The *Glycine-Glomus-Bradyrhizobium* symbiosis. XI. Nodule gas exchange and efficiency as a function of soil and root water status in mycorrhizal soybean

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Soybean [*Glycine max* (L.) Merr. cv. Hobbit] plants were inoculated with a HUP⁻ strain of *Bradyrhizobium japonicum* (Nitragin 61A118) and either colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* (Nicol & Gerd.) Gerd. and Trappe or fertilized with KH_2PO_4 (nonVAM). They were grown for 50 days in a growth chamber and harvested over a 4-day drought period during which available soil water decreased to 0. Nodule P concentrations and P-use efficiency declined linearly with soil and root water content during the harvest period in both VAM and nonVAM plants. Nitrogenase activity, estimated from H_2 evolution and C_2H_2 reduction data, was also a linear function of declining nodule P concentrations and CO_2-exchange rates and showed similar patterns in both treatments. Hydrogen evolution and the relative efficiency of N_2 fixation, on the other hand, reacted differently to increasing drought in VAM and nonVAM plants. Differences in the responses of nodule activity in VAM and nonVAM plants to drought are interpreted in terms of demand for nodule P and carbohydrates and of the effects of dehydration on O_2 diffusion through nodule tissue.

Key words – *Bradyrhizobium japonicum*, carbon dioxide exchange rate, drought stress, *Glomus mosseae*, *Glycine max*, nitrogen fixation, phosphorus nutrition, phosphorus-use efficiency, vesicular-arbuscular mycorrhiza.

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Introduction

Plants have excess capacity to supply their VAM and N_2-fixing endophytes with reduced C (Paul and Kucey 1981) when not under stress. When under drought stress, complex changes in intersymbiont relationships occur, since drought affects not only the availability of water, but also that of P (Mouat and Nes 1986) and carbohydrates (Smith and Gianinazzi-Pearson 1988). The supply of all three substances directly affects N_2 fixation and is influenced by VAM-fungal activity (Barea and Azcón-Aguilar 1983).

Biological N_2 fixation by the O_2-labile enzyme nitrogenase requires large amounts of ATP and reducing power derived from oxidative phosphorylation and from the products of photosynthesis (Phillips 1980). The

nodule must therefore be able to control its internal pO_2 to achieve optimum rates of respiration without impairing nitrogenase activity (Hunt et al. 1987). While high respiration rates are important in regulating internal pO_2, there is also evidence for a barrier to diffusion of gas into the nodule (Witty et al. 1987). The exact nature of the barrier is not known (Hunt et al. 1987), but it is thought to be under rapid biological control (Sheehy et al. 1983). This control of diffusion resistance is of particular interest during periods of stress, when changes in any factor affecting the flux of O_2 (the supplies of P, reduced C and ATP, respiration, and the barrier itself) may influence nodule activity.

The factors regulating pO_2 in the nodule are modified by VAM fungi, which compete with the nodule for carbohydrates under C stress (Bayne et al. 1984) but

Tab. 1. Plant parameters in vesicular-arbuscular mycorrhizal (VAM) and P-fertilized (nonVAM) soybean. Statistical evaluation by Student's *t*-test (NS, $P > 0.05$; *, $0.01 < P < 0.05$; **, $P < 0.01$). Specific nodule mass is total nodule mass divided by nodule number, and is an indicator of relative size. The nodule/root ratio shows nodule mass as a percentage of root mass. The leaf N/P ratio is an indicator of nutrient balance, with N/P = 13.5 for soybean plants near optimal growth. Colonization by the VAM fungus is presented as percentage of root length. N and P concentration were previously reported erroneously as a percentage but with concentration as units (Bethlenfalvay et al. 1990, Tab. I).

