NITROGENASE ACTIVITY AND VEGETATIVE REGROWTH OF ALFALFA GROWN ALONE AND IN MIXTURE WITH GRASS AFTER SUCCESSIVE HARVESTS[1]

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ABSTRACT - A field experiment was conducted with the purpose of establish the behavior of the nitrogenase activity and nodule mass of alfalfa plants and the effect that some grass species may have on the rate of legume N_2-fixation during many successive harvest cycles, when both grown in mixed stands. The results has shown that nitrogenase activity of alfalfa (*Medicago sativa* L.) is not significantly affected when grown in association with timothy (*Phleum pratense* L.) or bromegrass (*Bromus inermis* Leyss.). It was concluded that mixed cultures of alfalfa with timothy or bromegrass have no effect on alfalfa N_2-fixation (acetylene reduction). The initial decline in nitrogenase activity within two days of harvest, and subsequent fast recovery of nodule activity after harvest associated with the maintenance of the nodule weight of alfalfa suggest that the alfalfa plants have an adaptive mechanism to the stress of shoot removal. This may explain why the capacity of N_2-fixation by alfalfa is only temporarily impaired with fast recovery rate in comparison to other forage legumes.

Index terms: *Rhizobium meliloti, Medicago sativa*, nitrogen fixation, acetylen reduction, timothy grass, *Phleum pratense*, bromegrass, *Bromus inermis*, association, specific nodule activity.

ATIVIDADE DA NITROGENASE E CRESCIMENTO VEGETATIVO DA ALFAFA EM MONOCULTIVO E EM MISTURA COM GRAMÍNEAS DEPOIS DE SUCESSIVOS CORTES

RESUMO - Foi conduzido um experimento de campo com o propósito de determinar o comportamento da atividade da nitrogenase e da massa nodular da alfafa (*Medicago sativa* L.) e a interação do efeito das gramíneas sobre a taxa de fixação de nitrogênio da leguminosa durante vários ciclos sucessivos de corte, quando ambos crescem em associação. Os resultados demonstraram que a atividade da nitrogenase da alfafa não é afetada significativamente quando cultivada em associação com capim-timóteo (*Phleum pratense* L.) ou capim-bromo (*Bromus inermis* Leyss.). Foi concluído que a associação de alfafa com capim-timóteo ou capim-bromo não tem efeito sobre a fixação de N_2 (redução do acetileno). O declínio inicial da atividade da nitrogenase em dois dias após o corte e a veloz recuperação da atividade nodular depois do corte associada com a manutenção dos nódulos sugere que as plantas de alfafa têm um mecanismo de adaptação ao estresse da remoção da parte aérea. Isto pode explicar por que a capacidade de fixação biológica de N_2 pela alfafa é temporariamente enfraquecida com uma recuperação rápida, em comparação com outras leguminosas forrageiras.

Termos para indexação: *Rhizobium meliloti, Medicago sativa*, fixação de nitrogênio, redução do acetileno, capim-timóteo, *Phleum pratense*, capim-bromo, *Bromus inermis*, associação, atividade dos nódulos.

INTRODUCTION

Symbiotic N_2-fixation by the Rhizobium-legume association is linked closely to the presence of

photosynthetically active leaves and requires a large expenditure of energy for nodule growth and function. Rates of apparent N_2-fixation, measured as C_2H_2 reduction, has been associated with photosynthate supply and partitioning in a number of legume species. Supplemental light (Lawn & Brun 1974), grafting of a second shoot (Streeter 1974) and CO_2 enrichment (Havelka & Hardy 1976) enhanced nitrogenase activity (NA) of soybeans (*Glycine max* L. Merr.). Floral debudding and the associated promotion of vegetative growth stimulated nodule growth and nitrogenase activity of pea (*Pisum sativum* L.) (Raponen & Virtanen 1968). The nitrogenase activity decreased after exposure of subterranean clover (*Trifolium subterraneum* L.) to low light intensity (Gibson 1976), after CO_2 deprivation in soybean plants (Huang et al. 1975) and continuous darking of pea (Wheeler & Lawrie 1976). The depression of NA by such competitive sinks as

seed formation, developing fruits (Bethlenfalvay & Phillips 1977) further indicates the importance of photosynthate to N_2-fixation.

The shoot removal of legumes, as is periodically done in alfalfa (Medicago sativa L.) and other forages, and the competition between vegetative regrowth and nodules for reserve carbohydrates may adverse affect the capacity of nodules to sustain N_2-fixation. Previous investigators reported a decline in nodule number following herbage removal for several forage species (Butler et al. 1959, Whiteman 1970). White clover (Trifolium repens L.) (Sinclair 1972) and alfalfa (Vance et al. 1979) exhibited a pattern of initial decline and eventual recovery in NA after harvesting. It has been suggested that perennial legumes lose their nodules after shoot harvest or grazing and that new ones form during shoot regrowth. Wilson (1942) reported that white clover lost about a third of its nodules after harvest. Butler et al. (1959) found that defoliation and shading of red clover (Trifolium pratense L.) and birdsfoot trefoil (Lotus corniculatus L.) caused a severe reduction in nodule number, while nodules of white clover apparently were not affected. Moustafa et al. (1969) reported that defoliation of white clover caused a sharp decrease in NA within 24 hours as compared to nondefoliated controls.

