization on N_2 fixing activity is well documented (Fred et al., 1932; Gibson, 1977; Munns, 1977), however in certain instances nodulation of alfalfa (Medicago sativa L.) grown under greenhouse conditions was increased by various levels of N fertilization (Fishbeck and Phillips, 1981; Richardson et al., 1957; Weber and Leggett, 1966). Fishbeck and Phillips (1981) also found that N fertilization increased dry matter yields and herbage N concentrations of alfalfa clones during the first two regrowth cycles. Application at later stages showed no benefit. Results from field studies examining this practice are inconclusive (Rhykerd and Overdahl, 1972), perhaps due to variability in soil N levels (Gibson, 1977) and in the symbiotic effectiveness of the nodule occupants.

The primary objective of the following experiments was to evaluate dry matter yield, herbage N concentration, and N_2 fixing activity of 10 week old, field-grown, seedling alfalfa as affected by ammonium nitrate (NH_4NO_3) fertilization at establishment. In the first experiment, data was collected which suggested that; 1) the

inoculation of the plants was inadequate, and 2) that the native population of Rhizobium meliloti Dang. was unable to fix sufficient N_2 to meet the requirements of the plants. Similiar problems with ineffective R. meliloti have been described elsewhere, and are typically associated with acid soil conditions (Lie, 1974; Weber and Leggett, 1966). Under such conditions, N fertilization of alfalfa at establishment may be used to provide an alternate source of combined N for the seedlings in the event of an inoculation failure. In subsequent experiments, inoculation methods were improved and other modifications were made to permit a comparison of the effects of N fertilization on the yield and N nutrition of both effectively- and ineffectively-nodulated seedling alfalfa.

MATERIALS AND METHODS

Site Description and General Treatment Applications

Field experiments were conducted over 3 years (1980, 1981, and 1982) on three different field sites at the Oregon State University Hyslop Crop Science field research facility at Corvallis, Oregon. The soil type of all three sites was a Woodburn silt loam (fine, silty, mixed, mesic Aquultic Argixeroll). Alfalfa had been grown on these sites previously, however during the 10 years prior to these experiments the soils had either been in fallow or in small grains. Soil organic matter and total soil N averaged 20.0 and 1.0 g kg⁻¹, respectively. The average pH of the soils was 5.7 prior to amendment. One month prior to planting, each site received a uniform application of 4.5 Mg ha⁻¹ of ground agricultural limestone which raised the soil pH to 6.2 at the time of planting. Additional fertilizer applications were made based on soil test results and current recommendations for alfalfa (Gardner et al., 1976), and in respective years were: 1981; 36 kg S ha⁻¹ as gypsum (160 g S kg⁻¹), 3.3 kg B ha⁻¹ as sodium borate, and 0.5 kg Mo ha⁻¹ as sodium molybdate, 1982; the same quantities of fertilizer were applied as in 1981 with the addition of 62 kg K ha⁻¹ as potassium chloride.

Weed control measures used all 3 years included applications of benefin (N-butyl-N-ethyl-trifluoro-2,6-dinitro-p-toluidine) incorporated preplant at 1.7 kg ha⁻¹, and 2,4-DB ester [4-(2,4 dichlorophenoxy)-butyric acid] applied 2 weeks postplant at 0.8 kg ha⁻¹.

Experimental Design and NH_4NO_3 Treatments

Experiment 1, 1980 The experimental design utilized in 1980 was a randomized complete block with six rates of NH_4NO_3 and four replications. Nitrogen treatments consisted of commercial NH_4NO_3 (330 g N kg^{-1}) applied using a 0.92 m gravity type spreader calibrated to deliver six rates (0, 45, 90, 134, 179, and 224 kg N ha^{-1}). The N treatments were raked into the top few centimeters of soil prior to planting.

Experiment 2, 1981 The same design was used, except that three additional lower rates of NH_4NO_3 ($6, 11, \text{ and } 22 \text{ kg N ha}^{-1}$) were added. Also, uninoculated trap plants were grown outside the experimental area to confirm the ineffectiveness of the native population of R. meliloti.

Experiment 3, 1982 To facilitate the incorporation of an inoculation variable, a split block experimental design was used, with inoculated and uninoculated seed as the main plot treatments. The same nine rates of NH_4NO_3 ($0\text{-}224 \text{ Kg N ha}^{-1}$) applied in Experiment 2 were used as the subplot treatments. This experimental design was used to duplicate the experimental conditions and confirm the results of year one, where ineffective nodulation was observed, and year two, where effective nodulation was observed.

Inoculation Methods

Experiment 1, 1980 The seeds were coated with a commercial R. meliloti peat inoculant using milk as an adhesive. The inoculant was obtained from a local supplier.

Experiment 2, 1981 In response to evidence that insufficient inoculant had been applied in Experiment 1, the inoculation procedure was modified. All seeds were initially surface-sterilized by standard procedures with acidified HgCl_2 (Vincent, 1970). Commercial R. meliloti peat inoculant was obtained directly from a manufacturer (Nitragin Co., Milwaukee, WI). The seeds were lime pelleted using peat inoculant with gum arabic as an adhesive (Vincent, 1970). This method was found to yield approximately 10^5 viable cells per seed as determined by a plate count method.

Experiment 3, 1982 Main plot treatments consisted of inoculated and uninoculated seed. The seeds were surface-sterilized and divided into two equal portions. One portion was pelleted using viable peat inoculant as described in Experiment 2, and the other portion was pelleted using peat which had been sterilized by exposure to gamma radiation (50 kGy from a ^{60}Co source). The sterility of the the irradiated peat was verified by monitoring the nodulation of seedlings from both inoculation treatments in aseptic plant tubes (Vincent, 1970).

Planting Methods

Certified 'Apollo' alfalfa was planted on 19 June 1980, 19 June 1981, and 25 May 1982 at a rate of 12 kg ha^{-1} using an Ojyord small plot, cone-type seeder set for 15 cm rows at 1 cm depth. Individual plots were 1.83 x 9.15 m. Each replication was surrounded by a border, 1.83 m in width. In Experiment 2 (1981) surface-sterilized seeds for trap plants were planted in four 1 m^2

blocks outside the experimental area. In the third experiment (1982), the inside of the planter was cleaned with ethanol, the uninoculated seeds were planted first, and all main plots (inoculation treatments) were surrounded with an uninoculated border to avoid rhizobial contamination of the uninoculated main plot controls. Irrigation was provided as necessary by line source sprinklers and averaged 5.8 cm biweekly.

Analytical Procedures and Measurements

Soil Tests for Nitrogen Immediately prior to the beginning of each experiment, available soil mineral N was measured in samples taken to a depth of 15 cm (Berg and Gardner, 1978). Nitrate and ammonium N concentrations each ranged from 4 to 6 mg kg⁻¹. Available soil mineral N also was measured 8 weeks after planting in 1980, and at 4 and 8 weeks at 2 different depths in 1982. Results from these measurements were summarized using linear regression analysis (Table 1).

Acetylene Reduction Assay Acetylene reduction (AR) assays were conducted on replications one through four, respectively, 63, 65, 67, and 69 days after planting. Intervening days were used to rinse soil from the root systems in preparation for nodule enumeration. A total of eight soil cores per treatment per year were assayed, and each soil core contained an average of three plants. The soil cores were removed between 0730 hours and 1200 hours by centering a 25 cm length of 8.5 cm diameter perforated

Table 1. Regression equations describing soil mineral N concentrations (Y) in response to increasing rates of ammonium nitrate application (X). Six rates of ammonium nitrate N were applied in 1980 and nine rates were applied in 1982 (0 to 224 kg N per ha).

| Year | Weeks Post-Planting | N Form | Sample Depth --cm-- | Regression Equation | r ² | |
|------|---------------------|------------------------------|------------------------------|---------------------|--------------------------------|--------------------|
| 1980 | † | 8 | NO ₃ ⁻ | 0-15 | Y [§] = 7.1 + 0.107 X | 0.70 ^{**} |
| | | | NH ₄ ⁺ | 0-15 | Y = 11.4 + 0.230 X | 0.26 ^{**} |
| 1982 | † | 4 | NO ₃ ⁻ | 0-10 | Y = 33.7 + 0.551 X | 0.97 ^{¶¶} |
| | | | | 10-25 | Y = 13.1 + 0.088 X | 0.74 |
| | | | NH ₄ ⁺ | 0-10 | Y = 23.9 + 0.145 X | 0.63 |
| | | | | 10-25 | Y = 18.4 + 0.065 X | 0.50 |
| | 8 | NO ₃ ⁻ | 0-10 | Y = 3.5 + 0.158 X | 0.91 | |
| | | | 10-25 | Y = 5.0 + 0.054 X | 0.49 | |
| | | NH ₄ ⁺ | 0-10 | Y = 7.1 + 0.631 X | 0.87 | |
| | | | 10-25 | Y = 8.4 + 0.092 X | 0.63 | |

* **, Significant at the 0.05 and 0.01 levels of probability, respectively.

† Equations developed using 4 replicate samples per treatment (n = 24).

‡ Equations developed using a single composite sample per treatment (n = 9).

§ Soil mineral N concentration in mg per kg.

¶ Due to absence of replicate samples, F tests for lack of fit were not conducted in 1982.

steel tubing directly over the plants, and then driving the tubing vertically into the soil around the plants using a hammer developed by ARTS Machine Shop, American Falls, Idaho. The design of the hammer permitted the removal of the intact plants while still embedded in the soil core. Shoot removal was avoided because it has been shown to reduce AR activity (Mederski and Streeter, 1977). The soil core and plant remained in the tubing throughout the assay. Immediately after removal, cores were placed in 15 x 43 cm Saran bags (W. R. Grace Co., Cedar Rapids, IA) with the plant shoots exposed. The method of bag closure was similar to that described by Burris (1974) with the exception that kneaded rubber was used to seal around the plant stems. The bag was secured around a 3 cm section of pressure tubing fitted with a serum stopper, which served both as a gassing portal and a physical support for the plant shoot. The soil core assembly was then placed in an incubation chamber at the field site consisting of a 30 cm length of drainage tile buried vertically in the ground. The advantage of this design was that the shoots and the roots of the plants were exposed to natural environmental conditions of light and temperature during the incubation with acetylene. Gas space in the bag was estimated by water displacement, while pore space within the soil core was estimated from measurements of the bulk density, particle density, and moisture status of the soil. Acetylene was generated from calcium carbide as described by Burris (1974), and a sufficient amount was injected into the core to provide an atmosphere of $109 \text{ g m}^{-3} \text{ C}_2\text{H}_2$. This concentration was verified by repeated gas chromatographic

comparisons to a C_2H_2 standard. Incubation with acetylene was initiated between 1200 hours and 1300 hours. Gas samples were removed at three consecutive 90 minute intervals and stored in vacutainer tubes. Rates of ethylene appearance were determined with a gas chromatograph equipped with a flame ionization detector using N_2 as a carrier gas in a column containing Porapak R. Acetylene reduction rates were linear over the incubation period, and were expressed as micromoles of ethylene formed gram root dry weight⁻¹ hour⁻¹. Cores without plants showed no AR activity.

Determination of Nodule Number and Isolate Effectiveness

After the acetylene reduction assay, soil was washed from the root system over a 1 mm screen, and roots were immediately stored at -20 °C. At a later date, root systems were removed from storage, thawed, and blotted gently. Because of the coralloid morphology of some of the nodules, it was difficult to distinguish between nodules with multiple lobes and adjacent individual nodules. For this reason, individual lobes of nodular tissue were counted as nodules during enumeration. Root samples were dried to a constant weight at 65 °C. Trap plant R. meliloti nodule isolates were obtained in Experiment 2 (1981). Isolates of R. meliloti were also obtained from plants taken from the four replications of Experiments 2 and 3 (1981 and 1982, respectively). Nodule isolates were purified and maintained on yeast extract mannitol agar (Vincent, 1970). Nodule isolate effectiveness tests were conducted on 15 randomly selected nodule isolates per inoculation treatment per year. A plant tube method was used to evaluate isolate effectiveness and is described

in detail elsewhere (Bottomley and Jenkins, 1983). Each tube contained one seedling, and tubes were arranged in a randomized complete block design with six replicates and grown in a greenhouse under natural light conditions (average midday light intensity of $800 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ measured with a LiCor quantum sensor) with a day/night temperature range of 30/20 °C, respectively. After five weeks of growth, plants were harvested, and the shoots were dried at 65 °C. Dry weights were determined, and compared to two groups of control plants. The first control group was nitrogen deficient in that it remained uninoculated and received no N in nutrient solution. The second control group received high concentrations of N (3 ml of 18 mM N as KNO_3) at 2 weeks and 4 weeks during the growth period.

Field Harvest Schedule and Plant Analysis Seventy-one days after seeding when the plants had reached the early bloom stage, a 0.92 x 4.58 m area was harvested from the center of each field plot to a height of 5 cm. The harvested material was dried to a constant weight at 65 °C in a forced air oven and weighed.

Subsamples were removed, ground to pass a 1 mm screen, and redried prior to analysis for Kjeldahl N and nitrate N (Johnson and Uhlrich, 1950). Dried herbage tissue from the 1980 and 1981 experiments also was analyzed for P, K, Ca, Mg, S, Fe, Mn, Cu, B, and Zn by direct reading spark emission spectroscopy (Chaplin and Dixon, 1974).

Climatological Data Weather data was recorded at an NOAA evaporation station located at the facility. Irradiance was

measured using a Robitzsch bimetallic pyranograph (Redmond et al., 1983).

Statistical Analysis

Computer regression analysis was conducted using the Statistical Interactive Programming System (Department of Statistics, Oregon State University). Polynomial regression models were developed using a stepwise procedure, and the curve was selected based on coefficient of determination and error mean square values. Regression functions from two or more years which were the same were described using a single response function which was developed for the trend. The line graphs show the response curves and the average values of the respective treatments for the consecutive years.

RESULTS AND DISCUSSION

Experiment 1, 1980 The application of NH_4NO_3 in 1980 had little effect on herbage dry matter yield of the 10 week old alfalfa seedlings (Fig. 2). A slight increase in yield was observed in response to the intermediate rates of NH_4NO_3 application, however the low coefficient of determination ($R^2 = 0.33$) indicated that only a small proportion of the variation in dry matter yield was explained by the NH_4NO_3 treatments. Both nodulation and acetylene reduction activity were diminished in a curvilinear fashion by all levels of applied NH_4NO_3 (Figs. 3 and 4). Plants receiving the highest rates of NH_4NO_3 (179 to 224 kg N ha⁻¹) generally lacked nodules and exhibited no acetylene reduction activity.

Nitrate N concentration in the herbage tissue was relatively low (0.2 to 0.5 g kg⁻¹) except at the highest NH_4NO_3 treatment rate, where an increase in nitrate concentration (0.5 to 1.7 g kg⁻¹) was observed (Fig. 5). These results suggest that the plants were unable to assimilate high concentrations of internal nitrate when fertilized with high rates of NH_4NO_3 . Increases in nitrate concentration in response to NH_4NO_3 treatments on established stands of alfalfa have been reported previously (Smith and Sund, 1965).

