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USGA Executive Summary

Donald Y. Kobayashi and Bruce B. Clarke. Identification of Parasitic Bacteria as Biological Control Agents Against Summer Patch Disease. Rutgers University, Department of Plant Pathology, New Brunswick, NJ 08903.

Executive Summary

A fungal trapping method and an enrichment culture method were used to isolate several hundred bacteria from turf and soil sources. These isolates were screened in a greenhouse/growth chamber assay for the suppression of summer patch disease, caused by *Magnaporthe poae*, on Kentucky bluegrass. At least eight bacterial isolates were identified that were capable of consistently suppressing summer patch symptom development by greater than 50% compared to fungal-inoculated control plants. All eight isolates expressed activity of one or more of the extracellular enzymes chitinase, glucanase, protease and lipase. In addition, all eight isolates were capable of colonizing and persisting in the rhizosphere of Kentucky bluegrass at significant populations.

Two bacterial strains, *Xanthomonas maltophilia* 34S1 (Xm34S1) and *Serratia marcescens* 9M5, were capable of suppressing summer patch by greater than 70% and 50%, respectively, compared to disease in untreated control plants over a 3 week period. The rates at which disease progressed in plants treated with bacteria were not different compared to untreated plants; however, disease onset was significantly delayed in bacteria-treated plants. These results were interpreted to reflect the activity of antagonistic bacteria in reducing pathogen colonization of turfgrass roots. Disease onset was delayed for an extended period when Xm34S1 was applied to plants on a repeated schedule. Rhizosphere populations of Xm34S1 indicated that disease suppression was associated with populations between $>10^5$ and $>10^7$ cfu/g rhizosphere sample.

Xm34S1 was applied to pathogen-inoculated field plots in 1994 and 1995. Summer patch suppression was not observed in field trials in either year. High disease pressures and insufficient population establishment of Xm34S1 were attributed to the lack of performance in the field. Extensive field population studies in 1995 indicated that populations of Xm34S1 were maintained above 10^5 cfu/g rhizosphere sample throughout the entire summer season, but did not reach populations above values of 10^7 cfu/g rhizosphere sample. These populations were clearly 10-fold lower than the critical population values established in growth chamber studies.

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Title: Identification of parasitic bacteria as biological control agents against summer patch disease.

Specific Research Objectives:

1) To isolate and identify bacteria which can colonize and parasitize the mycelia of *Magnaporthe poae*, the causal agent of summer patch disease.

2) To screen bacteria isolated in objective one for disease control potential using controlled growth chamber and field studies.

INTRODUCTION

Summer patch disease is caused by the ectotrophic, root-infecting fungus, *Magnaporthe poae*. The disease is extremely damaging to turfgrass, affecting cool-season varieties under conditions of high soil temperature and high water potential. Disease development is enhanced by conditions that contribute to turfgrass root stress, such as low mowing heights or compacted soil. Symptoms of the disease are observed as patches of foliar necrotic areas, which will coalesce under severe disease conditions. In addition to aesthetic problems, summer patch is also problematic due to its damaging effect in playability on recreation turf. These problems become of greater economical importance when diseased areas are rapidly colonized by resistant, less desirable species of grass or weeds.

Demands for high quality turf results in control measures for summer patch and other diseases that rely heavily on regular applications of fungicides. Alternative control methods, such as genetic resistance, has not yet been developed in desirable grass varieties. Some management practices are known to alleviate symptom development, and strategies to integrated these into current management practices are in progress. Nonetheless, alternative disease control approaches are warranted, but have not been thoroughly investigated. We have initiated an intense investigation to determine the potential use of bacteria as biological pesticides for the control of summer patch. Several strategies for the identification of antagonistic bacteria were taken, including studying bacteria that expressed antibiotics, bacteria capable of colonizing and persisting in the turfgrass rhizosphere, and bacteria that had been previously described as biocontrol agents of agronomic diseases similar to summer patch on turfgrass (Thompson et al. 1995a; Thompson et al. 1995b). Suppression of summer patch by bacteria in pathogen-inoculated field plots were demonstrated in two separate studies (Thompson et al., 1995a,

Thompson et al., 1995b), providing encouragement for the use of bacteria as biocontrol agents in controlling summer patch disease.

