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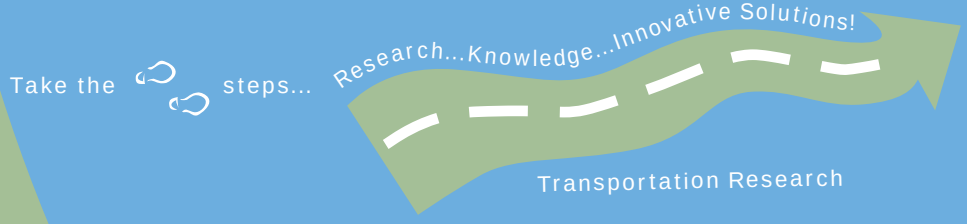
Biological Control of Canada Thistle in Wetland Prarie Restoration



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Pseudomonas syringae pv. *tagetis* (Pst), a phytopathogenic bacterium, was evaluated as natural biological control agent for Canada thistle [*Cirsium arvense* (L.) Scop.]. Canada thistle patches exhibiting symptoms of Pst infection commonly occur along roadsides in association with perennial grasses and a grass litter layer. Field experiments were conducted to determine if grass and litter provide an environment that supports Pst infection of Canada thistle or if grass, litter, and soil collected from infected Canada thistle patches act as inoculum sources for Pst infection of Canada thistle. This experiment provides evidence that grass and litter are important components of the landscape that support the natural Pst infection of Canada thistle, and perennial grass competition has potential to manage Canada thistle in roadside rights-of-way and wetland restoration sites. A previously published Pst specific primer set was determined to require high Pst populations for detection.

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Biological Control of Canada Thistle in Wetland Prairie Restoration

Final Report

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Executive Summary

Pseudomonas syringae pv. *tagetis* (Pst), a phytopathogenic bacterium, has the potential to be a natural biological control agent for Canada thistle [*Cirsium arvense* (L.) Scop.]. Canada thistle patches exhibiting symptoms of Pst infection commonly occur along roadsides in association with perennial grasses and a grass litter layer. To determine the role grass and litter play in supporting Pst infection of Canada thistle, an experiment consisting of two grass treatments (removed and intact) and two litter treatments (removed and intact) was initiated on Pst naturally infected Canada thistle patches along roadsides in east central Minnesota and was repeated for five years. The removal of either grass, litter, or grass and litter reduced disease incidence as compared to the grass and litter intact treatment. In 2006, the Canada thistle environment where grass and litter were present had higher relative humidity and temperature range as compared to areas where grass and litter had been removed. This experiment provides evidence that grass and litter are important components of the landscape that support the natural infection of Canada thistle with Pst.

Field experiments were conducted to determine if grass, litter, and soil collected from infected Canada thistle patches or cultured Pst act as inoculum sources for Pst infection of Canada thistle and if environments created by perennial grasses and grass litter can increase Pst infection of Canada thistle in areas with introduced or natural Pst infection. Grasses from naturally infected Canada thistle patches acted as inoculum sources for Pst on Canada thistle when transplanted into Canada thistle populations without Pst disease symptoms. However, Canada thistle in the non-treated controls also had low levels of Pst disease incidence (DI), suggesting that a natural population of Pst was present in the experiments or that Pst dispersal occurred between plots. Therefore, the low levels of Canada thistle DI observed in our experiments may not be entirely attributed to the inoculum source in that treatment, but may have occurred through natural infection. Although the grass and litter environments were established for at least one year in all experiments these environments did not increase DI to levels that were large enough to impact Canada thistle growth and development. A reduction in Canada thistle height and density in this study may be attributed to competition with the perennial grasses. This experiment indicates that dense stands of perennial grasses should be included as part of an integrated management system for Canada thistle in roadside rights-of-way and wetland restoration sites. More information is needed to determine how Pst infects Canada thistle and whether the perennial grasses and litter are inoculum sources or create environments that support Pst infection of Canada thistle.

Pseudomonas syringae pv. *tagetis* (Pst) specific primers which amplify regions of genes specific to tagetitoxin were used to develop a polymerase chain reaction (PCR)-based detection method for Pst in grass and litter, potential inoculum sources for Pst infection of Canada thistle (*Cirsium arvense* (L.) Scop). Sensitivity of a previously published primer set suggests high Pst populations are needed for detection and this test may not be reliable for environmental samples which could have low Pst populations. To improve sensitivity, a nested PCR protocol was developed with Pst specific primers from a different gene specific to tagetitoxin than the previous primer set. Nested primers potentially increased sensitivity to 10 to 100 cfu/ml, but false positives in the negative controls could not be overcome and this sensitivity could not be verified. If problems with the nested protocol can be resolved, this PCR-based detection method

could be used to further studies on Pst population dynamics and inoculum sources in natural systems.

More information is needed on how Pst infects Canada thistle and whether grass and litter are inoculum sources or environmental factors that contribute to disease. To fully understand the role of grass and litter in Pst natural infection on Canada thistle a reliable test for Pst in the environment is needed. In the future, creating an environment that is conducive for Pst infection has potential to improve the integrated management system for Canada thistle along roadsides and in prairie restoration areas.

CHAPTER 1

Introduction

Canada thistle (*Cirsium arvense* L. (Scop.)) is one of the most troublesome noxious weeds in the United States and Canada and it is on the noxious weed list of 43 states including Minnesota (1). It is a non-native, invasive, perennial species characterized by persistent vegetative growth from an extensive horizontal root system (2). Canada thistle invades cropland, pastures, roadsides, natural areas and must be controlled in these areas (3). It is an aggressive weed which outcompetes many native plant species and thrives in areas with little or no competition for light or nutrients (2).

Canada thistle is common in upland areas restored wetlands prairie restorations, CRP lands, roadsides, and non-disturbed areas containing mixtures of perennial grasses and legumes (4, 5, 6) Current Wetland Conservation Act (WCA) guidelines for restored or mitigated wetlands require a five year vegetation management plan which includes control of noxious weeds (7). Prairie restorations require soil disturbance which opens areas and reduces plant competition which may increase colonization and spread of invasive species (8). Restorations with native grasses and forbs planted from seed are often slow growing leaving open areas ideal for colonization by Canada thistle. New strategies that are not damaging to native species are needed for control of Canada thistle in prairie wetland areas.

Common Canada thistle management practices include integration of tillage, mowing, burning, and application of herbicides. These control methods may be costly and damaging to native plants (9). Alternative approaches for control of Canada thistle are needed in the perennial environments of roadside rights-of-way and wetland prairie mitigation areas. There is increased interest in developing a biological control agent that can provide selective control for Canada thistle. Biological control may be more cost effective than traditional methods for long term management of Canada thistle in perennial systems (10). Insect biocontrol agents, stem weevil, bud weevil, and stem gall fly for Canada thistle are commercially available, but their effectiveness at control in Minnesota is still relatively unknown (3). Biological controls, which manage but do not eradicate weeds, may be an effective management tool in perennial systems of roadside rights-of way and restored wetland areas.

Pseudomonas syringae pv. *tagetis* (Pst), a bacterial pathogen naturally infecting Canada thistle in Minnesota has been identified as a potential biological control agent.
Pseudomonas syringae pv. *tagetis* (Pst) is a plant pathogenic bacterium infecting several species in the Asteraceae family (11). It was originally isolated from African marigold (*Tagetes erecta*) and has been isolated from a variety of other Asteraceae species in nature including; Common ragweed (*Ambrosia artemisiifolia* L.), sunflower (*Helianthus annus* L.), Jerusalem artichoke (*Helianthus tuberosus* L.) and Canada thistle (*Cirsium arvense* (L.) Scop.) (12, 13, 14, 15, 16, 17). Symptoms of Pst infection include apical chlorosis which leads to stunting and reduced flowering and seed production (14). Pst infection symptoms are caused by the production of tagetitoxin, a toxin that is translocated to the plant apex (18, 19). In plants tagetitoxin inhibits

RNA polymerase III which disrupts the synthesis of chloroplasts in plant tissue causing chlorosis in infected tissue (20).

An initial study, examining Pst as a biological control agent, demonstrated almost complete control of Canada thistle after five applications of Pst in conventional soybean (16, 17). However, two applications of Pst were not enough to effectively control Canada thistle in organic soybean (21). Repeated applications of Pst are too costly to be an effective alternative to traditional control methods for Canada thistle in annual cropping systems. Integrated management including a competitive soybean variety and Pst application resulted in a reduction in survival, growth, and reproduction in Canada thistle with multiple applications of Pst, but there was no increase in effects with the competitive soybean variety (22). There were also no improvements in control of Canada thistle when Pst was combined with an fungal protein Nep 1 when compared to applying either alone (23). A recent study examined a different method of Pst application in which sap from naturally infected Canada thistle plants was applied with a surfactant to non-infected Canada thistle plants in an undisturbed area to evaluate natural colonization and spread (24). There were indications that the introduced Pst became a long term component of the community (24), however, it was not determined why this occurred. For Pst to be used in an effective control strategy, new techniques are required that focus on sustaining high populations of Pst, and more information is needed on what controls Pst populations dynamics and infection in natural systems with Canada thistle. Previous studies with Pst did not examine the environment around Canada thistle, so studying environmental components important to Pst infection in undisturbed areas may provide a better understanding of factors needed to improve Pst as a biological control agent.

Pst occurs naturally on infected Canada thistle plants in Minnesota. Typically Canada thistle plants naturally infected with Pst occur in roadsides, pastures, and natural areas. Studies have not previously been done on the efficacy of Pst on naturally infected Canada thistle populations, and the distribution and frequency of naturally infected Canada thistle plants is unknown. More information is needed on the biology of Pst in natural perennial systems and which factors influence Pst populations and toxin production. Pst infection on Canada thistle is particularly prevalent in roadside right of ways which contain perennial grasses and a thick litter layer. Infected populations of Canada thistle have high disease incidence and infection is maintained for subsequent growing seasons. For Pst to be part of an effective control strategy for Canada thistle techniques must be developed that sustain high populations of Pst and integrated management system based on the natural Pst infection on Canada thistle may be the appropriate approach in perennial systems. Two hypothesis for Pst naturally infected Canada thistle environment are perennial grass and litter are inoculum sources or create the environmental conditions necessary for Pst population growth and toxin production.

Pst is capable of surviving and overwintering in debris of marigold (25). Pst may have similar survival and overwintering capabilities on grasses or litter present in the Canada thistle patch. Plants and plant litter or debris can be important sources of inoculum or reservoirs for pathogens (26, 27). There are many examples of plant pathogens surviving on grasses and debris of non-hosts and serving as inoculum sources for infection on host plants (28, 29, 30, 31).

The phyllosphere environment has profound effects on bacterial populations. Grass and litter may contribute to changes in the phyllosphere environment by providing a protective environment or changing humidity and temperature which affect bacteria populations and toxin production. Litter creates moist conditions increasing plant susceptibility to disease, (32) and humidity is an essential component to the infection system in diseases caused by bacteria (33). Colonization and survival of bacterial pathogens is generally enhanced in protected environments with stable temperature, light and moisture (34). Grass and litter may provide the necessary phyllosphere environment for Pst populations to successfully cause and maintain disease on Canada thistle. Perennial grasses also act as competitors with Canada thistle and cause increased stress on Canada thistle plants, which could increase severity of infection (42). Grass competition with thistle species leading to reduced survival and growth has been demonstrated in several studies (34, 35, 36).

There is need to develop integrated management systems for Canada thistle in prairie wetland restoration areas that do not rely on the use of herbicides. This study was designed to determine if grass, litter, and the phyllosphere they create affect Pst infection of Canada thistle. Understanding the environment associated with Pst naturally infected Canada thistle will allow us to identify the components necessary for long term establishment and improved disease incidence. Previous observations suggest that perennial grasses and a grass litter layer are common to these areas with high natural Pst infection of Canada thistle. Our objectives are to:

(1) Determine if grass and litter are necessary for sustained Pst infection of Canada thistle by characterizing the effect on Canada thistle disease incidence, growth, and potential reproduction when grass, litter, or grass and litter are removed from naturally infected areas.
(2) Determine if perennial grasses and grass litter impact the phyllosphere environment and can be used to create environments that increase Pst infection of Canada thistle.
(3) Evaluate the potential of grass and litter to act as inoculum sources for Pst infection of Canada thistle.
(4) Develop a PCR-based detection method for Pst on potential inoculum sources.
(5) Evaluate the impact and effectiveness of incorporating grass and litter into the Canada thistle management system in perennial systems.

Chapter 2 pertains to objective 1 and 2 and describes an experiment conducted along roadside right-of ways in a seven county area in east central Minnesota on Canada thistle patches naturally infected with Pst. This experiment was designed to determine if grass, litter, or grass and litter are necessary for sustained infection of Pst on Canada thistle. We evaluated the importance of grass and litter in Pst natural infection on Canada thistle by characterizing the effect on Canada thistle disease incidence, growth, and potential reproduction when grass, litter, or grass and litter are removed. We will also evaluate the importance of grass and litter on environmental differences influencing initial infection and infection development of Pst on Canada thistle.

Chapter 3 pertains to objectives 2 and 3 in which two separate field experiments were set up at Cheri Ponds Minnesota Department of Transportation (Mn/DOT) wetland mitigation area in four separate areas with dense Canada thistle patches. We evaluated Canada thistle management

systems that utilized perennial grasses and grass litter to create environments that are highly competitive with Canada thistle and have been shown to support Pst infection of Canada thistle. We designed experiments to determine if perennial grasses and grass litter can be used to create environments that increase Pst infection of Canada thistle from grass, litter, and soil collected from infected Canada thistle patches or cultured Pst inoculum sources. Chapter 4 relates to objectives 3 and 4 with development of a polymerase chain reaction (PCR)-based detection method which would be used to determine if grass and litter were potential inoculum sources of Pst infection of Canada thistle. The objectives of this experiment were to use Pst specific primers to test grass and litter as natural inoculum sources for Pst infection of Canada thistle and as a potential substrate for survival of cultured Pst in the field.

Chapter 5 includes a summary of conclusions from the studies conducted and also a list of recommendations for practices that can be implemented for Canada thistle management and areas of future research.

CHAPTER 2

Effects of grass and litter on *Pseudomonas syringae* pv. *tagetis* natural infection of Canada thistle (*Cirsium arvense*)

ABSTRACT

Pseudomonas syringae pv. *tagetis* (Pst), a phytopathogenic bacterium, has the potential to be a natural biological control agent for Canada thistle [*Cirsium arvense* (L.) Scop.]. Canada thistle patches exhibiting symptoms of Pst infection commonly occur along roadsides in association with perennial grasses and a grass litter layer. A study was designed to determine the role grass and litter play in supporting Pst infection of Canada thistle. An experiment consisting of two grass treatments (removed and intact) and two litter treatments (removed and intact) was initiated on Pst naturally infected Canada thistle patches along roadsides in east central Minnesota and was repeated for five years. The removal of either grass, litter, or grass and litter reduced disease incidence (DI) as compared to the grass and litter intact treatment. Where grass and litter were present DI ranged from 48 to 68%, while in the grass and litter removed treatment DI ranged from 4 to 14% over all years of the experiment. In 2006, the Canada thistle environment where grass and litter were present had a daily maximum and minimum relative humidity 4 to 5 % higher and a 3 C higher daily air temperature range as compared to areas where grass and litter had been removed. This experiment provides evidence that grass and litter are important components of the landscape that support the natural infection of Canada thistle with Pst. Creating an environment that is conducive for Pst infection has potential to improve the integrated management system for Canada thistle along roadsides and in prairie restoration areas.

Nomenclatures: Canada thistle, *Cirsium arvense* (L.) Scop. CIRAR
Key Words: Canada thistle, *Pseudomonas syringae* pv. *tagetis*, grass, and litter

INTRODUCTION

Canada thistle [*Cirsium arvense* (L.) Scop.] is one of the most troublesome noxious weeds in the United States and Canada (1). It is a non-native, invasive, perennial species characterized by persistent vegetative growth from an extensive horizontal root system and is commonly found in agricultural lands, along roadsides, and in natural areas where it competes with native species (6, 34). The Minnesota Department of Transportation (Mn/DOT) is restoring native prairie mixes to cool-season grass dominated roadsides, however these efforts often result in increases in Canada thistle in these areas until native plants become established. The Minnesota Noxious Weed Law Section 18.88 requires control or eradication of all state prohibited noxious weeds including Canada thistle. Currently the management of Canada thistle on roadsides and wetland restoration sites involves mowing and herbicide application which requires repeated applications for several years and is costly and damaging to native forbs (9). Alternatively biological control may offer an environmentally safe and cost effective method for the long term management of Canada thistle in perennial grass systems (10). One potential biological control agent, *Pseudomonas syringae* pv. *tagetis* (Pst), is a pathogenic bacterium which occurs naturally on several species in the Asteraceae family including Canada thistle.

Pst was originally isolated from African marigold (*Tagetes erecta* L.) and has also been isolated from common ragweed (*Ambrosia artemisiifolia* L.), sunflower (*Helianthus annus* L.), Jerusalem artichoke (*Helianthus tuberosus* L.) and Canada thistle (11, 12, 13, 14, 15, 16, 17). Pst causes severe apical chlorosis due to production of tagetitoxin, a toxin which is translocated to the plant apex (18, 19). In plants, tagetitoxin inhibits RNA polymerase III, thereby disrupting the synthesis of chloroplasts in plant tissue, which causes a characteristic apical chlorosis in all new tissue in infected plants (20). Pst has been shown to reduce survivorship, growth, and reproduction in several aster species including Canada thistle (10, 11, 14, 21). For this reason Pst has been studied as a potential biological control agent on Canada thistle.

An initial study, examining Pst as a biological control agent, demonstrated almost complete control of Canada thistle after five applications of Pst in conventional soybean (16, 17). In conservation tillage soybean, Pst application caused a reduction in survival, growth, and reproduction in Canada thistle with multiple applications of Pst (22). However, two applications of Pst were not enough to effectively control Canada thistle in organic soybean (21). Repeated applications of Pst, although they may be effective, are too costly to be considered an alternative to traditional control methods in annual cropping systems.

A recent study examined a different method of Pst application in which sap from naturally infected Canada thistle plants was applied with a surfactant to non-infected Canada thistle plants in an undisturbed area to evaluate natural colonization and spread (24). There were indications that the introduced Pst became a long term component of the community (24), however, it was not determined why this occurred. For Pst to be used as an effective control strategy, new techniques need to be developed that will sustain high populations of Pst for extended periods of time. More information is needed to determine what regulates Pst population dynamics and infection of Canada thistle in natural systems. Previous Pst studies did not examine the micro-climate associated with Pst infected Canada thistle, so studying environmental components

important to Pst infection in undisturbed areas may provide a better understanding of the conditions needed to improve Pst as a biological control agent.

Natural infection of Canada thistle with Pst is particularly prevalent in roadside rights-of-way which are characterized by perennial grasses and a thick litter layer which consists of plant material from associated perennial grasses. Perennial grasses and litter found in association with roadside Canada thistle patches may provide surfaces for Pst survival and act as inoculum for Pst infection on Canada thistle or affect the phyllosphere environment providing conditions that support Pst populations, infection, or toxin production. The efficacy of Pst on naturally infected Canada thistle populations is unknown, because Pst is difficult to isolate and quantify from naturally infected Canada thistle plants in the field using standard culturing techniques. So, in most studies it was assumed that Canada thistle shoots exhibiting apical chlorosis symptoms contained populations of Pst.

Plants and plant litter or debris can be important sources of inoculum or reservoirs for pathogens (26, 27). Pst is capable of surviving and overwintering in debris of marigold (25) and Pst may have similar survival capabilities in Canada thistle patches. One possibility is Pst survives in Canada thistle debris present in the litter layer. There have been numerous studies demonstrating pathogens surviving in litter and debris which served as inoculum sources (26, 27, 30, 31). In one study, *Pseudomonas syringae* pv. *maculicola's* survival was related to the longevity of the litter material with bacteria in aboveground debris having the longest persistence (31). Pst may survive on the grass litter which breaks down at a slower rate than forb litter (37). Another possibility is that Pst survives on living tissue such as grasses. Bacterial pathogens surviving on non-hosts which act as inoculum for infection have been documented (28), and *Pseudomonas syringae* pathovars have the ability to survive epiphytically on non-hosts (29). Pst may have similar survival capabilities on plants or litter present in Canada thistle patches.

The presence of grasses and litter may contribute to environmental differences that are beneficial to Pst population survival and infection of Canada thistle. The phyllosphere environment has profound effects on bacterial populations. Grass and litter may contribute to changes in the phyllosphere environment by providing a protective environment or changing humidity and temperature which affect bacteria populations and toxin production. Litter creates moist conditions increasing plant susceptibility to disease, (32) and humidity is an essential component to the infection system in diseases caused by bacteria (33). Colonization and survival of bacterial pathogens is generally enhanced in protected environments with stable temperature, light, and moisture (38). Grass and litter may provide the necessary phyllosphere environment for Pst populations to successfully cause and maintain disease symptoms on Canada thistle.

This study was designed to determine if grass, litter, and the phyllosphere they create affect Pst infection of Canada thistle. Understanding the environment associated with Pst naturally infected Canada thistle plants will allow us to identify the components necessary for long term establishment and improved disease incidence. Previous observations suggest that perennial grasses and a grass litter layer are common to these areas with high natural Pst infection of Canada thistle. The objective of this study was to determine if grass, litter, or grass and litter are necessary for sustained infection of Pst on Canada thistle. We will evaluate the importance of

grass and litter in Pst natural infection on Canada thistle by characterizing the effect on Canada thistle disease incidence, growth, and potential reproduction when grass, litter, or grass and litter are removed. We will also evaluate the importance of grass and litter on environmental differences influencing initial infection and infection development of Pst on Canada thistle.

MATERIALS AND METHODS

Site Description

The experimental sites consisted of Pst naturally infected Canada thistle patches located on county and state roadside rights-of-way in a seven county area in east central Minnesota (Appendix A). Canada thistle patches varied in size from 5 m^2 to 0.5 ha with populations of Canada thistle ranging from 5 to 30 plants m^{-2}. Canada thistle patches were in various positions in relationship to the roadway, but were normally outside the mowing zone with the majority on up slope or down slope positions. The Canada thistle patches had developed in association with perennial non-native cool season grasses; Smooth brome grass (*Bromus inermis* Leyss.), Kentucky blue grass (*Poa pratensis*), Quack grass (*Elytroid repens*), and Reed canary grass (*Phalaris arundinacea*). The Canada thistle patches had a 2 to 5 cm deep litter layer which consisted of dead plant material from associated perennial grasses. Canada thistle patches with 80 to 90% Pst infection were identified in the summer prior to initiation of experimental treatments in the fall.

Individual Pst infected areas of Canada thistle patches were used for each replicate of each treatment with the treatment area defined as the entire infected area within a Canada thistle patch. The treatment areas varied from 3 to 10 m^2 with Canada thistle densities ranging from 4 to 30 plants m^{-2}. In this study a Canada thistle plant is defined a separate aboveground shoot as the root systems connections were not examined in this study.

Experimental Design

This study was designed to determine if grass, litter, and the phyllosphere they create affect Pst infection of Canada thistle through an experiment conducted in Canada thistle patches with natural Pst infection. The experiment consisted of four treatments applied to Pst infected Canada thistle patches; grass removed, litter removed, grass and litter removed, and grass and litter intact. The treatments were placed in a completely randomized design with four replicates for a total of 16 separate plots each year. The study was conducted in 2001, 2002, 2003, and 2005 with separate treatment areas used each year. The same treatment areas were used in 2005 and 2006 to study the long term effect of treatments. In 2006, environmental sensors were established in grass and litter intact and grass and litter removed treatments to evaluate the impact that grass and litter have on the environmental conditions that could influence initial infection and disease development of Pst on Canada thistle.

Plot Establishment and Maintenance

The grass and litter intact treatment consisted of a Canada thistle patch infected with Pst in a dense stand of cool season perennial grasses and a grass litter layer which were not altered throughout the duration of the experiment. In the litter removed treatment, the litter was removed in the fall by mowing the perennial grasses with a trimmer and then raking the entire plot to remove all aboveground biomass including the litter layer. No litter was present when Canada thistle plants emerged the following spring, and the plot was maintained litter free

throughout the duration of the experiment. The perennial grasses were not disturbed after the initial litter removal in the fall. In the grass removed treatment, the grasses were removed in the spring when the grasses first emerged by treating with quizalofop at 0.05 kg ai ha^{-1} plus crop oil concentrate at 0.3% [v/v]. The herbicide was applied with a hand held sprayer at 207 kPa and repeated weekly as needed throughout the growing season to control all grasses in the plot. The litter layer was left intact, so the Canada thistle plants were exposed to litter, but not to grass throughout the season. In the grass and litter removed treatment, the litter was removed in the fall and grass was controlled in the spring as described earlier. Canada thistle plants in the grass and litter removed treatment were not exposed to either grass or litter for the entire season.

