

Vesicular-arbuscular Mycorrhizal Fungi in Nitrogen-fixing Legumes: Problems and Prospects

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I. Introduction

The "rationale of mycorrhiza formation" for plants is the uptake of mineral nutrients, declared Stahl (1900) 90 years ago. But (so argued Stahl, all in one sentence), "though one must not infer from the occurrence of both bacteriotrophy and mycotrophy in the same plant that the meaning of the two symbioses for the host is different, this assumption is still probable, since otherwise it would be unlikely that two so strange and in their end effect equivalent mechanisms should have been acquired by the Papilionaceae in their struggle for survival".

Nonetheless, he concluded, it seems most likely that the rationale is valid for the legumes also, and this is the prevailing view on the subject to this day.

What Stahl did not know, and what Jones (1924) and Asai (1944) pointed out later, was the special, nutritional relationship between the two microsymbionts: the high phosphorus requirement of the nitrogen-fixing root nodule, the high nitrogen requirement of the chitin-walled vesicular-arbuscular mycorrhizal fungus, and the high carbon requirement of both. Since each symbiont can supply the other's needs in excess, the endophytes can bring about synergistic growth responses in the host plant when the association is grown in nutrient-deficient soil (Schenck and Hinson, 1973; Mosse, 1977). Such observations led to a view of the legume microsymbionts as biological substitutes for fertilizers (Azcón-Aguilar *et al.*, 1979).

But how do the symbionts of the tripartite legume association relate to each other when the growth medium is not deficient, such as in moderately fertilized field soils, a condition often found in modern agriculture? Or under experimental conditions, where scientific insight into the biology of plant symbioses rather than productivity is of interest? One of the reasons why this question is difficult to answer experimentally, is the lack of adequate, non-symbiotic controls in the quest for the nature of "symbiotic response". Without controls, one is reduced to using what appears to be the most appropriate comparison plant (another treatment) for whatever the objective of the experiment happens to be. For the legume association, this comparison treatment is necessarily a double approximation. What follows is an attempt to illustrate the problems encountered in such whole-plant experiments with mycorrhiza in nodulated legumes.

II. Different growth effects, same symbiosis

Organisms that have come to depend on one another (symbiosis) show this dependence by a range of symbiotic responses. When both symbionts benefit, we call the symbiosis mutualistic; when one benefits at the expense of the other, the former is known as a parasite. When neither seems to benefit, the commensal partners may still provide hidden advantages to one another, or to outsiders. The vesicular-arbuscular mycorrhizal fungus, an obligate biotroph (Warner and Mosse, 1980), always benefits, and it is the cost-benefit ratio to the host plant that determines its role in the partnership.

A. Mutualism

Many workers in mycorrhiza think of the vesicular-arbuscular mycorrhizal symbiosis exclusively as mutualistic. This is not the case. Growth depression, though transient, has also been observed in vesicular-arbuscular mycorrhizal plants (Tinker, 1978; Stribley *et al.*, 1980). Although the response of a plant to colonization by vesicular-arbuscular mycorrhizal fungi depends on many biotic and environmental factors (Barea and Azcón-Aguilar, 1983; Abbott and Robson, 1984; Hayman, 1987), the most important is phosphorus availability (Hayman, 1987). Host plant growth is enhanced when the benefits of increased phosphorus nutrition to the host outweigh the disadvantage of carbohydrate loss to the endophyte (Harley, 1969). Experimentally, one can follow the development of the mutualistic effect in the plant by relating changes in available phosphorus in the growth medium with changes in the vesicular-arbuscular mycorrhizal plant relative to non-vesicular-arbuscular mycorrhizal comparison grown under the same phosphorus regime (Bethlenfalvay *et al.*, 1982b). This can best be done in a synthetic, phosphorus-free medium, using nutrient solutions or slow-release phosphorus sources, such as hydroxyapatite. Determinations of the development of both macro- and microsymbiont are possible if sequential, time-course measurements are made (Bethlenfalvay *et al.*, 1982a).