Funding by the USGA was used to support studies investigating bacteria capable of lysing or parasitizing the summer patch causal agent, *M. poae*, as biocontrol agents. This particular approach is unique from the other strategies described above in that parasitic bacteria are proposed to function in disease suppression by directly reducing pathogen inoculum.

RESULTS

Isolation procedure and characterization of bacteria.

Two methods were used in attempts to isolate bacteria with lytic or parasitic capabilities to *M. poae*. The first procedure used mycelia of *M. poae* as bait in soils to "trap" bacteria that attach to the fungus (Kobayashi, et al., 1995). The second method involved the development of an enrichment culture procedure (Kobayashi and El-Barrad, 1995). This procedure involved culture growth in a minimal medium supplemented with the mycelium of *M. poae* as the sole carbon source. As bacterial growth increased, an aliquot was transferred to fresh medium, also supplemented with *M. poae* mycelium, enriching for isolates capable of utilizing the fungus as a carbon source. Bacteria capable of growing in these cultures were suspected as antagonists of *M. poae*.

Over 1000 bacterial isolates were isolated and tested in a greenhouse/growth chamber assay for the suppression of summer patch disease. At least 8 candidate bacteria were identified that were capable of consistently suppressing summer patch by at least 50% compared to fungal-inoculated control plants (Kobayashi et al., 1995; Kobayashi and El-Barrad, 1995). Seven of the eight isolates were identified as common soil bacteria belonging to the genera, *Bacillus* (1 isolate), *Serratia* (4 isolates), *Klebsiella* (1 isolate) and *Xanthomonas* (1 isolate). One isolate (N4-7) could not be identified by conventional bacteriological tests or by commercial tests with database searches using Biolog (Microlog, Inc., Hayward, CA) or fatty acid analysis (MIDI, Newark, DE). Sequencing of 16s ribosomal region of N4-7 also proved negative for a conclusive species match with previously characterized organisms, suggesting that this isolate is a newly described bacterium. Based on similarity values from fatty acid analysis and 16s ribosomal sequencing, N4-7 appears to be closely related to the genera, *Xanthomonas* or *Flexibacter*.

The eight bacterial isolates were further characterized for their ecological competence within the turfgrass rhizosphere, as well as the expression of traits antagonistic to the fungal pathogen. All eight isolates were capable of colonizing the turfgrass rhizosphere at significant populations. Most isolates were capable of maintaining populations above 10^4 cfu/g tissue (fresh weight) up to two weeks after application (Kobayashi et al., 1995; Kobayashi and El-Barrad, 1995). The bacteria were also characterized for in vitro antifungal activity against *M. poae*, and the production of extracellular degradative enzyme activities. Seven of the eight isolates produced chitinase activity, as detected by diffusion clearing zones when colonies were plated

on colloidal chitin. One isolate, N4-7, also produced ß-1,3-glucanase activity. These enzymes degrade the substrates chitin and ß-1,3-glucans, which are the major cell wall components of *M. poae*.

Progression of summer patch disease in growth chamber assays.

Root mass vs. foliar symptom development. Infection of turfgrass roots by the summer patch pathogen occurs when the fungus penetrates the root surface, grows through the cortex and colonizes the vascular tissue. Although infection occurs in the roots, summer patch disease is usually evaluated by ratings based on levels of foliar symptom production. We are currently in the process of developing a better disease quantifying assay by assessing infection on roots. However, to satisfy the question of the relationship between foliar symptoms and root mass, a direct correlation between turfgrass root mass and foliar symptom development was established. This was determined using typical containers of Kentucky bluegrass set up with varied amounts of inoculum of *M. poae* to insure a wide spectrum of disease symptom production. Plants were allowed to incubate under normal conditions established as part of the growth chamber assay as previously described (Kobayashi et al., 1995). After 4 weeks incubation under disease conducive conditions, disease was recorded in each container. Total roots were then recovered, thoroughly washed and placed in an drying oven prior to recording the dry weight. Regressions were performed between root weight and foliar disease symptom ratings. A linear relationship was observed for studies using two different *M. poae* isolates, indicating a direct, inverse relationship between foliar symptom development and root mass (Fig. 1).

