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Title: Evaluation of Rhizobium Strains With Three Mediterranean Forage Legumes for Biological Nitrogen Fixation

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Centuries of continuous grain cropping in northern Africa have reduced N levels in soils to a degree such that agricultural production is now largely dependent upon nitrogen fertilizer. Through the identification of highly effective legume and Rhizobium strain combinations, the production of protein-rich livestock forage and green manure can be substantially increased. Three common Mediterranean legumes, Lupinus albus, Medicago truncatula, and Trifolium alexandrium were chosen to be tested with various strains of Rhizobium. Greenhouse and growth chamber experiments were conducted to evaluate these forage legumes with commercially available Rhizobium inoculum, nodule isolates collected in Tunisia, and nodule isolates from other sources. Strain effectiveness was determined following 6 weeks of growth in plant tubes containing nutrient agar or modified Leonard jars containing nutrient solution. Each symbiotic system was evaluated for shoot, root, and nodule dry weight, nodule number, nodule acetylene reduction activity, and total plant N.

For L. albus, four single strains, 96A5, 96A19, 96B15, and 96B23, and one commercially available multiple strain 'H', produced acetylene reduction activity from 100 to 190 u moles ethylene evolved.$h^{-1}.g^{-1}$ dw of nodules. Plant dry weights and total plant N values were comparable to the +N treatment. All five strains were therefore recommended for further evaluation under field conditions.

Two single strains for M. truncatula, 102D6 and 102B11, produced acetylene reduction values from 800 to 900 u moles ethylene evolved. $h^{-1}.g^{-1}$ dw of nodules (1.5-5.1 umoles.h^{-1}.$plant^{-1}$). Plant dry weights and total plant N values were higher than the +N treatment. Both strains were recommended for further evaluation under field conditions.

Strains WCI-1 and 162X95 for T. alexandrium produced acetylene reduction activity from 900 to 1000 u moles ethylene evolved.$h^{-1}.g^{-1}$ dw of nodules (1.9-11.4 umoles.h^{-1}.$plant^{-1}$). Plant dry weights were 50%, and total plant N 80%, of the +N treatment. Both strains were recommended for further evaluation under field conditions.

Further evaluation of these strains in field trials is recommended. These trials should be performed in the area of eventual use or with soil and climatic conditions typical of the production area.

Evaluation of *Rhizobium* Strains with Three Mediterranean Forage Legumes for Biological Nitrogen Fixation

by

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EVALUATION OF *RHIZOBIUM* STRAINS WITH THREE MEDITERRANEAN FORAGE LEGUMES FOR BIOLOGICAL NITROGEN FIXATION

INTRODUCTION

Forage legumes have long been valued for their capacity to provide their nitrogen needs through biological nitrogen fixation (BNF). This capacity is the result of a symbiotic association of the bacteria, *Rhizobium* spp., with a legume host. Nodules, containing these bacteria in their bacteroid form, develop on the roots of leguminous plants. Within the nodules, the bacteroids convert atmospheric nitrogen to ammonium, a form usable by the plant. For effective and productive symbiosis to occur, the legume must be infected with a compatible strain of *Rhizobium*.

Effectively nodulated leguminous crops may fix up to three hundred kilograms of nitrogen per hectare per year (Vincent, 1974). N_2 fixation varies among geographic areas as well as among species with values often between 150 and 200 kg N as NH_3 per hectare per growing season. Under optimum growing conditions, N fixation of alfalfa generally exceeds that of red clover, with white clover or birdsfoot trefoil often ranking third (Heichel, 1985). A summary of N fixation data from the United States in 1975 showed pure stands of alfalfa and clover to fix 128 to 300 and 104 to 220 kg N per hectare per year, respectively. Fixation rates as high as 150 kg N per hectare per year have been measured in Canada in soybeans (Hume, 1978).

In addition to supplying nitrogen for the current leguminous crop, BNF has also been valued for providing nitrogen to succeeding rotation crops. In legume-nonlegume rotations, the amount of fertilizer N replaced by legume N depends upon the quantity of residue returned to the soil, the proportion of symbiotically fixed N in the residue, and the rate at which that N becomes available to the succeeding non-legume crop (Heichel, 1985).

Green manure crops are frequently forage legumes grown in association with compatible rhizobia and can provide a portion of the succeeding crops' nitrogen requirement in addition to improving soil structure. The improvement of soil structure is the result of many interactions within the soil (Barnes and Taylor, 1985), including the deep rooting of plants, organic matter returned to the soil following the death of the plants, the presence of macro- and micro-organisms necessary for the decomposition of organic matter, and soil conditions such as pH, ion exchange capacity, and water holding capacity.

These two characteristics of forage legumes - reducing fertilizer nitrogen needs and improving soil structure - are of particular importance to developing countries where agricultural production often is limited by an economical supply of nitrogen fertilizer (Burton, 1981). In a large portion of northern Africa, interruption of traditional migration patterns with resultant concentrated usage, overgrazing, and monoculture cereal cropping practices have led to the deterioration of natural rangelands, soil compaction, low soil ferti-

lity, and erosion of cropping lands. In these areas, leguminous species may be of value in renewing the rangelands and adding organic matter to the soil (F.A.O. Tunisia, 1964; Burton, 1981).

In pre-Roman times, the broad river valleys of Tunisia and the Great Plains of northern Algeria and Morocco were noted for their high fertility. Under Caesar's rule, all suitable land in northwest Africa was put under the plow to feed the urban population of Italy, producing nearly fifty thousand tons of grain every year. A century later, this area was producing 500 thousand tons per year; and continued to do so for many centuries. By the first century A.D. however, overcropping was beginning to exhaust the soil, dictating the spread of agriculture to less fertile land. With the increasing demand by Rome for olive oil, olive trees were planted and began to replace, or at least share prominence with wheat. Uncultivated hillsides were cleared, terraced, and gradually planted (Raven, 1984).

The traditional concept of open grazing (based on tribal versus individual ownership of tracts of land) and the interruption of normal patterns of migration (i.e. development of cities) have resulted in overgrazing of the available rangelands as each owner of livestock attempts to feed his animals (American University, 1979). The soil, having been stripped of a large proportion of its herbage as a result of this prolonged overgrazing, is exposed to compaction by trampling, since the flocks have to cover large distances to obtain their daily feed. This causes compaction of the soil, favoring waterlogging after rain and consequent erosion of the arable layers, frequently laying bare the rock or the relatively infertile subsoil. These phenomena

have been observed in Central Tunisia where ever the land is on a slope; this is particularly so on piedmonts, which are generally reserved for natural pastures (F.A.O. Tunisia, 1964).

The use of effectively nodulated forage legumes, both for the production of nitrogen-rich forage for livestock and as green manure crops in crop rotations, is increasing in Tunisia as agriculturalists are recognizing the problems associated with overgrazing and continuous grain cropping. Assistance is needed, however, in identifying highly effective legume species and Rhizobium strain combinations for BNF.

This project was initiated to identify effective Rhizobium strains for various forage legumes selected for trial in Tunisia. The legumes reported in this thesis are Lupinus albus (white lupin), Medicago truncatula (Syn. M. tribuloides Desr.), and Trifolium alexandrium (berseem clover).

SIGNIFICANCE OF DINITROGEN FIXATION TO THIRD WORLD DEVELOPMENT

In 1974, the world's population was 3.6 billion and projections were that it would reach 7 billion and stabilize. A decade later the population was 4.76 billion, and now the projected ceiling has been raised to around 10 billion. According to Hardy and Havelka (1975), in both the more and less developed areas of the world, the application of nitrogen fertilizer remains the single most important agronomic input, particularly for cereal cultivation. The greatest population growth, however, is in developing countries where dependence on imported industrially-fixed N fertilizer is creating a barrier to optimum agricultural production.

The developing countries consume just over one third of the total world fertilizer production, with their share increasing at a rate greater than that of the industrialized nations (Halliday, 1985). Except in those countries endowed with natural gas resources necessary for the production of N fertilizer, availability of imported N will be dependent upon a countries' financial resources and its ability to distribute this fertilizer to its areas of agriculture production.

Nitrogen is more limiting for more crops in more places than any other plant nutrient. According to Winteringham (as cited in Atkins, 1986), improved soil nitrogen management emerges as the single most important factor in dealing with the problems of agricultural development into the year 2000. Yet most farmers in developing areas remain unaware of the nitrogen fixing ability of legumes, despite the fact

that traditional and modern farming systems in the tropics almost invariably include legumes. Biological nitrogen fixation can be of significance to tropical agriculture and other developing countries, but only to the extent that it is an economically feasible alternative to N fertilizers.

It can be said that BNF technology is made up of two aspects: 1) deliberate use of legumes for their BNF abilities, and 2) use of agronomic practices to maximize BNF. The deliberate use of legumes in cropping systems to derive the benefits of their nitrogen fixing abilities includes crop rotation and intercropping, legume-based pastures, and green manures. The intentional use of practices designed to maximize the quantity of nitrogen fixed by the cropping system includes such things as inoculation and soil amendments to maximize BNF (Halliday, 1985). The implementation of these two aspects of BNF technology in developing countries holds the promise of alleviating much of their dependence on increasingly expensive imported N fertilizer.

OBJECTIVES

The objective of this study was to evaluate strains of Rhizobium for effective nitrogen fixation with three forage legumes: Lupinus albus, Medicago truncatula, and Trifolium alexandrium.

This work was performed as part of a USDA-CSRS research project entitled "Maximizing N_2 Fixation and Yield of Forage Legumes Grown in Tunisia". The objectives of the overall project were: 1) to evaluate the N_2 fixation potential of various forage legume/Rhizobium combinations in greenhouse and growth chamber trials, 2) to evaluate N_2 fixation and production capacity of selected forages in Tunisian field trials, 3) to determine the efficiency of P and S fertilizer use on selected species, and 4) to demonstrate in field trials the effectiveness of N_2 fixation in meeting total forage nitrogen needs.

The research conducted at OSU involved the evaluation of several Rhizobium strains to determine their degree of effectiveness on forage legumes commonly grown in Tunisia and to identify the superior strains among the currently available materials. The forage species evaluated in this study were chosen by the Tunisian cooperator and are species commonly utilized in Tunisia, representing a cross section of forage legume types.

REVIEW OF LITERATURE

THE NITROGEN FIXATION PROCESS

Within the plant kingdom, legumes are unique in their ability to satisfy their large demand for nitrogen either through absorption of inorganic N from the soil solution or from the atmosphere through the symbiotic process of dinitrogen fixation (Hardy and Havelka, 1975; Wych and Rains, 1978). This symbiosis provides bacteria of the genus Rhizobium with carbon, or energy, in the form of simple sugars which are translocated from their production site in the green parts of the plant through the phloem to the bacteria-containing nodules. Nitrogenous compounds from fixation in the nodules are made available to the plant after translocation through the xylem of the host plant.

Nitrogenous compounds vary according to legume species, but include glutamine, asparagine, allantoin, ureides, and a variety of amino acids (Sprent, 1979). These compounds are directly or indirectly used for protein synthesis and the resultant growth and maintenance of the legume.

Rhizobium bacteria occur as free-living heterotrophs in soil, but are unable to independently fix atmospheric nitrogen. After they have infected the root hair, entered the root cortex, and been transformed to bacteroids, they are able to fix atmospheric nitrogen and exhibit true symbiosis. Rhizobium bacteroids are able to protect their nitrogen-fixing enzyme system from O_2 damage and create an O_2-free

environment within the nodule where fixation is free to occur (Heichel, 1985). Although often grown for their ability to fix atmospheric nitrogen, legumes preferentially use N from carryover fertilizer or from decomposition of soil organic matter before commencing symbiotic fixation (Heichel, 1985). If mineral N is available from either soil or fertilizer N, even effectively nodulated temperate legumes may commonly obtain 50% or less of their N requirements from symbiotic N_2 fixation (Rennie, 1985).

ENERGY COST OF FIXATION

Leguminous plants are able to achieve the reduction of gaseous N_2 to ammonia at ambient temperatures and pressures in the nodule through enzymatic reactions and the use of energy and reductants from the oxidative phosphorylation of photosynthetically produced sugars. The ATP required to split the N_2 molecule and the electrons required for reduction of N_2 to ammonium are both produced by the electron transport system of the bacteroid during the respiration of imported photosynthate. Approximately 27 to 30 moles of ATP are directly consumed for each 2 moles of ammonia formed from dinitrogen (Imsande, 1981). The utilization of ATP by the nitrogenase reaction can be expressed in the formula:

$$N_2 + 8e^- + 8H^+ + 16ATP \xrightarrow[Mg^{2+}]{\text{Nitrogenase}} 2NH_3 + 16ADP + 16PO_4^{3-} + H_2$$

($8e^-$ is approximately equivalent to 12 ATP)

The enzyme which catalyses N_2 fixation is nitrogenase. As described by Postgate (1982), the purified Klebsiella nitrogenase consists of two components, one of which is a small (5-6 X 10^4 daltons) Fe protein and the other is a larger (2-2.5 X 10^5 daltons) Fe-Mo protein, both carrying a labile sulphur moiety. From a reduced ferridoxin, the Fe protein, with energy from Mg-ATP, gathers electrons which are subsequently transferred to the Mo-Fe protein. About 4 ATPs are used for the transfer of every two electrons. The Mo-Fe protein combines with N_2 which is then reduced stepwise to gaseous $2NH_3$, utilizing six electrons in the process. In addition to reducing N_2 to $2NH_3$, nitrogenase simultaneously reduces $2H^+$ to H_2 (Postgate, 1982). While this concomitant reaction has been viewed by many to be wasteful, experimental data has shown some rhizobia capable of chemolithotrophic growth through the coupling of the oxidation of this H_2 to ATP synthesis (Evans et al., 1987).

N_2 fixation also requires the formation and maintenance of the nodule, an energy-costly and highly specialized structure. The average allocation of carbon to nodule formation and functioning is 10 percent of the total photosynthate generated during the growth of the plant, and is generally regarded as a drain on the plant's resources and a significant competitive sink for plant assimilates (Atkins, 1986).

Although BNF is an energy demanding process consuming three to four times the amount of C necessary for the assimilation of soil N (Atkins (1984) estimated that 3 to 6 grams of C are used for each gram of N fixed, compared to 1 to 2.5 grams of C for each gram of N assimi-

lated from the soil), it has not been demonstrated that an effectively nodulated legume is less productive than the same legume given optimum fertilizer N. There is considerable evidence that alfalfa and soybeans, legumes capable of fixing substantial amounts of N_2, do not produce more forage or beans when fertilizer N is applied (Hume, 1978). This suggests that a legume receiving adequate levels of other inputs will produce assimilate in sufficient quantity to provide for BNF as well as for normal growth and seed production.

For an adequate comparison of costs in a particular country or economic area, it would be necessary to evaluate the cost of producing or importing commercial N fertilizer and its distribution, as well as the reduction in crop yield (and cost, if any) caused by utilizing BNF rather than fertilizer N.

INDUSTRIAL FERTILIZER PRODUCTION

Natural gas is required to produce ammonia for the fertilizer industry through the reduction of atmospheric N_2 (the Haber/Bosch process). From this natural gas, hydrogen is transferred to the N_2 molecule under high temperatures (300-600 C) and pressures (20-80 M Pa). The energy required to cause this reduction reaction is equivalent to 100 liters of gasoline per 60 kg of inorganic N fertilizer formed (Stoskopf, 1981). Fossil fuels are a limited resource. As prices escalate, this source of fixed N will become less available, especially in developing countries where funds for crop production inputs may be severely limited.