Data Collection

Canada Thistle Disease Incidence

Disease incidence (DI) was visually measured in each plot as the percentage of Canada thistle plants exhibiting symptoms of apical chlorosis on a scale of 0 (no disease) to 100 (all plants exhibiting symptoms). Evaluation of DI began in the spring, at the first indication of Pst infection of Canada thistle in the untreated control areas (April 27 to June 10) and continued every two weeks until senescence in the fall for a total of four to five assessments each season.

Canada Thistle Plant Height and Flower Heads

Evaluation of Canada thistle plant height began in the spring at the same time as disease incidence evaluation and continued every two weeks until senescence in the fall for a total of four to five assessments each season. Canada thistle height was evaluated on each date by measuring the heights of ten randomly selected Canada thistle plants in each plot.

Since Canada thistle is dioecious, with male and female flowers on separate plants, there was variation between plants and patches for seed production, so the number of flower heads per plant was used as an indicator of potential Canada thistle reproduction. A Canada thistle flower head consists of a 1 to 2 cm diameter compact cluster of flowers arranged so that the whole gives the effect of a single flower. The number of flower heads per plant was counted on ten randomly selected Canada thistle plants in each plot when Canada thistle was in the post anthesis stage of development.

Data Analysis

Canada thistle disease incidence, height, and flower heads per plant were normally distributed and equal variance was assessed with Levene's test of equality of variances. Each year was analyzed separately, because there was too much variation between years to combine all years of data. The data from Canada thistle DI and height were analyzed using SPSS univariate repeated measures analysis of variance (ANOVA) with between subject factor as treatment and within subject factor as date of data collection. Means of treatment differences were separated with Fisher's Protected LSD test at $P = 0.05$. The flower heads per plant data were analyzed with an ANOVA and treatment means separated with Fisher's Protected LSD at $P = 0.05$.

Environmental Monitoring and Data Analysis

To characterize environmental differences between treatments in Pst infected Canada thistle areas, environmental data loggers (Watchdog Data Loggers) that measure temperature, relative humidity (RH), and leaf wetness were set up in three replicates of the grass and litter intact treatment and three replicates of the grass and litter removed treatments, in 2006. Measurements were recorded every hour from May 1, 2006 when Canada thistle was emerging, until July 12, 2006 when Canada thistle was in the post anthesis stage of development. The collection period was divided into two periods: early-symptom phase and late-symptom phase. The early-symptom phase was from May 1 to May 31. During this period of time Canada thistle shoots emerged and developed without Pst infection symptoms for the first three weeks, however, Pst infection symptoms developed during the fourth week when Canada thistle was in the rosette to early bolting stage. The late-symptom phase was June 1 to July 12 which included Canada thistle early budding to post anthesis stages and the period of time in which there was maximum expression of Pst induced chlorosis.

Data collected included air temperature, relative humidity (RH), and leaf wetness which were recorded at 30 cm above the soil surface, and soil temperature which was recorded at 2 to 3 cm below the soil surface. Summaries of maximum, minimum, and range of daily air temperature, daily soil temperature, and RH were calculated using Specware Software[6]. Leaf wetness duration was calculated as the daily number of hours that leaf wetness was above 6 on a scale of 0 to 15 (dry to wet) and RH duration was calculated as the daily number of hours above 80% RH. An ANOVA was conducted on both time periods to determine treatment effects for each environmental parameter.

RESULTS

Canada Thistle Disease Incidence

Disease incidence varied between treatments and was dependent on the date of collection in 2001, 2002, and 2003 (Table 2.1). In 2005 and 2006, DI varied by treatment but was not dependent on the date of collection. The average DI in the grass and litter intact treatment ranged from 48 to 68% across all years, and DI was higher in the grass and litter intact treatment than all other treatments in all years of the experiment (Table 2.2 and Figure 2.1).

In 2001, 2002, and 2003, the grass and litter intact treatment had higher DI across all sampling dates as compared to treatments with grass, litter, or grass and litter removed (Figure 2.1). In 2001, the grass removed treatment had higher DI than litter removed or grass and litter removed with the exception of the last sampling date where no difference was detected between grass removed, litter removed, and grass and litter removed treatments. In 2002 and 2003, the litter removed treatment had higher DI than grass and grass and litter removed treatments with the exception of the second sampling date in 2002 (Table 2.1). In 2005 and 2006, grass and litter intact had higher DI than all other treatments (Table 2.2). There was no difference in DI between grass removed, litter removed, or grass and litter removed treatments in 2005 and 2006.

Canada Thistle Plant Height

Canada thistle plant height varied between treatments and was dependent on the date of collection in 2002 and 2003 (Table 2.3). In 2002, the Canada thistle plants were shorter in the grass and litter intact and grass removed treatments as compared to the litter removed and grass and litter removed treatments (Figure 2.2) with the exception of the first sampling date. In 2003, the Canada thistle plants in the grass and litter intact treatment were shorter as compared to all other treatments with the exception of the first sampling date (Figure 2.2). In 2002 and 2003, Canada thistle plants in the grass and litter removed treatment were taller than those in all other treatments (Figure 2.2). In 2005, Canada thistle plants were shorter in the grass removed treatment as compared to the grass and litter intact treatment. In 2006, Canada thistle plants in the grass and litter removed treatment were shorter than in all other treatments (Table 2.2).

Canada Thistle Flower Heads

In 2001 and 2005, the grass and litter intact treatment had fewer flower heads per Canada thistle plant than the grass and litter removed treatment (Figure 2.3). In 2002, the grass and litter intact treatment had fewer flower heads per plant than the litter removed treatment. In 2003, the grass and litter intact treatment had fewer flower heads per plant than all other treatments. In 2001, 2003, and 2005 there were more flower heads per plant in the grass and litter removed treatment than in the grass and litter intact treatment (Figure 2.3).

Environmental Monitoring

In the early-symptom phase, there were differences between treatments for RH and temperature. The grass and litter intact treatment had 4 to 5 % higher daily maximum and minimum RH than the grass and litter removed treatment (Table 2.4). The range in daily air temperature was 3 C higher in the grass and litter intact treatment, but the maximum daily air temperature was not different between treatments. The daily maximum soil temperature was 3 C higher in the grass and litter removed treatment as compared to the grass and litter intact treatment (Table 2.4).

In the late-symptom phase, the grass and litter intact treatment had a 4% higher daily maximum and minimum RH than the grass and litter removed treatment (Table 2.4). The daily duration of RH above 80% was two hours more in the grass and litter intact treatment. Maximum daily air temperature was 1 C higher and the minimum daily air temperature was 2 C lower in the grass and litter intact treatment and had a higher range in daily air temperature of 3 C (Table 2.4). The daily maximum soil temperature was 2 C lower and daily minimum soil temperature was 1 C lower in the grass and litter intact treatment.

Leaf wetness duration was not different between treatments in either time period, but leaf wetness duration was higher in the late-symptom phase than the early-symptom phase (Table 2.4). In the early-symptom phase, duration of RH above 80% was also less than the late-symptom phase.

DISCUSSION

In this study we demonstrated that the removal of grass, litter, or grass and litter caused a reduction in DI on Canada thistle as compared to the grass and litter intact treatment, suggesting that both play an important role in natural infection of Pst on Canada thistle. These reductions in DI were consistent for all five years of the experiment. Furthermore, the treatment differences in DI occurred during initial Pst infection of Canada thistle and were maintained throughout the season, suggesting that grass and litter are important in all stages of infection (Figure 2.1). This experiment demonstrated that grass and litter play an important role in supporting the infection of Canada thistle with Pst. We also expected DI to impact Canada thistle plant height and the number of flower heads per plant.

In 2002 and 2003, early season Canada thistle heights were similar in all treatments, but treatment differences occurred as Canada thistle developed later in the season (Figure 2.2). High levels of Pst infection have been shown to cause a reduction in Canada thistle height (21, 22). So, in this experiment when DI was reduced by removing grass, litter, or both we expected Canada thistle height to increase as compared to plants in the grass and litter intact treatment with higher DI. This was demonstrated in our experiment in 2002 and 2003 where DI was higher in the grass and litter intact treatment as compared to treatments where grass, litter, or grass and litter had been removed. Consequently, Canada thistle plants were shorter in the grass and litter intact treatment which had higher DI (Table 2.2). Conversely, in 2005 and 2006, Canada thistle plants were actually taller in the grass and litter intact treatment even though they had high DI, as compared to at least one other removal treatment with lower DI (Table 2.4). These height differences between years of the experiment may be explained by the stage of the Canada thistle development when infection occurred, because the stage of the Canada thistle development determines the degree to which plant growth is affected. Gulya et al. 1982 (14), showed that young sunflower seedlings infected with Pst were more severely stunted, when compared to older plants infected with Pst. Similarly, in our study in 2003, Pst disease symptoms developed earlier in the season and Canada thistle plants remained shorter throughout the season (Figure 2.1, Figure 2.2). Conversely, in 2005 and 2006, Pst infection occurred later and there were fewer differences in Canada thistle height between treatments. Canada thistle height may not be a reliable indicator of disease severity, because time of infection affects growth of Canada thistle and can interfere with the expected relationship between Canada thistle DI and height.

High levels of Pst infection are known to cause a reduction in flower head and seed head production of Canada thistle (21, 22), so when DI was reduced by removing grass, litter, or both we expected Canada thistle flower heads per plant to increase as compared to flower heads per plant in grass and litter intact treatments with higher DI. In 2001, 2003, and 2005, the grass and litter intact treatment had fewer flower heads per plant than the grass and litter removed treatment. Accordingly, the corresponding DI in the grass and litter intact treatment was also higher as compared to the grass and litter removed treatment (Figure 2.3 and Table 2.2). The differences in flower heads per plant between treatments with grass and litter alone were not as clear (Figure 2.3), suggesting that other factors may be influencing flower head production, such as competition for light or nutrients. The results were not consistent for all years, so flower heads

per plant may not be a reliable measure of disease severity. Furthermore, the number of flower heads per plant may not be an accurate predictor of reproduction potential as other measurements like seed head biomass, seeds per plant, and seed viability. In some patches Canada thistle plants with high disease incidence produced a large number of flower heads, but the flower heads were often small and may not have produced a significant amount of seed.

Other factors biotic or abiotic may have contributed to the differences in growth and reproductive potential of Canada thistle in this experiment. There are many factors that influence Canada thistle height and reproduction that were not measured in this study, such as nutrients, soil type, and light availability. Other factors such as grass competition with Canada thistle can also cause a reduction in growth and reproduction (39).

In this study, grasses that were competing with Canada thistle may have supported Pst survival and also enhanced its ability to cause disease by reducing the resiliency of the Canada thistle. In 2003, treatments with grass had higher DI as compared to treatments with grass removed (Figure 2.1). There are many examples of grasses acting as competitors in biological control systems that have increased the efficacy of biocontrol agents (36, 39, 40, 41). Grass competition with Canada thistle has been documented to reduce growth, reproduction, and spread of Canada thistle (34, 35, 39). Grass competition with Canada thistle occurred in our study, but more measurements of Canada thistle growth and development are needed to determine the complete affects of grass competition on Canada thistle and infection with Pst.

One possible explanation for the reduction in DI when grass, litter, or grass and litter are removed is that grass and litter are inoculum sources for Pst infection on Canada thistle. When infection first occurred in the spring, the DI levels between treatments were already differentiated (Figure 2.1). This suggests the removal of grass and litter may have reduced the amount of inoculum that was present in the system resulting in a reduction in DI. Plants and plant litter or debris can be important sources of inoculum or reservoirs for pathogens (26, 27). Pst is capable of surviving and overwintering in debris of marigold (25). Similarly, Pst may be surviving on debris in the litter in our experiment, because when litter was removed there was a reduction of Pst DI on Canada thistle (Figure 2.1). There have been numerous studies with other pathogens surviving in litter and debris which serve as inoculum sources for infection (26, 27, 30, 31), so this is a likely explanation for what is occurring in our experiment. The litter material in our study consisted of dead grasses which tend to break down at a slower rate than forb litter (37). In a study by Zhao et al. 2002 (31), *Pseudomonas syringae* pv. *maculicola's* survival was related to the longevity of the litter material with bacteria in aboveground debris having the longest persistence. Therefore, the longevity of the grass litter may be related to the survival of Pst in this experiment. The perennial grasses present in our study may also act as a survival and dispersal mechanism for Pst on Canada thistle, because when grasses were removed DI was reduced (Figure 2.1). A study showed that bacterial pathogens survived on non-hosts which acted as inoculum sources for infection (28) and *Pseudomonas syringae* pathovars can survive on non-hosts (29). Pst is difficult to isolate and quantify from naturally infected Canada thistle plants in the field using standard culturing techniques. In order to assess if grass and litter are inoculum sources, an accurate detection method needs to be developed to test these asymptomatic materials for the presence of Pst.

Another possible explanation for the importance of grass and litter in Pst infection of Canada thistle is that grass and litter may influence the microclimate around Canada thistle that supports Pst infection. Grass and litter contribute to changes in the phyllosphere environment such as temperature and humidity which are known to affect bacteria populations and may have influenced Pst infection of Canada thistle in this study. The environmental data collected in 2006, established that there were relative humidity and temperature differences between the grass and litter intact and grass and litter removed treatments, which may contribute to the DI differences observed.

Relative humidity is an important component of bacterial survival and growth (33). In our study, average daily maximum and minimum RH were 4 to 5 % higher in the grass and litter intact treatment as compared to the grass and litter removed treatment in both early-symptom phase and late-symptom phase (Table 2.4). In the original study which identified Pst on marigold, denser plant spacing caused higher humidity and more disease transmission, while wide spacing of plants caused a drier atmosphere and stopped transmission of disease providing evidence that high humidity caused by plant density can lead to higher disease levels (12). This is similar to our experiment, in which the grasses developed a high plant density which increased the microclimate humidity by 4 to 5% (Table 2.4) which may have increased disease transmission. Thus, the removal of the grass reduced plant density which reduced RH and potential disease transmission. In the late-symptom phase, the duration of RH above 80% was two more hours a day in the grass and litter intact treatment as compared to the grass and litter removed treatment (Table 2.4), suggesting that grass and litter also affect the duration of high levels of RH which may be important for Pst population development. Duration of RH has not been previously studied in this system. Relative humidity can influence Pst dispersal and may explain the differences in Pst infection of Canada thistle.

Temperature is also important in the regulation of Pst growth and toxin production. In 2006, when Pst symptoms occurred on Canada thistle in the early-symptom phase, the average daily maximum temperature was 22 to 24 C, which is close to optimal temperatures for Pst growth. The original study of Pst on marigold established optimal temperatures for Pst growth are 25 to 28 C and reduced growth and survival of Pst occurred at extreme temperatures of 33 to 35 C and 5 to 15 C (12). In 2006, DI in the grass and litter intact treatment was 60% and DI in the grass and litter removed treatment was 14% (Table 2.2) and these differences occurred with initial infection and throughout the season. The range in daily air temperature was 3 C higher in the grass and litter intact treatment as compared to grass and litter removed in both early-symptom and late-symptom phases and may have influenced the rate of Pst growth (Table 2.4). In the early-symptom phase, there were no differences in daily maximum and minimum air temperatures, but in the late-symptom phase the maximum daily air temperature was 1 C higher and the minimum daily air temperature was 2 C lower in the grass and litter intact treatment. This indicates that temperature differences in the late-symptom phase may play a role in maintaining Pst infection and expression of symptoms. The maximum soil temperature was 2 to 3 C greater in grass and litter removed treatment as compared to grass and litter intact treatment which may have led to reduced Pst survival (Table 2.4). One study indicated that high soil temperatures can reduce *Pseudomonas fluorescens* populations (42). Overwintering temperature

may be another interesting measurement for future experiments; since Pst can survive on media for over six months at -20 C (12) litter may act as media for survival and buffer cold temperatures in winter leading to successful overwintering.

A study by Giesler et al. 2000 (43), demonstrated additional hours of leaf wetness can aid in bacterial dispersal. In our experiment, leaf wetness duration was not different between treatments in either time period (Table 2.4). Leaf wetness may have been directly related to precipitation which would have been similar in both treatments. Tichich and Doll 2006 (24), discovered rainfall in the periods following Pst application in the field led to more successful establishment of Pst on Canada thistle, and in another study Tichich et al. 2006 (44) determined that rain events increased Pst populations and facilitated Pst development in Canada thistle leaves. Rainfall amount was not measured in our study, but the amount of time of leaf wetness and amount of rainfall are both important to consider in future studies. Rainfall data does exist for the region of our experiment, but does not exist at the treatment level, so it could not be used to explain the treatment differences in infection of Canada thistle.

Temperature and moisture conditions produced by grass and litter may enhance colonization and survival of bacterial pathogens. Thus, removing either grass or litter may disrupt these conditions and reduce survival and infection of Pst on Canada thistle. However, it is still unknown if the magnitude of the differences in temperature and relative humidity we observed in our study are enough to affect the treatment differences in DI. Additional years of environmental data would provide a better understanding of the relationships between disease incidence and temperature and relative humidity. We also need to examine the individual effects of grass and litter on the environment. In addition, other environmental factors, such as light or soil moisture, may contribute to Pst infection of Canada thistle and should be included in future studies.

Studying the long term effects of these grass and litter treatments on Pst infected Canada thistle will provide a better understanding of the effects of Pst infection on Canada thistle and importance of the environment over time. In the 2005 and 2006 experiments, the same experimental areas were used and high DI was maintained in grass and litter intact treatments for two years (Figure 2.1), suggesting this system is persistent. More long term data is needed to determine the effects of Pst infection on Canada thistle populations that are infected for several consecutive years to determine if repeated disease stress effectively reduces Canada thistle populations. Long term studies of these naturally infected patches could lead to prediction models for Canada thistle control induced by Pst infection.

This study provided evidence of a reduction in disease incidence in treatments with grass, litter, or grass and litter removed when compared to the grass and litter intact treatment. Also, there was a reduction in Canada thistle height and flower heads per plant in the grass and litter intact treatment in most years of the experiment. We recognize that differences were not solely due to disease severity and other factors may have influenced Canada thistle height and flower heads per plant. Environmental data provided evidence that the presence of grass and litter affected the environment in Canada thistle patches indicating that RH and temperature may be important factors contributing to Pst survival and symptom development in Canada thistle. Grass and litter may also serve as inoculum sources for Pst providing surfaces for survival and aiding in

dispersal. Ultimately, grass and litter may encourage Pst infection of Canada thistle through the creation of ideal environmental conditions for infection, inoculum sources, and competition increasing Pst efficacy on Canada thistle.

The addition of grass and litter to the Canada thistle control system may provide the environmental conditions necessary for Pst infection of Canada thistle. More studies are needed to determine why grass and grass litter are so important to the Pst infection system, and to obtain a better understanding of how Pst infects and survives on Canada thistle. Future studies are needed to establish if the grass and litter serve as inoculum sources, alternate hosts for the bacteria, attract insects for vectoring or wounding, or create other environmental conditions beneficial to Pst infection. Understanding the role of grass and litter in the Pst infection system on Canada thistle may help inform the development of integrated management systems that have the potential to manage Canada thistle without herbicides along roadsides and in prairie restoration areas.

Tables

Table 2.1. Repeated measures analysis of variance for disease incidence of *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN with between subject factor as treatment and within subject factor as date of data collection with separate analysis for each year 2001 to 2006.

	2001				2002			
Source of Variation	df[a]	MS	F	P	df	MS	F	P
Between-Subject								
Treatment	3	8082	114	<0.001	3	9016	43	<0.001
Error	12	71			12			
Within-Subject								
Date	2	1976	56	<0.001	2	2069	24	<0.001
Date x Treatment	7	407	11	<0.001	7	338	4	0.006
Error	28	35			27	88		

	2003				2005			
Source of Variation	df	MS	F	P	df	MS	F	P
Between-Subject								
Treatment	3	15468	264	<0.001	3	5998	6	0.01
Error	12	59			12	1004		
Within-Subject								
Date	3	575	12	<0.001	1	262	0.916	0.38
Date x Treatment	9	239	5	<0.001	4	500	1.7	0.19
Error	35	47			16	286		

	2006			
Source of Variation	df	MS	F	P
Between-Subject				
Treatment	2	8075	4.667	0.045
Error	8	1730		
Within-Subject				
Date	2	56	0.065	0.936
Date x Treatment	4	586	0.674	0.618
Error	16	869		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 2.2. Mean disease incidence and Canada thistle plant height (cm) of *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN in 2005 and 2006 with mean separation[a] of treatments.

	Disease Incidence [b]				Canada Thistle Height [c]			
Treatment	2005		2006		2005		2006	
	----------%----------				----------cm----------			
Grass and litter intact	51	a	60	a	80	b	69	b
Litter removed	11	b	35	b	77	ab	68	b
Grass removed	15	b	ND[c]		52	a	ND[d]	
Grass and litter removed	12	b	14	b	59	ab	52	a

[a] Mean separation was determined by Fisher's LSD ($\alpha = 0.05$), values with the same letter for a given column indicate they are not significantly different.
[b] Mean disease incidence averaged across each year.
[c] Mean height of Canada thistle plants averaged across each year.
[d] No data. All replicates in treatment were destroyed by mowing or spraying.

Table 2.3. Repeated measures analysis of variance for Canada thistle plant height in *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN with between subject factor as treatment and within subject factor as date of data collection with separate analysis for each year 2002 to 2006.

	2002				2003			
Source of Variation	df[a]	MS	F	P	df	MS	F	P
Between-Subject								
Treatment	3	4331	79	<0.001	3	3456	62	<0.001
Error	12	55			12	55		
Within-Subject								
Date	2	8449	93	<0.001	2	4739	218	<0.001
Date x Treatment	6	946	10	<0.001	7	337	16	<0.001
Error	22	91			29	22		
	2005				2006			
Source of Variation	df	MS	F	P	df	MS	F	P
Between-Subject								
Treatment	3	3638	3	0.096	2	1224	0.773	0.493
Error	10	1309			8	1584		
Within-Subject								
Date	2	5694	59	<0.001	2	7706	48	<0.001
Date x Treatment	7	42	0.433	0.115	3	52	0.325	0.816
Error	22	97			13	161		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 2.4 Summary[a] of environmental data collected from *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN with early-symptom phase[b] from 5/1/06 to 5/31/06 and late-symptom phase[c] from 6/1/06 to 7/12/06.

	Early-Symptom Phase			
Data type	Grass and Litter Intact	Grass and Litter Removed	Difference[d]	P-value[e]
Relative Humidity, %				
Maximum[f]	97	93	4	< 0.001
Minimum[g]	44	38	5	0.021
Duration[h]	6	4	1	0.11
Air Temperature, C				
Maximum[i]	24	22	1	0.263
Minimum[j]	7	8	1	0.126
Range[k]	17	14	3	0.004
Soil Temperature, C				
Maximum[l]	16	19	3	< 0.001
Minimum[m]	11	12	1	0.264
Range[n]	4	7	3	< 0.001
Leaf Wetness Duration[o]	3	2	0.4	0.455

	Late-Symptom Phase			
Data type	Grass and Litter Intact	Grass and Litter Removed	Difference	P-value
Relative Humidity, %				
Maximum	99	94	4	< 0.001
Minimum	37	34	4	0.042
Duration	9	7	2	0.001
Air Temperature, C				
Maximum	32	31	1	0.004
Minimum	11	13	2	0.011
Range	21	18	3	< 0.001
Soil Temperature, C				
Maximum	21	23	2	< 0.001
Minimum	16	17	1	< 0.001
Range	5	6	1	< 0.001
Leaf Wetness Duration	5	4	1	0.247

a. Mean daily summaries averaged across three replicates for each treatment for the entire collection period. **b.** Early-symptom phase: 5/1/06 to 5/31/06 during this time Canada thistle shoots emerged and developed during the first three and *Pseudomonas syringae pv. tagetis* (Pst) symptoms developed during the fourth week when Canada thistle was in the rosette to early bolting stage. **c.** Late-symptom phase: 6/1/06 to 7/12/06 included Canada thistle early budding to post anthesis stages and the period of time in which there was maximum expression of Pst induced chlorosis. **d.** Difference between treatments for mean daily summaries. **e.** P-values from ANOVA for treatment across the collection periods. **f.** Mean daily maximum RH. **g.** Mean daily minimum RH. **h.** Mean daily hours of relative humidity (RH) above 80% **i.** Mean daily maximum air temperature. **j.** Mean daily minimum air temperature. **k.** Mean daily range in air temperature. **l.** Mean daily maximum soil temperature. **m.** Mean daily minimum soil temperature. **n.** Mean daily range in soil air temperature. **o.** Mean daily hours of leaf wetness above 6 on a scale of 0 to 15 (dry to wet).