Parameter	Plant		Nodule	
	VAM	nonVAM	VAM	nonVAM
Dry mass (g)	5.3*	6.0	0.4**	0.6
N concentration (mg g^{-1})	34.9 NS	34.6		
P concentration (mg g^{-1})	1.3**	1.7	2.9**	3.9
Nodule number			124 NS	143
Specific mass (mg)			3.1*	3.8
Nodule/root ratio (%)	32*	43		
VAM colonization (%)	68			

enhance the supply of water and P under drought stress (Bethlenfalvay et al. 1988, Nelson and Safir 1982). The impact of individual effects of the VAM condition on N_2 fixation, and their contribution to a net effect, is not known. Since drought effects on stomatal behavior and net C assimilation rates are controlled by root or soil water status (Turner et al. 1985), a comparison of the effects of changes in the factors controlling leaf gas exchange on nodule activity is of interest.

The purpose of our experiment was to investigate VAM effects on nitrogenase activity in comparison to nonVAM plants not under P stress, and to relate this activity to nodule P status and decreasing root and soil water contents.

Abbreviations – ASW, available soil water; CER, CO_2-exchange rate; NPUE, nodule phosphorus-use efficiency; RE, relative efficiency of nitrogen fixation; VAM, vesicular-arbuscular mycorrhizal.

Materials and methods

Soybean [*Glycine max* (L.) Merr. cv. Hobbit] plants were grown in a growth chamber in association with the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe and the diazotrophic bacterium *Bradyrhizobium japonicum*, strain Nitragin 61A118 (originally obtained from USDA-ARS, Beltsville, MD, as USDA strain 138) lacking active uptake hydrogenase (Lim 1978). The plants were grown in a heavy silt loam soil (Typic Xerorthent) deficient in N and P. Leaf gas exchange by these plants and details of growth conditions were described previously (Bethlenfalvay et al. 1990). NonVAM comparison plants received KH_2PO_4 in a nu-

trient solution to alleviate P stress and to make them more comparable to the VAM plants in size (Bethlenfalvay et al. 1990). After 50 days of growth, water was withheld. VAM and nonVAM plants for evaluation were selected from the larger initial random design over a 4-day harvest period on the basis of gypsum block soil moisture sensor readings. The readings indicated the status of the slowly drying soil, and permitted selection of plants over the range of ASW. The experiment was terminated upon reaching zero ASW. The length of the drought period permitted the evaluation of 13 VAM and 12 nonVAM plants.

Total ASW was determined by measuring soil water content at -0.1 MPa (the highest water content that could be measured reliably with these sensors, see Bethlenfalvay et al. 1990) and at the permanent wilting point of plant leaves. The water content of the soil in each experimental pot was determined at harvest and expressed as a percentage of total ASW. Root water content was calculated from the difference between fresh and dry weights, and expressed as a percentage of fresh weight. The measurement of fresh weight after a rapid but thorough washing and dry dabbing of roots is permissible, since rehydration of decapitated roots is small in the absence of externally applied pressure (Fiscus 1987). This phenomenon is ascribed to the absence of a negative leaf-to-root vapor pressure gradient and the impairment of the root hairs in the washing process. Nodule P concentration was determined by standard analytical methods, and VAM colonization by the grid-line-intersect method.