The mechanisms controling nodule degeneration, nodule loss and nodule activity after harvest are not understood for any forage legume. Since alfalfa is often grown in mixture with perennial grasses and is frequently subjected to shoot removal by animal or mechanical harvesting and the rates of symbiotic N_2-fixation for alfalfa is greater than the most other legume species (Vance 1978), we thought that is was important to understand the effects of harvest on N_2-fixation. The purpose of the present study were to establish the behavior of the nitrogenase activity, nodule mass and the effect grass species may have on the rate of alfalfa N_2-fixation during a cycle of vegetative growth and regrowth, when both grown in mixed stands.

MATERIALS AND METHODS

Field experiment was established at Ottawa Research Station, Agriculture Canada, Ottawa, Ontario on a moderately drained sand loam soil during Spring of 1983. 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. However, the initial soil reaction was pH 5.1 (in water) and the K level was medium (98 ppm). Potassium and lime were applied at a rate of 30 kg.ha^{-1} and 3,000 kg.ha^{-1} as muriate of potash (KCl) and dolomitic lime (CaMg (CO$_3$)$_2$), respectively. Alfalfa (Medicago sativa L.) seeds cv. '520' were inoculated with a preparation of Rhizobium meliloti strains (L-26, L-6 and 102F20, Nitragin Co., Milwaukee, Wisconsin) and were sown at the rate of 13 kg.ha^{-1} in monoculture, and in mixed culture, at the rate of 11 kg.ha^{-1} with timothy Phleum pratense L. cvs. 'Salvo' and 'Climax' at 6 kg.ha^{-1}, and bromegrass (Bromus inermis Leyss.) cv. 'Tempo' at 9.0 kg.ha^{-1}. The same grass seeding rates in mixed stand were utilized in pure stands. When the seedlings of alfalfa and grasses were well established, 26 days after sowing (May 27, 1983) the following measurements were evaluated: alfalfa acetylene reduction rate (AR), nodule fresh weight, dry matter (DW) yields of root and top growth and total nitrogen (TN). Thereafter alfalfa and grass plant were sampled, when alfalfa reached 50% bloom stage (initial harvest on 23 July 1983) and after harvest: 2, 9, 15, 25 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 shoot removal on 23 June 1984. Grass plants were also analyzed for DW yield and total N concentration. Nitrogenase activity (NA) of intact alfalfa roots was determined by the AR technique. Plants from each treatment and from each replication were removed between 10.0 and 12.0 h.. Two 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 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 & Peterson (1982). After 1 hour incubation at room temperature (25°C $\pm$ 1°C), four 0.5 ml samples of the gas were withdrawn from each jar by syringes and injected into a GC-9700 BASIC-Tm gas chromatograph equipped with a 0.4 cm x 180 cm Poropak N column. The injector temperature was 72°C $\pm$ 1°C and the carrier gas was N$_2$ at a flow rate of 30-35 cc/min. Rate of ethylene (C$_2$H$_4$) production from acetylene (C$_2$H$_2$) was computed and was compared to a 0.1% ethylene standard curve that was established daily. Total N concentration was determined with a Tecator-Kjeltec designed to performed N measurements based on Kjeldahl method. Total N was determined from plant DW and percentage N calculated.

Analyses of variance were performed according to the appropriate design. 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 was used to determine which treatments means were significantly different.

