The Effect of Mycorrhizae on Nitrogen-Fixing Bacteria Associated with the Salt Marsh Grass *Spartina patens*

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ABSTRACT

Soil microbes, notably nitrogen-fixing bacteria found within the rhizosphere as well as vesicular arbuscular mycorrhizae (VAM), may play an important role in salt marsh communities by enabling plants to overcome nitrogen stress and, in so doing, structuring the plant community to favor species that form microbial symbiotic associations. We explored the effect of VAM on the community of microbial nitrogen fixers associated with the rhizosphere and soil of a common high marsh grass species, *Spartina patens*. DNA extracted from rhizosphere and soil samples was used as template for PCR amplification of *nif*H, the gene that codes for the key enzyme nitrogenase, which has a phylogenetic significance and is commonly used in the analysis of uncultured populations of nitrogen-fixing bacteria. In the presence of VAM, populations of nitrogen-fixing bacteria in the rhizosphere were found to be different from populations in bulk soil at every depth (2.5, 5.0, and 7.5 cm from the surface). In the presence of VAM, population patterns in the rhizosphere were different from treatments where VAM was excluded. Suppression of VAM altered rhizosphere populations such that they came to resemble microbial populations encountered in adjacent soil, and lowered the overall genetic heterogeneity of the N-fixing communities encountered in the rooting zone of *S. patens*.

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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 (Bertness and Callaway 1994, Bertness 1992). Fertilizer amendment studies have shown that one of the most singular stresses encountered by salt marsh plants is a functional nitrogen deficiency (Chalmers 1979, Valiela and Teal 1974) brought about by soil saturation that accompanies tidal flooding (Adam 1990, Bertness 1992, Valiela and Teal 1979). Although rising tidal waters saturate marsh soil and foster N-limited conditions, these waters also import N to the marsh surface and are an important source of available N for marsh plants. The significance of the tidal N subsidy decreases with increasing distance from tidal creeks, and thus, high salt marsh usually receives less tidally imported N (Adam 1990, Bertness 1992, Chalmers 1979). This frequently results in a zone of stunted "short form" *S. alterniflora* that marks the transition between low and high marsh (Bertness 1992, Chalmers 1979). The N-budget of plants within this zone depends more on nitrogen recycled within the plant and N mineralized from soil organic matter than on tidal import (DeLaune et al. 1989, Hopkinson and Schubauer 1984, White and Howes 1994).

N-fixation is another important component of the nitrogen budget of nutrient limited, high marsh systems. N-fixation by rhizosphere bacteria may also provide as much N as tidal import (DeLaune et al. 1989, White and Howes 1994). N-fixation by diazotrophic bacteria associated with the rhizosphere, root surface and root cortex of *S. alterniflora* is fueled largely by carbon leaked from the plant root which provides the microbes with a readily available carbon source (Boyle and Patriquin 1981, Patriquin and Keddy 1978, Whiting et al. 1986). Bacterial activity for some phylogenetic groups, such as sulfate reducers, is highest for bacteria physically associated with plant root surfaces, underlining the importance of root exudation to the activity of soil micro-flora (Hines et al. 1999).

Nitrogen fixation has been found to be phosphorus limited (Hayman 1986, Mikola 1986). Diazotrophic bacteria have a high demand for P that can easily exceed the requirements of the plants with which they associate. So the bacteria plants rely upon to

provide available nitrogen are P-limited, even though the plants do not appear so limited themselves. There is evidence that the P-demand of diazotrophs can be satisfied by the activity of vesicular arbuscular mycorrhizae (VAM) associations in plant roots (Hayman, 1986, Mikola 1986). Mycorrhizae are effective at acquiring and supplying P from the soil to the host plant (Hayman 1986). VAM also transport N, usually in the form of ammonium, from the soil to the host plant (Ames et al. 1983). Although salt marshes are not considered phosphorous limited, a functional P-limitation may exist for N-fixing rhizosphere bacteria, and this may underlie the apparent N-limitation in salt marsh systems. This functional limitation may be a consequence of both high bacterial demand for P as well as the exclusion of P by iron sheaths or plaques that form on salt marsh plant roots. VAM may therefore serve a similar role to that found in legume systems, where mycorrhizae indirectly increase N-fixation by eliminating the P- limitation of the associated diazotrophic bacteria (El Ghandour et al. 1996, Hamel et al. 1991, Huang et al. 1985). Since distance from tidal flooding may increase the reliance on bacterial fixation as a N-source, high marsh plants should be more likely to host a fungal symbiont.

VAM are commonly found in many salt marshes (Cooke et al. 1993, Hoefnagels et al. 1993); however, low marsh plant species, such as *Spartina alterniflora*, are typically found without mycorrhizae possibly due to resistance to VAM infection (Hoefnagels et al. 1993, Van Duin et al. 1989). On the other hand, high marsh species such as *Spartina patens* and *Distichlis spicata* are usually encountered with VAM infection (Cooke et al. 1993). The greater competitive ability of *S. patens* over *S. alterniflora* in high marsh environments (Bertness 1991) may be due, in part, to the ability of the species to form effective VAM associations. VAM formation could improve plant performance when tidal N subsidy is low and anaerobic conditions make roots more susceptible to plaque formation. The effect of VAM on the population of P-limited diazotrophic bacteria may, therefore, be critical to successful plant activity in this region of a salt marsh. The goal of this research was to explore the interaction of vesicular arbuscular mycorrhizae with nitrogen fixing organisms associated with the roots and rhizosphere soil of a common high marsh grass species, *Spartina patens*. We used molecular techniques to determine the structural similarity between root and soil associated N-fixing microbial communities. These findings establish critical, primary

linkages between plant, fungal, and microbial aspects important in nutrient cycling and community structure in salt marsh systems.