Nitrogenase activity in intact, nodulated root systems was measured in closed incubation vessels as H_2 evolution and C_2H_2 reduction, using gas chromatography as reported previously (Bethlenfalvay and Phillips 1977). Because the plants were grown in a heavy silt-loam soil of low gas permiability, it was not possible to measure gas exchange in undisturbed roots with a flow-through system. Nitrogenase activity (N_2 reduced) of VAM vs nonVAM plants was estimated as (C_2H_2 reduced/3) $\times$ RE, where RE = $[1 - (H_2$ evolved/C_2H_2 reduced$)]$ (Schubert and Evans 1976). Problems of interpretation of C_2H_2-reduction data to estimate N_2-fixation rates in closed incubation vessels have been discussed by Upchurch (1987), with the conclusion that the procedure is adequate for comparative purposes, as was the case here. Since our *Bradyrhizobium* strain has no uptake hydrogenase activity, H_2 evolution was a function of nitrogenase activity only. The RE was therefore assumed to be a valid measure of the electron allocation coefficient of nitrogenase (Edie and Phillips 1983). Nodule phosphorus-use efficiency (NPUE) was calculated as N_2 reduced plant^{-1} h^{-1} divided by total nodule P. Carbon dioxide exchange rates (CER) were determined by infrared gas analysis, utilizing an incubation chamber that permitted measurement of leaf gas exchange in the entire shoot of a single plant (Bethlenfalvay et al. 1990).
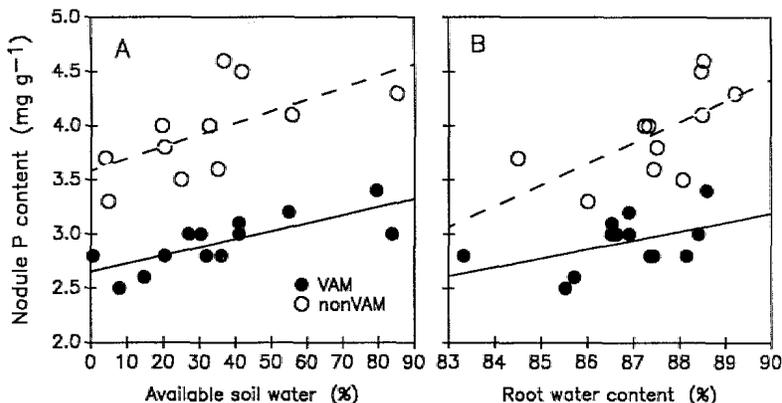
Fig. 1. Nodule P concentration as a function of available soil water (A) and root water content (B) in vesicular-arbuscular mycorrhizal (VAM) and P fertilized (nonVAM) soybean plants. Regression analysis for the individual data sets are: A. VAM, $r^2 = 0.58$, $P = 0.002$, $y = 0.007x + 2.655$; nonVAM, $r^2 = 0.43$, $P = 0.02$, $y = 0.01x + 3.57$. B. VAM, $r^2 = 0.23$, $P = 0.1$, $y = 0.08x - 4.03$; nonVAM, $r^2 = 0.40$, $P = 0.03$, $y = 0.2x - 11.2$.

The response of VAM and nonVAM nodules to changing soil and root water status was evaluated by regression analysis within and between the VAM and nonVAM data sets. Differences between nodule parameters of VAM and nonVAM plants were established by two-tailed Student's t-test.

Results

Both our VAM and nonVAM plants were P-deficient (Tab. 1) in comparison to plants grown under optimal conditions (deMooy 1973). Previous work (Brown and Bethlenfalvay 1988) with plants grown under similar conditions (but not drought-stressed at harvest) showed higher P-use efficiency in the more P-deficient VAM plants. As seen in previous work on P fertilization (Mul-

len et al. 1988) and microsymbiont competition (Bethlenfalvay et al. 1985), nodule mass was greater and nodule P concentration higher in nonVAM plants, although the total number of nodules was not significantly different ($P > 0.05$) (Tab. 1). Nodules on VAM roots were smaller (specific mass) and had a smaller share of the total below-ground plant mass (Tab. 1).

