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ASPECTS IN THE EPIDEMIOLOGY AND CONTROL OF BACTERIAL WILT OF GERANIUM

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ABSTRACT

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By

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Seed geraniums were shown to be as susceptible as cutting geraniums to bacterial wilt of geraniums caused by Xanthomonas campestris pv. pelargonii (Xcp). Ten of 63 seed geranium varieties tested were found to have significant levels of tolerance to leaf infection. In survival studies, Xcp was found to overwinter on plant debris for up to 9 months. On seeds, Xcp survived up to 1 year. Enzyme-Linked Immunosorbent Assay (ELISA) was developed as a potentially accurate and rapid method of detecting Xcp in the greenhouse. In disease control experiments, oxytetracycline provided only minimal control of the disease. Three bacteria from sewage, Pseudomonas flourescens, Alcaligenes faecalis, Acinetobacter lwoffi were inhibitory to Xcp in vitro and also exhibited moderate control in greenhouse studies. These three bacteria may be useful as biocontrol agents for bacterial wilt of geranium.

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INTRODUCTION

The genus Pelargonium belongs to the Geraniaceae family. Its namesake and related genus, Geranium, are common plants which grow throughout the world. Even though it belongs to a different genus, the cultivated Pelargonium has been mistakenly called a geranium for many years. When used in the following text, the name geranium refers to the genus Pelargonium.

The geranium has an illustrious history (5,6,7,8,18, 36). The zonal or common garden geranium is a product of centuries of breeding. Pelargoniums originated in South Africa where they were discovered by the Cape Colony Dutch who introduced them into Europe in 1609. These geraniums were more fragile than currently grown varieties of geraniums and had to be maintained in hothouses in the great mansions of that time. Their hothouse existence caused them to be called an aristocratic flower for many years. Pelargonium zonale was the first species introduced to the public and was soon found in many gardens in Europe. Other species were introduced to Europeans when the British took control of the Cape Colony in the last decade of the 18th century. England was then deluged with many different species and cultivars of the Pelargonium. Plants that were grown in greenhouses hybridized interspecifically on their

own and many cultivars far different from the original wild species were produced. Historical accounts of some of the aristocratic gardens in Europe describe the many different types of geraniums grown. By the middle of the 19th century, the geranium was a very popular plant for gardens all over the European continent and breeding for new traits became serious work.

The geranium was introduced into the United States around 1750 but the plant did not become popular until the end of the 19th century when new species and cultivars were introduced. Fuel rationing during World War I forced greenhouses in this country and in Europe to curtail production of all plants, including the popular geranium. After World War I, geranium production continued but the popularity of the plant did not significantly increase again until the end of World War II. This increase was due in part to the publication of the first book on geraniums written in this century (7). Published in England, the book became very popular with garden writers and the geranium became a highly publicized plant. The production and cultivation of geraniums expanded rapidly and their popularity has been on the increase since that time.

Recent books (5,24) published on Pelargoniums list a large number of species available but the one commonly grown is the zonal geranium, Pelargonium X hortorum Bailey (PXh). Due to poor record keeping of the early geranium breeders, the original parents of this hybrid are unknown, but the primary parents are believed to be Pelargonium zonale and

Pelargonium inquinans. The plant's common name, zonal geranium, comes from the characteristic zonal leaf pattern. Pelargonium inquinans, contributed the brightly colored flower petals that makes the hybrids so popular.

Due to the complex nature of the PXh genome which can cause great variability in seed lines, geraniums traditionally are propagated from vegetative cuttings. Today, after years of selection, most cutting geraniums are tetraploids and, if crossed, produce seed with low viability. Diploid pelargoniums are used in the production of seed geraniums. This type of geranium has high variability which made seed geraniums desirable only to breeders. However, in 1965, the commercial production of seed geraniums was made practical with the introduction of 'Nittany Lion Red', the first F-1 hybrid seed geranium that bred true (28). This type of geranium was still a PXh, but it shared only a few traits with the cutting geranium.

A census in 1979 reported that total sales of geraniums in this country were greater than $86,100,000, a 300% increase over sales in 1970 (34). This accounted for 7% of the total floriculture industry sales of 1.234 billion dollars (includes bedding plant, cut flower, pot flower, cut green, and foliage plant sales). Geraniums have been the fastest growing floriculture crop in total sales in the last decade, and it has the potential for further growth (34). The increase in sales is linked to greater production of cutting geraniums and the introduction of the seed geranium. An estimated 90-100 million hybrid seed geraniums are

produced in this country annually. Future increases by the geranium will probably be linked to the increased production and increased public acceptance of the seed geranium.

Bacterial Wilt of Geranium

Bacterial wilt of geranium, caused by Xanthomonas campestris pv. pelargonii (Brown) Dye (X. c. pelargonii), has been a serious disease in the culture of geraniums around the world for almost a century. Localized symptoms of the disease include 2 to 3 mm diameter water-soaked lesions on the lower leaf surface, often surrounded by a yellow halo. These lesions rarely coalesce and eventually turn brown to black and desiccate. Infected leaves wilt and die.

Leaf symptoms from systemically infected plants are characterized by water-soaked V-shaped lesions bounded by the veins. The lesions become necrotic with chlorotic edges. Leaves of infected plants may wilt and die while still attached to a firm petiole.

The disease was first reported in 1891 as a stem rot of geranium in Washington D.C. (17). Fifty percent of the geraniums in that area were lost due to the disease. Cuttings taken from the infected plants developed a black rot at their cut base. An unidentified bacterium was isolated from the rotted tissue. The disease was spread by knife cuts made into diseased tissue followed by cuts made in healthy tissue. Geraniums growers were warned of the

problem on their plants.

In 1898, Stone (31) reported the occurrence of a leafspot on geraniums in Massachusetts. It was thought to be a fungal infection but upon isolation, Stone found only bacteria. He assumed that the bacteria he isolated was not actually causing the disease and that the problem would not reoccur. Two years later, Stone was proven wrong when the disease spread throughout the eastern United States during a long period of wet weather (32). He then concluded that a bacteria was causing the disease of the geraniums but was unable to culture the bacterium to further characterize it.

A leaf disease similar to the one that infected geraniums in the Eastern States occurred in Texas in the early 1900's. The symptoms of this disease were leaf spots with indistinct margins whereas the leaf spots occurring in Massachusetts geraniums had distinct margins. Lewis (22), in 1914, characterized the Texas bacterium and named it Bacterium erodii which was changed to Pseudomonas erodii. It was thought that the Texas bacterium was responsible for the Massachusetts disease until Brown (1) isolated the Massachusetts bacterium in 1923. Using the same tests as those used by Lewis, she proved she had a different bacterium and named it Bacterium pelargonii.

Work by Dodge and Swift with the bacterium suggested that the stem rot stage seen by Galloway and the leafspot stage seen by Stone were caused by the same pathogen (9). Bacteria were isolated from leafspots and from stem rots. Rotting resulted from stem inoculations made with either

isolate. Bacteria were reisolated but not indentified. The researchers were still uncertain if both symptoms were caused by the same bacterium and they stressed that more culture work with the bacterium was needed.

In the late 1930's, the nomenclature of plant pathogenic bacteria was in a turmoil and many bacteria were moved into a new genus, Phytomonas (11,15). This genus included many pseudomonads and other bacteria that had similar traits. The change in nomenclature caused many complaints (16) and Dowson (12) tried to set up a standard nomenclature. He proposed to place many species back into the Pseudomonas genus and make a new genus for some of the Pseudomonas-like bacteria. This new genus called Xanthomonas which included the yellow pseudomonads, excluded white to green pigmented pseudomonads. The new nomenclature was accepted in 1941 (4,29) and the bacterium which caused geranium leaf spot was renamed Xanthomonas pelargonii.

During the same period, a new disease was found on the wild geranium (Geranium sangeunium). Burkholder (3) isolated the causal bacterium which was similar to Phytomonas pelargonii but would not infect Pelargonium species. This became Phytomonas geranii. In 1955, Starr and others found that this bacterium was identical to X. c. pelargonii. According to the rules of bacterial nomenclature, the name of this bacterium was changed to X. pelargonii (30).

A change in nomenclature of the Xanthomonads was proposed once more in the 1960's, when Dye noted that many

of the bacteria in this genus were separated by only the host plant on which each were pathogenic (13). He suggested that these be refered to as nomenspecies (14). In 1978, the pathovar system was devised and accepted by plant pathologists (37). Today, Xanthomonas campestris pathovar pelargonii (Brown) Dye, is the causal agent of bacterial wilt of geraniums.

In the mid 1950's, Hellmers first successfully linked the stem rot and leaf spot to the same bacterium (19). His research was the first in depth study of the bacterium since Brown's work. He conducted the study at a time when bacterial wilt was near epidemic levels in this country. Hellmer examined different Pelargonium species for resistance to X. c. pelargonii and he found that all species of geranium tested were susceptible except for P. graveolens. Hellmer rated the species on leaf spot symptoms only. He did not test for stem rots or vascular infections.