MATERIALS AND METHOD

Sampling Site. Cores of intact plants and soil were collected from the Piermont Marsh, (41° 02' 30'' N Latitude, 73° 55' 00" W Longitude) located along the western bank of the Hudson River approximately 18 km north of New York City. Situated within Tallman Mountain State Park and part of the National Oceanic and Atmospheric Administration's (NOAA) Hudson River National Estuarine Research Reserve (NERR), Piermont Marsh is a 500 ha tidal salt marsh that experiences a tidal range of 1 m and salinity between 0-15 ppt. The marsh is characterized by *Typha* sp. and *Phragmites australis*, but large patches of *S. patens* exist in the center of the marsh

Experimental Set-up. Soil cores (10x10x10cm) weighing approximately 1 kg fresh weight, were collected on June 7, 1999. Cores were transported back to a greenhouse at Rutgers University-Newark, fitted into plastic pots and placed in an individual pail containing 5 ppt artificial seawater (Instant Ocean® Mentor, OH, USA). Pails containing potted cores were arranged randomly on a greenhouse bench and water was added to each pail so that standing water was present 5 cm below the top of the core. This was the level of water observed in holes remaining in the field after core removal. After a 1 week acclimation period, pots were randomly exposed to one of three treatments: the fungicide benomyl (50% WP; Bonide Products, Inc. Yorkville, NY, USA) dissolved in 100 ml of tap water was applied to 8 cores at a rate of 0.1 g per kg of soil, an additional 8 cores were treated with the same benomyl solution but also received a 100 ml standard Hoagland's phosphorous solution (0.35 g KH_2PO_4 per L), and 8 cores served as controls and were treated only with tap water. Benomyl has been shown to suppress VAM colonization in previous studies (Hetrick et al. 1994) and was meant to exclude VAM from two treatments so that mycorrhizal effects could be noted on the bacterial populations. Phosphorous treatments were meant to assess microbe effects in the absence of VAM but with higher soil P levels. Benomyl-phosphorous treatments received

additional phosphorous every 2 weeks at the rate described above. Benomyl was applied only once, at the start of the study.

Soil Sampling. Soil and root samples were collected from each pot using a cork corer (1 cm diameter). During sample extraction, pH, temperature and redox potential were measured using an Oakton® pH/mv meter (Cole-Parmer Instrument Company, Vernon Hills, ILL, USA). Samples were collected from 2.5, 5.0, and 7.5 cm below the surface of each core. Each sample was divided into 3-1 g portions, placed in sterile Eppendorf tubes and kept on ice until preparation. One sample collected from each depth was preserved in paraformaldehyde and reserved for biomass estimates of phylogenetic groups by *in situ* hybridization. A second sample was frozen at -80°C and kept for bacterial population analysis, and another sample was also frozen and retained for pore-water separation and analysis.

Pore Water Chemistry. Concentrations of Cl^- and SO_4^{2-} were determined by a Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column using an eluent of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ (Hess et al., 1996; Hess et al., 1997).

Plant Characteristics. To assess plant physiological status Fv/Fm ratios, a chlorophyll fluorescence measurement of photosystem II efficiency and a reliable indicator of physiological stress, were measured on all treatments using a Hansatech FMS-2 pulse amplitude modulated fluorimeter. Plant samples were dark adapted for a minimum of 15 min prior to estimation of Fv/Fm. Measurements were made 2 weeks after treatment initiation and again 4 weeks later. After 6 weeks of growth, declines in Fv/Fm ratios for all treatments were noted and coincided with a visible decline in the greenness of plant tissue. Sampling of soil cores began at this time.

Leaf tissue was harvested from a subset (15 plants) of the soil cores based upon the determined effectiveness of fungicide treatment. Five leaves were collected from each selected plant and leaf area was measured using a Li-Cor LI3000 leaf area meter (LiCOR Instruments, Ltd., Lincoln, NE, USA). Leaf dry weight was determined after drying for 4 days at 90°C. Specific leaf area was calculated by dividing leaf dry weight by leaf area. Root density was estimated from samples used for pore water analysis. After removal of pore water, gentle washing in distilled water separated the sediment and

root material that remained. Root and soil material was then dried as indicated above and the density of both fractions determined by dividing dry weight by sample volume. Water weight of the samples, the difference between the samples original wet weight and the sum of soil and root dry weights, was used as an estimate of core saturation.

Mycorrhizae. Additional samples at each depth were collected for determination of VAM colonization. Roots for VAM estimation were stained with Trypan Blue in lactic acid using a modification of the procedure outlined by Kormanick and McGraw (1982). We used a no heat method that employed 5% KOH for de-staining. Colonization was determined using a slide mount method where 30 roots were examined for each slide. VAM colonization was determined by detecting the presence of either arbuscules or hyphal coils in sample roots. Since we were interested in the effect VAM might have on the microbial populations, samples used for population estimates were selected so that substantial differences in the extent of mycorrhizal colonization existed between the cores.

Nitrogen-fixing Microbial Populations. Rhizosphere and bulk soil samples were used to extract DNA after lyzing bacteria through bead beating (Widmer et al. 1999). DNA served as template for PCR amplification. Studies on nitrogen-fixing bacteria were based on the analysis of PCR amplified structural genes for the key enzyme nitrogenase (*nif*H) which has phylogenetic significance (Aguilar et al. 1998). Several regions in the *nif*H gene code for highly conserved protein stretches and have successfully been used as target sites for PCR primers. For highly specific amplification, a nested PCR based on three primers has been described and used in molecular ecology studies (Widmer et al. 1999). The resulting amplification products were used to generate restriction fragment length polymorphism (RFLP) patterns with two restriction endonucleases (*Hae*III, *Rsa*I). After gel electrophoretic separation (7.5% polyacrylamide gels) of the different fragments, the gels were stained with silver nitrate following the procedure outlined in Bassam et al (1991). Stained gels were scanned with a Hewlett Packard ScanJet 4200C (Hewlett-Packard, Palo Alto CA, USA) and the obtained images analyzed using the GelManager 1.5 program (Biosystematica). GelManager 1.5 employs the Pearson product moment coefficient (r) to generate a band similarity matrix using information about the apparent molecular weights of the bands and band spacing, the results of which

are used to construct UPGMA (unweighed pair group method with arithmetic average) dendograms. These analyses allowed us to assess shifts in the genetic heterogeneity of microbial populations, either associated with treatment or different environmental conditions.

