NITROGEN FIXATION, TRANSFER AND COMPETITION IN ALFALFA-GRASS MIXTURES

BY

HELIO ALMEIDA BURITY

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Department of Plant Science
Macdonald College of
McGill University
Montreal, Quebec
Canada

Short title

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H.A.BURITY

ABSTRACT

Ph.D. HELIO ALMEIDA BURITY Plant Science (Agronomy)

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The productivity of pastures in the temperate regions of the world is limited largely by the availability of Nitrogen (N). Nitrogen fixing legumes have been widely used as sources of N both for improving the soil and for improving forage production. However, very few measurements have been undertaken to assess the beneficial effect of legume on the development of grasses in mixtures. This study was carried out to characterize the contribution of N_2 fixation by alfalfa (Medicago sativa L.) and N transfer to associated grasses timothy (Phleum pratense L.), bromegrass (Bromus inermis Leyss.), orchardgrass (Dactylis glomerata L.) and tall fescue (Festuca arundinacea Schreb.) when grown in mixture, using the ^{15}N dilution technique under greenhouse and field conditions.

The percentage of alfalfa N derived from atmosphere (% Ndfa) increased throughout the growing season and ranged from 69.0% to 83.0% (field) and from 95.0% to 98.0% (greenhouse). The total amount of N_2 fixation presented a pattern similar to % Ndfa with slight increases in the absolute amount of N_2 fixed by alfalfa in mixed stands. It was concluded that mixed cultures of alfalfa did not reduce the apparent activity of alfalfa N_2 fixation. Indeed, the presence of a grass may have stimulated alfalfa N_2 fixation.

N transfer from alfalfa to an associated grass was evident, and contributed 26, 46 and 38% of the total annual N yield of grass and represented an absolute amount of about 5.0, 20.0 and 19.0 kg N/ha during the first, second and third year, respectively. The gradual and consistent percentage N transfer that occurred before initial harvest indicated that this transfer is not primarily due to the decomposition of nodulated roots after shoot removal, but involves a considerable excretion of N compounds during the period before and after harvest.

The ^{15}N dilution technique provided a useful method to measure N_2 fixation and N transference. The results showed that all grass species benefitted similarly from N transfer from alfalfa, although earlier maturing species with greater competitive ability are more slightly responsive.

Field and greenhouse estimates of N_2 fixation using the N difference method and ^{15}N dilution method were fairly similar for the two techniques and larger than N_2 fixation values estimated by acetylene reduction. It is concluded that ^{15}N dilution technique is the method of choice when precise measurements of N_2 fixation are required, and N difference may be used when resources for isotope technique are limiting.

RESUME

Ph.D. **HELIO ALMEIDA BURITY** Phytotechnie (Agronomie)

LA FIXATION ET LE TRANSFERT D'AZOTE ET LA COMPETITION DANS DES MELANGES LUZERNE-GRAMINEE

La productivité des herbages dans les zones tempérées du monde est limitée en grande partie par la disponibilité de l'azote (N). Les légumineuses sont souvent utilisées comme source d'azote pour l'amélioration du sol et pour l'augmentation de la production fourragère. Cependant, il n'existe que peu d'études concernant l'effet bénéfique de la légumineuse sur les graminées cultivées en mélange. Cette étude porte sur la fixation de l'azote (N_2) par la luzerne (Medicago sativa L.) et le transfert de N aux graminées cultivées en association, telles la fléole (Phleum pratense L.), le brome (Bromus inermis Leyss), le dactyle (Dactylis glomerata L.) et la fétuque élevée (Festuca arundinacea Schreb.). On a utilisé la technique de dilution de ^{15}N en serre et en champ.

Le pourcentage d'azote dans la luzerne provenant de l'atmosphère (% Ndfa) a augmenté avec le temps de coupe et varié de 69.0% à 88.0% dans le champ, et de 95.0% à 98.0% dans la serre. On a observé une relation entre la quantité totale d'azote fixé et % Ndfa avec de légères augmentations de la quantité absolue d'azote fixé quand la luzerne est cultivée en association avec une graminée. On a conclu que l'association luzerne-graminée n'a pas réduit la fixation d'azote par la luzerne comparé au semis pur. Il semble même que la présence d'une graminée a peut-être stimulé la fixation d'azote par la luzerne.

Il est évident qu'il existe un transfert d'azote entre la luzerne et la graminée associée. Ce transfert a contribué pour 26, 46 et 38% du rendement annuel total de N des graminées, soit 5.0, 20.0 et 19.0 kg N/ha pour les trois premières années. Le transfert graduel et proportionnellement constant d'azote observé avant la première récolte indique que ce transfert n'est pas principalement dû à la décomposition, après coupe, de racines nodulées, mais à l'excrétion d'une quantité considérable de composés azotés avant et après la récolte.

La technique de dilution de ^{15}N s'est avérée un méthode efficace pour quantifier la fixation de N_2 et le transfert de N. Les résultats montrent que toutes les graminées ont bénéficié de façon similaire du transfert de N de la luzerne, quoique les espèces plus hâtives et plus compétitives répondent mieux.

Les deux méthodes utilisées pour estimer la quantité de N_2 fixé en champ et en serre, les méthodes dites de différence de N et de dilution de ^{15}N, ont donné des valeurs semblables et supérieures à la quantité estimée par la réduction d'acétylène. De préférence, la technique de dilution de ^{15}N devrait être utilisée quand la fixation de N_2 doit être évaluée de façon précise, alors que la méthode de différence d'azote suffira dans les cas où le manque de ressources empêche l'utilisation de la technique de l'isotope.

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1. INTRODUCTION

Nitrogen is the nutrient that most frequently limits productivity in most ecosystems of the world. Production may be increased with addition of nitrogen fertilizer but this requires high inputs of energy at escalating costs. The association of bacteria of the genus Rhizobium (rhizobia) with leguminous plants is unique, and is the phenomenon in which N_2 from the atmosphere is fixed into NH_3 by the enzyme nitrogenase. This needs to be more widely utilised as an alternative to reduce the dependency of forage and crop production on fertilizer N.

In range systems, legume-grass mixtures present a good strategy to increase forage production and quality. Most pertinent literature agrees that the presence of legumes in pastures not receiving fertilizer N increases the total herbage yield and protein content as well as the yield of the grass component. In fact, these studies have shown that mixtures of legumes and grasses often have greater total yields than expected on the basis of the yield of the component species, when grown in pure stands.

Alfalfa (Medicago sativa L.), timothy (Phleum pratense L.), bromegrass (Bromus inermis Leyss.), orchardgrass (Dactylis glomerata L.) and tall fescue (Festuca arundinacea Schreb.) have a long and significant history as farm crops and are very important in forage production in North America. They are well

adapted to a range of climatic and edaphic conditions. Previous research has shown that alfalfa in association with timothy, bromegrass, and other grass species has remarkable potential in improving forage production and quality. In addition, alfalfa is very compatible when seeded in mixtures with grasses. These species were used to conduct all experiments in the present study.

The nature of the mechanism of forage crop interaction has been studied and inferred from various observations. However, a better understanding of N_2 fixed and released into the soil and of interactions that occur among forage species when grown in association is required. Soil N uptake by legumes and grasses in mixtures has been extensively investigated in recent years using the isotope dilution technique and the difference method. Competition between legume and grass for soil N depends on the levels of soil N and fixed N and the interactions between the two species. High levels of soil N provide superior growth potential to the grass and result in lower N_2 fixation rates by the legume. However, at low levels of soil N, higher N_2 fixation rates promote higher relative growth rates by the legume in comparison to grass.

Many researchers have tried to quantify N transfer from legume to grass in mixtures. There is considerable controversy about their results, which showed that 0 to 8% of the N_2 fixed by the legume was transferred to the companion grass. This

controversy is mainly due to the methods used to measure N_2 fixation and N transfer in each trial and to the poor understanding of the mechanism of transfer. It is important to understand these aspects as well as the effect that competition from grass species may have on the rate of legume N_2 fixation, when seeded in association.

The present study was conducted to achieve the following objectives:

1. To evaluate the amount of N transfer from N_2 fixing alfalfa to associated grass under field and greenhouse conditions, using the ^{15}N dilution technique.

2. To measure N_2 fixation rates of alfalfa grown alone and in mixture with grass, using isotope dilution, difference method and acetylene reduction assay.

3. To quantify competitive ability for N of different grass species and alfalfa, when seeded in mixed culture.

4. To investigate the behavior of the nitrogenase activity of alfalfa grown alone and in mixture with grass, before and after successive harvests.

2. LITERATURE REVIEW

Literature pertinent to legume-grass mixtures will be reviewed in this section. Nitrogen fixation by alfalfa, transfer of N from legumes to grasses, general N balance, competition between plant species and stability in the mixture, and methods for measuring N_2 fixation will be emphasized.

2.1 Nitrogen fixation by *Medicago sativa* L.

Many members of the family leguminoseae possess the ability to enter into symbiosis with bacteria of the genus *Rhizobium*. Within this relationship the bacterium fixes atmospheric N_2 into biologically available ammonia and the macrosymbiont (host) supplies the necessary energy in the form of sucrose from which electrons and generated ATP (adenosine triphosphate) drive the N_2 fixation process. This arrangement gives the legume access to the large pool of N present in the atmosphere as N_2, an extraordinary advantage under conditions of N limitation (Stewart 1982). Legumes able to nodulate and subsist on biologically fixed N can do so with varying degrees of efficiency (LaRue and Patterson 1981). *Medicago sativa* L. is a forage legume species often considered to have high ability to form an adequate symbiosis with *Rhizobium meliloti*. Early workers have shown this with different species and varieties of *Medicago* (Brockwell and

Hely 1961, 1966; Erdman and Ura Mae 1953; Gibson 1962). Burton (1975) studied the strain-variety reactions when eight varieties of alfalfa were tested against 13 strains of R. meliloti. The results confirmed that all varieties were nodulated by the strains of rhizobia tested, but the amount of N_2 fixed varied due to rhizobial strain effectiveness.

The data on amount of symbiotic N_2 fixation by alfalfa are few and vary widely. Lyon and Bizzell (1933) cultured alfalfa continuously over a 10 years period and reported symbiotic N_2 fixation of 273 kg N/ha per year. Estimates for alfalfa came from an 11 year lysimeter study by Karraker et al. (1950). The authors studied the N balance in a continuous cropping with alfalfa and other legumes and Kentucky bluegrass (Poa pratensis L.) as a control. The annual average of N_2 fixation calculated by the difference method indicated that alfalfa plants were more effective in fixing N_2 than other forage legumes. Averages of 212, 128, 154 kg N/ha per year were obtained for alfalfa, white clover (Trifolium repens L.) and red clover (Trifolium pratense L.), respectively. Heichel et al. (1981) used isotope dilution and 'An-value' to estimate N_2 fixation in two populations of alfalfa selected for high nitrogenase activity. They reported an average of 148 kg N/ha in the establishment year with 43% of total N yield derived from fixation. However, the amount of N_2 fixed and percentage of N derived from biological fixation varied over the growth season. In an extensive study of alfalfa

conducted by Salver and Hardy (1975) the rate of N_2 fixed was 40 kg N/ha inthe seeding year, when alfalfa was not inoculated and the soil was deficient in P or K, but the rate of N_2 fixation increased to 90 kg N/ha in the establishment year, when the soil was deficient in nitrogen only. Bell and Nutman (1971) reported a rate of N_2 fixation of 220 kg N/ha per year for effectively inoculated alfalfa plants, with up to 78% of plant N derived from the atmosphere.

More conservative estimates range from 83 to 100 kg N/ha (Erdman and Ura Mae 1953). In 1973, Eskew and associates reported a high activity of nitrate reductase in the shoots of the alfalfa plants, documenting the utilization of fixed nitrogen in high levels. Since alfalfa is a perennial and can produce seven or eight cuttings in one climate, and only two or three cuttings in another, it is normal that estimates of N_2 fixed should vary widely (Burton 1975).

There have been many studies of the influence of fertilizer on symbiotic N_2 fixation in alfalfa and other legumes. It is established that large amounts of applied N reduce root-hair infection (Munns 1968a; Dazzo and Brill 1978), nodule number and nodule mass (Dart and Mercer 1965; Summerfield et al. 1977), and the N_2 fixing activity of nodulated roots (Allos and Bartholomew 1959; Gibson 1974). Allos and Bartholomew (1959) indicated that small amounts of combined N, up to 80 mg of N per pot, increased growth and symbiotic N_2 fixatiop in alfalfa, but high rates did

not increase the growth rate and replaced symbiotic N_2 fixation in large degree. Richardson et al. (1957) found similar results, nevertheless they reported in no instance N_2 fixation was completely replaced by fertilizer N.

Using ^{15}N labelled fertilizer, McAuliffe et al. (1958) obtained the opposite results. In 10 weeks growth of alfalfa in a N poor soil, symbiotic N_2 fixation dropped from 58 to 17% when N fertilizer level was increased from 22 to 88 Kg/ha. These conflicting results should be explained by other factors that influence directly the N_2 fixation process. Dart and Wildon (1970) and Eaglesham et al. (1983) reported on the form of the N compound that inhibits N_2 fixation. Other authors published that the degree of inhibition varied with legume species (Allos and Bartholomew 1959; Dean and Clark 1980), cultivar (Gibson 1974), strain of Rhizobium (Pate and Dart 1961), season (Pate and Dart 1961), light intensity (Dart and Mercer 1965), temperature (Gibson 1974) and nutritional conditions (Pankhurst 1981). However, small amounts of combined N is usually recommended to alleviate the N-stress in the initial phase of nodulation (Gibson and Nutman 1960; Munns 1968a, b; Dart and Wildon 1970; Gibson 1974; Lawn and Brun 1974; Dean and Clark 1980; Heichel et al. 1981; Eaglesham et al. 1983).

2.2 Nitrogen transfer from legume to grass

Attention has been focused on the question of the existence of transfer of N from legume to grass during associated growth. In the 1930's a review of evidence by Virtanen (1933) and Nicol (1934) concluded that the transfer of N from legumes to associated non-legumes can be taken as well established. They reported that gramineae growing in mixed cultures together with legumes can obtain their nitrogen supply with the aid of legumes, and that one pea plant is capable of supplying sufficient N for the growth of two oat plants. It has been suggested also that transference may occur at an early growth stage, approximately three months or less from planting (Thornton and Nicol 1934). Lyon and Bizzell (1911) found that the N content of a non-legume grown under field conditions could reach a higher value when in association with a legume. Their results were based on analyses rather than on absolute quantities and the material was obtained under field conditions which permitted considerable variation. Stallings (1926), concluded that N was transferred as ammonia and his results indicated that wheat derived substantial amounts of N from soybeans grown in association. However, no references were given and Stallings provided no justification for his conclusion.

The investigation of Virtanen (1933) appears to have been most comprehensive, although carried out with little replication or statistical control. However, the differences were so large and consistent and the various aspects of the problem were

so completely covered, that there appears to be no doubt that under his conditions nitrogen was gained by grasses from associated legumes. Nevertheless, the quantities of N derived from the legumes, seem to be surprisingly high (Tumble and Strong 1937). The work conducted by Thornton and Nicol (1934) appears to support the N transfer hypothesis. The authors found that Italian ryegrass grown in association with lucerne, contained considerably more N than was supplied in the fertilizer added to sand cultures. They concluded that this amount had been derived from lucerne with evidence for transference at 12 weeks after planting.

The results of these workers apparently did not lead to further research of the problem subsequently, until approximately 20 years later, when Butler and Bathurst (1956) reported a significant amount of N transferred, and stated that the underground transference of N from legumes to associated grasses may involve at least two different mechanisms. First, direct excretion of soluble N compounds by the intact root system of the growing legume, may be stimulated by the associated growth of two plant species. Second, the sloughing off and decomposition of legume nodules and root tissues. According to recent evidence of Faris and Ta (1985), the excretion of nitrogen compounds is mainly in the form of ammonia, glutamate, aspartate and alanine. However, due to the apparent operation of the excretion mechanism when the plants is subjected to stress through shading, low

temperature or when forage legumes reach advanced maturing stage, Butler and Bathurst (1956); Dilz and Mulder (1962); Henzell (1962); Simpson (1965; 1976) concluded that a more important pathway of transference in a legume-grass pasture involves the decomposition of legume nodulated roots.

In the legume-grass mixture the quantity of N transferred from legume to grass varied from 0 to 25% of the N_2 fixed by the legume (Seerge 1961; Dilz and Mulder 1962; Henzell 1962; Simpson 1965; Vallis et al. 1967; Henzell et al. 1968; Haystead and Marriott 1979; Faris and Ta 1985). N transfer varies for different legume species under different experimental conditions. The differences can be partially explained by the methods used to measure N transfer in the legume-grass mixture. Dilz and Mulder (1962) separated the legume and the grass plants by a glass plate in the control pots, where no transfer of N was allowed, and compared grass total N in pots without the glass plate and the grass only control. They found 1 to 8% of fixed N_2 to be transferred from legume to grass. Seerge (1961) and Simpson (1965) measured transfer of N as the quantity of N excreted by the legume into plant nutrient solution, and found 1 to 4% of the fixed N_2 was transferred to the grass. Faris and Ta (1985) used labelled ^{15}N to measure N transfer from alfalfa to grass, and estimated between 12 to 18% of fixed N_2 under field conditions was transferred to associated grasses.

Henzell (1962) used the difference method to investigate N

transfer from some tropical legumes, Indigofera spicata Forsk., Lotonis bainesii Bak., Desmodium unicatum (Jacq) D.C., and Stylosanthes bojeri Vogel, in comparison with white clover (Trifolium repense L.) and alfalfa (Medicago sativa L.) to Paspalum commersonii Lam., and only 0.6 to 1.7% of the total N_2 fixed was transferred from legume to the grass. Ross et al. (1964) used gas lysimeters to estimate the quantity of N transferred, and reported that only 0.1 to 2.3% of fixed N_2 was transferred to associated grass.

Vallis et al. (1967) used the isotope dilution method to assess N transfer from legume to grass as the difference between total N uptake and soil N uptake of the grass (control). They found no significant transfer of N from Townsville lucerne (Stylosanthes humilis H.B.K.) to Rhodesgrass (Chloris gayana Kunth.) in a pot experiment. Henzell et al. (1968) reported little transfer of N from siratro (Phaseolus atropurpureus D.C.) to Rhodesgrass, but Haystead and Marriott (1979) used the isotope dilution method and found evidence of N transfer of 1.7% of fixed N_2 from white clover to ryegrass (Lolium perenne L.).

Broadbent et al. (1982) reported extensive transfer of fixed N_2 to ryegrass. Up to 80% of the N in ryegrass in mixed culture was derived from the fixation process in white clover. However, they found little transfer of N in a relatively short term and suggest that a time of several months is involved in the gradual mineralization of dead roots and nodule tissues from the legume through microbial activity. Recently, Faris and Ta (1985)

demonstrated the importance of N transfer from alfalfa to timothy in mixed stands. This transfer increased with times of clipping and contributed to about 3, 8 and 25% of total N yield of timothy in the first, second and third cut, respectively. The authors also stated that the ^{15}N dilution technique proved to be suitable to measure N transfer.

2.3 Competition between legumes and grasses in mixtures

2.3.1. Limiting factors

Competition begins when immediate supply of a single necessary factor falls below the combined demands of the plants. Then competition can be defined as the combined demands of associated species for a limiting factor (Donald 1963). The definition does not apply to the legume-grass mixture because N is not limiting for the legume which takes it up from the soil and the atmosphere, but is limiting for the grass. Hall (1978) defined competition for soil N in the legume-grass mixture by two processes: competitive and non-competitive relationships for soil N. The definition is appropriate in the legume-grass mixture because non-competitive relationship for soil N is possible and can be explained by the fact that the legume can fix its N requirement from the air. Harper (1977) defined competition as the capture of nutrients in the soil volume shared by other

plants which are different in size and rooting habits. This definition facilitates interpretation of some of the interaction between the legume and the grass from the standpoint of the underground component.

Competition between legume and grass when seeded in mixture has been investigated extensively by previous researchers. Clover, siratro and alfalfa compete favorably when grown together with grass (Siewerdt and Holt 1974; Cook and Dolby 1981). McBratney (1981) found that red clover production was higher in the mixture than in the pure stand four years after seeding. Ta and Faris (1985) reported that mixing timothy with alfalfa increased both quality and quantity of herbage production. Although timothy had a dominant competitive ability over alfalfa in later harvests, the productivity of the alfalfa-timothy mixed culture, expressed by land equivalent ratio (LER) was always equal to or greater than 1.0, showing that maximum dry matter yield per land unit was obtained from the mixtures.

The success of a component species in competition for a limiting soil factor usually leads to its enhanced absorption of other soil factors. If the availability of the latter factors is not high, competition may then occur for them also. This situation is often found in mixed pastures and mixtures of legume and non-legume species. On low N soils, the non-legume is frequently suppressed or has little advantage, but on high N soils the strong growth responses of the non-legume usually cause

it to dominate the legume by shading it. The vigorously growing non-legume takes up large amounts of nutrients such as P, K and S, the last two sometimes with luxury comsumption, and the legume may suffer deficiency in soils low in these nutrients (Trenbath 1974). Competition between legume and grass for light, P and K can be reduced by successive clippings and appropriate fertilizer applications, respectively (Trenbath 1974; 1976).

Earlier reports have shown that mixtures of legumes and grasses often have greater advantage than expected on the basis of the component species, when grown in pure stands. Although several explanations have been suggested for this, the most obvious reasons are that the legume and grass use different N sources and have spatial differences in the use of resources, i.e., various rootings depths (Martin and Snaydon 1982).

2.3.2 Nitrogen balance and stability in the legume-grass mixture

N balance and stability in the legume-grass mixture depends on levels of soil N, N_2 fixed by the legume, persistence of the two species and the competition between legume and grass (Camlin 1981; Siewerdt and Holt 1974; Vallis 1978). Applied N fertilizer affects mixture stability by inhibiting fixation and favoring grass growth (Stern and Donald 1962). Craig de Anda and co-workers (1981) concluded that mixed cultures of alfalfa and

red clover with orchardgrass and timothy have no detrimental effect on legume specific nodule activity (SNA), and may stimulate nodule activity after clipping. Craig de Anda et al. (1981) have suggested that during the normal growth and harvest of the alfalfa plants some death of secondary roots and nodule tissue occurs with a consequent release of N to the soil. This may inhibit either additional initiation of alfalfa nodules or the nitrogenase activity of nodules already present. Grasses grown in association with alfalfa may absorb the newly released N and thus reduce the soil N which may inhibit alfalfa N_2 fixation. This may be responsible for the stimulation of alfalfa N_2 fixation with time in the mixed cultures and consequently increase the stability of the alfalfa-grass mixed stand.

Shade increased leaf area index (LAI) and concentration of N in green-panic grass, but decreased dry weight, LAI and nodulation of siratro (Rhodes and Stern 1978; Wong and Wilson 1980; Eriksen and Whitney 1982).

2.3.3 Soil nitrogen uptake by legume and grass

The recovery of fertilizer N by legumes and the effect of fertilizer N on N_2 fixation has interested many researchers because of its economic importance (Richards and Soper 1979; Eaglesham et al. 1983; Vasilas and Ham 1984). To determine soil N uptake by legume and the grass in association, the construction

of a complete N balance is necessary (Walker et al. 1956; Vallis et al. 1967; Simpson 1976; Bergersen 1980).

Feigenbaun and Hadas (1980) used materials labelled with ^{15}N to investigate N uptake by the legume and grass in mixtures. They found that the legume and the grass grown alone took up the same amount of ^{15}N from the soil, but when grown together, 33% of the ^{15}N was recovered by the legume and 66% by the grass. The recovery of ^{15}N by the plants (atom % ^{15}N/plant) decreased exponentially with time, and changes in the proportions of the legumes and grasses in the mixtures had no effect on uptake of ^{15}N by the legume and grass. They also found that the grass took up the same amount of N when seeded alone or in mixture with the legume. However, the legume took up 80% of its soil N during the first four weeks after seeding. The uptake of soil N in early stage of growth by the legume is explained in terms of an alleviation of the N-stress in the initial phase of nodulation (Gibson and Nutman 1960; Munns 1968a, b; Dart and Wildon 1970; Gibson 1974; Lawn and Brun 1974; Dean and Clark 1980; Heichel et al. 1981; Eaglesham et al. 1983).

2.4 Methods for measuring dinitrogen fixation

2.4.1 Acetylene reduction

Dilworth (1966) and Schollhorn and Burris (1966) discovered

that the enzyme nitrogenase, which is responsible for the reduction of N_2 to NH_3, also reduced acetylene (C_2H_2) to ethylene (C_2H_4); so far, it is the only biological agent to do so (LaRue and Patterson 1981). This discovery provided the insight to assay the activity of nodules by measuring the rate of ethylene (C_2H_4) production. It is a simple and inexpensive method to estimate N_2 fixation (Hardy et al. 1973). An H_2 flame ionization gas chromatograph is used to measure the C_2H_4 produced. The acetylene reduction assay has the advantages of sensitivity and speed. A detection limit of pmoles C_2H_4/ml gas permits estimation of nitrogenase enzyme activity even when only a few nodules are formed (LaRue and Patterson 1981).

A principal assumption in the method involves the ratio of acetylene reduced to N_2 fixed. The reduction of N_2 to NH_3 uses six electrons, while the reduction of C_2H_2 to C_2H_4 uses two electrons. The ratio C_2H_4/N_2 was assumed to be equal to three, i.e., a mole of C_2H_4 was equivalent to 1/3 mole of N_2 reduced. However, the theoretical ratio of C_2H_4/N_2 of three to one (3:1) is variable because protons are also reduced by nitrogenase to hydrogen gas which may or may not be metabolized by hydrogenase in different species of rhizobia, and may miss detection. So values greater than the theoretical conversion have been observed and reported by Schollhorn and Burris (1967); Hardy et al. (1973); Schubert and Evans (1976).

Due to the non availability of a method for calibrating C_2H_4

formation with N_2 fixation, Hardy and associates (1973) recommended the conversion factor of four to one (4:1) for forage legumes as a more adequate ratio. In addition to the variation in the theoretical conversion (C_2H_4/N_2), other errors may arise due to diurnal variation in N_2 fixing activity (Bergersen 1970; Carran et al. 1982), plant to plant variability, partial harvests of the nodules and the plants used are usually destroyed (single measurement). These factors make this technique less precise with frequent over-estimation of N_2 fixed (LaRue and Patterson 1981; Martensson and Ljunggren 1984). Rennie et al. (1978) have stated that acetylene reduction technique is a short-term kinetic measurement and any extrapolation to total N_2 fixed over a growing season is questionable. Therefore an estimate of fixation over an entire growing season requires a mathematical summation of many frequently obtained assays during the season.

2.4.2 Nitrogen difference method

Measurement of N_2 fixation by the N difference method requires growing a legume and a non-N_2-fixing plant separately under the same conditions and analyzing the total N in both plants. The difference in total reduced N yield between the plants is the contribution of N_2 fixation by the legume. Three versions of the difference method are commonly used (LaRue and Patterson 1981):

1. Comparison of a legume with a non-legume; the control plants can be a graminaceous species (Wagner 1954).
2. Comparison of a legume with a non-nodulating legume; in this approach the control is a legume genetically incapable of forming root nodules (Williams and Lynch 1954; Ruschel et al. 1979).
3. Comparison of inoculated and uninoculated legumes; the difference method is a comparison of single cultivars grown on inoculated or uninoculated soil (Bezdicek et al. 1978).

It is assumed that the legume and the reference plant remove identical amounts of N from the soil. To ensure that this is the case, plants should be grown for the same period of time under similar conditions. This method is used extensively because it is relatively inexpensive and simple. However, soil and natural biological variability, within and among species, can induce major errors (LaRue and Patterson 1981) that often underestimate the quantity of N_2 fixed (Hardy et al. 1973).

2.4.3 Isotopic methods

The stable heavy isotope ^{15}N became commercially available in the early 1970's. This development encouraged the use of ^{15}N-enriched compounds in the field. The natural abundance of isotopes in the soil N and atmosphere may be preferred because it is a stable condition and does not change during the period of experiment. According to Rennie et al. (1978) and Ruschel et al.

(1979), the measurement of ^{15}N relative to ^{14}N is a highly sensitive and precise technique. Detection of ^{15}N incorporation into plant material is 1000 times more sensitive by this method than by measuring differences by the Kjeldahl method (Hardy et al. 1973), and gives definitive evidence of N_2 fixation (Ruschel et al. 1979). Thus, the stable isotope ^{15}N has excellent applications.

2.4.3.1 Isotope dilution methods

2.4.3.1.1 Enriched method

In this method, the fixing crop and a non-fixing control are grown in the soil to which a small amount of ^{15}N was added as labelled nitrate or ammonium. The percentage of total N derived from fixation is calculated as:

$$\% \text{ N derived from atmosphere} = \left[1 - \frac{(\text{atom \% } ^{15}N \text{ excess fixing system})}{(\text{atom \% } ^{15}N \text{ excess non-fixing system})} \right] \times 100$$

The control plant is used to measure the ^{15}N content of the soil and can be a non-legume or a non-nodulating isoline of a legume. The plant obtaining parts of its N from the atmosphere will have less of its N as ^{15}N, expressed as the atom % ^{15}N.

The dilution method is based on the assumption that the roots of the legume and the control plant should exploit the same soil zone and have equal access to ^{15}N label and the other sources of N (LaRue and Patterson 1981; Rennie et al. 1982; Boddey et al. 1983). This method also assumes insignificant discrimination between ^{15}N and ^{14}N in N uptake (Fried and Middelboe 1977).

The dilution technique is very useful because a single sampling can show significant treatment effects on N_2 fixation in pasture and rangeland ecosystems over a period of time. To show the same effect with acetylene reduction would require multiple samples with questionable assumptions made about the ratio of C_2H_4 produced per unit of N_2 fixed (McAuliffe et al. 1958; Phillips and Bennett 1978). However, Goh et al. (1978) and Broadbent et al. (1982) expressed reservations concerning the use of labelled N techniques for measuring N_2 fixation in the legume-grass mixture because of the error encountered in transfer of N from legume to grass or from the legume to the soil.

Plant material labelled with ^{15}N has been used as a source of N in the soil (Vallis 1983). The release of N depends on the rate of mineralization. Since the mineralization process is progressive during an extended period of time, it allows a continual release of ^{15}N to the plants during the period of the experiment. However, labelled N incorporated into the soil N does not result in a constant source of labelled N because of the

complexity of the soil N pool (Henzell et al. 1968).

2.4.3.1.2 Natural abundance method

The natural abundance of ^{15}N in the air is 3663 ppm ^{15}N (Rennie et al. 1978). The effect of various soil microbiological reactions on the fractionation process of N isotopes induces an increase in the ^{15}N abundance of soil N as compared with the atmospheric N (Hauck and Bremner 1976). The difference in ^{15}N abundance of the soil and atmosphere can be used to measure N_2 fixation by using the dilution technique (Rennie et al. 1978; LaRue and Patterson 1981).

The abundance method is based on the assumption that there are very slight isotope effects during biological processes and the non-fixing plants that obtain all N from soil will have a slightly enriched ^{15}N relative to the atmosphere. A plant obtaining N from symbiosis will have a lower ^{15}N composition. A better understanding of plant and soil isotope discrimination factors is important for the used of the natural abundance method (Bremner 1965). The ratio of $^{15}N/^{14}N$ in biological tissues differs considerably from those expected theoretically because the kinetic fractionation depends on the relative speed of the chemical reactions of ^{29}N and ^{28}N. Shearer et al. (1980) reported ^{14}N and ^{15}N discrimination between roots and shoots in soybean. Rennie et al. (1976) and Knowles (1980) reported that

^{14}N is slightly favored in N_2 fixation reactions, and if this is not included in the calculations, the level of fixation will appear to be higher than it is.

The advantage of this method is that it does not necessitate the purchase of isotopic N to added to soil, ^{15}N is in equilibrium with all sources of N in the soil and natural abundance values are within analytical precision. However, small variations in the ^{15}N natural abundance values in the legume and the control plant will correspond to a large difference in N_2 fixation. Therefore, the ^{15}N analysis in this method should be done very accurately, using an expensive and time consuming technique - mass spectrometry.

2.4.3.2 ^{13}N method

Some researchers have employed ^{13}N to measure short-term N_2 fixation and subsequent metabolism (Rubens et al. 1940). This isotope of N has the disadvantage of being unstable and radioactive. For this reason few studies have been done with ^{13}N. However, the radiographic technique can be used to indicate ^{13}N uptake and to identify the compounds into which it has been incorporated. Due to the short half-life of about 10 minutes (Bergersen 1980) the use of ^{13}N to assess symbiotic N_2 fixation has severe limitations (Rennie et al. 1978).

2.4.4 Ureide method

The ureides such as allantoin and allantoic acid have been recognized as major transport or storage form of fixed N_2 in some species of grain legume. This compound move by xylem transport from nodules to shoot, where they are metabolized (Matsumoto et al. 1977; Streeter 1979). The biosynthetic reactions leading to these compounds are not fully characterized, nevertleless uric acid is important intermediate (Woo et al. 1980).

Recently it has been demonstrated that some legumes of the tribe Phaseoleae export N from nodule to the shoot predominantly in ureides, and from non N_2 fixing root tissue chiefly as amides (McClure and Israel 1979; Streeter 1979; Sprent and Embrapa 1980). So the close relationship between ureides and N_2 fixation in some legumes suggests that ureides might serve as a useful indicator of N_2 fixation in the field (Herridge 1982a; Patterson and LaRue 1983b).

Since some nitrate N taken up by soybeans is reduced in the root and transported as ureides in the xylem, a more accurate indication of N_2 fixation based on ureides analysis should include an adjustment for plant N derived from nitrate. Herridge (1982a, b) proposed "relative abundance of ureides" as a more accurate index of symbiotic N_2 fixation than an estimate based on ureides alone, because the index accounts for the contribution of nitrate from the growth medium.

The ureide level in tissues such as that of leaves might be used as a quick non-destructive assay for N_2 fixation. This would be especially useful to plant breeders attempting to select genotypes with ability of N_2 fixation (Patterson and LaRue 1983a). Otherwise, the relative abundance of ureides index proposed by Herridge (1982a, b) would be especially important to detect genotypes with high capacity to utilize soil N. However, the accumulation of ureides as N storage pools in various tissues has made this technique less useful and it seems to be premature to use this as a quantitative measure of N_2 fixation. Furthermore, alfalfa roots do not synthesize ureides, but produce organic N chiefly as asparagine (Ta et al. 1985a, b).