After manipulating several parameters, consistency in summer patch development in Kentucky bluegrass var. Baron was achieved in growth chamber assays. Foliar symptom development, when plotted as percent necrosis over time, followed a sigmoidal-shaped curve typical of diseases caused by pathogens that follow an exponential growth pattern. As part of efforts to develop consistency in growth chamber assays, three factors were found to greatly influence disease development. These included depth of fungal inoculum placement, seed density, and fungal inoculum density.

Fungal Inoculum. The influence of *M. poae* inoculum density and foci number on disease was studied. Increasing *M. poae* inoculum density or foci did not influence the rate at which summer patch disease progressed. However, disease onset increased as inoculum density increased (Fig. 2). Disease onset also increased with increasing inoculum foci (Fig. 2). These results were interpreted to reflect the influence of increased inoculum density and foci on pathogen colonization of turfgrass roots, which in turn resulted in faster foliar symptom development.

Depth of inoculum placement. The depth at which *M. poae* inoculum was placed greatly affected disease development. Similar to fungal inoculum concentrations, the rate at which disease progressed did not change as depths at which inoculum was placed were varied. However, as the distance between fungal inoculum and seeds were increased, delays in disease onset were increased (Fig. 3). These observations were interpreted to reflect that closer

placement of fungal inoculum to seeds allowed for faster colonization of roots by the pathogen, and thus rapid disease development.

Seed density. Seed density also affected summer patch development. In general, disease appeared more consistently and rapidly when seed densities were increased (Fig. 4). These observations, which are common for soilborne diseases, were interpreted to reflect the influence of increased root densities (resulting from higher seed densities) on rapid colonization of roots by *M. poae* due to the higher probabilities of initiating contact with the fungal inoculum in the soil.

Results from inoculation density, depth of inoculum, and seed density studies indicated that disease appeared to be contingent upon colonization of roots by the fungus. These factors influenced disease not by altering the rate of disease progression, but by changing the time of disease onset. Consequently, strong evidence is provided that the prevention of *M. poae* colonization of turfgrass roots prior to infection should significantly reduce summer patch disease.

Characterization of bacterial agents.

The primary selective trait of all bacteria identified in this study was the production of extracellular enzymes that could degrade the cell wall and outer membrane of the fungal pathogen. However, all bacteria capable of suppressing summer patch disease were also capable of colonizing the turfgrass rhizosphere in high populations, regardless of the source from which the bacteria originated (Kobayashi et al., 1995; Kobayashi and El-Barrad, 1995). These observations provided evidence that support the importance of rhizosphere colonization by biocontrol agents in summer patch suppression.

Two bacteria isolated for their summer patch suppressive abilities were *Xanthomonas maltophilia* 34S1 (Xm34S1) and *Serratia marcescens* 9M5 (Sm9M5). In growth chamber assays, the biocontrol ability of Xm34S1 was more effective than Sm9M5 (Kobayashi et al., 1995). The effect of different concentrations of these bacteria on disease suppression was studied (Kobayashi et al., 1995). Greater disease control was observed with increasing concentrations of Xm34S1. Surprisingly, the optimal concentration of Sm9M5 for disease suppression was not the highest concentration of bacteria tested, suggesting a deleterious effect on disease control with too high a bacterial concentration. Similar to factors influencing summer patch symptom development, comparisons of disease progression curves indicated that effective bacterial cell concentrations did not change the rate of disease progression, but instead delayed the onset of the disease, most probably by reducing fungal inoculum density and thus preventing initial colonization of turfgrass roots by the pathogen.

