DETECTION OF *PUCCINIA PELARGONII-ZONALIS* AND MANAGEMENT OF GERANIUM RUST IN THE GREENHOUSE

by

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(Under the Direction of James W. Buck)

ABSTRACT

Geranium rust, caused by the fungus *Puccinia pelargonii-zonalis,* can result in unmarketable plants and economic losses to commercial growers. *P. pelargonii-zonalis* is a microcylic, autoecious rust pathogen that infects zonal geranium (*Pelargonium* × *hortorum*) (Doidge 1926; Rytter 1993). Current management of geranium rust includes scouting, use of clean stock plants, and prophylactic applications of fungicides. If rust is discovered, either neighboring plants in a 1-m^2 area are destroyed if disease pressure is low or all plants are destroyed if disease pressure is high. A real-time polymerase chain reaction (qPCR) assay using the specific primer pair GRF and GRust-R2 was developed to detect urediniospore DNA. GRF and GRust-R2 consistently amplified *P. pelargonii-zonalis* DNA at 1 ng and 100 pg in a conventional polymerase chain reaction (PCR) assay and at 1 pg using qPCR, and the primer pair detected 100 urediniospores ml^{-1} using qPCR. Urediniospore lysis by beating with glass beads or by freezing in liquid nitrogen and then grinding for subsequent PCR was affected by the volume of 0.1% Tween 20 used to suspend urediniospores. Volumes of 50-600 µl of Tween 20 resulted in >95% of urediniospores macerated but increasing the volume to 750-900 µl reduced maceration to <40%. Plants with sporulating lesions produced 1,580 urediniospores over a 24-h

period. A linear relationship was observed between atmospheric concentrations of urediniospores and distance from an inoculum source. At greenhouse bench level, 20 urediniospores cm^{-2} were deposited 1.8 m from the source plants producing inoculum after 9 h. The presence of geranium plants reduced movement of urediniospores along the bench. DNA was amplified by qPCR from urediniospores washed from a single inoculated leaf but urediniospores obtained from mixtures of one inoculated leaf among healthy leaves were not detectable using qPCR. Integrated disease management (IDM) strategies, specifically removing senescent flowers and han- watering, impacted urediniospore movement from plant to plant. A combination of no fungicide application, removal of senescent flowers, and hand-watering resulted in more than twice the number of urediniospores (40 urediniospores cm^{-1}) moving among plants than any other combination of IDM treatments ($\leq$ 20 urediniospores cm^{-1}). Therefore, timely discovery of infected geranium plants is essential to limit urediniospore spread in the greenhouse. My results suggest that removal of a 1-m^2 block of plants around an inoculum source may not be sufficient to eliminate all of the potentially contaminated plants.

INDEX WORDS:Rust, urediniospores, real-time PCR, integrated disease management

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DEDICATION

To my parents, for always being my cheerleaders throughout life and never giving up on me when times were tough. Without your love and support, I would not be the woman I am today. Thanks for letting me make my mistakes, so that I could learn from them. To my brother Chris, thanks for being my shoulder to cry on during those incredibly horrible times and my fan during the best of times. I am forever indebted to you for truly being a great brother and a wonderful human being.

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INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The presence of rust on floriculture crops can result in unmarketable plants and large monetary losses to growers. Adequate detection methods and proper management strategies must be implemented in a timely manner to minimize infection and spread of the pathogen. In greenhouses, spread of this disease can occur in a short period of time resulting in either crop loss due to quarantine measures or the need for expensive curative fungicide sprays. The research in this proposal will utilize geranium rust caused by *Puccinia pelargonii-zonalis*, as a model system for the detection of rust spores associated with geranium leaves, and the influence of routine greenhouse management practices on spread of rust in greenhouse production.

LITERATURE REVIEW

In 2009, the floriculture industry was valued at $3.83 billion of which herbaceous annual bedding and flats accounted for 35.7% (USDA 2010). Floriculture crops are considered by the United States Department of Agriculture (USDA) to be foliage plants, flowering plants, cut flowers or bedding and garden plants. Vegetative geranium cuttings used for bedding plants, flats and hanging baskets were valued at $26 million among a 15-state survey of growers (USDA 2010). More than half of U.S. floriculture sales are accredited to varieties of bedding plants such as *Pelargonium* (geraniums), *Impatiens* and *Petunias*; and of those bedding plants.

Wild geranium is thought to have originated in South Africa and there are now approximately 300 *Pelargonium* species and many cultivars that currently exist throughout the world (Strider 1985). Due to interspecific crosses between genera, *Pelargonium* species are grouped into four categories: zonal, ivy, regal, and scented-leaf geraniums. The most cultivated of these groups are the zonal geraniums, which are popular among landscapes and are sold in flats, pots, or hanging baskets. Ivy-leaved geraniums are predominantly used in hanging baskets and window boxes because of the trailing habit of the ivy-like leaves (Oglevee 1985). Regal geraniums are considered the show geraniums with large, brightly colored flowers and elaborate leaf markings (Oglevee 1985). Many of these varieties were secretly bred in the mid-1800s and by the early 1920s Carl Faiss of Germany introduced many of the varieties most popular today (Oglevee 1985). Scented-leaf geraniums are sold based not on flowers, but on the scent of the leaves. Many of these varieties are true *Pelargonium* species and only a select few are hybrids. The four most common scents are lemon, lime, orange and strawberry (Oglevee 1985).

Geranium varieties are bred for aesthetic characteristics such as plant height, flower color, and leaf markings. Zonal geraniums are characterized by their distinct leaf markings and colorful flowers. Prominent primary colors include pink, white, red, or salmon; however, there are varieties available that showcase hues of these primary colors. The red geranium is the most popular among U.S. consumers, which are traditionally planted in cemeteries on Memorial Day (Oglevee 1985).

Zonal Geranium Propagation

Geraniums can be propagated from seed or vegetative cuttings, whichever is more economically advantageous to the grower. For example, two major U.S. growers, Oglevee Ltd.

and Goldsmith Seeds, propagate geraniums from cuttings and seeds, respectively. Growers start by selecting a growing media that has optimal physical, chemical, and biological characteristics (Bethke 1993). Physical characteristics include porosity, bulk density and water holding capability. The bulk density of growing media is determined as media weight per unit volume at a specific moisture content (Bethke 1993). Plant stability is important when transporting and watering geraniums; therefore, stability is dependent on the bulk density to provide that structural support.

Growing media and fertilizer directly influence root zone pH. A pH of 5.8-6.5 is recommended for geranium propagation (Bethke 1993). Biernbaum et al. (1988) observed that low pH (< 5.8) resulted in increased iron and manganese toxicity, resulting in speckling and/or strippling of mature geranium leaves. Typically, manganese toxicity is prevalent in steam pasteurized soil, but with the use of soilless potting media, growers are experiencing manganese toxicity due to a low pH (< 5.0), poor drainage, or by using large quantities of ammonical nitrogen fertilizer (Biamonte et al. 1993). To achieve proper nutrition and pH, a fertilization program utilizes water soluble and controlled release fertilizer with the growing medium. This program allows for optimal nutrient uptake over time and uses less of both fertilizers with timed watering (Biamonte et al. 1993).

Propagating geranium cuttings from stock plants is a popular practice among commercial growers, especially when the majority of the cuttings are certified pathogen-free. Many growers use the terminal leaf end of the geranium stock plant for propagation. The last 2-4 inches are cut and placed in a rooting medium comprised of light soil mixes, inert growing media or rock wool blocks (Rogers 1993). Many growers prefer placing the cuttings directly into cell packs, Jiffy strips (Jiffy; Lorain, OH) or rock wool blocks and then sell those rooted cuttings or transplant into a 4-inch pot for retail sales. Although geraniums root readily, rooting hormones, such as

Hormodin, can be applied to the cuttings (Rogers 1993). Once the cuttings are placed into containers, spacing the plants is important so that the leaves have adequate air flow to reduce the incidence of grey mold (*Botrytis cinerea*). Many growers still utilize mist irrigation for cuttings. When using mist irrigation, root temperature is important because evaporation cools the rooting medium. The recommended temperatures for cuttings is a night temperature of 15°C and bottom heat (root zone) temperature of 21-23°C (Rogers 1993). To achieve rapid rooting, the cutting needs as much light as tolerable; however too much light will result in wilting (Rogers 1993). Stock geranium plants are typically watered three times on the first day of planting with a 10% leachate (minimal water leaching through the soil), which improves root-soil contact and relative humidity, while reducing the temperature around the plant and water stress (Oglevee 1985). Typically, there are no visible symptoms when moderate water stress is occurring; however severe water stress can cause wilting, leaf curl, changes in leaf color, and lower leaf yellowing (Leith and Burger 1993). Once the propagated cutting is transplanted to a 4 inch container (4 weeks), watering is recommended once a day for the next 3 days, and then every other day thereafter. Fertilizers can be applied after 3 days of continuous watering.

Geraniums are also propagated by seeds. Seeds are sown in 21 inch flats that contain a germinating mix that is porous, sterile and free of excess salts, and maintained at 23ºC with with adequate moisture (Oglevee 1985). Seedlings will emerge approximately 7-10 days after sowing. Depending on the grower, the seedlings are transferred to a Jiffy-7 or cell packs or 1½ – 2 inch pots (Oglevee 1985). At this stage a fertilizer is applied (20-20-20 mix at 100 ppm) with irrigation. Once the seedlings are mature, the plants are transplanted into 4 inch pots with a soil media such as peatlite or the Cornell mix (Oglevee 1985). A fungicide drench is usually applied

when transplanting the plug. Irrigation can be applied as hand watering or drip irrigation according to the scale of propagation.

Many irrigation methods can be utilized in geranium propagation. Depending on labor rates, manual watering of geraniums can be advantageous by applying the appropriate amounts of water without waiting for water to soak into the soil mix as with many automated systems. Another popular method of watering is drip irrigation, which employs a supply line routed on the greenhouse bench with weighted drip lines that direct a slow flow of water from the line to the container (Leith and Burger 1993). When developing a watering schedule other factors, such as temperature and light intensity, must be considered for the developmental rate of zonal geranium.

Typically, optimal temperatures for geranium growth in greenhouses range from 18-23°C during daylight hours and should be kept around 13-16°C at night (Olgevee 1985). Differences between day and night temperatures affect stem elongation; as the differences of day temperature minus night temperature (DIF) increases plant height and internode length increases (Erwin 1991; Heins et al. 1989). Growers can control plant height not only by DIF, but also by applying growth retardants such as chlormequat (Cycocel; OHP Inc., Mainland, PA), ancymidol (A-Rest; SePro, Carmel, IN), or daminozide (B-Nine; Fine Americas Inc., Walnut Creek, CA).
The rate of flower bud formation increases as temperatures increase from 10-22°C, but temperatures above 22°C can slow the formation of buds (Erwin and Heins 1993). To prepare stock plants for vegetative cuttings, flower bud development is arrested by applying ethephon (Florel) (Southern Agricultural Insecticides Inc.; Palmetto, FL). Ethephon is composed of ethylene in a commercial formulation that promotes rapid development of axillary shoots (Oglevee 1985).

Light influences the development of geraniums at different growth stages. Photoperiod is not as important to morphological characteristics as light intensity (Erwin and Heins 1993). For example exposure to low irradiance (<500 foot-candles) will reduce branching, while high irradiance (>3000 foot-candles) can cause plant stunting (Erwin and Heins 1993). Plants grown under incandescent light are more likely to develop tall, sparsely branched and lighter green. Plants grown under fluorescent lamps appear shorter, more branched and darker green. To achieve the desirable compact and stocky growth, light intensities of $\geq$ 2,000 foot-candles must be maintained.

Floriculture Diseases

All economically important floriculture crops can have problems with diseases when environmental conditions are conducive. Diseases such as gray mold (*Botrytis cinerea*), root rots (*Pythium* spp., *Phytophthora* spp., *Rhizoctonia* spp.), and viruses (Impatiens Necrotic Spot Virus, Tomato Spotted Wilt Virus) have a broad host range among floriculture crops. Other diseases such as bacterial vascular wilts (*Xanthomonas campestris, Ralstonia solanacearum*) and rusts (*Puccinia* spp.) have a more narrow host range. Many floriculture plants have recurrent problems with rust pathogens (Table 1.1). Rust diseases are named for the rust-colored pustules comprised of massive amounts of urediniospores that develop on the host foliage. More than 125 species of rust causing fungi occur on 56 ornamental crops (Chase 2002).

Rusts (Mycota: Basidiomycetes) are classified as autoecious (single host) or heterecious (multiple hosts) obligate parasites that can have up to 5 spore stages in their life cycle (Hiratsuka and Sato 1982). In a generalized rust life cycle, the spermagonial (stage 0) and aecial (stage I) stages would be present on an alternate host. The aeciospores are disseminated by air or other means onto the

primary host, which then develops uredia and urediniospores. The uredinial stage (II) is the asexual, repetitive stage that infects the same plants or other plants within a field or greenhouse. When temperatures decrease, the telial stage (III) can develop and teliospores are formed on plant tissue. Teliospores are overwintering structures produced by the fungus to endure harsh conditions. Once temperature and environmental conditions become conducive, teliospores germinate to form basidia and basidiospores (basidial stage; IV). The basidiospores are disseminated by wind or rain splash and land on the alternate host to perpetuate the cycle to repeat again.

Geranium rust caused by *Puccinia pelargonii-zonalis* Doidge, was first described on *Pelargonium spp.* in South Africa by Doidge in 1926 (Doidge 1926). Geranium rust then spread to Europe, New Zealand and Australia presumably on infected plants. Geranium rust was first reported in the U.S. in 1967 in New York followed by many other states during the 1970's (Dimock et al. 1967; Nichols and Forer 1972; Wehlburg 1970). Currently, geranium rust is endemic in California and considered a quarantine pathogen in Ohio (Ohio Dept. Ag., 2005). *P. pelargonii-zonalis* (Order Pucciniales) is a microcyclic, autoecious rust with an asexual stage and a rarely observed sexual stage. The asexual stage consists of the production of brown urediniospores within geranium leaf tissue. As pustules erupt and lesions age a pattern of concentric brown rings occurs (Rytter 1993; Fig. 1.1A). Urediniospores are ovate, light brown, thin-walled, and consist of two equatorial germ pores that can give rise to only one germ tube (Strider 1985). Rare occurrence of the sexual stage results in teliospore production. The optimal temperature range needed for spore germination is speculated to be 16-21°C, while temperatures higher than 25°C inhibited spore germination (Harwood and Raabe 1979). Urediniospore germination occurs in 2-4 h in the presence of moisture. Appressoria usually form 5-6 h after

inoculation and penetration occurs through the stomata on upper and lower leaf surfaces (Strider 1985).

Signs of infection first appear 7-10 days post-inoculation as small white flecks, commonly on the upper surface of the leaf. White pustules initially form on the underside of the leaf (Fig. 1.1A) (McCoy 1975; Strider 1985). As pustules enlarge the plant epidermis ruptures releasing the reddish-brown urediniospores (Fig.1.1B) approximately 10-14 days post-inoculation (McCoy 1975) depending on environmental conditions and cultivar (Buck 2007). Infected leaves can become chlorotic (Fig. 1.1C) and eventually defoliate. Older lesions are often characterized by a halo of green host tissue in the dying leaves.

Integrated Pest Management

Integrated pest management (IPM) is broadly interpreted as the use of multiple tactics to control pests of different classes by use of economical and ecological means (Ehler 2006). IPM efforts in the early 1960s were largely focused on insect pests and the idea of reducing insecticides use. The need for reducing insecticides was due to the development of resistance in target populations and resurgence of primary pests, the decline of natural enemies' populations, an increase populations of secondary pests, and the potential effects on non-target organisms and humans (Ehler 2006). By the 1970s, many disciplines of agriculture in the U.S. were focusing on an integrated approach to managing cropping systems, instead of using one management practice (i.e. chemical management).

IPM can be implemented with far greater precision in greenhouses than in a field cropping systems because a greenhouse is a contained, semi-controlled environment. Greenhouses are constructed of scaffolding covered by materials including plastic, sheet glass or fiber glass, which

are designed to capture the maximum amount of sunlight and heat (Jarvis 1992). Environmental conditions, including light quantity and quality, temperature and humidity can be manipulated. The greenhouse environment consists of warm, humid climates that are ideal for many insect pests and plant pathogens (Jarvis 1992). IPM strategies to slow or eliminate disease spread should incorporate a rotation of different fungicide chemistries at optimum timing in combination with scouting, use of resistant cultivars (when available), sanitation, drip or hand irrigation, adequate air circulation and use of certified stock plants. In floriculture production, resistant cultivars are not widely available because the plants are primarily bred for horticultural characteristics, such as flower color or foliage.

A study conducted by Buck (2007) on commercial geranium varieties of two large producers, revealed six varieties that had > 10% of infected leaves (Orbit Glow, Patriot Cherry Rose, Candy White Splash Ice, Patriot Bright Pink, Candy Rose Splash, and Stardom Red) and one variety (PAC Fox) with no infected leaves when inoculated with a mixture of two *P. pelargonii-zonalis* isolates. Although there are cultivars with differing levels of resistance to rust available, consumer demand determines the varieties propagated, and, therefore, susceptible varieties will still be propagated and sold.

Disease prevention through sanitation and pathogen exclusion is important cultural control steps in the management of plant pathogens in the pre-planting phase. Pathogen exclusion begins with the use of certified or culture-indexed stock. Specifically in geranium propagation, certified cultured indexed stock (pathogen free) should only be used for vegetative cuttings (Dreistadt 2001; O'Donovan 1993; Olgevee 1985). Sterilization methods, such as using a common disinfectant (e.g. sodium hypochlorite), are routinely used to disinfest benches, tools, and containers, while soil is usually steam sterilized (Dreistadt 2001; Jarvis 1992). However, disease

management strategies can aid in dispersal of inoculum throughout greenhouses. Increased movement of inoculum throughout the greenhouse by grower activity can negatively affect management strategies and or eradication steps. *B. cinerea* causes blight on stems, leaves and flowers of ornamental crops (Hausbeck and Moorman 1996) and peak atmospheric conidial concentrations of *B. cinerea* increased to >50 m^{-3}/h^{-1} when growers were either planting, placing geranium cuttings on benches, irrigating, fertilizing or spraying pesticides (Hausbeck and Pennypacker 1991). Watering poinsettias altered the relative humidity in greenhouses, which caused an increase in atmospheric conidial concentrations of *Odium* sp. 3 h after watering (Byrne et al. 2000). A large amount of conidia were collected (>100 conidia m^{-3}) in one hour increments indicating conidial release events (CRE) had occurred due to the altering relative humidity (RH) in the greenhouse after irrigation. When temperatures exceeded 25ºC for more than 20 d, CRE were reduced by 75-80% compared to previous months. Therefore, environmental and grower activity can cause movement and release of spores throughout the greenhouse that can influence the amount of disease present.