3. MATERIALS AND METHODS

Five experiments were conducted to accomplish the proposed objectives. Experiments I and II were under greenhouse conditions using N-free substrate (vermiculite and sand) with ^{15}N label or without any N fertilizer. Their purpose was to examine stability, competition and transfer of N from alfalfa to associated grass, under low N conditrons. In order to measure N_2 fixation, soil N uptake, transfer of N from alfalfa to grass and the effect of grass species on the rate of alfalfa N_2 fixation under field conditions, experiments III, IV and V were conducted in the field. Two included enrichment with ^{15}N and one was run in the normal soil N.

3.1 Greenhouse studies

The experiments were conducted in a greenhouse at the Ottawa Research Station, Agriculture Canada, Ottawa, Ontario between July 1982 and August 1984. Experiment I was carried out between July 1982 and January 1983. Alfalfa cv.' Saranac ', timothy cv. ' Salvo ', bromegrass cv.' Tempo ' and orchardgrass cv.' Kay ' seedlings were grown in plastic sleeves 5x5x20 cm embedded in vermiculite on a greenhouse bench. Each sleeve was sown with two seeds in monoculture or two seeds (one alfalfa and one grass) in mixed culture. Each experimental unit consisted of 150 sleeves

that were seeded with the four monocylture and the three mixed culture in a randomized complete block design with two replicates.

The photoperiod was 16 hours and consisted of natural light supplemented with fluorescent light at 350 uE. m^{-2}. sec^{-1} intensity at the top of the plants. The temperature was approximately 24 $\pm$ 1°C during the day and 20 $\pm$ 1°C during the night. The relative humidity was held constant at 80%. The experiment was hand seeded on 27 July 1982 and irrigated after seeding and weekly during the experiment with N-free Hoagland's solution (Table 1). The alfalfa seedlings were inoculated with a preparation of Rhizobium meliloti strains L-26, L-6, C7-Balzac and 102f70 (Nitragin Co. Milwaukee, Wisconsin). However, N fertilizer at 5 mg per sleeve (20 kg/ha) was added to the grass monocultures 2 weeks after seeding in order to improve the establishment of the grass and to maintain the grass plants in monoculture in normal growth in relation to grass in association.

When alfalfa plants reached early bud stage and flower formation (7 and 28 September, respectively), plants from two sleeves in each treatment and in each replicate were removed and acetylene reduction assays (ARA) were made on the detached nodulated alfalfa roots. The dry weight (DW) and total nitrogen concentration (TN) yields of the roots and tops were determined for grass as well as alfalfa plants. The rest of the experiment was cut 2 to 3 cm above the bench and was sampled 6 and 10 weeks

TABLE 1. Nutrient composition of nitrogen-free Hoagland solution used to water the plants in greenhouse experiments, Experiment I and II.

Stock solution	Chemical	Concentration	Quantity of stock used per liter
1	KH_2PO_4	34.00 g/l	1.0 ml
2	$MgSO_4.7\ H_2O$	123.00 g/l	1.0 ml
3	K_2SO_4	65.00 g/l	1.0 ml
4	$CaCl_2.2\ H_2O$	147.00 g/l	4.5 ml
5	$FeCl_3$ NaH_2 EDTA	0.84 g/l 1.70 g/l	1.0 ml
6	KCl H_3BO_3 $MnCl_2.4\ H_2O$ $ZnSO_4.7\ H_2O$ $CuSO_4.5\ H_2O$ $H_2MoSO_4.\ H_2O$	0.75 g/l 124.00 mg/l 67.00 mg/l 46.00 mg/l 10.00 mg/l 2.00 mg/l	1.0 ml

The plant nutrient solution is basically that of Munns (1968a) and Macdowall (1982). Adjusted to pH 6.5 by adding 1 N KOH.

(3 December and 3 January, respectively) after initial harvest (13 October 1982 when alfalfa reached later bloom stage), and the same determinations were performed again.

Experiment II was initiated on 14 February 1984, to investigate the benefit of N_2 fixed by alfalfa to companion grasses using the isotope dilution technique, with legume-grass grown in mixed stands. The growing medium was a mixture of vermiculite (70% v/v) and sterilized sand (30% v/v). Alfalfa cv.' 520 ', timothy cv.' Climax ', bromegrass cv.' Tempo ', orchardgrass cv.' Kay ' and tall fescue cv.' Kentucky 31 ' pre-germinated seedlings were sown in 15 cm plastic pots. Each pot was seeded with eight pre-germinated seeds in monoculture or eight pre-germinated seeds in mixed culture (50% legume and 50% grass) under ambient conditions similar to Experiment I. The experiment was irrigated after seeding and weekly during the experiment with N-free Hoagland's solution (Table 1). One week after seeding, a mixture of Rhizobium meliloti strains L-6, L-26, C7-Balzac and 102F70 (Nitragin Co. Milwaukee, Wisconsin) was added to all pots. At the same time, each pot received a basal fertilization of 10 mg of N (20 kg/ha), 10 mg of available phosphoric acid (P_2O_5) (20 kg/ha) and 10 mg of soluble potash (K_2O) (20 kg/ha) to assist the establishment of the alfalfa and grass seedlings. When the seedlings of alfalfa and grasses were well established, 2 weeks after sowing, 5 mg of ammonium sulphate ($(^{15}NH_4)_2SO_4$) enriched with 99% atom ^{15}N excess dissolved in

50 ml deionized water was applied into the surface of each pot. An additional 2.0 mg of ammonium sulphate (99% atom ^{15}N excess) dissolved in 50 ml deionized water was added to each pot, after harvest 2, in order to maintain the level of % atom ^{15}N sufficient for detection in the plant tissue.

The experimental units (6 pots) were harvested when the alfalfa plants reached 50% bloom stage, every 4 to 5 weeks. Four harvests were obtained during the course of the experiment. At each harvest, one pot was sacrificed and the following measurements were done: acetylene reduction of alfalfa (nmoles or umoles $C_2H_4.pl.^{-1}\ h.^{-1}$), nodule number, nodule fresh weight (mg), plant height (cm), roots and tops growth DW (g/pl.). Grass plants were also analyzed for DW yields of roots and tops growth, tiller number and total nitrogen concentration. Total N concentration was performed by the Kjeldahl method (Bremmer 1965) and atom % ^{15}N in the plant material was analyzed by an emission spectroscopy procedure (Preston et al. 1981) as described below.

The experiment consisted of five monocultures and four mixed cultures in a randomized complete block design with three replicates, in a total of 162 pots.

3.2. Field studies

Field experiments were established at Ottawa Research Station, Agriculture Canada, Ottawa, Ontario on a moderately drained sand loam soil during Spring of 1983 (Experiments III and

IV) and Spring of 1984 (Experiment V). In the previous year the experimental area was cultured with barley. Following harvest the stover was ploughed down. The soil had adequate levels of Mg and P (Table 2) based on soil test. However, the initial soil reaction was pH 5.1 (in water) and the K level was medium. Potassium and lime were applied 1 month prior to the planting at a rate of 30 kg/ha and 3,000 kg/ha as muriate of potash (KCl) and dolomitic lime ($CaMg(CO_3)_2$), respectively. Experiment III was initiated on 27 May 1983, to examine nitrogenase enzyme activity of alfalfa grown alone and mixed culture, during vegetative growth and regrowth, and to determine the pattern of nodule activity of alfalfa in association before and after successive harvests.

Alfalfa seeds cv.' 520 ' inoculated with Rhizobium meliloti strains 102F70 and L-26 (Nitragin Co. Milwaukee, Wisconsin) were sown at the rate of 13 kg/ha in monoculture and in mixed culture at the rate of 11 kg/ha with timothy cvs.' Salvo and Climax ' at 6.0 kg/ha and bromegrass cv.' Tempo ' at 9.0 kg/ha. The same grass seeding rates in mixed stand were utilized in pure stands (Field Crop Recommendations 1982) under dry land conditions. Each experimental unit consisted (1.60 x 6.00 m) of eight rows 6.0 m long and 0.20 m apart that were planted with four monocultures and three mixed cultures in a randomized complete block design with five replicates.

When the seedlings of alfalfa and grasses were well

TABLE 2. Chemical analysis of sand loam soil at Ottawa Research Station, Agriculture Canada, including five essential elements, two micronutrients and pH, Spring of 1983 and 1984.

	EXPERIMENTS III and IV		EXPERIMENT V	
Nutrient	Concentration	Level	Concentration	Level
	1983		1984	
Nitrogen	0.09 ±0.14 %	Low	0.12 ±0.2 %	Medium
Phosphorus	96.7 ±7.27 ppm	High	66.6 ±14.5 ppm	High
Potassium	98.0 ±8.25 ppm	Medium	125.9 ±28.4 ppm	Medium
Magnesium	55.0 ±10.9 ppm	Medium	119.7 ±5.2 ppm	High
Calcium	>2000 ppm	Medium	>2000 ppm	Medium
Zinc	30.2 ±1.83 ppm	Adequate	32.2 ±5.49 ppm	Adequate
Manganese	39.0 ±2.45 ppm	Adequate	45.5 ±2.19 ppm	Adequate
pH (in water)	5.1 ±0.22	Acid	5.6 ±0.33	Acid

Soil samples were taken from each of the replicate plots. The standard error is given for the mean of the twenty replicate samples.

established, 36 days after sowing, the following measurements were evaluated: nitrogenase enzyme activity, nodule fresh weight, dry weight of roots and tops growth and total N concentration. Thereafter alfalfa and grass plants were sampled, when alfalfa reached 50% bloom stage (initial harvest on 23 July 1983) and after harvest: 2, 9, 15, 23 and 30 days after initial harvest in the seeding year. In the following year, the plants were sampled again 7, 20, 28, 39 and 50 days after a initial harvest (23 June 1984). Grass plants were also analyzed for tiller number and DW yield of roots and tops growth and total N concentration.

Experiments IV and V were initiated on 27 May 1983 and 23 May 1984, respectively. Both experiments had the same characteristics and they were conducted to evaluate the amount of N transfer from alfalfa to grass, soil N uptake by alfalfa and grass and to measure N_2 fixed by alfalfa in mixed swards under field conditions. In order to increase the accuracy of measuring N transfer and N uptake, the isotope dilution technique was utilized to enrich the percentage atom ^{15}N in the soil.

The experiments were conducted on a sandy loam soil which contained a low level of available N. Soil test results are shown in Table 2. The treatments consisted of monocultures of alfalfa, timothy and bromegrass and two mixture cultures of alfalfa-grass. An additional grass species, tall fescue, was introduced in Experiment V. The experimental design was a randomized complete block with two and four replicates for

Experiment IV and V, respectively. Alfalfa seeds cv.' 520 ' inoculated with a commercial preparation of Rhizobium meliloti strains 102F70 and L-26 (Nitragin Co., Milwaukee, Wisconsin) were planted at the same seed rates of Experiment III. The grass species tall fescue cv.' Kentucky 31 ' was sown at a rate of 10 Kg/ha in both stands. Each experimental unit (1.60 x 6.0 m) consisted of eight rows 6.0 m long and 0.20 m apart. In addition, one microplot (1.0 x 1.0 m) was established approximately in the center of each plot. A solution of 1g ammonium sulphate $(^{15}NH_4)_2SO_4$ with 99% atom ^{15}N excess dissolved in 1.0 l deionized water was applied to the surface of the soil in each microplot (1 m^2). The amount of N (2.1 kg/ha) added was very limited to maintain the normal soil process, without disturbance. An additional 0.25 g of ammonium sulphate (99% atom ^{15}N excess) in solution was added to each microplot annually in the Spring, to keep the level of % atom ^{15}N sufficient to detect in the plant tissues.

Plots were harvested when alfafa reached 50% bloom stage. In Experiment IV two cuts were obtained during the seeding year, and subsequent year, but three cuts were performed in the third year. In Experiment V only one cut was obtained during the 1984 season and three cuts during the 1985 growing season. At each harvest, plants were cut 3 cm above the ground level, dried at 80°C for three days, weighed and then ground for total N concentration and ^{15}N analyses.

3.3 Method of total N concentration analysis

Harvested plant materials were dried at 80°C for at least three days to a constant weight and ground in a mill with a 0.5 mm screen (40 mesh). Total N concentration was made with a Tecator Kjeltec designed to perform N measurements based on the Kjeldahl method. After grinding, 500 mg legume and grass plant materials were placed into separate 250 ml digestion tubes, 12 ml of concentrated sulfuric acid (H_2SO_4) and 2 kjeltabs of a mixture of selenium (Se) and potassium sulphate (K_2SO_4) were added, in order to increase the rate of digestion of organic matter. The tubes were heated for 1 hour at 425 $\pm$ 5°C on a Tecator Digestion System 20. They were removed from the digester, cooled for 6 minutes approximately and diluted with distilled water to 75 ml volume.

Diluted samples were connected to a steam distillation unit, Kjeltec Auto 1030, and 40 ml of 40% sodium hydroxide solution (NaOH) was added to neutralize the H_2SO_4 and to release the ammonium (NH_3). The resulting NH_3 in solution was distilled into 25 ml of 1% boric acid (H_3BO_3) and then titrated with 0.1 N hydrochloric acid (HCl) and percentage N calculated. Duplicate determinations were carried out on each group of 20 samples to check the precision of the digestions and distillation procedures. Samples of 500 mg were used instead of the usual 100 mg in order to minimize sample error. Total reduced N was determined from plant DW and percentage N calculated.

3.4 Acetylene reduction technique

Nitrogenase enzyme activity of the intact alfalfa roots (Turner and Gibson 1980; Knowles 1980) was determined by the acetylene reduction assay (ARA) in both greenhouse and field experiments. Plants from each treatments and from each replication were removed between 10.00 and 12.00 hours. Under greenhouse conditions, the tops were excised and the vermiculite or vermiculite/sand medium was separated from the root system by gently shaking. For the field experiments, soil cores were dug around each plant root system, approximately 20 cm deep. The cores were submerged in the water for several minutes to allow separation of the root system from the soil with minimum loss of nodules and minimum damage to the roots.

The root system was placed in a 1.0 l reaction jar. One hundred cubic centimeters of air in the jar was replaced by the same volume of freshly prepared acetylene to provide a partial pressure of 0.10 atm. Acetylene was generated *in situ* by the reaction of calcium carbide (CaC_2) with tap water, as described by Sirois and Peterson (1982). After one hour incubation at room temperature (25 $\pm$ 1°C), four 0.5 ml samples of the gas were withdrawn from the jar by syringes, and the points of the syringe needles were placed into large rubber stoppers to prevent leakage. The amount of ethylene was measured with a Gas Chromatograph 9700-Tm at 72 $\pm$ 1°C equipped with a flame

ionization detector. Compounds were separated on a poropak N column and N_2 was the carrier gas. Sample peak height was compared to a 0.1% ethylene standard, similar to that described by Turner and Gibson (1980). The ethylene standards were performed with samples which contained known amounts of acetylene.

Total nitrogenase activity (TNA) is expressed on the basis of nmoles or umoles of ethylene (C_2H_4) produced per plant per hour (nmoles or umoles $C_2H_4.pl.^{-1}\ h.^{-1}$). The activity when measured per g nodule fresh weight is designated specific nodule activity (SNA) and is expressed as nmoles or umoles of (C_2H_4) produced per g nodule fresh weight per hour (nmoles or umoles $C_2H_4.mg\ nodule^{-1}\ h.^{-1}$).

3.5 ^{15}N analysis

^{15}N was analyzed by optical emission spectroscopy, following conversion of the sample N to N_2 gas according to the Dumas method. The principle of this technique was based on the fact that the bands in the band spectra of N_2 have different wavelengths for different isotopic composition of the N_2 molecule. The spectrometer is designed to register the intensities of the electronic emission, when the $^{14}N^{14}N$ molecule has a wavelength of 297.7 nm, $^{14}N^{15}N$ 298.3 nm, and $^{15}N^{15}N$ 298.9 nm (Preston et al. 1981). The variation of light intensity with

different wavelength was finally recorded on a strip chart recorder and the peak height was used as a direct measure of the intensity. The percentage atom ^{15}N was then calculated:

$$\text{atom \% } ^{15}N = \frac{100}{2R + 1} \qquad [1]$$

where R = the ratio of peak height for the ^{28}N molecule and ^{29}N molecule.

Dried plant material containing approximately 7 ug N was introduced to a pyrex tube (6 mm O. D. and 30 cm long), sealed at one end. After adding a small amount of calcium oxide (CaO) and a few pieces of cupric oxide (CuO), preheated at 950°C and 550°C, respectively, the tube was connected to a vacuum system and evacuated to 10^{-3} Torr, heated by flame to about 500°C to remove humidity and then treated with a spark from a Tesla coil to remove absorbed traces of gaseous impurities. When the vacuum system reached 10^{-4} Torr, the tube was sealed off. The tube was then heated in a furnace at 550°C to convert organic N of sample to N_2 gas by the presence of CuO. Any traces of H_2O or CO_2 that arose from plant material were absorbed by CaO and the tube was cooled to room temperature.

N_2 gas in the tube was excited by a Tesla coil, when tube was set on the electrode of high voltage, and a purplish light was emitted from the excited N_2 molecule. The peak height of $^{14}N^{14}N$ and $^{14}N^{15}N$ was recorded and calculated as described above.

All ^{15}N concentrations were referred to a standard of natural abundance, and isotope terminology was according to LaRue and Patterson (1981) and Rennie and Kemp (1984). Atom percent ^{15}N excess (atom % ^{15}N exc.) in plant material was derived from:

atom % ^{15}N exc. = atom % ^{15}N in the sample - atom % ^{15}N in the atmosphere [2]

where, the ratio $^{14}N:^{15}N$ in the atmosphere is 273 $\pm$ 3, yielding 0.3663 $\pm$ 0.0004 atom % ^{15}N.

3.5.1 ^{15}N dilution technique to estimate the amount of N_2 fixed and N transferred from alfalfa to associated grass

The isotope dilution technique was carried out as follows: the soil was amended with ^{15}N labelled fertilizer in a low concentration so that symbiotic N_2 fixation was not inhibited. The percent plant N derived from atmosphere (%Ndfa) was estimated based on the following assumptions:

1. The fixing plant and the non-fixing control take up N from the soil at the same ^{15}N-label; in other words, alfalfa and grass roots absorb the same proportion of soil N (mineralized) and applied labelled ^{15}N during the growing period;

2. Any input from outside the soil system, such as N in rainfall, dust or from free living organisms contributed to both plant species equally.

The total nitrogen of alfalfa was calculated on the basis that there are two sources of N available; soil N and N from atmosphere in form of N_2 gas. Therefore, N_2 fixed by the plant originated from unlabelled atmospheric N_2 that diluted the labelled ^{15}N.

$$\text{Total N (alfalfa)} = \text{N-fixed from atmosphere} + \text{N uptake from soil} \qquad [\ 3\ ]$$

$$\text{Total N (alfalfa)} = F(a) + [\ 1 - F(a)] \qquad [\ 4\ ]$$

where, F(a) is the fraction of N in alfalfa plants originated from fixation process. Using the ^{15}N dilution technique to distinguish the soil N uptake and N_2 fixed, the equation [4] is expressed as:

$$(\text{atom \% } ^{15}N \text{ exc. alfalfa}) = [\ \text{atom \% } ^{15}N \text{ exc. atmosphere} \times F(a)\ ] + ([\ 1 - F(a)] \times \text{atom \% } ^{15}N \text{ exc. soil}) \qquad [\ 5\ ]$$

Since normally atom % ^{15}N exc. in the atmosphere is zero, and the atom % ^{15}N exc. in the soil is also taken up by the control

(non-fixing system), then atom % ^{15}N exc. in the soil = atom % ^{15}N exc. in the control.

$$[\ 1 - F(a)\ ] = \frac{\text{atom \% } ^{15}\text{N exc. alfalfa}}{\text{atom \% } ^{15}\text{N exc. control}} \qquad [\ 6\ ]$$

$$F(a) = \left(1 - \frac{\text{atom \% } ^{15}\text{N exc. alfalfa}}{\text{atom \% } ^{15}\text{N exc. control}}\right) \qquad [\ 7\ ]$$

$$\%\ \text{Ndfa} = \left[\ 1 - \frac{\text{atom \% } ^{15}\text{N exc. alfalfa}}{\text{atom \% } ^{15}\text{N exc. control}} \times 100\ \right] \qquad [\ 8\ ]$$

The N_2 fixed was calculated,

$$N_2 \text{ fixed} = \frac{\%\ \text{Ndfa}}{100} \times \text{Total reduced N alfalfa} \qquad [\ 9\ ]$$

The amount of N transferred from alfalfa to grass (Nt), when both were grown in association, was estimated from the difference of isotopic composition of grass plant tissues in mixed culture, with that in pure stand as reference value. Since grass plants do not fix N_2, it was assumed that any N supplied to grass species in mixed culture originated from the soil N uptake and from the associated legumes. Due to the very similar conditions of grass plants grown in both mono and mixed culture, the contribution of N fixed by free living organism or perhaps by organism associated with grass roots are negligible in this estimation.

Total N (grass in mixed stand) = N uptake from soil
+
N transferred from alfalfa [10]

Total N (grass in mixed stand) = F(t) + [1 - F(t)] [11]

where, F(t) is the fraction of N in grass plants originating from alfalfa, when both grown in association. Using the ^{15}N dilution technique to distinguish the soil N uptake and the N transfer from alfalfa, the equation [11] was expressed as:

(atom % ^{15}N exc. grass in mixed stand)=
(atom % ^{15}N exc. alfalfa in mixture) x F(t)
+
(atom % ^{15}N exc. soil x [1 - F(t)] [12]

Since atom % ^{15}N exc. in the soil is uptake by the control (grass in pure stand), then atom % ^{15}N exc. soil = atom % ^{15}N exc. control.

(atom % ^{15}N exc. grass in mixed stand) =
atom % ^{15}N exc. alfalfa mixture x F(t)
+
[1 - F(t)] x (atom % ^{15}N exc. control) [13]

(atom % ^{15}N exc. grass in mixed stand) =
[atom % ^{15}N exc. alfalfa mixture x F(t)]
+
(atom % ^{15}N exc. control)
-
[F(t) (atom % ^{15}N exc. control)] [14]

(atom % ^{15}N exc. grass in mixed stand - atom % ^{15}N exc. control)
=
F(t) (atom % ^{15}N exc. alfalfa mixture - atom % ^{15}N exc. control)
[15]

$$F(t) = \frac{(\text{atom \% } {}^{15}N \text{ exc. grass in mixed stand}) - (\text{atom \% } {}^{15}N \text{ exc. control})}{(\text{atom \% } {}^{15}N \text{ exc. alfalfa mixture}) - (\text{atom \% } {}^{15}N \text{ exc. control})} \quad [16]$$

$$\% F(t) = \frac{(\text{atom \% } {}^{15}N \text{ exc. grass in mixed stand}) - (\text{atom \% } {}^{15}N \text{ exc. control})}{(\text{atom \% } {}^{15}N \text{ exc. alfalfa mixture}) - (\text{atom \% } {}^{15}N \text{ exc. control})} \times 100 \quad [17]$$

The N transferred (Nt) was calculated :

$$Nt = \frac{\% F(t)}{100} \times \text{Total reduced N grass in mixed stand} \quad [18]$$

The remaining amount of N was estimated as soil N uptake by grass in mixture culture.

Nitrogen benefit (Nb) was defined as the difference between total N yield of grass in mixed culture and pure stand on a plant basis.

3.6 N difference method

Estimates of N_2 fixation by the N difference method were based on the following equation;

$$N_2 \text{ fixed} = (\text{Total N alfalfa}) - (\text{Total N control}) \quad [19]$$

The same nonlegume treatments (grass in monoculture) were used as reference plants to estimate N_2 fixed by alfalfa in pure stand. For mixtures of alfalfa and a companion grass, the N_2 fixed was estimated by first adding the total N in the grass in mixed stand to that of alfalfa in mixture culture, then subtracting the total N contained in the grass pure stand. However, under greenhouse conditions the total N yield per sleeve or pot (alfalfa plants in monoculture and alfalfa plus grass plants in mixture) was used to be an estimate of N_2 fixed, due to the fact they grew in N free medium (vermiculite and vermiculite-sand) and received only basal fertilization in order to improve the establishment of the species.

3.7 Concepts used in the investigation of legume-grass mixtures

In this section most of the pertinent concepts used to investigate competition in a legume-grass association are discussed.

3.7.1 Relative crowding coefficient

De Wit and Van den Bergh (1965) defined the relative crowding coefficient (RCC) as a measure of the aggressiveness of one species toward the other. RCC is a ratio of relative yields of mixed cultures to pure stand per plant. In this case, RCC

represents the ratio of alfalfa plant yield to grass in mixture versus alfalfa pure stand.

$$RCC = \frac{Yij/Yji}{Yii/Yjj} \qquad [20]$$

Where, Yii = mean yield per plant of alfalfa in pure stand;
Yij = mean yield per plant of alfalfa in mixture;
Yjj = mean yield per plant of grass in pure stand;
Yji = mean yield per plant of grass in mixture.

The RCC has a practical meaning to predict the yield of the mixture from the yield of the pure stands.

3.7.2 Competitive ratio

Competitive ratio (CR) is defined as the ratio of the relative area of monoculture that would be required to produce the yield obtained by mixtures for each component of the mixed culture, after correcting the proportions in which the species were sown initially. The CR value greater than unity demonstrates that one species is more competitive for a factor than the other species in the mixed culture (Faris et al. 1983). This ratio is particularly useful when comparing the competitive ability (degree of competition) of different species and in identifying which plant characters are associated with poor or good competitive ability for one limiting factor (Willey and Rao 1980).

The competitive ratio is expressed as

$$CR = \frac{Yij/Yii \times Zab}{Yji/Yjj \times Zba} \qquad [\ 21\ ]$$

where, Z_{ab} is the proportion of alfalfa in the mixture culture
Z_{ba} is the proportion of grass in the mixture culture.

3.7.3 Relative yield and relative yield total

Relative yield (RY) relates the yield of each species to its pure stand. RY is used to determine the stability of the mixture and the competitive relationships of two species (De Wit et al. 1966). The RY of each species in association is calculated as:

$$RY = \frac{\text{yield of a species in mixture}}{\text{yield of a species in pure stand}} \qquad [\ 22\ ]$$

Relative total yield (RTY) is the total of the relative yields of the two species in association. This parameter is used to evaluate the performance of the mixture through time (De Wit and Van den Bergh 1965).

$$RTY = \text{Relative yield alfalfa} + \text{Relative yield grass} \qquad [\ 23\ ]$$

3.8 Statistical analysis

Data collected in the experiments described above were analysed through the computing facilities of Forage Crop Section,

Ottawa Research Station, Agriculture Canada utilizing the Statistical Analysis System (SAS).

Analyses of variance were performed appropriate to the design of experiments. For each analysis, when a significant treatment effect was found ($p \leq 0.05$), a least significant difference (LSD) and Duncan's multiple range test were calculated to determine which treatments means were significantly different. The orthogonal comparisons were made within each analysis according to Steel and Torrie (1980).

Data on nitrogenase enzyme activity and growth variables in Experiment III appeared to be non normally distributed, i. e. the variances were not homogeneous (Appendices 1, 2, 3, and 4), according to the Bartlett test (Daniel 1978; Steel and Torrie 1980). In order to normalize the data, natural logarithm transformations were applied before analyses. In some case transformation produced data sets containing both positive and negative values, inflating the coefficients of variation for these sets. In these cases, the data sets were multiplied by 1000 and reanalysed to avoid having negative values in the transformed data. For display in figures the means of the transformed data were retransformed. As the data actually analysed were natural logarithm data, the differences are proportional and not absolute.

4. RESULTS AND DISCUSSION

4.1 Greenhouse studies

4.1.1 Effect of grass species on nitrogenase enzyme activity

Experiment I (1982) was the first and simplest of this series of experiments to examine the effects of grasses on alfalfa nitrogenase activity and the benefit of legumes in mixture. In this experiment the isotope dilution technique was not included as a method to assess N_2 fixation, so that only the acetylene reduction assays were performed and used as an estimate of N_2 fixation and nitrogenase enzyme activity.

Total nitrogenase enzyme activity (TNA) in this investigation (Tables 3 and 4) were less than or similar to those previously reported for various forage legumes (Vance et al. 1979; Aparicio-Tejo et al. 1980). TNA of alfalfa seedlings in mono and mixed cultures did not differ significantly before harvest at early bud stage and initial flower formation (Table 3). However, in some cases the TNA of alfalfa grown in monoculture was significantly higher when compared to alfalfa in association with grasses (Table 4). In all the determinations, at 50% bloom stage, alfalfa in association had a higher TNA value. A marked exception was the trend towards lower activity of alfalfa in association with orchardgrass in the later

Table 3. Total nitrogenase activity (TNA) of alfalfa for the various mono and mixed cultures at four determinations after planting July 27, under greenhouse conditions (Experiment I).

TREATMENT OR COMPARISON	Total Nitrogenase Activity (umoles C_2H_4. $hour^{-1}$ $plant^{-1}$) Determinations+ 1	2	3	4
Alfalfa	0.85a	1.38a	1.77ab	1.51a
Alfalfa with timothy	1.07a	1.99a	1.84a	1.29a
Alfalfa with bromegrass	1.08a	1.20a	1.28c	1.12a
Alfalfa with orchardgrass	0.99a	1.68a	1.51bc	0.81a
Mean++	1.00b	1.56a	1.60a	1.18b
±SE	0.16	0.21	0.08	0.52
Alfalfa vs. Alfalfa with grasses	NS	NS	NS	NS

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Determination #1 performed 7 September 1982 at early bud stage; determination #2 performed 28 September 1982 at initial flower formation; determination #3 performed 3 December 1982, six weeks after initial harvest on 13 October; determination #4 performed 3 January 1983.

NS, No significant difference.

++Means compared horizontally

Table 4. Total nitrogenase activity (TNA) of alfalfa for the various mono and mixed cultures when clipped periodically after planting February 14 (Experiment II).

TREATMENT OR COMPARISON	Total Nitrogenase Activity (umoles C_2H_4 plant^{-1} hour^{-1})			
	Harvest+			
	1	2	3	4
Alfalfa	0.95b	1.17a	0.94b	1.28a
Alfalfa with Timothy	1.47ab	1.81a	1.74a	1.36a
Alfalfa with Bromegrass	1.30ab	1.38a	1.67a	1.85a
Alfalfa with Orchardgrass	2.41a	1.86a	0.94b	0.97a
Alfalfa with Tall Fescue	1.43ab	1.85a	1.49ab	1.29a
Mean++	1.51a	1.62a	1.319a	1.33a
± SE	0.61	0.54	0.53	0.53
Alfalfa vs. Alfalfa with grass	*	NS	NS	NS

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

*, Significant different at the 0.05 level of probability,
NS, No significant difference.

++Means compared horizontally

assessments in both experiments.

The TNA of alfalfa grown in mono and mixed cultures increased significantly between early bud stage and initial flower formation (Table 3, determination #1 and #2) that was performed before initial harvest; but the TNA declined considerably between initial flower formation (Table 3, determination #3) and the later bloom stage of alfalfa after initial harvest (Table 3, determination #4). The depression of TNA is probably a result of a competitive sink for photosynthate exhibited at bloom stage, and indicates the importance of photosynthate supply to the N_2 fixation process (Bethlenfalvay and Phillips 1977; Herridge and Pate 1977). Vance et al (1979) and Cralle and Heichel (1981) found that specific nodule activity (SNA) of alfalfa increased up to 40-60 days after planting (i. e. until early flower stage). However, SNA declined 88% within 24 h after cutting and remained very low during the next 15 days as compared to control plants (unharvested). After the 18th day, the rate of SNA recovery was fast, and in the 20th day of regrowth the nodule number and nitrogenase activity of the alfalfa plants were similar to the control. The decline in nodule activity after shoot removal is similar to reports with other legume species (Wilson 1942; Butler et al. 1959; Moustafa et al. 1969; Whiteman 1970). Moreover alfalfa maintains nodule structure and funotion which suggests that the capacity of N_2 fixation is only temporarily impaired for a few days (Cralle and

and Heichel 1981). In the present study, the time of regrowth was sufficient to allow complete recovery of TNA. However, after 10 weeks of regrowth (Table 3, determination #4), the TNA significantly declined about 25% below that of 6 weeks of regrowth (Table 3, determination #3). This reduction supports the previous explanation of the competitive sink for photosynthate between seed formation and nodules N_2 fixation during later stages of alfalfa.

Differences in TNA of alfalfa alone versus in association were significant only at harvest 1 in Experiment II (Tables 3 and 4). These observations agree with the results published by Craig de Anda et al. (1981) who also found that grasses have no detrimental effects on legume SNA. It has been reported by Whitehead (1970) that during normal growth and harvest of alfalfa plants some death of secondary roots and nodule tissue occurs with a consequent release of N to the soil. This may inhibit either additional initiation of new nodules or the nitrogenase activity of nodules already present. Grasses grown in association with legume may absorb the newly released N and thus reduce the soil N mediated inhibition of legume N_2 fixation. This may explain the increase in alfalfa TNA with time in the mixed cultures.

The specific nodule activity (SNA) was determined by measuring the amount of C_2H_4 produced per mg of fresh nodule (Table 5). The nodule effectiveness was almost equal for alfalfa

Table 5. Specific nodule activity (SNA) of alfalfa for the various mono and mixed cultures when clipped periodically after planting February 14 (Experiment II).

TREATMENT OR COMPARISON	Specific Nodule Activity ($nmoles\ C_2H_4\ mg.\ nodule^{-1}\ hour^{-1}$) Harvest+ 1	2	3	4
Alfalfa	13.7ab	9.5b	12.9a	13.1a
Alfalfa with timothy	15.2a	14.5ab	12.3ab	14.2a
Alfalfa with bromegrass	10.4b	13.1b	16.9a	13.8a
Alfalfa with orchardgrass	20.7a	22.8a	8.9b	8.8b
Alfalfa with tall fescue	13.9a	14.8ab	13.4a	11.8ab
Mean++	14.8a	14.7a	12.8a	12.2a
±SE	6.9	4.1	4.8	4.1
Alfalfa vs. Alfalfa with grasses	NS	NS	NS	NS

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

NS, No significant difference.

