1993 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.

Summer patch disease, caused by the root-infecting fungus *Magnaporthe poae*, is a devastating disease of cool-season grasses. The disease is especially prevalent within the northeastern, midwestern and western United States. Current control methods include systemic fungicides used on a preventive basis, combined with intensive management practices that reduce conditions conducive for the disease. We are interested in selecting bacteria, parasitic to *M. poae*, as a biological complement to the use of chemical pesticides to control summer patch. The bacteria were obtained from soils known to harbor natural populations of *M. poae*. Bacteria that possessed traits suggestive of a parasitic relationship with this pathogen were selected and screened to determine their potential as biocontrol agents. The methods used to isolate bacteria involved a fungal baiting method and enrichment cultures designed to enhance the probability of identifying parasitic bacteria. The baiting method involved burying the fungus in soil thought to contain bacteria of interest. After incubating for one to three days, the fungus was recovered, vigorously washed and plated onto agar media to promote growth of bacteria which had attached to the fungus. Bacteria obtained by this method were indicative of isolates that may be parasitic to *M. poae*. The enrichment culture method utilized a liquid growth medium supplemented with the fungus as the nutrient source. Bacteria able to grow in this medium were suggestive of isolates that can specifically utilize the fungus as a growth substrate, and thus should represent parasitic organisms. For both methods, the ability of bacteria to express enzymes capable of degrading chitin, the major cell wall component of *M. poae*, was used as a secondary selection parameter. This provided an additional trait in the selection of potential parasitic organisms. Bacteria identified by these criteria were evaluated in growth chamber studies for their ability to suppress the development of summer patch disease on Kentucky bluegrass. The bacteria were applied as soil drenches to turf that had previously been inoculated with *M. poae*. Several isolates were identified that significantly reduced summer patch symptom development. Two isolates, a *Xanthomonas maltophilia* and a *Serratia marcescens*, suppressed foliar symptom development by 70% and 55%, respectively. Populations of these isolates were not found to persist in soil for long periods of time; however, both isolates were found to colonize the turfgrass roots at significantly high levels. This suggests that these bacteria persist in the region where infection occurs. Several other bacteria identified as potential disease suppressors are being tested for their ability to suppress summer patch symptom development in field experiments.

1993 USGA Annual Report

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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.

RESEARCH TO DATE

Isolation of Potential Bacterial Antagonists

Fungal Baiting Method. A previously described fungal baiting method was modified and used for the isolation of bacteria potentially antagonistic to *M. poae*. The baiting method utilized growth of the fungus on a membrane with inert properties such as nylon mesh. However, attempts to grow the fungus on nylon mesh were unsuccessful; consequently, identification of an alternative membrane was necessary. The use of cellophane proved useful, since the membrane was easily sterilized, nutrients from agar plates diffused through the membrane readily to allow growth of the fungus, and the membrane remained intact when incubated in soil. Several different soil sources, ranging geographically from New Jersey and West Virginia, were utilized for the isolation of potential antagonistic bacteria. All soil sources were known to harbor the fungal pathogen either at the time of sampling, or in recent years. Many of these soil sources were suppressive to the disease. Soil suppressiveness is a natural phenomenon where the pathogen is present but disease does not occur. Soils of these types are suggestive of active microbial populations that function to debilitate or out compete the pathogen, thus preventing the growth of the pathogen and the occurrence of disease. These types of soils provide ideal sources for isolating bacteria that are potentially antagonistic to the fungus.

