Effects of a Vesicular-Arbuscular Mycorrhizal Fungus on Nitrate Reductase and Nitrogenase Activities in Nodulating and Non-Nodulating Soybeans

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ABSTRACT

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The root systems of soybeans can be infected by vesicular-arbuscular (VA) endomycorrhizal fungi and by nitrogen-fixing bacteria. Both microorganisms are beneficial to the plant and the possibility of a direct interaction between the fungus and bacterium was considered. Nodulating and non-nodulating soybean isolines were treated with various combinations of *Glomus fasciculatus, Rhizobium japonicum*, and phosphate fertilizer in an attempt to evaluate this interaction. Dually-infected nodulating soybean plants showed increases in total dry weight and nodule dry weight, as well as higher levels of nitrogenase and nitrate reductase activities over singly or noninfected plants. When phosphorus was substituted for mycorrhizal infection, similar growth and enzyme activity increases were observed. This suggests that VA endomycorrhizal fungi, which can assist legumes in the uptake of phosphorus, do not interact directly with nitrogen-fixing bacteria.

Mutualistic symbiotic relationships are established between legumes and two distinct categories of microorganisms: bacteria and fungi. Nitrogen-fixing bacteria in concert with legumes fix atmospheric nitrogen which is then made available to the infected plant (9). Vesicular-arbuscular (VA) endomycorrhizal fungi assist legumes, as well as many other plants, in the absorption of phosphorus and other inorganic nutrients from the soil (12).

References have been made to an interaction between nitrogen-fixing bacteria and VA fungi: Asai (2) reported that several legume species failed to nodulate in autoclaved soil unless they were mycorrhizal, and Crush (3) indicated that infection by VA mycorrhizal fungi stimulated nodulation and growth of several legumes grown in phosphorus-deficient soil. Daft and El-Giahmi (4) reported increased growth, number and weight of nodules, leghemaglobin, and acetylene reduction rates in *Phaseolus vulgaris* inoculated with both *Rhizobium phaseoli* and *Endogone macrocarpa* var. *geospora*. Daft and El-Giahmi (5) and Mosse et al (13) later reported similar results with other legumes. It was concluded that increases in the concentration of host plant phosphorus, which result from mycorrhizal infection, account for enhancement of nodulation and nitrogen fixation. We have studied the effect of infection by VA mycorrhizal fungi on nitrogen fixation and host nitrogen metabolism by measuring nitrogenase and nitrate reductase activities in nodulating and non-nodulating isolines of soybean [*Glycine max* (L.) Merr.] treated with various combinations of *Rhizobium japonicum* (Kirchner) Buchanan, *Glomus fasciculatus* (Thaxt. sensu Gerdemann)

Gerdemann and Trappe and phosphate fertilizer.

MATERIALS AND METHODS

Plastic 10-liter pots were filled with a sand-soil mixture (3:1, v/v) which had an organic matter content of 0.8 percent, pH 6.2, and initial concentrations (kg/ha) of the following elements: Bray I P_2O_5 = 134, Bray II P_2O_5 = 238, Ca = 1,460, Mg = 168, and K = 101. Potassium chloride (1.575 g/kg) was added to each pot to increase available potassium.

Nodulating (T202) and non-nodulating (T201) isolines of soybean described by Weber (20) were used as host plants. *Rhizobium japonicum* does not form nodules on T201 plants. Both nodulating and non-nodulating plants were separately exposed to the following five treatment combinations:

1. Non-sterilized soil + *R. japonicum* = NSR
2. Sterilized soil + *G. fasciculatus* + *R. japonicum* = SGR
3. Sterilized soil + phosphorus fertilizer + *R. japonicum* = SPR
4. Sterilized soil + *R. japonicum* = SR
5. Sterilized soil = S

Soil was sterilized with Dowfume MC-2 (98% methyl bromide and 2% chloropicrin) (Dow Chemical Company, Midland, MI 48640). Five g of *Rhizobium japonicum* (proprietary strains 5708, 5706, 5708-6, Research Seeds, Inc., St. Joseph, MO 65402) were blended into the top 3 cm of soil. Two hundred g of *G. fasciculatus* inoculum, produced in soybean pot culture and consisting of a root-soil-fungus mixture containing 500-600 chlamydospores, was blended into the central third of the sand-soil mixture. Concentrated superphosphate (0.355 g

phosphorus per pot) was added to raise the available phosphorus concentration.