Fungicides

Fungicides are an important component of an IPM program in ornamental cropping systems. Unlike agricultural crops, ornamental plants are purchased primarily for aesthetics; therefore, signs and symptoms of disease are detrimental and can reduce profits. Fungicide applications must also take into consideration problems with residues that reduce aesthetic qualities. Current fungicides labeled for *P. pelargonii-zonalis* are listed in Table 1.2. Rotation of fungicides with different modes of action is important to minimize development of fungicide resistance in target populations can be minimized. Jeffers and Luszcz (1998) reported

excellent control of geranium rust control with two broad spectrum protectants (chlorothalonil and mancozeb) as well as with a demethylation inhibitor (myclobutanil) on artificially inoculated plants. Mueller et al. (2004) reported that azoxystrobin, chlorothalonil, myclobutanil and triadimefon were effective against *P. pelargonii-zonalis* when applied 5 days, 1 day, and 1 h before inoculation. Propiconazole, azoxystrobin, myclobutanil and triadimefon were effective when applied 1 to 7 days post-inoculation. These studies have closely evaluated the importance of application timing; however, the most effective application method (dips, sprays, drench) must be determined empirically.

Quarantine

The introduction of exotic pests and pathogens to new geographical locales often occurs via commercial trade. World-wide movement of ornamental plants increases the potential for introduction of new plant pathogens. Quarantines and eradication measures have had mixed success in eliminating invasive species from the U.S. Limited success in quarantine and eradication with many of the rust pathogens, is primarily due to the easy dispersal of spores to other plants (Wise et al. 2004). Rusts such as gladiolus rust (*Uromyces transversalis*), chrysanthemum white rust (*Puccinia horiana*), and geranium rust (*P. pelargonii-zonalis*) are significant quarantine pests. Imported commodities into U.S. are carefully monitored by APHIS (Schumann 1991) for the presence of these fungi.

Gladiolus rust was first discovered in Hawaii on cut flowers from Manatee County, FL, in 2006 (APHIS 2007). This disease was also reported on a gladiolus farm in Hendry County, FL, and at three residential areas in San Diego County, CA. All infected plant materials were destroyed to eradicate the pathogen; however, the threat of infected plant material entering the

country via commercial propagative material from Central and South American countries still exists (APHIS 2007). Eradication efforts ultimately were proven successful in eliminating the pathogen from commercial farms in Florida.

Greenhouses with rust-infected plant material can result in implementation of federal or state quarantine measures. To date, geranium rust is still a quarantined pathogen in Ohio (ODA 2005); however, *P. pelargonii-zonalis* is endemic in California where landscape geraniums are present year-round. Ohio state law requires a certificate of pathogen-free cuttings or plants imported from California (ODA 2005). Ohio has the majority of the U.S. geranium producers with an estimated whole-sale value of $405,000 for geranium cuttings in 2007 (USDA 2008). Daylily rust (*Puccinia hemerocallidis* Thüm) and soybean rust (*Phakopsora pachyrhizi* Sydow) are also examples of pathogens that failed to be eradicated from the U.S. The constant movement of daylilies between hobbyists hindered the eradication efforts. Additionally the temperate climate in the Southeastern U.S, specifically Florida, which allows for year-round daylily production and associated rust have hampered eradication efforts. Soybean rust was not eradicated because of a broad host range, which includes many weeds. For example, in the Southeastern U.S., kudzu is a ubiquitous weed that can become infected by this fungus and thereby act as a source of inoculum. Of these examples, soybean rust has been monitored in fields by real-time polymerase chain reaction (qPCR) assays (Frederick et al. 2002) that can be a valuable tool to aide in the timing of fungicide applications.

Molecular Diagnostic Techniques

Molecular techniques are becoming popular diagnostic tools for detection of various plant pathogenic organisms (Martin et al. 2000; Mumford et al. 2006; Schaad and Frederick 2002).

Puchta and Sanger (1989) first reported the detection of viroid RNA by the use of conventional polymerase chain reaction (PCR). Many more molecular detection methods by conventional PCR, especially for viroids and phytoplasmas, have since been published (Mumford et al. 2006). Throughout the 1990's conventional PCR coupled with sequence analysis and southern hybridization were not quickly adopted as diagnostic tools because these tests were labor intensive and took several days for results (Mumford et al. 2006). However, this changed with the design of fungal-specific internal transcribe region (ITS) primers (Gardes and Bruns 1993; Redecker 2000; White et al. 1990), a better understanding of phylogenetics of fungi (Berbee and Taylor 1999; Wingfield et al. 2004), and an increase in available fungal sequence databases; all of which helped increase the effectiveness and efficiency of molecular diagnostics.

The advent of molecular diagnostics began in the early 1990's with the development of Taqman® chemistry (Applied Biosystems; Foster City, CA) (Holland et al. 1991; Mumford et al. 2006) and portable real-time thermocyclers (Schaad and Frederick 2002). The major advantage of utilizing real-time PCR as an accurate and high throughput test is the ability to quantify target DNA in plant tissue, soil, and water or air samples. Real-time PCR can detect multiple templates by using multiple primers in one mixture (multiplexing) with different fluorescent probes. This approach is beneficial for detecting multiple pathogens in field samples that may be present in a single cropping system (Schaad and Frederick 2002).

Real-time PCR (qPCR) has been used to distinguish species of fungal pathogens and to monitor inocula spread in soybean and wheat. Soybean rust is caused by *Phakopsora meibomiae* or the more devastating *P. pachyrhizi*, which also has a larger host range (Frederick et al. 2002). Monitoring inocula spread and species differentiation are based on qPCR assays. The fungus was collected in rain samples at 124 National Atmospheric Deposition Program/National Trends

Network sites (NADP/NTN) and then subjected to qPCR (Barnes et al. 2006; Krupa et al. 2006). Soybean rust has been monitored in fields by qPCR assays (Frederick et al. 2002), which has improved fungicide timing.

In wheat cropping systems, *Puccinia graminis*, *P. striiformis* and *P. triticina* can infect wheat at the same time; however, *P. graminis* and *P. striiformis* have a very broad host range that includes many wild grass species, which can impact disease management strategies (Leonard and Szabo 2005; Stubbs 1985). Real-time PCR was used to distinguish these pathogens with DNA detection levels ≤ 1.0 pg, which is equivalent to the DNA in less than 5 urediniospores (Barnes and Szabo 2007). The ability to rapidly detect fungal DNA with qPCR can be utilized to better manage fungicide applications or detect potentially damaging rust pathogens in greenhouse cropping systems.

Research objective

There is little knowledge of geranium rust epidemiology, but management strategies include eradicating plants around disease foci. Because many of the vegetative cuttings are being imported from Central and South America and some parts of California, which P. pelargonii-zonalis is endemic, the potential for accidental introduction of urediniospores into growers greenhouses in the Southeastern U.S. is plausible. Therefore, determining the ureidniospore production and dispersal throughout a greenhouse needs to be addressed to determine if this pathogen follows a typical epidemiological model, which can provide a better recommendation of eradication zones. Furthermore, molecular detection of asymptomatic plant material would aid in reducing accidental introduction of this pathogen in greenhouses. By using qPCR, the presence

of urediniospores can be detected before an epidemic occurs. However, once introduced into the greenhouse, which grower activities may move any inoculum within the greenhouse.

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Table 1.1 Examples of rust disease caused by pathogens on floriculture crops.

Host Plant(s)	**Fungus**
Chrysanthemum	*Puccinia horiana*
Snapdragon	*Puccinia antirrhini*
Gladiolus	*Uromyces transversalis*
Daylily	*Puccinia hemerocallidis*
Begonia	*Pucciniastrum boehmeriae*
Daisy	*Puccinia distincta*

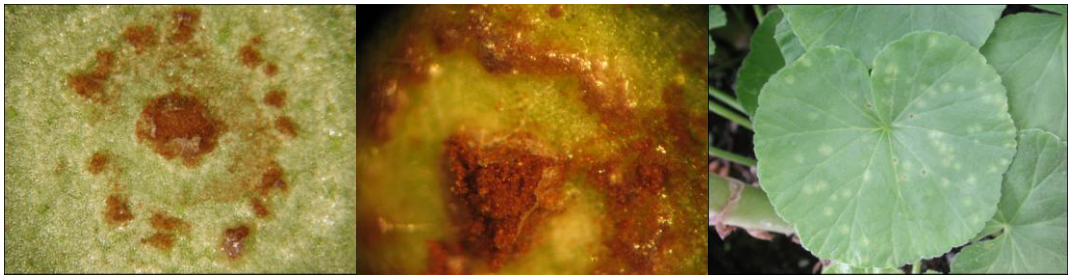
Table 1.2. Fungicides used for control of *P. pelargonii-zonalis* (**Williams-Woodward 2008**).

Fungicide	**Trade Name**	**Mode of Action**
triadimefon	Bayleton, Strike	Systemic
ferbam	Carbamate WDG	Contact
chlorothalonil	Daconil 2787, Daconil Ultrex, Thalonil	Contact
mancozeb	Dithane, Fore, Mancozeb, Protect T/O	Contact
thiophanate methyl+mancozeb	Duosan, Zyban	Contact and Systemic
flutolanil	Contrast	Contact
myclobutanil	Eagle, Systhane	Systemic
azoxystrobin	Heritage	Systemic
chlorothalonil+thiophanate methyl	Spectro 90	Contact and Systemic

FIGURE 1.1

SIGNS AND SYMPTOMS OF GERANIUM RUST ON ZONAL GERANIUM. (A) GERANIUM RUST PUSTULE ON THE UNDERSIDE OF A ZONAL GERANIUM LEAF. (B) UREDINIOSPORES ERUPTING FROM A PUSTULE. (C) CHLOROTIC SPOTS CAUSED BY THE RUST ON THE TOP OF LEAVES.

A B C



CHAPTER 2

DETECTION OF *PUCCINIA PELARGONII-ZONALIS* USING REAL-TIME PCR

To be submitted to *Plant Disease*.

Abstract

The presence of geranium rust (*Puccinia pelargonii-zonalis*) in commercial greenhouses can result in unmarketable plants and large financial losses. The purpose of this research was to develop a real-time polymerase chain reaction (PCR) assay for rapid detection of *P. pelargonii-zonalis* urediniospores on greenhouse-grown geraniums. The primers GRF and GRust-R2 were designed using ITS sequence data from three isolates of *P. pelargonii-zonalis*. GRF and GRust-R2 amplified a 131-bp product from each isolate of *P. pelargonii-zonalis* but did not amplify product from12 other rust fungi or four non-rust, plant pathogenic fungi. *P. pelargonii-zonalis* DNA was amplified consistently from solutions with 1 ng and 100 pg ml^{-1} of DNA in conventional PCR and at 1 pg ml^{-1} using real-time PCR. The detection threshold was 10^2 urediniospores ml^{-1} for real-time PCR and 10^4 urediniospores ml^{-1} for conventional PCR. Urediniospores were lysed by beating with glass beads or freezing in liquid nitrogen. These techniques resulted in amplified DNA by conventional PCR from suspensions with 10^5 and 10^4 urediniospores ml^{-1}, respectively. The volume of Tween 20 used in the bead beating technique affected urediniospore lysis. All urediniospores were lysed and DNA subsequently was amplified using 50-300 µl of Tween 20. Lysis of urediniospores was least in volumes greater than 700 µl and DNA was not amplified. Leaf washes were successful in liberating urediniospores from leaf surfaces. DNA was amplified by real-time PCR from urediniospores washed from a single inoculated leaf, but urediniospores obtained from combinations of one inoculated leaf with 5, 10, 25, and 50 healthy leaves were not detectable. Early detection of *P. pelargonii-zonalis* using real-time PCR has the potential to allow the implementation of more timely strategies for disease management in commercial settings.

Key words: urediniospores, primers, ITS, rust

Introduction

In 2009, the floriculture industry in the USA was valued at $3.83 billion, and 35.7% of this was accounted for by herbaceous annual bedding plants (USDA 2010). Vegetative geranium cuttings used to produce bedding plants, which are sold as flats or hanging baskets were valued at $26 million among a 15-state survey of growers (USDA 2010). The presence of geranium rust, caused by *Puccinia pelargonii-zonalis* Doidge, in commercial greenhouses can result in unmarketable plants and major economic losses. *P. pelargonii-zonalis* is a microcyclic, autoecious rust with an asexual stage consisting of reddish-brown urediniospores. Signs of infection first appear 7-10 days post-infection as small chlorotic flecks commonly on the adaxial surface of the leaf while white lesions initially form on the abaxial surface of the leaf (McCoy 1975; Strider 1985). As lesions enlarge, the epidermis ruptures and releases urediniospores approximately 10-14 days after initial infection (McCoy 1975)—depending on environmental conditions and geranium cultivar (Buck 2007).

The first report of geranium rust in the USA occurred in 1967 in New York followed by occurrences in many other states during the 1970s (Dimock et al. 1967; Nichols and Forer 1972; Wehlburg 1970). Currently, geranium rust is endemic in California and is considered a quarantine pathogen in Ohio; any geranium plant material shipped from California to Ohio must be certified disease-free (Ohio Dept. Ag. 2010). Occasional outbreaks also have occurred in the southeastern USA (Jeffers 1998). Unintentional introductions of exotic fungus pathogens, including chrysanthemum white rust and gladiolus rust, are of major concern to growers of ornamental plants in the USA (APHIS 2008 and 2009). Mandatory federal quarantine and eradication measures to eliminate these fungi are expensive and occasionally fail. This occurred with daylily rust; the federal quarantine in the USA eventually failed in part due to rapid,

widespread movement of infected plant material that resulted in dissemination and establishment of the fungus over a large geographical area (Wise et al. 2004). In 2006, 878 million units of unrooted plant cuttings for propagation of annual bedding plants were imported to the U.S. from Costa Rica, Guatemala, and Mexico (USDA 2007). With such a large amount of propagative material being imported, phytosanitary issues are of paramount concern. Screening for plants infected by *P. pelargonii-zonalis* can be difficult because the initial stages of infection do not result in symptoms. A molecular assay to detect this pathogen in asymptomatic tissues or pustules would be helpful for determining if the pathogen was present in large quantities of plants or planting material.

Molecular tools such as the real-time polymerase reaction (PCR) using pathogen-specific primer pairs can be used for rapid detection of quarantine-significant rust fungi and to monitor inoculum movement. Real-time PCR detection assays have been reported for soybean rust, cereal rusts, chrysanthemum white rust, and seed-borne pathogens (Barnes and Szabo 2007; Barnes et al. 2009; Calderon et al. 2001; Chilvers et al. 2007; Coolong et al. 2008; Frederick et al. 2002; Guillemet et al. 2004; Pedley 2009; Williams et al. 2001). With soybean and cereal rusts, real-time PCR is used on field samples to monitor inoculum levels and discriminate between species that differ in their ability to damage crops. For example, with soybean rust, real-time PCR can be used to determine if *Phakopsora meibomiae* or the more problematic *Ph. pachyrhizi* is present (Frederick et al. 2002). PCR also can be used to distinguish between rust and the early stages of bacterial pustule, caused by *Xanthomonas axonopodis* pv. *glycines*, which can mimic soybean rust symptoms (Frederick et al. 2002). Specific primers, Ppm1/Ppa2 and Ppm1/Pme2, were designed for discrimination and confirmation tests that could be conducted on infected soybean leaves in 5 h. In wheat cropping systems, *P. graminis*, *P. striiformis*, and *P. triticina* can infect wheat at the

same time; however, *P. graminis* and *P. striiformis* have a broad host range that includes many wild grass species that can impact management strategies (Leonard and Szabo 2005; Stubbs 1985). Real-time PCR was used to differentiate between these fungi with DNA detection thresholds of ≤ 1.0 pg (Barnes and Szabo 2007), which is theoretically equivalent to the DNA in less than five urediniospores (Backlund and Szabo 1993). With this rapid approach to pathogen confirmation, management strategies can be specifically aimed at reducing the amount of inoculum within a field and the spread of the pathogen to neighboring fields. Real-time PCR detection assays are not available to monitor most quarantine-significant rusts ornamental plants with the exception of chrysanthemum white rust (*Puccinia horiana* Henn.) (Pedley 2009). Specific *P. horiana* ITS region primers detected 1 ng ml^{-1} and 1 pg ml^{-1} of template DNA from infected plant tissue in conventional and real-time PCR, respectively.

A real-time PCR detection assay for *P. pelargonii-zonalis* would enable a rapid, precise confirmation of the pathogen on propagative plant material coming into the country. Currently, inspection of plant material is solely based on disease symptoms and/or signs; thus, latent infections are not detected. The objective of this study was to develop species-specific primers for use in a real-time PCR assay to detect *P. pelargonii-zonalis*.

Materials and Methods

Maintenance of plants and fungi. A local isolate of *P. pelargonii-zonalis* originally recovered from diseased plants in Georgia was maintained on zonal geranium plants (*Pelargonium ×hortorum* cv. Maverick Red) growing in Metro-Mix 360 soilless potting medium (The Scotts Co.; Marysville, OH) in 19-L containers in a greenhouse with average night and day temperatures of 19 and 21°C, respectively (Buck 2007). Plants were grown from vegetative

cuttings obtained from a local commercial producer. Urediniospores were collected from actively-sporulating 3-wk-old pustules using a vacuum spore collector, immediately suspended in 0.05% Tween 20 (J. T. Baker, Phillipsburg, NJ), and filtered through five layers of sterile cheesecloth (Mueller and Buck 2003). Urediniospores of two additional isolates of *P. pelargonii-zonalis* recovered from diseased plants growing in California and South Carolina (isolates Plz-California-1 and Plz-South Carolina-1, respectively) were stored in 95% EtOH in a screw-top container. With the exception of primer design, all other experiments were conducted using the Georgia isolate of *P. pelargonii-zonalis*.

