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AN AMERICAN PERSPECTIVE OF
CHRYSANTHEMUM WHITE RUST
CAUSED BY PUCCINIA HORIANA

A Dissertation in
Plant Pathology
by
Grace O’Keefe

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The dissertation of Grace O'Keefe was reviewed and approved* by the following:

Donald D. Davis  
Professor of Plant Pathology and Environmental Microbiology  
Dissertation Adviser  
Chair of Committee

David M. Beyer  
Professor of Plant Pathology and Environmental Microbiology

John Pecchia  
Assistant Professor of Plant Pathology and Environmental Microbiology

Dennis R. Decoteau  
Professor of Horticulture and Plant Ecosystem Health

Michael Fidanza  
Professor of Plant and Soil Science  
Special Committee Member

David E. Geiser  
Professor of Plant Pathology and Environmental Microbiology  
Interim Head of the Department of Plant Pathology and Environmental Microbiology

*Signatures are on file in the Graduate School.
ABSTRACT

Chrysanthemums are perennial flowering plants that were illustrated in Chinese literature as early as the 15th Century B.C. and have been cultivated as an herb for more than 3000 years. Chrysanthemums arrived in Japan during AD386 where highly prized cultivars were developed within a short period of time. Since the earliest recognition of chrysanthemums in China, popularity of this flower has continued to grow throughout the world.

Chrysanthemum White Rust (CWR), caused by *Puccinia horiana* Henn., is an autoecious, microcyclic rust that is pathogenic on many chrysanthemum species (*Chrysanthemum* spp.) and close relatives within the Asteraceae family. Chrysanthemum white rust is economically important due to its ability to infect florist chrysanthemum (*Chrysanthemum x morifolium*) cultivars used as cut flowers and potted plants throughout the world. *Puccinia horiana* can cause serious damage to chrysanthemums in commercial greenhouses as well as at homeowner sites. Many countries, including the United States, have established Phytosanitary Quarantines against CWR, due to financial loss from CWR and difficulty of eradicating the disease. Currently *P. horiana* is not considered indigenous in the United States.

The objectives of this study include determining the ability of *P. horiana* to overwinter in Pennsylvania, and to illustrate morphological details of *P. horiana* within infected chrysanthemum leaves. Although we previously reported the presence of *P. horiana* in the roots and stems of asymptomatic chrysanthemum plants, we had not provided detailed descriptions of the morphology, intercellular colonization, and intracellular growth of *P. horiana* in chrysanthemums until this paper. Images produced
using compound microscopy and SEM in this paper provide additional, detailed illustrations regarding *P. horiana* colonization of chrysanthemum stems and leaves. Also, anastomosis between two adjacent strands of *P. horiana* hyphae is illustrated for the first time.

Another objective was to determine the economic impact of CWR based on incidence and severity of *P. horiana* on selected varieties at one location in central Pennsylvania, and to develop a molecular screening technique sensitive enough to detect latent, hidden infections of CWR. Federal regulations state that, for a chrysanthemum plant to be considered positive for *P. horiana*, it must exhibit visible teliospores on or in the infected plant. Thus, chrysanthemum plants that are determined to be positive for *P. horiana* via molecular screening do not legally have be eradicated, if teliospores or telial sori are not visible. This need for visualization of morphological symptoms may cause a serious time delay in recognition of diseased plants. This delay in identification of CWR increases the financial costs, since later eradication may involve digging and removing larger established plants. Sensitive and accurate molecular screening of chrysanthemum plants by using molecular methods, as demonstrated by our research, employed prior to dispersal of asymptomatic, yet *P.horiana*-positive, chrysanthemum plants could greatly reduce cost of production for wholesale and retail horticultural businesses, as well as homeowners.

The final objective of this study was to genotype *P. horiana* isolates collected throughout the United States, and to compare the genotypes of the United States isolates with previously collected worldwide isolates. This phase of the study considered 101 isolates, including 61 collected within the United States. The 61 United States isolates
were collected from seven states. Six multilocus genotypes were identified within the United States, as compared to 28 for the worldwide collection. We determined there are four main genotypes in the United States, with one newly discovered in this study. This study suggests that five or six separate introductions into the United States have occurred. Supported by confirmation of *P. horiana* overwintering in the United States, this study indicates that *P. horiana* is now endemic within the United States.
# TABLE OF CONTENTS

List of Tables .................................................................................................................... vii  
List of Figures .................................................................................................................. viii  
Acknowledgements ............................................................................................................ xi  

## Chapter I. LITERATURE REVIEW: AN AMERICAN PERSPECTIVE OF CHrysanthemum WHITE RUST CAUSED BY PUCCINIA HORIANA ................................................................. 1  
  Abstract ...........................................................................................................1  
  Taxonomy of Chrysanthemums (Chrysanthemum sp.) .................................2  
  History of Chrysanthemums ...........................................................................3  
  Economical Value ...........................................................................................9  
  Commercial Chrysanthemum Production .....................................................10  
  Chrysanthemum Diseases and Insect Pests in the United States ..............21  
  Rust Fungi .....................................................................................................22  
  Taxonomy of *P. horiana* ...........................................................................25  
  History of *P. horiana*, causal agent of chrysanthemum white rust ....25  
  Disease Cycle of *P. horiana* ......................................................................27  
  Detection of *Puccinia horiana* ...................................................................32  
  Pathotypes .....................................................................................................36  
  Genotyping of *Puccinia horiana* .................................................................36  
  High Resolution Melting (HRM) Analysis ...................................................40  
  Hosts .............................................................................................................42  
  Economic losses due to *P. horiana* ...............................................................42  
  Bibliography .................................................................................................44  
  Tables and Figures ........................................................................................77  

## Chapter II. Morphology of *Puccinia horiana* .......................................................... 99  
  Abstract .........................................................................................................99  
  Introduction ..................................................................................................100  
  Material and Methods ..................................................................................104  
    Study Sites .................................................................................................104  
    Evaluating ability of *P. horiana* to overwinter in Pennsylvania ......105  
    Determine morphological details of *P. horiana* ..................................106  
  Results .........................................................................................................109  
    Overwintering of *P. horiana* in Pennsylvania ....................................109  
  Discussion ....................................................................................................111  
    Morphological details of *P. horiana* .......................................................112  
    Colonization of *P. horiana* ..................................................................113  
    Anastomosis in *P. horiana* ....................................................................115  
    Overwintering of *P. horiana* in Pennsylvania ....................................116  
  Literature Cited ............................................................................................118
Chapter III. Detection of Chrysanthemum White Rust .................................................132

Abstract ..................................................................................................................132
Introduction ..............................................................................................................133
Material and Methods ............................................................................................135
Study Sites ..............................................................................................................135
Determining the incidence and severity of CWR on 10 mum varieties growing on the PSU campus in fall of 2012 ............................................135
Determining the incidence and severity of CWR on 17 mum varieties in 2013 ........................................................................................137
Development of molecular screening protocol ..................................................139

Results ......................................................................................................................140
Determining the incidence and severity of CWR on 10 mum varieties growing on the PSU campus in fall 2012 ............................................141
Determining the incidence and severity of CWR on 17 mum varieties in 2013 ........................................................................................142
Development of molecular screening ...............................................................142

Discussion ...............................................................................................................144
Determining the incidence and severity of CWR mum varieties growing on the PSU campus ..............................................................144
Molecular screening protocol ............................................................................147