Munnecke worked on symptomatology and epidemiology of geranium leaf spot in California in the 1950's (25-27). The bacterium was found to be a serious limitation in the field production of cutting geraniums in that state. Resistance studies, performed concurrently as those of Hellmers, found no PXh variety resistant to the stem rot stage and only a few resistant to the leaf spot stage (25). These results substantiated those of Hellmers. The ivy geraniums (P. peltatum) were highly susceptible to leaf spot and stem rot infection and Regal or Martha Washington geraniums (P. X domesticum) were resistant.

The bacterium was found in the vascular system of symptomless infested PXh plants (25). Munnecke found vascular infection in cuttings taken from infected mother plants, planted in an infested soil, or cut with an infested knife. He found that X. c. pelargonii could survive in soil at least 6 months. Plants could also easily pick up the bacterium by direct contact or water splashing. This appeared to be the method of plant to plant spread in the field. Recommended controls at that time were field rotation with a one year fallow period, use of disinfected knives for cutting, and the use of clean stock plants in sterile mother blocks to avoid contaminating cuttings.

In the late 1950's, Kivilaan and Scheffer (20) also studied the factors affecting the development of the stem rot stage of the disease. Their published research also stressed the importance of symptomless, infected plants. In a random survey comprised of 600 plants and six varieties, 26% were found to be infected with the bacterium. A host range study performed on 93 species belonging to 40 families revealed that the bacterium can not survive on plants outside the geranium family. None of the plants inoculated contained detectable amounts of X. c. pelargonii after 60 days. Their research suggested that greenhouse and field plants and weeds could not serve as possible sources of contamination.

Kivilaan and Scheffer (20) also studied environmental factors that affected the disease. They found that plants that were fertilized above or below optimum levels developed

more severe symptoms than those fertilized properly; symptoms were rapidly expressed in plants grown at a temperature of 27°C while at cooler temperatures, disease developed slowly; and experiments with cut tissue demonstrated that bacteria must be in contact with the parenchyma tissue for infection to occur. Bacteria in the vascular tissue alone did not produce symptoms.

In a study conducted by Bugbee and Anderson (2), whiteflies (Trialeuroides vaporariorum) were examined as possible vectors of X. c. pelargonii. They observed that bacterial wilt occurred in sterile mother blocks set up by commercial geranium growers. Upon examining the plants, whiteflys were noted on the undersides of many of the leaves. In their study, whiteflys were collected from diseased plants and X. c. pelargonii was isolated from them. They also found that after a 24 hour feeding period on infected leaves, previously non-infested whiteflies could pass the disease to healthy plants. It was concluded that proper insect control plays an important role in preventing the spread of the disease to healthy plants.

The same researchers histologically examined leafspot formation. The lesions began as small blisters on the leaf surface caused by the enlargement of mesophyll cells. The blister continued to rise above the surface of the leaf until all the cells died. Cork cells were noted in the blisters and it was believed that these cells prevented the spread of the bacterium.

A resistance trial using a wide range of Pelargonium

species and varieties was carried out by Knauss and Tammen in 1967 (21). In this study, both leafspot and stem rot symptoms were rated. They also evaluated bacterial populations in some of the varieties tested. The Pelargonium species fell into 3 categories; highly resistant, susceptible or highly susceptible. The P. X domesticum hybrids fell within the first group. These hybrids did not develop symptoms under conditions ideal for infection but the bacterium was found to survive in the vascular system. This data is contrary to Munnecke's report of immunity in these hybrids (25). The survival of bacteria in the vascular systems of P. X domesticum hybrids may cause these plants to serve as symptomless carriers for susceptible PXh varieties.

It was hoped that a resistant PXh variety could be indentified, however all varieties tested of this hybrid were susceptible. Hope for finding resistance in this hybrid was reduced when the probable PXh parents, P. zonale, P. inquinans, and P. scandens were not found to be resistant. The ivy geranium (P. peltatum) was also shown to be highly susceptible. Pelargonium graveolens, a species that Hellmers found to be immune, was susceptible in studies by Knuass and Tammen. The fact that Hellmer only rated leaf spots and not stem rot may possibly account for the difference in the findings.

Wainwright and Nelson (35) in 1972 investigated the differences between resistant and susceptible species of Pelargonium using histopathological techniques. They found

that in both species, the initial spread of the bacterium in the plant is the same. The bacteria travel through the xylem vessels from the point of entry. Vessels containing bacteria did not show any morphological changes when compared to non-infested vessels. In susceptible plants, the bacteria moved out laterally from the protoxylem, parenchyma cell walls started to break down and the bacterium could be found in intercellular and intracellular areas. Bacteria rarely moved out of the xylem in resistant plants. The numbers of bacteria in susceptible plants increased as more tissue broke down. The cells began to collapse and the bacteria would fill all vessel elements. Lesions were seen on the exterior of the stem before it collapsed.

Histochemical examination of plant tissue revealed that suberin-like and tannin-like substances formed at or near the infections site. Since these substances were first found around the infection site, it was thought that this was a plant defense reaction. They concluded that this response was secondary because the resistant species did not form any of the suberin-like compounds. Tanin-like materials were found in both types of species but the compounds produced by the susceptible and resistant species appeared to be different based on color differences in stained tissue. They concluded that further work needed to be done to identify the compounds in geraniums.

In 1974, Daughtery (10) investigated the dissemination of the bacterium in the greenhouse and possible chemical

controls for the disease (10). Disease spread was rapid when tightly spaced pots were watered from overhead but, the disease did not spread when in-pot irrigation tubing was used. The disease was also found to spread from splashing water when infected cuttings were propagated under mist. In another experiment, plants showing only leafspots symptoms were found to have bacteria throughout their vascular systems after three weeks. As noted previously, they showed that symptomless plants can yield cuttings that contain X. c. pelargonii.

The major objective of Daughtery's research was to look at possible chemical controls. Streptomycin and cupric hydroxide provided some control of the disease but the levels required caused phytotoxicity. The chemicals were bacteriostatic and not bactericidal and after weekly sprays were concluded, the disease appeared. Control with other experimental chemicals was attempted but proved to be ineffective. The researchers concluded that the only effective control of bacterial wilt was the exclusion of the pathogen with the use of culture-indexed stock.

The movement and distribution of X. c. pelargonii was examined by McPherson and Preece (23). Following artificial stem innoculations, they discovered that the bacterium could be found in the lower 5 cm of a 7 cm plant in 15 minutes. After 2 to 3 weeks, the numbers of bacteria in the stem were very high and the stem started to blacken and rot with typical leaf wilt symptoms following. After 33 days, high numbers of bacteria could only be found in the apical 3 cm

portion of the plant. The bacteria were also found for the first time in the apical meristem. The bacterial titer declined rapidly in portions of the plant showing disease symptoms. The data showed that the bacteria preceeded symptom development by a number of days.

McPherson and Preece also tested an avirulent isolate of X. c. pelargonii using artificial stem inoculations. This isolate did not move throughout the plant as the virulent X. c. pelargonii isolate used previously but remained around the inoculation point. Bacterial titer started to decline three days after inoculation and continued to do so until the experiment was terminated. A cork barrier, which was thought to be a defensive reaction similar to a hypersensitive response, formed in the plant. They speculated that this barrier may have prevented the avirulent isolate of X. c. pelargonii from spreading in the vascular system.

Strider in 1982 studied the susceptibility of a few PXh varieties to X. c. pelargonii and Pseudomonas solanacearum (southern bacterial wilt) (35). Using 16 cutting and 4 seed varieties, Strider found no variety resistant to P. solanacearum. Tests with X. c. pelargonii also demonstrated no resistance in PXh varieties but only in 11 cutting varieties and no seed varieties were tested.

With the introduction of culture-indexed geraniums in the 1960's, the incidence of bacterial wilt declined. Since that time, many growers have become less concerned about this disease. However the disease persists at a low level

and small epidemics occur each year throughout the country. In three of five cases diagnosed at the M.S.U. Plant Diagnostic Clinic in the beginning of 1984, the disease had spread thoughout the greenhouses involved resulting in great losses. In one case, culture-indexed stock was grown among contaminated stock and the disease spread unchecked resulting in the loss of 40,000 plants.

Many varieties of cutting geraniums have been examined for resistance but new cultivars are constantly being introduced and these have not been checked for disease resistance. Seed geraniums have not been assayed for susceptibilty because it was thought that bacterial wilt would not be a problem. There has also been little work done on possible use of chemical or biological control measures. The main objectives of this study were to: 1), describe the symptomatology of bacterial wilt on the seed geranium; 2), evaluate previously untested varieties of geraniums for resistance or tolerance to bacterial wilt; 3), look for possible chemical or biological controls that may protect the plant from infection or eradicate the disease; and 4), devise a rapid detection method for identifying plants infested with *X. c. pelargonii*.