DNA extraction, amplification, and ITS sequence analysis. A suspension of 10^5 urediniospores ml^{-1} of each of the three isolates of *P. pelargonii-zonalis* was placed in an 1-ml screw top tube (Sarstedt, Inc; Newton, NC) with 150 µl of 0.1% Tween 20 and approximately 100 mg zirconia/silica beads (0.5 mm; BioSpec Products, Inc.; Bartlesville, OK). Urediniospores were subjected to bead beating for 5 min 28 Hz (Qiagen Tissuelyser II;Valencia, CA) to mechanically lyse urediniospores. After bead beating, a Qiagen DNeasy Plant Mini Kit was used to isolate genomic DNA according to manufacturer's instructions. A portion of the ITS region was amplified using the primer pair ITS1-F and ITS6R2, specific for basidiomycetes (Gardes and Bruns 1993; Wingfield et al., 2004). Amplification used the following thermocycler conditions: 95ºC for 5 min, followed by denaturation at 95ºC for 30 s, annealing at 50ºC for 30 s, and elongation at 72ºC for 60 s then a final elongation step at 72ºC for 120 s. The PCR product was separated on a 1% agarose gel containing ethidium bromide in 1× TAE buffer, and the amplicon was excised and purified using an Omega E.Z.N.A Gel Extraction Kit (Omega Bio-Tek; Norcross, GA). The 427-bp product was cloned into pCR®8/GW/TOPO vector using a TOPO TA Cloning Kit (Invitrogen; Carlsbad, CA) and transformed into TOPO 10 chemically competent *E.*

coli according to manufacturer's instructions. Plasmid DNA from 10 clones from each isolate of *P. pelargonii-zonalis* was extracted using a Qiagen Plasmid Mini Kit (Valencia, CA) according to manufacturer's instructions. The selected plasmid DNA was sequenced with an Applied Biosystems 3730xl 96-capillary DNA Analyzer (IBL Laboratories; Athens, GA) using primers supplied in the TOPO TA cloning kit. Sequences were aligned using ClustalW, and a consensus sequence was used in a BLAST search of the NCBI nucleotide database.

Primer design and specificity. A primer pair, GRF (5' – TTA TAC TTG TGT TGA TTC – 3') and GRust-R2 (5' – TGT CTT TTT TAT AAG TGC – 3'), was designed for specific detection of *P. pelargonii-zonalis*. Primer pairs were designed from the ITS sequence based on non-homologous regions of other fungal ITS sequences (*P. obscura*; 89% similarity) obtained in a BLAST search (NCBI). The oligonucleotide sequences were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Using plasmid DNA with an ITS sequence insert from *P. pelargonii-zonalis*, the primers were optimized using a BioRad gradient thermocycler (Hercules, CA) with the following conditions: 1 cycle at 95ºC for 5 min; 30 cycles at 95ºC for 30 s with annealing temperatures of 42.7ºC, 43.6ºC, 45.5ºC, 47.9ºC, 50.6ºC, 53.0ºC, and 56.5ºC) for 30 s and 72ºC for 60 s; and a final extension step of 72ºC for 2 min. An annealing temperature of 53ºC was chosen based on intensity of the amplicon at the optimal temperature that yielded the brighter band in the agarose gel. PCR was conducted in 20-µl reaction volumes containing 0.625 units of Promega Go Taq Flexi DNA polymerase, 1× of Go Taq Green Buffer, 1 mM of $MgCl_2$, 0.2 mM of dNTPs, 1.25 µM of each primer, and 5 ng of template DNA using an GeneAmp PCR system 9700 thermocycler (ABI; Carlsbad, CA) at the following conditions: one cycle at 95ºC for 120 s, 34 cycles at 95ºC for 30 s, 53ºC for 30 s, 72ºC 120 s, and one cycle at 72ºC for 5 min.

The primer pair was tested twice for specificity against DNA from 12 rust fungi, two other basidiomycete fungi, and two ascomycete fungi (Table 2.1). Water was used as a negative control, and *P. pelargonii-zonalis* template DNA was used as a positive control. Three replicates of serial dilutions (1 ng, 100 pg, 10 pg, 1 pg and 100 fg ml^{-1}) of template *P. pelargonii-zonalis* DNA were used in a sensitivity assay using conventional PCR with the following conditions: one cycle at 95ºC for 120 s, 34 cycles at 95ºC for 30 s, 53ºC for 30 s, 72ºC for 120 s, and one cycle at 72ºC for 5 min.

Urediniospore mechanical lysis assays. Liquid nitrogen and bead beating techniques were evaluated and compared to determine the most effective method to disrupt urediniospores of *P. pelargonii-zonalis* so DNA could be extracted. A serial dilution of urediniospores (10^5 to 10^1 urediniospores ml^{-1}) was used for each lysis technique. For bead-beating, 1 ml of each spore dilution was placed into a 1-ml screw top tube with 150-µl of 0.1% Tween 20 and approximately 100 mg (0.5-mm diameter) zirconia/silica beads. Urediniospores were subjected to bead beating for 5 min at 28 Hz to lyse spores. For the liquid nitrogen technique, a 1-ml aliquot of the urediniospore suspension was added to a 1 ml centrifuge tube and spores were pelleted by centrifugation (14,000 rpm). The urediniospore pellet and 20 ml of liquid nitrogen were combined in a mortar, and a sterile pestle was used to grind the urediniospores. The ground urediniospores were collected in a 50-ml BD Falcon tube by washing the mortar with approximately 10-ml of liquid nitrogen after which the liquid nitrogen was allowed to evaporate. After the bead beating or liquid nitrogen treatments, a Qiagen DNeasy Plant Mini Kit was used to isolate genomic DNA following manufacturer's instructions. The extracted DNA from both techniques was subjected to PCR with the primer pair GRF and GRust-R2 using the previously

described thermocycler conditions, and products were run on a 1% agarose gel with ethidium bromide. The experiment was conducted three times.

The effect of the volume of 0.1% Tween 20 solution on efficiency of the bead beating technique to lyse spores was investigated. A 1-ml aliquot of spore suspension (10^5 urediniospores ml^{-1}) was placed in a 1-ml screw-top tube, and spores were pelleted by centrifugation (14,000 rpm) (Fisher Table Top Centrifuge). The liquid was decanted, and volumes of 0, 50, 150, 300, 450, 600, 750, or 900 µl of 0.1% Tween 20 solution were added to individual tubes. Approximately 100 mg of zirconia/silica beads (0.5 mm) were added to each tube, and spore-bead-Tween 20 mixtures were beaten for 5 min at 28 Hz. Two 10-µl samples from each treatment (50-µl of Tween 20 was added to the 0-µl volume treatment after beating) were observed at 100× magnification, and the numbers of ruptured and intact urediniospores were counted. The treatments were then subjected to DNA extraction using a Qiagen DNeasy Plant Mini Kit. Treatments were subjected to conventional PCR using the primers GRF and GRust-R2, and the previously described thermocycler conditions, and products were separated on a 1% agarose gel. The experiment was conducted twice.

Real-time PCR of DNA obtained from urediniospores. Real-time PCR assays were conducted using a Cepheid SmartCycler (Sunnyvale, CA) in 20-µl reaction volumes. The 20-µl volume reactions contained 2-5 ng ml^{-1} of template DNA, 2× of BioRad Sybr Green, 3.25 µM of the forward (GRF) and reverse primers (GRust-R2), and HPLC-grade water (Fisher Chemical; Pittsburgh, PA) to bring to final volume. All reactions were conducted using the following conditions: 1 cycle of 95ºC for 120 s, 40 cycles of 95ºC for 30 s, 53ºC for 30 s, and 72ºC for 60 s with an amplification threshold set at 15 cycles. Aliquots from a ten-fold serial dilution (10^5 to 10^1 spores ml^{-1}) of urediniospores was subjected to bead-beating using 150 µl of 0.1% Tween 20

solution. DNA was extracted as described previously and then subjected to real-time PCR using the primer pair GRF and GRust-R2. The DNA concentration of each sample was determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Inc.; Wilmington, DE). Data was analyzed statistically using a simple linear regression model (PROC REG; SAS ver. 9.2, 2009). The experiment was conducted three times.

Real-time PCR of DNA obtained from urediniospores washed from leaves. Leaves were collected from healthy cv. Maverick Red geranium plants located in a separate greenhouse not exposed to rust. Two sets of five leaves (average size = 56 cm^2) were inoculated with a different concentration of urediniospore suspension (Trial 1: 5.0×10^5 and 5.0×10^3 urediniospores ml^{-1}; Trial 2: 3.1×10^5 and 3.1×10^3 urediniospores ml^{-1}). Urediniospore suspensions were applied using a spray bottle until leaves were uniformly wet (~ 1.5 ml/leaf), and then leaves were placed in a laminar flow cabinet for 1 h to dry. For each urediniospore concentration, one inoculated leaf was placed into a 500-ml Erlenmeyer flask with 0, 5, 10, 25, or 50 non-inoculated leaves and 250 ml of 0.1% Tween 20. Leaves were agitated by shaking by hand for 30 s then placed on a table top shaker for 1 h at 120 rpm to dislodge the urediniospores. Leaves were removed, the leaf wash was transferred into a 50-ml BD Falcon centrifuge tube, and urediniospores were pelleted by centrifugation at 14,000 rpm for 5 min. The supernatant was discarded, and the urediniospores were resuspended in 1-ml of 0.1% Tween 20. Each sample was pipetted into a separate well of a BD Falcon (San Jose, CA) 24-well tissue culture plate and urediniospores were enumerated using an inverted microscope (200× magnification). Urediniospores in each sample then were pipetted into a screw-top tube and centrifuged at 14,000 rpm. The supernatant was discarded and the urediniospore pellet was resuspended in 150 µl of 0.1% Tween 20. Cellular contents were mechanically lysed by bead beating and DNA was

extracted and then subjected to real-time PCR using the GRF and GRust-R2 primers as previously described. The experiment was conducted twice.

Results

Primer specificity and sensitivity. Primers GRF and GRust-R2 were designed to amplify a portion of the ITS region of *P. pelargonii-zonalis*. The sequences from the three isolates of *P. pelargonii-zonalis* were similar except for an additional base pair (adenine) located at the 233-bp position of the California isolate (Plz California-1) sequence (Fig. 2.1). An optimal annealing temperature of 53ºC for the primer set was based on band intensity (Fig. 2.2) and was used in both conventional and real-time PCR for all reactions. Increasing the annealing temperature to 54ºC resulted in no amplification by real-time PCR. GRF and Grust-R2 did not produce an amplicon by conventional PCR from the 12 additional rust fungi and four non-rust fungi (Table 2.1). A 131-bp product was amplified using template DNA from the three *P. pelargonii-zonalis* isolates (Fig 2.3). *P. pelargonii-zonalis* DNA was amplified consistently at 1 ng and 100 pg concentrations in conventional PCR and at 1 pg using real-time PCR (melt curve 78ºC). There was a linear relationship between log DNA concentrations and Ct values (P=0.0210; $y = 36 – 0.007x$; $R^2 = 0.73$) (Fig. 2.4). The detection threshold for real-time PCR was 10^2 urediniospores; however, the relationship between urediniospore serial dilutions and Ct values was not linear (P=0.2119; $y = 32 – 7\times10^{-6}x$; $R^2 = 0.45$) (Fig. 2.5).

Effect of urediniospore lysis techniques on efficiency of PCR. DNA was consistently amplified by conventional PCR after urediniospores in suspensions of 10^5 and 10^4 urediniospores ml^{-1} were lysed by beating with glass beads or by grinding after freezing with liquid nitrogen (Fig. 2.6 A and B). However, differences were observed between the two lysis techniques at lower concentrations of urediniospores. The expected DNA amplicon was obtained when spores

in a dilution of 10^3 urediniospores ml^{-1} was beaten with beads but not when ground after freezing in liquid nitrogen. No amplicon was produced from spores in the 10^2 or 10^1 urediniospore concentrations after lysis by either bead beating or liquid nitrogen freezing (Fig 2.6A and B). The volume of 0.1% Tween 20 used in the bead beating procedure significantly affected urediniospore lysis (Fig. 2.7). One hundred percent of the urediniospores were lysed when suspended in 50 to 300 µl of Tween 20, and 95% of the urediniospores samples were lysed when suspended in 450 and 600 µl of Tween 20 (Fig. 2.7). Addition of either 700 or 900 µl of Tween 20 reduced the lysis of urediniospores to below 40% of the total number of spores observed. Using conventional PCR, DNA amplification was observed in samples that contained 50, 150, 300, 450, and 600 µl of 0.1% Tween 20 (data not shown).

Real-time PCR of DNA obtained from urediniospores washed from leaves. The leaf washing protocol I used successfully liberated into suspension the urediniospores inoculated onto geranium leaves. Significantly more urediniospores were washed from leaves sprayed with the 10^5 urediniospore ml^{-1} than with the 10^3 urediniospore ml^{-1} (Fig. 9). The recovery of urediniospores from the 10^5 urediniospore ml^{-1} dilution demonstrated that a substantial amount of urediniospores were recovered off of one inoculated leaf by itself. When an inoculated leaf was mixed with 5, 10, 25, and 50 non-inoculated leaves, there was a steep decline in the number of liberated urediniospores in leaf-wash suspensions (Fig. 2.8). When inoculated with 10^3 urediniospore suspension, all treatments (1 leaf alone, 1 in 5, 1 in 10, 1 in 25, and 1 in 50 non-inoculated leaves) resulted in ≤ 100 urediniospores ml^{-1} recovered (Fig. 2.8). When leaves that were inoculated with 10^5 urediniospores ml^{-1} were subjected to real-time PCR, the DNA sample from a single inoculated leaf was amplified in both trials while the other samples were not amplified (Table 2.2). DNA extracted from urediniospores liberated from leaves inoculated with

10^3 urediniospore ml^{-1} (25-100 urediniospores) was below the threshold for amplification by real-time PCR.

Discussion

Molecular detection assays have been developed and used for many plant pathogens (Barnes and Szabo 2007; Frederick et al. 2002; Schaad and Frederick 2002; Pedley 2009). The oligonucleotide primers designed for this study, GRF and GRust-R2, consistently and specifically amplified a 131-bp portion of the ITS region from three isolates of *P. pelargonii-zonalis* obtained from the Southeastern USA and California. Although there was an 89% similarity between the ITS sequences from *P. pelargonii-zonalis* and *P. obscura*, the primers were specific to *P. pelargonii-zonalis* based on assays with other basidiomycete and ascomycete fungi. Some of the fungi screened are commonly found in the greenhouse or were different species of *Puccinia*. With such specificity, positive Ct values indicated amplification of *P. pelargonii-zonalis* DNA.

The primers GRF and GRust-R2 amplified 1 pg ml^{-1} of *P. pelargonii-zonalis* DNA using real-time PCR, which was ten-fold more sensitive than conventional PCR. Similar results were reported with specific primers designed for detection of *P. horiana* DNA (Pedley 2009). Serial dilutions of template DNA demonstrated a linear relationship between Ct value and DNA concentrations. This linear relationship indicated that reduced DNA concentrations corresponded to higher Ct values and an average threshold of 1 pg ml^{-1} of template DNA was amplified consistently. Although urediniospore concentration and Ct value did not display a linear relationship, there was amplification of *P. pelargonii-zonalis* DNA suggesting the assay may be useful for detection but not quantification. The lack of a linear relationship could be due to urediniospore hydrophobicity, which makes physical disruption of urediniospores a challenge.

Adjusting the volume of 0.1% Tween 20 used to suspend urediniospores prior to disruption by bead beating resulted in >95% efficiency in mechanical lysis of urediniospores, presumably with associated release of cytoplasm without the need to germinate the urediniospores, which has been done with other rust pathogens (Frederick et al. 2002). Extraction of DNA from *Ph. meibomiae* and *Ph. pachyrhizi* consisted of placing urediniospores on petri dishes overnight to allow germination to occur followed by using a mortar and pestle to grind the tissue (Frederick et al. 2002). The formation of germ tubes from urediniospores resulted in a greater surface area of the pathogen and a germ tube cell wall that is thinner than that of the urediniospore. These tissues would be more susceptible to mechanical or physical disruption. The DNA extraction protocol developed for *P. pelargonii-zonalis* in this study can be completed in less than a day. A similar DNA extraction technique using bead beating was reported for samples of airborne spores of *Botrytis squamosa* using a Taqman real-time PCR assay that detected 2-116 conidia m^{-3} (Carisse et al. 2009). However, conidia are easier to lyse than the hydrophobic and thick-walled urediniospores of *P. pelargonii-zonalis*.

The leaf washing procedure I employed was successful at recovering urediniospores from inoculated geranium leaves, and spores subsequently were identified by microscopy and real-time PCR. Leaf washing was most efficient at removing urediniospores from a single inoculated leaf; fewer spores were washed from a mixture of one inoculated leaf combined with 5, 10, 25, and 50 non-inoculated leaves. There was a ten-fold difference in the number of urediniospores collected from one inoculated leaf compared to the combination of inoculated and non-inoculated leaves. Therefore, the possibility that the urediniospores are being intercepted by the leaves during the washing process is likely. Geranium leaves are known to be pubescent, and the bundant amount of leaf-surface trichomes may have retained urediniospores (Oglevee 1985). Since leaf washing

was more efficient at removing urediniospores from a single leaf, detection of urediniospores from a sample of many leaves may be accomplished best by washing leaves individually and then pooling the wash suspensions before real-time PCR. All treatments, except for the single leaf inoculated with 10^5 urediniospores ml^{-1}, were below the detectable real-time PCR threshold of 10^2 urediniospores. Pedley (2009) reported that detection of *P. horiana* only occurred when heavily-infected leaf tissue with visible disease symptoms was used for DNA extraction. Our assay can detect non-germinated urediniospores from asymptomatic plant leaves using qPCR. However, in all treatments involving combinations of inoculated and non-inoculated leaves, urediniospores were detected and enumerated directly by microscopy. This may provide another tool for detecting this plant pathogen on leaf tissue when visible symptoms are below detection thresholds.

Only approximately 10^3 urediniospores were recovered from a single leaf inoculated with a 10^5 urediniospore ml^{-1} suspension. DNA of the lysed urediniospores was amplified in real-time PCR at an average Ct value of 29.57. However, urediniospores that were recovered from all other treatments were not amplified in qPCR, but the numbers recovered were within the detection threshold. Lack of detection by qPCR could be due to the presence of other non-specific DNAs from debris associated with the leaves. We observed insect parts, other fungal spores, and plant cells in the urediniospore suspensions washed from leaves. In contrast, urediniospore suspensions used in the primer pair and threshold assays were prepared with spores collected from lesions and suspended in sterile 0.1% Tween 20 solution; the spore suspensions then were filtered through cheesecloth to eliminate any foreign debris that may have inhibited PCR. Li et al., (2008) reported that a cetyltrimethylammonium bromide (CTAB) extraction technique was more efficient than a DNeasy Plant Kit at extracting DNA from *Ralstonia solanacearum* and removing

PCR inhibitors from infected geranium samples. Microscopy can be used to detect rust urediniospores in leaf washings samples, but the real-time PCR assay must be further optimized to confirm and verify that the urediniospores present in leaf washings are actually those of *P. pelargonii-zonalis*. This assay has a threshold of 10^3 urediniospores ml^{-1}, but there is a zero tolerance for this pathogen in the greenhouse.

