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Effect of heavy metals on interactions between vesicular arbuscular mycorrhizal fungi (AMF) and *Spartina patens* in a restored urban salt marsh in the Hackensack Meadowlands

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1. Summary

High salt marsh plants such as the grasses *Spartina patens* and *Distichlis spicata* commonly form associations with vesicular arbuscular mycorrhizae (AMF) fungi within their roots. AMF have been shown to enhance plant growth, increase water uptake, improve the capture of limiting soil nutrients such as nitrogen (N) and phosphorus (P), and also interfere with the growth of competing non-mycorrhizal plant species. Since nutrient enrichment or heavy metal contamination can reduce AMF colonization and thus the effectiveness of plant-AMF associations, environmental contamination can impact microbial interactions that drive ecosystem functions such as plant diversity, productivity and variability. We proposed to study AMF associated with *S. patens*, a common Northeastern native salt marsh grass, in soil cores from Harrier Meadow (North Arlington) that were artificially contaminated with nickel (Ni). Since AMF cannot be obtained in pure culture, the abundance of AMF in Harrier Meadow was determined by analyzing root colonization, and by assessing abundance of spores in soil. In addition diversity of AMF was analyzed by molecular methods. The analysis did not display significant differences in AMF colonization in soils or on roots of *S. patens* in Ni-amended and non-amended cores. With on average 40 % of roots colonized by fungi, root length colonized was generally high compared to other studies suggesting the prominent abundance of saprophytic fungi. Differences in abundance of AM fungi between Harrier Meadow and other sites (e.g. Piermont Marsh), however, are gradual and most likely due to differences in matrix rather than differences in heavy metal concentrations. Spore numbers were generally below 1 per cm^3 with no significant differences between treatments suggesting that Ni-amendment had no negative effect on AMF spores in soil. These results, however, remain preliminary since only 2 replicate samples were analyzed and standard errors were large. Molecular studies on uncultured Glomales using PCR amplification with specific primers suggested that only members belonging to the *Glomus mossea*/*intraradices* group were present since sequences were retrieved from all samples (non-amended and Ni-amended), while no sequences for members of the *Glomus occultum*/*brasilianum* and *Acaulospora gerdemannii*/*trappei* group, the *Glomus etunicatum*/*claroideum* group, and the *Acaulosporaceae sensu stricto* group were not detectable. Comparative sequence analysis of 12 clones supposedly representing members of the *Glomus mossea*/*intraradices* group, however, did not retrieve any sequences that could be assigned to the Glomales. They rather resembled yeast-like organisms, even though none of the sequences was closely related to any cultured fungi. Although these data indicate the presence of a functional mycorrhizal community in both non-amended and Ni-amended soil cores from Harrier Meadow, additional studies are needed to verify data on root colonization or to address the methodological drawbacks that might have impacted analyses on spore content and diversity in soil.

2. Introduction

Salt marshes are physically harsh environments in which facilitation among species is known to be an important mechanism influencing plant succession and tolerance of abiotic conditions (3, 5, 10). The prominence of these interactions for plant success grows with the severity of the abiotic environment. Mycorrhizal root networks are often encountered in plant communities of this type. Mycorrhizae have been shown to enhance plant growth, increase water uptake, improve the capture of limiting soil nutrients such as nitrogen (N) and phosphorus (P), and also interfere with the growth of competing non-mycorrhizal plant species. It has been suggested that turf compatibility, the persistence of plants of relatively equal competitive ability in a community, is really a matter of compatibility with mycorrhizal fungi that hinder the growth of non-mycorrhizal plants while improving the growth of those plants which are suitable mycorrhizal hosts. However, despite the importance of mutualistic plant-fungal interactions in many natural communities, few studies have examined the functional role of mycorrhizae in salt marsh communities. High salt marsh plants such as the grasses *Spartina patens* and *Distichlis spicata* commonly form associations with vesicular arbuscular mycorrhizae (AMF) fungi within their roots (12, 23). AMF are highly effective at acquiring and supplying limiting nutrients such as P and N from the soil to the host plant (1, 15). In low salt marshes, where tidal N import is high, dominant species such as *S. alterniflora* are typically found without AMF (16, 28). The greater competitive ability of *S. patens* over *S. alterniflora* in high marsh environments (4) may therefore depend in part on the ability of *S. patens* to form AMF associations to increase N acquisition when tidal N subsidy is low.