HOST PLANT/RHIZOBIA RECOGNITION AND NODULE DEVELOPMENT

The nodule is the focal point of reaction between the Rhizobium and its host plant. When a susceptible, leguminous plant and a compatible strain of rhizobia are brought together under conditions favorable for growth and infection, a nodule (a highly organized mass of tissue derived from the root cortical cells) will form (Heichel, 1985). The establishment of a nodule and its subsequent development and morphogenesis involves complex interactions between the host plant and it's bacterial symbiont. Intricate structural and biochemical changes in each partner occur, resulting in a symbiotic association capable of fixing molecular nitrogen.

The infection of the legume host's roots by a bacterium of a specific Rhizobium species involves interaction of both symbionts and is host specific (Dazzo et al., 1985). Specificity is determined by a microsymbiont's ability to effectively fix nitrogen in a particular species of legume, and that species only. The surface chemistry of the Rhizobium and of the root hair partly determines the compatibility of the two symbionts and whether or not a nodule will form (Lim and Burton, 1982).

The ability of a Rhizobium strain and its legume symbiont to recognize each other prior to infection was described in the early work of Hamblin and Kent (1973) and Bohlool and Schmidt (1974). Dazzo et al. (1978) found that a white clover glycoprotein lectin called

trifoliin A accumulated on the surface of root hairs at the growing tip and appeared to function as a cell recognition molecule, complementary to saccharide receptors or 'binding sites' on the surface of the bacterial symbiont. This phenomonon became known as 'the lectin hypothesis' which suggested that plant lectins (carbohydrate binding proteins) are involved in the binding and/or bridging of Rhizobium bacteria to the host root surface (Graham, 1981).

Upon recognizing the specific legume host, rhizobia bacteria become attracted and attached to root hairs, causing them to become twisted, curled, or otherwise deformed (Newcomb, 1981). At the site where rhizobia penetrate the host cell wall, the infection thread forms and grows toward the base of the root hair cell. At the same time, the cortical cells are induced to undergo mitosis and are then invaded by the infection threads.

In the final step of infection, bacteria are released from the infection threads into the host cytoplasm where they undergo a change from motile rods to a non-motile form called bacteroids, capable of N_2 fixation. Bacteroids are enclosed in membranes of host plant origin, sometimes singly and sometimes in groups. The bacteroid-containing cells enlarge and develop into nodules which become the sites of nitrogen fixation and amino acid synthesis (Newcomb, 1981 and Kleczkowska et al., 1968). It is within fully functioning bacteroids that the N_2-fixing enzyme nitrogenase is found.

Within the nodule is also found an oxygen-binding, red pigment protein, leghemoglobin, which maintains the optimum oxygen concentra-

tion necessary for N_2 fixation (Heichel, 1985). Leghemoglobin has a high affinity for O_2 and serves as a reservoir of oxygen, providing the bacteroids with a level of O_2 necessary for proper oxidative metabolism, but low enough to protect the nitrogenase system from oxidative damage.

The type of nodulation which results in nitrogen fixation is called "effective" nodulation. The nodules tend to be large, concentrated on the upper root system, and usually have reddish interiors from the leghemoglobin, which characterizes an effective nodule. When nodules develop, but fix little or no nitrogen they are called "ineffective". These nodules are usually small, numerous, and widely scattered throughout the root system (Lim and Burton, 1982). Eardly et al. (1985a) have shown that some indigenous Rhizobium strains, while still able to infect and cause nodulation, are ineffective at N fixation.

FACTORS AFFECTING NODULATION AND OPTIMUM N FIXATION

SOIL CONDITIONS

Soil conditions have a marked effect on Rhizobium in terms of their survival and ability to infect root hairs. Different Rhizobium species exhibit different temperature optima at which infection and fixation successfully occur. Some are able to perform fixation at temperatures as low as 5 C, while others may fix at temperatures as high as 40 C. For the majority of temperate-climate legumes, optimal

rates of fixation occur at temperatures of 25 to 28 degrees C. Although some plants are unable to form nodules at low pH, this is likely to be due to the inability of root hair infection to occur rather than the rhizobia's sensitivity in acidic soils (Atlas and Bartha, 1981).

Rhizobium generally achieve optimum N_2 fixation at soil moisture levels that are most beneficial to the host plant, usually at or near field capacity (Graham, 1984). The soil must be moist enough to meet the needs of both the plant and the rhizobia, yet not so moist as to inhibit nodule respiration. As rhizobia are aerobic organisms, the anoxic condition of flooded soils has a detrimental effect on their survival, although there is variation in tolerance between species (Eaglesham and Ayanaba, 1984). It has been demonstrated (Graham, 1984) that flooding not only reduces fixation, but in plant species intolerant of flooded conditions, nodules may be shed after only 1 to 2 days of inundation.

SOIL NUTRIENT CONCENTRATIONS

The levels of specific ions in the soil have been shown to have an affect on N fixation. Nitrate and nitrite ions inhibit nodule formations at relatively low concentrations. Harper and Gibson (1984) showed that concentrations of these nitrogen ions as low as 4.0 mM adversely affected nodulation of Trifolium subterraneum and were in-

hibitory to the formation of nodules on Medicago truncatula. Results of field experiments by Eardly et al. (1985b) showed that both nodulation and acetylene reduction activity were diminished in a curvilinear manner by all levels of applied NH_4NO_3 (5 to 225 kg ha^{-1}).

As early as 1954, Nicholas and Nason demonstrated the need for molybdenum as an electron carrier in nitrate reduction. Work by Payne (1973) and Notton (1983) showed Mo to be a component of a cytochrome necessary for the respiration of certain bacteria under anaerobic conditions which enable the organism to use nitrate as a terminal oxidant in place of O_2. Mo is well known as a component of the Mo-Fe protein complex of nitrogenase and is essential for its activity (Mengel and Kirby, 1982).

Cobalt deficient nodules in sweet lupin (Lupinus angustifolius) were reported by Dilworth et al. in 1979. This trace mineral deficiency resulted in lowered bacteroid densities within the nodules and a 50 to 80% decrease in acetylene reduction activity. Klueds et al. (1983) also found that trace amounts of nickel are necessary for hydrogen-dependent growth of Rhizobium japonicum and for urease activity expression in soybean leaves.

OTHER SOIL ORGANISMS

An indirect and beneficial relationship exists between Rhizobium and mycorrhizal root fungi which form mutualistic relationships with plants. Mycorrhizal associations occur with many plant species, including most agricultural crops. In this association, the mycelium of the fungi forms a loose network in the soil and functions as an extension of the root hairs, resulting in increased uptake of phosphate and other ions. In tropical soils subject to extensive leaching or in soils poor in phosphorus and other nutrients, the presence of mychorrhizae may be beneficial (Atlas and Bartha, 1981).

Abbott and Robson (1977) observed a marked increase in the growth and phosphorus content of Trifolium subterraneum plants inoculated with a mycorrhizal fungus. It was also noted that nodulation responses closely paralleled responses in growth. This increase in nodulation may be due to the increased vigor and subsequent increased demand for nitrogen by the plant as it is benefited by its mycorrhizal association.

Other potentially beneficial relationships may develop in the presence of particular soil organisms. Agrobacterium rhizogenes populations have been found which are capable of producing organic chemicals which are able to stimulate growth of root hairs and other plant tissue (Atlas and Bartha, 1981). This increase in root hairs could increase the number of potential infection sites for rhizobia.

In addition to beneficial relationships, there are antagonistic relationships between various soil fungi and bacteria (such as some parasitic Bdellovibrio) and Rhizobium. Some bacteriophages (bacterial viruses) have been isolated and found to be specific for Rhizobium (Atlas and Bartha, 1981). Parasitism of Rhizobium by these organisms, however, has not been observed when Rhizobium is in its bacteroid form in the nodule. Additionally, concentrations of these bacteriophages in soil rarely reach a level high enough to decrease the Rhizobium population to affect initial infection and nodulation of the legume host.

TEMPERATURE EFFECTS

Rhizobium species typically grow and fix nitrogen best at 25 to 30 C (Vincent, 1970), although there is considerable variation among species. A range of 20 to 40 C has been seen for cowpea and its associated rhizobia and 15 to 25 C for Pisum sativum (Sprent, 1979). Within a species, temperatures below and above the optimum range tend to limit fixation. Limited survival of Rhizobium is observed at extremely high temperatures. When temperatures were 70 to 80 C, survival of strains of R. trifolii, R. japonicum, and R. lupini was limited to five or six hours (Eaglesham and Ayanaba, 1984).

LIGHT QUALITY AND QUANTITY

Although light quality and quantity predominantly affect the growth of the legume host, the quantity of photosynthate available for nodule growth and maintenance and for N_2 fixation must also be considered. Light is necessary for the synthesis of nitrogenase, and for the production of ATP and reductant for nitrogenase (Sprent, 1979).

In their work with Medicago truncatula, Ruegg and Alston (1978) found that acetylene reduction activity was closely correlated with dry weight and photosynthetic leaf area. They demonstrated an increase in acetylene reduction activity during the growing season following a pattern similar to that of plant growth. They also found that the rate of N_2 fixation generally decreased with the onset of flowering. This may be due to hormonal effects associated with flowering or to the high demand for photosynthate by the flower during pollination and seed set, leading to deprivation of assimilate at the nodule. Defoliation and shading studies gave further evidence of the importance of light and photosynthate for N_2 fixation, as these treatments resulted in a substantial decrease in nodule acetylene reduction activity.

Plant pigments absorb light with wavelengths between 380 and 700 nm, with blue light (440 nm) being approximately twice as efficient at capturing light energy as red light (680 nm) (Hoober, 1984). In addition to the effect of light quality on photosynthesis, plants are also subjected to variable lengths of daily light and dark periods, with plants in the upper latitudes more affected than those near the

equator (McCloud and Bula, 1985). For optimum plant growth, and subsequent availability of assimilate to the nodule, a plant must receive light of the proper wavelength as well as for a period of time adequate for that plant's photosynthetic needs.

MATERIALS AND METHODS

SPECIES SELECTED FOR STUDY

LUPINUS ALBUS

Lupinus albus (white lupin) is an upright, long-day winter annual with a coarse stem, medium-size digitate leaves, and large attractive white flowers growing to 120 cm. Lupins are of the tribe Genisteae, subtribe Lupininae and are mainly adapted to temperate regions. Inoculated seed is planted in the fall in areas where temperatures do not fall below -9 degrees C (15 F) (Martin et al., 1976).

Lupins are generally tolerant of soil infertility and volunteer readily in open places where the soil is loose or has been disturbed (Gladstones, 1982). L. albus is found native in acid soils and grows well on mildly acid and slightly calcareous sandy loams.

Although L. albus requires more fertility and nutrients than either L. angustifolius or L. luteus, it is also the most winter-hardy of the three lupines. L. albus requires at least a five month period with mean monthly temperatures of 5 to 25 C and annual rainfall of 400 to 800 mm. It is reported to tolerate annual precipitation of 360 to 1780 mm, annual mean temperature of 5.7 to 26.2 C, and pH of 4.8 to 8.2 (Reed, 1981).

White lupin is thought to have been first cultivated in the Balkan Peninsula where wild types still exist. It is now widely cultivated in Mediterranean countries, the Canary Islands, Madeira, and the Upper Nile (Gladstones, 1976).

Sweet (alkaloid-free) strains of L. albus were selected during the 1930's by van Sengbusch and others and are grown for grazing as well as for green manure. Fresh cut forage has been shown to contain approximately 2.8% nitrogen and 2.7% crude fiber. Yields range from 5 to 7.5 metric tons dry herbage per hectare, containing 250 to 450 kg of N per hectare (5-6% N)(Reed, 1981).

MEDICAGO TRUNCATULA

Medicago truncatula (Syn. M. tribuloides Desr.) is an annual medic of the tribe Trifolieae native to the arid zones of North Africa but found throughout the Mediterranean region. Its growth habit is ascending or procumbent, branching from near the base, and normally growing 15 to 30 cm. In Israel, it is generally found as a component of dwarf-shrub formations growing on terra rossa and basaltic and alluvial soils. Data from other countries indicates a similar habitat (Heyn, 1963). M. truncatula is used for rotational farming in semi-arid zones with annual rainfall of 300 to 600 mm and annual mean temperatures of 6.0 to 26.5 C. (Le Houerou, 1979).

Jemalong barrel medic is a cultivar used extensively in Australia for pasture improvement. It has persisted on coarse textured soils in most trials conducted in southwest Australia, but generally does not grow well where the soil is acidic in reaction or poorly supplied with lime (Trumble, 1939). Jemalong medic has been observed to set a high percentage of hard seed. This characteristic imparts a high degree of drought tolerance but also requires scarification to ensure adequate germination (Trumble, 1939; Alchin, 1974).

The chromosome number (2n) of M. truncatula is 16, being half that of M. sativa at 32 (Lesins and Gillies, 1972).

TRIFOLIUM ALEXANDRIUM

Trifolium alexandrium (berseem clover) has been grown in Egypt since ancient times, is apparently of Egyptian origin, and is a well-known fodder crop in the Mediterranean area. Three types of berseem have been distinguished; Fahl, Miscawi, and Saidi (Bogdan, 1977).

Berseem clover is an annual crop of the tribe Trifolieae with stems of 30 to 60 cm. It is predominately self pollinated, with warm spring weather stimulating flowering and seed formation. It is particularly valued for its rapid growth under cool conditions of subtropical or tropical winters (Bogdan, 1977).

Under dryland conditions only one, or occasionally two cuts of the Fahl type cultivars can be taken (Bogdan, 1977). These cultivars produce herbage in the spring and are well adapted for semi-arid areas, but are unable to regenerate after harvesting and must therefore be reseeded.

Miscawi is an early summer type that regenerates readily after each of 4 to 6 harvests (Reed, 1981). Production, palatability, and longevity of berseem clover are considered excellent. Early yields are superior to those of white clover (Trifolium repens), alfalfa (Medicago sativa), or sweet clover (Melilotus spp.). For the first two harvests, dry matter contents of multicut cultivars are generally about 10 to 12% with fresh weight yields of 25 to 37.5 metric tons per hectare. When harvested pre-bloom, crude protein content of Mescawi

is reported to be 18.3 to 26.6% and approximately 16.6% for Fahl. Berseem is reported to have about one-half the crude fiber and more carbohydrate and fat than alfalfa (Reed, 1981).

According to Kretschmer (as cited in Bogdan, 1977), berseem clover can withstand 2 to 3 degrees C below the freezing point and basal parts of adult plants can survive at -7 degrees C. It responds well to soil moisture and irrigation but does not tolerate water-logging. According to Reed (1981), berseem clover is reported to tolerate annual precipitation of 380 to 1660 mm, annual mean temperatures of 7.0 to 26.7 degrees C, and pH values of 4.9 to 7.8. It grows on various soils, although heavier soils are more suitable than light, sandy soils. Tolerance to soil alkalinity and salinity for berseem clover is well established (Bogdan, 1977).

SEED SOURCE

Lupinus albus seeds of the cultivar "Ultra" were provided by the Clyde Robbin Seed Co. (Castro Valley, CA) and had a germination of 80%. Seeds of M. truncatula cultivar "Sbiba" lot 83-057-15 were collected in Tunisia and exhibited an average post-scarification germination percentage of 81. Seeds of T. alexandrium were collected by the Institut National Agronomique Tunisie (INAT) and exhibited germination of 93%.

