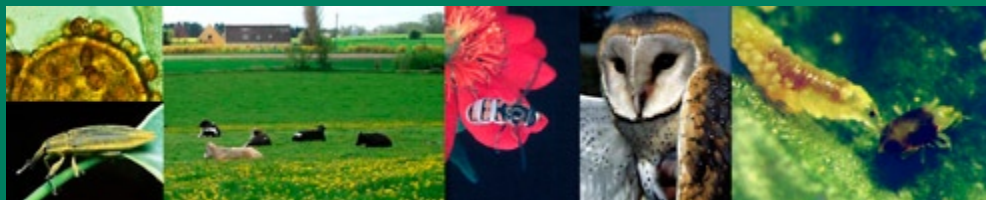


Implementation of Biocontrol in Practice in Temperate Regions - Present and Near Future

Proceedings of the International Workshop at Research Centre Flakkebjerg, Denmark on November 1 to 3, 2005

Lise Stengård Hansen, Annie Enkegaard, Tove Steenberg, Sabine Ravnskov and John Larsen





**“Implementation of Biocontrol in Practice in
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Biocontrol in the mycorrhizosphere

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Abstract: The mycorrhizal association of fungi with the roots of land plants in natural, undisturbed ecosystems logically includes associations with other functional groups of soil microbes, including a myriad of rhizobacteria, other rhizosphere fungi, and diverse fauna that contribute to successful plant growth and health. We have attempted to characterize qualitative changes in populations of rhizobacteria associated with plants with mycorrhizae in what is called the "mycorrhizosphere". Microbial populations in the mycorrhizosphere can change dynamically over time and are influenced by what microbes are present in the background soil or growth medium and by the process of selective enrichment of specific functional groups of microbes from that medium due to root exudation and arbuscular mycorrhizal (AM) fungus hyphal exudates. The mycorrhizosphere phenomenon includes specific roles that some rhizobacteria might play in combination with mycorrhizal fungi, especially in relation to plant growth enhancement and increased antagonism against soilborne pathogens. Plant diseases are rare in undisturbed ecosystems compared to disturbed agroecosystems where they often cause serious economic loss. Disease suppressive soils occur naturally or due to specific management practices, and are thought to involve soil type and specific bacteria, fungi, or actinomycetes. However, I believe that mycorrhizae play a significant role as well. In our research, we have explored factors that affect AM formation and have determined that AM formation causes an increase in levels of antagonistic bacteria, provided the background soil contains effective antagonists to be selectively increased. This has led me to describe a new mycorrhizosphere paradigm that is a microbial hierarchy wherein roots attract mycorrhizal fungi and the latter attract bacterial associates. The result is a "team" system that functions to support plant growth and health. The microbial components of the system must come from inoculation (mycorrhizae) or selection of bacterial associates from the bulk soil or potting medium. Optimization of any production system comes from having microbes, such as bacterial antagonists, selected from a medium with high microbial diversity, that are efficacious and compatible and therefore can function in tandem. This mycorrhizosphere paradigm involving plants forming AM that select specific bacterial associates can explain the success of the AM symbiosis in supporting plants for some 460 million years.

Key words: Mycorrhiza, rhizosphere, hyphosphere, mycosphere, plant growth-promoting rhizobacteria (PGPR), biological control, antagonistic potential, arbuscular mycorrhizal fungi (AMF)

Introduction

My interest in understanding the rhizosphere has always been from the perspective of controlling soilborne diseases through some manipulation of the microbial populations

therein. My assumptions or beliefs are that root health is the product of microbial activities in the rhizosphere, and that above-ground plant growth is a reflection of the health of the root system. A parallel assumption, based on my observations and those of others, is that root disease is rare in natural ecosystems, due to microbial support systems in the rhizosphere soil associated with plant roots. My goal has always been to characterize the microbial systems involved in normal healthy growth of plants and to incorporate that knowledge into agricultural systems as a means of improving crop productivity and health. This has led me to believe that among the rhizosphere microbial populations with the greatest influence, arbuscular mycorrhizal (AM) fungi are the most important, but only in combination with bacterial associates in what we now call the “mycorrhizosphere”.