Eight seeds were planted and thinned to three plants per pot after emergence. Plants were maintained in the greenhouse under 14-hr daylight, supplemented by fluorescent, incandescent, and high-intensity metal halide lamps. Temperatures ranged from 28-32 C in the daytime to 16-20 C at night. Bi-weekly harvests (three pots with three plants per pot) were made from 4 to 14 wk and plants were evaluated for nitrate reductase activity (NRA), nitrogenase activity, percent mycorrhizal fungus infection, percent nitrogen, percent phosphorus, and total plant dry weight. Nitrogen and phosphorus determinations were not begun until the 6-wk harvest, due to the small amount of tissue available. By the 14th week, all plants had reached maturity.

Nitrogenase activity.—Nitrogenase activities were determined according to the methods of Fishbeck et al (8). The soil-less nodulating root systems were placed in 490-ml glass jars and, after the jars were sealed, 50 ml of air was extracted and 50 ml of acetylene was added through a rubber serum stopper, yielding an internal atmosphere of 10% acetylene. Samples were incubated for 30 min at room temperature. One ml gas samples then were collected from each jar with evacuated glass tubes (vacutainers) and stored for analysis. Samples were analyzed on a Varian Aerograph Series 1400 chromatograph equipped with a flame ionization detector. A 2.1 m × 2 mm aluminum column packed with Porapak P was maintained at 55 C and nitrogen (20 ml/min) was the carrier gas. Ethylene was quantified from a standard curve.

Nitrate reductase (NR).—Following nitrogenase activity measurement, nodule NR determinations were made by the methods of Jaworski (11). All nodules were separated from the roots, washed, cut into 1-mm slices and placed in an incubation medium of 10 mM Tris (hydroxy methyl aminomethane) buffer, 50 mM potassium nitrate, 10 μg chloramphenicol, and 0.5% (v/v) Neutronyx 600 (Onyx Chemical Company, Jersey City, NJ 07302) and adjusted to pH 7.5. The samples were incubated in the dark at 30 C for 30 min. A 0.4-ml sample of the incubated solution was mixed with 1.0 ml of 1% sulfanilamide in 1.5 N HCl, and 1.6 ml of distilled water. Samples then were centrifuged for 5 min at 100 rpm. The optical density of this solution at 540 nm was recorded and nitrate was determined from a standard curve. Total nodule NR was calculated from these measurements.

Leaf NR was determined in a similar manner (11). Plant tops were cut at the soil surface and placed on ice. Leaf disks (7 mm diameter) totaling 200 mg fresh weight were cut and placed in 5 ml of incubation solution consisting of 5 mM potassium phosphate buffer, 100 mM potassium nitrate, and 0.5% (v/v) Neutronyx 600, adjusted to pH 7.5. Incubation and nitrate determination were as described for nodules, except that samples were not centrifuged prior to the measurement of optical density. Total leaf NR was calculated from these determinations.

Mycorrhizal infection.—The percentage of the root systems infected by mycorrhizal fungi was determined by preparing roots according to the method of Phillips and Hayman (14). A 5-mm-wide cross section was cut across each root system 8 cm below the root crown. The

segments were placed in a 10% (w/v) potassium hydroxide solution and then autoclaved for 10 min. Cleared segments were then stained by steaming for 5 min in hot lactophenol containing 0.05% trypan blue. Excess stain was removed by soaking the segments in pure lactophenol overnight. The segments were examined under a binocular microscope at ×150 and estimates of mycorrhizal infection levels were made to the nearest 10% of the total root length in the sample.

Dry weight, nitrogen, and phosphorus determinations.—Root and shoot tissue remaining after completion of other analyses were oven-dried at 50 C to a constant weight. Following dry weight determination, samples were ground and blended for mineral analysis. Nitrogen and phosphorus determinations were made by the Missouri Agricultural Experiment Station Analytical Chemistry Laboratory according to the method of Wall and Gehrke (18, 19).