Statistical Analysis. Two tailed t-tests were employed to assess whether differences between treatments existed for environmental parameters. Pearson product moment coefficients calculated within the GelManager 1.5 program completed statistical analysis for comparison of microbial populations patterns. Band similarities greater than 0.7 indicate that there are no significant differences between adjacent patterns, which are then assumed to be the product of the same population. Multiple analysis of variance (MANOVA) was utilized to determine whether treatment differences existed among plants for chlorophyll fluorescence measurements.

RESULTS

Pore Water Chemistry. Mean soil pH ranged from 6.0 to 6.6 for the three treatments and no significant differences were found either between treatments or with depth (Figure 1). Soil temperature (Figure 1) for the benomyl-phosphorous cores was found to be lower than the benomyl and non-treated cores but these differences were not significant. Soil temperature remained relatively constant with depth, probably the consequence of growing plants on a greenhouse bench where solar radiation affected all sides of the core. Redox potential was found to decline (Figure 1) with depth for all treatments, the result of greater core saturation. The non-treated cores were found to have significantly lower redox potentials ($p<0.05$) at 5.0 (-160.8 ± 38.5) and 7.5 cm (-234.0 ± 22.8) as compared to both benomyl (-50.3 ± 46.4; -132.8 ± 36.7) and benomyl-phosphorous cores (23.1 ± 64.9; -156.8 ± 36.9).

Ion analysis found no significant differences between treatments for either chloride or sulfate concentrations in pore water (Figure 2) except for chloride at the 5 cm depth where non-treated pots had lower chloride concentrations than benomyl-treated pots ($p<0.02$). Chloride concentrations ranged from 7.6 ($\pm$ 1.1) to 9.1 ($\pm$ 1.0) g/l at the

Figure 1: Soil Environmental Conditions. Symbols represent the following: non-treated ■, benomyl ⊙, and benomyl phosphorous ●. VAM colonization represents the percentage of roots that contained hyphal coils indicating active mycorrhizae.