A number of mechanisms have been proposed whereby mycorrhizae can reduce the incidence and/or severity of diseases. Such proposed mechanisms are based on research using experimental systems that vary between researchers, and the results do not allow for comparisons or final conclusions. Thus, it may be that multiple mechanisms are involved overall, and that it might not be impossible to point to any one as the most likely. However, among those proposed, including mycorrhiza-enhanced nutrition, competition for nutrients and infection sites, morphological changes, changes in chemical constituents of plant tissues, alleviation of abiotic stress, and microbial changes in the mycorrhizosphere (Linderman, 1994, 2000), the latter seems to be the most likely and has been the focus of my research.

The mycorrhizosphere concept

The rhizosphere phenomenon, as described by Hiltner (1904), described the increase in microbial populations in the soil next to roots, induced initially by nutrients released from the roots. The realization that mycorrhizae altered the microflora in the rhizosphere led to the expanded concept of the mycorrhizosphere (Linderman, 1988) in which mycorrhizae significantly influence, qualitatively and quantitatively, the microflora due to altered root physiology and exudation (Ames *et al.*, 1984; Bagyaraj, 1984; Fitter and Garbaye, 1994; Meyer and Linderman, 1986; Secilia and Bagyaraj, 1987; Gryndler, 2000). But the paradigm of the mycorrhizosphere, as initially described (Oswald and Ferchau, 1968; Rambelli, 1973; Linderman, 1988), is not complete, both temporally and spatially, and in terms of the dynamic processes that occur. Following the initial enrichment by root products that are specific to the plant species, the dynamic process is influenced by the age of the plant, the nature and treatment of the soil, foliar applications, environmental factors, fertilizer applications and host nutrition, and last, but not least, by the microbial interactions that occur therein. Because they establish a persistent interface between the host root and the soil, mycorrhizae become perhaps the only stable microbial system in the rhizosphere. While increases and decreases in the abundance of certain types of microorganisms have been reported, how and when those changes occur has not been determined fully. Further, descriptions of qualitative changes in microbial populations with potential functional activity have only inferred that such activity would occur because of the increased numbers of microbes with that potential. Measurement

of actual *in situ* activity, such as antagonistic activity against a specific pathogen, has not been documented.

Consideration of the microbial shifts that can be induced by the formation of mycorrhizae requires examination of the sources of nutrient enrichment within the mycorrhizosphere: (a) root tissue exudates and sloughed cells, and (b) AM fungal hyphal exudates (Figure 1). Both can have qualitatively specific chemical components that favor some microbes and not others (Andrade *et al.*, 1997, 1998a, b; Olsson *et al.*, 1996; Vancura *et al.*, 1989). When considering the microbial composition of the mycorrhizosphere, the sum of the two sources must be included. **Thus, rhizosphere soil is soil adjacent to roots and influenced by root exudates, while mycorrhizosphere soil is soil adjacent to mycorrhizae and influenced by exudates from both the root tissue and the fungal hyphae. Both have increased populations of specific microbes selected from the bulk soil.**



Figure 1. Bacterial growth on the hyphal surface of an arbuscular mycorrhizal fungus on agar in response to nutrients exuded from the hypha. (Photo from R. P. Schreiner).

Recent studies have physically separated AM fungal (AMF) hyphae from roots or roots + AMF hyphae by means of mesh that restricts root growth but allows AMF hyphae to pass through, and have distinguished microbial changes induced directly by the hyphae due to their specific exudates (Andrade *et al.*, 1997, 1998a, b; Filion *et al.*, 1999; Vancura *et al.*, 1989). Others have examined the interactions of the AMF hyphae with other microbes in a two-compartment *in vitro* system that also separates hyphae from host roots (Fortin *et al.*, 2002). The *in vitro* system, of course, eliminates the dynamic interactions that occur from having different hosts, different AMF symbionts, changing environmental conditions, and from having a myriad of other microbes that would be present in a soil system. Nonetheless, there is information derived from both culture-based and non-culture-based systems that sheds light on what the mycorrhizosphere phenomenon is and how it relates to microbial shifts that could affect plants. Our research has been strictly culture-based.

Rhizosphere/mycorrhizosphere microbial composition

A myriad of microbes can be present and functioning in the rhizosphere of plants, including rhizobacteria, rhizosphere fungi, fauna, and mycorrhizal fungi. How these microbes may interact and function in relation to plant growth and health is of great interest and relevance. Our research focus, however, has been limited to bacteria-mycorrhiza interactions.