A number of methods are available to determine both the internal and external portion of vesicular-arbuscular mycorrhizal fungal mycelium (Bethlenfalvay and Ames, 1987; Bethlenfalvay *et al.*, 1981; Giovanetti and Mosse, 1980). All of these methods involve estimates, to some extent. Recently, techniques have been developed to estimate also the viability of the external (extraradical) hyphae of vesicular-arbuscular mycorrhizal fungi (Sylvia, 1988), adding little to the cumbersomeness, but a great deal to the precision and validity of mycorrhizal work. The difficulty of quantifying vesicular-arbuscular mycorrhizal soil hyphae generally prevents workers from including statements in their reports on this important organ of the fungus-root, even though the tenuous nature of the relationship between root colonization and the growth effect is well-known (Graham *et al.*, 1982).

B. Parasitism

Growth depression in vesicular-arbuscular mycorrhizal plants is different from pathogenic parasitism, in that neither irreversible tissue damage nor necrosis is observed as a result of vesicular-arbuscular mycorrhizal

colonization. In the vesicular-arbuscular mycorrhizal association, where the host plant and its fungal endophyte live together in an intimate, balanced relationship, symptoms of symbiotic (non-pathogenic) parasitism occur when the balance is disturbed (Gerdeman, 1974). The effects of disturbance can be dramatic (Bethlenfalvay *et al.*, 1982b). Experimentally, the conditions imposed to produce the parasitic response involve a manipulation of phosphorus levels in the medium (Bethlenfalvay *et al.*, 1982d, 1983). When phosphorus is extremely limiting, competition for it by the symbionts results in growth depression of the host. If phosphorus availability is increased slightly, vesicular-arbuscular mycorrhizal roots gain an advantage over non-vesicular-arbuscular mycorrhizal roots in exploiting this limiting resource, due to the phosphorus-uptake efficiency of the vesicular-arbuscular mycorrhizal soil mycelium (Sanders *et al.*, 1977). This is the stage of mycotrophic growth, which is limited to a range of suboptimal soil phosphorus concentrations (Ross, 1971; Bethlenfalvay *et al.*, 1983) and is evaluated as growth enhancement relative to non-vesicular-arbuscular mycorrhizal plants suffering from the same limitations. Growth inhibition also occurs at levels of phosphorus availability above those which result in mycotrophy. At such levels, there is sufficient phosphorus for the plant to supply its needs without its (now parasitic) symbiont, but not enough to inhibit colonization. Thus, the fungus can proliferate, and may divert significant amounts of carbon from the host (Buwalda and Goh, 1982). Eventually, with increasing phosphorus additions, colonization is eliminated.

Responses to phosphorus availability modified by vesicular-arbuscular mycorrhizal fungi appear to be aggravated in nodulated soybean plants (Bethlenfalvay *et al.*, 1982e), where inhibition of host-plant growth is more pronounced, apparently as a result of increased competition for carbohydrates by the nodules and the high energy requirement of nitrogen fixation.

III. Is there a best endophyte?

Co-evolution of vascular plants with vesicular-arbuscular mycorrhizal fungi over geological time is likely to have produced combinations best adapted for survival within the biotic communities of their origin (Trappe, 1987; Pirozynski and Dalpé, 1989). An exotic crop plant is not likely to encounter a symbiotic mycoflora best suited for its requirements, when introduced to a new area (Hall, 1987). This deficiency is of particular interest for the highly mycotrophic legume plant, and led to

research evaluating the merit of native versus introduced vesicular-arbuscular mycorrhizal fungi in the field (Hall, 1987).

Root systems are typically colonized by more than one vesicular-arbuscular mycorrhizal fungus. Mutual exclusion is not observed, but success in occupancy varies and is not necessarily related to host response (Daft, 1983; Lopez-Aguillon and Mosse, 1987). Host response can differ with fungal species (Graw *et al.*, 1979; Wilson, 1988), and its variability may be due to seasonal development (Daft *et al.*, 1981) or to varying uptake or exclusion capabilities of different fungi for different elements (Sieverding and Toro, 1988; Bethlenfalvay and Franson, 1989). Multiple colonization by mixed inocula containing fungi with different host effects and environmental adaptations might therefore be more consistently beneficial to the host plant (Daft, 1983) than one (ideal, but non-existent) endophyte. Disturbance, agrochemicals, management practices and cultural and environmental stress conditions are likely to influence the composition of the vesicular-arbuscular mycorrhizal mycoflora, whose components may possess different levels of tolerance for conditions imposed by unsustainable agriculture (see Trappe, 1982; Trappe *et al.*, 1984; Jeffries, 1987). Utilization of the endophytes in agricultural situations (Dagoberto *et al.*, 1986; Hayman, 1987) therefore not only poses the problem of selecting one "best" organism, but a collection of organisms which are compatible with each other, with the native microflora, with new host plants and with the new host soil.