Microbial interactions can drive ecosystem functions such as plant diversity, productivity and variability. Nutrient enrichment, for example, has been shown to reduce AMF colonization and the effectiveness of plant-AMF associations (13), thereby reducing the vigor of typical turf forming grass species like *S. patens*, especially under stressful conditions (14). AMF have also been shown to improve plant tolerance to heavy metal stress in polluted environments and to bind heavy metals (18, 26, 29). However, in highly contaminated environments, the presence of AMF might result in the opposite effect, i.e. a decrease in plant biomass due to increased metal toxicity (19, 24). Heavy metals might also impact AMF colonization and diversity. However, few and variable results exist on the significance of AMF in soils contaminated with heavy metals (30).

Reduction in AMF could result in lower productivity and growth of native salt marsh grasses, a situation found to increase the success of invasive plant species such as *Phragmites australis* (2, 21). Since the functioning and stability of terrestrial ecosystems are basically determined by plant biodiversity, reductions in AMF and thus plant growth performance could have broad implications for ecosystem functioning in general and the cycling of nutrients and energy in salt marsh ecosystems in particular. Maintenance of plant biodiversity in many environments, however, is not only dependent on overall colonization of roots by AMF, but also affected by the diversity of AMF (27). AMF-species richness is positively correlated to plant biodiversity, nutrient capture and productivity emphasizing “the need to protect AMF and to consider these fungi in future management practices in order to maintain diverse ecosystems” (27).

The aim of this research was therefore to determine the principal interactions between *S. patens*, a common Northeastern native salt marsh grass, with associated AMF that may limit productivity at increasing heavy metal concentrations in soils/sediments. Our study focused on Harrier Meadow (North Arlington), an urban salt marsh in the Hackensack Meadowlands, approximately 5 km north of Newark, New Jersey, and 10 km west of New York City, thus part of the larger New York/New Jersey Harbor Estuary. Harrier Meadow that was subject of wetland restoration work by the New Jersey Meadowlands Commission (NJMC), was used as disposal site for shot rock from construction of U.S Route 280 in the past. In sediments of this marsh, a recent baseline study conducted in our laboratory and supported by MERI (01/01/2000-12/31/2000) found moderate levels of heavy metal contamination still below but close to regulatory action levels (25 ppm Ni, 45 ppm Cr, 3 ppm Cd, 72 ppm Cu, 270 ppm Pb, 670 ppm Zn). Harrier Meadow that experiences a tidal range of 0.5 m and a salinity between 5-15 ppt, is characterized by *P. australis*, but large patches of *S. patens* exist in the center of the marsh. In this marsh, reduction in AMF on *S. patens* could result in lower productivity and growth of this native salt marsh grass, and increase the chances on displacement by invasive plant species such as *P. australis*.

2. Basic experimental setup

The analyses performed used soil cores with *S. patens* (10 x 10 x 10 cm, n = 20) weighing approximately 1 kg fresh weight, that were collected at the end of February 2000 similar to a previous study (9). Cores were transported from Harrier Meadow (Fig. 1) back to a greenhouse at Rutgers University-Newark, fitted into plastic pots and placed in individual pails containing 5 ppt artificial seawater (Instant Ocean? Mentor, OH, USA) amended with 5 ppm NH_4^+ and PO_4^{3-}, respectively. Pails containing potted cores were arranged randomly on a greenhouse bench and artifical seawater was added to each pail so that standing water was present 5 cm below the top of the core. This correlated to the level of water observed in holes remaining in the field after core removal. In one half of the cores the availability of Ni was artificially increased through amendments with $NiSO_4$ (50 mg kg^{-1} core material). The second half of the cores received NaCl and served as controls for heavy metal contaminated cores.