Rhizobacteria. Bacteria that occupy the rhizosphere/mycorrhizosphere soil can have various functions in relation to plant growth and health. We know that some of those bacteria can be antagonistic to soilborne pathogens, based on *in vitro* tests showing inhibition due to the production of antibiotics or other inhibitors. What is often not appreciated, however, is that many, if not most, of the antagonists are also plant growth-promoting rhizobacteria (PGPR) (Mahaffee and Kloepper, 1994; Pieterse *et al.*, 2003). We have confirmed this in tests with petunia using a range of bacterial or actinomycete antagonists to inoculate young seedlings. All of the antagonists stimulated plant growth and flowering, and thus would be classified as PGPR (Linderman, 1993) (Figure 2). Of course, other bacteria, such as symbiotic or free-living nitrogen fixing bacteria, can also be considered as PGPR (Bashan *et al.*, 2004). We should not forget, too, that some of the rhizobacteria might have deleterious effects on plant growth (deleterious rhizobacteria, DRB), presumably due to the production of toxic materials that retard plant growth (Nehl *et al.*, 1997; Suslow and Schroth, 1982).

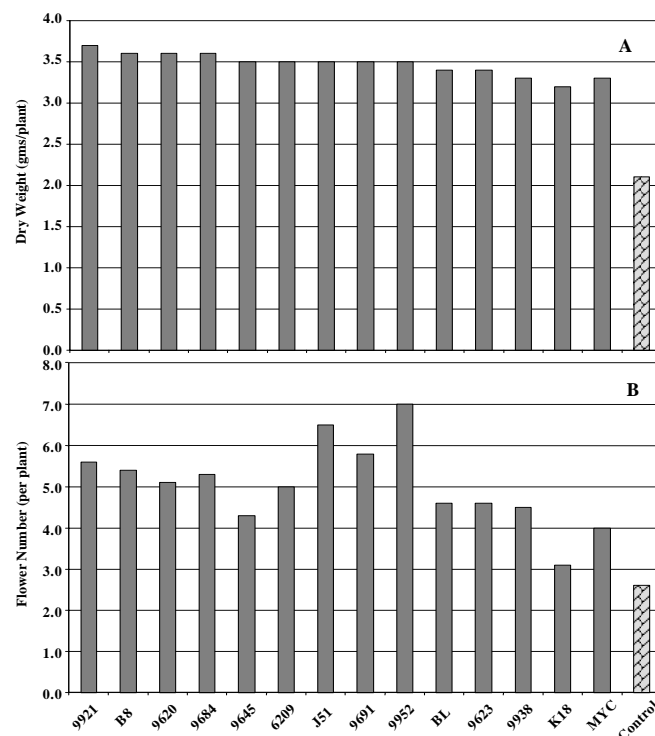


Figure 2. Experimental data showing that rhizobacteria antagonistic toward soilborne pathogens can function as plant growth-promoting rhizobacteria (PGPR) in enhancing the growth (A) and flowering (B) of inoculated petunia plants compared to the water control (Linderman, 1993).

Arbuscular mycorrhizae (AM). We know of many benefits of AM to plant growth and health, due to the unique capacity of AMF to colonize host plant roots internally as well as externally into the surrounding soil. The soil hyphae and spores provide a source of inoculum for new infections as well as uptake of water and nutrients from the soil (Smith and Read, 1997). Exchange of materials within the root takes place by means of the arbuscules. The symbiotic relationship that is established is reported in many publications documenting many benefits to plants. Those benefits include improved plant growth under nutrient (especially P) deficient conditions (Figure 3), improved tolerance to soil toxicity from heavy metals and salinity, improved transplant success, improved crop uniformity, improved root development on cuttings and transplants, improved drought tolerance, and improved disease tolerance. Benefits to plant growth can also be the result of improved soil structure by means of enhanced formation of water-stable aggregates resulting from the entanglement and binding of microaggregates into macroaggregates (Tisdale *et al.*, 1997; Wright and Upadhyaya, 1996). Such aggregates are significant sites within the mycorrhizosphere, providing conditions for microbial activity within the aggregates, such as phosphate solubilization (Andrade, 1998b) as well as the production of other bacterial metabolites and substances that hold the aggregates together. The point to remember, however, is that the microbial products within the aggregates would be immediately available for uptake by the AMF hyphae and translocation to the plant root. Those microbial products may contribute significantly to the overall effects of the mycorrhizae on plant growth and health (Bethlenfalvay and Linderman, 1992).



