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Phosphorus regulation of nitrogen fixation in a traditional Mexican agroecosystem

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Abstract. Although nitrogen is considered to be the nutrient that most commonly limits production of natural and managed terrestrial ecosystems, I propose that phosphorus may regulate productivity in many continuously cultivated agroecosystems that do not receive applications of synthetic fertilizers. One way P may limit agroecosystem productivity is by controlling nitrogen fixation of legume crops, thus affecting nitrogen availability in the overall agroecosystem. I tested this hypothesis in two studies by examining the effect of phosphorus nutrition on nitrogen fixation of alfalfa in traditional Mexican agroecosystems. All farms used in the research relied on alfalfa as the primary nitrogen source for maize cultivation and other crops, and had minimal or no reliance on synthetic fertilizers.

In one study, I used the natural abundance of ^{15}N to estimate nitrogen fixation in five alfalfa plots with soils representing a wide range of P fertility. I found a correlation of $r = 0.85$ between foliage P concentrations and nitrogen fixation in the alfalfa plots. Mean nitrogen fixation in alfalfa plots ranged between 232–555 kg ha^{-1} yr^{-1} as estimated by the ^{15}N-natural abundance method.

In a second study, I sampled soils from alfalfa plots on traditional farms located in 5 different physiographic regions of Mexico. Half of each soil sample was augmented with phosphorus in a greenhouse experiment. I grew alfalfa on the fertilized and unfertilized soils from each site and then determined nitrogenase activity (acetylene reduction) of the *Rhizobium* on the plant roots. Nitrogenase activity increased in the alfalfa grown on all soils with added phosphorus, with two of the five differences being statistically significant at $P < 0.01$, and one at $P < 0.05$. Foliage P concentrations and nitrogenase activity were positively correlated ($r = 0.81$, $P < 0.01$).

Introduction

Low nitrogen availability is thought to limit production in many natural and managed terrestrial ecosystems (White-Stevens 1977; Gutschick 1981; Tilman 1988; Vitousek & Howarth 1991). The possible reasons for nitrogen limitation in natural terrestrial systems are complex and numerous, but two of the more prevalent ideas are that (1) nitrogen is extremely

mobile and leaves ecosystems through more avenues and in greater quantities than most other nutrients; and (2) for reasons not well understood, nitrogen-fixing organisms are often unable to make up the nitrogen deficits that terrestrial ecosystems tend to incur (Vitousek & Howarth 1991).

In response to low nitrogen availability, plants in natural communities have undergone considerable selection to conserve and sequester nitrogen. Adaptations such as retranslocation of N (Lajtha 1987), luxury uptake and storage of N (Mooney & Rundel 1979), uptake of amino N by mycorrhizal fungi (Harley & Smith 1983), and reduction of leaf turnover rates (Chapin 1980) have allowed plant species in many natural communities both to minimize nitrogen losses and to function on cycled rather than newly acquired nitrogen (Gutschick 1981; Chapin 1980; Chapin et al. 1987).

Agroecosystems experience all of the same avenues of nitrogen loss as natural ecosystems, but the losses tend to be greater. Tillage of fallowed or previously uncultivated soils has been shown to increase soil organic nitrogen mineralization (Schimel 1986), nitrification, and subsequent nitrate leaching (Huber et al. 1977). Irrigation can have similar effects on soil nitrogen dynamics (Myers et al. 1982; Pratt 1984). Raising soil pH through liming has been shown to increase ammonia volatilization (Freney & Black 1987). Erosion also is commonly much greater in agroecosystems, often resulting in removal of N-rich, fine, surface organic matter (Slater 1942; Schimel et al. 1985). In addition to these avenues of N loss that occur to a lesser extent in natural ecosystems, considerable quantities of N are removed from agroecosystems in food harvests. Only in agroecosystems where human manure is used as a soil amendment is part of the harvest-N retained.

High losses of N in agroecosystems deplete the soil-nitrogen as compared to analogous uncultivated soils (Hill 1954; Haas 1957; Smith & Young 1975; Tiessen et al. 1984). Continually cultivated agroecosystems, therefore, depend less on cycling and more on new inputs to maintain a viable nitrogen budget. This is clearly illustrated on the scale of the global nitrogen budget where approximately 57% of the nitrogen fixed per year in terrestrial ecosystems (both biologically and industrially fixed N) is appropriated by croplands, which comprise roughly 11% of land surface (Hardy & Burns 1975; Atjay et al. 1979; FAO 1990).

In this century, use of synthetic N fertilizers has largely eliminated nitrogen limitation in agroecosystems of industrialized nations. Although effective, synthetic fertilizers are also the single most energy intensive input to modern agricultural production, accounting for approximately 68% of on-farm commercial energy use in less developed countries and 40% in more developed countries (Mudahar & Hignett 1987). The more

immediate negative consequences of a dependence on nitrogen fertilizers are nitrate contamination of groundwaters (NRC 1989), coastal eutrophication (Howarth 1988), and increased production of NOx greenhouse gases (McKenney et al. 1980; Anderson & Levine 1987).

In 1981, Cole & Heil postulated that the biologically-active phosphorus in many terrestrial ecosystems is ultimately more responsible than nitrogen in controlling plant biomass production. They argued that critical nitrogen dynamics are controlled by the rates of dissolution of inorganic mineral phosphorus, as well as processes of mineralization and immobilization of soil organic phosphorus. Free-living and symbiotic nitrogen fixation, nitrogen mineralization from organic matter, and the ability of plants to recover mineral nitrogen from the soil are processes that are strongly influenced and potentially limited by the biologically active pool of phosphorus.

That biologically available phosphorus limits nitrogen and carbon accumulation in ecosystems is not a recent idea. The basis for this hypothesis is found in the aquatic literature where Redfield (1958) drew attention to the relatively constant ratios of P, N and C in marine phytoplankton biomass, and implicated P as being a critical element controlling productivity. Under laboratory conditions, Stewart & Alexander (1971) showed the positive effect of increasing phosphorus availability on nitrogenase activity of blue-green algae. Under field conditions, Schindler (1977, 1978) demonstrated the strong regulating effect of P availability on phytoplankton productivity in temperate freshwater lakes. In addition to Cole & Heil (1981), other workers have offered a terrestrial version of this hypothesis (Walker & Adams 1958; Walker & Syers 1976; Tate & Salcedo 1988), but it has proven difficult to test primarily because of methodological limitations in measuring the biologically-active phosphorus of an ecosystem, as well as in precisely measuring rates of biological nitrogen fixation and other processes of the nitrogen cycle in natural communities.