Fig. 1 Sampling location in Harrier Meadow (red-marked spot)

Each core and treatment was divided into three zones. The first zone was sampled with a cork corer (2 cm diameter) at the beginning (May, largest root exudation correlated to fastest plant growth; vegetative growth), the second in the middle (July, reduced exudation correlated to flowering; reproduction) and the third at the end (October, no exudation due to senescence) of the vegetation period. Soil and root samples were separated. Samples (n=3) were collected between surface and 1.5 cm (referred to as 0.7 cm), 1.5 and 3.5 cm (2.5 cm), 4.0 – 6.0 cm (5 cm), and 6.5 – 8.5 cm (7.5 cm) below the surface of each core as in our preliminary study (9). Each of these samples was divided into six-1 g portions. One portion served for the analysis of AMF colonization, a second retained for pore-water separation and analysis and sediment and root dry weight determination, and a third was frozen at -80?C and kept for DNA extraction. The other three portions were evaluated for heavy metal concentrations in the plant roots, associated sediment and pore water. All cores were destructively harvested in May of the second year, and stored separated in slices of the respective depth intervals.

3. Abundance of AMF on roots

Since AMF cannot be obtained in pure culture, two approaches were taken in order to get an estimate on the abundance of AMF in Harrier Meadow. The first approach was based on the determination of root colonization by AMF, and the second on that of abundance of spores in soil. Roots for AMF estimation were only analyzed from samples at a depth of 2.5 cm since previous experiments had shown significant colonization at this depth only and not at depths of 5 and 7.5 cm (7) which was attributed to a reduction in oxygen availability as evidenced by decreasing redox potential with depth, a situation consistent with natural marsh soil and one that we desired in an effort to approximate field conditions. These values suggested increasing oxygen limitation in all cores with depth and the use of alternative electron acceptors in microbial metabolism. This assumption is supported by pore water analysis that revealed declines in sulfate concentrations with depth corresponding to decreasing redox potential, suggesting increasing activity of sulfate-reducing bacteria with depth.

Roots were stained with Trypan Blue in lactic acid using a modification of the procedure outlined by Kormanick and McGraw (20) that employed incubation in 5% KOH for 6 hours at room temperature instead of heat for root clearing. Trypan Blue is often used in studies of salt marsh plant colonization (12, 23) and was used here to allow equivalent comparison of our results with those of other investigators. Root samples were mounted on microscope slides and colonization was determined using the slide mount method of McGonigle et al. (22). A total of 100 intersections were scored for each slide (slides=24). The root length colonized by mycorrhizal hyphae, arbuscules and vesicles was determined according to the method of Brundrett et al. (6) where hyphae are considered mycorrhizal only if visually connected to vesicles or arbuscules. Since arbuscules and vesicles are transitory structures variably distributed, this method of assessing AMF colonization can underestimate the actual colonization of plant roots.

The analysis did not display significant differences in AMF colonization on roots of *S. patens* in Ni-amended and non-amended cores. With on average 40 % of roots colonized by fungi, root length colonized (Fig. 2) was generally high compared to other studies suggesting the prominent abundance of saprophytic fungi. In North Carolina marshes, for example, root length colonized

typically ranged from 26-52%, but levels as low as 5% have been reported from marshes in Connecticut (12, 16). In studies on Piermont Marsh, we have found that root length colonized by AM varies seasonally in *S. patens* ranging between 20 and 25% in non-treated cores between vegetative and reproductive growth stages in greenhouse studies, levels comparable to those encountered under field conditions (7, 8). The suggestion of high abundance of saprophytic fungi on roots of *S. patens* growing in either Ni-amended or non-amended cores was supported by slightly lower numbers of AMF as indicated by % arbuscular and vesicular colonization (Fig. 2) as found in our previous studies (7, 8). Both colonization by saprophytic and AM fungi decreased during the growing season similar to results in our previous studies (7). Differences in abundance of saprophytic and AM fungi between Harrier Meadow and other sites (e.g. Piermont Marsh), however, are gradual and most likely due to differences in matrix rather than differences in heavy metal concentrations.