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Chapter 1

Symptomatology and reaction of seed geraniums to *Xanthomonas campestris* pv. *pelargonii*

Introduction

Bacterial wilt, also known as bacterial stem rot and leaf spot, is caused by Xanthomonas campestris pv. pelargonii (Brown) Dye (X. c. pelargonii), and has been recognized since 1923 as an important disease in the United States on florists geraniums (Pelargonium X hortorum Bailey) (1). This disease can produce serious losses at all stages of plant production. Previous research (2,4,5,7) has reported a diverse range of symptoms associated with the disorder on cutting geraniums. Localized symptoms include 2 to 3 mm diameter water-soaked lesions on the lower leaf surface, often surrounded by a yellow halo. These lesions rarely coalesce, eventually turning brown to black and desiccating. Infected leaves wilt and die.

Systemic leaf symptoms are characterized by water-soaked, V-shaped lesions bounded by the veins. The lesions became necrotic with chlorotic edges. Leaves of infected plants may wilt and die while still attached to a firm petiole.

This disease has been traditionally associated with cutting and ivy geraniums and has not been considered a problem in the seed geranium industry. The bacterium is easily spread during asexual propagation and the use of geraniums grown from seed was thought to be a method of

avoiding this disease. However, during the past 3 years, the pathogen has been detected in seedling geraniums in Michigan (S. K. Perry, Plant Diagnostic Lab, Michigan State Unversity, personal communication). In 1984, bacterial wilt has been frequently found on all types of geraniums in commercial greenhouses and in retail plantings. With the growing popularity of seedling geraniums, concerns have risen regarding the pathogen and its possible effect on the seedling industry. The breeding of resistant varieties is an important disease control strategy since current chemical controls are ineffective (2). Differences in resistance may exist in seed geranium varieties due to the great variability and recombination occurring in the breeding of these diploid plants. The tetraploid cutting geraniums is always propagated from cuttings and little recombination takes place. This makes finding differences in resistance less likely in this type of geranium. The purpose of this research was to: 1) determine whether or not resistance to the disease is present in available geranium varieties and 2) describe the symptomatology of Xanthomonas campestris pv. pelargonii on the seedling geraniums.

Materials and Methods

Geranium production

Geranium seeds (Ball Seed Co., West Chicago, IL 60185, and Dept. of Horticulture, Michigan State University, East Lansing, MI 48824) were sown onto moist potting media (Sunshine Mix, Blend 1. Fisons-Western Corp., Vancouver, BC, Canada) in 18 x 13 x 6 cm trays, 20 seeds per tray, and germinated at 24°C with misting as needed to prevent the soil from drying. The seedlings were transplanted into 4 inch diameter clay pots approximately 35 days after planting.

Cutting and ivy geraniums were obtained from a grower of CVI (culture virus indexed) geraniums. These were re-indexed for the presence of *X. c. pelargonii* and propagated by vegetative cuttings. Lateral branches were snapped off to prevent possible infection with contaminated knives. The cuttings were planted in a porous soilless mix (2 parts vermiculte, 2 parts peat moss, 1 part perlite). Cuttings were misted daily with a fertilizer mixture (100 ppm of N with a 20% N: 20% P_2O_5: 20% K_2O fertilizer) till roots formed. Rooted plants were potted into 4 inch clay pots and grown with the seed geraniums.

All geraniums were grown under 20 hours of high pressure sodium (HPS) light (58.56 $umol.s^{-1}m^{-2}$) with a

constant liquid feed of 200 ppm of N with a 20% N: 20% P_2O_5: 20% K_2O fertilizer at 24°C day and 21 night temperature until the plants were ready for inoculation. Plants 45 days after transplant and older were used for tests.

Inoculum production

The bacteria were stored in 0.85% w/v NaCl at 4°C until needed, then grown in petri plates containing a complete agar medium (6). Forty-eight hours after culturing X. c. pelargonii isolate AP-10, one colony was aseptically transfered to a 125 ml flask containing 50 ml of complete broth (6). The flask was shaken on a rotary shaker at 180 rpm, and after 3 days, the culture was diluted to a concentration of 1 X 10^9 colony forming units per ml as determined by standard dilution plating and turbidimetric assays, and used as inoculum. Isolate AP-10 did not differ in virulence from isolates of X. c. pelargonii collected from around the country when tested on various seed and cutting geranium varieties.

Inoculation

Plants were disbudded prior to inoculation to prevent Botrytis sp. infections from occurring in the mist chamber. The plants were sprayed to run off with a pneumatic hand sprayer containing the bacterial inoculum and placed in a mist chamber at 24°C. Control plants were sprayed to run off with a diluted complete broth mixture. The plants were misted to keep the leaf surfaces moist which insured the

best possible conditions for infection. After 3 days, the plants were placed on a greenhouse bench at 29 day and 21 night temperatures and watered as described. Symptoms were observed after 21 days.

Disease Rating Scale

The level of infection was based on a scale of one to four where a one equalled no visible infection on inoculated plants, two equalled 25% or less of the leaf area infected, three equalled less than 50% of the leaf area infected, and four equalled more than 50% of the leaf area infected. Plant death was noted at six weeks on all plants. All surviving plants at that time were tested for vascular infection using the methods of Yount and Rhoads (9).

Treatments

Preliminary tests with the seed geranium varieties 'Pinwheel Salmon' and 'Salmon Express' were conducted to determine if plant age, chlormequat chloride (American Cyanamide Co., Wayne, NJ 07470) (CCC) application, or Hps lighting would effect plant reactions to X. c. pelargonii. The effects of plant age and floral development were evaluated by inoculating plants between the age of 10 days after transplant and full flower development (approximately 70 days).

Chlormequat chloride, a common growth retardant used by the greenhouse industry to control geranium plant size, was applied to 35 days old plants at a rate of 1500 ppm while a

water control was applied to others. Plants at 0,10,20,30,and 40 days after application were inoculated as described before and symptom development was observed.

The effect of HPS lighting was tested by illuminating plants for either 20 hours a day with 58.56 umol.$s^{-1}m^{-2}$ of light or giving normal photoperiod (12 hours) with natural light. These plants were treated the same as all other inoculated plants.

Variety trials were then performed in which 63 seed varieties were spray inoculated 45 days after transplanting. Thirty-three varieties obtained from the M.S.U. Department of Horticulture initially exhibited CCC injury but were not inoculated until all symptoms of CCC injury had disappeared. The other 30 varieties were grown without CCC application. Symptom development was observed every day following inoculation and up to 21 days after inoculation.

A set of cutting and ivy geraniums were spray inoculated to test for leaf infections. This test was done in conjunction with the seed geranium trials. Another set of cutting geraniums were tested for susceptibility to vascular infections using methods similar to the one developed by Nichols (8). Terminal growing points of culture indexed geraniums were removed to allow the maximum amount of lateral shoot proliferation. Lateral shoots greater than 3 inches long were removed from the plant and were placed in a test tube of water. Cuttings were taken just prior to inoculation and were kept in the test tubes as much as possible to prevent the cuttings from drying.

Inoculations were made with a syringe filled with the same bacterial suspension used in the leaf spot inoculations. A drop of the suspension was hung from the tip of a 27 gauge needle. The needle was then pushed through the stem. Another drop was hung and the needle was withdrawn. Five injections were made at various locations in a 1 inch verticle area of the stem. Inoculated cuttings were placed back into the the test tubes and were covered with plastic bags. The tubes were kept in a laboratory at $24^{o}C$ with 18 hours of fluorscent light (10 $umol.s^{-1}m^{-2}$) provided each day. Stem lesion development was rated in 2 weeks by measuring the total length of the lesion on the inside of the stem.

Results and Discussion

There were no significant differences in infection levels (Figure 1) on plants older than 40 days after transplant. Plants were inoculated at 10 and 20 days were not included in the data in Figure 1 because they were to small to accurately rate. The plants inoculated at 30 days were growing rapidly and the increase in total leaf area after the time of inoculation may have offset disease development. Plants older than 70 days were discarded due to infection of flower parts by Botrytis sp. which made rating difficult.

In preliminary tests, plants inoculated 20 days or later after CCC application exhibited similar levels of infection as plants that received no CCC (Figure 2). Chlorotic leaf margins, a typical symptom of CCC injury, were visible on plants 10 days after application of CCC, but the plants quickly recovered. The level of infection by X. c. pelargonii was higher on injured plants. Twenty days after application the plants had almost recovered from the CCC injury and infection levels dropped.