++Means compared horizontally

in pure stand and for alfalfa in association with grass. The highest SNA was obtained on alfalfa plants grown with orchardgrass (harvests 1 and 2), but the activity declined considerably in later measurements (havests 3 and 4). The data suggest that alfalfa grown without competition of grass in the early stage of growth and regrowth plants reached the 50% bloom stage with all nodules receiving enough supply of photosynthate to support normal activity. The reduction in SNA of alfalfa/orchardgrasss mixture, seems to be related to the growth habits (early maturing species) and competitive ability of orchardgrass.

4.1.2 N_2 fixation, herbage and N yield of alfalfa

Atom % ^{15}N excess data of the alfalfa and grasses were collected from four harvest times (Tables 6 and 7). Atom % ^{15}N excess in the alfalfa was significantly lower than the atom % ^{15}N excess of the grasses, and nearly equal to the level of natural abundance indicating vigorous N_2 fixation by alfalfa. To quantify the amount of N_2 fixed by alfalfa, the isotope dilution method was used in the Experiment II. Data showed that during the entire experiment most if not all alfalfa N requirements came from the N_2 fixation process (Table 8). The proportion of N derived from the atmosphere (%Ndfa) was frequently observed to be higher than 95%, and remained relatively constant with slight

Table 6. Atom % ^{15}N excess in shoots of alfalfa plants for the various mono and mixed cultures when clipped periodically after planting February 14 (Experiment II).

TREATMENT	Harvest+ 1	2	3	4
	(atom % ^{15}N excess)			
Alfalfa	0.17a	0.13ab	0.19a	0.18a
Alfalfa with timothy	0.07b	0.09ab	0.13a	0.09b
Alfalfa with bromegrass	0.07ab	0.15a	0.09b	0.13ab
Alfalfa with orchardgrass	0.11ab	0.11ab	0.11ab	0.12ab
Alfalfa with tall fescue	0.10ab	0.07b	0.08b	0.06b
Mean++	0.10a	0.11a	0.12a	0.12a
±SE	0.04	0.04	0.05	0.04

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

++Means compared horizontally

Table 7. Atom % ^{15}N excess in shoots of grass plants for the various mono and mixed cultures when clipped periodically after planting February 14 (Experiment II).

TREATMENT	Harvest+ 1	2	3	4
	(atom % ^{15}N excess)			
Timothy with alfalfa	3.43b	3.23a	4.03a	3.78a
Bromegrass with alfalfa	3.54b	3.12a	3.66a	5.29a
Orchardgrass with alfalfa	6.10ab	4.12a	3.94a	7.58a
Tall Fescue with alfalfa	5.39ab	4.62a	4.02a	4.09a
Timothy	5.58ab	7.39a	6.26a	5.85a
Bromegrass	4.45b	6.03a	5.80a	6.27a
Orchardgrass	9.04a	5.21a	5.80a	7.16a
Tall Fescue	5.60ab	5.63a	5.17a	6.54a
Mean++	5.38a	4.83a	4.84a	5.57a
±SE	2.07	2.65	1.83	1.87

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were perfotmed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

++Means compared horizontally

Table 8. Percent of nitrogen derived from atmosphere (%Ndfa) of alfalfa for various mono and mixed cultures when clipped periodically after planting February 14 (Experiment II).

TREATMENT	Percent Nitrogen Derived from Atmosphere Harvest+				
	1	2	3	4	Mean
Alfalfa	97.1a	95.5a	96.5a	96.8a	96.5
Alfalfa with Timothy	98.2a	97.3a	97.4a	95.7a	97.1
Alfalfa with Bromegrass	97.4a	98.0a	95.5a	96.9a	96.9
Alfalfa with Orchardgrass	98.2a	97.3a	98.0a	96.2a	97.4
Alfalfa with Tall Fescue	98.1a	97.3a	98.8a	97.2a	97.9
Mean++	97.8a	97.1a	97.3a	96.6a	97.2
±SE	1.0	1.5	1.6	1.7	1.5

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

++Means compared horizontally

variations, through the entire experimental period. These values were much higher than those of Heichel et al. (1981) where the % N fixed in pure stands of seedling year alfalfa was approximately 43% in the first and fourth harvests and 65% in the second and third harvests. However, these results in the present investigation generally agree with those from mixtures containing alfalfa or other forage legume species, which indicate that from 80 to 100% of the legume shoot N can be derived from N_2 fixation when growing with grass (Haystead and Lowe 1977; Vallis et al. 1977; Edmeades and Goh 1978; Bergersen and Turner 1983; Phillips et al. 1983; West and Wedin 1985). The high values of %Ndfa in this experiment reflected the low N content (nearly 0) of the medium used where alfalfa plants relied only on their ability to fix N_2.

There was a significant difference in the atom % ^{15}N excess from alfalfa grown in mixtures as compared with that in pure stand, specifically when grown with timothy (harvests 1 and 4) and with tall fescue (harvests 2, 3 and 4). However, when the data were expressed as %Ndfa this difference disappeared.

These data demonstrated the usefulness of the isotope dilution method to measure N_2 fixation in mixed swards. In this report, isotope dilution leads to an estimation of N_2 fixation that exceeded in an average of 0.4 to 1.4%, when N_2 fixed was measured in mixed cultures in comparison with N_2 fixed by alfalfa in monoculture. This overestimation was not as severe as

that claimed by Broadbent et al. (1982) who concluded that the ^{15}N dilution method cannot be used with a legume-grass mixture because of underground transfer of N from legume to grass. This leads to erroneous estimates of N_2 fixation, if comparisons are made between legume and non-legume in the mixture. However, Bole and Rennie (1983) claimed that Broadbent et al. (1982) overstated the seriousness of this error. Recent studies reviewed by Chalk (1985) have shown that the isotope technique can be applied to the measurement of N_2 fixation in associated pastures because grass and legumes roots are intimately mixed, and therefore sample the same soil N pool. Talbott et al. (1982) and Fried et al. (1983) also emphasized this aspect with respect to intercropping. However, a suitable reference plant in pure stand is required to estimate the relative contribution of indigenous and soil N to the N nutrition of the fixing plant. Therefore, mixing alfalfa with grass did not affect significantly the N_2 fixation by alfalfa. As a matter of fact, it slightly favored the fixation process by alfalfa (Tables 3, 4 and 8, and Figures 1 and 2). This is related to a reduction of soil N available to legumes through more efficient N uptake by associated grasses (Craig de Anda et al. 1981; Faris and Ta 1985). Also, grasses may excrete some biologically active substances leading to a stimulation of legume N_2 fixation. Wahua and Miller (1978) suggested that there is a delay in the senescence of legume nodules in mixed swards.

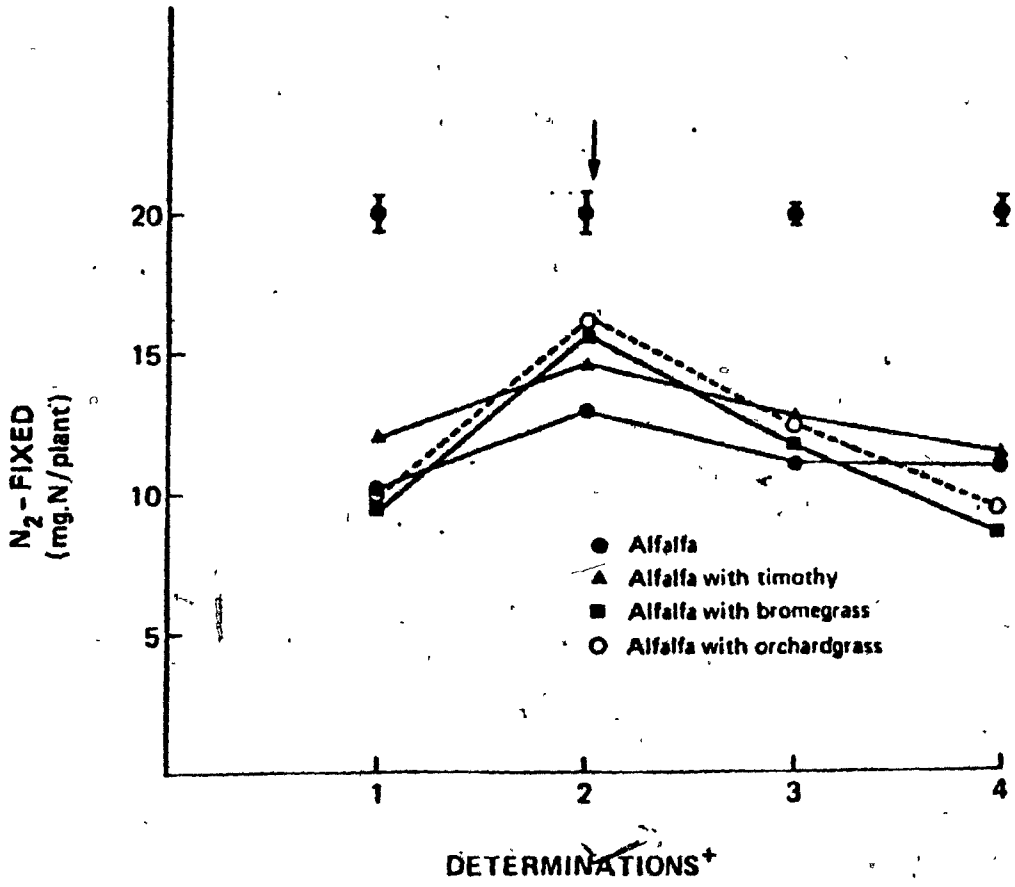

Figure 1. Dinitrogen fixation by alfalfa in mono and mixed cultures from 27 July 1982 to 3 January 1983 (Experiment I). Each point is the mean of four observations and the bar represents standard error (±SE) of the mean. Arrow indicates time of harvest.
+Determination #1 performed 7 September 1982 at early bud stage; determination #2 performed 28 September 1982 at initial flower formation; determination #3 performed 3 December 1982, six weeks after initial harvest on 13 October; determination #4 performed 3 January 1983.

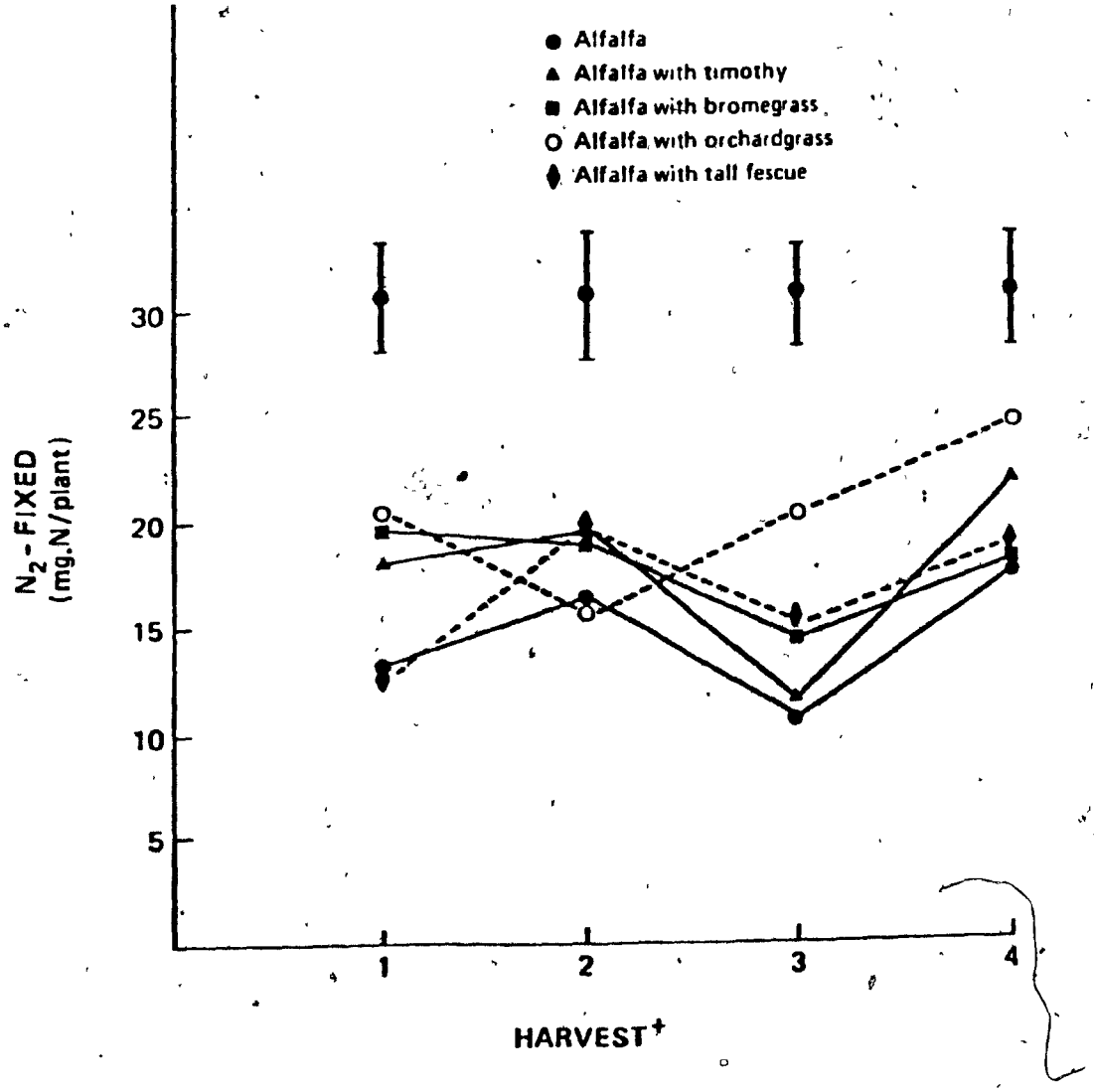

Figure 2. Dinitrogen fixation by alfalfa in mono and mixed cultures from 14 February 1984 to 24 August 1984 (Experiment II). Each point is the mean of three replicates and the bar represents standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

On a plant basis, the absolute amount of N in alfalfa derived from N_2 fixation in mono and mixed cultures from Experiments I and II, respectively are shown in Figures 1 and 2. The amount of N_2 fixed by alfalfa in both cultural systems varied slightly during the experimental period in patterns similar to those of %Ndfa. During the entire experimental period, alfalfa plants fixed an average of 55 and 71 mg N/plant in pure and mixed stands, respectively. Since the proportion of %Ndfa was relatively constant through time, the main determinants of the amount of N_2 fixed were % N concentration and dry matter accumulation, both of which were not affected significantly when alfalfa grown in mixture. Heichel et al.(1981) observed that the rate of N_2 fixation was closely linked to the growth rate of alfalfa.

The association of alfalfa with grasses did not have a detrimental effect on dry weight (DW) and N yield per plant (Figures 3 and 4). Nevertheless, the DW and N yield accumulation of alfalfa in association showed a trend of improvement over alfalfa in pure stand. This lesser DW of alfalfa plants in pure stand may be a response to intraspecific competition among the legume plants. In fact, it is generally accepted that intraspecific competition is more intensive than interspecific competition, and both result in a reduction of DW of the plants (McCloud and Mott 1953). Dubbs (1971) also concluded that alfalfa plants received more competition from other alfalfa

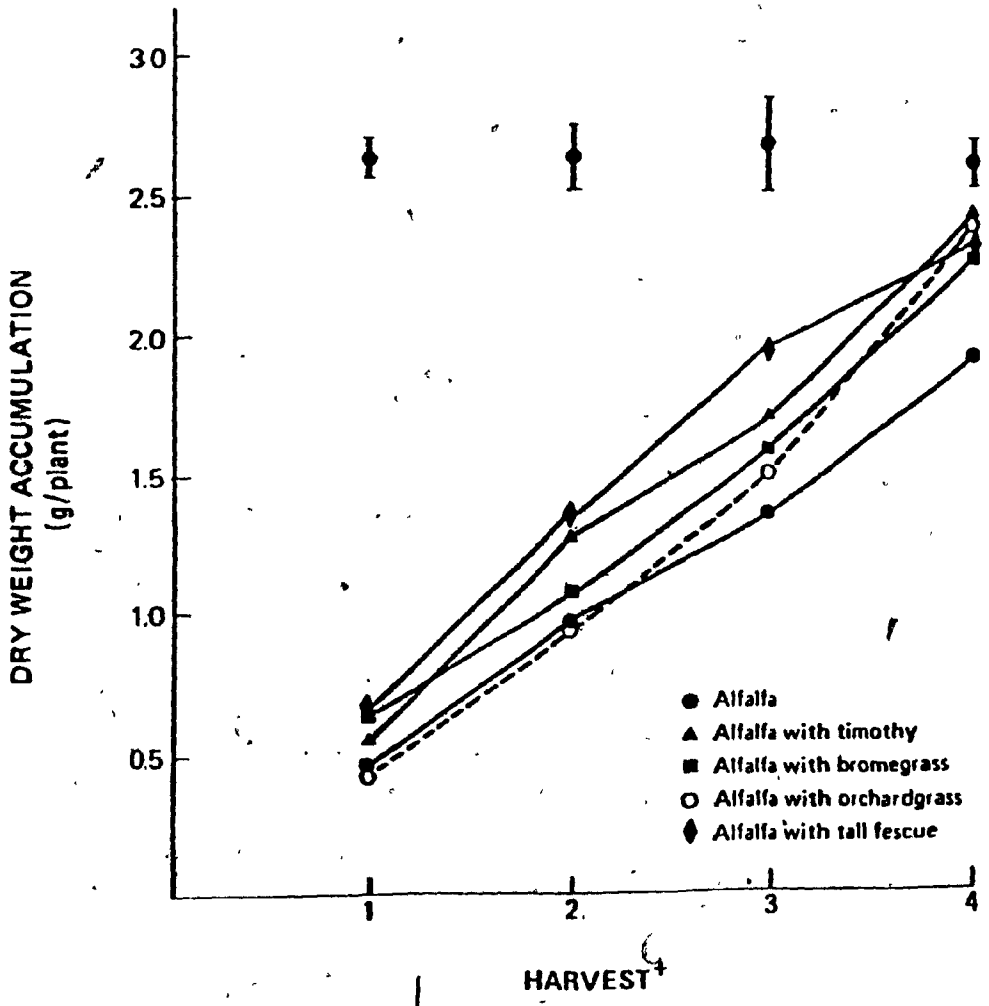

Figure 3. Accumulation of dry weight of shoots alfalfa plants grown in mono and mixed cultures and clipped periodically after planting February 14 (Experiment II). Each point is the mean of three replicates and the bar represents standard error (±SE) of the mean.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

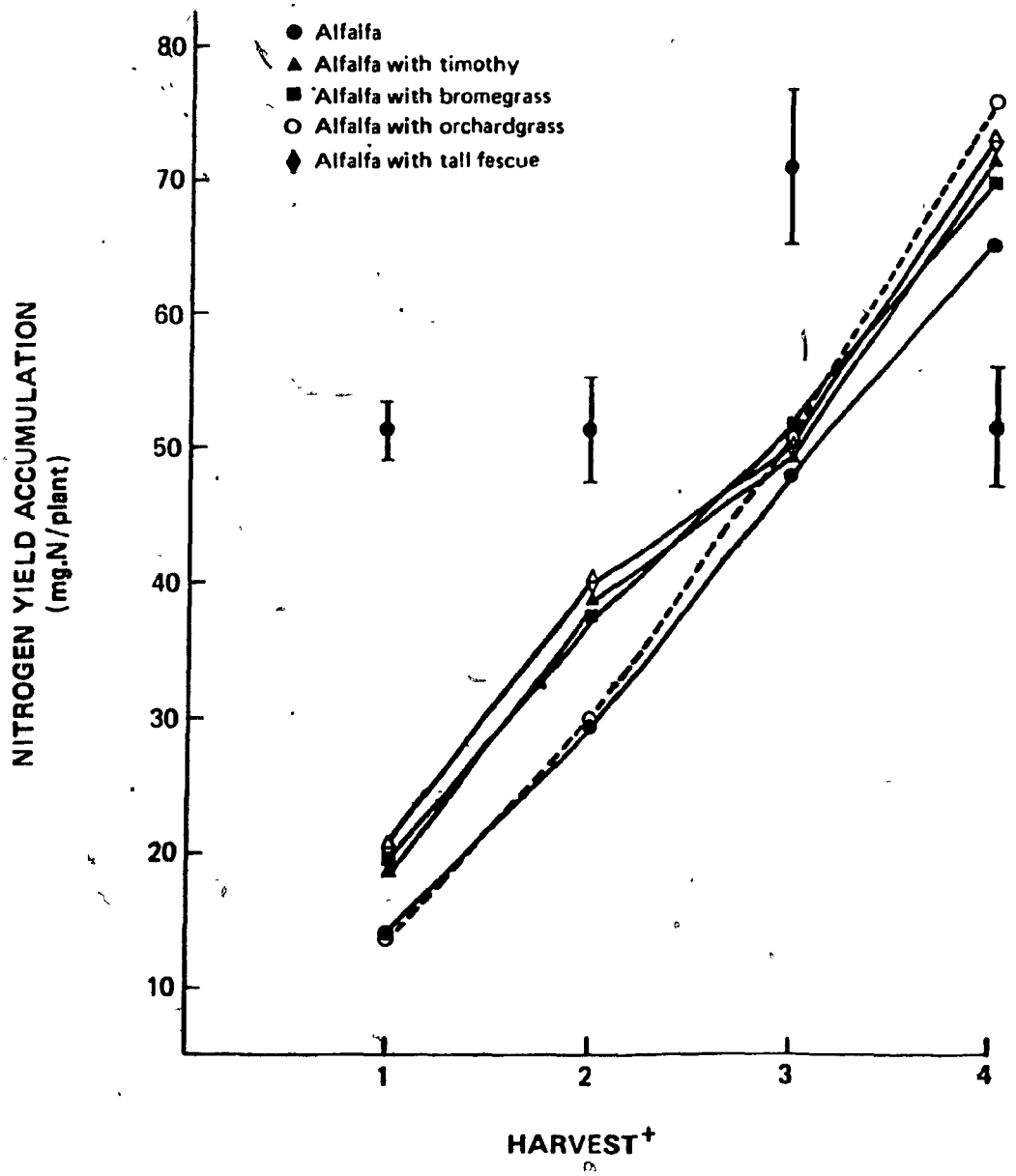

Figure 4. Accumulation of nitrogen yield of shoot alfalfa plants grown in mono and mixed cultures and clipped periodically after planting February 14 (Experiment II). Each point is the mean of three replicates and the bar represents standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

plants than from grass species when grown in mixed stand. Under these experimental conditions, it is impossible to separate effects of each kind of competition, but the intraspecific competition could be observed through the reduction of number of alfalfa plants in mixed stand (8 alfalfa plants/pot in monoculture versus 4 alfalfa plants/pot in mixed culture).

4.1.3 N transference, herbage and N yield of grass

The amount of N transferred from alfalfa to grass at the four determinations is shown in Figure 5 (Experiment I). As the grass plants in association grew in N free medium, the N yield in grass shoots is considered to be the result of N transference from alfalfa to the grasses. These calculations ignore any N in the seed or the root. The three grass species were similar in N transference although orchardgrass showed a slight advantage at the earlier regrowth stage. However, in general all grass species benefitted from N transfer from alfalfa during the entire period. Up to an average of 24% of the N_2 fixed by alfalfa in mixed stand was transferred to the associated grass. The quantity of N transferred declined at 6 weeks after cut, moreover 10 weeks later it showed signs of increase.

The ^{15}N dilution technique showed that (Experiment II) atom % ^{15}N excess of grass (Table 7) in the mixed stand was lower than the pure stand, demonstrating that some of the N_2 fixed by

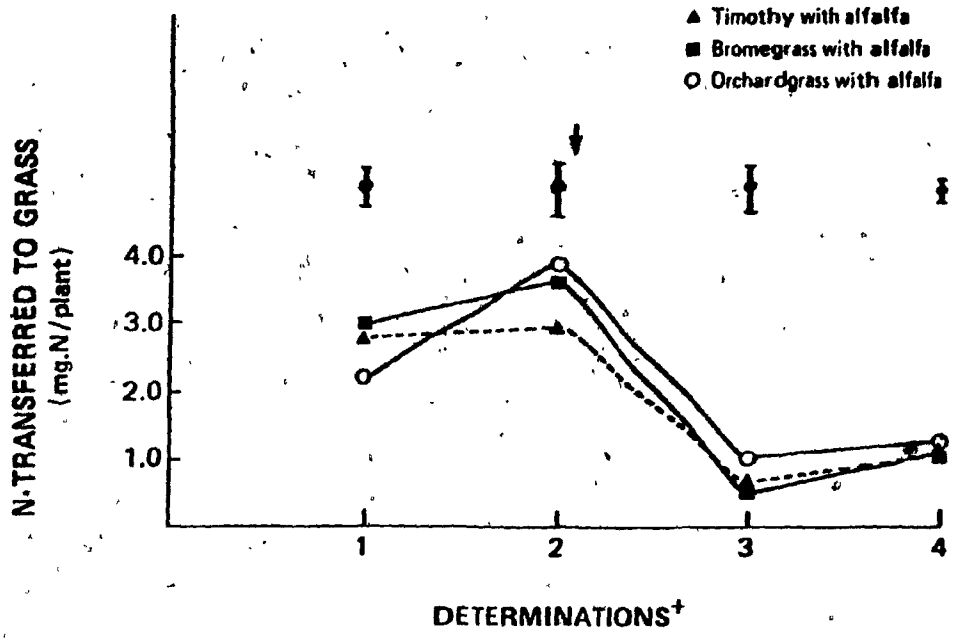

Figure 5. Nitrogen transferred to the grass at each determination from 27 July 1982 to 3 January 1983 (Experiment I). Each point is the mean of four observations and the bar represents standard error (±SE) of the mean. Arrow indicates time of harvest.

+Determination #1 performed 7 September 1982 at early bud stage; determination #2 performed 28 September 1982 at initial flower formation; determination #3 performed 3 December 1982, six weeks after initial harvest on 13 October; determination #4 performed 3 January 1983.

alfalfa was transferred to associated grass. The proportion in the grass of N transferred (Table 9) varied significantly with clipping time. The % N transferred was significantly lower during the first regrowth after initial harvest. However, in later assessments the % N transferred increased substantially and reached its maximum in harvest 4. These results suggest that the supply of N from excretion is low after defoliation due to the limitation of carbohydrates which support the normal nitrogenase enzyme activity, and the contribution of N from decay of nodules and death of roots was enough to alleviate the initial period of N stress in the associated grass. This is in agreement with the results of the amount of N transfer after 10 weeks of regrowth (Experiment I, determination #4) and in harvests 3 and 4 (Experiment II), where the process of mineralization was able to increase the total amount of N transferred (Figures 5 and 6). The substantial transference of N observed after second harvest suggests that the root and nodule tissue is not totally decomposed at 10 weeks. This is in agreement with the conventional view of gradual mineralization of dead roots and nodule tissues from the legume through microbial activity (Broadbent et al. 1982).

Data from Table 9 and Figures 5 and 6 show a consistent transference of N to associated grass even during the early stages of cultivation (Experiment I, determinations #1 and #2; Experiment II, harvest 1). This agrees with a direct excretion

Table 9. Percentage of nitrogen in grasses transferred from associated alfalfa from 14 February 1984 to 24 August 1984 (Experiment II).

TREATMENT	Nitrogen Transfer Harvest+			
	1	2	3	4
	%			
Timothy with alfalfa	27.4a	21.6a	54.7a	65.1a
Bromegrass with alfalfa	31.8a	19.6a	47.3b	65.0a
Orchardgrass with alfalfa	20.7a	16.1a	52.8ab	56.5b
Tall Fescue with alfalfa	18.4a	15.6a	58.7a	55.3b
Mean++	24.6c	18.2d	53.4b	60.5a
±SE	6.6	4.8	5.7	5.4

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

++Means compared horizontally

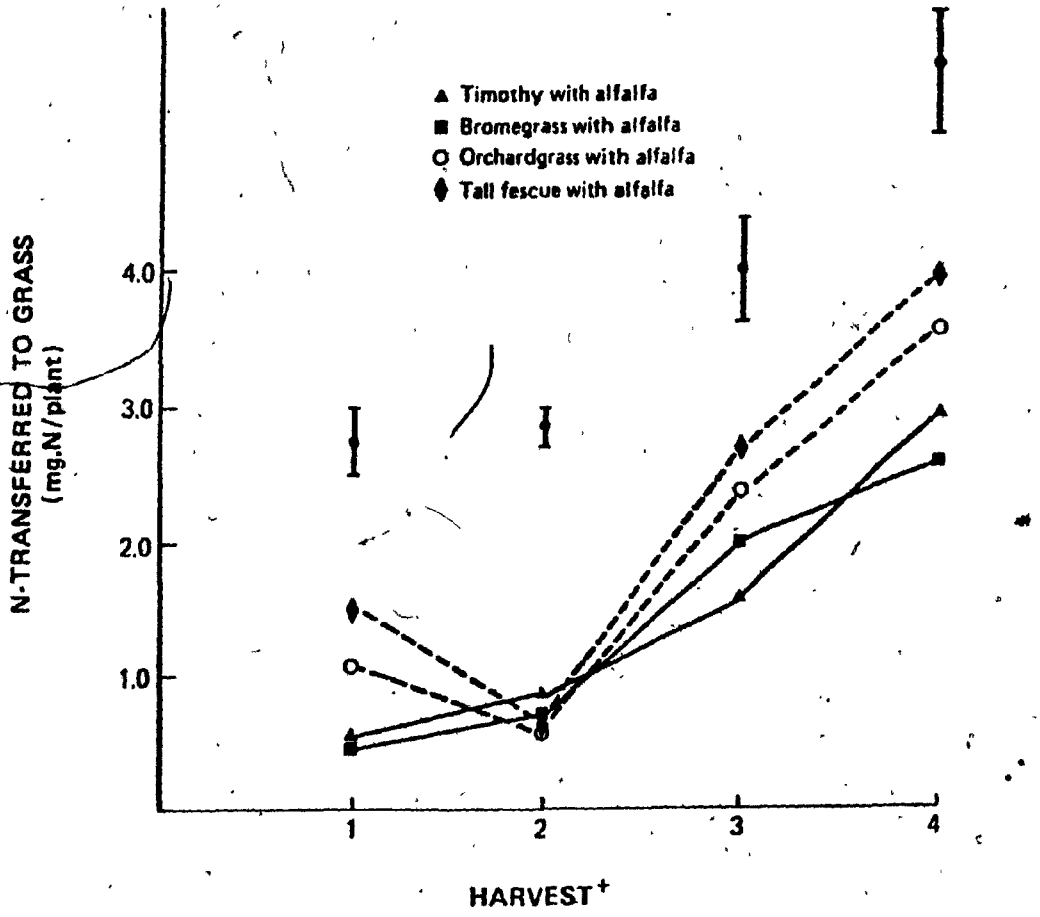

Figure 6. Nitrogen transferred to the grass at each harvest from 14 February 1984 to 24 August 1984 (Experiment II). Each point is the mean of three replicates and the bar represents standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

of N compounds from living alfalfa root systems (Faris and Ta 1985). Chujo and Daimon (1984) reported that the growth acceleration of grass species raised in association with red clover in the early stage of growth is probably due to the absorption of N excreted directly from the root system of the legume. The roots of alfalfa and the grasses in these experiments were tightly intertwined in the sleeves or pots, and any N compounds excreted by, or decomposed from the legume root and nodule debris, would be adjacent to the grass roots.

Values of % total N in each harvest from Experiment II are shown in Table 10. These values were not affected by the basal fertilization applied in the first week after seeding. The initial harvest of the grass in mixtures had a significantly higher N content than in pure stand. This supports the conclusion of beneficial effect of alfalfa to grass species when both were grown together rather than with only the application of N fertilizer to the grass. The presence of alfalfa caused an average increase of N content in grass of 42, 55, 66 and 80% over the grass control for timothy, bromegrass, orchardgrass and tall fescue, respectively. This may be explained by N released from alfalfa through either direct excretion and/or decomposition of nodules and alfalfa plant tissues. Similar results were also reported by Virtanen et al (1937); Butler and Bathurst (1956) Butler et al. (1959); Henzell et al. (1968); Simpson (1976); Vallis (1978); Haystead and Marriott (1978); Broadbent et al

TABLE 10. Total nitrogen concentration (%) in shoots of grass plants for the various mono and mixed cultures when clipped periodically after planting February 14 (Experiment II).

TREATMENT	Total Nitrogen Concentration Harvest+ 1	2	3	4	Mean++
	%				
Timothy with alfalfa	1.29bc	1.87a	1.77a	1.41bc	1.58a
Bromegrass with alfalfa	1.56b	1.91a	1.53ab	1.75ab	1.69a
Orchardgrass with alfalfa	2.00a	1.78a	1.41abc	1.61abc	1.70a
Tall Fescue with alfalfa	1.48b	1.74a	1.71a	1.94a	1.73a
Timothy	0.97dc	1.09b	1.15bc	1.23dc	1.11b
Bromegrass	1.17bcd	1.14b	1.16bc	0.91d	1.09b
Orchardgrass	0.97cd	0.90b	1.02c	1.19cd	1.02b
Tall Fescue	0.81d	0.99b	1.13bc	0.89d	0.96b
Mean	1.28	1.43	1.35	1.36	1.36
±SE	0.18	0.22	0.19	0.21	0.21

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

++Means compared vertically

(1982); Ta and Faris (1985). On the other hand, alfalfa N content was not affected by the presence of the grass as compared to that of alfalfa grown alone, demonstrating the absence of a negative interaction of the alfalfa-grass mixture on N content.

The absolute amount of N transferred from alfalfa to grass ranged from 1.03 mg N/plant (harvest 1) to 3.34 mg N/plant (harvest 4) equivalent to 5.7 and 15.5% of the amount of N_2 fixed by alfalfa in the initial and later harvests, respectively. Transfer of N from legume to grass is important for the growth of the grass, under low N conditions. Visual observations during present study showed that grass in mixed stand grew more vigorously than that in pure stand. However, the transfer of N did not allow the grass plants to grow to their full potential. Dilz and Mulder (1962) quantified transfer of N and found 1 to 8% (first cut) and 6 to 22% (second cut) of the fixed N_2 was transferred to the associated grass. Simpson (1965) found 1 to 4% of N_2 fixed by the legume was transferred to the grass. Faris and Ta (1985) reported considerable N transference from alfalfa to timothy, the amount being equivalent to 12% of the total N_2 fixed at the early stage of growth. Thus, the quantity of N transferred from alfalfa to the associated grass, as estimated by isotope dilution technique in this experiment was within the reasonable range obtained by other workers.

