AN ABSTRACT OF THE THESIS OF

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Title: Effect of Sulfur on Dinitrogen Fixation of Alfalfa (Medicago sativa L.)

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Field experiments were established in 1982 and 1983 on a Woodburn silt loam soil (Aquultic Argixeroll) to examine the effect of S fertilizer on dinitrogen fixation and dry matter yield of 10 week-old seedling alfalfa (Medicago sativa L. cv. 'Apollo').

Six levels of S fertilizer (0 to 67.2 kg S/ha) were applied as powdered gypsum. The experimental design was a split-block with one half of the seeds inoculated with commercial inoculum, and the other half non-inoculated.

Inoculation effects were highly significant in both years, indicating that the indigenous population of Rhizobium meliloti at the experimental sites was ineffective in N_2-fixation. Dry matter yields were higher in inoculated than in non-inoculated treatments. Inoculated plants also showed a significantly higher acetylene reduction rate, N concentration, and total tissue N and S. No significant differences in any of these parameters were detected for the different S treatments.

Sulfur fertilization increased the S concentration of non-inoculated plants more than inoculated plants, and decreased

the N:S ratio in the forage by increasing tissue S content. The highest value of N_2-fixed by the inoculated plants was obtained from the 44.8 kg S/ha treatment.

Greenhouse experiments were performed to evaluate the effect of varied nutrient solution concentrations of sulfate on the yield, nodulation, dinitrogen fixation, N and S concentrations, and partitioning of N and S into shoots and roots of six week-old alfalfa seedlings. Four levels of S (0, 1, 2.5, and 25 mg S/L) were applied in a randomized complete block design, with three replications. Seeds were inoculated with commercial inoculum, planted in plastic containers of acid-washed sand, and irrigated with nutrient solution for one minute, at 2 h intervals.

The addition of 2.5 mg S/L to the nutrient solution resulted in the highest total dry matter, acetylene reduction rate, total N content, percent S recovery, and percent increase in N due to dinitrogen fixation. N:S ratios were 50% higher in shoots (16:1) than roots (9:1), with S fertilization decreasing the N:S ratios.

Data from field and greenhouse experiments support the conclusion that S fertilization will increase seedling alfalfa yield when S levels in the plant are below 2.5 mg S/g (0.25%). In inoculated plants S fertilization increased both total N and S, demonstrating the importance of S in symbiotic N_2-fixation and the quality of forage produced.

EFFECT OF SULFUR ON DINITROGEN FIXATION OF ALFALFA (*Medicago sativa* L.)

by

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Typed by Gerardo Lopez-Jurado for Gerardo Lopez-Jurado

DEDICATED TO:

my wife Margoth for her love, understanding, help, and encouragment, and to our sons Andres, Mario, Javier, and Denisse, for their help, patience, and sharing of the burdens of completing my graduate program that made my objective our objective.

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INTRODUCTION

Nitrogen (N) is an essential element for all forms of life (Brill, 1977; Evans, 1969). As a component of amino acids, proteins, and nucleic acids (Goodwin and Mercer, 1983), N is essential to plant and animal nutrition (Emerich and Evans, 1980). Higher plants contain an average of 30 mg N/g (3%) on a dry matter basis, while microbes contain 80 mg N/g (8%), and animals often contain 100 mg N/g (10%) in the dry tissues (Evans, 1969).

Following water, N is the most frequently encountered factor limiting crop production (Stoskopt, 1981). The atmosphere provides a vast reservoir of molecular N_2 (79 to 80% N by volume), but this free atmospheric N_2 is not available to most plants and animals. Molecular N_2 can be converted to a usable form (ammonia) by alfalfa (*Medicago sativa* L.) and other legumes when growing in symbiotic association with appropriate *Rhizobium* species (Atlas and Bartha, 1981; Brill, 1977; Evans, 1975).

The scarcity of appropriate sources of energy, high costs for the manufacture of N fertilizers, and other problems associated with world food production have stimulated a renewed worldwide interest in biological nitrogen fixation (BNF)(Emerich and Evans,

1980; Hardy and Havelka, 1975; Quispel, 1974). The development of the acetylene reduction (AR) assay as an index of dinitrogen fixation (N_2-fixation) has permitted substantial progress in laboratory and controlled environmental investigations, providing a means of estimating the contribution of fixed N_2 by many systems (Emerich and Evans, 1980; Evans and Barber, 1977; Hardy et al., 1975; Heichel et al., 1981; Westermann and Kolar, 1978). These techniques have allowed attention to be given to the enhancement of N_2-fixation in legume cultivars and rhizobial strains with superior nodulation and/or N_2-fixation rates (Duke et al., 1980).

For centuries the ability of leguminous plants to improve soil productivity has been recognized. It is known that this property is associated with the symbiotic legume-Rhizobium association which converts atmospheric N_2 into ammonia (NH_3) (Evans, 1969). About 18,000 species of the family Leguminosae have been described, and aproximately 10% of these have been examined for nodulation. Nodulation has been found in more than 90% of the plants examined in the subfamilies Mimosoideae and Papilionoideae, but in only about 30% of Caesalpinoideae (Allen and Allen, 1976).

Nodulated legumes grown for pasture, grain, hay, and other agricultural purposes account for almost half, 8×10^{10} kg (80×10^6 metric tons) of the total N_2 fixed by biological systems each year (Brill, 1977; Hardy and Havelka, 1975). In the United States alone, leguminous crops have been estimated to fix 5,500 million kg of N (5.5 million metric tons) per year (Burris, 1976). It is

clear, therefore, that leguminous plants, growing in symbiosis with the appropriate Rhizobium species, are of great economic importance in the conversion of atmospheric N_2 to a form that can be used efficiently for the nutrition of living things.

Alfalfa (Medicago sativa L.), "the queen of forages", is grown in both temperate and subtropical regions. On a global basis it is not only the most widely used forage, but also the oldest. Alfalfa is grown on nearly 15 million ha of production in North America and 33 million ha on a world scale, and it has been a major crop in the United States for more than 100 years (Walton, 1983). Annual rates of N_2-fixation in alfalfa have been reported to vary from 150 to 600 kg/ha (Hanson and Barnes, 1980; Hoffaman and Melton, 1981).

Sulfur (S) is one of the elements essential for the life of all organisms: plants, microorganisms, and animals (Anderson, 1978). Sulfur is required for the production of the amino acids cystine, cysteine, and methionine; these S containing amino acids make up 90% of the total S content of plants (Allaway and Thompson, 1969). Sulfur has been shown to be necessary in maintaining forage quality and yield (Drlica and Jackson, 1979; Tisdale, 1977). Sulfur fertilization has an appreciable effect on the N content of many leguminous plants (Pumphrey and Moore, 1965a, 1965b). The effect of sulfate on symbiotic N_2-fixation in alfalfa and associated metabolic reactions, however, has not been examined in detail, and may provide significant information pertaining to alfalfa growth, development, and the quality of the

product produced.

Objectives

The general objectives of this study were:

1. To determine the quantity of sulfate required for optimal seedling growth, nodule development, and N_2-fixation of alfalfa.
2. To examine the distribution of N and S under varied levels of sulfate.
3. To determine the N:S ratios present in roots and shoots during optimal alfalfa seedling development and nodule function.
4. To determine the effect of commercial inoculum on alfalfa seedling growth under limiting and non-limiting S conditions.

REVIEW OF LITERATURE

Biological Nitrogen Fixation

Biological nitrogen fixation, the process whereby certain free-living or symbiotic bacteria and blue-green algae convert atmospheric N_2 to a form that plants can use, is a process that is fundamental to world agriculture (Hardy et al., 1975; Postgate, 1982). An important feature of symbiotic N_2-fixation is that the energy for conversion of atmospheric N_2 to ammonia comes from sunlight. Legumes utilize photosynthetic products to supply plant nodules with energy for BNF. One of the major advantages of BNF over N fertilization is that BNF is maximal during pod and seed development, at which time soil N availability and plant root absorption is declining (Hardy et al., 1975).

From an agricultural standpoint, the most important N_2-fixers are those bacteria which fix N symbiotically in association with plants. The principal N_2-fixing systems useful in world agriculture are legumes: for example alfalfa, clovers, soybeans, and beans, all of which involve plant associations with the bacterial genus Rhizobium. Symbiotic N_2-fixation provides N to the plant directly, and indirectly through decomposition of nitrogenous materials formed as a result of N_2-fixation.

Symbiotic N_2-fixation is enhanced in legumes when effective and highly competitive strains of Rhizobium successfully nodulate host plants. Legume seed inoculation can be beneficial in soils

in which the specific rhizobia are absent, or sparse, or where indigenous rhizobia are ineffective or submaximal in their N_2-fixing capacity (Vincent, 1974).

Early Experiments

Boussingault in 1837 showed the essentiality of N, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and S, and concluded that nitrate (NO_3^-) was a major source of N for plants. From field experiments he observed, in 1838, that legumes fixed N_2, but when legumes were grown in sterilized soils they failed to grow and fix N_2. Lachman, in 1858, observed nodules on the roots of legumes. He believed that the nodules were associated with N_2-fixation but no conclusive proof was provided. The capability of leguminous plants to fix atmospheric N_2 was not fully appreciated, however, until the results of the classical experiments of Hellriegel and Wilfarth were published in 1888. Their main conclusions were as follow: nodules on peas are formed as a result of plant root infection by Rhizobium; nodules are necessary for the fixation of atmospheric N_2; non-sterile soils may contain effective Rhizobium, but sterilization of the soil kills the Rhizobium and prevents nodulation (Burris, 1979; Evans, 1969).

Winogradsky, in 1890, established that certain free-living anaerobic clostridia (Clostridium pasteurianum) fixed N_2. Beijerinck, 1888-1891, isolated Rhizobium and demonstrated that the aerobic Azotobacter chroococcum also had the capability to fix

N_2. In 1892, Schlosing and Laurent demonstrated that N_2 fixed by legumes as measured by N content of tissues was equal to the loss of N_2 gas around plants (see review by Burris, 1979; Evans, 1969).

Rhizobium-Legume Symbiosis

Legumes are unique among crop plants in their ability to satisfy their large demand for N either through absorption and assimilation of inorganic N from the soil solution (and obtain N from fertilizer applied to the soil), or from the atmosphere through symbiotic N_2-fixation (Hardy et al., 1975; Wych and Rains, 1978). For more than 85 years it has been known that bacteria of the genus Rhizobium infect legume roots and form structures called nodules. By definition, both the bacteria and the host legume benefit from this symbiotic relationship. The bacteria obtain energy and a protected environment from the legume root while converting gaseous atmospheric N_2 to inorganic forms of N which are available to plants. Under most circumstances, neither the plant nor the rhizobia fix N_2 individually (Allen, 1980). The NH_3 produced by the bacteria is then used to make amino acids, which are the building blocks of proteins (Brill, 1977; Goodwin and Mercer, 1983; Lehninger, 1982).

Rhizobia are generally present in soils. Nevertheless, inoculation usually is recommended to insure nodulation, and to provide large numbers of an effective N_2-fixing strain (Brill, 1977). Infection of the plant root and production of a nodule does not guarantee vigorous N_2-fixation. A delicate balance

governs an effective symbiosis between plant and bacteria, and this is reflected in the phenomena of strain variation and host plant specificity (Burris, 1976). Plants can be nodulated, but the bacterial plant relationship can support poor fixation in some instances and good fixation in others. These differences in effectiveness are poorly understood (Burris, 1976), but they are known to be influenced by environment (Sprent, 1979).

The association between rhizobia and plant roots is very specific, and it has been the subject of a great deal of research: the plant is thought to produce attractants for rhizobia which respond with plant-directed taxis; the rhizobia then produce auxin-like substances which initiate root-hair curling; the mucigel at the top of the growing root provides a favorable site for rhizobial attachment; lipopolysaccharides have been implicated in the infection process, and lectins have been proposed as recognition substances involved in specificity (Atlas and Bartha, 1981; Bal et al., 1978; Brill, 1977; Dazzo et al., 1978; Postgate, 1982; Sprent, 1979; Vincent, 1982).

Rhizobia show a degree of specificity to their host plants: Rhizobium japonicum from soybean nodules, for example, do not colonize alfalfa. Host specificity has formed the basis of a classification known as cross-inoculation groups (Postgate, 1982). Rhizobia have been divided into fast-growing types (having doubling times at 30° C of 2 to 5 h on conventional culture media), and slow-growing types (doubling about every 12 to 24 h). Rhizobium meliloti, the species of Rhizobium which under

appropriate conditions infects roots of alfalfa, and is responsible for the initiation of root nodules, is a fast-growing type (Postgate, 1982).

Infection and Nodule Development

Prior to infection of a root hair, rhizobia must be present in the rhizosphere (Atlas and Bartha, 1981; Sprent, 1979). Rhizobia enter the legume, in most cases, through root hairs or during the emergence of lateral roots, and grow within modified parts of the plant roots called nodules (Vincent, 1982). Infection may occur as early as 4 to 12 days after seed germination. The original infection rapidly develops into visible nodules 3 to 5 weeks after plants emerge, depending on the plant species and its growth rate. The multiplying rhizobia form unusually shaped cells called bacteroids. During transformation of normal Rhizobium cells into bacteroids, the bacterial nuclear material degenerates, and at one time was argued to eliminate the capacity of bacteroids for independent multiplication. The bacteroid cell contains active nitrogenase which is the enzyme system responsible for BNF (Eady and Postgate, 1974). The rhizobial bacteroids within the nodule perform the fixation of atmospheric N_2 (Atlas and Bartha, 1981).

All N_2-fixing organisms contain nitrogenase which does not vary significantly in structure from one species to another (Brill, 1977). Nitrogenase consists of two components: one that contains molybdenum (Mo), iron (Fe), and S and is designated the

Mo-Fe protein, Component I, or Protein 1, and another that contains Fe and S, designated the Fe-protein, Component II, or Protein 2 (Brill, 1977; Goodwin and Mercer, 1983). A special characteristic of all nitrogenase systems is that both protein components of the enzyme are denatured by contact with free molecular oxygen (O_2). The O_2 barrier about which the most is known, is found in Rhizobium-legume symbiosis. Oxygen is trapped before it can reach the bacteria by an O_2-binding protein, leghemoglobin. This protein is synthesized by plant tissue in the root nodules. As a result, Rhizobium can use an efficient aerobic metabolism while still protecting nitrogenase from O_2 (Brill, 1977).

Vincent (1982) summarized the steps in establishing the symbiosis, as follows: 1) colonization of the rhizosphere by rhizobia; 2) entrance of rhizobia via root hairs resulting in the formation of infection threads; 3) commencement of a persistent nodule meristem; 4) release of rhizobia from the infection thread; 5) multiplication of rhizobia within membrane envelopes of the nodule host cell; 6) conversion of rhizobia to nodule bacteroids; 7) deposition of leghemoglobin synthesized by the host in the membrane envelope; 8) establishment and continuance of a shared metabolism between plant and bacterium. This intimate association between Rhizobium and the host requires all aspects of the relationship to be mutually acceptable for effective N_2-fixation. The differentiation of the bacteria into bacteroid and leghemoglobin production is accompanied by the onset of

N_2-fixation capability (Beevers, 1981).

The nodules formed in legume roots by effective bacteria are larger, and the interiors have a red or pink color when compared with the smaller, more pale, ineffective nodules. The red or pink color is due to leghemoglobin, a reddish protein (Bergersen et al., 1973). The development of highly effective nodules by effective strains of Rhizobium is necessary; otherwise, roots may be nodulated by ineffective strains of Rhizobium (Emerich and Evans, 1980). Leghemoglobin is present in very high concentrations (150 to 300 uM) in effective nodules (Postgate, 1982). Leghemoglobin has been shown to facilitate the diffusion of O_2 in aqueous media (Burns and Hardy, 1975). About 10% of the leghemoglobin appears to reside within the bacteroid envelopes. The remainder is outside, presumably in the cytoplasm of the plant cells which were colonized (Postgate, 1982).

Nutritional Effects on Nodulation and Nodule Function

Soil conditions have a marked effect on rhizobia survival and ability to infect root hairs (Andrew, 1976; Date, 1981). Nitrate and nitrite ions inhibit nodule formation (Atlas and Bartha, 1981; Burns and Hardy, 1975; Lang and Collins, 1981). One explanation is that the effect of combined N on infection is due to a change in the surface chemistry of the root hairs such that fewer lectins are available for binding the rhizobia, whereas the effects on nodule development and nitrogenase activity are related to a lower level of carbohydrates in the roots (Gibson, 1981). Eardly et al.

(1985) demonstrated that application of ammonium nitrate at the time of alfalfa seeding resulted in a significant reduction of nodule numbers, nodule weight, and in BNF, as measured by acetylene reduction. Similar results were reported by Heichel et al. (1981).

Legumes as a group do not differ greatly from non-legumes either in their qualitative or quantitative requirments for mineral nutrients. Apart from those nutrients required specifically for symbiotic N_2-fixation (cobalt (Co), Mo), nutrients influence N assimilation through effects on host legume growth. For most nutrients, however, the requirement for nodule function is less than for plant metabolism elsewhere in the plant (Robson, 1978).

Acidity, Ca deficiency, and excess aluminum (Al) and manganese (Mn) tend to occur together in soils and to interact in their effect on nodulation and plant growth (Munns, 1977). Interaction of Ca and pH on nodulation has been demonstrated with alfalfa, soybeans, and clover (Loneragan and Dowling, 1958; Loos and Louw, 1965; Munns, 1970; Vincent, 1965). Nodulation has been shown to require more Ca and higher pH than does N_2-fixation and growth of plants with already established nodules (Munns, 1970; Vincent, 1965). In Medicago the critically sensitive stage of the nodulation process occurs within 1 to 3 days after inoculation, and corresponds with the stage of plant development when root hairs curl and infection begins. Root growth, root hair development, infection thread elongation, and nodule growth are

all less sensitive than the initiation of the infection thread (Munns, 1968, 1970). After initiation of infection, nodule development can proceed at Ca concentrations even lower than those required for host growth (Munns, 1970). An interpretation of this observation is that acidity and Ca shortage diminish the association of Ca with cell walls, membranes, or enzymes, thereby preventing essential biochemical processes in the rhizosphere, such as the pectolysis that may be needed in the initiation of infection (Munns, 1969).

Calcium is required in greater amounts for nodule function than for plant metabolism (Robson, 1978). Calcium also moderates toxic effects of manganous ions in leguminous plants (Robson and Loneragan, 1970). Vose and Jones (1963), working with Trifolium repens in solution cultures found that increasing Ca from 0.4 to 2.0 mM ameliorated the adverse effect of 200 uM Mn on plant growth, nodule number, and nodule size.

Specific roles in the nodule are amply established for P, as a constituent of nucleotides; for S as a constituent of the Fe-S proteins; and for K for its osmotic regulation and enzyme activation (Evans and Russell, 1971; Evans and Sorger, 1969; Epstein, 1972). Deficiencies of P, S, and K also severely and frequently limit N_2-fixation by limiting the growth of the host plant. Although there are no clear demonstrations in controlled conditions that they directly limit nodulation or N_2-fixation, there are data suggestive of such effects in soil experiments (Munns, 1977; Robson, 1978).