Fungal mycelia grown on cellophane membranes were cut into squares and buried in various soil sources. After 24-72 h of incubation, the mycelial squares were recovered and washed in distilled water for several hours to remove soil and loosely adhering bacteria. The squares were then rewashed in sterile distilled water and

placed, mycelium side down, onto rich agar plates to allow the growth of bacteria still remaining attached to the mycelium. Such bacteria are indicative of isolates that can strongly adhere to the fungus, and thus are potentially parasitic to the organism. After 24 hours of incubation, the mycelial squares were removed and incubation of the plates were continued until single bacterial colonies could be easily identified and transferred to a new agar plate. Over 150 individual bacteria were isolated using this method. Each of these isolates were then tested for the ability to grow on a minimal agar medium supplemented with chitin, which is the major cell wall component of *M. poae*. The utilization of chitin as a sole carbon source was selected as a second screen to increase the potential of identifying bacteria that can destructively parasitize the fungus. Of the tested isolates, 27 were found to grow on the chitin plates. All 27 formed clearing zones in the chitin-amended agar. These clearing zones represented the activity of chitinase, for which evidence provided by several studies have indicated its role in the biological control of many plant diseases. Each of the 27 isolates were also tested for their ability to suppress summer patch symptom development. Two isolates, 21C6 and 9M5, were found to significantly suppress summer patch symptom development in growth chamber assays. The Biolog redox chemistry identification system was use to identify isolate 21C6 as *Xanthomonas maltophilia* and isolate 9M5 as *Serratia marcescens*. Characteristics of these isolates are given in Table 1.

Enrichment Culture Method. The fungal baiting method yielded two potentially useful bacteria for disease suppression capabilities, as demonstrated in growth chamber studies. However, isolation of bacteria using this method often proved difficult in the identification and selection of single unique colonies. The potential to miss significant, yet slower-growing or lower populating isolates was possible. Therefore, an alternative method was devised to identify potentially antagonistic bacteria. This method combined the fungal baiting method with enrichment cultures commonly used to select for bacteria that can utilize xenobiotic or recalcitrant compounds (Fig. 1). The fungal baiting method was carried out to the step of washing of the mycelium, as described above. After washing, mycelial squares were transferred to sterile water, vigorously shaken and then sonicated to release bacteria adhering to the fungus. The resulting solution was then used to inoculate liquid cultures containing a minimal medium supplemented with *M. poae* mycelium as the sole carbon and energy source. After extended periods of incubation, a small aliquot of the culture was transferred to a new culture supplemented with fresh mycelium. Continued transfers were made to new cultures for several weeks. Growth in these types of cultures were assumed to 'enrich' for bacteria that were parasitic to the fungus.

Ten different soil sources were utilized for the isolation of bacteria using the enrichment culture procedure. The enzyme chitinase catalyzes the breakdown of chitin, the major cell wall component of many fungi including *M. poae*, to its monomeric units. Therefore, chitinase was selected as a specific antagonistic trait to

monitor the percentage of bacteria within cultures that express the ability to degrade the fungus. Similar to the fungal baiting method, chitinase was also used as a secondary screen for the selection of potential biocontrol agents. Over 1000 bacteria were screened for chitinase production from enrichment cultures initiated from the 10 different soil sources. Isolates were selected from several stages within enrichment cultures, in efforts to monitor the appropriate times to recover the appropriate isolates. Frequencies of chitinase producers in a given population within a specific culture ranged from 0% to 85% (Fig. 2). In all tested cases using various soil sources, the frequency of chitinase-producing bacteria were increased from initial percentages by enrichment cultures transfers. However, with prolonged incubations and transfers, frequencies of chitinase producers often fluctuated and sometimes decreased over time (Fig. 2). Several chitinase-positive bacteria from these isolates were then selected for testing in growth chamber assays for the suppression of summer patch symptom development.