SEED GERMINATION

L. albus and T. alexandrium seeds required no pre-treatment to enhance germination. M. truncatula seeds were subjected to scarification due to prescarification germination percentages of less than 50%. Scarification was achieved by spinning 1 g of seeds in a metal cylinder (5.1 cm long by 6.35 cm in diameter) with air pressure (3.45 X 10^5 Pa) for 20 seconds. The inside of the cylinder was lined with #7220 grade emery cloth. The equipment used was provided by the USDA Seed Processing Laboratory at Oregon State University.

All work prior to transfer of seedlings to the growth chamber or greenhouse was performed using sterile solutions and equipment under a laminar flow hood (Pure Aire, model 720C+30). Following surface sterilization in 95% ethanol (15 seconds) and 1% sodium hypochlorite (5-8 minutes), seeds were transferred to sterile disposable petri dishes (Falcon 1029, 100 X 15 mm) containing 20 ml of cooled 1.5% water-agar (Agar-agar No. A-7002, Sigma Chemical Co.). For the small seeded species, 50 seeds were germinated per dish. This number was reduced to 25 per dish for L. albus. Dishes were covered, sealed with "Parafilm" (American Can Company, Greenwich, CT), and placed upside down in a drawer at room temperature (18-22 C) until radicles attained a length of 15-20 mm.

RHIZOBIUM STRAINS

All Rhizobium strains (isolates) were provided by the Nitragin Co. (Milwaukee, WI) unless otherwise indicated. Strains were in the form of agar slants or peat inoculum. Strains utilized, species from which strains were isolated, the form used, and source (or site of collection) are shown in Tables 1-3.

Nodule isolates were obtained using the method described by Kahn, (1984). Nodules were cut from roots of the host plant, soaked in 5% H_2O_2 for 30 seconds, and then rinsed in four successive washes of distilled H_2O. Small nodules were crushed and then streaked onto Petri dishes containing Yeast Extract Mannitol (YEM). Large nodules were cut in half and then the center of the nodule was probed 2-4 times with a disecting needle and streaked onto a dish with the tip of the needle. This procedure was more effective in reducing contamination than crushing nodules. Dishes were incubated at 30 C for five days.

RHIZOBIUM SOLUTIONS

Rhizobium strains were grown in yeast mannitol broth (Vincent, 1970). For strains in slant form, a small amount (one transfer loop) of material was swirled into a flask containing 50 ml of broth, capped, and placed in a warm (26 C), dark oven. Flasks were swirled 3-4 times per day to maximize growth. When the solution was milky (usually five days) indicating a Rhizobium density of approximately

Table 1. Strain identification, origin of isolate, form of micro-symbiont, and the source or collection site of Rhizobium used for Lupinus albus experiments.

Strain	Origin of isolate	Form	Source
96A5	L. albus	slant	Nitragin Co.
96A19	L. albus	slant	Nitragin Co.
96B15	L. angustifolius	slant	Nitragin Co.
96B23	L. angustifolius	slant	Nitragin Co.
H	Lupinus spp. mix	in peat	Nitragin Co.
FWR-4	L. polyphyllus	nodule isolate	Benton Co., Ore.
FWR-LA	L. albus	nodule isolate*	Ore. State Univ.

* - Taken from L. albus grown in soil collected from rhizosphere of nodulated L. polyphyllus.

Table 2. Strain identification, origin of isolate, form of micro-symbiont, and the source or collection site of Rhizobium used for the Medicago truncatula experiment.

Strain	Origin of isolate	Form	Source
102B11	M. hispida	slant	California
102N1	M. arborea	slant	Neftza, Tunisia
102D6	M. orbicularis	slant	North Carolina
102A12	M. arabica	slant	South Carolina

Table 3. Strain identification, origin of isolate, form of micro-symbiont, and the source or collection site of Rhizobium used for the Trifolium alexandrium experiment.

Strain	Origin of isolate	Form	Source
162X95	T. alexandrium	in peat	Nitragin Co.
LX937	T. alexandrium	in peat	Tunisia (Nitragin Co.)
LX684	T. fragiferum	nodule isolate*	Oregon State Univ.
WCI-1	T. repens	nodule isolate	Corvallis, OR

* - Taken from T. alexandrium inoculated with LX684 peat. LX684 peat received from Nitragin Co.

1 X 10^7 cells per ml (Vincent, 1970), inoculations were performed. For strains in peat form, 0.15-0.2 g of material was mixed into 50 ml of broth and allowed to incubate in the same manner as the slant form. When sufficient growth was apparent, 1 ml of this solution was transferred to 50 ml of fresh broth and again allowed to incubate as described.

SEEDLING TRANSFER AND INOCULATION

M. truncatula and T. alexandrium seedlings were transferred to their respective growth media when radicles reached 15-20 mm. Using Gibson's method for a partly enclosed system (Vincent, 1970), seedlings were transfered to 25 X 250 mm tubes containing 60 ml of solution plus 1.5% agar. Prior to cooling of the agar solution, tubes were inclined to create a long growing surface.

A hot disecting needle was used to melt a hole into the agar long enough to accomodate the radicle. Holding the seedling with forceps, the radicle was slipped into the hole in the agar. One ml of Rhizobium broth solution was pipetted around the radicle and onto the surface of the agar.

Seedlings were capped with Kaputs (Belco Glass Co.), and kept in the dark for 2 days to allow for etiolated stem elongation. Following etiolation, caps were removed and foil caps with a hole large enough to fit over the cotyledons were placed on the tubes and secured with

rubber bands. The area around the stem in the holes was carefully filled with loose cotton. All replicates were labeled, randomly placed in tube racks, and transferred to growth chambers.

Germinated L. albus seeds were transfered to modified Leonard Jars (Vincent, 1970), inoculated, and covered with 7 to 10 mm of paraffined sand. Inoculation was performed by pipetting 1 ml of Rhizobium broth solution directly onto the root and the surrounding growth media. In the first experiment, Leonard jars were constructed of new 20.32 cm (8 inch) clay pots set into number 10 tin cans lined with 25.4 X 38.1 cm (10 X 15 inch) 'Clavies' (Bel-Art F-13182, VWR Scientific) autoclavable bags.

A second lupin experiment was conducted for verification of results. In this second experiment, previously used 15.24 cm (6 inch) pots were used and were lined with clavies to prevent possible leaching of nutrients from earlier work.

Growth media contained 50% vermiculite and 50% acid (3N HCl) washed silica sand. Prior to use, sand was rinsed with excess amounts of tap water with a final rinse with glass distilled H_2O. Cheese-cloth wicks were used to transport nutrient solution from the can into the media. Nutrient solution was poured onto the media as well as into the can.

Completed pots and cans were covered with aluminum foil and autoclaved prior to use. Following sterilization and cooling, the aluminum foil was carefully opened and five germinated seeds were placed in each pot and inoculated. Plants were thinned to three per pot after adequate establishment (9 days).

NUTRIENT SOLUTION

A modified, 20% Jensen's solution (Vincent, 1970) was used for all three species. For +N treatments, KNO_3 was added to 5 mM. Additional autoclaved nutrient solution was added to each replicate as needed throughout the growing period.

GROWTH CHAMBER CONDITIONS

M. truncatula seedlings were grown in a growth chamber (Conviron, model E8VH) on a 16 hour day with a light intensity of 300 umol m^{-2} s^{-1} (Dazzo et al., 1978) and 25/20 C day/night temperature reqime.

T. alexandrium seedlings were also grown in a growth chamber but initially started on a 12 hour day, 20/16 C day/night temperature, and a light intensity of 100 umol m^{-2} s^{-1} utilizing fluorescent light and no incandescent light. Day length was increased 1 hour and light intensity was increased 75 umol m^{-2} s^{-1} weekly until 14 hour days and a light intensity of 250 umol m^{-2} s^{-1} were provided.

GREENHOUSE CONDITIONS

L. albus seedlings were grown in pots placed on greenhouse benches under natural and fluorescent lighting (250 umol m^{-2} s^{-1}) with a 16 hour day. Temperatures averaged 21 and 16 C for day and night, respectively.

SUPPLEMENTARY *LUPINUS ALBUS* EXPERIMENT

In the initial *L. albus* experiment, 'H', a commercially available inoculum, was ineffective in establishing nodules. As 'H' is usually considered highly effective, it was necessary to repeat the experiment. In the supplementary experiment, two strains (96A19 and 96B23) were included which had not been used in the initial experiment. Two locally collected strains (FWR-4 and FWR-LA) included in the initial experiment, but shown to be ineffective, were not tested in the supplementary experiment.

ANALYTICAL PROCEDURES AND MEASUREMENTS

ACETYLENE REDUCTION

Acetylene reduction was evaluated when nodulated plants had attained a size and robust nature indicative of successful nitrogen fixation, usually 5-6 weeks following seedling transfer. Roots of intact plants were subjected to a 10% acetylene concentration with 5 cc samples withdrawn at 30, 60, and 90 minute intervals. During this procedure, the plants were left in the temperature and light conditions under which they had been growing. The three lupines growing in each pot were treated as a single unit.

CHAMBERS FOR ACETYLENE REDUCTION

For small seeded legumes, acetylene reduction analyses were performed in chambers of 40 mm inside diameter polyvinyl chloride (pvc) pipe cut to a length which would accommodate 250 cc of air. A #9 (45 mm top, 37 mm bottom, 25 mm long) rubber stopper was placed in one end of the pipe. The stem of a plant, just above the root, was wrapped with kneaded rubber (Eberhard Faber Inc.) and placed in a 8 mm hole cut in the center of a second #9 stopper. This stopper, split from the center out with a razor blade, was placed in the other end of the pipe. A thin sheet of kneaded rubber was placed in the split to prevent gas leakage. Acetylene was injected into the chamber through a rubber septum secured in a hole drilled into the side of the pvc pipe. Similar chambers were made for large seeded legumes using larger diameter pvc pipe, for a total volume of 1400 cc. This procedure was described by Monaco et al. (1981) for use with Alnus rubra seedlings.

GAS CHROMATOGRAPHY

Samples drawn during acetylene reduction by the root nodules were injected into a Carle Analytical Gas Chromatograph (model 311) for the quantification of ethylene produced using a standard curve of purified ethylene analyzed by the instrument under indentical conditions. Samples from the L. albus supplementary experiment were

injected into a Hewlett Packard, Model 5710 A, gas chromatograph. Data for all experiments were expressed as umoles of ethylene produced per g of nodule dry weight per hour.

DRY WEIGHTS OF PLANTS AND NODULES

Shoots, roots, and nodules for each tube or pot were dried at 68 C for 24 hours and weighed. Nodules were counted when removed from roots and weighed separately following drying. Size, shape, and color of the nodules were also noted.

KJELDAHL NITROGEN

For determination of total plant N, roots and shoots for each tube or pot were ground and analyzed for Kjeldahl nitrogen at the Plant Analysis Laboratory of Oregon State University. Due to the small amount of plant material available from the M. truncatula and T. alexandrium experiments, treatment replications were pooled and analyzed as a single unit. L. albus plants provided sufficient material for replicate analyses.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

Plants were arranged in a completely randomized block design for each species experiment. The initial L. albus experiment consisted of 7 treatments (5 Rhizobium strains, uninoculated +N, and uninoculated) with 6 replications each. The supplementary experiment also consisted of 7 treatments but with 5 replications each. For both M. truncatula and T. alexandrium, there were 4 Rhizobium treatments, an uninoculated +N, and an uninoculated treatment, with 8 replications each. Analysis of variance was used to evaluate treatment effects. Separation of treatment means was performed using Fisher's protected LSD value (Snedecor and Cochran, 1980).

PROBLEMS ENCOUNTERED

During preliminary experiments, Jensen's solution was used at the strength described in Vincent, (1970). This proved to be too concentrated for these selected legumes and seedling death resulted. Upon consultation with personnel from the Nitrogen Fixation Laboratory, the concentration was reduced to 1/5 strength. This resulted in good seedling response.

T. alexandrium seedlings developed a reddish-purple color and exhibited minimal growth in the growth chamber when subjected to the same light and temperature conditions as used with M. truncatula.

Earlier greenhouse experience indicated that high light intensities seemed to bring about this condition in T. alexandrium seedlings as well as mature plants. The +N treatment seedlings, however, showed no signs of discoloration and continued to grow vigorously.

RESULTS

LUPINUS ALBUS INITIAL EXPERIMENT

Shoot dry weight

Shoot dry weight of 43 day-old plants was greatest with the +N treatment (Figure 1) and produced 16% and 22% greater weight than strains 96B15 and 96A5, respectively. There was no significant difference between strains 96B15 and 96A5. All other treatments produced weights approximately 50% less than the +N treatment. Shoot dry weight mean values are shown in Table 4.

Root dry weight

The +N treatment also produced the greatest root dry weight value, producing 43% greater weight than strain 96B15 (Figure 1). All other treatments produced weights more than 50% less than the +N treatment. No significant differences were noted among the Rhizobium and uninoculated treatments. Root dry weight mean values are shown in Table 4.

Nodule morphology, number, and mass

Treatments 96B15 and 96A5 were the only treatments to display significant nodule formation, with nearly equal mean numbers for each (Table 4). These nodules were red to dark reddish-brown in color, round in shape, and found encircling the main root just below the junction of shoot and root.

The mean nodule dry weight for strain 96B15 was significantly (29.91%) higher than for 96A5. All other treatments were found to be essentially void of nodules (Table 4).

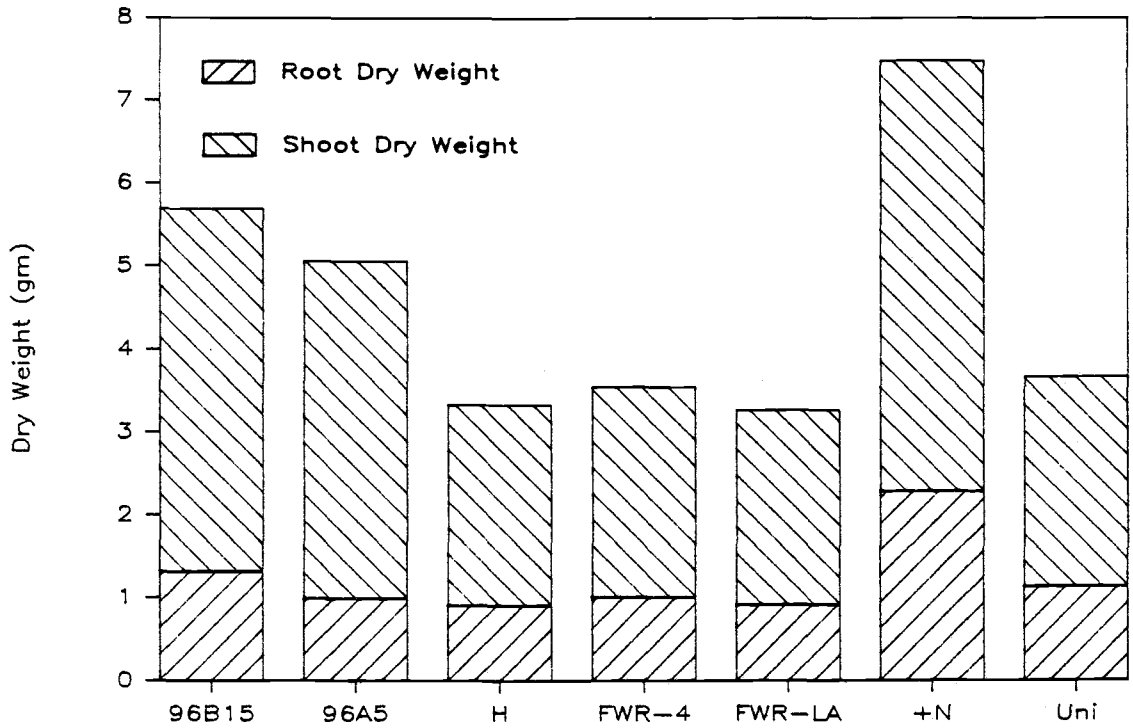

Figure 1. Mean shoot and root dry weight (g) of 43 day-old Lupinus albus plants as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatments.