RESULTS

At the first harvest (4 wk after planting) root systems of both nodulating and non-nodulating soybean plants grown in sterile soil inoculated with *G. fasciculatus* (SGR) were heavily colonized. In succeeding harvests, average colonization exceeded 90% of the total root length sampled. Nodulating and non-nodulating plants grown in nonsterilized soil (NSR) became infected to a somewhat lesser degree, averaging 70% after the 6 wk harvest. In spite of the low levels of indigenous VA fungus inoculum in this nonsterile soil treatment (NSR), plants became heavily infected. All other treatments (SPR, SR, and S) remained free of infection throughout the experiment. Nodules never contained mycorrhizal infections.

Large, dry weight increases were observed in nodulating plants fertilized with phosphorus (SPR), inoculated with *G. fasciculatus* (SGR) or grown in nonsterilized soil (NSR). Similar increases were not observed in the non-nodulating isoline (Fig. 1). Phosphorus concentrations in *R. japonicum* inoculated (SR) and noninoculated (S) treatments were equal to or less than 0.11% in both nodulating and non-nodulating plants. Concentrations of phosphorus in both nodulating and non-nodulating plants receiving other treatments (NSR, SGR, SPR) were greater than SR and S with some non-nodulating treatments (SGR, NSR) attaining final concentrations above 0.3% (Fig. 2). Nitrogen accumulation was much higher in nodulating than in non-nodulating plants since compensatory nitrogen was not applied to the latter (Fig. 3). Although the difference between isolines in treatment S was greater than expected, this may have been accentuated by limited nodule formation and a low nitrogen fixation capacity in nodulating plants (Fig. 4, 5) arising from *Rhizobium* contamination which would not have been reflected in the non-nodulating isoline. Close correspondence of S and SR curves for the nodulating isoline (Fig. 3, 4, 5) tends to support this interpretation. Other treatments had no apparent effect on nitrogen concentration in plant tissues within isolines (Fig. 3).

Nitrogen-fixing capacity of nodulating plants increased in response to phosphorus fertilization (SPR) and VA fungal infection (SGR, NSR), as indicated by

increases in nodule dry weight and acetylene reduction. Dry weight of nodules from plants that had received such treatments was five-fold greater than that from plants in the other two treatments (SR, S) (Fig. 4). Acetylene reduction differences between the same two groups of treatments were of an even larger magnitude (Fig. 5), and showed a typical activity peak at mid-pod fill.

Nitrate reductase was measured in leaf tissue of non-nodulating plants and in leaf and nodule tissue of nodulating plants. In the leaves of both isolines (Fig. 6), similar activity patterns were observed. Activity decreased rapidly from a peak at 4 wk, and was at a relatively constant low level thereafter. No significant differences in leaf NR were observed, neither due to treatment nor to soybean isoline. Nodule NR was highest at final harvest. Fungus infection (SGR, NSR) and phosphorus fertilization (SPR) resulted in significantly higher NR in nodules (Fig. 7).

DISCUSSION

Results of these experiments support the contention (1, 3, 4, 5, 13) that infection by VA mycorrhizal fungi stimulates soybeans to produce greater plant mass and, in concert with nitrogen-fixing bacteria, to produce a greater nodule mass and to fix larger quantities of nitrogen. However, application of phosphate fertilizer produces a similar response, indicating that the VA fungus in all probability has no direct effect on the nitrogen-fixing bacterium, but brings about the above mentioned increases by improving the nutrition of the host. It has been well documented that mycorrhizal fungi assist plants in accumulating higher concentrations of phosphorus (12), and phosphorus is also known to have a positive effect (6) on both nodulation and nitrogen fixation (7, 17).