There was no difference in infection levels in plants grown under either HPS lighting or natural light. The plants grown under the HPS light matured faster and had larger, more uniform leaves which made rating infection

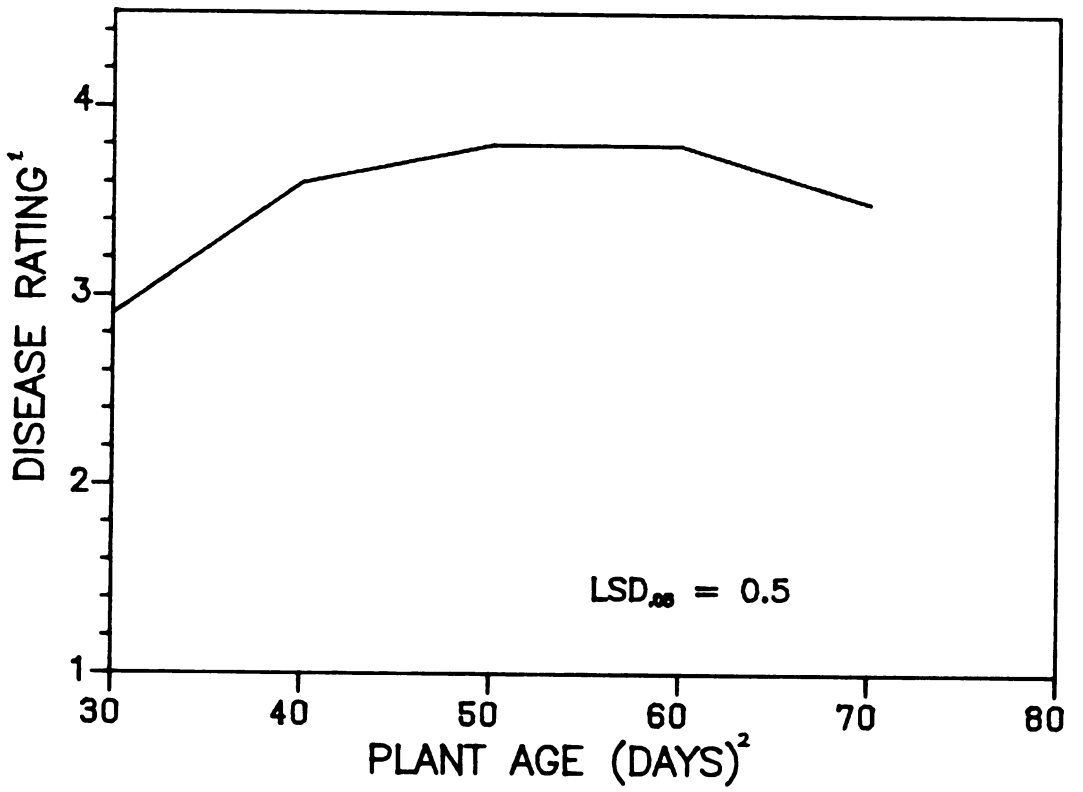

Figure 1. Leaf inoculation of the seed geranium varieties 'Pinwheel Salmon' and 'Salmon Express' with Xanthomonas campestris pv. pelargonii at different plant ages.

1. Numbers followed by the same letter are not significantly different at P = 0.05 using an LSD test. 1 = no visible infection; 2 = 25% or less leaf area infected; 3 = 50% or less leaf area infected; 4 = greater than 50% leaf area infected.

2. Age of plants in days after transplanting

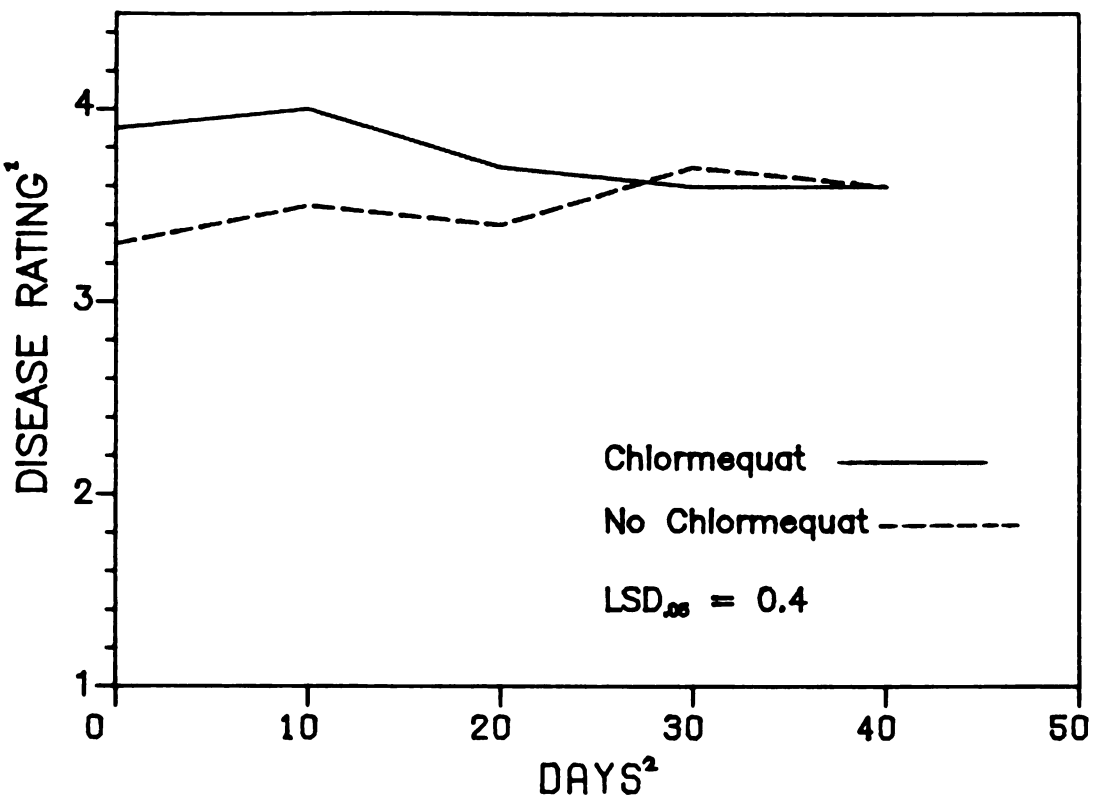

Figure 2. Leaf inoculation of the seed geranium varieties 'Pinwheel Salmon' and 'Salmon Express' with *Xanthomonas campestris* pv. *pelargonii* at various times following application of chlormequat chloride.

1. Numbers followed by the same letter are not significantly different at P = 0.05 using an LSD test. 1 = no visible infection; 2 = 25% or less leaf area infected; 3 = 50% or less leaf area infected; 4 = greater than 50% leaf area infected.

2. Inoculation time in days after chlormequat chloride application.

easier.

The symptoms expressed by the seed geraniums are similar to those shown on cutting geraniums. Small round necrotic lesions developed on infected leaves. These lesions grew to a maximum of 3 mm in diameter and were surrounded by a chlorotic halo. In rare cases, the lesions coalesced to form large necrotic areas on the leaf surface, a symptom which is not seen on cutting or ivy geraniums. Another type of leaf symptom observed was V-shaped necrotic lesions which developed on leaves and indicated that a vascular infection had occurred. The vascular infection was usually accompanied by a leaf wilt with petioles remaining rigid. As the infection progressed, the entire plant wilted and a rotting of the stem tissue occurred. Infections of flower parts by Botrytis sp. became a problem after this stage.

No variety tested was resistant to infection (Table 1). However, ten varieties were significantly less susceptible than the others. All plants that received a rating of four died within seven weeks. Of the plants that received a rating of three, only 50% had died within 7 weeks. Individual plants among the varieties with a rating of two or less did not die. One variety, 'Carefree Crimson', had an 80% survival rate. Six of the 10 tolerant varieties dropped the leaves with the leaf spots on them. This response left the plant with fewer leaves but permitted plant survival. No bacteria could be detected in any of the plants in two of these six varieties. All other plants had

Table 1.

Disease severity response of seed geraniums to leaf inoculation with *Xanthomonas campestris* pv. *pelargonii*

Seed Geranium Variety	Mean Disease Rating[2]
*Carefree Crimson[1]	2.4 a
Exp. Rose PAC	2.5 ab
*Marathon	2.8 abc
PAC Andretta	2.8 abc
**Cherry Diamond	3.0 abcd
*Delta Queen	3.0 abcd
Orbit Red	3.0 abcd
**Ringo Dolly	3.0 abcd
Smash Hit Red	3.0 abcd
*Orbit White	3.0 abcd
Cameo	3.1 bcde
Exp. Scarlet PAC	3.2 cdef
Encounter Salmon	3.2 cdef
Gala Sunbird	3.2 cdef
Ringo Salmon	3.2 cdef
Exp. F-1 Zoned Red 6X1027	3.2 cdef
Smash Hit Salmon	3.2 cdef
Ringo Rouge	3.3 cdefg
Exp. F-1 White	3.3 cdefg
Merlin	3.3 cdefg
Orbit Appleblossom	3.3 cdefg
Ice Queen	3.3 cdefg
PAC Quix Improved	3.3 cdefg
Red Express	3.3 cdefg
Smash Hit Rose Pink	3.3 cdefg
Exp. F-1 Zoned Red 6X891	3.4 cdefgh
Red Elite	3.4 cdefgh
Cherry Glow	3.4 cdefgh
Snowden	3.4 cdefgh
Gala Flamingo	3.5 defgh
PAC Sitta Improved	3.5 defgh
Ringo Rose	3.5 defgh
Rosita Improved	3.5 defgh
Showgirl	3.5 defgh
Smash Hit White	3.5 defgh
Pinwheel Salmon	3.6 defgh
Cherrie Improved	3.6 defgh
Ringo Scarlet	3.6 defgh
Salmon Express	3.6 defgh
Hollywood Star	3.7 efgh
Exp. Salmon PAC	3.7 efgh
Hollywood Red	3.7 efgh
Hollywood Salmon	3.7 efgh
Jackpot	3.7 efgh
Orbit Pink	3.7 efgh

Table 1 (cont'd)

Piccaso	3.7	efgh
Orbit Cherry Improved	3.8	fgh
Encounter Red	3.8	fgh
Exp. Scarlet PAC	3.8	fgh
Capri Deep Red	3.8	fgh
Exp. Holywood White	3.8	fgh
Gala Amaretto	3.8	fgh
Rosita "80"	3.8	fgh
Tiffany Red	3.8	fgh
Heidi	3.9	gh
Mustang	3.9	gh
Orbit Scarlet	3.9	gh
Sprinter Scarlet	3.9	gh
Sprinter White Type	3.9	gh
Cherie "80"	4.0	h
Exp. White PAC	4.0	h
Gala Redhead	4.0	h
Sprinter Salmon	4.0	h

1. * = Plants from which infected leaves abscise; ** = same as * except no bacteria were found in the vascular system of the plants that survived.