RESULTS AND DISCUSSION

There were no significant differences in the seasonal variation of nitrogenase activity (NA) between alfalfa in mono and mixed stands throughout the experimental period (Fig. 1). The average NA alfalfa plants was 383 μ moles
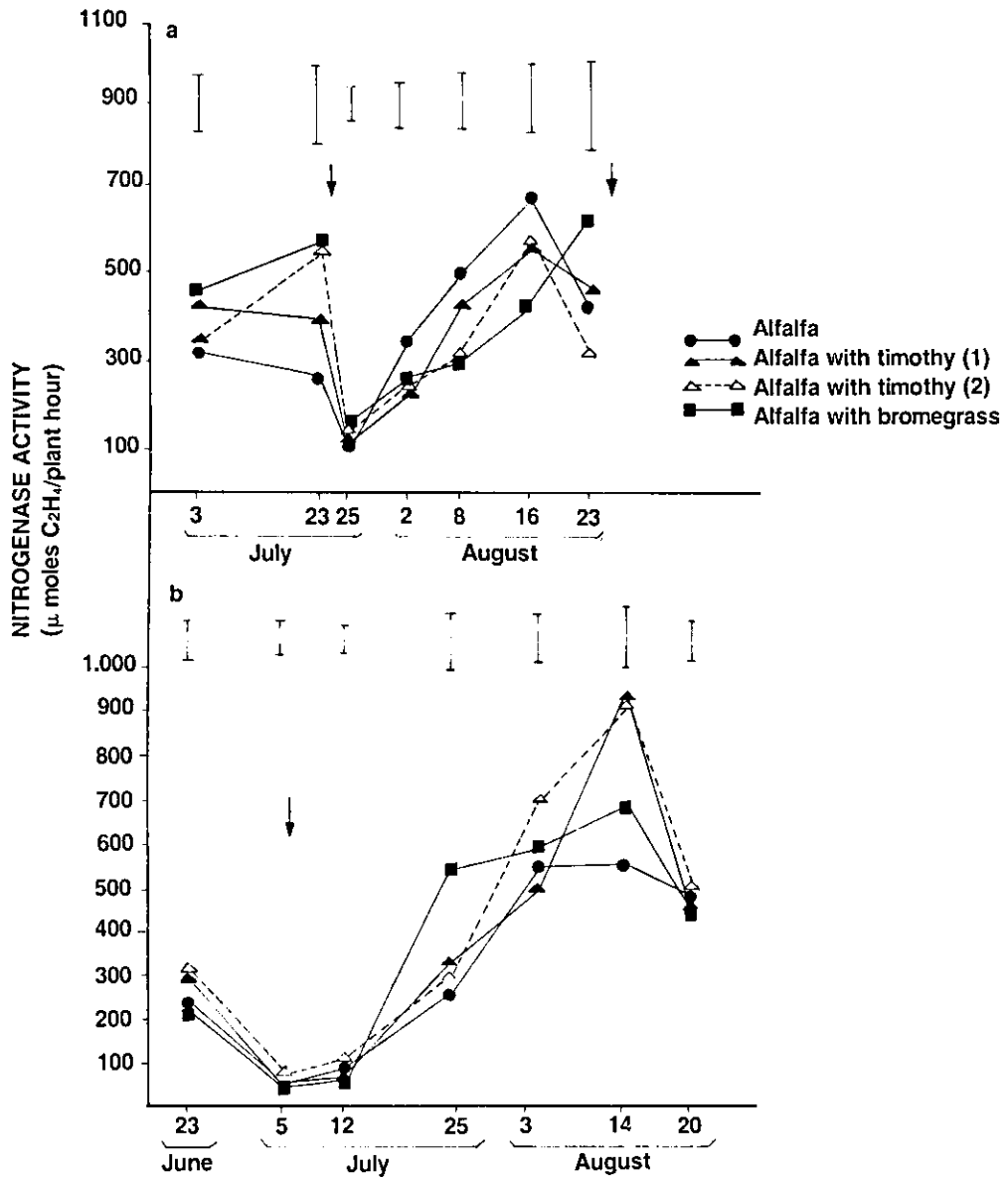
FIG. 1. Seasonal changes in nitrogenase activity of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth. Each point is the mean of five replicates and the bars represent Least Significance Difference (LSD) at the 5% level of probability. Arrows indicate 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.

C_2H_4/h. plant for the first 36 days, and increased to 432 μ moles C_2H_4/h. plant by day 50. Moreover, the NA declined about 70% within 48 hours after harvest for both years. There was a major recovery of NA from day 9 to 15, after shoot removal and by day 23 the activity rose to a maximum of 550 μ moles of C_2H_4/h. plant. In two - years old alfalfa plants, the NA measured in Spring (June 23) averaged 273 μ moles C_2H_4/h. plant, but declined significantly to 28.5 μ moles C_2H_4/hour plant within 24 hours after harvest. However, 20 days after shoot harvest the nodule activity had returned to the previous levels as measured on June 23 (Fig. 1b). 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 NA in the later was faster than in the seeding year and reached higher levels.

The decline of NA of alfalfa with herbage removal and its 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 & Havelka 1976), 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 (Vance et al. 1979, Cralle & Heichel 1981), and illustrates the interdependence of N_2-fixation and canopy photosynthate capacity. The rapid decline in NA of alfalfa plants support the substantial evidence that shoot removal causes temporary senescence of the nodule and that the recovery of nodule activity depends upon vegetative regrowth (Fig. 1). The reduction of NA 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 μ moles C_2H_4/h. plant and 60.3 μ moles C_2H_4/h. plant in the first and second year, respectively), point 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 NA was faster in the second year of alfalfa than in the first year, demonstrating that larger root system of the older alfalfa had greater ability to supply reserve carbohydrates to support vegetative regrowth.