There was no significant difference among grass species on the amount of N transferred. The four grasses behaved alike,

although tall fescue and orchardgrass showed a slight advantage in the later harvest. According to Henzell (1962) the increase of N transference may be a function of the competitive ability of the grass component in the association. Thus, the advantage could be attributed to a greater competitive ability of these two earlier maturing species. Bromegrass and timothy, which are intermediate and late maturing species respectively, benefitted from the N transference process to a lesser extent than tall fescue and orchardgrass.

Total herbage yield of both grass alone and in mixture with alfalfa (Figure 7) indicated that all grass species in mixed stand produced higher yield than that of grass grown in pure stand. The advantage of adding a legume in mixtures to increase forage production is in agreement with that reported by Henzell (1962); Dilz and Mulder (1962); Hamilton et al. (1969); Haystead and Marriott (1978, 1979); Belzile et al. (1983). However, there was a significant difference among grass species. Tall fescue and orchardgrass were the most responsive species to N transference with significant increases of herbage yield. Bromegrass and timothy benefitted when grown in mixed stand, but both species were less productive than tall fescue and orchardgrass.

Total N yields of grasses were similar in pattern to herbage yields and followed an identical trend (Figure 8). The largest cumulative N was obtained from tall fescue and

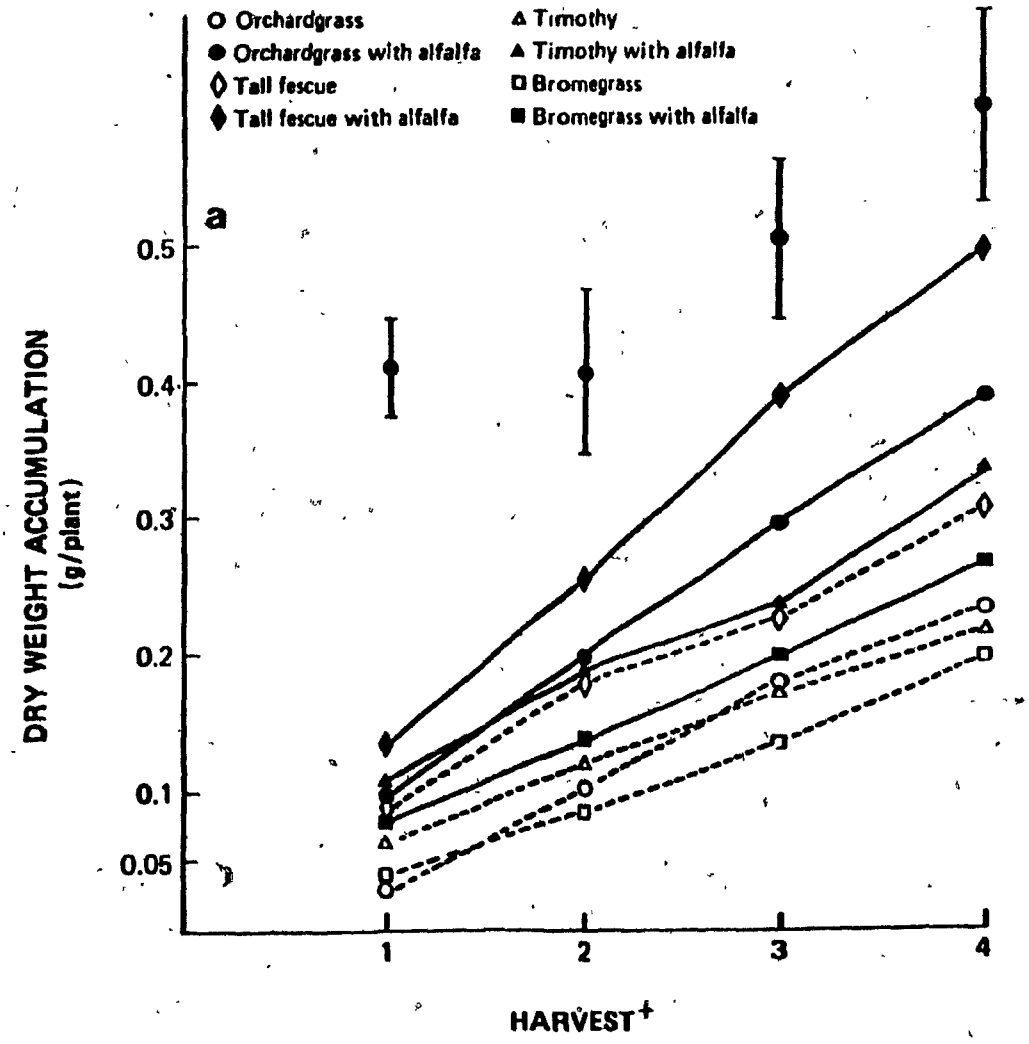

Figure 7. Accumulation of dry weight of grass plants in mono and mixed cultures, clipped periodically after planting February 14 (Experiment II). Each point is the mean of three replicates and the bar represents standard error (±SE) of the mean.
a. accumulation of dry weight of shoot
b. accumulation of dry weight of root + crown
c. accumulation of dry weight of whole plant
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

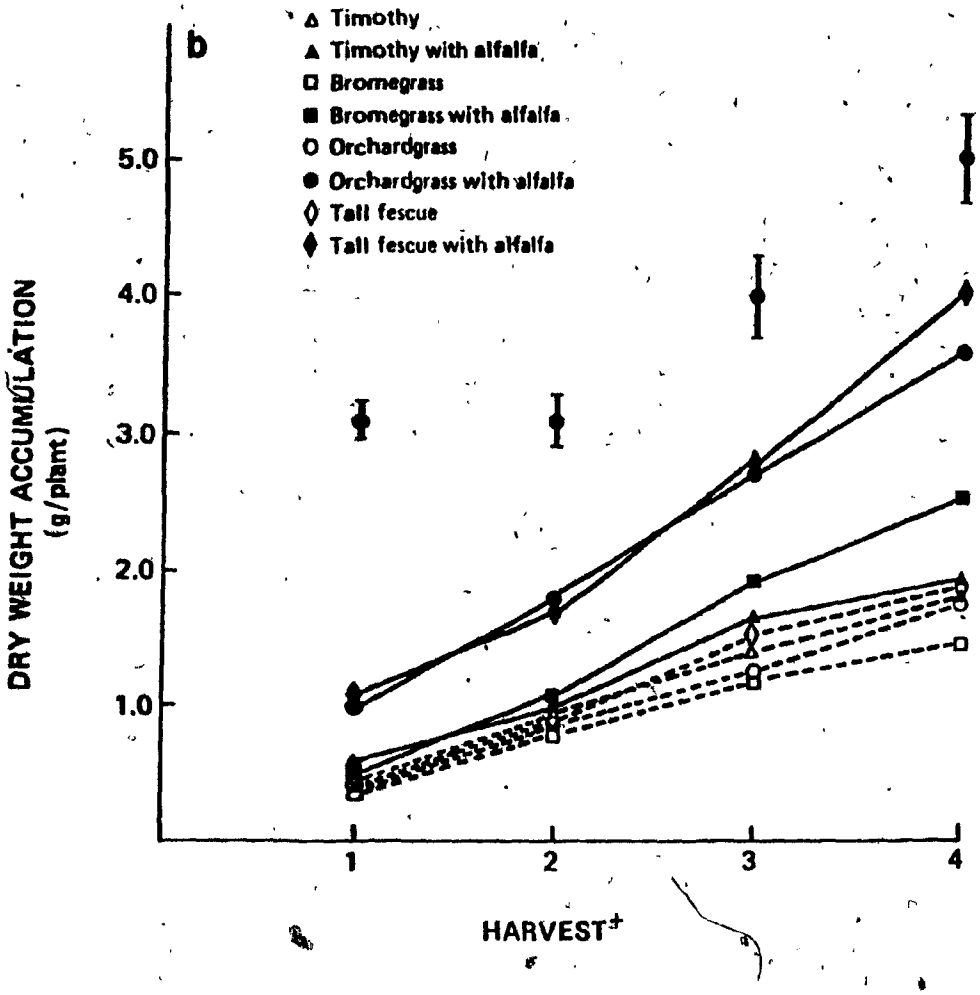

Figure 7. b. accumulation of dry weight of root + crown

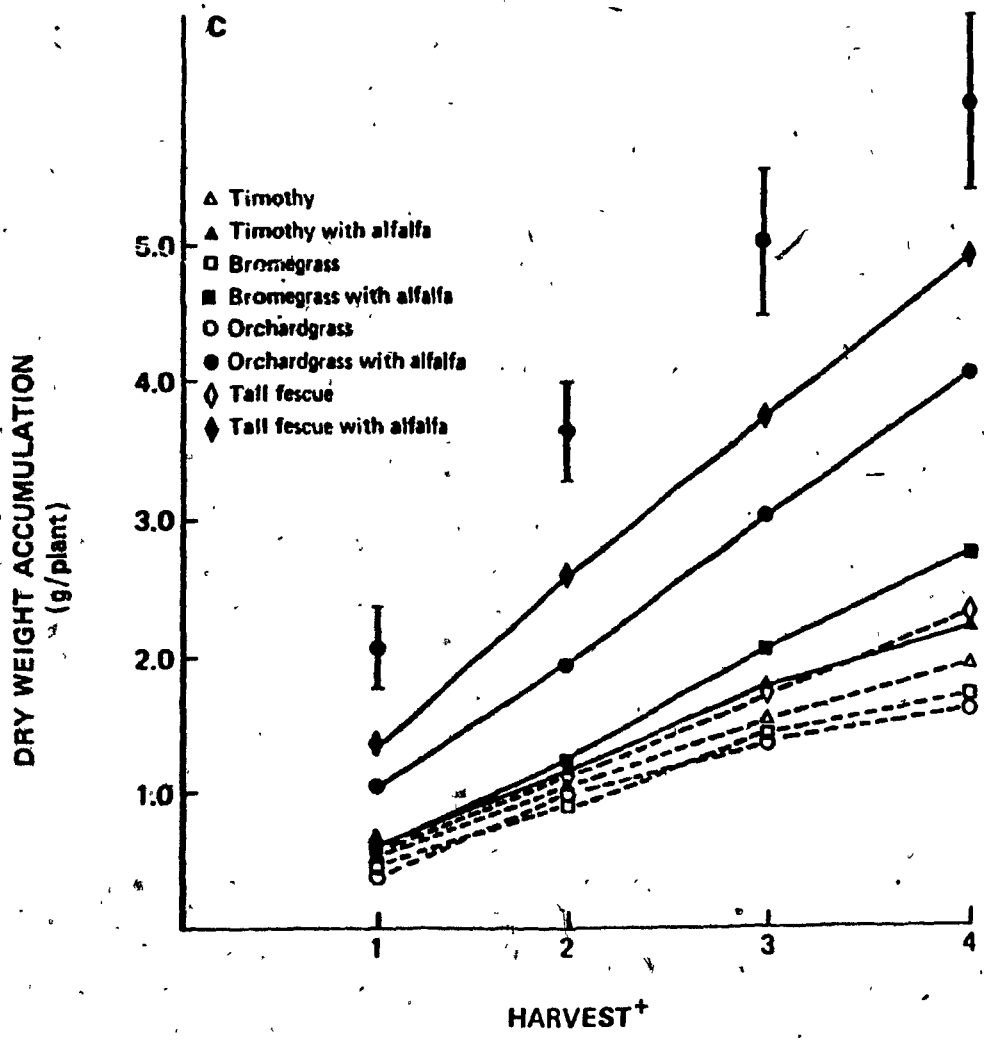

Figure 7. c. accumulation of dry weight of whole plant

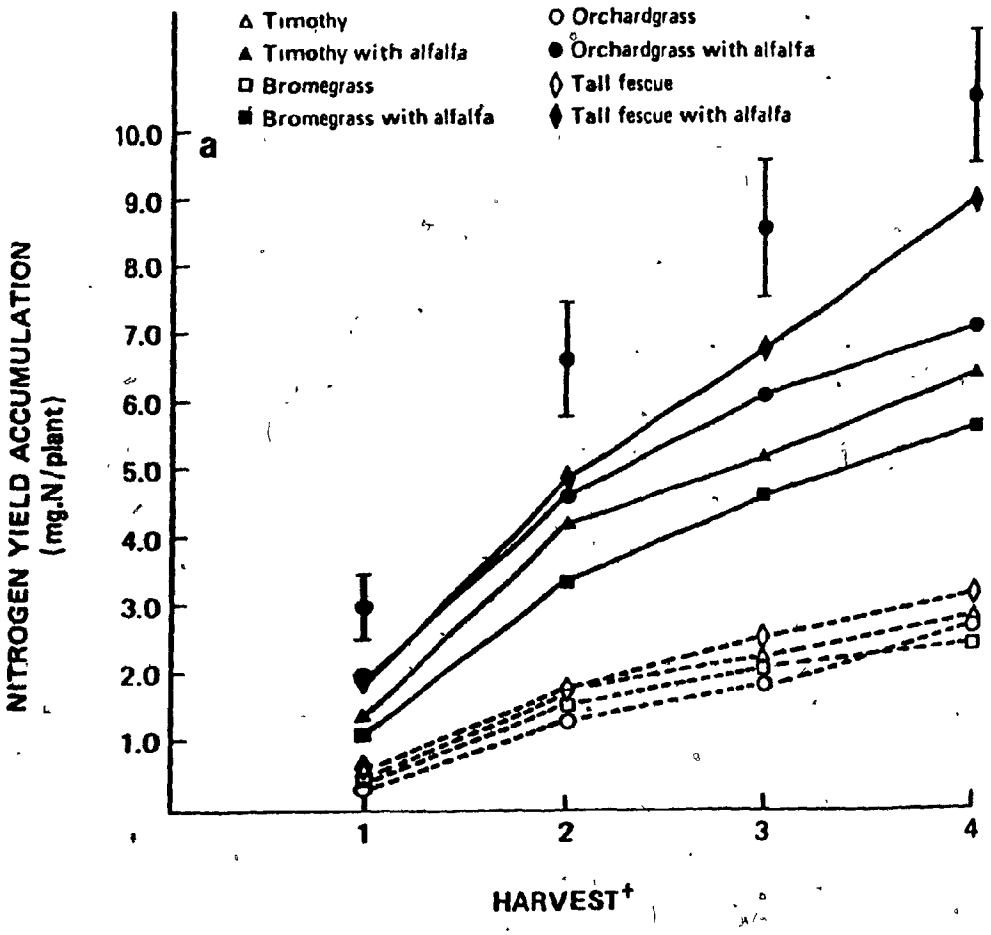

Figure 8. Accumulation of nitrogen yield of grass plants in mono and mixed cultures, clipped periodically after planting February 14 (Experiment II). Each point is the mean of three replicates and the bar represents standard error (±SE) of the mean.
a. accumulation of nitrogen yield of shoot
b. accumulation of nitrogen yield of root + crown
c. accumulation of nitrogen yield of whole plant
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

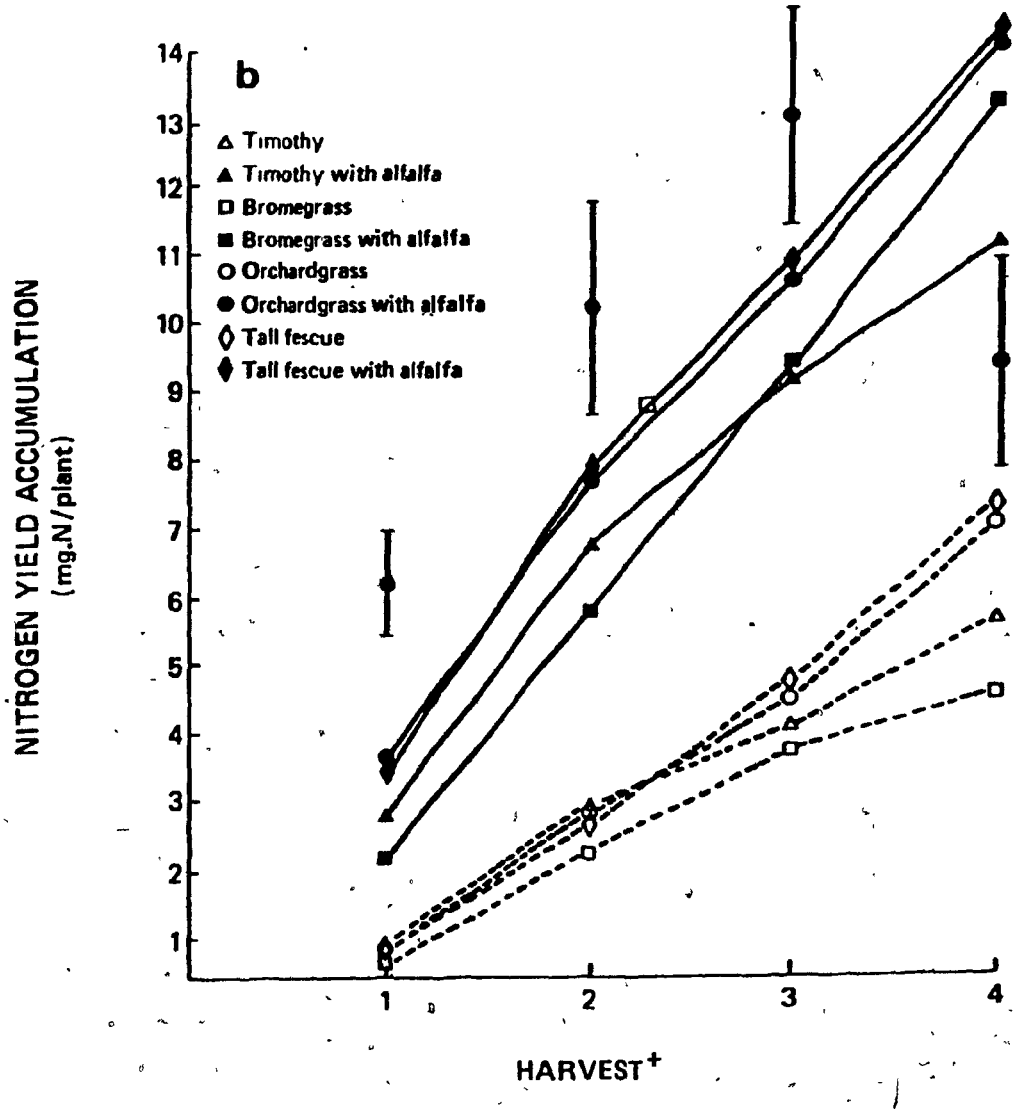

Figure 8. b. accumulation of nitrogen yield of root + crown

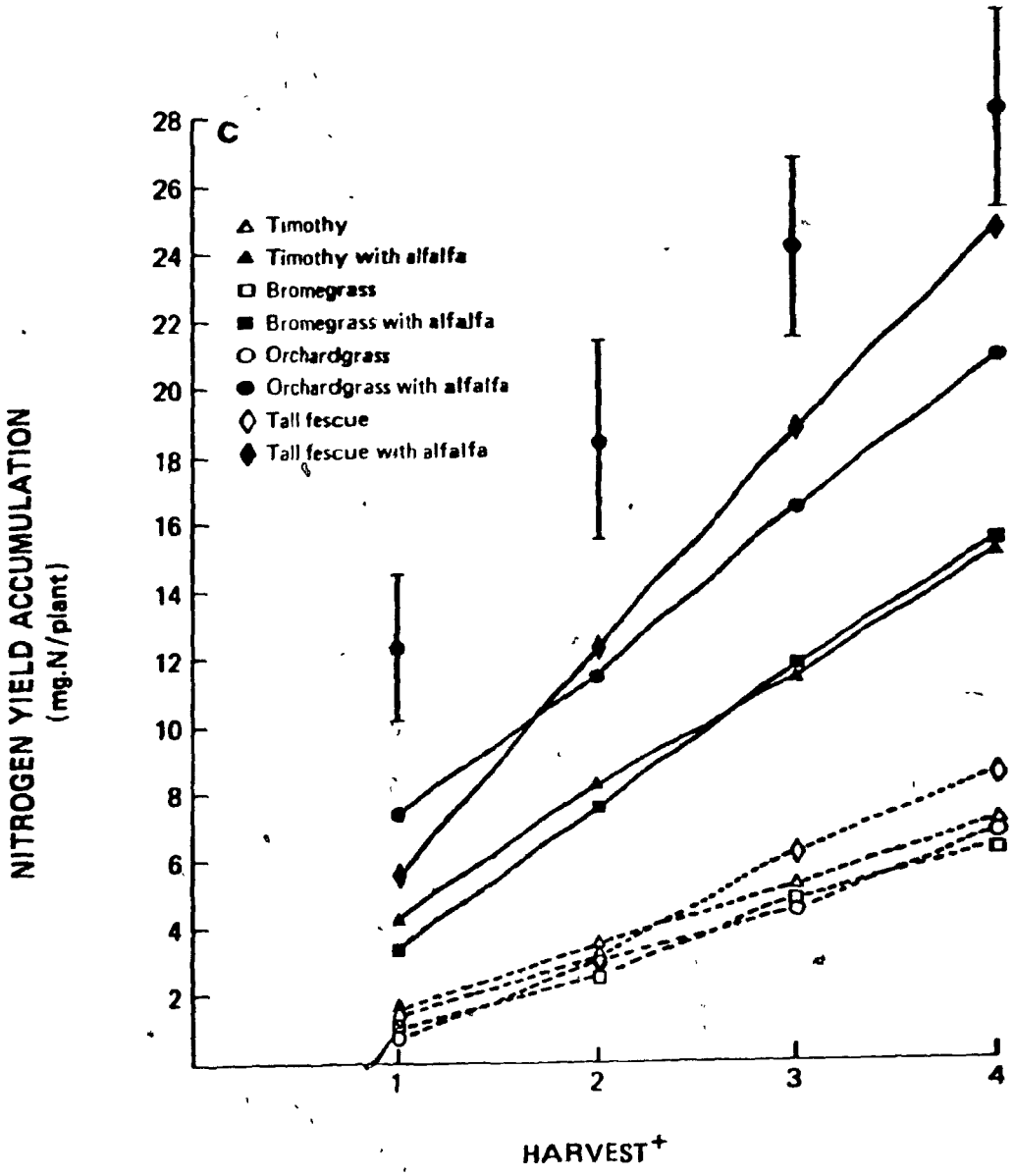

Figure 8. c. accumulation of nitrogen yield of whole plant

orchardgrass in mixture, and the smallest from timothy and bromegrass. This difference is a reflection of the slow growth and regrowth of timothy and bromegrass due to the fact that both grass species were harvested before heading. In clipping the experiment periodically, when alfalfa reached 50% bloom, the earlier grasses were more mature, and benefitted more in the mixed stand. Thus, the productivities of the grass species used in this study were different and appeared to be related to the growth habits and competitive abilities of the species.

4.1.4 Competition between alfalfa and grass

The competitive effects were conducted with objectives to evaluate the changes in each component species during the growing time, under greenhouse conditions. Then, any changes in the competitive ability of the species may not be related to the field experiments.

The competitive effects of alfalfa on grass, and vice-versa, expressed as the relative crowding coefficient (RCC) are shown in Figures 9 and 10. The competitive effect of alfalfa with respect to grass decreased significantly with harvest time (Figure 9). In contrast, the RCC of grass in comparison to alfalfa increased with time of clipping (Figure 10). The greater competitive ability of alfalfa in the early harvest was due mostly to the capacity of alfalfa to fix N_2 and the very low growth rate of the grass species under poor N medium. The

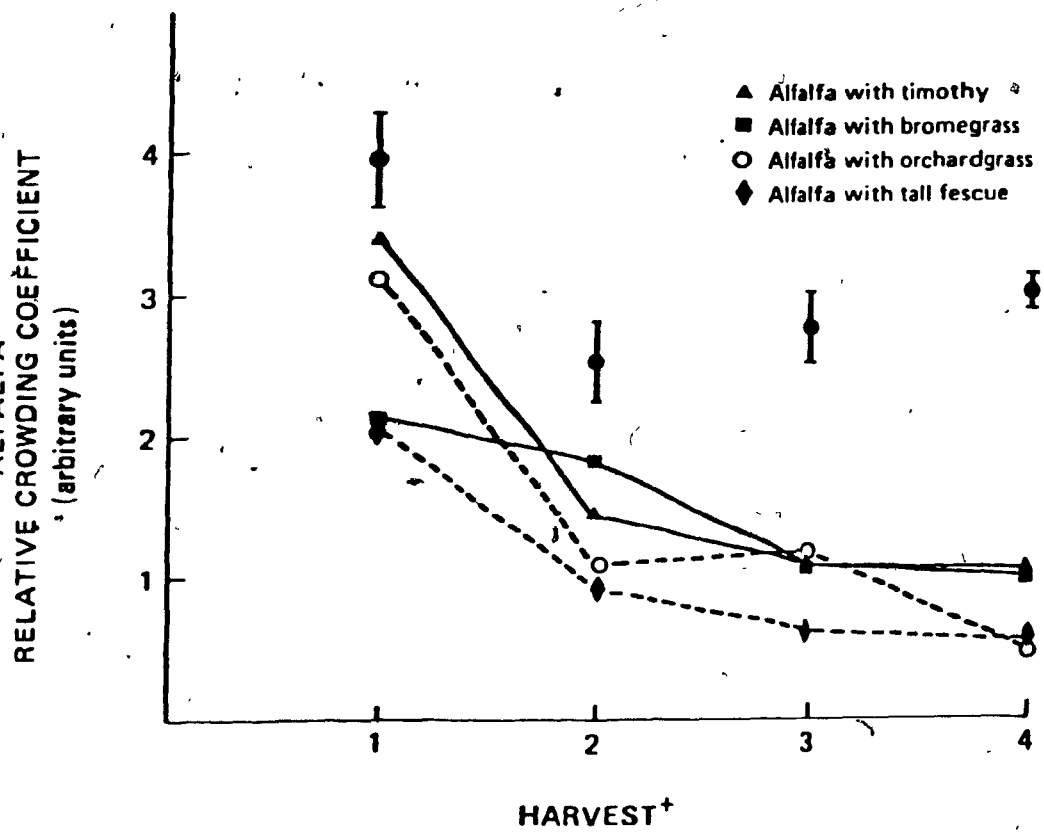

Figure 9. Changes in the relative crowding coefficients of alfalfa on grass at four harvests. Each point represents the mean of three replicates and the bar is standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

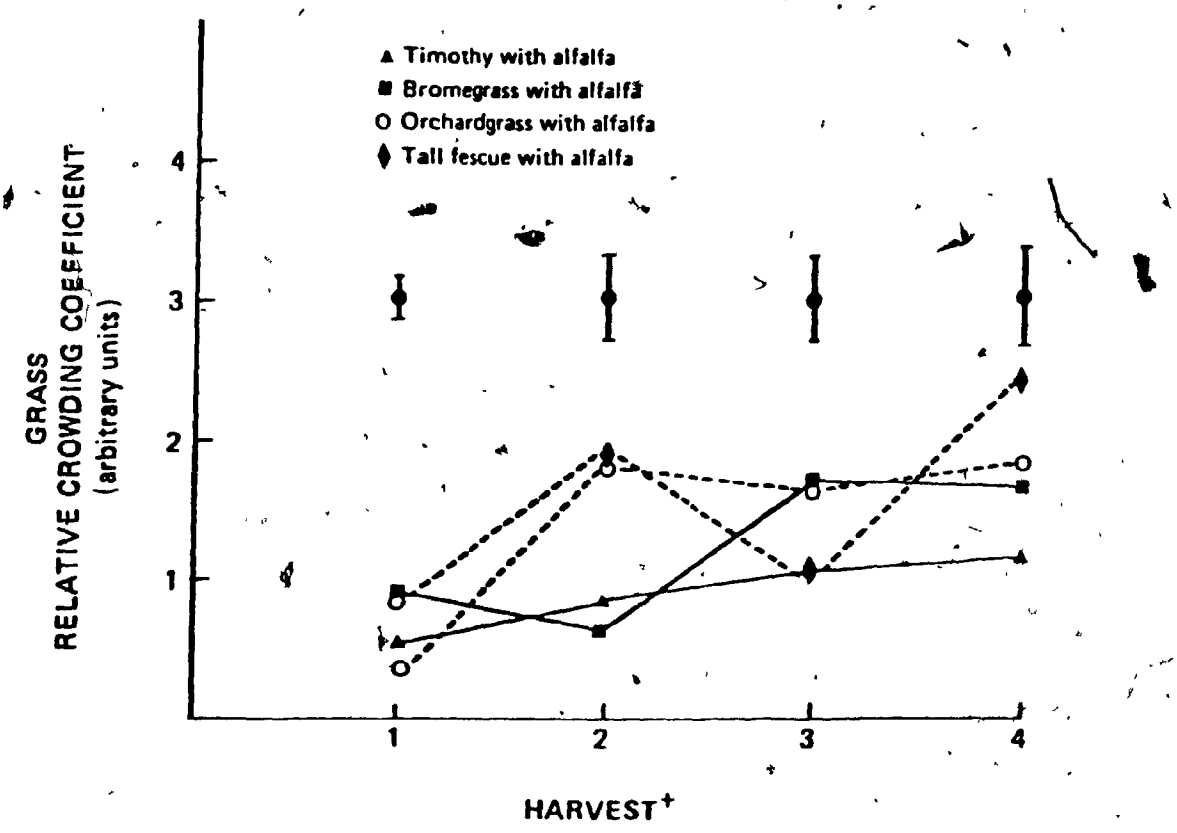

Figure 10. Changes in the relative crowding coefficients of grasses on alfalfa at four harvests. Each point represents the mean of three replicates and the bar is standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

increase of the competitive ability of grass after harvest 2 supported the conclusion that the grass species benefitted increasingly from the N transference process. It appears that clipping increased the competitive ability of grass when grown in mixed culture.

These differences can indeed be explained in terms of the effects of clipping on the two species. Both alfalfa and grass make demands on the N resources. The alfalfa plants however, are able to fix almost all their N requirements from the atmosphere until the energy requirements of this process from photosynthesis are cut off (Table 8). So clipping in this case produced favourable conditions for the growth of grass plants through the availability of the N compounds from any sloughed off nodules and root tissues (Butler et al. 1959) but more likely from leached alfalfa root N. Thus clipping decreased the competition of alfalfa against grass and increased that of grass on alfalfa. Chujo and Daimon (1984) reported that growth acceleration of the grass species increased in association with the legume even during the early stage of cultivation. Faris and Ta (1985) recently found a considerable N transfer during the initial clipping of the legume plants and this process increased with subsequent harvest times.

The enhancement of the competitive effect of grass species varied between harvests. Orchardgrass and tall fescue in harvest 4 tended to compete aggressively with alfalfa and almost

dominated the mixture, which is expressed with high RCC. These two grasses were slightly more responsive species to N transference (Figure 6), which corresponds to their accumulation of dry weight and N yield (Figures 7 and 8). Thus, the increase in competitiveness is related likely to the amount of N transferred from alfalfa and the efficiency of both grasses in utilizing the available N in the medium. Timothy was a species with an insignificant increase in competitive effect during the experiment, and it showed less productivity with clipping time.

The competitive ratio (CR) values presented in Figures 11 and 12 represent the degree of competition between alfalfa and grass in mixed culture. A CR value equal to 1 indicates that this crop has a higher competitive ability when compared with the other species in the mixture (Willey and Rao 1980; Faris et al. 1983). CR values followed the same pattern as the relative crowding coefficient values. In general, alfalfa was more competitive than the grasses specially at the early harvest as compared with the other three harvests. Nevertheless, in later harvests when the grass species benefitted from N transference, the competitive ability of grasses increased significantly. As a result, alfalfa decreased in its competitive ability.