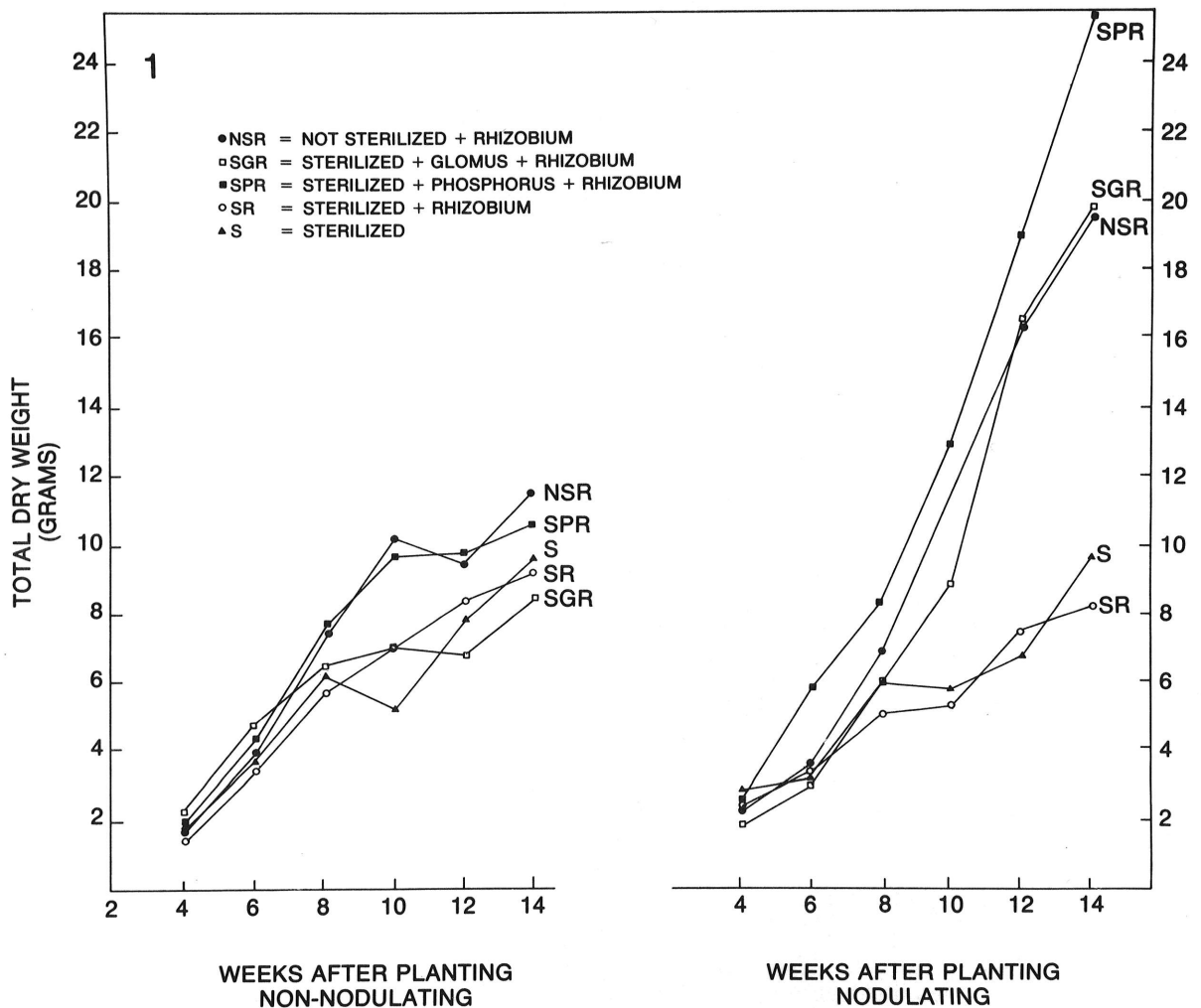The percent of phosphorus in mycorrhizal (SGR,

Fig. 1. Total dry weight of nodulating and non-nodulating soybean isolines grown in soil treated with various combinations of *Glomus fasciculatus, Rhizobium japonicum,* and phosphorus fertilizer.

NSR) and phosphorus-fertilized (SPR) plants was higher than in plants from the other two treatments (SR, S) in both nodulating and non-nodulating soybeans (Fig. 2). The percent of phosphorus increased because a larger quantity of phosphorus was made available to these plants either by VA fungus infection (SGR and NSR) or phosphate fertilization (SPR). In nodulating plants that received additional phosphorus (SGR, NSR, and SPR), the percent of phosphorus in total plant tissue was maintained at a moderate and relatively constant level (.14 to .21%) throughout the experiment, because of the concurrent increase in the dry weight of these plants (Fig. 1). The quantity of available phosphorus also was increased in non-nodulating plants by these treatments (SGR, NSR, and SPR), but corresponding increases in dry weight did not occur (Fig. 1). Although there was no dry weight increase in non-nodulating plants that had received additional phosphorus (SGR, SPR, and NSR),

larger quantities of phosphorus were absorbed resulting in higher concentrations (.27 to .31%) in their tissues.