Imposition of drought stress resulted in a loss of P from the nodules (Fig. 1A). Loss of P was significantly correlated with decreasing ASW in VAM and nonVAM plants. The rates of loss were not significantly different, as indicated by the regression lines, which had the same slopes ($P = 0.40$) but different y intercepts ($P < 0.001$). The pattern was similar for the stress response of the relationship between nodule P concentration and root water content. However, here neither the slopes ($P =
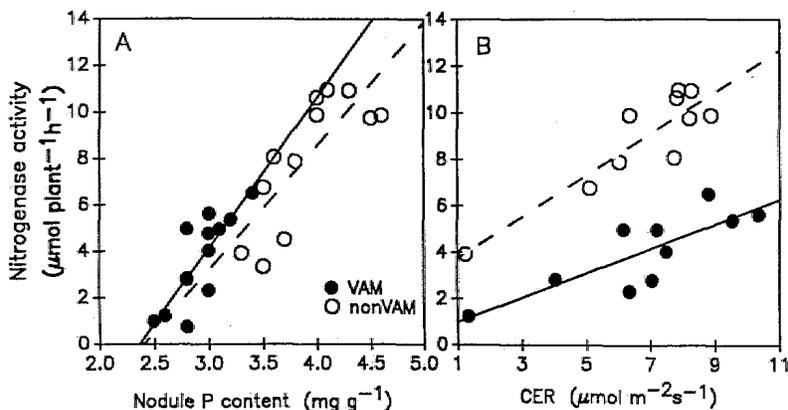

Fig. 2. Nitrogenase activity as a function of nodule P concentration (A) and CO_2 exchange rate (CER) (B) in vesicular-arbuscular mycorrhizal (VAM) and P-fertilized (nonVAM) soybean plants. Nitrogenase activity (N_2 reduced) was estimated as: (C_2H_2 reduced/3) × RE, where RE = 1 − (H_2 evolved/C_2H_2 reduced). Regression analyses for the individual data sets are: A. VAM, $r^2 = 0.66$, $P = 0.0007$, $y = 6.5x - 15.3$; nonVAM, $r^2 = 0.2$, $P = 0.14$, $y = 2.9x - 3.5$. B. VAM, $r^2 = 0.67$, $P = 0.04$, $y = 0.5x + 0.5$; nonVAM, $r^2 = 0.29$, $P = 0.1$, $y = 0.6x + 4.3$.
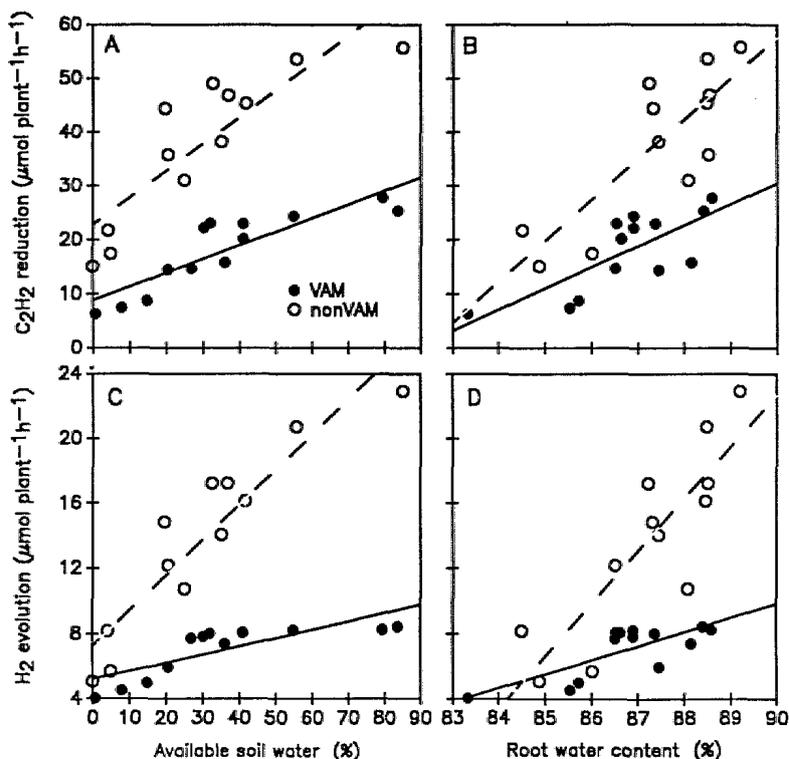
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Fig. 3. Acetylene reduction and hydrogen evolution as a function of available soil water (A and C) or root content (B and D) in vesicular-arbuscular mycorrhizal (VAM) or P-fertilized (nonVAM) soybean plants. Regression analyses for the individual data sets are: A. VAM, $r^2 = 0.76$, $P = 0.0001$, y = 0.3x + 8.8; nonVAM, $r^2 = 0.55$, $P = 0.009$, y = 0.4x + 24.7. B. VAM, $r^2 = 0.57$, $P = 0.003$, y = 3.4x − 320.3; nonVAM, $r^2 = 0.41$, $P = 0.03$, y = 5.7x − 464.8. C. VAM, $r^2 = 0.63$, $P = 0.001$, y = 0.05x + 5.23; nonVAM, $r^2 = 0.82$, $P = 0.0001$, y = 0.2x + 7.1. D. VAM, $r^2 = 0.58$, $P = 0.003$, y = 0.9x − 67.0; nonVAM, $r^2 = 0.40$, $P = 0.04$, y = 2.5x − 201.0.
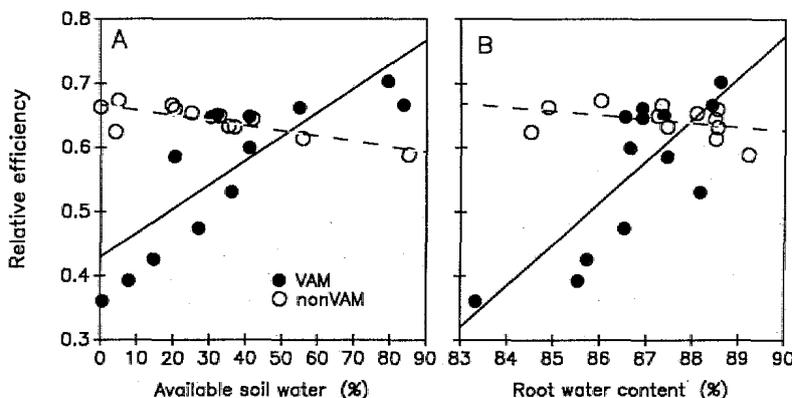