Application of Xm34S1 and Sm9M5 to turfgrass appeared to shift disease progress curves, resulting in a delay of disease onset (Kobayashi et al., 1995). Based on these observations, it was predicted that repeated applications of bacteria in effective concentrations should continuously delay disease onset, and thus result in extended high level disease

suppression. To test this hypothesis, Xm34S1 was applied to plants every week and every two weeks throughout an 8 wk period, and were compared to a single application two weeks after planting. High levels of summer patch suppression was extended to times when disease values reached close to maximum in fungal-inoculated controls (Fig. 5). Plants treated with Xm34S1 every two weeks showed less than 30% necrotic areas eight weeks after planting, compared to untreated control plants, which had reached maximum disease levels. Surprisingly, plants treated on a weekly schedule enhanced disease, indicating that high concentrations of bacteria resulted in a deleterious or phytotoxic effect. These results are consistent with deleterious effects of high concentrations of bacteria, as mentioned above with Sm9M5. Populations of Xm34S1 in the rhizosphere of turfgrass indicated that values were continuously raised above 10^7 cfu/g (fresh weight) rhizosphere sample in treatments receiving repeated application. In comparison, populations of Xm34S1 resulting from standard inoculations decreased to between 10^5 and 10^6 cfu/g sample (Fig. 6). These observations indicated that Xm34S1 populations need to be established above 10^7 cfu/g tissue for disease suppression to occur.

Results from field studies.

Disease suppression studies using various bacterial biocontrol agents in pathogen-inoculated field plots have been conducted since 1990. Although yearly adjustments have been made to improve disease induction, optimal conditions for field studies still have not been determined. For example, disease did not occur in field plots during the summer of 1993. It was apparent that data from the summer of 1990 indicated that high fungal inoculum contributed to high disease pressures that made control by biological agents extremely difficult. However, a superior method for rating summer patch disease in pathogen-inoculated field plots has been developed, and field suppression of summer patch has been demonstrated using bacterial isolates obtained in previous studies (Thompson et al., 1995a; Thompson et al., 1995b).

Xm34S1, Sm9M5 and a *Serratia* sp. isolate N2-4 (described in Kobayashi and El-Barrad) were applied to pathogen-inoculated field plots in 1994. These plots were located in low management, but high disease pressure, areas of the Rutgers Turfgrass Farm in New Brunswick, NJ. No disease suppression was observed when any bacteria-treated plots were compared with fungal-inoculated field plots. Although sufficient bacterial population data was not obtained for 1994, it was suspected that high disease pressure and insufficient bacterial populations contributed to the lack of disease suppression by the bacteria. These observation were supported by previous field studies (e.g., Thompson et al., 1995), which strongly suggested that high disease pressure resulted from first year pathogen inoculations. The high disease pressure is thought to be due to the high density of pathogen inoculum placed at a single site. Subsequent field studies conducted at sites inoculated with the pathogen in previous years appear more true to field situations, since the pathogen inoculum is presumed to originate from diseased tissue from previous years.

Application of Xm34S1 to field plots were repeated in 1995. In attempts to improve turfgrass rhizosphere populations of Xm34S1, the bacterium was applied on a weekly schedule and were compared to applications every other week. Data has not yet been fully analyzed,

although initial observations suggest that no disease suppression occurred in treated plots compared to pathogen-inoculated control plots. Bacterial populations monitored in the field during the 1995 summer season revealed that, although populations could be maintained between 10^4 cfu/g and 10^7 cfu/g (fresh weight) rhizosphere sample throughout the summer (Fig. 7), it is apparent from growth chamber studies that populations are 10-fold lower that levels necessary to achieve disease suppression (Fig. 6).