Development of Growth Chamber Assay

The growth chamber assay used for disease suppression of summer patch was previously developed in our laboratory; however, several modifications were necessary to achieve consistency in results. Uniformity was achieved by standardizing the optimum fungal inoculum, seeding and bacterial inoculum. 0.1 grams of *M. poae*, as agar plugs grown on PDA agar medium for two weeks, were inoculated in 9 inch containers partially filled with a 4:1 sand:peat mix used as golf course top-dressings. The fungal inoculum was then covered with approximately 1 inch of top-dressing, and 50 seeds of Kentucky bluegrass var. baron were then sown on the surface. The containers were then allowed to stand on greenhouse benches with overhead watering. After approximately 2 weeks, 50 ml of bacterial suspensions at concentrations of 10^9 cells/ml were inoculated as soil drenches onto the turf plants in the containers, which remained on greenhouse benches an additional two weeks. Incubation periods on greenhouse benches and number of bacterial treatments sometimes varied, depending upon specific experiments. After incubation on greenhouse benches, the turf plants were moved to a growth chamber set at conditions of 30°C, 14 hour light intensity of 500 uE and 70% relative humidity. Plants were cut to a 1 inch height on a regular basis and were rated weekly or more frequently upon the first appearance of foliar symptoms. A minimum of 5 replications for each bacterial treatments were used. Ratings utilized a 5 or 10 point scale based on percentage of foliar symptom expression. The 5 point scale was based on the following percentage of foliar symptom development: 1 = 0% disease; 2 = 25% disease; 3 = 50% disease; 4 = 75% disease; 5 = 100% disease. The 10 point scale was also based on percentage of foliar area showing symptoms, where 0 = 0-10% and 9 = 90-100% of area showed disease.

Isolation of Potential Antagonists

As previously mentioned, *X. maltophilia* and *S. marcescens*, isolated from the fungal baiting technique, significantly suppressed foliar symptom development. *X. maltophilia* was observed to suppress foliar symptom development by 70%, while *S. marcescens* suppressed symptoms by 55% (Fig. 3). General characteristics of these isolates are shown in Table 1.

Several isolates were identified using the enrichment culture method that suppressed symptom development at various levels. All soil sources yielded some isolates that suppressed symptom development at statistically significant levels compared to untreated controls. An example of disease suppression obtained with three bacterial isolates, originating from soil obtained from Mill River Golf Course in New York, is shown in Fig. 4. The level of suppression obtained from these isolates appear substantial, reducing symptom development upwards of 85% relative to total amount of disease caused by the fungal-inoculated controls. Several additional isolates have been identified to perform at levels similar to the bacterial isolates from Mill River, and are currently being further characterized (Table 1).

Populations Dynamics of Bacteria

Survival of *X. maltophilia* and *S. marcescens* was determined in soil and on roots to assess the general population dynamics of the isolates relative to the observed disease symptom suppression. A steady decrease in the survivability of the bacterial strains in soil was observed over time (Fig. 5a). No significant difference was observed in the populations of either strain, suggesting that soil survival of the bacteria was not a contributing factor to the differences observed in disease suppression between the two isolates (Fig. 3).

Both *X. maltophilia* and *S. marcescens* are two bacterial species that have been previously reported as rhizosphere competent on other plant hosts. Therefore, it was not surprising that both strains were able to colonize the roots of turf in significantly high populations. Populations on roots were monitored over a 24 day period, with samplings at 9 days after inoculations (one day before moving into the growth chamber), and at 24 days after inoculation (15 days after moving into the growth chamber) (Fig. 5b). Nine days after inoculation, populations of *X. maltophilia* on roots were observed at levels of 2×10^8 CFUs/gram tissue, while *S. marcescens* populations on roots were observed at 2×10^7 CFUs/gram tissue. This small but statistically significant difference may reflect a relevant difference in the initial colonization of roots by the bacteria immediately following inoculation treatments. Such differences may have bearing on the observed differences in disease symptom suppression observed between the two isolates (Fig. 3). Although populations for both bacteria fell to levels of 5×10^6 CFUs/gram tissue 24 days after inoculation, these levels represent significant populations of the bacteria in the turf rhizosphere.

Such rhizosphere competence may contribute to persistence of the bacterium, and thus prolonged protection against infection by the pathogen.

PROPOSED RESEARCH AND PROJECTED RESULTS FOR FORTHCOMING YEAR.