Fig. 4. Relative efficiency of nitrogen fixation (RE) as a function of available soil water (A) and root water content (B) in vesicular-arbuscular mycorrhizal (VAM) and P-fertilized (nonVAM) soybean plants, with RE = 1 − (H_2 evolved/C_2H_2 reduced). Regression analyses for the individual data sets are: A. VAM, $r^2 = 0.65$, $P = 0.0009$, y = 0.004x + 0.429; nonVAM, $r^2 = 0.38$, $P = 0.04$, y = −0.002X + 0.681. B. VAM, $r^2 = 0.61$, $P = 0.002$, y = 0.07x − 5.07; nonVAM, $r^2 = 0.40$, $P = 0.58$, y = −0.008x + 1.324.
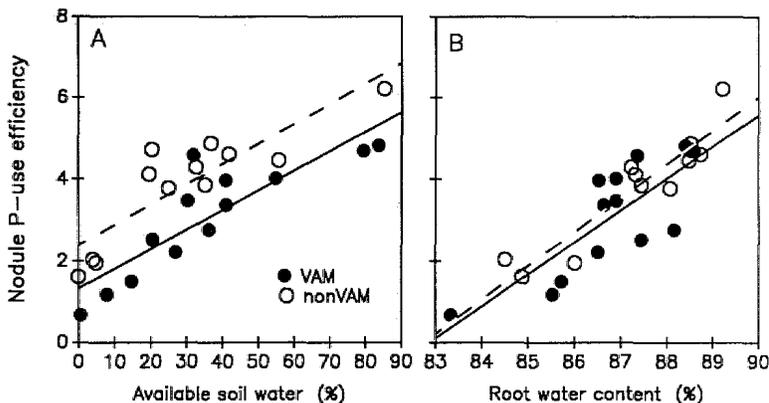
Fig. 5. Nodule phosphorus-use efficiency (NPUE, N_2 reduced plant^{-1} h^{-1} divided by total nodule P) as a function of available soil water (A) and root water content (B) in vesicular-arbuscular mycorrhizal (VAM) or P-fertilized (nonVAM) soybean plants. NPUE was caluclated as nitrogenase activity per total nodule N content. Regression analyses for the individual data sets are: A. VAM, $r^2 = 0.45$, $P = 0.01$, $y = 0.04x + 1.59$; nonVAM, $r^2 = 0.45$, $P = 0.02$, $y = 0.03x + 2.77$. B. VAM, $r^2 = 0.37$, $P = 0.03$, $y = 0.7x - 56.2$; nonVAM, $r^2 = 0.71$, $P = 0.001$, $y = 0.7x - 58.5$.