Total Plant Nitrogen

Rhizobium strain treatments 96A5 and 96B15 exhibited the highest values for total plant nitrogen, although the value for the +N treatment was not significantly less (Figure 2). All other treatments exhibited values significantly less than the +N treatment (Table 4).

Acetylene reduction

Of the four strains evaluated for effectiveness, only strains 96A5 and 96B15 showed significant acetylene reduction activity (Figure 3). Strain effectiveness, expressed as rate of ethylene evolution, is shown in Table 4.

Table 4. Mean shoot, root, and nodule dry weight (g), nodule number, total plant N (mg), and acetylene reduction activity (umole ethylene evolved.h^{-1}.g^{-1} dw of nodules) per three plants of 43 day-old Lupinus albus plants as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

Treatment	Dry weight (g)			Nodule	Total Plant	Acetylene
	shoot	root	nodule	number	N (g)	reduction
96A5	4.38ab	1.32b	.1320a	105.5a	.1482a	80a
96B15	4.07b	1.49b	.1042b	102.8a	.1486a	131a
H	2.43c	0.91b	0.0c	0.0b	.0434b	0b
FWR-4	2.55c	1.01b	0.0c	0.0b	.0442b	0b
FWR-LA	2.36c	0.92b	.0021c	0.17b	.0430b	0b
+N	5.20a	2.29a	0.0c	0.0b	.1411a	0b
Unin.	2.54c	1.14b	0.0c	0.0b	.0456b	0b
LSD	0.93	0.81	.0247	21.7	.0319	29

Means followed by different letters are significantly different at the .05 probability level.

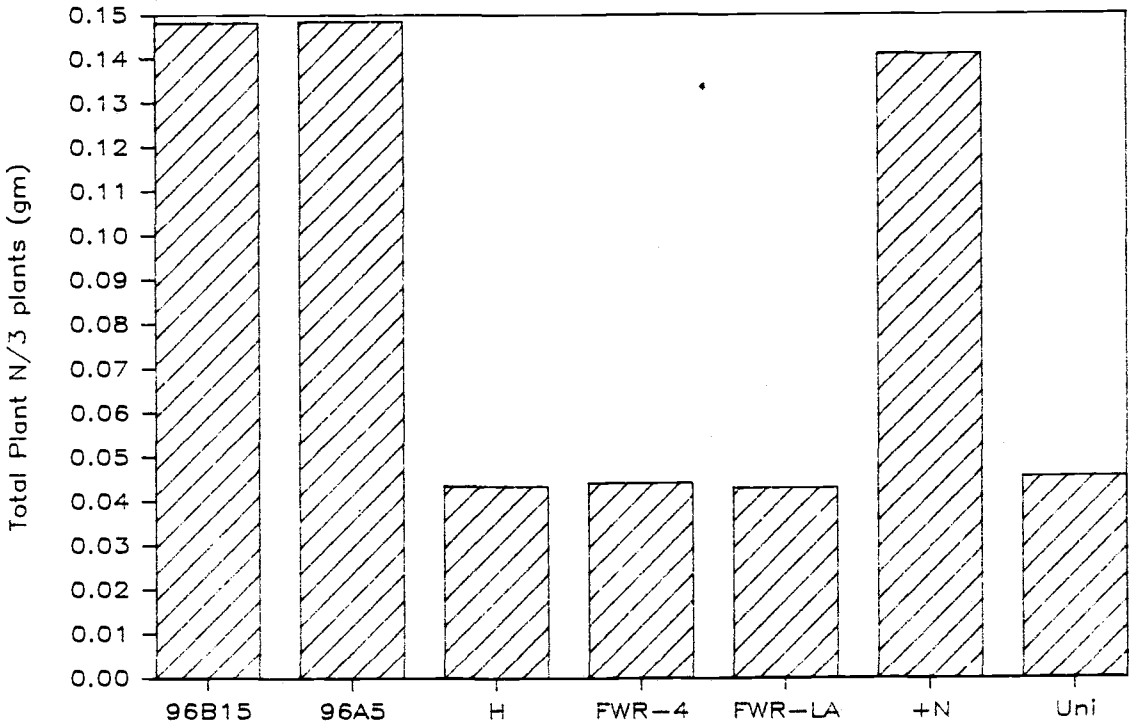

Figure 2. Mean total plant N (g) of 43 day-old Lupinus albus plants as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

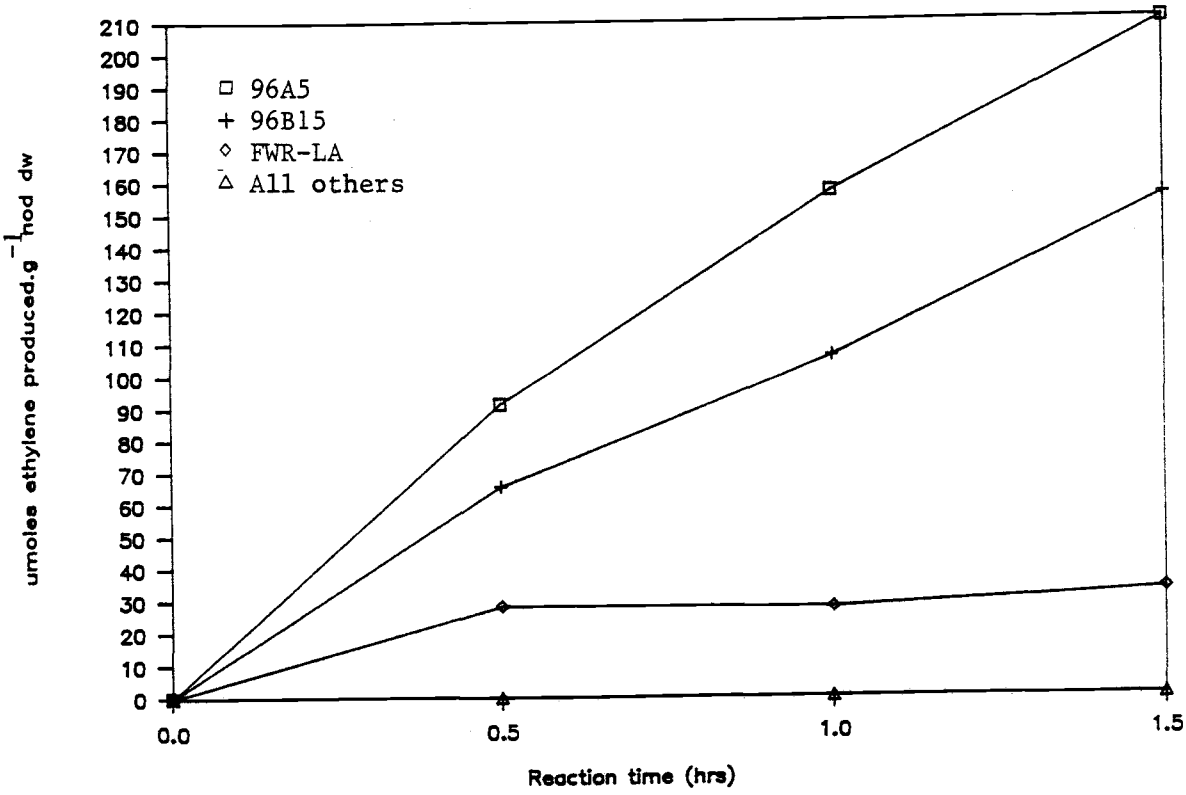

Figure 3. Mean acetylene reduction activity (umole ethylene evolved. $h^{-1}.g^{-1}$ dw of nodules) per three plants of 43 day-old Lupinus albus plants as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

LUPINUS ALBUS SUPPLEMENTARY EXPERIMENT

Shoot dry weight

Shoot dry weight of 42 day-old plants was greatest with Rhizobium strains 96B23 and 96B15, although the other three strain treatment values were not significantly less (Figure 4). The +N and uninoculated treatments exhibited similar weights, were significantly less than strains 96B23 and 96B15, but were not significantly less than the other Rhizobium strains. Shoot dry weight mean values are shown in Table 5.

Root dry weight

Root dry weight of 42 day-old plants was greatest with the +N treatment and Rhizobium strain 96B15, although all other Rhizobium strain treatments produced weights which were not significantly less (Figure 4). Root dry weight mean values are shown in Table 5.

Nodule morphology, number, and mass

All five Rhizobium treatments displayed significant nodule formation, ranging from 161 nodules for strain 96B23 up to 204 nodules for strain 96A5 (Table 5). All nodules were red to dark reddish brown in color, round in shape, and found encircling the main root just below the junction of shoot and root. Two of the +N treatment replicates developed a few nodule-like bodies that were white, irregular in shape, and found on the main root. The other three +N treatment replicates produced no nodules.

Mean nodule dry weight was similar for all Rhizobium strains with no significant difference between their means (Table 5).

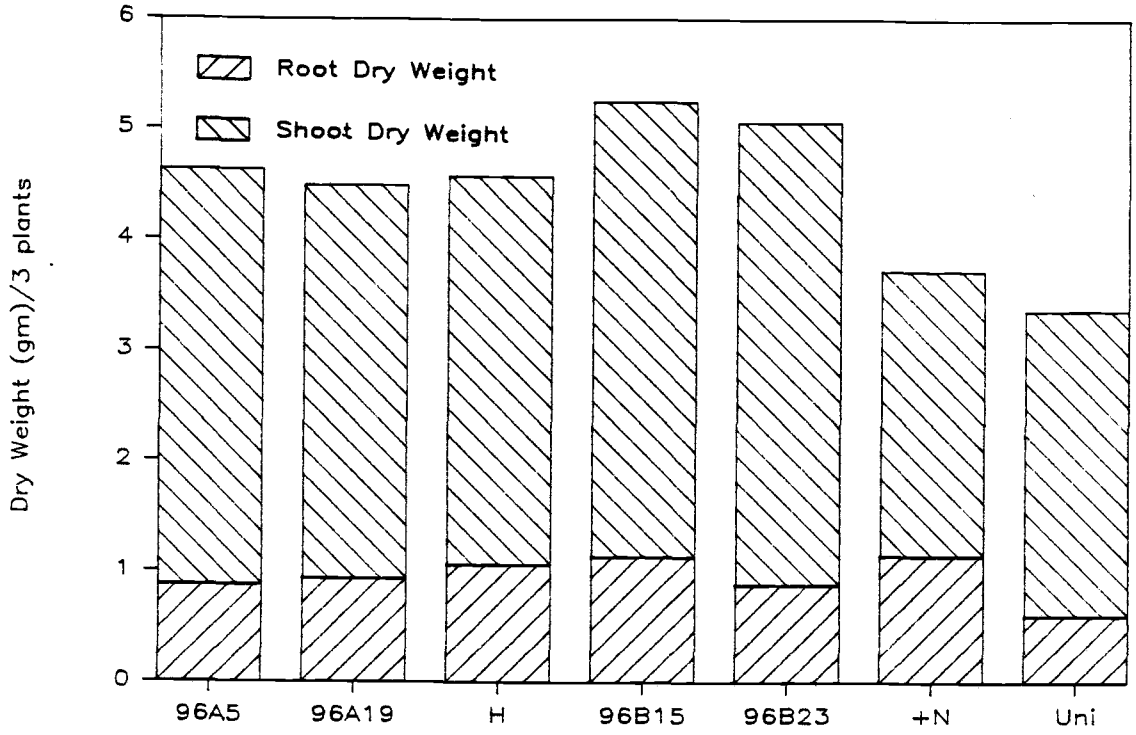

Figure 4. Mean shoot and root dry weight (g) of 42 day-old Lupinus albus plants (supplementary experiment) as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

Total Plant Nitrogen

All Rhizobium strains exhibited a similar level of total plant nitrogen (Figure 5). Both the +N and uninoculated treatment values were significantly less than the lowest Rhizobium strain (H) (Table 5).

Acetylene reduction

All five Rhizobium strain treatments exhibited high levels of acetylene reduction, with no significant differences noted between strains (Figure 6). The +N treatment exhibited a small and significantly lower amount of activity. Strain effectiveness, expressed as rate of ethylene evolution, is shown in Table 5.

Table 5. Mean shoot, root, and nodule dry weight (g), nodule number, total plant N (g), and acetylene reduction activity (umole ethylene evolved.h^{-1}.g^{-1} dw of nodules) per three plants of 42 day-old Lupinus albus plants (supplementary experiment) as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

Treatment	Dry weight (g)			Nodule	Total Plant	Acetylene
	shoot	root	nodule	number	N (g)	reduction
96A5	3.76ab	.88a	.2423a	204.4a	.1688a	169a
96A19	3.56ab	.94a	.1971a	125.8b	.1669a	139a
H	3.52ab	1.06a	.2097a	179.8a	.1660a	128a
96B15	4.12a	1.14a	.2474a	164.4ab	.1910a	133a
96B23	4.19a	.88a	.2740a	161.0ab	.1830a	151a
+N	2.58b	1.15a	.0061b	8.0c	.0545b	1.25b
Unin.	2.76b	.60b	.0000b	0.0c	.0452b	0.0b
LSD	1.35	0.49	.0891	46.7	.0580	55

Means followed by different letters are significantly different at the .01 probability level.