Orchardgrass and tall fescue had a high CR in the later assessment, as compared with the other grasses. The increase in competitive ability and dry weight production by those grasses seems to be related to their growth habits associated with the

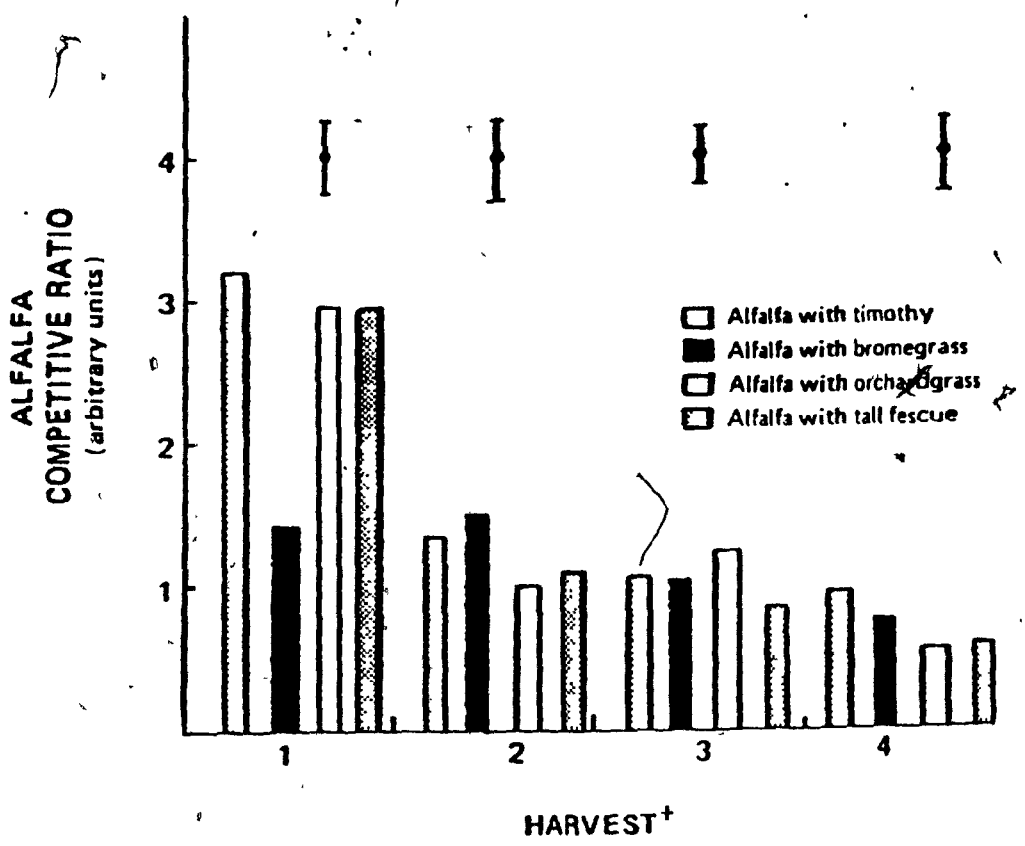

Figure 11. Alfalfa shoot competitive ratio in mixture with grass at four harvests. Each bar represents the standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

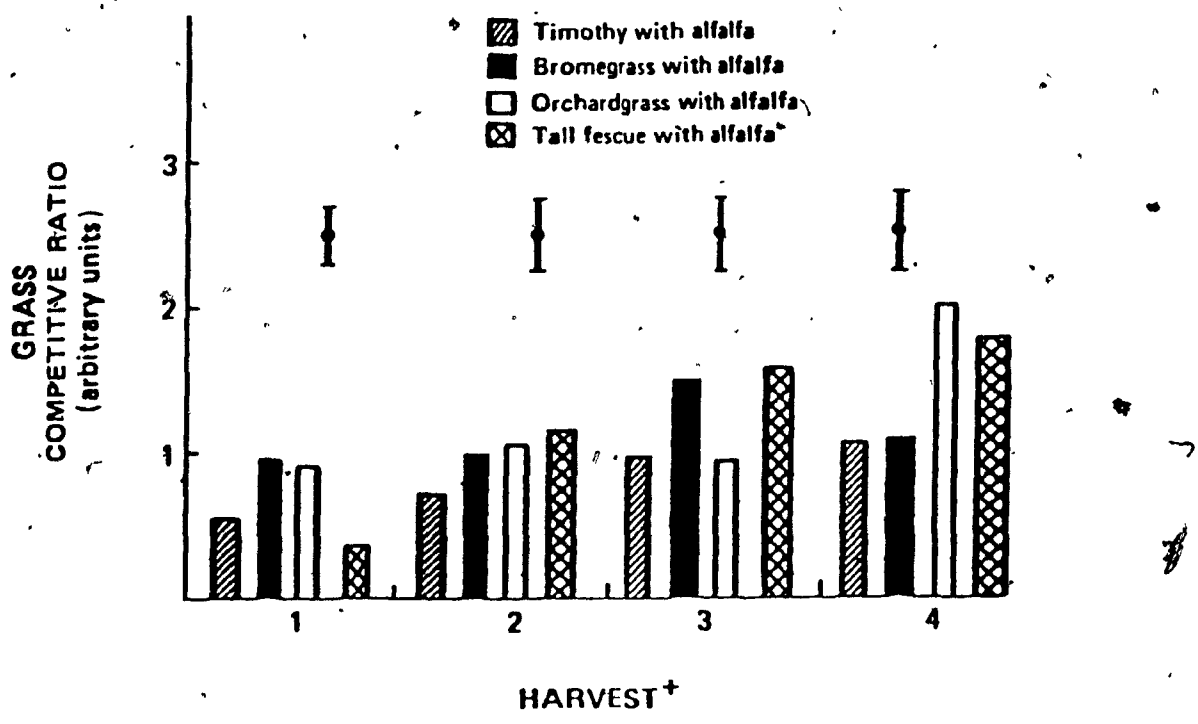

Figure 12. Grass shoot competitive ratio in mixture with alfalfa at four harvests. Each bar represents the standard error (±SE) of the mean.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

greater access to N from alfalfa. Bromegrass and timothy, which are intermediate and late maturing species, respectively, varied the competitive performance during the season.

The productivity of the alfalfa-grass mixed culture relative to the pure stand is shown in Figure 13. A relative yield total (RYT) greater than 1 indicates the yield advantage of a mixed culture over a monoculture (De Wit and Van de Bergh 1965). RYT was greater than 1 in all mixed systems. However, the composition of RYT differed between crops and among harvests. At the earliest harvest the relative yield (RY) values of timothy, orchardgrass and tall fescue were less than 1, and those of alfalfa were greater than 1. The main reason for the small grass RY value was the poor competitive ability of the grass. However, after the second harvest the RY of grass was always higher than alfalfa, demonstrating the beneficial effect of alfalfa to the development of grass in mixed cultures.

4.1.5 Comparison of methods for estimating N_2 fixation by alfalfa

Estimates of N_2 fixation by acetylene reduction assay (ARA), difference method (DM) and ^{15}N dilution technique (ID) are given in Table 11. As the interaction between measurement techniques and cultural systems was not significant, the values presented in Table 11 are the main effects of both. DM and ID technique gave a high estimate and ARA the lowest. DM and ID gave estimates of

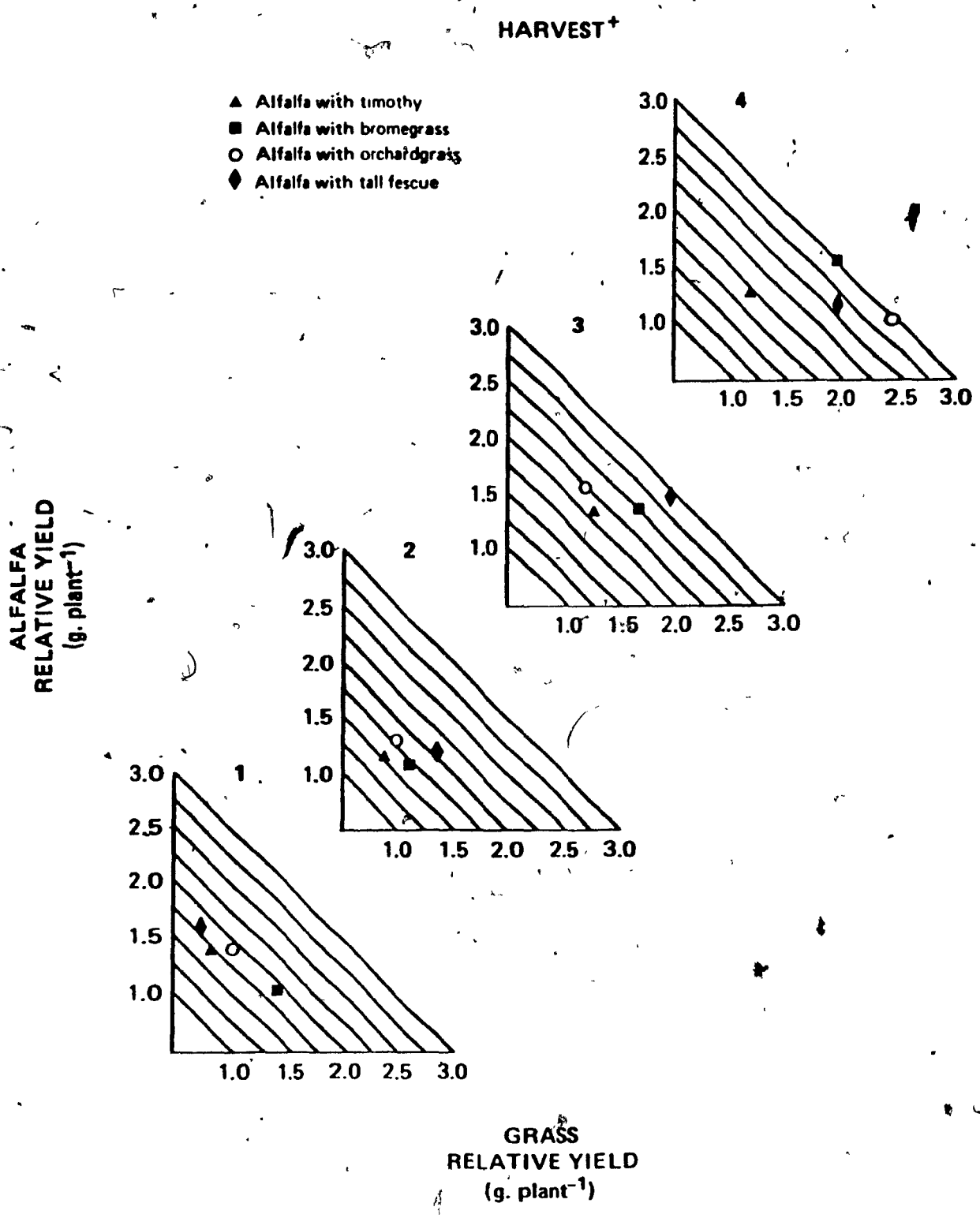

Figure 13. Yield advantage of alfalfa and grasses in mixed cultures at four harvests. Data expressed in relative total yield units.
+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

Table 11. Dinitrogen fixed by alfalfa in mono and mixed cultures when clipped periodically after planting February 14, measured by three techniques (Experiment II). Values in parentheses are the standard error of the mean for each technique.

Measurement Technique or Cultural System	N_2 fixed Harvest+ 1	2	3	4	Mean+++
	mg N/plant				
Acetylene Reduction	9.37b (3.22)	6.01b (2.23)	5.54b (2.09)	4.46b (1.72)	6.35b
Isotope Dilution	16.20a (2.23)	16.22a (2.12)	15.50a (2.02)	21.26a (2.07)	16.54a
Difference Method	16.20a (2.92)	17.42a (2.20)	17.63a (3.62)	18.74a (2.90)	17.49a
Cultural System					
Alfalfa alone	11.74b	11.30b	11.24a	12.02b	11.57b
Alfalfa with grass	16.09a	15.13a	12.53a	17.62a	15.34a
Mean++	13.91a	13.21ab	11.89b	14.82a	13.46
±SE	1.70	1.53	2.22	2.6	2.72

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Harvest 1 performed 23 May 1984; harvest 2 performed 15 June 1984; harvest 3 performed 17 July 1984 and harvest 4 performed 24 August 1984. All harvests were performed at 50% bloom stage, 4 to 5 weeks after initial harvest on 23 May 1984.

++Means compared horizontally
+++Means compared vertically

N_2 fixation that were not different significantly. Although, the assessments of N_2 fixation by DM exceeded those produced by ID by an overall average of 5.7%. This suggests that the DM will provide a representative N_2 fixation value where the the medium conditions permit proper development of control plants. For example, at harvest 1, ID and DM produced essentially the same values (16.20 mg N/plant), independent of the cultural systems.

These findings concur with those reported by several other researchers, but conflict with certain other results. Legg and Sloger (1975) after incorporating ^{15}N labelled fertilizer N into the soil, found excellent agreement between the DM and ID throughout the season. Williams et al. (1977) determined the relation between the two methods and found that they are strongly linear (r=0.98). Previous values cited for N_2 fixation measured by the DM for annual legumes, however, may have been underestimated by about 40% (Holland et al.1969). Martensson and Ljunggren (1984) compared the derived total N_2 fixation values from DM and ID, and concluded the ID gave a significantly low value of N_2 fixed. Coale et al. (1985) compared ID and DM to quantify the %Ndfa and the amount of N_2 fixed. These comparisons showed that there was no difference between methods. Nevertleless, there was a difference in the precision of the methods. The ID consistently had a lower standard deviation than DM for both parameters. Rennie (1984) stated that DM was a reliable method in experiments in which soil N level

was low so that non-fixing plants showed signs of N deficiency. He concluded that DM can be used with reasonable confidence to estimate N_2 fixation in field grown legumes, when the fertilizer use efficiency of the fixing system is identical to that of the non-fixing system. However, the ID method offers greater sensitivity compared to the DM (Ruschel et al. 1979; Talbott et al. 1982; Broadbent et al. 1982), which is an advantage when measuring relatively low rates of N_2 fixation. Several authors have also commented on the higher precision of ID estimates of %Ndfa compared to yield-dependent estimates based on total N difference (Ruschel et al. 1979; Talbott et al. 1982; Rennie and Rennie 1983; Rennie 1984).

The estimates by ARA were always significantly lower than those by the other two methods. A picture of the acetylene reduction through the growing season is shown in Table 4. The amount of N_2 fixed was calculated by converting the rates of acetylene reduction to rates N_2 reduction with a conversion factor of 3:1, and integrating the daily values for the season and adjusting for circadian variation of the nitrogenase enzyme function. The total amount of N_2 fixed was estimated to be an average 4.46 to 9.37 mg N/plant. Thus, ARA method gave the lowest N_2 fixation values and the error was notable due to the great variation of nitrogenase activity among alfalfa plants. These variations may be explained by the facts that a mixture of Rhizobium strains was used in association with the genetical

variations that occur within the alfalfa population (Tough and Crush 1979). In spite of the variations already mentioned, the ARA provides a useful test for studying the nitrogenase enzyme activity. However, many precautions are necessary when using the ARA for estimating total N_2 fixed by alfalfa, and it is possible that still further precautions are required.

Estimates of N_2 fixation from alfalfa grown in mixed culture was always significantly greater than that grown alone. Under these conditions, the results suggest that grass may stimulate N_2 fixation of alfalfa when both are grown in association. This is likely due to the utilization by the grass of N excreted into the medium by the alfalfa. A comprehensive review of alfalfa-grass mixture research showed that legumes secrete nitrogeneous materials that are used efficiently by associated grasses. Thus, if the grass removes this N from the medium, there will be no inhibitory effect of these nitrogenous compounds on the N_2 fixation process.

Our data are consistent with the results of others (Ruschel et al. 1979; Broadbent et al. 1982; Talbott et al. 1982; Henson and Heichel 1984; Vasilas and Ham 1984; Coale et al. 1985) and suggest that the ID technique is the method that provides the best estimates compared to the ARA and DM, when the most precise field measurements of N_2 fixation are needed. However, the similarity of the results between the DM and ID techniques suggest that the former may be adequate when resources for

isotope experiments are limiting. Also, the results indicated that mixed cultures of alfalfa with grass have no detrimental effect on the ability of alfalfa to fix N_2, indeed the presence of the grasses may increase alfalfa N_2 fixation.

4.2 Field studies

4.2.1 Experiment III

Experiment III was conducted over a two year period to examine the seasonal changes in nitrogenase enzyme activity of alfalfa in pure and mixed cultures. The other growth variables dry matter, plant height, N yield and N benefit through the vegetative growth and regrowth in successive harvests were also examined.

4.2.1.1 Seasonal changes in the growth and nitrogenase activity of alfalfa

The inclusion of grasses in the mixtures did not have any significant effect on dry weight per plant of alfalfa (Figure 14). However, in the second year, dry weights in mixture tended to be higher than in pure stand later in the season. After harvest, shoots of the plants regrew slowly up to 9 days. From day 9 to 20 there was a rapid increase in shoot weight which

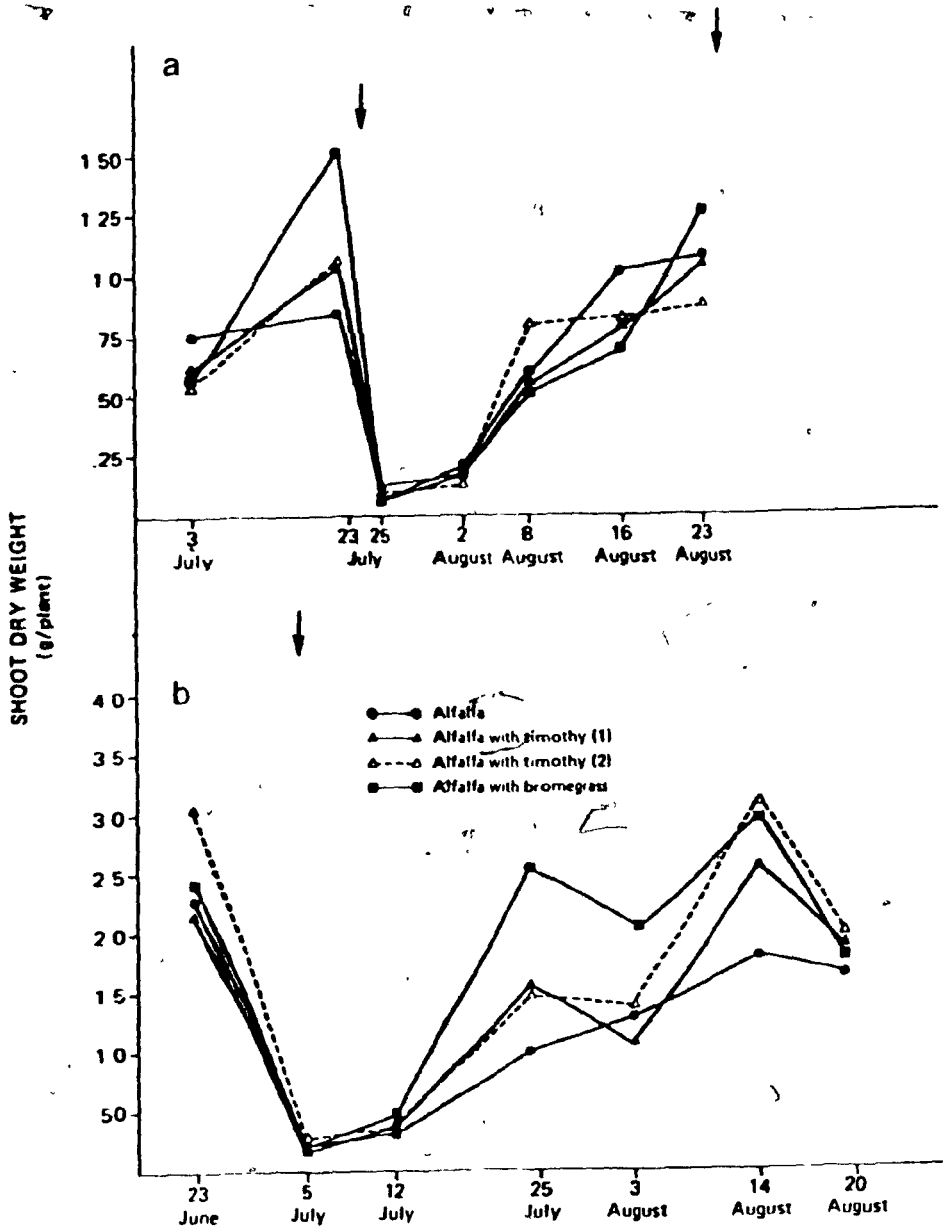

Figure 14. Seasonal changes in shoot dry weight of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. seeding year, 1983
b. subsequent year, 1984
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

reached the initial values by day 30 in the seeding year and by day 40 in the second year. Dry weight of alfalfa plants in the second year after harvest was almost twice as high as in the first year.

There were no significant differences in the seasonal variation of total nitrogenase activity (TNA) between alfalfa in mono and mixed stands throughout the experimental period (Figure 15). The average TNA of alfalfa plants was 383 nmoles C_2H_4/h. plant for the first 36 days and increased to 432 nmoles C_2H_4/h. plant by day 50. Moreover, the TNA declined about 70% within 48 h. after harvest for both years (Figure 15). There was a major recovery of TNA from day 9 to 15, after harvest and by day 23 the activity rose to a maximum of 550 nmoles of C_2H_4/h. plant. In 2 year old alfalfa plants, the TNA measured in spring (June 23) averaged 273 nmoles C_2H_4/h. plant, but declined significantly to 28.5 nmoles C_2H_4/h. plant within 24 h. after shoot removal. However, 20 days after harvest the nodule activity had returned to the previous levels as measured on June 23 (Figure 15b). Despite the similar proportional decrease of 71 and 79% in nodule activity after cutting in the seeding year and in the second year plants, respectively, the recovery rate of alfalfa TNA in the later was faster than in the seeding year and reached higher levels.

The decline of TNA of alfalfa with herbage removal and its

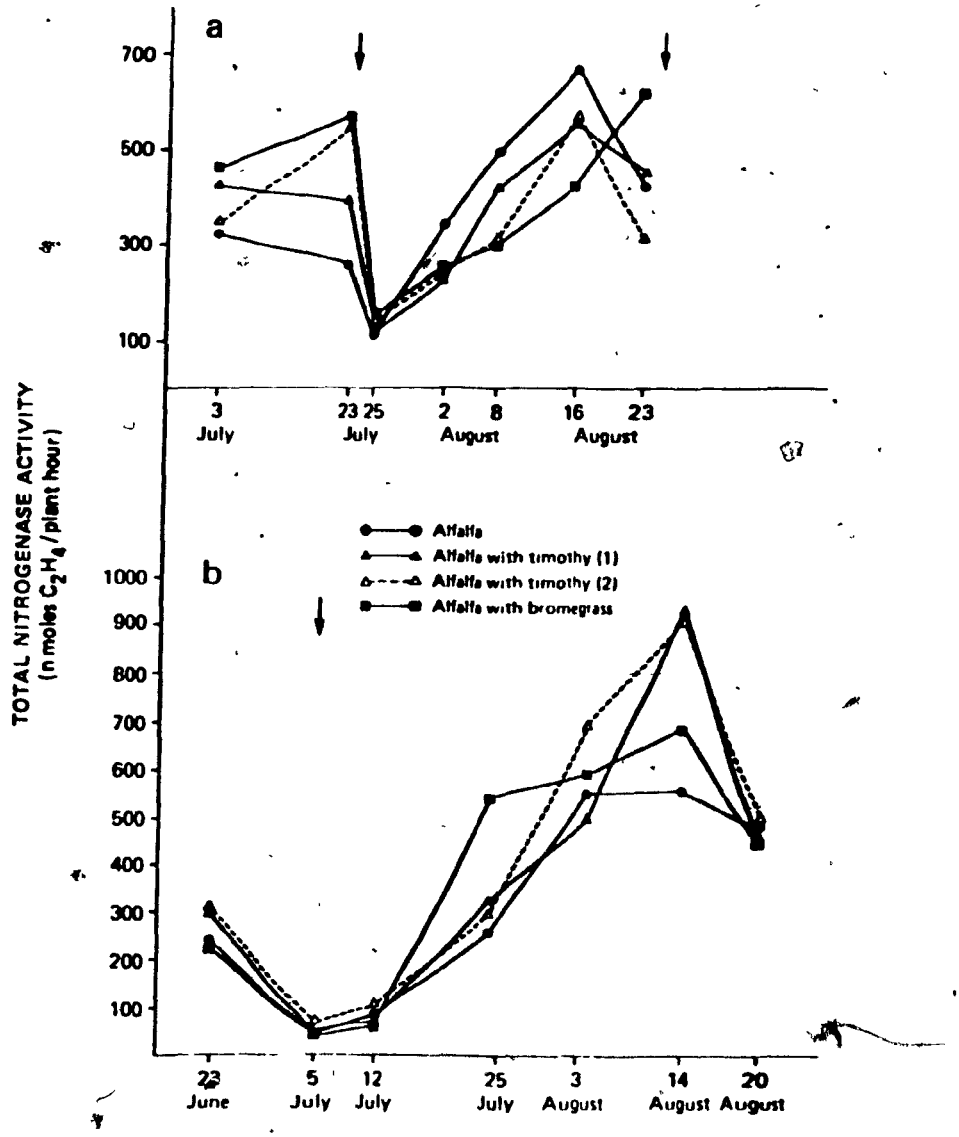

Figure 15. Seasonal changes in total nitrogenase activity of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. seeding year, 1983
b. subsequent year, 1984
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

recovery with the onset and extent of the vegetative regrowth were similar to that observed in white clover (Moustafa et al. 1969; Sinclair 1972) and similar to the effect of stem girdling in soybean (Hardy and Havelka 1975) and shading in peas (Raponen 1970). In fact, removal of stems and leaves interrupts the supply of current photosynthate that are essential for nodule formation, function (Butler et al. 1959; Whiteman 1970), maintenance (Moustafa et al. 1969; Vance et al. 1979; Cralle and Heichel 1981), illustrates the interdependence of N_2 fixation and canopy photosynthate capacity. The rapid decline in TNA of alfalfa plants supports the substantial evidence that shoot removal causes temporary senescence of the nodule and that the recovery of nodule activity depends upon vegetative regrowth (Figures 15). The reduction of TNA after harvest is in agreement with data obtained from other legume species (Wilson 1942; Butler et al. 1959; Whiteman 1970). However the maintenance of nodule activity at low levels (123.9 nmoles C_2H_4/h. plant and 60.3 nmoles C_2H_4/h. plant in the first and second year, respectively) points out the role of reserve carbohydrate in the root, vascular system or nodule in sustaining nitrogenase activity, so that the capacity for N_2 fixation is only temporarily impaired. The recovery of TNA was faster in the second year of alfalfa than in the first year, demonstrating that the larger root system of the older alfalfa had greater ability to supply reserve carbohydrates to support vegetative regrowth.

The specific nodule activity (SNA) (Figure 16) showed a pattern parallel to that observed for TNA. The nodule effectiveness, however, was almost equal for alfalfa in pure stand and for alfalfa in association with grass. Harvest caused a significant decrease of SNA in the seeding year. However, in 2 year old alfalfa, there was a slight increase 7 days after harvest, although the SNA values at spring (June 23) were lower as compared with those after harvest (Figure 16a). This could be explained by the effect of carbohydrate competitive sink for seed formation in late bloom stage of alfalfa. This agrees with the evidence presented by Cralle and Heichel (1981) that nodules are weak sinks compared with other tissues. Also, the chilling injury may limit the N_2 fixation capability after the nodules were exposed to cold winter temperatures.

The rate of recovery of SNA in seedlings and in older plants of alfalfa was proportional to the rate of herbage regrowth (Figure 14). Although the recovery of SNA was fast in both cultural systems, the nodule activity in older alfalfa plants reached higher values than in young plants, and the same trend was also observed for N yield (Figure 17). This is consistent with the fact that older plants usually fix more nitrogen by symbiosis than younger ones. Sheehy et al. (1980) reported that any alteration in the photosynthetic rate over a sufficiently long period of plant growth associated with nodule mass will be reflected in the nitrogen accumulation from the fixation process.

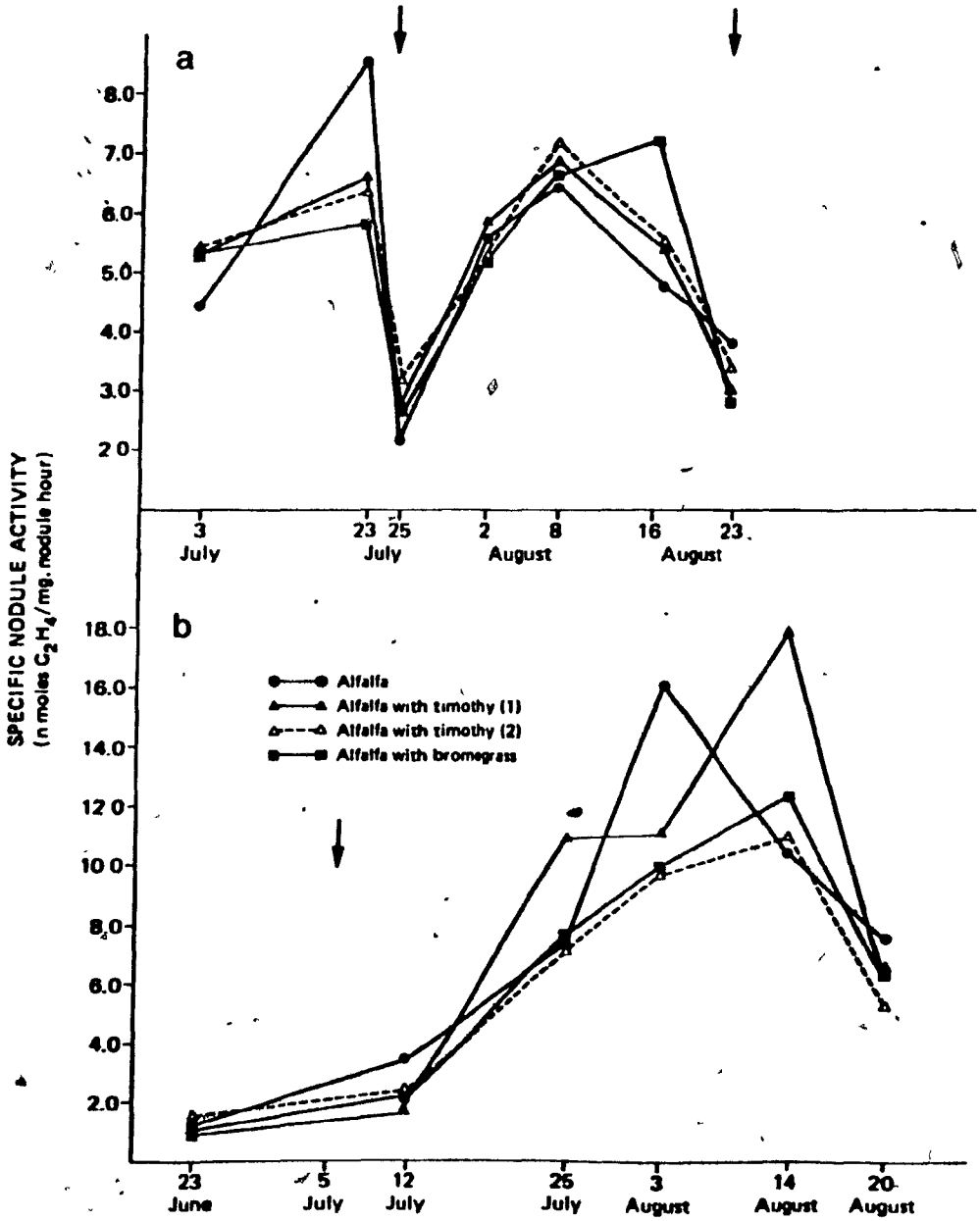

Figure 16. Seasonal changes in specific nodule activity of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. seeding year, 1983
b. subsequent year, 1984
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

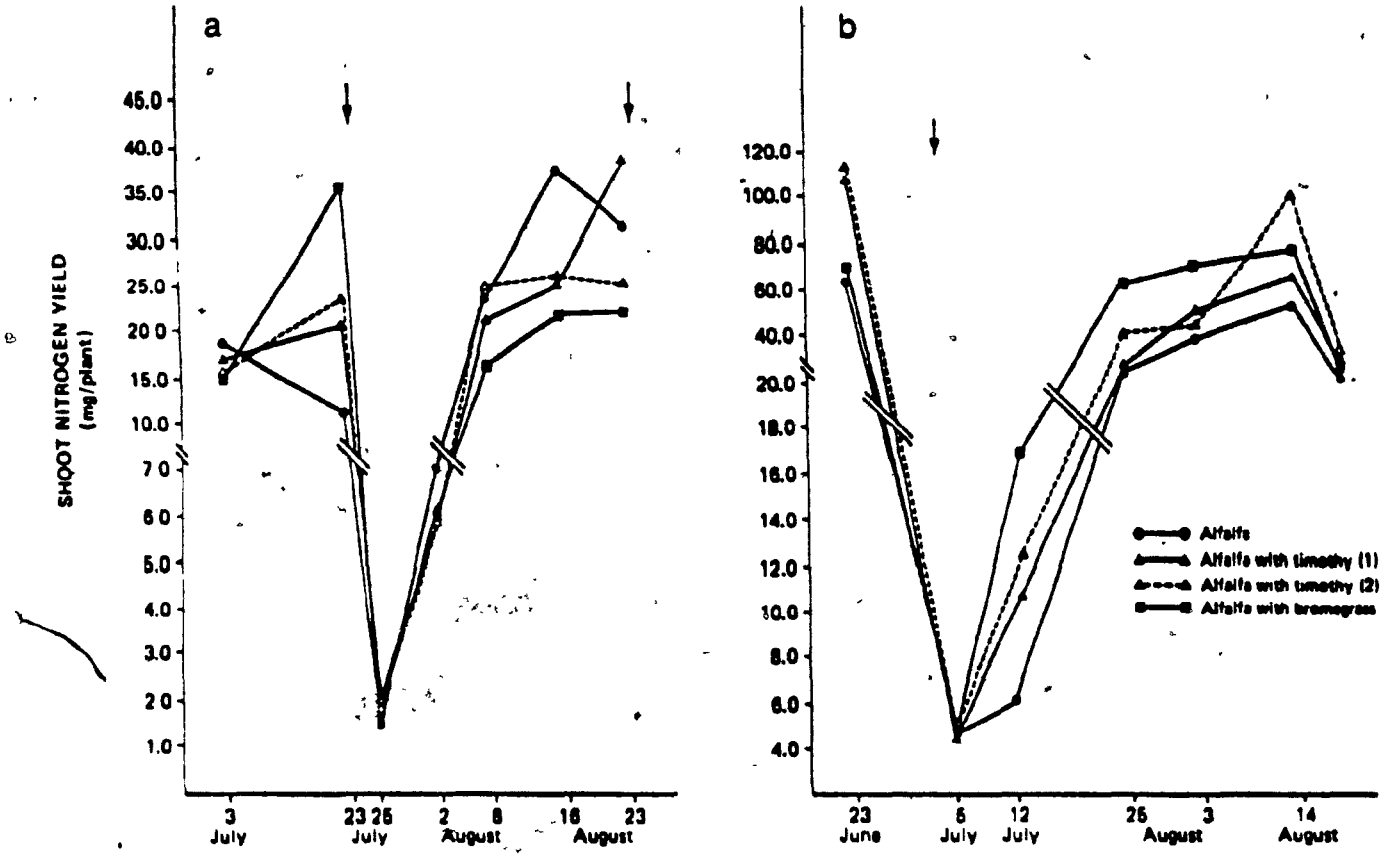

Figure 17. Profiles of nitrogen yield of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. seeding year, 1983
b. subsequent year, 1984
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

The significant decrease of SNA assessments conducted at August 23 in the seeding year, and at August 20 in the second year could be attributed to decreasing temperatures and to the advanced stage of alfalfa plants. Extreme temperatures are known to reduce nitrogenase activity in nodules of both annual and perennial legumes (Day and Dart 1969; Masterson and Murphy 1976; Munns et al. 1977). Using intact nodules of alfalfa, Day and Dart (1969) found a linear increase of nitrogenase activity from 5 to 25°C. While Cralle and Heichel (1982) who plotted log nodule activity versus absolute temperature, found a non-linear increase in nitrogenase activity between 20 and 35°C. Similar patterns of increase have been reported for the nitrogenase activities of several legumes species (Hardy et al. 1968). The chilling injury of nodules may be related to the biological change of nitrogenase enzyme (Moustafa et al. 1969) or to rapid change in the membrane permeability of the nodules (Levitt 1972). Undoubtedly, the responses of nitrogenase enzyme activity to temperature vary with the ontogeny of the plant (Cralle and Heichel 1982).

4.2.1.2 Seasonal changes in nodule weight of alfalfa

Shoot removal caused no significant loss of nodule weight in the seeding year, however a significant loss of nodule weight was observed in 2 year old alfalfa after harvest (Figure 18).