Scocco et al., (Chapter 3) observed an average of 23 urediniospores cm^{-2} on leaves sampled from plants directly next to plants with sporulating lesions after five days. This is below the real-time PCR detection threshold for spores washed from leaves, which require 1,000 urediniospores for a positive amplification of *P. pelargonii-zonalis* DNA. Therefore, if the inoculum present on the geranium leaves is low, sampling needs be modifies to include a greater number of leaves per sample. Development of a more rigorous protocol for washing leaves to ensure a high percentage of recovery of the urediniospores present on infected or infested leaves would allow for a more sensitive detection procedure overall.

Real-time PCR assays for pathogen detection and confirmation can improve both management of endemic pathogens and eradication efforts targeting exotic pathogens with zero tolerance. The assay developed in this study can be another tool for diagnostic laboratories, especially for cases in which several rust pathogens may be present and spore morphology is indistinguishable. Using a combination of PCR and microscopic examination, *P. pelargonii-zonalis* urediniospores can be detected when inoculum is present on leaf tissue with little or no signs or symptoms of disease present. Therefore, an enhanced pathogen detection procedure would be a great advantage to geranium growers and should improve management and eradication of the geranium rust pathogen—especially in Ohio where a quarantine still is present.

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FIGURE 2.1

CLUSTALW COMPARISON OF ITS SEQUENCES FROM ISOLATES OF *PUCCINIA PELARGONII-ZONALIS* FROM GEORGIA, SOUTH CAROLINA, AND CALIFORNIA AMPLIFIED WITH ITS1F AND ITS6R2 TO ITS SEQUENCES FROM *P. HEMEROCALLIDIS* AND *P. OBSCURA* ITS REGIONS.

```
GA-plz       -TCAT--GACACTCAAACAGG---TGTACCTTTTGGAATACCAAAAGGTGCAAGGTGCGT 54
CA-plz       -TCAT--GACACTCAAACAGG---TGTACCTTTTGGAATACCAAAAGGTGCAAGGTGCGT 54
SC-plz       -TCAT--GACACTCAAACAGG---TGTACCTTTTGGAATACCAAAAGGTGCAAGGTGCGT 54
Daylily rust ATCATTGAATCTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACA---CCTGT 57
P. obscura   ATCATTGAATCTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACA---CCTGT 57

GA-plz       TCAAAGATTCAATGATTCACTGAATTCTGCAATTCACATTACTTATCACATTTCACTGTG 114
CA-plz       TCAAAGATTCAATGATTCACTGAATTCTGCAATTCACATTACTTATCACATTTCACTGTG 114
SC-plz       TCAAAGATTCAATGATTCACTGAATTCTGCAATTCACATTACTTATCACATTTCACTGTG 114
Daylily rust TTGAGTGTC--ATGA------AAATCTCTCA--TCAAATTAATTTTTGGTGGATGTTGAG 107
P. obscura   TTGAGTGTC--ATGA------AAATCTCTCA--TCAAATTAATTTTTGGTGGATGTTGAG 107

GA-plz       TTCTTCATCGATATGAGAGCCTAGAGATCCATTGTTAAAAGTTATTTTTATAATAATTAA 174
CA-plz       TTCTTCATCGATATGAGAGCCTAGAGATCCATTGTTAAAAGTTATTTTTATAATAATTAA 174
SC-plz       TTCTTCATCGATATGAGAGCCTAGAGATCCATTGCTAAAAGTTATTTTTATAATAATTAA 174
Daylily rust TGCTGCTGTTATCTAGCTCACTTTAAATATAT------AAGTCATTTT----ATGA--AT 155
P. obscura   TGCTGCTGTTATCTAGCTCACTTTAAATATAT------AAGTCTTTTT----CTAATTAT 157

GA-plz       TTTTTACATTCTTGGTTTA-TACTTGTGTTGATTCAAAAAAAAAAAAA-TGAGGTGGGGG 232
CA-plz       TTTTTACATTCTTGGTTTA-TACTTGTGTTGATTCAAAAAAAAAAAAAAATGAGGTGGGGG 233
SC-plz       TTTTTACATTCTTGGTTTA-TACTTGTGTTGATTCAAAAAAAAAAAAA-TGAGGTGGGGG 232
Daylily rust CTTGGATTGACTTGGTGTAATATTT--TTTGAT-CATCAAGGAAAGTAGCAATACTTGCC 212
P.obscura    GTTGGATTGACTTGGTGTAATAATTATTTTGAT-CATCAAGGAAAGTAGCAATACTTGCC 216

GA-plz       GACAATGTAACATAACTGTTACGTATACCTGAGTGCAATGGCACTTATAAAAAAGACACC 292
CA-plz       GACAATGTAACATAACTGTTACGTATACCTGAGTGCAATGGCACTTATAAAAAAGACACC 293
SC-plz       GACAATGTAACATAACTGTTACGTATACCTGAGTGCAATGGCACTTATAAAAAAGACACC 292
Daylily rust AATA-TTTATTTTC---------------------------------------------- 225
P. obscura   AATG-TTTTGGACA---------------------------------------------- 229

GA-plz       ACACAAACAAGCTTTAGGGTATAATTGTATAAAAGGTTCAGAGCCACAATGAAGTGCACT 352
CA-plz       ACACAAACAAGCTTTAGGGTATAATTGTATAAAAGGTTCAGAGCCACAATGAAGTGCACT 353
SC-plz       ACACAAACAAGCTTTAGGGTATAATTGTATAAAAGGTTCAGAGCCACAATGAAGTGCACT 352
Daylily rust ------------------------------------------------------------
P. obscura   ------------------------------------------------------------

GA-plz       CCTTTTGTATGAATAATGATCCTTCTGCAGGTTCACCTACAGAAACCTTGTTACGACTTT 412
CA-plz       CCTTTTGTATGAATAATGATCCTTCTGCAGGTTCACCTACAGAAACCTTGTTACGACTTT 413
SC-plz       CCTTTTGTATGAATAATGATCCTTCTGCAGGTTCACCTACAGAAACCTTGTTACGACTTT 412
Daylily rust ------------------------------------------------------------
P.obscura    ------------------------------------------------------------

GA-plz       TACTTCCTCTAAATGACCAAG 433
CA-plz       TACTTCCTCTAAATGACCAAG 434
SC-plz       TACTTCCTCTAAATGACCAAG 433
Daylily rust ---------------------
P.obscura    ---------------------
```

FIGURE 2.2

ANNEALING TEMPERATURE OPTIMIZATION OF THE PRIMERS GRF AND GRUST-R2 USING *PUCCINIA PELARGONII-ZONALIS* TEMPLATE DNA (LANE 1: 60.2 ºC, LANE 2: 58.6 ºC, LANE 3: 56.5ºC, LANE 4: 53.0ºC, LANE 5: 50.6ºC, LANE 6: 47.9ºC, LANE 7: 45.5ºC, LANE 8: 43.6ºC, LANE 9: 42.7ºC, LANE 10: DNA LADDER)

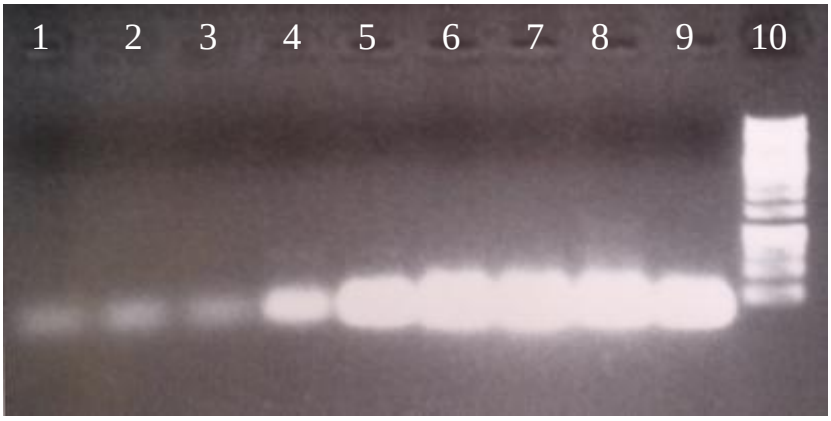
1
2
3
4
5
6
7
8
9
10

FIGURE 2.3

SPECIFICITY OF PRIMER PAIR GRF AND GRUST-R2 FOR PCR AMPLIFICATION OF A 131-BP PRODUCT FROM *PUCCINIA PELARGONII-ZONALIS* DNA. LANE: (1) *USTILAGO MAYDIS*, (2) *BOTRYTIS CINEREA*, (3) *RHIZOCTONIA SOLANI*, (4) *FUSARIUM* SP., (5) *P. SORGHI*, (6) *P. OXALIS*, (7) *P. HEMEROCALLIDIS*, (8) ASTER RUST PATHOGEN, (9) *PUCCINIASTRUM VACCINII*, (10) *P. ANTIRRHINI*, (11) *P. IRIDIS*, (12) *P. STRIIFORMIS*, (13) *P.TRITICINA*, (14) GERANIUM PLANT (15) *P.PELARGONII-ZONALIS* SC ISOLATE, (16) *P. PELARGONII-ZONALIS* CA ISOLATE, (17) *P. PELARGONII-ZONALIS* GA ISOLATE, (18) NEGATIVE CONTROL (NO DNA), AND (19) 100 BP LADDER

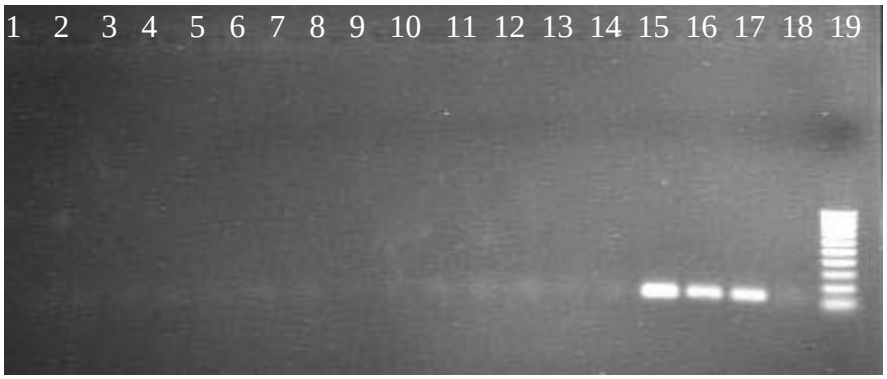
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

FIGURE 2.4

AMPLIFICATION OF TARGET *P. PELARGONII-ZONALIS* DNA CONCENTRATIONS IN REAL-TIME PCR USING PRIMER PAIR GRF AND GRUST-R2 (LOG DNA CONCENTRATION PLOTTED AGAINST CT VALUE)

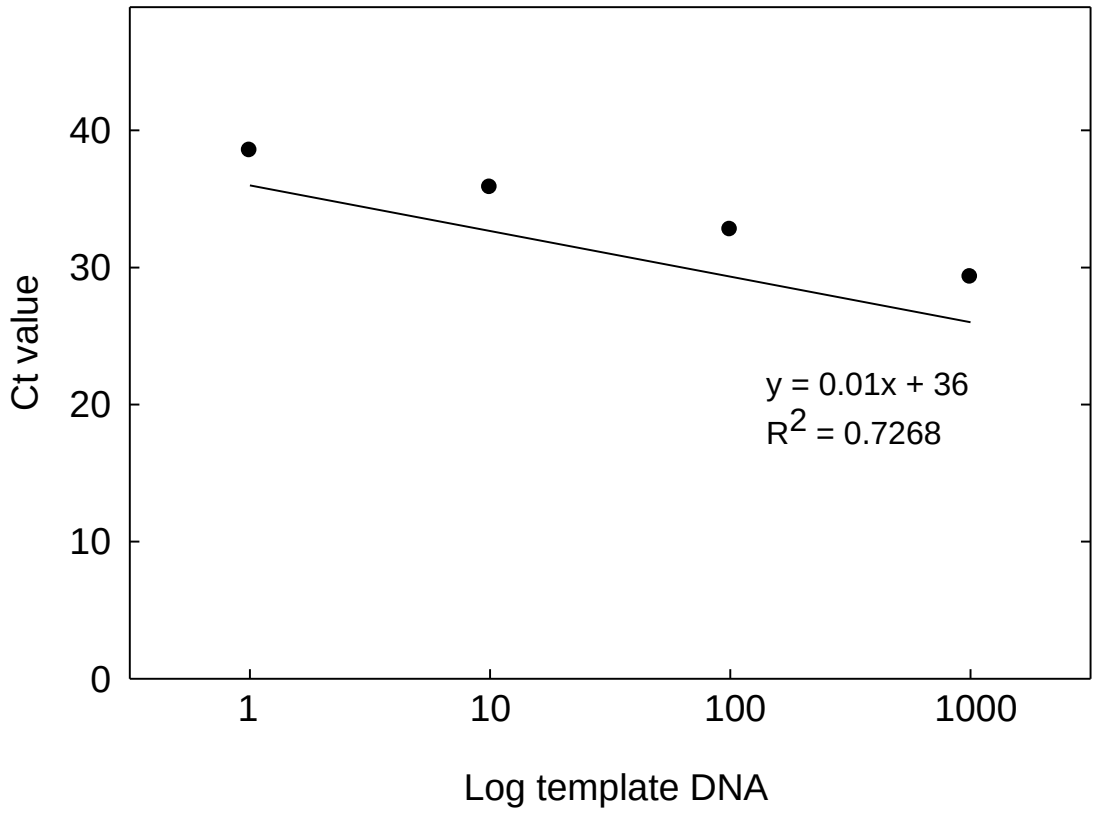
40
30
20
10
0
Ct value
1
10
100
1000
Log template DNA
y = 0.01x + 36
R2 = 0.7268

FIGURE 2.5

DESCENDING CT VALUES INDICATED MORE DNA PRESENT IN SERIAL DILUTIONS OF UREDINIOSPORES ML^{-1} (LOG UREDINOSPORES WERE PLOTTED AGAINST CT VALUES IN RELA-TIME PCR USING PRIMER PAIR GRF AND GRUST-R2)

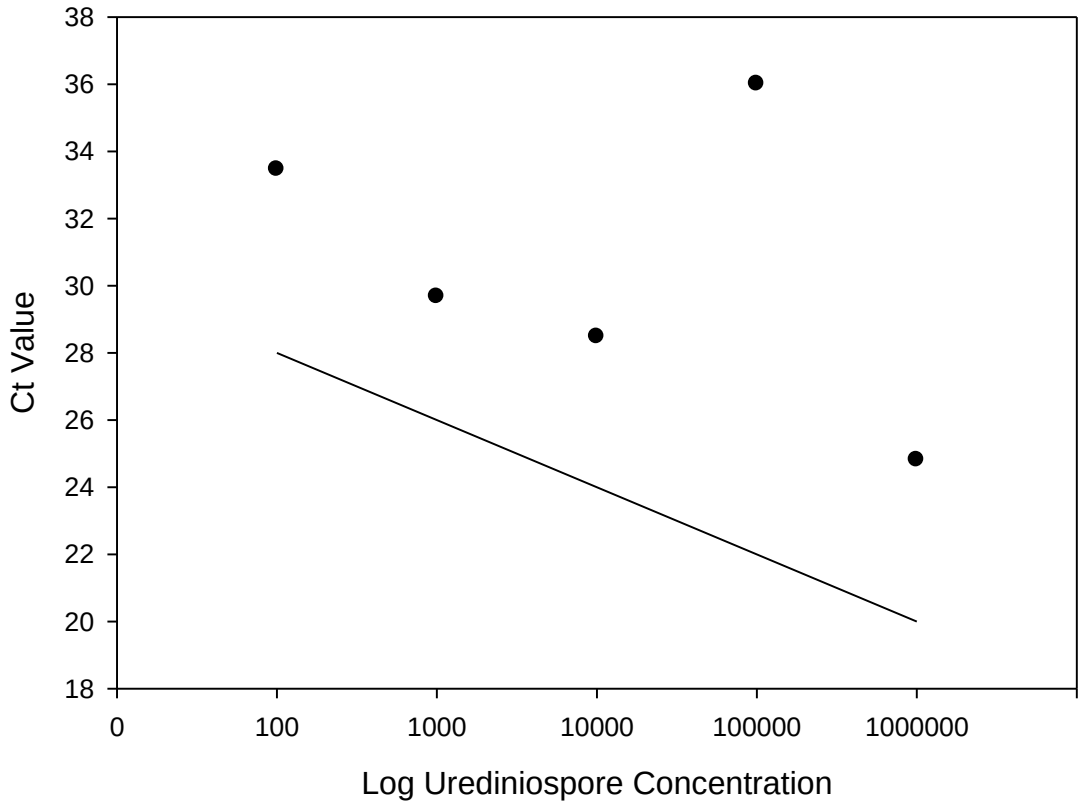
38
36
34
32
30
28
26
24
22
20
18
Ct Value
0
100
1000
10000
100000
1000000
Log Urediniospore Concentration

FIGURE 2.6

PCR PRODUCTS USING PRIMER PAIR GRF AND GRUST-R2 AND DNA FROM *PUCCINIAPELARGONII-ZONALIS* UREDINIOSPORES OBTAINED BY (A) BEAD BEATING OR (B) LIQUID NITROGEN: LANES 1 AND 8: DNA LADDERS, LANE 2: CONTROLS (NEGATIVE-A AND POSITIVE-B), LANE 3: 100, LANE 4: 1000, LANE 5: 10000, LANE 6: 100000, LANE 7: 1000000 UREDINIOSPORES)