Figure 3. Growth enhancement of redwood seedlings (left) and lavender (right) inoculated or not with *Glomus intraradices* and grown in P-deficient growth media.

Effects of AM on diseases

Root disease management strategies: Among the strategies for managing soilborne diseases (including chemical, cultural, and biological strategies), I have always favored the biological

approaches, including microbial antagonism and organic amendments. I have a keen interest in understanding the basis for natural disease suppressive systems, with the hope of transferring the key components into agricultural production systems. In analyzing effective suppressive soil systems, such as the Ashburner system for controlling *Phytophthora* root rot of avocado in Australia, I accept the involvement of bacteria or actinomyces, increased in population due to the build-up of an organic layer on the soil, in the disease suppression, but reason too that the roots had to be colonized by AM fungi as well. So, understanding the factors that affect AM formation and how they could contribute to the disease suppression has been a personal goal.

AM formation and benefits: Arbuscular mycorrhizae are best known for their effects on plant growth due to increased capacity of colonized roots to absorb water and nutrients from the soil, especially under P-limiting conditions. Other benefits as mentioned above, however, are not well understood. An underlying need in all cases, however, is to determine how to incorporate mycorrhizae into the production systems in order to realize the benefits, especially under environmental stress conditions.

The general consensus of mycorrhiza researchers has been that mycorrhizae function primarily as scavengers of nutrients from the soil, but they also induce significant physiological changes in their host plant, one of which is to alter the quantity and quality of root exudates (Graham *et al.*, 1981). The result of those changes is a shift in the microbial composition in the mycorrhizosphere soil. In defining the mycorrhizosphere, however, one must consider the processes and components that are involved in establishing mycorrhizae in the first place, including the soil or substrate; the microbial dynamics in the rhizosphere over time; and inputs of fertilizers as well as organic matter amendments to soil or to soilless potting media. A myriad of microbes occur in the bulk soil, and every soil or soilless medium has a different composition of microbes and is physically and chemically different, depending on the parent material, geographic origin, and cropping history or plant cover. In artificial substrates or other soilless media, these traits are generally very distinct from those of soil. The substrate variability can, in my opinion, significantly affect the formation and function of AM, thus explaining in part why different studies under different conditions yield different results.

We have investigated the effect of different components of soilless plant growth media used in the nursery industry on the establishment and function of the AM symbiosis. If we hope to employ AM on plants to suppress soilborne plant diseases, or any other beneficial function for that matter, we must first evaluate the most commonly used materials in soilless media to determine which favor and which suppress AM formation. Our work has been a continuation of the work of Menge *et al.* (1982), who showed that organic matter in soilless nursery media inhibited the establishment of AM. We investigated different peat mosses to determine if they were responsible for the inhibition and found that some inhibited but did not completely suppress AM formation (Linderman and Davis, 2003a). We examined the use of coconut fiber (coir) as a soilless medium component and found that it did not adversely affect AM formation (Linderman and Davis, 2003b). We then examined the use of different com-

mercial organic and inorganic fertilizers to determine which were more compatible with AM. In general, we found that organic fertilizers were more compatible with AM formation, presumably because they require microbial breakdown and thus more slowly release bound nutrients. However, inorganic fertilizers were compatible if the P content was kept low (Linderman and Davis, 2004). Currently we are investigating the amendment of soilless media with different composts to determine their influence on AM formation. In general, different composts inhibit AM formation in soilless media, presumably due to their high P content. Composts do not inhibit AM formation in soil, however (Linderman *et al.*, 2003). Nonetheless, composts in general add to soilless media a more diverse microbial community, some of which could have significant effects on AM formation and function, both negative (Hetrick *et al.*, 1986) and positive. Some may provide microbes that are “helpers” in the formation of AM (Garbaye, 1994).

Disease suppressive systems: There are numerous examples of disease suppressive soils, such as the Ashburner system for controlling root rot of avocado caused by *Phytophthora cinnamomi* (Linderman *et al.*, 1983). Ashburner was a farmer who sought to transfer what appeared to be natural pathogen suppression in the adjacent rain forest into his avocado orchard. He deduced that the key was to create a layer of organic matter around the trees that would simulate the accumulated litter layer in the forest. The intense microbial activity that occurred in the decomposition of the organic matter appeared to be responsible for the disease suppression that he observed. The roots that grew into the decomposing organic matter were free of the pathogen and thus were able to support normal growth of the trees. Work by Australian scientists showed that heat-tolerant bacteria or actinomycetes were involved in the observed pathogen suppression (Figure 4). The component of the microbial community that was not considered by them, however, was the AM fungi that surely had colonized those roots.