An increase in Kjeldahl N concentration with increasing NH_4NO_3 rates (Fig. 6) indicated that N_2 fixation was probably not meeting the N requirements of the plants. A light green coloration typical of N deficiency was observed preharvest (6 to 10 weeks) on plants receiving low rates of NH_4NO_3 (0 to 90 kg N ha⁻¹). Plants receiving higher rates of NH_4NO_3 were dark green. This observed color

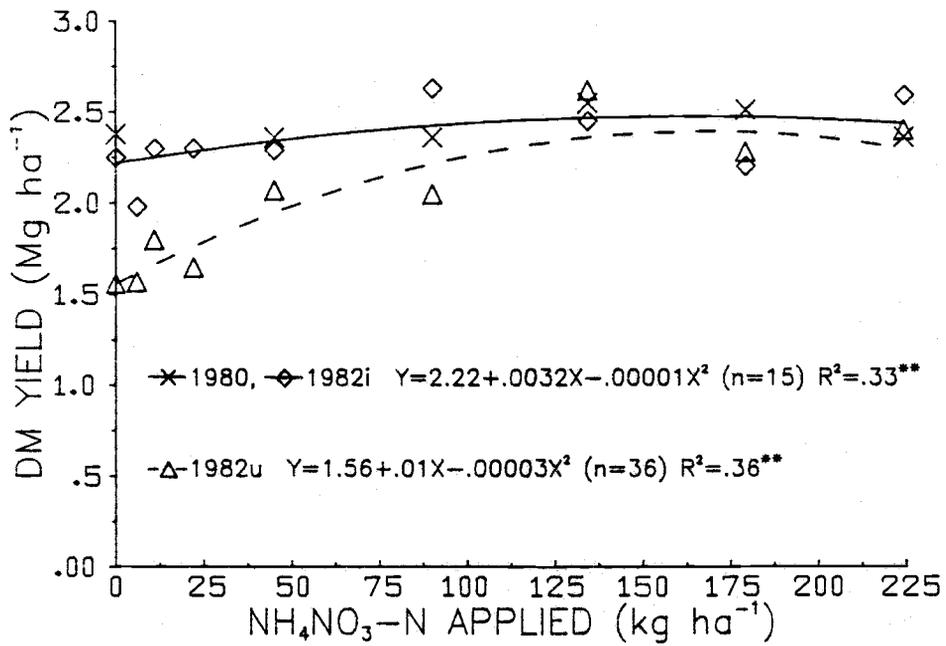


Figure 2. Dry matter yield of ten-week-old alfalfa seedlings as affected by ammonium nitrate fertilization at establishment; i = inoculated main plot treatment; u = uninoculated main plot treatment.

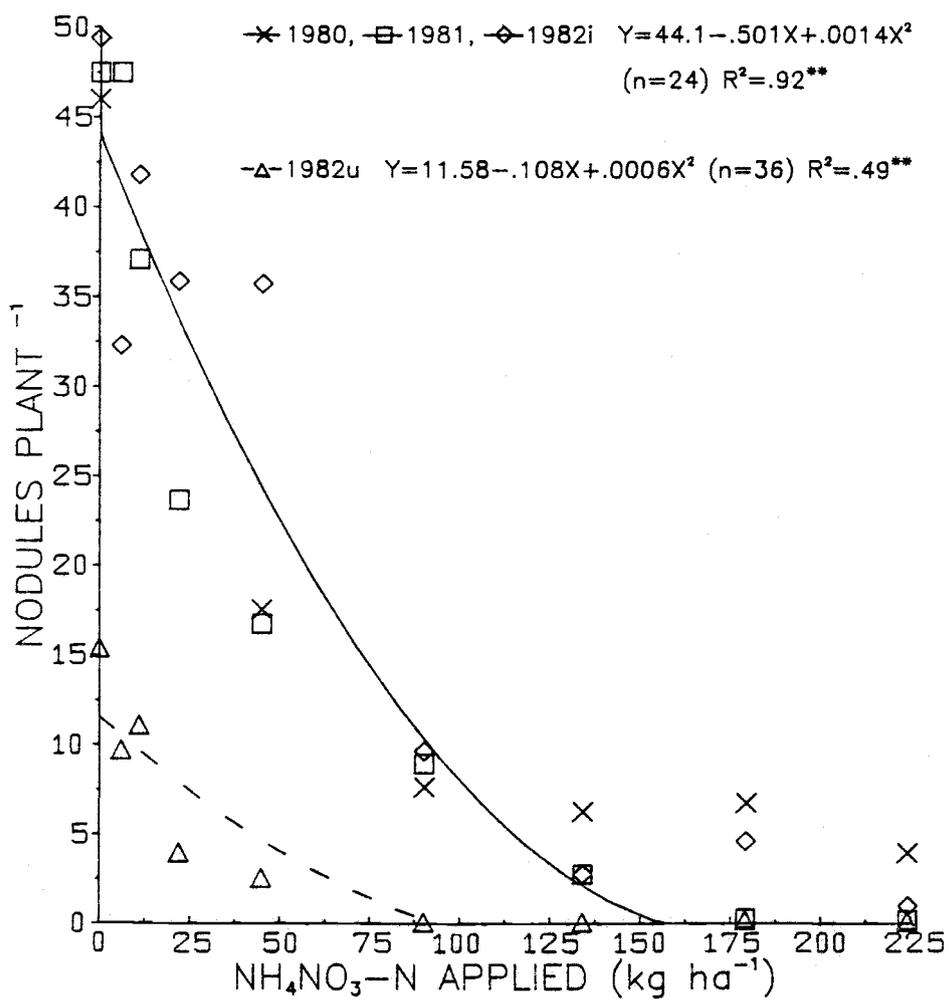


Figure 3. Nodules per plant of ten-week-old alfalfa seedlings as affected by ammonium nitrate fertilization at establishment; i = inoculated main plot treatment; u = uninoculated main plot treatment.

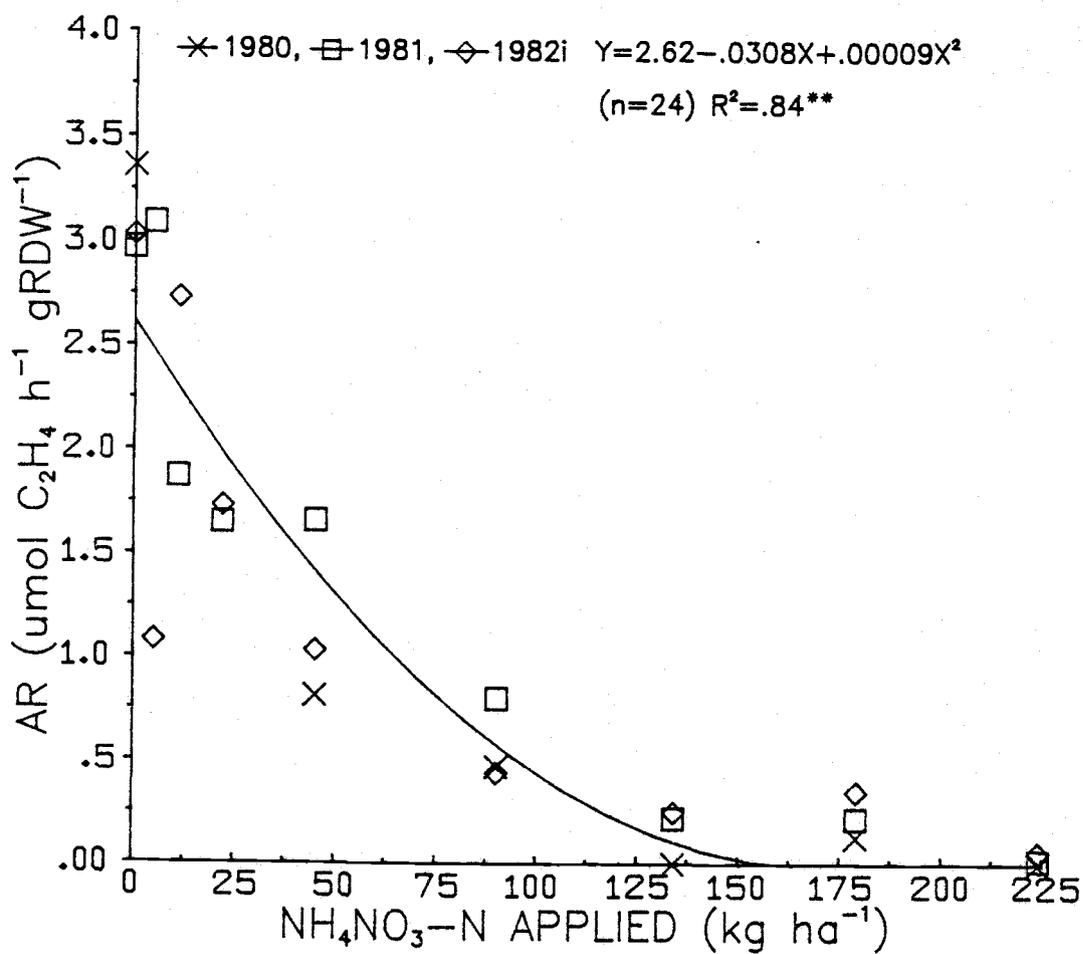


Figure 4. Acetylene reduction activity of ten-week-old alfalfa seedlings as affected by ammonium nitrate fertilization at establishment; i = inoculated main plot treatment.

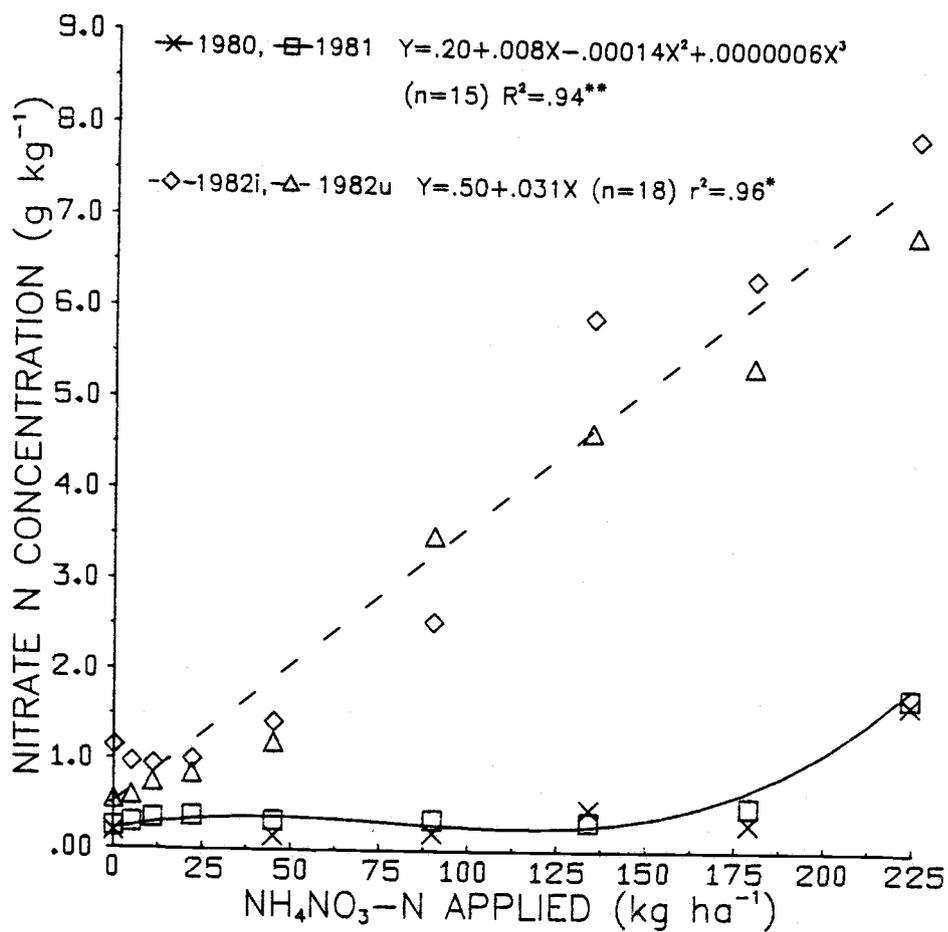


Figure 5. Herbage nitrate N concentration of ten-week-old alfalfa seedlings as affected by ammonium nitrate fertilization at establishment; i = inoculated main plot treatment; u = uninoculated main plot treatment.

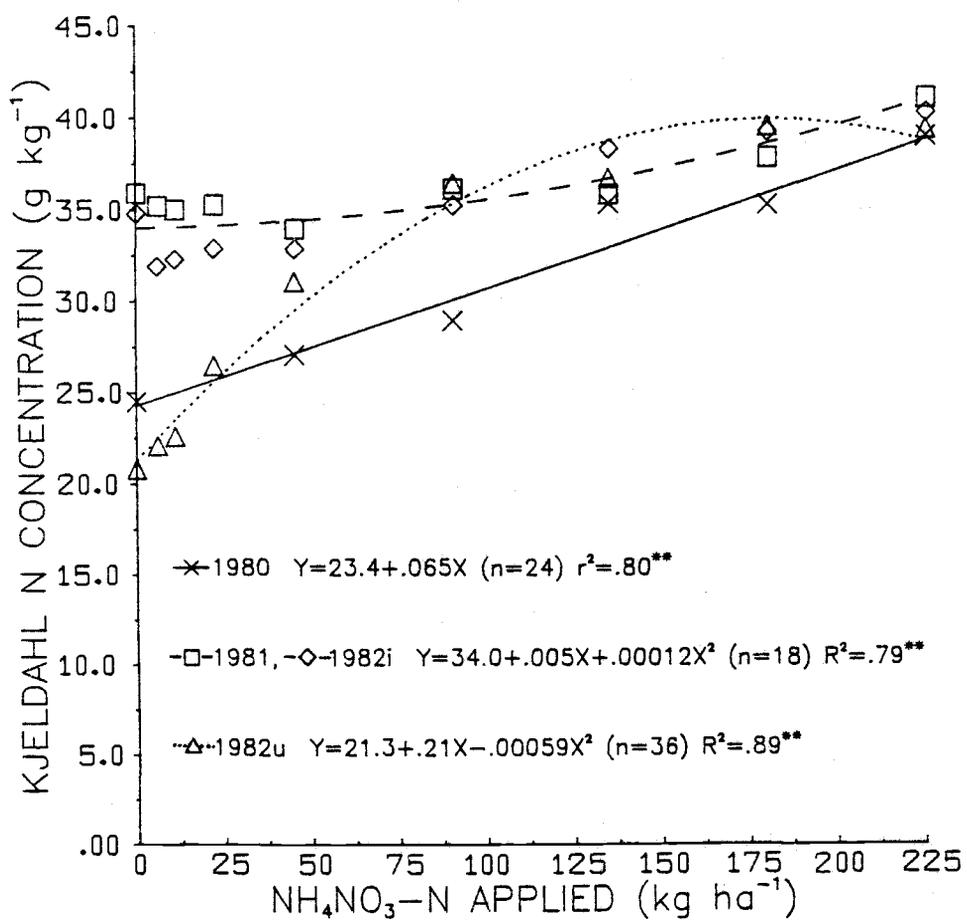


Figure 6. Herbage Kjeldahl N concentration of ten-week-old alfalfa seedlings as affected by ammonium nitrate fertilization at establishment; i = inoculated main plot treatment; u = uninoculated main plot treatment.

difference disappeared in the regrowth. An explanation for the pre-harvest response to combined N was not obvious, since nodules exhibited a normal pink coloration at 10 weeks. Also, acetylene reduction rates, nodule numbers per plant, and dry matter yields of these plants were equivalent to values in subsequent years where N deficiencies were not evident (Figs. 2, 3, and 4). Analysis of herbage tissue indicated that no other mineral nutrient deficiencies were present (data not shown) when compared to levels considered sufficient for alfalfa in the early bloom stage (Rhykerd and Overdahl, 1972). It was concluded that the N deficiency of the herbage was probably due either to low numbers of effective inoculant cells per seed at planting or to slow development of effective nodules. It is possible that the nodules had only recently become fully functional at the time of sampling for acetylene reduction and nodule enumeration. This would explain the high rates of acetylene reduction immediately preharvest and the disappearance of the N deficiency symptoms in the regrowth. Factors such as the number of viable R. meliloti in the peat inoculant may have influenced the rate of functional nodule development on these seedlings.