It is likely that the quantity of available nitrogen was a limiting factor to the growth of non-nodulating plants, since foliar nitrogen deficiency symptoms were evident and nitrogen concentrations were extremely low (Fig. 3). It also is apparent that infection by mycorrhizal fungi had no effect on nitrogen uptake, which is in agreement with an earlier report by Schenck and Hinson (15).

The NR determinations were restricted to leaf and nodule tissue because preliminary tests indicated that they were the primary centers of activity. Leaf NR in both nodulating and non-nodulating plants was highest at the initial harvest followed by much lower activity levels in later harvests (Fig. 6). Streeter (16) reported similar results with field-grown soybeans in which the NR peak preceded flowering. The uniform low level of NR in leaves of maturing plants in all treatments with both nodulating
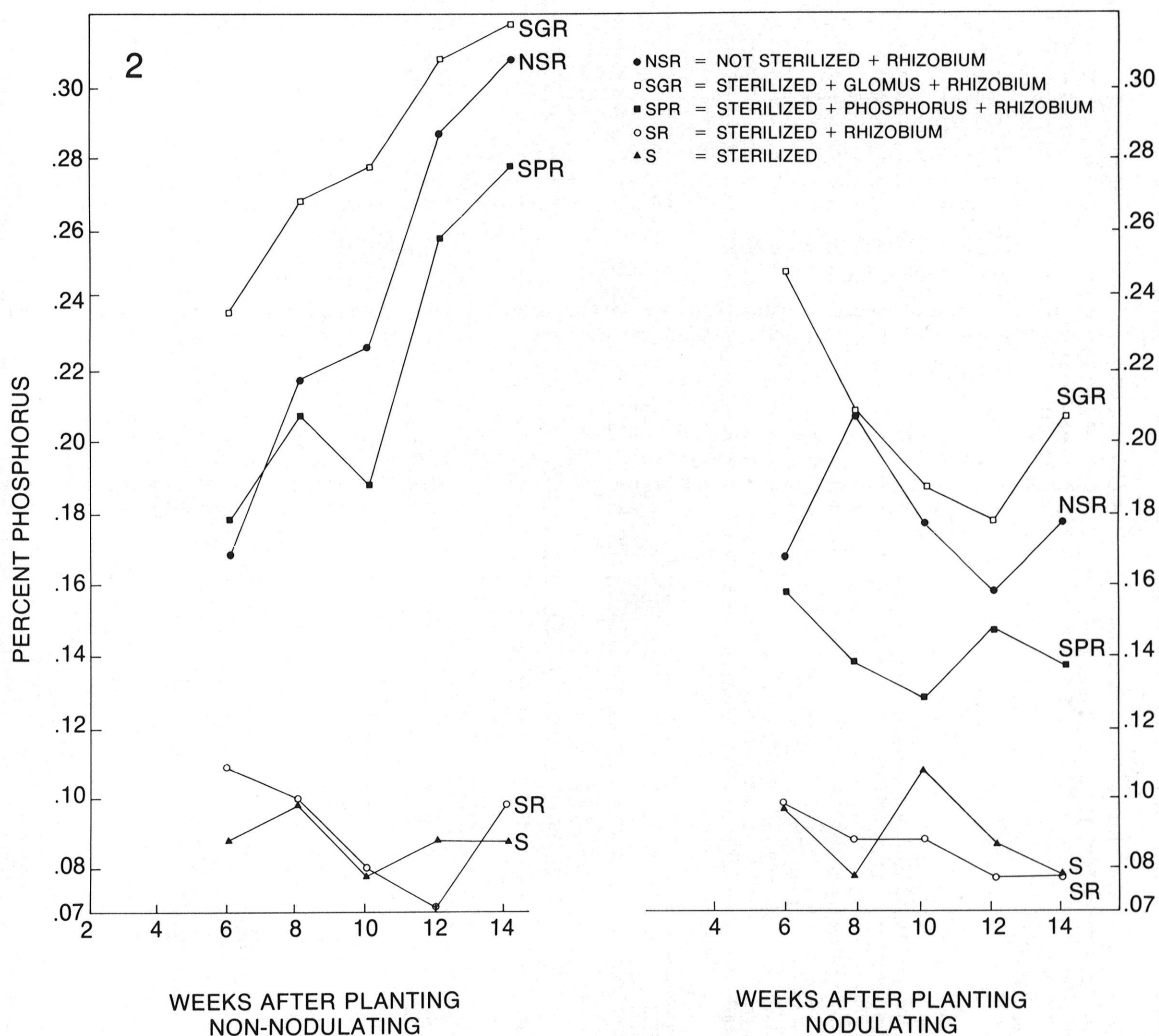