Figures

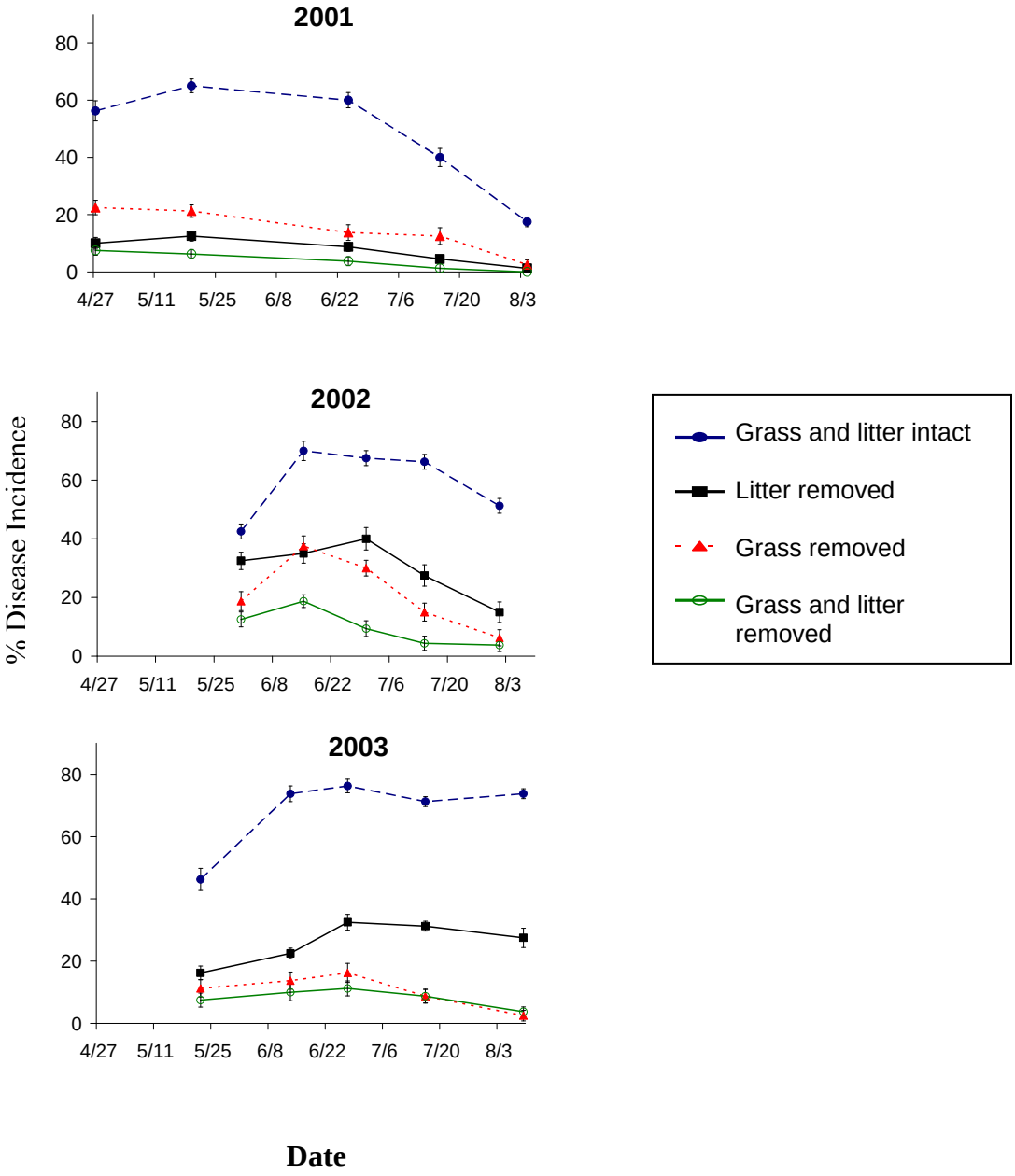

Figure 2.1. Mean disease incidence on *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN averaged across four replicates for each treatment with bars representing ±SE for 2001 to 2003.

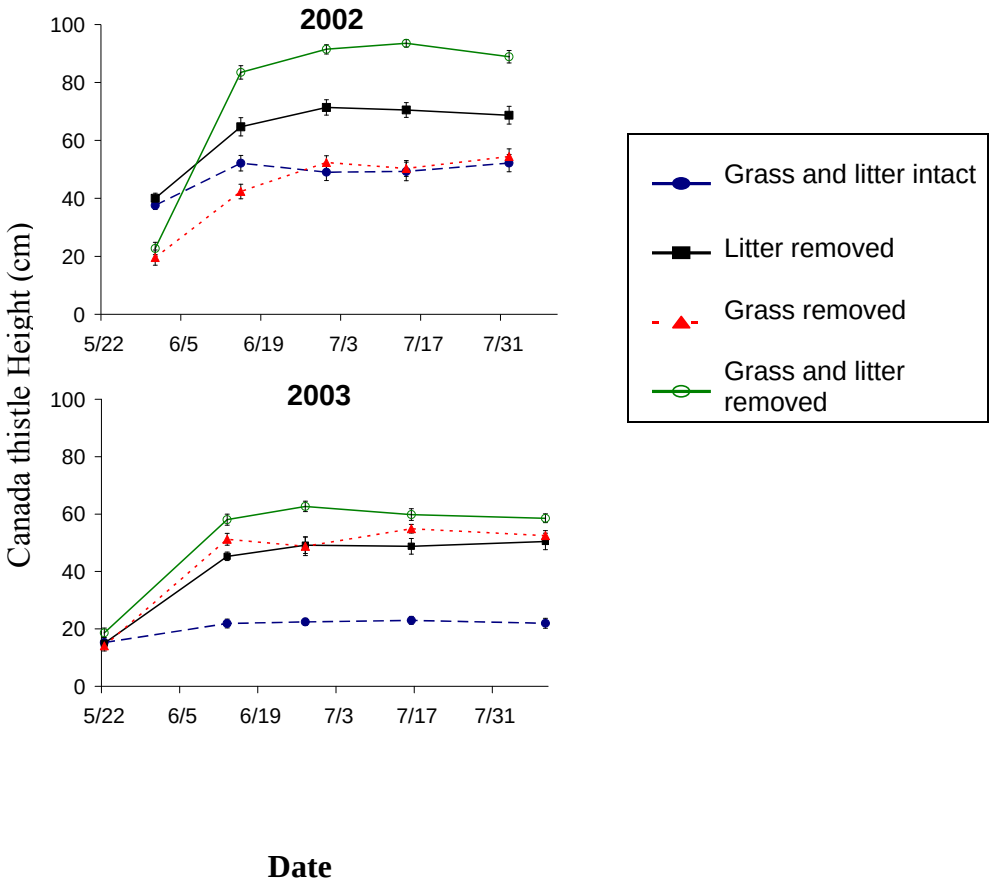

Figure 2.2. Mean Canada thistle plant height (cm) in *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN averaged across four replicates for each treatment with bars representing ±SE for 2002 and 2003.

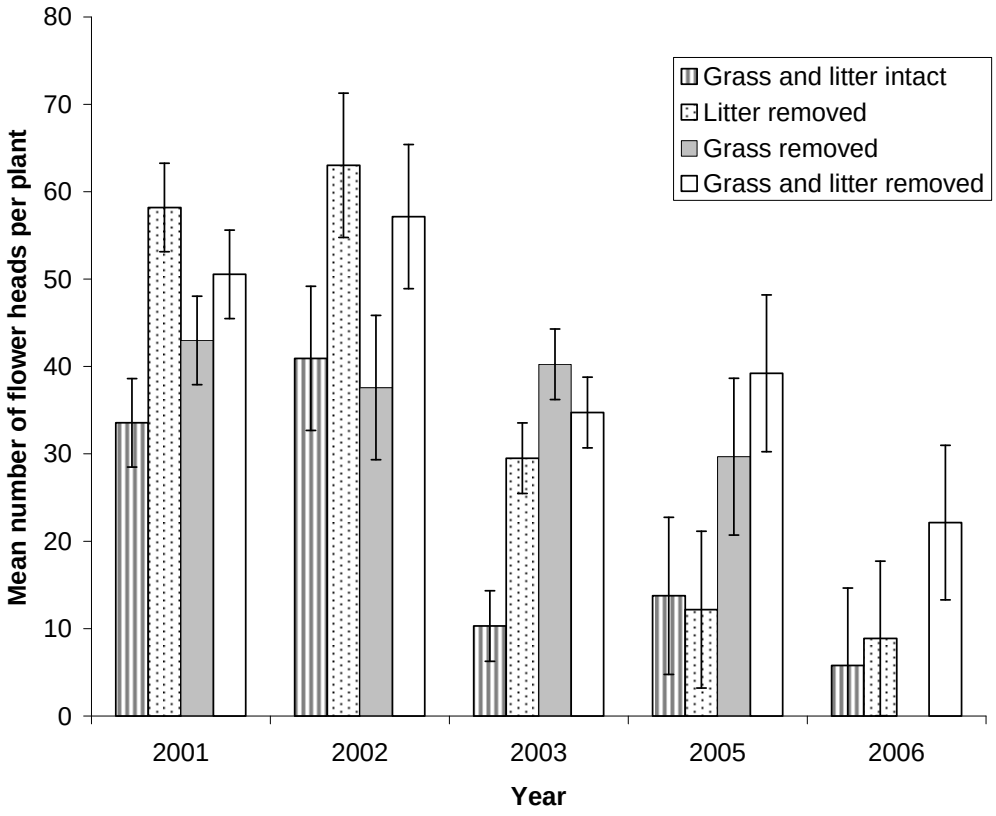

Figure 2.3. Mean flower heads per Canada thistle plant in *Pseudomonas syringae pv. tagetis* naturally infected Canada thistle patches along roadsides in east central MN averaged across four replicates of each treatment for 2001 to 2006. Bars represent Fisher's LSD values at P=0.05.

CHAPTER 3

Effects of grass and litter as part of an integrated management system with *Pseudomonas syringae* pv. *tagetis* for Canada thistle (*Cirsium arvense*) in restored wetland prairie

ABSTRACT

Field experiments were conducted to determine if grass, litter, and soil collected from infected Canada thistle patches or cultured *Pseudomonas syringae* pv. *tagetis* (Pst) act as inoculum sources for Pst infection of Canada thistle and if environments created by perennial grasses and grass litter can increase Pst infection of Canada thistle in areas with introduced or natural Pst infection. Grasses from naturally infected Canada thistle patches acted as inoculum sources for Pst on Canada thistle when transplanted into Canada thistle populations without Pst disease symptoms. However, Canada thistle in the non-treated controls also had low levels of Pst DI, suggesting that a natural population of Pst was present in the experiments or that Pst dispersal occurred between plots. Therefore, the low levels of Canada thistle DI observed in our experiments may not be entirely attributed to the inoculum source in that treatment, but may have occurred through natural infection. Although the grass and litter environments were established for at least one year in all experiments these environments did not increase DI to levels that could impact Canada thistle management. However, more time may be required for Pst populations to establish and increase to levels that result in significant infection of Canada thistle. Canada thistle height and density reductions observed in this study may be attributed to competition with the perennial grasses. Cool season perennial grasses effectively established ground coverage and had the highest impact on Canada thistle density. This experiment indicates that dense stands of perennial grasses should be included as part of an integrated management system for Canada thistle in roadside rights-of-way and wetland restoration sites. More information is needed to determine how Pst infects Canada thistle and whether the perennial grasses and litter are inoculum sources or create environments that support Pst infection of Canada thistle.

INTRODUCTION

Canada thistle (*Cirsium arvense* (L.) Scop) is the most widespread noxious weed in the United States and Canada and requires control in agricultural land, natural areas, and roadside rights-of-way (1, 3). It is a non-native, invasive, perennial species characterized by persistent vegetative growth from an extensive horizontal root system. It also reproduces by seed which can persist in the seed bank for up to 20 years (2, 34). Canada thistle thrives in areas with little or no competition (2), such as prairie restorations, CRP lands, roadsides and non-disturbed areas containing mixtures of perennial grasses and legumes (5, 6). Prairie restorations typically involve disturbance, a reduction in plant competition, and slow growing native grasses and forbs planted from seed which all provide ideal conditions for colonization by Canada thistle (8). A survey of CRP lands in Minnesota found Canada thistle was one of the most common noxious weeds and was on 65 to 75% of all lands surveyed (5). Canada thistle is also common in upland areas of natural and restored wetlands (4).

Wetlands are an essential part of the Minnesota landscape because they provide essential ecosystem services such as, stabilizing the hydrology of watersheds and providing habitat for 40% of Minnesota's rare plant and animal species (45). For these reasons, wetlands need to be maintained and managed properly. Section 404 of the Federal Clean Water Act requires no net loss of wetlands and describes the necessary mitigation to offset wetland losses. In the event a wetland is destroyed then another of equal value must be created, enhanced, or restored (46). Current Wetland Conservation Act (WCA) guidelines for restored or mitigated wetlands require a five year vegetation management plan which includes control of noxious weeds (7). Canada thistle is a common problem in establishing prairie wetlands, because it out competes native plants. Management practices for Canada thistle in non-crop land areas currently include integration of mowing, burning, tillage, and application of herbicides; however these methods can be costly and damaging to some native species (9). Therefore, there is increasing interest in developing an integrated management system for Canada thistle that can provide selective control. A unique management system must be developed for diverse perennial plant systems that contain grass and forb mixtures and can be used in wetland prairie restorations. An integrated management system which includes a biological control agent may offer an environmentally safe and cost effective alternative for long term management of Canada thistle in perennial systems.

Pseudomonas syringae pv. *tagetis* (Pst), a bacterial pathogen that naturally infects Canada thistle has been identified as a potential biological control agent (16,17). Pst occurs naturally on several species in the Asteraceae family causing severe apical chlorosis due to production of tagetitoxin, a toxin which is translocated to the plant apex (18,19). In plants, tagetitoxin inhibits RNA polymerase III, thereby disrupting the synthesis of chloroplasts in plant tissue, which causes a characteristic apical chlorosis in all new tissue in infected plants. Pst has been shown to reduce survivorship, growth, and reproduction in several aster species including Canada thistle (10, 11, 14, 21). For this reason, Pst has been studied as a potential biological control agent for Canada thistle.

An initial study, examining Pst as a biological control agent, demonstrated almost complete control of Canada thistle after five applications of Pst in conventional soybean (16,17). However, two applications of Pst were not sufficient to effectively control Canada thistle in organic soybean (21). Repeated applications of Pst, although they may be effective, are too costly to be considered an alternative to traditional control methods for Canada thistle in annual cropping systems.

Integrated management including a competitive soybean variety and Pst application resulted in a reduction in survival, growth, and reproduction in Canada thistle with multiple applications of Pst, but there was no increase in effects with the competitive soybean variety (22). In another study, Pst was combined with an fungal protein Nep 1, but there were no improvements in Canada thistle control when comparing the combination to applying either alone (23). Although previous integrated management systems with Pst have not improved control of Canada thistle, one based on the natural Pst infection of Canada thistle may be the appropriate approach in perennial systems.

The natural distribution and frequency of Pst infection of Canada thistle is not well described and neither is its impact on Canada thistle growth and development in natural systems. Pst infection on Canada thistle is particularly prevalent in roadside rights-of way which are characterized by perennial grasses and a thick litter layer which consists of dead plant material from associated perennial grasses. In a study of naturally infected Canada thistle patches along roadsides, the presence of both grass and litter was necessary for high Pst disease incidence on Canada thistle (Chapter 2). That study also provided evidence that grass and litter altered relative humidity and temperature in areas with naturally infected Canada thistle which may increase Pst infection of Canada thistle. An increase in Pst infection of Canada thistle may be achieved by creating the appropriate environment around Canada thistle with grass and litter and introducing Pst into the management system. A recent study examined a different method of Pst application in which sap from naturally infected Canada thistle plants was applied with a surfactant to non-infected Canada thistle plants in an undisturbed area. Disease incidence ranged from 25 to 40%, which was below that required to affect control of Canada thistle. However, there were indications that Pst became a long term component of the community, but it was not determined why this occurred (24). For Pst to be used as an effective control strategy, new techniques need to be developed that will sustain high populations of Pst for extended periods of time. More information is needed to determine what regulates Pst populations dynamics and infection of Canada thistle in natural systems. Pst is difficult to isolate and quantify from naturally infected Canada thistle plants in the field using standard culturing techniques. So, in most studies it is assumed that Canada thistle shoots exhibiting apical chlorosis symptoms have high populations of Pst. Creating natural environments that support initial infection and sustain infection over time are key components in the development of an integrated management system for Canada thistle.

Plants and plant litter or debris can be important sources of inoculum or reservoirs for pathogens (26, 27). Pst is capable of surviving and overwintering in debris of marigold (25), and may have similar survival capabilities in Canada thistle patches. One possibility is Pst survives in Canada thistle debris present in the litter layer. There have been numerous studies demonstrating

pathogens surviving in litter and debris which serve as inoculum sources (26, 27, 30, 31). In one study, *Pseudomonas syringae* pv. *maculicola's* survival was related to the longevity of the litter material with bacteria in aboveground debris having the longest persistence (31). Pst may survive on the grass litter which breaks down at a slower rate than forb litter (37). Another possibility is that Pst survives on living tissue such as grasses. Bacterial pathogens surviving on non-hosts and acting as inoculum for infection have been documented (28, 29). Bacterial pathogens can be recovered from soil that contains infected plant debris, but soil sources are of minor importance as inoculum (30, 47). Pst may have similar survival capabilities on plants, litter, or debris present in Canada thistle patches.

The phyllosphere environment has profound effects on bacterial populations. The presence of perennial grasses and litter increases relative humidity and temperature range in Pst naturally infected Canada thistle patches and these environmental components may support Pst populations and infection of Canada thistle (Chapter 2). Litter creates moist conditions increasing plant susceptibility to disease, (32) and humidity is an essential component to the infection system in diseases caused by bacteria (33). Colonization and survival of bacterial pathogens is generally enhanced in protected environments with stable temperature, light, and moisture (38). Grass and litter may provide the necessary phyllosphere environment for Pst populations to successfully cause and maintain disease on Canada thistle.

Perennial grasses are effective competitors with Canada thistle (34). In a greenhouse study with two perennial grasses used in this study, Canada wild rye and smooth brome, grasses effectively competed with Canada thistle causing increased stress, reduced survival, and enhanced control (35). In another study, perennial grasses were effective in inhibiting the growth of two thistle species *Carduus nutans* L. and *Cirsium vulgare* L., but the effect was not dependent on the type of grass (36). Allelopathic interactions with roots and root competition with perennial grasses have been shown to decrease thistle survival and persistence (36). Following control of Canada thistle, beneficial competitors, such as native plants, may be needed to the fill the niche vacated by the target weed to inhibit new weeds from establishing (48).

Combining grass competition with biological control agents of Canada thistle has been previously studied. The insect biological control agent, *Cassida rubiginosa* Muller, a defoliator of Canada thistle was studied in combination with grass competition, and the combination was more effective than the insect alone at reducing Canada thistle growth (41). The same biocontrol agent was also studied in combination with annuals and perennials which were seeded to 100% vegetation cover causing a decrease in thistle populations (40). A shoot boring weevil, *Apion anonpordi* Kirby, was studied in combination with grass competition and a synergy between the weevil and competition occurred which reduced growth and reproduction of Canada thistle (39). There is evidence that combining biocontrol agents with grass competition can increase the establishment of the biocontrol agent and increase control of Canada thistle. The combination of grass competition and Pst have not been previously studied, but given that perennial grasses and Canada thistle infected with Pst naturally occur this may be an appropriate approach for Canada thistle control in perennial systems.

There is need to develop integrated management systems for Canada thistle in prairie wetland restoration areas that do not rely on the use of herbicides. We evaluated Canada thistle management systems that utilized perennial grasses and grass litter to create environments that are highly competitive with Canada thistle and have been shown to support Pst infection of Canada thistle. We designed experiments to determine if perennial grasses and grass litter can be used to create environments that increase Pst infection of Canada thistle from grass, litter, and soil collected from infected Canada thistle patches or cultured Pst inoculum sources. We pursued the following objectives through field studies: 1. Determine if perennial grass and grass litter can be used to create environments that increase Pst infection of Canada thistle by assessing Canada thistle disease incidence (DI), height, density, and reproductive potential. 2. Evaluate grass, litter, soil, and cultured Pst as inoculum sources for Pst infection of Canada thistle by assessing Canada thistle disease incidence, height, density, and reproductive potential. 3. Evaluate the impact of Pst and competitive perennial grasses on Canada thistle management.

MATERIALS AND METHODS

Site Description

The Cheri Ponds, Minnesota Department of Transportation (Mn/DOT) mitigation wetland area in Carver County, MN was selected as the field research site. Before restoration began in 2002, the area was conventionally farmed and supported large patches of Canada thistle that were in close proximity to a wetland area. In preparation of the site, soil was redistributed within the landscape which moved the Canada thistle roots and seed bank throughout the site. The site was seeded with a wetland prairie seed mix for the wetland fringe and a mesic prairie seed mix for the upland area. The native species were slow to develop because of drought in 2002 and 2003, preventing much of the seed from germinating until 2004. Established Canada thistle patches were mowed once in late July of 2003 after Canada thistle seed production. The Mn/DOT mitigation area was selected for this research, because it contained dense, uniform Canada thistle patches. The four Canada thistle patches selected for our experiments were located in distinctly different landscape positions ranging from non-disturbed to highly disturbed and varied in slope and Canada thistle density. Patch 1 was flat with a Canada thistle density of 52 plants m^{-2} and was in an non-disturbed area, patch 2 was on an east facing slope with a Canada thistle density of 34 plants m^{-2} in a disturbed area, patch 3 was on a southeast facing slope with a Canada thistle density of 19 plants m^{-2} in a disturbed area, and patch 4 was on a west facing slope with a Canada thistle density of 45 plants m^{-2} in a disturbed area (Appendix B). In this study a Canada thistle plant is defined a separate aboveground shoot as the root systems connections were not examined in this study.

In early spring 2004 and 2005, when Canada thistle plants were emerging and three weeks before initiating the experiments, the four experimental areas were treated with glyphosate at 0.386 kg ha^{-1} and dicamba at 0.702 kg ha^{-1} applied with a boom sprayer at 242 kPa to reduce weed competition.

Introduced Infection Experiment 1

Experimental Design

Three areas with uniform Canada thistle (Patches 1, 2, 3) were selected at Cheri Ponds Mn/DOT mitigation area as replicates for this experiment (Appendix B). Canada thistle patches with no symptoms of Pst infection were selected in the growing season prior to initiation of the experiment. The experiment consisted of 26 treatments in a randomized complete block design with three replications. The 26 treatment combinations were designed to create an environment with perennial grass and litter that could potentially increase Pst infection of Canada thistle from Pst inoculum sources grass, litter, soil, or cultured Pst (Table 3.1, Appendix C). The experiment was initiated in 2004, and plot size was 1.5 x 1.5 m with 0.5 m alley between plots.

Grass Treatments

In this experiment, perennial grass treatments included cool season native, warm season native, cool and warm season native mix, and a cool season exotic. The perennial grass treatments were designed to determine if early season grass coverage, late season grass coverage, or continuous grass coverage are most beneficial for Pst establishment on Canada thistle. The cool season native grass, Canada wild rye (*Elymus canadensis*) and the warm season native grass, Indian grass (*Sorghastram nutans*) were chosen based on frequency in prairie mixes and history of establishment in previous Mn/DOT wetland restorations (Bob Jacobson). Smooth brome grass (*Bromus inermis)*, a non-native cool season grass, was included because it is one of the most common grasses associated with naturally infected Canada thistle patches on road rights-of-way.