Many reviews on the subject of plant disease suppression by mycorrhizae (Azcon-Aguilar and Barea, 1996; Caron, 1989; Dehne, 1982; Fillion *et al.*, 1999; Hooker *et al.*, 1994; Jalali and Jalali, 1991; Linderman, 1992, 1994, 2000; Linderman and Paulitz, 1990; Zak, 1964) have focused on the mechanisms of interaction such as (a) enhanced nutrition, (b) competition for nutrients and infection sites, (c) morphological changes, (d) changes in chemical constituents in plant tissues, (e) alleviation of abiotic stress, and (f) microbial changes in the mycorrhizosphere. Depending on the disease and the environmental situation, any or all mechanisms could be involved, but change in microbial populations in the mycorrhizosphere seems to be the best explanation, yet the least studied.

We believe that mycorrhiza formation establishes a selective pressure on microbes in the background soil, and that the greater the diversity of microbes, the greater the chance that antagonists would be increased. Our hypothesis is that changes in antagonist populations induced in the mycorrhizosphere can influence the incidence and severity of plant diseases (Figure 5). We developed an *in vitro* method of assessing the antagonistic potential of bacterial populations that occur in the rhizosphere soil of plants with or without AM against a range of soilborne, root pathogens. We define the antagonistic potential as the sum of the potential of bacteria to suppress any specific pathogen, and the antagonistic potential index



Figure 4. Biological suppression of *Phytophthora cinnamomi* due to activity of specific microbes from Ashburner's avocado orchard soil, demonstrated by means of heat treatment using aerated steam to establish specific temperatures at (left to right) ambient, 120°F, and 212°F for 30 min. Each flat was inoculated with the pathogen and seeded to susceptible jacaranda. Heat tolerant microbes, such as spore-forming bacteria or actinomycetes, were shown to be responsible for the suppression. Photo by P. Broadbent as presented in Linderman *et al.*, 1983.



Figure 5. Pythium root rot on snapdragon plants not inoculated with AM fungi. Inoculated plants were disease free.

(API) as the number generated by summing the widths of the *in vitro* zones of inhibition against a pathogen by all the bacterial antagonists isolated. Bacteria are isolated from dilution plates of rhizosphere or mycorrhizosphere soil extracts. Our results show that, in general, when AM are formed, there is an increase in the number and proportion of bacteria from the mycorrhizosphere soil that can inhibit specific pathogens *in vitro*, compared to those from rhizosphere soil from non-mycorrhizal plants (Figure 6).

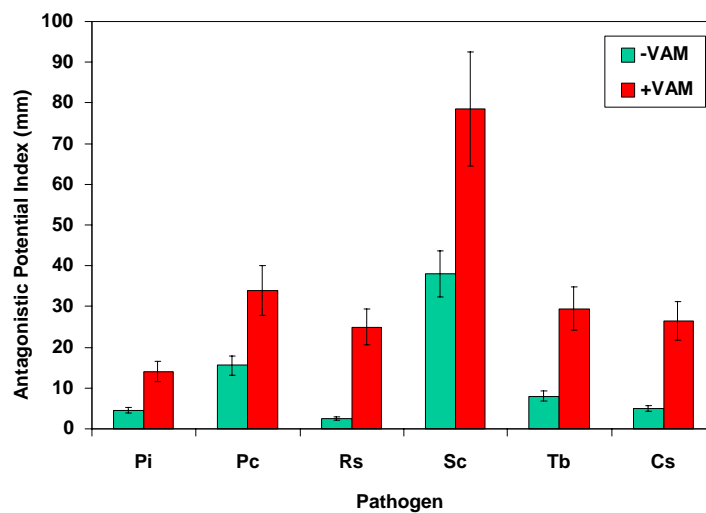


Figure 6. Antagonistic potential index (API) of rhizobacteria from rhizosphere soil around roots of plants with or without AM (VAM) against the soilborne pathogens *Pythium irregulare* (Pi), *Phytophthora cinnamomi* (Pc), *Rhizoctonia solani* (Rs), *Sclerotium cepivorum* (Sc), *Thielaviopsis basicola* (Tb), and *Cylindrocladium scoparium* (Cs) (Linderman, 2000).