In the present study, I examine the relationship between biologically available phosphorus and nitrogen fixation by the alfalfa/*Rhizobium* symbiosis. Many traditional farmers in central and northern Mexico rely on alfalfa (*Medicago sativa*, L.) as the primary nitrogen source for their maize crops. Alfalfa is a perennial legume which, in symbiosis with the bacteria *Rhizobium* spp., has been reported to fix between 0—463 kg nitrogen ha^{-1} yr^{-1} (Bell & Nutman 1971; Nutman 1976; LaRue & Patterson 1981; Heichel 1987). These estimates come largely from temperate regions where average alfalfa yields range between 5.7 and 8.4 Mg dry weight ha^{-1} $year^{-1}$ (Michaud et al. 1988).

In many traditional Mexican agroecosystems, including those discussed

in this study, alfalfa plots are cut 6—10 times per year, and the fodder is fed to work and dairy animals. Manure from these animals is collected, stored, and applied to the maize fields either before planting or as a sidedress. In this way a portion of the nitrogen fixed by the alfalfa is made available to the maize. In addition, after 3—5 years, the farmers plow a given alfalfa plot under and sow maize. This also results in a transfer of alfalfa-fixed N to subsequent maize crops.

Phosphorus availability greatly affects nitrogen fixation and production of legumes, including alfalfa (Truesdell 1917; Griffith 1978; Barea & Azcón-Aguilar 1983; Marschner 1986). It is debated whether the effect of increased P availability directly enhances the *Rhizobium*'s capacity to fix nitrogen, or whether it mainly improves the photosynthetic capacity of the host plant, which in turn benefits the *Rhizobium* symbiont (Hardy & Havelka 1976). Nitrogen fixation is known to be a P-intensive process (Gutschick 1980; Anderson et al. 1978). Barea et al. (1988) report that *Rhizobium* nodules often contain 2 to 3 times more P per unit of dry matter than the root on which they are formed. Numerous investigators have measured an increase in either nitrogen fixation, nodule weight, nodule nitrogen content, or overall N content of legumes when fertilized with phosphorus (Gates 1974; Graham & Rosas 1979; Singleton et al. 1985; Israel 1987; Pereira & Bliss 1987). In this study I searched for a relationship between nitrogen fixation and plant phosphorus status in the field. Then in a greenhouse experiment, I grew alfalfa on five different Mexican soils, in which half of each soil was fertilized with P, and tested for responses of nitrogen fixation to the P treatments.

Methods

Natural abundance of ^{15}N background

The $\partial^{15}N$ method of measuring N fixation is possible in many terrestrial ecosystems because microbial and physical fractionation processes tend to increase the natural abundance of the ^{15}N isotope in soil nitrogen as compared to atmospheric nitrogen. The atmosphere has an ^{15}N concentration of 0.3663 atom % which is almost uniform planet-wide (Shearer & Kohl 1986). Denitrification, ammonia volatilization and nitrification followed by leaching of nitrate are processes that tend to preferentially remove ^{14}N from the soil pool (Turner et al. 1983; Shearer & Kohl 1986). Consequently, the soil becomes relatively enriched in ^{15}N. In a survey of the total N of 124 surface soils in the US, Shearer et al. (1978) found the mean natural abundance of ^{15}N to be 9.2% (SD = 2.1).

To estimate the extent to which a particular plant/microbe N-fixing symbiosis obtains nitrogen from the soil or through biological nitrogen fixation, one compares the $\partial^{15}N$ value of N in the plant tissue with those of the atmosphere and the plant-available N in the soil. The $\partial^{15}N$ of the plant-available N in the soil is generally estimated by measuring the $\partial^{15}N$ of a non-fixing reference plant that approximately occupies the same soil volume as the N-fixer being examined. If the nitrogen fixer derives the majority of its nitrogen from the atmosphere, it will have a $\partial^{15}N$ value close to zero, whereas if the N-fixer is taking up most of its nitrogen from the soil, then it will have a $\partial^{15}N$ value similar to that of the non-fixing reference plant (Shearer & Kohl 1986, 1989). If the N-fixing symbiosis releases N into the rhizosphere, as was detected in *Prosopis* by Lajtha & Schlesinger (1986), the difference in $\partial^{15}N$ of the soil, and atmosphere will narrow. This should not affect the calculation of N fixation rates, however, as long as the reference plant(s) access the same pool of soil nitrogen as the fixer.

The natural abundance of ^{15}N is expressed in ∂ units, which are defined as

$$\partial^{15}N‰ = \frac{^{15}N/^{14}N\ (\text{sample}) - {}^{15}N/^{14}N\ (\text{standard})}{^{15}N/^{14}N\ (\text{standard})} \times 1000 \qquad (1)$$

where atmospheric N_2 or a nitrogen compound such as ammonium sulfate, with a known $\partial^{15}N$, is used as the standard. The fraction of nitrogen in the leaf tissue of a nitrogen fixing plant that was derived from biological nitrogen fixation is calculated as:

$$\%N\ \text{fixed} = \frac{\partial^{15}N_r - \partial^{15}Nf}{\partial^{15}N_r - \partial^{15}N_h} \qquad (2)$$

where $\partial^{15}N_r$ is the $\partial^{15}N$ value of the non nitrogen-fixing reference plant(s) that represent the soil $\partial^{15}N$ label. $\partial^{15}N_f$ is the $\partial^{15}N$ value of the nitrogen fixing plant being studied that relies on atmospheric and/or soil sources of N. $\partial^{15}N_h$ is the $\partial^{15}N$ value of the same nitrogen fixing plant species as $\partial^{15}N_f$, grown hydroponically without N in solution. This parameter gives an estimate of the extent of rhizobial or other symbiont $^{15}N/^{14}N$ fractionation.

Field sites for $\partial^{15}N$ analysis

I sampled alfalfa and reference plant foliage for the $\partial^{15}N$ estimation of nitrogen fixation from four farms in Southwestern Tlaxcala, a small state

80 km east of Mexico City (Fig. 1). Soils of this area are Entisols and Inceptisols, tentatively classified as Ustipsamments and Tropaquepts in the USDA soils classification system (Soil Survey Staff 1990) or as Fluvisols and Gleysoles using the FAO soil classification (Werner 1988) (Table 1). The soils originated by alluvial deposition of materials transported from the Atoyac and Zahuapan river watersheds during the Holocene (Werner 1988). The parent material of the entire region is volcanic. Variation in phosphorus fertility of agricultural fields can be attributed to local variation in pedogenic factors, and to differences in farmer management practices.