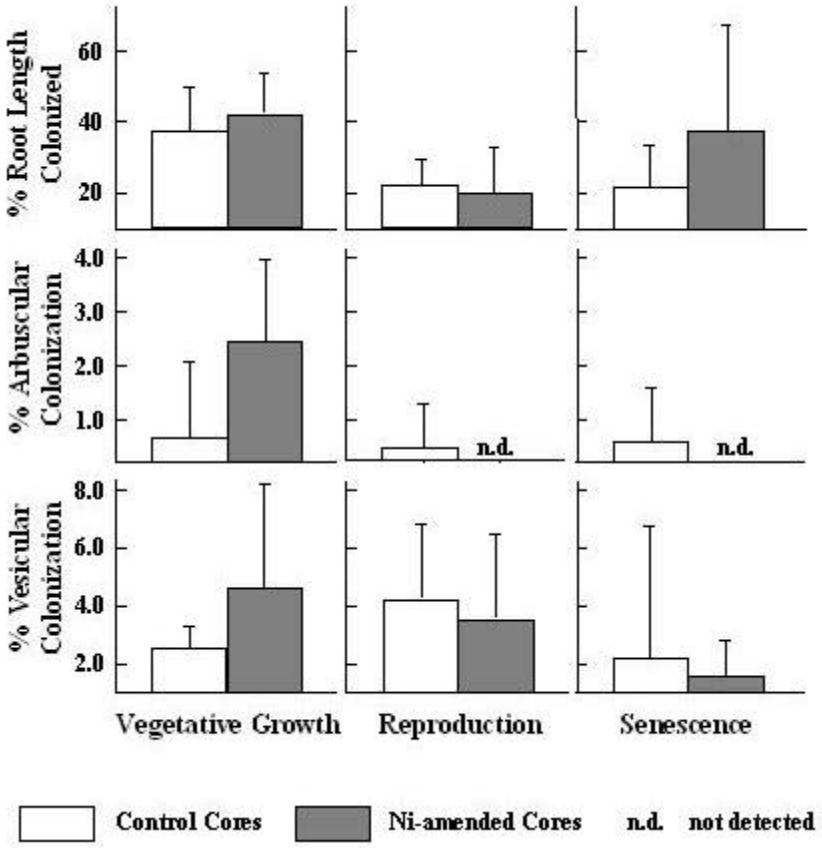

Fig. 2 Colonization of roots of S. patens at a depth of 2.5 cm by AMF at three stages during the growing season

4. Abundance of AMF in soil

For the analysis of spore densities, spores were separated from 25 g of soil sample from the depth of 2.5 cm (n=2 for each treatment). The samples were stirred in 100 ml distilled water in a 500 ml beaker for 10 to 15 minutes. Soil clumps were subsequently homogenized in a mortar taking care to prevent damage to the spores held within that sample. The homogenized soil solution was sieved through a series of sieves with pore sizes measuring 750, 250, 106, and 50

µm and washed constantly with tap water. Residues on the 106-µm-sieve were collected in a 50 ml centrifuge tube, tubes filled with distilled water to the 50 ml mark and centrifuged for 5 minutes at 2000 rpm in order to remove floating debris and organic material from the sample. The pellet was subsequently re-suspended in 50 % sucrose and centrifuged for 1 minute at 2000 rpm to separate spores from the remaining soil matrix. The supernatant containing the spores was finally filtered through a 50-µm-sieve to remove sucrose. Spores remaining on the sieve were collected individually in microfuge tubes containing 15 - 20 µl of sterilized distilled water using a dissecting microscope (15 x magnification) and stored at -20°C.

Spore numbers retrieved were generally very low with less than 1 spore per cm^3 (the volumetric measure was preferred to gravimetric measurements (grams) since the density of the soil was extremely different at different depths). However, no significant differences were obtained between treatments suggesting that Ni-amendment had no negative effect on AMF spores in soil. These results, however, remain preliminary since only 2 replicate samples were analyzed and standard errors were large (Fig. 3).

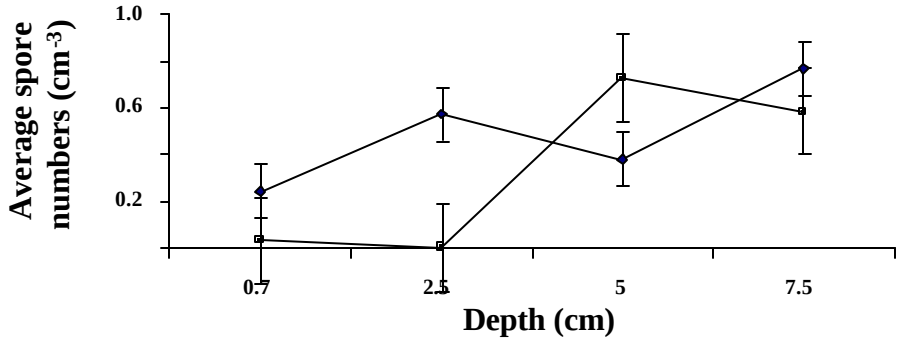

Fig. 3 Average spore numbers in soil (cm^{-3}) with (?) and without (¦) Ni-amendment