2. Numbers followed by the same letter are not significantly different at P = 0.05 using an LSD test. LSD = 0.6. 1 = no visible infection; 2 = 25% or less leaf area infected; 3 = 50% or less leaf area infected; 4 = greater than 50% leaf area infected. Plants rated 3 weeks after inoculation with broth culture of Xanthomonas campestris pv. pelargonii.

detectable amounts of bacteria present in their vascular system.

Cutting and ivy geranium varieties demonstrated significant differences in susceptibility to leaf infections but not to the extent of the seed geraniums (Table 2). None of these geraniums showed a low level of infection. Bacteria were detected in the vascular systems of all plants tested.

There were no significant differences between cutting geranium varieties when tested for vascular infections (Table 2). All varieties had a brown necrotic water soaked area above and below the point of the injection on the interior of the stem. Exterior symptoms consisted of only a slight browning around the point of injection. From this data, none of the varieties were tolerant to infection.

Tolerance may be a useful trait to seed geranium breeding programs. Continuous inbreeding with tolerant varieties may produce a resistant P. X hortorum cultivar. Currently only P. X domesticum varieties are resistant to X. c. pelargonii. Interspecific crosses between P. X hortorum and P. X domesticum may produce hybrid plants with resistance to the disease and also have traits of the desirable P. X hortorum. Standard crossing techniques have failed in the past (3). Perhaps new techniques with in vitro pollination and embryo culture may provide the much desired hybrids.

Table 2.

Cutting and Ivy Geranium Susceptibility to *Xanthomonas campestris* pv. *pelargonii*.

Geranium Type and Variety	Mean Leaf Infection Rating	Vascular Infection[2] in mm
Cutting		
Sincerity	3.0 a[1]	28
Pink Camelia	3.0 a	21
Cherry Blossom	3.0 a	24
Snowmass	2.8 ab	30
Red Irene	2.8 ab	27
Aurora	2.5 ab	27
Penny Irene	2.5 ab	24
Sringfield Violet	2.3 ab	19
Wendy Anne	2.2 b	33
Picardy	2.2 b	29
Ivy		
Yale	2.6	ND
Balcon Royale	2.5	ND
Sybil Holmes	2.1	ND
Beauty of Eastborne	2.3	ND

1. Numbers followed by the same letter are not significantly different at P = 0.05 using and LSD test. LSD = 0.7 (Cutting), 0.5 (Ivy). 1 = no visible infection; 2 = 25% or less leaf area infected 3 = 50% or less leaf area infected; 4 = greater than 50% leaf area infected. Mean leaf rating of 8 replicates, cutting: 5 replicates, Ivy, after spray inoculation with *X. c. pelargonii*.

2. ND = Not Determined. Mean length of internal vascular browning 2 weeks after stem injections with *X. c. pelargonii*, 6 replicates

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Chapter 2

Survival of *Xanthomonas campestris* pv. *pelargonii* in stock beds and on seeds

Introduction

Avoiding the introduction of Xanthomonas campestris pv. pelargonii (Brown) Dye into the greenhouse remains the most successful control of bacterial wilt of either cutting or seed geraniums. Studies have demonstrated that the bacterium is introduced into greenhouses on plants with latent infections.

Many growers use culture index stock to insure that they do not introduce this disorder into their greenhouse range. However, many growers, in order to save money, will grow their best plants after sales for outdoor stock. In the fall, the grower will bring the healthiest plants into the greenhouse for stock beds. Cuttings may be taken from these plants and planted as stock for the next bedding plant season.

Growers of seed geraniums are limited to the introduction of the pathogen on infested seed or from cutting geraniums grown near the seed geraniums. Since seed geraniums are not resistant to the disorder, it is possible that seed harvested from infected plants could be a carrier of the bacterium.

The objectives of this study were to determine if Xanthomonas campestris pv. pelargonii can survive in the soil overwinter in infested stock beds and to evaluate the

potential infested seeds may have in introducing the organism into the greenhouse.

Materials and Methods

Stock Bed Survival

Fifty day old plants of the cutting geranium varieties Sincerity and Red Irene were infected in the greenhouse by taking a suspension of Xanthomonas campestris pv. pelargonii (X. c pelargonii) (1 X 10^{10} cfu/ml) and applying it with a pneumatic hand sprayer until leaf run-off. A rifampicin resistant mutant of X. c. pelargonii suspended in complete broth (1) was used as inoculum. This isolate was marked in order to easily recover the pathogen from the soil and decaying plant material. The virulence of this mutant was identical to its parent strain. To obtain plants that remained symtpomless, the inoculated plants were maintained under conditions unfavorable for disease symptom development. Plants were kept symptomless to simulate conditions in which a grower might select particular plants to use as oversummering material for a stock bed.

The tissue of plants was ground and incorporated into soil to simulate tilling. Another group was left above ground during the winter. The plant material was placed outside in December before the first major freeze. Samples were taken at bimonthly intervals throughout the winter until April and then at 6 and 9 months. Samples with

yellow, Gram negative colonies forming on rifampicin media were rated as a positive for the presence of X. c. pelargonii. Rifampicin media consisted of 100 ug/ml of rifampicin and 25 ug/ml of cycloheximide added to autoclaved complete agar media (1).

Survival on infested seed

Seed geranium varieties Mustang and Pinwheel Salmon were spray inoculated using the same proceedure described for the cutting geraniums. To avoid plant death, conditions unfavorable for symptom expression were maintained. After the plants produced seed, the seeds were harvested. Plants were allowed to dry and the seeds were removed. Any remains of the plants on the seeds were removed with forceps. No scarification of the seeds were performed. The seeds were assayed for the presence of X. c. pelargonii by grinding them in 0.85% w/v NaCl and plating the solution on complete agar media. Seeds which yielded yellow Gram negative, mucoid colonies were rated as positive for X. c. pelargonii.

To test for survival of the bacterium on geranium seeds over time, a group of seeds were soaked in a 24 hour old complete broth culture of X. c. pelargonii for 10 minutes. The seeds were removed and allowed to air dry. Five seeds were assayed for X. c. pelargonii using the assay described before at 1,4,8, and 16 days, and then every other month after that.

Results and Discussion

Stock bed survival

The bacterium survived the winter without any decrease in population in infected stock beds (Table 1). There was nearly 100% survival in samples taken during the first 4 months of winter. It was not until the warmer weather in April (month 4) that a decrease in the survival of the bacterium was seen. This decrease continued until the experiment was terminated in the summer. It is known that the bacterium survives in association with plant tissue. It is likely that the drop in survival can be correlated with the biological break down of plant tissue. Even with the decrease in population, the bacterium survived on some samples in both the tilled and whole plants through to the summer when a grower would normally replant a stock bed. These results indicate that geranium plants free of bacterial wilt could be infected by being planted into a stock bed previously containing infected plant material (2).

Table 1.

Survival period of Xanthomonas campestris pv. pelargonii in stock beds on infected plant material.

	Months								
	1	1.5	2	2.5	3	3.5	4	6	9
Whole plants[2]	5[1]	5	5	5	5	3	4	3	2
Tilled plants[3]	5	5	5	5	4	5	4	2	1

1. Number out of five samples testing positive for X. campestris pv. pelargonii.

2. Whole infected plants were subjected to winter conditions and portions were sampled at the designated intervals

3. Infected plants were ground and incorporated with soil. This was subjected to winter conditions and samples of plants and soil were taken at designated intervals.

Survival on infested seed

Six percent of geranium seeds harvested from infected plants had detectable levels of X. c. pelargonii present. All seeds artificially inoculated were found to have the bacterium present immediately after inoculation. The artificially inoculated seed retained the bacterium for up to one year (Table 2). Levels of X. c. pelargonii detected remained high for the first 3 months. After this period, the number of seeds with detectable levels of bacteria declined. After 12 months, 20% of the seeds were still infested with the bacterium. These data indicate that seed from geraniums could be a source of greenhouse contamination and ultimately plant infection. If seed producers begin to grow cutting geraniums with seed stock, screening of the seeds for X. c. pelargonii may become necessary.