Screening of Bacterial Isolates. Several hundred bacteria have been screened in growth chamber assays for their ability to suppress summer patch symptom development. We plan to continue screening additional isolates, albeit on a lower scale. Our direction of focus for selecting isolates will include screening non-chitin degrading strains found in enrichment cultures. It is unknown why these strains continue to grow in the enrichment cultures containing the fungus as a sole carbon source. Although a likely possibility is that these bacteria utilize nutrients released from the fungus upon initial degradation by chitinase producers, another possibility is that these strains express other significant traits that contribute to the degradation or lysis of the fungus. Such traits may also function as mechanisms of antagonism to *M. poae*, and thus these strains may function as good biocontrol agents for the disease. Isolates that are selected as effective disease suppressors, as well as those previously selected, will be identified to the genus and hopefully to the species level using the Biolog redox chemistry identification system as well as by simple biochemical tests.

Population Dynamics of Bacteria. We are currently investigating the effects of different inoculum densities of *X. maltophilia* 21C6 and *S. marcescens* 9M5 on the suppression of summer patch. This study will provide information on any toxic effects that high dosages of bacteria may have on the turf plants. It will also provide information on the optimal dosage of bacteria on disease suppression. However, all strains identified as potential biocontrol agents will be tested for population dynamics in soil and on roots (rhizosphere competence). The major objective for this study is to correlate population threshold levels of the bacteria with disease suppression. That is, at what population level is disease suppression lost? A second question that this study may shed light on is, at what time does protection occur? That is, does protection occur prior to conditions conducive for disease or during disease conducive conditions? The answer to these questions will provide a better understanding of the pathogenesis of the organism, and better strategies for disease control.

Field Studies. Field trials were initiated during the summer season in 1993. Several bacteria showing potential in growth chamber studies were used as bacterial treatments. These included *X. maltophilia* 21C6 and 12 other bacterial isolates obtained from enrichment cultures. Disease did not appear on treated plots until September; consequently, this data has not been analyzed and could not be included in this report. Once the analysis is complete, isolates showing promise in the field will

be repeated during the 1994 season. In addition, the best isolates identified in continued growth chamber screens will also be used in field studies in 1994.

Additional Relevant Studies. Little is known about the pathogenesis of *M. poae*, and thus it has become difficult to evaluate and determine the mechanisms involved in the disease symptom suppression resulting from treatment with the various bacterial isolates. As a consequence, we are currently attempting to improve growth chamber assays by developing two different methods for evaluating disease suppression. The first method is to correlate pathogen inoculum density with the percentage of foliar disease development. This study will hopefully provide a disease percentage scale for use in comparisons with bacterial treatments. Since the strategy for this project is directed at reducing the fungal inoculum, such a percentage scale will hopefully provide a method of correlation to compare reduction of fungal inoculum with bacterial treatments within a given study.

The second method in which we are currently developing involves a root assay for the assessment of percentage of root infection by *M. poae*. This would allow a much more direct assessment of disease, and hopefully a much more sensitive and accurate assay for assessing the control of disease by potential bacterial antagonists. Development of a more sensitive assay involving the roots is necessary, since root infection by the fungus appears to occur prior to the influence of conditions conducive to disease. Information gained by these studies should strongly influence the strategy for the use of bacterial antagonists in control of the disease.

Table 1. Characteristics of bacteria that suppress summer patch symptom development on Kentucky Bluegrass var. Baron in growth chamber assays.

Isolate	Characteristics	Source
Xanthomonas maltophilia 21C6	chitinase +; root colonizer	New Brunswick, NJ
Serratia marcescens 9M5	chitinase +; root colonizer	New Brunswick, NJ
N3-17 (unknown bacterium)	chitinase +; Gram negative	leaf compost mix
N3-13 (unknown bacterium)	chitinase +; Gram positive	leaf compost mix
Serratia sp. N4-5	chitinase +	Mill River, NY
N4-7 (unknown bacterium)	chitinase +	Mill River, NY
Serratia sp. N4-11	chitinase +	Mill River, NY
Pseudomonas sp. N6-18	chitinase +; fluorescent	Adelphia, NJ