A B

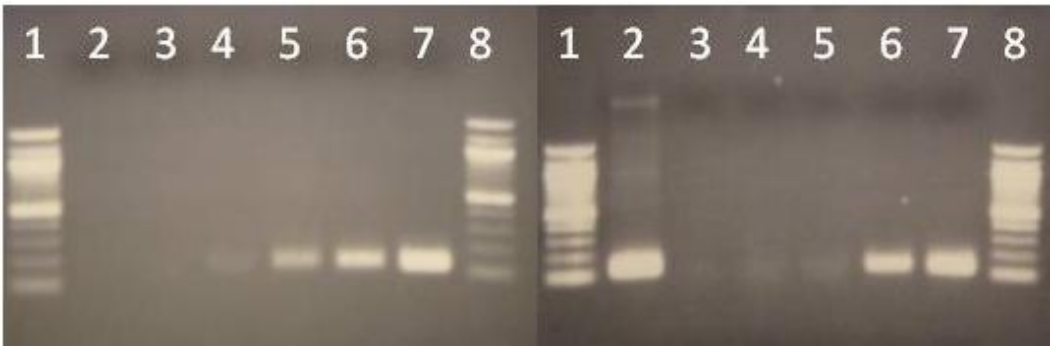

FIGURE 2.7

EFFECT OF THE VOLUME OF 0.1% TWEEN 20 ON EFFICENCY OF BEAD BEATING FOR MECHANICALLY DISRUPTING UREDINIOSPORES OF *PUCCINIA PELARGONII-ZONALIS* (ERROR BARS ARE STANDARD ERRORS)

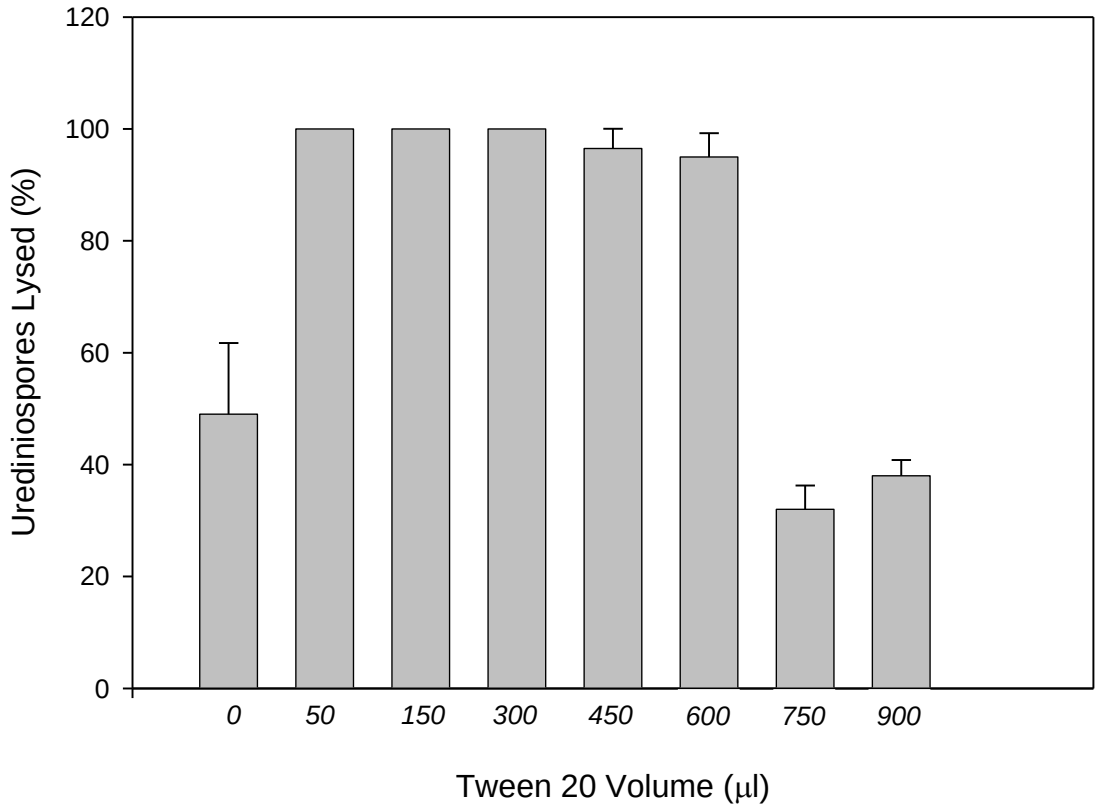
120
100
80
60
40
20
0
Urediniospores Lysed (%)
0
50
150
300
450
600
750
900
Tween 20 Volume (μl)

FIGURE 2.8

RECOVERY OF UREDINIOSPORES OF *PUCCINIA PELARGONNI-ZONALIS* FROM A LEAF INOCULATED WITH 10^5 OR 10^3 SPORES ML^{-1} AND THEN COMBINED WITH 0, 5, 10, 25, OR 50 NON-INOCULATED LEAVES

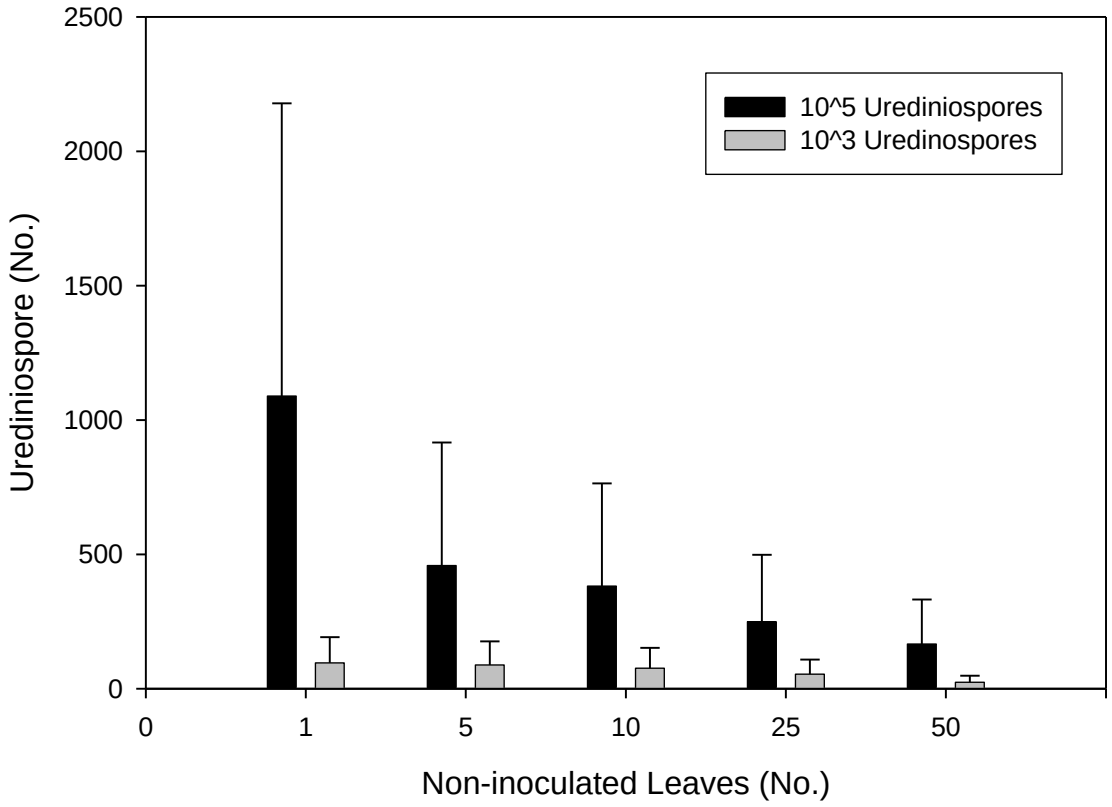
2500
2000
1500
1000
500
0
Urediniospore (No.)
10^5 Urediniospores
10^3 Uredinospores
0
1
5
10
25
50
Non-inoculated Leaves (No.)

Table 2.1. Pathogenic fungi assayed to assess specificity of the primers GRF and GRust-R2, which were selected for specificity to DNA from *Puccinia pelargonnii-zonalis*.

Fungus pathogen	Disease
Puccinia triticina	Wheat leaf rust
Unknown rust fungus	Aster rust
Uromyces phaseoli	Bean rust
P. striiformis	Wheat stripe rust
P. hemerocallidis	Daylily rust
P. iridis	Iris rust
P. helianthus	Sunflower rust
P. sorghi	Corn rust
P. antirrhini	Snapdragon rust
P. oxalis	Oxalis rust
Pucciniastrum vaccinii	Azalea rust
Phakopsora pachyrhizi	Soybean rust
Ustilago maydis	Corn smut
Fusarium sp.	Fusarium Wilt
Rhizoctonia solani	Root rot
Botrytis cinerea	Gray mold

Table 2.2. Cycle threshold values from real-time PCR using primer pair GRF GRust-R2 for a leaf washing assay (10^5 urediniospore ml^{-1}).

Leaf Ratio (inoculated:non-inoculated)[a]	Ct Value[b]			Urediniospores (no.)[c]		
	Rep 1	Rep 2	Average (±SE)	Rep 1	Rep 2	Average (±SE)
1	30.0	29.2	29.67±0.4	1054	1123	1088.5±34.5
1 in 5	nr	nr	nr	478	437	457.5±20.5
1 in 10	nr	nr	nr	353	410	381.5±28.5
1 in 25	nr	nr	nr	237	261	249.0±12.0
1 in 50	nr	nr	nr	172	159	165.5±6.5

a the number of non-inoculated leaves that one inoculated leaf was added to.
b the real-time PCR cycle threshold for each treatment. Nr indicates no amplification.
c The number of urediniospores counted for each treatment using an inversion microscope before extracting DNA.

CHAPTER 3

PRODUCTION OF UREDINIOSPORES BY *PUCCINIA PELARGONII-ZONALIS* AND THEIR DISPERSAL WITHIN A GREENHOUSE

To be submitted to *Plant Disease*.

Abstract

Geranium rust caused by *Puccinia pelargonii-zonalis* can result in significant economic losses to commercial greenhouse operators. Production and movement of urediniospores throughout the greenhouse requires immediate management such as fungicide applications or crop removal. The purpose of this research was to quantify urediniospore production by *P. pelargonii-zonalis* and to track airborne movement of these spores under greenhouse conditions. Two to three week-old sporulating pustules on Maverick Red geraniums were assessed for urediniospore production over 24-h intervals for 3 days. An average of 1580 urediniospores were produced per pustule every 24 h. Movement of urediniospores from infected plants along a greenhouse bench and down rows of geranium plants was assessed in separate experiments. Atmospheric urediniospore concentrations and urediniospore deposition were measured at varying distances from source plants. After 8 h, urediniospores were detected in the atmosphere up to 1.8 m at bench level (deposition) and 2.1 m at pot height from source plants. Cultural practices that resulted in significant dissemination of urediniospores were those that included a lack of fungicide application, plant manipulation, and either drip or hand watering treatments. A minimal amount of inoculum moved to plants furthest from the sporulating plants while most of the inoculum remained close to the sporulating plants. Therefore, eradication of plants in a contaminated greenhouse should be placed on those closest to disease foci rather than on all plans.

Key words urediniospore dispersal, deposition, rotorods, Power Law, negative exponential

Introduction

The presence of geranium rust, caused by *Puccinia pelargonii-zonalis* Doidge, in commercial greenhouses can result in total crop loss for growers. *P. pelargonii-zonalis* is a macrocyclic, autoecious basidiomycete fungus first described on zonal geraniums (*Pelargonium* ×*hortorum*) in the United States in 1967 (Dimock et al. 1968). On the abaxial surface of the leaves, lesions initially appear as chlorotic spots that later develop into sporulating lesions as disease progresses. If the disease is present on plants in a greenhouse, subsequent urediniospore dissemination throughout the greenhouse can have a significant impact on crop value. Many popular cultivars of zonal geraniums are susceptible to rust—including Maverick Red, Salmon and White series; however, resistance is present in some cultivars (Buck 2007). Typically, consumer demand for flower color or foliage, rather than disease resistance, dictates which cultivars are propagated commercially.

Integrated disease management (IDM) is an environmentally sound and economically sustainable form of management utilizing all possible options—e.g., chemical, cultural, and biological controls as well as host plant resistance. Current IDM strategies include using certified disease-free plants, proper sanitation, host resistance (if available), proper irrigation, and preventative fungicide applications (Daughtery and Benson 2005). Specific IDM strategies for geranium cultivation also include removal of senescent flower blossoms to limit the spread and infection of *Botrytis cinerea* (Hausbeck and Moorman 1996) and the feeding and reproduction of the western flower thrips (*Frankliniella occidentalis* Pergande) that can transmit tospoviruses to other herbaceous plants being cultivated by growers (Daughtery et al. 1997). Several chemical classes of fungicides are registered for and have efficacy against rusts on ornamental crops. These include the QoI's (e.g., azoxystrobin), sterol biosynthesis inhibitors (e.g., myclobutanil,

propiconazole), and broad-pectrum protectants (e.g., chlorothalonil, mancozeb) (Jeffers and Luszcz 1998; Jeffers et al. 2001; Mueller et al. 2004).

Grower activity can increase movement of fungus spores that serve as inoculum throughout the greenhouse and negatively affect management strategies. The necrotrophic ascomycete fungus *B. cinerea* causes blight on stems, leaves, and flowers of ornamental crops (Hausbeck and Moorman 1996). Peak atmospheric conidium concentrations of *B. cinerea* increased to >50 conidia m^{-3} h^{-1} when growers were either planting, placing geranium cuttings on benches, irrigating, fertilizing, or spraying pesticides (Hausbeck and Pennypacker 1991). Watering poinsettias altered the relative humidity in greenhouses, which caused an increase in atmospheric concentrations of conidia of *Odium* sp. 3 h after watering (Byrne et al. 2000). A large number of conidia were collected (>100 conidia m^{-3}) in one-hour increments after watering, indicating events that caused conidia to bereleased had occurred.

P. pelargonii-zonalis is endemic in California and is a quarantine pathogen in Ohio (Ohio Dept. of Ag. 2010). Currently, rusts on ornamental crops that are subjected to federal quarantine and eradication include chrysanthemum white rust (*Puccinia horiana* Henn.) (APHIS 2008) and gladiolus rust (*Uromyces transversalis* Thum.) (APHIS 2009). Eradication efforts for chrysanthemum white rust in commercial greenhouses include destruction of all plant material within a 1-m^2 area around the source of inoculum (APHIS 2008). Eradication of gladiolus rust in field plantings requires disposal of all plants in a 1600-m^2 area around symptomatic plants as well as subsequent flooding of fields (APHIS 2009). These eradication strategies are to prevent the presumptive movement of basidiospores of *P. horiana* and the urediniospores of *U. transversalis* from the disease loci.

If geranium rust is found in a commercial greenhouse, current management recommendations include eradication of either 1-m^2 block of plants around the sporulating plants if disease pressure is low (personal correspondence, Syngenta Flowers) or destruction of all plants within a greenhouse if disease pressure is high (personal correspondence Oglevee LTD). The speed and distance of dispersal of urediniospores in greenhouses will directly affect the success of quarantine and eradication measures. However, there is no published information on the movement of urediniospores of *P. pelargonii-zonalis* within a greenhouse crop to determine if these guidelines are practical. The objectives of this study were to quantify the number of urediniospores produced per pustule (i.e., inoculum pressure), investigate the movement and deposition of urediniospores in a greenhouse, and determine the affect of IDM strategies on the movement of urediniospores among greenhouse-grown geraniums.

Materials and Methods

Maintenance of plants and fungi. An isolate of *P. pelargonii-zonalis* from geraniums grown in Georgia was maintained on Maverick Red geraniums (*Pelargonium ×hortorum*) plants (Buck 2007). Plants were grown in a soilless container mix, Metro-Mix 360 (The Scotts Co.; Marysville, OH), in 19-liter containers in a greenhouse with average night and day temperatures of 19 and 21°C, respectively. Experimental plants were inoculated with urediniospores collected with a vacuum spore collector from actively-sporulating 3-week-old pustules. To prepare inoculum, urediniospores were suspended in 0.05% Tween 20 (J. T. Baker; Phillipsburg, NJ), and filtered through five layers of sterile cheesecloth (Mueller et al. 2003). Urediniospores in suspension were enumerated using a hemacytometer and diluted to 1 to 3×10^5 urediniospores

ml^{-1} of 0.05% Tween 20. All urediniospore suspensions were sprayed on plants within 30 min of preparation.

For sporulation and germination assays, 3-month-old rooted cuttings of Maverick Red geraniums in 1.2-liter pots were inoculated, and, for all other experiments, 6-month-old Maverick Red geraniums in 19-liter containers were spray-inoculated. Geraniums were watered immediately prior to inoculation, sprayed with the urediniospore suspension until runoff, placed in plastic bags to maintain high relative humidity, and stored in the dark at 20°C (Mueller and Buck 2003). After 24 h, bags were removed and geraniums were placed in a greenhouse with average night and day temperatures of 19 and 21°C. Plants were fertilized with100 mg $liter^{-1}$ of Scotts Peters Geranium Special 15-15-15 (N-P-K) (Scotts Co.; Marysville, OH) every 2 weeks for the length of the experiment. Foliage was kept dry during each experiment to promote lesion development.

All urediniospore movement and deposition experiments were conducted in a hoop-house (30 m × 9 m) on the University of Georgia, Griffin Campus. Two fans, 10 m apart, were suspended from the ceiling at a 30º angle on each side of the house. Wind speed at the inoculum source was 0.2-0.3 m $second^{-1}$ at plant height for all benches.

Urediniospore quantification. Production of urediniospores by individual pustules was estimated using 3-week old pustules on three leaves from each of 20 infected plants (60 total leaves). Using a vacuum apparatus, urediniospores were collected from the three leaves on each plant into a 20-ml glass vial every 24 h for 3 days. A volume of 1 ml of 0.1% Tween 20 was added to each vial and samples were vortexed for 1 min to suspend the spores. Urediniospores were enumerated in each sample using a hemacytometer at 100× magnification. The number of urediniospores per pustule was calculated by dividing the total number of urediniospores

collected per plant by the number of pustules vacuumed on that plant. The mean number of spores per plant then was calculated as the average for the 20 plants. This experiment was conducted three times.

Atmospheric urediniospore concentrations and urediniospore deposition from an inoculum source along a greenhouse bench. An inoculum source consisting of three geraniums in 19-liter containers with 3-week-old pustules and an average severity of six pustules cm^{-2} was placed on one end of a galvanized steel bench 3 m long and 1 m wide. Urediniospore deposition at bench level was determined by placing two glass slides coated with petroleum on the bench at 0.6-m intervals up to 2.4 m from the source plants. To investigate dispersal of urediniospores at plant height (0.5 m), rotorods samplers (Multidata LLC, St. Louis Park, MN) containing two rods were placed at 0.3, 0.9, 1.5, and 2.1 m from the source plants. Deposition and air dispersal of urediniospores was assessed over 9-h periods (9 am to 6 pm) for five successive days. Samples were collected daily at 6 pm. Urediniospores were enumerated on the glass slides and rotorods by microscopy at 100× magnification. Data on dispersal at plant height (rotorods) were converted to average number of urediniospores m^{-3} air using the following calculation: average number of urediniospores counted/1.01 m^3, where 1.01 m^3 equals the amount of air sampled based on the length of the rod and time (Multidata LLC). The experiment was conducted twice.