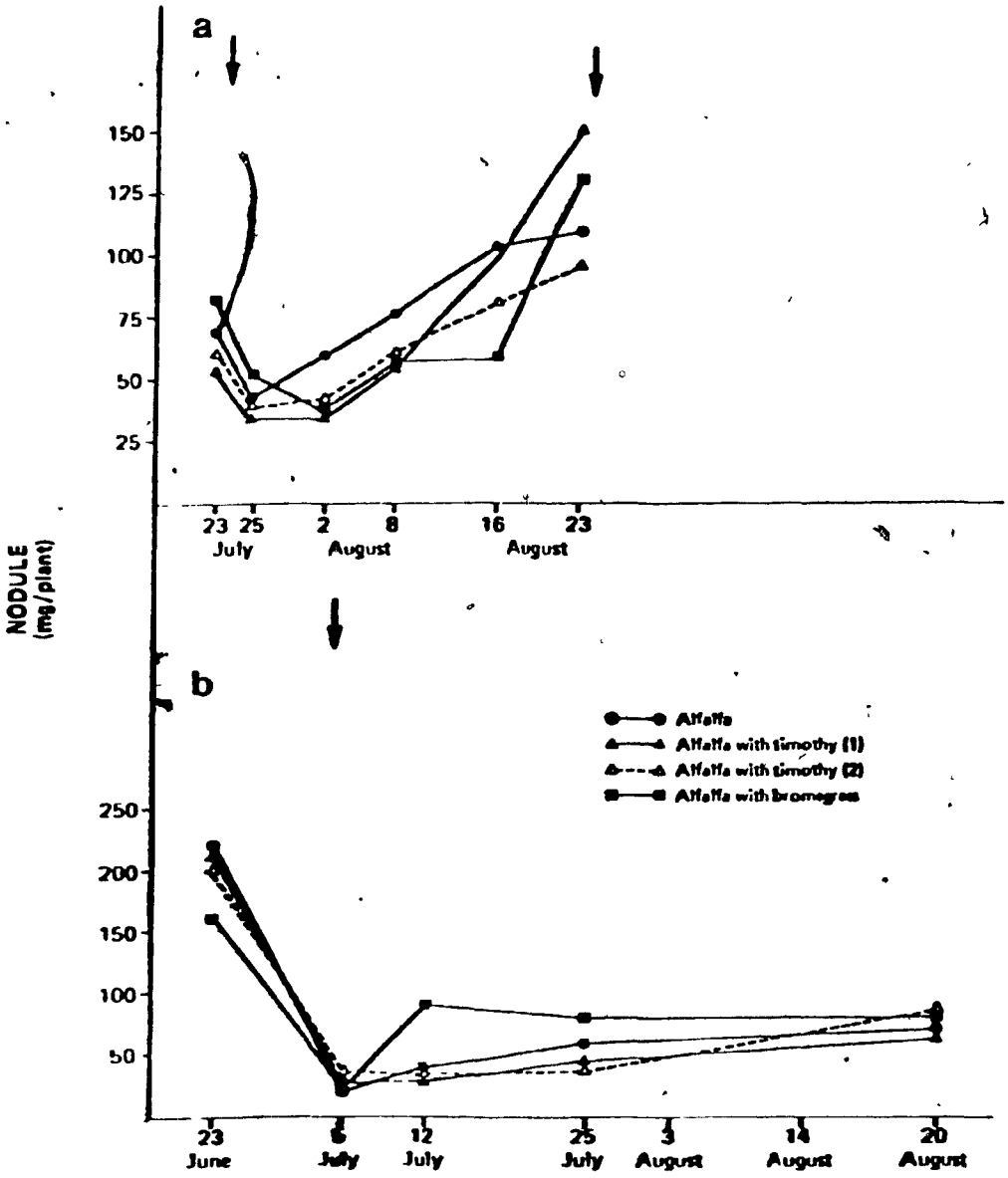

Figure 18. Seasonal changes in nodule weight of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. seeding year, 1983
b. subsequent year, 1984
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

The nodule fresh weight after initial harvest declined slightly and remained constant from day 2 to 10 and then increased between days 10 to 30 (Figure 18a). Nodule weight of 2 year old alfalfa plants decreased from about an average of 200 mg per plant to 30 mg per plant, after shoot removal. Thereafter it increased slightly until the end of the experiment. In other forage legumes, (red clover, white clover and birdsfoot trefoil) defoliation or grazing has been shown to cause shedding of nodules with apparent reinfection and formation of new nodules (Wilson 1942; Butler et al. 1959; Whiteman 1970). The data from seeding year alfalfa plants in the present study suggest that alfalfa have the ability to adapt to the stress of shoot removal by maintaining an unchanged or slightly reduced nodule weight. Vance et al. (1979) reported that the maintenance of nodule mass by alfalfa plant after harvest was attributable to ability of the nodules to continue growth and the prolonged survival of a functional apical meristem on the elongated nodule. This may be important in explaining why the capacity of N_2 fixation by alfalfa is only temporarily impaired (Figure 15), and why alfalfa has a rapid regrowth potential as compared to other forage legumes. The decrease of nodule weight (Figure 18) encountered in 2 year old alfalfa after harvest could be attributed to the senescence or to the decay of older nodules that had overwintered.

4.2.1.3 Profile of the effect of alfalfa on grass species

The association of alfalfa with grasses increased grasses total nitrogen (TN) concentration before and after initial harvest during the seeding year (Tables 12 and 13) and the subsequent year (Tables 14 and 15). However, the increase of % TN in the second year was more marked than the first year. The enhancement of grass N content and crude protein by association with legumes has previously been reported (Dilz and Mulder 1962; Birch and Dougall 1967; Hamilton et al. 1969; Dubbs 1971; Chestnutt 1974; Haystead and Lowe 1977; Haystead and Marriott 1978, 1979; Craig de Anda et al. 1981; Kroth et al. 1982; Belzile et al. 1983).

In the seeding year, TN content of grasses grown in association did not vary in the same extent as grasses grown in pure stand, but these values tended to decrease during the growing season. The result indicated that in the earlier stage of development, there was a relative abundance of available soil N to support the initial phase of the growth. However, at the end of season the depletion of soil N, mainly under grasses in monoculture resulted in the reduction of TN. The inclusion of alfalfa significantly increased the TN of grasses in almost all estimates, with the exception at July 3 in the seeding year. Although this increase did not correspond to the increase in N yield (Figure 19). This may be due to the competition between

Table 12. Total nitrogen concentration (%) in shoots of grasses for the various mono and mixed cultures sampled before harvest and during herbage regrowth in the seeding year, 1983 (Experiment III)+.

TREATMENT	Determination 1 3 July	2 23 July	3 25 July	4 2 Aug.	5 8 Aug.	6 16 Aug.	7 23 Aug.
	(Total Nitrogen Concentration %)						
Timothy (1) with alfalfa	3.06a	2.26ab	2.09b	2.24b	2.19a	1.94b	2.10a
Timothy (2) with alfalfa	2.91a	2.31ab	2.24a	2.45a	1.80b	2.35a	2.08a
Bromegrass with alfalfa	3.08a	2.36a	2.29a	2.49a	1.79b	1.89bc	2.09a
Timothy (1)++	3.02a	1.74d	1.86c	2.17b	1.90b	1.77dc	1.82b
Timothy (2)	2.92a	2.04bc	1.99bc	2.20b	1.48c	1.73d	1.75bc
Bromegrass	2.80a	1.98cd	1.95bc	1.89c	1.55c	1.70d	1.72c
Mean	2.97	2.12	2.07	2.24	1.79	1.89	1.92
± SE	0.28	0.19	0.11	0.10	0.14	0.09	0.06

Mean in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+ Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983.

++ Timothy (1) cultivar 'climax'
Timothy (2) cultivar 'salvo'

Table 13. Total nitrogen concentration (%) in roots of grasses for the various mono and mixed cultures sampled before harvest and during herbage regrowth in the seeding year, 1983 (Experiment III)+.

TREATMENT	Determination 1 3 July	2 23 July	3 25 July	4 8 Aug.	5 16 Aug.	6 23 Aug.
	(Total Nitrogen Concentration %)					
Timothy (1) with alfalfa	0.72a	0.71b	0.67ab	0.72b	0.64c	0.75b
Timothy (2) with alfalfa	0.70a	0.82ab	0.73ab	0.89a	0.92a	0.93a
Bromegrass with alfalfa	0.73a	0.80ab	0.79a	0.60c	0.78bc	0.81b
Timothy (1)++	0.68a	0.70b	0.65bc	0.56c	0.52dc	0.42d
Timothy (2)	0.59b	0.60c	0.66b	0.49d	0.53d	0.38d
Bromegrass	0.58b	0.57c	0.54c	0.40e	0.55d	0.60c
Mean	0.67	0.70	0.67	0.60	0.65	0.64
±SE	0.06	0.06	0.08	0.05	0.06	0.06

Mean in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+ Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983.

++ Timothy (1) cultivar 'climax'
Timothy (2) cultivar 'salvo'

Table 14. Total nitrogen concentration (%) in shoots of grasses for the various mono and mixed cultures sampled before harvest and during herbage regrowth in the subsequent year, 1984 (Experiment III)+.

TREATMENT	Determination 1 23 June	2 5 July	3 12 July	4 30 July	5 3 Aug.	6 14 Aug.	7 20 Aug.
	(Total Nitrogen Concentration %)						
Timothy (1) with alfalfa	1.88a	0.88a	2.15b	2.47b	2.50b	2.42b	3.00c
Timothy (2) with alfalfa	1.55b	0.92a	2.12b	2.63b	2.67b	2.57ab	3.93b
Bromegrass with alfalfa	1.58b	0.80a	2.69a	2.98a	3.16a	2.80a	4.38a
Timothy (1)++	1.00d	0.35b	0.76d	1.50c	1.60c	1.35c	1.60d
Timothy (2)	1.00d	0.37b	1.12c	1.04d	1.47c	1.08c	1.60d
Bromegrass	1.28c	0.40b	1.03b	1.61c	1.50c	1.27c	1.70d
Mean	1.38	0.62	1.98	2.04	2.15	1.91	2.71
±SE	0.09	0.09	0.16	0.22	0.18	0.20	0.23

Mean in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+ Harvest 3 performed 5 July 1984

++ Timothy (1) cultivar 'climax'
Timothy (2) cultivar 'salvo'

Table 15. Total nitrogen concentration (%) in roots of grasses for the various mono and mixed cultures sampled before harvest and during herbage regrowth in the subsequent year, 1984 (Experiment III)+.

TREATMENT	Determination 1 23 June	2 5 July	3 12 July	4 30 July	5 3 Aug.	6 14 Aug.	7 20 Aug.
	(Total Nitrogen Concentration %)						
Timothy (1) with alfalfa	1.20a	1.01a	1.02a	1.35a	1.31a	1.02ab	1.08b
Timothy (2) with alfalfa	1.07b	1.03a	1.17a	1.18b	1.09ab	1.18a	1.16b
Bromegrass with alfalfa	0.90c	0.88b	0.95c	1.01c	0.92b	0.86b	1.51a
Timothy (1)++	0.29e	0.33c	0.36de	0.40d	0.58c	0.47c	0.53c
Timothy (2)	0.28e	0.34c	0.39d	0.40d	0.48c	0.42c	0.61c
Bromegrass	0.38d	0.38c	0.30e	0.42d	0.51c	0.48c	0.47c
Mean	0.69	0.66	0.70	0.79	0.81	0.73	0.89
±SE	0.05	0.06	0.05	0.08	0.07	0.13	0.19

Mean in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+ Harvest 3 performed 5 July, 1984

++ Timothy (1) cultivar 'climax'
Timothy (2) cultivar 'salvo'

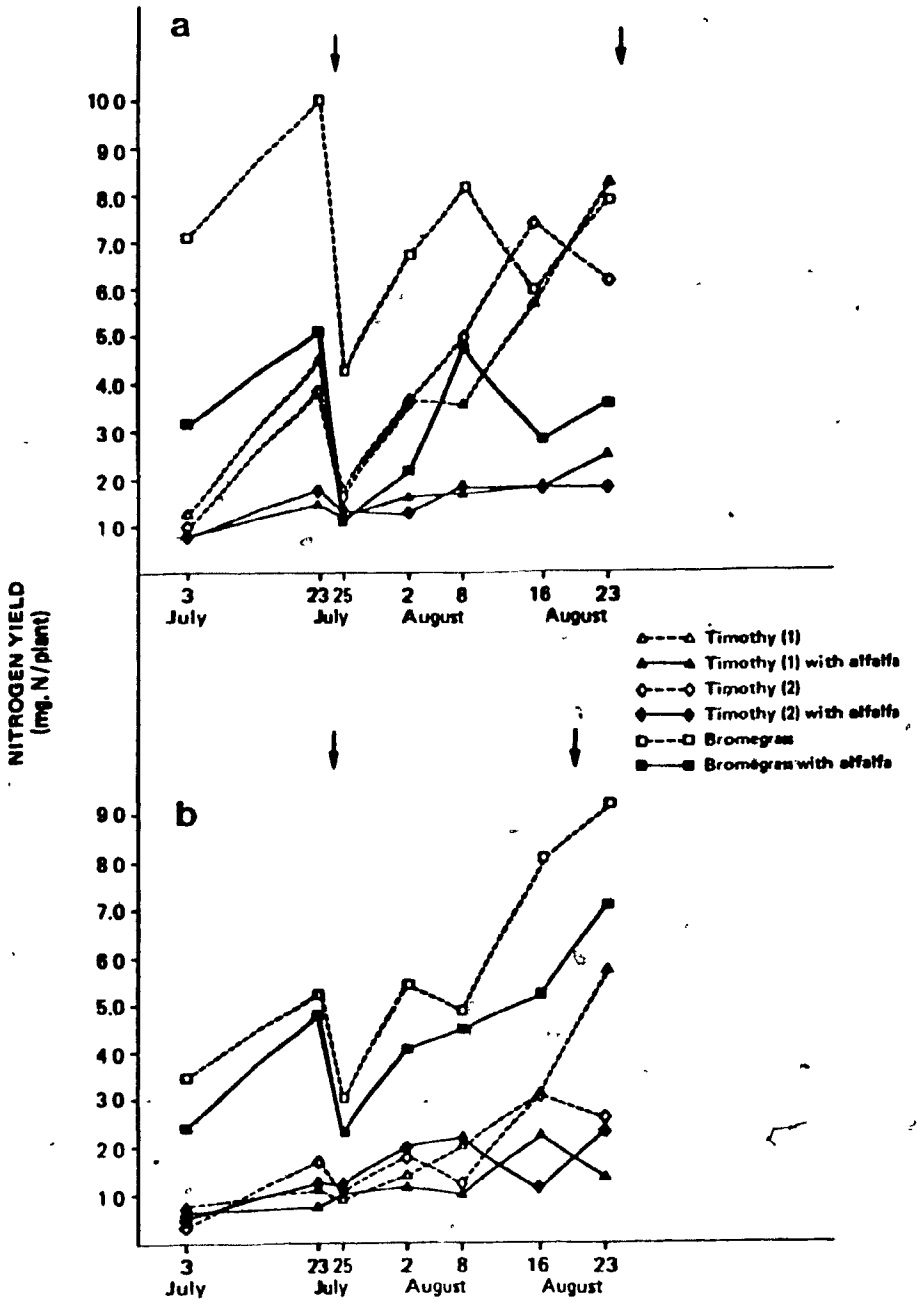

Figure 19. Profiles of nitrogen yield of grasses for the various mono and mixed cultures sampled before harvest and during herbage regrowth in the seeding year, 1983 (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. nitrogen yield of shoot
b. nitrogen yield of root + crown
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

the two species, where alfalfa was the dominant one. The competitive dominance of legume is usually reported for legume-grass mixtures, when the soil N level is low (Trenbath 1974, 1976; Haynes 1980). Haynes (1980) reported that at low levels of soil N, the N_2 fixing legumes benefitted from high rates of N_2 fixation that promotes higher growth rates in comparison to grasses, which rely on available soil N.

At the second year, the difference of TN concentration among grasses in association and those in monoculture was more pronounced (Tables 14 and 15). This beneficial effect due to alfalfa could be attributed to the additional contribution of the decomposition of nodules and roots during alfalfa late stages besides the continuous excretion of N compounds from living alfalfa roots during the growing stage.

Table 16 shows the effect of the alfalfa on the total nitrogen content of the soil during the experimental period. The total N content in the soil where alfalfa was grown alone or with grass increased significantly during the experiment, but the concentration of nitrogen in the plots where grass was grown alone declined significantly. Therefore, alfalfa affected the soil N content probably with the formation of a surface organic layer which effectively contributed to the nitrogen status of the soil. Birch and Dougall (1967) also reported that alfalfa increased N abundant organic matter in the soil, which effectively contributed to the nitrogen status of the soil

Table 16. Total nitrogen concentration (%) in the soil under different treatment during the experimental period, from April 1983 to May 1984 (Experiment III).

PERIOD	1983		1984	
	April (control)	September	April	September
TREATMENT	(Total Nitrogen Concentration %)			
Alfalfa alone	0.0927 (±0.0145)	0.1105* (±0.0220)	0.1100* (±0.0240)	0.1170* (±0.0007)
Alfalfa with grass	0.0927 (±0.0145)	0.1050 (±0.0160)	0.1040* (±0.0130)	0.1130* (±0.0180)
Grass alone	0.0927 (±0.0145)	0.0850 (±0.0120)	0.0830 (±0.0100)	0.0770* (±0.0008)

Each value is the mean of the ten observations ± the standard error of the particular mean value.
*Significantly different from control (April 1983) $P \leq 0.05$.

and leads to an increase in the dry matter and N yield of the associated grass.

The grasses in association showed excellent development during the spring of 1984 with high TN. This is expressed in significant increase in the N yield over grasses in monoculture (Figure 20). However, in the measurement conducted before harvest (July 5, Table 14), when the grasses reached advanced heading stage, the TN concentration declined significantly reaching very low level. This reduced markedly the N yield in both cultural systems. These results may be due to the fact that the chemical composition of grasses change significantly with advance in maturity. Phillips et al. (1954) studied these changes in chemical composition of grass during the spring, and reported a continuous decline of protein content and soluble ash with a significant increase in lignin.

TN concentration and N yield of grasses grown alone changed significantly over time. However the change observed was not as large, as would be expected due to depletion of soil N by grass grown in monoculture (Table 16). The maintenance of the TN content in grass alone (Tables 14 and 15) which is supported by N yield in the second year (Figure 20), could be attributed to mineralized soil N and possible input of N from outside of the soil system, such as N in rainfall, N_2 fixed by free living organisms or possibly by organisms associated with grass roots as suggested by many reseachers (Dobereiner and Day (1975); De Polli

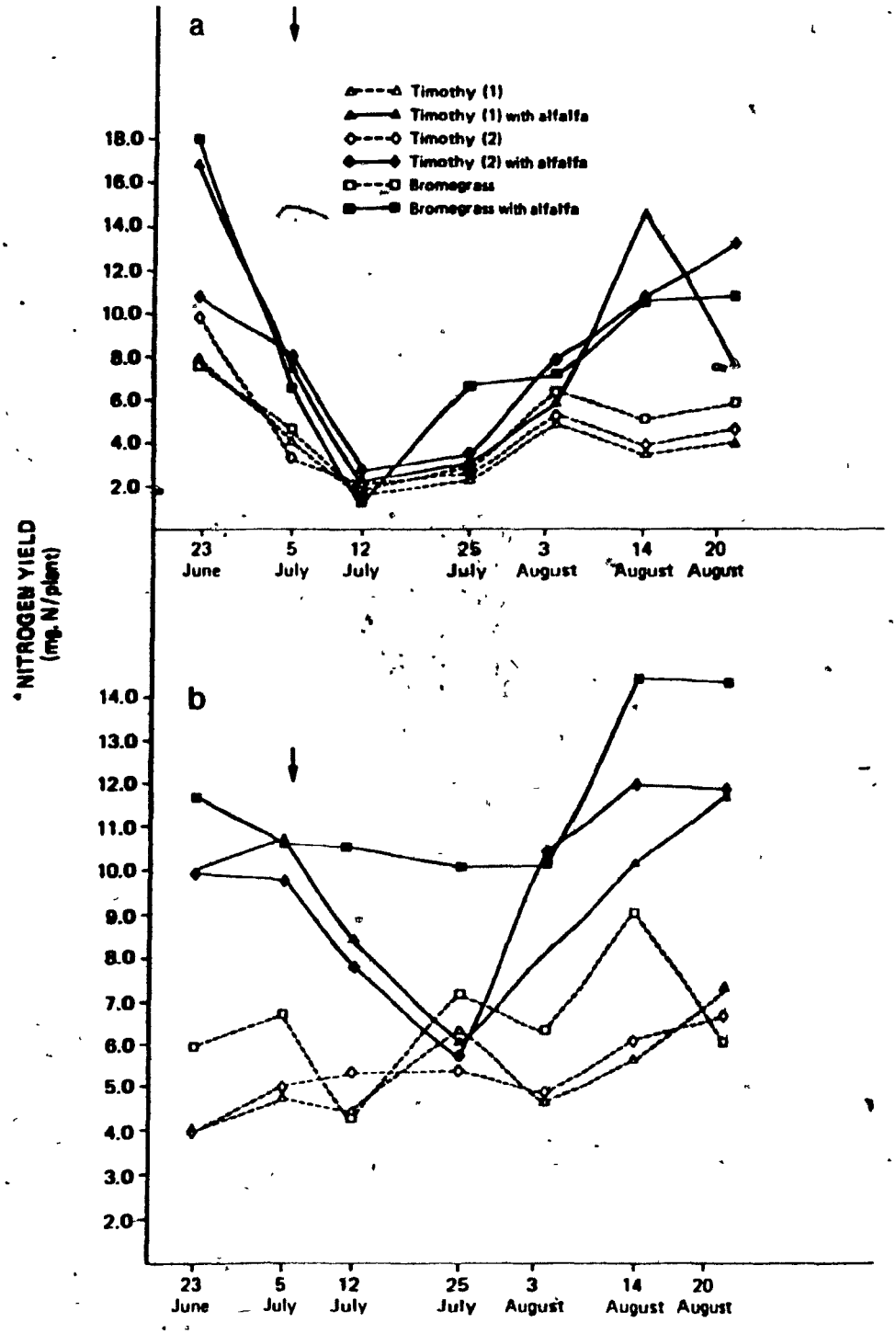

Figure 20. Profiles of nitrogen yield of grasses for the various mono and mixed cultures sampled before harvest and during herbage regrowth in the subsequent year, 1984 (Experiment III). Values shown are the retransformed natural logarithm of the means for each treatment. Each point is the mean of five replicates. Arrows indicate time of harvests.
a. nitrogen yield of shoot
b. nitrogen yield of root + crown
Harvest 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

et al (1977); Neyra and Dobereiner (1977); Van Berkum and Bohlool (1980); Van Berkum and Day (1980); Van Berkum (1980); Boddey et al. (1983); Wani et al. (1983); Rennie et al. (1983); Vose (1983). However, this speculation on associative N_2 fixation in temperate grass needs more investigation.

No significant differences in N yield were found among grass species grown in association during both years, but there was a difference among grass species in association with respect to % TN (Tables 12 and 14). This difference was not translated to an increase in dry weight and N yield. The effect of the associated alfalfa on the N concentration of grass was evident from the early stage in the seeding year. However this enhancement did not cause an increase in yield. The beneficial effect on the grass N yield first became apparent in the determination performed in 23 June 1984, and continued through all of the subsequent assessments.

All grasses in the mixtures were taller and more vigorous than those grown alone (Table 17). The possible explanation for this result is due to the formation of fertile tillers (seed stalks) in the grass in mixed culture because there was more N available for growth. When grass was grown alone fertile tillers did not normally form, and were replaced by production of secondary tillers which were small and less vigorous than fertile ones. Table 17 also shows that the grass in association had fewer tillers than that in monoculture, but the tillers were more

Table 17. Plant height, number and weight of tillers of grasses for the various mono and mixed cultures sampled in the subsequent year, 1984 (Experiment III).

TREATMENT	Character: Plant height 23 June (cm)	Plant height 14 August (cm)	Tiller+ 23 June number (no/sample)	Tiller+ 23 June weight (g/sample)
Timothy (1) with alfalfa++	76.7a	45.7ab	11.0c	0.30b
Timothy (2) with alfalfa	73.7ab	43.0b	16.0bc	0.48b
Bromegrass with alfalfa	71.2b	50.5b	18.0abc	0.68a
Timothy (1)	44.0c	20.0c	28.2a	0.45b
Timothy (2)	42.7cd	19.0c	27.2a	0.41b
Bromegrass	38.0d	19.5c	23.0ab	0.47b
Mean	57.7	33.0	20.6	0.47
±SE	1.10	4.00	6.8	0.12

Mean in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+ Number and weight of tiller per sample
++ Timothy (1) cultivar 'climax'
Timothy (2) cultivar 'salvo'

vigorous and responsible for increases in yield. Other greenhouse and field clipping studies have shown various effects on the growth and chemical composition of forage plants. Increased height of the plants at the time of clipping led to higher dry matter yields of tops and roots (Davis 1960; Langille and Warren 1962; Dubbs 1971) and fewer numbers of tillers per plant (Baker 1957).

4.2.2 Experiments IV AND V

4.2.2.1 N_2 fixation, herbage and N yield of alfalfa

Experiments IV and V were conducted with objectives to evaluate the amounts of N transferred from alfalfa to grass, the soil N uptake by alfalfa and grass, and to measure N_2 fixed by alfalfa in mixed swards under field conditions. In order to increase the accuracy of assessing the parameters cited above, the isotope dilution technique was utilized to enrich the percentage of atom ^{15}N in the soil.

Atom % ^{15}N excess data of the alfalfa and grasses were collected from two experiments at several harvest times (Tables 18, 19, 20 and 21). Percent ^{15}N excess in the alfalfa was significantly lower than that of the grasses and demonstrates that the legume was fixing N_2 from the air. However, the atom % ^{15}N excess in the alfalfa plants in both cultural systems was

Table 18. Atom % ^{15}N excess of alfalfa grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV).

YEAR	TREATMENT	Harvest 1	2	3
		(Atom % ^{15}N excess)		
1983	Alfalfa	0.3292ab	0.2975a	
	Alfalfa with timothy(1)+	0.3875a	0.2449a	
	Alfalfa with timothy(2)	0.2670b	0.1990a	
	Alfalfa with bromegrass	0.3230ab	0.2847a	
	Mean++	0.3266a	0.2565b	
	± SE	0.0320	0.0333	
1984	Alfalfa	0.2133a	0.0870a	
	Alfalfa with timothy(1)	0.1953b	0.1016a	
	Alfalfa with timothy(2)	0.1799c	0.0951a	
	Alfalfa with bromegrass	0.1488c	0.0947a	
	Mean++	0.1843a	0.0945b	
	± SE	0.0035	0.0049	
1985	Alfalfa	0.0923a	0.0910a	0.0810a
	Alfalfa with timothy(1)	0.0871a	0.0793a	0.0530a
	Alfalfa with timothy(2)	0.1266a	0.0803a	0.0590a
	Alfalfa with bromegrass	0.1342a	0.0605a	0.0790a
	Mean++	0.1101a	0.0777b	0.0657b
	± SE	0.0298	0.0106	0.0097

Means in a column, followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax',
Timothy(2) cultivar 'salvo'
++Means compared horizontally

Table 19. Atom % ^{15}N excess of alfalfa grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V).

YEAR	TREATMENT	Harvest 1	2	3
		(Atom % ^{15}N excess)		
1984	Alfalfa	0.1790a		
	Alfalfa with timothy	0.1783a		
	Alfalfa with bromegrass	0.1770a		
	Alfalfa with tall fescue	0.1723a		
	Mean	0.1766		
	± SE	0.0123		
1985	Alfalfa	0.1042a	0.0690a	0.0778a
	Alfalfa with timothy	0.0957b	0.0703a	0.0675b
	Alfalfa with bromegrass	0.0937b	0.0683a	0.0598bc
	Alfalfa with tall fescue	0.0927b	0.9723a	0.0580c
	Mean+	0.0966a	0.0699b	0.0657c
	± SE	0.0022	0.0037	0.0052

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally

Table 20. Atom % ^{15}N excess of grasses grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV).

YEAR	TREATMENT	Harvest 1	2	3
		(Atom % ^{15}N excess)		
1983	Timothy(1) with alfalfa	0.7726cd	0.6660c	
	Timothy(2) with alfalfa	0.6273e	1.0234abc	
	Bromegrass with alfalfa	1.0950b	0.9549bc	
	Timothy(1)+	0.8453c	0.8323c	
	Timothy(2)	0.6985dc	1.4839ab	
	Bromegrass	1.2350a	1.5437a	
	Mean++	0.8789b	1.0480a	
	±SE	0.0538	0.2037	
1984	Timothy(1) with alfalfa	0.7950bcd	0.2338b	
	Timothy(2) with alfalfa	0.7143cd	0.3536b	
	Bromegrass with alfalfa	0.5868d	0.3654b	
	Timothy(1)	1.0498abc	0.3378b	
	Timothy(2)	1.1740a	0.6986a	
	Bromegrass	1.0973ab	0.6978a	
	Mean++	0.9028a	0.4478b	
	±SE	0.1402	0.0560	
1985	Timothy(1) with alfalfa	0.2964b	0.3122abc	0.2687cd
	Timothy(2) with alfalfa	0.4292a	0.2415bc	0.1788e
	Bromegrass with alfalfa	0.3622b	0.2350c	0.2435de
	Timothy(1)	0.4163a	0.3675a	0.3955ab
	Timothy(2)	0.5833a	0.3303ab	0.3400bc
	Bromegrass	0.4481a	0.3433a	0.4400a
	Mean++	0.4225a	0.3049a	0.3110a
	±SE	0.0237	0.0343	0.0281

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally

Table 21. Atom % ^{15}N excess of grasses grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V).

YEAR	TREATMENT	Harvest 1	2	3
		(Atom % ^{15}N excess)		
1983	Timothy with alfalfa	0.3320b		
	Bromegrass with alfalfa	0.3343b		
	Tall fescue with alfalfa	0.3255b		
	Timothy	0.3532a		
	Bromegrass	0.3560a		
	Tall fescue	0.3500a		
	Mean	0.3418		
	± SE	0.0076		
1984	Timothy with alfalfa+	0.4437b	0.3320b	0.3735b
	Bromegrass with alfalfa	0.4440b	0.3343b	0.3343bc
	Tall fescue with alfalfa	0.4463b	0.3255b	0.3255c
	Timothy	0.5123a	0.4030a	0.5528a
	Bromegrass	0.5143a	0.4060a	0.4915a
	Tall fescue	0.5113a	0.4115a	0.4885a
	Mean+	0.4786a	0.3687b	0.4276b
	± SE	0.0195	0.0082	0.0175

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally

higher than the level of natural abundance, showing that the alfalfa plants took up soil N and competed slightly with the grass for mineral N mainly in the seeding year in both experiments (Tables 18 and 19). In the second and third year (Experiment IV) and second year (Experiment V) the uptake of label ^{15}N nearly obtained the level of natural abundance, indicating a substantial N_2 fixation from the atmosphere by alfalfa plants at those times.

The ^{15}N enrichments (atom % ^{15}N excess) of the grasses and alfalfa exhibited approximately parallel declines throughout the years in both experiments, with the exception of harvest 2 (Experiment IV). The decline reflected the effect of plant uptake, immobilization into soil organic N fractions, and gaseous and leaching losses as well as release of non-labelled N from soil organic matter. The trends in reduction of atom % ^{15}N excess of the grass and legume components can be explained by the relatively constant percentage of alfalfa N derived from fixation over the harvest times in each trial. The similarity of the enrichment trends indicates that the seasonal soil N uptake patterns by the two species were not different enough to violate the assumption of the ^{15}N dilution technique to measure N_2 fixation. These results provide preliminary evidence that the grasses used in this study were a satisfactory control.

The proportion of N derived from the atmosphere (% Ndfa) ranged from 62 to 78% (average of harvest) in Experiment IV

(Table 22) and Experiment V (Table 23), respectively. There were usually only slight seasonal variations through the entire period. The crop obtained the greatest proportion of N from symbiosis after the seeding year, with an annual average about 80% fixed N during the second year and third year. In the seeding year in experiment IV, the % Ndfa of alfalfa plants grown in pure and mixed stands contained 62.78% fixed N in the initial harvest, while in the Experiment V the proportion of N from symbiosis reached an average of 78.32% N fixed. This difference was probaly related to the availability of readily mineralizable N in the soil during the initial phase of nodulation. TN available ranged from 0.10 to 0.14% in the profile at establishment (Table 2). It is well established that large amounts of available N reduce N_2 fixation drastically (Dart and Mercer 1965; Munns 1968b; Gibson 1974; Summerfield et al. 1977; Dazzo and Brill 1978). However, small amounts of available N are often found to alleviate the N-stress in the initial phase of establishment of the symbiotic process (Gibson and Nutman 1960; Dart and Wildon 1970; Gibson 1974; Lawn and Brun 1974; Dean and Clark 1980; Heichel et al. 1981; Faglesham et al. 1983).

The proportions of N derived from the air in these studies were much higher than those reported by Heichel et al. (1981), where the percentage N fixed was approximately 43% in the first and fourth harvests and 65% in the second and third harvests. The values in the present trials ranged from an annual average of

Table 22. Percent of nitrogen derived from atmosphere (% Ndfa) of alfalfa grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV)

YEAR	TREATMENT	Percent Nitrogen derived from atmosphere Harvest 1	2	3	Mean
		(%)			
1983	Alfalfa	60.78b	64.30b		62.54
	Alfalfa with timothy(1)+	53.94b	71.61b		62.77
	Alfalfa with timothy(2)	61.78b	86.58a		74.18
	Alfalfa with bromegrass	74.64a	81.35a		77.99
	Mean++	62.78b	75.96a		69.37
	± SE	3.16	3.00		2.86
1984	Alfalfa	79.68a	74.26b		76.95
	Alfalfa with timothy(1)	81.68a	69.89b		75.65
	Alfalfa with timothy(2)	84.07a	86.01a		85.04
	Alfalfa with bromegrass	86.41a	86.41a		86.41
	Mean++	82.89a	79.14b		81.01
	± SE	2.67	2.72		2.53
1985	Alfalfa	77.18a	75.35a	79.47a	77.56
	Alfalfa with timothy(1)	79.09a	75.94a	89.09a	81.38
	Alfalfa with timothy(2)	81.18a	75.64a	82.26a	79.70
	Alfalfa with bromegrass	70.16a	82.35a	82.04a	78.18
	Mean++	77.07b	77.33b	83.21a	79.20
	± SE	4.43	2.93	1.93	3.56

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax,
Timothy(2) cultivar 'salvo'
++Means compared horizontally

Table 23. Percent of nitrogen derived from atmosphere (% Ndfa) of alfalfa grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V)

YEAR	TREATMENT	Percent Nitrogen derived from atmosphere Harvest 1	2	3	Mean
		(%)			
1984	Alfalfa	77.64b			77.64
	Alfalfa with timothy	77.89ab			77.89
	Alfalfa with bromegrass	78.38ab			78.38
	Alfalfa with tall fescue	79.37a			79.37
	Mean	78.32			78.32
	± SE	0.92			0.92
1985	Alfalfa	79.60b	82.07b	85.94b	82.56
	Alfalfa with timothy	81.31a	82.57b	87.79a	83.89
	Alfalfa with bromegrass	81.77a	83.19ab	87.85a	84.26
	Alfalfa with tall fescue	82.09a	84.26a	88.12a	84.82
	Mean+	81.21c	83.02b	87.42a	83.89
	± SE	0.55	0.92	0.94	0.82

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally

69 to 81% and 78 to 83% in Experiments IV and V respectively. These values are similar to those of other studies of mixtures containing alfalfa or other forage legume species, which indicate that from 70% to almost 100% of the legume shoot N can be derived from N_2 fixation when growing with grass in mixture (Haystead and Lowe 1977; Vallis et al. 1977; Edmeades and Goh 1978; Bergersen and Turner 1983; Phillips et al. 1983; Faris and Ta 1985; West and Wedin 1985).