These large standard errors might at least in part be due to methodological problems of the analysis. One of the main reasons for discrepancies in the final number of spores obtained in a particular soil sample when compared to another may be due to the loss of many spores during the process of sieving. To remedy this, several precautions should be considered: i) avoid shaking the sieve too vigorously during the initial filtration period which will decrease the amount of water that is lost due to clogging and overfilling of the sieve plate during the decanting process. ii) instead of using a smaller pore size sieve plate to flush out the sucrose solution, vacuum filtration can be used during this step. There is also a tendency for spores to become trapped on the outer edges of the microcentrifuge tube during the final step of this procedure. In addition, spores can be caught on the tools used to pick them up from the moist filter paper. To avoid these situations it may be necessary to: i) rotate the tool used to pick up spores under the microscope, and ii) to examine the outer edges of the microcentrifuge tube for loose spores that have not made it into the storage medium. Finally, damaged spores may increase the potential for error when looking for viable spores within a certain soil type at a certain depth. To avoid this situation, it may be necessary to slightly rotate the spore under the microscope for viewing purposes.

5. Diversity of AMF in soil

In order to determine diversity of uncultured AMF in soil, DNA was isolated from either single spores or the overall yield from one extraction at a depth of 2.5 cm (89 spores) using a

method based on a bead beating protocol (17). This protocol was also useful for the extraction of nucleic acids from roots. Released nucleic acids were purified by phenol/chloroform extraction and subsequent Sephadex-200 column chromatography as described in (11).

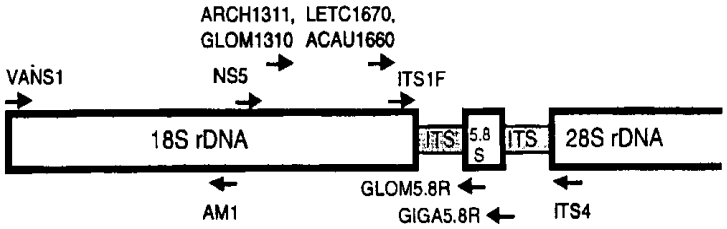

Fig. 4 Schematic presentation of ribosomal RNA genes with annealing sites of primers (25)

Purified DNA was used as template in a nested PCR targeting internal transcribed spacers (ITS) of ribosomal DNA (25). A hot-start PCR protocol with primers NS5 and ITS4 (31) amplifying about half of the 18S rDNA, the 5.8S rDNA as well as two ITS regions between 18S and 28S rDNA (amplicon of about 1.8 kb) of fungi (Fig. 4). A small portion of the amplicon (1%) was subsequently used in a nested PCR using specific primers ARCH1311, LET1670, GLOM1310, and ACAU1660 (25), respectively, with ITS4 to detect AMF of the *Glomus occultum/brasilianum* and *Acaulospora gerdemannii/trappei* group, the *Glomus etunicatum/claroideum* group, the *Glomus mossea/intraradices* group and the *Acaulosporaceae sensu stricto* group as described by Redeker (25) (Fig. 5).

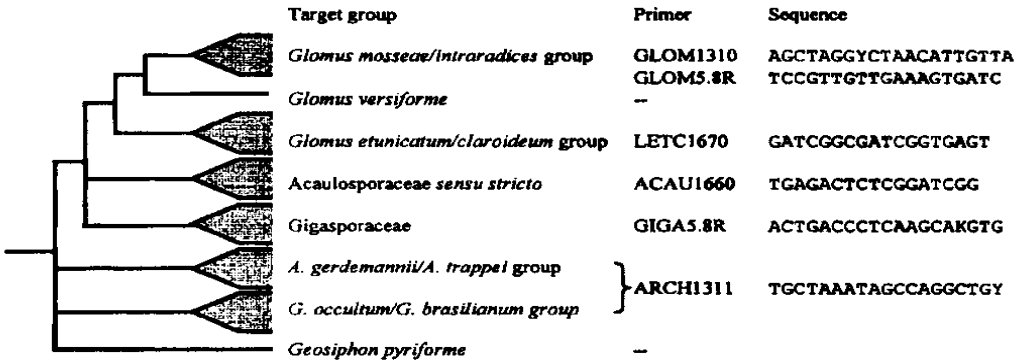

Fig. 5 Specific primers for AMF targeting phylogenetic groups within the Glomales (25)