Table 2.

Survival over time of *Xanthomonas campestris* pv. *pelargonii* over time on geranium seeds.

	Days				Months				
	1	4	8	16	1	3	5	7	12
Seeds with bacteria present[2]	5[1]	5	5	5	5	5	2	1	1

1. Number of seeds out of five testing positive for *X. campestris* pv. *pelargonii*

2. Seeds were artificially inoculated by soaking in broth culture of *X. c. pelargonii*, and air drying. The seeds were stored in a dark, dry petri dish unitl needed.

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CHAPTER 3

Detection of Bacterial Wilt of Geranium

Introduction

Bacterial wilt caused by Xanthomonas campestris pv. pelargonii (Brown) Dye is an important disease of cutting geraniums (Pelargonium X hortorum) and can result in serious losses in the greenhouse. Previous research (3,7,8,9,10, 12,14,17) has reported a diverse range of symptoms associated with the disorder on cutting geraniums. Localized symptoms include 2 to 3 mm diameter water-soaked lesions on the lower leaf surface, often surrounded by a yellow halo. These lesions rarely coalesce and eventually turn brown to black and desiccate. Infected leaves wilt and die.

Systemic leaf symptoms are characterized by water-soaked, V-shaped lesions bounded by the veins. The lesions become necrotic with chlorotic edges. Leaves of infected plants may wilt and die while still attached to a firm petiole.

This disease not only causes the direct loss of plant material but the bacterium can survive and be carried into the greenhouse on symptomless plants. Splashing water can spread the bacterium, Xanthomonas campestris pv. pelargonii (X. c pelargonii), from the symptomless infected plants to healthy, non-infected geraniums. The spread of the disease can occur rapidly through overhead irrigation.

The disease can also spread via infected propagation material. Geraniums are traditionally propagated from cuttings. Knives used to make the cuttings can transfer the bacterium between plants as easily as water splashing (14).

Ease of spread of the disease make control strategies critical. Chemical controls, used in other bacterial plant diseases, are ineffective (8). Environmental control can slow symptom development (12) but this will not stop disease spread or cure infected plants. Currently, the only practical control is to avoid the introduction of infected material into the greenhouse.

A rapid and accurate method of diagnosis is needed to aid geranium growers in detecting infected plant material. Many other systems have been developed to identify plant pathogenic bacteria including selective media (16), phage typing (5), and serology (15,20). Current methods of assaying plants for the pathogen consist of isolation and identification of the bacterium. This process is very time consuming with identification taking 4-5 days. Time and special requirements make this method impractical for greenhouse use. To be useful, a detection method must be quick, simple, and inexpensive. There are several new serological detection methods which are promising and fulfill these requirements.

One test commonly used in phytobacteriology is the Ouchterlony double diffusion test. Bacteria involved with plant disease have been successfully detected with this test. Potato black leg, caused by Erwinia carotovora, has

been detected and differentiated from non-pathogenic strains of E. carotovora (18,21). Ring rot of potato, Rhizobium galls, and the various strains of Agrobacterium have also been identified from plant tissue using Ouchterlony tests (6,11). This test is quite useful, but samples require special treatment.

Two serological tests that meet all the requirements previously mentioned are the Enzyme-Linked Immunosorbent Assay (ELISA) and the Latex Bead Agglutination test (LBA). These tests can be used with ground plant tissue and the results are easily read with minimal equipment requirements. ELISA has been used successfully in identifying plant pathogenic bacteria (20). Latex bead agglutination tests are new in plant pathology and have not been used extensively in bacteriology (2). The objective of this research is to examine the potental use of ELISA and the Latex Bead Agglutination test for detecting bacterial wilt of geraniums.

Materials and Methods

Antisera Production

Antisera were prepared against glutaraldehyde fixed cells and live cells of several pathogenic isolates of X. c. pelargonii. Several isolates were used in an attempt to eliminate the possiblity of antigenic differences between different isolates. Cultures of these bacteria were grown in complete broth (13) at 27°C for three days. Bacteria harvested from these cultures were washed three times in phosphate buffered saline (PBS) [.01 M Potassium Phosphate Buffer (pH 7.2) and 0.15 M NaCl] and were suspended in PBS. This bacterial solution was stored at 4°C. Half of this solution was used as a live immunogen and the other half used as described below.

The glutaraldehyde immunogen was prepared by dialyzing washed bacteria against 2% glutaraldehyde for 4 hours at room temperature, then dialyzing against PBS at 4°C for 20 hours with three changes of PBS at 2 hour intervals with a final change overnight (1). Both bacterial preparations were diluted with PBS to a final concentration of 1 X 10^9 cells per ml.

The immunogens were prepared for injection by emulsifying 1.5 ml of the appropriate bacterial suspension

with 1.5 ml of Freund's incomplete adjuvent (Difco Laboratories, Detroit MI 48232). Three month old female New Zealand White rabbits received, intramuscularly, 2 ml of the emulsion weekly for nine weeks from a 2 ml glass syringe with a 20 gauge needle. Disposable plastic syringes would not work well with the adjuvent.

Blood was collected for normal sera before injections began, and then every week starting 6 days after the fourth injection. The procedure used in the bleeding were adapted from Weir (22).

Sterile plastic centrifuge tubes coated with petroleum jelly on the interior were used for blood collection. After collection, the tubes were left undisturbed for 4 hours then refrigerated overnight. The serum was drawn off from the clotted blood and centrifuged at 1800 g for 15 minutes to remove cellular components of the blood. The crude antisera were stored in sterile glass vials at -15°C.

After each bleeding, the agglutination titer of the serum was determined by the micro-agglutination test adapted from Ball (2). The bottoms of plastic petri dishes were scored to form a grid. Dilutions of the test serum were made in PBS and 0.01 ml drops were placed onto the grids, followed by 0.01 ml drops of the previously prepared live bacterial immunogen. The plates were covered, sealed with parafilm, and incubated at room temperature for 2 hours. The reaction was read with a dissection microscope using indirect lighting against a dark background. After 7 bleedings, the serum with the highest titer was retained for

use.

The gamma globulin portion of the antiserum was fractionated using methods from Clark and Adams (4) except that DE 23 cellulose was used to further purify the protein. All gamma globulin preparations were stored either for short terms at 4°C or for long terms at -20°C.

Enzyme-Linked Immunosorbent Assay

Gamma globulin was conjugated with alkaline phosphatase (#5521, Sigma Chemical Co. St. Louis MO, 63178) using the methods of Clark and Adams (4). Using a grid scheme of dilutions, the alpha optimum of the coating and conjugate gamma globulin was determined. Flat bottomed microtitration plates (Immulon 1, #011-010-3350, Dynatech Laboratories, Inc. Alexandria, Va 22314) were used for the direct double antibody sandwich ELISA technique (4). The techniques were modified to work with bacteria by allowing the antigen to incubate at 37°C for 20 hours rather than the 4 hours used for virology ELISA. The substrate, 2-nitrophenyl phosphate (Sigma 104 phosphate substrate, Sigma Chemical Co.), was added at a concentration of 1 mg per ml to the wells after the conjugate was washed out. Readings were made every 20 minutes for 1 hour with a Dynatech ELISA minireader. Positives were determined to be any absorbance three times greater than the highest control absorbance. Visual observations were made at the same time as the machine readings.

Latex Bead Agglutination

The LBA test was carried out using the methods of Van Regenmortel (19) in microtitration plates. Latex beads (Latex 0.81, Difco Laboratories, Detroit MI 48232) were sensitized with different dilutions of gamma globulin to find a working concentration of antibody and latex beads. Once this concentration was found, 25 ul of the sensitized beads were mixed with 50 ul of antigen. The mixture was oscillated at 120 rpm for 15 minutes. The reactions were read with a dissection microscope using a dark background and indirect lighting. The presence of many floccules was interpreted as a positive reaction.

Antigen Preparation

Samples tested included pure bacterial cultures grown on complete agar medium or infected and non-infected plant material. Bacterial cultures were prepared for testing by growing lawns of the test bacteria on complete agar. Each lawn was flooded with 5 ml of PBS and left for 5 minutes. The bacteria were scraped loose from the plates with sterile glass rods and the resulting bacterial suspensions were pipeted into sterile test tubes.

Plant tissue was prepared for testing by taking a small amount of the tissue and titurating it in a buffer. The type of buffer depended on the test. The ELISA buffer was

the PBS-Tween buffer descibed by Clark and Adams (4) except 2% w/v polyvinylpyrolidone was added as a stabilizer. Tween-HCL PVP buffer descibed by Van Regenmortel was used in the latex bead agglutination tests. The ground samples were allowed to soak in the buffer approximately 1 hour to allow the bacteria to leave the tissue.