RESULTS OF RELATED STUDIES

Mechanisms of biological control

In addition to basic biological characterization of bacterial antagonists, studies involving the molecular characterization of biocontrol traits in Xm34S1 and N4-7 have been in progress. Both Xm34S1 and N4-7 are proposed to function in suppressing summer patch primarily by reducing *M. poae* inoculum through the expression of extracellular enzymes. Particular genes of interest include chitinases, ß-1,3-glucanases, proteases and lipases, all of which are capable of degrading different cell wall components of *M. poae*. The genetic characterization of each of these genes are essential to evaluate the role of each enzyme in the biocontrol activity of the organism. Genes for chitinase, protease and at least two different lipase activities have been cloned from a genomic library of Xm34S1. Molecular characterization of the cloned chitinase gene, designated *chiX*, from Xm34S1 indicated the gene encoded a protein product of a predicted length of 535 amino acids. An insertional mutation, created in a cosmid clone carrying this gene, was reintroduced into Xm34S1 as a site-directed mutation. Complete loss of chitinolytic activity was obtained by the mutant strain, as determined by complete loss of diffusion clearing zones on chitin-amended media, providing strong evidence that the *chiX* gene is completely responsible for chitinolytic activity observed by Xm34S1. Disease studies indicated that the *chi*⁻ mutant isolate, C5, did not suppress summer patch to the same level as the wildtype (Fig. 8), indicating that the chitinase activity produced by Xm34S1 is important in suppression of summer patch. However, C5 was still capable of suppressing disease at significant levels compared to pathogen-inoculated control plants, indicating that there are clearly other traits involved in biocontrol by this organism. Root colonization ability along with protease and lipase activities detected in Xm34S1 may clearly be involved with these observations. Each of these activities have been cloned from the genomic library of Xm34S1. Molecular and biochemical characterization of lipase activity suggests at least two forms of activity based on the reactions when screened on the substrate Tween 80. These activities have been separately cloned, and the nucleotide sequence of the gene with major lipase activity is currently being determined. A protease activity was also cloned from Xm34S1, in which five different cosmid clones were recovered from the genomic library of 34S1. Restriction map analysis of the five clones indicated a similar banding pattern that suggested all five clones harbor the same gene. No other protease activity was observed. Marker exchange mutagenesis will be performed using this clone.

In addition to Xm34S1, molecular characterization of lytic enzymes produced by N4-7 is also in progress. Three cosmid clones expressing lipase activity have been identified from

a genomic library of N4-7. The 3 clones differed according to their restriction patterns. In addition, a single clone expressing protease activity, and several clones expressing at least 3 different ß-1,3-glucanase activities have been cloned from N4-7. Similar experiments to those on Xm34S1 will be performed on N4-7.

We are currently in the process of conducting an extensive study on the role of chitinase in Xm34S1 on soil survival. These types of ecological studies will also be conducted with mutants of protease and lipase activities in efforts to identify conditions important in ecological fitness and biocontrol ability. Understanding of the roles of each of these traits, and the conditions by which they are expressed, will provide valuable information into manipulating the environment to achieve better performance by biocontrol agents.

REFERENCES

Kobayashi, D.Y., Guglielmoni, M. and Clarke, B. 1995. Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. Soil Biol. Biochem. 27:1479-1487.

Kobayashi, D.Y. and El-Barrad, N. 1995. Selection of bacterial antagonists using enrichment cultures for the control of summer patch disease of Kentucky bluegrass. Cur. Microbiol. In press.

Thompson, D.C., Clarke, B., and Kobayashi, D.Y. 1995a. Evaluation of bacterial antagonists for the reduction of summer patch symptoms in Kentucky bluegrass. Plant Dis. 32 p. (accepted with revisions).

Thompson, D.C., Kobayashi, D.Y., and B.B. Clarke. 1995b. Isolation of turfgrass rhizosphere competent bacteria for the suppression of summer patch and their establishment on Kentucky bluegrass. submitted to Soil Biology and Biochemistry.

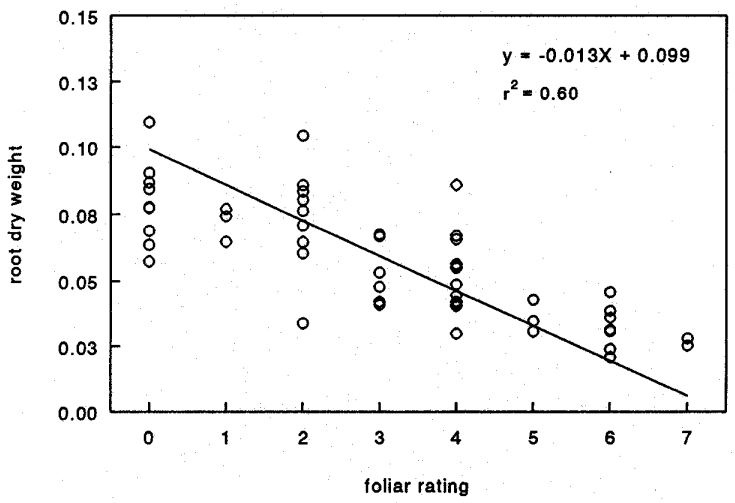

Figure 1. Relationship of summer patch foliar symptoms and root mass of infected Kentucky bluegrass cultivar Baron. The equation for the regression line is given. Foliar rating is based on a nine point rating scale described in the text. Root dry weight is the total weight of roots from a single container of 8 week old plants.