A. Selection of the endophytes

As major characteristics for the selection of vesicular-arbuscular mycorrhizal fungi, Abbott and Robson (1985) listed the ability to infect rapidly and extensively, to form an effective soil mycelium for the uptake of phosphorus, and to produce a large number of propagules. Their scheme included criteria for the collection and culture of the fungi under different edaphic conditions, and for response evaluation. Recently, they further extended their discussion of the field management of vesicular-arbuscular mycorrhizal fungi and proposed new methods to evaluate their effects in terms of plant growth and phosphorus nutrition (Abbott and Robson, 1990).

Selecting the right organism to help solve the right problem is further complicated by differences in plant responses not only to vesicular-arbuscular mycorrhizal fungal species but also to their geographical isolates ("edaphotypes", *sensu* Bethlenfalvay *et al.*, 1989). Furthermore, host effects are not restricted to those of phosphorus nutrition, but are

pervasive in both plant form and function. Therefore, utilization of vesicular-arbuscular mycorrhizal fungi in the field on more than a hit-or-miss basis will necessitate a complex screening and selection phase. Thus, problem identification is an important first step prior to launching the screening process. The uses for vesicular-arbuscular mycorrhizal fungi are manifold in terms of desirable host plant or host soil responses: drought resistance, tolerance to metal toxicity, mineral nutrient uptake from the soil, organic nutrient transport to the soil biota, effective nitrogen fixation, and others. With the objectives identified, isolates collected from specific sites can be tested for a specific requirement. Site selection for collecting the fungi is important, because useful adaptations are presumably site-specific. Alternatively, starting with a given set of available inocula, one may test for a wide range of their possible capabilities.

The "common garden" technique long employed in experimental botany (Heslop-Harrison, 1964) to equalize conditions for response testing, is a good approach for pre-screening vesicular-arbuscular mycorrhizal fungi in the greenhouse or growth chamber. It imposes uniform conditions (same new soil, same new host) for the screening of fungi that hail from diverse places. However, to make the comparisons between host responses to different fungal species or edaphotypes meaningful, the infectivity of the inocula must be carefully determined (Franson and Bethlenfalvay, 1989). This is a time-consuming procedure, and a step that is often omitted.

Recently the Diagnosis and Recommendation Integrated System (DRIS), long used as a diagnostic tool for fertilizer application (Walworth and Sumner, 1987), has been tested for its applicability to the ranking of vesicular-arbuscular mycorrhizal effects on plant nutrition (Bethlenfalvay *et al.*, 1990). Analysis by DRIS is based on nutrient balance ratios, and its use minimizes morphogenic and genotypic effects on the accuracy of deficiency diagnoses. It also predicts which nutrient is most limiting to yield (Hallmark *et al.*, 1987). Thus, plant nutritional responses to vesicular-arbuscular mycorrhizal fungi in a given soil may be evaluated for each nutrient. Since vesicular-arbuscular mycorrhizal fungi may affect the uptake of each nutrient differently, resulting in both growth enhancement and inhibition (see Ames and Bethlenfalvay, 1987), DRIS promises to become a particularly valuable tool in vesicular-arbuscular mycorrhizal screening efforts.