PCR amplification resulted in products only if primer combination GLOM1310 and ITS4 were used. This suggests that the *Glomus mossea/intraradices* group was present in all samples (non-amended and Ni-amended), whereas the *Glomus occultum/brasilianum* and *Acaulospora gerdemannii/trappei* group, the *Glomus etunicatum/claroideum* group, and the *Acaulosporaceae sensu stricto* group were not detectable. Amplicons generated with primer combination GLOM1310 and ITS4 were subsequently cloned and a part sequenced. Comparative sequence analysis of 12 clones, however, did not retrieve any sequences that could be assigned to the Glomales. They rather resembled yeast-like organism, even though none of the sequences was closely related to any cultured fungi. Similar to the analysis of spores in soil, these results might

be impacted by methodological constrains. Nested PCR that involves two separate amplification reactions is used to increase sensitivity and to overcome potential inhibition by contaminating substances (Redecker et al., 2000). This procedure confirmed the presence of Glomalean fungi within the target sample. However, the difficulty in obtaining sterile spore isolates makes it very hard to later use PCR as a means of identifying various mycorrhizal fungi. Also, Glomales cannot be cultivated axenically as their spores harbor numerous other organisms. This results in frequent contamination during PCR with both universal and fungal specific primers. It may be necessary to treat the intact spores with a sterilizing agent and later isolate them for a second time for PCR analysis.

6. Conclusions

The data assembled on AMF in Harrier Meadow indicate the presence of a functional mycorrhizal community in both non-amended and Ni-amended soil cores from Harrier Meadow with no significant differences in AMF colonization on roots of *S. patens* in Ni-amended and non-amended cores. However, additional studies are needed to verify data on root colonization and to address the methodological drawbacks that might have impacted analyses on spore content and diversity in soil. Also, long-term studies and/or comparative analyses with pristine (and subsequently contaminated) salt marsh sediments analyzed over time could be useful additions to the studies presented.

6. References

1. **Ames, R.N., C.P.P. Reid, L.K. Porter, and C. Cambardella.** 1983. Hyphal uptake and transport of nitrogen from two N-labelled sources by *Glomus mosseae*, a vesicular-arbusuclar mycorrhizal fungus. New Phytol. **95:** 381-396.
2. **Amsberry, L., M.A. Baker, P.J. Ewanchuk, and M.D. Bertness.** 2000. Clonal integration and the expansion of *Phragmites australis*. Ecol. Appl. **10:** 1110-1118.
3. **Bertness, M.D.** 1992. The ecology of a New England marsh. American Scientist **80:**260-268.
4. **Bertness, M.D.** 1991. Zonation of *Spartina patens* and *Spartina alterniflora* in a New England salt marsh. Ecology **72:** 138-148.
5. **Bertness, M. D., and R. Callaway.** 1994. Positive interactions in communities. *Trends Ecol. Evol.* **9:** 191-193.
6. **Brundrett, M.C., L. Melville, and R.L. Peterson.** 1994. Practical methods in mycorrhiza research. Mycologue Publications, Waterloo, Ontario, Canada.
7. **Burke, D., E.P. Hamerlynck, and D. Hahn.** 2002. Effect of arbuscular mycorrhizae on soil microbial populations and associated plant performance of the salt marsh grass *Spartina patens*. Plant Soil **239:** 141-154.
8. **Burke, D., E.P. Hamerlynck, and D. Hahn.** 2002. Interactions among plant species and microorganisms in salt marsh sediments. Appl. Environ. Microbiol. **68:** 1157-1164.
9. **Burke, D., E.P. Hamerlynck, and D. Hahn.** 2003. Interactions between the salt marsh grass *Spartina patens*, arbuscular mycorrhizal fungi and sediment bacteria during the growing season. Soil Biol. Biochem. **35:** 501-511
10. **Callaway, R.M.** 1997. Positive interactions in plant communities and the individualistic-continuum concept. Oecologia **112:** 143-149.
11. **Chatzinotas, A., R.-A. Sandaa, W. Schönhuber, R.I. Amann, F.L. Daae, V. Torsvik, J. Zeyer, and D. Hahn.** 1998. Analysis of broad-scale differences in microbial communities of two pristine forest soils. System. Appl. Microbiol. **21:** 579-587.