Chapter IV. Genotyping and Analysis of 61 P. horiana Isolates from the U.S.........157

Abstract ..................................................................................................................157
Introduction ..............................................................................................................158
Genotyping of P. Horiana ......................................................................................160
High Resolution Melting (HRM) ..........................................................................164
Material and Methods ............................................................................................165
Genotyping using SNP markers ............................................................................167
SNP data analysis ..................................................................................................168
Recombination and pathotypes correlation analysis ......................................169
Results ......................................................................................................................169
Discussion ...............................................................................................................171
Genotyping data analysis ......................................................................................171

Table of Contents
LIST OF TABLES

Table 1.1. Current taxonomy of chrysanthemums…………………………………….66
Table 1.2. List of important dates and authorities involved in production of chrysanthemums in the western world…………………………………….67
Table 1.3. Value of chrysanthemums in the United States during 2011 and 2012. Data collected for hardy/garden varieties, potted patio and florist varieties, and pom-pom varieties; data collected from 15 program states………………..68
Table 1.4. Top 10 producers of chrysanthemum cuttings …………………………….69
Table 1.5. Common chrysanthemum insects and symptoms. .........................70
Table 1.6. Common diseases found on chrysanthemums……………………………71
Table 1.7. Taxonomic classification of P. horiana to species…………………….73
Table 1.8. Plant hosts of Puccinia horiana, causal agent of CWR…………………74
Table 3.1. PCR primers, probes and nucleotide sequences, location and reference…………………………………………………………………..140
Table 3.2. Frequency of CWR incidence among 10 chrysanthemum varieties in the field during 2012 …………………………………………………………141
Table 3.3. Mean real-time CT values obtained using protocols of Pedley, Alaei et al., and a protocol we modified……………………………………………….142
Table 3.4. Chrysanthemum plants from three varieties putatively rated positive with molecular assay during 2013……………………………………………..143
Table 4.1. Isolate information code, isolate name, collection location, collection year and source…………………………………………………………………..167
Table 4.2. List of primers and probes used for HRM analysis of P. horiana isolates.170
Table 4.3. Average melt temperature (°C) for each SNP and Δ melt....................171
Table 4.4. HRM results using 32 single-nucleotide polymorphisms (SNPs) and one simple-sequence repeat (SSR) for 55 US isolates.................................172
Table 4.5. Comparison of HRM results using 32 single-nucleotide polymorphisms (SNPs) and one simple-sequence repeat (SSR) for 55 US isolates ………173
LIST OF FIGURES

Figure 1.1. Wild chrysanthemum soup made from Ye Juhua chrysanthemums.........75
Figure 1.2. ShZu the Shih tzu “Chrysanthemum” dog.................................76
Figure 1.3. Worldwide chrysanthemum production, 2006..............................77
Figure 1.4. Pom-pom and incurve type chrysanthemums..........................78
Figure 1.5. Distribution of chrysanthemum species studied in relation to chromosome number.................................................................79
Figure 1.6. Kinds and sequence of spores and spore-producing structures in rust fungi and nuclear condition of each...............................................80
Figure 1.7. Distribution map of P. horiana..................................................81
Figure 1.8. Life Cycle of Puccinia horiana................................................82
Figure 1.9. Teliospores of Puccinia horiana.................................................83
Figure 1.10. Infection schematic diagram summary of infection process of a Puccinia spp..............................................................84
Figure 1.11. Schematic illustration of recombination in Puccinia horiana........85
Figure 1.12. Dissociation curve using unlabeled probe SNP mismatch, match and amplicon peaks.................................................................86
Figure 1.13. CWR interceptions in the United State and Canada since 1977........87
Figure 2.1. Overwintered volunteer chrysanthemum plant with P. horiana telial sori.................................................................111
Figure 2.2. A. Germinating P. horiana basidiospores and germ tubes on chrysanthemum leaf epidermis; (B) Intracellular P. horiana hypha in symptomatic chrysanthemum stem. (C) Intracellular P. horiana teliospores in symptomatic chrysanthemum stem; (D) P. horiana teliospore with promycelium developing from the basal cell. Teliospore is intracellular within a symptomatic chrysanthemum stem. P. horiana basidiospores and germ tubes on chrysanthemum leaf epidermis.................................................................112
Figure 2.3  E. Intracellular P. horiana hypha growing in a symptomatic chrysanthemum stem; (F) Intracellular P. horiana hypha growing within an asymptomatic chrysanthemum stem; (G) Germinating P. horiana teliospores erupting through chrysanthemum leaf epidermis; (H) Germinating P. horiana teliospores erupting through chrysanthemum leaf epidermis. P. horiana hypha grown in a symptomatic chrysanthemum stem

Figure 2.4. I. Germinating P. horiana teliospores erupting through chrysanthemum leaf epidermis germ tube emanating from teliospores; (J) Germinating P. horiana teliospore with initial germ tube; (K) Anastomosis occurring between hyphal strands of P. horiana; (L) Intracellular P. horiana mycelia with M haustoria as viewed under compound microscope. P. horiana teliospores erupting through chrysanthemum leaf epidermis germ tube emanating from teliospores

Figure 2.5. M Basidiospores (a) germinating on promycelium (b) developing from teliospores; (N) Intracellular mycelium in association with chrysanthemum mesophyll cells; (O) P. horiana telia sori containing germinating basidiospores (a), conical shaped promycelium (b), teliospore pedicles (c) and teliospores (d); (P) Intracellular P. horiana mycelium in chrysanthemum mesophyll cells (a, b) and xylem (c); germinating on promycelium developing from Teliospores

Figure 2.6. Germinating P. horiana basidiospores with elongating germ tubes

Figure 2.7. P. horiana mycelium within xylem in chrysanthemum stem

Figure 2.8. A. Promycelium developing from both apical and basal cells of a P. horiana teliospore B. Conical shaped sterigmata and P. horiana basidiospores at tips of promycelium

Figure 2.9. Q. Thin section of P. horiana sori with basidiospores; R. Thin section of P. horiana with basidiospores and germinating teliospores

Figure 3.1. Frequency of CWR among 10 chrysanthemum varieties, as rated from 0-3

Figure 4.1. Dissociation curve using unlabeled probe SNP mismatch, match and amplicon peaks

Figure 4.2. Harr’s Uni-Core tool used for collecting P. horiana pustules from herbarium samples

Figure 4.3. A. Dendrogram showing cluster relationship of 61 US Puccinia horiana isolates based on the Gower similarity measure and paired group algorithm
method using 32 SNPs and 1 SSR. B. Alleles for the 32 SNPs and 1 SSR for each isolate. C. Mean probability for each isolate assigned to 6 populations (K=6) based on 20 Markov chains determined with STRUCTURE program analysis.

Figure 4.4. Rendered tree illustrating relationship of 61 United States isolates of *P. horiana*.

Figure 4.5. A. Dendrogram showing cluster relationship of 101 *Puccinia horiana* isolates based on the Gower similarity measure and paired group algorithm method using 32 SNPs and 1 SSR. B. Alleles for the 32 SNPs and 1 SSR for each isolate. C. Mean probability for each isolate assigned to 5 populations (K=5) based on 20 Markov chains determined with STRUCTURE program analysis.

Figure 4.6. Rendered tree illustrating the relationship of 101 worldwide isolates of *P. horiana*. The United States isolates are highlighted in yellow.