0.28) nor the intercepts ($P = 0.34$) were different (Fig. 1B).

The rates of nitrogenase activity declined with decreasing nodule P concentration (Fig. 2A). The regression lines expressing this relationship for VAM and nonVAM plants were not significantly different (slope, $P = 0.13$; intercept, $P = 0.17$), and indicated a continuous decline of nitrogenase activity with decreasing nodule P concentration regardless of the presence or absence of the fungus. Nitrogenase activity also declined with decreasing CER in the leaves (Fig. 2B). The response pattern of nitrogenase activity to change in CER in VAM and nonVAM plants was similar to the response to change in P concentration (Fig. 2A); the slopes of VAM and nonVAM regression lines were not significantly different ($P = 0.88$). Nodules were still active at 0% ASW, while leaf gas exchange in the same plants (as reported earlier, Bethlenfalvay et al. 1990) was no longer measurable at 10% ASW. Thus, nodule activity ceased at a higher level of stress (as indicated by ASW) than leaf gas exchange.

Individual components of nodule gas exchange, C_2H_2 reduction and H_2 evolution, were also functions of decreasing ASW and root water content (Fig. 3). However, while the slopes of the regression lines for C_2H_2 reduction vs soil (Fig. 3A) and root (Fig. 3B) water contents were not significantly different for the VAM and nonVAM data sets (ASW, $P = 0.30$; root, $P = 0.48$), the corresponding slopes for H_2 evolution (Fig. 3C, D) were different (ASW, $P = 0.0007$; root, $P = 0.05$). Even greater differences were found in the RE between VAM and nonVAM plants, both in slope and y intercept relative to ASW (Fig. 4A) and root water content (Fig. 4B) (ASW: slope, $P = 0.0007$, intercept, $P = 0.00002$; root: slope, $P = 0.002$, intercept, $P = 0.002$). Thus, shifts in drought-dependent electron allo-

cation by nitrogenase to different substrates were apparently influenced by the VAM condition of the root. On the other hand, the dependence of nitrogenase activity on total P available in the nodules was not affected by the presence of VAM fungi. The NPUE, expressed as a measure of nodule P accumulation necessary for a given level of nitrogenase activity, declined similarly with both ASW (Fig. 5A) and root water content (Fig. 5B) (ASW: slope, $P = 0.63$, intercept, $P = 0.14$; root water: slope, $P = 0.93$, intercept, $P = 0.93$).

Discussion

The effect of root colonization by VAM fungi on nodule activity is generally regarded to be stimulatory (Barea and Azcón-Aguilar 1983). When grown under continuous drought stress, VAM legumes have higher levels of nodulation and P content than P-fertilized plants, although the latter perform better in the absence of water stress (Azcón et al. 1988). Since experimentation with VAM fungi usually seeks to exploit the improved P-uptake capability of the mycorrhiza under conditions of P deficiency, the VAM effect on nodule development and function tends to be thought of as a P effect. This interpretation is understandable in view of the high dependence of nodules on P (Israel 1987), but tends to obscure those relationships between the microsymbionts of the legume association that are not explained by P nutrition.