Greenhouse grown perennial grass seedlings were transplanted in the experiment to ensure success of establishment. Seedlings of Canada wild rye, Indian grass, and smooth brome (Appendix D) were grown for six weeks in the greenhouse in a 1:1 mixture of sterile greenhouse soil and Sunshine potting medium in 1 cm diameter, 15 cm deep plastic cones under a 16 hour photoperiod. In late May, the 15 cm tall grass seedlings were transplanted into the field in a 15 cm grid spacing for a total of 80 plant m^{-2}. To transplant the seedlings, a dibble bar was used to create a hole the size of the cone and then the seedling with entire root mass was placed in the hole and watered immediately after transplanting. The cool and warm season grass mix treatment was planted at a 1:1 cool and warm season grass ratio. Grass transplanting occurred over a three week period. Due to low germination of Indian grass in 2004, there were not enough seedlings for transplant, so seedlings of native warm season grass big bluestem (*Andropogon gerardi*) were used to supplement the warm season grass treatments. Big bluestem was obtained from two year old stands on St. Paul Campus field plots by removing 10 cm cores and dividing into individual plants which were transplanted in a 1:1 mixture with Indian grass. Therefore, in 2004, the warm season grass treatment was a mix of Big bluestem and Indian grass. Seedlings were watered every other day for three weeks following transplant to ensure plant survival. The Canada thistle in all plots was mowed biweekly to the height of the transplanted grasses until transplants were established in July 2004.

A non-treated control was included in the experiment that consisted of the naturally occurring vegetation in the plots, which was mainly annual grasses such as Giant foxtail (*Setaria faberi*) and native grasses from the original prairie mixture. The grass density in these control plots was lower than treatments with transplanted grasses.

Some treatments contained no grass, so in order to clearly differentiate between treatments with and without grass an herbicide treatment of quizalofop at 0.05 kg ai ha^{-1} plus crop oil concentrate at 0.3% [v/v] was applied with a hand sprayer at 207 kPa to control naturally occurring grasses in the plot. Herbicide treatment began in the spring, when grasses first emerged and was repeated biweekly to control all grasses as needed.

Litter Treatment

Litter consisting of fine dead grasses was collected from a Canada thistle patch with 7 plants m^{-2} without Pst symptoms (non-infected) for application in the experimental plots. The litter was collected from a Canada thistle patch located within 5 miles of the Cheri Ponds mitigation area (Appendix D). In mid-August, litter was removed from an area equal in size to the experimental litter treatment area by first mowing and removing all living plant material above the litter level. Then the litter was loosened with a gas trimmer, collected using rakes, thoroughly mixed, and stored in 75 liter plastic containers overnight at 22 C before being applied to experimental plots. Litter was applied by hand to the ground around the plants at 0.67 kg m^{-2} to yield a layer 2 to 5 cm thick. In 2005, the same amount of litter was reapplied in mid-August to ensure that the litter layer was maintained.

Sources of Pst Inoculum

Grass

To test grasses as a possible source of Pst inoculum, grass plants were collected from a roadside Canada thistle patch exhibiting symptoms of Pst infection located within 5 miles of the Cheri Ponds mitigation area (Appendix D). The primary perennial grasses associated with Canada thistle at this site were cool season grasses mainly Smooth brome grass and Kentucky blue grass (Appendix D) and will be referred to as exotic grass from an infected site. In May 2004, at the same time the greenhouse grasses were transplanted, exotic grasses from the infected site were collected using a 10 cm coring device and planted the same day in the plots on a 30 cm grid spacing for a total of 9 cores m^{-2}. These grass transplants contained intact roots and soil which were transplanted with the grasses in the plots.

Litter

To test litter as a possible source of Pst inoculum, litter consisting of fine dead grasses was collected from a Canada thistle patch with 27 plants m^{-2} and forty percent Pst disease incidence (infected site) for application in experimental plots. The litter was collected from a roadside Canada thistle patch located within 5 miles of the Cheri Ponds mitigation area (Appendix D). In August 2004 and 2005, litter was collected and applied using the same methods as described earlier for litter from a non-infected site.

Soil

Following litter collection from the Pst infected Canada thistle patch, soil was collected as a source of Pst inoculum (Appendix D). The top 5 cm of soil was removed from a 1.5 x 3 m area, shredded, mixed with a mechanical soil shredder, and then sifted by hand through a 0.5 cm grid to remove roots and debris. Soil was stored in 18 liter plastic containers over night at 22 C and applied to experimental plots the following day. The sifted soil was spread over the experimental plots at 5 liters m^{-2} in a 1 cm layer, which fully covered the ground. In treatments with both soil and litter, the soil was applied first and the litter was placed on top of the soil layer.

Cultured Pst

Pst cultured in the laboratory was applied to plots as a source of inoculum. Pst strain 1-502a, originally isolated from Canada thistle in Minnesota was the strain used for all applications (16,17). Pst was grown in 2.5 liter Fernbach flasks by inoculating one liter of tryptic soy broth with 100 mg of Pst cells which was incubated on a rotary shaker at 300 rpms for 18 hours at 23 C. The resulting culture was centrifuged for 10 minutes at 5,000 rpms and the pellet was resuspended in sterile deionized water to a concentration of $1x10^9$ colony forming units per milliliter (cfu/ml).

In August 2004 and 2005, liquid Pst culture was mixed with non-infected litter in a ratio of 500 ml of liquid Pst culture to 1 kg litter. The amount of litter for each plot was measured out and mixed with liquid Pst culture by hand. Then the Pst amended litter was applied at 0.67 kg m^{-2} to the plots using the same litter application methods as described earlier. In the Pst culture treatment without litter 333 ml m^{-2} of liquid Pst culture was evenly sprayed with a hand held sprayer directly onto the ground avoiding contact with Canada thistle plants.

Maintenance of Experimental Site

The 0.5 m alley between each plot was mowed biweekly throughout the duration of the experiment to allow access to the plots for data collection and application of treatment components. In 2005, litter, soil, and Pst were reapplied to appropriate treatments in the fall to ensure inoculum sources were present. Following reapplication of litter, the plots were mowed to allow more uniform application of litter. The mowed plant materials and the reapplied litter resulted in a total litter layer of 10 to 15 cm. In the no litter treatments, plant materials were mowed and removed by raking the materials off the plots.

Data Collection

Canada Thistle Disease Incidence

Disease incidence (DI) was visually estimated in each plot as the percentage of Canada thistle plants exhibiting symptoms of apical chlorosis ranging from 0 (no disease) to 100 (all plants exhibiting symptoms). Evaluation of DI began in early June, at the first indication of Pst infection of Canada thistle in the untreated control areas and continued every two weeks until senescence in the fall for a total of four to five assessments each season.

Canada Thistle Plant Height

Evaluation of Canada thistle plant height began at the same time as disease incidence data collection and continued every two weeks until senescence in the fall for a total of four to five assessments each season. In 2005 and 2006, Canada thistle height was evaluated on each date by measuring the heights of three randomly selected Canada thistle plants in each plot.

Canada Thistle Flower Heads

Since Canada thistle is dioecious, male and female flowers are on separate plants, there was variation between plants and patches for seed production, so the number of flower heads per plant was used as an indicator of potential Canada thistle reproduction. A Canada thistle flower head is a 1 to 2 cm diameter compact cluster of flowers arranged so that the whole gives the effect of a single flower. In 2005 and 2006, the number of flower heads per plant was counted on three randomly selected Canada thistle plants in each plot when Canada thistle was in the post anthesis stage of development.

Canada Thistle Density

In 2005 and 2006, Canada thistle density was measured by counting all Canada thistle plants in each plot in the spring and fall to evaluate change in Canada thistle density.

Grass and Forb Percent Cover
In 2006, percent coverage of grasses and forbs was assessed in the spring and fall. The percent of ground covered by grasses and by forbs was visually estimated in each plot. Forbs assessments did not include Canada thistle.

Data Analysis

Introduced Infection Experiment 1 had two years of data which were analyzed separately. All statistical analysis was conducted with the SPSS statistical package. A factorial repeated measure analysis of variance (ANOVA) was conducted on Canada thistle disease incidence and height by comparing the different factors in the experiment block, grass, litter, soil, Pst, and the appropriate interactions with time of data collection. LSD multiple comparisons were conducted on factors with more than two treatments comparing the different treatments within each factor. Mean DI was averaged across time of sampling and block. Mean Canada thistle plant height was averaged across samples, time of sampling, and block. A factorial ANOVA was conducted for Canada thistle density, flower heads per plant, and percent coverage of grass and forbs. LSD multiple comparisons were conducted on factors with more than two treatments comparing the different treatments within each factor. Mean Canada thistle flower heads per plant, density, and grass and forb coverage were averaged across samples and blocks.

Introduced Infection Experiment 2

Experimental Design

A modified duplication of the Introduced Infection Experiment 1 was initiated in 2005 adjacent to the Introduced Infection Experiment 1 in an area with a heavy, uniform Canada thistle population with no symptoms of Pst infection in the growing season prior to initiation of the experiment. This experiment included replicates of ten treatments from the Introduced Infection Experiment 1. In this experiment, perennial grass treatments included a mixture of cool and

warm season native grasses transplanted from the greenhouse to create an environment with continuous grass coverage. The experiment consisted of 17 treatments in a randomized complete block design with three replications (Table 3.2). The 17 treatment combinations were designed to create an environment with perennial grass and litter that increased Pst infection of Canada thistle from grass, litter, soil, or cultured Pst inoculum sources (Table 3.2). Plot size was 1.5 x 1.5 m with 0.5 m alley between plots.

Grass Treatment

In May 2005, the cool season native grass, Canada wild rye (*Elymus canadensis*) and the warm season native grass, Indian grass (*Sorghastram nutans*) (Appendix C) were grown in the greenhouse and planted in a 1:1 mixture in the field as described in the Introduced Infection Experiment 1. A no grass control treatment was included in the experiment as described in the Introduced Infection Experiment 1.

Litter Treatment

To test if litter creates an environment for Pst infection, in August 2005, litter from the same Canada thistle patch without symptoms was collected and applied to plots as described in the Introduced Infection Experiment 1.

Sources of Pst Inoculum

Grass

To test grasses as a possible source of Pst inoculum, in May 2005, grasses were collected from a roadside Canada thistle patch exhibiting symptoms of Pst infection adjacent to the collection area in the Introduced Infection Experiment 1. Exotic grasses from the infected site were obtained and planted as described in the Introduced Infection Experiment 1.

Litter

To test litter as a possible source of Pst inoculum, in August 2005, litter was collected from a Canada thistle patch with symptoms of Pst infection adjacent to the litter collection area in the Introduced Infection Experiment 1 and applied using the same methods.

Soil

Following litter removal from the Pst infected Canada thistle patch, soil was collected as a source of Pst inoculum. Soil was collected, processed, and applied to experimental plots as described in the Introduced Infection Experiment 1 (Appendix C).

Cultured Pst
Pst was cultured in the laboratory as described in the Introduced Infection Experiment 1 to produce liquid culture which was applied in combination with grass, litter, and soil as inoculum sources for Pst infection of Canada thistle.

In May 2005, a high concentration of Pst (1 x 10^9 cfu/ml) was applied with a hand held sprayer at 333 ml m^{-2} on cool and warm season mix grass seedlings after planting as described in the Introduced Infection Experiment 1. This treatment will be referred to as cool and warm season grass mix with Pst.

In August 2005, litter was collected from the same non-infected Canada thistle patch as described earlier and liquid Pst culture was mixed with litter in a ratio of 500 ml of liquid culture to 1 kg litter separately by hand for each plot and then this modified litter was applied at 0.67 kg m^{-2} to the plots using the same litter application methods as described in the Introduced Infection Experiment 1.

In August 2005, following litter removal in the non-infected Canada thistle patch (Appendix C) the top 5 cm of soil was removed from a 1.5 x 3 m area and was processed as described earlier. Soil was mixed in a ratio 333 ml of Pst (1x10^9 cfus/ml) to 5 liters of soil by hand separately for each plot and then this modified soil was applied to plots at 5 liters m^{-2} as described in the Introduced Infection Experiment 1.

Maintenance of Experimental Site

The 0.5 m alley between each 1.5 x 1.5 m plot was mowed biweekly throughout the duration of the experiment to allow access to the plots for data collection and application of treatment components.

Data Collection

In 2006, Canada thistle disease incidence, height, flower heads per plant, density, and percent ground cover of grasses and forbs were measured as described earlier in the Introduced Infection Experiment 1.

Data Analysis

The Introduced Infection Experiment 2 had one year of data which was analyzed as described in the Introduced Infection Experiment 1.

Enhanced Infection Experiment

Experimental Design

The Enhanced Infection Experiment was set up in an area at Cheri Ponds with a dense uniform stand of Canada thistle that had light (5%) uniform symptoms of Pst infection in the growing season prior to initiation of the experiment (Patch 4 Appendix B). The objective of this experiment was to determine if perennial grasses and grass litter can be used to create environments that increase Pst infection on Canada thistle that previously exhibited symptoms of Pst infection. The experiment consisted of 7 treatments in a completely randomized design with three replications. The treatments consisted of a mixture of cool and warm season native grasses, exotic grass, and litter from a non-infected site applied alone and in combination with the grass treatments (Table 3.3). Plot size was 1.5 x 1.5 m with 0.5 m alley between plots. The Enhanced Infection Experiment was established in 2004 and repeated in an adjacent area in 2005.

Grass Treatments

To test if perennial grasses enhance Pst infection, grasses were transplanted as seedlings to ensure success of establishment. Perennial grass treatments consisting of a 1:1 mixture of Canada wild rye (*Elymus canadensis*) and Indian grass (*Sorghastram nutans*) and an exotic grass smooth brome (*Bromus inermis)* (Appendix C) were grown in the greenhouse and planted in plots as described in the Introduced Infection Experiment 1. A no grass treatment was included in the experiment as described in the Introduced Infection Experiment 1.

Litter Treatment

To test if litter enhances Pst infection, litter was collected from a Canada thistle patch without Pst symptoms. In August, litter was collected from the same location and applied to plots as described in the Introduced Infection Experiment 1 (Appendix C).

Experiment Maintenance

The 0.5 m alley between each 1.5 x 1.5 m plot was mowed biweekly throughout the experiment duration to allow access to the plots for data collection and application of treatment components. In 2005, litter was reapplied to appropriate treatments in the fall as described in the Introduced Infection Experiment 1.

Data Collection

In 2005 and 2006, evaluation of experimental treatments began in early June at the first signs of infection in naturally infected areas. Canada thistle DI, height, flower heads per plant, density, and percent cover of grass and forbs were evaluated as described in the Introduced Infection Experiment 1.

Data Analysis

All statistical analysis was conducted with the SPSS statistical package. A factorial repeated measure analysis of variance (ANOVA) was conducted on Canada thistle DI and height data by comparing the different factors (grass and litter) in the experiment and the appropriate interactions with time of data collection. LSD multiple comparisons were conducted comparing grass treatments. Mean DI was averaged across time of sampling and replicate. Mean Canada thistle plant height was averaged across samples, time of sampling, and replicate. A factorial ANOVA was conducted for Canada thistle density, flower heads per plant, and percent coverage of grass and forbs. Mean Canada thistle flower heads per plant, density, and grass and forb coverage were averaged across samples and replicate.

RESULTS

Disease Incidence

Introduced Infection Experiment 1

In 2005, there was an interaction between grass and litter for DI of Pst on Canada thistle (Table 3.4). In 2005, treatments with exotic grass from an infected site and litter from an infected site had higher DI (23%) than other treatments (Table 3.5). In 2006, there were no differences in DI between treatments. In both years DI varied by date of sampling, but there was no interaction between date and any treatment factors.

Introduced Infection Experiment 2

In 2006, there were interactions between grass and date of sampling and litter and date of sampling for DI of Pst on Canada thistle (Table 3.6). There was a peak in DI on the second sampling date in 2006. Mean DI was higher in treatments with exotic grass from an infected site as compared to all grass treatments on the second sampling date (Figure 3.1). Treatments with no litter had higher DI than treatments with litter on the second sampling date (Figure 3.2).

Enhanced Infection Experiment

There were no differences in DI between grass and litter treatments in either 2005 or 2006 (Table 3.7). DI varied by date of sampling in both years of this experiment, but there was no date by treatment interaction.

Canada Thistle Height

Introduced Infection Experiment 1
Canada thistle height varied between grass treatments in 2006 (Table 3.8). In 2006, mean Canada thistle height was lower in treatments with no grass and natural grass as compared to all other grass treatments (Table 3.9).

Introduced Infection Experiment 2

Canada thistle height varied between grass treatments and there was an interaction between soil and date of sampling in 2006 (Table 3.10). Mean Canada thistle height was lower in all treatments with grass as compared to treatments with no grass (Table 3.11). The effects of soil treatments on Canada thistle height were unstable overtime (Figure 3.3).

Enhanced Infection Experiment

There was no variation between Canada thistle height in 2005 and 2006 (Table 3.12).

Canada Thistle Flower Heads

Introduced Infection Experiment 1

In 2005, there was no variation in Canada thistle flower heads per plant for treatment in either 2005 and 2006 (Table 3.13).

Introduced Infection Experiment 2

There were no differences in flower heads per plant for grass, litter or soil treatments in 2006 (Table 3.14).

Enhanced Infection Experiment

The number of Canada thistle flower heads per plant varied between litter treatments in 2005 (Table 3.15). In 2005, treatments with no litter had more flower heads per plant (27 plant^{-1}) as compared to treatments with litter (20 plant^{-1}) (Table 3.16).

Canada Thistle Density

Introduced Infection Experiment 1

In 2005 and 2006, there was no variation in Canada thistle density for treatments (Table 3.17).

Introduced Infection Experiment 2

In 2006, Canada thistle density varied between grass treatments (Table 3.18). Mean Canada thistle density was lower in all treatments with grass as compared to treatments with no grass (Table 3.19).

Enhanced Infection Experiment

Canada thistle density varied between grass treatments in 2006 (Table 3.20). In 2006, mean Canada thistle density was lower in treatments with cool and warm season grass mix (13 plants m^{-2}) and exotic grass (15 plants m^{-2}) as compared with treatments with no grass (18 plants m^{-2}) (Table 3.21).

Percent Coverage of Grass and Forbs

Introduced Infection Experiment 1

Grass and forb coverage varied between grass and litter treatments (Table 3.22). Mean grass coverage was higher in treatments with cool and warm season grass mix and exotic grass as compared to treatments with warm season grass, exotic grass from an infected site, and natural

grass (Table 3.23). Mean forb coverage was higher in exotic grass from an infected site, natural grass, and no grass as compared to all other grass treatments (Table 3.23).

Introduced Infection Experiment 2

There were differences in grass treatments for grass and forb coverage in 2006 (Table 3.24). Mean grass coverage was higher in treatments with cool and warm season grass mixed with Pst (69%) as compared to treatments with natural grass (40%) (Table 3.25). Mean forb coverage was higher in treatments with no grass as compared to all treatments with grass (Table 3.25).

Enhanced Infection Experiment

Grass and forb coverage varied between grass treatments (Table 3.26). Mean grass coverage was higher in treatments with the cool and warm season grass mix (78%) and exotic grass (76%) as compared to treatments with natural grass (20%) (Table 3.27). Mean forb coverage was higher in treatments with no grass (48%) and natural grass (45%) as compared to treatments with cool and warm season grass mix (10%) and exotic grass (8%) (Table 3.27).

DISCUSSION

Field experiments were conducted to determine if grass, litter, soil, or cultured Pst act as inoculum sources for Pst infection of Canada thistle and if environments created by perennial grasses and grass litter can increase Pst infection of Canada thistle in areas with introduced or natural Pst infection. Pst was introduced in areas that previously had no infection, but it was difficult to distinguish which treatments were more successful at introducing Pst. Pst was present in all experiments, but it may take more time for Pst populations to establish and infection may be increased as the grass and litter environments continue to mature. Although there were differences in DI between treatments in some years, these differences were not large enough to impact Canada thistle growth and development. The grass and litter environments had been established for two years in the Introduced Infection Experiment 1 and one year in the Introduced Infection Experiment 2, so some differences in results between experiments may be explained by the differential periods of time that the treatments had been established. We expect the environment created by grass and litter treatments to evolve and may support Pst infection of Canada thistle with time.

An objective of this study was to determine if grass, litter, soil, or cultured Pst are sources of inoculum for infection of Canada thistle. Grasses were tested as a source of Pst inoculum by transplanting grasses collected from a naturally infected Canada thistle patch into treatments and litter was tested by adding a litter layer with litter collected from naturally infected Canada thistle patches. In the Introduced Infection Experiment 1, in 2005, treatments with exotic grass from an infected site and litter from an infected site had higher DI (23%) (Table 3.5) as compared to other treatments indicating grass and litter are potential inoculum sources for Pst infection of Canada thistle. In the Introduced Infection Experiment 2, treatments with exotic grass from an infected site had higher DI (37%) than all other grass treatments at the second sampling date in 2006 (Figure 3.1). Also at the second sampling date treatments with no litter had higher DI (33%) than treatments with litter (Figure 3.2). The role of litter as an inoculum source is not clear because in the Introduced Infection Experiment 1 litter may be a source of Pst inoculum for infection of Canada thistle or it may contribute to the environment, but in the Introduced Infection Experiment 2 treatments with no litter had higher DI. A test needs to be developed to determine if litter contains Pst. However, these experiments indicated that grasses transplanted from a Pst infected Canada thistle patch may be a source of inoculum for Pst infection of Canada thistle, because of higher DI in both Introduced Infection Experiments 1 and 2. Grasses are known to be symptomless hosts for bacterial pathogens, and *Pseudomonas syringae* strains have the ability to survive epiphytically on non-hosts (28, 29). Pst was most likely present in the exotic grasses transplanted from an infected site. However, it is not known if the soil, debris, roots, or seeds that were brought in with the transplants contained Pst. Characteristics of these grass transplants need to be examined more closely, so a test needs to be developed that can accurately determine if these materials contain Pst.

Soil and Pst cultured in the laboratory were also tested as potential sources of inoculum for Pst infection of Canada thistle. Bacterial pathogens can be recovered from soil that contains infected plant debris (47), so soil was also tested as a possible source of Pst inoculum for infection of Canada thistle. There was no evidence that soil acted as an inoculum source for Pst infection of

Canada thistle in this study. Soil is often not considered a good source of inoculum (30). Pst cultured in the laboratory was tested as inoculum for Pst infection of Canada thistle in Introduced Infection Experiment 1 and 2, but it did not cause an increase in DI over other treatments. A test for Pst in grass, litter, and soil materials is needed to determine if Pst survives in these materials over time. Determining what vectors Pst into the plant is important to understand when developing further Pst research.

The non-treated controls had low levels of Pst DI on Canada thistle in the Introduced Infection Experiment 1 2005 (7 %) (Table 3.5) and the Introduced Infection Experiment 2, 2006, (13% at second sampling date) (Figure 3.1), suggesting a natural population of Pst was present in experiments or Pst dispersal occurred between plots. Therefore, the low levels of DI seen in our experiment may not be attributed to the inoculum source in that treatment, but may have occurred through natural infection. More information is needed on Pst dispersal in natural environments, how Pst is vectored into the plant, and where Pst is located in the environment. It is not known if Pst infection spreads through wind, water, insects or the root system, but the close proximity of plots, only 0.5 m apart, may be a factor in Pst dispersal to control plots. Another factor to consider is, Canada thistle plants in separate plots may be connected through the root system (2). Pst can be transported through the vascular system into tubers in Jerusalem artichoke (15, 49). A similar transport system in Canada thistle may occur where Pst or tagetitoxin are transported through the root system to other Canada thistle plants. Therefore, a plant infected in one plot may be connected to a plant in another plot that is also exhibiting symptoms. More information on the biology of Pst infection of Canada thistle in the field is needed before further conclusions can be drawn from the experiments.

In the Introduced Infection Experiment 2, there was a peak in DI on the second sampling date, but these levels were not maintained in the following sampling dates (Figures 3.1 and 3.2). In the Introduced Infection Experiment 1, DI varied by date of sampling indicating that Pst infection cycles vary with time and may be influenced by environmental factors. More data on they infection cycles and factors that contribute to severity of Pst disease on Canada thistle would be beneficial for future studies. Although inoculum sources may be present Pst populations or toxin production was not maintained in this experiment which indicated the environmental components may need more time to develop.

The Enhanced Infection Experiment was designed to determine if perennial grasses and litter can be used to create environments that increase Pst infection on Canada thistle in areas that had previously exhibited symptoms of Pst infection. In the Enhanced Infection Experiment, there were no differences in DI between grass and litter treatments throughout the duration of this experiment (Table 3.7), which suggests that the addition of grass and litter does not increase Pst infection on Canada thistle plants naturally infected with Pst or that it may take more than two years for increases in infection to occur. We expected that grass and litter would increase Pst infection of Canada thistle, because previous research has shown that grass and litter play a key role in supporting high levels of DI on Pst naturally infected Canada thistle along roadsides (Chapter 2). Pst DI varied by date of sampling, but not by treatment indicating the environmental conditions may contribute to Pst infection cycles. Canada thistle patches with grass and litter had higher humidity and range in daily air temperature, as compared to Canada

thistle patches with grass and litter removed. These environmental differences may explain higher DI in Canada thistle patches with grass and litter (Chapter 2). So, in our study, we hypothesized that grass and litter would change phyllosphere conditions such as relative humidity and temperature and would lead to an increase in DI on Canada thistle. Humidity is an essential component to the infection system in diseases caused by bacteria (33) and litter creates moist conditions increasing plant susceptibility to disease (32). Grass and litter may provide the necessary phyllosphere environment for Pst populations to successfully cause and maintain disease on Canada thistle, but the proper balance was not achieved with this experiment or more time is needed for the environmental to establish.