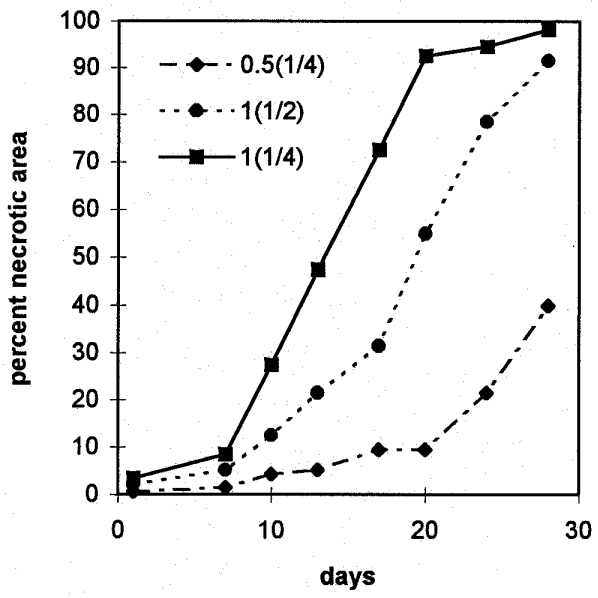

Figure 2. Disease progression curves of summer patch in containers inoculated with different concentrations and inoculum foci of *Magnaporthe poae*. Initial inoculum consisted of a 4mm plug of *M. poae* grown on potato dextrose agar cut into quarters (1/4) or halves (1/2). 0.5(1/4), is an inoculum density of a half agar plug cut into two quarters for two inoculum foci. 1(1/2), is an inoculum density of 1 plug, cut into 2 half pieces as inoculum foci; 1(1/4) is an inoculum density of 1 plug, cut into 4 pieces as inoculum foci. Days refer to the length of time plants were incubated in the growth chamber under disease conducive conditions.

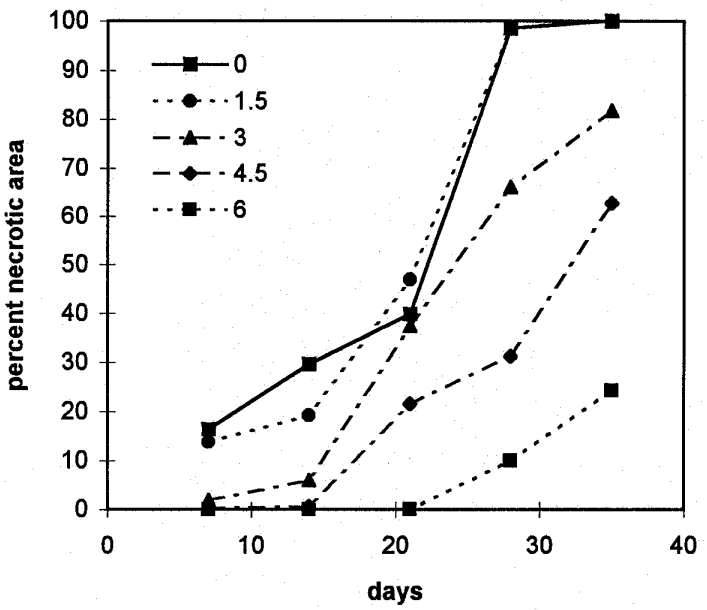

Figure 3. The effect of the depth of inoculum placement on summer patch disease development. *Magnaporthe poae* inoculum was placed at the level at which Kentucky bluegrass var. Baron seeds were sown (0); 1.5 cm below seeds (1.5); 3 cm below seeds (3); 4.5 cm below seeds (4.5); and 6 cm below seeds (6). Disease was rated in plants based on the percentage of foliar necrosis in each container (percent necrotic area) over a 5 week period once plants were moved to the growth chamber (days).