Specificity of serological tests to bacterial isolates or diseased tissue

Three sets of specificity tests were conducted. In the first, the specificity of the serological tests to various bacterial isolates was determined. Dilutions of the bacterial isolates were made with PBS. These were plated out to get standard count and were then used for the serology.

The second major set of tests involved determining the specificity of the serological tests to diseased plant tissue. Geraniums were tested that had varying levels and types of infection from bacterial wilt. Plant tissue was prepared as described above.

A related experiment was conducted using geraniums infected with Botrytis sp. and tomatoes infected with Pseudomonas syringae pv. tomato. Botrytis sp. produces symptoms on the geraniums similar to bacterial wilt. Pseudomonas syringae pv. tomato produces a leaf spot on tomatoes similar to the bacterial leaf spot on the geraniums.

Seed and cutting geraniums were used for the initial tests. Both types were spray inoculated to induce leaf spots. A set of cutting geraniums were also stem inoculated to produce stem rot symptoms. Inoculated plants were maintained at 24°C, a temperature favorable for infection. Another set was maintained at cooler temperatures to prevent symptom expression. All plants were assayed for the presence of X. c. pelargonii before the serological tests.

Plants suspected of being infected were collected from the M.S.U. Plant Diagnostic Clinic and from growers with geranium that were suspected to be infected by X. c. pelargonii. These were tested with the serological tests and were isolated from to detect any bacteria.

Results and Discussion

The serum from bleed four of the live immunogen injected rabbit had a titer of 1024. Bleedings before and after this had substantially lower titers. Blood sera from the glutaraldehyde immunogen injected rabbit never approached the titer of the sera prepared from the live immunogen (Table 1). After the titer drop in bleed 5, the amount of bacteria in the sixth injection was increased to 1 X 10^{11} bacteria per ml for both types of immunogens. This did not lead to an increase in titer and the extra bacteria adversely affected the rabbits. Blood serum 4 from the live immunogen injected rabbits and blood serum 5 prepared from the glutaradehyde immunogen (titer = 512) were purified for use in the serological tests.

The microagglutination test was used to determine which gamma globulin had the best titer and specificity. Serum 4 was chosen on the basis of having the highest titer. The ELISA alpha optimum of the conjugated versus the normal gamma globulin was determined to be 1:800 and 1:200 respectively. These numbers are lower than expected for standard ELISA but a strong fast reaction was desired. At this time, it was discovered that better reactions occurred if the bacteria were left in the microplate wells longer. Further testing revealed that 20 hours of incubation of the

Table 1

Titer of antisera made to different types of *Xanthomonas campestris* pv. *pelargonii* immunogens

Immunogen type[3]	Bleed Number[1]						
	1	2	3	4	5	6	7
Live	64[2]	256	512	1024	512	512	64
Glutaraldahyde fixed	64	128	256	256	512	128	256

1. Designates the number of weeks starting 6 weeks after the first injection that blood was drawn.

2. Titer determined by standard microagglutination tests.

3. Live bacteria were harvested from broth by centrifugation and stored in saline. Fixed bacteria were harvested in the same manner and fixed with 2% glutaraldahyde.

bacteria in the microplate wells gave the best results. Strong readings could be visually observed in 10 to 15 minutes.

The LBA test required that the gamma globulin be bound to the latex beads. The working concentration of gamma globulin to beads was determined to be between 1:50 and 1:100. Both dilutions gave equal responses. A 1:25 dilution gave no reactions and a dilution of 1:200 gave numerous false positives. All beads used in further tests were sensitized at a 1:100 dilution.

Only xanthomonads were detected when both tests were used on pure bacterial isolates (Table 2). All non-xanthomonads gave negative results when used at concentrations similar to those of the Xanthomonads. ELISA produced strong readings at a wide range of X. c. pelargonii dilutions. The other Xanthomonads produced high absorbance values but only Xanthomonas campestris pv. vesicatoria isolate 1 and X. c. pv. campestris isolate 2 gave a reading that could be confused for a positive. Visually, the yellow reaction produced by all the Xanthomonads would have been rated as positive. These false positives are not troublesome because all the bacteria in this genus are similar but most are host specific. Therefore, only X. c. pelargonii would be expected to be on geraniums. The water saprophytes tested, which may be found on geraniums, gave negative results with both tests.

The latex bead agglutination test also detected the pure cultures of X. c. pelargonii. Other Xanthomonads gave

Table 2

Serological tests with different genera and species of pathogenic and saprophytic bacteria

Bacterial isolate	log CFU/ml	ELISA[2] O.D.	Latex Bead Agglutination
Control	0.0	0.45[4]	-[3]
Acinetobacter lwoffi*[1]	8.0	0.12	-
Agrobacterium radiobacter	9.5	0.05	-
Agrobacterium tumefaciens k27	9.8	0.02	-
Agrobacterium rhizogenes	ND	0.07	-
Agrobacterium tumefaciens B6	9.5	0.10	-
Alcaligenes faecalis*	6.3	0.05	-
Corynebacterium michiganense	9.5	0.05	-
Erwinia amylovora	9.0	0.65	-
Pseudomonas fluorescens*	8.0	0.63	-
Pseudomonas phaseolicola 15	8.3	0.07	-
Pseudomonas phaseolicola 35	8.9	0.08	-
Xanthomonas campestris pv. campestris 1	7.6	1.22	-
Xanthomonas campestris pv. campestris 2	8.7	1.51	+/-
Xanthomonas campestris pv. pelargonii (Xcp)			
Xcp isolate UL3[5]	6.3	2.44	+/-
Xcp isolate PG3[5]	10.3	2.44	++
Xcp isolate OLD[5]	8.9	2.44	++
Xcp isolate AP10[5]	7.9	2.44	+
Xanthomonas campestris pv. vesicatoria 1	5.0	1.82	+/-
Xanthomonas campestris pv. vesicatoria 2	10.6	1.15	-

1. * represents saprophytic bacteria

2. Average absorbance value (A_{405}) after 20 minutes of plate incubation, average of 4 trials. An O.D. value must be at least 3 X greater than the control to be considered a positive.

3. ++ = strong agglutinations, + = Mild agglutinations, +/- = variable, - = no reaction.

4. Control ELISA absorbance is the highest value of all controls, ranging from 0.07-0.40

5. Isolates used as imunogen

agglutinations but not as many as a positive test. Rating agglutinations was difficult to do. The extracellular polysaccharide of the Xanthomonads seemed to cause the beads to string together. This gave the variable responses indicated in the data.

ELISA was more accurate than the LBA in detecting X. c. pelargonii on infected geraniums (Table 3). The LBA test gave a false positive among the controls and detected only 56% of the infected geraniums. Using ELISA, 100% of the infected geraniums were detected and there were no false positives in the control geraniums. Visual readings coorrelated with the machine readings.

Neither test falsely detected the Botrytis sp. infected geraniums. When both tests were used with P. syringae pv. tomato infected tomato leaves, ELISA gave a false positive. The LBA did not give any reaction.

Both tests were very quick and easy to perform but ELISA was the most accurate. Because the disease is rapidly spread, all infected plants must be found. The LBA test detected far too few plants to make it a viable test. False positives for both tests were insignificant. Rating ELISA was easier and more objective than rating the latex bead agglutinations. Because many samples can be tested rapidly and efficiently, ELISA may be a good test for the greenhouse industry to use.

Table 3.
Serological detection of *Xanthomonas campestris* pv. *pelargonii* on geraniums and comparisons with other infected plant material

	number of plants	number with bacteria isolated	number ELISA positive	number LATEX positive
Non-inoculated geraniums	10	0[2]	0[3]	1
X. c. pelargonii inoculations				
Plants with symptoms	30	30	30	17
Symptomless plants	10	9	9	5
Natural infections	17	17	17	10
Botrytis spp. on geraniums	7	0	0	0
Pseudomonas syringae pv. *tomato* on tomato	5	5[1]	1	0

1. *Pseudomonas syringae* pv. *tomato* isolated.
2. Standard isolations and tests performed to identify *Xanthomonas campestris*.
3. A positive was any absorbance (A_{405}) which was three times that of the highest healthy control.

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Chapter 4

Chemical and Biological Control of *Xanthomonas campestris* pv. *pelargonii*

Introduction

Bacterial wilt caused by Xanthomonas campestris pv. pelargonii (Brown) Dye is an important disease of cutting geraniums, (Pelargonium X hortorum) and can result in serious losses in the greenhouse. Previous research (2,7,8,9,11) as reported a diverse range of symptoms associated with the disorder on cutting geraniums. Localized symptoms include 2 to 3 mm diameter water-soaked lesions on the lower leaf surface, often surrounded by a yellow halo. These lesions rarely coalesce and eventually turn brown to black and desiccate. Infected leaves wilt and die.

Systemic leaf symptoms are characterized by water-soaked, V-shaped lesions bounded by the veins. The lesions became necrotic with chlorotic edges. Leaves of infected plants may wilt and die while still attached to a firm petiole.