One possible reason the grass and litter treatments did not increase DI is because of experimental plot location and design. In the roadside Pst naturally infected Canada thistle study (Chapter 2) the treated area was the entire infected area in a Canada thistle patch, where as in this set of experiments the Canada thistle patch was a replicate and was subdivided into many smaller plots (1.5 x 1.5 m). Plots were separated by a 0.5 m alley, which may increase air flow between plots and cause different environmental responses. In the future, environmental sensors should be installed in experiments to determine if there are environmental differences between plots with different grass and litter treatments. Experiments were conducted at one wetland restoration area, so the environmental conditions such as rain and temperature may be similar in all experimental areas and may have influenced Pst DI more than the individual treatments. Soil type, nutrients, water availability, and light availability may have also influenced Pst infection of Canada thistle.

In the first two years of the experiments, DI was low, and it may take more time for Pst populations to establish and increase as the grass and litter environment continues to develop. Grass competition is known to increase efficacy of biocontrol agents (39, 40, 41), but this did not occur during the first two years of our experiments. We expected Pst infection of Canada thistle to impact Canada thistle height, flower heads per plant, and density, but with such low levels of DI that did not occur. However, grass competition is one possible explanation for treatment differences in Canada thistle height, flower heads per plant, and density observed in these experiments.

In the Introduced Infection Experiment 2, Canada thistle plants were shorter in all treatments that contained grass as compared to treatments with no grass, indicating that grass competition may be reducing Canada thistle height (Table 3.11). Reduced height of Canada thistle plants in treatments with grass may be due to resource competition with grasses for light and nutrients (32, 39). However, there were no differences in Canada thistle heights between treatments with grass suggesting that the presence of grass in high density has more of an affect on Canada thistle height than grass type. In the Introduced Infection Experiment 1 in 2006, Canada thistle plants in treatments with no grass were shorter as compared to treatments with perennial grass transplants (Table 3.9), so grass competition did not reduce Canada thistle height in this experiment. Competition with grasses and nutrient availability from litter may have impacted Canada thistle height.

There were no differences in the number of flower heads per Canada thistle plant between treatments in the Introduced Infection Experiment 1 or 2. In the Enhanced Infection Experiment treatments with litter had fewer flower heads per plant than treatments with no litter. The number of flower heads per plant were not influenced by grass competition in these experiments.

Canada thistle density and grass coverage may be related because perennial grasses are known to be effective competitors with Canada thistle (34, 35). In the Introduced Infection Experiment 2, Canada thistle density was reduced in all treatments with grass as compared to treatments with no grass (Table 3.19). This suggests that grasses may play a role in reducing Canada thistle density, but the type of grass may not be as important as the ability to effectively establish ground coverage. This also occurred in another experiment in which grasses effectively competed with thistle and were not dependant on grass type (36). In 2006, in the Enhanced Infection Experiment, Canada thistle density was reduced in all treatments with transplanted perennial grasses as compared to treatments with no grass (Table 3.21). This provides additional evidence of grass competition decreasing Canada thistle density. Included in these grass treatments were the cool season native grass Canada wild rye and cool season exotic grass smooth brome grass which are known to be effective competitors with Canada thistle (35). The results of this experiment support a grass competition hypothesis in which grasses can compete and effectively reduce Canada thistle density. The dense perennial grasses introduced into the Canada thistle patch may provide improved control of Canada thistle populations over the natural system.

Observations from the first two years of our studies indicated that cool season grasses provide the greatest vegetation cover. Cool season grasses emerged in the spring before Canada thistle and shaded developing Canada thistle plants. Warm season grasses may take longer to establish. Planting seedlings into Canada thistle patches achieved a high grass density in a short time and reduced the risks of seeding. The combination of a dense stand of perennial native grasses and a biological control agent, such as Pst, may be key to effectively controlling Canada thistle in restored wetland prairie.

Grass and litter environments were established in all experiments, but these environments did not increase DI to levels that could impact control of Canada thistle. Pst was introduced in areas that previously had no infection, but it may take more time for Pst populations to establish and increase as the grass and litter environment continues to develop. The Introduced Infection Experiments showed that exotic grasses from infected Canada thistle patches caused greater DI and may serve as a source of Pst inoculum. Although DI levels were low, competition from perennial grasses has potential to increase stress on Canada thistle plants which may increase Pst infection of Canada thistle over time. More information is needed on Pst dispersal in natural environments, how Pst is vectored into the plant, and where Pst is located in the environment. A reliable test is needed to measure Pst in natural inoculum sources and to determine if grass and litter promote Pst survival. Perennial grass transplants in this experiment caused a reduction in Canada thistle density, so future integrated management of Canada thistle in restored wetland prairies should include dense planting of native perennial grasses, which may outcompete and suppress Canada thistle populations. Long term monitoring of Canada thistle populations in

these experiments may provide valuable information on best management practices using perennial grasses and Pst.

Tables

Table 3.1. Introduced Infection Experiment 1 treatments for Canada thistle management in restored wetland prairie in Carver County, MN.

Introduced Infection Experiment 1

1. Cool season grass[a] (non-infected[b]) + Litter (non-infected)
2. Cool season grass (non-infected) + Litter (non-infected) + Soil (infected site[c])
3. Cool season grass (non-infected) + Litter (non-infected) + Pst[d]
4. Cool season grass (non-infected) + Litter (infected site)
5. Warm season grass[e] (non-infected) + Litter (non-infected)
6. Warm season grass (non-infected) + Litter (non-infected) + Soil (infected site)
7. Warm season grass (non-infected) + Litter (non-infected) + Pst
8. Warm season grass (non-infected) + Litter (infected site)
9. Cool and warm season grass mix[f] (non-infected) + Litter (non-infected)
10. Cool and warm season grass mix (non-infected) + Litter (non-infected) + Soil (infected site)
11. Cool and warm season grass mix (non-infected) + Litter (non-infected) + Pst
12. Cool and warm season grass mix (non-infected) + Litter (infected site)
13. Exotic grass[g] (non-infected) + Litter (non-infected)
14. Exotic grass (non-infected) + Litter (non-infected) + Soil (infected site)
15. Exotic grass (non-infected) + Litter (non-infected) + Pst
16. Exotic grass (non-infected) + Litter (infected site)
17. Exotic grass (infected site) + Litter (non-infected)
18. Exotic grass (infected site) + Litter (non-infected) + Soil (infected site)
19. Exotic grass (infected site) + Litter (non-infected) + Pst
20. Exotic grass (infected site) + Litter (infected site)
21. No grass[h] + Soil (infected site)
22. No grass + Pst
23. No grass + Litter (infected site)
24. No grass + Exotic grass (infected site)
25. No grass + No Litter
26. Control[i] (Natural system)

See Appendix C for treatment layout maps

[a]Cool season grass: Canada wild rye (*Elymus canadensis*) transplanted from the greenhouse.
[b]Non-infected: grasses planted in greenhouse and litter collected from a roadside Canada thistle patch which had no symptoms of Pst infection.
[c]Infected site: collected from a roadside Canada thistle patch which was exhibiting symptoms of Pst infection.
[d]Pst: *Pseudomonas syringae* pv. *tagetis* strain 1-502a cultured in the laboratory.
[e]Warm season grass: Indian grass (*Sorghastram nutans*) and Big bluestem (*Andropogon gerardi*) transplanted from the greenhouse.
[f]Cool and warm season grass mix: 1:1 ratio of cool and warm season grass season mix transplanted from the greenhouse.
[g]Exotic grass: Smooth brome grass (*Bromus inermis*) grass transplanted from the greenhouse
[h]No grass: grasses were removed with an herbicide treatment.
[i]Control (Natural system): non-treated control treatment contained vegetation that occurred naturally, which included annual grasses such as Giant foxtail (*Setaria faberi*) and native grasses from the prairie mixture.

Table 3.2. Introduced Infection Experiment 2 treatments for Canada thistle management in restored wetland prairie in Carver County, MN.

Introduced Infection Experiment 2

1. Cool and warm season grass mix[a] (non-infected[b]) + Litter (non-infected)
2. Cool and warm season grass mix (non-infected) + Litter (infected site[c])
3. Cool and warm season grass mix (non-infected) + Litter w/Pst[d]
4. Exotic grass (infected site) + Litter (non-infected site)
5. Cool and warm season grass mix w/Pst culture + Litter (non-infected)
6. Cool and warm season grass mix (non-infected) + Litter (non-infected) + Soil (infected site)
7. Cool and warm season grass mix (non-infected) + Litter (non-infected site) + Soil w/Pst
8. Exotic grass (infected site) + Litter (infected site)
9. Cool and warm season grass mix w/Pst culture + Litter w/Pst
10. Exotic grass (infected site)
11. Cool and warm season grass mix w/Pst
12. No grass[e] + Litter (infected site)
13. No grass + Litter w/Pst
14. No grass + Soil (infected site)
15. No grass + Soil w/Pst
16. No grass + No Litter
17. Control[f] (Natural system)

See Appendix C for treatment layout maps

[a]Cool and warm season grass mix: 1:1 ratio of cool and warm season grass season mix transplanted from the greenhouse.
[b]Non-infected: grasses planted in greenhouse and litter collected from a roadside Canada thistle patch which had no symptoms of Pst infection.
[c]Infected site: collected from a roadside Canada thistle patch which was exhibiting symptoms of Pst infection.
[d]Pst: *Pseudomonas syringae* pv. *tagetis* strain 1-502a cultured in the laboratory.
[e]No grass: grasses were removed with an herbicide treatment.
[f]Control (Natural system): non-treated control treatment contained vegetation that occurred naturally, which included annual grasses such as Giant foxtail (*Setaria faberi*) and native grasses from the prairie mixture.

Table 3.3. Enhanced Infection Experiment treatments for Canada thistle management in restored wetland prairie in Carver County, MN.

Enhanced Infection Experiment

1. Cool and warm season grass mix[a]
2. No grass[b] + Litter[c]
3. Cool and warm season grass mix + Litter
4. Exotic grass[d]
5. Exotic grass + Litter
6. No grass + No Litter
7. Control[e] (Natural System)

See Appendix C for treatment layout maps

[a]Cool and warm season grass mix: 1:1 ratio of cool and warm season grass season mix transplanted from the greenhouse.
[b]No grass: grasses were removed with an herbicide treatment.
[c]Litter: collected from a roadside Canada thistle patch which had no symptoms of Pst infection.
[d]Exotic grass: Smooth brome grass (*Bromus inermis*) grass transplanted from the greenhouse.
[e]Control (Natural system): non-treated control treatment contained vegetation that occurred naturally, which included annual grasses such as Giant foxtail (*Setaria faberi*) and native grasses from the prairie mixture.

Table 3.4. Analysis of variance of repeated measures factorial for disease incidence of *Pseudomonas syringae* pv. *tagetis* (Pst) on Canada thistle among treatment factors for the Introduced Infection Experiment 1 in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Disease Incidence							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Between-Subject								
Block	2	4433	10.443	< 0.001	2	285	1.698	0.195
Grass (G)	6	95	0.224	0.967	6	90	0.536	0.778
Litter (L)	2	41	0.96	0.909	2	54	0.32	0.728
Soil (S)	1	413	0.974	0.328	1	57	0.338	0.564
Pst (P)	1	822	1.936	0.17	1	7	0.04	0.842
G x L	5	856	2.016	0.092	5	147	0.879	0.503
G x L x S	5	263	0.621	0.685	5	236	1.408	0.240
G x L x P	5	160	0.377	0.862	5	225	1.342	0.265
Error	50	425			44	168		
Within-Subject								
Date[b] (D)	2	929	6.792	0.001	1	4093	11.536	< 0.001
Block x date	5	459	3.351	0.009	3	82	0.232	0.864
G x D	14	109	0.796	0.672	9	237	0.667	0.728
L x D	5	37	0.273	0.918	3	65	0.182	0.900
S x D	2	137	0.998	0.382	1	52	0.147	0.790
P x D	2	164	1.2	0.309	7	130	0.367	0.622
G x L x D	12	198	1.445	0.158	7	393	1.109	0.369
G x L x S x D	12	56	0.411	0.954	7	209	1.288	0.270
G x L x P x D	12	57	0.415	0.953	63	355	0.59	0.764
Error	117	137						

a. Abbreviations: df, degrees of freedom; MS, mean square.
b. Date represents data collections made five times biweekly throughout the growing season.

Table 3.5. Canada thistle *Pseudomonas syringae* pv. *tagetis* disease incidence for grass and litter treatments in Introduced Infection Experiment 1 in 2005 in restored wetland prairie in Carver County, MN.

Litter Treatment	Litter from non-infected site		Litter from infected site		No litter	
	Mean	SE	Mean	SE	Mean	SE
Grass Treatment	----------%----------					
Cool Season	10	± 4	10	± 6	n/a	n/a
Warm season	14	± 4	3	± 6	n/a	n/a
Cool and warm mix	11	± 4	8	± 6	n/a	n/a
Exotic grass	3	± 4	11	± 6	n/a	n/a
Exotic grass from infected site	4	± 4	23	± 6	9	± 6
Natural Grass	n/a	n/a	n/a	n/a	7	± 6
No grass	n/a	n/a	9	± 6	12	± 4

Table 3.6. Analysis of variance of repeated measures factorial for disease incidence of *Pseudomonas syringae* pv. *tagetis* (Pst) on Canada thistle among treatment factors for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Disease Incidence			
	2006			
Sources of Error	df[a]	MS	F	P
Between-Subject				
Block	2	564	5.231	0.011
Grass (G)	4	412	3.828	0.012
Litter (L)	3	586	5.441	0.004
Soil (S)	2	41	0.377	0.689
G x L	5	109	1.016	0.424
G x L x S	2	20	0.184	0.833
Error	32	108		
Within-Subject				
Date[b] (D)	2	3333	15.541	< 0.001
Block x date	3	2015	8.790	< 0.001
G x D	6	503	2.195	0.055
L x D	5	563	2.456	0.048
S x D	3	100	0.437	0.739
G x L x D	8	98	0.429	0.898
G x L x S x D	3	197	0.859	0.474
Error	51	229		

a. Abbreviations: df, degrees of freedom; MS, mean square.
b. Date represents data collections made five times biweekly throughout the growing season.

Table 3.7. Analysis of variance of repeated measures factorial for disease incidence of *Pseudomonas syringae* pv. *tagetis* (Pst) on Canada thistle among treatment factors for the Enhanced Infection Experiment in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Disease Incidence							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Between-Subject								
Grass (G)	3	132	0.178	0.909	3	8.9	0.06	0.981
Litter (L)	1	817	1.105	0.314	1	56	0.376	0.543
G x L	2	199	0.269	0.769	2	140	0.934	0.402
Error	12	739			35	149		
Within-Subject								
Date[b] (D)	3	2020	7.947	< 0.001	2	4807	22.124	< 0.001
Grass x D	9	252	0.992	0.464	5	173	0.795	0.547
Litter x D	3	287	1.128	0.351	2	86	0.397	0.616
G x L x D	6	106	0.415	0.864	3	230	1.059	0.375
Error	36	254			53	217		

a. Abbreviations: df, degrees of freedom; MS, mean square.
b. Date represents data collections made five times biweekly throughout the growing season.

Table 3.8. Analysis of variance of repeated measures factorial for Canada thistle height among treatment factors for the Introduced Infection Experiment 1 in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Height							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Between-Subject								
Block	2	42757	51.338	< 0.001	2	68083	75.05	< 0.001
Grass (G)	6	769	0.924	0.486	6	1774	1.956	0.097
Litter (L)	2	21	0.026	0.975	2	110	0.122	0.886
Soil (S)	1	599	0.719	0.4	1	1.4	0.002	0.969
Pst (P)	1	654	0.785	0.38	1	395	0.436	0.513
G x L	5	439	0.527	0.755	5	1293	1.426	0.238
G x L x S	5	326	0.392	0.852	5	501	0.553	0.735
G x L x P	5	355	0.426	0.828	5	649	0.716	0.616
Error	50	833			37	907		
Within-Subject								
Date[b] (D)	2	11804	52.972	< 0.001	3	1807	15.508	< 0.001
Block x date	5	2834	12.719	0.349	6	188	1.609	0.157
G x D	14	249	1.117	0.746	18	79	0.681	0.813
L x D	5	118	0.531	0.62	6	64	0.547	0.759
S x D	2	119	0.535	0.618	3	49	0.417	0.727
P x D	2	120	0.538	0.986	3	32	0.273	0.83
G x L x D	12	70	0.315	0.662	15	71	0.611	0.85
G x L x S x D	12	176	0.788	0.562	15	66	0.564	0.886
G x L x P x D	12	198	0.887		15	54	0.461	0.948
Error	120	223			111	117		

a. Abbreviations: df, degrees of freedom; MS, mean square.
b. Date represents data collections made five times biweekly throughout the growing season.

Table 3.9. Effect of grass treatments on Canada thistle height for the Introduced Infection Experiment 1 in 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Height[a]	
	2006	
Grass Treatment	--------cm--------	
Cool season	72	b
Warm season	69	b
Cool and warm season mix	63	b
Exotic grass	63	b
Exotic from infected site	61	b
Natural grass	60	a
No grass	47	a

a Mean Canada thistle height values were averaged from three plants measured in each plot biweekly for five data collections. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.10. Analysis of variance of repeated measures factorial for Canada thistle height among treatment factors for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Height			
	2006			
Sources of Error	df[a]	MS	F	P
Between-Subject				
Block	2	44192	76.836	< 0.001
Grass (G)	4	3010	5.233	0.002
Litter (L)	3	46	0.079	0.971
Soil (S)	2	77	0.133	0.876
G x L	5	251	0.437	0.819
G x L x S	2	1763	3.066	0.061
Error	31	575		
Within-Subject				
Date[b] (D)	3	4283	41.804	< 0.001
Block x date	6	819	7.990	< 0.001
G x D	12	107	1.047	0.414
L x D	9	104	1.019	0.431
S x D	6	239	2.329	0.039
G x L x D	15	127	1.24	0.257
G x L x S x D	6	159	1.551	0.170
Error	93	102		

a. Abbreviations: df, degrees of freedom; MS, mean square.
b. Date represents data collections made five times biweekly throughout the growing season.

Table 3.11. Effect of grass treatments on Canada thistle height for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Height[a]	
	2006	
Grass Treatment	-----cm-----	
Cool and warm season mix	60	a
Cool and warm season mix w/Pst	59	a
Exotic from infected site	54	a
Natural grass	56	a
No grass	76	b

a Mean Canada thistle height values were averaged from three plants measured in each plot biweekly for five data collections. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.12. Analysis of variance of repeated measures factorial for Canada thistle height among treatment factors for the Enhanced Infection Experiment in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Height							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Between-Subject								
Grass (G)	3	576	2.495	0.102	3	292	0.714	0.551
Litter (L)	1	226	0.98	0.339	1	185	0.453	0.505
G x L	2	123	0.535	0.597	2	137	0.335	0.718
Error	14	231			34	409		
Within-Subject								
Date[b] (D)	4	11259	218.103	< 0.001	2	4474	70.363	< 0.001
G x D	12	69	1.327	0.23	7	48	0.759	0.626
L x D	4	5	0.104	0.981	2	143	2.256	0.102
G x L x D	8	47	0.904	0.52	5	46	0.719	0.604
Error	56	52			81	64		

a. Abbreviations: df, degrees of freedom; MS, mean square.
b. Date represents data collections made five times biweekly throughout the growing season.

Table 3.13. Analysis of variance for Canada thistle flower heads per plant among treatment factors for the Introduced Infection Experiment 1 in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Flower Heads per Plant							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Block	2	6469	48.301	< 0.001	2	1552	22.751	< 0.001
Grass (G)	6	195	1.454	0.213	5	54	0.795	0.559
Litter (L)	2	115	0.86	0.429	2	43	0.623	0.54
Soil (S)	1	109	0.815	0.371	1	0.22	0.003	0.955
Pst (P)	1	148	1.105	0.298	1	82	1.198	0.279
G x L	5	244	1.82	0.126	5	113	1.653	0.165
G x L x S	5	77	0.577	0.717	5	51	0.749	0.591
G x L x P	5	65	0.488	0.784	5	18	0.266	0.929
Error	50	134			47	68		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.14. Analysis of variance for Canada thistle flower heads per plant among treatment factors for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Flower Heads per Plant			
	2006			
Sources of Error	df[a]	MS	F	P
Block	2	1887	22.312	<0.001
Grass (G)	4	102	1.203	0.329
Litter (L)	3	47	0.553	0.650
Soil (S)	2	41	0.485	0.620
G x L	5	62	0.739	0.600
G x L x S	2	93	1.105	0.344
Error	32	85		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.15. Analysis of variance for Canada thistle flower heads per plant among treatment factors for the Enhanced Infection Experiment in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Flower Heads per Plant							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Grass (G)	3	56	2.097	0.147	2	6	0.48	0.623
Litter (L)	1	150	5.663	0.032	1	19	1.48	0.232
G x L	2	13	0.496	0.619	2	5	0.356	0.703
Error	14	27			36	13		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.16. Effect of litter treatments on Canada thistle flower heads per plant for the Enhanced Infection Experiment in 2005 in restored wetland prairie in Carver County, MN.

	Flower Heads per Plant[a]	
	2005	
Litter Treatment		
Litter	20	a
No litter	27	b

a. Mean number of flower heads on each plant was averaged across three plants in each treatment and across three replicates. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.17. Analysis of variance for Canada thistle density among treatment factors for the Introduced Infection Experiment 1 in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Density							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Block	2	9092	85.852	<0.001	2	4282	65.574	<0.001
Grass (G)	6	125	1.185	0.33	6	114	1.746	0.13
Litter (L)	2	41	0.382	0.684	2	41	0.627	0.593
Soil (S)	1	19	0.181	0.673	1	0.431	0.007	0.936
Pst (P)	1	15	0.138	0.711	1	13	0.206	0.652
G x L	5	142	1.341	0.263	5	114	1.744	0.142
G x L x S	5	79	0.748	0.591	5	63	0.971	0.445
G x L x P	5	35	0.328	0.894	5	23	0.356	0.876
Error	50	106			50	65		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.18. Analysis of variance for Canada thistle density among treatment factors for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Density			
	2006			
Sources of Error	df[a]	MS	F	P
Block	2	3194	17.212	<0.001
Grass (G)	4	1196	6.446	0.001
Litter (L)	3	38	0.207	0.891
Soil (S)	2	326	1.754	0.189
G x L	5	356	1.92	0.118
G x L x S	2	728	3.923	0.030
Error	32	186		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.19. Effect of grass treatments on Canada thistle density for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Density[a]	
	2006	
Grass Treatment	-----plant m^{-2}----	
Cool and warm season mix	17	b
Cool and warm season mix w/Pst	13	b
Exotic from infected site	13	b
Natural grass	21	b
No grass	32	a

a. Mean Canada thistle density per square meter averaged across three replicates for each treatment and two assessments each year. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.20. Analysis of variance for Canada thistle density among treatment factors for the Enhanced Infection Experiment in 2005 and 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Density							
	2005				2006			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Grass (G)	3	47	0.887	0.433	3	70	3.359	0.030
Litter (L)	1	3	0.049	0.827	1	3	0.121	0.730
G x L	2	14	0.257	0.777	2	9	0.45	0.641
Error	14	53			35	21		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.21. Effect of grass treatments on Canada thistle density for the Enhanced Infection Experiment in 2006 in restored wetland prairie in Carver County, MN.

	Canada Thistle Density[a]	
	2006	
Grass Treatment	----------plants m^{-2}----------	
Cool and warm season mix	13	a
Exotic grass	15	a
Natural grass	16	ab
No grass	18	b

a. Mean Canada thistle density per square meter averaged across three replicates for each treatment and two assessments each year. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.22. Analysis of variance for grass and forb coverage among treatment factors for the Introduced Infection Experiment 1 in 2006 in restored wetland prairie in Carver County, MN.