Experiment 2, 1981 Failure of weed control measures resulted in a weed infestation during the latter part of Experiment 2 (7 to 10 weeks after planting). Visual examination of the weed infestation indicated that it was positively correlated with the level of NH_4NO_3 fertilization. Because of the weed problem, dry matter yield determinations were not made, however areas were hand-weeded to allow measurement of acetylene reduction, nodulation, herbage

nitrate and Kjeldahl N concentrations. The increased weed density in the N fertilized plots has been observed in previous investigations which concluded that N fertilization increased the competitiveness of the weed population (Peters and Stritzke, 1970). These observations were interpreted as circumstantial evidence that the site of the 1981 experiment was deficient in mineralizable N, and therefore suggests that alfalfa is dependent on N_2 fixation for the major portion of its N requirement.

Trends of herbage nitrate concentration, nodulation, and acetylene reduction in response to NH_4NO_3 fertilization were similar to those observed in 1980 (Figs. 3, 4, and 5). Low rates of NH_4NO_3 application consistently resulted in decreased nodulation and acetylene reduction activity in comparison to untreated controls.

Inoculated plants maintained a uniform dark green coloration throughout Experiment 2 (1981). In contrast to data obtained from Experiment 1 (1980), Kjeldahl N concentration after 10 weeks was unaffected by moderate rates of NH_4NO_3 , and increased from 34 to 40 $g\ kg^{-1}$ at higher rates of NH_4NO_3 fertilization (Fig. 6). The lack of increased herbage N concentration in response to moderate rates of fertilizer N indicated that the inoculation procedure and/or inoculant strains had resulted in effective nodulation of seedlings by the R. meliloti in the inoculant. After 10 weeks of growth (early bloom), the uninoculated trap plants in areas outside the experiment exhibited the persistent light green coloration typical of N deficiency. The nodules on these plants were small and white, the typical color of ineffective nodules. Most native R. meliloti

isolates produced white nodules on plants in tube culture, in comparison with the pink nodules produced by isolates from inoculated plants. Results from effectiveness tests indicated that shoot dry weights of plants receiving native R. meliloti isolates were not significantly different from the N deficient, uninoculated control plants. In contrast, shoot dry weights of plants receiving inoculated plant isolates were similar to the control plants receiving KNO_3 . These results support the hypothesis that insufficient numbers of effective rhizobia were initially present in Experiment 1 (1980), and that the insufficiency resulted in the temporary response to N fertilizer during establishment.

Experiment 3, 1982 After 10 weeks of growth, the dry matter yields of the field grown inoculated main plots (1982i) exhibited a similar response to NH_4NO_3 fertilization as was seen in 1980 (Fig. 2). Yields from the uninoculated plots (1982u) were not different from their inoculated counterparts, however a NH_4NO_3 rate by inoculation interaction was evident (Table 2). Incremental yield increases arising from low rates of NH_4NO_3 on uninoculated plants were evident in comparison to the slight yield increases resulting from equivalent rates of NH_4NO_3 applied to inoculated plants.

Nitrate-N concentration increased markedly (0.5 to 7.7 g kg^{-1}) with increased NH_4NO_3 in Experiment 3, 1982 (Fig. 5). This response, which differed from 1980 and 1981, may have been the result of insufficient light energy for optimal nitrate reduction (Fig. 1). In 1980 and 1981 normal levels of irradiance were observed, and these levels averaged $2.0 \times 10^7 \text{ J m}^{-2} \text{ day}^{-1}$. In 1982, cloudy

Table 2. Summary of Experiment 3, 1982 main plot (inoculation) and interaction (inoculation x NH_4NO_3 -N rate) effects.

| Treatment | Herbage Kjeldahl-N Concentration | Dry Matter Yield | Acetylene Reduction Activity | Nodules per Plant | Herbage Nitrate-N Concentration |
|----------------------|--|---------------------------|---|-------------------------|---------------------------------------|
| | -- g kg ⁻¹ -- | -- Mg ha ⁻¹ -- | umol C ₂ H ₄ .h ⁻¹ .gRDW ⁻¹ | - no. - | -- g kg ⁻¹ -- |
| Inoculated | 35.0 | 2.3 | 1.47 | 27 | 3.1 |
| Uninoculated | 30.0 | 2.0 | 0.03 | 5 | 2.7 |
| Main plot LSD (0.05) | 2.0 | NS | 0.39 | 7 | NS |
| Interaction | * | ** | ** | NS | NS |

*,** Significant at the 0.05 and 0.01 levels of probability, respectively.

conditions persisted and levels of irradiance were below normal, especially during the 2 weeks prior to harvest when levels averaged $1.5 \times 10^7 \text{ J m}^{-2} \text{ day}^{-1}$. Beevers and Hageman (1972) cited many instances where light was shown to stimulate the activity of nitrate reductase. Light intensity has been implicated as a factor in nitrate accumulation in various crops (Bathurst and Mitchell, 1958; Crawford et al., 1961).

As in 1981, Kjeldahl N concentration in the inoculated plants was unaffected by low rates of NH_4NO_3 (0 to 45 kg N ha^{-1}), and was increased slightly by higher rates of NH_4NO_3 (Fig. 6). Kjeldahl N concentrations of the uninoculated, ineffectively nodulated plants increased in response to increasing rates of NH_4NO_3 up to 90 kg N ha^{-1} . The different trends for the inoculated and uninoculated plants are substantiated by an inoculation by NH_4NO_3 rate interaction (Table 2). The increase in Kjeldahl N of the uninoculated plants in response to increasing rates of NH_4NO_3 resembled the response of the ineffectively nodulated plants during the 1980 experiment.

Nodule numbers of uninoculated plants were more variable than their inoculated counterparts. A large proportion of this variation in nodules per plant arose from factors other than the NH_4NO_3 treatments ($R^2 = 0.49^{**}$). In this context, nodule enumeration data indicated that uninoculated plants from one of the replications had fewer nodules than the other replications, suggesting an uneven distribution of the native R. meliloti population over the field site. At 10 weeks, nodules on field grown plants from the

uninoculated main plots were small and white, similar to those on the trap plants in Experiment 2 (1981). In contrast, nodules on plants from the inoculated main plots were larger and pink. The plant tube method was again used to assess the effectiveness of selected nodule isolates representing the indigenous population of R. meliloti. As in 1981, shoot dry weights of plants grown in tube culture inoculated with indigenous isolates of R. meliloti were not significantly different from the uninoculated, N deficient controls.

Curvilinear decreases in nodulation and acetylene reduction rates of inoculated plants in 1982 were similar to the previous 2 years (Figs. 3 and 4). Using the fitted response curve (Fig. 3) to estimate the influence of NH_4NO_3 on nodulation, it was concluded that an application of 44 kg N ha^{-1} reduced nodulation by 45% (44 to 24 nodules plant^{-1} on inoculated plants, and 11 to 5 nodules plant^{-1} on uninoculated plants). Acetylene reduction rates of inoculated plants were also reduced 45% (2.63 to $1.42 \text{ umol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \text{gRDW}^{-1}$) by the application of 45 kg N ha^{-1} (Fig. 4). This rate of N fertilizer is commonly recommended by seed companies for the establishment of alfalfa. In contrast, previous greenhouse experiments indicated a stimulation of nodulation of effectively nodulated plants by low levels (2mM) of NH_4NO_3 in nutrient solution (Fishbeck and Phillips, 1981). The absence of similar increases in these experiments may be explained by variation in levels of available soil N under field conditions, since soil test results indicated that appreciable mineralization of N was observed at four weeks (Table 1). This mineralization may have been due to liming the soil (Ishaque and

Cornfield, 1972), which possibly enhanced the inhibitory effect of the applied NH_4NO_3 on nodulation.

Acetylene reduction activity of the uninoculated plants was generally negligible (Table 2), although detectable rates occurred randomly in a few of the uninoculated cores analyzed. Even though nodule numbers of the uninoculated plants were lower than their inoculated counterparts (Table 2), they still exhibited a curvilinear decrease in response to increasing rates of NH_4NO_3 (Fig 3). This inhibition of nodulation in response to low levels of NH_4NO_3 (0 to 45 kg N ha^{-1}) was of interest because the same plants were exhibiting symptoms of N deficiency (Fig 6). Similar results were obtained in Experiment 1 (1980). These results support the conclusion by Munns (1977) that nodulation is not directly influenced by the N nutrition of the host plant. These results also suggest that the nodulation response to applied NH_4NO_3 is independent of the effectiveness of the soil Rhizobium population.

It was concluded from these studies that NH_4NO_3 fertilization of seedling alfalfa under conditions similar to those described will not result in economical increases in the yield or quality of effectively nodulated seedling alfalfa. In contrast, NH_4NO_3 fertilization of ineffectively nodulated seedling alfalfa will probably increase herbage Kjeldahl N concentration, however possible undesirable consequences, such as increased herbage nitrate concentration and increased weed competition, may result.

CHAPTER II

Postharvest Nitrogen Fixation and Yield of Field-Grown Seedling
Alfalfa as affected by Ammonium Nitrate Fertilization

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ABSTRACT

Nitrogen fertilization of perennial forage legumes at establishment is a common yet controversial practice. Two field experiments were conducted in consecutive years (1981 and 1982) on a Woodburn silt loam soil (Aquultic Argixeroll) containing ineffective Rhizobium meliloti Dang. to examine the effects of preplant N fertilization and inoculation on the N nutrition and yield of seedling alfalfa during the regrowth period following the first harvest at ten weeks (early bloom). In 1981, treatments consisted of 4 rates of $\text{NH}_4\text{NO}_3\text{-N}$ (0, 45, 90, and 179 kg N ha⁻¹) applied prior to planting 'Apollo' alfalfa in a randomized complete block design. In 1982, a split block experimental design was used with 3 rates of $\text{NH}_4\text{NO}_3\text{-N}$ (0, 45, and 90 kg N ha⁻¹) as subplots and inoculated or uninoculated seed as main plots. Acetylene reduction (AR) and nodulation were evaluated at 4, 8, 12, 16, and 20 days postharvest in 1981 and at 2, 8, 18, 35, and 43 days postharvest in 1982. In the 1982 experiment, 2 subsequent harvests were also made (one in 1982 and one in 1983) to assess residual treatment effects on herbage dry matter yield and total herbage N assimilated. Nodules of uninoculated plants had very low levels of AR activity and, unlike inoculated plants, showed no evidence of postharvest senescence. Acetylene reduction rates of inoculated plants were high (3.0 $\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ g root dry weight}^{-1}$) immediately preharvest, and then declined an average of 68% at 2 to 4 days postharvest in both years. In comparison to untreated controls, the plants receiving the NH_4NO_3 treatments had lower postharvest AR activity levels and

fewer nodules plant⁻¹ during the initial 8 days postharvest. For example, at 8 days postharvest, 45 kg N ha⁻¹ decreased AR specific activity and nodulation of inoculated plants an average of 30% and 53%, respectively, in comparison to plants receiving no N. Over subsequent harvests, the NH₄NO₃ treatments increased herbage dry matter yield and total N content of uninoculated plants, but not to levels comparable to the inoculated plants. Since the inoculated alfalfa plants showed little herbage yield or total N response to the NH₄NO₃-N rates, it was concluded that during the establishment year, managerial emphasis should be placed on promoting N₂ fixation rather than N fertilization to obtain a consistent, economical source of N for herbage protein and dry matter production.

Additional Index Words: Medicago sativa L.; Rhizobium meliloti

Dang.; NH₄NO₃; Nodulation; Nitrogen fixation; Acetylene reduction.

INTRODUCTION

The application of fertilizer N in the establishment of pure stands of legumes was recommended by approximately half the states surveyed in a previous study (Hojjati et al., 1978). The lack of consensus on this subject reflects the inconclusive results in the body of literature in which this practice was examined (Tesar and Jackobs, 1972). Proponents of the use of N fertilization during the establishment of pure legume stands suggest that periods of N deficiency exist prior to the development of N_2 fixation in effective nodules (Lie, 1974). These periods of deficiency are especially critical if levels of available soil N are low and if effective Rhizobium meliloti Dang. are not present in sufficient numbers (Eardly et al., 1985). Nitrogen deficiency in perennial legumes such as alfalfa (Medicago sativa L.) may not be limited strictly to the period prior to the development of functional root nodules. In a greenhouse study using alfalfa clones, Fishbeck and Phillips (1981) found that an effective M. sativa - R. meliloti symbiosis was unable to provide sufficient reduced N for optimal growth during the first few regrowth cycles.

Several studies have examined acetylene reduction (AR) activity in greenhouse-grown alfalfa during regrowth and found that harvesting typically resulted in a 72% to 88% decline in AR activity from preharvest levels within the first few days after harvest, followed by a 2 to 3 week recovery period (Cralle and Heichel, 1981; Fishbeck and Phillips, 1982; Groat and Vance, 1981; Vance et al., 1979). Vance et al. (1979) measured the activity of nodule nitrate

reductase during early regrowth and found that it was inversely proportional to AR activity. Because of this inverse relationship, they suggested that increased nitrate reductase activity could be an alternate mechanism for supplying nitrogen to the seedling after harvest. While these results do not indicate that supplemental N is necessary for alfalfa establishment, they do suggest a possible explanation for the beneficial effects of the supplemental combined N in the experiments of Fishbeck and Phillips (1981).