I selected the farms for study in Southwest Tlaxcala using the following criteria: (1) the farmers presently and historically applied little or no exogenous fertilizer in their entire cropping system; (2) the farmers grew alfalfa, and the alfalfa plants present had been under cultivation for three years; (3) the alfalfa plots were irrigated; (4) the soils selected represented a wide range in phosphorus availability; and (5) the farms were located within a narrow climatic regime.

Fig. 1. Location of field sites: 1, 2, 6-Santa Cruz Porvenir, Tlaxcala; 3, 4-San Felipe Ixtacuixtla, Tlaxcala; 5-Santa Ines Tecoexcomac, Tlaxcala; 7-Dolores Hidalgo, Guanajuato; 8-Patzcuaro, Michoacan; 9-Nuevas Casas Grandes, Chihuahua; 10-Actopan, Hidalgo.

Table 1. Alfalfa field sampling site locations in Mexico and soil characteristics.

Site #	Municipality	Soil order (USDA)	Soil class (FAO)	Geograph. form.	Soil temp.	Soil moist.
Sites sampled for the $\partial^{15}N$ natural abundance analysis						
1	Santa Cruz Porvenir Tlaxcala	Entisol	Fluvisol	neo-volcanic corridor (east)	isothermic	ustic
2	Santa Cruz Porvenir Tlaxcala	Entisol	Fluvisol	neo-volcanic corridor (east)	isothermic	ustic
3	San Felipe Ixtacuixtla Tlaxcala	Entisol	Fluvisol	neo-volcanic corridor (east)	isothermic	ustic
4	San Felipe Ixtacuixtla Tlaxcala	Entisol	Fluvisol	neo-volcanic corridor (east)	isothermic	ustic
5	Santa Ines Tecoexcomac Tlaxcala	Inceptisol	Gleysol	neo-volcanic corridor (east)	isothermic	ustic
Sites sampled for the acetylene reduction experiment						
6	Santa Cruz Porvenir Tlaxcala	Entisol	Fluvisol	neo-volcanic corridor (east)	isothermic	ustic
7	Dolores Hidalgo Guanajuato	Molisol	Kastanozem	Mesa Central	thermic	ustic
8	Patzcuaro Michoacan	Andosol	Andosol	neo-volcanic corridor (west)	isothermic	udic
9	Nuevas Casas Grandes Chihuahua	Aridisol	Xerosol	Sierra Madre Occidental	thermic	aridic
10	Actopan Hidalgo	Entisol	Xerosol	Sierra Madre Oriental	isothermic	ustic

Sources: Soil Survey Staff (1990); Van Wambeke (1992, 1987); FAO (1975); Werner (1988).

Sample collections and analyses of $\partial^{15}N$

I sampled alfalfa leaves in May, 1990, from six plants in 33-m^2 plots, at each of the four farms. One farm (Porvenir) had two study plots because of notable variation in available phosphorus in two separate alfalfa fields. The plots from which the samples were taken had been cut to within 4 cm of the soil surface, 28 days earlier. I removed leaves from the tops of non-flowering plants that stood roughly 75 cm tall, and dried the leaves at 60 °C in a drying oven within two hours of their harvest. First, I ground the samples with a mortar and pestle, and then used a ball mill for fine grinding.

I took considerable care in collecting the reference plant material so that it would provide a representative estimate of the soil $\partial^{15}N$ label that the alfalfa plants were likely to encounter. I excavated alfalfa plants to examine rooting patterns in each study plot. In all cases, I found fine alfalfa roots to be most abundant in the top 20 cm and less abundant below 50 cm. In order to approximate the alfalfa plants' rooting distributions, I selected two non-nitrogen fixing weedy species as reference plants that grew amidst the alfalfa in every study plot. The reference plants displayed two rooting morphologies: *Rumex* sp. was rooted to 30 or 40 cm while *Bromus* sp. had a rooting depth that rarely exceeded 15 cm. I dried the reference leaf material at 60 °C, pooled equal weights of the two species, and ground the samples following the same procedure described for alfalfa.

Immediately after collecting alfalfa and reference leaf material, each plot was cut to within 4 cm of the soil surface. Alfalfa biomass from each plot was weighed wet, and a subsample was dried at 60 °C and reweighed for dry-weight determination. I analyzed triplicates of each alfalfa sample and associated non-fixing plant reference samples for ^{15}N atom percentage on a Europa Scientific Tracermass Stable Isotope Analyzer, using ammonium sulfate calibrated with NBS ammonium sulfate (N-1) as the standard.

I determined foliage phosphorus contents by combusting ~ 2.5 mg of leaf material at 550 °C for two hours, followed by digestion of the ash in 0.17 N HCl for 2 hours at 105 °C (Allen 1989). Using standard acid molybdate procedures (Allen 1989), I analyzed the samples by spectrophotometer. The precision of this method was ± 0.01% P for NBS Citrus leaves (# 1572). Foliage concentrations of other elements (K, Ca, Fe) were measured by ICP at the Cornell Fruit and Vegetable Sciences analytical lab. Digestions of leaf material for ICP analysis were carried out using a procedure similar to that outlined above with original ashing performed at 450 °C, followed by an addition of 30% H_2O_2 and re-ashing at 450 °C.

I estimated soil organic matter by loss on ignition at 550 °C. I estimated $CaCO_3$-C by adding 0.5 M HCl to soils, leaving them for one hour, and back-titrating samples with 0.5 M NaOH (Allen 1989). Soil inorganic carbon values were below 0.1% of soil dry weights except for Tecoexcomac, which has a value of 0.4%. I measured soil pH with a Radiometer probe after mixing 2 g of soil with 4 mL of water and allowing one hour for equilibration.

Field sites for acetylene reduction (AR) assay

In July, 1991, I collected soils from alfalfa fields near the towns of Dolores Hidalgo, Guanajuato; Patzcuaro, Michoacán; Actopan, Hidalgo; Porvenir, Tlaxcala; and Neuvas Casas Grandes, Chihuahua. I chose these regions for soil collections because of the diverse climate, parent material, and pedogenic factors that influence soil phosphorus dynamics (Fig. 1, Table 1). The criteria employed for selecting alfalfa fields were identical to those described above for Tlaxcalan fields.

I collected soils by excavating one 30 cm × 30 cm × 50 cm cube of soil from the center of each of the five sites. My intent with this sampling approach was to gather large soil samples from various parent materials with differing pedogenetic histories, and not to represent the soil variability across the specific alfalfa fields. After sampling, I allowed soils to air dry until approximately 10% of the soil-mass was water and stored them at approximately 12 °C for one month before they were used in the experiment.