Phosphorus application which increases growth commonly increases nodule number, nodule volume, and nodule weight (Munns, 1977). This effect generally can be explained by indirect effects of P on nodulation associated with growth responses by the legume (Robson, 1978). Phosphorus concentrations in nodules may greatly exceed those in either shoots or roots. However, nodule function has not been shown to have a higher internal requirement for P than host plant growth (Robson, 1978).

Munns (1977) reported that nodule formation is prevented by boron (B) deficiency, but it is affected little and inconsistently by deficiencies of the other micronutrients unless the deficiency is severe enough to injure several other phases of the symbiosis. Boron deficiency in legumes produces symptoms common to all plants, such as a characteristic meristematic failure. This suggests that the B requirements for growth and development of the host plant are similar to the requirements for nodule development (Munns, 1977).

Copper (Cu) is required in greater amounts for nodule function than for plant metabolism (Robson, 1978). Copper deficient nodules of subterranean clover (Trifolium subterraneum) incorporated ^{14}C into amino acids and proteins more slowly, and had fewer bacteroids, more starch, and less cytochrome c oxidase than nodules from Cu-sufficient plants. Correction of Cu deficiency has been observed to alter the distribution of nodules in solution-grown subterranean clover (Cartwright and Hallworth, 1970).

Zinc (Zn), Mn, chlorine (Cl), Fe, and Co have not been observed to affect nodulation significantly, although they are all needed for growth of the host, the rhizobia, or both (Munns, 1977). Molybdenum and Co are nutrients whose requirements for nodule function far exceed their requirements elsewhere in the plant (Robson, 1978). Molybdenum is a constituent of nitrogenase, and may have no other major role in plant and bacterial nutrition except as a constituent of nitrogenase and nitrate reductase (Evans and Russell, 1971). Molybdenum deficient plants often have small nodules, sometimes in abnormally large numbers (Anderson, 1956; Mulder et al., 1959). The role of Co within the nodule appears to be associated with it being a component of cobamide coenzymes, which are required for at least three enzyme systems: methyl malonyl CoA mutase, ribonucleotide reductase, and methionine synthetase. The primary effect of Co on nodule function operates through effects on ribonucleotide reductase (Robson, 1978).

Iron is a constituent of leghemoglobin, which is important for nodule function (Bergersen, 1971; Bergersen et al., 1973). In the Fe-S form, Fe is intimately involved as a constituent of both components of nitrogenase and of a bacterial ferredoxin which may function as a reductant of nitrogenase (Bergersen, 1971). Despite these specific requirements within the nodule, limitation of nodule function does not appear to be the major effect of either Fe or S deficiencies. This indicates, according to Robson (1978) that requirements for Fe and S for metabolism outside the nodule

are greater than those within the nodule.

Symptoms of nutrient deficiency are diagnostically useful, but their usefulness in extrapolation to function is limited. Observations on nodulation are informative, where small green nodules may indicate limitation of N_2-fixation by combined N, or deficiencies of Mo, P, or S (Anderson, 1956). Absence or extreme sparsity of nodules may indicate high soil nitrate concentration, soil acidity, or B deficiency, as well as lack of infective rhizobia. Less extreme variations in nodule number, however, may have no bearing on N_2-fixation, since fixation depends more on the mass or volume of nodules and their leghemoglobin content (Anderson, 1956).

Soil Effects on Nodulation

Soil components including gallic and tannic acids, and certain leaf and root exudates have been found to limit nodulation in some cases (Burns and Hardy, 1975; Jensen et al., 1981). Other factors such as temperature and light (Dart, 1981; Gibson, 1977), water stress and waterlogging (Gibson, 1977; Minchin, 1981), mycorrhizal interactions (Smith, 1981), and root health (Minchin, 1981) also influence nodulation and N_2-fixation under field conditions.

Adverse soil and planting conditions can be partially overcome by application of a larger number of rhizobia to increase the probability that enough bacteria will survive until roots are developed and infection can occur. Commercially prepared

inoculant cultures are available for specific crops. The correct culture must be used when inoculating each type of legume seed.

Methods for Measuring Dinitrogen Fixation

Several methods have been utilized for estimating the N_2-fixing ability of crops. These include N accumulation, difference methods, isotopic methods, acetylene reduction, and indirect methods. Detection of small N changes in natural systems in the field is difficult and requires sensitive measuring techniques.

Nitrogen Accumulation

The simplest estimate of N_2-fixation is by total N accumulation of the crop. This is based on the assumption that the crop derives all its N via symbiotic fixation (LaRue and Patterson, 1981). The standard procedure for N analysis is the Kjeldahl determination (Nelson and Summers, 1973); it has been widely applied for measurement of N_2-fixation (Hardy and Holsten, 1977).

Difference Methods

An adjusted measure of fixation by the N accumulation technique is obtained when the contribution of soil N to the total N of legumes is estimated. This procedure is known as the difference method (Williams et al., 1977), and has three versions: 1) comparison of a legume with a non-legume, 2) comparison of a legume with a non-nodulating legume, and 3) comparison of inoculated and non-inoculated legumes (LaRue and Patterson, 1981).

Isotopic Methods

Fixation of $^{15}N_2$, a direct method, remains the method of choice for checking the validity of other estimates of fixation (Burris, 1974), and has been utilized in many investigations (Ham, 1978; Hardy and Holsten, 1977; Knowles, 1981; LaRue and Patterson, 1981, Rennie et al., 1978). Dinitrogen fixation also can be estimated by isotope dilution. In this method the fixing crop and a non-fixing control are grown in soil to which a small amount of ^{15}N has been added as labeled nitrate or ammonium (Heichel et al., 1981). Other methods are based on natural isotope abundance (LaRue and Patterson, 1981).

Indirect Methods

Several indirect methods have been used for estimating the N_2-fixing ability of legumes such as index of nodulation, number of nodules, fresh or dry weight of nodules, and leghemoglobin concentration in nodules, or amount per plant (Bordeleau et al., 1981; LaRue and Patterson, 1981; Masterson, 1977).

Acetylene Reduction Method

The acetylene reduction (AR) method, which has the advantage of sensitivity, speed and economy, is based on a universal and specific property of nitrogenase, the catalysis of the formation of ethylene from acetylene. No other biological system catalyzes this reaction (Postgate, 1982). The rate of ethylene production is a measure of nitrogenase (Atlas and Barta, 1981). Several variations of this method have been described (Burris, 1974; Hardy and Holsten, 1977; Hardy et al., 1968, 1973; LaRue and Patterson,

1981).

Hardy et al. (1968, 1973) provided a detailed description of the methodology, and applications of AR for the estimation of BNF. The AR technique uses nodules, or decapitated intact root systems, or the root systems of intact plants which are enclosed in a gas tight container. Gas samples are then withdrawn over a period of several hours to determine a rate of AR. Most early applications of AR for investigating legume N_2-fixation employed excised nodules, nodulated root segments, or soil cores (Wych and Rains, 1978). Preferably, intact plants should be used, since studies have shown that intact plants have AR rates five times higher than detached nodules, and twice as large as a decapitated root system, indicating an adverse effect of plant mutilation on AR rates (Mederski and Streeter, 1977). Studies on nitrogenase activity have been greatly enhanced by development of the AR method. This method has been utilized in crops such as soybeans and beans to evaluate the N_2-fixing activity of nitrogenase.

Alfalfa in particular has been the subject of many investigations utilizing the AR assay (Bordeleau et al., 1981; Collins and Duke, 1981; Duke and Doehlert, 1981; Duke et al., 1980; Eardly et al., 1985; Hardarson et al., 1981; Hoffman and Melton, 1981; Tan, 1981).

Energy for Biological Dinitrogen Fixation

The fixation of N_2 requires not only nitrogenase, but also energy in the form of adenosine 5'-triphosphate (ATP), reduced

ferredoxin, or reduced flavodoxin (Atlas and Bartha, 1981; Hardarson et al., 1981; Hardy and Havelka, 1975; Koch et al., 1970). The ATP and reductant needed to support N_2-fixation in symbiotic associations is derived from the photosynthate produced by the plant. The conversion of one molecule of N_2 into two molecules of NH_3 requires about 24 molecules of ATP (Brill, 1977; Emerich and Evans, 1980; Gibson, 1966; Hardy and Havelka, 1975; Mulder, 1975; Phillips, 1980; Pate et al., 1981; Postgate, 1982).

Part of the energy required for BNF is used to break the very stable triple bond of N_2. Experiments with nitrogenase from various organisms have shown that approximately 75% of the electron flow through nitrogenase is utilized in the reduction of N_2 to NH_3, with the remaining 25% used in the evolution of H_2 (Brill, 1977; Emerich and Evans, 1980; LaRue and Patterson, 1981). In the absence of N_2 or any other added reducible substrate, all the electron flow through nitrogenase is utilized in the reduction of protons to H_2, in an ATP-dependent process (Emerich and Evans, 1980). At 0.101 MPa N_2 (1.01 bars), proton reduction continues at approximately 35% of the maximum value obtained in the absence of N_2. Between 13 and 23% of the total electron flow through nitrogenase is lost as H_2 evolution, even at infinite N_2 concentration (Rivera and Burris, 1975). At least one mole of H_2 is evolved for every mole of N_2 reduced at 0.101 MPa N_2 (1.01 bars) (Emerich and Evans, 1980). This loss of H_2, which requires about 4 ATP molecules per mole of H_2, is important because there is evidence that the amount of photosynthate available to the

nodule may be a primary factor limiting N_2-fixation (Gutschick, 1980; Hardy and Havelka, 1975; Minchin and Pate, 1973; Pate, 1977).

Importance of Sulfur

Sulfur has been known to be essential for plant growth for more than 100 years (Eaton, 1966). Of the considerable number of compounds that have been found in plants, only a few have been recognized as required for normal cell function. These vital compounds include the S containing amino acids: cysteine, cystine and methionine. Sulfur also is a constituent of glutathione, S-adenosyl methionine, thiamine, biotin, lipoic acid, and coenzyme A. Nitrate reductase (NR), the enzyme regulating the conversion of NO_3-N to protein, is a sulfhydryl-dependent enzyme (Pal et al., 1976). Sulfur is a constituent of the nitrogenase enzyme system (Eady and Postgate, 1974; Tisdale and Nelson, 1975), as well as a constituent of other proteins required for biochemical reactions by the N_2-fixing bacteria. Plants normally synthesize all organic S compounds from inorganic sulfate ions absorbed by plant roots (Thompson et al., 1970).

Soil Sulfur

The normal origin of S for plant growth is soil (Anderson, 1976). Sulfur occurs in soil in organic and inorganic forms. The relative proportions of the various forms of S in the soil can vary depending on the physical and chemical properties of the

soil, seasonal conditions, the vegetation that it supports, and whether fertilizers containing S have been supplied. Only 7% of the total S in the top 25 cm of soil is available to plants at any one time in most of the well-drained soils used for agricultural purposes. Approximately 60-70% of the total S is permanently unavailable. The remainder is mostly associated with organic matter in the soil (Ludecke, 1967). The organic S compounds in the soil become available to plants only after mineralization by microorganisms (Anderson, 1978; Ludecke, 1967).

Inorganic soil S is mainly SO_4^{2-}, and its absorption increases as soil pH is reduced (Mengel and Kirkby, 1982). Plants absorb S from soil mainly in the form of sulfate, and its uptake is an active process (Nissen, 1971; Schief and Frankhauser, 1981). The uptake of sulfate is accomplished by a series of specific carrier proteins located in the plasma membrane (Anderson, 1978). Some sulfate is reduced in root cells but most of it is transported acropetally in the xylem to the leaves where it enters the chloroplasts; the capability of higher plants to move S basipetally is relatively poor (Mengel and Kirkby, 1982). After entering the chloroplasts, sulfate reacts with ATP, in the presence of ATP sulfurylase, to form adenosine 5'-phosphosulfate (APS) and pyrophosphate (PPi). The APS formed is then reduced to sulfite (or thiosulfate) and sulfide, in a complex series of reactions in which ferredoxin, generated by the light reactions, serves as the reductant. Finally, the sulfide is incorporated into O-acetyl serine to form cysteine. Cysteine, in turn, is the

starting point for the synthesis of most other S-containing compounds (Anderson, 1978; Schiff and Frankhauser, 1981; Schiff and Hodson, 1973).

Plants can utilize atmospheric SO_2 as part of their S supply. Once SO_2 is absorbed through the stomata, it is distributed throughout the entire plant and has been detected in various fractions such as protein-S, amino acid-S, and sulfate-S (Mengel and Kirkby, 1982). The SO_2 absorbed by the soil can be readily oxidized to SO_4 by chemoautotrophic organisms making the S available to plants (Anderson, 1978).

Sulfur Deficiency and Toxicity

Sulfur has been called the "neglected element" (Hanley, 1972), and little attention has been given to S deficiency, as compared to deficiencies of other nutrients. As a result, S deficiency symptoms are not commonly recognized. The similarity of S deficiency to N deficiency symptoms further complicates identification. Under conditions of continuously low supplies of either S or N, plant appearance is not an adequate means of differentiating between deficiencies of the two elements (Hanley, 1972). In S deficient plants the sulfate-S levels are very low, whereas amide-N and nitrate-N accumulate. This contrasts markedly with N deficiency where soluble N levels are depressed and sulfate-S levels are normal (Mengel and Kirkby, 1982). Sulfur deficiency results in accumulation of nitrate and free amino acids (Dijkshoorn and van Wijk, 1967).

In plants suffering from S deficiency the rate of plant growth is reduced. Generally, the growth of the shoots is more affected than root growth. Frequently the plants are rigid and brittle, and the stems remain thin. In contrast to N deficiency, chlorotic symptoms occur first in the younger, most recently formed leaves. In alfalfa, S deficiency symptoms appear first at the top of the plant. The leaves turn from light green to light yellow, which is often followed by pronounced general yellowing (Anderson, 1978; Ulrich et al., 1967).

Sulfur deficiency is known to retard protein synthesis, and as a consequence adversely affects both nodulation and N_2-fixation of legumes (Adams and Sheard, 1966; Smith, 1982; Zaroug and Munns, 1979). Severe deficiency reduces the rate of protein synthesis more than the rate of N_2-fixation, and leads to accumulation of non-protein N (Spencer, 1959). Moderate deficiency limits protein synthesis and N supply from the nodule about equally. Jones et al. (1971) suggested that non-protein N need not accumulate in nodulated legumes when the S deficiency is moderate (20% yield reduction with Stylosanthes). Sulfur deficiency also has been reported to significantly lower plant protein yield without reducing plant growth (Anderson, 1952; Jones et al., 1971; Spencer, 1959).

The number and weight and nodules is reduced on S deficient plants (Smith, 1982). However, Oke (1969), and Spencer (1959) reported that reduced nodule number, or nodule size, when it occurs, is probably a consequence of poor N nutrition and growth

of the host plant. Spencer (1959), and Anderson and Spencer (1950), working with nodulated clovers, found that inhibition of N_2-fixation from S deficiency was indirect. Sulfur deficiency primarily inhibited protein synthesis, as it does in non-legumes, and since N_2-fixation was less sensitive to the deficiency than protein synthesis, S deprived plants accumulated non-protein N compounds.

Plants are comparatively insensitive to high sulfate concentrations in the nutrient medium. Only in cases where sulfate concentrations approach 50 mM, which may occur in some saline soils, is plant growth adversely affected (Mengel and Kirkby, 1982).

In the absence of industrial activity, the concentration of SO_2 in the atmosphere is typically 1 to 3 ug SO_2-S/m^3 (0.001 to 0.003 ppm) (Anderson, 1978). The critical concentrations of SO_2 in the atmosphere above which toxic effects in plants are observed is in the range of 500 to 700 ug SO_2-S/m^3 (0.5 to 0.7 ppm). High SO_2 concentrations result in necrotic symptoms in the leaves (Mengel and Kirkby, 1982). Most healthy human subjects exposed to SO_2 concentrations greater than 3000 ug SO_2-S/m^3 (3 ppm) show a detectable physiological response. However, most plants are more sensitive than man (Anderson, 1978).

Yield and Quality Response to Sulfur Application

Sulfur nutrition of alfalfa is important since its application not only increases yields but also improves the

quality of the product (Hanley, 1972; Tisdale, 1977). In general, plants form reduced S compounds from sulfate, and animals form sulfates from reduced S compounds. Within the animal the reduced S compounds perform many essential functions prior to their oxidation and excretion as sulfates. Plants growing with adequate S concentrations generally contain more S-amino acids and are presumably of better nutritional quality than are S-deficient plants. Thus when the yield of a forage is increased through the use of S fertilization, an improvement in its nutritional quality for ruminants coincides with increased yield (Allaway and Thompson, 1966; Tisdale, 1977). This key role of S in the production of high quality protein is now attracting more research attention (Hanley, 1972).

Research in Oregon toward evaluation of the relationships between results of S analysis of soils, responses of alfalfa to S fertilization, and the S content of plants, showed that increases in yield due to S application varied according to soil type (Harward et al., 1962). According to this work, the difference in S content between treatments for those soils with significant yield responses was greater in the first two cuttings and became smaller as the growth periods progressed. These data indicated a close relationship between S and N content of alfalfa. It was suggested that part of the effect of S applications may be indirect and related to N relationships of the legume. Harward et al. (1962), in a greenhouse experiment with alfalfa, obtained a highly significant correlation ($r = 0.79$) between percentage yield

and S content of the plant. Pumphrey and Moore (1965b) showed that significant yield increases in alfalfa occurred only when the S content of the plant was less than 2.2 mg S/g (0.22%).

Westermann (1974) and Pumphrey and Moore (1965a) showed that S fertilization significantly increased forage yield of alfalfa. Collins and Duke (1981) reported that shoot and root weight per alfalfa plant, and the total weight were influenced primarily by soil S levels. Nodule number per plant was higher in S fertilized than in S unfertilized treatments. Meyer and Marcum (1980) found that S fertilized alfalfa yielded significantly more dry matter than the control in response to surface applications of high rates (220 kg S/ha) of gypsum and elemental S, but there was no difference between the two sources. Hoeft and Walsh (1975) found significant yield responses of alfalfa to S applications. Spring applied potassium sulfate (K_2SO_4) was more effective than a fall application, and K_2SO_4 applied at a rate of 28 kg S/ha each year over a 2-year period was more effective than a single application of 56 kg S/ha made at the beginning of the 2-year period. Aulakh and Dev (1978) showed that the application of S with and without applied Ca significantly increased dry matter production of alfalfa. Andrew (1977) found that alfalfa responded positively to application of sulfate, and that dry matter yields were not depressed at the highest sulfate treatment (30 kg S/ha as $CaSO_4$).

Sulfur Fertilizers

The most important S containing fertilizers are gypsum (calcium sulfate), ammonium sulfate ($(NH_4)_2SO_4$), potassium sulfate (K_2SO_4), single superphosphate, and triple superphosphate. Elemental S, and S coated fertilizers also contribute to the S supply of plants.