Figure 4.7. A. Anastomosis occurring between hyphal strands of *P. horiana*; B. Schematic illustration of recombination in *Puccinia horiana* (A) “Tip-to-Toe” anastomosis as also described by Wang and McCallum (2009) between vegetative mycelium or germ tubes from two different genotypes (illustrated with black versus white nuclei) of *P. horiana* eventually results in heterozygous mycelium with two haploid nuclei. (B) During the telial stage karyogamy occurs with a rearrangement of the chromosomes resulting in a recombinant genotype (illustrated with gray nuclei). After the second somatic meiotic division in the promycelium, two identical haploid nuclei migrate in each of the two basidiospores, which give rise to homozygous recombinant mycelium. The nuclear status is indicated for each stage in the rectangular insets: c represent the number of chromosomes, n represents the ploidy number.

Figure 4.8. Distribution of 61 *P. horiana* isolates and 6 genotypes within 8 US States.
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Chapter 1

Literature Review: Chrysanthemum White Rust caused by *Puccinia horiana* - An American Perspective

**ABSTRACT**

Chrysanthemums (mums) are perennial flowering plants that were illustrated in Chinese literature as early as the 15th Century B.C. and have been cultivated as an herb for over 3000 years. (Hackett, 2013) Chrysanthemums arrived in Japan during AD386 where, within a short period of time, highly prized cultivars were developed (NCS, 2013). Chrysanthemums were introduced to Europe via France during the 17th century. During 1753, Linnaeus combined the Greek words “chrysos” (gold) and “anthemom” (flower) for describing illustrations of the small, yellow daisy-like flowers. Mums were brought to North America during colonial times, as well as to Africa, Australia, Central America, and South America (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013). Since the earliest recognition of chrysanthemums in China, popularity of this flower has continued to grow throughout the world. Today, due to their easy cultivation, ability to bloom on time, variety of long lasting flower form and color, the chrysanthemums is one of the most commercially valuable flowers in the United States. While some European countries associate chrysanthemums with funerals, in the United States they are considered “Queen of the Fall Flowers” (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013).

Chrysanthemum White Rust (CWR), caused by *Puccinia horiana* Henn., is an autoecious, microcyclic rust that is pathogenic on many chrysanthemum species
(Chrysanthemum spp.) and close relatives within the Asteraceae family. Chrysanthemum white rust is economically important due to its ability to infect florist chrysanthemum (Chrysanthemum × morifolium) cultivars used as cut flowers and potted plants throughout the world (Baker, 1967; Dickens, 1990; Firman and Martin, 1968; Horst and Nelson, 1997). The first detection of CWR in the United Kingdom occurred in 1963 on chrysanthemum plants in a nursery in Essex, England. During 1964, more than 50 CWR interceptions occurred on cuttings arriving into England, Denmark, Norway, and Holland from South Africa (Baker, 1967). CWR was first intercepted in the United States during 1977 in New Jersey and Pennsylvania (Nelson and Longenecker, 1978; Nichols, et al. 1978; Peterson et al., 1978; Seymour, 1977). Puccinia horiana has since been reported in most regions where chrysanthemums are commercially produced (EPPO/CABI, 2004; Water, 1981).

Puccinia horiana can cause serious damage to chrysanthemums in commercial greenhouses as well as at homeowner sites (Anon, 2010). Many countries, including the United States, have established Phytosanitary Quarantines against CWR, due to financial loss and difficulty of eradicating CWR (USCFR, 2012). Official USDA APHIS interception reports for the United States indicate that although eradication has been attempted, unconfirmed observations suggest that the rust pathogen may be able to overwinter in volunteer plants (Kim et al., 2011). In fact, “CWR is known to overwinter in Europe where chrysanthemums overwinter (average minimum temperatures ranging from -10°F to 10°F)” (Anon, 2010).

Taxonomy of chrysanthemums (Chrysanthemum spp.)
Historically, the taxonomic classification of chrysanthemums has changed numerous times. Various chrysanthemum species generally have been placed into two genera, *Chrysanthemum* and *Dendranthema*. The most recent change, determined in 1991 by the International Code of Botanical Nomenclature, states that “*Chrysanthemum* is now conserved in the sense of *Dendranthema*” (USDA ARS GRIN, 2013). The same decision established the genus *Glebionis*, which is composed of species once placed within *Chrysanthemum*, including *G. carinata* (syn. *C. carinatum*) = tricolor daisy, *G. coronaria* (syn. *C. coronarium*) = crown daisy, and *G. segetum* (syn. *C. segetum*) = corn marigold. Also segregated were the genera *Argyranthemum*, *Leucanthemopsis*, *Leucanthemum*, *Rhodanthemum*, and *Tanacetum* (USDA ARS GRIN, 2013). Many hybrids of chrysanthemums have been developed. “The most important hybrid is *Chrysanthemum × morifolium* (syn. *C. × grandiflorum*), derived primarily from *C. indicum* but also involving other species” (Hamilton, 2013). Table 1.1 illustrates the current taxonomic classification of chrysanthemums (USDA NRCS Plants Database, 2013).

**History of chrysanthemums**

Chrysanthemums were grown in China as an herb as early as the 15\(^{th}\) century BC. Confucius documented mum cultivation around 500 BC. Known as “Chu,” mums, believed to provide the power of life, were used for medicinal and culinary as well as decorative purposes. On ancient Chinese pottery, chrysanthemums appear similar to some varieties grown today, since modern day hybrids utilizing *C. indicum* and *C. sinense* can trace parentage back to early China. In Chinese and East Asian art, mums represent autumn as one of the “Four Gentlemen” plants used to depict the seasons of the year. The
city of Chu-Hsien (Chrysanthemum City), China was named in honor of chrysanthemums (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013).

Chrysanthemums were introduced to Japan in the 8th century. By the 9th century, the Japanese Emperor Uda had cultivars grown specifically for the Japanese Imperial Gardens, some of which are still used in present day exhibitions as well chrysanthemum flower shows. The Japanese embraced the chrysanthemum in many aspects of their life and included a single flower variety called "Ichimonjiginu" as the official crest and seal of the Emperor. The sphere in the Japanese flag is actually the heart of a chrysanthemum despoiled of its petals, not the rising sun as often thought (Flores del Oriente, 2013).

Prominent Japanese families continue to use chrysanthemums called “Kikumon” as part of their crest ("Kiku" means chrysanthemum and "Mon" means crest in Japanese). The Imperial Order of the Chrysanthemum is the highest Order of Chivalry in Japan and a Festival of Happiness celebrates National Chrysanthemum Day (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013).

Chrysanthemums were not referenced in Europe until 1689 when the botanist Bregnius noted their introduction. In 1753, the Swedish botanist Karl Linnaeus, combined the Greek words χρυσός chrysous, for “golden” color and ἄνθεμον -anthemon, meaning “flower”. The word accurately described ancient species that, according to illustrations and notations, were small in size with yellow daisy-like flowers. Although many introductions were made from Asia, it was more than 100 years until chrysanthemums became successfully cultivated in Europe. Pierre Louis Blancard returned to France from China in 1789 with three cultivars; but, only one, known as “Old Purple,” survived. According to the Botanical Magazine of 1796, cuttings from the one survivor were
eventually brought to Kew Gardens in London, England. The Old Purple variety was the first large flowering chrysanthemum known in modern times. The Horticultural Society of London encouraged growers to import additional cultivars from Asia. By the early part of the 19th century, chrysanthemums had quickly become one of the most popular flowering plants in Europe. Between 1798 and 1808, eight new varieties were imported into England, and in 1802, a sport from Old Purple was discovered. During 1823, an additional 17 varieties were introduced from Asia, and by 1822, more than 40 additional varieties were imported. Over the following years, the number of varieties continued to grow. However, until 1824, chrysanthemums were identified by their flower form and color as the translation of the Chinese varietal names were not always clear (Barlow, 2013; Hackett, 2013; NCS, 2013; Payne, 1892; Smith, 2013).