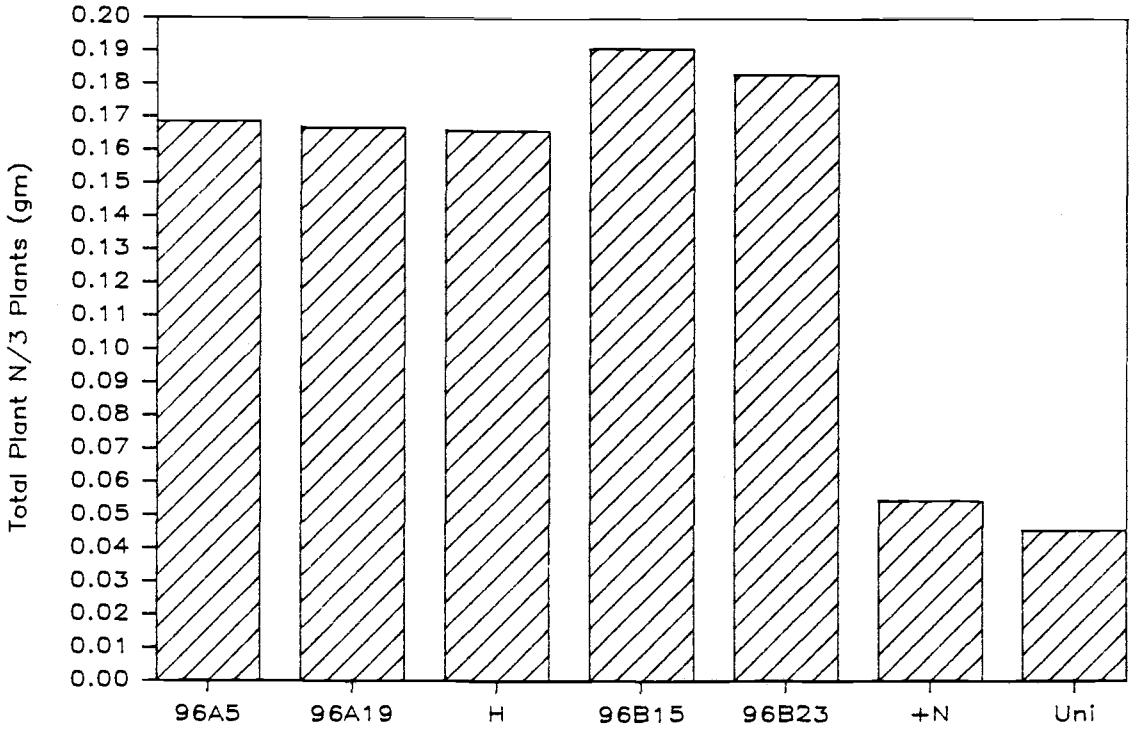

Figure 5. Mean total plant N (g) of 42 day-old Lupinus albus plants (supplementary experiment) as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

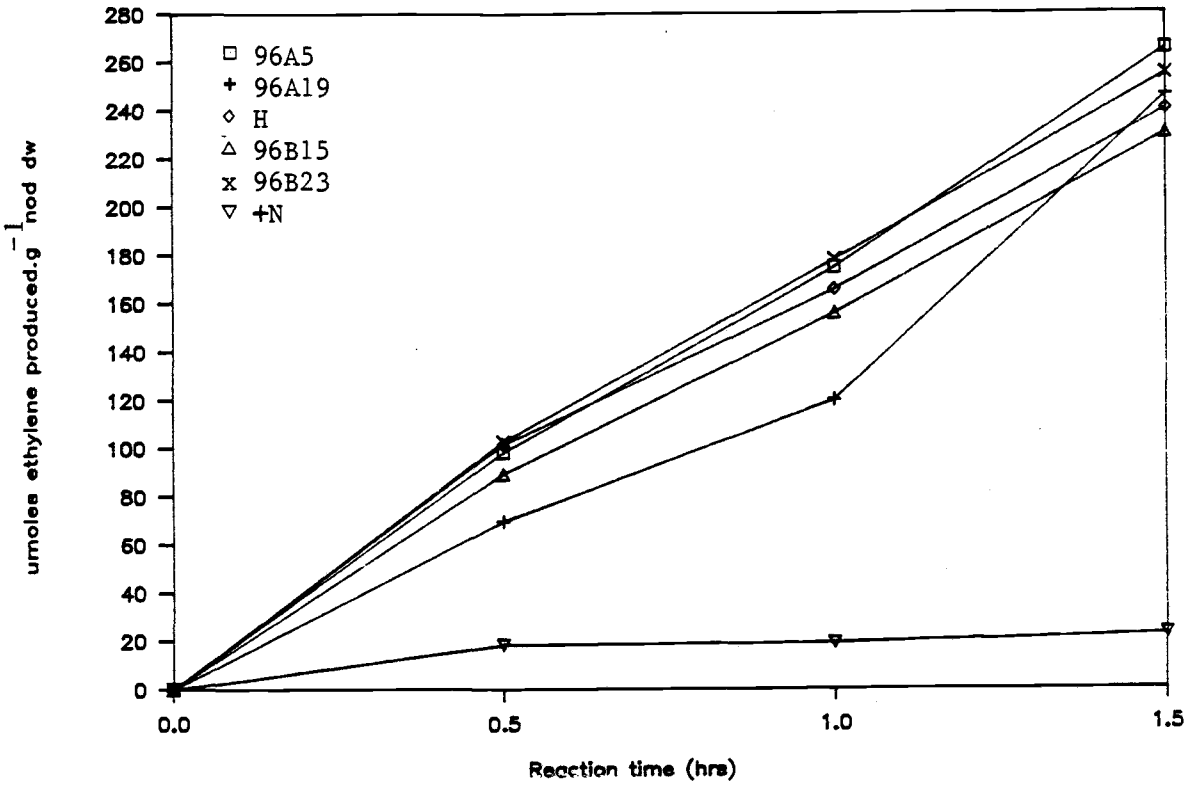

Figure 6. Mean acetylene reduction activity (umole ethylene evolved. $h^{-1}.g^{-1}$ dw of nodules) per three plants of 42 day-old Lupinus albus plants (supplementary experiment) as affected by different treatments. Treatments consisted of inoculation with five Rhizobium strains, a +N treatment, and an uninoculated treatment.

MEDICAGO TRUNCATULA EXPERIMENT

Shoot and Root Dry weights

Shoot dry weight for 45-day old plants was greatest with strain 102D6 (Figure 7). The next greatest weight (although not significantly different from 102D6) was observed with strain 102B11, which produced 28% less shoot weight than 102D6. No significant mean root weight differences were found between the +N treatment and strain treatments 102D6, 102A12, and 102B11. The +N treatment produced 63% less shoot weight and 32% less root weight than strain 102D6. Mean shoot and root dry weights are shown in Table 6.

Nodule morphology, number, and mass

Treatments 102D6 and 102B11 produced the highest mean number of nodules (Table 6). Treatments 102A12 and 102N1 produced significantly lower nodule numbers, with no nodules observed with the +N and uninoculated treatments.

Mean nodule dry weight values are shown in Table 6. Mean nodule numbers for 102D6 and 102B11 were significantly greater than for all other treatments, while mean nodule dry weight values were not significantly different among the four Rhizobium strains.

Nodules were primarily oblong in shape, with some lobed nodules. Nodules produced from treatment 102D6 were generally pink in color, with some tending more toward white. Nodules from treatment

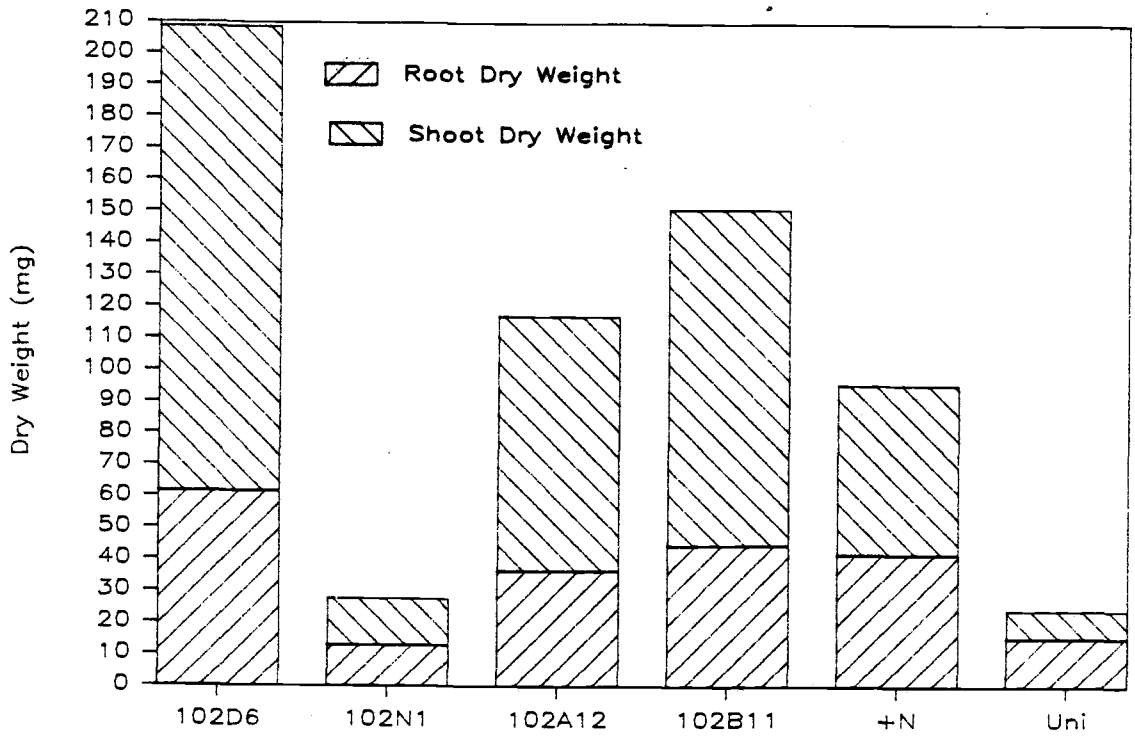

Figure 7. Mean shoot and root dry weight (mg) of 45 day-old Medicago truncatula plants as affected by different treatments. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

102B11 were more reddish, with some tending toward pink and white. Treatment 102A12 nodules were generally white, with some tending toward pink, while those of treatment 102N1 were nearly all white. Nodules were found throughout the root mass, with larger, more mature nodules on older roots.

Table 6. Mean shoot, root, and nodule dry weight (mg), nodule number, total plant N (mg), and acetylene reduction activity (umole ethylene evolved.h^{-1}.g^{-1} dw of nodules) of 45 day-old Medicago truncatula plants as affected by different treatments. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

Treatment	Dry weight (mg)			Nodule	Total Plant	Acetylene
	shoot	root	nodule	number	N (mg)	reduction
102D6	147.0a	62.6a	5.94a	38.5a	8.97	1009a
102N1	14.8de	1.3c	3.15ab	17.8b	0.41	85c
102A12	68.2bc	39.0b	3.24ab	19.1b	5.05	571b
102B11	106.5ab	44.7ab	5.54a	38.5a	6.76	944a
+N	54.0cd	41.9b	0.0b	0.0c	3.24	0.0c
Unin.	8.9e	15.4c	0.0b	0.0c	0.13	0.0c
LSD	41.6	18.6	3.31	12.8		

Means followed by different letters are significantly different at the .05 probability level.
Mean separation (.05 level) by independent t-test due to unbalanced data.

Total Plant Nitrogen

For total plant nitrogen (Table 6), replications within each treatment were analyzed together due to the small amount of material. Rhizobium strain 102D6 exhibited the highest level of total plant N: 8.97 mg. Strain 102B11 was 75% of 102D6 (6.76 mg), strain

102A12, 56% (5.05 mg), and the +N treatment 36% (3.24 mg). Strain 102N1 was only 4.57% of 102D6 (0.41 mg) while the uninoculated treatment was 1.45% (0.13 mg)(Figure 8).

Acetylene reduction

Treatments 102D6, 102A12, and 102B11 exhibited the highest rates of acetylene reduction activity and are significantly higher (P=.05) than strain 102N1 (Figure 9). Treatment values for ethylene evolution are shown in Table 6.

TRIFOLIUM ALEXANDRIUM EXPERIMENT

Shoot and Root Dry weights

The greatest shoot and root dry weight for 42 day-old plants (Figure 10) was produced by the +N treatment, with approximately 54% greater shoot weight and 74% greater root weight than the highest Rhizobium treatments (WCI-1 and 162X95). No significant difference was noted between treatments WCI-1 and 162X95. Shoot and root dry weights mean values are shown in Table 7.

Nodule morphology, number, and mass

Strain WCI-1 had the highest nodule number, although not significantly higher than strains LX684 and 162X95 (Table 7).

WCI-1 exhibited the greatest nodule dry weight and was significantly greater than 162X95, which was significantly greater than LX684 (Table 7).

Nodules from strains WCI-1 and 162X95 were red to reddish-

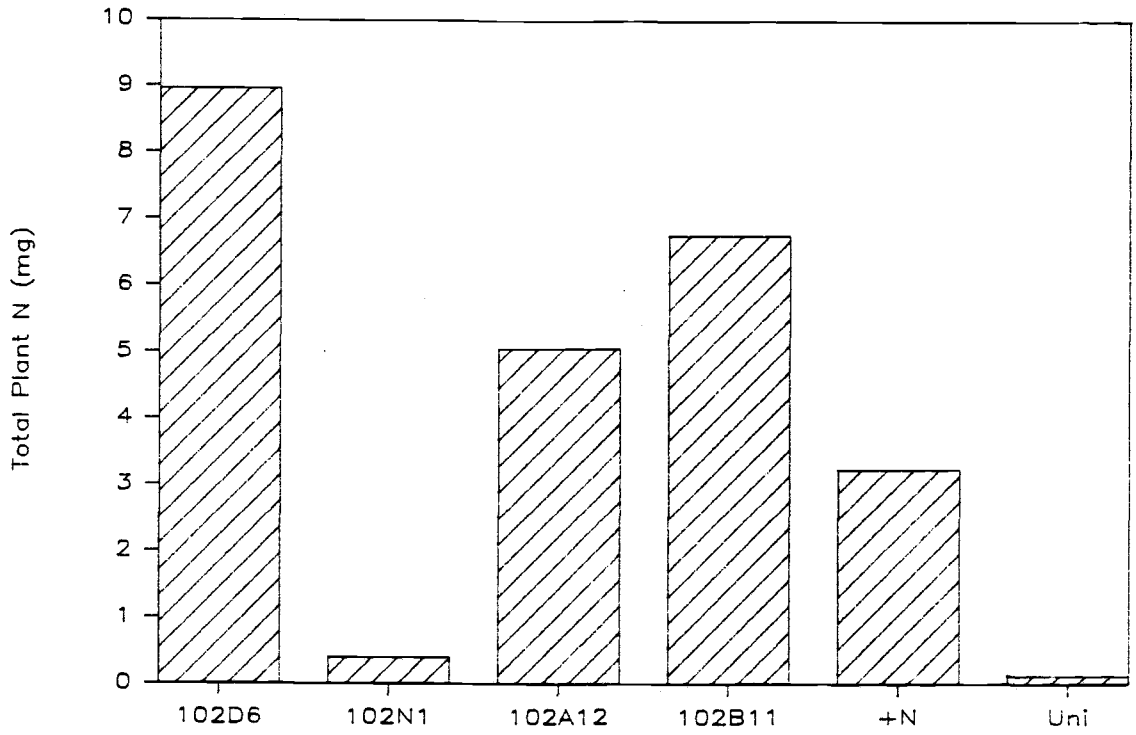

Figure 8. Mean total plant N (mg) of 45 day-old Medicago truncatula plants as affected by different treatments. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

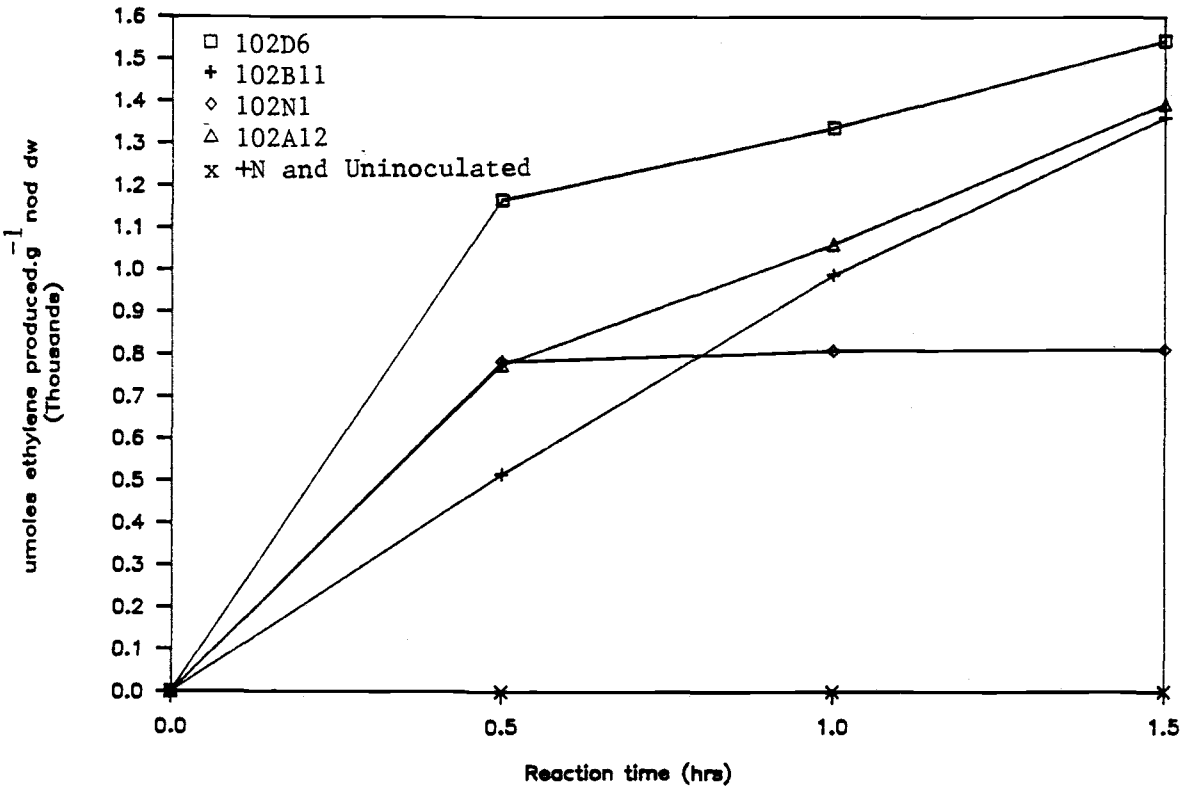

Figure 9. Mean acetylene reduction activity (umole ethylene evolved. $h^{-1}.g^{-1}$ dw of nodules) of 45 day-old Medicago truncatula plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treat ment.