Gypsum ($CaSO_4.2H_2O$), a neutral salt, is used for direct appplication from which both S, 190 mg S/g (19%) and Ca, 230 mg Ca/g (23%) are readily available. In low-leaching environments, gypsum has been reported to be equal or superior to elemental S (Walker, 1964). Application of gypsum is often used where soils are severely deficient in S (Mengel and Kirkby, 1982). The rates generally applied are in the range of 10 to 50 kg S/ha. Gypsum gives a very rapid plant response after application because the S applied is in the sulfate form, and is immediately available.

Single superphosphate, considered the traditional phosphate fertilizer used in agriculture, is manufactured by the addition of sulfuric acid to phosphate rock, and contains 95 mg P/g (9.5%), and 115 mg S/g (11.5%) (Palmer et al., 1983). Triple superphosphate, produced by the reaction of sulfuric acid with phosphate rock to produce phosphoric acid, and then mixed with additional rock phosphate, contains 196 mg P/g (19.6%), and only 10 mg S/g (1%) (Palmer et al., 1983).

Ammonium sulphate, probably the oldest synthetic fertilizer, is a byproduct of the coal industry, and of various metallurgical processes which produce sulfur dioxide. Sulfur dioxide is

converted to sulfuric acid and then neutralized with ammonia in fertilizer manufacture (Palmer et al., 1983).

Potassium sulfate is manufactured from potassium chloride by reaction with sulfur dioxide or sulfuric acid. It contains 170 to 200 mg S/g (17 to 20%) (Palmer et al., 1983).

Elemental S, at 1 g S/g (100%) nutrient, has a higher analysis than any other fertilizer. The material is insoluble in water and larger quantities of elemental S are necessary to attain adequate yields, because bacterial oxidation is necessary before elemental S can be utilized (Meyer and Marcum, 1980; Palmer et al., 1983).

Effects of Sulfur on Symbiotic Dinitrogen Fixation

Increased N content of various legumes has resulted from the use of S-containing fertilizers on S deficient soils (Anderson and Spencer, 1950b). In 1919 Miller suggested that the increase in N content of the legume was due to the sulfates stimulating the action of the N_2-fixing bacteria. Neller, in 1926, as reported by Anderson and Spencer (1950b) further suggested that sulfate had an indirect effect upon legumes through its direct action or effect upon the N_2-fixing organisms. Pitz (1916) demonstrated that gypsum had a stimulative effect on nodule-forming bacteria of red clover roots. Bacteria were from 2 to 3 times as numerous in culture media to which $CaSO_4$ had been added compared to controls. Duley (1916) showed that S and gypsum increased the number of nodules on red clover roots in certain Missouri soils. Reimer and

Tartar (1919) observed than on some soils the nodules on the roots of alfalfa plants from the fertilized plots were far more numerous than on those from the unfertilized plots.

In 1915 and 1917, however, Wilson obtained results indicating that sulfates, in relatively low concentrations, inhibited nodulation. Gaw and Soong in 1942 reported improved nodulation and yield of peas with Ca and Fe sulfates; improved nodulation but no increase in yield with sodium (Na) and Zn sulfates; and decreased nodulation with other sulfates, including potassium sulfate. Ammonium sulfate increased the yield but not nodulation, perhaps due more to the effect of NH_4^+ than SO_4^{2-}, as reported by Anderson and Spencer (1950b).

With legumes, the effect of S is doubly important, because an adequate supply of S in the rooting system is essential to the rhizobial fixation of N, as well as for the subsequent synthesis of protein by the host plant (Tisdale, 1977). Anderson and Spencer (1950b) found that the increase in yield of clover due to S was associated with an increase in both the number and size of nodules. A late application of sulfate caused a rapid increase in N_2-fixation without any increase in the number of nodules. Sulfur also increased total N and improved nodulation in clover plants; however, the S deficient clover plants were not deficient in N for protein formation or growth.

Nitrogen-Sulfur Relationships

The partitioning of N and S contents in plants has been studied because of the very close association of N and S in the synthesis of proteins. Nitrogen may occur in plants in two main forms, protein N and non-protein N. The form in which N exists in plant tissue reflects the overall metabolism of the plant. Sulfur may have profound effects on the composition of proteins and hence on the metabolism of plants (Adams and Sheard, 1966). A large accumulation of amide-N in alfalfa, and amino acid, amide, and nitrate-N in beans under limited supply of S has been reported (Adams and Sheard, 1966; Rendig and McComb, 1961).

Nitrogen-sulfur relationships in soil organic matter and plant tissues are useful in predicting when S deficiency may be limiting plant growth. A useful guideline, described by Stewart (1969) is that about 1 part of S is released from soil organic matter for every 10 parts of N. Sulfur deficiencies are unlikely if soil organic matter is the chief source of N. However, when large amounts of N are supplied by legumes or through N fertilization, the supply of S from soil organic matter will not be sufficient, since a wide variety of field crops require about 1 part of S for every 15 parts of N for maximum yields and quality (about 14:1 for grasses, and 17:1 for legumes); in these conditions other natural sources of S such as sulfate in rainfall, inorganic salts in the soil, subsoil, and irrigation water should be evaluated. If these sources are minimal, fertilizer S should be added (Stewart, 1969).

Nitrogen:Sulfur Ratios, and Critical Sulfur Concentrations

Total S, sulfate S, and the nitrogen:sulfur (N:S) ratio have been used as indices of the S status of plants. Total S has been utilized because plant S content is directly related to S supply (Cairns and Carson, 1961; Cressman and Davis, 1962; Jones, 1962; Rendig, 1956). Because of the accumulation of sulfate-S after S demands for protein synthesis have been satisfied, several researchers have proposed that sulfate-S is as good an indicator of the S status of the plant as total S (Dijkshoorn et al., 1960; Jones, 1964; Noggle, 1979; Walker and Bently, 1975; Westermann, 1975). The N:S ratio in the plant is a much more reliable measure of S adequacy that the absolute level of S (Thompson et al., 1970).

Westermann (1975) found that the N:S ratio of a specific protein is constant, since the sequence and number of amino acids in the polypeptide chain are determined by genetic information. Therefore, the N:S ratio of proteinaceous material of a plant varies only when changes occur in the relative proportions of the individual proteins formed.

Dijkshoorn and van Wijk (1967) proposed than when the total N:total S ratio, (N:S), exceeds 16:1 deficiency may be expected, and protein formation is limited. Pumphrey and Moore (1967a) reported that for alfalfa an N:S ratio of 11:1 or below indicated an adequate S supply and produced maximum yield. Aulakh et al. (1976), in greenhouse conditions, found that an N:S ratio of about 11:1 obtained with the application of 20 ug S/g (20 ppm) indicated

an adequate supply of S for alfalfa.

Dow (1982) suggested using a critical nutrient range rather than critical nutrient concentration, and defined it as "that range of concentrations above which one is reasonably sure the crop is amply supplied and below which one is reasonably sure the crop is deficient." The critical nutrient range of S has been determined by plotting the dry matter yield against the percentage of S in the plant tops, and estimating the S concentration corresponding to 90% maximum dry matter yield (Andrew, 1977). This method has been used to establish the critical concentrations of P (Andrew and Robins, 1969a), and K (Andrew and Robins, 1969b) in a number of legumes. In alfalfa, the critical concentrations of S are between 2.0 and 2.8 mg S/g (0.20 and 0.28%). Andrew (1977) calculated a value of 2.0 mg S/g (0.20%). Harward et al. (1962), Pumphrey and Moore (1965a), Rendig (1956), and Tisdale et al. (1950) established a value of 2.2 mg S/g (0.22%). Critical nutrient range values of S in alfalfa, however, are dependent upon stage of development (Pumphrey and Moore, 1965b).

Typically concentrations of S from 2.0 to 2.2 mg S/g (0.20% to 0.22%) in whole tops of different legumes at early bloom have been found to be required for optimal growth and development (Drlica and Jackson, 1979; Kiemnec et al., 1981; Westermann, 1975). For comparison, the typical concentrations for deficiency in whole plant tops of pasture and forage legumes before flowering for other elements are: 1.7 to 2.5 mg P/g (0.17 to 0.25%); 8 to 15 mg K/g (0.8 to 1.5%); 0.5 ug Mo/g (0.5 ppm); 10 to 20 ug for B,

Zn, and Mg/g (10 to 20 ppm); and 2 to 5 ug Cu/g (2 to 5 ppm), on a dry weight basis (Andrew and Hegarty, 1969).

MANUSCRIPT I

EFFECT OF $CaSO_4$ ON DINITROGEN FIXATION IN FIELD GROWN SEEDLING ALFALFA

EFFECT OF $CaSO_4$ ON DINITROGEN FIXATION IN FIELD GROWN SEEDLING ALFALFA

ABSTRACT

Two field experiments were established to examine the effect of different levels of S fertilizer on dinitrogen fixation and dry matter yield of 10 week old seedling alfalfa (Medicago sativa L.). 'Apollo' alfalfa seeds were planted during 1982 and 1983 on a limed Woodburn silt loam soil (fine, silty, mixed, mesic Aquultic Argixeroll), of pH 6.2, and initial SO_4-S levels (mg SO_4-S/kg) of 3.3 at 15 cm in 1982, and 7.2 at 30 cm in 1983.

Six levels of S fertilizer, as powdered gypsum (0 to 67.2 kg S/ha) were applied and lightly incorporated before planting. The experimental design was a split-block in which one half of the seeds were inoculated with commercial inoculum, and the other half non-inoculated. Nodule number, nodule fresh and dry weights, root fresh and dry weights, dry matter yield, percent N and S, and acetylene reduction were determined.

All S treatments increased the dry matter yield above the check for both inoculated and non-inoculated treatments in both 1982 and 1983, although no significant response was observed from the application of S, among inoculated or non-inoculated plants. Dry matter yield was significantly increased in both years by inoculation, averaging 53% increase over non-inoculated treatments. Inoculation also significantly increased nodule number (307%), acetylene reduction rate (444%), N concentration

(79%), total tissue N (172%), total tissue S (16%), and the N:S ratio (140%) over the non-inoculated treatments.

In both years the highest value for N_2-fixed by the inoculated plants was obtained from the 44.8 kg S/ha treatment, which gave values of 60 and 92 kg N/ha/10 weeks.

The percent yield increase with respect to the check plants, and the percent tissue S were significantly higher in non-inoculated plants. Total tissue N and S were increased with increased S levels, while forage N:S ratios decreased from 11.3 to 9.1 with increasing S fertilization. Percent utilization efficiency of S was decreased with increased S levels, but no significant differences were observed between non-inoculated and inoculated plants, at the different S levels.

These data support the conclusion that S fertilization increases seedling alfalfa yield when S levels in the plant are below 2.2 g S/kg. In inoculated plants, S fertilization increased total N and S, supporting the importance of S in symbiotic N_2-fixation and the quality of the forage produced.

Additional index words: Acetylene reduction, Gypsum, Inoculation, Medicago sativa L., N_2-fixation, N:S ratio, S fertilization.

INTRODUCTION

Alfalfa (Medicago sativa L.), the most important forage legume in many parts of the world, is grown in various areas of the United States, accounting for nearly 15 million hectares of production (Walton, 1983). It provides high quality, high protein feed for many classes of livestock. A proper balance of nutrients, and control of other factors like pH and effective strains of Rhizobium inoculum, which influence the capacity of alfalfa to fix atmospheric N_2 into plant proteins, are important in maintaining highly productive stands. Annual rates of N_2-fixation in alfalfa have been reported to vary from 150 to 600 kg/ha (Hanson and Barnes, 1980; Misshustin and Shil'nikova, 1971).

The availability of essential mineral nutrients may limit N_2-fixation in alfalfa, and other legumes, by affecting the growth of the plant itself, growth and survival of Rhizobium, infection and nodule development, or nodule function (Robson, 1978). Although rhizobia generally are present in soils, inoculation usually is recommended to insure nodulation, and to provide large numbers of an effective N_2-fixing strain (Brill, 1977).

Sulfur, recently rated the fourth most important plant nutrient (Platou and Irish, 1982), is required in relatively large amounts for proper growth and development of alfalfa (Radet, 1966). Many researchers have claimed that S is specifically involved in N_2-fixation in legumes (Adams and Sheard, 1966; Masterson, 1977; Walker and Adams, 1958; Zaroug and Munns, 1979).

These claims are based on the observations that alleviating S deficiency in these legumes increased yield and N concentrations of the forage.

Yield responses of alfalfa to S fertilization have been reported under field conditions (Cairns and Carson, 1961; Fox et al., 1964; Hoeft and Walsh, 1975; Meyer and Marcum, 1980; Pumphrey and Moore, 1965a, 1965b), and under greenhouse conditions (Adams and Sheard, 1966; Aulakh and Dev, 1978; Martel and Zizka, 1977).

Sulfur deficiencies in plants are becoming more common because higher purity fertilizers are more often used, or types in which S is not a component (Beaton et al., 1974; Tisdale, 1977). Sulfur deficiencies are common in many parts of the United States, Africa, Asia, Australasia, Canada, and Central and South America (Platou and Irish, 1982). Sulfur deficiencies are unlikely if soil organic matter is the chief source of N. However, when N supply is increased either through use of fertilizer-N, or N_2-fixation, and if these conditions coincide with low inputs of S from external sources the risk of S deficiency is high (Probert and Jones, 1977), and fertilization with S is needed (Stewart, 1969). Gypsum ($CaSO_4$) often is applied to soils deficient in S. The rates generally applied are in the range of 10 to 50 kg S/ha (Meyer and Marcum, 1980).

Total S, sulfate S, and the N:S ratio have been used as indices of S status in alfalfa plants (Andrew, 1977; Dijkshoorn and van Wijk, 1967; Dijkshoorn et al., 1960; Gardner, 1974;

Pumphrey and Moore, 1965a, 1965b; Stewart, 1969; Westermann, 1975). The relative requirements of S for symbiotic growth, and growth of the host alone, however, have not been adequately assessed.

Previous experiments (Eardly et al., 1985) had shown that the indigenous population of Rhizobium meliloti at the sites used in these experiments was ineffective in N_2-fixation. This provided an opportunity to determine the quantity of sulfate fertilizer required for optimal yield, seedling growth, nodule development, stand establishment, and N_2-fixation of seedling alfalfa, and to examine the effect of commercial strains of rhizobia as compared to indigenous strains on N and S nutrition.

MATERIALS AND METHODS

Experimental Sites

Field experiments were conducted during 1982 and 1983 on two different experimental sites at the Oregon State University Hyslop Crop Science field research facility at Corvallis, Oregon. The soil type of both experimental sites was a Woodburn silt loam (fine, silty, mixed, mesic Aquultic Argixeroll).

The experimental sites were selected primarily on the basis of a history of non-legumes culture for 8 to 10 years before the experiments. During this period the soils had been either in fallow or in small grains. The pH of the soil before amendment was 5.4 in 1982, and 5.5 in 1983; the SO_4-S (mg SO_4-S/kg) was 3.3 at 15 cm in 1982, and 7.2 at 30 cm in 1983.

Fertilization and Weed Control

Several weeks prior to planting, the entire area of each site was uniformly limed with 2,800 kg dolomitic limestone/ha, to increase the pH to 6.2. Fertilization included 56 kg K/ha, as KCl; 3.35 kg B/ha, as Borax, and 0.56 kg Mo/ha, as sodium molybdate. The day before planting 1.68 kg Balan[1]/ha was incorporated for weed control.

[1] Mention of a trademark, proprietary product, or company name does not constitute a guarantee or warranty of the product and does not imply endorsement of the product by the authors or Oregon State University.

Experimental Design and Sulfur Treatments

The experimental design was a split-block with 6 rates of S fertilization, 2 inoculation treatments, and 4 replications. The main plot treatments were inoculated and non-inoculated seeds; subplot treatments were the 5 rates of gypsum, plus the control. Main plots in both years were 10.98 x 9.14 m; subplots were 1.83 x 9.14 m. A non-inoculated 3.66 m border separated inoculated and non-inoculated treatments, with a 6.10 m border surrounding the entire experiment.

Sulfur treatments consisted of powdered gypsum (laboratory grade, 180 mg S/g) applied by use of a gravity feed spreader 0.91 m wide, which was calibrated to deliver 5.6, 11.2, 22.4, 44.8, and 67.2 kg S/ha. The gypsum was applied to the soil surface and lightly incorporated into a previously prepared seedbed. The single S application utilized simulates common field practice where fertilizer S customarily is added to the soil at the beginning of the growing season.

Inoculation

Alfalfa seeds used in both 1982 and 1983 were pelleted with lime as described by Vincent (1970). Seeds were surface sterilized using a solution of commercial bleach (80 ml H_2O + 20 ml sodium hypochlorite) followed by 10 sterile water washes, and then dried in sterile paper towels.

Two thirds of the seeds (10.45 kg) were pelleted with 1.2 g of laboratory grade powdered calcium carbonate ($CaCO_3$) and 0.086 g

of peat. The peat was previously sterilized by gamma radiation ($5x10^6$ MR from a ^{60}Co source). The remaining seeds (5.25 kg) were pelleted with $CaCO_3$ and a fresh batch of commercial inoculant from Nitragin[1], Milwakee, Wis., at a rate of 0.086 g inoculant per g of seed. Gum arabic solution (82 ml H_2O + 35.5 g powdered gum arabic) was used as an adhesive in both experiments.

In 1983 the seeds were pelleted with commercial grade powdered $CaCO_3$ and no peat was applied to the non-inoculated treatment.

Planting

Certified 'Apollo' alfalfa (provided by North American Plant Breeders[1]) was planted on 21 June 1982, and 30 June 1983, at a rate of 15.7 kg/ha, using a small plot cone-type seeder set for 15 cm rows at 1 cm depth. To prevent rhizobial contamination of the non-inoculated seed, the inside of the planter was cleaned with 95% vol/vol ethanol, and the non-inoculated seed was planted first. Both experiments were watered as needed with overhead irrigation.

Acetylene Reduction Analyses (Nitrogenase activity)

Acetylene reduction (AR) assays were performed on alfalfa seedlings 67, 69, 73, and 75 days after planting, using an intact core method (Eardly et al., 1985). This method involved the use of a 25 cm length and 8.5 cm diameter cylinder of metal pipe centered directly over 1 to 3 plants and driven into the soil

around the alfalfa seedlings, using a hammer developed by ARTS Machine Shop[1], American Falls, Idaho. The cores were then removed intact, placed in a 15 x 43 cm Saran bags (W.R. Grace & Co[1], Cedar Rapids, Iowa), fitted with a gas-tight seal around the alfalfa stem and placed in a incubation chamber.

The incubation chamber consisted of a 30 cm length of drainage tile buried vertically in the ground, with a wooden support placed in the bottom of the chamber to bear the soil core. The whole plant was exposed to natural environmental conditions during incubation with acetylene. The soil core samples were taken between 0730 and 1130. A total of 8 cores per treatment were assayed per year.

A 100 ml/m^3 (10%) C_2H_2 atmosphere was injected via syringe and septum into the Saran bag. Gas space in the Saran bag was estimated by water displacement. Pore space within the soil core was estimated from bulk density, particle density values and soil moisture of the soil. Acetylene was generated from calcium carbide as described by Burris (1974). Incubation with acetylene was initiated at 1130 and gas samples were removed after 90, 180, and 270 min, and stored in Vacutainer tubes (Vacutainer Systems Rutherford[1], N.J.), 75 x 13 mm, with a 5.6 ml volume.