The specific nodule activity (SNA) showed a pattern parallel to that observed for NA (Fig. 2). The nodule effectiveness, however, was almost equal for alfalfa in pure stand and for alfalfa in association with grass. Herbage removal caused a significant decrease of SNA in the seeding year. Moreover, in two-years old alfalfa plants, there was a slight increase seven days after harvest, although the SNA values at Spring (June 23) were lower as compared with those after harvest (Fig. 2a). 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 & 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 recovery of SNA was fast in both cultural systems, but the nodule activity in older alfalfa plants reached higher values than in young plants, and the same trend was also observed for N yield (Fig. 3). 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. 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 advanced stage of alfalfa plants. Extreme temperature are known to reduce NA in nodules of both annual and perennial legumes (Day & Dart 1969, Masterson & Murphy 1976, Munns et al. 1977). Using intact nodules of alfalfa, Day & Dart (1969) found a linear increase of NA from 5 to 25°C. While Cralle & Heichel (1982) who plotted log nodule activity versus absolute temperature, found a non-linear increase in NA between 20°C and 35°C. Similar patterns of increase have been reported for the NA 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 & Heichel 1982).

Shoot removal causes no significant loss of nodule weight in the seeding year, however a significant loss of nodule weight was observed in 2-years
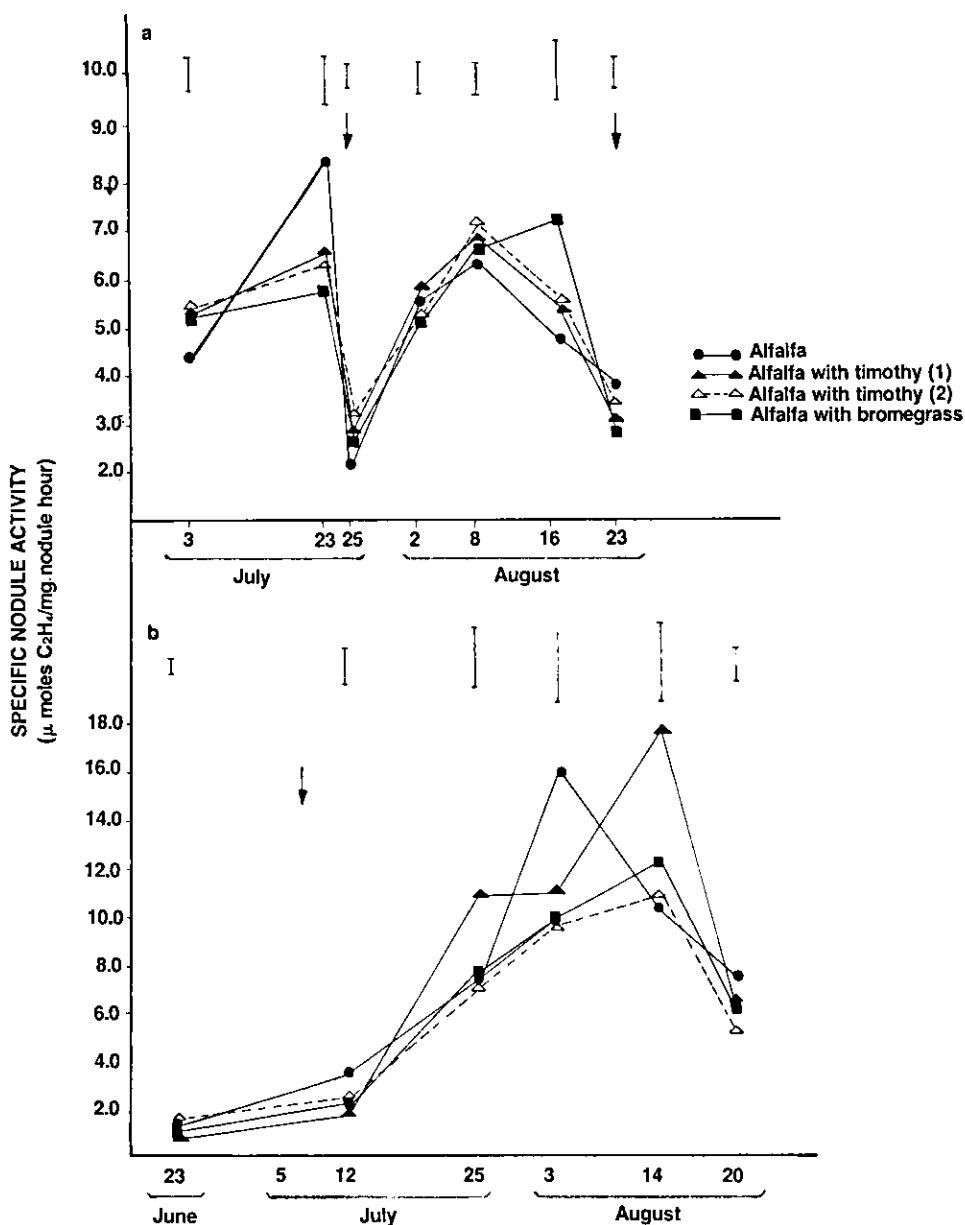
FIG. 2. Seasonal changes in specific nodule activity of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth. Each point is the mean of five replicates and the bars represent Least Significance Difference (LSD) at the 5% level of probability. Arrows 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.
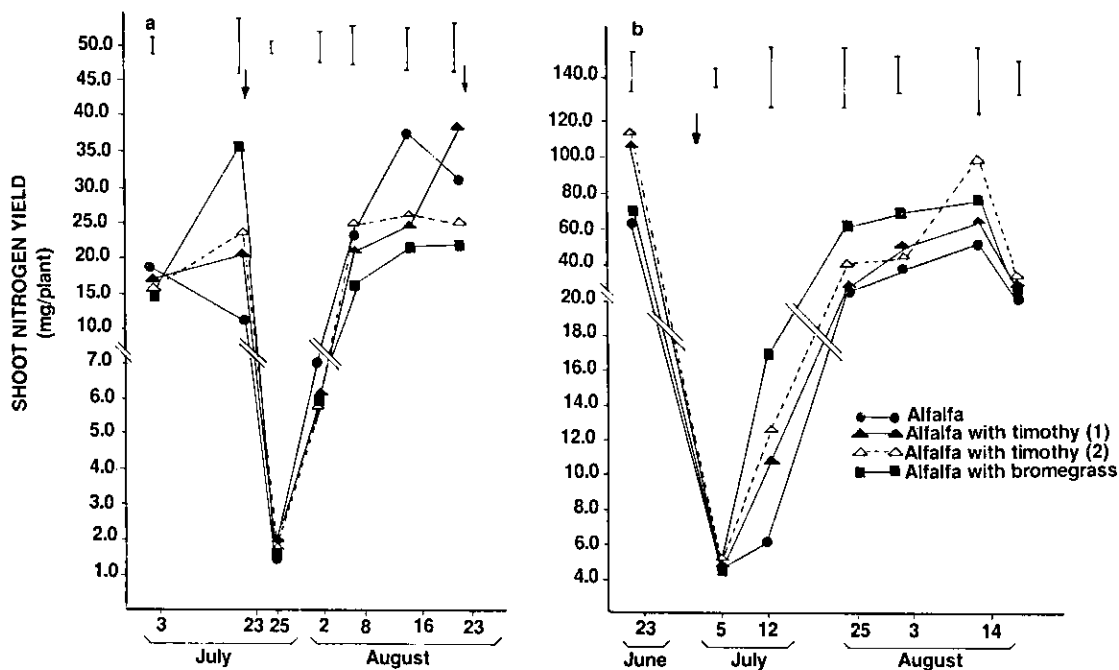
FIG. 3. Profiles of nitrogen yield of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth. Each point is the mean of five replicates and the bars represent Least Significance Difference (LSD) at the 5% level of probability. Arrows indicate time of 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.