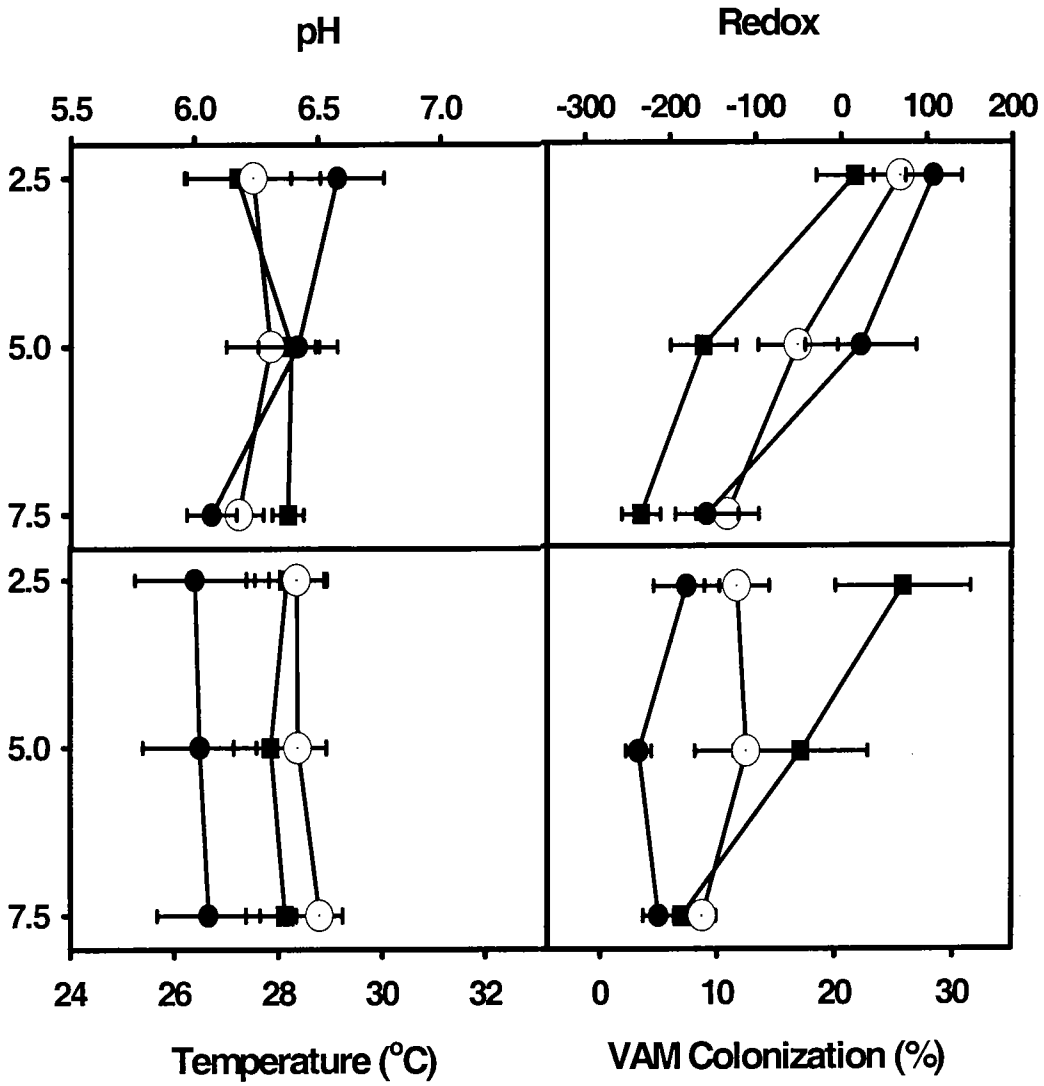
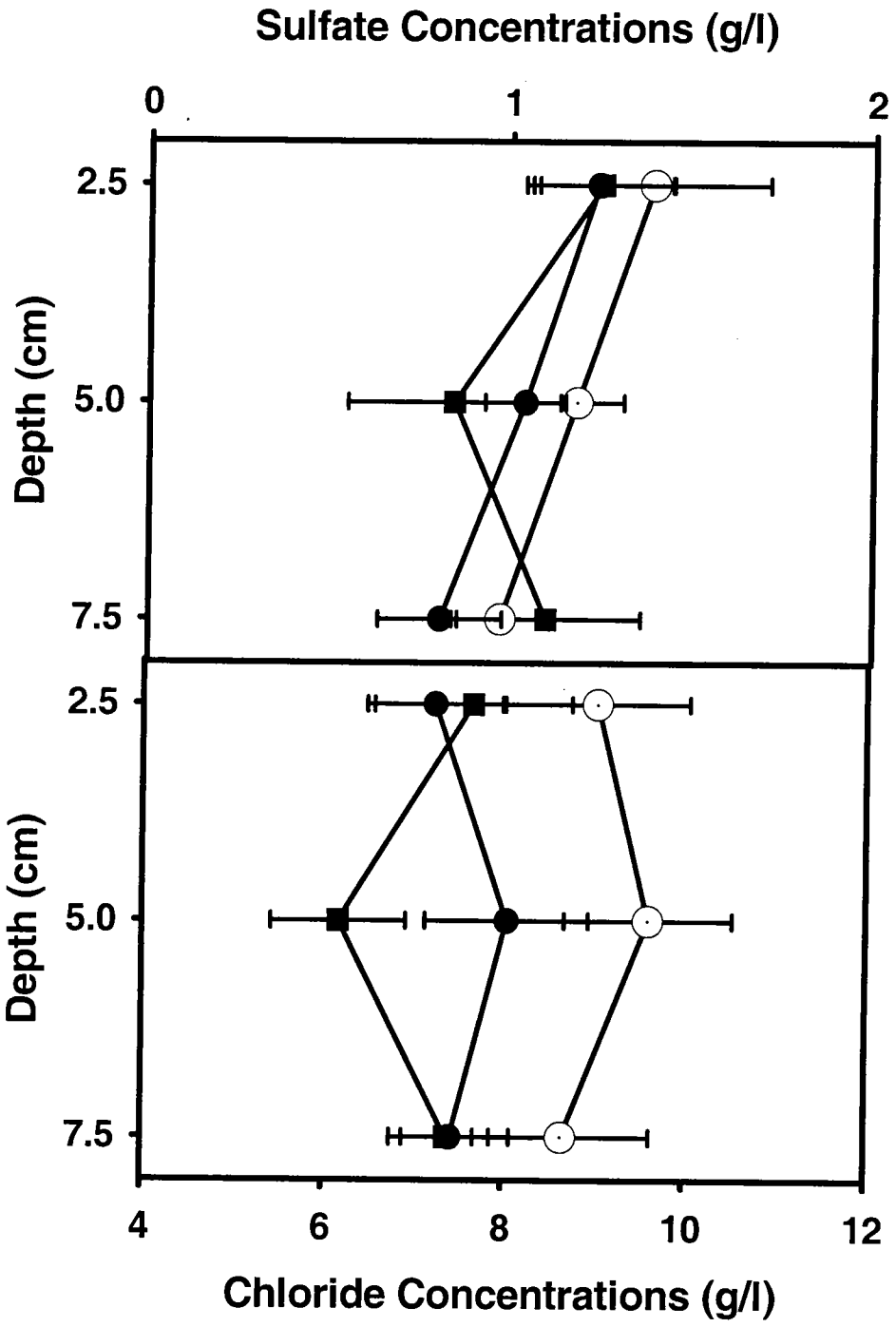


Figure 2: Pore Water Ion Analysis. Concentrations of chloride and sulfate in pore water expressed in g/l as measured by ion chromatography. Symbols represent the following: non-treated ■, benomyl ⊙, and benomyl phosphorous ●.



2.5 cm depth to 7.4 (± 0.5) to 8.7 (± 1.0) g/l at the 7.5 cm depth. No significant differences either between treatments or with depth were observed for sulfate concentrations although sulfate did decline slightly with increasing depth (Figure 2). Sulfate concentrations ranged between 1.3 (± 0.2) and 1.4 (± 0.3) g/l at the 2.5 cm depth to 1.1 (± 0.3) and 0.8 (± 0.2) g/l at the 7.5 cm depth.

Plant Characteristics. Chlorophyll fluorescence measurements revealed no significant differences between treatments for photosystem II efficiency (Figure 3). Treatment did not appear to impose any photo-oxidative stress upon the plants. However, significant declines in efficiency were noted between dates for all treatments ($p<0.01$ non-treated, benomyl; $p<0.05$ benomyl-phosphorous). This decline corresponded with a visible loss in greenness and onset of yellowing in the test plants. Analysis of specific leaf mass also showed no significant differences exist between treatments (Figure 3). Yet, both non-treated and benomyl-phosphorous treatments had higher specific leaf masses than did the benomyl treatment. Rooting density was also not affected by treatment (Figure 4). Root density was not found to vary either between treatments or with depth.