There were significant differences in the atom % ^{15}N excess of alfalfa grown in mixtures as compared with that in monoculture, specifically in harvest 1 (Experiment IV) performed in 1984 (Table 18) and harvests 1 and 3 (Experiment V) in 1985 (Table 19). A similar trend was also observed, when the data were expressed as % Ndfa (independent yield criteria). When the data were converted to the amount of N_2 fixed (Tables 24 and 25) almost all significant differences disappeared. The only exception observed was in harvest 1 (Table 24) when alfalfa was grown with the timothy cultivar 'Climax', the total amount of N_2 fixed was significantly lower than in pure stands. Therefore, growing alfalfa in association with the grass did not reduce the activity of N_2 fixation by alfalfa.

The few significant differences detected in the atom % ^{15}N excess and proportion of %Ndfa from alfalfa grown in mixtures as compared with that in pure stand in the field and greenhouse conditions showed the validity of the isotope dilution technique

Table 24. Amount of nitrogen fixed by alfalfa plants when grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV).

YEAR	TREATMENT	N_2 fixed Harvest 1	2	3 (kg/ha)	Total+++
1983	Alfalfa	42.75ab	40.65a		83.40b
	Alfalfa with timothy(1)+	34.59b	46.17a		80.76b
	Alfalfa with timothy(2)	40.41ab	51.19a		91.60ab
	Alfalfa with bromegrass	56.17a	60.27a		116.44a
	Mean++	43.48a	49.57a		93.05b
	±SE	6.12	8.53		8.41
1984	Alfalfa	154.01a	115.61a		269.62a
	Alfalfa with timothy(1)	123.38a	123.68a		247.06a
	Alfalfa with timothy(2)	137.39a	131.83a		269.22a
	Alfalfa with bromegrass	121.44a	124.56a		246.00a
	Mean++	134.05a	123.94a		258.00a
	±SE	25.29	35.49		40.81
1985	Alfalfa	122.44a	63.97a	44.30a	230.71a
	Alfalfa with timothy(1)	133.14a	53.60a	65.01a	251.75a
	Alfalfa with timothy(2)	118.04a	60.52a	45.40a	223.96a
	Alfalfa with bromegrass	102.66a	55.55a	42.15a	200.36a
	Mean++	119.06a	55.91b	49.21b	226.70a
	±SE	26.30	13.85	10.95	41.38

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'

++Means compared horizontally

+++Total annual (means) compared vertically.

Table 25. Amount of nitrogen fixed by alfalfa plants when grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V).

YEAR	TREATMENT	N_2 fixed Harvest 1 (kg/ha)	2	3	Total++
1984	Alfalfa	40.69a			40.69a
	Alfalfa with timothy	44.79a			44.79a
	Alfalfa with bromegrass	43.27a			43.27a
	Alfalfa with tall fescue	49.56a			49.56a
	Mean	44.58			44.58b
	±SE	6.55			6.55
1985	Alfalfa	124.73a	70.06a	46.44a	241.23a
	Alfalfa with timothy	114.63a	74.26a	46.22a	235.11a
	Alfalfa with bromegrass	127.58a	64.18a	52.27a	244.03a
	Alfalfa with tall fescue	135.20a	85.28a	47.24a	267.72a
	Mean+	125.53a	73.44b	48.04c	247.03a
	±SE	22.79	16.45	14.36	40.79

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally
++Total annual (means) compared vertically.

to assess N_2 fixation in legume-grass mixtures, when both dependent and independent criteria of yield were utilized to assess N_2 fixation.

Broadbent et al. (1982) reported severe restrictions to the use of the isotope dilution method in mixed swards for the measurement of N_2 fixation when comparisons are made between the legume and non-legume in the mixture. Recent studies reviewed by Chalk (1985) have shown that the isotope technique can be applied to the measurement of N_2 fixation in associated pastures. However, a suitable reference plant in pure stand is required to estimate precisely the contribution of indigenous and soil N to the N nutrition of the fixing plant. In the present study, mixing alfalfa with grass did not lead to erroneous estimates of N_2 fixation because suitable controls were utilized with close uptake of labelled ^{15}N (Tables 20 and 21), and thus, any significant differences were not found when the amounts of N_2 fixed were calculated.

The absolute amounts of N_2 fixed by alfalfa in mono and mixed cultures from Experiments IV and V, are shown in Tables 24 and 25. In general, there was seasonal variation in the amount of N_2 fixed. During the seeding year, alfalfa plants fixed (an average per harvest) from 40.67 to 42.75 kg N/ha and 34.59 to 60.27 kg N/ha in pure and mixed stands, respectively. In the second and third year, the alfalfa plants fixed large amounts of N_2 in the initial harvest. Thereafter, the

amount declined significantly during the season and reached low values comparable to the seeding year in harvest 3 (Experiment IV, 1985 and Experiment V, 1985). However, there was only slight variation when the N_2 fixed by alfalfa in mono and mixed cultures were compared.

The annual pattern of N_2 fixation showed that alfalfa fixed 44.58 to 93.05 kg N/ha during the year of establishment, and as much as 224.19 to 258.00 kg N/ha during the second and third year stands in both experiments. The published data on amount of N_2 fixed by alfalfa are few and very widely. Bell and Nutman (1971) reported a rate of N_2 fixation of 220 kg N/ha per year for effectively inoculated alfalfa plants with up to 78% of plant N derived from the atmosphere. Heichel et al. (1981) found an average of 148 kg N/ha in the establishment year with 43% of the total N yield derived from fixation. More conservative estimates range from 83 to 100 kg N/ha (Erdman and Ura Mae 1953). Recently West and Wedin (1985) reported an annual amount of N_2 fixed of 70 kg N/ha with a range of 15-136 kg N/ha in the seeding year and 15-122 kg N/ha in the 2 year old alfalfa in mixtures with orchardgrass. Since alfalfa is a perennial and can produce seven or eight cuttings in one climate, and only two or three cuttings in another, it is normal that estimates of N_2 fixed vary widely (Burton 1975). Also, the amount of N_2 fixed is highly correlated with the percentage of alfalfa in the sward and with alfalfa dry matter yield (West and Wedin 1985), and it is closely linked to

growth rate in alfalfa (Heichel et al. 1981).

The results in this investigation suggest that the amount of N_2 fixed and recovered in the alfalfa shoots in mono and mixed cultures varied among harvests in patterns similar to changes in alfalfa dry matter (Tables 26 and 27) and N yield (Tables 28 and 29). As the proportion of Ndfa remained relatively constant through the year with slight variation within the year (Tables 22 and 23), the major determinant of the amount of N_2 fixed was alfalfa DW yield.

The association of alfalfa with grasses did not have a detrimental effect on total dry weight (DW) and N yield per hectare. The DW and N yield of alfalfa in pure stand, however, showed a trend of improvement over alfalfa in mixed stands (Tables 26, 27, 28 and 29). The values in parentheses give the contribution of alfalfa to total DW and N yield harvested, as percentage of total herbage and N harvested. Without exception the amount of DW and N yield from alfalfa to total herbage and N harvested was well maintained throughout the years, demonstrating a dominant competitive ability of alfalfa over associated grass in environments with low to medium available soil N. When alfalfa was grown with timothy, the contributions of alfalfa DW and N yield to the total annual were superior to that when grown with bromegrass. These differences were always small and none reached significant proportions. Nevertheless, when alfalfa was grown in association with bromegrass, the total legume

Table 26. Herbage production (DW) of alfalfa grown in mono mixed cultures at different harvests, 1983 to 1985 (Experiment IV). Values in parentheses are the percentages of the total annual herbage harvested.

YEAR	TREATMENT	Dry Weight (DW) Harvest 1	2	3	Total+++ (t/ha)	
1983	Alfalfa	3.15a	2.18ab		5.33a	(100.0)
	Alfalfa with timothy(1)+	2.79ab	1.87c		4.66b	(85.2)
	Alfalfa with timothy(2)	2.65b	2.04bc		4.69b	(86.7)
	Alfalfa with bromegrass	3.02a	2.29a		5.31a	(84.4)
	Mean++	2.91a	2.10b		5.01	
	±SE	0.26	0.15		0.29	
1984	Alfalfa	7.32a	4.23a		11.55a	(100.0)
	Alfalfa with timothy(1)	6.64a	3.96a		10.60b	(83.9)
	Alfalfa with timothy(2)	6.90a	3.81a		10.71b	(83.9)
	Alfalfa with bromegrass	6.59a	3.88a		10.47b	(80.4)
	Mean++	6.86a	3.96b		10.83	
	±SE	0.64	0.63		0.52	
1985	Alfalfa	5.40a	3.24a	2.38a	11.02a	(100.0)
	Alfalfa with timothy(1)	4.86a	2.78a	2.42a	10.06a	(80.8)
	Alfalfa with timothy(2)	4.48a	2.82a	2.38a	9.68a	(78.5)
	Alfalfa with bromegrass	4.62a	2.46a	1.94a	9.02a	(76.9)
	Mean++	4.84a	2.83b	2.28c	9.95	
	±SE	0.80	0.64	0.79	1.50	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally
+++Total production (means) compared vertically

Table 27. Herbage production (DW) of alfalfa grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V). Values in parentheses are the percentages of the total annual herbage harvested.

YEAR	TREATMENT	Dry weight (DW) Harvest 1 (t/ha)	Harvest 2 (t/ha)	Harvest 3 (t/ha)	Total++ (t/ha)	
1984	Alfalfa	2.13a			2.13a	(100.0)
	Alfalfa with timothy	1.99a			1.99a	(93.8)
	Alfalfa with bromegrass	1.83a			1.83a	(85.5)
	alfalfa with tall fescue	1.99a			1.99a	(94.3)
	Mean	1.98			1.98	
	±SE	0.45			0.45	
1985	Alfalfa	5.48a	2.52a	1.44a	9.44a	(100.0)
	Alfalfa with timothy	4.40a	2.57a	1.51a	8.48a	(87.9)
	Alfalfa with bromegrass	5.22a	2.47a	1.64a	9.33a	(79.8)
	Alfalfa with tall fescue	5.26a	2.98a	1.47a	9.71a	(90.0)
	Mean+	5.09a	2.63b	1.51c	9.24	
	±SE	0.81	0.55	0.35	1.10	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally
++Total production (means) compared vertically

Table 28. Nitrogen yield of alfalfa grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV). Values in parentheses are the percentages of the total annual nitrogen harvested.

YEAR	TREATMENT	N Yield Harvest 1 (kg/ha)	2	3	Total+++	
1983	Alfalfa	64.55a	62.35a		126.90b	(100.0)
	Alfalfa with timothy(1)+	57.64b	62.49a		120.13b	(87.1)
	Alfalfa with timothy(2)	61.57ab	65.60a		127.17b	(89.3)
	Alfalfa with bromegrass	72.06a	71.04a		143.10a	(86.7)
	Mean++	63.95a	65.40a		129.33	
	±SE	7.90	6.59		10.21	
1984	Alfalfa	185.11a	165.73a		350.84a	(100.0)
	Alfalfa with timothy(1)	146.35b	162.71a		308.06ab	(87.6)
	Alfalfa with timothy(2)	170.60a	130.41a		301.01b	(85.2)
	Alfalfa with bromegrass	148.65a	123.56a		272.21b	(80.7)
	Mean++	162.67a	145.35b		308.03	
	±SE	21.02	29.81		32.21	
1985	Alfalfa	143.38a	91.14a	75.74a	310.26a	(100.0)
	Alfalfa with timothy(1)	146.04a	66.30b	75.82a	288.16a	(85.9)
	Alfalfa with timothy(2)	142.72a	74.84ab	68.36a	285.92a	(85.7)
	Alfalfa with bromegrass	135.44a	62.68b	69.86a	267.98a	(80.5)
	Mean++	141.89a	73.74b	70.94b	288.08	
	±SE	19.54	16.46	25.43	44.31	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally
+++Total production (means) compared vertically

Table 29. Nitrogen yield of alfalfa grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V). Values in parentheses are the percentages of the total annual nitrogen harvested.

YEAR	TREATMENT	N Yield Harvest 1	2	3	Total++	
		(kg/ha)				
1984	Alfalfa	52.50a			52.50a	(100.0)
	Alfalfa with timothy	59.53a			59.53a	(91.4)
	Alfalfa with bromegrass	55.32a			55.32a	(86.1)
	Alfalfa with tall fescue	62.61a			62.61a	(95.2)
	Mean	56.98			56.98	
	±SE	8.24			8.24	
1985	Alfalfa	156.65a	85.34a	53.93a	295.92a	(100.0)
	Alfalfa with timothy	141.36a	90.03a	52.70a	284.09a	(91.2)
	Alfalfa with bromegrass	156.00a	77.15a	59.45a	292.60a	(84.3)
	Alfalfa with tall fescue	164.68a	101.25a	53.59a	319.53a	(92.2)
	Mean+	154.67a	88.44b	54.92c	298.03	
	±SE	28.02	19.78	16.34	48.75	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally
++Total production (means) compared vertically

contribution to DW and N yield decreased moderately in the third year (Experiment IV).

4.2.2.2 N transference, herbage and N yield of grass.

The ^{15}N dilution technique was utilized to assess the proportion of N transferred from alfalfa to grass when both were grown in association. Both species received N from the soil pool, which was labelled with ^{15}N. However, alfalfa plants also obtained N_2 from the atmosphere by fixation and grasses in mixture benefitted from N released by the associated alfalfa. This additional N source will dilute the labelled ^{15}N where grass is grown in mixture. Assuming that the ratio of the isotope (^{15}N) of soil-derived N is similar to both crops, the percentage of N transferred was calculated from the difference of isotopic composition of grass plant tissues in mixed culture, with those in pure stand as reference values.

Percent atom ^{15}N excess in the alfalfa, in pure stand and mixture was superior to the normal abundance level, showing that the alfalfa took up soil N and competed with the associated grass for mineral N in the establishment year (Tables 18 and 19), but in the subsequent years the legume contributed some fixed N_2 to the grass. In almost all instances, grass atom % ^{15}N excess was lower in the mixtures than in pure stand (Tables 20 and 21) which indicates that N_2 fixed by alfalfa was transferred from legume

to associated grass.

The ^{15}N enrichments of the grasses exhibited increases and declines in both experiments through the season and years. The observed increases or reductions reflected the additional applications of label ^{15}N in the spring and the losses of ^{15}N from the available soil N pool via shoot removal (uptake) and other process (immobilization and leaching). However, the similarity of the enrichment trends indicates that the seasonal soil N uptake patterns of the grass species used in this study were similar with only slight variations. The atom % ^{15}N excess for the grass in pure stand provides satisfactory evidence of this fact.

The proportion of N transferred to the grass (Tables 30 and 31) varied significantly with clipping time in 1983 and 1984. In 1985, the differences among harvests were always small and none reached significance. The % of N transferred (% Nt) ranged from an average of 16% in initial harvest to 48% in the final harvest (Experiment IV), while in Experiment V, the proportion Nt ranged from an average of 8.0% in the seeding year to 37.0% in the last harvest in 1985. In Experiment V, the % Nt was lower than that of Experiment IV. These variations could be attributed to the difference in available soil N in each experimental site. The analyses of soil prior to the initiation of the trials showed a % total available N of 0.09% ($\pm$ 0.14) and 0.12% ($\pm$ 0.20) for Experiments IV and V, respectively.

Table 30. Percentage of nitrogen in grasses transferred from associated alfalfa at different harvests, 1983 to 1985 (Experiment IV).

YEAR	TREATMENT	Nitrogen Transfer Harvest 1 (%)	Harvest 2 (%)	Harvest 3 (%)
1983	Timothy(1) with alfalfa+	16.11a	30.84b	
	Timothy(2) with alfalfa	16.47a	33.32ab	
	Bromegrass with alfalfa	15.48a	46.90a	
	Mean++	16.02b	37.02a	
	± SE	5.50	8.31	
1984	Timothy(1) with alfalfa	29.79a	43.91b	
	Timothy(2) with alfalfa	44.71a	55.38a	
	Bromegrass with alfalfa	50.80a	56.85a	
	Mean++	41.76b	52.05a	
	± SE	6.48	5.10	
1985	Timothy(1) with alfalfa	35.94a	23.70a	35.55a
	Timothy(2) with alfalfa	26.92a	34.36a	56.62a
	Bromegrass with alfalfa	35.94a	37.70a	54.72a
	Mean++	32.64a	31.92a	48.97a
	± SE	4.50	10.84	13.41

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally

Table 31. Percentage of nitrogen in grasses transferred from associated alfalfa at different harvests, 1984 to 1985 (Experiment V).

YEAR	TREATMENT	Nitrogen transfer Harvest 1 (%)	Harvest 2 (%)	Harvest 3 (%)
1984	Timothy with alfalfa	7.35a		
	Bromegrass with alfalfa	7.78a		
	Tall fescue with alfalfa	8.83a		
	Mean	8.12		
	± SE	0.87		
1985	Timothy with alfalfa	16.47a	25.27a	36.93a
	Bromegrass with alfalfa	16.88a	21.24a	36.71a
	Tall fescue with alfalfa	15.57a	25.33a	37.86a
	Mean+	16.24c	22.61b	37.13a
	± SE	5.20	3.12	2.60

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally

The % Nt of the three grass species were similar, although timothy cultivar 'Climax' showed a significant disadvantage in the later regrowth in harvest 2 at 1984 (Table 30). In general, all grass species benefitted from N transfer from alfalfa during the entire period. Up to 50% (Experiment IV) and 37% (Experiment V) of the total nitrogen of grass in mixed swards was derived from the N_2 fixation process of alfalfa. The % N transferred was significantly lower during the first growth (harvest 1) in both experiments, but it increased substantially thereafter. The results did indicate that some N transference did occur before first cutting even in the seeding year. Thus the transference of N from alfalfa was not primarily due to the sloughing off of the nodules from alfalfa, nor to a decay of its root system after harvest as suggested by Butler and Bathurst (1956); Dilz and Mulder (1962); Simpson (1965). It may have involved a considerable degree of N excretion during the growing season. The % Nt observed in both trials (16.02% and 8.12%) before shoot removal in the seeding year is in agreement with an excretion mechanism. Faris and Ta (1985) reported that 12% of total N_2 fixed by alfalfa was transferred to associated grass prior to shoot removal. This porportion contributed 3.0% of the total N yield of grass, which on an area basis is equivalent to 1.75 kg N/ha. Under hydroponic culture conditions, Ta and Faris (1985) found that there is considerable release of soluble organic N compounds, mainly in the form of ammonia, glutamate, aspartate

and alanine into the medium by the living alfalfa root nodule system. Chujo and Daimon (1984) reported that a growth acceleration of grass species in association with red clover in the early stage of development is probably due to the absorption of N compounds excreted directly from the root system of the legume. However due to the apparent operation of the excretion mechanism mainly when the plants are subjected to stress, Butler and Bathurst (1956); Dilz and Mulder (1962); Henzell (1962); Simpson (1965, 1976); Haystead and Marriott (1978, 1979) concluded that a more important pathway of transference in a legume-grass pasture involves the sloughing off and decomposition of legume nodules and root tissues.

On the basis of the present results, it was not possible to distinguish the contribution from direct excretion or N released from decomposition of sloughed off nodules and dead root tissues. The data from Experiment III suggest, however, that alfalfa plants have the ability to adapt to the stress of shoot removal by maintaining an unchanged or slighlty reduced nodule weight. Vance et al. (1979) reported similar results, and concluded that the maintenance of nodule mass by alfalfa after harvest was attributable to the nodules ability to continue growth with the prolonged survival of the apical meristem in the nodule. It is thus suggested that N transference in alfalfa-grass mixtures is not mainly due to the death of roots and nodules tissue, but involves N excretion during the period

before and after harvest.

N transfer from alfalfa to grasses in mixed stands was calculated as the difference between the isotopic composition of grass in mixture and pure stand under the same soil conditions, therefore, the contribution of N fixed from free-living, fixing organisms or other input of N from outside of the soil system was considered to be negligible in this investigation. The results in Tables 32 and 33 clearly showed the evidence of the benefit of fixed N_2 obtained from alfalfa through underground transfer. On the area basis, the amount of N transferred ranged from an annual total average of 4.54, 20.16 and 18.64 kg N/ha for the first, second and third year, respectively (Experiment IV). In Experiment V, the amount of N transferred from alfalfa to the associated grass varied from an average annual amount of 0.39 to 7.39 kg N/ha for the first and second year, respectively (Table 33). The first values (Experiment IV) are equivalent to 4.9, 7.8 and 8.1% of the total annual amount of N_2 fixed by the alfalfa and equal to 26.5, 46.9 and 37.8% of total N yield of grasses in association. In Experiment V, the total annual amount of N transferred was less than that calculated in the previous trial. This could likely be explained by the fact that the site where Experiment V was established showed a greater availability of soil N.

Transfer of N from legume to grass is important for the growth of the grass, under low N conditions. This process

Table 32. Amount of nitrogen transferred to the associated grass at different harvests, 1983 to 1985 (Experiment IV).

YEAR	TREATMENT	Amount N transferred Harvest 1	2	3 (kg/ha)	Total+++
1983	Timothy(1) with alfalfa+	1.22a	2.24b		3.46a
	Timothy(2) with alfalfa	1.61a	1.90b		3.51a
	Bromegrass with alfalfa	1.38a	5.28a		6.66a
	Mean++	1.40a	3.14a		4.54
	±SE	0.49	1.72		2.56
1984	Timothy(1) with alfalfa	3.05b	10.68b		13.73b
	Timothy(2) with alfalfa	4.73ab	14.91ab		19.64ab
	Bromegrass with alfalfa	5.72a	21.40a		27.12a
	Mean++	4.50a	15.66b		20.16
	±SE	0.58	7.59		8.17
1985	Timothy(1) with alfalfa	11.72ab	1.37b	1.81b	14.90b
	Timothy(2) with alfalfa	8.31b	3.75a	3.47ab	15.53b
	Bromegrass with alfalfa	15.57a	5.89a	4.03a	25.49a
	Mean++	11.86a	3.67b	3.10b	18.64
	±SE	2.02	1.19	0.43	2.91

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'

++Means compared horizontally

+++Total transference (means) compared vertically.

Table 33. Amount of nitrogen transferred to the associated grass at different harvests, 1984 to 1985 (Experiment V).

YEAR	TREATMENT	Amount N transferred Harvest 1	2	3 (kg/ha)	Total++
1984	Timothy with alfalfa	0.21b			0.21b
	Bromegrass with alfalfa	0.70a			0.70a
	Tall fescue with alfalfa	0.27b			0.27b
	Mean	0.39			0.39
	±SE	0.11			0.11
1985	Timothy with alfalfa	3.46ab	1.01a	0.43b	4.90b
	Bromegrass with alfalfa	5.46a	3.29a	2.41a	11.16a
	Tall fescue with alfalfa	2.15b	2.13a	1.82a	6.10ab
	Mean+	3.69a	2.14a	1.55b	7.39
	±SE	1.74	0.89	0.77	2.76

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally
++Total transference (means) compared vertically.

represents a small quantity of the N_2 fixed by the alfalfa and allows the grass plants to grow at a rate similar to grass in monoculture. Dilz and Mulder (1962) quantified transfer of N and found that 1 to 8% (first cut) and 6 to 22% (second cut) of the N_2 fixed was transferred to the associated grass. Seerge (1961) and Simpson (1965) measured transfer of N as the amount of N excreted by the legume into medium and found that 1 to 4% of the fixed N_2 was transferred to the grass. Henzell (1962) reported N transfer from tropical legume to grass, and found only 0.6 to 1.7% of the total N_2 fixed was transferred from legume to the grass. Haystead and Marriott (1979) used the isotope dilution method to quantify N transfer and indicated that at least 1.7% of fixed N_2 by white clover was transferred to ryegrass.

Broadbent et al. (1982) reported extensive transfer of fixed N_2 to grass. Up to 80% of the N in ryegrass in mixed culture was derived from the symbiotic process in ladino clover. However, they found little transfer of N in a relatively short term and suggest that several months is involved in the gradual mineralization of dead roots and nodule tissues from the legume through microbial activity. Recently, Faris and Ta (1985) demonstrated the importance of N transfer from alfalfa to timothy in mixed stands. This transfer increased with times of clipping and contributed 3, 8 and 25% of total N yield of timothy in the first, second and third cut, respectively. Thus, the amount of N transferred from alfalfa to the associated grass, as estimated by

isotope dilution technique in this study was within the range obtained by other researchers.

Evidence of N transfer was observed for all grass species. However, there were differences among grass species in the amount of N transferred from the alfalfa. Bromegrass was the most responsive species to N transference with significant increases in the total N concentration (Tables 34 and 35). This may be due to the fact that bromegrass has a well developed root system, better adapted to drought conditons. Abnormal dry conditions that prevailed through to the summers 1984 and 1985, decreased the rate of the growth of the grass species less adapted to a drought environment (timothy and tall fescue) and influenced significantly their ability to take up available N.

Good contact between roots and large occupation of soil volume make more efficient use of N released from associated legumes and soil N. Craig de Anda et al. (1981) and Chujo and Daimon (1984) reported that the roots of grasses and legumes were tightly intertwined when they are grown together, and any N compounds excreted by legume root nodules would be rapidly absorbed by grass roots. Henzell (1962) suggested that an increase of N transfer may result from better competitive ability of the grass components in the association. The advantage observed by bromegrass in this experiment could be related to its greater root competitive ability and greater adaptability to drought conditions. Timothy and tall fescue, which are more

Table 34. Total nitrogen concentration (%) of grasses grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV).

YEAR	TREATMENT	Total nitrogen concentration (%) Harvest 1	2	3
1983	Timothy(1) with alfalfa+	2.28ab	2.10a	
	Timothy(2) with alfalfa	2.34a	2.08a	
	Bromegrass with alfalfa	2.36a	2.10a	
	Timothy(1)	1.72d	1.74b	
	Timothy(2)	2.02b	1.78b	
	Bromegrass	1.98cd	1.70b	
	Mean++	2.12a	1.92b	
	±SE	0.21	0.06	
1984	Timothy(1) with alfalfa	0.91ab	2.98c	
	Timothy(2) with alfalfa	0.97a	3.90b	
	Bromegrass with alfalfa	0.82b	4.34a	
	Timothy(1)	0.35c	1.58d	
	Timothy(2)	0.39c	1.60d	
	Bromegrass	0.40c	1.54d	
	Mean++	0.64b	2.66a	
	±SE	0.06	0.21	
1985	Timothy(1) with alfalfa	1.78ab	2.47a	3.08b
	Timothy(2) with alfalfa	1.68b	1.96b	3.11b
	Bromegrass with alfalfa	1.95a	2.68a	3.81a
	Timothy(1)	1.14d	1.60c	1.42d
	Timothy(2)	1.00d	1.29d	1.35d
	Bromegrass	1.40c	1.76bc	1.80c
	Mean++	1.49	1.96	2.43
	±SE	0.14	0.23	0.28

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally

Table 35. Total nitrogen concentration (%) of grasses grown in mono and mixed cultures at different harvest, 1984 to 1985 (Experiment V).

YEAR	TREATMENT	Total nitrogen concentration (%) Harvest 1	2	3
1984	Timothy with alfalfa	2.02b		
	Bromegrass with alfalfa	2.77a		
	Tall fescue with alfalfa	2.61a		
	Timothy	1.95b		
	Bromegrass	2.09b		
	Tall fescue	2.04b		
	Mean	2.25		
	±SE	0.19		
1985	Timothy	2.36a	2.46b	2.91b
	Bromegrass	1.95b	2.99a	3.76a
	Tall fescue	2.45a	2.68b	2.70b
	Timothy	1.15d	1.43d	1.73d
	Bromegrass	1.55c	1.86c	2.13c
	Tall fescue	1.48cd	1.87c	1.83d
	Mean	1.83	2.22	2.50
	±SE	0.24	0.16	0.19

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.

susceptible species to drought conditions, benefitted from the N transference process to a lesser extent than bromegrass.

Total herbage yield of both grass alone and in mixture with alfalfa (Tables 36 and 37) indicated that all grass species in pure stands produced higher yields than those of grasses grown in mixed stands, principally in the first and second year. In the third year stand (Experiment IV), however, yields in mixtures were equal to pure stands. As the experiment did not receive any N fertilizer, these results perhaps indicate that in the establishment year and early stage of development in the second year there was a relative abundance of available soil N to support the growth of the grasses in pure stand. However, the decline in the soil N availability by the end of the season in the second year, produced the decrease of the herbage yield in the third year. Trenbath (1976) and Haynes (1980) reported a competitive dominance of legume under low soil N availability, where the grass is frequently suppressed or has little advantage. However, under high N levels the strong responses of the non-legume usually causes decreases in the legume growth. From the results shown in Tables 36 and 37, the depletion of soil N resulted in the reduction in the herbage yield of grass grown in monoculture, but did not affect yields of grasses in mixtures.

Because total N yield (Tables 38 and 39) was obtained from herbage yield X total N concentration, it followed the same trend as N content and herbage yield. As expected, grass in

Table 36. Herbage production (DW) of grasses grown in mono mixed cultures at different harvests, 1983 to 1985 (Experiment IV). Values in parentheses are the percentages of the total annual herbage harvested.

YEAR	TREATMENT	Dry Weight (DW) Harvest 1	2	3	Total+++ (t/ha)	
1983	Timothy(1) with alfalfa+	0.33c	0.50c		0.83c	(14.8)
	Timothy(2) with alfalfa	0.38c	0.34c		0.72c	(13.3)
	Bromegrass with alfalfa	0.44c	0.54c		0.98c	(15.6)
	Timothy(1)	1.43ab	0.96b		2.39b	(100.0)
	Timothy(2)	1.17b	0.94b		2.11b	(100.0)
	Bromegrass	1.79a	1.43a		3.22a	(100.0)
	Mean++	0.92a	0.78b		1.70	
	±SE	0.31	0.22		0.46	
1984	Timothy(1) with alfalfa	1.13c	0.88ab		2.01c	(16.0)
	Timothy(2) with alfalfa	1.15c	0.89ab		2.04c	(16.1)
	Bromegrass with alfalfa	1.50c	1.07a		2.57c	(19.6)
	Timothy(1)	3.48b	0.64b		4.12b	(100.0)
	Timothy(2)	2.82b	0.76b		3.58b	(100.0)
	Bromegrass	4.49a	0.50b		4.99a	(100.0)
	Mean++	2.43a	0.79b		3.22	
	±SE	0.54	0.27		0.51	
1985	Timothy(1) with alfalfa	1.75a	0.31ab	0.16b	2.22a	(19.2)
	Timothy(2) with alfalfa	1.86a	0.51a	0.20ab	2.57a	(21.5)
	Bromegrass with alfalfa	2.04a	0.50a	0.21a	2.75a	(23.1)
	Timothy(1)	2.42a	0.29b	0.05c	2.76a	(100.0)
	Timothy(2)	1.98a	0.37ab	0.04c	2.39a	(100.0)
	Bromegrass	2.38a	0.43ab	0.06c	2.87a	(100.0)
	Mean++	2.07a	0.40b	0.12c	2.60	
	±SE	0.56	0.14	0.03	0.59	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally
+++Total production (means) compared vertically

Table 37. Herbage production (DW) of grasses grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V). Values in parentheses are the percentages of the total annual herbage harvested.

YEAR	TREATMENT	Dry weight (DW) Harvest 1	2	3	Total++	
		(t/ha)				
1984	Timothy with alfalfa	0.14bc			0.14bc	(6.2)
	Bromegrass with alfalfa	0.32bc			0.32bc	(14.5)
	Tall fescue with alfalfa	0.12c			0.12c	(5.7)
	Timothy	0.57b			0.57b	(100.0)
	Bromegrass	1.15a			1.15a	(100.0)
	Tall fescue	0.57b			0.57b	(100.0)
	Mean	0.47			0.47	
	±SE	0.16			0.16	
1985	Timothy with alfalfa	0.96b	0.16b	0.04b	1.16b	(12.1)
	Bromegrasss with alfalfa	1.69b	0.51b	0.18b	2.38b	(20.2)
	Tall fescue with alfalfa	0.57b	0.32b	0.17b	1.06b	(10.0)
	Timothy	4.89a	0.99a	0.67b	6.55a	(100.0)
	Bromegrass	5.84a	0.96a	0.65b	7.45a	(100.0)
	Tall fescue	5.80a	1.28a	1.16a	8.25a	(100.0)
	Mean+	3.28a	0.71b	0.48b	4.47	
	±SE	1.00	0.20	0.17	1.17	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally
++Total production (means) compared vertically

Table 38. Nitrogen yield of grasses grown in mono and mixed cultures at different harvests, 1983 to 1985 (Experiment IV). Values in parentheses are the percentages of the total annual nitrogen harvested.