old alfalfa after harvest (Fig. 4). 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 (Fig. 4). Nodule weight of 2-years 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 result from seeding year alfalfa plants 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 decreased (Fig. 1), and why alfalfa has a rapid regrowth potential as compared to other forage legumes. The decline of nodule fresh weight (Fig. 4b) encountered in 2-years old alfalfa after harvest could be attributed to the senescence or to the decay of older nodules that had overwintered.

The profile of the effect of alfalfa on grass species are shown in Table 1. The association of alfalfa with grasses increased grasses total nitrogen (TN) concentration before and after initial harvest during the seeding year and subsequent year. However, the increase of % TN in the second year was more marked than the first year. The enhancement of grass TN content and crude protein by association with legumes has previously been reported (Birch & Dougall 1967, Dubbs 1971, Haystead & Lowe 1977, Haystead & Marriott 1979,
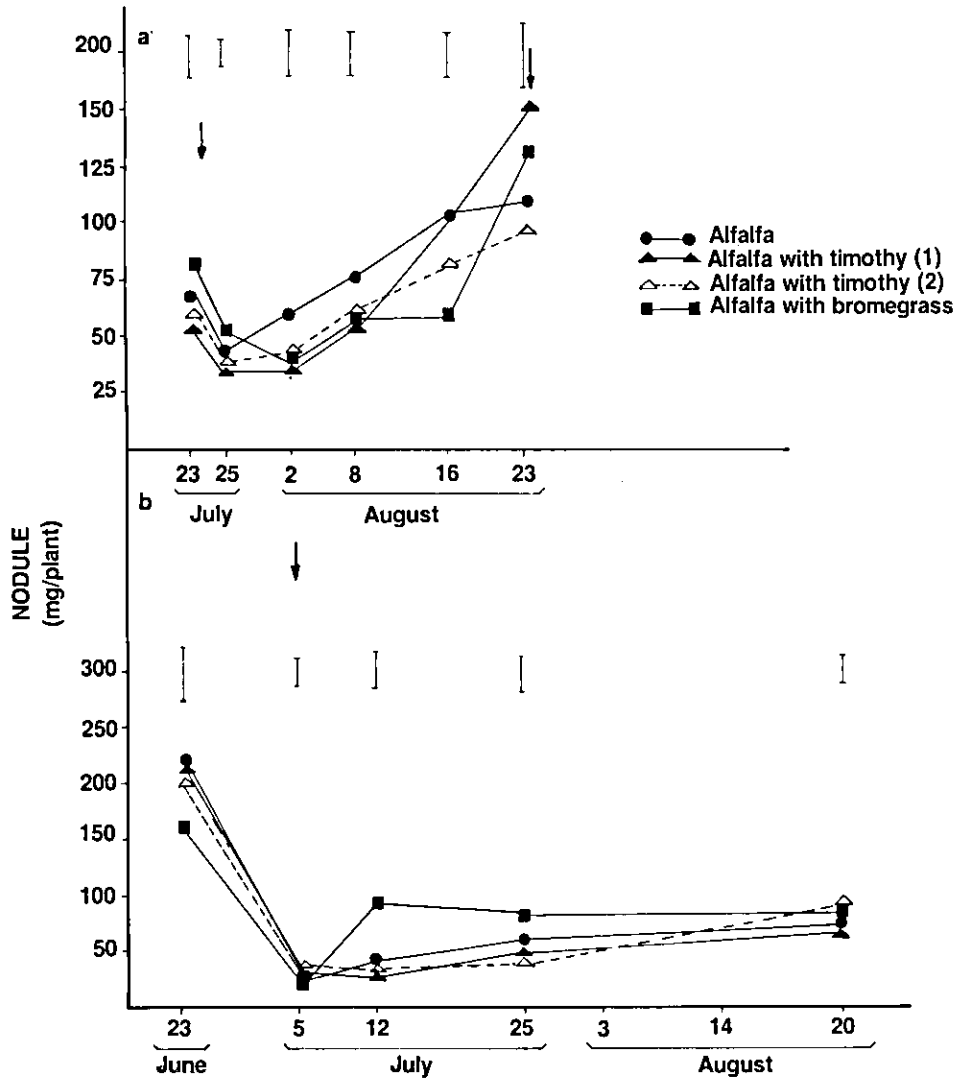
FIG. 4. Seasonal changes in nodule weight of alfalfa plants grown in mono and mixed cultures sampled before shoot removal and during herbage regrowth. Each point is the mean of five replicates and the bars represent Least Significance Difference (LSD) at the 5% level of probability. Arrows indicate time of harvests.
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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, 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. Moreover, at the end of season the depletion of soil N, mainly under grasses in pure stands resulted in the reduction of TN.

Evidence of N benefit was observed for all grass species, although there were differences among grass species in the enhances of TN concentration. Bromegrass was the most responsive species to N benefit with significant increases in the % TN, mainly in the second year. This may be due to the fact that bromegrass cv. Tempo has a greater competitive ability of this earlier maturing cultivar. Timothy cvs. Salvo and Climax, which are intermediate and late maturing cultivars respectively, were benefitted from the associated legume with a lesser extent than bromegrass. According to Henzell (1962), the increase of N benefit perhaps is a function of the competitive ability of the grass component in the association. Good contact between

roots and large occupation of soil volume make more efficient use of N release from associated legumes and soil N (Chujo & Daimon 1984).

The inclusion of alfalfa significantly increased the TN of grasses in almost all estimates, in the seeding year. Although this increase did not correspond to the increase in N yield. This may be due to the competition between 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). Haynes (1980) reported that at low levels of soil N, the N_2-fixing legume 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 content among grasses in association and those in monoculture was more pronounced. This beneficial effect due to alfalfa could be attributed to the contribution of the decomposition of nodules and roots debris during alfalfa late stages, besides the continuous excretion of soluble N compounds from living alfalfa roots during the growing stage. Therefore, alfalfa affected the soil N content probably with the formation of a surface organic layer which effectively benefitted the associated grass.