Rates of ethylene appearance were determined by analyzing 0.5 ml gas samples with a HP 5830A gas chromatograph[1], and were expressed as umole C_2H_4/mg nodule dry wt/h.

Nodule Number and Plant Weight

Following AR measurements, tap water was used to wash the soil from the root system over a 1 mm screen. Roots were immediately stored at -20° C. Plants were separated into shoots, roots, and nodules for counting, and fresh and dry weight determinations. Nodules were counted 2 weeks later. Samples of shoots, roots, and nodules were dried separately at 60° C for 12 hours. In 1983 shoot determinations (number of shoots, and fresh and dry weights) were not performed.

Yield

For each treatment, yield was measured at the intermediate pre-flowering stage by taking a 0.97 x 3.05 m swath through the middle of each plot, with a sickle bar mower type harvester. The area used for yield determinations had not been used for soil core samples. In 1982 the yield was determined 82 days after planting. In 1983 yield was recorded 76 days after planting. A plant sample (0.4 to 0.8 kg) from each treatment was taken for dry matter determinations. The samples were dried to a constant weight at 60° C in a forced air oven.

Chemical Analyses

Subsamples of harvested plant tops were oven dried at 60° C for 12 hours, and ground to pass a 0.5 mm screen. Ground samples were stored in air tight plastic bags and redried before weighing for analysis. Nitrogen was analyzed by an automated micro-

Kjeldahl apparatus (Schuman et al., 1973). The level of S was determined following a procedure which is a modification of that reported by Tabatabai and Bremner (1970). The plant material was digested in a beaker with 2 ml of ethanol and 3 ml of saturated $Mg(NO_3)_2$ solution, ashed in a muffle furnace, cooled to room temperature, and 10 ml of 3 M HCl added. The sulfate content of an aliquot of the digest was determined turbidimetrically as $BaSO_4$, by a barium chloride-gelatin procedure. Phosphorus, K, Ca, Mg, Fe, B, Zn, Mn, Cu, Co, and Mo were determined using a Jarrel-Ash Inductively Coupled Argon Plasma spectrometer (ICAP-9000), manufactured by Allied Analytical Systems[1].

RESULTS AND DISCUSSION

During the first 3 to 4 weeks after planting, alfalfa seedlings from inoculated and non-inoculated treatments had a normal green color. In subsequent weeks, visual differences in color and growth of alfalfa plants occurred. At 9 weeks, inoculated alfalfa plants were darker green than non-inoculated plants. Although alfalfa seedlings from the non-inoculated treatments were yellowish, they did not exhibit the extreme chlorotic condition often associated with N-deficient or S-deficient plants. The pale color remaining at harvest, however, indicated the possibility of an inadequate legume-indigenous rhizobia symbiosis. These visual differences in color between alfalfa grown in the non-inoculated and inoculated plots were observed in both years, being more pronounced in 1982.

Effect of Sulfur on the Yield of Alfalfa

An increase in dry matter yield above the check treatment for the different rates of applied S was observed for both inoculated and non-inoculated treatments in both 1982 and 1983 (Fig. I-1). No significant response in dry matter yield was observed, however, from the application of S (Table I-1).

The lack of yield response with S application, for both the inoculated and the non-inoculated plants, in both 1982 and 1983, indicates that the experimental sites were not sufficiently deficient in S to limit forage yield during the establishment

year. Higher content of available sulfate (present in lower soil horizons) than that detected by soil analysis, or stimulation of S mineralization by lime may have further contributed to the absence of a significant yield response to S application.

Inoculation treatment effects were highly significant in both years. Dry matter yields were higher in the inoculated than in the non-inoculated treatments, and were much higher (40%) in 1983 than in 1982 (Fig. I-1).

The percent yield increase, expressed as the yield of the fertilized treatment minus the yield of the check, and divided by the yield of the unfertilized treatment times 100, is shown in Table I-2. Although the percent yield increase varied between 1982 and 1983, S clearly increased the percent yield more in the non-inoculated than in the inoculated plants. For the non-inoculated treatments the highest percent increase in yield was obtained at a rate of 67.2 kg S/ha in 1982, and at 44.8 kg S/ha in 1983, and varied from 20 to 36% for 1982 and 1983, respectively. For the inoculated treatments the percentage increase in yield varied from 7 to 17% (for 1983 and 1982, respectively), at a rate of 44.8 kg S/ha.

Nodules

Nodules of plants randomly selected from each plot indicated no morphological differences in nodulation due to S levels. In contrast, significant differences were observed between inoculated and non-inoculated treatments at all S levels in both years.

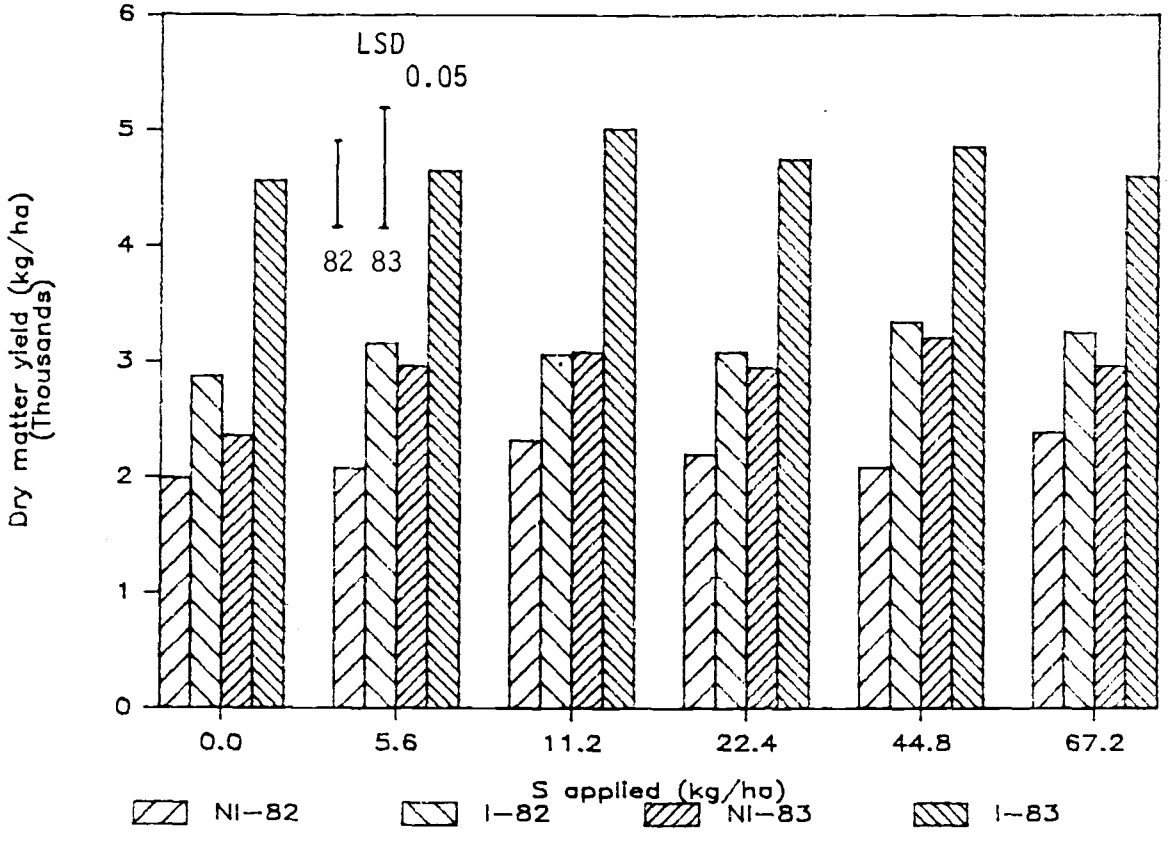

Figure I-1. Dry matter yield (kg/ha) of non-inoculated (NI) and inoculated (I) field grown seedling alfalfa plants, at different S levels.

Table I-1. Dry matter yield, percent N, and total N of non-inoculated and inoculated treatments, at different S levels. Each value is the mean of four replications.

Experiment	S applied (kg/ha) 0.0	5.6	11.2	22.4	44.8	67.2
1982			Inoculated			
Yield[+]	2881^{*}_{*}	3162^{*}_{*}	3070^{NS}_{*}	3092^{*}_{*}	3355^{*}_{*}	3264^{*}_{*}
% N	2.65	2.53	2.61	2.53	2.55	2.68
Total N[++]	76.3	80.0	80.1	78.2	85.6	87.5
			Non-inoculated			
Yield	2001	2084	2326	2203	2093	2399
% N	1.27	1.36	1.40	1.24	1.22	1.30
Total N	25.4	28.4	32.6	27.3	25.5	31.2
1983			Inoculated			
Yield	4573^{**}_{**}	4658^{*}_{**}	5028^{**}_{**}	4761^{*}_{**}	4875^{*}_{**}	4616^{*}_{**}
% N	3.18	2.92	2.90	3.05	3.07	3.07
Total N	145.4	136.0	145.8	145.2	149.7	141.7
			Non-inoculated			
Yield	2369	2968	3090	2957	3212	2976
% N	1.86	1.71	1.98	1.99	1.79	2.11
Total N	44.1	50.8	61.2	58.8	57.5	62.8

*,** Denote significance between inoculated and non-inoculated plants, at the 0.05 and 0.01 levels of probability for values in the same column and year.
NS Not significant.
+ Dry matter yield (kg/ha).
++ Total N (kg/ha) = dry matter yield x % N.

Table I-2. Percent yield increase of non-inoculated (Non-inoc) and inoculated (Inoc) field grown seedling alfalfa plants, at different S levels.

S applied (kg/ha)	Percent yield increase+			
	1982		1983	
	Non-inoc	Inoc	Non-inoc	Inoc
5.6	4.15	9.75	25.28	1.86
11.2	16.24	6.56	30.43	9.45
22.4	10.10	7.32	24.82	4.11
44.8	4.60	16.45	35.58	6.60
67.2	19.90	13.29	25.62	0.94

+ Percent yield increase = (yield fertilized treatment - yield unfertilized treatment)/yield unfertilized treatment x 100.

Nodules from the inoculated plants were well formed, pink pigmented, small, but higher in number than nodules from non-inoculated plants which were greater in size, but lower in number, coralline, and whitish.

In 1983, inoculated plants had a significantly higher number of nodules, and higher mass expressed as fresh or dry weight, than did non-inoculated plants (Table I-3). In the inoculated plants nodule fresh weight, nodule dry weight, and the number of nodules appeared to be decreasing as S levels increased; however, no significant differences were detected. In non-inoculated plants, nodule number, nodule fresh weight, and nodule dry weight showed a

trend of increasing as S levels were increased, although no significant differences were observed.

Table I-3. Nodule number, nodule fresh weight, and nodule dry weight of non-inoculated (Non-inoc) and inoculated (Inoc) field grown seedling alfalfa plants, at different S levels, in 1983. Each value is the mean of eight observations.

Inoculation Treatment	S applied (kg/ha)					
	0.0	5.6	11.2	22.4	44.8	67.2
	Nodule (number/plant)					
Non-inoc	11*	10**	11**	13*	4**	20*
Inoc	56	86	71	44	59	40
	Nodule fresh weight (mg/plant)					
Non-inoc	12.85*	9.78*	16.25*	13.82*	10.55*	72.57NS
Inoc	200.26	190.97	154.14	130.35	142.28	96.64
	Nodule dry weight (mg/plant)					
Non-inoc	2.26*	1.49*	2.56*	2.10*	1.54*	10.55NS
Inoc	24.00	25.69	17.58	17.39	19.11	11.76

*, ** Denote significance at the 0.05 and 0.01 levels of probability, respectively, for values in the same column.
NS Not significant.

Acetylene Reduction Rate

Significant differences for acetylene reduction were observed between inoculated and non-inoculated plants in 1982. Acetylene reduction rates in 1983 were 2 to 3 times higher than those found in 1982 (Figure I-2), suggesting a greater level of nitrogenase activity; however, significant differences were detected only at the 0.10 level of probability. Although significant differences for AR rates were observed in 1982, between inoculated and non-inoculated plants, no significant differences were noted in either year for the different S levels.

Nitrogen Concentration

Tissue N concentration (expressed on a dry matter basis) was significantly higher in the inoculated plants than in the non-inoculated plants in both 1982 and 1983, indicating that symbiosis was enhanced in the inoculated plants (Table I-1). No significant differences, however, were detected in either year for tissue N concentration, as affected by S application (Table I-1).

Total tissue N content (dry weight yield x % N) showed a tendency to increasing with increased S levels in both years. In the inoculated treatments N values were higher than the non-inoculated treatments. An increase in N content in legumes from S fertilization has been reported by several researchers (Harward et al., 1962; Rendig, 1956). This effect was attributed to an indirect effect of increased symbiotic N_2-fixation.

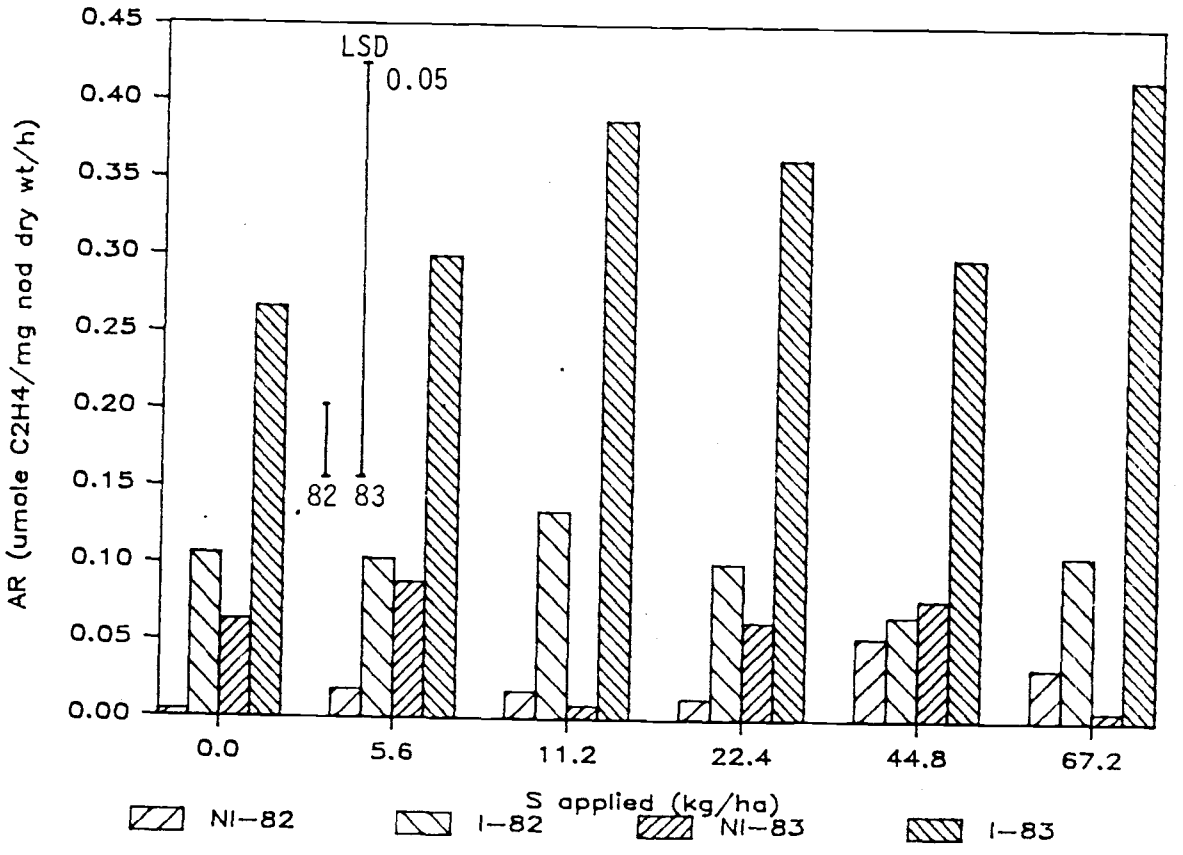

Figure I-2. Acetylene reduction (AR) rate in non-inoculated (NI) and inoculated (I) field grown seedling alfalfa plants, at different S levels.

The increase in N_2-fixed by the inoculated alfalfa plants was calculated by subtracting the total N present in the non-inoculated treatments from the corresponding inoculated treatments (Table I-1). The range of N_2-fixed, calculated by this method, varied from 48 to 60 kg N/ha in 1982, and from 80 to 101 kg N/ha in 1983. This difference between years is explainable since higher yields were obtained in 1983 than in 1982. With the exception of the high value in the check treatment in 1983, the highest values for N_2-fixed were obtained from the 44.8 kg S/ha treatment, which gave values of 60 and 92 kg N/ha, in 1982 and 1983, respectively.

Percent Sulfur in Alfalfa Tissue

The effect of gypsum application on the S concentration of field grown seedling alfalfa plants, is shown in Table I-4. Significant differences were detected between non-inoculated and inoculated plants, at all S levels applied in 1982, while significant differences occurred between non-inoculated and inoculated treatments, at the three highest levels of S, in 1983.

Sulfur fertilization resulted in a larger increase in the S concentration of non-inoculated plants than inoculated plants at different S levels, indicating that more S was used in roots and nodules of inoculated plants than non-inoculated plants, thereby decreasing the quantity of S in shoots (Hanley, 1977; Tisdale, 1977). Sulfur concentration of the non-inoculated plants varied from 3.5 to 4.4 g S/kg (0.35 to 0.44%) in 1982, and from 3.3 to

Table I-4. Percent S, and total S of non-inoculated and inoculated field grown seedling alfalfa plants, at different S levels. Each value is the mean of four replications.

Experiment	S applied (kg/ha)					
	0.0	5.6	11.2	22.4	44.8	67.2
1982						
			Non-inoculated			
% S	0.35^{NS}	0.35^{NS}	0.40*	0.40*	0.41*	0.44*
Total S^{+}	6.90	7.36	9.44	9.17	8.46	10.45
			Inoculated			
% S	0.27	0.26	0.27	0.27	0.26	0.30
Total S	7.80	8.20	8.58	8.17	8.61	9.52
1983						
			Non-inoculated			
% S	0.37*	0.35^{NS}	0.33^{NS}	0.38*	0.37*	0.38*
Total S	8.72	10.30	9.75	11.01	11.65	11.20
			Inoculated			
% S	0.28	0.30	0.30	0.31	0.28	0.31
Total S	12.79	14.00	14.73	14.55	13.68	14.27

*, ** Denote significance between inoculated and non-inoculated plants at the 0.05 and 0.01 levels of probability, for % S values in the same column and year.
NS Not significant.
+ Total S (kg/ha) = Dry matter yield (from Table I-1) x % S.