Mycorrhizae. Benomyl application significantly reduced mycorrhizal colonization (Figure 1). At the 2.5 cm depth, non-treated cores had significantly greater colonization (25.8% ± 5.7%; $p\leq0.05$) than either the benomyl (11.7% ± 7.3%) or benomyl-phosphorous (6.7% ± 2.8%) treatment. Colonization differences between cores declined with depth, however, so that at 7.5 cm there were no differences between treatments. However, colonization varied greatly within treatments and standard deviations were high. Since we were interested in the effect VAM might have on microbial populations, samples used for population estimates were selected so that substantial differences in the extent of mycorrhizal colonization existed between the cores. One non-treated, one benomyl, and one benomyl phosphorous core were selected and examined. VAM colonization rates for the non-treated pot were 53%, 27%, and 7% at the 2.5, 5.0 and 7.5 cm depth respectively. Colonization for both benomyl treatments was 7% at each of the three depths examined. *Spartina patens* in this study were found to form VAM with hyphal coils (Figure 5). Arbuscules were not detected in these plants and few vesicles were noted.

Figure 3: Effect Of Treatment On Plant Performance.
Chlorophyll fluorescence measurements were made twice over the course of 6 weeks and time is represented on the x-axis. Symbols represent the following: non-treated ■, benomyl ⊙, and benomyl phosphorous ●.

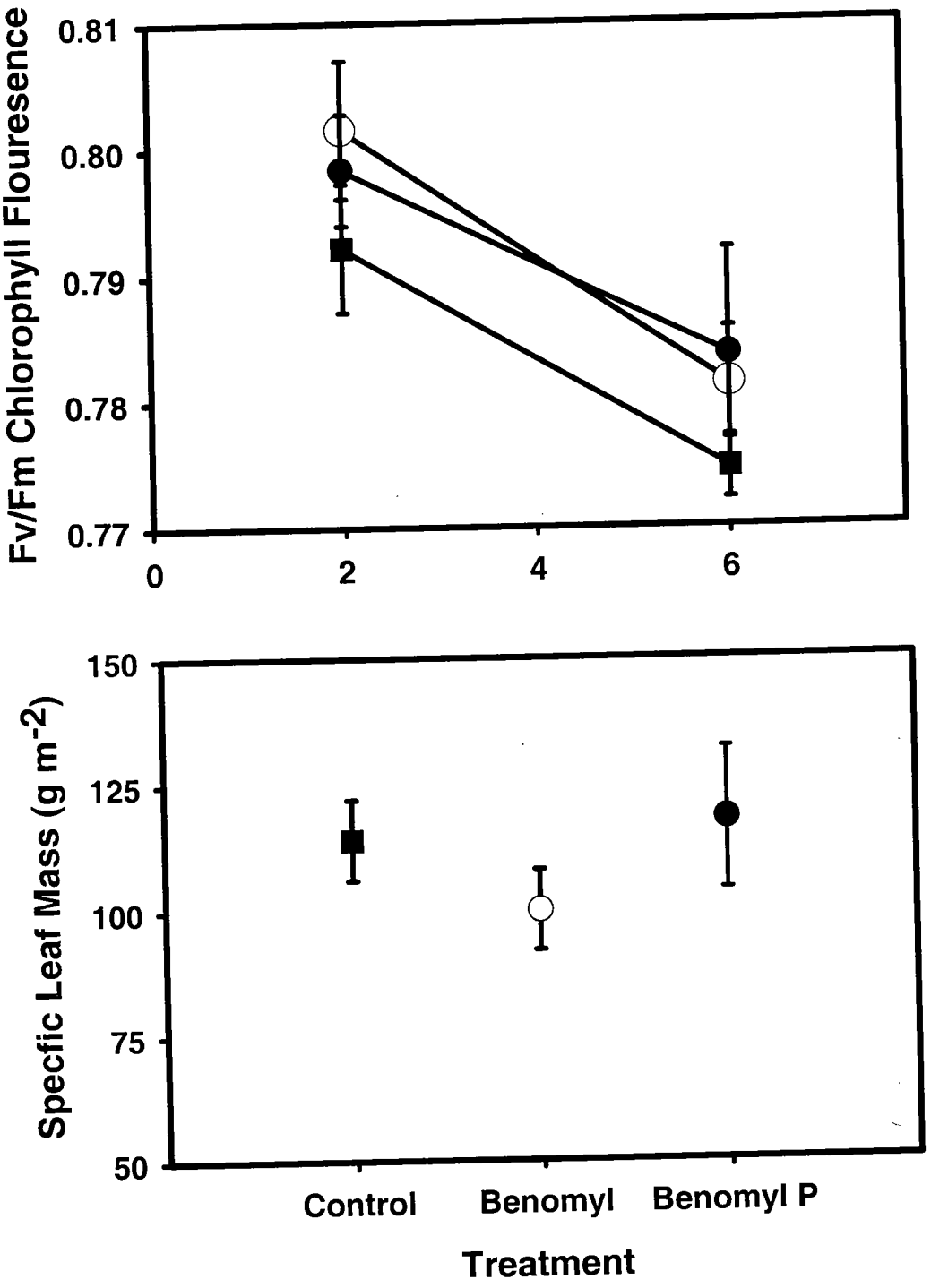


Figure 4: Root and Soil Distribution In Test Plants. Root and soil weights are reported in mg dry weight per cm^3. Water weight is reported in mg of water per cm^3 of soil collected as determined through removal by centrifugation.