	Grass Coverage				Forb Coverage			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Block	2	2347	5.516	0.007	2	2642	9.958	<0.001
Grass (G)	5	3849	9.044	<0.001	5	2516	9.482	<0.001
Litter (L)	2	962	2.26	0.115	2	615	2.316	0.109
Soil (S)	1	1046	2.459	0.123	1	12	0.044	0.835
Pst (P)	1	1046	2.459	0.123	1	72	0.273	0.604
G x L	5	204	0.479	0.790	5	72	0.273	0.926
G x L x S	5	180	0.422	0.831	5	181	0.682	0.639
G x L x P	5	226	0.532	0.751	5	200	0.753	0.587
Error	51	426			51	265		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.23. Effect of grass treatments on grass and forb coverage for the Introduced Infection Experiment 1 in 2006 in restored wetland prairie in Carver County, MN.

	Grass Coverage[a]		Forb Coverage	
Grass Treatment	----------%----------			
Cool season	47	bc	18	a
Warm season	45	b	16	a
Cool and warm season mix	65	c	14	a
Exotic grass	64	c	18	a
Exotic from infected site	44	b	37	b
Natural grass	33	b	40	b
No grass	0	a	64	c

a Mean grass and forb coverage averaged across three replicates for each treatment. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.24. Analysis of variance for grass and forb coverage among treatment factors for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

		Grass Coverage				Forb Coverage		
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Block	2	1325	3.411	0.020	2	1987	12.242	<0.001
Grass (G)	4	7272	24.201	<0.001	4	1471	8.663	<0.001
Litter (L)	3	279	0.927	0.439	3	186	1.148	0.345
Soil (S)	2	339	1.128	0.336	2	404	2.49	0.099
G x L	5	395	1.314	0.283	5	57	0.353	0.877
G x L x S	2	339	1.128	0.336	2	393	2.442	0.105
Error	32	300			32	162		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.25. Effect of grass treatments on grass coverage and grass and soil on forb coverage for the Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

	Grass Coverage[a]		Forb Coverage	
Grass Treatment	----------%----------			
Cool and warm season mix	60	bc	13	a
Cool and warm season mix w/Pst	69	c	11	a
Exotic from infected site	47	bc	21	a
Natural grass	40	b	13	a
No grass	0	a	37	b
Soil Treatment				
Soil from an infected site			23	a
Soil mixed w/Pst			19	a
No soil			21	a

a Mean grass and forb coverage averaged across three replicates for each treatment. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Table 3.26. Analysis of variance for grass and forb coverage among treatment factors for the Enhanced Infection Experiment in 2006 in restored wetland prairie in Carver County, MN.

	Grass Coverage				Forb Coverage			
Sources of Error	df[a]	MS	F	P	df	MS	F	P
Grass (G)	2	24662	150.478	< 0.001	2	6105	51.967	< 0.001
Litter (L)	1	12	0.074	0.787	1	170	1.451	0.236
G x L	2	288	1.759	0.187	2	92	0.787	0.463
Error	36	164			36	117		

a. Abbreviations: df, degrees of freedom; MS, mean square.

Table 3.27. Effect of grass treatments on grass and forb coverage for the Enhanced Infection Experiment in 2006 in restored wetland prairie in Carver County, MN.

	Grass Coverage[a]		Forb Coverage	
Grass Treatment	----------%----------			
Cool and warm season mix	78	c	10	a
Exotic grass	76	c	8	a
Natural grass	20	b	45	b
No grass	0	a	48	b

a Mean grass and forb coverage averaged across three replicates for each treatment. Values with the same letter for a given column did not differ significantly at P = 0.05 according to Fisher's LSD.

Figures

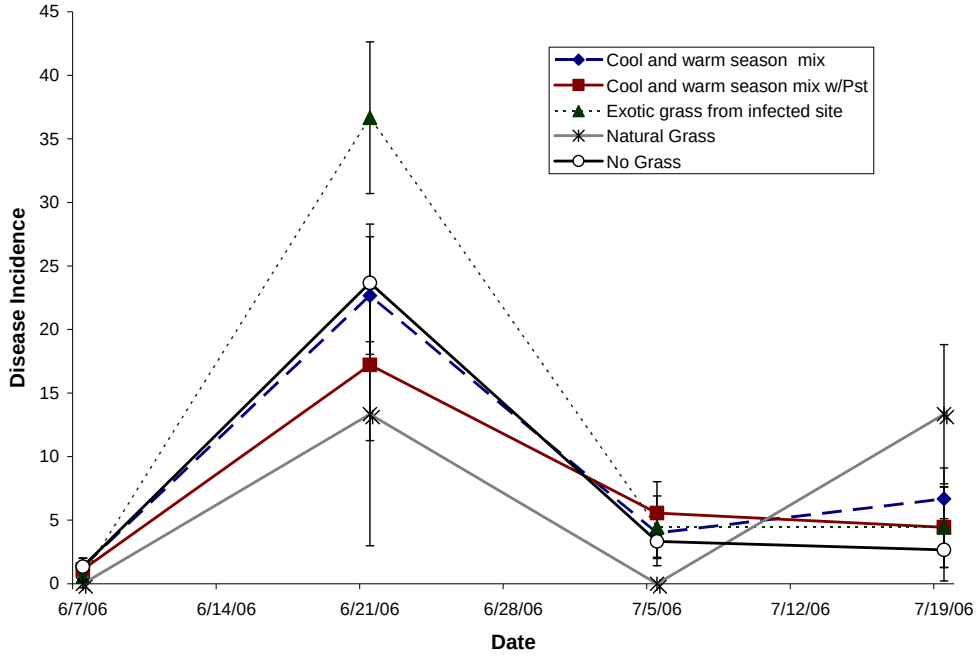

Figure 3.1. Canada thistle *Pseudomonas syringae* pv. *tagetis* disease incidence for grass treatments in Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

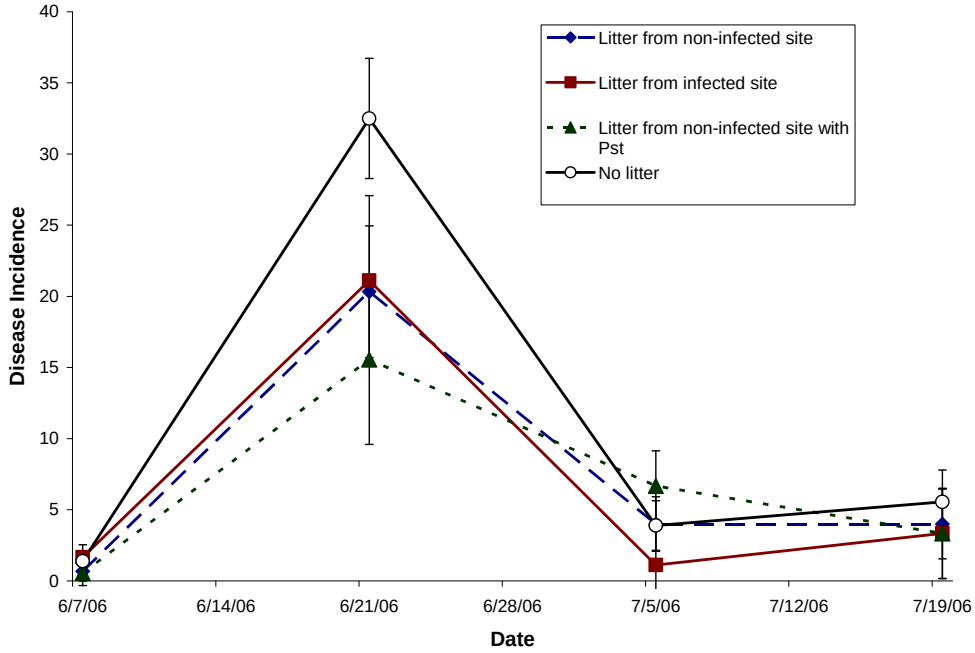

Figure 3.2. Canada thistle *Pseudomonas syringae* pv. *tagetis* disease incidence for litter treatments in Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

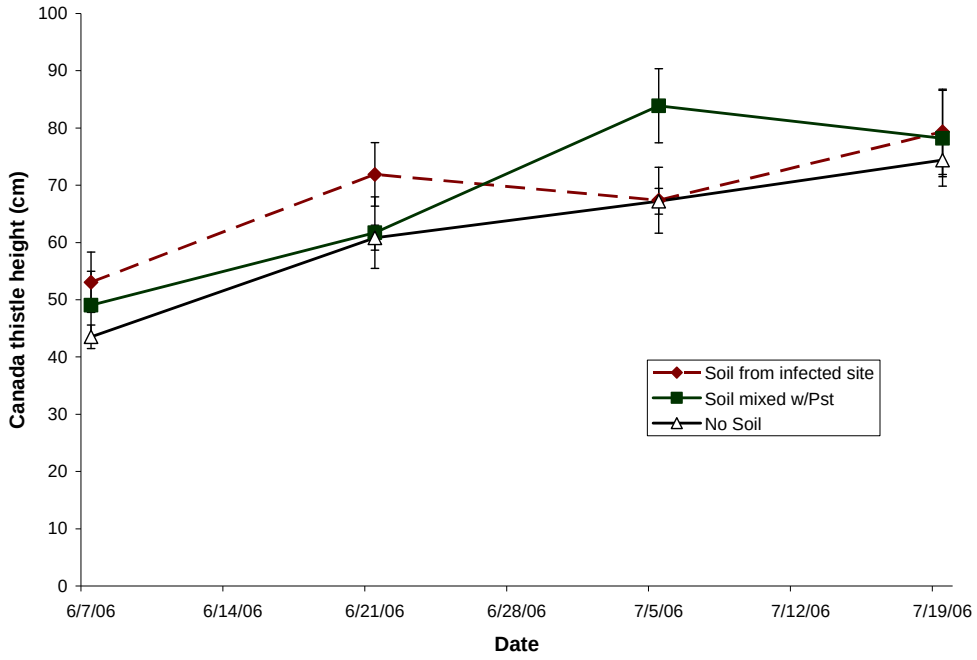

Figure 3.3. Canada thistle height for soil treatments in Introduced Infection Experiment 2 in 2006 in restored wetland prairie in Carver County, MN.

CHAPTER 4

Development of PCR-based detection method for identification and monitoring of *Pseudomonas syringae* pv. *tagetis,* a potential biological control agent for Canada thistle

ABSTRACT

Pseudomonas syringae pv. *tagetis* (Pst) specific primers which amplify regions of genes specific to tagetitoxin were used to develop a PCR-based detection method for Pst in grass and litter, potential inoculum sources for Pst infection of Canada thistle (*Cirsium arvense* (L.) Scop). Sensitivity of a previously published primer set specific to Pst was determined to be 1 x 10^5 to 1 x 10^6 cfu/ml. This suggests high Pst populations are needed for detection and this test may not be reliable for environmental samples which could have low Pst populations. This PCR protocol successfully detected Pst in litter from the field 12 days after Pst inoculation. However, Pst was also cultured on media from the same sample and there were high populations of Pst (1 x 10^7 cfu/ml) present in the sample. In order to improve sensitivity, a nested PCR protocol was developed with Pst specific primers from a different gene specific to tagetitoxin than the previous primer set. Nested primers potentially increased sensitivity to 10 to 100 cfu/ml, but false positives in the negative controls could not be overcome and this sensitivity could not be verified. If problems with the nested protocol can be resolved, this PCR-based detection method could be used to further studies on Pst population dynamics and inoculum sources in natural systems. The ability to monitor Pst in the environment could improve integrated management systems for Canada thistle along roadsides and in prairie restoration areas.

INTRODUCTION

Pseudomonas syringae pv. *tagetis* (Pst) is a plant pathogenic bacterium, which has been identified as a potential biological control agent producing symptoms of apical chlorosis, stunting, and reduced flowering on members of the Asteraceae family (11). Pst causes severe apical chlorosis due to production of tagetitoxin, a toxin which is translocated to the plant apex (18,19). In plants, tagetitoxin inhibits RNA polymerase III, thereby disrupting the synthesis of chloroplasts in plant tissue, which causes a characteristic apical chlorosis in all new tissue in infected plants (20). Pst was originally isolated from African marigold (*Tagetes erecta* L.) and has also been isolated from common ragweed (*Ambrosia artemisiifolia* L.), sunflower (*Helianthus annus* L.), Jerusalem artichoke (*Helianthus tuberosus* L.) and Canada thistle (*Cirsium arvense* (L.) Scop.) (11,12, 13, 14, 15, 16, 17).

Canada thistle is a perennial noxious weed that has a need for improved control methods in natural systems, especially in roadsides and restored wetland prairie areas where control methods such as frequent mowing and herbicides can be damaging to native plants (9). Alternatively biological control may offer environmentally safe and cost effective control for long term management of Canada thistle in perennial systems (10). Pst has previously been evaluated as a biological agent on Canada thistle (10, 16, 17, 21, 22, 24).

An initial study, examining Pst as a biological control agent, demonstrated almost complete control of Canada thistle after five applications of Pst in conventional soybean (16, 17). In conservation tillage soybean, Pst application caused a reduction in survival, growth, and reproduction in Canada thistle with multiple applications of Pst (22). However, two applications of Pst were not enough to effectively control Canada thistle in organic soybean (21). Repeated applications of Pst, although they may be effective, are too costly to be considered an alternative to traditional control methods in annual cropping systems.

A recent study examined a different method of Pst application in which sap from naturally infected Canada thistle plants was applied with a surfactant to non-infected Canada thistle plants in an undisturbed area to evaluate natural colonization and spread (24). There were indications that the introduced Pst became a long term component of the community (24), however, it was not determined why this occurred. For Pst to be used as an effective control strategy, new techniques need to be developed that will sustain high populations of Pst for extended periods of time. More information is needed on Pst population dynamics and infection of Canada thistle in natural systems.

Natural infection of Canada thistle with Pst is particularly prevalent in roadside rights-of-way which are characterized by perennial grasses and a thick litter layer which consists of plant material from associated perennial grasses. Perennial grasses and litter found in association with roadside Canada thistle patches may provide surfaces for Pst survival and act as inoculum for Pst infection of Canada thistle or affect the phyllosphere environment providing conditions that support Pst populations, infection, or toxin production. The efficacy of Pst on naturally infected Canada thistle populations is unknown, because Pst is difficult to isolate and quantify from naturally infected Canada thistle plants in the field using standard culturing techniques. It is not

known where Pst is located in the plant or if it is in a non-culturable form. So a new method that can accurately detect Pst in this system is needed. There is a need for basic biology regarding Pst population levels, location in the plant, transmission of Pst, and potential inoculum sources of Pst infection of Canada thistle.

In a study of Pst naturally infected Canada thistle patches along roadsides, the presence of both grass and litter was necessary for high Pst disease incidence on Canada thistle (Chapter 2). In an integrated management study examining grass and litter as potential inoculum sources for Pst infection of Canada thistle, Pst infection was introduced in treatments with materials from naturally infected patches, but it could not be verified if Pst was actually present in materials or if natural dispersal was occurring (Chapter 3). The ability to detect and monitor Pst populations in the field and test potential sources of natural inoculum could lead to development of better integrated management systems with Pst and Canada thistle.

Development of a polymerase chain reaction (PCR) protocol with oligonucleotide primers specific to Pst may provide a method of identifying Pst in non-symptomatic grass and litter that are potential inoculum sources for Pst infection of Canada thistle. PCR amplifies specific regions of DNA or genes of an organism by utilizing primers specific to the particular region of interest (50). PCR does not require the target organism to be cultured or viable to be detected (50) and PCR can detect specific pathogens, strains, or group of pathogens. PCR-based detection methods specific to toxins produced by other *Pseudomonas* bacteria have been developed (31) and a PCR-based detection method has also been used to detect strains of *Pseudomonas* from environmental samples (51) and asymptomatic materials (52). PCR has been used in biocontrol systems to differentiate target biocontrol organism from other organisms on the plant (53) and has also been used in studies with applications of biocontrol agents in soil (54, 55). Kong et al. 2004 (56), developed primers specific to Pst from genes required for tagetitoxin production, distinguishing Pst from other toxin producing bacteria. These primers will be used in our study to develop a PCR-based detection method for Pst in potential inoculum sources for Pst infection of Canada thistle. Asymptomatic materials will be tested, so a there is a need for a highly sensitive method.

Nested PCR is a method of increasing sensitivity of PCR to detect lower thresholds of target DNA. Nested PCR involves two sets of primers, an outer and inner, in which the reaction with the outer primers is used as the template in a second reaction with the inner primers. This method increases the product and leads to higher sensitivity than a single PCR reaction. In asymptomatic tissue of *Xanthomonas fragariae* on strawberry, nested PCR improved sensitivity from 10^4 to 10^5 cfu/ml in a single round of PCR to 18 cfu/ml in nested PCR reactions (57, 58). Another study with nested PCR detected 1 cfu/ml of *Pseudomonas savastanoi* pv. *savastanoi* in asymptomatic olive (59). Nested PCR is typically conducted in a separate tube for each reaction, but nested PCR in a single tube reduces contamination risks and improves sensitivity (50, 60). For nested PCR in a single tube, the outer and inner reactions take place in one tube and reactions are separated by different annealing temperatures in two consecutive PCR cycles which are conducted at appropriate annealing temperatures for the primer set (60).

The objectives of this experiment were to use Pst specific primers to test grass and litter as natural inoculum sources for Pst infection of Canada thistle and as a potential substrate for survival of cultured Pst in the field. The TAGTOX-9 and TAGTOX-10 primers previously developed from genes specific to tagetitoxin production (56) were utilized in this study. Sensitivity of these primers was determined to establish the accuracy of the test, and field samples of grass and grass litter were tested for Pst. Since asymptomatic materials were studied a highly sensitive test was needed. So new primers were developed and used in a nested PCR protocol in an attempt to increase sensitivity. In this paper, we present results of work to develop a PCR-based detection method for Pst in potential inoculum sources.

MATERIALS AND METHODS

Pst Culture

Pst strain 1-502a, originally isolated from Canada thistle in Minnesota (16, 17) and strain EB037, isolated from ragweed, were used in this study. Both strains were maintained on King's media B (KB) (61) at 4 C. For all applications, Pst was grown in 2.5 liter Fernbach flasks by inoculating one liter of tryptic soy broth with 100 mg of Pst cells which was incubated on a rotary shaker at 300 rpms for 18 hours at 23 C. The resulting culture was centrifuged for 10 minutes at 5,000 rpms and the pellet was resuspended in sterile deionized water to a concentration of $1x10^9$ colony forming units per milliliter (cfu/ml).

PCR-Based Detection Method with TAGTOX-9 and TAGTOX-10 Primers

Sensitivity Tests

For the sensitivity test, ten-fold serial dilutions of Pst culture were made from 1×10^9 to 1 cfu/ml, and 100 ul of each dilution was plated on KB media to verify the number of colonies in each dilution. Sensitivity tests were performed with the PCR protocol which utilized TAGTOX-9 and TAGTOX-10 with direct PCR on serial dilutions of bacteria culture and DNA isolated from the serial dilutions. Strain 1-502a and EBO37 were used in sensitivity tests and all tests were repeated at least three times.

Field Material Collections

Field plots at Cheri Ponds mitigation wetland area in Carver County, MN (Chapter 2, Eichstaedt 2007) were collection sites for samples used to test the survival of cultured Pst on grass and litter (Appendix E). Seedlings of Canada wild rye (*Elymus canadensis*) and Indian grass (*Sorghastram nutans*) were grown for six weeks in the greenhouse in a 1:1 mixture of sterile greenhouse soil and Sunshine potting medium in 1 cm diameter, 15 cm deep plastic cones under a 16 hour photoperiod. In late May, the 15 cm tall grass seedlings were transplanted into the field in a 15 cm grid spacing for a total of 80 plant m^{-2} in 1.5 x 1.5 m plots. For seedling planting, a dibble bar was used to create a hole the size of the cone and then the seedling with entire root mass was placed in the hole and watered immediately after transplanting. Canada wild rye and Indian grass were planted at a 1:1 ratio. After planting, a high concentration of Pst (1×10^9 cfu/ml) was applied with a hand held sprayer at 333 ml m^{-2} onto grass seedlings. In August 2005, litter was collected from a non-infected Canada thistle patch as described in Chapter 3, and liquid Pst culture was mixed with litter in a ratio of 500 ml of liquid culture to 1 kg litter separately by hand for each plot and then this modified litter was applied at 0.67 kg m^{-2} to the 1.5 x 1.5 m plots (Chapter 3). Six samples each of grass and litter were collected 12 days after Pst application and again the following spring (Appendix E). Grass and litter were also collected from Pst naturally infected Canada thistle patches along roadsides to test as natural sources of inoculum (Appendix E).

Bacterial Culturing from Samples

All field samples tested with the PCR-based method were also cultured on media to verify PCR results. Field samples were tested by using 100-200 mg of tissues which were surface sterilized with 70% ethanol for 30 seconds, then rinsed in sterile water to remove epiphytic bacteria and macerated in 1.5 ml of sterile water. Macerate was serially diluted before plating onto KB media, and colonies were used directly in PCR reaction by diluting in 20 µl of sterile deionized water.

DNA Isolation

DNA was isolated from 100 to 200 mg of grass and litter material from field samples using the FastDNA Kit and the FastPrep Instrument (Qbiogene, Inc. CA). DNA was isolated directly from plant material using the bacteria specific cell lysis solution (CLS-TC, Qbiogene, Inc. CA), to include any non-culturable forms of Pst and to remove PCR inhibitors. DNA isolations were also made from pure Pst culture and serial dilutions.

PCR Amplification

A PCR-based detection method specific to Pst was developed to test field materials for the presence of Pst. The primers TAGTOX-9 and TAGTOX-10 were previously developed from two genes required for tagetitoxin production by Kong et al. 2004 (56). DNA from field samples and direct PCR on single colonies were used in PCR reactions. Positive controls were developed using Pst strain 1-502a and EB037 single colonies or DNA. The following primers and protocol were used as described by Kong et al. 2004 (56). The TAGTOX-9 FP1 21-mer oligonucleotide with sequence 5' –CCCGCAGTGCTGGCTTACAAC and TAGTOX-9 RP1 19-mer 5'-TGAGCAACGCGGCCATAGC produced a 507-bp amplicon. TAGTOX-10 FP1 19- mer 5'-TACCCGTGAGGCAGTGGCA and a 22-mer TAGTOX-10 RP1 primer with sequence 5'-TTTGAACTTGCCGGGGATACGG produced a 733-bp amplicon. PCR amplifications were performed in a 25 µl reaction, and reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 µM each primer, 200 µmol each dNTP, 1 U AmpliTaq Gold (Perkin-Elmer, Norfolk, CT), and 40 ng of bacterial DNA or 4 µl of bacteria cell suspension. Reactions were performed in Perkin-Elmer GeneAmp PCR System 9600 with denaturation at 95 C for 10 min for the first cycle and 94 C for 30 s for each subsequent cycle. Annealing and elongation were at 50 C for 30 s and 72 C for 30 s, respectively for 5 cycles adding 2 s to the annealing time per cycle. Annealing and elongation for an additional 25 cycles were at 50 and 72 C for 30 s adding 1 s to the annealing and extension times per cycle. Reactions were stopped after a final 5 min elongation at 72 C. The amplicons in the reaction mix were separated by 1% gel electrophoresis and stained with ethidium bromide (56).

Development of Nested PCR Detection Method

Primer Development

Nested PCR primers were developed (Table 4.1) from TOX-15 (Appendix F), a region of Pst DNA that has low homology to other Pseudomonads and is required for tagetitoxin production. Two outer and inner primer sets were developed and used for nested PCR in two separate reaction tubes. For nested PCR two tube method, the same PCR amplification protocol was used as describe earlier for both rounds of PCR, except in the second round 1µl of first reaction was used as the template in the second reaction (Table 4.1). The amplicons in the reactions were separated by 1% gel electrophoresis and stained with ethidium bromide.

In the nested PCR protocol in a single tube, nested primers were separated by annealing temperature, so the two reactions can occur in one tube. In the single tube method, the same PCR amplification protocol was used as described earlier, except the primer amount was reduced to 0.4 µM for the outer and inner primer sets. The set of outer primers was tested with the three different sets of inner primers (Table 4.1). The reactions cycles were run twice with annealing and elongation temperatures adjusted for each round of PCR (Table 4.1).

For nested PCR in a single tube, in the first round of PCR with outer primer set denaturation was at 95 C for 10 min for the first cycle and 94 C for 30 s for each subsequent cycle. Annealing and elongation were at 70 C for 30 s and 72 C for 90 s, respectively for 5 cycles adding 2 s to the annealing time per cycle. Annealing and elongation for an additional 25 cycles were at 70 and 72 C for 90 s adding 1 s to the annealing and extension times per cycle. Reactions were stopped after a final 5 min elongation at 72 C.