3.8 g/kg (0.33 to 0.38%) in 1983. For the inoculated plants the S content varied from 2.6 to 3.0 (0.26 to 0.30%), and from 2.8 to 3.1 g/kg (0.28 to 0.31%) in 1982 and 1983, respectively. In both years, the total average tissue S value was the same (0.33%), and

exceeded the 2.2 g/kg (0.22%) established as the critical S concentration for optimal growth and development of alfalfa plants (Harward et al., 1956; Pumphrey and Moore, 1965a, Rendig, 1956; Tisdale et al., 1950). Critical nutrient concentration of S in alfalfa, however, is dependent upon stage of development (Pumphrey and Moore, 1965a). For the present study, it is clear that any attempt to use a critical level of S in alfalfa as a diagnostic tool must be limited to the specific stage of growth examined, in this case 10 weeks after establishement.

Total S content (dry matter yield x % S, Table I-4) showed an increasing trend in both years as the S levels increased, for both non-inoculated and inoculated alfalfa plants. Inoculated plants were higher in total S than non-inoculated plants. The largest increase in total tissue S per quantity of S applied resulted from the 67.2 kg S/ha treatment.

A sulfur concentration higher than 2.2 g/kg (0.22%) at the early bloom stage of growth, or an N:S ratio less than 11:1 indicates an adequate supply of S (Pumphrey, 1967). Values below 2.2 g/kg did not occur in these experiments, and no yield response to S was found, thus supporting a critical value above which little response to S will be observed. The higher tissue S values observed in non-inoculated plants (0.44%), however, have been previously reported in field experiments (Delas et al., 1970; Martin and Walker, 1966).

N:S Ratios

Sulfur fertilization decreased the N:S ratio in the forage by increasing tissue S levels. Nitrogen to sulfur ratios (N:S) were wider in the inoculated plants than in the non-inoculated plants (Table I-5). In 1983, inoculated plants showed wider N:S ratios than in 1982, although in only one case did ratios approach or exceed the 11:1 value that has been reported as a critical value, indicating an adequate supply of S (Pumphrey, 1967) in the plants of these experiments.

Data from Table I-4 suggest a differential partitioning of S in the inoculated and non-inoculated plants. These data were supported by subsequent greenhouse experiments (see Manuscript II). This differential partitioning allowed a greater quantity of S to be available for symbiotic N_2-fixation, as demonstrated by increased N levels in alfalfa shoots (Table I-1). Thus, the N:S ratio in the forage became larger as the need for S was increased (Table I-5).

Percent Utilization Efficiency of Sulfur

The percent utilization efficiency of S for the different S treatments is shown in Table I-6. It was calculated as follows: the total S (kg/ha) in treated plants was divided by the total S in check plants (Table I-4) plus the applied S, and multiplied by 100.

Although the total S in both non-inoculated and inoculated treatments was increased with increased S levels (Table I-4), the

percent utilization efficiency of S decreased for both non-inoculated and inoculated field grown seedling alfalfa plants (Table I-6). No significant differences were observed in percent utilization efficiency of S between non-inoculated and inoculated plants, at the different S levels.

Table I-5. Nitrogen to Sulfur (N:S) ratios of non-inoculated (Non-inoc) and inoculated (Inoc) field grown seedling alfalfa plants, at different S levels. Each value is the mean of four replications.

S applied (kg/ha)	N:S ratios			
	1982		1983	
	Non-Inoc	Inoc	Non-Inoc	Inoc
0.0	3.7	10.0**	5.1	11.3**
5.6	3.9	9.9**	4.9	9.9**
11.2	3.5	9.6**	6.2	9.9*
22.4	3.1	9.5**	5.3	9.9**
44.8	3.1	9.9**	4.9	11.0*
67.2	3.1	9.9**	6.6	9.9*

*, ** Denote significance at the 0.05 and 0.01 levels of probability, for values in the same row and year.

Table I-6. Percent utilization efficiency of S in non-inoculated (Non-inoc) and inoculated (Inoc) field grown seedling alfalfa plants, at different S levels.

Experiment	S applied (kg/ha)					
	0.0	5.6	11.2	22.4	44.8	67.2
	Percent utilization efficiency of S+					
1982						
Non-inoc	100.0	58.9	52.2	31.3	16.4	14.1
Inoc	100.0	61.2	45.2	27.1	16.4	12.7
1983						
Non-inoc	100.0	71.9	49.0	35.4	21.8	14.8
Inoc	100.0	76.1	61.4	41.4	23.8	17.8

+ Percent utilization efficiency of S = (kg S/ha in treatment plants)/(kg S/ha in check plants + S applied) x 100.

The highest utilization of fertilizer S at lower levels of applied S may be the consequence of more stress exerted on roots for absorption of this nutrient. Conversely, the decrease of fertilizer S utilization at the higher levels of S application may be due to available S in excess of the actual needs of the plants (Aulakh and Dev, 1978).

The percent utilization efficiency for the highest rate of applied S (67.2 kg S/ha) varied between 13 and 14% in 1982, and from 15 and 18%, in 1983. For the lowest S rate (5.6 kg S/ha) the values were relatively high (59 to 76%); however these values represent small amounts of total S recovered. For the present

study, the addition of 5.6 kg S/ha was sufficient to satisfy the total demands for S, as estimated by comparing the different S treatments with the highest values for total S obtained in the check treatment.

CONCLUSIONS

Inoculation effects were highly significant in both years, indicating that the indigenous population of Rhizobium meliloti at the two experimental sites was ineffective in N_2-fixation on 'Apollo' alfalfa. This conclusion has been supported by further experiments on parallel sites.

Dry matter yields were higher in inoculated than non-inoculated alfalfa seedlings. Inoculated alfalfa seedlings had a significantly higher acetylene reduction rate, N concentration, and total N and S contents.

Sulfur fertilization increased the S concentration of non-inoculated plants more than inoculated plants, at the different S levels. Sulfur fertilization decreased the N:S ratio in the forage by increasing tissue S levels, with inoculated plants having wider N:S ratios.

Percent utilization efficiency of S was decreased with increased S levels. No significant differences were detected in percent S utilization efficiency, between inoculated and non-inoculated alfalfa seedlings, at the different S levels. The highest increase in N_2-fixed by the inoculated plants was obtained from the 44.8 kg S/ha treatment.

The lack of yield response with S application for both the inoculated and non-inoculated alfalfa seedlings, indicates that the experimental sites were not sufficiently deficient in S to limit forage yield.

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MANUSCRIPT II

SULFUR NUTRITION EFFECTS ON DINITROGEN FIXATION OF SEEDLING ALFALFA

SULFUR NUTRITION EFFECTS ON DINITROGEN FIXATION OF SEEDLING ALFALFA

ABSTRACT

Greenhouse experiments with alfalfa (Medicago sativa L. cv. 'Apollo') were performed to evaluate the effect of varied nutrient solution concentrations of S on the yield, nodulation, dinitrogen fixation, N and S concentration, and to determine the partitioning of N and S into shoots and roots.

Sulfur treatments consisted of four levels (0, 1, 2.5, and 25 mg S/L) of added S. The experimental design was a randomized complete block, with three replications. Seeds were inoculated with commercial inoculum, planted in plastic containers with acid-washed sand, and irrigated with nutrient solution for one minute, at 2 h intervals.

Sulfur application increased the yield of all treatments. The results demonstrated that the addition of 2.5 mg S/L to the nutrient solution, besides providing the highest total dry matter yield (12 g/72 plants), showed the highest percent yield increase (19%), acetylene reduction rate (0.426 umole ethylene/mg nodule dry wt/h), total N content (306 mg/72 plants), percent recovery of S (3.8%), and percent increase in N due to dinitrogen fixation (32%).

N:S ratios obtained were different for shoots and roots, and S fertilization decreased the N:S ratios. The N:S ratios of 16:1

(shoots), and 9:1 (roots) obtained in the 2.5 mg S/L treatment were found to be adequate for normal growth and development. These data indicate that the 2.5 mg S/L treatment (2.7 mg total S/L) is optimal for alfalfa seedling development.

Key words: Medicago sativa L., N_2-fixation, N:S ratio, Sulfur.

INTRODUCTION

Soils are normally deficient in N, and commonly deficient in P, S, or K (Munns, 1977). For leguminous forage crops, N supply can be increased either through the use of fertilizer-N or N_2-fixation. If conditions of high N supply coincide with low inputs of S from external sources, the risk of S deficiency is high (Probert and Jones, 1977).

Sulfur, the fourth most important plant nutrient (Platou and Irish, 1982) is required in relatively large amounts for proper growth and development of alfalfa (Medicago sativa L.)(Radet, 1966). Many authors have claimed that S is specifically involved in N_2-fixation in legumes (Adams and Sheard, 1966; Masterson, 1977; Walker and Adams, 1968; Zaroug and Munns, 1979). These claims are based on the observations that alleviating S deficiency in the legumes increase yield and N concentration of the forage. Sulfur deficiency can limit both nodulation and N_2-fixation (Oke, 1969; Spencer, 1959). Masterson (1977) reported the effect of S on symbiotic N_2-fixation on white clover (Trifolium repens L.) and red clover (Trifolium pratense L.) in greenhouse experiments. When soil S was low additions of S containing fertilizers gave highly significant increases in plant yield, N_2-fixation, nodule weight per plant, nodule weight per unit weight of root, and N_2-fixation per unit weight of nodule. Adams and Sheard (1966) found that S deficiency of alfalfa retarded protein synthesis and, as a consequence, reduced nodulation.

Tissue concentrations of S needed to express the potential yield varies between crops, but there is little information on the external concentration required by plants, expressed as the concentration in the soil solution or in nutrient culture. Spencer (1975) cited data that 3 to 5 mg S/L (3 to 5 ppm) is adequate for the growth of many species; however, alfalfa needed 8 mg S/L (8 ppm).

The objectives of the experiments described here were to evaluate the effect of varied nutrient solution concentrations of S on growth, nodulation, N_2-fixation, and mineral composition of alfalfa seedlings, and to determine the partitioning of N and S into roots and shoots under limiting and non-limiting S concentrations.

MATERIALS AND METHODS

Experiments were conducted during 1983 and 1984 in greenhouse facilitites at Oregon State University in Corvallis. The alfalfa plants (Medicago sativa L. cv. 'Apollo') were grown under a 16 h photoperiod, and a day/night temperature of 25/18° C. Natural illumination was supplemented with Sylvania 1000 Metalarc R[1] lamps (M/1000/E/V) placed at 1.10 m intervals along the greenhouse bench, 1 m above canopy level, and with Sylvania[1] 100 W lamps (projector flood), placed 1 m apart, and 1 m above the canopy. Photosynthetically active radiation at plant height was 500 to 525 $uE/m^2/s$.

Experimental Design and Sulfur Treatments

The experimental design was a randomized complete block, with 4 treatments and 3 replications. Six observations were made for each treatment. Sulfur treatments consisted of four S levels: 0, 1, 2.5, and 25 mg S/L of added S (0, 1, 2.5, and 25 ppm). Twenty four containers, with three plants per container were utilized for each treatment.

[1] Mention of a trademark, proprietary product, or company name does not constitute a guarantee or warranty of the product and does not imply endorsement of the product by the authors or Oregon State University.

Plastic Containers

Black plastic containers, 6.2 cm (top), and 5.2 cm (bottom) in diameter, and 24 cm deep, with five holes near the base, were utilized. A circular plastic disk with a drainage hole was cut to fit the lower diameter of the container (5.2 cm). This disk was supported 5 cm above the bottom of the container and was covered by a fine plastic mesh screen. The containers were filled with acid (3N HCl)-washed sand (0.02 mm) to within 0.5 cm of the top. The sand was rinsed with deionized water 10 times to remove residual acidity. The S content of the sand was less than 0.06 ug/g sulfate S (0.06 ppm), and the total N content less than 0.1 mg N/g (0.01%).

Inoculation

Alfalfa seeds were surface-sterilized by exposure for three minutes with 200 ml/L (20%) commercial bleach (sodium hypochlorite), followed by 10 sterile water washes. Seeds were then dried in sterile paper towels.

Alfalfa seeds at planting were pelleted with lime as described by Vincent (1970). The pellet mixture included a recently purchased fresh preparation of commercial inoculant from Nitragin[1], Milwaukee, Wis., at a rate of 100 mg inoculant per g of seed, and laboratory grade $CaCO_3$ (0.01% sulfate), at 1.2 g per g of seed. Gum arabic solution (82 ml H_2O + 35.5 g powder gum arabic, obtained from Sigma Chemical Co[1]., St Louis, MO) was used as an adhesive.

Nodules were first visible on the primary roots 9 to 10 days after planting. Further observations of the extent of nodulation were made at the time the acetylene reduction (AR) assays were conducted.

Planting

Five to six inoculated 'Apollo' alfalfa seeds were planted in the plastic containers, containing acid-washed sand, and irrigated with nutrient solution for one minute, at 2 h intervals. Eight to 10 days after germination seedlings were thinned, on the basis of visual uniformity, to three per container.

Nutrient Solution Composition and Application

Nutrient solutions were prepared according to Wych and Rains (1978) and following the procedure described by Steiner (1961) for preparing nutrient solutions of a desired composition. Reagent grade chemicals were utilized in all experiments.

The macronutrient stock solution, expressed in meq/L, is shown in Table II-1, and the concentration of the micronutrient stock solution is provided in Table II-2. All treatments also received 0.05 ml of $CoCl_2.6H_2O$ (50 mM); 1 ml Fe EDDHA (12 mM), and 1 ml of micronutrient stock solution/L final solution. Sulfur was applied as $MgSO_4.4H_2O$. An initial application of 0.19 meq N/L as $Ca(NO_3)_2$ was applied to all treatments to provide a small amount of N for vigorous growth until symbiotic N_2-fixation was established.

Table II-1. Macronutrient stock solution concentrations, utilized in the preparation of the nutrient solutions.

Salt source	meq/L of nutrient solution					
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.62		4.62		
K_2HPO_4			4.62	4.62		
$CaCl_2$	5.54				5.54	
$MgCl_2$	3.69					3.69

Three days after initiation of germination, the solutions were deprived of N. Nitrogen-free solutions were imposed for the duration of the experiment.

Nutrient solutions were placed in 20 liter plastic containers, and the solutions were applied automatically with an irrigation system. The solutions remained at pH 6.3 ± 0.2 for one week, and were completely renewed at weekly intervals.

Deionized water, obtained from a mixed cation/anion exchange resin (Culligan, Aqua Summa System[1]) was used for preparing nutrient solutions and for cleaning glassware, including solution storage bottles, and plastic containers. Plants were grown for 6 weeks in the nutrient solutions containing different S concentrations (Table II-3). The experiment was terminated at the end of the 6 weeks when plants were 28 to 33 cm.

Table II-2. Micronutrient stock solution concentrations, utilized in the preparation of the nutrient solutions.

Salt source	Concentration of stock solution (mM)
H_3BO_3	25.0
$MnCl_2.4H_2O$	2.0
$ZnCL_2.2H_2O$	2.0
$CuCl_2.2H_2O$	0.5
$Na_2MoO_4.2H_2O$	0.5

Acetylene Reduction Analyses

Six weeks after germination six containers with three plants per container were selected at random for the AR assays. The three plants in each container were gently removed from the sand, and put immediately in a 15 x 43 cm Saran bag (W. R. Grace & Co[1]., Cedar Rapids, Iowa), fitted with a air-tight seal around the alfalfa stems, and placed in a paper bag to avoid continued exposure to light. The plants were then replaced to their original growing location on the experimental bench where they were incubated with acetylene.

Acetylene reduction assays were performed between 0800 and 1200 hours and were initiated by adding acetylene with a syringe. Sufficient acetylene was injected through a septum fitted in the bag to obtain 100 ml/m^3 (10%) acetylene in the atmosphere around the plant. Acetylene was generated by the addition of calcium

carbide to distilled water as described by Burris (1974).

Table II-3. Nutrient solution composition for sulfur treatments, with and without N. Sulfur treatments are designated S0, S1, S2.5, and S25 which represent 0, 1, 2.5, and 25 mg S/L.

Ions	Sulfur treatments							
	With NO_3^-				Without NO_3^-			
(meq/L)	S0	S1	S2.5	S25	S0	S1	S2.5	S25
Cl^-	9.05	8.90	8.92	7.69	9.23	9.18	9.10	7.88
$H_2PO_4^-$	4.62	4.62	4.63	4.74	4.62	4.62	4.63	4.74
$HPO_4^=$	4.62	4.62	4.63	4.74	4.62	4.62	4.63	4.74
NO_3^-	0.19	0.19	0.19	0.19	0.00	0.00	0.00	0.00
$SO_4^=$	0.00	0.06	0.16	1.59	0.00	0.06	0.16	1.59
K^+	9.23	9.24	9.26	9.48	9.23	9.24	9.26	9.48
Ca^{++}	5.54	5.54	5.55.	5.68	5.54	5.54	5.55	5.68
Mg^{++}	3.69	3.70	3.70	3.79	3.69	3.70	3.70	3.79

At the conclusion of each incubation interval of 30, 60, and 90 minutes (times selected from preliminary experiments), the air-acetylene mixture in the Saran bag was mixed by repeatedly pumping a 5-ml hypodermic syringe inserted in the Saran bag through the septum. Gas samples were then withdrawn and stored in 75 x 13 mm, 5.6 ml evacuated glass tubes (Vacutainer Systems[1] Rutherford, N. J.).

The ethylene content of each gas sample was determined by analyzing a 0.5 ml gas sample with a Hewlet Packard 5830A gas chromatograph[1]. The amount of ethylene produced and the dry weight of the nodules were used for calculation of the AR rate (nitrogenase specific activity).

Harvest

After the AR assay, plants were separated into roots, shoots, and nodules. Roots were thoroughly rinsed, nodules separated from the roots and each component weighed. Roots, shoots, and nodules were dried at 60° C for 24 h in a forced air oven, cooled to ambient temperature, and weighed. The remaining plants in each treatment were harvested for total yield, and for chemical analyses.

The percent yield increase was defined as the yield of the S fertilized treatment minus the yield of the unfertilized treatment divided by the yield of the S unfertilized treatment, and multiplied by 100.

Chemical Analyses

Shoot and root samples were taken for chemical analyses at the same time of yield determinations (45 days after planting). The samples were washed with distilled water, dried to a constant weight in a forced air oven at 60° C, and ground to pass a 0.5 mm screen. Ground samples were stored in air tight plastic bags and redried before weighing for analysis.

Nitrogen was analyzed by an automated micro-Kjeldahl apparatus (Schuman et al., 1973). Total S was determined following a procedure modified from Tabatabai and Bremner (1970). The plant material was digested in an erlenmeyer flask with 2 ml of ethanol and 3 ml of saturated $Mg(NO_3)_2$ solution; ashed in a muffle furnace, cooled to room temperature, and suspended in 10 ml of 3 M HCl. The sulfate content of an aliquot of the digest was determined turbidimetrically as $BaSO_4$, by a barium chloride gelatin procedure. Phosphorus, K, Ca, Mg, Fe, Mn, Mo, Co, B, Zn, and Cu, were analyzed using a Jarrel-Ash Inductively Coupled Argon Plasma spectrometer (ICAP-9000) manufactured by Allied Analytical Systems[1].

The percent utilization efficiency of S was defined as the ratio of the total S in treatment plants divided by the total S available, and multiplied by 100. The percent recovery of applied S was defined as the difference between the amount of S contained in the tops and roots of plants which received S, minus the amount of S contained in the check plants, and divided by the total amount of S available times 100 (Holford, 1971).