A number of factors can influence the potential and magnitude of disease suppression due to mycorrhizosphere microbial populations. One significant factor is the microbial diversity as affected by the amendment of soil or potting mix with composted materials (Figure 7). The host species or genotype within the species can also affect the nature of root exudation and the specifics of the AM association. Any change in the combination of host and fungal endophyte can alter the energy supply to the microbial associates in the mycorrhizosphere. As mentioned before, the soil or growth medium can provide different numbers and kinds of microbes that become AM associates, and different soils have different AMF to form the AM association. It is also important to consider the temporal aspects of AM formation in relation to infection by pathogens: time to establish the mycorrhizal association, to effect physiological change, and to establish a fully functional extraradical mycelial network will affect the effectiveness of the mycorrhizosphere microbial community to suppress root pathogens. For many annual crop plants, time required for disease onset is often too short for AM to become established. This

fact strongly suggests the need for establishing AM and their antagonistic associates as early in the production cycle as possible, even by preinoculating transplants before outplanting into the field.

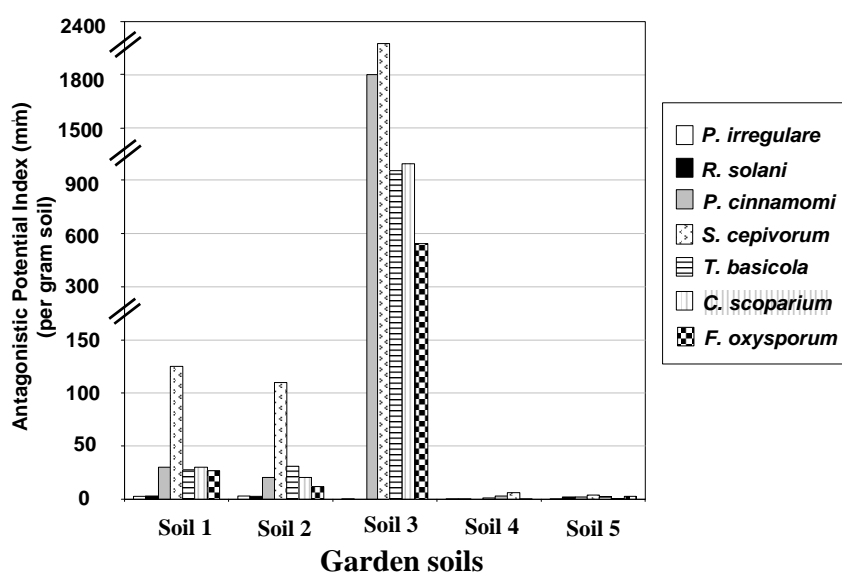


Figure 7. Antagonistic potential of garden soils amended with composts for 1 year (soils 1 and 2), 3 years (soil 3), or non-amended (soils 4 and 5). The antagonistic potential index (API) was determined against a series of soilborne pathogens: *Pythium irregulare*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Sclerotium cepivorum*, *Thielaviopsis basicola*, *Cylindrocladium scoparium*, and *Fusarium oxysporum*.

In our studies, inoculating marigold seedlings with the AMF *Glomus intraradices* and transplanting them into soil, amended or not with compost, increased the API dramatically only on plants with AM (Figure 8).