B. Selection of endophyte combinations

The dependence of nodulation on vesicular-arbuscular mycorrhizal colo-

nization when phosphorus is limiting is well-documented (Mosse, 1977; Barea and Azcón-Aguilar, 1983; Barea *et al.*, 1987; Smith and Gianinazzi-Pearson, 1988). Effects of root nodules on the development of vesicular-arbuscular mycorrhizal fungi, on the other hand, are little-known (Bethlenfalvay *et al.*, 1985a). The management of the bacterial and fungal endophytes of the tripartite legume association is important for the production of these crops (Daft *et al.*, 1985). Compatibility of such endophyte combination should therefore be determined, because it affects co-endophyte effectiveness (Bayne and Bethlenfalvay, 1987). Determinations should be made utilizing nutrient-sufficient plants, otherwise nutrient-deficiency effects will mask symbiotic effects. Before evaluating tripartite relationships, one must first determine the effects of each endophyte on the host individually, in the simpler, bipartite associations. This is necessary, since the evaluation of one of the two endophytes of the tripartite association must be made against the bipartite association colonized by the co-endophyte and supplemented nutritionally with nitrogen or phosphorus (Bayne and Bethlenfalvay, 1987).

Generally, factors which are easily determined in characterizing compatibility and effectiveness of all three symbionts are their developmental characteristics (extent of vesicular-arbuscular mycorrhizal colonization, nodule mass, and plant mass). When nutrient-sufficient comparison plants are employed in such studies, antagonistic (Bethlenfalvay *et al.*, 1985a), as well as stimulatory relationships are revealed between the microsymbionts. Plant and endophyte activities can also be determined, by leaf gas-exchange (Brown and Bethlenfalvay, 1987), nitrogenase activity (Brown *et al.*, 1988) and vesicular-arbuscular mycorrhizal hyphal viability (Sylvia, 1988) measurements, and by nutritional analysis (Jarrel and Beverly, 1981). Stable isotope (^{13}C , ^{15}N) labelling (Rundel *et al.*, 1988) can be a useful tool, but is as yet little used in studies to determine the complex source-sink relationships (Bayne *et al.*, 1984) between the symbionts.

IV. The vesicular-arbuscular mycorrhizal legume associated with non-legumes

Nutrient transfer between the roots of associated plants is enhanced when the roots are colonized by vesicular-arbuscular mycorrhizal fungi (Francis and Read, 1984; van Kessel *et al.*, 1985). This has profound implications for our understanding of plant community structure (Fitter,

1985). The concept of resource distribution optimized by the movement of nutrients along concentration gradients between vesicular-arbuscular mycorrhizal donor and receiver plants (Read *et al.*, 1985) is of particular interest in plant associations including legumes, because of the role of legumes as sources of nitrogen. Practices, such as soil disturbance (Evans and Miller, 1988) or fallowing (Thompson, 1987), which directly or indirectly disrupt the integrity of the vesicular-arbuscular mycorrhizal soil mycelium, have been shown to be detrimental to plants. A continuous hyphal system, on the other hand, can enhance seedling establishment (Read and Birch, 1988) and nutrient uptake (Fairchild and Miller, 1988). While quantitatively important, vesicular-arbuscular mycorrhizal-mediated nutrient fluxes between plants in the field have not yet been reported (Newman, 1988), significant nutrient transfer between soybean and corn plants has been demonstrated under controlled conditions (G. J. Bethlenfalvay *et al.*, unpubl. res.).

For an unambiguous demonstration of nutrient transfer by vesicular-arbuscular mycorrhizal hyphae, a root-free zone must be created between the root systems of the donor and receiver plants (Camel *et al.*, 1991). This can be achieved by delimiting a soil bridge between donor and receiver plants by means of fine screens permeable by hyphae, but not by roots. Such an arrangement eliminates nutrient transfer by the "soil pool pathway" (such as exudation, diffusion of ions, root anastomosis, and access to biologically bound nutrients following mineralization, see Newman and Ritz, 1986). The direction of lateral fluxes of water between the soils of the bridge and adjacent root compartments can be controlled by watering schedules which keep soil water content slightly lower in the donor-plant soil. With water flux, if any, moving from receiver to donor, nutrient-ion counterfluxes become unlikely. In studies utilizing nitrogen fertilizers enriched or depleted in ^{15}N , the soils of donor, bridge and receiver compartments can be tested for ^{15}N abundance to check for nitrogen diffusion.