RESULTS AND DISCUSSION

The alfalfa plants grown in the nutrient solutions developed normally, and showed visible S deficiency symptoms only in the control S treatment. Due to the presence of some sulfate in the chemicals used in the preparation of the nutrient solutions, the check nutrient solution contained 0.205 mg S/L nutrient solution/week. All S treatments were increased by this amount (0.205 mg S/L/week).

Growth of nodules in the plants of all treatments was vigorous. The average number of nodules was between 29 and 34 per plant, at the time of the AR assay. More numerous and vigorous nodules were observed in the treatment with 2.5 mg S/L (2.5 ppm); however, no significant differences were detected for the different S treatments.

Yield

Harvest of plants was made five days after the AR assay was performed, and plant were separated into shoots and roots (roots + nodules), and weighed.

The dry weight yield response to S application was similar for shoots, roots and nodules, and the total plant (Table II-4). Sulfur application increased the yield of all treatments, compared with that of the control, with the 2.5 mg S/L treatment resulting in highest yields. Although significant differences were detected for dry weight yield of roots + nodules, and for the total plant,

no significant differences were observed for dry weight yield of shoots.

Table II-4. Dry matter yield of shoots, roots (roots + nodules), and total yield, and percent yield increase from inoculated greeenhouse grown seedling alfalfa plants, at different S levels.

S applied (mg/L)	Dry matter yield (g/72 plants)			% yield increase+		
	Shoots	Roots	Total	Shoots	Roots	Total
0.0	5.20^{a++}	4.96^{bc}	10.16^{bc}	-	-	-
1.0	5.31^{a}	5.20^{ab}	10.51^{b}	2.11	4.84	3.44
2.5	6.15^{a}	5.90^{a}	12.05^{a}	18.26	18.95	18.60
25.0	5.61^{a}	4.60^{c}	10.21^{bc}	7.88	-7.26	0.49

\+ % yield increase = (Yield fertilized treatment - yield unfertilized treatment)/yield unfertilized treatment x 100.
++ Means in any vertical column followed by the same letter are not significantly different at the 0.05 level of probability.

Fresh weight yield responses of shoots, roots + nodules, and total plant, to the different S treatments were similar to those found in the dry weight determinations (data not shown). Average shoot yields were increased at all rates of S application. In the corresponding analysis of variance, significant differences were found for the effect of S on fresh weight yield of roots + nodules, and the entire plant, but not for shoot fresh weight.

The highest percent yield increase for shoots, roots +

nodules, and for the total plant (18.26, 18.95, and 18.60, respectively) was observed in the 2.5 mg S/L treatment (Table II-4), and was higher for roots than for shoots.

Acetylene Reduction Rate

The AR rate, expressed as umole of ethylene (C_2H_4)/mg nodule dry weight/h, showed no significant differences between the control and the lowest and highest rate of S applied (Table II-5). Ethylene production, however, was significantly higher in the 2.5 mg S/L treatment, in which the production of ethylene was more than 60% greater than the control.

Table II-5. Acetylene reduction (AR) rate of six week-old alfalfa seedlings growing in nutrient solution, at different S levels.

S applied (mg/L)	Acetylene reduction rate (umole ethylene/h)		
	AR/plant	AR/g nod fresh wt	AR/mg nod dry wt
0.0	0.735^{ab+}	0.014^{b}	0.266^{b}
1.0	0.467^{bc}	0.012^{bc}	0.232^{bc}
2.5	0.782^{a}	0.029^{a}	0.426^{a}
25.0	0.283^{c}	0.011^{bc}	0.139^{bc}
$LSD_{0.05}$	0.282	0.012	0.154
$LSD_{0.01}$	0.375	0.016	0.205

+ Means in any vertical column followed by the same letter are not significantly different at the 0.05 level of probability.

Acetylene reduction rate also was expressed as umole C_2H_4/g nodule fresh weight/h, and as umole of C_2H_4/plant/h (Table II-5). The results were similar as when expressed on a dry weight basis. Significant increases in AR rate were detected with 2.5 mg S/L treatment (2.5 ppm S).

Nitrogen Concentration

Increasing the nutrient solution concentration of S did not result in significant increases in N concentration either in shoots or in roots (Table II-6). Percent N content of roots was, however, approximately one third lower than in shoots.

Table II-6. Nitrogen concentration (% dry matter), total N (mg), and percent increase of N due to N_2-fixation from inoculated greeenhouse grown seedling alfalfa plants, at different S levels.

S applied (mg/L)	Concentration N (%)		Total N^+ (mg)			Increase over control (mg N)
	Shoots	Roots	Shoots	Roots	Total	
0.0	3.45[a]	1.05[a]	179.4	52.1	231.5	-
1.0	3.58[a]	1.54[a]	190.1	80.1	270.2	38.7
2.5	3.50[a]	1.54[a]	215.3	90.9	306.2	74.7
25.0	3.57[a]	1.54[a]	200.3	70.8	271.1	39.6
$LSD_{0.05}$	0.43	0.64				
$LSD_{0.01}$	0.65	0.97				

+ Total N (mg) = N(%) x dry matter yield (Table II-4) x 1000/100.
++ Percent increase of N due to N_2-fixation.

The total N content (mg dry weight x % N) in shoots and roots of alfalfa seedlings, presented in Table II-6, demonstrated that S fertilization significantly increased the total N content. The highest total N content was observed in the 2.5 mg S/L treatment in which total N content was lower in roots than in shoots.

The percent increase in N, which was assumed to be due to N_2-fixation and as a consequence of S application, varied from 17 to 32% (Table II-6). Therefore, the application of 2.5 mg S/L to the nutrient solution increased N_2-fixation more than 30% over the control and represents 75 mg of increased N.

Percent Sulfur in Alfalfa Tissue

Increasing the S supply increased the S concentration in both shoots and roots of six week-old alfalfa seedlings (Table II-7). At all levels of S applied, S concentration was 40% higher in shoots than in roots. In the roots, the highest S value was obtained at the highest rate of S applied (25 mg S/L); however, no significant differences were detected between treatments with 25 and 2.5 mg S/L, between 2.5 and 1.0, or between 1.0 and the control.

Total S (mg dry weight x % S) was increased as nutrient solution S was increased. Highest total S was observed at the highest rate of S applied (25 mg S/L).

Table II-7. Sulfur concentration (% dry matter), and total S (mg) of shoots and roots (roots + nodules) from inoculated greenhouse grown seedling alfalfa plants, at different S levels.

S applied (mg/L)	S concentration (%)		Total S+ (mg)	
	Shoots	Roots	Shoots	Roots
0.0	0.16[d]	0.07[c]	8.3	3.5
1.0	0.20[c]	0.11[bc]	10.6	5.7
2.5	0.22[b]	0.18[ab]	13.5	10.6
25.0	0.25[a]	0.22[a]	14.0	10.1
$LSD_{0.05}$	0.001	0.07		
$LSD_{0.01}$	0.022	0.10		

+ Total S (mg) = S (%) x dry matter yield (Table II-4) x 1000/100.

N:S Ratios

The N:S ratio in shoots was higher than that in roots at all levels of solution S (Table II-8). Increased solution S decreased the N:S ratio in both shoots and roots. The N:S ratio indicates the partitioning of N and S in shoots and roots. This ratio decreased with increased S levels demonstrating that high quantities of S can accumulate in alfalfa seedlings. The N:S ratio of 22:1 and 18:1 found in the shoots of the control and 1 mg S/L treatment plants, respectively, indicated a severe deficiency of S.

Table II-8. Nitrogen to sulfur (N:S) ratio of shoots and roots (roots + nodules) from inoculated greenhouse grown seedling alfalfa plants, at different S levels.

S applied (mg/L)	N:S ratio	
	Shoots	Roots
0.0	22	20
1.0	18	14
2.5	16	9
25.0	14	7

An N:S of 16:1 for shoots was found to be adequate for normal growth and development of alfalfa seedlings, and resulted in highest yield. A 14:1 ratio found in shoots of the 25 mg S/L treatment were found to be in excess of requirements indicating absorption and translocation of S in excess of need; a "luxury uptake" of S.

Percent Utilization Efficiency of Sulfur

Although the total S for shoots and roots was increased with increased S levels (Table II-7), the percent utilization efficiency of S decreased for both shoots and roots (Table II-9). Percent utilization efficiency of S was higher in shoots than in roots, at all levels of S.

The percent utilization efficiency (a measure of the amount of total S present in the corresponding tissue), for the highest

rate of S applied (25 mg S/L) varied between 0.33 and 0.46%, for roots and shoots, respectively. The value for the total plant was 0.79%. Although the control treatment values were relatively high; 14, 34, and 48% for roots, shoots, and total plant, respectively; they represent only small amounts of total S.

For the present experimental conditions, the addition of 2.5 mg S/L to the nutrient solution was adequate for optimal yield, satisfied the total demand for S, and resulted in a higher percentage of utilization efficiency as compared with the lowest and the highest rate of S applied.

Table II-9. Percent S recovery, and percent utilization efficiency (UE) of S from inoculated greenhouse grown seedling alfalfa plants, at different S levels.

S applied (mg/L)	Total S[+] (mg)	% S recovery[++]			% UE of S[+++]		
		Shoots	Roots	Total	Shoots	Roots	Total
0.0	24.6	-	-	-	33.7	14.3	48.0
1.0	144.6	1.6	1.5	3.1	7.3	3.9	11.2
2.5	324.6	1.6	2.2	3.8	4.2	3.3	7.5
25.0	3024.6	0.2	0.2	0.4	0.5	0.3	0.8

\+ Total S = (S applied + S from all contaminants) x 20 L x 6 weeks.

++ % S recovery = (Total S in treatment - total S in check) (Table II-5)/total S available x 100.

+++ % Utilization Efficiency S = weight S in treatment (Table II-5)/weight S available in treatment x 100.

Percent Sulfur Recovery

Quantitative recovery of S by six week-old seedling alfalfa plants was based on the difference between S uptake from the control and S added treatments (Table II-9). Highest percent S recovery and highest yield corresponded with the 2.5 mg S/L treatment.

The percent S recovery for the total plant varied from 0.4 to 3.8%. The lowest percent S recovery value was observed with the highest rate of S applied. With the 25 mg S/L treatment only 12.4 mg of S were taken up by the plants from the 3025 mg of S available in the nutrient solution, over the six week period. Almost the same amount of S (12 mg S) was absorbed by plants growing in the 2.5 mg S/L treatment. Since the amount of available S was less (325 mg S), the percent recovery was higher (3.8% compared to 0.4%).

Chemical Analyses

The concentration of S in shoots, and concentration of S, P, K, and Mg in roots (Table II-10), showed a common response to increased solution S; concentrations were higher at all rates of S applied than those of the control (Table II-7). Percent S in shoots was significantly higher for all treatments from that of the control; however, significant differences were detected in roots only among the control, medium and high levels of S applied (Table II-10).

In roots, concentrations of P, K, and Mg were higher than

that of the control at all rates of S applied. Those concentrations were significantly higher in the 1.0 mg S/L treatment, but no differences were detected among the different S treatments.

In contrast to other minerals, the concentration of Mo was decreased significantly with each increment of S applied, in shoots and in roots (Table II-10).

Table II-10. Effect of S supply on the chemical composition of shoots and roots of six week-old greenhouse grown seedling alfalfa plants, at different S levels.

S applied (mg/L)	Mineral elements in tissue						
	Shoots		Roots				
	S+	Mo++	S	P	K	Mg	Mo
0.0	0.16^{a}	18.26^{a}	0.07^{c}	0.66^{c}	2.63^{d}	0.18^{c}	15.26^{a}
1.0	0.20^{a}	11.12^{b}	0.11^{bc}	1.03^{a}	3.90^{a}	0.27^{ab}	13.97^{ab}
2.5	0.22^{a}	7.60^{c}	0.18^{ab}	0.96^{ab}	3.70^{ab}	0.25^{b}	10.86^{abc}
25.0	0.25^{a}	3.82^{d}	0.22^{a}	0.90^{abc}	3.52^{abc}	0.25^{b}	6.34^{c}
$LSD_{0.05}$	0.001	1.05	0.07	0.25	0.85	0.06	4.97
$LSD_{0.01}$	0.022	1.59	0.10	0.37	1.29	0.09	7.53

+ Sulfur, P, K, and Mg expressed as percent dry matter.
++ Molybdenum, expressed as mg/kg (ppm).

The lowest value (6.34 ppm Mo) was obtained with the highest rate of S applied. In shoots, significant differences were observed among the different S treatments and the control. In roots, however, differences were detected only between the control and the treatment with 25 mg S/L.

The inhibitory effects of sulfate on Mo, according to Pal et al. (1976) occur primarily during the absortion process, with some antagonism involved during translocation from roots to shoots.

CONCLUSIONS

The results of this study demonstrate that the 2.5 mg S/L treatment (2.7 mg total S/L) gave the highest yield, showed the the highest percent yield increase, acetylene reduction rate, total N content, percent utilization efficiency of S, percent recovery of S, and percent increase in N due to N_2-fixation. In addition, the N:S ratios of 16:1 for shoots and 9:1 for roots, obtained in the 2.5 mg S/L treatment, were found to be adequate for normal growth and development. Thus, the 2.5 mg S/L treatment (2.7 mg total S/L of nutrient solution) is considered optimal for growth and development of alfalfa seedlings.

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CONCLUSIONS

1. In greenhouse experiments S application increased the total dry matter yield of alfalfa seedlings.
2. Inoculation treatment effects were highly significant, indicating that the indigenous population of Rhizobium meliloti at the experimental sites was ineffective in dinitrogen fixation of 'Apollo' alfalfa. These data are supported by experiments on parallel sites.
3. Significant differences for acetylene reduction rate were observed between inoculated and non-inoculated field grown alfalfa seedlings, but not with the different levels of S fertilizer applied. However, in greenhouse experiments inoculated alfalfa seedlings in the 2.5 mg S/L treatment showed a 60% higher production of ethylene than the control.
4. Tissue N concentration was significantly higher in inoculated than in non-inoculated plants, indicating that symbiosis was enhanced by commercial inoculum. However, tissue N concentration was not affected by S application.
5. Total tissue N content showed a tendency of increasing with increased S levels. In the inoculated treatments N values were higher than in the non-inoculated plants. This effect was attributed to an indirect effect of increased symbiotic dinitrogen fixation.

6. A 30% increase in N was observed in the nutrient solution with 2.5 mg S/L treatment, as compared to the control. This was assumed to be due to dinitrogen fixation, and as a consequence of S application.
7. Sulfur fertilization resulted in a larger increase in the S concentration of non-inoculated than inoculated alfalfa seedlings. This may be the result of more dry matter being accumulated in inoculated plants. Sulfur concentration was 40% higher in shoots than in roots, indicating differential partitioning of this element for varied metabolic needs.
8. Total S content showed an increasing trend as the S levels increased, for both non-inoculated and inoculated plants. Inoculated plants, however, were higher in total S than non-inoculated plants. In both field and greenhouse experiments it was observed that the largest increase in total tissue S always resulted from the highest rate of applied S.
9. Sulfur fertilization decreased the N:S ratio in the forage by increasing tissue S levels. Nitrogen to sulfur ratios were wider in inoculated than in non-inoculated plants, and were wider in shoots than in roots. This differential partitioning allowed a greater quantity of S to be available for symbiotic dinitrogen fixation, as demonstrated by increased N levels in alfalfa shoots.

10. Although the total S in both inoculated and non-inoculated alfalfa seedlings was increased with increased S levels, the percent utilization efficiency of S decreased. No differences were observed between inoculated and non-inoculated alfalfa seedlings in total uptake of S.
11. Sulfur fertilization changed the chemical composition of alfalfa seedlings. In addition to changes in N and S, K, P, and Mg increased with increased levels of S fertilizer. Molybdenum, conversely, decreased significantly with each increment of S applied, in shoots and in roots, probably due to antagonism by sulfate on Mo absorption.

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APPENDIX

Appendix Table 1. Alfalfa nodule number per plant of non-inoculated and inoculated treatments, at different S levels in 1982. Each value is the mean of eight observations.

S applied	Nodule number	
	Non-inoculated	Inoculated
kg/ha	Number/plant	
0.0	26	126^{*}
5.6	21	61^{NS}
11.2	11	94^{*}
22.4	34	74^{NS}
44.8	29	86^{NS}
67.8	35	61^{NS}

* Denotes significance at the 0.05 level of probability for values in the same row.
NS Not significant.

Appendix Table 2. Analysis of variance for alfalfa nodule number per plant of non-inoculated and inoculated treatments, at different S levels in 1982.

Analysis of variance Split Block Design

Source var	df	Sum Square	Mean Square
Reps	3	4685.58	1561.86
Inoc	1	39790.10	39790.10
Reps*Inoc	3	4234.92	1411.64
Slev	5	5587.40	1117.48
Reps*Slev	15	18801.90	1253.46
Inoc*Slev	5	8210.90	1642.18
Inoc*Reps*Slev	15	13877.10	925.14
Total	47		

F (Inoc)	=	28.19^{*}
F (Slev)	=	0.89^{NS}
F (Inoc*Slev)	=	1.78^{NS}

* Denotes significance at the 0.05 level of probability.
NS Not significant.
Grand mean = 55 CV = 55.30%
$LSD_{0.05}$ = 60 $LSD_{0.01}$ = 110

Appendix Table 3. Acetylene reduction rate of non-inoculated and inoculated alfalfa plants, at different S levels in 1982. Each value is the mean of eight observations.

S applied	Acetylene reduction rate	
	Non-inoculated	Inoculated
kg/ha	umole ethylene/mg nod dry wt/h	
0.0	0.005	0.107^{**}
5.6	0.019	0.104^{**}
11.2	0.018	0.135^{**}
22.4	0.014	0.102^{NS}
44.8	0.054	0.068^{*}
67.2	0.035	0.108

*, ** Denote significance at the 0.05 and 0.01 levels of probability for values in the same row.
NS Not significant.

Appendix Table 4. Analysis of variance for acetylene reduction rate of non-inoculated and inoculated treatments, at different S levels in 1982.

Analysis of variance	Split Block Design		
Source var	df	Sum square	Mean square
Reps	3	0.00225	0.00075
Inoc	1	0.07640	0.07640
Reps*Inoc	3	0.00189	0.00063
Slev	5	0.00255	0.00051
Reps*Slev	15	0.02145	0.00143
Inoc*Slev	5	0.01285	0.00257
Inoc*Reps*Slev	15	0.02625	0.00175
Total	47		
F (Inoc)	=	121.26^{**}	
F (Slev)	=	0.36^{NS}	
F (Inoc*Slev)	=	1.47^{NS}	

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 0.064 CV = 65.36%
$LSD_{0.05}$ = 0.040 $LSD_{0.01}$ = 0.073

Appendix Table 5. Alfalfa dry matter yield of non-inoculated and inoculated treatments, at different S levels in 1982. Each value is the mean of four replications.