Spatial dispersal of urediniospores from an inoculum source down a row of geranium plants. Ten rows of three healthy geranium plants (cv. Maverick Salmon) in 1.2-liter containers were placed on a galvanized steel bench that was 3 m long and 1 m wide. An inoculum source of three geraniums each in a 19-liter pot with 3 week-old sporulating pustules (7 pustules cm^{-2} in trial 1; 9 pustules cm^{-2} in trial 2) was placed at one end of the bench. Urediniospore dispersal from the inoculum source was tracked by sampling one leaf from each

plant in each row daily for five days. Leaves were placed in separate 50-ml centrifuge tubes (BD Falcon; San Jose, CA) with 15 ml of 0.1% Tween 20. Leaf samples were agitated by vortexing for 30 s and then placed on a table-top shaker for 1 h at 120 rpm min^{-1} to dislodge urediniospores from leaf surfaces. The leaf was removed from each tube and leaf area was measured with a CI-202 Portable Area Meter (CID Bio-science; Camas, WA). The dislodged urediniospores were pelleted by centrifugation (5 min at 14,000 rpm), the Tween 20 solution was discarded, and urediniospores were resuspended in 1 ml of 0.1% Tween 20. Each sample was transferred into a separate well of a 24-well tissue culture plate (BD Falcon; San Jose, CA), and urediniospores in each well were enumerated at 200× magnification using an inverted microscope (Olympus model CKX31; Center Valley, PA). Data were converted to urediniospores cm^{-2} of leaf surface for each row sampled by day. To measure disease development due to inoculum movement, after the fifth day plants from each row were placed in plastic bags to maintain high relative humidity (~85%) and incubated at 20^{o}C in the dark for 24 h (Mueller and Buck 2003). Plants were removed from bags and moved to the greenhouse to maintain high relative humidity. The percentage of infected leaves per plant was recorded 14 days later.

Effect of cultural practices on movement of urediniospores among geranium plants in a greenhouse. The effect of eight treatments (Table 3.1) that consisted of combinations of four cultural and chemical management strategies on urediniospore movement along a row of geranium plants was evaluated. The four management strategies used in these combinations were: foliar fungicide (azoxystrobin, Heritage; 0.15 g a.i./liter) applied with a CO_2- pressurized sprayer using a TeeJet 11002VS spray nozzle at 25 psi; plant manipulation—leaves on the inoculum source plants were handled and then leaves on each successive plant in the row were handled; hand watering down the row of plants; and drip irrigation provided by drip-tubes placed into each

plant container at 2 h intervals. Plant manipulation mimicked grower activities that involved removal of senescing flowers and leaves. Plant manipulation and hand watering were first done at the source plant and then successively down the rows of healthy plants. Fungicide treatments were applied once at the beginning of the experiment while all other treatments were conducted every day for 7 d throughout the experiment.

Experiments were conducted on galvanized steel tables that were 3 m long and 1 m wide; tables were placed parallel with the long axis of the greenhouse. Each combination of treatments was conducted on a single table and replicated on a table located on the opposite side of the greenhouse. For each treatment combination, a 3-week-old diseased Maverick Red geranium plant was placed at one end of the bench and eight healthy Maverick Red and Maverick Salmon geranium plants were placed approximately 2.5 cm apart in a row leading away from the inoculum source plant. One leaf on each plant was sampled on days 1, 3, 5 and 7, and each leaf was placed in a 50-ml centrifuge tube with 15 ml of 0.1% Tween 20. Leaf samples were vortexed for 30 s and then placed on a table-top shaker (New Brunswick; Edison, New Jersey) for 1 h at 120 rpm. For each sample, the leaf was taken out of the tube and leaf area was recorded. Urediniospores were pelleted by centrifugation at 14,000 rpm, and the supernatant was discarded. Urediniospores were resuspended in 1 ml of 0.1% Tween 20; suspensions were transferred to a 24-well tissue culture plate; and spores were counted using an inverted microscope (200×). Data were converted to urediniospores cm^{-2} by dividing the number of urediniospores recovered from each leaf by the area of that leaf.

Statistical analyses. Data were subjected to ANOVA using a multivariate mixed model procedure (PROC GLIMMIX; SAS 2009, Cary, NC) with days used as a repeated measure for all experiments. Data on urediniospore dispersal and production, which were recorded as number of

urediniospores per pustule, were analyzed as a randomized complete block design. All experiments were repeated and means were separated using the Tukey-Kramer method (P<0.01). Data on both atmospheric and deposition of urediniospores were fitted to a simple linear regression model using PROC REG and two negative exponential models, Power Law ($y = as^{-b}$) and Exponential ($y = a^{-bs}$) using PROC NLIN (SAS 2009). Linear regression models were considered best fit at if significant (P<0.05) and a high R^2 value. Negative exponential models were deemed a best fit based on significance at P<0.05, a high F-value, and evenly distributed residuals. A correlation between urediniospore deposition on leaves and disease incidence was analyzed using PROC CORR ($P< |r|$ under H_0: Rho = 0). Data from individual trials of each experiment were combined if there was no significant interaction between the trials.

Results

Urediniospore production. Urediniospore production was not significantly different (P<0.92) for each day that was sampled over a three day period. The mean number of urediniospores produced per pustule per day was 1,580 and ranged from 1,466 to 1,734 on the susceptible cultivar Maverick Red. Calculating urediniospore production allowed for estimation of total spore load (total lesions × urediniospores lesion^{-1}) for the infected plants. The average daily spore load was 154,810 (day 1), 86,394 (day 2), and 58,783 (day 3) urediniospores per plant, respectively.

Atmospheric concentrations and depositions of urediniospores along a greenhouse bench from an inoculum source. Data from both trials on concentrations of urediniospores in the greenhouse atmosphere were combined because there was no trial by treatment interaction (P<0.2304), and days were used as repeated measures. As expected, concentrations of urediniospores in the atmosphere (i.e., urediniospores m^3) showed a decreasing trend as the

distance from the source increased (Fig. 3.1). Rotorods placed 0.3 m from the source plants collected a significantly greater number of urediniospores than rotorods placed at all other distances from the source plants. Regression analysis of the atmospheric concentrations of urediniospores was significant ($P < 0.0100$) for a simple linear regression ($R^2 = 0.14$; $y = -10x+29$) (Fig. 3.1A). The Power Law model ($y = 20x^{-0.4}$) was significant ($P<0.0001$) with an F-value of 24.20, but a low correlation coefficient (r = -0.6) (Fig. 3.1B). The negative exponential model ($y = 32e^{-0.6x}$) was also significant ($P<0.0001$; F-value 24.79) with a strong correlation coefficient ($r = 0.8$) (Fig. 3.1C). Therefore, the negative exponential model best described the relationship between atmospheric urediniospore concentrations and distance from the source plant. These results indicated that the greatest amount of inoculum in the atmosphere is closest to the plants with sporulating pustules (i.e., the source of inoculum) and this inoculum eventually can be deposited onto leaves of plants on greenhouse benches; however, as distance from the inoculum source increases, the concentration of inoculum in the air decreases.

Deposition of urediniospores on glass slides indicated that there are significantly more urediniospores at 0 m next to the source (240 urediniospores cm^{-2}) and less than 20 urediniospores at 2.4 m. All three models explained these data ($P<0.0001$), but one was better than the other two. The simple linear regression model ($y = -24x + 168$) had a low R^2 value (0.26) (Fig. 3.2A). The Power Law model ($y = 31x^{-0.9}$) had an F-value of 122 and a high correlation coefficient ($r = -0.9$) (Fig. 3.2B). In the negative exponential model ($y = 244e^{-0.8x}$), the F-value was 117 and a low correlation coefficient ($r = 0.2$) (Fig. 3.2C). Therefore, the Power Law model best described spore deposition data; there was a great amount of variability with data points closest to the inoculum source and the amount of inoculum deposited decreased as the distance from the source increased (Fig. 3.2B).

For dissemination of urediniospores on plants, spore loads calculated in trials 1 and 2 were 398,160 and 540,360 urediniospores per plant per day, respectively. As expected, plants in the row closest the inoculum source had significantly more urediniospores than plants in all other rows over a 5-d period (Table 3.2). Row and day interactions were not significant (Table 3.2). Disease incidence was highest (25%) on plants in row one than any other row, and there were no pustules that developed on plants in row 10 (Fig. 3.3). There was a positive correlation between number of urediniospores deposited on plants and disease incidence ($r = 0.7$). So, disease incidence was less on plants that received fewer numbers of spores—which is logical.

Effect of integrated management practices on urediniospore dispersal within geranium plants. Plant manipulation, no fungicide application, and drip irrigation (treatment 2) , no fungicide application, plant manipulation, and hand watering (treatment 5), and no fungicide application, no plant manipulation, and hand watering resulted in the greatest number of urediniospores (10 urediniospores cm^{-2}) than any other treatment (Table 3.3). All other treatments were not significantly different; however, treatments 7 (fungicide, no plant manipulation and drip irrigation) and 8 (fungicide, no plant manipulation and hand irrigation) moved the least amount of urediniospores (Table 3.3). Most of the urediniospores dispersed from sporulating plants were confined to plants at 0 and 0.2 m from the inoculum source for all days sampled and all treatments. Over a 7-day period, plants significantly more urediniospores were deposited on foliage on Days 3 and 5 than on other days (Table 3.3). In a 3-way analysis of variance of these data, urediniospore dispersal was affected by management treatments, distance from the inoculum source, and length of time that the source plant lesions sporulated; there were no significant interactions among these factors (Table 3.3).

Discussion

Determining the inoculum load and movement of *P. pelargonii-zonalis* urediniospores in a greenhouse can aid in current eradication and management strategies employed by growers. Initial inoculum of newly emerged *P. pelargonii-zonalis* urediniospores produced on plants was 1,580 urediniospores per pustule, and inoculum movement was concentrated closest to the sporulating plants. Urediniospore dispersal depended greatly on initial spore load produced by diseased plants and air movement within a greenhouse, which can vary considerably from greenhouse to greenhouse—depending on presence, placement, and speed of fans as well as other disturbances that cause air movement. Integrated disease management strategies, both cultural and chemical, are important for managing diseases like rust during geranium cultivation. However, the choice of management strategy can affect urediniospore movement and thus infestation of clean stock from an inoculum source. All of the combinations of management practices employed in this study affected urediniospore dispersal from an inoculum source down rows of healthy plants.

Maximum urediniospore production in a greenhouse has been observed during morning hours for *P. pelargonii-zonalis* (Pady 1971) and *P. triticina* (Kramer and Pady 1966). In my study, production of urediniospores during a 24-h period demonstrated that plants with relatively low disease severity (10-100 pustules) can produce 15,800-158,000 urediniospores and plants exhibiting relatively high disease severity (500-1000 pustules) potentially can produce more than a million urediniospores. My data suggest that removal of plants in a - m^2 radius around an infected plant will not remove all infested plants. While the majority (approximately 95%) of urediniospore dispersal was concentrated on the two plants closest (ca. 0.2 m) to the inoculum source with high disease severity (540,360 urediniospores/plant) in the 9-h period, urediniospores

were deposited almost the entire length of the greenhouse bench during this timeframe. However, our study focused on initial inoculum produced on a single plant; however, over time, the polycyclic nature of this disease eventually will result in additional inoculum build-up. Therefore, rapid discovery and removal of diseased plants is important for managing urediniospore dispersal and disease development.

In this study, urediniospores moved almost the entire length of the greenhouse bench in one day. So, quarantining new vegetative cuttings or plants that might be infected would reduce the possibility of urediniospore dispersal onto healthy geranium plants. Newly-arrived plants then could be scouted daily for disease symptoms and signs without the risk of contaminating other plants in the greenhouse. If diseased plants were detected, growers could rogue these plants and initiate a fungicide program to inhibit urediniospore dispersal germination (Mueller et al., 2004; Mueller et al., 2005). However, 90-95% of urediniospores were concentrated at immediately around diseased plants, which was best explained by a negative exponential model. Furthermore, the direct correlation between urediniospore concentration in the air and disease confirmed that less disease was observed on plants as the number of urediniospores present decreased and distance from the inoculum source increased.

The eradication zones of either 1 m^2 or removal of all plants in a greenhouse, which are employed by two commercial growers when infected geraniums are present in a greenhouse, are based on presumed movement and dispersal of urediniospores. Long distance movement of rust inoculum is well documented—e.g., the Puccinia pathway described by Stakman et al. (1923**)** relied on wind to liberate and move urediniospores into the upper atmosphere. Plant-to-plant movement within a greenhouse requires little atmospheric disturbance. Wind speed will vary from greenhouse to greenhouse, but a minimal wind speed of 0.2-0.3 m^{-2} in the current study liberated

urediniospores of *P. pelargonii-zonalis* from the source plants. We observed a high concentration of urediniospores at canopy height—i.e., 0.5 m above the greenhouse bench—but the majority of the urediniospores in the atmosphere were concentrated closest to the inoculum source. Presumably greater concentrations would be detected in the air further away from the inoculum source with higher wind speeds.

Urediniospore dispersal was impeded by the presence of plants, which presumably acted as physical barriers. Urediniospores were deposited farther from the inoculum source along a bench without plants that when plants were present. When urediniospores were averaged over the 5-d sampling period, there were significantly more urediniospores concentrated around the inoculum source. The mean number of urediniospores at 2.4 m from the source was 21 urediniospores cm^{-2} (bare bench) compared to 2.5 urediniospores cm^{-2} on geranium leaves. This suggests that geranium plants present on a greenhouse bench, while becoming infested with urediniospores of *P. pelargonii-zonalis* from adjacent infected plants, serve as filters to intercept spores and, therefore, reduce the amount of inoculum available for dispersal to neighboring plants farther from the disease source.

Routine greenhouse management practices that disturb plant foliage resulted in dispersal of urediniospore from the inoculum source. Minimizing plant disturbance and applying fungicides is a strategy recommended to be used by geranium growers to prevent spread of *B. cinerea* (Hausbeck and Pennypacker 1991). This recommendation also should minimize dispersal of urediniospores of *P. pelargonii-zonalis*. Hausbeck and Pennypacker (1991) concluded that grower activities such as fertilization, sanitation, planting, pesticide application, and cutting plants, resulted in peak conidium concentrations in the air (>50 conidia/m^3/hr) of *B. cinerea*.

Although grower activity in a greenhouse results in inoculum dispersal, routine greenhouse practices—including IDM strategies—should not be abandoned but practiced with caution.

Mueller et al. (2005) reported that chlorothalonil, mancozeb, azoxystrobin, and copper sulfate pentahydrate greatly reduced or prevented urediniospore germination. Furthermore, when fungicide treated urediniospores were inoculated onto geranium seedlings, infection efficiency was reduced dramatically. Therefore, the following recommendations can be made to minimize spread of inoculum when *P. pelargonii-zonalis* is found in a greenhouse: application of fungicides that are toxic to urediniospores, removal and destruction of diseased plants, employment of an eradication zone of at least 3 m^2 around diseased plants, and minimize human activity in the greenhouse.

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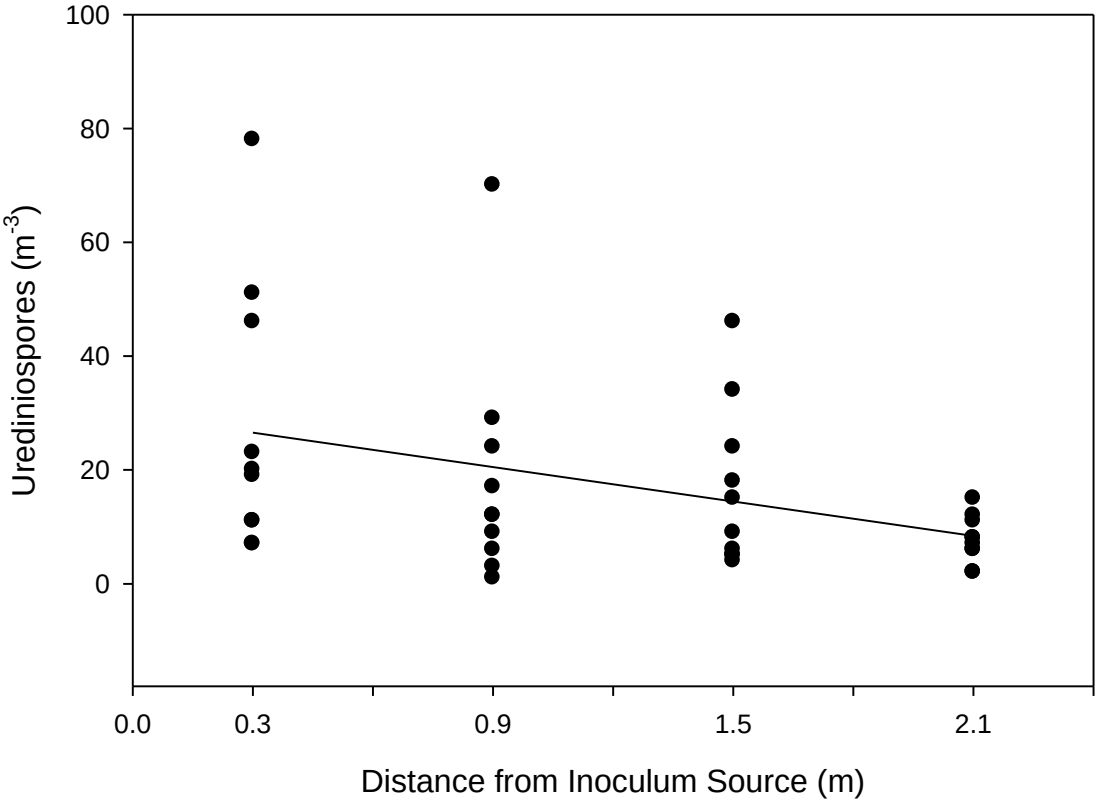
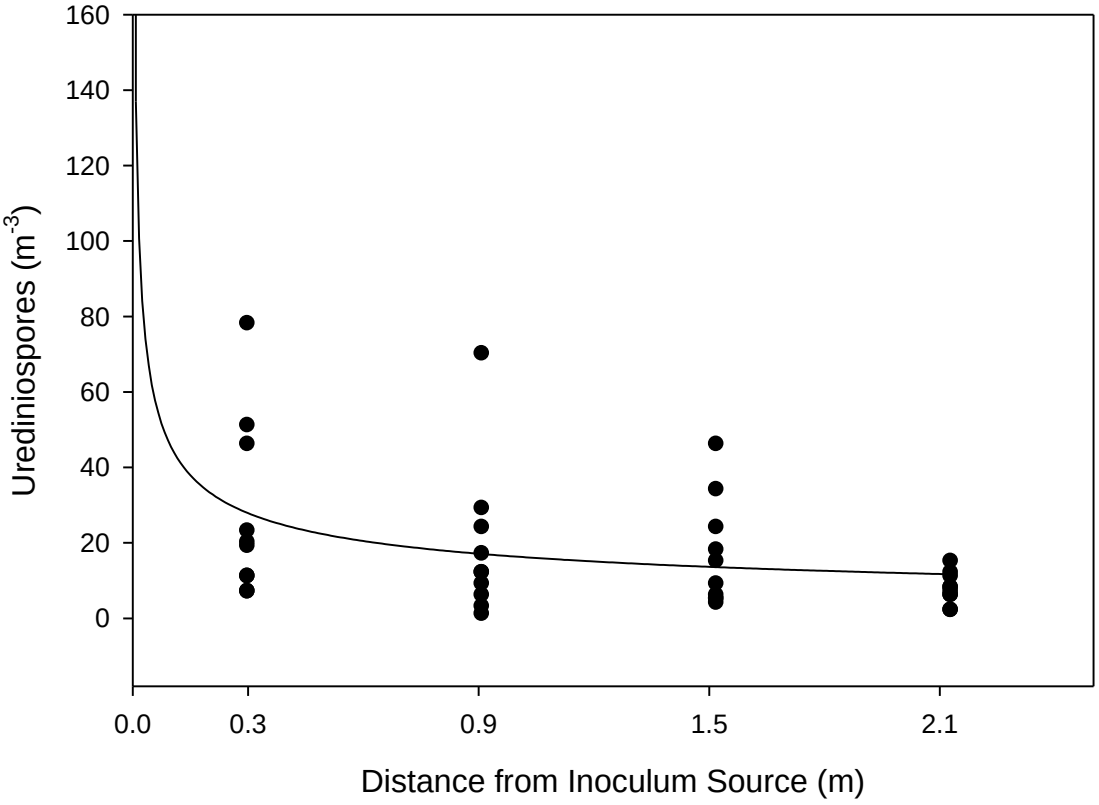
FIGURE 3.1

ATMOSPHERIC CONCENTRATIONS OF UREDINIOSPORES AT VARIOUS DISTANCES FROM AN INOCULUM SOURCE MODELED USING (A) LINEAR REGRESSION, (B) POWER LAW, AND (C) NEGATIVE EXPONENTIAL.