Design and procedure of greenhouse experiment

I gently broke up aggregates of the soil samples with a mortar and pestle, passed the loose soil through a 2-mm sieve, then autoclaved the 2-mm soil fraction at 121 °C for ninety minutes to eliminate the mutualistic effects of VA mycorrhizal fungi and the parasitic effects of soil nematodes on phosphorus availability and alfalfa growth. I thoroughly mixed sterile sand with each type of soil in a ratio of 2:5, sand:soil, to improve soil drainage properties. The sand had a nominal 0.7 μg resin-P gdw^{-1}, as compared to the field soils which had between 5 and 93 μg resin-P gdw^{-1} soil. I then divided each of the five soils into two subgroups. I fertilized one of the subgroups with 0.13 mg P gdw^{-1} soil as $CaHPO_4$, a fertilization level roughly equivalent to a field application of 100 kg P ha^{-1}. Seven pots of each of the five unfertilized and fertilized soils were filled with 600 mL of soil/sand mixtures.

I inoculated alfalfa seeds of the Oneida variety with commercial alfalfa

inoculant (AGWAY) and sowed them on moistened, sterile vermiculite. Eight days after germination, I transplanted one seedling/pot into the seventy prepared pots. I immediately replaced the 10% of the seedlings that died within the first week.

I raised the alfalfa plants in a greenhouse under artificial and natural light conditions. Artificial light was delivered 16 hours per day. The light flux that was provided by Sylvania Metalarc metal halide bulbs at 100 cm above the pots was 446 μE m^{-2} s^{-1}. On days with full sun, light levels reached 1335 μE m^{-2} s^{-1}. Diurnal temperatures in the greenhouse ranged between 18—28 °C. Alfalfa pots were watered with deionized water.

I allowed the plants to grow until all were between the late vegetative and early flower stage of maturation (cf. Fick & Mueller 1989), then cut them to within 2 cm of the soil and allowed them to regrow. These harvests were made 50 and 72 days after germination. After the second cut, I once again allowed plants to reach late vegetative/early flower maturation stages, and then measured nitrogenase activity (acetylene reduction) on the harvested root balls.

Acetylene reduction assay

On day 106 after germination, I cut the tops of the potted alfalfa plants, removed the soil and roots from the pots, gently shook the intact root balls from the soil, and sieved the soils through a 2-mm sieve to recover roots that had detached from the root balls. All roots collected from a given plant, still covered with a veneer of soil, were placed in 500-ml Nalgene polycarbonate bottles with modified polypropylene lids to allow for gas sampling. I had modified the lids by drilling holes and fitting them with Wheaton 20-mm rubber flange stoppers sealed with Permatex # 2B gasket sealant. I used teflon tape to form a gas-tight seal between the lids and bottles. The average ratio of bottle volume space (ml) to wet root weight (g) was 130.

I injected 25 ml of acetylene through the rubber stopper into each sample bottle, then mixed the gas for 15 seconds by gently shaking the roots and two teflon beads inside the bottles. After mixing, I inserted a needle through the rubber stopper to release positive pressure from inside the bottle, placed incubating samples laterally on a shaker table and agitated them every 30 minutes for 60 seconds. After two hours, I removed 4 ml of gas from each sample bottle and injected it into a Varian 3300 gas chromatograph to analyze for ethylene production. The gas chromatograph was set up with a 0.5 mL gas sampling loop and a 2-m Porapak N (80—100 mesh) column.

One week before the acetylene reduction assay was to be undertaken

on the entire population of potted alfalfa, I destructively sampled one replicate pot of each soil treatment and performed several analyses to optimize the AR assay procedure. From this I ascertained the following information: no detectable ethylene or acetylene was sorbed or off-gassed by the polycarbonate bottle assemblies; I could not detect any losses of ethylene from the polycarbonate bottle assemblies when I injected the bottles with 5 ml of 1% ethylene, and allowed them to incubate for 24 hours; when I incubated roots in the bottle assemblies for two hours with no gases added, I found the roots and associated root microbes to produce negligible quantities of ethylene (0.003 μmol plant^{-1} hour^{-1} $\pm$ 40%); when I incubated roots with 5 ml of 1% C_2H_4, I detected no ethylene consumption (i.e. the variation in ethylene standards was greater than any difference in root + C_2H_4 incubations).

I determined foliar P concentrations of greenhouse alfalfa samples using the method described above. I measured resin extractable phosphorus of 0.5 g soil samples with 80 mesh, 3.5 × 5 cm polyester bags containing 0.4 g of 1—4X, 20—50 mesh, Biorad anion exchange resin in the Cl^- form (after Sibbesen 1977). Soils, resin bags and 50 mL of deionized water were shaken for 16 hours. I then extracted P from the resin bags using 0.2 M NaCl, developed color in the NaCl matrix using standard acid molybdate reagents (Olsen & Sommers 1982) and analyzed the samples spectrophotometrically.

All statistical analyses were carried out using the JMP software package (SAS 1990). Differences in acetylene reduction of *Rhizobium* on alfalfa roots grown on soils with and without added P were determined using a one tailed t-test. Use of a one-sided test for significance was justified since the null hypothesis was that added P would induce no differences between treatments (Campbell 1989).

Results and discussion

$\partial^{15}N$ and leaf tissue analyses

The percent of total plant N contributed by nitrogen fixation ranged from 45—65% between the five study plots (Table 2). The mean $\partial^{15}N$ values measured for leaf tissue of alfalfa (a) and reference plants (r) at each study site were as follows: Porvenir-1, a = 0.85, r = 1.21; Porvenir-2, a = 1.35, r = 2.42; Ixtacuixtla-3, a = 1.43, r = 2.88; Ixtacuixtla-4, a = 0.92, r = 2.67; and Tecoexcomac-5, a = 1.75, r = 3.48. To estimate rates of nitrogen fixation at the different sites over the 28-day growth period, I first estimated %N concentration of herbage (leaves + stems) from %N of

Table 2. Aboveground alfalfa harvest measurements, % leaf nitrogen, and N-fixation from Tlaxcalan plots.