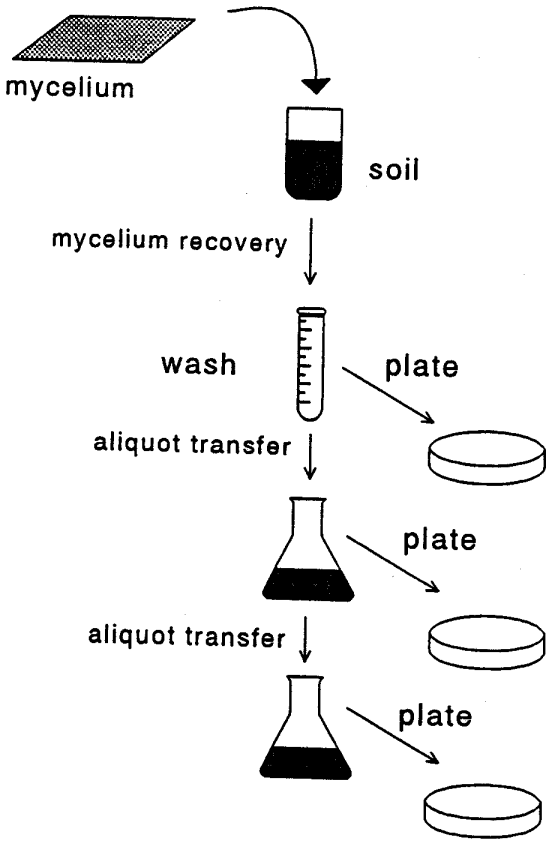

Figure 1. Schematic drawing of the enrichment culture procedure used to isolate bacteria antagonistic to *Magnaporthe poae*. Cellophane squares containing *M. poae* were buried in soil for 24-72 hours. Squares were recovered and washed in distilled H_2O to remove loose soil and loosely adhering bacteria. The squares containing *M. poae* mycelia were then vigorously shaken and sonicated in sterile distilled H_2O. 100 μl aliquots were then transferred to 100 ml of minimal medium broth cultures supplemented with *M. poae* as the sole carbon source. Each culture was shaken at 30°C for 1-2 weeks, depending upon the experiment, at which time 100 μl was transferred to a new flask. At each transfer, the culture was serially diluted and plated for the selection of single bacterial colonies. Culture enrichments were continued for a minimum of 4 transfers.