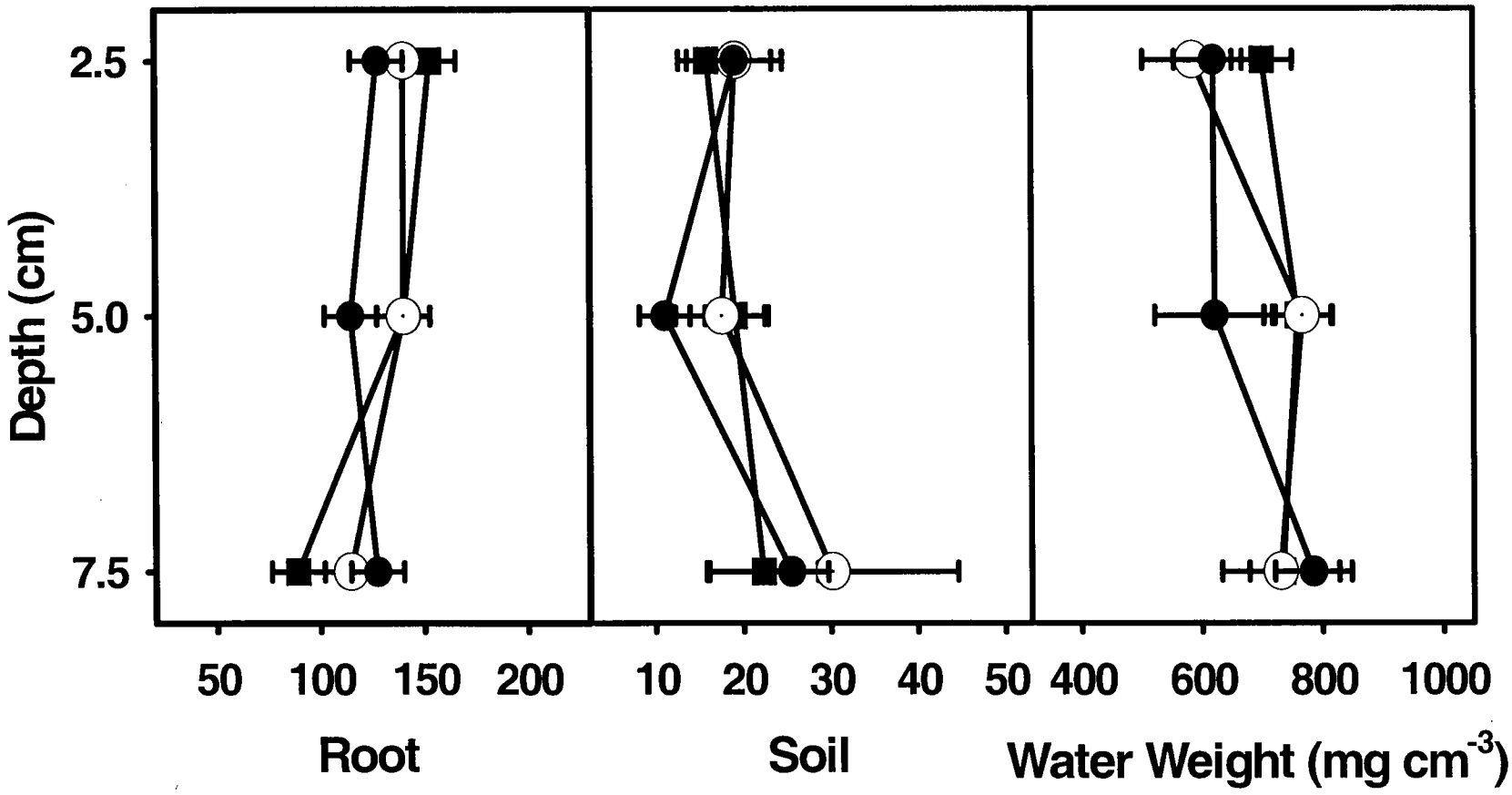


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Figure 5: VAM Colonization of *Spartina patens*. Typical colonization of *Spartina patens* roots. Hyphae on outer root surface are visible and indicated by ▶▶, while ▶ denotes hyphal coiling within root cortical cells.

Nitrogen-fixing Microbial Populations. Using restriction enzyme *Rsa*I, differences in the RFLP patterns of *nif*H amplification products were not detected within pots. Rhizosphere and soil samples were not found to be different within pots, and no differences were noted with depth. Examination between pots also found no differences between treatments, either for rhizosphere or soil populations of nitrogen-fixing organisms. However, differences both within pots and between benomyl and non-treated pots were found using restriction enzyme *Hae*III (Figure 6). For the benomyl treatments, two basic patterns were found within each pot. The first pattern was affiliated with soil at depth 2.5 cm, and the second was associated with rhizosphere material and soil at depths of 5.0 and 7.5 cm. Both benomyl treatments were similar in respect to banding patterns, suggesting that the application of phosphorous did not alter the microbial communities. Other than the observed difference between soil at 2.5 cm and the other soil samples, RFLP patterns of nitrogen-fixing bacteria in soil below 2.5 cm were not affected by depth and were similar to patterns obtained from rhizosphere bacteria. Differences in the intensity of the bands indicate that shifts in the microbial populations may occur (Figure 7), but that only one community is associated with root and soil at the lower depths. Additional patterns were noted in the non-treated pot (Figure 6), and unlike the benomyl treatments, rhizosphere material and soil were associated with different patterns. Nitrogen-fixing bacteria in the rhizosphere at the 7.5 cm depth, displayed RFLP patterns that were unlike those in the rhizosphere at the more shallow depths where colonization was greater.

Examination of differences between treatments confirmed that rhizosphere material from the non-treated pot was associated with a different RFLP banding pattern of nitrogen-fixing bacteria than rhizosphere material from either of the benomyl treatments. Bacteria in the rhizosphere in the non-treated pot at the 2.5 and 5.0 cm depth, where mycorrhizal colonization was greatest, possessed a pattern that was distinct from that of bacteria in the rhizosphere in the benomyl treatments at corresponding depth. At the 7.5 cm depth, where no differences were found between treatments in terms of VAM colonization, RFLP patterns associated with rhizosphere material were more similar to one another, regardless of treatment, than to other rhizosphere material.

Figure 6: Similarity Of N-Fixing Microbial Populations. Pattern analysis using GelManager 1.5 program (Biosystematica). Information about apparent molecular weights of the bands and band spacing were used to construct UPGMA dendograms. Results are for patterns produced with *Hae*III. Similarities between patterns greater than 0.7 are not considered to be different.