S applied	Alfalfa dry matter yield	
	Non-inoculated	Inoculated
kg/ha	kg/ha	
0.0	2001	2881*
5.6	2084	3162*
11.2	2326	3070NS
22.4	2203	3092*
44.8	2093	3355*
67.2	2399	3264*

* Denotes significance at the 0.05 level of probability for values in the same row.
NS Not significant.

Appendix Table 6. Analysis of variance for alfalfa dry matter yield of non-inoculated and inoculated treatments, at different S levels in 1982.

Analysis of variance Split Block Design

Source var	df	Sum square	Mean square
Reps	3	933309	311103
Inoc	1	10892600	10892600
Reps*Inoc	3	397545	132515
Slev	5	675720	135144
Reps*Slev	15	4595760	306384
Inoc*Slev	5	343730	68764
Inoc*Reps*Slev	15	1389015	92601
Total	47		

F (Inoc)	=	82.20**
F (Slev)	=	0.44NS
F (Inoc*Slev)	=	0.74NS

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 2661 CV = 11.44%
$LSD_{0.05}$ = 819 $LSD_{0.01}$ = 1504

Appendix Table 7. Nitrogen concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1982. Each value is the mean of four replications.

S applied	N concentration	
	Non-inoculated	Inoculated
kg/ha	N (% dry matter)	
0.0	1.27	2.65*
5.6	1.36	2.53*
11.2	1.40	2.61*
22.4	1.24	2.53*
44.8	1.22	2.55*
67.2	1.30	2.68

* Denotes significance at the 0.05 level of probability for values in the same row.

Appendix Table 8. Analysis of variance for N concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1982.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	0.432	0.144
Inoc	1	20.008	20.008
Reps*Inoc	3	0.801	0.267
Slev	5	0.105	0.021
Reps*Slev	15	0.240	0.016
Inoc*Slev	5	0.075	0.015
Reps*Inoc*Slev	15	0.300	0.020
Total	47		
F (Inoc)	=	74.94^{**}	
F (Slev)	=	1.32^{NS}	
F (Inoc*Slev)	=	0.75^{NS}	

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 1.94 CV = 7.28%
$LSD_{0.05}$ = 0.82 $LSD_{0.01}$ = 1.51

Appendix Table 9. Sulfur concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1982. Each value is the mean of four replications.

S applied	S concentration	
	Non-inoculated	Inoculated
kg/ha	S (% dry matter)	
0.0	0.35	0.27^{NS}
5.6	0.35	0.26^{NS}
11.2	0.40	0.27^{*}
22.4	0.40	0.27^{*}
44.8	0.41	0.26^{*}
67.2	0.44	0.30^{*}

* Denotes significance at the 0.05 level of probability for values in the same row.
NS Not Significant.

Appendix Table 10. Analysis of variance for S concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1982.

Analysis of variance	Split Block Design		
Source var	df	Sum square	Mean square
Reps	3	0.0243	0.0081
Inoc	1	0.1789	0.1789
Reps*Inoc	3	0.0054	0.0018
Slev	5	0.0215	0.0043
Reps*Slev	15	0.0285	0.0019
Inoc*Slev	5	0.0075	0.0015
Reps*Inoc*Slev	15	0.0195	0.0013
Total	47		
F (Inoc)	=	99.39^{**}	
F (Slev)	=	2.26^{NS}	
F (Inoc*Slev)	=	1.15^{NS}	

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 0.33 CV = 12.85%
$LSD_{0.05}$ = 0.10 $LSD_{0.01}$ = 0.18

Appendix Table 11. Nitrogen to sulfur (N:S) ratios of non-inoculated and inoculated alfalfa plants, at different S levels in 1982. Each value is the mean of four replications.

S applied	N:S ratios	
	Non-inoculated	Inoculated
kg/ha	% N:% S	
0.0	3.7	10.0**
5.6	3.9	9.9**
11.2	3.5	9.6**
22.4	3.1	9.5**
44.8	3.1	9.9**
67.2	3.1	9.1

** Denotes significance at the 0.01 level of probability for values in the same row.

Appendix Table 12. Analysis of variance for N:S ratios of non-inoculated and inoculated alfalfa plants, at different S levels in 1982.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	4.419	1.473
Inoc	1	481.650	481.650
Reps*Inoc	3	1.416	0.472
Slev	5	3.940	0.788
Reps*Slev	15	7.965	0.531
Inoc*Slev	5	1.240	0.248
Reps*Inoc*Slev	15	6.210	0.414
Total .	47		

F (Inoc)	= 1020.44**
F (Slev)	= 1.48NS
F (Inoc*Slev)	= 0.60NS

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 6.54 CV = 9.84%
$LSD_{0.05}$ = 1.5 $LSD_{0.01}$ = 2.8

Appendix Table 13. Alfalfa nodule number per plant of non-inoculated and inoculated treatments, at different S levels in 1983. Each value is the mean of eight observations.

S applied	Nodule number	
	Non-inoculated	Inoculated
kg/ha	Number/plant	
0.0	11	56*
5.6	10	86**
11.2	11	71*
22.4	13	44**
44.8	4	59*
67.2	20	40

*, ** Denote significance at the 0.05 and 0.01 levels of probability for values in the same row.

Appendix Table 14. Analysis of variance for alfalfa nodule number per plant of non-inoculated and inoculated treatments, at different S levels in 1983.

Analysis of variance	Split Block Design		
Source var	df	Sum square	Mean square
Reps	3	1328.49	442.83
Inoc	1	27265.30	27265.30
Reps*Inoc	3	386.49	128.83
Slev	5	2349.75	469.95
Reps*Slev	15	13923.75	928.25
Inoc*Slev	5	4085.90	817.18
Inoc*Reps*Slev	15	10769.25	717.95
Total	47		

F (Inoc)	=	211.63**
F (Slev)	=	0.51NS
F (Inoc*Slev)	=	1.14NS

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 35 CV = 76.01%
$LSD_{0.05}$ = 18 $LSD_{0.01}$ = 33

Appendix Table 15. Alfalfa nodule fresh weight of non-inoculated and inoculated treatments, at different S levels in 1983. Each value is the mean of eight observations.

S applied	Nodule fresh weight	
	Non-inoculated	Inoculated
kg/ha	mg/plant	
0.0	12.85	200.26*
5.6	9.78	190.97*
11.2	16.25	154.14*
22.4	13.82	130.35*
44.8	10.55	142.28*
67.2	72.55	96.64NS

* Denotes significance at the 0.05 level of probability for values in the same row.
NS Not significant.

Appendix Table 16. Analysis of variance for nodule fresh weight of non-inoculated and inoculated treatments, at different S levels in 1983.

Analysis of variance Split Block Design

Source var	df	Sum square	Mean square
Reps	3	3256.17	1085.39
Inoc	1	202197,00	202197.00
Reps*Inoc	3	14462.55	4820.85
Slev	5	7222.95	1444.59
Reps*Slev	15	99614.40	6440.96
Inoc*Slev	5	34758.75	6951.75
Inoc*Reps*Slev	15	88028.40	5688.56
Total	47		

F (Inoc)	=	41.94**
F (Slev)	=	0.22NS
F (Inoc*Slev)	=	1.22NS

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 87.54 CV = 86.16%
$LSD_{0.05}$ = 110.47 $LSD_{0.01}$ = 202.78

Appendix Table 17. Alfalfa nodule dry weight of non-inoculated and inoculated treatments, at different S levels in 1983. Each value is the mean of eight observations.

S applied	Nodule dry weight	
	Non-inoculated	Inoculated
kg/ha	mg/plant	
0.0	2.26	24.00*
5.6	1.49	25.69*
11.2	2.56	17.58*
22.4	2.10	17.39*
44.8	1.54	19.11*
67.2	10.55	11.76NS

* Denotes significance at the 0.05 level of probability for values in the same row.
NS Not significant.

Appendix Table 18. Analysis of variance for alfalfa nodule dry weight of non-inoculated and inoculated treatments, at different S levels in 1983.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	91.89	30.63
Inoc	1	3009.92	3009.92
Reps*Inoc	3	235.55	78.52
Slev	5	108.00	21.60
Reps*Slev	15	1392.51	92.83
Inoc*Slev	5	644.81	128.96
Inoc*Reps*Slev	15	1245.96	83.06
Total	47		

F (Inoc)	=	38.33**
F (Slev)	=	0.23NS
F (Inoc*Slev)	=	1.55NS

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 11.34 CV = 80.37%
$LSD_{0.05}$ = 14.10 $LSD_{0.01}$ = 25.88

Appendix Table 19. Acetylene reduction rate of non-inoculated and inoculated alfalfa plants, at different S levels in 1983. Each value is the mean of eight observations.

S applied	Acetylene reduction rate	
	Non-inoculated	Inoculated
kg/ha	umole ethylene/mg nodule dry wt/h	
0.0	0.064^{+}	0.267^{NS}
5.6	0.089	0.300^{NS}
11.2	0.009	0.389*
22.4	0.064	0.364^{NS}
44.8	0.079	0.300*
67.2	0.007	0.417

* Denotes significance at the 0.10 level of probability for values in the same row.
NS Not significant.

Appendix Table 20. Analysis of variance for acetylene reduction rate of non-inoculated and inoculated treatments, at different S levels in 1983.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	0.22533	0.07511
Inoc	1	0.99044	0.99044
Reps*Inoc	3	0.34869	0.11623
Slev	5	0.01275	0.00255
Reps*Slev	15	0.30375	0.02025
Inoc*Slev	5	0.08220	0.01644
Reps*Inoc*Slev	15	0.43035	0.02869
Total	47		
F (Inoc)	= 8.52*		
F (Slev)	= 0.12^{NS}		
F (Inoc*Slev)	= 0.57^{NS}		

* Denotes significance at the 0.10 level of probability.
NS Not significant.
Grand mean = 0.200 CV = 84.69%
$LSD_{0.10}$ = 0.278 $LSD_{0.05}$ = 0.541

Appendix Table 21. Alfalfa dry matter yield of non-inoculated and inoculated treatments, at different S levels in 1983. Each value is the mean of four replications.

S applied	Alfalfa dry matter yield	
	Non-inoculated	Inoculated
kg/ha	kg/ha	
0.0	2369	4573**
5.6	2968	4658**
11.2	3090	5028**
22.4	2957	4761**
44.8	3212	4875**
67.2	2976	4616**

** Denotes significance at the 0.01 level of probability for values in the same row.

Appendix Table 22. Analysis of variance for alfalfa dry matter yield of non-inoculated and inoculated treatments, at different S levels in 1983.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	13788240	4596080
Inoc	1	39896900	39896900
Reps*Inoc	3	630228	210076
Slev	5	1828775	365755
Reps*Slev	15	7004490	466966
Inoc*Slev	5	470820	94164
Inoc*Reps*Slev	15	1969080	131272
Total	47		

F (Inoc)	=	189.92**
F (Slev)	=	0.78NS
F (Inoc*Slev)	=	0.72NS

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 3840 CV = 9.43%
$LSD_{0.05}$ = 1031 $LSD_{0.01}$ = 1893

Appendix Table 23. Nitrogen concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1983. Each value is the mean of four replications.

S applied	N concentration	
	Non-inoculated	Inoculated
kg/ha	N (% dry matter)	
0.0	1.86	3.18^{**}
5.6	1.71	2.92^{**}
11.2	1.98	2.90^{*}
22.4	1.99	3.05^{**}
44.8	1.79	3.07^{**}
67.2	2.11	3.07^{*}

*, ** Denote significance at the 0.05 and 0.01 level of probability for values in the same row.
NS Not significant.

Appendix Table 24. Analysis of variance for N concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1983.

Analysis of variance	Split Block Design		
Source var	df	Sum square	Mean square
Reps	3	0.276	0.092
Inoc	1	15.176	15.176
Reps*Inoc	3	0.285	0.095
Slev	5	0.370	0.074
Reps*Slev	15	0.810	0.054
Inoc*Slev	5	0.280	0.056
Reps*Inoc*Slev	15	0.690	0.046
Total	47		

F (Inoc)	$= 159.74^{**}$
F (Slev)	$= 1.37^{NS}$
F (Inoc*Slev)	$= 1.22^{NS}$

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 2.47 CV = 8.68%
$LSD_{0.05}$ = 0.58 $LSD_{0.01}$ = 1.06

Appendix Table 25. Sulfur concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1983. Each value is the mean of four replications.

S applied	S concentration	
	Non-inoculated	Inoculated
kg/ha	S (% dry matter)	
0.0	0.37	0.28^{*}_{NS}
5.6	0.35	0.30^{NS}_{NS}
11.2	0.33	0.30^{*}_{*}
22.4	0.38	0.31^{*}_{*}
44.8	0.37	0.28^{*}_{*}
67.8	0.38	0.31^{*}

* Denotes significance at the 0.05 level of probability for values in the same row.
NS Not significant.

Appendix Table 26. Analysis of variance for S concentration of non-inoculated and inoculated alfalfa plants, at different S levels in 1983.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	0.0240	0.0080
Inoc	1	0.0514	0.0514
Reps*Inoc	3	0.0099	0.0033
Slev	5	0.0065	0.0013
Reps*Slev	15	0.0090	0.0006
Inoc*Slev	5	0.0040	0.0008
Reps*Inoc*Slev	15	0.0105	0.0007
Total	47		
F (Inoc)	=	15.57^{*}_{NS}	
F (Slev)	=	2.16^{NS}_{NS}	
F (Inoc*Slev)	=	1.14^{NS}	

** Denotes significance at the 0.05 level of probability.
NS Not significant.
Grand mean = 0.33 CV = 8.02%
$LSD_{0.05}$ = 0.06 $LSD_{0.01}$ = 0.11

Appendix Table 27. Nitrogen to sulfur (N:S) ratios of non-inoculated and inoculated alfalfa plants, at different S levels in 1983. Each value is the mean of four replications.

S applied	N:S ratios	
	Non-inoculated	Inoculated
kg/ha	% N:% S	
0.0	5.1	11.3**
5.6	4.9	9.9*
11.2	6.2	9.9**
22.4	5.3	9.9**
44.8	4.9	11.0*
67.2	5.6	9.9

*, ** Denote significance at the 0.05 and 0.01 levels of probability for values in the same row.

Appendix Table 28. Analysis of variance for N:S ratios of non-inoculated and inoculated alfalfa plants, at diffferent S levels in 1983.

Analysis of variance Split Block Design			
Source var	df	Sum square	Mean square
Reps	3	4.347	1.499
Inoc	1	297.505	297.505
Reps*Inoc	3	4.233	1.411
Slev	5	3.565	0.713
Reps*Slev	15	12.435	0.829
Inoc*Slev	5	9.445	1.889
Reps*Inoc*Slev	15	12.420	0.828
Total	47		
F (Inoc)	= 210.84**		
F (Slev)	= 0.86NS		
F (Inoc*Slev)	= 2.28NS		

** Denotes significance at the 0.01 level of probability.
NS Not significant.
Grand mean = 7.81 CV = 11.65%
$LSD_{0.05}$ = 2.4 $LSD_{0.01}$ = 4.4

Appendix Table 29. Root fresh weight of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Root fresh weight (g/sample)
0.0	23.07^{c+}
1.0	27.90^{b}
2.5	29.03^{a}
25.0	21.96^{c}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 30. Analysis of variance for root fresh weight of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	5.702	2.851	
Slev	3	109.968	36.656	11.58**
Reps*Slev	6	18.990	3.165	
Total	11			

** Denotes significance at the 0.01 level of probability.

Grand mean = 25.49 CV = 6.97%

$LSD_{0.05}$ = 3.55 $LSD_{0.01}$ = 5.38

Appendix Table 31. Roots + nodules dry weight of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Root dry weight (g/sample)
0.0	4.96^{bc+}
1.0	5.20^{ab}
2.5	5.90^{a}
25.0	4.60^{c}

\+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 32. Analysis of variance for roots + nodules dry weight of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.272	0.136	
Slev	3	2.733	0.911	6.56*
Reps*Slev	6	0.834	0.139	
Total	11			

* Denotes significance at the 0.05 level of probability.

Grand mean = 5.17 CV = 7.21%

$LSD_{0.05}$ = 0.74 $LSD_{0.01}$ = 1.13

Appendix Table 33. Total fresh weight yield of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Total fresh weight yield (g/sample)
0.0	47.27^{bc+}
1.0	52.88^{b}
2.5	59.43^{a}
25.0	47.65^{bc}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 34. Analysis of variance for total fresh weight yield of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	12.016	6.008	
Slev	3	291.276	97.092	10.60**
Reps*Slev	6	54.930	9.155	
Total	11			

** Denotes significance at the 0.01 level of probability.

Grand mean = 51.81 CV = 5.84%

$LSD_{0.05}$ = 6.04 $LSD_{0.01}$ = 9.16

Appendix Table 35. Shoot dry weight yield of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Shoot dry weight yield (g/sample)
0.0	5.20[a+]
1.0	5.31[a]
2.5	6.15[a]
25.0	5.61[a]

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 36. Analysis of variance for shoot dry weight yield of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.122	0.061	
Slev	3	1.602	0.534	1.82[NS]
Reps*Slev	6	2.358	0.293	
Total	11			

NS Not significant.

Grand mean = 5.57 CV = 9.72%

$LSD_{0.05}$ = 1.08 $LSD_{0.01}$ = 1.63

Appendix Table 37. Total dry weight yield of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Total dry weight yield (mg/sample)
0.0	10.16^{bc+}
1.0	10.51^{b}
2.5	12.05^{a}
25.0	10.21^{bc}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 38. Analysis of variance for total dry weight yield of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.556	0.278	
Slev	3	7.152	2.384	5.96*
Reps*Slev	6	2.400	0.400	
Total	11			

* Denotes significance at the 0.05 level of probability.

Grand mean = 10.73 CV = 5.89%

$LSD_{0.05}$ = 1.54 $LSD_{0.01}$ = 2.34

Appendix Table 39. Acetylene reduction rate (umole ethylene/plant/h) of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of 18 observations.

S applied (mg/L)	Acetylene reduction rate (umole ethylene/plant/h)
0.0	0.735^{ab+}
1.0	0.467^{bc}
2.5	0.782^{a}
25.0	0.283^{c}

+ Means followed by the same letter are not significantly different at 0.05 level of probability.

Appendix Table 40. Analysis of variance for acetylene reduction rate (umole ethylene/plant/h) of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of Variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.358	0.179	
Slev	3	2.973	0.991	5.51^{**}
Reps*Slev	6	3.306	0.551	
Error	60	10.800	0.180	
Total	71			

** Denotes significance at the 0.01 level of probability.

Grand mean = 0.567 CV = 74.83%

$LSD_{0.05}$ = 0.282 $LSD_{0.01}$ = 0.375

Appendix Table 41. Acetylene reduction rate (umole ethylene/g nodule fresh weight/h) of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of 18 observations.