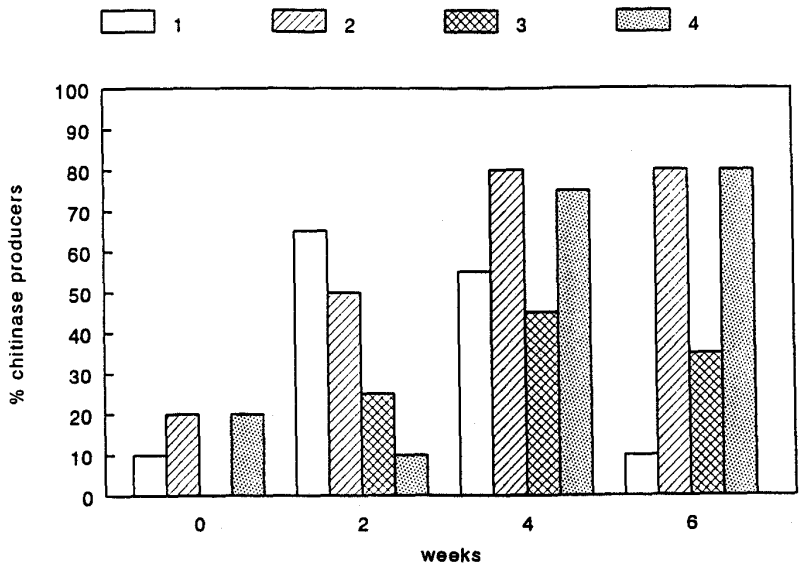

Figure 2. Estimated percentage of chitinase producers from enrichment cultures originating from soil samples. Bacterial isolates were chosen from dilution plates of enrichment cultures extending over six weeks using a cross-hatched grid pattern for random selection. Bacterial colonies appearing or touching cross-hatches were selected. A minimum of 20 colonies per culture was selected, and plated onto a chitin-amended minimal medium to determine chitinase activity. Activity was determined by growth and clearing zones around the colony. The percentage of chitinase producers among selected isolates were taken as a representation of the total population within each culture flask. Graph represents values obtained from cultures where inocula originated from four different soil sources. 1 = leaf compost mix; 2 = brewers compost mix; 3 = Mill River Country Club, Long Island, NY; 4 = Rutgers turf farm, New Brunswick, NJ.

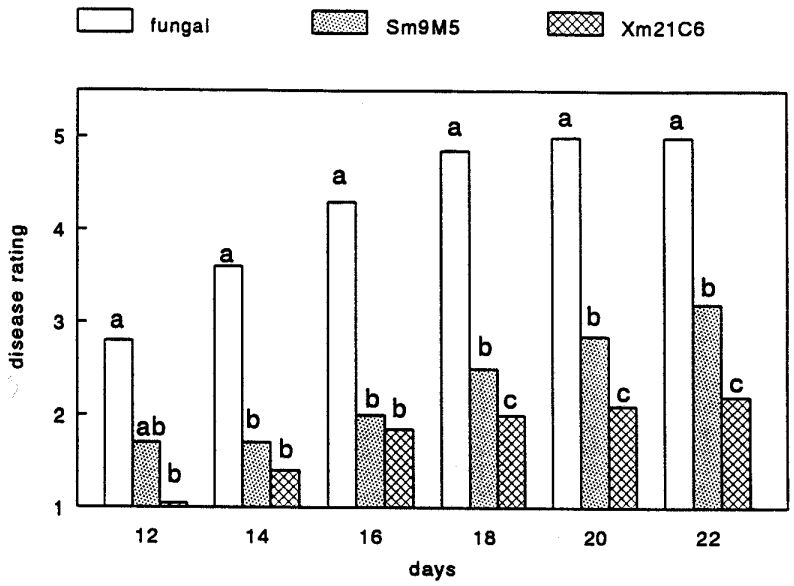

Figure 3. Summer patch symptom suppression by *Xanthomonas maltophilia* and *Serratia marcescens*. Containers of Kentucky bluegrass var. Baron infected with *Magnaporthe poae* and treated with *X. maltophilia* 21C6 (Xm21C6) and *S. marcescens* 9M5 (Sm9M5) compared to fungal inoculated, untreated control plants (fungal). Plants were rated by a 5 point rating scale described in the text, over a 22 day period under growth chamber conditions conducive to disease development (see text). Values are the mean of seven replications. Different letters represent significant differences according to Duncan's Multiple Range Test (P = 0.05).