Site	Plot harvest $g\ m^{-2}\ day^{-1}$	Plot harvest $Mg\ ha^{-1}\ yr^{-1}$	% Leaf N	% of N fixed	N fixed $mg\ m^{-2}\ day^{-1}$	N fixed $kg\ ha^{-1}\ yr^{-1}$
1-Porvenir	5.4	12.1	5.4 (0.12)	55 (16)	103 (41)	232 (93)
2-Porvenir	8.8	19.5	4.7 (0.30)	45 (17)	119 (74)	266 (167)
3-Ixtacuixtla	9.6	21.4	5.5 (0.18)	53 (18)	178 (89)	397 (200)
4-Ixtacuixtla	10.3	23.1	5.7 (0.13)	65 (10)	246 (29)	550 (67)
5-Tecoexcomac	10.9	24.4	5.8 (0.16)	54 (10)	219 (30)	488 (68)

Values in parentheses = 1 S.E., $n = 6$; Plot harvest $ha^{-1}\ yr^{-1}$ and nitrogen fixation $ha^{-1}\ yr^{-1}$ values are extrapolations from plot data. Harvest data are dry weight values.

leaf tissue data using a correction factor of 0.37 (SE ± 0.007) leaf dry weight/total herbage dry weight derived from data reported by Brown & Tanner (1983) and Sharratt & Baker (1986). The %N of stem tissue was estimated using the regression: stem %N = 0.470 + 0.346* (leaf %N) (S.E. of predicted Y = ± 0.0022—0.0024), derived from data of Lee & Smith (1972). The independent correction factor errors and the correlated errors of % leaf nitrogen and % nitrogen fixed were propagated using a second order Taylor series expansion (Beers 1957).

The estimates for N-fixation m^{-2} day^{-1} were calculated as the N-fixation rates determined over the 28-day growth period, divided by 28. I extrapolated the rates of N-fixation over the 28-day growth period (one harvest) to N fixed $hectare^{-1}$ $year^{-1}$, assuming eight harvests (Table 2). This is a conservative estimate of annual fixation and production since the alfalfa plots are typically harvested between 8 and 10 times per year in this region. Moreover, this estimate does not include fixed nitrogen or production in belowground structures. All extrapolated estimations of biomass production and nitrogen fixation rates for Tlaxcala (Table 2) are high for alfalfa, and the specific values for Ixtacuixtla-2 and Tecoexcomac are among the highest reported (Bell & Nutman 1971; Nutman 1976; LaRue & Patterson 1981; Heichel 1987; Michaud et al. 1988). The sustained productivity of these traditional agroecosystems appears to be made possible in part by high rates of nitrogen fixation by alfalfa.

Table 3 presents a correlation matrix between nitrogen fixation rates (Table 2, column 6) and a range of soil available elements and charac-

Table 3. Simple linear correlation coefficient matrix between alfalfa N-fixation rates (from $\partial^{15}N$), % leaf N, and production vs. alfalfa foliage element concentrations and soil characteristics of five study plots in Tlaxcala, Mexico.

Factor	N-fixation rate	% leaf N	Biomass prod.
N-fixation rate	1.00	0.75	0.85+
% leaf N	0.75	1.00	0.39
biomass production	0.85+	0.39	1.00
% leaf P	0.85+	0.72	0.87+
% leaf K	−0.68	−0.16	−0.92*
% leaf Ca	0.62	0.82+	0.40
% leaf Fe	−0.22	−0.78	0.21
soil pH[a]	0.35	0.65	0.54
soil % org matter[a]	0.70	0.73	0.93+
soil avail. NO_3^-[a]	−0.16	−0.36	−0.01

Correlation coefficient significance levels: + $p < 0.10$, * $p < 0.05$; [a] $n = 4$, no data for one of two Porvenir plots.

teristics known to affect rates of nitrogen fixation (Marshner 1986; Vance et al. 1988). The correlation between % leaf P and nitrogen fixation (r = 0.85; P = 0.07) is notably higher than that of any other element, and suggests that plant phosphorus nutrition may have regulated nitrogen fixation in these soils (Fig. 2). Bickoff et al. (1972) reports that P deficiencies in alfalfa should be evident at foliage concentrations less than 0.2%. Here, P tissue concentrations appeared to have some effect on N-fixation and productivity at levels up to 0.5%, well above the estimated deficiency level. The high correlation coefficient between % leaf P and aboveground biomass production (r = 0.87; p < 0.10) suggests that P may have in part controlled photosynthesis or allocation in the alfalfa plants. It is plausible, therefore, that N-fixation by rhizobia was limited by photosynthate supplies, and not by P directly (Hardy & Havelka 1976). In contrast, low correlation coefficients suggest that plant-availability of K, Ca and Fe did not systematically control nitrogen fixation rates in the plots studied.

Although known to affect rates of nitrogen fixation, the elements molybdenum, cobalt and aluminum are not included in the correlation matrix because leaf tissue values of these elements were not meaningful.

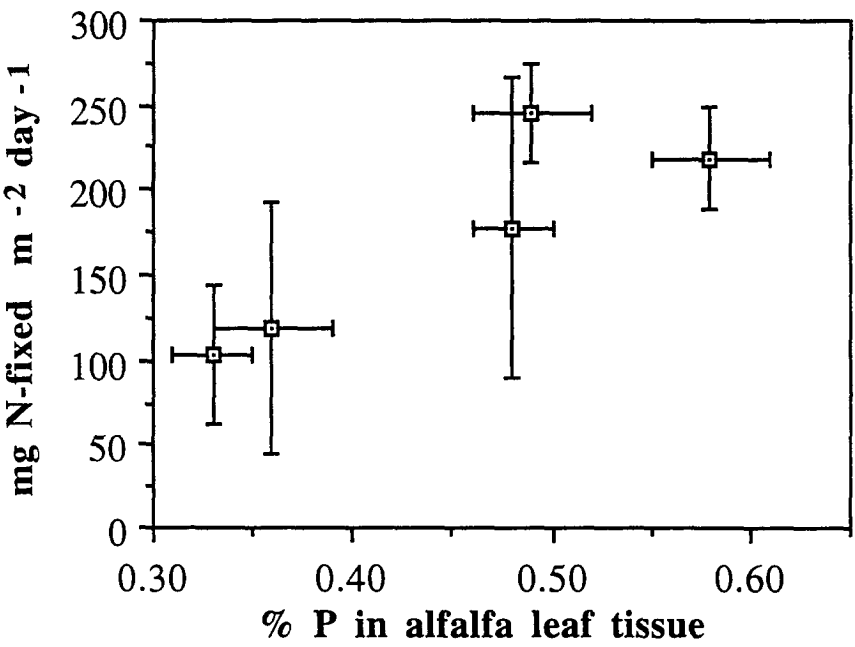


Fig. 2. Correlation of alfalfa nitrogen fixation rates and % of foliage phosphorus concentrations. Error bars = 1 S.E., $r = 0.85$, $p < 0.07$.