It is not clear why root nodules demand the high P concentrations that are generally observed, and why P stress inhibits nodulation while still permitting root growth. The high ATP requirement of nitrogenase has been assumed to be a plausible reason (Phillips 1980). Evidence for the localization of large amounts of P_i in the vacuoles of uninfected cortical cells (Rolin et al.

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1989a), and stress-related redistribution of P_i (Mitsumori et al. 1985, Rolin et al. 1989b) suggests that the infected nodule cells require a reservoir of readily available P_i, perhaps as a stress reserve. It is possible that the layer enriched in P_i in the nodule cortex is associated with part of the O_2 barrier that modulates the influx of molecular oxygen (P. E. Pfeffer, personal communication), and that changes in this P, as observed by us as a result of drought (Fig. 1), affect that barrier.

VAM fungi did not influence the rates of stress-related declines of nodule P concentration (Fig. 1), and those of nitrogenase activity with decreasing nodule P concentration (Fig. 2A), or NPUE (Fig. 5). The fungi are therefore seen to be involved in P acquisition rather than subsequent P utilization, and the changes in nodule P concentration are likely to be a consequence of P redistribution rather than C accumulation in view of the drought-related limitation on fresh C supplies. However, nodule activity (H_2 evolution) and the RE were affected by VAM colonization (Figs 3 and 4) in ways apparently not related to P nutrition. From what is known about VAM effects on root physiology (Sheehy et al. 1983), we suggest that VAM fungi, which do not normally penetrate the root nodule, affect nitrogenase activity indirectly, as described below.

When plants are under carbohydrate stress, induced directly through shading (Bethlenfalvay and Pacovsky 1983) or defoliation (Bayne et al. 1984), or indirectly through drought (Bethlenfalvay et al. 1990), reducing power becomes limiting and the fungi compete with the nodules for it. The fungi thus modify the nodule's ability to regulate O_2 tension through respiration (Sheehy 1987).

When under drought stress, the fungi can alter plant water status (Bethlenfalvay et al. 1990, Cooper 1984), and therefore may influence the fluxes of water and solutes to and from the nodules through phloem and xylem, and symplastic flow through the parenchyma (Raven et al. 1989). Likewise, they may also influence resistance to diffusion of gases (Pankhurst and Sprent 1975, Sprent and Gallacher 1976) by varying the length of water-filled spaces in the nodule apoplast (Minchin et al. 1985). VAM fungi may mediate a further effect on the barrier, whose closure is thought to be triggered by the cessation of NH_4^+ production (Witty et al. 1986), because of their role as strong sinks for N, preferably as NH_4^+ (Davies et al. 1990). Finally, there is a minimum diameter for effective nodules (Raven et al. 1989). This, in turn, affects diffusion and accumulation of gases such as H_2 that may competitively inhibit nitrogenase activity (Dixon et al. 1981). The more gradual change in H_2 evolution with stress in the VAM-plant relative to the larger nonVAM-plant nodules may be influenced by any of the purported functions of the barrier.

Of course, the process of uprooting and the concomitant disruption of the hyphal connections between root and soil prior to the measurement of nodule activity has unknown consequences on nodule function. Nevertheless, nitrogenase activity, isolated as it is from events occurring in the extraradical hyphae by four membrane systems, is not likely to be affected differentially for VAM or nonVAM plants during the brief time span between uprooting and incubation.

Our data show that nodulation and nodule activity in VAM and nonVAM plants are affected by soil and root water status in similar ways. Nodule P concentrations (Fig. 1) and NPUE (Fig. 5) are altered by soil and root water status, but are not influenced by the presence of VAM fungi. Nodule activity (Fig. 3) and RE (Fig. 4), however, are affected by the root's VAM status, and the effect may be brought about by changes in nodule respiration and in the parameters of gas diffusion through the nodule.

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