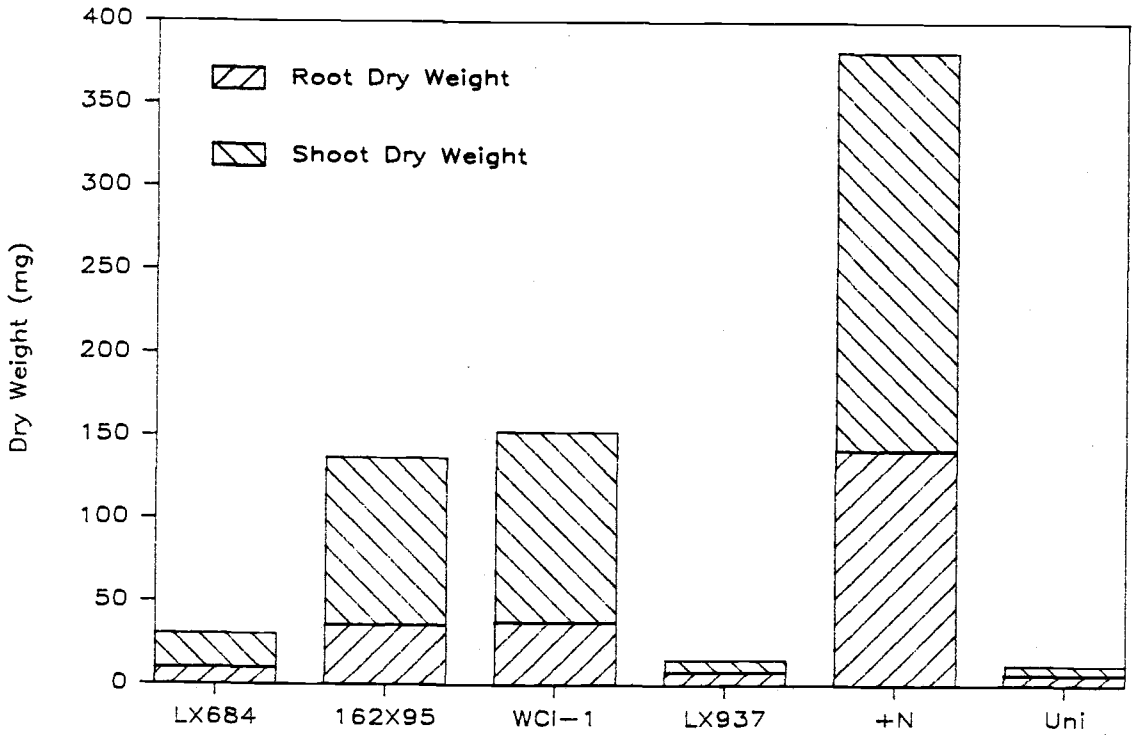

Figure 10. Mean shoot and root dry wt (mg) of 42 day-old Trifolium alexandrium plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

pink in color and oblong with many lobes. Those from LX684 tended to be smaller, round, and white to pink in color. Only one replicate of the LX937 treatment produced nodules, those nodules were small, round, and white.

Table 7. Mean shoot, root, and nodule dry weight (mg), nodule number, total plant N (mg), and acetylene reduction activity (umole ethylene evolved.h^{-1}.g^{-1} dw of nodules) of 42 day-old Trifolium alexandrium plants as affected by different treatments. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

Treatment	Dry weight (mg)			Nodule	Total Plant	Acetylene
	shoot	root	nodule	number	N (mg)	reduction
LX684	20.6c	10.2c	2.48c	31.63a	0.44	528a
162X95	100.9b	36.2b	6.34b	26.88a	4.70	791a
WCI-1	115.1b	37.7b	12.36a	37.13a	5.24	818a
LX937	7.4c	8.2c	0.05d	0.13b	0.04	3.4b
+N	240.3a	141.9a	0.00d	0.00b	5.96	0.0b
Unin.	5.9c	6.5c	0.00d	0.0b	0.03	0.0b
LSD	35.0	14.3	2.058	9.59		475

Means followed by different letters are significantly different at the .05 probability level.

Total Plant Nitrogen

Total plant nitrogen (Table 7) was determined on pooled replicates of each treatment due to the small amount of material. The +N treatment exhibited the highest level of total plant N: 5.96 mg. Strain WCI-1 was 88% of +N (5.24 mg), and strain 162X95, 79% (4.7 mg). Strain LX684 was only 7% of +N (0.44 mg), while strain LX937 and the uninoculated treatment were 0.7% (0.04 and 0.03 mg respectively) (Figure 11).

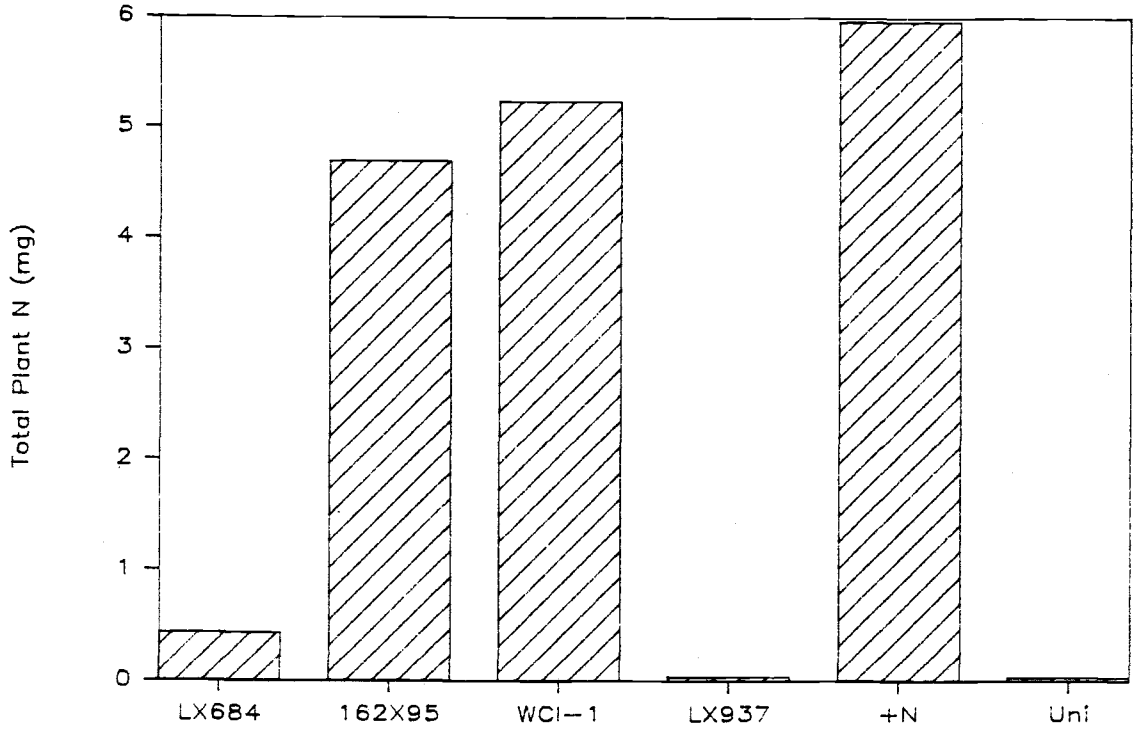

Figure 11. Mean total plant N (mg) of 42 day-old Trifolium alexandrium plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

Acetylene reduction

Rhizobium strains 162X95, WCI-1, and LX684 exhibited the greatest acetylene reduction activity, with no significant differences between their rates (Figure 12). The rate for strain LX937 was significantly lower than for the other three strains. Treatment rate values for ethylene evolution are shown in Table 7.

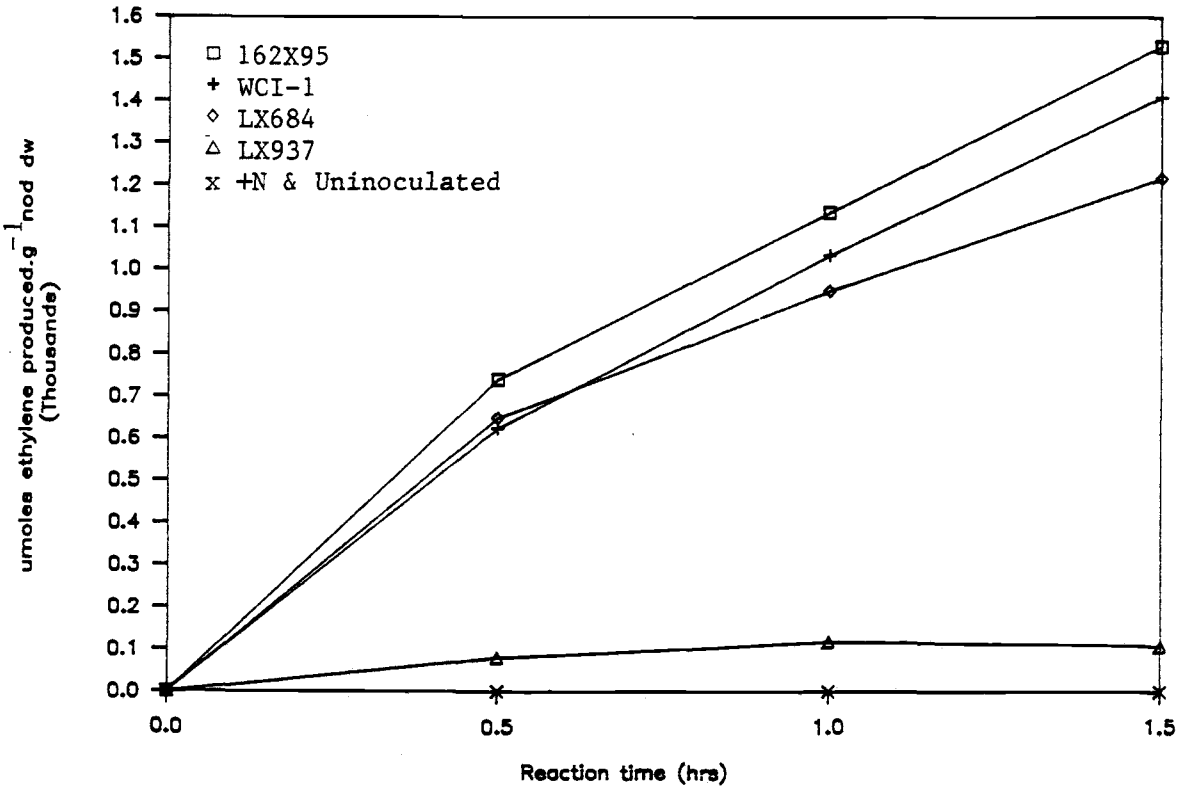

Figure 12. Mean acetylene reduction activity (umole ethylene evolved. $h^{-1}.g^{-1}$ dw of nodules) of 42 day-old Trifolium alexandrium plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

DISCUSSION

Lupinus albus Initial Experiment

Shoot growth with Rhizobium strain 96B15 was less than that of the +N treatment, but not significantly less, indicating that this strain was able, through BNF, to supply the total N required by white lupin. Strain 96A5 resulted in slightly less shoot dry matter than the +N treatment but not significantly less than strain 96B15. Root growth was highest in the +N treatment and was significantly higher than all other treatments.

Strains 96B15 and 96A5 were well nodulated and exhibited significant acetylene reduction activity, with strain 96A5 approximately 33% higher than 96B15. These two strains and the +N treatment had comparably high levels of total plant N. These characteristics of 96B15 and 96A5 (good shoot and root growth and high rates of acetylene reduction) suggest that these strains are good candidates for further testing in field environments where L. albus is grown.

Strain FWR-4, a nodule isolate from L. polyphyllus found growing locally (Corvallis, Oregon), was not effective in establishing nodulation of L. albus. It cannot be concluded from this one isolate that there are no strains found in conjunction with L. polyphyllus that are effective on L. albus. This particular indigenous isolate, however, was ineffective, possibly related to an inability to infect the root hairs.

FWR-LA also was a poor strain, forming a total of only three nodules in the 6 replications. FWR-LA was a nodule isolate from a six week-old L. albus plant grown in the greenhouse in soil collected from around the L. polyphyllus plant. Although several nodules (8-10) were found on the L. albus plant from which the isolate was made, that number is still considerably fewer than the number found on effectively nodulated plants (105) from this experiment. This poor degree of nodulation may also be due to an impaired ability to infect the root hairs of L. albus. No serologic tests were conducted to determine if FWR-LA and FWR-4 were the same or different indigenous strains.

In the initial experiment, commercial inoculum 'H', a composite of several R. lupini strains, was ineffective in nodule establishment on L. albus. Since 'H' is recognized as an effective inoculum source, the experiment was repeated using a different source of 'H'. These results are discussed under 'Lupinus albus Supplementary Experiment'.

Because of the storage reserve in the large seeds of lupin, seedlings are able to develop for several weeks without added nitrogen. Thus, even the uninoculated and ineffective Rhizobium treatment lupin plants survived until harvest.

Lupinus albus Supplementary Experiment

In this trial, strains FWR-4 and FWR-LA were replaced by strains 96A19 and 96B23 which were provided by the Nitragin Co. New inoculum for commercial strain 'H' was also provided by the Nitragin Co. All Rhizobium strains were highly effective in all criteria. For shoot,

root, and nodule dry weights, total plant N, and acetylene reduction, no significant differences were noted between strains. Nodule number varied between 126 for strain 96A19 to 204 for strain 96A5. Upon depletion of the initial KNO_3 in the nutrient solution of the +N treatment (as evidenced by poor plant performance), additional KNO_3 was provided. Plant response to this addition was slow, however, and resulted in sub-optimal growth at harvest and a poor comparison for optimal plant growth.