Availability of the elements molybdenum and cobalt are known to affect rates of nitrogen fixation through their roles in nitrogenase activity and leghemoglobin synthesis, respectively (Marschner 1986). Neither of these elements, however, affects N-fixation rates by influencing photosynthate production in plant leaves; rather, they affect N-fixation rates at the nodules. Nodule Mo or Co concentrations would therefore be the appropriate factors to correlate with N-fixation. In acid soils, aluminum toxicity also can affect nitrogen fixation rates of alfalfa (Lanyon & Griffith 1988). Aluminum was not included in the correlation matrix, however, because foliar Al concentration is not considered an accurate indicator of Al toxicity (Bouma et al. 1981).

The non-significant correlation between % soil organic matter and N-fixation rates ($r = 0.67$) is interesting when contrasted with the high correlation between % organic matter and plant biomass production ($r = 0.92$; $P < 0.10$). One might expect a negative correlation between soil organic matter and nitrogen fixation as mineralization of soil organic matter would release inorganic nitrogen and potentially depress rates of nitrogen fixation. Here no significant correlation was found between soil available nitrate and either nitrogen fixation ($r = -0.16$) or % leaf N ($r = -0.36$), implying that nitrogen was not being mineralized from soil organic matter at rates sufficient to strongly influence nitrogen fixation. Soil organic matter may affect soil water storage capacity and the availability of nutrients other than nitrogen, either of which could directly affect biomass production. Alternatively, it is possible that the effect of soil organic matter content on soil microfaunal populations could have direct consequences on alfalfa production. For example, soil parasitic nematode populations have been found to be suppressed in soils with high organic matter content in several traditional agroecosystems (Thurston 1992). Parasitic nematodes can depress alfalfa production (Leath et al. 1988) and are common in Tlaxcalan alfalfa fields (Alejandro Tovar, personal communication).

Sources of possible error

In this field study, I was not able to control for possible variation in rhizobia strains or alfalfa varieties between the fields, nor was I able to account for possible differences in soil moisture between fields even though all were irrigated during the study. It is clear from the work by Steele et al. (1983) that different strains of rhizobia in symbiosis with the same legume species have different patterns of nitrogen isotope fractionation. Steele et al. inoculated single legume species with different strains of rhizobia and measured the $\partial^{15}N$ values of above and belowground plant

parts. They observed the $\partial^{15}N$ values of stems + leaves to vary from −1.2 and 0.8 for *Lotus pedunculatus* (rhizobia $n = 4$), −2.6 to −1.4 for soybeans (rhizobia $n = 2$) and −2.6 to −1.0 for white clover (rhizobia $n = 2$) depending on rhizobia inoculum used.

Shearer & Kohl (1989) recommended that investigators grow the symbiosis in hydroponic culture without N to gain a precise estimate of the fractionation that occurs during nitrogen fixation (the $\partial^{15}N_h$ parameter in equation 2). This approach was not practical in this experiment because of the difficulties involved in isolating the specific field strains of rhizobia for use of an inoculant in the hydroponic culture. Moreover, it is likely that hydroponic growing conditions would not represent the rhizobia fractionation patterns that occur in undisturbed soil. Therefore, to calculate %N fixed in this study, I assumed the $\partial^{15}N_h$ to be 0. This is consistent with findings by Yoneyama et al. (1984), Steele et al. (1983) and Rasmussen et al. (1989) who grew *Rhizobium* inoculated alfalfa hydroponically and measured $\partial^{15}N$ leaf tissue values of 0.1, 0.0, and −0.3 respectively. Turner & Bergersen (1983), however, obtained a $\partial^{15}N$ of 3.69 for hydroponically grown alfalfa. To test the sensitivity of the $\partial^{15}N_h$ parameter in the alfalfa N-fixation estimates presented here, I calculated kg of N fixed ha^{-1} yr^{-1} using $\partial^{15}N_h$ values of +0.5 and −0.5. With the positive $\partial^{15}N_h$ value, calculated rates of N-fixation decreased by an average of 71 kg ha^{-1} yr^{-1}, (S.E. = 7) compared to rates calculated when $\partial^{15}N_h = 0$ (Column 7, Table 2). The negative $\partial^{15}N_h$ value of −0.5 increased rates by an average of 116 ha^{-1} yr^{-1} (S.E. = 24) compared to rates calculated when $\partial^{15}N_h = 0$. N-fixation rates estimated using a $\partial^{15}N_h$ value of 0 ranged from 232—550 kg ha^{-1} yr^{-1}.

Greenhouse experiment

In the phosphorus addition study, I measured greater nitrogenase activity in all alfalfa plants grown on soils that received P additions than in plants grown on soils at field fertility (Fig. 3). Mean ethylene production (μmoles C_2H_4 $plant^{-1}$ $hour^{-1}$), increased with soil phosphorus additions from 1.3 to 1.7 on the Porvenir soil, 0.1 to 1.9 on the Patzcuaro soil, 0.4 to 1.8 on the Dolores Hidalgo soil, 1.4 to 1.7 on the Nuevas Casas soil and 1.8 to 3.3 on the Actopan soil.

Much higher rates of ethylene production have been measured elsewhere by alfalfa grown under greenhouse conditions. For example, Fishbeck & Phillips (1982) measured C_2H_4 production of 7.0 and 9.3 μmoles $plant^{-1}$ $hour^{-1}$, Tan (1981) measured C_2H_4 production of 1.86 to 5.04 μmoles $plant^{-1}$ $hour^{-1}$ and Barta (1978) measured between 12 and 32 μmoles C_2H_4 $plant^{-1}$ $hour^{-1}$; all well above the range of 0.1 to 3.3 μmoles

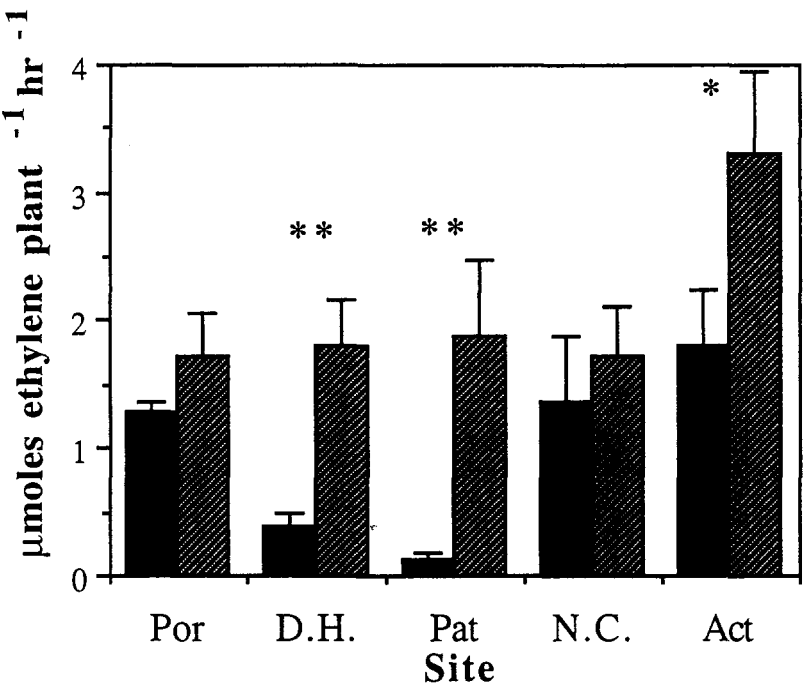
C_2H_4 plant^{-1} hour^{-1} reported here. One likely reason is that in every case, the growth medium was sand with macro and micro nutrients provided through solution or initial fertilization. In a sense, therefore, these rates represent potential nitrogenase activity of various alfalfa and rhizobia strains under optimal nutrient conditions.