Fig. 2. Phosphorus expressed as a percent of total plant dry weight of nodulating and non-nodulating soybean isolines grown in soil treated with various combinations of *Glomus fasciculatus, Rhizobium japonicum,* and phosphorus fertilizer.
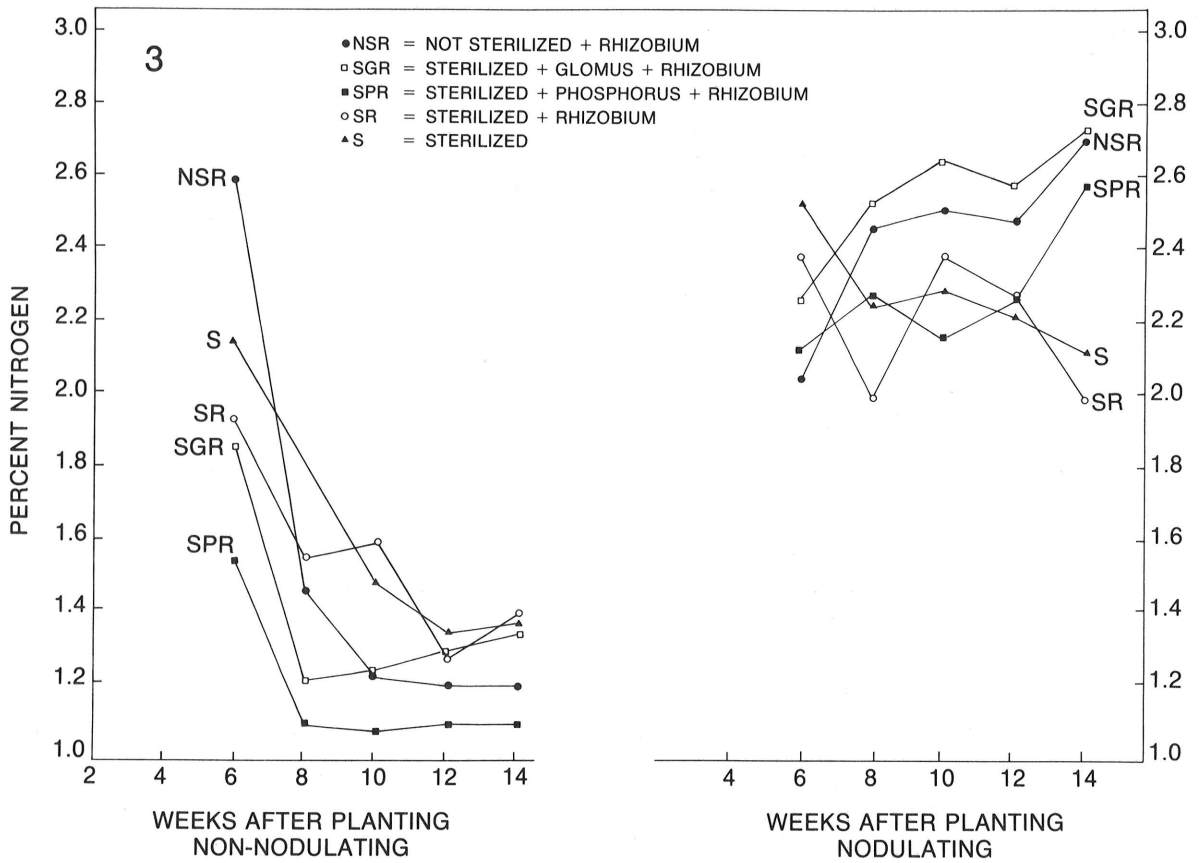