After many failed attempts, chrysanthemum seed was produced in England in 1827. During 1836, an amateur grower from Jersey, the Channel Islands, United Kingdom, put considerable time and effort into producing chrysanthemum seedlings. His endeavors succeeded, resulting in marked improvements in the chrysanthemum varieties he produced. During 1838, John Salter, an English seed distributor known as the “Father of the Chrysanthemum” (Hibbard, 1890), moved to Versailles, France where the weather was considered conducive to establish a chrysanthemum nursery. By 1840, he was cultivating 300 to 400 varieties and by 1860, his collection contained more than 750 varieties. Amateur gardeners in Portugal, France, Ireland and the United States also developed new varieties from seed, but, according to Shirley Hibberd of the Royal Horticulture Society of London, the best growers in the world “…have never yet equaled the natives of Japan in their culture of this flower from seed.” (Barlow, 2013; Hackett,
2013; Hibbard, 1890; NCS, 2013; Payne, 1892; Smith, 2013). The important dates and authorities involved in production of chrysanthemums in the western world are shown in Table 1.2.

Chrysanthemums were introduced to North America during colonial times, but unfortunately, the dates and facts regarding this first introduction were not recorded. The 1937 USDA Yearbook of Agriculture (Emsweller et al., 1937) notes that in the 1828 William Prince catalog, John Stevens introduced a dark purple chrysanthemum to Hoboken, New Jersey from Europe in 1798. According to James Morton (Morton, 1891), “... a variety of the small yellow chrysanthemum was common in...” Virginia, North Carolina and South Carolina around 1811, but, it wasn’t as popular as in Europe. By 1835, Hovey’s American Gardener’s Magazine and Register noted more than 50 distinct varieties available in the United States. During this time, most chrysanthemums in the United States were propagated by cuttings, which reduced breeding of new types and may explain why so few varieties were available in this country. Although mums were not routinely grown in United States greenhouses until after 1860, Robert Kilvington exhibited the garden variety “William Penn” at the Pennsylvania Horticultural Society in 1841. The first record of new seedlings being produced in the United States was in 1879 by Dr. H.P. Walcott, Massachusetts, who exhibited his new varieties at the Massachusetts Horticultural Society show, but they received little attention. Nevertheless, Dr. Walcott helped to improve the nomenclature of chrysanthemums by assigning short common names to his varieties (Barker, 1895; Emsweller et al., 1937).

In 1886, John Thorpe, known for his chrysanthemums in both the United States and Europe, wrote “How to Grow Chrysanthemums,” the first book about
chrysanthemums published in the United States (Barker, 1895). During 1889, Thorpe helped establish the National Chrysanthemum Society of America and became its first president.

By 1891, chrysanthemums were grown throughout the United States. Due to the conducive climate in southeastern Pennsylvania, most chrysanthemums were produced near Philadelphia. Although many chrysanthemums were also produced in Indiana and California, Morton reported that “…the chrysanthemum raising section of America does not extend over an area of two hundred square miles” from Boston, Massachusetts, including parts of New York and New Jersey, to Philadelphia (Morton, 1891).

Due to easy cultivation, quality, and diversity of its long-lasting blooms, popularity of chrysanthemums has steadily grown over time. Chrysanthemums can be trained to develop into different growth forms such as bonsai or cascading fans. An artificial classification system, based on bloom and growth type patterns, is also used to categorize and identify mums. The 2012 edition of the United States National Chrysanthemum Society Classification Handbook contains “A comprehensive list of cultivars including class, size, color, response and year introduced, plus a separate list of 2012 introductions and classification changes” (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013). While Americans consider mums the “Queen of the Fall Flowers,” Europeans consider mums the “Death Flower” due to its extensive use in memorial gardens on graves (Hackett, 2013; NCS, 2013).

Whereas perennial varieties are capable of overwintering in the ground in northern locations, “Exhibition Varieties” are used for florist arrangements and as prized display plants and blooms at garden exhibitions and are not winter hardy. Hardy garden varieties
require little maintenance while producing an abundance of small blooms. Exhibition Varieties require considerable maintenance, including artificial lightning and pruning. However, they are known for their showy, larger blooms, spray forms, topiary, bonsai, cascades and standard tree forms (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013).

Chrysanthemums not only are utilized for aesthetic value, they can also be used as comestibles due to their “soft and refreshing taste” (http://www.cultural-china.com/chinaWH/html/en/Kaleidoscope2197bye5707.html) in addition to their sweet aroma. Mums can be eaten in a variety of ways including fresh, dry, raw or cooked. In China, Chrysanthemum tea has been used as a medicinal herb for thousands of years. In addition to garden varieties being used in various foods, Ye Juhua (Wild Chrysanthemum), with its sweet aroma but bitter taste, can be used in teas, extracts, soups (Figure 1.1) and broth decoctions. In China, chrysanthemums commonly grow wild on hillsides, in bushes, or by the road. For more details on edible chrysanthemums, go to: http://www.cultural-china.com/chinaWH/html/en/Kaleidoscope2197bye5707.html.

Chrysanthemums have the unique distinction of being associated with Shih Tzu dogs (Figure 1.2). Lady Brownrigg brought the first Shih Tzu to England from Peking, China during 1928. Shih Tzu dogs became known as the Chrysanthemum Dog when the breed’s face was described by Lady Brownrigg as looking “like the chrysanthemum flower, with hair sprouting in all directions like flower petals” (http://dogtime.com/dog-breeds/shih-tzu).

Pyrethrum, the dried flower head of *Chrysanthemum cinerariifolium* (syn. *Pyrethrum cinerariifolium*, *Tanacetum cinerariifolium*), as well as *Chrysanthemum coccineum* (syn. *Pyrethrum coccineum*, *Tanacetum coccineum*), produces the active
insecticide ingredient pyrethrin. The earliest known record of pyrethrum use was more than 2000 years ago during the Chou Dynasty in China. The flowers were grown in the Dalmatian region and then traded along the Silk Road. French soldiers, during the Napoleonic Wars (1804 to 1815), controlled fleas and lice by making “Dalmatian Flea Powder” from crushed flower heads. Pyrethrum was initially brought to the United States in 1860 as a powder. In 1881, pyrethrum production was introduced to England from Japan, who became the major supplier of the flowers during World War I. Production of the flowers was brought to Kenya in 1928. By 1940 Kenya and other neighboring East African countries dominated the production of pyrethrum extract. Between 1990 and 1998, Australia, using modern agricultural practices, started supplying crude pyrethrum extract for refining. Supplies of refined pyrethrum dropped off dramatically from Kenya during 2006 to 2008 due to adverse climatic conditions, economic and social issues, allowing Australia to become the largest supplier. Today, self-sustaining pyrethrum production occurs in Australia, East Africa, and Papua New Guinea (Greenhill, 2007; Kumar et al., 2005; http://www.pyrethrum.com/About_Pyrethrum/History.aspx).