Although the negative effects of nitrogen fertilization on N_2 fixation are well established (Fred et al., 1932), the influence of fertilizer N during seedling regrowth under field conditions needs to be clarified. Under conditions of low soil N, applications of fertilizer N at early stages of development may serve to complement, rather than negate the symbiotic potential of the plant (Allos and Bartholomew, 1959; Gibson, 1977). It is possible that if sub-standard inoculant is applied, or if faulty inoculation technique is used, N fertilizer may serve to sustain the legume crop until an effective population of Rhizobium can develop (Bell and Nutman, 1971).

The objectives of this study were to examine the effects of ammonium nitrate (NH_4NO_3) applied at planting on the postharvest AR activity, nodule number, herbage N concentration, and dry matter yield of seedling alfalfa under field conditions. Experiments were conducted in 1981 and 1982 as a continuation of an earlier series of experiments which examined the effects of NH_4NO_3 fertilizer on seedling alfalfa at 10 weeks after planting (Eardly et al., 1985).

Results from early experiments indicated that the native population of R. meliloti at the field site was ineffective in N₂ fixation. These conditions provided an opportunity in 1982 to compare the effects of ammonium nitrate (NH₄NO₃) fertilization on effectively and ineffectively nodulated plants by the inclusion of an inoculation variable.

MATERIALS AND METHODS

Site Description and General Treatment Applications

Field experiments were initiated during the summers of 1981 and 1982 at the Oregon State University Hyslop Crop Science Field Research Facility at Corvallis, Oregon. The soil at the 2 experimental sites for the 2 years was a Woodburn silt loam (fine, silty, mixed, mesic Aquultic Argixeroll). The pH of the soil was 5.7 prior to amendment. Total soil N and organic matter averaged 1 and 20 g kg⁻¹, respectively, in samples taken to a depth of 15 cm. Available nitrate N and ammonium N averaged 4 to 6 mg kg⁻¹ of each form prior to planting in the spring. Each site received lime, fertilizer supplements, and weed control as described previously (Eardly et al., 1985). In both years, field sites were irrigated with 5 to 7 cm of water 5 days prior to harvest. A similar irrigation treatment was applied at 20 days postharvest in 1982.

Experimental Design and Treatment Applications

1981 The experimental design was a randomized complete block with four replications. Commercial NH₄NO₃ was applied at 4 rates (0, 45, 90, and 179 kg N ha⁻¹) to plots 1.83 x 9.15 m using a 0.5 m gravity type spreader. The NH₄NO₃ treatments were applied and raked into the top few centimeters of soil the day before planting. Certified 'Apollo' alfalfa seeds were lime pelleted with gum arabic and fine CaCO₃ as described by Vincent (1970) using a commercial peat inoculant containing R. meliloti obtained from the Nitragin

Co., Milwaukee, WI. The seeds were planted on 19 June, 1981 using 14 kg ha⁻¹ in 15 cm rows at approximately 1 cm depth using a 10 row, small plot, cone-type planter.

1982 Based on previous evidence that the native population of R. meliloti was ineffective in N₂ fixation (Eardly et al., 1985), a split block experimental design was used to compare the effect of fertilizer N on effectively and ineffectively nodulated seedling alfalfa. Main plot treatments consisted of inoculated and uninoculated lime pelleted seed which was planted on 25 May, 1982. Subplots consisted of 3 rates of NH₄NO₃-N (0, 45, and 90 kg N ha⁻¹) applied the day before planting.

Analytical Procedures and Measurements

Field Harvest All harvests were made when the plants were in the early bloom stage of development. The regrowth time course commenced at 71 days after planting when plots were harvested with a sickle-bar mower to 5 cm from a 0.92 x 4.58 m area in the center of each plot. In the 1982 experiment, two subsequent harvests were made; one at the termination of the seedling regrowth time course on 19 September, 1982; and a second the following spring on 24 May, 1983. The purpose of the subsequent harvests was to assess residual treatment effects on yield, N concentration, and total N content of the herbage. Immediately after harvest the herbage was dried in a forced air oven at 65 °C, weighed, and subsamples were ground to pass a 1 mm screen for Kjeldahl N analysis.

Acetylene Reduction Assay and Nodule Enumeration Evaluation

of N_2 fixation consisted of an in situ AR assay followed by nodule enumeration, and is described in detail elsewhere (Eardly et al., 1985). In 1981, N_2 fixation was evaluated at 4, 8, 12, 16, and 20 days after the initial harvest. In 1982, N_2 fixation was evaluated over a longer time course at 2, 8, 18, 35, and 43 days after the initial harvest. On each sampling date, one core per treatment per replication was evaluated for N_2 fixation activity. Each cylindrical soil core was 8.5 x 25.0 cm and contained 2-3 intact plants. For comparative purposes, AR activity ($\mu\text{mol h}^{-1}$) of control plants in 1982 was expressed on a per plant basis and on a per gram of root dry weight (RDW) basis (specific activity). Immediately after the AR assay, the soil was gently rinsed from around the roots over a 1 mm screen in preparation for nodule enumeration. Because of the coralloid morphology of some of the nodules, each individual lobe of nodule tissue was counted as an individual nodule during enumeration.

Statistical Analysis To determine the type of response to the $\text{NH}_4\text{NO}_3\text{-N}$ rates on each sampling date, a regression analysis for each date was conducted. The observed trend is indicated in columnar form in Tables 1 through 5. To permit comparison of means between dates over the regrowth time course, the standard error of the mean (SE) was calculated for each treatment combination on each sampling date using the values of the 4 replicate samples.

RESULTS

During both years, the AR activity of inoculated seedlings declined within 2 to 4 days after harvest (Fig. 7, Tables 3 and 4). The postharvest decline in AR specific activity (per gram root dry weight) of inoculated control plants dropped an average of 68% from preharvest levels. Levels of AR activity per plant in 1981 (data not shown) were similar to values shown for 1982 (Fig. 7). The rate of recovery of AR activity during regrowth was greater when expressed on a per plant basis than when expressed on a per gram root dry weight basis (Fig. 7, Table 4). In the inoculated control plants (1982) recovery of AR activity per plant was most rapid between 8 and 18 days postharvest when rates increased from 0.32 to 0.67 $\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ plant}^{-1}$ (Fig. 7). Rates of AR per plant then declined prior to the second harvest. In contrast, when AR was expressed on a per gram root dry weight basis, the specific activity of inoculated plants during regrowth remained low and increased relatively slowly during the regrowth period, and did not reach preharvest levels (Tables 3 and 4). The contrasting trends between AR per plant and AR per gram of root dry weight is due to the increase in root dry weight during the initial 18 days of the regrowth period (Fig. 7).

On each sampling date, the NH_4NO_3 rates resulted in a linear decrease in AR specific activity through day 8 of the regrowth time course (Tables 3 and 4). Acetylene reduction per plant followed similar trends, however variation arising from normal differences in seedling size under field conditions prevented the use of those

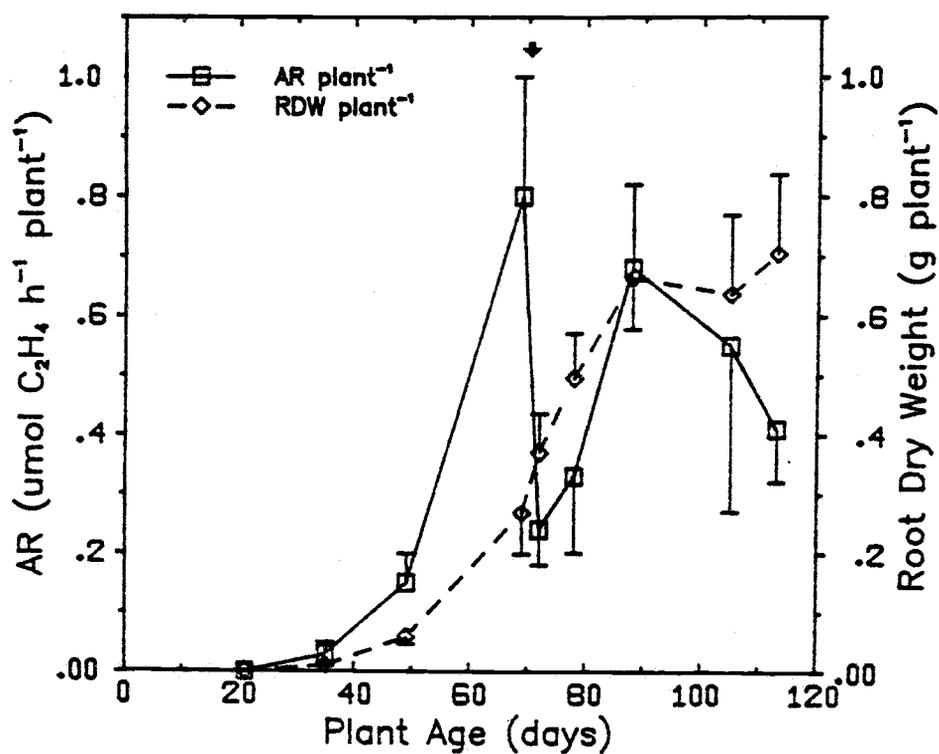


Figure 7. Acetylene reduction per plant (\pm SE) and root dry weight per plant (\pm SE) of seedling alfalfa over time in 1982. Seeds were planted at time zero and the arrow denotes first harvest.

Table 3. Acetylene reduction specific activity (\pm SE) of seedling alfalfa over the regrowth time course in 1981 in response to 4 rates of ammonium nitrate N applied at planting.

| N Rate | Pre-harvest † | Days Postharvest | | | | |
|----------------------------|---------------|--|-------------|-------------|-------------|-------------|
| | | 4 | 8 | 12 | 16 | 20 |
| kg ha ⁻¹ | | umol C ₂ H ₄ h ⁻¹ g RDW ⁻¹ | | | | |
| 0 | 2.97 ± 0.41 | 1.35 ± 0.32 | 1.48 ± 0.51 | 1.09 ± 0.27 | 1.54 ± 0.99 | 2.07 ± 0.29 |
| 45 | 2.56 ± 0.90 | 0.90 ± 0.27 | 1.25 ± 0.54 | 2.54 ± 0.39 | 0.54 ± 0.13 | 2.80 ± 0.54 |
| 90 | 0.80 ± 0.59 | 0.69 ± 0.24 | 0.25 ± 0.12 | 1.29 ± 0.67 | 0.52 ± 0.22 | 1.08 ± 0.42 |
| 179 | 0.27 ± 0.09 | 0.13 ± 0.05 | 0.07 ± 0.04 | 1.16 ± 0.60 | 0.26 ± 0.10 | 0.70 ± 0.37 |
| <u>Source of Variation</u> | | ----- level of significance ‡ ----- | | | | |
| linear | - | ** | * | NS | NS | * |
| quadratic | - | NS | NS | NS | NS | NS |
| residual | - | NS | NS | NS | NS | NS |

* , ** Significant at the 0.05 and 0.01 levels of probability, respectively.

† Results reported previously (Eardly et al., 1985).

‡ Response trends pertain only to data within the same column.

Table 4. Acetylene reduction activity specific activity (\pm SE) of seedling alfalfa over the re-growth time course in 1982 in response to inoculation (Inoc) and 3 rates of ammonium nitrate N applied at planting. Treatment code; I = inoculated seed; U = uninoculated seed.

| Inoc | N Rate | Pre-harvest [†] | Days Postharvest | | | | |
|------|---------------------|--|------------------|-----------------|-----------------|-----------------|-----------------|
| | | | 2 | 8 | 18 | 35 | 43 |
| | kg ha ⁻¹ | ----- umol C ₂ H ₄ h ⁻¹ g RDW ⁻¹ ----- | | | | | |
| I | 0 | 3.03 \pm 0.46 | 0.49 \pm 0.09 | 0.59 \pm 0.17 | 0.94 \pm 0.24 | 0.83 \pm 0.25 | 0.80 \pm 0.25 |
| | 45 | 1.03 \pm 0.39 | 0.36 \pm 0.12 | 0.33 \pm 0.13 | 1.25 \pm 0.54 | 0.85 \pm 0.14 | 1.09 \pm 0.42 |
| | 90 | 0.45 \pm 0.19 | 0.16 \pm 0.07 | 0.09 \pm 0.05 | 0.35 \pm 0.24 | 0.58 \pm 0.10 | 0.61 \pm 0.16 |
| U | 0 | 0.00 | 0.00 | 0.00 | 0.03 \pm 0.02 | 0.00 | 0.22 \pm 0.17 |
| | 45 | 0.00 | 0.00 | 0.00 | 0.12 \pm 0.10 | 0.07 \pm 0.05 | 0.20 \pm 0.18 |
| | 90 | 0.00 | 0.00 | 0.00 | 0.01 \pm 0.01 | 0.01 \pm 0.01 | 0.14 \pm 0.10 |

Source of Variation

| | ----- level of significance ^{††} ----- | | | | | |
|----------------|---|----|----|----|----|----|
| Inoculation | - | ** | * | * | * | * |
| Response to N; | | | | | | |
| linear | - | * | * | NS | NS | NS |
| residual | - | NS | NS | NS | NS | NS |
| Interaction | - | * | * | NS | NS | NS |

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

[†] Results reported previously (Eardly et al., 1985).

^{††} Response trends pertain only to data within the same column.

values for comparison of N rate effects. Acetylene reduction activity of uninoculated plants in 1982 was not evident until 18 days postharvest (13 weeks postplant) and was not influenced by the NH_4NO_3 rates. Neither inoculation nor NH_4NO_3 application rates had a significant effect on root dry weights over the time course (data not shown), however root dry weights were generally smaller in 1981 than in 1982 (295 ± 39 vs. 598 ± 75 mg RDW per plant, respectively). Thus, postharvest AR specific activity levels (per g RDW) were lower in 1982 than in 1981 (Tables 3 and 4).

Harvesting had little effect on the number of nodules per plant (Tables 5 and 6). The number of nodules per plant remained relatively stable during the 8 to 12 days of the initial regrowth period, followed by a large increase at 16 to 18 days postharvest. The influence of the 45 and 90 kg N ha^{-1} rates of NH_4NO_3 on nodulation was evident only through 18 to 20 days postharvest (13 weeks postplant) in both years. Plants which received the highest rate of $\text{NH}_4\text{NO}_3\text{-N}$ (179 kg ha^{-1}) maintained only a few nodules per plant throughout the time course (Table 5).