Fig. 3. Nitrogen expressed as a percent of total dry weight of nodulating and non-nodulating soybean isolines grown in soil treated with various combinations of *Glomus fasciculatus, Rhizobium japonicum,* and phosphorus fertilizer.
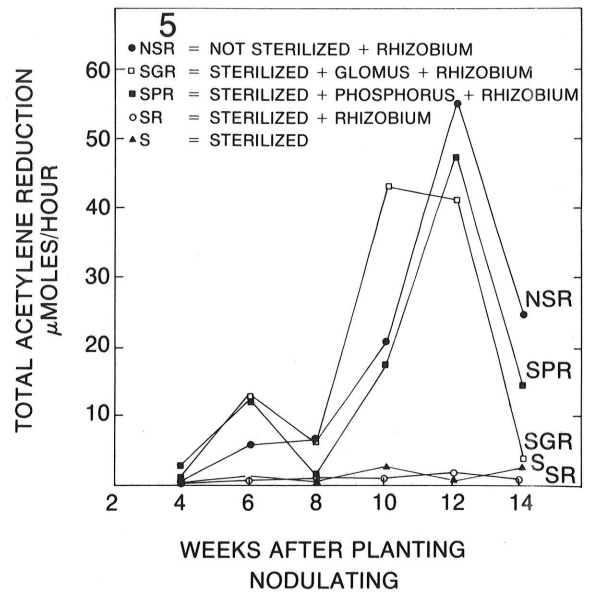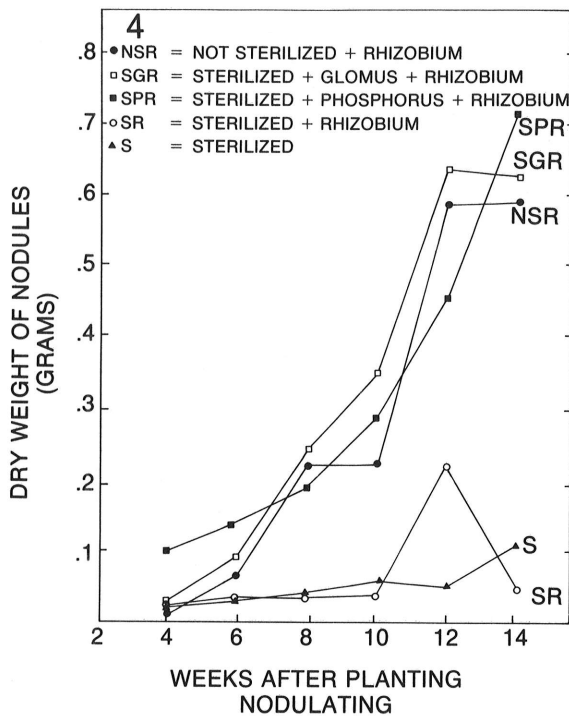