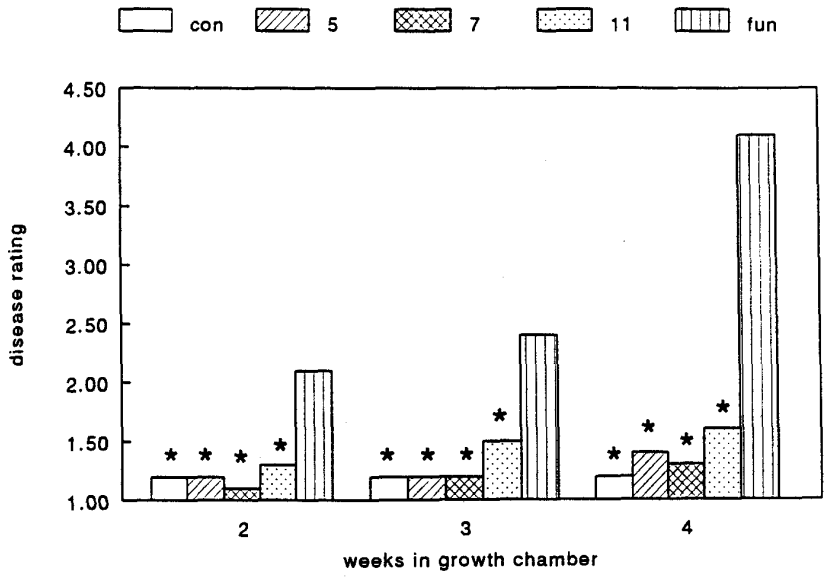

Figure 4. Suppression of summer patch symptom development by bacteria isolated from enrichment cultures. Containers of Kentucky bluegrass var. Baron infected with *Magnaporthe poae* and treated with chitinase-producing bacteria originating from soil obtained from Mill River Country Club, NJ. Con = uninoculated control plants; 5 = *Serratia* sp. N4-5; 7 = unknown bacterium N4-7; 11 = *Serratia* sp. N4-11; fun = fungal inoculated, untreated control. Asterisks represent statistically different values according to (P = 0.05).

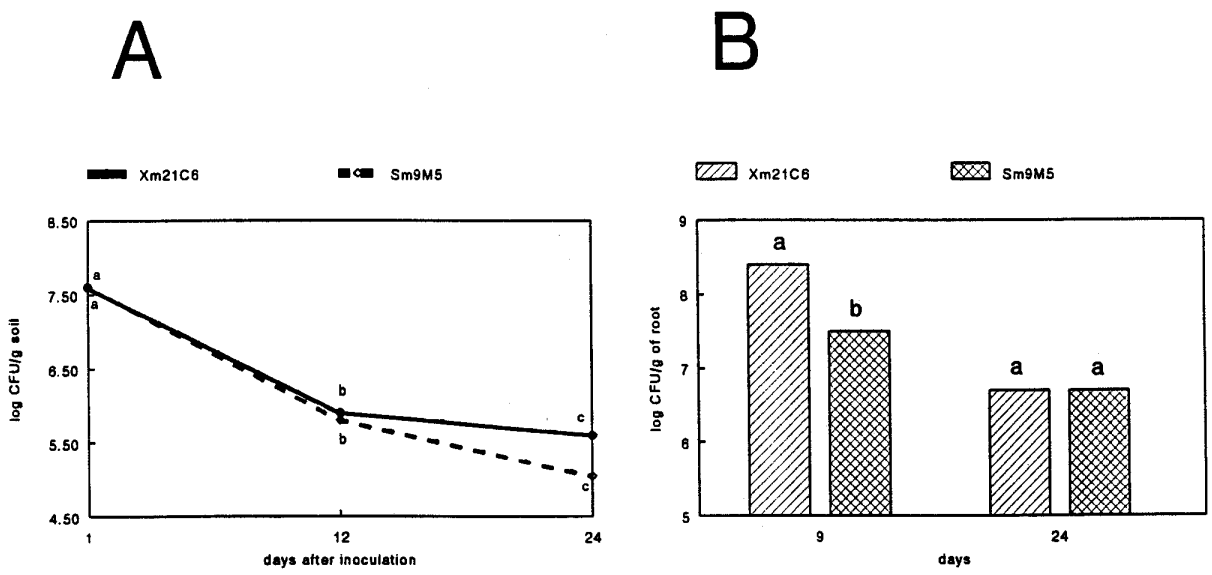

Figure 5. Populations of *Xanthomonas maltophilia* and *Serratia marcescens*. Populations of *X. maltophilia* 21C6 (Xm21C6) and *S. marcescens* 9M5 (Sm9M5) were monitored over a 24 day period in a 4:1 sand:peat mix (A) and on the roots of Kentucky bluegrass var. Baron (B), as described in the text. Similar letters represent no statistical differences according to Analysis of Variance (P = 0.05). Values are the means of four (A) or three (B) replications.