It was observed during nodule enumeration on the first sampling date in both 1981 and 1982 that a green senescent region had developed at the proximal end of nodules on the inoculated plants, a phenomenon which has been described previously (Vance et al., 1980). The distal portion of these nodules exhibited the red coloration of leghemoglobin. There was no evidence of this senescent region on any of the nodules of the uninoculated plants in 1982, which were white and apparently ineffective in N_2 fixation both preharvest and

Table 5. Nodules per plant (\pm SE) of seedling alfalfa over the regrowth time course in 1981 in response to 4 rates of ammonium nitrate applied at planting.

| N Rate | Pre-harvest † | Days Postharvest | | | | |
|----------------------------|---|------------------|-------------|-------------|-------------|-------------|
| | | 4 | 8 | 12 | 16 | 20 |
| kg ha ⁻¹ | ----- nodules plant ⁻¹ ----- | | | | | |
| 0 | 48 \pm 20 | 30 \pm 11 | 30 \pm 16 | 33 \pm 13 | 66 \pm 18 | 62 \pm 15 |
| 45 | 17 \pm 7 | 19 \pm 6 | 17 \pm 7 | 18 \pm 6 | 16 \pm 6 | 43 \pm 4 |
| 90 | 9 \pm 6 | 14 \pm 7 | 5 \pm 3 | 15 \pm 7 | 27 \pm 10 | 10 \pm 5 |
| 179 | 0 | 2 \pm 1 | 5 \pm 2 | 5 \pm 2 | 4 \pm 2 | 6 \pm 2 |
| <u>Source of Variation</u> | ----- level of significance ‡ ----- | | | | | |
| linear | - | ** | NS | * | ** | ** |
| quadratic | - | NS | NS | NS | * | NS |
| residual | - | NS | NS | NS | * | NS |

* **, Significant at the 0.05 and 0.01 levels of probability, respectively.

† Results reported previously (Eardly et al., 1985).

‡ Response trends pertain only to data within the same column.

Table 6. Nodules per plant (\pm SE) of seedling alfalfa over the regrowth time course in 1982 in response to inoculation (Inoc) and 3 rates of ammonium nitrate N applied at planting. Treatment code; I = inoculated seed; U = uninoculated seed.

| Inoc | N Rate | Pre-harvest † | Days Postharvest | | | | |
|------|---------------------|---------------|-----------------------------|---------|----------|----------|----------|
| | | | 2 | 8 | 18 | 35 | 43 |
| | kg ha ⁻¹ | | nodules plant ⁻¹ | | | | |
| I | 0 | 49 ± 14 | 52 ± 8 | 64 ± 8 | 112 ± 21 | 89 ± 32 | 113 ± 24 |
| | 45 | 36 ± 16 | 20 ± 8 | 24 ± 8 | 59 ± 9 | 163 ± 36 | 93 ± 21 |
| | 90 | 10 ± 5 | 10 ± 4 | 9 ± 4 | 31 ± 10 | 138 ± 51 | 75 ± 15 |
| U | 0 | 15 ± 5 | 13 ± 4 | 23 ± 15 | 30 ± 9 | 107 ± 44 | 154 ± 39 |
| | 45 | 3 ± 2 | 0 | 2 ± 1 | 19 ± 6 | 146 ± 45 | 91 ± 35 |
| | 90 | 0 | 0 | 1 ± 1 | 8 ± 3 | 45 ± 13 | 31 ± 14 |

| Source of Variation | level of significance ‡ | | | | | |
|---------------------|-------------------------|----|----|----|----|----|
| Inoculation | - | * | * | * | NS | NS |
| Response to N; | | | | | | |
| linear | - | ** | ** | ** | NS | NS |
| residual | - | * | NS | NS | NS | NS |
| Interaction | - | * | ** | * | NS | NS |

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

† Results reported previously (Eardly et al., 1985).

‡ Pertains only to data within the same column.

immediately postharvest. At 35 and 43 days postharvest, however, some of the larger nodules on the uninoculated plants developed a uniform light pink coloration, which corresponded with detectable levels of AR activity at that time. At the end of the regrowth time course in September, 1982, the yield and total herbage N of the uninoculated plants increased linearly with NH_4NO_3 application, while inoculated plants were unaffected by the NH_4NO_3 application (Table 7). This response confirmed the lack of effective native R. meliloti at the field site. These results also indicated that the application of 90 kg N ha^{-1} at establishment was not sufficient to meet the N requirements of the seedlings during their first regrowth cycle. Dry matter yields and total herbage N values of plants the following spring indicated that the inoculation effects were persistent, and that the fertilizer N effects had diminished, with the exception of a slight linear increase in herbage N concentration of the inoculated plants.

Table 7. Residual effects of 3 ammonium nitrate N rates and inoculation (Inoc) on herbage dry matter yield (\pm SE) and Kjeldahl N concentration (\pm SE) of second and third harvests of seedling alfalfa. Treatment code; I = inoculated seed; U = uninoculated seed.

| Inoc | N Rate | Second harvest September 1982 | | | Third harvest May 1983 | | |
|----------------------------|---------------------|--|--------------------------|-------------------------|---------------------------|--------------------------|---------------------------|
| | | Dry Matter Yield | Kjeldahl N Concentration | Total Herbage N | Dry Matter Yield | Kjeldahl N Concentration | Total Herbage N |
| | kg ha ⁻¹ | Mg ha ⁻¹ | - g kg ⁻¹ - | - kg ha ⁻¹ - | Mg ha ⁻¹ | - g kg ⁻¹ - | -- kg ha ⁻¹ -- |
| I | 0 | 2.42 \pm 0.35 | 32.4 \pm 1.1 | 78.4 \pm 9.9 | 6.50 \pm 0.46 | 23.8 \pm 0.1 | 154.7 \pm 11.3 |
| | 45 | 2.50 \pm 0.22 | 32.4 \pm 0.2 | 81.0 \pm 7.0 | 6.05 \pm 0.16 | 24.9 \pm 0.5 | 150.6 \pm 2.0 |
| | 90 | 2.38 \pm 0.26 | 30.7 \pm 0.8 | 73.1 \pm 6.2 | 6.05 \pm 0.22 | 26.1 \pm 0.5 | 157.9 \pm 5.0 |
| U | 0 | 0.78 \pm 0.12 | 25.0 \pm 0.7 | 19.5 \pm 2.7 | 4.30 \pm 0.89 | 23.2 \pm 1.2 | 99.8 \pm 22.1 |
| | 45 | 1.23 \pm 0.14 | 21.9 \pm 1.0 | 26.9 \pm 3.5 | 4.04 \pm 0.62 | 23.5 \pm 1.6 | 95.0 \pm 20.0 |
| | 90 | 1.70 \pm 0.10 | 24.7 \pm 0.8 | 42.0 \pm 3.1 | 3.98 \pm 0.43 | 22.9 \pm 1.5 | 91.1 \pm 4.7 |
| <u>Source of Variation</u> | | ----- level of significance [†] ----- | | | | | |
| Inoculation | | * | ** | * | ** | NS | * |
| Response to N; | | | | | | | |
| linear | | * | NS | NS | NS | NS | NS |
| residual | | NS | * | NS | NS | NS | NS |
| Interaction | | ** | NS | ** | NS | ** | NS |

* **, Significant at the 0.05 and 0.01 levels of probability, respectively.

[†] Pertains only to data within the same column.

DISCUSSION

Postharvest decreases in AR activity of greenhouse-grown alfalfa have been reported previously (Cralle and Heichel, 1981; Fishbeck and Phillips, 1982; Groat and Vance, 1981; Vance et al., 1979). The results reported here are of importance since they confirm the response under field conditions. As was found with greenhouse conditions, levels of AR activity were high prior to harvest, dropped noticeably at 2 to 4 days postharvest, and then increased at 18 to 20 days postharvest. In general, postharvest levels of AR per gram of root dry weight were noticeably lower in 1982 than in 1981. This may have been due to the lower root dry weights of samples taken in 1981, since AR rates were expressed on a per gram root dry weight basis, and since levels of AR per plant were similar both years. It is also possible that variation in environmental conditions, eg. soil moisture, may have influenced AR specific activity (Gibson, 1977).

Decreased nodulation of mature greenhouse-grown alfalfa in response to harvesting has been reported previously (Cralle and Heichel, 1981), however the current field experiments did not reveal this phenomenon consistently. This may have been due in part to the immaturity of the plants in this study, which were still undergoing rapid root development during the early portion of the regrowth time course. Nodule development of the inoculated plants during seedling regrowth coincided with the recovery of AR activity per gram root dry weight, in that both increased noticeably at 16 to 18 days postharvest. Previous studies using older plants, showed that

resumption of AR activity following harvest was correlated with expansion in leaf area and carbon exchange rates (Fishbeck and Phillips, 1982). The current study suggests that increased nodule numbers corresponding with increased seedling root development may also be an important factor in the recovery of N_2 fixation activity during seedling regrowth under field conditions.

Although soil N levels were not measured during the regrowth period, it was apparent from the diminished N_2 fixation activity and the consistent herbage total N values in response to the NH_4NO_3 application rates that the plants initially utilized the fertilizer N and then developed N_2 fixation potential to replace the depleted fertilizer N source. This interpretation is supported by the nodulation results which showed that the plants receiving high rates of supplemental N had fewer nodules and lower AR activity over time. In contrast to previous greenhouse work with alfalfa clones (Fishbeck and Phillips, 1982), these results suggest that under field conditions the combined contributions of seed N, soil N, and fixed N in effectively nodulated plants are sufficient to attain high herbage yields and N concentrations during seedling regrowth, and that high rates of fertilizer N ($>90 \text{ kg N ha}^{-1}$) would be required to compensate for inadequate contributions from the latter 2 sources.

Because AR activity is decreased by harvesting, and nodule nitrate reductase activity has been reported to increase post-harvest, it was of interest to determine if fertilizer N would complement the N_2 fixation process during early regrowth by providing an alternate source of combined N for shoot development.

These results suggest that there was no benefit to applying combined N, since fertilizer N decreased the AR specific activity and nodulation of inoculated plants during regrowth, with no concomitant increase in dry matter yield or total N in subsequent harvests. Rather than complementing N_2 fixation, fertilizer N apparently replaced N_2 fixation during the regrowth phase of development. Similar conclusions were drawn in previous greenhouse experiments where 40 and 80 kg N ha⁻¹ as KNO₃ were applied at harvest (Groat and Vance, 1981; Vance and Heichel, 1981). In one of these studies it was determined that increased nodule nitrate reductase activity in alfalfa following harvest resulted in only a minor contribution of combined N to the plant during early regrowth, and that reduced nitrogenase activity after shoot removal does not limit vegetative regrowth (Vance and Heichel, 1981).

The use of NH₄NO₃ to sustain ineffectively nodulated seedling alfalfa was also examined. Previous experiments by Bell and Nutman (1971) concluded that under the conditions of their experiments, a small native population of effective R. meliloti could eventually proliferate and dominate an otherwise ineffective population. In the current experiments, the uninoculated plants remained ineffectively nodulated throughout the duration of the experiment, although low levels of AR activity were observed late in the time course. Since all of the uninoculated plants in 1982 were N deficient during regrowth, and since nodulation of these plants was initially decreased and later unaffected by the NH₄NO₃-N treatments, it was concluded that supplemental fertilizer N had no beneficial

role in promoting N_2 fixation potential of ineffectively nodulated plants. Indeed, the evidence suggests that fertilizer N inhibits nodulation, even under conditions where the level of mineral N is below levels necessary for optimum growth.

The delayed development of AR activity in the nodules of the uninoculated plants in 1982 is unusual, however a similar phenomenon has been reported previously with ineffectively nodulated Phaseolus vulgaris (Baird et al., 1983). These observations suggest a common explanation for the lack of nodule effectiveness which is occasionally observed in these two plant species, i.e. a lack of coordinated nodule development conditioned by ineffective microsymbionts (Baird et al., 1983).

In summary, the results of these experiments verified the existence of a postharvest decline in AR activity in field-grown seedling alfalfa, which probably occurred due to competition for limited photosynthate between the regrowing plant and N_2 fixation in the nodules. The results also indicated that supplemental N as NH_4NO_3 applied preplant had no complementary effect on N_2 fixation potential, total herbage N, or dry matter yield of effectively nodulated seedling alfalfa during early regrowth. In contrast, the total herbage N and dry matter yield of ineffectively nodulated alfalfa seedlings did respond to NH_4NO_3 applications, however rates as high as 90 kg N ha^{-1} were unable to provide sufficient combined N for optimal plant growth. Additionally, the results suggested that the use of NH_4NO_3 to temporarily sustain ineffectively nodulated plants was of no benefit in establishing an effective symbiotic

relationship. These results indicate that an effective symbiotic relationship in alfalfa relies primarily on the presence of a population of effective microsymbionts, and not on the presence of combined N. From these results, it was concluded that during the establishment year, managerial emphasis should be placed on inoculation rather than on N fertilization to obtain a consistent, economical source of N for herbage protein and dry matter production.

Chapter III

Characteristics of Alfalfa Nodule Isolates Ineffective
in Nitrogen Fixation

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ABSTRACT

The widely grown forage legume, alfalfa (Medicago sativa L.) relies on a symbiotic relationship with Rhizobium meliloti Dang. for most of its N requirements. The high protein content and yield of alfalfa make it a particularly desirable crop, however its range of adaptation is limited by the sensitivity of its microsymbiont, R. meliloti, to moderately acid soil conditions (pH 5.0 to 6.0). The intolerance of R. meliloti to acid soils is not well understood. However, it is known that R. meliloti will not proliferate in acid soils, and ineffectiveness in R. meliloti has been correlated with increasing soil acidity. This study was initiated to characterize an indigenous population of ineffective Medicago sativa nodule isolates in an effort to explain their persistence in moderately acid soils. Field isolates were characterized by analysis of growth rate, acid production on conventional growth media, and their symbiotic performance. Thirty-one out of thirty-two randomly selected isolates were found to be ineffective in N_2 fixation. Most isolates contained one or more anomalous characteristics, including either slow growth rate, lack of acid production on conventional media, or both. It was determined that the slow growth was probably not due to inadequacies of the growth medium. Infectiveness tests on a broad range of legume hosts revealed an unusual cross-infective relationship between the native M. sativa nodule isolates and Phaseolus vulgaris. These results provided circumstantial evidence that in some situations, ineffective nodulation of M. sativa may be

due to the presence of promiscuous, native Rhizobium species.

Additional Index Words; Medicago sativa L.; Rhizobium meliloti
Dang.; Nitrogen fixation.

INTRODUCTION

In describing symbiotic N_2 fixation potential between Rhizobium bacteria and their respective plant hosts, the terms effective and ineffective are frequently used. Effective symbiotic associations are capable of supplying sufficient reduced N to the plant host for optimal plant development, while ineffective associations provide either insufficient amounts of reduced N, or in some cases none at all. Ineffective symbiotic relationships can occur under various situations. For example, if a plant host is infected by an incompatible species or strain of Rhizobium, ineffective nodules may result (Nutman, 1965). Either the host or the microsymbiont can be responsible for the incompatibility (Vance and Johnson, 1981).

Environmental factors such as soil acidity may also influence the effectiveness of symbiotic relationships, particularly through its effect on Rhizobium species which are known to be acid-sensitive, such as R. meliloti (Graham and Parker, 1964). It has been established that moderate acidity (pH 5.5) in nutrient solution has a negative effect on the rhizobial infection process in alfalfa (Munns, 1968a). Laboratory and field studies suggest that acidity may also have negative effects on the survival and multiplication of R. meliloti in soils (Barber, 1980; Lowendorf et al., 1981; Rice et al., 1977). In an Australian greenhouse study using Medicago truncatula as the plant host, Robson and Loneragan (1970) suggested that, in terms of relative impact on N_2 fixation activity, the adverse effects of soil acidity on the growth and survival of R. meliloti were more important than its adverse effects on the

infection process. In another greenhouse study, Rice (1975) found that as soil acidity increased from pH 5.4 to 4.9, the fresh weight of ineffective nodules on M. sativa increased proportionately.