Fig. 4-5. 4) Nodule dry weight and 5) acetylene reduction rates of nodulating soybean isolines grown in soil treated with various combinations of *Glomus fasciculatus, Rhizobium japonicum,* and phosphorus fertilizer.
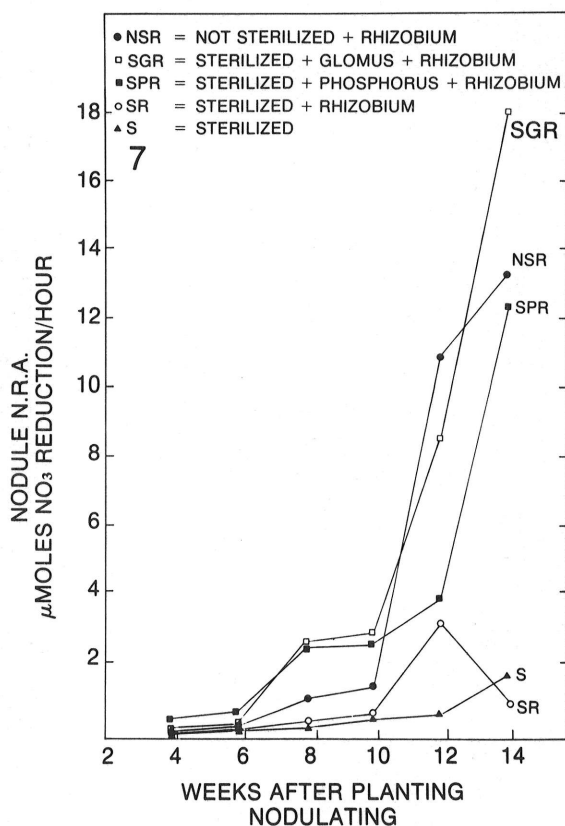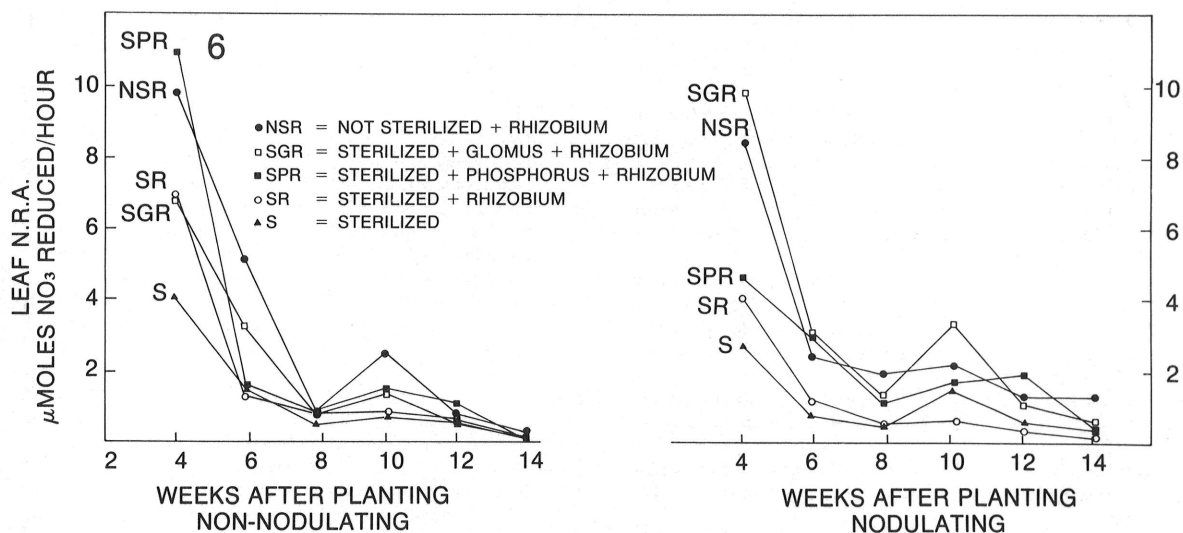
Fig. 6-7. Nitrate reductase activity (NRA) in: 6) leaves in nodulating and non-nodulating isolines of soybean grown in soil treated with various combinations of *Glomus fasciculatus, Rhizobium japonicum,* and phosphorus fertilizer; 7) nodule NRA from the nodulating soybean isoline.

fixing capacity of the plant have little effect on NR in the soybean leaves.

The NR was higher in the nodule tissue of nodulating plants than in the leaves of either isoline. Levels of NR in the nodules of mycorrhizal fungus-infected (SGR, NSR) and phosphorus-fertilized (SPR) plants were ten times the NR in the other treatments (SR, S) (Fig. 7). Mycorrhizal fungi possess nitrate reductase (10), but such activity would not be involved here since only nodule tissue was evaluated. Our observations agree with those of Crush (3) who reported that mycorrhizal fungi do not invade nodules.

In summary, total plant and nodule dry weight, as well as nitrate reductase and nitrogenase activities, are increased significantly in mycorrhizal nodulating plants. The fact that phosphate fertilization is able to produce a similar effect, however, suggests the absence of a direct interaction between the VA mycorrhizal fungus and the nitrogen fixing bacterium. A more reasonable explanation appears to be that by improving the nutritional environment for the plant, each microorganism complements the stimulatory effect that the other has on growth of the host plant.

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