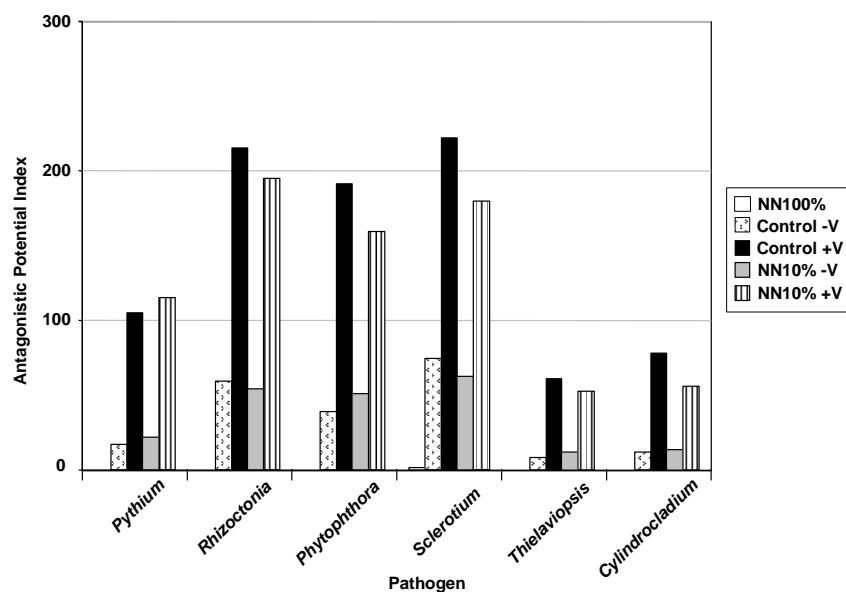


Figure 8. Antagonistic potential index (API) of soil amended or not with compost (10% Natures Needs Compost (NN) or non-amended control) and inoculated or not with the AMF *Glomus intraradices* (V). The data indicate that the API increases dramatically against all pathogens in mycorrhizosphere soil compared to rhizosphere soil from non-mycorrhizal marigold plants. Pathogens used were: *Pythium irregulare*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Sclerotium cepivorum*, *Thielaviopsis basicola*, and *Cylindrocladium scoparium*.

Other roles of AM bacterial associates

While our studies have focused on antagonistic bacterial associates of AM in the mycorrhizosphere, we should consider other possible roles that bacterial associates may play in plant health. Bacterial associates of mycorrhizae could also alter the normal life cycle stages of the pathogens involved. For example, root infections by species of *Phytophthora* normally produce sporangia that release zoospores that initiate root infections. Sporangia production is stimulated by metabolites of soil bacteria, but some bacteria can also inhibit sporangia production. We are currently characterizing populations of rhizobacteria for their capacity to inhibit sporangia production. This is a follow-up of earlier work (Meyer and Linderman, 1986) where we demonstrated that an extract of rhizosphere soil from the roots of mycorrhizal plants inhibited sporangia production of *P. cinnamomi*, compared to an extract of rhizosphere soil from a non-mycorrhizal plant. If we could increase populations of sporangia inhibitors in soil, we could prevent root infections from being initiated, much as was demonstrated in the Ashburner system in Australia, and has been demonstrated recently (Sultana *et al.*, unpublished results) by testing sporangia production in response to bacteria isolated from the roots of *Phytophthora*-infected avocado roots. They found a high population of sporangia stimula-

tors and fewer sporangia inhibitors. We also have developed some preliminary results indicating that antagonistic bacteria isolated from compost can suppress sporangia production by *Phytophthora ramorum* in a bioassay.

Another approach to creating suppressive soils would be to increase populations of cellulose-producing microbes that could lyse the mycelium of *Phytophthora* as was demonstrated by Downer *et al.* (2001). Mulching avocado trees with organic matter increased populations of cellulase-producing microbes that decreased the population density of the pathogen, leading to improved tree growth and health.

Summary, conclusions, and future prospects for disease management

Formation of an effective AM symbiosis in production agriculture can be important under a number of stressful situations, including the growth-limiting effect of P deficiency, soil salinity, drought stress, and disease pressure. Several management strategies must be considered in order to assure AM formation and the prospect of having any effect on plant performance in early growth stages or after transplanting. Preinoculation of transplants seems to be a logical approach in order for AM to effectively address any future stresses. Nursery practices for production of transplants with AM should include organic fertilizers or inorganic fertilizers with low P, could include peat or coir as an amendment to the soilless growth media commonly used, and could include the use of compost to increase the microbial diversity of the medium that could contribute to potential disease suppression. Without that diversity, there might be too few of the needed bacterial associates to complete the “team”, the members of which function in tandem to support or enhance plant growth and health. This means that the mycorrhizosphere paradigm is actually a hierarchy wherein the plant roots select and allow formation of AM, and the extraradical hyphae, along with modified host root exudates changes (Graham *et al.*, 1981; Lynch and Whipps, 1990), select specific bacterial associates and sustain them, in part, by means of specific hyphal exudates (Bago *et al.*, 1996; Bansal and Mukerji, 1994). The specificity of AM function that we see could be explained in terms of quality and completeness of the mycorrhizosphere team that can vary with different AM fungi and the soil/growth medium and the microbial populations contained therein. I believe that all soils contain microbial components capable of performing needed functions that aid “normal” plant growth. This mycorrhizosphere paradigm could explain the success of the AM system for some 460 million years (Remy *et al.*, 1994; Smith and Read, 1997; Taylor *et al.*, 1995; Simone *et al.*, 1993).

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