Field observations from various locations in the northwestern U. S. and western Canada have indicated that the persistence of an ineffective R. meliloti in moderately acid soils is a common, yet unexplained phenomenon (Barber, 1980; Bottomley and Jenkins, 1983; Eardly et al., 1985; Rice et al., 1977; Weber and Leggett, 1966). Managerial practices which have proven to be useful in addressing this problem consist of liming the soil to a pH of 6.0 or greater in conjunction with the application of large quantities of effective R. meliloti inoculants (Burton, 1972). In certain areas of the northwestern U.S. however, there has been a reluctance to increase soil pH above 5.8 in crop rotations, since higher pH's have been associated with an increased severity of take-all root rot disease of winter wheat caused by the fungus Gaeumannomyces graminis var. tritici (Jackson et al., 1983). The sensitivity of alfalfa to lower pH's thus limits its acceptability as a crop for use in rotation with wheat. Recent investigations on the use of acid-tolerant strains of R. meliloti suggest that the proliferation of these strains under acid soil conditions is dependent upon the presence of the host plant (Lowendorf and Alexander, 1983). In contrast, there is little information on characteristics of R. meliloti which may influence its ability to persist and maintain symbiotic effectiveness traits under acid soil conditions when no host is present. In a survey of R. meliloti isolates obtained from diverse locations

within the U.S., Ellis and Barnes (1983) concluded that acid-tolerance of R. meliloti was apparently related to geographic origin of the host cultivar.

This investigation was initiated in response to results obtained from a field study where it was determined that a field site having moderately acid soil conditions (pH 5.7) contained only ineffective, native R. meliloti. Since alfalfa had not been grown on these sites for at least ten years, it was of interest to determine if the native population of R. meliloti possessed other unusual traits which were associated with its persistence and ineffectiveness. It was noted that when grown in pure culture, these organisms exhibited an unusually slow growth rate in comparison to the strains of R. meliloti which formed effective symbiosis. An investigation was initiated to characterize selected members of this native population. The objective of these studies was to verify that the native Rhizobium were indeed R. meliloti, and to provide an explanation for their anomalous growth rate and ineffectiveness.

MATERIALS AND METHODS

Source of Strains

Medicago sativa (cv. 'Apollo') nodule isolates were obtained from 2 field sites located at Corvallis, Oregon in 1981 and 1982, respectively. The sites were located at the Hyslop Crop Science field research facility. Alfalfa had been grown at both sites previously, however during the past 10 years the plots had been in either fallow or wheat. There were no indigenous Medicago species in the region, however M. lupulina has been observed growing as an introduced turfgrass weed (W. S. McGuire, pers. comm.). The soil at the sites was a moderately acid (pH 5.5 to 5.7) silt loam (Aquultic Argixeroll), which was limed to pH 6.2 one month prior to planting. Seeds for trap plants were surface-sterilized by standard procedures (Vincent, 1970) using a brief ethanol rinse followed by a 3 minute immersion in acidified 0.2% HgCl₂. The seeds were then rinsed in sterile water, dried, and lime pelleted with sterile peat (exposed to 5 MR from a ⁶⁰Co source). For comparative purposes, a similar batch of seed received Nitragin 'A' peat inoculant. Each seed treatment was then planted at four separate locations within each field site. Seeds from each treatment were also germinated in sterile closed plant tubes (3.0 x 20 cm) containing 20 mL of a modified Jensen's seedling agar (Bottomley and Jenkins, 1983; Vincent, 1970) to assess seed sterilization and inoculation procedures. The seedlings in plant tubes were grown in a greenhouse with a typical day/night temperature range of 30/20 °C under an average midday

light intensity of $800 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$. Detailed analysis of N_2 fixation activity and herbage N composition of the field-grown plants is described elsewhere (Eardly et al., 1985).

Effectiveness and infectiveness of native Rhizobium isolates was evaluated by removing sixteen plants from each inoculation treatment. The sequence of experiments using isolates from these plants is summarized in Fig. 8. Two nodules were randomly selected and removed from each plant. The nodules were surface-sterilized by a brief immersion in 95% ethanol followed by a 3 min immersion in 0.1% acidified HgCl_2 (Vincent, 1970). Nodules were then rinsed, squashed and streaked onto plates containing yeast-extract mannitol (YEM) agar (Vincent, 1970). Isolates were identified using a 3 digit code (001-200). Plates were incubated at $30 \pm 1 \text{ } ^\circ\text{C}$ for 4 to 7 d. Single colonies were picked and restreaked 3 times prior to storage on YEM slants at $4 \text{ } ^\circ\text{C}$. Isolates were also obtained from nodules on plants grown in plant tubes. Slants of all strains were removed from storage and restreaked every 3 to 4 months. Alfalfa (cv. 'Apollo') seeds were surface-sterilized and germinated on sterile 10% water agar plates for 48 h. The closed plant tube method mentioned earlier was used to grow the plants aseptically. One seedling was transferred to each cotton-stoppered plant tube. These were then transferred to the greenhouse. Individual field isolates were grown in 50 mL of sterile YEM broth medium in 250 mL Erlenmeyer flasks for 2 to 7 days at $30 \pm 1 \text{ } ^\circ\text{C}$ on a rotary shaker (200 rpm). Five days after planting, seedlings were inoculated (ca. 10^8 cells/seedling) with native M. sativa nodule isolates. Fifteen

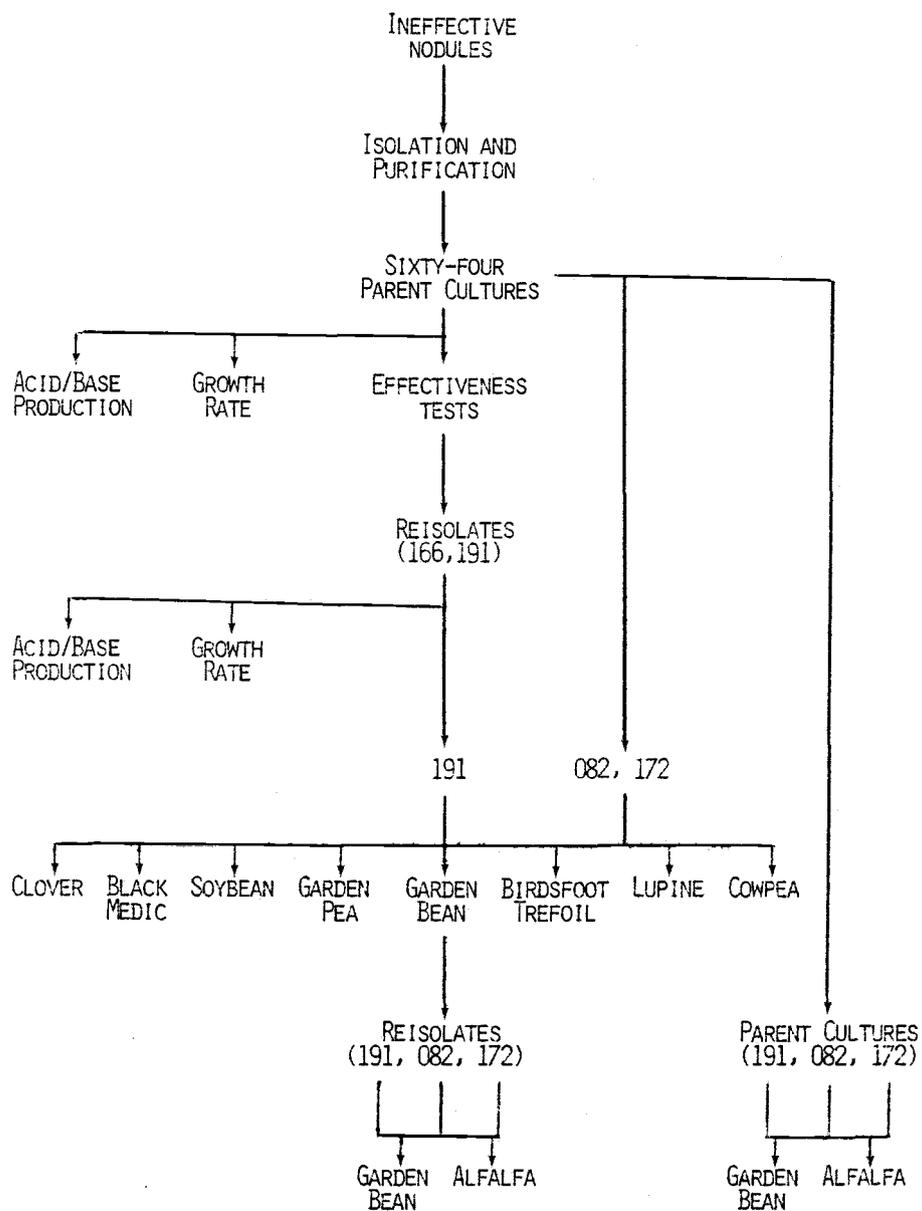


Figure 8. Flow scheme of native Rhizobium characterization experiments.

isolates from each field inoculation treatment were evaluated using a completely randomized experimental design with 6 replications. Control treatments were either uninoculated or received 3 mL of 18 mM KNO_3 at 2 and 4 weeks after germination. After 5 weeks, plants were removed, the shoots were harvested, and dried at 65 °C.

Acid Production of Nodule Isolates

Production of acid during growth was evaluated by streaking isolates onto pH 7.0 YEM agar plates containing bromthymol blue (Norris, 1965; Vincent, 1970). Color change was noted after 2, 5, and 9 days.

Mean Generation Time

Eight representative native field isolates were selected based on differences observed for nodule characteristics, apparent growth rates, and acid production. The mean generation times of the 8 native isolates and 2 control strains (R. meliloti, 102F34, obtained from R. S. Smith, Nitragin Co.; R. japonicum, USDA 110, obtained from the collection of Dr. H. J. Evans) were evaluated. Three replicate flasks for each isolate were inoculated with 0.05 mL of a refrigerated suspension containing approximately 10^7 cells in YEM broth media. Mean generation time was determined turbidimetrically in 50 mL of YEM broth media in 300 mL sidearm flasks placed on a reciprocal shaker (200 rpm) at 30 ± 1 °C. Turbidity was measured with a Bausch and Lomb Spectronic 20 during the exponential growth phase. For comparative purposes, the growth rates of 2 isolates, (166 and

191) were also measured after plant passage, i.e. using isolates which had been reisolated from nodules of plants used in the infectiveness tests.

Carbon Source Growth Stimulation

The relative growth rate of three of the native Rhizobium isolates (082, 172, and 191) was also tested in various carbon sources (glucose, fructose, galactose, arabinose, mannitol, and succinate). The isolates were selected based on diverse growth rates, nodule characteristics, and acid production. The basal broth medium contained yeast extract and mineral salts (Vincent, 1970), plus a vitamin (Chakrabarti et al., 1981) and trace metal (Evans, 1974) mixture, and was adjusted to pH 7.0. The carbon source was autoclaved separately, and added to the basal medium to provide 2 g L⁻¹. Relative growth rate was also compared using TY broth and Luria broth (Canon, 1980). Cultures were inoculated and grown in 50 mL of broth medium in 250 mL Erlenmeyer flasks on a reciprocal shaker at 30 °C. Flasks were inoculated with 0.05 mL of a refrigerated YEM suspension containing approximately 10⁷ cells. Uninoculated flasks and flasks containing no carbon source were included as controls. Turbidity of the broth cultures was visually ranked daily for 5 days after inoculation.

Production of 3-ketolactose

The same 3 strains (082, 172, and 191) used in the carbon source growth stimulation test were also evaluated for their ability

to convert lactose to 3-ketolactose (Bernaerts and DeLey, 1963), a trait common to Agrobacterium tumifaciens.

Alfalfa Cultivar x Native Rhizobium Strain Effectiveness Test

The closed plant tube method was used to determine if the ineffectiveness of the native Rhizobium was a function of the alfalfa cultivar used. A 5 x 6 factorial treatment combination consisted of 5 cultivars (Vernal, Saranac, Buffalo, Apollo, and DuPuits), and 6 native Rhizobium field isolates having diverse characteristics; 3 from the 1981 field site (081, 082, and 084), and 3 from the 1982 field site (166, 172, and 191). Cultivars were selected based on several criteria, including adaptation to local conditions (Apollo), previous planting history on the field site (DuPuits), and diverse varietal origin (Buffalo, Saranac, and Vernal)(Barnes et al., 1977). The experiment was replicated six times, and was conducted in a growth chamber under conditions described earlier.

Acetylene Reduction Activity of Native Rhizobium

Acetylene reduction (AR) of plants inoculated with either native Rhizobium isolate 191 or an effective commercial strain 102F34 was evaluated after 3, 4, and 5 weeks of growth. 'Apollo' alfalfa plants were grown using an open plant tube method described by Vincent (1970). Plant tubes contained a 40 mL slant of Jensen's seedling agar, and seeds were surface-sterilized, germinated, and inoculated as described earlier. The plants were grown in a growth chamber, with 14/10 h day/night cycle, at 22/20 °C. Fluorescent and

incandescent lights in the chamber provided a light intensity of $500 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$. Under laboratory conditions, six plants of each treatment were detopped, and the roots were transferred to 23 mL serum bottles which contained 1 mL H_2O . The vials were partially evacuated, and C_2H_2 was added to provide a final 10% atmosphere (v/v). One mL gas samples were removed at 15, 30, and 45 minutes, and quantities of C_2H_4 in the samples were measured by gas chromatography.