Economical value

Today, chrysanthemums are one of the most commercially produced flowers and the most widely grown potted plants. Cut flowers, grown in plastic tunnels or greenhouses, are produced in Japan, the Netherlands, Italy and Colombia (De Backer, 2012; Spaargaren, 2002). Potted plants are grown in greenhouses as spray flowers or disbudded to only one flower per stem. Garden mums are grown in greenhouses or fields (Anderson, 2006; De Backer, 2012). Figure 1.3 illustrates the major countries that commercially
produce chrysanthemums.

In Japan, production of cut chrysanthemum flowers exceeded 2 billion stems per year during 1993 (Anderson 2006, De Backer, 2012). In Europe, Flora-Holland ranked chrysanthemums second after roses in 2012, with a total of 1,154 million stems, and potted chrysanthemums ranked as fifth with 41 million pots sold (Flora-Holland, 2012). During 2012, the USDA listed chrysanthemums as one of the top ten horticultural crops grown in the United States with a wholesale value of $14,156 million for cut flowers, $21,548 million for flowering potted plants and $123,655 million for garden chrysanthemums (USDA NASS, 2014). Table 1.3 shows the value of chrysanthemums in the United States during 2011 and 2012 from data collected for hardy/garden varieties, potted patio and florist varieties, and pom-pom varieties from 15 program states.

**Commercial chrysanthemum production**

Most commercial chrysanthemums crops are started as unrooted cuttings in off-shore production facilities in Africa and South America. Unrooted cuttings produced in South Africa, Tanzania, Uganda, Kenya and Ethiopia are usually destined for the European market, whereas 70% of those produced in Costa Rica, Guatemala, Honduras, El Salvador, Mexico and Brazil are shipped to rooting facilities in the United States. Table 1.4 lists the top ten cutting producers in 2009, as well as quantity of chrysanthemums produced.

Off-shore producers who export chrysanthemum cuttings to the United States are required to follow a disease and pest certification program by the USDA. After 6-month post entry quarantine, the unrooted cuttings are grown into either rooted pre-finished plugs or finished crops at wholesale facilities. Rooted pre-finished plugs are distributed to
other wholesale growers or commercial producers, where they are then potted and grown into full size plants. During 1995, the Yoder Brothers facility in Pendleton, South Carolina, reported selling over 52 million cuttings annually (Pertuit, 1995).

**Chrysanthemum breeding**

The center of origin for all Chrysanthemum species used in breeding is China, but the centers of diversity are primarily China, Japan, and Korea in Asia, as well as Algeria and the Canary Islands in the Mediterranean region. Numerous species have spread from China and Japan to areas across Eurasia. New species were introduced to the Americas as exotics, with the exception of *Tanacetum* spp., *Dendranthema arcticum*, *D. arcticum*, *D. angustifolium*, *D. camphoratum*, and *D. cespitosum* (Anderson, 2006). The majority of chrysanthemum species are herbs while the rest are perennial plants (Anderson, 2006; Dowrick, 1952).

Chrysanthemums were first cultivated in Japan around 386 A.D. when they arrived via Korea from China. At that time, chrysanthemums grown in gardens were most likely a “single many-flowered variety similar to *C. indicum*,” which still grows naturally in China, Japan, and Korea (Dowrick, 1953). The original small, yellow daisy-like flowers were produced on perennial herbaceous plants, which, over time, have been cultivated and hybridized into many colors, and, shapes and can grow in excess of 4 feet tall.

In Europe, a small flowered variety was introduced into the United Kingdom from the Netherlands during 1754. Although this variety was lost, the larger flowered variety “Old Purple” was later introduced during 1790 (Payne, 1892; Hibbard, 1890). This variety
of *C. indicum* is similar to many present day varieties. By 1826, after more introductions of chrysanthemums from China had occurred, 48 varieties were being grown. Although bred in China and Japan centuries before being brought to Europe, the first recorded hybridization and selection of “superior seedlings” wasn't published until 1827 by M. Bernet (Emsweller, et al., 1937). By 1827, new forms, hybridized from the original 48 varieties, were developed and gave rise to the incurved types grown today. During 1832, the first vegetative sport of chrysanthemum was recorded. Figure1.4 shows “Pom-pom” and “Incurve-type” chrysanthemums.

Between 1843 and 1846, Robert Fortune imported two “small flower” varieties to England, termed “Pom-pom” and “Chusan Daisy.” These two small flower types were not well received until they were brought to France where breeders improved these varieties as well as others. Since then, many other varieties have been introduced and bred around the world; many hybrids grown today can be traced back to improved cultivars developed in France during the late 1800s (Anderson, 2006; Dowrick, 1953; Jones, 1967).

As garden chrysanthemums became popular in the United States, breeding programs started including varieties developed by Alex Cumming, Jr., Yoder Brothers, Inc., and the New York Botanical Garden. Alex Cumming, Jr., a chrysanthemum breeder located in Bristol, Connecticut, bred garden-variety chrysanthemums during the early 1900s, helping to develop and increase the United States germplasm stock. A private breeding program developed by Yoder Brothers, Inc. in Barberton, Ohio, enhanced Cumming’s efforts. During the same time, other germplasm was collected and stored at the New York Botanical Garden. The United States Department of Agriculture in conjunction with many Land Grant Universities conducted active breeding programs
during the early 1900s. By 2004, the only remaining public sector breeding program remaining in the United States was located at the University of Minnesota, St. Paul.

Although most present day varieties of chrysanthemums can be traced back to varieties grown in Japan over 1000 years ago, ancient growers might not recognize the wide range of floral colors, shapes, and sizes of chrysanthemums grown today due to extensive cultivation and hybridization (Dorwick 1953). Some modern varieties still resemble daisies, while others, such as the “Pom-Pom” varieties, are showier. Flower colors now include pink, purple, red, bronze, orange, and white, as well as yellow. In addition, some varieties have been developed to include different colors between the disc and ray florets while others have bi-colored ray florets (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013). Over time, breeding and cultivation resulted in changes including the development of three types of florets. From the original small, five-tooth disc floret, a broad flat ray floret, an elongated, quilled or tasseled floret and an anemone-centered floret were developed (Dorwick, 1953).

Chrysanthemum blooms, although they appear to be a single flower, are comprised of many single small florets. Chrysanthemums have two types of florets. Ray florets, where the outer parts of the bloom are the florets, are found on daisy-type mums. Disc type florets are those where the center of the bloom is composed of the florets. Both types of florets are found in all chrysanthemum classes. Disc florets, which contain both male and female parts, are the only structures able to reproduce and produce seed. However the disc florets are not always apparent, so breeders must uncover them for pollination to take place (http://www.mums.org/history-of-the-chrysanthemum). Other changes due to early breeding and cultivation efforts include atrophy of the male sex organs involving the
reduction of the anther size. In other varieties, only the male filaments remains, while in
still other varieties, the filament has completely disappeared (Dowrick 1953).