12. **Cooke, J.C., R.H. Butler, and G. Madole.** 1993. Some observations on the vertical distribution of vesicular arbuscular mycorrhizae in roots of salt marsh grasses growing in saturated soils. Mycologia **84:** 547-550.

13. **Egerton-Warburton, L.M., and E.B. Allen.** 2000. Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecol. Appl. **10:** 484-496.

14. **Francis, R., and D.J. Read.** 1994. The contributions of mycorrhizal fungi to the determination of plant community structure. Plant Soil **159:** 11-25.

15. **Hayman, D.S.** 1986. Mycorrhizae of nitrogen-fixing legumes. MIRCEN J. **2:** 121-145.

16. **Hoefnagels, M.H., S.W. Broome, and S.R. Shafer.** 1993. Vesicular-arbuscular mycorrhizae in salt marshes in North Carolina. Estuaries **16:** 851-858.

17. **Hönerlage, W., D. Hahn, and J. Zeyer.** 1995. Detection of mRNA of *npr*M in *Bacillus megaterium* ATCC 14581 grown in soil by whole cell hybridization. Arch. Microbiol. **163:** 235-241.

18. **Joner, E.J., and C. Leyval.** 1997. Uptake of ^{109}Cd by roots and hyphae of *Glomus mosseae/Trifolium subterraneum* mycorrhiza from soil amended with high and low concentrations of cadmium. New Phytol. **135:** 253-260.

19. **Kilham, K., and M.K. Firestone.** 1983. Vesicular-arbuscular mycorrhizal meditation of grass response to acidic and heavy metal deposition. Plant Soil **72:** 39-48.

20. **Kormanick, P.P., and A.C. McGraw.** 1982. Quantification of vesicular-arbuscular mycorrhizas in plant roots, p. 37-45. *In* N. C. Schenck (ed.), Methods and Principals of Mycorrhizal Research. The American Phytopathological Society, St Paul, Minnesota.

21. **Levine, J.M., J.S. Brewer, and M.D. Bertness.** 1998. Nutrients, competition and plant zonation in a New England salt marsh. J. Ecol. **86:** 285-292.

22. **McGonigle, T.P., M.H. Miller, D.G. Evans, G.L. Fairchild, and J.A. Swan.** 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. **115:** 495-501.

23. **Patriquin, D.G., and C. Keddy.** 1978. Nitrogenase activity (acetylene reduction) in a Nova Scotian salt marsh: its association with angiosperms and the influence of some edaphic factors. Aquatic Botany **4:** 227-244.

24. **Pawlowska, T.E., J. Blaszkowski, and A. Ruehling.** 1996. The mycorrhizal status of plants colonizing a calamine spoil mound in southern Poland. Mycorrhiza **6:** 499-505.

25. **Redeker, D.** 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza **10:** 73-80.

26. **Tonin, C., P. Vandenkoornhuyse, E.J. Joner, J. Straczek, and C. Leyval.** 2001. Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover. Mycorrhiza **10:** 161-168.

27. **van der Heijden, M.G.A., J.N. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I.R. Sanders.** 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature **396:** 69-72.

28. **Van Duin, W.E., J. Rozema, and W.H.O. Ernst.** 1989. Seasonal and spatial variation in the occurrence of vesicular-arbuscular mycorrhizae in salt marsh plants. Agricult., Ecosyst. Environ. **29:** 107-110.

29. **Weissenhorn, I., C. Leyval, G. Belgy, and J. Berthelin.** 1995. Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*Zea mays* L.) in pot culture with contaminated soil. Mycorrhiza **5:** 245-251.

30. **Weissenhorn, I., M. Mench, and C. Leyval.** 1995. Bioavailability of heavy metals and arbuscular mycorrhiza in a sewage-sludge-amended sandy soil. Soil Biol. Biochem. **27:**287-296.

31. **White, T.J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (eds.), PCR protocols, a guide to methods and applications. Academic Press, San Diego.