Legume Host x Native Rhizobium Strain Infectiveness Test

The infectivity of 3 native Rhizobium isolates used earlier (82, 172, and 191) was evaluated by inoculating 7 representative legume hosts of the commonly used cross-inoculation groups (Vincent, 1974)(Table 8). The experiment was conducted in a growth chamber using a 3 x 7 factorial treatment combination with 3 replications in a completely randomized design. Except for black medic (Medicago lupulina L.), seeds were surface-sterilized by a brief 95% ethanol rinse followed by a 3 min immersion in 2% NaClO . Black medic seed, which was collected locally and apparently hard-seeded, was surface-sterilized using a 10 min immersion in concentrated HCl , which promoted germination substantially. All seeds were transferred to sterile water agar (10%) for germination, and planted immediately after radicle emergence. Time for germination varied, therefore seed sterilization was staggered to allow the planting and inoculation of all host species at the same time. Germinated seedlings (1 per tube) were grown in modified open plant tubes containing coarse

Table 8. Legume hosts and control inoculant strains used in native Rhizobium infectiveness tests.

| Legume Host | | Microsymbiont | |
|---|------------------------------|-------------------------|-------------|
| Common Name (Genus Species) | Cultivar | Genus Species | Strain |
| Red Clover (<u>Trifolium pratense</u> L.) | Florie | <u>R. trifolii</u> | RT162P46 † |
| Black medic (<u>Medicago lupulina</u> L.) | local ecotype | <u>R. meliloti</u> | 102F34 † |
| Garden Bean (<u>Phaseolus vulgaris</u> L.Savi.) | Aurora | <u>R. phaseoli</u> | 127-K-12b † |
| Garden Pea (<u>Pisum sativum</u> L.) | Little Marvel | <u>R. leguminosarum</u> | 128-C-53 † |
| Soybean (<u>Glycine max</u> Merr.) | Cutler | <u>R. japonicum</u> | USDA 110 † |
| Birdsfoot trefoil (<u>Lotus corniculatus</u> L.) | Granger | <u>R. sp.</u> | 19BFT3 † |
| Lupine (<u>Lupinus polyphyllus</u> L.) | Little Lulu | <u>R. lupini</u> | ATCC10318 † |
| Cowpea (<u>Vigna unguiculata</u> L. Walp.) | California Blackeye no. 5 | <u>R. sp.</u> | 32H1 † |

† Strains from the collection of Dr. H. J. Evans.

‡ Strains supplied by Dr. R. S. Smith (Nitragin Co.).

washed vermiculite. The method is described elsewhere (Vincent, 1970), however several additional precautions were taken to avoid contamination of the root system with foreign Rhizobium. These precautions included; performing all manipulations with the tubes (seeding, inoculation, etc.) under a clean air hood; limiting the duration of the experiment to 4 weeks to avoid supplemental watering and thus unnecessary exposure of the roots; doubling the protective covering at the stem-root junction; and doubling the number of uninoculated control plants for a better assessment of contamination. The plant tubes (3.0 x 20 cm) were filled with coarse, washed vermiculite, and capped with aluminum foil which was secured with a rubber band. The capped tubes were then loosely covered, autoclaved, and cooled. The caps were then removed, and 60 ml of 1/2 strength Jensen's mineral salts media was added to each tube. A single germinated seedling was placed in the vermiculite, the cap was replaced, and small flaps were cut in the cap with a sterile scalpel to allow emergence of the shoot. The upper 2/3 of the capped tube was then covered with a sterile 8 x 18 cm Whirl-pak plastic bag, which was securely fastened around the lower section of the tube. The tubes were then transferred to a growth chamber, and plants were grown under the conditions described above. Temperature extremes were avoided due to the diverse temperature requirements of the plant species. Five days after planting, each seedling was inoculated with approximately 10^8 cells of a native Rhizobium field isolate. Inoculated control strains listed in Table 8 were applied as individual treatments to their respective host species.

Immediately after inoculation, the flapped hole surrounding the stem was packed with sterile cotton wool, the plastic bag was replaced over the tube, and seedlings were returned to the growth chamber. At 10 d after planting, the ends of the plastic bags covering the large seeded legume species (eg. soybean, garden bean) were cut off to allow shoot development, and the cut portion of the remaining bag was fastened closely around the stem. Because growth conditions appeared to be restrictive for development of the large seeded legumes after 3½ weeks, assessments of rhizobial effectiveness were not made.

Subsequent tests were conducted to confirm the infectiveness of native Rhizobium on the garden bean. Using only garden beans as plant hosts, the first infectiveness experiment was repeated, using 6 replicates and the same native Rhizobium isolates and control treatments that were used in the previous experiment. An additional experiment was conducted to assess possible contamination of the native Rhizobium cultures, and to determine if reciprocal infectivity existed for native Rhizobium isolates. A 2 x 6 factorial treatment combination was used with 3 replications. The treatments were; 2 host species (alfalfa, cv. Apollo, and garden bean, cv. Aurora), each of which received one of 6 inoculation treatments. The 6 inoculation treatments consisted of the 3 parent cultures used for the initial infectivity test and the respective field bean nodule reisolates of these strains. Nodule reisolates were obtained from bean plants in the initial infectiveness experiment. Additional control treatments included effective commercial strains for each

plant species (R. meliloti strain 102F34 on alfalfa and R. phaseoli strain 127K12b on garden bean), as well as uninoculated plants. For alfalfa only, this experiment was duplicated using the closed plant tube method mentioned earlier.

RESULTS

In the preliminary field experiments, all uninoculated plants appeared light green, and possessed only white nodules on their roots, while the inoculated plants appeared dark green, and had only pink nodules on their root systems. Branched or coralloid nodule morphology was evident on plants of both inoculation treatments. During the initial purification of the native Rhizobium isolates, it was observed that many of the native isolates produced small (1 mm), rough colonies which are typical of the slow-growing species of Rhizobium (Vincent, 1974). A few isolates, however, produced large (2 to 4 mm), gummy colonies in 3 days, typical characteristics of the fast-growing Rhizobium.

The results of preliminary effectiveness tests in plant tubes indicated that 2 isolates out of a total of 34 would not nodulate alfalfa, and that 31 of the 32 of the remaining isolates were totally ineffective in N_2 fixation in terms of dry matter yield (Appendix Tables 11 and 12). Nodules on half of the plants receiving the native field isolates were white, and representatives of this group (eg. isolate 172) contained undifferentiated rod-shaped bacterial cells. In contrast, a few isolates, typically those with the fast-growing characteristics, (eg. 166 and 191) produced pink nodules, with a small brown senescent zone at the proximal end. These nodules typically contained branched bacteroids, which were similar to, but more compact than bacteroids of nodules formed by effective inoculant strains. These observations are similar to those reported previously (Vance and Johnson, 1981). Low, but

detectable rates of AR activity were observed for root systems containing these prematurely senescent nodules, however this activity was not translated into significant yield increases (Table 9).

The native field isolates varied in their influence on media pH (Table 10). Unlike the typical acid reaction observed for the fast-growing R. meliloti control strain (102F34), many native field isolates had little effect on the pH of the YEM agar. No correlation between symbiotic effectiveness and acid production was apparent (Appendix Tables 11 and 12).

The mean generation times of the native Rhizobium isolates were longer and more variable than the R. meliloti control strain (102F34). Growth curves of 2 native Rhizobium isolates (166 and 191) were identical both before and after plant passage. The relative growth of the native Rhizobium strains were not influenced by carbon source or growth medium. Although no stimulation of growth occurred, some carbon sources gave better growth than others. Their relative ranking from most growth to least growth was ranked in 3 groups; mannitol = glucose = fructose > galactose = succinate = tryptone > arabinose. None of the native Rhizobium isolates examined were able to convert lactose to 3-ketolactose, thus it was concluded that these strains were not members of the genus Agrobacterium.

To determine if the field isolate ineffectiveness was conditioned by alfalfa cultivar, the effectiveness of 30 host cultivar x native Rhizobium strain combinations (5 cultivars x 6 strains) was evaluated using the closed plant tube method. Nodule color and

Table 9. A comparison of shoot dry weight (SDW), acetylene reduction activity (AR), and nodule fresh weight (NFW) of a commercial *R. meliloti* inoculant strain (102F34) and a native *Medicago sativa* nodule isolate (191).

| Seedling Age | Strain or Isolate | | | | | |
|---------------|-------------------|--|------------|------------|--|------------|
| | 102F34 | | | 191 | | |
| | SDW | AR † | NFW | SDW | AR † | NFW |
| --- weeks --- | ---- mg --- | umol.h ⁻¹ .pl ⁻¹ | --- mg --- | --- mg --- | umol.h ⁻¹ .pl ⁻¹ | --- mg --- |
| 3 | 5.9 ± 0.7 | 124.6 ± 30.8 | 5.8 ± 0.4 | 6.4 ± 0.8 | 13.0 ± 11.7 | 4.8 ± 0.8 |
| 4 | 25.2 ± 2.5 | 516.6 ± 103.1 | 14.1 ± 1.1 | 6.9 ± 1.1 | 76.9 ± 36.7 | 9.9 ± 2.8 |
| 5 | 47.4 ± 11.7 | 907.8 ± 20.6 | 18.3 ± 4.1 | 7.5 ± 1.4 | 94.9 ± 53.8 | 11.9 ± 3.0 |

† umol C₂H₄ per hour per plant

Table 10. Cultural and symbiotic effectiveness characteristics of several native Medicago sativa nodule isolates and two reference strains.

| Source of Isolate or Strain | Identification Code | Acid Production † | Mean | Shoot Dry Weight |
|--------------------------------|---------------------------|----------------------|--------------------------------------|--|
| | | | Generation Time ----- hours ----- | (effectiveness test) ----- mg ----- |
| 1981 Field Isolate | 81 | + | 8.7 ± 0.9 | 4.5 ± 0.3 |
| " | 82 | + | 8.2 ± 0.6 | 4.3 ± 0.4 |
| " | 84 | - | 5.9 ± 0.7 | 5.1 ± 0.2 |
| 1982 Field Isolate | 160 | - | 9.9 ± 1.4 | 4.8 ± 0.6 |
| " | 166 | - | 3.4 ± 0.1 | 5.2 ± 0.5 |
| " | 172 | + | 7.7 ± 0.9 | 4.5 ± 0.2 |
| " | 188 | - | 8.5 ± 0.2 | 4.9 ± 0.7 |
| " | 191 | - | 3.7 ± 0.2 | 5.4 ± 0.3 |
| Nitragin Co. | 102F34 (R. meliloti) | + | 1.5 ± 0.2 | 19.8 ± 0.7 |
| Private Collection ‡ | USDA 110 (R. japonicum) | - | 5.4 ± 0.2 | NM§ |
| NA ¶ | Potassium Nitrate Control | NA | NA | 19.9 ± 0.4 |
| NA | Uninoculated Control | NA | NA | 5.4 ± 0.2 |

† Code; (+) denotes acid production; (-) denotes no acid production.

‡ Collection of Dr. H. J. Evans

¶ Not applicable

§ Not measured

morphology was generally consistent for each strain, however some variation occurred between nodules on the same plant. Seedling dry matter yield, the primary determinate of effectiveness, did not differ across cultivars or native Rhizobium strains. Thus, seedling shoot dry weights for plants receiving a given isolate were averaged across cultivars and are listed in Table 10. In general, shoot dry weights obtained in the closed tube systems which were grown in the greenhouse (Table 10) were less than half of the weights obtained from plants grown using the open tube system in the growth chamber (Table 9). This discrepancy was probably due to the compound effects of CO₂ limitation in the closed tube system (Gibson, 1962) and the more favorable temperature regime which was obtainable in the growth chamber. Nevertheless, significant differences due to symbiotic effectiveness were obtained in both environments.

Since some of the native Rhizobium isolates exhibited anomalous characteristics in pure culture (no acid production on YEM growth media and slow growth rate), it was of interest to verify that these strains were indeed R. meliloti. Therefore, various legumes were inoculated with one of the 3 native Rhizobium isolates used in previous tests. All legume hosts were nodulated by their respective control strains, and none of the uninoculated controls were nodulated. Thus, it was concluded that growth conditions were appropriate for nodulation of the various hosts, and that rhizobial contamination was not responsible for the results obtained. The only legumes which were nodulated by the native Rhizobium isolates were black medic (M. lupulina) and garden bean (Phaseolus vulgaris).

All plants of these two species which were inoculated with native isolates developed at least 15 nodules per plant. Nodule color observations of bean plants revealed the same response that was observed when alfalfa was nodulated with these isolates; strain 191 produced pink nodules, while the other two isolates gave smaller nodules with less pigmentation, i.e. either white or light brown. No evidence of a senescent region was observed on the garden bean nodules, which had the normal spherical shape of determinate nodules. Because the garden bean is not typically nodulated by R. meliloti (Graham and Parker, 1964) the experiment was repeated using the same inoculation treatments with additional replication and the garden bean as the only plant host. Again, none of the uninoculated controls nodulated, and all plants receiving the native field isolates did nodulate.

To test for reciprocal infectivity of the native Rhizobium isolates, and to investigate the possibility of contamination of the inoculant cultures with R. phaseoli, isolates were obtained from garden bean nodules taken from plants which had originally received the 3 native Rhizobium parent cultures. The inoculation of alfalfa and field bean with either the parent cultures or the reisolates resulted in prolific nodule development. No nodulation was observed on uninoculated controls. Thus, reciprocal infectivity of the native Rhizobium isolates between alfalfa and field bean was indicated. Alfalfa grown using the closed plant tube method gave similar results.

DISCUSSION

The results of these experiments provided novel information on the taxonomic and symbiotic characteristics of several field isolates of Rhizobium found to be ineffective in N_2 fixation with M. sativa. Isolates of R. meliloti, like other fast-growing Rhizobium, eg. R. trifolii, R. leguminosarum, and R. phaseoli, usually have a generation time of 2 to 4 hours, and are acid producers on YEM growth medium (Vincent, 1974). In contrast, typical slow-growing Rhizobium, eg. R. japonicum, R. lupini, have a generation time of greater than 6 hours, and usually produce an alkali reaction when grown on conventional media. Interestingly, the ineffective nodule isolates from M. sativa examined in this study exhibited characteristics of both groups, by showing atypical combinations of growth rate and acid production characteristics when grown on conventional media. The common practice of categorizing Rhizobium based on their growth rate and reaction in conventional media has been useful and generally consistent within certain cross-inoculation groups (Graham and Parker, 1964; Norris, 1965; Vincent, 1974). However, exceptions are becoming increasingly common (Dakora and Vincent, 1984; Hernandez and Focht, 1984; Keyser et al., 1982; Lowendorf and Alexander, 1983; Stowers and Eaglesham, 1983) thus decreasing the utility of these methods and at the same time expanding the diversity of Rhizobium.

The relatively slow growth rate of field isolates used in these experiments is an interesting phenomenon. The cause for slower growth in some species of Rhizobium is obscure (Hernandez and

Focht, 1984). Since the native isolates in this study infected only hosts of fast-growing Rhizobium, it was initially thought that the slow growth rate of these organisms was due to some peculiar inadequacy of the YEM growth medium. The experimental results however, suggest that the slow growth rate of the ineffective isolates is not readily explained by consideration of growth medium composition.

It has been well documented that ineffectiveness or partial effectiveness in a M. sativa - R. meliloti symbiosis may be conditioned by the host cultivar used (Burton, 1972; Erdman and Means, 1953; Gasser et al., 1972; Gibson, 1967; Leach, 1968). Results of this study suggest that the alfalfa cultivars used had no influence on symbiotic effectiveness expressed by the M. sativa nodule isolates. The consistent symbiotic ineffectiveness expressed by the native isolates with the varieties tested is suggestive of a common relationship between the native isolates, in that diverse native R. meliloti populations usually contain a few individuals which show some degree of effectiveness (Barber, 1980; Bottomley and Jenkins, 1983).