The Rhizobium strains evaluated in this experiment were collected from L. albus and L. angustifolius, with 'H' being a L. spp. mix. This would suggest that either; 1) Rhizobium strains from these two lupin species are cross-infective or that, 2) these strains are the same, are able to infect both lupin species, and elicit slightly different plant response.

Data for strains 96B15 and 96A5 from the initial L. albus experiment were confirmed in the supplementary experiment; these strains were effective in supporting optimal tissue growth and N fixation. Although further trials under conditions more similar to those found in Tunisian production areas may produce differences in plant response (due to differing strain tolerances of pH, soil temperature and moisture, and competitiveness with rhizosphere microorganisms including indigenous Rhizobium strains), these experiments have identified a subset of highly effective strains worthy of further testing.

Medicago truncatula Experiment

Strain 102D6 exhibited the highest overall effectiveness for Medicago truncatula, as evidenced by shoot, root, and nodule dry weight and nodule number. Strain 102B11 showed slightly lower effectiveness. Strain 102D6 was highest in total plant N and was 25% higher than the next highest strain, 102B11. Strain 102A12 was 44% less than 102D6, while the +N treatment was 64% less. No significant differences (P=.05) were noted between values for Rhizobium strain treatments 102D6, 102A12, and 102B11 for acetylene reduction activity. Values for strain 102N1 were significantly lower than for the other three strains.

In these experiments, acetylene reduction activity was expressed as umoles of ethylene produced per hour per gram of nodule dry weight. Due to the small nodule dry weight of even very well-nodulated small-seeded legumes, the calculated rates are quite high (100 to 1,000). When acetylene reduction activity was calculated on a per plant vs. per nodule dry weight basis, however, values ranged from 1.54 umoles/plant/hour for strain 102N1 to 5.05 for strain 102B11. These values are comparable to those reported by Smith and Baltensperger (1983) in their work with effectively nodulated 12 week-old M. truncatula. They recorded acetylene reduction activity values of approximately 3 umoles/plant/hour. Hopmans et al. (1982) and Alston and Graham (1982) found similar values (3.0 to 5.6) in their work with M. truncatula.

Since data reported for acetylene reduction were not based on total umoles of ethylene produced, but rather on the rate of fixation (production per gram nodule dry weight per hour), strains resulting in small nodule dry weights can be comparable in acetylene reduction activity to well nodulated strains. This suggests that some factor(s) other than a strain's ability to reduce dinitrogen is limiting the plant's ultimate response. This could include less than optimal infection and nodule formation or a temperature or pH requirement that differs from more effective strains.

None of the Rhizobium strains evaluated in these experiments was collected from M. truncatula, but from other annual medics; strain 102D6 was collected from M. orbicularis, 102B11 from M. hispida, and strain 102A12 from M. arabica; strain 102N1 was collected in Tunisia from M. arborea. This may not be a significant factor, but further trials should evaluate at least one strain collected from M. truncatula, whether indigenous to Tunisia or from some other source.

If field trials in Tunisia confirm that strain 102N1 is only marginally effective for M. truncatula, strains other than this native strain will need to be used for inoculation purposes. If ineffective, it would also need to be determined, before inoculation production begins, if this strain is commonly found in the soils of the major forage production areas. If it is present in high numbers and is also a highly competitive strain, it would preferentially bind to the root hairs of M. truncatula at the expense of other, more effective strains. If this were to occur, optimum N_2 fixation and subsequent plant performance would be limited.

In the M. truncatula experiment, the +N treatment was not the optimal comparison treatment for shoot and root dry weight, since several Rhizobium strains exceeded this treatment. This indicates that additional N was needed in the +N experiment. Nitrate as KNO_3 was not added separately to the +N treatment, but rather as part of the overall nutrient solution. At the recommended rate of 5 mM, the plants apparently depleted the solution of available N before additional solution could be added. Design of future growth chamber experiments should allow for providing additional N, by using a higher concentration or adding N more frequently.

Trifolium alexandrium Experiment

In contrast to the M. truncatula experiment, the +N treatment for T. alexandrium was a more optimal growth treatment, providing a better comparison for the Rhizobium strains.

Rhizobium strains WCI-1 and 162X95 demonstrated a high effectiveness for T. alexandrium in all evaluation criteria, while strains LX684 and LX937 were considerably less effective. Strain LX684 produced an acetylene reduction rate comparable to WCI-1 and 162X95, but exhibited a significantly lower nodule dry weight. When calculated on a per plant vs. per nodule dry weight basis, acetylene reduction activity values ranged from 1.94 umoles/plant/hour for strain LX684 to 11.37 for strain WCI-1. Hopmans et al. (1982) recorded values near 5.5 umoles/plant/hour for effectively nodulated 10

week-old T. subterraneum plants. Thus, acetylene reduction values obtained for T. alexandrium are comparable to previous Trifolium spp. experiments.

Total plant N was highest with the +N treatment while strains WCI-1 and 162X95 produced values 88% and 79% of +N. All other treatments produced values of only 7% of the +N treatment or less.

Rhizobium strains 162X95 and LX937 were both collected from T. alexandrium. Strain 162X95 was very effective, while LX937 was nearly completely ineffective. This is a puzzling anomaly, as LX937 was collected in Tunisia from native T. alexandrium and would be expected to be an effective strain. As with M. truncatula strain 102N1, strain LX937 may be common in Tunisian soils and could preferentially bind to the root hairs of T. alexandrium at the expense of other, more effective strains. Additional nodule collection and strain identification experiments are needed to evaluate this possibility.

Strain LX684 was collected from T. fragiferum while strain WCI-1 was locally collected (Corvallis, OR) from T. repens. WCI-1 was highly effective. A slant has been sent to the Nitragin Co. to determine whether it is unique or can be identified as a known strain. In either case, it has potential for future use with T. alexandrium.

RECOMMENDATIONS

Based on the evaluation methods utilized and considering the growing conditions (temperature, lighting, growth media, and nutrient solution) to which plants were subjected, the following Rhizobium strains are recommended for further evaluation under field conditions: 1) 96A5, 96A19, 96B15, 96B23, and H for Lupinus albus, 2) 102D6 and 102B11 for Medicago truncatula, and 3) WCI-1 and 162X95 for Trifolium alexandrium.

In accordance with the stated objectives of 'Maximizing N_2 fixation and yield of forage legumes grown in Tunisia', further greenhouse/growth chamber studies should be instituted. Studies to evaluate which Rhizobium strains elicit the highest protein and energy content, and the content of other nutrients necessary for animal gain from the forage would be desirable. Studies should also be designed to evaluate strains for maximum plant N as it relates to improvement of soil fertility through green manuring or root N as it relates to stubble incorporation. Other valuable greenhouse/growth chamber studies would incorporate temperature and soil moisture and temperature conditions comparable to those of various Tunisian growing areas.

Experiments to be conducted in Tunisian field trials should evaluate forage dry matter response for each chosen strain under irrigated vs. non-irrigated conditions, response on different soil types commonly found in the major forage areas, and competitiveness

(infectiveness and effectiveness) of chosen strains with indigenous strains. The studies already mentioned for the greenhouse and growth chamber should be verified in field experiments.

Since the results of the experiments described are from a relatively small number of Rhizobium strains for each legume species, additional strains should be collected and evaluated. Sources could include additional strains indigenous to Tunisia, other commercially available strains, and strains already evaluated in other research programs.

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APPENDIX

STATISTICAL METHODS AND RATIONALE

For most argricultural experiments, data tend to depart from the laws of exact causality. It is nearly impossible to precisely predict the results of an experiment due to chance variations resulting from a multitude of uncontrollable variables. It is the purpose of statistical science to provide an objective basis for the analyses and evaluation of experimental data. Three principles important to all experimental designs are; 1) replication of treatments, 2) randomized assignment of treatments to experimental units and, 3) local control which allows for certain restrictions on randomization to reduce experimental error.

The statistical procedure for comparing means from two or more treatments uses an assumption called the 'null hypothesis', which assumes that the treatments have no effect. If analysis indicates that the observed differences would rarely occur in random samples drawn from a population with equal means and variances, the 'null hypothesis' can be rejected with the conclusion that at least one treatment had a real effect. If the probability is 5% or less that the observed variation among means could occur by chance, the means are said to be 'significantly different'.

Treatment effects for these experiments were evaluated using analysis of variance as it is the only simple and reliable method of determining the appropriate pooled error variance 's^2'. ANOVA tables were prepared to determine the error mean square and 'F

value'. A calculated F value equal to or greater than the F value at a specified probability, i.e. 5%, from an F table indicates that the null hypothesis is rejected.

Separation of treatment means was performed using 'Fisher's protected LSD value' ($LSD = t_{.05}*((2*MSE)/r)^{1/2}$). This method is used as for 'ranking' the means when F is significant. A difference between means greater than the LSD value indicates a significant difference at the indicated level (i.e. 0.05).

A completely randomized design (CRD) was used as it is most useful in experiments where there are no identifiable sources of variation, i.e. soil, light, nutrient, temperature, among the experimental units other than treatment effects. All the experiments conducted for this project were performed either in a greenhouse or growth chamber with controlled environments. The CRD is the most flexible design with regard to the physical arrangement of experimental units, maximizes the degrees of freedom available for estimating experimental error, and minimizes the F value required for statistical significance.

Table 8. Lupinus albus initial experiment shoot dry weight (g) data table and ANOVA of 43 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96B15	96A5	H	FWR-4	FWR-LA	+N	Unin.
Reps							
1	4.23	5.42	2.21	2.46	2.82	7.27	2.12
2	3.57	3.59	2.40	2.50	2.39	4.59	2.26
3	4.25	3.25	1.80	2.59	1.67	5.26	2.67
4	4.55	4.10	2.94	2.46	2.98	5.08	2.55
5	4.87	3.86	2.68	2.38	1.97	4.27	2.95
6	4.78	4.20	2.56	2.90	2.32	4.75	2.68
Means	4.38	4.07	2.43	2.55	2.36	5.20	2.54

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	41	61.09		
Treatment	6	48.79	8.13	23.14
Error	35	12.30	0.35	

† $F_{.01} > 3.37$

$t_{.01} = 2.724$

$LSD = 2.724*((2*0.35)/6)^{1/2} = 0.9323$

Table 9. *Lupinus albus* root dry weight (g) data table and ANOVA of 43 day-old plants as affected by treatment. Treatments consisted of inoculation with four *Rhizobium* strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96B15	96A5	H	FWR-4	FWR-LA	+N	Unin.
Reps							
1	1.43	1.30	0.93	1.06	1.09	2.30	1.25
2	0.84	0.72	0.82	0.75	0.76	2.13	0.37
3	1.07	0.62	0.85	1.48	0.68	1.88	1.02
4	1.51	4.03	0.97	0.89	1.27	1.48	1.32
5	1.51	1.27	1.13	0.99	0.90	1.40	1.28
6	1.54	1.01	0.74	0.90	0.81	4.53	1.59
Means	1.32	1.49	0.91	1.01	0.92	2.29	1.14

ANOVA

Source	df	Sum of Squares	Mean Square	F †
Total	41	25.25		
Treatment	6	8.52	1.42	2.97
Error	35	16.73	0.48	

† $F_{.05} > 2.37$

$t_{.05} = 2.030$

$LSD = 2.030*((2*0.48)/6)^{1/2} = 0.8103$

Table 10. Lupinus albus nodule number data table and ANOVA of 43 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96B15	96A5	H	FWR-4	FWR-LA	+N	Unin.
Reps							
1	79	151	0.00	0.00	1	0.00	0.00
2	90	96	0.00	0.00	0.00	0.00	0.00
3	96	83	0.00	0.00	0.00	0.00	0.00
4	103	92	0.00	0.00	2	0.00	0.00
5	157	100	0.00	0.00	0.00	0.00	0.00
6	108	95	0.00	0.00	0.00	0.00	0.00
Means	105.50	102.83	0.00	0.00	0.50	0.00	0.00

ANOVA

Source	df	Sum of Squares	Mean Square	F †
Total	41	99497.83		
Treatment	6	92850.00	15475.00	81.47
Error	35	6647.83	189.94	

† $F_{.01} > 3.37$

$t_{.01} = 2.724$

$LSD = 2.724*((2*189.94)/6)^{1.2} = 21.67$

Table 11. Lupinus albus nodule dry weight (g) data table and ANOVA of 43 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96B15	96A5	H	FWR-4	FWR-LA	+N	Unin.
Reps							
1	.1035	.1676	.0000	.0000	.0058	.0000	.0000
2	.1124	.0874	.0000	.0000	.0000	.0000	.0000
3	.1547	.0735	.0000	.0000	.0000	.0000	.0000
4	.1216	.1154	.0000	.0000	.0144	.0000	.0000
5	.1602	.0906	.0000	.0000	.0000	.0000	.0000
6	.1393	.0905	.0000	.0000	.0000	.0000	.0000
Means	.1320	.1042	.0000	.0000	.0034	.0000	.0000

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	41	.1291		
Treatment	6	.1205	.0201	81.87
Error	35	.0086	.0002	

† $F_{.01} > 3.37$

$t_{.01} = 2.724$

$LSD = 2.724*((2*.0002)/6)^{1/2} = 0.0247$

Table 12. Lupinus albus total plant N (g) data table and ANOVA of 43 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96B15	96A5	H	FWR-4	FWR-LA	+N	Unin.
Reps							
1	2.40	2.93	1.38	1.14	1.23	1.79	1.23
2	2.87	2.74	1.29	1.35	1.27	1.78	1.55
3	2.81	2.59	1.37	1.11	1.46	2.06	1.31
4	2.27	2.64	1.19	1.23	1.29	2.06	1.21
5	2.62	2.55	1.28	1.24	1.36	2.27	1.15
6	2.73	2.51	1.34	1.40	1.34	1.56	1.11
Means	2.62	2.66	1.31	1.25	1.33	1.92	1.26

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	41	15.84		
Treatment	6	14.87	2.48	88.88
Error	35	0.98	0.03	

† $F_{.01} > 3.37$

$t_{.01} = 2.724$

$LSD = 2.724*((2*.03)/6)^{1/2} = 0.2618$

Table 13. *Lupinus albus* acetylene reduction activity (umole ethylene evolved.$h^{-1}.g^{-1}$ dw of nodules) data table and ANOVA of 43 day-old plants as affected by treatment. Treatments consisted of inoculation with four *Rhizobium* strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
Reps	96B15	96A5	H	FWR-LA	FWR-4	+N	Unin.
1	92.83	143.32	.00	-41.41	.00	.00	.00
2	72.66	73.29	.00	.00	.00	.00	.00
3	89.02	115.47	.00	.00	.00	.00	.00
4	40.82	158.19	.00	33.36	.00	.00	.00
5	86.97	118.36	.00	.00	.00	.00	.00
6	98.86	176.95	.00	.00	.00	.00	.00
Sums	481.16	785.58	.00	-8.05	.00	.00	.00
Means	80.19	130.93	.00	-1.34	.00	.00	.00

Source	df	Sum of squares	Mean square	F †
Total	41	138848.19		
Treatment	6	127058.50	21176.42	62.87
Error	35	11789.70	336.85	

† $F_{.01} > 3.37$

$t_{.01} = 2.724$

$LSD = 2.724*((2*336.85/6)^{1/2} = 28.7$

Table 14. Lupinus albus supplemental experiment shoot dry weight (g) data table and ANOVA of 42 day-old plants as affected by treatments. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96A5	96A19	H	96B15	96B23	+N	Unin.
Reps							
1	3.18	4.71	2.59	3.02	3.59	3.24	3.63
2	5.11	3.92	3.54	3.88	4.84	2.47	2.77
3	4.16	3.95	3.44	5.22	4.29	2.25	2.50
4	4.29	2.45	4.44	3.60	4.14	2.26	2.72
5	2.07	2.78	3.58	4.89	4.10	2.68	2.20
Sums	18.80	17.80	17.59	20.61	20.95	12.90	13.82
Means	3.76	3.56	3.52	4.12	4.19	2.58	2.76