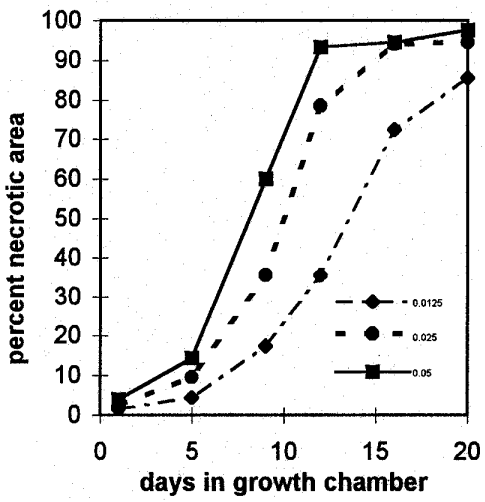

Figure 4. The effect of Kentucky bluegrass var. Baron seed density on summer patch development. Kentucky bluegrass seed densities of 0.0125 g/container (ca. 30 seeds), 0.025 g/container (ca. 60 seeds) and 0.05 g/container (ca. 120 seeds) were sown into containers inoculated with *Magnaporthe poae*. Disease was rated once symptoms began to appear and continued until disease reached maximum values.

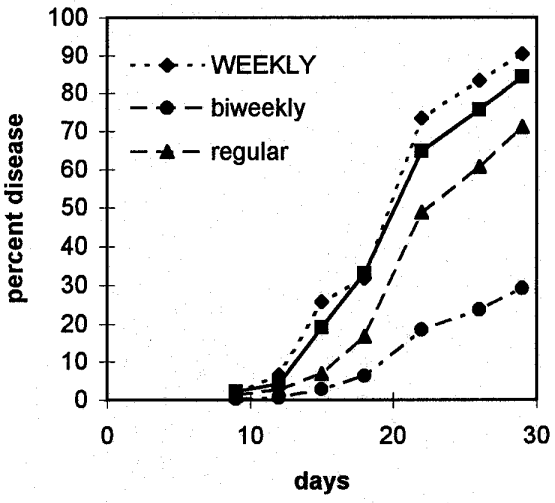

Figure 5. The effect of repeated application of *Xanthomonas maltophilia* 34S1 on summer patch suppression in Kentucky bluegrass var. Baron. *X. maltophilia* 34S1 was inoculated as a single inoculation (regular), once a week (weekly), and once every two weeks (biweekly) over an eight week period. Containers were moved to the growth chamber 4 weeks after seeding to disease conducive conditions, and disease was rated twice a week until control plants reached maximum values.

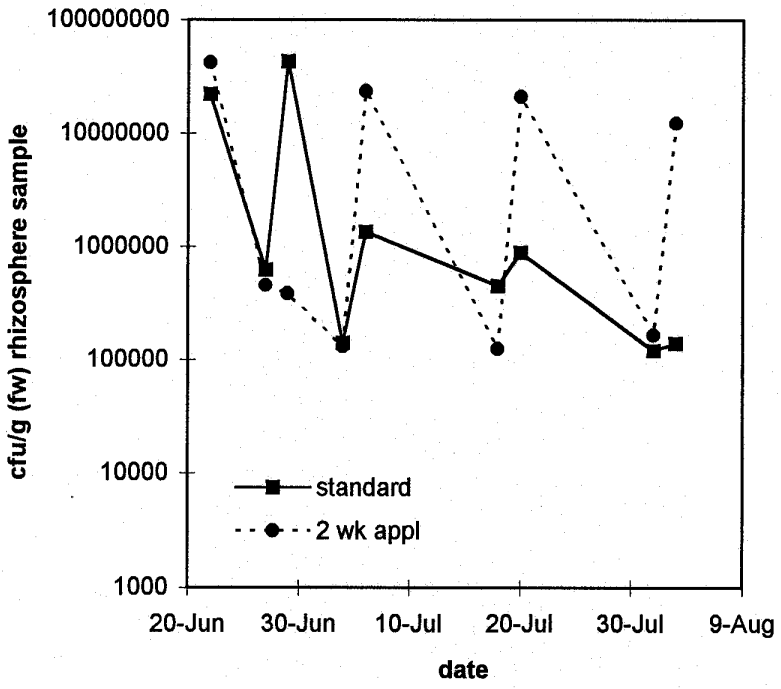

Figure 6. Rhizosphere populations of *Xanthomonas maltophilia* 34S1 repeatedly applied to Kentucky bluegrass var. Baron. *X. maltophilia* 34S1 (Xm34S1) populations applied on weeks 2 and 3 after seeding were compared to populations of Xm34S1 in the rhizosphere of Kentucky bluegrass treated every two weeks (2 wk appl).