Application of ^{13}C as CO_2 is also a useful tool in vesicular-arbuscular mycorrhizal nutrient-transfer studies. Exposure of corn plants to labelled CO_2 resulted in a rapid movement of ^{13}C to associated soybean plants, where it was shown to enhance nitrogenase activity significantly. Comparative work with nodulated and unnodulated soybean showed that the nodules are a strong sink for phosphorus, which they may obtain from their vesicular-arbuscular mycorrhizal companion plant by mycorrhizal hyphal transport (G. J. Bethlenfalvay *et al.*, unpubl. res.). It appears that mycorrhiza-mediated nutrient fluxes are controlled by source-sink relationships and tend to balance nutrient supply and demand between interdependent plants.

V. Growing experimental tripartite legume associations

Perhaps the most difficult aspect of experimentation with the legume symbiosis is the lack of adequate controls. The well-known nutritional effects of the microsymbionts are most conspicuous when the plants are grown under conditions limiting in nitrogen, or phosphorus, or both. However, the non-symbiotic plants grown under such nutrient regimes do not provide meaningful comparisons in applied or basic studies, since such legumes are unlikely to occur or survive long in the field, while they are too stunted to serve as physiological "controls" in experimentation (Bethlenfalvay *et al.*, 1987).

A. The symbiotic test plant

It is possible to grow well-nodulated soybean plants with root colonization by vesicular-arbuscular mycorrhizal fungi in excess of 50% within three weeks after planting. Rapid establishment of the symbiotic condition is important in experiments with plants grown in pot cultures, since the vesicular-arbuscular mycorrhizal effect is dependent on root density and tends to diminish or disappear as the roots become pot-bound (Baath and Hayman, 1984). However, the factors contributing to the growth of the association must be favourable to attain rapid symbiotic development. Some of these factors are temperature (Sieverding, 1988), light intensity (Diederichs, 1982a), day length (Diederichs, 1982b), soil conditions (Bethlenfalvay *et al.*, 1985b), viability (Sylvia, 1988), and dormancy (Tommerup, 1983) of the vesicular-arbuscular mycorrhizal inoculum, effectiveness of the Rhizobia (Barea and Azcón-Aguilar 1983), intersymbiont compatibilities, and the little-known effects of the syndrome known as "suppressive soil" (Wilson *et al.*, 1988). The agents of the latter may be introduced into the usually sterilized growth medium with the non-sterile vesicular-arbuscular mycorrhizal soil inoculum, and may be responsible, to a large extent, for variation between vesicular-arbuscular mycorrhizal experiments.

Depending on the nature of the medium utilized, the available and bound nutrient content of which should be known to the experimenter, nutrients added in soluble or slow-release capsule form should be calculated to maximize the information to be gained from the experiment. For instance, if the uptake of one specific nutrient ion is of interest, none of the other nutrients should be limiting. In the tripartite association, there is a delicate balance in the demand (*Rhizobium*) and tolerance (vesicular-arbuscular mycorrhizal fungus) of the microsymbiont.

bionts for phosphorus. This balance shifts during the ontogeny of the association (Bethlenfalvay *et al.*, 1982c). Adequate phosphorus supplies are essential for nodulation in the early stages of plant and nodule development, but excess phosphorus will inhibit vesicular-arbuscular mycorrhizal colonization (Barea and Azcón-Aguilar, 1983). In soybean, grown in soil severely deficient in available phosphorus, an initial concentration of 0.2 mM KH_2PO_4 in nutrient solution applied as a drip twice a week until nodules became functional, generally produced a rapid rate of vesicular-arbuscular mycorrhizal colonization. With the establishment of colonization, phosphorus may be withdrawn since the fungi then assume the role of supplying the nodulated plant with its phosphorus requirement. An initial nitrogen supplement (≈ 1 mM NH_4NO_3) helps the nodules to develop rapidly, and prevents transient growth depression of the host during nodule development (Bethlenfalvay *et al.*, 1978).

B. The non-symbiotic comparison plant

Different aspects of a plant's form and function react differently to nutrients supplied by its symbiotic partners or by fertilization (Bethlenfalvay *et al.*, 1989). Success in growing symbiotic test plants and non-symbiotic comparison plants that are equivalent in more than one physiological or morphological parameter is therefore unlikely. Hence, the experimenter must decide which of the many possible growth responses he will seek to equalize. If leaf gas exchange is of interest, for example, adjustments of nitrogen and phosphorus regimes in the comparison plant may produce leaf areas equivalent to those of the test plant. However, the root-shoot ratios and leaf nutrient concentrations, factors which also have an impact on water fluxes through the plant, will be different (Brown *et al.*, 1988).