TABLE 1. **Total nitrogen concentration (%) in shoots and in roots of grasses for the various mono-and-mixed cultures sampled before and during herbage regrowth in 1983 and 1984.**

Treatment	Determination							
	1983				1984			
	Shoot		Root		Shoot		Root	
	3 July [+]	23 August	3 July	23 August	23 June	20 August	23 June	20 August
Timothy (1) with alfalfa	3.06 a	2.10 a	0.72 a	0.75 b	1.88 a	2.42 b	1.20 a	1.08 b
Timothy (2) with alfalfa	2.91 a	2.08 a	0.70 a	0.93 a	1.55 b	2.57 ab	1.07 b	1.16 b
Bromegrass with alfalfa	3.08 a	2.09 a	0.73 a	0.81 b	1.58 b	2.80 a	0.90 c	1.51 a
Timothy (1) [++]	3.02 a	1.82 b	0.68 a	0.42 d	1.00 d	1.35 c	0.29 e	0.53 c
Timothy (2)	2.92 a	1.75 bc	0.59 b	0.38 d	1.00 d	1.08 c	0.28 e	0.61 c
Bromegrass	2.80 a	1.72 c	0.58 b	0.60 c	1.28 c	1.27 c	0.38 d	0.47 c
Mean	2.97	1.92	0.67	0.64	1.38	1.91	0.69	0.89
± SE	0.28	0.06	0.06	0.06	0.09	0.20	0.05	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 1 performed 23 July 1983; harvest 2 performed 26 August 1983; harvest 3 performed 5 July 1984.

[++] Timothy (1) cultivar 'Climax'
    Timothy (2) cultivar 'Salvo'

CONCLUSIONS

1. This study provides evidences that harvest induced little changes in nitrogenase activity and nodule weight of alfalfa.

2. The results suggest that alfalfa plants have an adaptive mechanism to maintain the most of their nodules 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 nitrogenase activity in few days.

3. Furthermore, evidence was obtained indicating that the presence of the grasses in the stand did not affect alfalfa nodule activity.

4. The results also show that all grasses were benefitted significantly with inclusion of alfalfa in the stand. Thus, alfalfa should be grown in mixtures with grass and managed for maximize N_2-fixation while maintaining grass to efficiently utilize available soil N.

REFERENCES

ANDA, L.G. de; WIEBOLD, W.J.; MCINTOSH, M.S. Nitrogen fixation rates of alfalfa and red clover grown in mixture with grass. **Agron. J.**, 73:996-8, 1981.

BELZILE, L.; PELLETIER, H.; AUGER, M.; DESJAR-DINS, R. **Productivity of alfalfa and timothy grown in pure and mixed stands at La Pocatie-re.** Ottawa, Canadex Report, 1983.

BETHLENFALVAY, G. & PHILLIPS, D.A. Ontogenic interactions between photosynthesis and symbiotic nitrogen fixation in legumes. **Plant Physiol.**, 60:412-8, 1977.

BIRCH, H.F. & DOUGALL, H.W. Effect of a legume on soil nitrogen mineralisation and percent nitrogen in grasses. **Plant Soil**, 27:292-6, 1967.

BUTLER, G.W.; GREENWOOD, R.D.; SOPER, K. Effects of shading and defoliation on the turnover of root and nodule tissue of plant of *Trifolium repens, Trifolium pratense* and *Lotus corniculatus.* **N. Zealand. J. Agric. Res.**, 2:415-26, 1959.

CHUJO, H. & DAIMON, H. Plant growth and fate of nitrogen in mixed cropping, intercropping and crop rotation. I. Growth acceleration of some temperate grasses in early stage of mixed cropping with red clover. **J. Crop. Sci.**, 53:213-21, 1984.

CRALLE, H.T. & HEICHEL, G.H. Nitrogen fixation and vegetative regrowth of alfalfa and birdsfoot trefoil after successive harvests or floral debudding. **Plant Physiol.**, 67:898-905, 1981.

CRALLE, H.T. & HEICHEL, G.H. Temperature chilling sensitivity of nodule nitrogenase activity of unharded alfalfa. **Crop Sci.**, 22:300-4, 1982.

DAY, J.M. & DART, P.H. **Light intensity, nodulation and nitrogen fixation;** report of the Rothamsted Experimental Station. Harpenden, England. Rothamsted Experimental Station, 1969. part 1, p.105.

DUBBS, A.L. Competition between grass and legume species on dryland. **Agron. J.**, 63:359-62, 1971.

GIBSON, A.H. Recovery and compensation by nodulated legumes to environmental stress. In: NUTMAN, P.S. ed. **Symbiotic nitrogen fixation in plants.** Cambridge, Cambridge Univ. Press, 1976. p.385-483.

HARDY, R.W.F.; HOLSTEN, R.O.; JACKSON, E.K.; BURNS, R.J. The acetylene-ethylene assay for N_2-fixation: laboratory and field evaluation. **Plant Physiol.**, 43:1185-207, 1968.

HARDY, R.W.F. & HAVELKA, U.D. Photosynthate as a major factor limiting nitrogen fixation by field grown legumes with emphasis on soybeans. In: NUTMAN, P.S. ed. **Symbiotic nitrogen fixation in plants.** Cambridge, Cambridge Univ. Press, 1976. p.421-39.

HAVELKA, U.D. & HARDY, R.W.F. Legume nitrogen fixation as a problem in carbon nutrition. In: NEWTON, W.E. & NYMAN, C.J. eds. International Symposium Nitrogen Fixation 1., **Proceedings...** Pullman, Washington State Univ. Press, 1976. v.2. p.456-75.