The ancestry of most garden type chrysanthemums is not known. While
researching various chrysanthemum species in relation to chromosome number, Dowrick
(1952) reported two main centers of diversity within the *Chrysanthemum* genus, one
center including China and Japan and one in the Mediterranean area including Algeria and
the Canary Islands. While most of the species found in the Mediterranean area are annuals
and diploid, the species found in Asia are mostly perennial and polyploid, while Siberian
species are entirely polyploid. This discovery led Dowrick to theorize that polyploidy in
the various chrysanthemum species was positively related to increase in northern latitude
(Anderson, 2006; Dowrick, 1952). Dowrick (1952) also determined that an increase in
chromosome number in polyploid species was associated with a decrease in chromosome
size (Dowrick, 1952). Figure 1.5 shows the distribution of chrysanthemum species studied
in relation to chromosome number. The majority of diploid species occur in the
Mediterranean region. A higher degree of polyploidy is found in China and Japan. The
European species are probably younger then those found in Asia (Dowrick, 1952).

Chrysanthemums reproduce in the wild asexually, via emergent and non-emergent
rhizomes in perennial species, or sexually as outcrossing species. In commercial
production, new garden and florist cultivars of chrysanthemums can be developed
sexually from seed or asexually from bud sports collected from vegetatively propagated
plants. Bud sports occurring from one cultivar can result in multiple varieties. The new
bud sport is a chimaera, which can revert to the previous/parental type. Some varieties
may continue to produce additional new bud sports, while others may remain stable for
years. Some varieties, after years of stability have produced new bud sports, so it is possible that all varieties are capable of producing chimaeras (Anderson, 2006; Dowrick, 1953).

In plants, self-incompatibility (SI) forces outcrossing which promotes the generation of new genotypes. In diploid plants, a single gene controls self-incompatibility, while polyploidy plants may have additional genes controlling self-incompatibility. Self-incompatibility was first reported in cultivated chrysanthemums in 1931 by Niwa (Anderson, 2006). Hexaploid chrysanthemums possess three genes that control self-incompatibility (Boase et al., 2010; Drewlow et al., 1973; Zagorski et al., 1983). Self-incompatibility occurs throughout all species and all ploidy levels in chrysanthemums. However, “genetic analysis of cultivated chrysanthemums revealed the lack of pollen (trinucleate) germination or stigmatic inhibition of pollen tubes and reciprocal crossing differences, indicating the existence of a sporophytic SI system” (Anderson, 2006; Drewlow et al., 1973).

Additional research conducted by Dowrick (1953) demonstrated that ancestral species of garden Chrysanthemums had chromosome numbers of 2n=6x=54, while modern varieties had chromosome numbers ranging between 47 to 63. He determined a correlation between inflorescence size and the chromosome number in both Japanese and English varieties. Dowrick also determined that variation in chromosome number within plants helps to determine origin of new varieties and vegetative sports, and that the amount of variation can also differ due to growing conditions. He demonstrated that varying chromosome numbers within families of sports could affect flower color, leaf shape and resistance to disease. Unfortunately, the similarity of chromosome size and
forms between some garden-variety species of chrysanthemums that Dowrick studied, reduced his ability to accurately determine ancestry. His research was further complicated due to extensive breeding and cultivation resulting in modern garden chrysanthemum varieties not resembling ancestral garden varieties (Dowrick, 1953).

According to Dowrick (1952), *C. indicum*, due to its yellow flowers, is the species most likely involved in chrysanthemum ancestry, but he was not certain if it was the only species involved. Dowrick (1953) reported that Hemsley, in 1889, considered *C. sinense* (*morifolium*), due to its vigorous growth, tomentose leaves and ray florets color difference to the disc, as a likely ancestor, and that Stapf, in 1933, considered ancestry probably included hybridization of *C. erubescens*, *C. ornatum*, *C. japonense* and *C. makinoi* species. All of these species are hexaploid (2n=6x=54) (Dowrick, 1953). Today, most garden and florist type chrysanthemums, bred for traits including flower color, shape, and disease resistance, are also “allohexaploid (2n=6x=54) with somatic chromosome numbers ranging from 2n=47-63” (Anderson, 2006).

Today, the most economically important chrysanthemum hybrid is *Chrysanthemum x morifolium* (syn. *Dendrathema x grandiflorum*). This hybrid was developed from crosses derived from 10 or more hexaploid species, but mainly including *C. indicum*, *C. japonicum*, and *C. sinense*. Wild populations of *C. morifolium* (syn. *D. grandiflorum*) with a single white, yellow or pink daisy like flower grows in Japan and China (Anderson, 2006; Crook, 1942). A double flower form appeared as early as 910 AD, which Crook attributed to either spontaneous mutation, or as the result of a directed breeding effort. Over time, several other species were integrated into the *C. morifolium* gene pool for genetic improvements such as winter hardiness, stronger stems and earlier flowering.
Unfortunately, the species used for hybridization are all susceptible to *Puccinia* species that cause rust diseases (De Backer, 2012; Dowrick, 1953).

Today, commercial chrysanthemums cultivar gene pools are hybridized for several traits attributed to specific genes not yet mapped on chromosomes (Anderson, 2006; De Backer, 2012;). Hybridization is often performed between garden-type chrysanthemums and florist-type chrysanthemum cultivar gene pools. However, hybridization becomes difficult as gene pools diverge. Desired traits include novel color, flower form, plant architecture, flowering time and disease resistance. Crosses can be accomplished in either direction but, unfortunately, undesirable traits are often transferred into new cultivars due to linked genes. In order to reduce the transfer of negative traits, “recurrent or congruity backcrosses of the hybrids with inbred or non-inbred parents” must occur, and florist and garden chrysanthemums demonstrate increased inbreeding depression after repeated self-pollination (Anderson, 2006). Due to self-incompatibility and inbreeding depression, breeding is accomplished via outcross pollination (Anderson et al., 1992; Anderson and Ascher, 2000). De Backer (2012) reported “mutation breeding using gamma or Rontgen radiation is performed to obtain cultivars that differ only in flower color.” After breeders develop new cultivars they are vegetatively propagated.

While florist chrysanthemums are related to hexaploid Asian species, English garden botanical species are mostly diploid (De Backer, 2012; Dowrick, 1952) Dowrick reported the basic chromosome number for chrysanthemums as nine (2n=6x=54). Dowrick also reported varying degrees of polyploidy within eight species of chrysanthemums, with most having 54 to 56 chromosomes and a peak of 2n=54.
According to Dowrick (1953), somatic chromosome numbers in different cultivars can range from $2n = 47$ to 63 between and within plants of both hexaploid and heptaploid types. Dowrick determined that an “increase in the chromosome number in the polyploidy series is accompanied by a decrease in the chromosome size”. He also reported a correlation between the chromosome number and inflorescence size (Dowrick, 1953). Dowick further suggested that variation in chromosome number accounted for origin of new varieties as vegetative sports and that the amount of variation differs among varieties, and that the variation in chromosome number also facilitates phenotypes determination, as well as the occurrence of visible sports in certain cultivars.

Meiosis in hexaploid species is complex compared to meiosis in diploid species. In chrysanthemums, meiosis is similar to diploids with 54 chromosomes where chrysanthemums form pollen by way of 27 bivalents and 27 chromosomes. The unbalanced bivalents form a minimum of irregularity because they do not form trivalents (Dowrick, 1953). Bivalent formation is associated with preferential pairing of homologous chromosomes during meiosis (Watanabe, 1977, 1983).