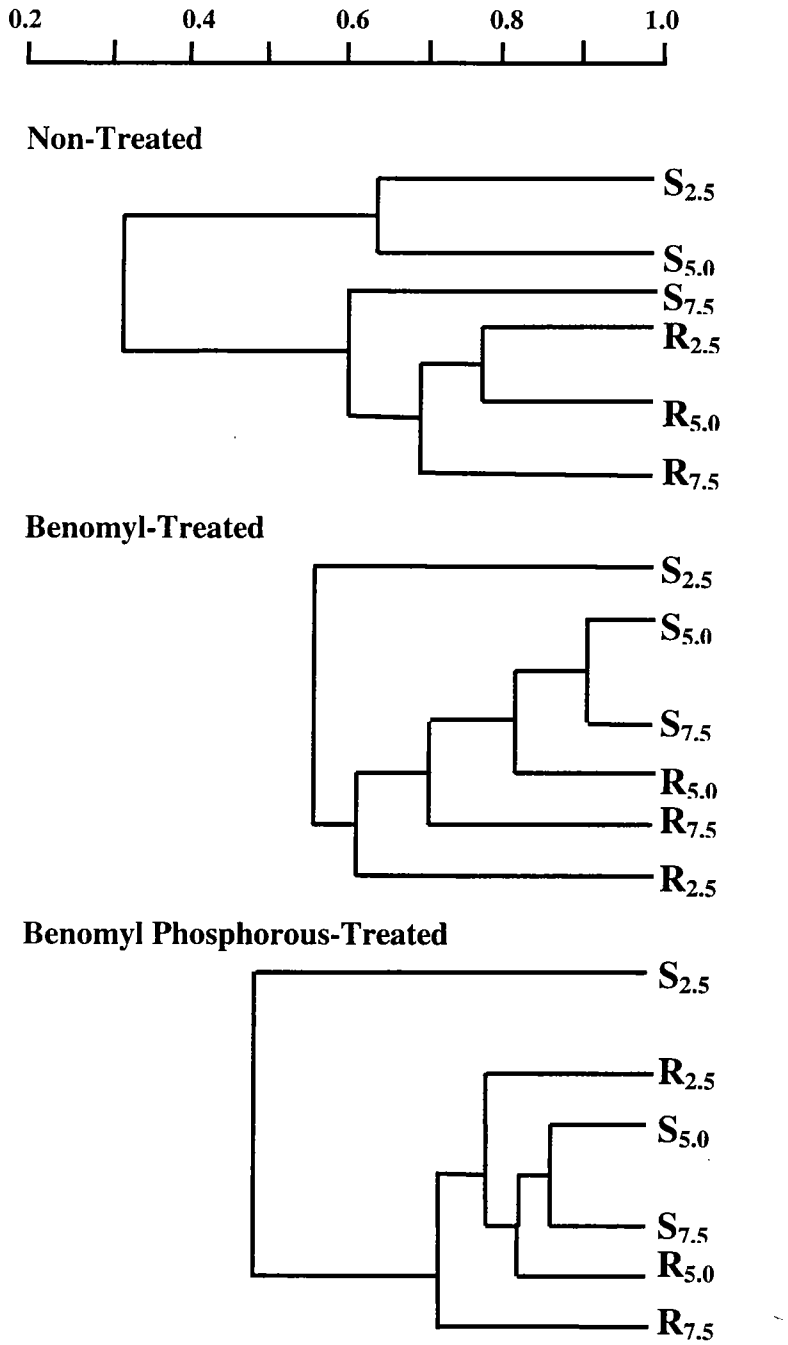
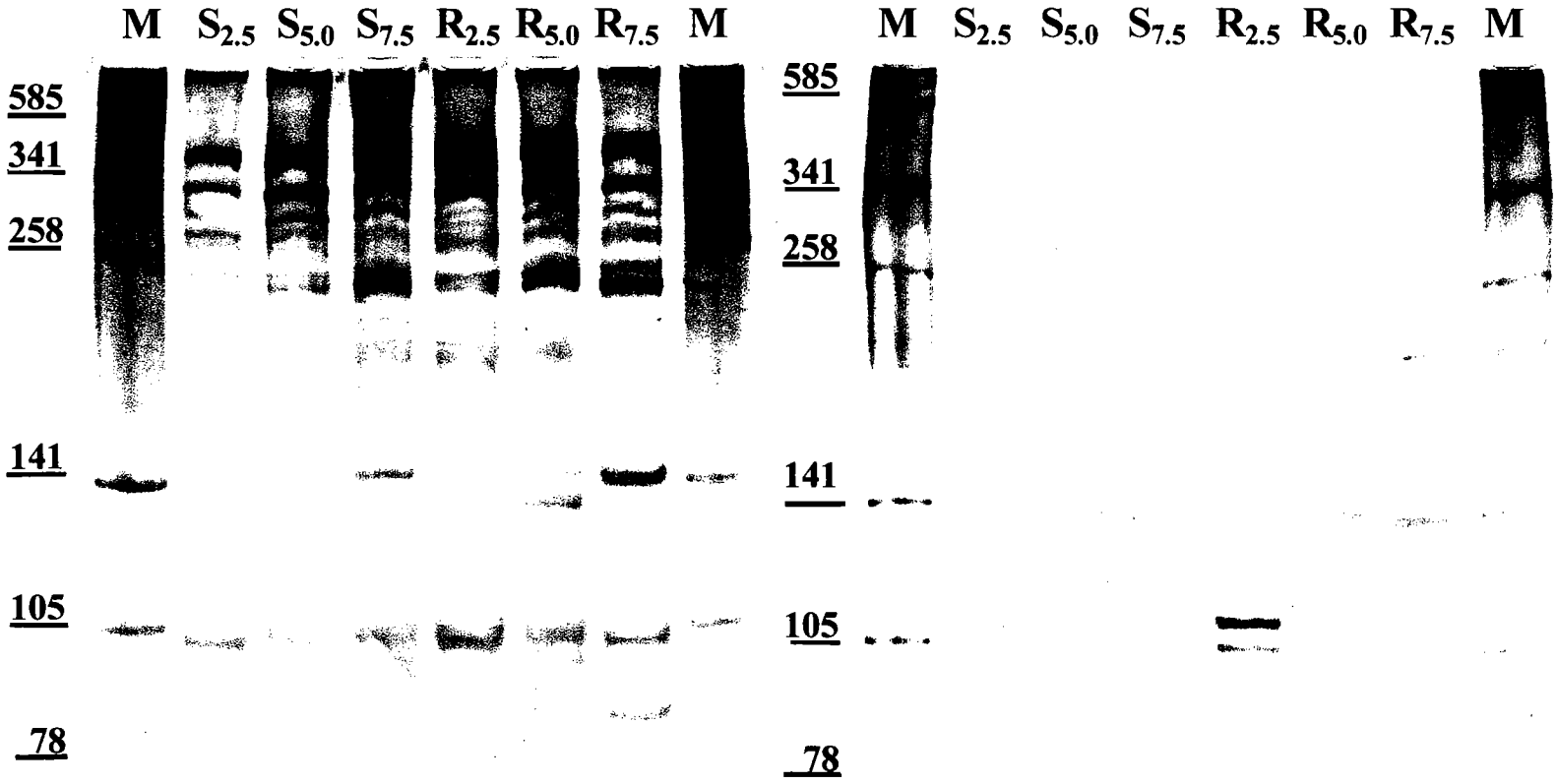