In contrast to studies with minimal or no nutrient limitation, Bouton et al. (1981) grew alfalfa in pots of highly weathered, acid soils, amended with 384 μg P gdw^{-1} soil, about one-third of the phosphorus I applied to the fertilized soil treatments in this study. They found C_2H_4 production to vary between 0.05 and 1.0 μmoles plant^{-1} hour^{-1} depending on alfalfa variety and soil pH. That the range of acetylene reduction rates measured by Bouton et al. was lower than the range I measured across soil treatments is logical, because I applied more phosphorus in the fertilized treatments, and the Mexican soils in this study were generally less weathered, and therefore were likely to have had higher native available P and other nutrients.

The greatest increases in alfalfa root nitrogenase activity with added phosphorus occurred in the Dolores Hidalgo and Patzcuaro soil treatments. The native soil available phosphorus of these two soils was quite low at 5 and 7 μg gdw^{-1} soil, respectively, as estimated by anion exchange resin extractions. The Porvenir soil also had a low P availability of 6 μg gdw^{-1} soil, but the alfalfa/rhizobia symbiosis growing on this soil did not show as sharp an increase in nitrogenase activity with added P. The Nuevas Casas and Actopan soils had much higher levels of resin-extractable, available P, 65 and 92 μg gdw^{-1} soil, respectively. It is notable that nitrogenase activity increased significantly with added P in the Actopan soil even though the native soil P level was high.

The effect of phosphorus additions on nitrogenase activity did not appear to be due to increased fixation per unit plant biomass. Expressed per unit root mass, ethylene production did not differ significantly between fertilized and unfertilized treatments (Fig. 4). Root:shoot ratios also remained nearly constant (Fig. 5). The significant increase in nitrogenase activity of the alfalfa/*Rhizobium* symbioses grown on Dolores Hidalgo and Patzcuaro soils, and to a lesser extent on the Actopan soils, appears to be proportional to an increase in plant biomass (Figs. 3 and 6). This finding suggests that the effect of phosphorus on nitrogen fixation resulted primarily from an increase in host plant biomass rather than a direct effect of phosphorus on *Rhizobium* fixation rates.

Alfalfa leaf tissue phosphorus concentrations reflected the soil phosphorus addition treatments. Mean foliar P concentrations (in μg gdw^{-1} leaf) increased with P fertilization as follows: Porvenir-2, from 0.15 to 0.24; Dolores Hidalgo, 0.17 to 0.26; Patzcuaro 0.13 to 0.31; Nuevas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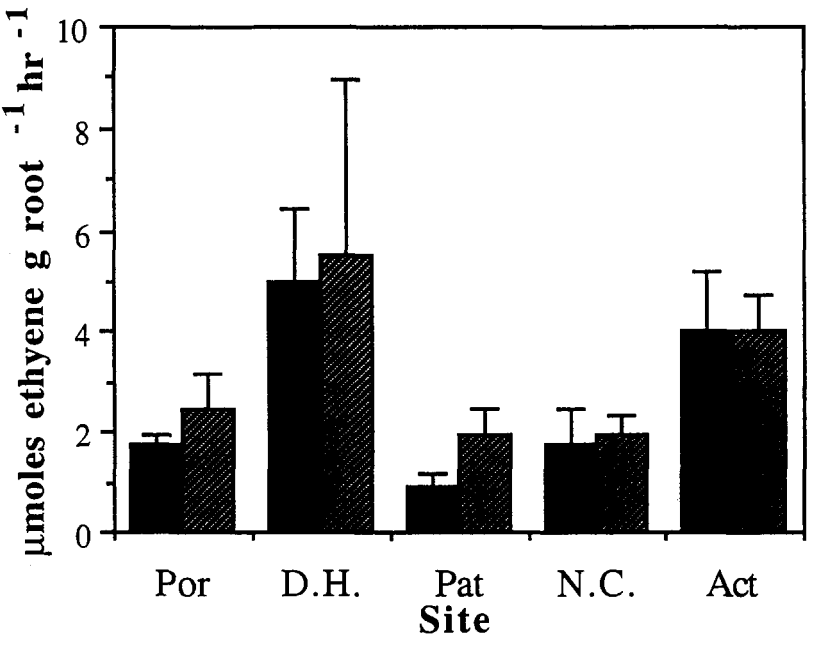
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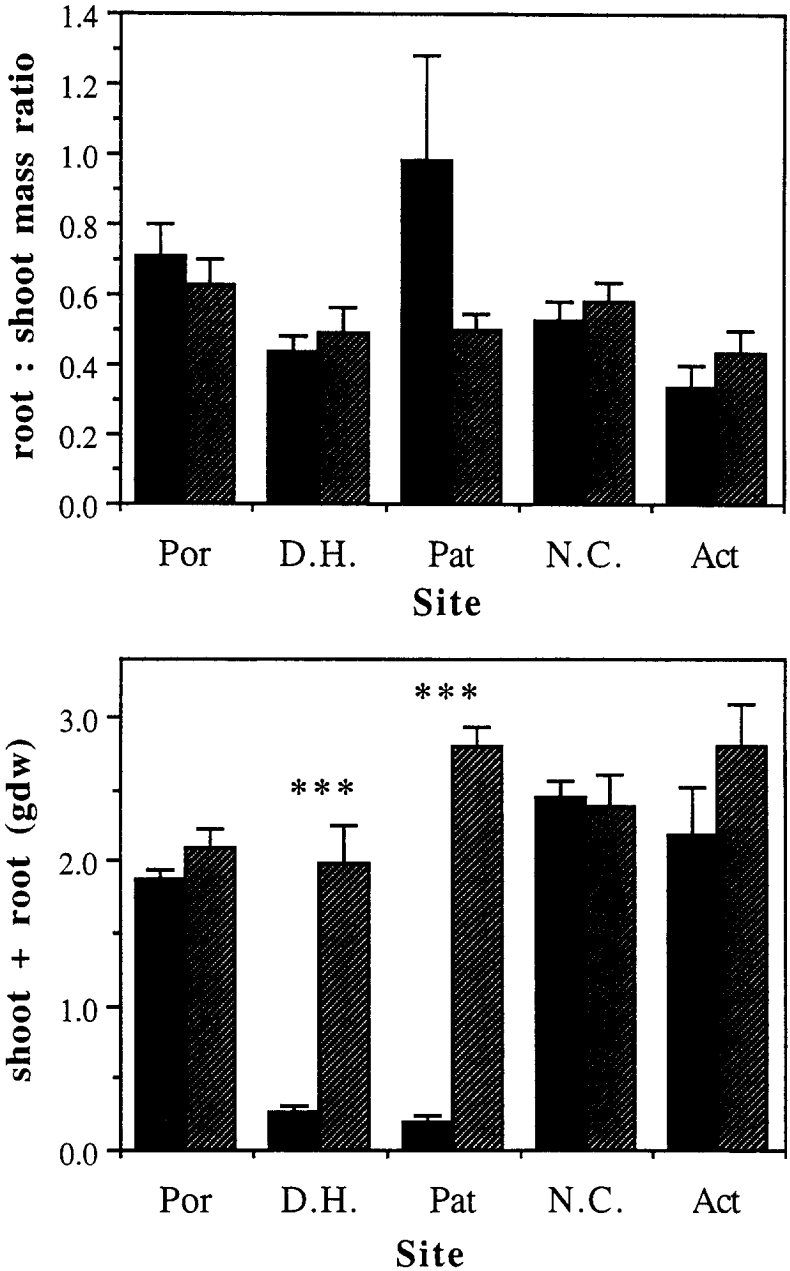