DeJong and Radenmaker (1986) reported on the segregation of $P. \text{ horiana}$ resistance in chrysanthemums. They describe four types of reactions resulting in three types of resistance; incomplete resistance with pustules that develop slowly, necrosis inhibiting spore formation, and susceptible plants that sporulate profusely. Although their results suggest a single dominant gene confers resistance, deviations from expected results did occur. These deviations were explained as the result of other components combined with monogenic resistance or possibly other components affecting the growth of the pathogen on the plant. Although their results appear to fit expected ratios of
preferential pairing, they also suggest that a combination of two pairing types can occur.

Langton (1988) observed hexasomic inheritance of carotenoid pigments in chrysanthemums, but his studies were not conclusive. His data regarding segregation of hexasomic inheritance in chrysanthemums better supports a single active zygomere, as theorized by Watanabe (1983), based on cytological studies. This aspect indicates the possibility of a marked homology that was present or developed between chromosomes of identical karyotype groups in ancestral species. Watanabe (1977) reported circumstantial evidence of such homology. Prior to Langton’s report, polysomic inheritance and bivalent formation had only been observed in a few species (Langton, 1989). DeJong and Rademaker’s result conflict with Langton’s, which may also indicate that two types of pairing may occur during meiosis. During 2012, De Backer reported the inheritance of resistance to *P. horiana* in chrysanthemums is much more complex than previously thought. De Backer’s study determined preferential pairing dominates during meiosis but random pairing could not be excluded. He suggests further studies of modulator genes in order to clarify their mode of action. He reported different dominant resistant genes located on a minimum of two loci modify resistance to pathotypes, which results in diverse resistance segregations for various pathotypes. As reported by Friedman and Baker (2007), clusters of resistance genes can have ramifications during co-evolution of host and pathogens by aiding the transformation of novel resistant susceptible phenotypes due to rearrangement of resistant genes. This suggests new phenotypes and resistant cultivars be integrated into bioassays for the selection of improved resistance. Development of specific markers for resistance genes would greatly aid selection of cultivars containing specific resistance genes already incorporated, allowing optimization
of chrysanthemum breeding.

**Commercial production of chrysanthemums**

There are production differences among chrysanthemum varieties grown as garden mums, potted plants, and as cut flowers. Before 1850, chrysanthemums were produced outside in fields. After 1850, breeding and selection of chrysanthemums for cut flower vs. garden varieties separated production into two types, field or greenhouse production. By 1894, more than 160 greenhouse cultivars had been developed (Anderson, 2006; Emsweller, et al. 1937). After the discovery of photoperiodism (1920) and its effects on flower initiation and development, numerous chrysanthemum breeding programs flourished as chrysanthemums could now be programed to flower year-round (Anderson, 2006).

All chrysanthemums require approximately 9.5 hours of non-interrupted dark night length in order to change from a vegetative mode to a reproductive mode. The flower buds, once initiated, then require at least 10.5 hours of night in order to develop. Chrysanthemum plants contain phytochrome, a blue, proteinaceous pigment that has two forms existing in the plant concurrently. Each form is able to convert to the other type. The quality of light determines which phytochrome pigment is most abundant. One form, known as “PR”, absorbs red light while the other form, known as “PFR”, absorbs far red light. If PFR absorbs far red light, it changes to PR, whereas if PR absorbs red light it changes to PFR. PFR is not stable and after 4 hours of darkness, automatically starts to convert to PR. PFR is considered the dominant active form of phytochrome because it inhibits the plant’s ability to initiate flowers. The reaction that triggers the plant to change
from the vegetative to reproductive happens after about 7.5 hours of non-interrupted darkness. Sunlight has essentially the same affect on phytochrome as red light, but artificial incandescent or fluorescent lighting can also be used to manipulate these pigments (Pertuit, 1995).

Commercial production of chrysanthemums in greenhouses began with cultivars that grew within 8 to 14 weeks in response to short days. Although commercially grown chrysanthemum plants are perennial, not all varieties are able to overwinter. Spaargaren, as cited in Van Der Ploeg and Heuvelink (2006) determined that in colder climates, year-round production could be achieved in greenhouses if temperatures were maintained between 18 to 20°C. Spaargaren also noted that flower production, initiated when daylight is less then 11 hours, and timing of flowering, which ranges between 6 to 11 weeks, depends on the cultivar (Anderson, 2006; De Backer, 2012).

Commercial garden chrysanthemums, the most popular type grown in the United States, are grown in either greenhouses or fields. Most garden cultivars are varieties that are produced under a 6 to 8 week, short-day response program and are sold as small plants during the spring or full grown during the autumn (Anderson 2006).

**Chrysanthemum diseases and insect pests in the United States**

Chrysanthemums are relatively trouble-free if grown in full sun, with fertile well-drained soil, and with adequate watering. However, commercial chrysanthemum production can be adversely impacted by diseases and insects, although plant vigor can be maximized using appropriate cultural practices as a disease and insect pest management strategy. Important insect pests (Table 1.5) in the United States include Chrysanthemum
aphids (*Macrosiphoniella sanborni*), two-spotted spider mites (*Tetranychus urticae*) and chrysanthemum leafminer (*Phytomyza sygenesiae*) (Pertuit, 1995). Control of these insects is generally achieved with pesticides, predatory insects, and sanitation.

In addition to insects, many diseases affect commercial chrysanthemum production in the United States (Table 1.6). Based on samples submitted for diagnosis to The Pennsylvania State University Plant Disease Clinic, bacterial blight (*Erwinia chrysanthemi*) on cuttings in greenhouses, bacterial leaf spot (*Pseudomononas cichorii*) on outdoor-grown potted plants, and root rot (*Pythium* spp.) on greenhouse-grown potted plants, are the three major chrysanthemum diseases in Pennsylvania (Moorman, personal communication). These diseases can be controlled with fungicides, sanitation and good cultural methods. Chrysanthemum White Rust, caused by *Puccinia horiana*, is an economically important disease of chrysanthemums in the United States and is classified as a regulated plant pathogen (USCFR, 2012).

**Rust fungi - general**

Many of the world’s most important plant diseases are caused by rust fungi. Comprised of approximately 7000 species in the monophyletic order Uredinales, rust fungi are specialized obligate plant parasites (biotrophs). Rust fungi tend to be very host specific. Many rust fungi infections result in leaf spots, some of which may become systemic. Many rust fungi appear on stems and leaves as orange, rusty colored spores erupting through the epidermis. Other rusts form galls or swellings (Agrios, 2005; Kolmer et al., 2009).

Diseases caused by rust fungi have been noted since antiquity. Aristotle (384–322
BC) recorded years of heavy damage due to rusts on grain crops caused by “warm vapors.” Theophrastus (371–287 BC) also recorded damage caused by rust on cereals. The Roman festival of “Robigalia”, celebrated on 25 April, was observed in order to please the rust god Robigo in the hopes of reducing the damage caused by rust. It was not until 1767 when Fontana and Tozzetti concluded that rusts were parasitic plants of grains and therefore, biological organisms (Agrios, 2005; Kolmer et al., 2009).

Many rusts have complex life cycles consisting of up to five spore stages (macro cyclic) occurring on two taxonomically unrelated hosts (heteroecious). Other rusts life cycles are simpler, consisting of two or three spore stages (microcyclic) on one host (autoecious). Different biologic forms of some rust species have the ability infect taxonomically different genera of various host plants. Other specific rust species, that are morphologically identical, are able to infect different genotypes of one specific host species (forma specialis) (Agrios, 2005; Kolmer et al., 2009).