Source	df	Sum of squares	Mean square	F †
Total	34	323.6		
Treatment	6	306.99	51.17	86.25
Error	28	16.61	.5932	

† $F_{.05} > 2.44$

$t_{.05} = 2.048$

$LSD = 2.048*((2*.5933)/5)^{1/2} = 1.00$

Table 15. Lupinus albus supplemental experiment root dry weight (g) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96A5	96A19	H	96B15	96B23	+N	Unin.
Reps							
1	.78	1.00	.67	.77	.72	1.37	.82
2	1.25	.91	.78	1.28	1.02	.80	.56
3	.93	1.27	.82	1.49	.93	1.39	.60
4	.96	.47	1.66	.93	.81	1.30	.61
5	.47	1.03	1.38	1.21	.91	.88	.43
Sums	4.38	4.68	5.31	5.69	4.39	5.74	3.01
Means	.88	.94	1.06	1.14	.88	1.15	.60

Source	df	Sum of squares	Mean square	F †
Total	34	24.63		
Treatment	6	22.44	3.74	47.75
Error	28	2.19	.0782	

†$F_{.05} > 2.44$

$t_{.05} = 2.048$

$LSD = 2.048*((2*.0782)/5)^{1/2} = .3622$

Table 16. Lupinus albus supplemental experiment nodule number data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96A5	96A19	H	96B15	96B23	+N	Unin.
Reps							
1	167	133	172	117	128	0	0
2	226	172	158	134	153	0	0
3	215	125	217	182	189	36	0
4	207	102	181	176	126	0	0
5	207	97	171	213	209	4	0
Sums	1022	629	899	822	805	40	0
Mean	204.4	125.8	179.8	164.4	161	8	0.0

ANOVA

Source	df	Sum of squares	Mean square	F†
Total	34	226646.74		
Treatment	6	206638.74	34439.79	48.2
Error	28	20008	714.57	

† $F_{.01} > 3.53$

$t_{.01} = 2.763$

$LSD = 2.763*((2*714.57)/5)^{/2} = 46.71$

Table 17. Lupinus albus supplemental experiment nodule dry weight (g) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96A5	96A19	H	96B15	96B23	+N	Unin.
Reps							
1	.1638	.2582	.1520	.1635	.2128	.0000	.0000
2	.3354	.2595	.2250	.2376	.3124	.0000	.0000
3	.2867	.1944	.2435	.2893	.3011	.0233	.0000
4	.2916	.1293	.2236	.2290	.2708	.0000	.0000
5	.1342	.1439	.2042	.3175	.2731	.0070	.0000
Sums	1.2117	.9853	1.0483	1.2369	1.3702	.0303	.0000
Mean	.2423	.1971	.2097	.2474	.2740	.0061	.0000

Source	df	Sum of squares	Mean square	F†
Total	34	.4719		
Treatment	6	.4005	.0068	26.17
Error	28	.0714	.0026	

† $F_{.01} > 3.53$

$t_{.01} = 2.763$

$LSD = 2.763*((2*.0026)/5)^{1/2} = .0891$

Table 18. Lupinus albus supplemental experiment total plant N (g) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
	96A5	96A19	H	96B15	96B23	+N	Unin.
Reps							
1	.1429	.2158	.1195	.1354	.1647	.0603	.0579
2	.2423	.1876	.1520	.2076	.1999	.0618	.0447
3	.1714	.1804	.1773	.2281	.1846	.0681	.0428
4	.1971	.1205	.2068	.1677	.1892	.0205	.0455
5	.0904	.1301	.1743	.2159	.1768	.0616	.0352
Sums	.8440	.8343	.8300	.9548	.9152	.2723	.2262
Mean	.1688	.1669	.1660	.1910	.1830	.0545	.0452

Source	df	Sum of squares	Mean square	F†
Total	34	.1470		
Treatment	6	.1148	.0191	16.69
Error	28	.0321	.0011	

† $F_{.01} > 3.53$

$t_{.01} = 2.763$

$LSD = 2.763*((2*.0011)/5)^{1/2} = .0580$

Table 19. Lupinus albus supplemental experiment acetylene reduction activity (umole ethylene evolved.$h^{-1}.g^{-1}$ dw of nodules) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS						
Reps	96A5	96A19	H	96B15	96B23	+N	Unin.
1	185.49	212.51	155.02	181.14	156.07	-4.33	.00
2	173.11	131.63	148.69	88.71	167.63	0	.00
3	146.70	90.57	152.62	93.13	124.89	0	.00
4	130.52	171.62	56.93	143.54	200.67	10.60	.00
5	206.88	86.39	127.12	157.80	103.49	0	.00
Sums	842.70	692.72	640.38	664.32	752.75	6.27	.00
Means	168.54	138.54	128.08	132.86	150.55	1.25	.00

ANOVA

Source	df	Sum of squares	Mean square	F†
Total	34	339108.38		
Treatment	6	304532.14	50755.36	41.10
Error	28	34576.24	1234.87	

† $F_{.01} > 3.37$

$t_{.01} = 2.724$

$LSD = 2.724*((2*1234.87)/6)^{1/2} = 55$

Table 20. Medicago truncatula shoot dry weight (mg) data table and ANOVA of 45 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	102D6	102N1	102A12	102B11	+N	Unin.
Reps						
1	248.0	17.3	9.2	91.3	33.5	7.9
2	173.2	1.6	124.5	104.8	56.4	3.3
3	157.6	13.0	37.0	110.0	55.6	9.9
4	101.4	23.2	52.3	67.4	42.0	17.6
5	3.8	6.5	137.3	16.9	61.0	9.9
6	166.8	25.6	61.9	170.8	61.8	6.7
7	199.2	17.4	98.5	129.5	51.0	3.9
8	126.1	14.0	25.2	161.0	70.3	11.8
Sums	1176.1	118.6	545.9	851.7	431.6	71.0
Means	147.0	14.8	68.2	106.5	54.0	8.9

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	185432.8		
Treatment	5	113845.7	22769.1	13.4
Error	42	71587.1	1704.5	

† $F_{.05} > 2.44$

$t_{.05} = 2.018$

$LSD = 2.0182*((2*1704.5)/8)^{1/2} = 41.7$

Table 21. *Medicago truncatula* root dry weight (mg) data table and ANOVA of 45 day-old plants as affected by treatment. Treatments consisted of inoculation with four *Rhizobium* strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	102D6	102N1	102A12	102B11	+N	Unin.
Reps						
1	94.50	6.80	8.00	35.90	38.90	8.40
2	96.50	18.00	50.90	29.30	40.60	27.50
3	68.10	10.30	24.80	36.80	30.80	12.50
4	33.20	8.00	28.80	34.50	54.30	10.00
5	16.70	10.00	60.00	17.30	47.70	3.80
6	67.90	30.50	31.70	102.30	44.60	27.50
7	69.50	10.50	38.90	50.70	34.70	15.30
8	46.00	10.00	68.70	50.70	43.50	18.30
Sums	492.40	104.10	311.80	357.50	335.10	123.30
Means	61.55	13.01	38.98	44.69	41.89	15.41

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	28040.68		
Treatment	5	13792.17	2758.43	8.13
Error	42	14248.51	339.25	

† $F_{.05} > 2.44$

$t_{.05} = 2.018$

$LSD = 2.018*((2*339.25)/8)^{1/2} = 18.58$

Table 22. Medicago truncatula nodule dry weight (mg) data table and ANOVA of 45 day-old plants as affected by treatment. Treatments consisted of four Rhizobium strains, a +N treatment, and an uninoculated treatment. Data expressed as mg.

	TREATMENTS					
	102D6	102N1	102A12	102B11	+N	Unin.
Reps						
1	8.50	.50	.20	12.60	.00	.00
2	5.30	1.10	5.00	4.60	.00	.00
3	7.00	.50	1.10	6.70	.00	.00
4	4.70	17.90	3.20	2.80	.00	.00
5	.10	.30	6.10	.60	.00	.00
6	7.20	2.50	1.90	6.80	.00	.00
7	11.10	.30	2.60	4.20	.00	.00
8	3.60	2.10	5.80	6.00	.00	.00
Sums	47.50	25.20	25.90	44.30	.00	.00
Means	5.94	3.15	3.24	5.54	.00	.00

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	717.58		
Treatment	5	265.15	53.03	4.92
Error	42	452.44	10.77	

† $F_{.05} > 2.44$

$t_{.05} = 2.018$

$LSD = 2.018*((2*.000012)/8)^{1/2} = 0.0035$

Table 23. Medicago truncatula nodule number data table and ANOVA of 45 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	102D6	102N1	102A12	102B11	+N	Unin.
Reps						
1	42	20	3	34	0	0
2	38	13	26	51	0	0
3	58	12	14	40	0	0
4	31	35	9	23	0	0
5	3	4	42	8	0	0
6	75	18	15	58	0	0
7	32	22	18	54	0	0
8	29	18	26	40	0	0
Sums	308	142	153	308	0	0
Means	38.50	17.75	19.13	38.50	0	0

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	18608.98		
Treatment	5	11872.60	2374.52	14.80
Error	42	6736.38	160.39	

† $F_{.05} > 2.44$

$t_{.05} = 2.018$

$LSD = 2.018*((2*160.39)/8)^{1/2} = 12.78$

Table 24. Medicago truncatula acetylene reduction activity (umole ethylene evolved.h^{-1}.g^{-1} dw of nodules) data table and ANOVA of 45 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	102D6	102N1	102A12	102B11	+N	Unin.
Rep						
1	299	0	634	332	0	0
2	1915		1154	745	0	0
3	942	0	461	303	0	0
4		35	218	906	0	0
5		0	624	1480	0	0
6	1119	76	668	1007	0	0
7	720	423	317	1661	0	0
8	1057	60	492	1121	0	0
Sums	6052	595	4569	7555	0	0
Means	1009	85	571	944	0	0

r = 8 p = 6
df total = 44 df treatment = 5 df error = 39

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	44	11862764		
Treatment	5	8069596	1613919	16.59
Error	39	3793168	97261	

† $F_{.05}>3.85$
Mean separation (.05 level) by independent t-test due to unbalanced data.

Table 25. *Trifolium alexandrium* shoot dry weight (mg) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four *Rhizobium* strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	LX684	162X95	WCI-1	LX937	+N	Unin.
Reps						
1	29.7	74.2	59.6	5.8	234.9	4.4
2	17.8	205.2	108.9	4.9	232.3	6.7
3	14.7	72.9	104.4	8.6	258.5	6.5
4	44.4	183.6	184.6	6.6	187.3	3.9
5	9.7	51.7	145.0	12.5	267.1	7.0
6	8.8	113.0	70.8	8.4	223.2	6.7
7	33.4	73.6	96.2	6.9	213.2	5.0
8	6.2	32.8	151.5	5.2	306.0	6.6
Sums	164.70	807.00	921.00	58.90	1922.50	46.80
Means	20.59	100.88	115.13	7.36	240.31	5.85

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	383691.04		
Treatment	5	333254.87	66650.97	55.50
Error	42	50436.17	1200.86	

† $F_{.01} > 3.49$

$t_{.01} = 2.6984$

$LSD = 2.6984*((2*1200.86)/8)^{1/2} = 46.75$

Table 26. Trifolium alexandrium root dry weight (mg) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	LX684	162X95	WCI-1	LX937	+N	Unni.
Reps						
1	14.70	26.60	19.50	6.50	138.60	4.80
2	8.70	73.60	35.70	5.50	137.10	7.50
3	7.20	26.10	34.20	9.50	152.60	7.20
4	21.90	65.90	60.40	7.20	110.50	4.30
5	4.80	18.50	47.50	13.80	157.70	7.80
6	4.40	40.50	23.20	9.30	131.80	7.40
7	16.50	26.40	31.50	7.70	125.90	5.50
8	3.10	11.70	49.60	5.80	180.70	7.30
Sums	81.30	289.30	301.60	65.30	1134.90	51.80
Means	10.16	36.16	37.70	8.16	141.86	6.48

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	115875.87		
Treatment	5	107390.14	21478.03	106.31
Error	42	8485.73	202.04	

† $F_{.01} > 3.49$

$t_{.01} = 2.6984$

$LSD = 2.6984*((2*202.04)/8)^{1/2} = 19.17$

Table 27. Trifolium alexandrium nodule dry weight (mg) data table and ANOVA of 42 day old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	LX684	162X95	WCI-1	LX937	+N	Unin.
Reps						
1	3.40	4.80	11.50	.00	.00	.00
2	1.60	10.50	9.80	.00	.00	.00
3	2.80	5.90	15.30	.00	.00	.00
4	3.60	12.70	13.00	.00	.00	.00
5	1.20	3.80	17.00	.00	.00	.00
6	1.30	6.50	9.00	.00	.00	.00
7	4.00	3.50	7.80	.00	.00	.00
8	1.90	3.00	15.50	.40	.00	.00
Sums	19.80	50.70	98.90	.40	.00	.00
Means	2.48	6.34	12.36	.05	.00	.00

ANOVA

Source	df	Sum of squares	Mean square	F†
Total	47	1166.9525		
Treatment	5	992.3200	198.4640	47.73
Error	42	174.6325	4.1579	

† $F_{.01} > 3.49$

$t_{.01} = 2.6984$

$LSD = 2.6984*((2*4.1579)/8)^{1/2} = 7.63$

Table 28. Trifolium alexandrium nodule number data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	LX684	162X95	WCI-1	LX937	+N	Unin.
Reps						
1	50	32	29	0	0	0
2	24	38	23	0	0	0
3	35	17	44	0	0	0
4	22	52	30	0	0	0
5	24	24	73	0	0	0
6	25	26	30	0	0	0
7	43	8	37	0	0	0
8	30	18	31	1	0	0
Sums	253	215	297	1	0	0
Means	31.63	26.88	37.13	.13	.00	.00

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	16377.92		
Treatment	5	12581.42	2516.28	27.84
Error	42	3796.50	90.39	

† $F_{.01} > 3.49$

$t_{.01} = 2.6984$

$LSD = 2.6984*((2*90.39)/8)^{1/2} = 12.8273$

Table 29. Trifolium alexandrium acetylene reduction activity (umole ethylene evolved.h^{-1}.g^{-1} dw of nodules) data table and ANOVA of 42 day-old plants as affected by treatment. Treatments consisted of inoculation with four Rhizobium strains, a +N treatment, and an uninoculated treatment.

	TREATMENTS					
	LX684	162X95	WCI-1	LX937	+N	Unin.
Reps						
1	774	1601	782	0	0	0
2	526	119	693	0	0	0
3	507	477	621	0	0	0
4	436	505	427	0	0	0
5	790	1247	855	0	0	0
6	0	758	2375	0	0	0
7	725	987	704	0	0	0
8	468	632	91	27	0	0
Sums	4226	6325	6546	27	0	0
Means	528	791	818	3	0	0

ANOVA

Source	df	Sum of squares	Mean square	F †
Total	47	11677588		
Treatment	5	6480878	1296176	10
Error	42	5196710	123731	

† $F_{.01} > 3.49$

$t_{.01} = 2.6984$

$LSD = 2.6984*((2*123731)/8)^{1/2} = 475$