HAYNES, R.J. Competitive aspects of the grass-legume association. **Adv. Agron.**, 33:227-61, 1980.

HAYSTEAD, A. & LOWE, A.G. Nitrogen fixation by while clover in hill pasture. **J. Br. Grassl. Soc.**, 32:57-63, 1977.

HAYSTEAD, A. & MARRIOTT, C. Transfer of legume nitrogen to associated grass. **Soil Biol. Biochem.**, 11:99-104, 1979.

HENZELL, E.F. Nitrogen fixation and transfer by some tropical and temperate pasture legumes in sand culture. **Aust. J. Exp. Agric. Anim. Husb.**, 2:132-40, 1962.

HUANG, C.Y.; BOYER, J.S.; VANDERHOEF, L.N. Limitations of acetylene reduction (nitrogen fixation) and metabolic activities of soybeans having low water potentials. **Plant Physiol.**, 56:228-32, 1975.

KROTH, E.M.; MEINKE, L.; MATTAS, R. **Effective nitrogen fixation by legumes in cool season grass mixtures.** Missouri, Missouri Agric. Exp. Sta. 1982. 26p. (Res. Bull., 1047).

LAWN, R.J. & BRUN, W.A. Symbiotic nitrogen fixation in soybeans. I. Effect of photosynthate source-sink manipulations. **Crop Sci.**, 14:11-6, 1974.

LEVITT, J. **Responses of plants to environmental stresses,** New York, Academic Press, 1972. 697p.

MASTERSON, C.L. & MURPHY, P.M. Application of the acetylene reduction technique to the study of nitrogen fixation by white clover in the field. In: NUTMAN, P.S. ed. **Symbiotic nitrogen fixation in plants.** Cambridge, Cambridge Univ. Press. 1976. p.299-316.

MOUSTAFA, E.; BALL, R.; FIELD, R.R.O. The use of acetylene reduction to study the effect of nitrogen fertilizer and defoliation on nitrogen fixation by field-grown white clover. **N. Zealand J. Agric. Res.**, 12:691-96, 1969.

MUNNS, D.N.; FOGLE, V.W.; HALLCK, B.G. Alfalfa root nodule distribution and inhibition of nitrogen fixation by heat. **Agron. J.**, 69:377-80, 1977.

RAPONEN, I.E. The effect of darkness on the leghemoglobin content and amino acid levels in the root nodules of pea plants. **Physiol. Plant.**, 23:452-60, 1970.

RAPONEN, I.E. & VIRTANEN, A.I. The effect of prevention of flowering on the vegetative growth of inoculated pea plants. **Physiol. Plant.**, 21:655-67, 1968.

SHEEHY, J.E.; FISHBECK, K.A.; PHILLIPS, D.A. Relationships between apparent nitrogen fixation and carbon exchange rate in alfalfa. **Crop Sci.**, 20:491-5, 1980.

SINCLAIR, A.G. Non-destructive acetylene reduction assay of nitrogen fixation applied to white clover plants growing in soil. **N. Zealand J. Agric. Res.**, 16:263-70, 1972.

SIROIS, J.C. & PETERSON, E.A. A rapid screening method for *Rhizobium meliloti* symbiotic nitrogenase activity. **Can. J. Microbiol.**, 28:265-8, 1982.

STREETER, J.G. Growth of two soybean shoots on a single root. **J. Exp. Bot.**, 25:189-98, 1974.

TRENBATH, B.R. Biomass productivity of mixtures. **Adv. Agron.**, 26:177-210, 1974.

VANCE, C.P. Nitrogen fixation in alfalfa; an overview. Annual Alfalfa Symposium, 8., **Proceedings...** St. Paul, University of Minnesota, 1978. p.34-42.

VANCE, C.P.; HEICHEL, G.H.; BARNES, D.K.; BRIAN, J.W.; JOHNSON, L.E. Nitrogen fixation, nodule development and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. **Plant Physiol.**, 64:1-8, 1979.

WHEELER, C.T. & LAWRIE, A.C. Nitrogen fixation in root nodules of alder and pea in relation to the supply of photosynthetic assimilates. in: NUTMAN, P.S. ed. **Symbiotic nitrogen fixation in plants.** Cambridge, Cambridge Univ. Press, 1976. p.239-54.

WHITEMAN, P.C. Seasonal changes in growth and nodulation of perennial tropical pasture legume in the field. II. Effects of controlled defoliation levels on nodulation of *Desmodium intortum* and *Phaseolus atropurpureus*. **Aust. J. Agric. Res.**, 21:207-14, 1970.

WILSON, J.K. The loss of nodules from legume roots and its significance. **J. Am. Soc. Agron.**, 34:460-71, 1942.