Figure 7: Comparison Of RFLP Patterns Obtained From Benomyl Pots. Restriction enzyme *Rsa*I was used to produce the RFLP on the left while *Hae*III was used to produce the RFLP on the right. The marker used was pUC19 DNA cleaved with *Sau3A*. Molecular weight of marker bands are indicated.



DISCUSSION

Environmental variability in regards to oxygenation of soil cores was found within the rooting zone of test plants as evidenced by both redox potential which declined with depth as well as VAM colonization (Figure 1). Anticipated declines in redox were evident in all treatments but were significantly greater in non-treated pots, probably the result of greater VAM colonization. Corresponding declines in VAM colonization and redox potential in the non-treated pots suggests that either VAM reduce redox potential through respiration or that declining redox lower the ability of the aerobic fungi to colonize root surfaces. Alternatively, the interaction between reduced aeration and fungal use of oxygen for respiration may place a lower limit on root colonization, a limit that we encountered at the 7.5 cm depth. Pore water analysis revealed declines in sulfate availability with depth and increasing redox potential (Figure 2), suggesting that the activity of sulfate reducers increases with declining redox potential. This was not unexpected. However, given the redox and the potential for sulfate reduction, we would have expected to find greater declines in sulfate with depth. The experimental cores were saline as evidenced by the chloride concentrations, a situation we desired in an attempt to replicate natural conditions, but this salinity was maintained in the pots through the application of artificial seawater on a weekly basis. This weekly application would also have provided a regular influx of sulfate into the pore water of the pots, and may partly explain the high sulfate concentrations we encountered.

Although application of the fungicide benomyl was effective at reducing overall mycorrhizal colonization in our test plants from a mean of 25% in the non-treated pots to 7% in the benomyl phosphorous pots (Figure 1), we did not observe any significant differences between treatments for those physiological parameters used. The absence of mycorrhizae did not affect Fv/Fm chlorophyll fluorescence (Figure 3) indicating that the elimination of VAM did not result in any photo-oxidative stress. Plants were grown under greenhouse conditions with low to moderate salinity, a situation that might be considered benign for salt marsh plants, and not likely to adversely affect photosystem II efficiency. These mild conditions are not likely to be encountered under field situations and further testing under more rigorous environmental conditions might reveal positive effects of VAM.

Consequently, it is not surprising that we failed to observe any effect of VAM suppression on plant growth measurements. VAM colonization did not alter the production of root biomass. VAM colonization often results in changes to root architecture, both in terms of the quantity of root biomass produced as well as in the branching pattern of those roots (Hetrick et al. 1988). Unfortunately, since plant cores were collected in June at the beginning of summer, much of the root growth that was to occur in our test plants may have already taken place during the spring in the presence of VAM. We did see suggestive, although not significant, differences in terms of specific leaf area (Figure 3). The benomyl treatment had lower specific leaf areas than did either the non-treated or benomyl-phosphorous plants, which were comparable. Since we did not measure microbial activity, it is speculative to say that increased phosphorous supply had the effect of increasing the activity of N-fixing bacteria in the root cores, therefore making N more available to the plant. In any case, changes in specific leaf area did not affect photosynthesis. A longer study duration begun prior to active plant growth would be more useful for answering this question.

Benomyl was effective in suppressing VAM colonization, especially at the 2.5 cm depth, but the extent of the suppression varied greatly between the pots within a given treatment (Figure 1). As a result of the variability in VAM colonization encountered within treatments, pots chosen for study of N-fixing populations were selected for comparable root zone environmental conditions but large differences in VAM presence. In this fashion, the effect of VAM upon those populations could be extracted and more easily disentangled. Although redox declined in the rooting zone of the non-treated plant, falling from –122.7 at 2.5 cm to –199.6 at 7.5 cm, similar declines were noted for the benomyl plants. In addition, declines in redox potential did not appear to strongly influence the N-fixing community as evidenced by the high similarities between patterns associated with rhizosphere and soil samples at 5.0 and 7.5 cm in the benomyl pots. If oxygenation of the rooting zone greatly influenced populations below the 2.5 cm depth, marked differences should have been observed between rhizosphere and soil populations since oxygen would have been more available to microbes physically attached to root surfaces. Changes were noted between the 2.5 cm depth and the others, indicating that redox potential did have some affect on the population structure of soil microbes. But the