The apparent promiscuity of the native Rhizobium isolates in terms of reciprocal infectiveness between the garden bean (P. vulgaris) and alfalfa (M. sativa) is of particular interest. Medicago sativa is rarely nodulated by Rhizobium from outside its own cross-inoculation group (Graham and Parker, 1964; Vincent, 1974; Wilson, 1944) and only a few exceptions have been reported (Trinick, 1965; Wilson, 1944). The observation that the 3 selected M. sativa

native isolates shared an infective relationship between M. sativa and P. vulgaris is important, because if these observations can be substantiated, then the specificity associated with the R. meliloti cross-inoculation group would be obscured. Although supporting evidence for the reciprocal infectivity between the two cross-inoculation groups is scant, some evidence of similar results can be found in the literature. In a study by Wilson (1944), it was observed that an isolate from M. sativa formed nodules on Phaseolus coccineus L. (scarlet runner bean). Indirect evidence, which also supports the unusual reciprocal infectivity observed, can be found. For example, Graham and Parker (1964) observed that out of 12 host species examined, P. vulgaris appeared to be the most promiscuous host, in that it was nodulated by the members of 6 of the 7 groups of root nodule bacteria examined. Incidentally, the R. meliloti group was the only one which did not nodulate P. vulgaris. Since P. vulgaris is an exceptionally receptive host, it would seem a likely candidate to participate in a cross-infective relationship with member of the highly specific R. meliloti cross-inoculation group. Nucleic acid hybridization studies also suggest a degree of similarity between R. meliloti and R. phaseoli (Gibbins and Gregory, 1972), thus providing a possible genetic explanation for the observed promiscuity. Other circumstantial evidence which can be used to implicate R. phaseoli as the promiscuous M. sativa microsymbiont in this study comes from previous work which has shown that variants of R. phaseoli can be either acid or base producers (Norris, 1965), and that they also may exhibit a higher degree of acid-tolerance than

the acid-sensitive R. meliloti group (Graham and Parker, 1964; Lowendorf and Alexander, 1983).

The cause of ineffective nodulation in alfalfa has traditionally been attributed to acid soil conditions adversely affecting either the multiplication of R. meliloti (Rice, 1975) or the retention of symbiotic effectiveness traits (Burton, 1972). Nodulation by an incompatible strain of Rhizobium from within the R. meliloti cross-inoculation group may also result in ineffective nodulation (Brockwell and Hely, 1966; Gibson, 1967). Since Rhizobium from outside the R. meliloti cross-inoculation group rarely nodulate species of Medicago (Graham and Parker, 1964; Vincent, 1974; Wilson, 1944), it is reasonable to assume that little concern was ever given to the possibility that ineffective nodulation in alfalfa may be due to nodulation by a promiscuous Rhizobium species from outside the R. meliloti group. The results of this study however, suggest that promiscuous Rhizobium which can ineffectively nodulate M. sativa may exist. If substantiated, these results would provide an alternative explanation for the reports of ineffective nodulation of alfalfa from various locations around the world (Burton, 1972).

CONCLUSIONS

This study provided information to evaluate the common management practice of N fertilization of seedling alfalfa at establishment, and the effect of fertilizer N on N_2 fixation. An inoculation failure, which was experienced in the first year of the study, indicated that the protein content and yield of the seedling alfalfa crop is heavily dependent on effective R. meliloti to provide reduced N for the plant. Further results indicated that the native R. meliloti population at the field site was ineffective at N_2 fixation. These results suggest that alfalfa growers in this region may be applying N fertilizer during establishment as insurance against similar inoculation failures.

A few previous reports have suggested that, even in the presence of symbiotically effective R. meliloti, N fertilization may enhance symbiotic N_2 fixation (Fishbeck and Phillips, 1981; Allos and Bartholomew, 1959; Gibson, 1977). The results of these field studies indicated that all N rates applied (5 to 225 kg N ha⁻¹) decreased acetylene reduction activity of seedlings through the third week of after the initial harvest. Nodulation of plants was also adversely affected, regardless of whether the plants were effectively or ineffectively nodulated. A commonly recommended rate of fertilizer N for establishment of alfalfa (45 kg N ha⁻¹) reduced both nodulation and acetylene reduction by 45% at 10 weeks after planting. From these results, it was concluded that it is unlikely that fertilizer N would be useful to complement N_2 fixation in alfalfa. In the 1981 experiment, the N rates were also correlated

with increased weediness, while in the 1982 experiment, the N rates resulted in high levels of nitrate accumulation in the herbage. Ammonium nitrate applications did increase dry matter yield and herbage N of ineffectively nodulated plants during the seeding year, however this effect was diminished the second year. In contrast, effective inoculant applied at establishment resulted in sustained high levels of herbage dry matter yield and herbage N through the second year of growth. In summary, these results suggest that if alfalfa seedlings receive effective inoculant at establishment, little is to be gained by the application of NH_4NO_3 .

Reasons for the lack of general agreement on this subject in the literature are unclear, however it is possible that the different methods in previous studies may have contributed to the differing results. With this in mind, one suggestion for a future study would be to conduct simultaneous experiments in controlled environments and under field conditions to allow a direct comparison of the rate of development of symbiotic N_2 fixation as a function of cultural methods and environmental conditions. Control plants, grown strictly on combined N in such studies, would be valuable for the observation of plant development under conditions where they are independent of N_2 fixation. These studies would reveal differences in the rates of N_2 fixation development under different conditions which undoubtedly would help to explain inconsistencies from previous experiments where diverse methods were used. Results of these studies would also be useful in the design and interpretation

of future experiments where the goal is to be able to predict field results based on conclusions from controlled environment studies.

The novel finding that promiscuous, alfalfa-nodulating Rhizobium may exist under moderately acid soil conditions provides ample opportunity for further research. Substantiation of the results reported in this study are recommended. One possible method for substantiation would be to conduct an exhaustive evaluation of the reciprocal infectiveness of several single colonies isolates derived from one of the ineffective parent cultures. More stringent bacterial identification methods (such as antibiotic resistance markers) may also be useful. If it is determined that the isolates do represent promiscuous Rhizobium, then other possible studies might include; 1) an examination of the serological properties of the native ineffective Rhizobium and, 2) elucidation of the catabolic pathways used by the native ineffective Rhizobium. Such studies may provide explanations for the anomalous characteristics of these bacteria as well as their apparent ability to tolerate moderately acid soil conditions.

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APPENDIX

Explanation of Statistical Methods

Two types of statistical analysis using standard analysis of variance methods were utilized in this study, to permit discrimination between treatment effects and other types of variation which are inherent in such field studies. The type of statistical analysis which was used in each case was dependent on the nature of the treatments applied. If the treatments consisted of a quantitative, graded series of a given material, eg. $\text{NH}_4\text{NO}_3\text{-N}$, then the data were analyzed using regression techniques to develop a response curve or trend resulting from the treatments applied. If on the other hand, the treatments consisted of one or several qualitative variables, eg., inoculated vs. uninoculated seed, or field isolate A vs. field isolate B vs. field isolate C, then the analysis of variance involved partitioning the variation into individual treatment effects. This method permits pair-wise comparisons between 2 means (eg. isolate a vs. isolate b) or several means. The latter techniques are used most commonly, and most researchers are familiar with their use and interpretation. However, they are frequently misused, in that they are sometimes employed to find pair-wise treatment differences between 2 levels of a graded, quantitative series of treatments (Petersen, 1977). In this case, a trend analysis using regression techniques would provide much more meaningful information. Since regression methods are less widely used, this discussion is provided to summarize the rationale for their use and the fundamental concepts used in their interpretation.

When treatments consist of a graded, quantitative series of a given variable, one expects to see a response to the increasing increments applied. By the use of regression analysis to examine the variation in the data, it is possible to discern if a significant response trend is present. When regression methods are used, it is common to fit various types of curves to the data, and then select the curve which best fits the obvious trend in the data, if it exists. Of course, a straight line can be fit through any 2 points (or treatment means), a curve through any 3 points, and 2 curves through any 4 points, etc. But as higher order polynomials are used to describe data, the interpretation of the results becomes more obscure. For example, in chapter II, 3 graded levels of $\text{NH}_4\text{NO}_3\text{-N}$ were used, and the response to these applications was evaluated on several individual dates. The use of regression analysis in the interpretation of the results on a single date permits one of three conclusions; 1) the trend follows a linear response, 2) the trend follows a nonlinear response (curvilinear), or 3) the variation in the data suggests no discernable treatment response.

The R^2 values (coefficients of determination) and regression equations reveal several points about the data in question. The R^2 values can be interpreted to indicate the percentage variation in Y (dependent variable) explained by the application of the treatments X (independent variable). The remaining percentage to total 100, is thus attributed to unexplained variation, which could be due to either experimental error or simply the lack of treatment effects.

For example, if $R^2 = .64$, then 64% of the variation in the data can be attributed to the treatments applied, and the remaining 36% of the variation can be attributed to other factors. It should be emphasized that a low R^2 value doesn't necessarily imply a large amount of experimental error, it could be simply an indication that the treatments do not result in a distinct response. The R^2 values in regression analysis are not to be confused with the correlation coefficients which are denoted in the same way. Regression analysis R^2 values imply a cause and effect relationship between the variables, whereas correlation coefficient R^2 values simply relate a common response between 2 independent variables.

The level of probability associated with R^2 values in regression analysis indicates the appropriateness of the curve selected to represent the data. The asterisks following the R^2 values in Chapter I and in the lower sections of tables in chapter II denote this probability. For example, if 2 asterisks are present, then there is a highly significant probability that the shape of the curve selected adequately describes the response trend. Recall however, that if enough curves are used, a highly significant R^2 value will inevitably result, even though the resulting convoluted trend may be difficult to interpret.

In summary, the regression equation and the R^2 value reveal 3 fundamental aspects of the response trend; 1) the order of the polynomial used in the regression equation provides an indication of the complexity of the response trend, 2) the R^2 value indicates how much of the variation in the data is a result of the treatments

applied, and 3) the level of probability of the R^2 value indicates how appropriate the shape of the curve is for the data.

The major advantage to the use of regression techniques for the analysis of graded, quantitative treatment responses lies in the amount of information obtained. Not only can one assess the variation explained and the appropriateness of the response curve, but it also may be used to make predictions on expected responses within a range of treatment levels. In essence, it provides statistical validity to the practice of 'connecting the dots' on a response curve. For example, given treatments; 1X, 2X, 3X, and 4X; by the use of a standard pair-wise comparison, one can compare only one treatment with another (eg. X compared with 2X, or X compared with 3x, etc). By the use of regression methods, differences over the whole range of X to 4X can be validly estimated at once, thus making it a much more powerful method for predicting responses to a given treatment.

Table 11. Summary of observations from plant tube effectiveness tests of field isolates obtained in 1981 (Hyslop field 2, range 8). Nodules evaluated on a scale of 1 to 5.

| Inoculation Treatment | Isolate ID no. | Nodule color [†] | Nodule size [‡] | Acid Production [§] | Shoot Dry Weight -- mg -- |
|-----------------------|----------------|---------------------------|--------------------------|------------------------------|------------------------------|
| inoculated | 003 | 5 | 5 | - | 9.4 |
| " | 005 | 3 | 5 | + | 9.3 |
| " | 007 | 4 | 5 | + | 12.5 |
| " | 010 | 5 | 4 | - | 11.2 |
| " | 021 | 5 | 5 | - | 10.8 |
| " | 023 | 3 | 5 | + | 10.0 |
| " | 029 | 5 | 5 | + | 11.7 |
| " | 032 | 5 | 5 | + | 12.9 |
| " | 043 | 5 | 4 | - | 9.3 |
| " | 044 | 5 | 5 | - | 11.5 |
| " | 049 | 5 | 5 | + | 9.9 |
| " | 051 | 5 | 5 | + | 10.9 |
| " | 065 | 3 | 5 | + | 8.9 |
| " | 070 | 5 | 5 | + | 12.4 |
| " | 072 | 5 | 5 | + | 11.7 |
| " | 075 | 4 | 4 | not measured | 10.5 |
| uninoculated | 078 | 2 | 3 | + | 3.6 |
| " | 080 | 2 | 2 | not measured | 4.1 |
| " | 081 | 1 | 1 | + | 2.8 |
| " | 082 | 1 | 1 | + | 3.4 |
| " | 083 | 2 | 2 | not measured | 3.1 |
| " | 084 | 3 | 3 | - | 3.3 |
| " | 095 | 1 | 1 | + | 3.4 |
| " | 097 | 1 | 1 | + | 4.1 |
| " | 099 | 3 | 2 | not measured | 4.4 |
| " | 000 | 3 | 3 | + | 2.8 |
| " | 103 | 2 | 2 | + | 3.7 |
| " | 105 | 3 | 2 | + | 4.2 |
| " | 107 | 3 | 2 | + | 3.6 |
| " | 108 | 1 | 3 | + | 3.5 |

[†] Color code; 1 = white, 5 = red.

[‡] Size code; 1 = small, 5 = large.

[§] Code; (+) denotes acid production; (-) denotes no acid production.

Table 12. Summary of observations from plant tube effectiveness tests of field isolates obtained in 1982 (Hyslop field 4, range 12). Nodules evaluated on a scale of 1 to 5.

| Inoculation Treatment | Isolate ID no. | Nodule color † | Nodule size ‡ | Acid Production § | Shoot Dry Weight -- mg -- |
|-----------------------|----------------|----------------|---------------|-------------------|------------------------------|
| inoculated | 145 | 4 | 4 | + | 8.7 |
| " | 146 | 5 | 5 | + | 20.4 |
| " | 147 | 3 | 3 | - | 11.3 |
| " | 173 | 5 | 5 | + | 18.3 |
| " | 174 | 2 | 3 | + | 6.9 |
| " | 175 | 5 | 5 | + | 13.8 |
| " | 193 | 5 | 4 | + | 14.2 |
| " | 194 | 4 | 4 | + | 10.4 |
| " | 203 | 4 | 4 | + | 12.9 |
| " | 204 | 4 | 4 | + | 9.4 |
| " | 205 | 5 | 4 | + | 13.5 |
| uninoculated | 155 | 3 | 4 | - | 10.5 |
| " | 160 | 1 | 1 | - | 5.9 |
| " | 163 | 2 | 3 | - | 5.3 |
| " | 164 | 2 | 1 | - | 5.0 |
| " | 165 | 3 | 2 | - | 5.7 |
| " | 166 | 3 | 3 | - | 5.6 |
| " | 170 | 2 | 3 | - | 7.2 |
| " | 172 | 1 | 2 | + | 6.0 |
| " | 184 | 3 | 2 | - | 6.2 |
| " | 185 | 2 | 3 | - | 6.7 |
| " | 186 | 1 | 2 | - | 5.7 |
| " | 187 | 4 | 3 | - | 6.1 |
| " | 188 | 1 | 2 | - | 5.6 |
| " | 191 | 5 | 4 | - | 5.9 |
| " | 221 | 3 | 2 | + | 6.0 |

† Color code; 1 = white, 5 = red.

‡ Size code; 1 = small, 5 = large.

§ Code; (+) denotes acid production; (-) denotes no acid production.