For the second round of PCR conditions were specific to inner primer set. Denaturation was at 95 C for 10 min for the first cycle and 94 C for 30 s for each subsequent cycle. Annealing and elongation were at 56 C for 30 s and 72 C for 30 s, respectively for 5 cycles adding 2 s to the annealing time per cycle. Annealing and elongation for an additional 25 cycles were at 56 C and 72 C for 30 s adding 1 s to the annealing and extension times per cycle. Reactions were stopped after a final 5 min elongation at 72 C. The amplicons in the reaction were separated by 1% gel electrophoresis and stained with ethidium bromide.

Sensitivity Tests

Sensitivity tests were performed with nested primers through direct PCR on ten-fold serial dilutions of bacteria culture and DNA isolation from these serial dilutions with EB037 and 1-502a strains. All sensitivity tests were repeated at least three times.

RESULTS

PCR-Based Detection Method with TAGTOX-9 and TAGTOX-10 Primers

Sensitivity Test

Sensitivity of the primers TAGTOX-9 and TAGTOX-10 was tested with direct PCR on ten-fold serial dilutions of Pst strain 1-502a and EB037. Primers were not highly sensitive, requiring a range of 1×10^5 to 1×10^6 cfu/ml (Figure 4.1), so this test is not reliable for samples with less than 1×10^5 cfu/ml. Attempts made to increase sensitivity by optimizing PCR protocol were unsuccessful.

Field Sample Testing

The PCR-based detection method with TAGTOX-9 and TAGTOX-10 primers successfully detected Pst on inoculated litter, which was collected from the field 12 days after inoculation (Figure 4.2). Pst was also cultured from the litter sample in which Pst was detected at high populations of 1×10^7 cfu/ml. However, Pst was not detected on grass or litter samples collected the following spring nine months after inoculation. Pst was also not detected in any of the grass and litter collected from Pst naturally infected Canada thistle patches (Appendix E). If fewer than 1×10^5 cfu/ml of Pst were present in the sample, false negatives would occur. DNA was isolated from all field materials, but may have included DNA from other organisms which would dilute the sample.

Nested PCR

Sensitivity Test

Nested primers developed from the TOX-15 gene (Appendix F) had similar sensitivity in the first round of PCR (1×10^5 cfu/ml) (Figure 4.3) as compared to sensitivity tests with TAGTOX-9 and TAGTOX-10 (Figure 4.1). There was a potential increase in sensitivity to 10 cfu/ml in the second round of the nested protocol (Figure 4.4). However, the second round of PCR was prone to false positives in the negative controls, so these sensitivity results are not reliable (Figure 4.4). Attempts to control the false positives such as, optimizing the PCR protocol, and eliminating sources of contamination were unsuccessful. When the product was sequenced, multiple sequence alignment in Clustal W did not match the expected sequence (data not shown), but contained multiple products. Single tube nested PCR with primers separated by temperature was attempted to control for possible cross contamination, but was also unsuccessful. Full specificity tests were not conducted on nested primer sets, but comparisons of the primers to sequences in the Blast database showed the primers were not similar to any other sequences.

DISCUSSION

The objective of this experiment was to develop an accurate PCR-based test for Pst in grass and litter potential inoculum sources for Pst infection of Canada thistle. Pst is difficult to isolate from naturally infected Canada thistle plants, plant residues and soil and an accurate detection method specific to Pst would greatly improve our understanding of the biology of the system. PCR-based detection methods developed in this study require increased sensitivity because the asymptomatic materials tested may have low Pst populations. Also, detection methods require accuracy to specifically test Pst from grass and litter which could contain other closely related bacteria. Neither one of the protocols examined, PCR with TAGTOX-9 and TAGTOX-10 primers nor nested PCR achieved both sensitivity and accuracy. These protocols need further optimizing before either can be used to accurately test grass and litter as potential inoculum sources for Pst infection of Canada thistle.

Sensitivity is important for detecting specific microorganisms from environmental samples with unknown population sizes. TAGTOX-9 and TAGTOX-10 primers were not highly sensitive, requiring a range of 1×10^5 to 1×10^6 cfu/ml (Figure 4.1), so this test is not reliable for samples with less than 1×10^5 cfu/ml. Primers with a high sensitivity of realistically about 10-100 cfu/ml would be needed to accurately test materials. Other potential problems with testing methods were some plant or other microorganism DNA may have been co-isolated in the DNA isolation process which would dilute the concentration of Pst DNA in the sample leading to false negatives. Attempts made to increase sensitivity by optimizing the PCR protocol were unsuccessful.

The PCR-based detection method with TAGTOX-9 and TAGTOX-10 primers successfully detected Pst on inoculated litter, which was collected from the field 12 days after inoculation (Figure 4.2). Pst was also cultured from the litter sample in which Pst was detected at high populations of 1×10^7 cfu/ml, so PCR was not an improvement over standard culturing techniques. Pst may not have successfully over wintered, because Pst was not detected on grass or litter samples collected the following spring, but Canada thistle plants in plots where samples were collected and tested negative for Pst did exhibit chlorosis symptoms later that same year, but it is difficult to explanation why this occurred without an accurate test for Pst (Chapter 3). Pst was also not detected in any of the grass and litter collected from Pst naturally infected Canada thistle patches (Appendix E). However, if fewer than 1×10^5 cfu/ml of Pst were present in the sample, false negatives would occur. So it is possible Pst was present in these samples, but could not be detected with this test. In this study, few samples were tested and sample methods may not be all inclusive, so results of this study are not reliable for making predictions about Pst survival. It is not known where Pst is located in the natural system and materials used in this experiment did not have identifiable areas which had high concentrations of bacteria populations, such as lesions on infected plants. Accurate tests for Pst inoculum sources for infection of Canada thistle cannot be conducted at this time using TAGTOX-9 and TAGTOX-10 primers.

Nested PCR can be a highly sensitive and accurate method for detecting pathogens from plant material with sensitivity as low as 1 cfu/ml (59). In our study, there was a potential increase in

sensitivity to 10 cfu/ml in the second round of the nested protocol (Figure 4.4). However, the second round of PCR was prone to false positives in the negative controls, so these sensitivity results are not reliable (Figure 4.4). The nested PCR protocol suggested here has potential, if false positive problems can be overcome by optimizing the PCR protocol or creating new primers that are both specific and highly sensitive. Nested PCR has improved sensitivity 100 to 1000 times more than a single round of PCR (60) and has been used on asymptomatic plants (52). Nested PCR could be used to determine secondary hosts or inoculum sources for Pst infection of Canada thistle if accuracy of the test can be improved.

Common problems with nested PCR that result in false positives are cross contamination from aerosols or reagents (57) or mis-priming, annealing of primers to alternate sequences. An attempt to control cross contamination was made by nested PCR in a single tube method, but this was unsuccessful at controlling false positives. Mis-priming may have occurred because when the product from the water control sample was sequenced the expected sequence was not present, but multiple smaller sequences occurred, suggesting the problem may be with the primer set from TOX-15 gene. Also, reaction conditions such as magnesium concentration or annealing temperatures may need to be optimized (50). One or more of these problems may be occurring in this experiment, and more work is needed to determine the cause of false positives in nested PCR.

Specificity is also needed in a PCR based detection method, because other closely related *Pseudomonas* bacteria are commonly found in the phyllosphere and may be misidentified if primers are not specific. Also, similar chlorosis symptoms can occur on Canada thistle by infections with other pathogens such as *Pseudomonas* spp. (62) and a fungal pathogen *Phoma macrostoma* (63). This highlights the importance of the need for a test to verify the causal organism for disease on Canada thistle in natural systems. The TAGTOX-9 and TAGTOX-10 primers are highly specific to Pst (56). A full specificity test was not conducted on the new nested primers, but comparisons in the Blast database suggest they are highly specific to Pst. The ability to accurately identify the causal organism for disease on Canada thistle and monitor Pst inoculated materials in the field is essential in continuing the understanding of how Pst survives and causes infection of Canada thistle in natural systems.

The goals for developing a PCR-based detection method were to detect Pst populations in potential inoculum sources and monitor survival on grass and litter in association with Canada thistle. This method should be an improvement over standard culturing procedures and could be used on potential non-culturable forms of Pst. More research is needed on improving the PCR-based tests described in this study to provide an identification and monitoring system for Pst in grass and litter which would provide insight into determining which materials are potentially acting as inoculum sources for Pst infection of Canada thistle. Monitoring the organism's survival in different environmental conditions and materials will provide important information on improving Pst's success as a biological control agent. A reliable test of Pst in environmental samples would be a very useful tool in understanding basic biology of the life cycle of Pst on Canada thistle including transmission and location on the plant. This information may lead to new approaches that could be used in integrated management systems to increase Pst infection of Canada thistle.

Table

Table 4.1 List of nested primers developed from TOX-15 a gene highly specific to *Pseudomonas syringae* pv. *tagetis*.

Nested Primers	Two tube method		
Primer Name	**Sequence**	**Annealing Temp**	**Product Size**
TOX15-OL1	CCTCACCGGGTAAGCAGTCG		
TOX15-OR1	GTAGCCGCCCGTGGCTTG	66	1307
TOX15-OL2	AGTTCTGCCTGCTGATCGTC		
TOX15-OR2	GGCCAACAATGGTGTAAAGG	60	1103
TOX15-IL1	CAGAGATCTACACCAGCTCACC		
TOX15-IR1	ACCGACAAAGCTGCAGAAGT	60	261
TOX15-IL2	TCATTATGAGGCTTGGAGGTG		
TOX15-IR2	GGATAACAAGCTTGGGGACA	60	178
	Single tube method		
Primer Name	**Sequence**	**Annealing Temp**	**Product Size**
TOX15-OL3	ATCAAGCGCCCGACCCGATT		
TOX15-OR3	GGTAGCCGCCCGTGGCTTG	70	1522
TOX15-IL3	CTTCCCTTCTACGGTACAGA		
TOX15-IR3	CGACAAAGCTGCAGAAGTC	56	275
TOX15-IL4	TAGGTCATCGTGAATTTTGC		
TOX15-IR4	AGTCCTGCAGGTCATTCTG	56	813
TOX15-IL5	AGGAAGTAGTCCATCGTTGC		
TOX15-IR5	GCACACCTAACCGCTCTAC	56	840

Figures

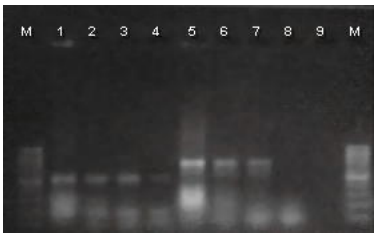

Figure 4.1. Sensitivity test of TAGTOX-9 507-bp and TAGTOX-10 733-bp primers with ten fold serial dilutions of Pst strain 1-502a. Lane 1: 1 x 10^8 cfu/ml with TAGTOX-9, Lane 2: 1 x 10^7 cfu/ml with TAGTOX-9, Lane 3: 1 x 10^6 cfu/ml with TAGTOX-9, Lane 4: 1 x 10^5 cfu/ml with TAGTOX-9, Lane 5: 1 x 10^8 cfu/ml with TAGTOX-10, Lane 6: 1 x 10^7 cfu/ml with TAGTOX-10, Lane 7: 1 x 10^6 cfu/ml with TAGTOX-10, Lane 8: 1 x 10^5 cfu/ml with TAGTOX-10. Detection threshold 1 x 10^5 to 1 x 10^6.

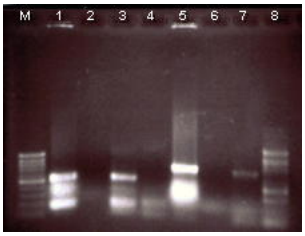

Figure 4.2. TAGTOX-9 507-bp and TAGTOX-10 733-bp test of DNA isolated from litter inoculated with Pst culture 1-502a tested 12 days after inoculation. Lane 1: Positive control with TAGTOX-9 primers Pst strain 1-502a DNA, Lane 3: DNA from litter isolation with TAGTOX-9 primers. Lane 5: Positive control with TAGTOX-10 primers Pst strain 1-502a DNA, Lane 7: DNA from litter isolation with TAGTOX-10 primers.

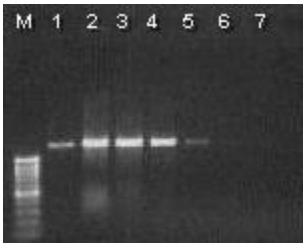

Figure 4.3 Sensitivity test of nested PCR with ten fold serial dilutions of Pst strain EB037. First round of PCR with TOX-15 O1 outer primers product size 1307. Bands are at ~ 1307 bp. Lane 1: Positive control Pst DNA. Lane 2: 1 x 10^8 cfu/ml, Lane 3: 1 x 10^7 cfu/ml, Lane 4: 1 x 10^6 cfu/ml, Lane 5: 1 x 10^5 cfu/ml, Lane 6: 1 x 10^4 cfu/ml, Lane 7: 1 x 10^3 cfu/ml. Detection threshold 1 x 10^5 cfu/ml.

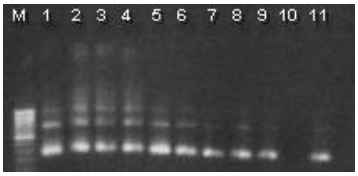

Figure 4.4. Sensitivity test for nested PCR primers. First round of PCR used TOX-15 O1 outer primers with ten fold serial dilutions of strain EB037. 1 µl of this product was used in second round with inner primers TOX-15 I1 with product size 261 bp with the lower band at ~ 261 bp. Lane 1: Pst DNA for positive control, Lane 2: 1 x 10^8 cfu/ml, Lane 3: 1 x 10^7 cfu/ml, Lane 4: 1 x 10^6 cfu/ml, Lane 5: 1 x 10^5 cfu/ml, Lane 6: 1 x 10^4 cfu/ml, Lane 7: 1 x 10^3 cfu/ml, Lane 8: 1 x 10^2 cfu/ml, Lane 9: 1 x 10^1 cfu/ml, Lane 10: 1 cfu/ml, Lane 11: water for negative control. Detection threshold 10 cfu/ml, but false positive occurred in negative control.

CHAPTER 5

Conclusions and Recommendations

Conclusions

Grass and litter are important components of the Pst naturally infected Canada thistle environment which maintains high levels of Pst infection. When either grass, litter, or grass and litter were removed from areas with naturally Pst infected Canada thistle there was a decrease in Pst infection symptoms. Understanding the role of grass and litter in the Pst infection system on Canada thistle and their influence on the environment may help inform development of systems that encourage infection leading to an integrated management system including Pst, grass, and litter which has potential uses in managing Canada thistle in roadsides and natural areas. This study examined the role of grass and litter in Pst infection of Canada thistle as either providing environmental conditions for Pst symptom development or acting as inoculum sources for Pst infection of Canada thistle. Environmental data provided evidence that the presence of grass and litter did affect the temperature and relative humidity surrounding Canada thistle and may be important factors contributing to Pst survival and symptom development in the field. More years of environmental data may be useful in establishing the relationship between the environment and Pst symptom development. One field experiment was conducted in an area that exhibited symptoms of natural Pst infection of Canada thistle. In this experiment perennial grasses and litter were added, but did not cause an increase in Pst infection symptoms that would lead to Canada thistle control. However, the dense perennial grass coverage that was established in this area may have led to a reduction in Canada thistle density.

Field experiments in Canada thistle patches in a restored wetland prairie were designed to determine if grass, litter, soil, or cultured Pst are inoculum sources for Pst infection on Canada thistle. In these experiments Pst was successfully introduced into Canada thistle patches, but disease incidence was not enough to influence control of Canada thistle and there was also some infection in the control plots. These experiments indicated grasses from naturally infected Canada thistle patches should be examined more closely as sources of inoculum. Observations from the first years of these studies indicate that cool season grasses provide the greatest vegetation cover and the role of warm season grasses may need more time to be determined. The combination of established native grasses at a high density and the introduction of biological control agent such as Pst may be the key to effectively controlling Canada thistle in restored wetland prairie. Before it can be used as part of a management strategy more information is needed on establishing and maintaining Pst populations in the environment. However, valuable information on reducing Canada thistle density with grass competition may be used as part of an integrated management system for Canada thistle in wetland prairie areas. Long term monitoring of Canada thistle populations in this experiment may provide more valuable information on best management practices.

Development of a test for Pst in grass and litter is needed to test materials as sources of inoculum for Pst. A PCR-based test was developed, but needs improvement before it can be used reliably. A reliable test of Pst in environmental samples would be a very useful tool in

understanding basic biology of the life cycle of Pst on Canada thistle including transmission and location on the plant. This information may lead to new approaches that could be used in a biocontrol system for Canada thistle.

Recommendations

Recommendations for Canada thistle management in restored wetland prairies.

1. Canada thistle management should include dense planting of native perennial grasses, which may outcompete and suppress Canada thistle populations.
2. Establish native prairie grasses in dense Canada thistle patches, by planting a high density of seedlings into Canada thistle patches which may increase success of establishment.
3. Cool season grasses were effective competitors in the first two years of this study, more years of monitoring of the experiment are needed to determine the role of warm season grasses.
4. Introducing a biocontrol agent such as Pst into this competitive grass environment may increase control on Canada thistle.

Recommendations for *Pseudomonas syringae* pv. *tagetis* as a biocontrol agent for Canada thistle.

1. Continue to study natural Pst infection of Canada thistle as a model for management in roadside and natural areas.
2. Continue to monitor introduced and enhanced infection field experiments, because it may require several years for treatment effects to differentiate.
3. Develop a test or new culturing method for Pst on naturally infected Canada thistle to improve the knowledge of basic biology of Pst infection of Canada thistle and to test potential inoculum sources.
4. Establish an environment with grass and litter in Canada thistle infested areas, because it affects humidity, air temperature range, and soil temperature which may contribute to maintaining Pst infection.

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Appendix A

Locations of *Pseudomonas syringae* pv. *tagetis* naturally infected Canada thistle patches along roadsides in east central MN.

Plot no.	Treatment	Year	Latitude	Longitude
103	Grass and litter intact	2001	44 50' 44. N	94 18' 23. W
201	Grass and litter intact	2001	44 44' 51. N	93 03' 16. W
301	Grass and litter intact	2001	45 03' 53. N	93 13' 04. W
401	Grass and litter intact	2001	45 04' 44. N	93 09' 14. W
102	Litter removed	2001	44 46' 46. N	94 17 '04. W
203	Litter removed	2001	44 47' 00. N	93 03' 52. W
303	Litter removed	2001	45 04' 44. N	93 09' 37. W
403	Litter removed	2001	44 43' 55. N	94 19' 09. W
104	Grass removed	2001	44 52' 24. N	94 21' 01. W
202	Grass removed	2001	44 46' 40. N	93 03' 42. W
302	Grass removed	2001	45 04' 21. N	93 11' 05. W
402	Grass removed	2001	44 45' 27. N	93 07' 23. W
101	Grass and litter removed	2001	44 46' 46. N	94 17' 05. W
204	Grass and litter removed	2001	44 44' 31. N	93 06' 55. W
304	Grass and litter removed	2001	45 12' 01. N	93 03' 09. W
404	Grass and litter removed	2001	44 50' 54. N	94 18' 45. W
104	Grass and litter intact	2002	44 49' 48. N	94 16' 39. W
201	Grass and litter intact	2002	44 52' 26. N	94 21' 06. W
304	Grass and litter intact	2002	44 46' 32. N	93 44' 36. W
404	Grass and litter intact	2002	44 46' 46. N	94 17' 09. W
103	Litter removed	2002	44 50' 51. N	94 18' 39. W
204	Litter removed	2002	44 37' 34. N	94 13' 24. W
301	Litter removed	2002	44 42' 47. N	94 13' 53. W
402	Litter removed	2002	44 43' 20. N	93 50' 56. W
101	Grass removed	2002	45 05' 33. N	94 19' 20. W
203	Grass removed	2002	44 33' 57. N	94 13' 34. W
303	Grass removed	2002	44 46' 30. N	93 45' 08. W
403	Grass removed	2002	44 52' 35. N	94 21' 22. W
102	Grass and litter removed	2002	44 50' 54. N	94 18' 45. W
202	Grass and litter removed	2002	44 45' 36. N	94 16' 28. W
302	Grass and litter removed	2002	44 46' 03. N	94 06' 27. W
401	Grass and litter removed	2002	44 46' 33. N	93 44' 20. W

Plot no.	Treatment	Year	Latitude	Longitude
104	Grass and litter intact	2003	44 48' 37. N	94 22' 31. W
201	Grass and litter intact	2003	44 52' 26. N	94 21' 06. W
301	Grass and litter intact	2003	44 47' 18. N	94 12' 00. W
404	Grass and litter intact	2003	44 47' 30. N	93 50' 56. W
102	Litter removed	2003	44 32' 09. N	94 29' 15. W
203	Litter removed	2003	44 53' 47. N	94 20' 09. W
304	Litter removed	2003	44 46' 05. N	94 06' 50. W
401	Litter removed	2003	44 43' 53. N	93 56' 11. W
101	Grass removed	2003	44 33' 08. N	94 29' 27. W
204	Grass removed	2003	44 46' 09. N	94 10' 23. W
302	Grass removed	2003	44 46' 06. N	94 10' 42. W
403	Grass removed	2003	44 43' 53. N	93 51' 44. W
103	Grass and litter removed	2003	44 42' 19. N	94 22' 32. W
202	Grass and litter removed	2003	44 34' 06. N	94 22' 32. W
303	Grass and litter removed	2003	44 39' 07. N	94 05' 28. W
402	Grass and litter removed	2003	44 43' 53. N	93 53' 48. W
101	Grass and litter intact	2005	44 47' 40 N	94 01' 56. W
204	Grass and litter intact	2005	44 46' 34. N	93 39' 20. W
303	Grass and litter intact	2005	44 51' 48. N	93 42' 07. W
402	Grass and litter intact	2005	44 46' 35. N	93 43' 29. W
103	Litter removed	2005	44 49' 49. N	93 53' 18. W
202	Litter removed	2005	44 48' 14. N	93 47' 23. W
301	Litter removed	2005	44 50' 01. N	93 41' 00. W
401	Litter removed	2005	44 46' 24. N	93 43' 29. W
104	Grass removed	2005	44 49' 40. N	93 39' 07. W
201	Grass removed	2005	44 46' 15. N	93 45' 55. W
302	Grass removed	2005	44 45' 32. N	93 49' 42. W
404	Grass removed	2005	44 47' 30. N	94 02' 25. W
102	Grass and litter removed	2005	44 49' 36. N	93 39' 07. W
203	Grass and litter removed	2005	44 44' 53. N	93 49' 44. W
304	Grass and litter removed	2005	44 48' 17. N	93 53' 21. W
403	Grass and litter removed	2005	44 50' 11. N	93 40' 59. W

Plot no.	Treatment	Year	Latitude	Longitude
101	Grass and litter intact	2006	44 49' 44. N	93 39' 07. W
204	Grass and litter intact	2006	44 49' 45. N	93 39' 11. W
303	Grass and litter intact	2006	44 51' 48. N	93 42' 07. W
402	Grass and litter intact	2006	44 46' 35. N	93 43' 29. W
103	Litter removed	2006	44 49' 49. N	93 53' 18. W
202	Litter removed	2006	44 48' 14. N	93 47' 23. W
301	Litter removed	2006	44 50' 01. N	93 41' 00. W
401	Litter removed	2006	44 46' 32. N	93 43' 25. W
104	Grass removed	2006	44 49' 40. N	93 39' 07. W
201	Grass removed	2006	44 46' 15. N	93 45' 55. W
302	Grass removed	2006	44 45' 32. N	93 49' 42. W
404	Grass removed	2006	44 47' 30. N	94 02' 25. W
102	Grass and litter removed	2006	44 49' 36. N	93 39' 07. W
203	Grass and litter removed	2006	44 44' 53. N	93 49' 44. W
304	Grass and litter removed	2006	44 48' 17. N	93 53' 21. W
403	Grass and litter removed	2006	44 50' 11. N	93 40' 59. W

Appendix B

Time series for environmental data collected in *Pseudomonas syringae* pv. *Tagetis* infected Canada thistle patches along roadsides in east central MN.