Figs. 3–6. Results of acetylene reduction assay of *Rhizobium* on alfalfa roots grown on five Mexican soils. Solid bars represent treatments with no added phosphorus and hatched bars represent treatments with added phosphorus. All weights reported are oven dry weights. Site abbreviations and site # s are Por = Porvenir (6), D.H. = Dolores Hidalgo (7), Pat = Patzcuaro (8), N.C. = Nuevas Casas Grandes (9), Act = Actopan (10). Error bars = 1 S.E., p values determined using unpaired, one-tailed t-test: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Casas, 0.25 to 0.31; and Actopan, 0.26 to 0.31. I correlated leaf tissue P with ethylene production plant^{-1} for all soil and phosphorus treatments in the experiment (Fig. 7). Analogous to the results in Fig. 2 from the ^{15}N field study, this result further supports the hypothesis that a positive relationship exists between biologically-available phosphorus and the rates of nitrogen fixation in alfalfa.

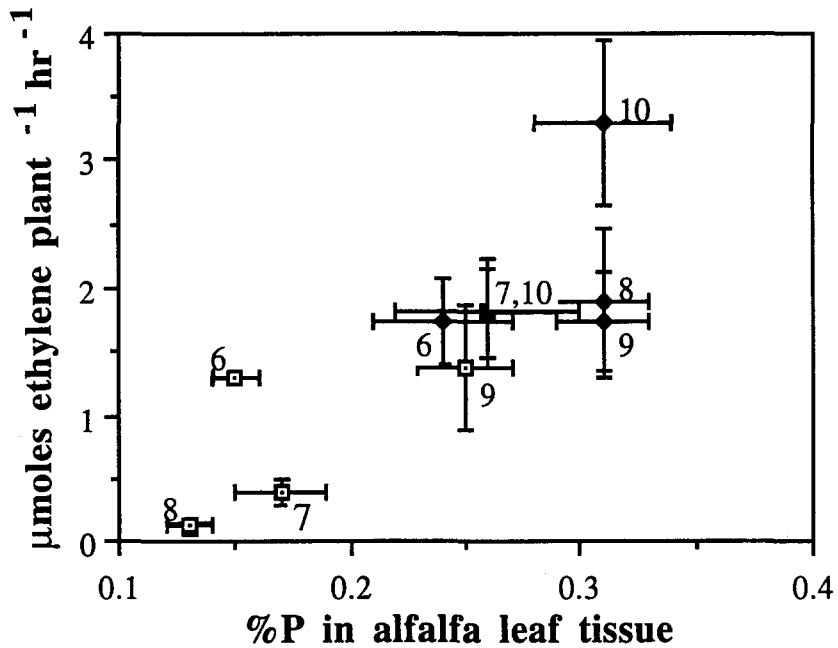

Fig. 7. Correlation of alfalfa/*Rhizobium* nitrogenase activity and foliage phosphorus concentrations. Open squares are values from treatments with no P additions; darkened squares are values from treatments with P additions. Treatments are identified by site numbers 6—10 (see Table 1). Error bars = 1 S.E, $r = 0.81, p < 0.01$.

Conclusion

The sustainability of an agricultural system hinges on the extent to which it can rely on, but not deplete, endogenous nutrient supplies (Crews et al. 1991). Nitrogen may be the most proximate nutrient limiting agricultural productivity, but of all nutrients, its limitation is the easiest to resolve through manipulation of nitrogen fixing species. The alfalfa plots in the Mexican cropping system described in this study provide the majority of nitrogen for associated maize-bean-squash polyculture fields. The results reported here suggest that factors controlling soil phosphorus availability

to the alfalfa may ultimately regulate the nitrogen flux to the polyculture fields.

Research on cropping systems and plant/microbe symbioses that maximize biological nitrogen fixation while relying on endogenous phosphorus supplies may lead to greater agroecosystem productivity in the short term. Two examples of such work are in understanding and optimizing the tripartate symbioses between legumes, rhizobia, and VA mycorrhizal fungi, in order to improve native phosphorus availability (Subba Rao & Krishna 1988; Azcón & Rubio 1989; Azcón et al. 1991), and research to select and improve legumes that perform well under conditions of low P availability (Pereira & Bliss 1989).

In a longer time frame, high rates of biological nitrogen fixation will only be maintained if the rate at which soil-P becomes available equals the rate of P loss from agroecosystems in harvests, leaching, erosion and formation of secondary minerals that occlude P. As appears to be the case in many natural systems, the native P supplying capacity of soils is among the most important factors controlling long-term productivity of continuously cropped, sustainable agroecosystems.

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