A


100
80
60
40
20
0
Urediniospores (m-3)
0.0
0.3
0.9
1.5
2.1
Distance from Inoculum Source (m)

B


160
140
120
100
80
60
40
20
0
Urediniospores (m-3)
0.0
0.3
0.9
1.5
2.1
Distance from Inoculum Source (m)

C

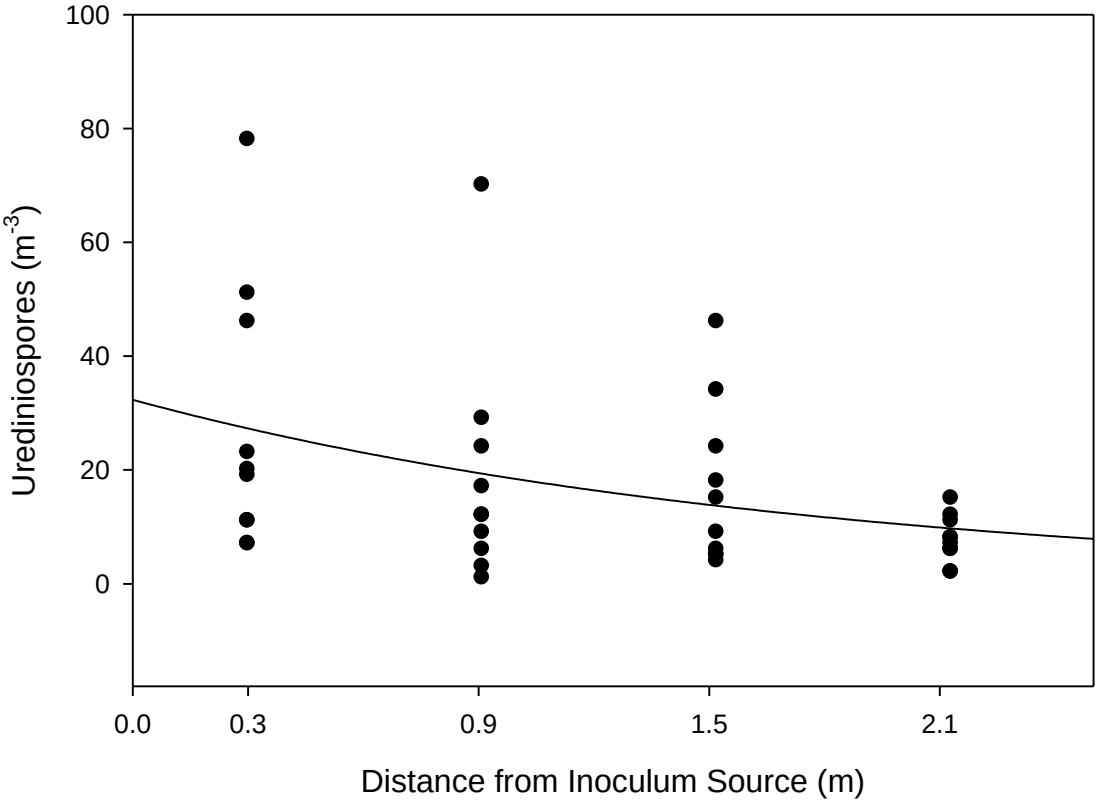
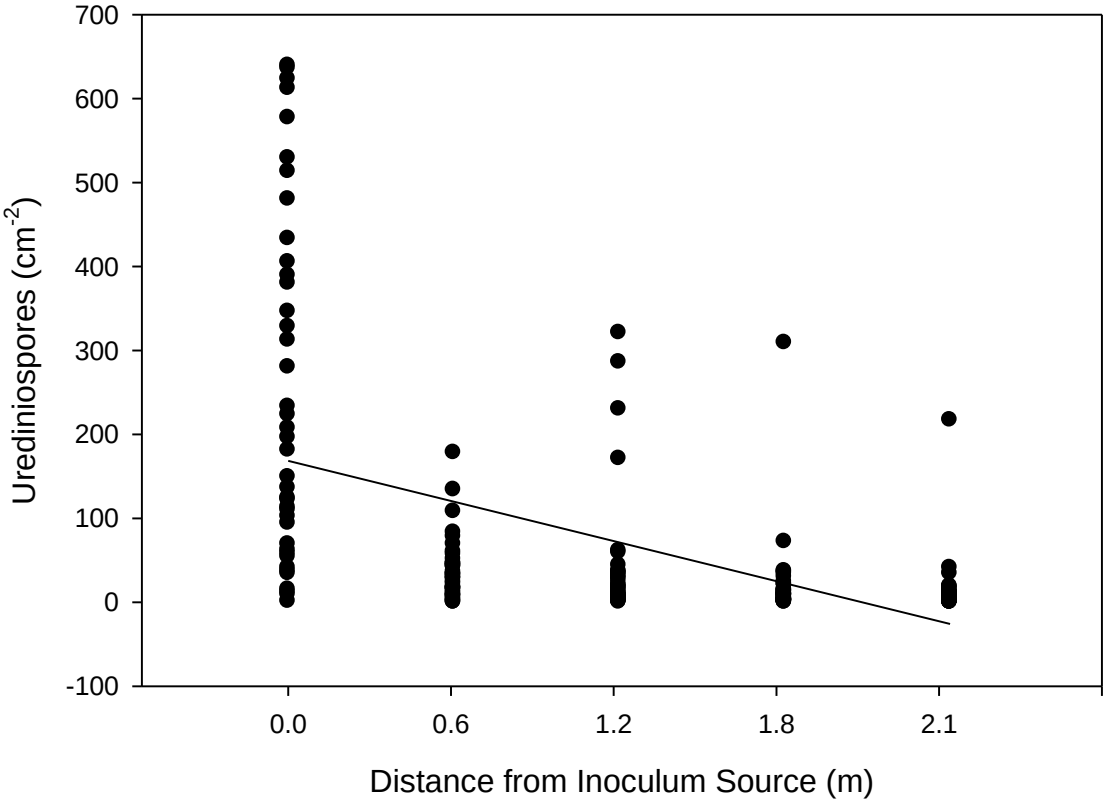

100
80
60
40
20
0
Urediniospores (m-3)
0.0
0.3
0.9
1.5
2.1
Distance from Inoculum Source (m)

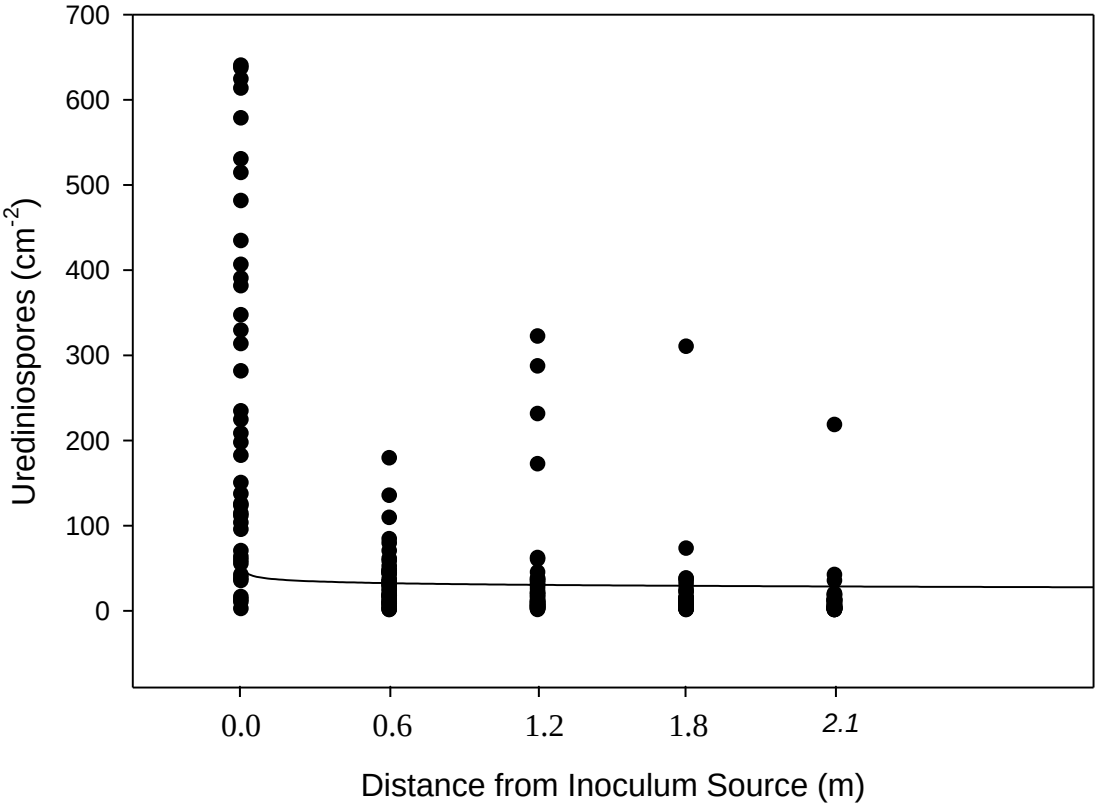
FIGURE 3.2

UREDINIOSPORE DEPOSITION AT BENCH LEVEL AT VARIOUS DISTANCES FROM AN INOCULUM SOURCE MODELED USING (A) LINEAR REGRESSION, (B) POWER LAW, AND (C) NEGATIVE EXPONENTIAL

A


700
600
500
400
300
200
100
0
-100
Urediniospores (cm-2)
0.0
0.6
1.2
1.8
2.1
Distance from Inoculum Source (m)

B


700
600
500
400
300
200
100
0
Urediniospores (cm-2)
0.0
0.6
1.2
1.8
2.1
Distance from Inoculum Source (m)

C

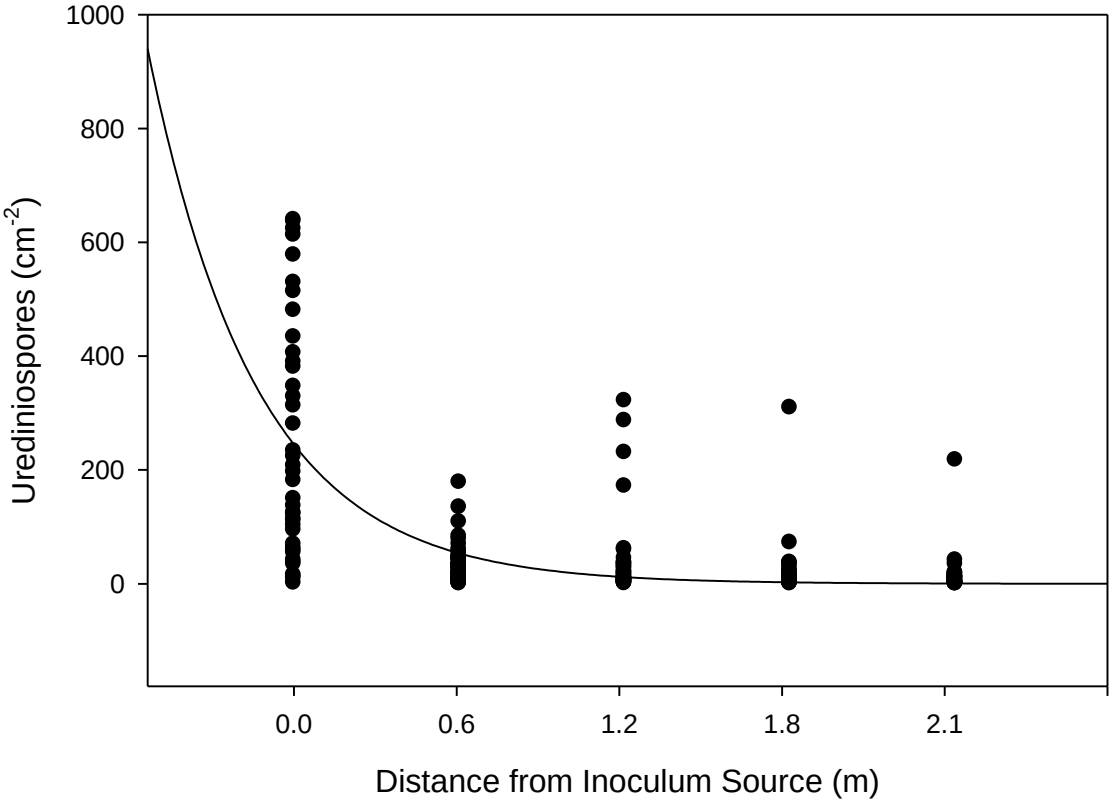

1000
800
600
400
200
0
Urediniospores (cm-2)
0.0
0.6
1.2
1.8
2.1
Distance from Inoculum Source (m)

FIGURE 3.3

DISEASE INCIDENCE ON GERANIUM PLANTS IN SEQUENCIAL ROWS FROM AN INOCULUM SOURCE AFTER EXPOSURE TO *PUCCINIA PELARGONII-ZONALIS*

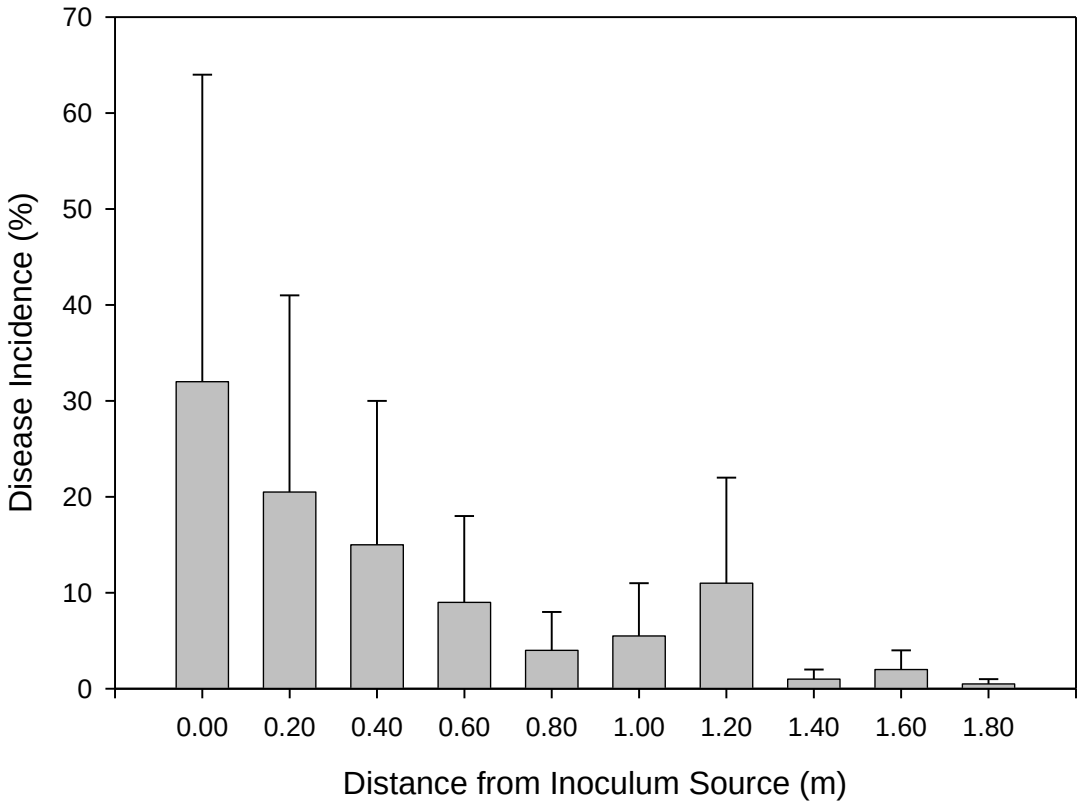

70
60
50
40
30
20
10
0
Disease Incidence (%)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
Distance from Inoculum Source (m)

Table 3.1. Combinations of management and cultural practices evaluated for effect on urediniospore dispersal within a greenhouse.