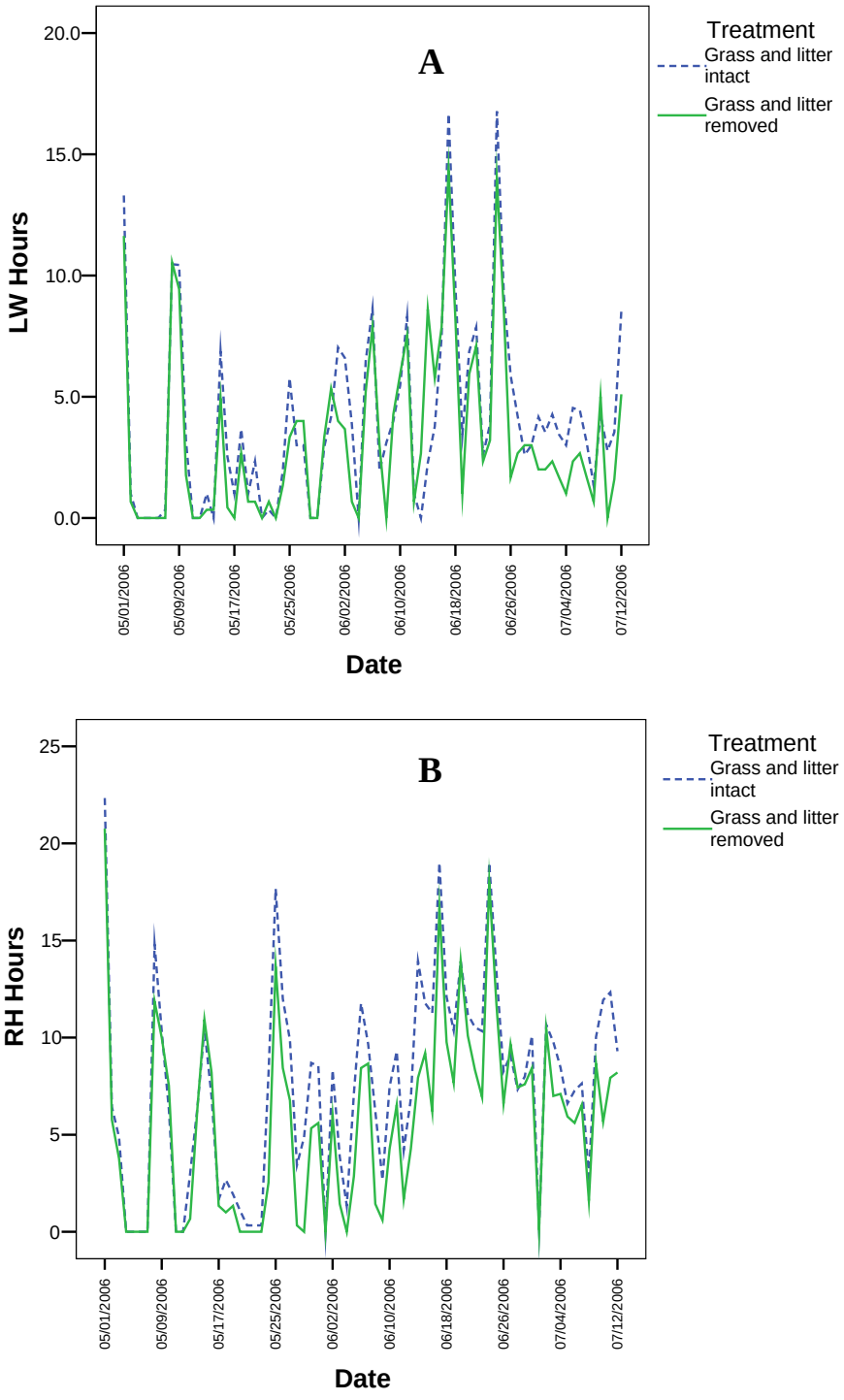

Figure 1. **A**. Total daily hours of leaf wetness (LW) above 6 on a scale of 0 to 15 (dry to wet). **B.** Total daily hours of relative humidity (RH) at or above 80%.

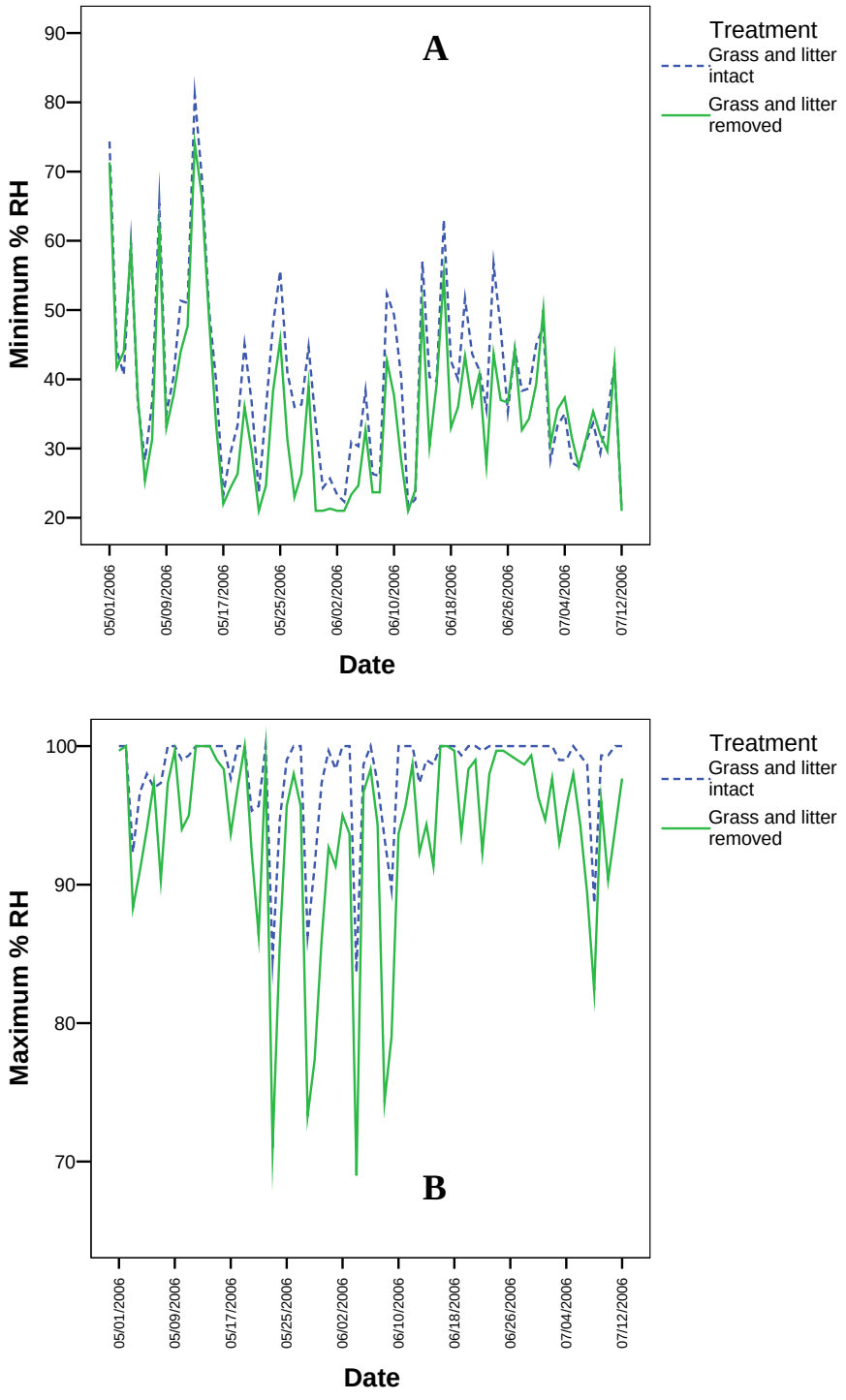

Figure 2. **A.** Daily minimum % relative humidity (RH) **B.** Daily maximum % RH.

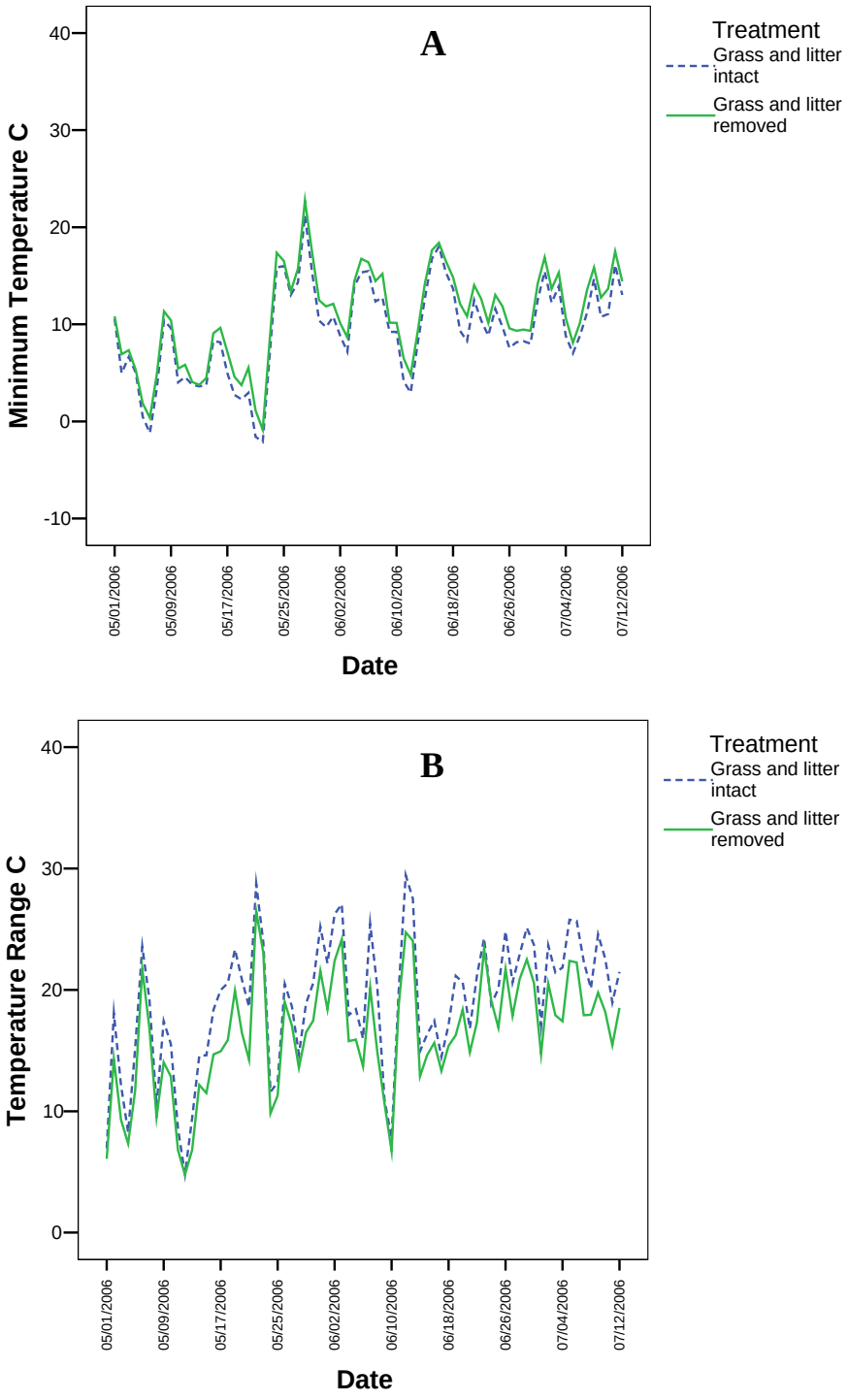

Figure 3. **A.** Daily minimum air temperature. **B.** Daily range in air temperature.

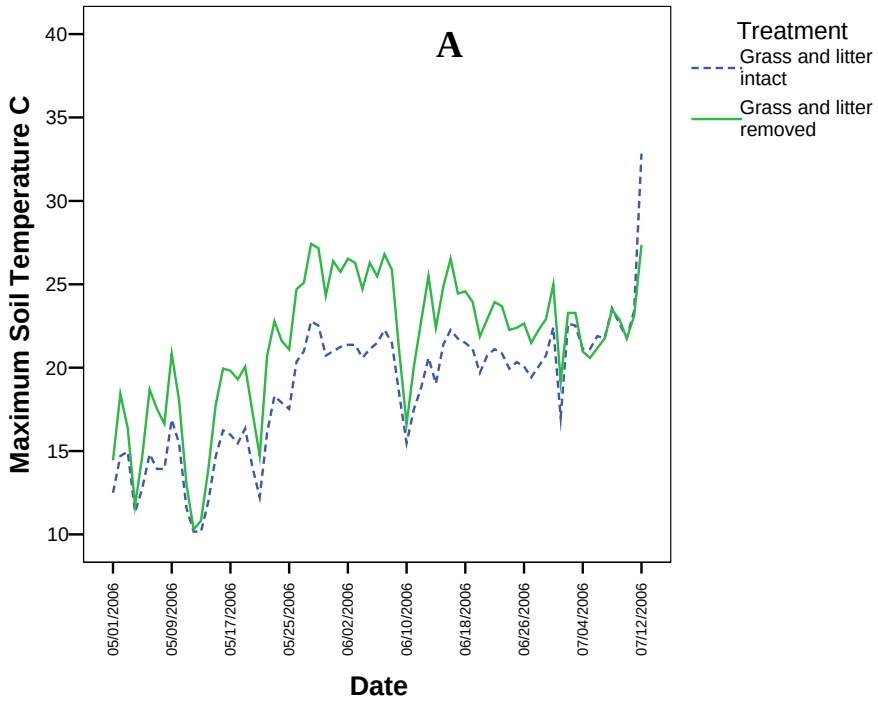

Figure 4. **A.** Daily maximum soil temperature.

Appendix C

Cheri Ponds (Mn/DOT wetland restoration area, Carver County, MN) experiment areas for the Introduced Infection Experiment and the Enhanced Infection Experiments.

Legend 1,2,3, represent replicates 1, 2, and 3 for Introduced Infection Experiments 1 and 2. 4 includes Enhanced Infection Experiments.

Appendix D

Layout of treatments for Cheri Ponds experiments

Site 1 (Rep 1) Introduced Infection Experiment (High thistle density, far west site, near tree line, west of farm road)

Introduced Infection Experiment 1

Introduced Infection Experiment 2

W
S + N
E

Tree Line

25 117	**8** 118	**18** 119	**11** 120	**24** 121	**23** 122	**13** 123	**7** 124	**15** 125	**26** 126
17 109	**22** 110	**14** 111	**19** 112	**9** 113	**3** 114	**1** 115	**2** 116		
		16 1014	**4** 1015	**11** 1016	**8** 1017	**12** 105	**10** 106	**16** 107	**5** 108
		17 1010	**1** 1011	**3** 1012	**9** 1013	**21** 101	**20** 102	**6** 103	**4** 104
					14 1005	**7** 1006	**15** 1007	**13** 1008	**5** 1009
						10 1001	**2** 1002	**12** 1003	**6** 1004

Site 2 (Rep 2) (Medium thistle density, slight slope)

Introduced Infection Experiment 1

Introduced Infection Experiment 2

W
S + N
E

24 214	**15** 215	**2** 216	**4** 217	**5** 218	**17** 219	**11** 220	**7** 221	**19** 222	**16** 223	**21** 224	**6** 225	**26** 226
1 201	**12** 202	**9** 203	**14** 204	**3** 205	**8** 206	**13** 207	**20** 208	**23** 209	**10** 210	**22** 211	**18** 212	**25** 213
				11 2009	**6** 2010	**13** 2011	**15** 2012	**8** 2013	**17** 2014	**7** 2015	**10** 2016	**2** 2017
				4 2001	**5** 2002	**12** 2003	**1** 2004	**3** 2005	**14** 2006	**16** 2007	**9** 2008	

Site 3 (Rep 3) (Low thistle density, steeper slope, south facing)

Introduced Infection Experiment 1

Introduced Infection Experiment 2

N
W + E
S

			16 3009	**2** 3010	**9** 3011	**6** 3012	**3** 3013	**4** 3014	**12** 3015	**10** 3016	**11** 3017	
			15 3001	**13** 3002	**5** 3003	**17** 3004	**7** 3005	**8** 3006	**1** 3007	**14** 3008		
			14 322	**7** 323	**20** 324	**22** 325	**18** 326					
			21 314	**4** 315	**17** 316	**5** 317	**26** 318	**3** 319	**6** 320	**1** 321		
12 301	**24** 302	**2** 303	**19** 304	**23** 305	**15** 306	**25** 307	**8** 308	**10** 309	**9** 310	**16** 311	**13** 312	**11** 313

Site 4 Enhanced Infection Experiment (Natural infection site, far east site, near Cheri Ponds main road)

E
N + S
W

1 4015	**2** 4016	**1** 4017	**6** 4018	**7** 4019	**6** 4020	**4** 4021	**7** 415	**4** 416	**3** 417	**6** 418	**4** 419	**1** 420	**1** 421
3 4008	**7** 4009	**3** 4010	**5** 4011	**4** 4012	**3** 4013	**5** 4014	**7** 408	**2** 409	**5** 410	**3** 411	**2** 412	**3** 413	**4** 414
2 4001	**6** 4002	**2** 4003	**1** 4004	**4** 4005	**7** 4006	**5** 4007	**7** 401	**1** 402	**2** 403	**6** 404	**5** 405	**5** 406	**6** 407

Wetland Edge

Set up in 2004

Set up in 2005

Appendix E

Sources of grass and litter for all experiments Introduced Infection 1 and 2 and Enhanced Infection

Grass Source for Treatments

Common Name	Scientific Name	Source
Canada wild rye	*Elymus canadensis*	Shooting Star Native Seed, Cool Season Native
Indian grass	*Sorghastram nutans*	Shooting Star Native Seed, Warm Season Native
Smooth brome grass	*Bromus inermis*	Allied Seed LLC, Cool Season Non Native Invasive
Big bluestem	*Andropogon gerardi*	St. Paul Campus Field Station, Warm Season Native
Smooth brome grass	*Bromus inermis*	
Kentucky blue grass	*Poa pratensis*	Non-native transplanted from infected Canada thistle patch

Grass Source	Latitude	Longitude
Grass from infected site	44 46' 37. N	93 44' 25. W

Litter Source		
Litter from non-infected site	44 46' 24. N	93 45' 35. W
Litter from infected site	44 46' 33. N	93 44' 22. W

Appendix F

Locations of grass and litter samples collected from a field experiment tested as a source of inoculum for Pst infection of Canada thistle. Results of PCR based protocol with TAGTOX-9 and TAGTOX-10 primers.

Samples Inoculated with Pst Culture

Sample type	Date of collection	Latitude	Longitude	Produced bands
grass	9/27/2005	44 48' 31. N	93 44' 28. W	-
grass	9/27/2005	44 48' 31. N	93 44' 28. W	-
grass	9/27/2005	44 48' 33.N	93 44' 27. W	-
grass	9/27/2005	44 48' 33.N	93 44' 27. W	-
grass	9/27/2005	44 48' 35. N	93 44' 20 W	-
grass	9/27/2005	44 48' 35. N	93 44' 20 W	-
litter	9/27/2005	44 48' 33.N	93 44' 27. W	-
litter	9/27/2005	44 48' 33.N	93 44' 27. W	+
litter	9/27/2005	44 48' 35. N	93 44' 20 W	-
litter	9/27/2005	44 48' 33.N	93 44' 27. W	-
litter	9/27/2005	44 48' 35. N	93 44' 20 W	-
litter	9/27/2005	44 48' 31. N	93 44' 28. W	-
litter	9/27/2005	44 48' 31. N	93 44' 28. W	-
litter	9/27/2005	44 48' 33.N	93 44' 27. W	-
litter	9/27/2005	44 48' 35. N	93 44' 20 W	-
grass	5/18/2006	44 48' 31. N	93 44' 28. W	-
grass	5/18/2006	44 48' 31. N	93 44' 28. W	-
grass	5/18/2006	44 48' 33.N	93 44' 27. W	-
grass	5/18/2006	44 48' 33.N	93 44' 27. W	-
grass	5/18/2006	44 48' 35. N	93 44' 20 W	-
grass	5/18/2006	44 48' 35. N	93 44' 20 W	-
litter	5/18/2006	44 48' 31. N	93 44' 28. W	-
litter	5/18/2006	44 48' 31. N	93 44' 28. W	-
litter	5/18/2006	44 48' 33.N	93 44' 27. W	-
litter	5/18/2006	44 48' 33.N	93 44' 27. W	-
litter	5/18/2006	44 48' 35. N	93 44' 20 W	-
litter	5/18/2006	44 48' 35. N	93 44' 20 W	-

Samples from Pst Naturally Infected Canada thistle Patches

Sample type	Date of collection	Latitude	Longitude	Produced bands
grass	6/23/2005	44 49' 40. N	93 39' 07. W	-
grass	6/23/2005	44 49' 40. N	93 39' 07. W	-
grass	6/23/2005	44 48' 36. N	93 44' 16 W	-
litter	6/23/2005	44 49' 40. N	93 39' 07. W	-
litter	6/23/2005	44 49' 40. N	93 39' 07. W	-
litter	6/23/2005	44 46' 33. N	93 44' 22. W	-

Appendix G

Tox-15 gene sequence from *Pseudomonas syringae* pv. *tagetis* (Pst) strain EB037 used to develop nested PCR primers specific to Pst.

Tox-15 data from T15 Analysis.wpd

Tn5 removed Tn5 inserted in the center of CTGA 1560 bp total; Tn5 inserted at 778

CGTTTGCGAACGCAGCCCCTCCGCCTGCATCAAGCGCCCGACCCGATTTCGCCCGCATGCCT
CTCCCAGCTCACGTAGGTCATCGTGAATTTTGCGATAGCCGTATACACCTCCGCTTTCCAGCC
AAGCGTGTTTGATCAAGCCAAGCAGACGTTGATCTTCTTTCGCTCGGGCAGATTGAGGCTCG
GCCAGCCAAGCGTAGTAGCCGCTGGCGTGCACCTTGAGGGTTTGGCAAAGGCGCCTCACCG
GGTAAGCAGTCGAGTGCTTCTTGATGAAGGCGTACTTCAGCCACACTCCTTGGCAAAGTACG
CGGCGGCCTTTTTTAAGATGTCTCGCTCTTCAGTCACGCGCTTAAGTTCAGCGTGTGGATTGC
GCAGTTCTGCCTGCTGATCGTCGTCTTGCTGACGCTGCTCTTGGGGCTTGCTGTAGACCTTGA
TCCAGGCGTAAAGGCTGTGCACGGACATGCCCAGGCGCTGGGCGACATCGGCGACAGGTTT
GCCTTTTTCGGTCACTTGCTTGACCGCTTCGATCTTGAATTCTTCGGGGTAACGCTGACGACT
CATGGCACCTCCTATTTGGGCCTCATTATGAGGCTTGGAGGTGTCTAGGAAACCAGGGACGA
TTCACTCTTCCCTTCTACGGTACAGAGATCTACACCAGCTCACCGCGCCATCCTCGCGTCGAG
GAAGTAGTCCATCGTTGCCGGGTGATCCACACAAAAAACTGGAAGGTAGCTTAGATGTCCCC
AAGCTTGTTATCCAACACCTGAGCGTCGGCTGACGGCTGCGGAGTTTCAGCACCTGGCTGCG
ATGCCTGCGGCAGTGGAGTGGTTCGCCAATATCGACAACCCACGCACGCGTCGTGCGTACCA
GAATGACCTGCAGGACTTCTGCAGCTTTGTCGGTCTGGCTGGCGCCGAGGAATTTCGCGCTG
TTACCCGATCTCACGTTTTAGCCTGGCGCGCACAGTTGGAACTGCGAGGCCTGGCCGGTGCC
ACGATCCGGCGCAAACTGGCGGCGCTGGCCAGCTTGTTCGATCATCTCCTGGAGAACAACGC
GGTCGCCGGCGGCAATCCCGTGCATGGCGTCAAACGGCCTCGCGTCGAGAGCAATGAAGGC
AAGACACCGGCCCTTGGTGATCACCAGGCCAAGCAGCTGCTCGATGCTCCGGACACTGAAA
CGCTCAAGGGTCTGCGCGACCGGGCAATTCTGGCCGTGCTGCTGTACCACGGCCTGCGTCGG
GAGGAAGCGGCGCAACTAAAGACCGGCGACCTGCAGGAGCGAGGTGGCATCAAACACCTG
CGGGTGCATGGCAAAGGCAGCAAGATCCGCTTTCTGCCGCTCCATCCAGTGGCCGCCGAGCG
TATCTAGAGCGGGATGTTGAACGAGACGCTGCGCCAGGTGCGCTGTTTCGCTCGATGCGTGG
GACCACCACAGGTGCTGGTATCACGGCGAACGGCCTTTACACCATTGTTGGCCAGTGGGCAC
GGGTGGCGGGCATTAAAGTAGAGCGGTTAGGTGTGCATGGTTTGCAAGCCACGGGCGGCTA
CCAACGCGCTGA