YEAR	TREATMENT	N Yield Harvest 1 (kg/ha)	2	3	Total+++	
1983	Timothy(1) with alfalfa+	7.36c	10.41c		17.77c	(12.9)
	Timothy(2) with alfalfa	8.99c	6.96c		15.96c	(10.7)
	Bromegrass with alfalfa	10.69c	11.25bc		21.94c	(13.3)
	Timothy(1)	24.54b	16.65b		41.19b	(100.0)
	Timothy(2)	23.65b	16.23b		39.88b	(100.0)
	Bromegrass	35.13a	24.44a		59.57a	(100.0)
	Mean++	18.39a	14.39b		32.78	
	±SE	6.67	4.17		9.39	
1984	Timothy(1) with alfalfa	10.22b	26.21b		36.43bc	(12.4)
	Timothy(2) with alfalfa	11.17b	35.12b		46.30ab	(14.8)
	Bromegrass with alfalfa	12.02b	46.57a		58.59a	(19.3)
	Timothy(1)	12.08b	9.63c		21.72d	(100.0)
	Timothy(2)	10.60b	12.37c		22.98cd	(100.0)
	Bromegrass	18.09a	7.61c		25.70cd	(100.0)
	Mean++	12.37b	22.92a		35.29	
	±SE	2.53	10.36		10.23	
1985	Timothy(1) with alfalfa	30.95ab	7.75bc	4.89b	43.59b	(14.1)
	Timothy(2) with alfalfa	31.41ab	9.90ab	6.08b	47.39ab	(14.3)
	Bromegrass with alfalfa	40.39a	13.28a	8.18a	61.85a	(19.5)
	Timothy(1)	27.8ab	4.82c	0.75c	33.37bc	(100.0)
	Timothy(2)	19.84b	4.82c	0.60c	25.26c	(100.0)
	Bromegrass	33.30ab	7.66bc	1.18c	42.15b	(100.0)
	Mean++	30.62a	8.04b	3.61c	42.27	
	±SE	10.55	2.78	1.30	11.42	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally
+++Total production (means) compared vertically

Table 39. Nitrogen yield of grasses grown in mono and mixed cultures at different harvests, 1984 to 1985 (Experiment V). Values in parentheses are the percentages of the total annual nitrogen harvested.

YEAR	TREATMENT	N Yield Harvest 1	2	3	Total++	
		(kg/ha)				
1984	Timothy with alfalfa	5.57c			5.57c	(8.6)
	Bromegrass with alfalfa	8.87c			8.87c	(13.9)
	Tall fescue with alfalfa	3.08c			3.08c	(4.8)
	Timothy	10.93bc			10.93bc	(100.0)
	Bromegrass	23.60a			23.60a	(100.0)
	Tall fescue	11.56b			11.56b	(100.0)
	Mean	10.13			10.13	
	±SE	4.14			4.14	
1985	Timothy with alfalfa	22.26c	3.87d	1.16d	27.29c	(8.8)
	Bromegrass with alfalfa	32.75bc	15.21bc	6.61dc	54.57bc	(15.7)
	Tall fescue with alfalfa	13.83c	8.55dc	4.69d	27.07c	(7.8)
	Timothy	55.82b	14.15bc	11.63bc	81.60b	(100.0)
	Bromegrass	91.68a	17.86ab	13.86b	123.40a	(100.0)
	Tall fescue	88.34a	24.05a	21.75a	134.13a	(100.0)
	Mean+	50.78a	13.95b	9.95b	74.68	
	±SE	21.17	4.63	3.98	24.08	

Means in a column followed by the same letter are not significantly different at the 5% level of probability, according to Duncan's Multiple Range Test.
+Means compared horizontally
++Total production (means) compared vertically

association yielded more than that of pure stands after the establishment year. The presence of alfalfa caused a significant increase of % N content of grass in mixed culture in contrast to the grass in pure stand which suffered from the decline in available soil N (Experiment IV). In Experiment V, however, the grass in pure stands yielded significantly more N than grass in mixtures. This could be attributed to the higher level of available nitrogen in the soil that supported a normal growth.

4.2.2.3 Dry matter and N yield of cultural systems

Annual dry weight (DW) yields are given in Tables 40 and 42 for each of the 7 cultural systems in each of the 3 years (Experiment IV) and 2 years (Experiment V). As the interaction between cultural systems and year were significant, the values of total annual dry weight and total annual N yield presented in Tables 40, 41, 42 and 43 are the secondary effects of both. DW harvested over all cultural systems averaged 4.32 t/ha in the first year, increased significantly to 8.95 t/ha in the second year and declined significantly to 7.98 t/ha in the third year (Experiment IV). The latter was due to the lower yields of DM of the three grasses in monoculture in the second and third harvest in 1985.

Alfalfa in pure stand and in alfalfa-grass mixtures gave higher DW yields than the grasses (Tables 40 and 42). Yields

Table 40. Herbage production (DW) harvested from different cultural systems at each harvest, 1983 to 1985 (Experiment IV).

YEAR	CULTURAL SYSTEM	Dry Weight (DW) Harvest 1	2	3	Total+++
		(t/ha)			
1983	Alfalfa	3.15	2.18		5.33
	Alfalfa with timothy(1)+	3.11	2.37		5.48
	Alfalfa with timothy(2)	3.03	2.37		5.40
	Alfalfa with bromegrass	3.47	2.83		6.30
	Timothy(1)	1.43	0.96		2.39
	Timothy(2)	1.17	0.94		2.11
	Bromegrass	1.79	1.43		3.22
	Mean++	2.45*	1.87		4.32
	LSD (5%)	0.48	0.27		0.58
1984	Alfalfa	7.32	4.23		11.55
	Alfalfa with timothy(1)	7.77	4.84		12.61
	Alfalfa with timothy(2)	8.05	4.69		12.74
	Alfalfa with bromegrass	8.06	4.94		13.00
	Timothy(1)	3.47	0.76		4.23
	Timothy(2)	2.82	0.64		3.46
	Bromegrass	4.50	0.50		5.00
	Mean++	6.00*	2.94		8.94
	LSD (5%)	0.96	0.85		0.81
1985	Alfalfa	5.42	3.24	2.41	11.07
	Alfalfa with timothy(1)	6.68	3.12	2.64	12.44
	Alfalfa with timothy(2)	6.38	3.34	2.61	12.33
	Alfalfa with bromegrass	6.60	2.94	2.18	11.72
	Timothy(1)	2.42	0.31	0.06	2.79
	Timothy(2)	1.98	0.38	0.05	2.41
	Bromegrass	2.36	0.44	0.07	2.87
	Mean++	4.54*	1.97	1.43	7.94
	LSD (5%)	0.92	0.65	0.78	1.69

*Denotes significant difference among harvest at the 5% level of probability, according to Least Significant Difference Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally
+++Total annual (means) compared vertically

Table 41. Nitrogen yield of different cultural systems at each harvest, 1983 to 1985 (Experiment IV).

YEAR	CULTURAL SYSTEM	N Yield Harvest 1	2	3	Total+++
		(kg/ha)			
1983	Alfalfa	64.56	63.32		127.88
	Alfalfa with timothy(1)+	64.98	72.90		137.88
	Alfalfa with timothy(2)	71.68	70.68		142.36
	Alfalfa with bromegrass	82.66	82.26		164.92
	Timothy(1)	24.52	16.66		41.18
	Timothy(2)	23.62	16.62		40.24
	Bromegrass	35.12	24.44		59.56
	Mean++	52.45	49.41		101.86
	LSD (5%)	11.65	6.62		13.19
1984	Alfalfa	185.10	157.12		342.22
	Alfalfa with timothy(1)	163.68	187.90		351.58
	Alfalfa with timothy(2)	183.72	165.58		349.30
	Alfalfa with bromegrass	160.68	176.74		337.32
	Timothy(1)	12.00	8.56		20.56
	Timothy(2)	10.58	12.32		22.90
	Bromegrass	18.06	7.60		25.66
	Mean++	105.40	102.30		207.70
	LSD (5%)	24.75	29.98		32.82
1985	Alfalfa	143.92	91.14	76.06	311.12
	Alfalfa with timothy(1)	178.44	74.46	82.42	335.32
	Alfalfa with timothy(2)	173.72	84.76	74.98	333.46
	Alfalfa with bromegrass	175.60	75.88	69.06	320.54
	Timothy(1)	28.46	5.10	1.79	35.35
	Timothy(2)	20.12	4.94	1.66	26.72
	Bromegrass	34.14	7.72	2.21	44.07
	Mean++	107.77*	49.14	43.60	200.51
	LSD (5%)	23.06	16.99	24.91	45.95

*Denotes significant difference among harvest at the 5% level of probability, according to Least Significant Difference Test.
+Timothy(1) cultivar 'climax'
Timothy(2) cultivar 'salvo'
++Means compared horizontally
+++Total annual (means) compared vertically

Table 42. Herbage production (DW) harvested from different cultural systems at each harvest, 1984 to 1985 (Experiment V).

YEAR	CULTURAL SYSTEM	Dry Weight (DW) Harvest 1	2	3	Total++
		(t/ha)			
1984	Alfalfa	2.13			2.13
	Alfalfa with timothy	2.12			2.12
	Alfalfa with bromegrass	2.14			2.14
	Alfalfa with tall fescue	2.11			2.11
	Timothy	0.57			0.57
	Bromegrass	1.15			1.15
	Tall fescue	0.60			0.60
	Mean	1.54			1.54
	LSD (5%)	0.53			0.53
1985	Alfalfa	5.48	2.52	1.44	9.44
	Alfalfa with timothy	5.36	2.73	1.55	9.64
	Alfalfa with bromegrass	6.90	2.98	1.81	11.69
	Alfalfa with tall fescue	5.83	3.30	1.64	10.77
	Timothy	4.89	0.99	0.67	6.55
	Bromegrass	5.84	0.96	0.65	7.45
	Tall fescue	5.80	1.28	1.17	8.25
	Mean+	5.73*	2.11	1.28	9.12
	LSD (5%)	1.91	0.74	0.47	1.70

*Denotes significant difference among harvest at the 5% level of probability, according to Least Significant Difference Test.
+Means compared horizontally
++Total annual (means) compared vertically

Table 43. Nitrogen yield of different cultural systems at each harvest, 1984 to 1985 (Experiment V).

YEAR	CULTURAL SYSTEM	N Yield Harvest 1	2	3	Total++
		(kg/ha)			
1984	Alfalfa	52.50			52.50
	Alfalfa with timothy	65.10			65.10
	Alfalfa with bromegrass	64.20			64.20
	Alfalfa with tall fescue	65.80			65.80
	Timothy	10.90			10.90
	Bromegrass	23.50			23.50
	Tall fescue	11.60			11.60
	Mean	41.94			41.94
	LSD (5%)	12.40			12.40
1985	Alfalfa	156.60	85.40	53.90	295.90
	Alfalfa with timothy	175.30	93.90	54.00	323.20
	Alfalfa with bromegrass	188.50	92.30	66.00	346.80
	Alfalfa with tall fescue	178.50	109.70	58.30	346.50
	Timothy	55.70	14.10	11.50	81.30
	Bromegrass	91.50	17.80	13.80	123.10
	Tall fescue	88.20	23.90	21.60	133.70
	Mean+	133.47*	62.44	39.87	235.78
	LSD (5%)	56.63	23.68	19.48	50.69

*Denotes significant difference among harvest at the 5% level of probability, according to Least Significant Difference Test.
+Means compared horizontally
++Total annual (means) compared vertically

from alfalfa-grass mixtures exceeded the yields of alfalfa grown alone, in majority of cuts. However, the differences were always small and were only significant for alfalfa grown in association with bromegrass in harvest 2 (1983, Experiment IV) and in harvest 3 (1985, Experiment V).

Among the three grasses grown in pure stand, bromegrass was significantly more productive in the initial two years, but in the third year this difference disappeared (Experiment IV). However, the total annual forage yield of this species was depressed by 69.5% during the first year, 48.6% in the second year and 4.5% in the third year of the experiment, when it was grown in mixture with alfalfa. On the other hand, in mixtures the contribution of bromegrass to annual dry weight yield increased from 15.6% in the first year to 23.1% in the third year (Table 36, Experiment IV).

In Experiment V, among the three grass species grown in pure stand, bromegrass was significantly more productive during the first year, but at the end of the second year tall fescue proved to be significantly more productive than bromegrass. The total annual forage yield from these two species, however, was very close. Moreover, the contribution of bromegrass to annual dry weight yield improved from 14.5% in the first year to 20.2% in the second year (Table 37).

N yield in alfalfa and alfalfa-grass mixtures were more than 3 to 4 fold as high as in grasses alone during the first year and

increased considerably in the following year (Table 41 and 43). Over the experiments, alfalfa-grass mixtures produced more N than alfalfa in monoculture. However, this difference was only significant in the first year in both harvests (Table 41). Moreover, among the alfalfa-grass mixtures there were only significant differences during the initial year.

The N contributions of the three grasses to the alfalfa-grass mixtures followed the same trend of dry weight production. The contribution of bromegrass to annual N yield was superior to the other grass species. Bromegrass contributed 13.3% of the total annual N yield in the first year and increased to 19.5% in the third year (Table 38, Experimnet IV).

The low N yield of these grasses in monoculture could be significantly improved by growing them in mixture with alfalfa, since the legume-grass mixtures reported here contained more than three times as much N yield as grasses grown alone. Such an improvement is mainly related to the high N content of the alfalfa, and also, in some cases to the release of N to grasses from the associated legume. The slight difference in N yield among the mixtures in comparison to alfalfa in monoculture reported here seemed to reflect seasonal variation in total dry weight production rather than N content of the species. However, the major differences in N yield among the mixtures as well as grasses in monocultures seemed to reflect differences in both dry weight production and % N content of the species. For a high and

nutritious forage yield under temperate conditions alfalfa should be grown in mixture with grasses because this cultural system often has greater advantage than is expected on the basis of performances in pure stand. Although several explanations have been suggested for this, the most obvious reasons are that the alfalfa and grass use different N sources, spatial differences in the use of resources (Martin and Snaydon 1982) and N transference from alfalfa to grass (Ta and Faris 1985).

2.2.3 Comparison of methods for estimating N_2 fixation by alfalfa

Nitrogen fixation by alfalfa grown in mono and mixed cultures was measured by three methods (Table 44). As there were significant differences among measurement techniques, cultural systems and the interaction of these two variables, the values presented in Table 44 are the two-way interaction and the main effects of both. Difference method (DM) and ^{15}N isotope dilution (ID) gave higher estimates than acetylene reduction assay (ARA). DM and ID gave estimates of N_2 fixation that were significantly different in the seeding year for both cultural systems. In the subsequent years, the estimates by these two methods were close, however, the ID showed lower standard error than DM, demonstrating that the ID was more precise to assess N_2 fixation. Generally the assessments of N_2 fixation by DM

Table 44. Significant two-way interaction and main effects of methods of estimating N_2 fixation and cultural systems at each year during 1983 to 1985. Values in parentheses are the standard error of the mean for each technique.

Cultural system x Measurement technique Interaction

System	Technique	N_2 fixed Year 1983	1984	1985
		(kg/ha)		
Alfalfa alone	ARA+	23.94	26.48	
	ID	83.95	266.90	229.85
	DM	67.77	288.85	213.86
Alfalfa with grass	ARA	24.51	23.79	
	ID	115.81	246.44	200.34
	DM	105.51	246.70	255.92
LSD (5%)		3.87	44.14	54.82

Main effects

Measurement technique or Cultural system	N_2 fixed Year 1983	1984	1985
	(kg/ha)		
ARA - Acetylene reduction	24.22	25.13	
	(4.9)	(4.7)	
ID - Isotope dilution	99.88	256.67	215.09
	(18.4)	(12.0)	(17.1)
DM - Difference method	86.64	267.78	234.89
	(21.8)	(32.8)	(33.97)
Alfalfa alone	58.55	194.07	221.85
Alfalfa with grass	82.11	172.31	228.13
LSD (5%)	3.87	44.14	54.82

+ARA = Acetylene reduction assay
ID = Isotope dilution technique
DM = Difference method

exceeded those produced by ID.

The DM assumes equal uptake of N from the soil by fixing and reference plants. In this study, during the seeding year, DM underestimated significantly N_2 fixation compared to the ID, while in the following years DM overestimated fixation. This could be due to a less efficient use of soil N by the fixing plants. As was observed in Tables (18 and 19) atom % ^{15}N excess in alfalfa plants in both cultures was higher than the level of natural abundance, showing that the fixing plant took up soil N and competed slightly with the grass for mineral N mainly in the seeding year. Bole and Rennie (1983) claimed that the N difference method has generally underestimated fixation in the field compared to the isotope dilution method. Martensson and Ljunggren (1984) compared the derived total N_2 fixation values from DM and ID, and concluded the ID gave a significantly lower value of N_2 fixed. Boddley et al. (1984) found that estimates of N_2 fixation by soybean were generally higher by the total N difference method, because the uptake by the nodulated plants was consistently higher than uptake by non-nodulated soybean. Wagner and Zapata (1982) also found a higher uptake of fertilizer N by nodulated soybean in the field compared to non-nodulated plants, with N_2 fixation being overestimated by total N difference. The relative uptake of fertilizer and/or soil N by fixing and reference plants will depend on soil, plant and environmental factors and management practices (Deibert et al. 1979; Vasilas

and Ham 1985).

Independent of the cultural system, the assessments of N_2 fixation by DM after the initial year exceeded those estimated by ID by an overall average of 4.15 and 8.43% in 1984 and 1985, respectively, nevertheless, these differences were not significants. This suggests that the DM will provide a representative N_2 fixation value where the environmental conditions permit proper development of control plants and there are no differences in uptake of N from soil or fertilizer between fixing and reference plants.

Although many comparisons of ID and DM estimates of N_2 fixation have been made, the results have been inconsistent. However, in a number of studies good agreement has been obtained, and this has lead some investigators (Broadbent et al. 1982; Phillips et al. 1983) to question if the ID offers clear advantages over the DM, and whether the extra cost associated with ID are warranted. Talbott et al. (1982) noted that individual observations of the amounts of N_2 fixed by soybean estimated by ID and DM were highly correlated, but individual observations of % Ndfa were not as closely related. Similarly, Rennie (1984) observed that significant relationships between ID and DM estimates were frequent, and generally of a higher statistical significance for the amounts of N_2 fixed than for % Ndfa. Coale et al (1985) compared ID and DM to quantify the % Ndfa and the absolute amount of N_2 fixed. These comparisons

showed some similarity, but there was a difference in the precisions of the methods. The ID consistently showed a lower standard deviation than DM for both dependent and independent yield criteria.

The ID offers greater sensitivity compared to the DM (Ruschel et al. 1979), which is an advantage when measuring low rates of fixation associated with forage grasses (Boddey et al. 1983) or cereal such as wheat (Rennie et al. 1983). Several authors have also commented on the higher precision of ID estimates based on total N yield difference (Ruschel et al. 1979; Talbott et al. 1982; Rennie and Rennie 1983; Rennie 1984). However, in the absence of evidence that fixing and reference plants up take the same proportion of N from soil and/or fertilizer, it cannot be assumed that the ID provides a more accurate estimate of N_2 fixed compared to the DM.

The estimates by ARA were always significantly lower than those of the other two techniques. The amount of N_2 fixed was calculated as already mentioned in Experiment II. The total amount of N_2 fixed ranged from 23.79 kg N/ha to 26.48 kg N/ha. In addition, the ARA estimate for alfalfa grown in pure stand was always close to that of alfalfa grown in mixed stand. Thus, ARA method gave the lowest N_2 fixation estimates and the variation was notable due to nitrogenase activity that was not normally distributed with no homogeneity of variance (Appendix 1). These variations and low values may be explained by the fact that a

mixture of Rhizobium strains was used in association with the genetical variations that occur within the alfalfa population (Tough and Crush 1979). As well the loss of nodules and damage to the roots during the process of separation of the root systems from the soil contributed to the variations.

Data from the greenhouse study (Table 11) indicated that the ARA for N_2 fixation underestimates the DM by an overall average of 64%. In this case, there was no control plant involved in the DM, therefore overestimation should not be a factor. This would imply a C_2H_2:N_2 ratio of near 1.1:1, which is almost the same as that measured for soybeans (Herridge 1982a). Application of such a ratio to the ARA estimate for field grown alfalfa would give an estimate of 66.05 kg N/ha and 68.53 kg N/ha in 1983 and 1984, respectively, still well below that of the DM estimates. In spite of the error of estimate for N_2 fixation, under field or greenhouse conditions and other limitations cited, the ARA provides a useful test for studying the relative activity of the nitrogenase enzyme complex at a specific point in time.

Estimates of N_2 fixation from alfalfa grown in mixed stands were significantly greater than for that grown alone in the seeding year. In the subsequent years, however, there were no significant differences between the amount of N_2 fixed by alfalfa plants grown in mono and mixed cultures. These data suggest that the grass has no detrimental effect on alfalfa N_2 fixation and may stimulate N_2 fixation of alfalfa when both are grown in

association. Craig de Anda *et al* (1981) reported that the inclusion of grass in legume mixtures caused an increase in the N_2 fixation of legumes. They explained that either less N is available to the legumes as a results of fast uptake by grasses, or a possible excretion of some active substances by the grass species may stimulate the legume N_2 fixation.

In summary, it can be concluded from the data presented in this report that under low soil N either the ^{15}N dilution technique or the N difference method is suitable for quantitative assessments of symbiotic N_2 fixation. These results are consistent with other findings (Ruschel *et al*. 1979; Talbott *et al*. 1982; Broadbent *et al*. 1982; Rennie 1984) and suggest that the ID is the method of choice when the most precise field measurements are needed. However, given the additional costs associated with the application and analysis of ^{15}N (atom % ^{15}N), the DM may be preferred in some situations.

5. SUMMARY AND CONCLUSIONS

This study investigated some aspects related to N_2 fixation by alfalfa and N transfer to associated grass when grown in mixture, using the ^{15}N dilution technique. The seasonal pattern of nitrogenase enzyme activity also was evaluated by acetylene reduction assay through the growth and regrowth time. Data were collected in five experiments; two were conducted under greenhouse conditions, using very low N level medium and the other three under field conditions.

The proportion of nitrogen derived from the atmoshpere (% Ndfa) showed that throughout the entire season(s) alfalfa plants were able to fix most of their total N requirement. The % Ndfa increased over the growing season and ranged from 69.0 to 83.0% (field studies) and from 95.0 to 98.0% (greenhouse studies). This was compatible with the reduction of atom % ^{15}N excess of alfalfa and grass during the experimental period. Also, this explained well the relatively small increases of % Ndfa and indicated that alfalfa and grass had an acceptable N uptake. In addition, the low variability associated with assessments of % Ndfa illustrated the usefulness of the ^{15}N dilution method in this study.

The total amount of N_2 fixed by alfalfa presented a pattern similar to % Ndfa with slight increases in the amount of N_2 fixed by alfalfa in association with grass. In other words, mixed cultures of alfalfa with grass did not reduce the apparent activity of legume N_2 fixation, and it appeared in some cases

that the presence of grass stimulated alfalfa N_2 fixation due to either less N available to legume or the excretion of biologically active substances that stimulated the legume N_2 fixation.

Transfer of N from alfalfa to the grass paralleled the N_2 fixing capacity of alfalfa and increased throughout the season. Although N transfer only accounted for 4.0 to 24.0% (greenhouse studies) and 5.0 to 8.0% (field studies) of the total amount of N_2 fixed by alfalfa it was significant in improving the production of grass when grown in mixture with alfalfa. During the initial season less than 5.0 kg N/ha was transferred from alfalfa to grass, but these values increased to 20.0 kg N/ha and 19.0 Kg N/ha in the second and at third year, and represented about 26.0, 46.0 and 38.0% of the total N yield of grass in association during those years. The consistent percentage N transfer that occurred before initial harvest, together with the requirement of maintenance of the nodule weight by alfalfa roots, indicated that this transfer process is not totally due to the death of roots and nodule tissue after shoot removal but may involve a considerable degree of N excretion during the period before and after harvest.

In general, the ^{15}N dilution technique provided a useful method to measure N transference and showed that all grass species benefitted similarly from N transfer from alfalfa during the entire period, although species with earlier maturity and greater competitive ability were more responsive. This suggests

that grass species with fast developing root systems should be used in alfalfa-grass mixtures to efficiently utilize available N.

The results on seasonal changes in nitrogenase enzyme activity that were associated with nodule weight of alfalfa suggest that alfalfa plants have the ability to maintain the most of their nodule weight after the stress of shoot removal. This information may explain why the capacity of N_2 fixation by alfalfa is only temporarily impaired, with fast recovery of nodule activity in few days.

Herbage and N yields from alfalfa-grass mixtures exceeded those produced by each component species grown in pure stands. Thus, alfalfa should be grown in mixtures with grass, and managed for high yield to maximize N_2 fixation, while maintaining grass to efficiently utilize available soil N.

Estimates of N_2 fixation, using the N difference method and ^{15}N dilution method were fairly similar and larger than N_2 fixation estimates by the acetylene reduction assay. Under low soil N levels either the ^{15}N dilution method or the N difference method is suitable for quantitative assessments of symbiotic N_2 fixation. The ^{15}N dilution technique is the method of choice when the most precise measurement of N_2 fixation is required. Moreover, due to the costs associated with the application and analysis of ^{15}N, and the similarity of the results obtained by these two techniques, the N difference may be preferred in many situations.

6. ORIGINAL CONTRIBUTIONS TO KNOWLEDGE AND IMPLICATIONS FOR FUTURE RESEARCH IN LEGUME-GRASS MIXTURES

Knowledge on the use of ^{15}N dilution method to measure N transfer from legume to associated grass over the life of perennial alfalfa-grass mixtures is practically non-existent. The present study is believed to be unique in that it is the first attempt to estimate the magnitude of the beneficial transfer of N from alfalfa to grass over time. The uniqueness of this investigation is also related to the fact that it describes measurement of N_2 fixation by alfalfa under mixed culture over several years.

2. This is the first report to compare the assessment of N_2 fixation by alfalfa grown in mono and mixed culture, utilizing isotope dilution techniques over several years with interpretation of long term isotope dilution study.

3. This is the first attempt to report the seasonal changes in nitrogenase enzyme activity of alfalfa grown alone and mixed with grass after successive harvests under field conditions.

4. It was shown that alfalfa plants have the ability to adapt to the stress of shoot removal by maintaining an unchanged nodule weight with the conclusion that the N_2 fixation process is impaired only immediately after harvest. This suggests that the N transfer mechanism is not only due to the decomposition or death of roots and nodule tissue after clipping. This was

supported by the finding that there was some N transferred before the first clipping in new stands. This is the first research that suggests that the N transference from alfalfa to grass involves a considerable degree of excretion of soluble N compounds by living roots.

The fundamental information found in this dissertation will contribute to a better understanding of N_2 fixed and released into the environment, and the interactions that occur within alfalfa-grass mixtures. However, several areas were identified that need further investigation. These areas can be summarized as follows:

(a) The associative N_2 fixation was not included in the objectives of this study. However, it was suggested from the results of Experiment III that associative N_2 fixation was contributing to the N nutrition of the grass. The associative N_2 fixation violates the assumption that the reference plant does not fix nitrogen from the atmosphere. To more accurately measure the N_2 fixation by the ^{15}N dilution method, associative N_2 fixation should be considered and included in the measurement.

(b) Transfer of N from alfalfa to grass was demonstrated and quantified. Further research is necessary to determine the proportion of this N which is obtained by the grass: (1) directly through absorption of excreted soluble N compounds; (2) indirectly through absorption of N from nodule and root breakdown; and (3) through associative N_2 fixation, perhaps

stimulated by the presence of the legume.

(c) Generally, all grass species benefitted from transfer of N from alfalfa in the present study. However the earlier maturing species and species with greater competitive ability were slightly more responsive to N transfer. Additional research is necessary to identify the effect of grass species and cultivars or genotypes within species on the amount of N transferred.

(d) Despite the extensive use of legumes as N sources in cropping systems, relatively few measurements on release of available N from the decomposition of legume residues are available. Research on this subject is needed to clarify the importance of this indirect pathway of N transfer from alfalfa to grass.

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8. APPENDICES

Appendix 1. Bartlett test for homogeneity of variance of alfalfa variables in the seeding year, 1983 (Experiment III).

Variable - Nodule fresh weight

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data+
Treatment (T)	3	552.7	68044.4	169884.8	7.81*	0.25 ns
Determination (D)	6		58577.2	213056.5	14.51*	2.16 ns
T in D	27		20183.7	211096.3	36.3 *	12.80 ns

Variable - Total nitrogenase activity

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	2.68	1.727	3.946	7.67*	1.86 ns
Determination (D)	6		0.383	4.047	35.69*	13.10*
T in D	27		0.175	19.901	43.05*	22.97 ns

Variable - Shoot dry weight

Source of Variation	D.F.	Overall Mean	Variance Minimun	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	4.52	9.574	14.855	2.94 ns	2.73 ns
Determination (D)	6		0.96×10^{-1}	16.322	126.39*	21.25*
T in D	27		0.37×10^{-1}	17.485	118.66*	48.56*

Cont...

Appendix 1. Continued ...

Variable - **Root dry weight**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	4.81	4.733	7.204	2.09 ns	2.98 ns
Determination (D)	6		1.395	8.568	21.83*	6.18 ns
T in D	27		0.497	9.847	34.21*	27.28 ns

Variable - **Shoot nitrogen yield**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	2.88	0.244	0.457	3.42 ns	2.11 ns
Determination (D)	6		0.525×10^{-1}	0.207	14.32*	8.70 ns
T in D	27		0.299×10^{-2}	0.348	63.69*	62.81*

Variable - **Root nitrogen yield**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	1.678	0.394×10^{-1}	0.957×10^{-1}	8.98*	1.46 ns
Determination (D)	6		0.957×10^{-2}	0.998×10^{-1}	29.18*	3.66 ns
T in D	27		0.200×10^{-2}	0.322	60.07*	27.60 ns

*, = significant difference at the 0.05 level of probability.
ns, = no significant difference.
+ Analysis of transformed data using natural logarithm.

Appendix 2. Bartlett test for homogeneity of variances of alfalfa variables in the subsequent year, 1984 (Experiment III).

Variable - Nodule fresh weight

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	378.61	1.12×10^5	3.22×10^5	11.5*	0.60 ns
Determination (D)	5		4.99×10^3	2.75×10^5	104.4*	13.10*
T in D	23		1.25×10^3	5.02×10^5	120.6*	33.70 ns

Variable - Total nitrogenase activity

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	2.225	3.73	9.29	7.6*	1.80 ns
Determination (D)	6		0.10	11.16	157.8*	13.00*
T in D	27		0.178×10^{-1}	17.49	161.8*	35.40 ns

Variable - Shoot dry weight

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual value	Bartlett test value transformed data
Treatment (T)	3	7.62	29.88	51.90	2.6 ns	1.20 ns
Determination (D)	6		0.16	35.03	152.6*	9.12 ns
T in D	27		0.23	80.25	161.8*	35.00 ns

Cont...

Appendix 2. Continued ...

Variable - **Root dry weight**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	8.19	8.27	12.95	1.80 ns	2.06 ns
Determination (D)	6		4.34	17.12	15.75*	10.80 ns
T in D	27		0.48	26.29	46.00*	33.34 ns

Variable - **Shoot nitrogen yield**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	3.02	0.11	0.41	14.71*	9.05*
Determination (D)	6		0.25×10^{-1}	0.29	36.65*	24.13 ns
T in D	27		0.29×10^{-2}	0.31	52.91*	30.48 ns

Variable - **Root nitrogen yield**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	3	1.63	0.26×10^{-1}	0.78×10^{-1}	17.42*	13.10*
Determination (D)	6		0.11×10^{-1}	0.74×10^{-1}	30.80*	36.57*
T in D	27		0.20×10^{-2}	0.23	75.84	32.93*

*, = significant difference at the 0.05 level of probability.
ns, = no significant difference.
+ Analysis of transformed data using natural logarithm.

Appendix 3. Bartlett test for homogeneity of variances of grass variables in the seeding year, 1983 (Experiment III).

Variable - Shoot dry weight

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	1.112	0.410×10^{-1}	3.124	179.5*	20.87*
Determination (D)	6		0.161	4.099	97.3*	6.90 ns
T in D	41		0.200×10^{-1}	19.76	335.9*	44.93 ns

Variable - Root dry weight

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual dat	Bartlett test value transformed data
Treatment (T)	5	2.331	0.912	6.762	92.38*	2.28 ns
Determination (D)	6		2.561	12.673	35.49*	8.09 ns
T in D	41		0.67×10^{-1}	13.027	160.13*	49.00 ns

Variable - Shoot nitrogen yield

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	2.143	0.735×10^{-1}	0.236	11.69*	1.96 ns
Determination (D)	6		0.375×10^{-1}	0.856×10^{-1}	9.10 ns	1.56 ns
T in D	41		0.199×10^{-2}	0.162	146.00*	33.55 ns

Cont...

Appendix 3. Continued ...

Variable - Root nitrogen yield

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	0.691	0.828×10^{-2}	0.276×10^{-1}	17.14*	6.20 ns
Determination (D)	6		0.895×10^{-2}	0.424×10^{-1}	24.86*	9.70 ns
T in D	41		0.199×10^{-2}	0.200×10^{-1}	54.30 ns	51.09 ns

*, = significant difference at the 0.05 level of probability.
ns, = no significant difference.
+ Analysis of transformed data using natural logarithm.

Appendix 4. Bartlett test for homogeneity of variances of grass variables in the subsequent year, 1984 (Experiment III).

Variable - **Shoot dry weight**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	1.96	0.479	10.937	81.1*	15.96*
Determination (D)	6		0.246	11.245	179.0*	9.95*
T in D	41		0.700×10^{-2}	8.455	179.6*	81.03*

Variable - **Root dry weight**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	6.56	1.228	32.680	115.7*	13.02*
Determination (D)	6		10.609	61.315	29.9*	12.04 ns
T in D	41		0.103	76.587	170.6	56.00 ns

Variable - **Shoot nitrogen yield**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	1.76	0.163	1.22	59.7*	5.08 ns
Determination (D)	6		0.733×10^{-1}	1.411	73.3*	11.77 ns
T in D	41		0.200×10^{-2}	0.108	165.5*	125.77*

Cont...

Appendix 4. Continued ...

Variable - **Root nitrogen yield**

Source of Variation	D.F.	Overall Mean	Variance Minimum	Variance Maximum	Bartlett test value actual data	Bartlett test value transformed data
Treatment (T)	5	0.754	0.635×10^{-2}	0.613×10^{-1}	62.9*	15.03*
Determination (D)	6		0.1096	0.191	4.1 ns	5.11 ns
T in D	41		0.200	0.167	127.0*	56.92 ns

*, = significant difference at the 0.05 level of probability.
ns, = no significant difference.
+ Analysis of transformed data using natural logarithm.