Treatment no.	Combination
1	Fungicide, plant manipulation, drip irrigation
2	No fungicide, plant manipulation, drip irrigation
3	No fungicide, no plant manipulation, drip irrigation
4	Fungicide, plant manipulation, hand watering
5	No fungicide, plant manipulation, hand watering
6	No fungicide, no plant manipulation, hand watering
7	Fungicide, no plant manipulation, drip irrigation
8	Fungicide, no plant manipulation, hand watering

Table 3.2. Daily deposition of urediniospores on geranium leaves (urediniospores cm^{-2}) on plants located at increasing distances (rows) from an inoculum source[a].

Row	Distance (m)[b]	Urediniospores cm^{-2}	Days[c]	Urediniospores cm^{-2}
1	0	18 a	1	1 c
2	0.2	11 b	2	4 abc
3	0.4	4 c	3	3 bc
4	0.6	2 c	4	6 ab
5	0.8	2 c	5	7 a
6	1.0	2 c		
7	1.2	1 c		
8	1.4	1 c		
9	1.6	1 c		
10	1.8	1 c		

a Means within columns followed by the same lower case letter are not significantly different (Tukey-Kramer LSD, $P<0.01$).
b The distance of each row of plants from inoculum source.
c Mean number of urediniospores for each day that leaves were sampled for enumeration of urediniospores.
P-values: row (0.0001), day (0.0001), row*day (0.3929)

Table 3.3. Effects of cultural control strategies on urediniospore dispersal down rows of geraniums on a greenhouse bench[a].

Management Treatments[b]			Distance from Inoculum Source[c]			Mean Urediniospore per Day[d]	
Treatment		Urediniospores cm^{-2}	Row	Distance (m)	Urediniospores cm^{-2}	Day	Urediniospores cm^{-2}
1	(F,P,D)	4 b	1	0	22 a	1	4 c
2	(P,D)	10 a	2	0.2	10 b	3	7 ab
3	(D)	4 b	3	0.4	4 c	5	8 a
4	(F,P,H)	4 b	4	0.6	3 c	7	5 cb
5	(P,H)	9 ab	5	0.8	3 c		
6	(H)	7 ab	6	1.0	2 c		
7	(F,D)	5 b	7	1.2	2 c		
8	(F,H)	5 b	8	1.4	2 c		

Factor[e]	*d.f.*[f]	*F* value	*P*>*F*
treatment	7	4.09	0.0004
row	7	34.79	<0.0001
day	3	6.00	0.0008
row*day	21	1.10	0.3441
treatment*day	21	1.34	0.1859
treatment*row	49	1.30	0.1616
treatment*row*day	147	0.55	0.9999

a Means within columns followed by the same lower case letter are not significantly different (Tukey-Kramer, $P<0.01$).
b Management practices used in a treatment: Fungicide application (F), Plant manipulation (P), Drip irrigation (D), and Hand watering (H).
c Average urediniospores for distances of each plant row.
d Average urediniospores for each day that leaf tissue was sampled.
e Three-way ANOVA of average urediniospores over all treatments, days, rows, and interactions.
f Degrees of freedom

CHAPTER 4

CONCLUSIONS

The studies described herein demonstrated that real-time PCR can be used to identify *P. pelargonii-zonalis* urediniospore DNA and described the epidemiology of the pathogen. These studies were conducted to aid in management strategies that are already in place. Furthermore, there is little known about the epidemiology of ornamental rusts within a greenhouse environment. Current management options include inspection of plants and certification as disease-free, prophylactic fungicide applications, and, if disease is present, eradication of all plant material in the greenhouse. Many operations will destroy all plant material if *P. pelargonii-zonalis* is detected in the greenhouse, which is typically accomplished by scouting plants for signs of the disease, and great economic loss occurs. However, these management strategies are simply conducted based solely on the idea that the urediniospores can spread throughout the entire greenhouse as would be expected from epidemiological studies of *P. graminis* f.sp. *tritici* urediniospores.

Previous research demonstrated that powdery mildew conidia have release events that occur in the greenhouse and can be aided by human interaction such as watering, replanting, or removal of dead leaves from plants. My research focused on the dispersal of urediniospores either in the atmosphere, deposited on leaves, or interactions with cultural control techniques (i.e., hand watering versus misting). The majority of the urediniospores were shown to remain close to the inoculums source. Concentrations of spores in the air were determined without other plants on

the greenhouse bench; so, my results are likely to be liberal. The presence of other plants probably will have an influence on the number of urediniospores in the air. However, this study established baseline urediniospore concentrations that could possibly be deposited on tissue. Typical cultural control techniques that are conducted in geranium-growing greenhouses were tested as possible means for urediniospore dispersal. Significant amounts of urediniospores were dispersed with combinations of no fungicide applications, flower removal, and both hand-watering and misting. Also, hand-watering and misting alone moved urediniospores.

My research included urediniospore deposition without and with plant material on a greenhouse table. Deposition of urediniospores on glass slides blown from sporulating pustules was best described by a exponential decrease, which indicated a significant amount of urediniospores were deposited closest to the inoculum source. Likewise, with the presence of geranium plants on the benches, the majority of the inoculum was deposited on leaves closest to the inoculum source. Furthermore, disease incidence was assessed on those plants and the highest amount of disease was on those plants closest to the inoculum source. Therefore, the current eradication zones for symptomless plants around diseased plants of 1 m^2 or 6 m^2 that are used commercially are either too small or too large. Based on my results, a recommendation of a 3 m^2 eradication zone is ideal. However, the correlation of the number of urediniospores on a single leaf and the number of pustules that form still must be addressed. Also, those urediniospores that are quiescent should be tested for viability, as they could become secondary inoculums over a period of time, by moving from a plant close to the inoculum source to other plants farther away, which is seen with *P. graminis* f.sp. *tritici* urediniospores in fields.

Many studies have demonstrated the usefulness of real-time PCR to detect and identify plant pathogens. Results from this study demonstrated *P. pelargonii-zonalis* can be detected on

geranium tissue by microscopy and identification can be confirmed using the primer pair GRF and GRust-R2. Plants that test positive for urediniospores then can be quarantined to prevent further dissemination of inoculum. The DNA extracted from spores washed from the 1:1 leaf ratio samples was amplified two out of two times by real-time PCR using the primers GRF and GRust-R2. Therefore, microscopy can be used for visual detection of urediniospores and then real-time PCR can be used to confirm that the urediniospores observed are those of *P. pelargonii-zonalis*.

Based on the research conducted, constant scouting is recommended when new plant material is introduced into a greenhouse. Further, quarantining new asymptomatic plants and planting stock will allow time for symptoms to develop if plants are infected, and these plants can be treated with fungicides that are toxic to urediniospores that may be infesting these plants or planting stock.

APPENDIX A

DRENCH APPLICATIONS OF FUNGICIDES FOR GERANIUM RUST, 2010.

To be submitted to *Plant Management Network: Fungicide and Nematicide Tests*

Three systemic fungicides, Heritage (azoxystrobin), Folicur (tebuconazole) and Eagle (myclobutanil) were evaluated as drench applications for management of geranium rust. The trial was completed in a greenhouse on the University of Georgia Griffin Campus located in Griffin, GA. All plants were maintained in 1.3 L containers. Three doses of fungicide (0.5 g active ingredient/pot, 0.25 g a.i./pot, and 0.125 g a.i./pot) were applied as a 120 ml drench. To test for toxicity, two leaves from each plant were sampled at pre-application and post-application and leaf area, weight, and internodes length recorded. To test for efficacy, plants were challenge-inoculated at 1 and 3 wk post-application with a 10^5 urediniospore ml^{-1} suspension in 0.01% Tween 20. All plants were sprayed to leaf wetness and then placed in plastic bags to maintain high relative humidity for 24 h. Plants were removed from bags and moved to the greenhouse. The average disease incidence (number of infected leaves divided by total leaves) was recorded 14 d post-inoculation. Four single plant replicates were used per treatment and placed in a complete randomized block design. Data was combined and analyzed using a general linear model PROC GLM using Fisher's lsd test ($P<0.05$). The experiment was repeated, and data from both trials were combined.

At 1-wk post-application of fungicides, no disease was observed with the 0.5 g doses all fungicides; however, Eagle had significantly more disease at the 0.125 g dose. Folicur and Heritage provided excellent control at 0.25 g and good control at 0.125 g dose. At 3-wk post-application, the lower doses of fungicide provided less control as more disease was evident. Folicur and Heritage at 0.5 g dose had significantly reduced the amount of disease incidence compared to the control and other treatments. No phytotoxicity was observed.

Table 4.1. Efficacy of three fungicides against post-applications of *P. pelargonii-zonalis* measured by percent disease incidence.

Treatment and Dose (A.I./pot)[b]	Disease Incidence (%)[c] 1 week post-app	3 week post-app
Folicur 3.6 F 0.5 g	0 c	1 e
Folicur 3.6 F 0.25 g	0 c	15 cde
Folicur 3.6 F 0.125 g	0 c	26 bcd
Eagle 20 EW 0.5 g	0 c	12 de
Eagle 20 EW 0.25 g	2 c	27 bcd
Eagle 20 EW 0.125 g	12 b	39 b
Heritage 50 WG 0.5 g	0 c	1 e
Heritage 50 WG 0.25 g	0 c	29 bc
Heritage 50 WG 0.125 g	0 c	26 bcd
Non-treated inoculated	100 a	100 a

a Means within columns followed by the same lower case letter are not significantly different (Fisher's lsd, P=0.05)
b Fungicide trade names and drench dosage of active ingredient per potted plant.
c Total percent disease incidence for each treatment at 1 week and 3 week post-application of *P. pelargonii-zonalis*.

APPENDIX B

FUNGICIDE RESITANCE AND DAYLILY RUST

Submitted to *The Daylily Journal*

Introduction

It has been almost a decade since *Puccinia hemerocallidis*-infected daylily plants were imported to the U.S. and rapidly spread throughout the country. Since that time the pathogen has become established in the southeastern U.S. and daylily enthusiasts have had to learn about managing rust. Remember, the best management option is always to avoid getting the disease! This can be accomplished by diligently scouting new plant material, using good management practices such as quarantining incoming stock and, if necessary, using a preventative fungicide program. Fungicides can prevent the establishment of rust but if you have disease present, fungicides can also be effective at limiting the spread of *P. hemerocallidis* (Buck and Williams-Woodward 2003; Buck and Youmans 2009a; Buck and Youmans 2009b; FRAC 2005). However, using fungicides incorrectly such as using improper rates or the wrong rotation of products, can lead to the development of fungicide resistance. To have a better understanding of fungicide resistance and ways to manage it, we must first understand what fungicides are and how they work.

What are fungicides?

Fungicides are compounds that either inhibit growth or kill fungi. Typically, a fungicide has a trade name – developed by the company marketing the product – and an active ingredient, which has both a chemical and common name (Table 5.1). Fungicides are placed into groups based on the way they kill fungi or mode of action which is important to know when choosing what fungicides to rotate in your spray program. Fungicides act by inhibiting cellular enzymes, damaging cell membranes, inhibiting cell division and other cellular functions that render the fungus incapable of causing disease. Fungicides are broadly characterized as either contact or systemic. Contact fungicides such as Daconil or Thalonil (both with the active ingredient

chlorothalonil) have a broad spectrum, multi-site mode of action and must be applied before spores of *P. hemerocallidis* come into contact with the plant surface. Systemic fungicides have protectant and curative properties and can move through plant tissues. The degree of movement is dependent on the chemical structure of the active ingredient – some systemic fungicides will only move a few cells layers in a plant. These fungicides have a site-specific mode of action and have a greater risk of resistance than do the contact fungicides (Table 5.2). An example is Heritage (azoxystrobin) which has a site-specific mode of action that targets only one area of the fungal cell (Table 5.1).

What is fungicide resistance and what causes it?

Fungicide resistance occurs when a fungus becomes less sensitive to a fungicide. The fungus can overcome sensitivity to the fungicide typically if a mutation occurs in its DNA that results in an altered target site so the fungicide cannot bind to it, synthesis of an alternate enzyme that can be substituted for the targeted enzyme, overproduction of the target site, the fungus pumping the fungicide out of the cells or reducing the amount it uptakes, or the fungus can breakdown the fungicide into a less toxic substance.

Fungicide resistance can be either quantitative or qualitative due to the level of resistance within a pathogen population (Vincelli 2002). Quantitative resistance is when the mutant strain is less sensitive to a fungicide than the original strain. Typically this mutant strain can still be controlled by applying a higher rate of the fungicide in question. Qualitative resistance is when the fungus is no longer sensitive to a fungicide – spraying the active ingredient provides no control and we have lost that fungicide from our arsenal of control options.

Selection pressure and fungicide resistance

Mutations are always occurring in fungi. Repeatedly spraying the same active ingredient will act as a selection pressure for the spores that are resistant to the fungicide. How does this happen? If one spore has a mutation that reduces sensitivity to fungicide, then every time you spray that fungicide you will kill the spores that are sensitive but NOT the mutant spores – this is selection pressure! If these mutant spores infect a plant, then all rust spores produced in those lesions will be resistant to the fungicide. If you spray the same active ingredient again (even in a different product) the mutant spore will not be affected. A change to a resistant population can occur quickly. We have observed that a single daylily rust lesion can produce a few thousand spores in a day. Even in a moderately infected planting there is the potential for hundreds of thousands of rust spores on the plants.

How can you avoid the development of fungicide resistance?

Fungicides that are considered at risk for the development of fungicide resistance in pathogen populations are monitored by the Fungicide Resistance Action Committee (FRAC). FRAC was organized in 1981 to employ methods to recognize and manage fungicide resistance and to reduce crop losses if resistance occurs (FRAC 2005). FRAC collaborates with pesticide companies, Universities and other non-profit agricultural organizations to manage and educate on fungicide resistance. Table 2 lists some of the fungicides that can be used for daylily rust and their potential risk for the development of fungicide resistance.

So how do you minimize the chance that fungicide resistance will develop in daylily rust?

First, never rely on fungicides as a sole management strategy. You should always consider using cultural management strategies, such as using resistant plant varieties (if possible) and stringent sanitation. To minimize the possibility of fungicide resistance developing in daylily

rust populations, you should rotate fungicide classes that contain different modes of action. When considering fungicide rotation, first read the label to make sure that product can be used on daylily. Second, look at the active ingredient and make sure it is in a different group of fungicides with a different mode of action than the previously used fungicide. For example, use a systemic product after a broad-spectrum fungicide. Broad-spectrum, low-risk fungicides can typically be used more often than systemic products. Third, if you have high levels of disease cut back infected foliage and discard (burn, bury or take off-site) prior to the application of a broad-spectrum fungicide. High risk fungicides such as azoxystrobin should not be applied when there is a lot of disease present in your daylilies to minimize the risk of resistance developing. With all fungicide applications you want to cover as much of the foliage as possible so don't cut corners when using systemic fungicides!

Read the label to determine what the active ingredient is in the fungicide product you want to spray – different trade names do not necessarily mean a different active ingredient! For example, Eagle and Spectracide Immunox contain myclobutanil as an active ingredient. Combination products are also available such as Pageant (pyraclostrobin and boscalid) and Zyban (thiophanate methyl and mancozeb) which typically include two fungicides with different modes of action. Always follow label directions to determine that the proper rates are being used. We have tested triadimefon and observed good activity against daylily rust. However, the labels for two products containing triadimefon (Bayleton and Strike) currently do not list daylily and do not provide a disclaimer about the user assuming responsibility for application to unlabelled plants. We have suggested to the manufacturers that daylily be added to those labels.

What should you do if you think you have fungicide resistance?

If you are spraying a fungicide that has managed rust in the past and see little or no effect, there is a chance that the population has developed resistance. First you need to confirm that you sprayed the product at the label rate and the plants received adequate coverage – that no mistakes were made in the fungicide application! If you sprayed a product with a low FRAC resistance risk such as chlorothalonil or mancozeb it is possible that there was an application error. Contact a county extension agent or an extension plant pathologist at your local Land Grant University and ask them to review your spray program. Fungicide resistance in daylily rust has not been verified to date.

If resistance develops in *Puccinia hemerocallidis* to a specific active ingredient it could result in reduced efficacy to products containing the closely related chemicals in the same group. For example, cross resistance has been observed between all members of the QoI group of fungicides (e.g azoxystrobin) (FRAC 2005). Thankfully, there are many different classes of products available and many fungicides will adequately control daylily rust (Buck and Williams-Woodward 2003). Proper use and rotation of fungicides will greatly reduce the risk of fungicide resistance in daylily rust populations and will ensure that you will have beautiful, rust-free daylilies for years to come!

References Cited

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FRAC. 2005. http://www.frac.info/frac/index.htm.

Mueller, D.S., Jeffers, S.N., and Buck, J.W. 2004. Effect of timing of fungicide applications on development of rusts on daylily, geranium, and sunflower. Plant Disease 88:657-661.

Vincelli, P. 2002. Q_oI (Strobilurin) Fungicides: Benefits and Risks. The Plant Health Instructor. DOI: 10.1094/PHI-I-2002-0809-02.

Table 5.1. An example of fungicide classification from mode of action to trade name.

Trade Name	Heritage
Common Name	azoxystrobin
Chemical Name	methyl (*E*)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate
Fungicide Class	QoI
Mode of Action	inhibits cellular respiration

Table 5.2. Fungicide resistance risk according to Fungicide Resistance Action Committee (FRAC). Daylily is not listed on the labels of some fungicides that show efficacy against *Puccinia* rusts of ornamentals. However, these labels state that the product should be tested on a small number of plants to ensure it is not harmful prior to large scale applications. User would then assume all risk arising from application to unlabelled plants.

Fungicide Group Name	**Product (active ingredient)**	**FRAC Code**	**Resistance Risk**
Methyl benzimidazole carbamates (MBC)	Cleary's 3336, Fungo Flo (thiophanate-methyl)	1	
Quinone outside inhibitors (QoI)	Heritage (azoxystrobin) Insignia (pyraclostrobin) Compass O (trifloxystrobin)	11	High
Succinate dehydrogenase inhibitors	Contrast, ProStar (flutolanil) Plantvax (oxycarboxin)	7	

Demethylation inhibitors (DMI)	Eagle, Spectracide Immunox (myclobutanil) Banner MAXX, Bonide Infusion (propiconazole) Bayer Advanced Disease Control (tebuconazole) Ortho Rose Pride (triforine)	3	Medium
Dithiocarbamates	Dithane, Fore (mancozeb) Ferbam Granuflo (ferbam)	M3	
Inorganic	Bonide Liquid Copper	M1	Low
Chloronitriles	Daconil, Thalonil (chlorothalonil)	M5	