Attempts to adjust nutrient regimes to make the comparison plants comparable to the symbiotic test plants are not always successful. Thus, it is possible, for instance, that decreasing the nitrogen supply to a non-nodulating legume to slow its growth to that of its nodulated counterpart, will achieve only a yellowing of its leaves instead. Similar scenarios occur when phosphorus input by fertilization versus vesicular-arbuscular mycorrhizal colonization are to be compared. It is therefore advisable, in working with such comparisons, to learn to grow both sets of plants satisfactorily, prior to starting time-consuming, large experiments.

VI. Vesicular-arbuscular mycorrhizal legumes in soil nutrition

Agriculture has been production-oriented since its early beginnings. Currently, however, a shift in emphasis is taking place to resource conservation, motivated by global erosion and desertification of agricultural lands (Beatty, 1982; Gibbs and Carlson, 1985; Healy *et al.*, 1986). Sustainable agriculture (Mosse, 1986; Sprent, 1986) utilizes practices which are equally concerned with crop productivity and soil conservation. Vesicular-arbuscular mycorrhizal legumes have a central role in this effort: since the soil mycelium of vesicular-arbuscular mycorrhizal fungi represents a significant portion of the soil microbial biomass (Hayman, 1978) and since the cell walls of its hyphae are composed mainly of the amino-sugar chitin (Weijman and Meuzelaar, 1987), the soil mycelium may be one of the most important vehicles for nitrogen and carbon input into the soil. The contribution of vesicular-arbuscular mycorrhizal legumes to soil organic matter, and as a consequence, to soil aggregate stability (Lynch and Bragg, 1984; Oades, 1984), therefore promises to become a new and exciting experimental field for research with the legume association.

The literature on the effects of vesicular-arbuscular mycorrhiza on soil aggregation has been reviewed by Thomas *et al.* (1986), and was brought into agro-ecological context by Bethlenfalvay and Newton (1990). The difficulties and challenges of experimentation with a system as complex as the soil microbiota were highlighted by Linderman (1988). Others (Tisdall and Oades, 1982; Gupta and Germinda, 1988) pointed out the role of micro-organisms and their organic products in the formation of aggregates at different levels of size and organization. An important new direction in soil-oriented, rather than plant-centred, vesicular-arbuscular mycorrhizal research involves the close association of vesicular-arbuscular mycorrhizal fungi and chitin-decomposing actinomycetes (Ames, 1989; Ames *et al.*, 1989) which are important for the formation of small aggregates. Experimentally, the effects of mycorrhizal roots, non-mycorrhizal roots, and mycorrhizal hyphae can be segregated by using split-root systems, inoculated or not inoculated with the fungi. The two parts of the root system can then be further separated by the use of fine screens, which permit passage only for hyphae (≈ 5 – 20 μm diameter) to adjacent soil compartments. The effect of each component of the fungus-root, with and without root nodules, can then be evaluated for aggregate formation.

VII. Conclusions

A fundamental role is ascribed to vesicular-arbuscular mycorrhizal fungi in the ecophysiology of nodulated legumes. The concept of cost-benefit relationships in plant symbioses recognizes the influence of changing conditions, such as nutrient or water availability, on the balance of nutrient supply and demand between the symbionts. Such changes may bring about transient shifts in the symbiotic condition, causing it to alternate between the mutualistic and parasitic modes. The carbon-nitrogen-phosphorus supply-demand relationship is a fundamental expression of the three symbionts as sources and sinks for one another's products within the closed system of an isolated plant. In the open system of inter-plant relationships within the plant-soil community, a major role of the legume association is envisioned as a nitrogen source, functioning not as a self-contained unit but as one involved in the fluxes of nutrients between the members of the community. The complexity of such a system is a challenge for each new experiment designed not only to gain basic insights into its workings, but also to put the findings to practical use.

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