Prevention is the most effective control of bacterial wilt. However, Xanthomonas campestris pv. pelargonii (X. c. pelargonii) is commonly carried over in infected but symptomless stock plants. It spreads rapidly from these infested plants to non-infected plants by splashing water or on infested propagating utensils. Therefore it would be extremely useful to develop control stategies for

greenhouses in which the disease is already present. Most of the chemical controls have been shown to be ineffective in controlling this bacterium (6). A control strategy is needed that will protect healthy plants from contracting this disease.

Biological control is an alternative control proceedure that has had some success in controlling plant disease. Perhaps the best known example of biological control of bacteria is the use of Agrobacterium radiobacter pv. radiobacter K84 to prevent infections of crown gall caused by A. radiobacter pv. tumefaciens (5). Other cases of biological control have been reported with varying degrees of success (4). Most of the current research in the area of biological control involves the use of supressive soils for combating soil-born pathogens. The use of biological control agents for foliar pathogens has not been explored extensively. In order to prevent foliar infection by X. c. pelargonii, an antagonist must be introduced onto the leaves of susceptible plants that will prevent the pathogen from reaching an infection site. Two antagonists that have been used for this purpose with other diseases have been bacteriophages and non-pathogenic bacteria (1,12). Both have shown some promising results but each antagonist seems to be specific for only one pathogen. The objectives of this study are to 1) examine the efficacy of new chemicals not tested previously for controlling bacterial wilt, and 2) isolate and test a biocontrol agent for this disease.

Materials and Methods

Chemical Control

Oxytetracycline was tested by applying it before, in conjunction with, and after the application of X. c. pelargonii inoculum. The rates of the chemical application were 0,100,200,400, and 800 ppm active ingredient. The chemical and the bacteria were applied with a pneumatic hand sprayer to leaf run-off. An evaluation of treated plants were made after 21 days.

Isolation of a biological control agent

Raw liquid sewage was collected from local sewage plants. Serial dilutions of the liquid were made and 0.01 ml aliquots were mixed with 2.5 ml of a soft agar medium (see below). Four drops of a 3 day old complete broth (10) culture of X. c. pelargonii were added to the soft agar. The contents were vortexed and overlayed into petri dishes containing hardened complete agar.

Soft agar medium contained the ingredients of complete broth (10) and 0.5% w/v of agar. The soft agar was sterilized and kept in small tests tubes at 50°C until needed.

After 2-3 days of incubations at room temperature (21°C), the plates were examined for the presence of clearing zones which would indicate that an inhibition of bacterial growth was occurring.

Portions of the clearing zones were transferred to fresh complete agar and were overlayed with soft agar containing X. c. pelargonii. Any clearing zones that developed on the transfer plates were treated in the same manner through two additional transfers in order to isolate the causal organism in pure culture.

Tests were conducted to determine the identity of each of the unknown bacteria. All tests were conducted as outlined in Bergey's Manual of Determinative Bacteriology (3).

In Vitro Tests

To determine if live antagonistic bacteria were needed for the inhibition to occur, pure cultures were placed in 1 cm spots on complete agar medium. After 4 days of growth, the culture plates were inverted over chloroform saturated filter paper for 30 seconds to kill the bacteria. Controls consisted of other non-antagonistic bacteria isolates which did not produce clearing zones. The plates were placed into a sterile area to allow the chloroform to evaporate. The plates were overlayed with X. c. pelargonii in soft agar. The diameter of the inhibition zone was measured after 3 days.

The specificity of the bacteria were tested using a similar procedure. Various pathogenic and saprophytic bacteria were overlayed onto the dead cultures of the biocontrol bacteria as before. The diameter of any inhibition zones produced was measured in 3 days. The size of these zones were compared with the size of the zones produced with X. c. pelargonii

The bacteria were tested for their ability to produce substances that were inhibitory to X. c. pelargonii in broth culture. Fifty ml of complete broth were inoculated with bacteria. After 3 days at 20°C on a rotary shaker, the cultures were centrifuged to remove the bacteria and the supernatant was filter sterilized. A few drops of the broth were placed onto 1 cm disks of sterile filter paper. After drying the disks were placed onto complete agar medium and this was overlayed with X. c. pelargonii. The diameter of any inhibition zones produced was measured in 3 days.

In Vivo Tests

Geraniums were seeded and grown for 60 days. The bacteria for the test (X. c. pelargonii and the biocontrol bacteria) were grown in complete broth until the desired bacterial concentrations were reached (1×10^8 for X. c. pelargonii and 1×10^{10} for biocontrol bacteria). The biocontrol bacteria were applied with a pneumatic hand sprayer to the geranium leaves until run off. While the leaves were still wet, X. c. pelargonii was sprayed on at a

rate of 20 ml per plant, the approximate amount needed to moisten dry leaves to run off. Control plants were sprayed with complete broth and *X. c. pelargonii*. All treated plants were maintained at 21°C and misted to maintain leaf wetness. After 4 days of misting, the plants were watered only as needed. Disease evaluations were made after 21 days.

Results and Discussion

Chemical Control

There were no significant differences between the oxytetracycline treated plants and the non-treated plants when both types were treated with *X. c. pelargonii*. Plants treated with 800 ppm of the chemical and *X. c. pelargonii* simultaneously were slightly less infected. However, phytotoxicity occured on all geraniums treated with this concentration of oxytetracycline. Edges of the leaves yellowed and turned necrotic. These results eliminated oxytetracycline from further consideration.

Isolation of Biological Control Agent

Numerous samplings were made from sewage treatment plants from 5 cities, seven bacterial isolates were found to inhibit the growth of *X. c. pelargonii*. No *X. c. pelargonii* specific bacteriophage were found. Five of the seven bacterial isolates were identified. Two were *Pseudomonas fluorescens*, two were *Acinetobacter lwoffi*, and one was *Alcaligenes faecalis*. The three species are common water saprophytes and were judged to be safe to use in other tests. Tests on the two remaining unknown isolates gave

conflicting results with known bacteria listed in Bergey's Manual of Determinative Bacteriology, therefore, the two cultures were not used in further studies.

In Vitro Tests

Pure cultures of the three biocontol bacterial species yielded similar results. The average zone of inhibition was approximately 4 cm for all 5 isolates (Table 1). Pseudomonas fluorescens had the largest clearing zones. Similar species had similar zone sizes therefore, only three of the different species were used in further tests.

The filter sterilized broth of the three isolates also produced clearing zones. The zones were not as distinct and were much smaller than the ones produced by the pure colonies. This may be due to lesser quantities of the antibacterial substance being present or different diffusion patterns produced by the filter paper. The pH of the broth was 7.5 before being applied to the filter paper. This demonstrated that the inhibition of the bacteria was not due to an improper pH. Some type of antibiotic may have been present and caused the inhibition.

The antibiotics from all three bacterial isolates were fairly specific for X. c. pelargonii in vitro. Clearing zones one half the normal size were produced with Xanthomonas campestris pv. campestris and Xanthomonas campestris pv. vesicatoria. No clearing zones were observed when Pseudomonas syringae pv. tomato and Corynebacterium

Table 1.

Zone of inhibition size in vitro produced by antagonistic bacteria when overlayed with *Xanthomonas campestris* pv. *pelargonii*

Isolate	Average zone size in cm
Pseudomonas fluorescens	4.5[1]
P. fluorscens	4.3
Alcaligenes faecalis	4.0
Acinetobacter lwoffi	3.7
A. lwoffi	3.7

1. Average diameter of the clearing zones produced by the antagonistic bacteria. Average of 5 replicates $LSD_{.05} = 0.8$

michiganense were used in overlays. The antibiotics also had no effect on their producing strains or the other two antibiotic producing bacteria.

In Vivo Tests

Bacterial wilt was reduced by spraying inoculated geraniums with the biocontrol bacteria. Of the three biocontrol bacteria, *Pseudomonas fluorescens* provided significantly better control over the other biocontrol bacteria (Table 2). Bacterial wilt was reduced but not completely controlled with this biocontrol agent. Artifically high levels of *X. c. pelargonii* inoculum were used in this study which may have unrealistically biased the results. The other two bacteria provided some control but not to the level of the *P. fluorescens*.

Attempts have been made at repeating these results. A more representative level of *X. c. pelargonii* inoculum have been applied to plants to determine if control can be otained under these conditions. Environmental conditions to date have hindered the production of good symptoms on control plants but attempts are continuing. With chemical control being unavailable, these biocontrol bacteria may provide the only method of preventing bacterial wilt of geraniums.

Table 2.

Control of Xanthomonas campestris pv. qpelargonii on geraniums using antagonistic bacteria

Isolate	Mean disease rating
Control	3.6 b[1]
Pseudomonas fluorescens	2.9 a
Alcaligenes faecalis	3.3 ab
Acinetobacter lwoffi	3.5 ab

LSD = 0.6 (p=0.05)

1. Mean of seven plants. 1 = no visible infection; 2 = 25% or less leaf area infected; 3 = 50% or less leaf areaa infected; 4 = greater than 50% leaf area infected. Plants were spray inoculated with the antagonistic bacteria followed by X. c. pelargonii. Number followed by the same letter are not significantly different at P = 0.05 using an LSD test.

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