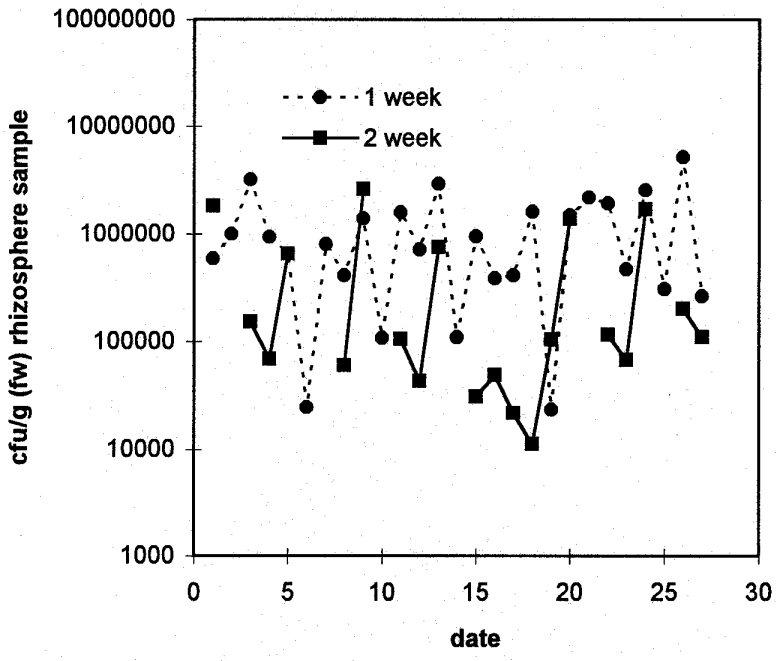

Figure 7. Rhizosphere populations of *Xanthomonas maltophilia* 34S1 in field plots of Kentucky bluegrass in 1995. Populations of weekly applications (1 week) were compared to applications every two weeks (2 week).

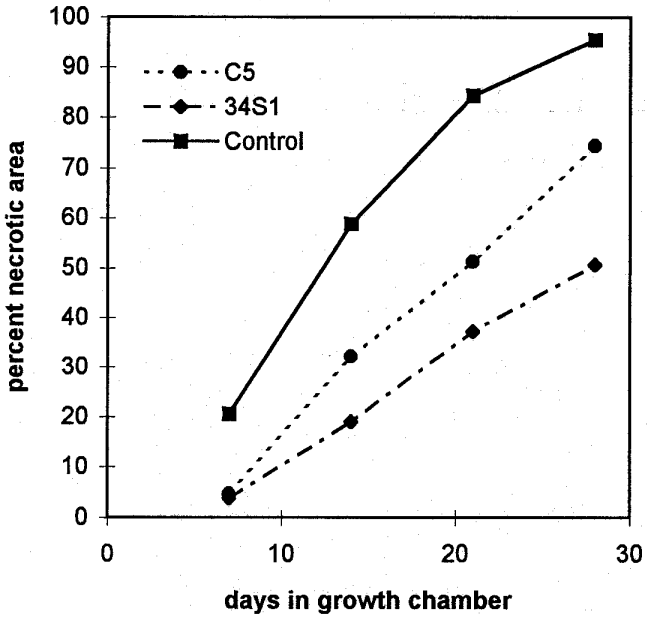

Figure 8. Summer patch suppression by *Xanthomonas maltophilia* 34S1 and the mutant C5 deficient in chitinase production on Kentucky bluegrass var. Baron. Plants were treated with the wildtype *X. maltophilia* 34S1 in standard growth chamber assays, and compared with the *chi*⁻ mutant C5 and untreated disease control plants in growth chamber assays.