S applied (mg/L)	Acetylene reduction rate (umole ethylene/g nod fresh wt/h)
0.0	0.014^{b+}
1.0	0.012^{bc}
2.5	0.029^{a}
25.0	0.011^{bc}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 42. Analysis of variance for acetylene reduction rate (umole ethylene/g nodule fresh weight/h) of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of Variance Randomized Complete Block Design

Source var	df	Sum squre	Mean square	F-test
Reps	2	0.00024	0.00012	
Slev	3	0.00372	0.00124	3.99**
Reps*Slev	6	0.00432	0.00072	
Error	60	0.01860	0.00031	
Total	71			

** Denotes significance at the 0.01 level of probability.

Grand mean = 0.0166 CV = 106.06%

$LSD_{0.05}$ = 0.012 $LSD_{0.01}$ = 0.016

Appendix Table 43. Acetylene reduction rate (umole ethylene/mg nodule dry weight/h) of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of 18 observations.

S applied (mg/L)	Acetylene reduction rate (umole ethylene/mg nod dry wt/h)
0.0	0.266^{b+}
1.0	0.232^{bc}
2.5	0.426^{a}
25.0	0.139^{bc}

\+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 44. Analysis of variance for acetylene reduction rate (umole ethylene/mg nodule dry weight/h) of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of Variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.050	0.025	
Slev	3	0.768	0.256	4.74**
Reps*Slev	6	0.942	0.157	
Error	60	3.240	0.054	
Total	71			

** Denotes significance at the 0.01 level of probability.

Grand mean = 0.266 CV = 87.36%

$LSD_{0.05}$ = 0.154 $LSD_{0.01}$ = 0.205

Appendix Table 45. Sulfur concentration of shoots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Sulfur concentration (%)
0.0	0.16^{d+}
1.0	0.20^{c}
2.5	0.22^{b}
25.0	0.25^{a}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 46. Analysis of variance for S concentration of shoots of alfalfa seedlings growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.001716	0.000858	
Slev	3	0.012600	0.004200	84.0**
Reps*Slev	6	0.000348	0.000058	
Total	11			

** Denotes signficance at the 0.01 level of probability.

Grand mean = 0.21 CV = 3.63%

$LSD_{0.05}$ = 0.001 $LSD_{0.01}$ = 0.022

Appendix Table 47. Sulfur concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (g/L)	Sulfur concentration (%)
0.0	0.07[c+]
1.0	0.11[bc]
2.5	0.18[ab]
25.0	0.22[a]

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 48. Analysis of variance for S concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.0078	0.0039	
Slev	3	0.0411	0.0137	12.45**
Reps*Slev	6	0.0066	0.0011	
Total	11			

** Denotes signficance at the 0.01 level of probability.

Grand mean = 0.14 CV = 23.69%

$LSD_{0.05}$ = 0.07 $LSD_{0.01}$ = 0.10

Appendix Table 49. Phosphorus concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (g/L)	Phosphorus concentration (%)
0.0	0.66^{c+}
1.0	1.03^{ab}
2.5	0.96^{b}
25.0	0.90^{b}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 50. Analysis of variance for P concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.2942	0.1471	
Slev	3	0.2295	0.0765	5.00^{**}
Reps*Slev	6	0.0918	0.0153	
Total	11			

** Denotes signficance at the 0.01 level of probability.

Grand mean = 0.89 CV = 13.90%

$LSD_{0.05}$ = 0.25 $LSD_{0.01}$ = 0.37

Appendix Table 51. Potassium concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (g/L)	Potassium concentration (%)
0.0	2.63^{c+}
1.0	3.90^{ab}
2.5	3.70^{b}
25.0	3.52^{b}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability

Appendix Table 52. Analysis of variance for K concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	3.690	1.845	
Slev	3	2.838	0.946	5.19*
Reps*Slev	6	1.092	0.182	
Total	11			

* Denotes signficance at the 0.05 level of probability.

Grand mean = 3.44 CV = 12.40%

$LSD_{0.05}$ = 0.85 $LSD_{0.01}$ = 1.29

Appendix Table 53. Magnesium concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (g/L)	Magnesium concentration (%)
0.0	0.18^{c+}_{ab}
1.0	0.27^{b}
2.5	0.25^{b}
25.0	0.25^{b}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 54. Analysis of variance for Mg concentration of roots of seedling alfalfa growing in nutrient solutions, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	0.0120	0.0056	
Slev	3	0.0150	0.0050	6.25*
Reps*Slev	6	0.0480	0.0008	
Total	11			

* Denotes signficance at the 0.05 level of probability.

Grand mean = 0.24 CV = 11.79%

$LSD_{0.05}$ = 0.06 $LSD_{0.01}$ = 0.09

Appendix Table 55. Molybdenum concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Molybdenum concentration (ug/g)
0.0	15.26^{ab+}
1.0	13.97^{bc}
2.5	10.86^{cd}
25.0	6.34^{d}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 56. Analysis of variance for Mo concentration of roots of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	96.604	48.302	
Slev	3	141.708	47.236	7.67*
Reps*Slev	6	36.948	6.158	
Total	11			

* Denotes signficance at the 0.05 level of probability.

Grand mean = 11.61 CV = 21.37%

$LSD_{0.05}$ = 4.97 $LSD_{0.01}$ = 7.53

Appendix Table 57. Molybdenum concentration in shoots of seedling alfalfa growing in nutrient solution, at different S levels. Each value is the mean of three replications.

S levels (mg/L)	Molybdenum concentration (ug/g)
0.0	18.26^{a+}
1.0	11.12^{b}
2.5	7.60^{c}
25.0	3.82^{d}

+ Means followed by the same letter are not significantly different at the 0.05 level of probability.

Appendix Table 58. Analysis of variance for Mo concentration in shoots of seedling alfalfa growing in nutrient solution, at different S levels.

Analysis of variance Randomized Complete Block Design

Source var	df	Sum square	Mean square	F-test
Reps	2	1.004	0.502	
Slev	3	339.720	113.240	411.78**
Reps*Slev	6	1.650	0.275	
Total	11			

** Denotes signficance at the 0.01 level of probability.

Grand mean = 10.20 CV = 5.14%

$LSD_{0.05}$ = 1.05 $LSD_{0.01}$ = 1.59

Appendix Table 59. Ratio and absolute values of ions required for preparing a nutrient solution with no S and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	49	49.0	9.04	9.04
$H_2PO_4^-$	25	25.0	4.62	4.62
$HPO_4^=$	25	12.5	4.62	2.31
NO_3^-	1	1.0	0.19	0.19
K^+	50	50.0	9.24	9.24
Ca^{++}	30	15.0	5.54	2.77
Mg^{++}	20	10.0	3.69	1.85
Total		162.5		30.02

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/162.5 = 0.185).

Appendix Table 60. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with no S and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L						
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	NO_3^-	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.62			4.62		
K_2HPO_4			4.62		4.62		
$CaCl_2$	5.35					5.35	
$Ca(NO_3)_2$				0.19		0.19	
$MgCl_2$	3.69						3.69
Total	9.04	4.62	4.62	0.19	9.24	5.54	3.69

Appendix Table 61. Ratio and absolute values of ions required for preparing a nutrient solution with 1.0 mg S/L and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	48.66	48.66	9.00	9.00
$H_2PO_4^-$	25.00	25.00	4.62	4.62
$HPO_4^=$	25.00	12.50	4.62	2.31
$SO_4^=$	0.34	0.17	0.06	0.03
NO_3^-	1.00	1.00	0.19	0.19
K^+	50.00	50.00	9.24	9.24
Ca^{++}	30.00	15.00	5.55	2.77
Mg^{++}	20.00	10.00	3.70	1.85
Total		162.33		30.01

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/162.33 = 0.185).

Appendix Table 62. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with 1.0 mg S/L and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L							
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	NO_3^-	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.62				4.62		
K_2HPO_4			4.62			4.62		
$CaCl_2$	5.36						5.36	
$Ca(NO_3)_2$					0.19		0.19	
$MgCl_2$	3.64							3.64
$MgSO_4$				0.06				0.06
Total	9.00	4.62	4.62	0.06	0.19	9.24	5.55	3.70

Appendix Table 63. Ratio and absolute values of ions required for preparing a nutrient solution with 2.5 mg S/L and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	48.16	48.16	8.92	8.92
$H_2PO_4^-$	25.00	25.00	4.63	4.63
$HPO_4^=$	25.00	12.50	4.63	2.31
$SO_4^=$	0.84	0.42	0.16	0.08
NO_3^-	1.00	1.00	0.19	0.19
K^+	50.00	50.00	9.26	9.26
Ca^{++}	30.00	15.00	5.56	2.78
Mg^{++}	20.00	10.00	3.70	1.85
Total		162.08		30.02

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/162.08 = 0.185).

Appendix Table 64. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with 2.5 mg S/L and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L							
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	NO_3^-	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.63				4.63		
K_2HPO_4			4.63			4.63		
$CaCl_2$	5.37						5.37	
$Ca(NO_3)_2$					0.19		0.19	
$MgCl_2$	3.55							3.55
$MgSO_4$				0.16				0.16
Total	8.93	4.63	4.63	0.16	0.19	9.26	5.56	3.71

Appendix Table 65. Ratio and absolute values of ions required for preparing a nutrient solution with 25 mg S/L and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	40.6	40.6	7.69	7.69
$H_2PO_4^-$	25.0	25.0	4.74	4.74
$HPO_4^=$	25.0	12.5	4.74	2.37
$SO_4^=$	8.4	4.2	1.59	0.80
NO_3^-	1.0	1.0	0.19	0.19
K^+	50.0	50.0	9.48	9.48
Ca^{++}	30.0	15.0	5.68	2.84
Mg^{++}	20.0	10.0	3.79	1.90
Total		158.3		30.1

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/158.3 = 0.190).

Appendix Table 66. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with 25 mg S/L and 0.19 meq N/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L							
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	NO_3^-	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.74				4.74		
K_2HPO_4			4.74			4.74		
$CaCl_2$	5.49						5.49	
$Ca(NO_3)_2$					0.19		0.19	
$MgCl_2$	2.20							2.20
$MgSO_4$				1.59				1.59
Total	7.69	4.74	4.74	1.59	0.19	9.48	5.68	3.79

Appendix Table 67. Ratio and absolute values of ions required for preparing a nutrient solution with no S, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	50	50.0	9.23	9.23
$H_2PO_4^-$	25	25.0	4.62	4.62
$HPO_4^=$	25	12.5	4.62	2.31
K^+	50	50.0	9.24	9.24
Ca^{++}	30	15.0	5.54	2.77
Mg^{++}	20	10.0	3.69	1.85
Total		162.5		30.02

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/162.5 = 0.185).

Appendix Table 68. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with no S, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L					
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.62		4.62		
K_2HPO_4			4.62	4.62		
$CaCl_2$	5.54				5.54	
$MgCl_2$	3.69					3.69
Total	9.23	4.62	4.62	9.24	5.54	3.69

Appendix Table 69. Ratio and absolute values of ions required for preparing a nutrient solution with 1.0 mg S/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	49.66	49.66	9.17	9.17
$H_2PO_4^-$	25.00	25.00	4.62	4.62
$HPO_4^=$	25.00	12.50	4.62	2.31
$SO_4^=$	0.34	0.17	0.06	0.03
K^+	50.00	50.00	9.24	9.24
Ca^{++}	30.00	15.00	5.54	2.77
Mg^{++}	20.00	10.00	3.69	1.85
Total		162.23		30.00

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/162.23 = 0.185).

Appendix Table 70. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with 1.0 mg S/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L						
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.62			4.62		
K_2HPO_4			4.62		4.62		
$CaCl_2$	5.54					5.54	
$MgCl_2$	3.63						3.63
$MgSO_4$				0.06			0.06
Total	9.17	4.62	4.62	0.06	9.24	5.54	3.69

Appendix Table 71. Ratio and absolute values of ions required for preparing a nutrient solution with 2.5 mg S/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	46.16	46.16	9.10	9.10
$H_2PO_4^-$	25.00	25.00	4.63	4.63
$HPO_4^=$	25.00	12.50	4.63	2.32
$SO_4^=$	0.84	0.42	0.16	0.08
K^+	50.00	50.00	9.26	9.26
Ca^{++}	30.00	15.00	5.55	2.78
Mg^{++}	20.00	10.00	3.71	1.85
Total		162.80		30.00

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/162.80 = 0.185).

Appendix Table 72. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with 2.5 mg S/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L						
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.63			4.63		
K_2HPO_4			4.63		4.63		
$CaCl_2$	5.55					5.55	
$MgCl_2$	3.55						3.55
$MgSO_4$				0.16			0.16
Total	9.10	4.63	4.63	0.16	9.26	5.55	3.71

Appendix Table 73. Ratio and absolute values of ions required for preparing a nutrient solution with 25 mg S/L, at 0.71 atmosphere osmotic pressure and 15° C (Steiner, 1961).

Ions	Ratio		Absolute values	
	Equiv %	Ions	meq/L*	mg ions/L
Cl^-	41.6	41.6	7.88	7.88
$H_2PO_4^-$	25.0	25.0	4.74	4.74
$HPO_4^=$	25.0	12.5	4.74	2.37
$SO_4^=$	8.4	4.2	1.59	0.80
K^+	50.0	50.0	9.48	9.48
Ca^{++}	30.0	15.0	5.68	2.84
Mg^{++}	20.0	10.0	3.79	1.90
Total		158.3		30.00

* Calculated by multiplying the ratio ion times a factor obtained as follows: total mg ions/L divided by total ratio ions (30/158.3 = 0.190).

Appendix Table 74. Milliequivalents per liter (meq/L) of various salts required for preparing a nutrient solution with 25 mg S/L, at 0.71 atmosphere osmotic pressure and 15° C.

Salt	meq/L						
	Cl^-	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	K^+	Ca^{++}	Mg^{++}
KH_2PO_4		4.74			4.74		
K_2HPO_4			4.74		4.74		
$CaCl_2$	5.68					5.68	
$MgCl_2$	2.20						2.20
$MgSO_4$				1.59			1.59
Total	7.88	4.74	4.74	1.59	9.48	5.68	3.79

Appendix Table 75. Chemical composition of 'Apollo' alfalfa seeds utilized in field and greenhouse experiments.

Element	Concentration	Element	Concentration
	(mg/g)		(mg/g)
N	-	Ca	2.3
K	10.0	Mg	2.1
P	7.1	S	11.0
	(ug/g)		(ug/g)
Fe	166	Sr	9
Na	52	Ba	5
Zn	43	Ni	1.56
Si	35	As	1.54
Mn	22	Mo	0.77
B	18	Co	0.09
Al	18	Cd	0.04
Cu	14	Se	0.02
Li	14		

Appendix Table 76. Sulfate content of the chemicals utilized in the preparation of nutrient solutions.

Chemical	Sulfate (mg/g)+	Sulfate (ug/g)
KCl	0.01	10
$Ca(NO_3)_2$	0.02	20
$MgCl_2$	0.02	20
KH_2PO_4	0.03	30
K_2HPO_4	0.05	50
$MnCl_2.4H_2O$	0.05	50
$CuCl_2.2H_2O$	0.05	50
$CaCl_2.2H_2O$	0.10	100
$CaCO_3$	0.10	100
$CoCl_2$	0.10	100
H_3BO_3	0.10	100
$ZnCl_2.2H_2O$	0.10	100
$Na_2MoO_4.2H_2O$	0.15	150

+ Values obtained from labels.

Appendix 77. Pelleting Procedure for Alfalfa.

Alfalfa Seed Sterilization

Place 500 g of alfalfa seed in a sterile beaker. Add sufficient 20% commercial bleach to cover seed, stir for 10 minutes and decant the bleach. Add sufficient sterile water to cover seed, stir for 2 minutes, and decant the water. Rinse at least 10 times with sterile water. Dry with hot air (hair dryer).

Gum Arabic Solution Preparation

Weigh out 35.5 g of powdered gum arabic. Place 82 ml of water in a beaker and slowly add the powdered gum arabic. Use magnetic stirring hot plate. Heat but do not boil. Cool before using.

Pelleting Procedure

Add 100 ml gum arabic solution to first beaker. Add 43 g peat/or inoculant to beaker and stir 2 to 3 minutes, or until there is a smooth slurry. Add 500 g of alfalfa seed to the gum-inoculum slurry (beaker # 1) while agitating until all seeds are coated. Promptly remove sticky seeds from beaker # 1 (spatula is helpful) and place in beaker # 2 which contains calcium carbonate. Stir seeds briskly in calcium carbonate attempting to coat all seeds uniformly with lime coating (rolling seeds around in flask or beaker helps to "firm-up" seed coat). Sieve seeds to remove clumps and excess calcium carbonate powder. Manually break up clumps and add extra calcium carbonate to seeds to coat. Refrigerate seeds if not planting inmediately.

Appendix 78. Procedure for the Determination of Total Sulfur in Plant Material.

Dry Ash Procedure

Weigh 0.5 g of plant material into 50 ml beakers. Add 2 ml of ethanol (70-95%), and 3 ml of saturated magnesium nitrate solution. Heat on low heat (30 to 50° C) to drive off ethanol. Ash in muffle furnace 3 to 5 h at 550° C. Cool to room temperature and add 10 ml of 3 M hydrochloric acid. Warm gently to aid disolution (hot plate at 125° C). Filter into 50 ml volumetric flasks and bring to volume with distilled water.

Preparation of Blanks

Prepare 5 blanks, and treat the same as other samples. Use one as a blank. Add an appropriate amount of S standard (before bringing to volume) to the other four to make 10, 20, 30, and 40 mg S/L. (10, 20, 30, and 40 ppm S).

Turbidity Solution Preparation

Heat 200 ml of distilled water to 65° C and add 0.6 g of gelatin (gelatin should be low in sulfate). Cool solution overnight and add 4 g of barium chloride crystals. Store in refrigerator when not in use; however, use at room temperature (turbidity solution may only be stored a couple of weeks).

Turbidity Procedure

Use a 10 ml aliquot of the sample solution and add 1 ml of the acid solution (50% hydrochloric acid + 50% acetic acid). Allow to stand at least 1 h. Add 1 ml of the turbidity solution and swirl 30 seconds (after adding the turbidity solution, the sample should stand at least 30 minutes but no more than 1 h before reading). After 30 minutes, swirl 15 seconds and read absorption (turbidity) with a spectrophotometer at 500 nm.

Appendix 79. Summary of Procedures for Nitrogen Analysis and ICAP Analysis.

Nitrogen Analysis

Weigh 0.4 g dried ground tissue into Folin-Wu digestion tubes, and 0.4 g of known tissue standard in another tube of the same type. Add a small scoop (approximately 1 g) of $NaSO_4$ and Se catalyst and 8 ml concentrated sulfuric acid to each tube. Mix thoroughly. Place entire rack into heater block. Heat 1.25 h at 120° C, and 4.25 h at 350° C. Cool to room temperature. Add 30 ml distilled water. Shake thoroughly. Add more distilled water to bring volume to 75 ml. Shake samples. Remove 4 ml aliquot from each tube and place in auto-sampler vials. Determine N in samples via an auto sampler-colorimeter. Convert values to percent N.

ICAP Analysis

Weigh 1 g dried ground tissue into crucibles. Ash samples at 550° C for 6 h. Cool to room temperature. Add 5 ml 20% nitric acid. Allow to stand for 2 to 4 h. Add 15 ml distilled water. Mix. Allow to settle overnight. Remove 4 to 5 ml and place in tube for ICAP analysis.