All rusts produce basidiospores and teliospores. Macro cyclic, heteroecious rusts produce five spore stages on two taxonomically unrelated host, while autoecious, micro cyclic rusts produce two to three spore stages on one host. Demi-cyclic rusts do not have the uredinia stage, while other rust life cycles are comprised of only the uredinia stage and are asexual due to the loss of the alternative host. Many scientists now believe that the more complex the rust life cycle is, the more evolutionary primitive it is, whereas shortened life cycles are evolutionally advanced.

With rusts, in general, germinating basidiospores can directly penetrate the epidermis, or penetrate through stomata, and initiate perennial mycelium in a host. In general, botrophic fungi are believed to release low but sufficient levels of cell wall
degrading enzymes at the host penetration site. Colonization is often via intercellular mycelium with intracellular haustoria and resulting colonization may spread from stem to leaves and from stem to roots (Larous and Losel, 1993) Penetration by rust fungal filamentous haustoria through host cell walls also indicates enzyme degradation. During primary phases of rust life cycles, the intracellular filamentous structures appear similar to intercellular hyphae (Larous and Losel, 1993). However, there have been few detailed descriptions regarding distribution of rust fungal structures within infected host tissue.

The uredinia stage is often the most economically important stage, especially in agricultural crops. Urediniospores are often disseminated by wind and rain. After landing on the host epidermis, if a film of free water is present, the spores absorb water, swell, and produce a germ tube. The germ tube elongates and forms an appresorium at a stoma, where it then forms a penetration peg that permeates the intercellular leaf space, prior to forming a substomatal vesicle. Infection hyphae develop from the substomatal vesicle, grow towards the mesophyll cells, and form a haustorial mother cell. A second penetration peg develops from the haustorial mother cell, which invaginates the extrahaustorial membrane and the host cell wall. Nutrients are absorbed across the extrahaustorial membrane. Seven to ten days later, mycelia forms sporogenous cells in the host tissue. Spore buds from these cells erupt though the host epidermis on which urediniopores form. Figure 1.6 illustrates the kind and sequence of spores and spore-producing structures in rust fungi and nuclear condition of each (Agrios, 2005).

Virulence in Puccinia spp. can occur due to three possible scenarios: sexual recombination, mutation and/or somatic hybridization due to anastomosis. Mutation, which a change of the nucleotide sequence of the genome of an organism, is thought to
be the major source of virulence in cereal rust as virulent phenotypes arising from the same clonal lineage of *Puccinia* species tend to vary in one or two attribute(s), but are otherwise genetically similar. It has been hypothesized that new races of cereal rusts can develop via mutation followed by selection of virulence against resistance genes already present in wheat cultivars (Wang and McCallum, 2009). Evidence of somatic hybridization has been demonstrated in various *Puccinia* spp. For example, *P. graminis* pathotypes 34-2, 11 was thought to originate via somatic hybridization between two pathotypes, 21-0 and 126-5, 6, 7, 11, due to nuclear exchange. The hybrid nature of *P. graminis* pathotypes 34-2,11 was demonstrated by analyzing the virulence and isozymic profiles of *P. graminis* pathotypes 34-2,11 and its possible parents. It has been hypothesized that somatic hybridization in cereal rusts is associated with anastomosis of hyphae prior to heterokaryosis or due to parasexuality and somatic meiosis (nuclear fusion and segregation).

**Rust fungi – specific: *Puccinia horiana*, causal agent of chrysanthemum white rust**

*Puccinia horiana* was first reported in 1895 in Japan (Hiratsuka 1957). The first taxonomic description of *P. horiana* was by Hennings in 1901, and was similar to the current taxonomy, as shown in Table 1.7 (Invasive.Org, 2012). The first report of *P. horiana* occurring outside of Asia was in 1963 when CWR was identified on chrysanthemum plants found at a nursery in Essex, England. The original introduction date could not be determined since the infected plants, hardy perennials identified as *C. sinense*, trade name “Konji-mum,” could only be traced back to one of several shipments of chrysanthemums arriving from Japan. Although eradication was attempted in 1964,
potentially infected cuttings were distributed throughout England and Wales, which may help explain the presence of infected plants during a CWR outbreak in Great Britain the following year. More than 50 additional interceptions occurred in 1964 on cuttings arriving from South Africa into England, Denmark, Norway and Holland (Baker 1967). By the late 1960s CWR was found in most regions where chrysanthemums were commercially produced, including Europe, Africa, Oceana, South America, and other parts of Asia (Barlow, 2013; Hackett, 2013; NCS, 2013; Smith, 2013).

In North America, CWR has been reported in the United States [NJ and PA (1977), OR and WA (1990), and CA (1991)], in Canada (1993) and in Mexico (1994). (Water 1981, EPPO/CABI, 2004). In 1977, a chrysanthemum hobbyist submitted a *P. horiana* infected chrysanthemum sample to Rutgers University for disease identification. After confirmation, additional infected cuttings purchased directly from Japan, were identified in “Exhibition-Type” varieties growing outdoors at 14 homeowner sites in NJ. Upon investigation, it was determined that CWR had spread among the sites due to the amateur growers exchanging infected rooted cuttings. Infected plants were also found at a homeowner site in PA in 1977. Because CWR was not considered to be systemic, eradication was attempted by simply cutting and removing the above-ground portion of infected plants. In 1978, during a subsequent survey, a new infection site was detected within a short distance of one of the original 14 sites in NJ. Infected plants were eradicated at the additional site and no further infections were identified (Nichols et al., 1978).

While interceptions of *P. horiana* occur annually at United States ports, until 2007 domestic interceptions remained sporadic [OR and WA (1990), CA (1991)]. After 2007,
concerns regarding possible changes within the biology of *P. horiana* surfaced, including its possible ability to overwinter in the United States, as reports of CWR interceptions dramatically increased. In 2007, CWR was identified at 40 commercial and residential sites in CA, CT, ME, MD, NY, and PA. In 2008, an additional 13 sites were identified in CA, DE, MA, and MI. Positive interceptions increased in 2009 when 16 locations were identified in NY and VA. California and MD reported 17 positive locations in 2010. In 2011, PA reported 139 CWR positive samples intercepted at 82 sites in 22 counties over 4 years. Increases in new interceptions of *P. horiana* combined with repeat infections identified at sites previously eradicated, lead to postulations that CWR may be able to overwinter in parts of North America (Anon 1992, 2003; Kim et al., 2011; NAPIS, 2013). The first confirmed report of *P. horiana* overwintering in Pennsylvania was reported in 2012 (OKeefe and Davis, 2012). Figure 1.7 illustrates the current worldwide distribution of CWR.

**Disease Cycle of *P. horiana***

*Puccinia horiana* morphology has been described in general terms by several authors (Firman and Martin, 1968; Kapooria and Zadoks, 1973; Punithalingam, 1968). *Puccinia horiana* is an autoecious, microcyclic rust and forms only two spore stages, teliospores and basidiospores. Figure 1.8 illustrates the CWR disease cycle and *P. horiana* life cycle.

Kapooria and Zadoks (1973) described the morphology and cytology of the promycelium and basidiospores formation of *P. horiana*, but presented only line drawings and images of low magnification images. EPPO/CABI (2004) presented more
detailed descriptions. Telia are white to pink, 2 to 4 mm in diameter, hard and compact, and can be distributed over an entire infected plant. Teliospores are 2-celled (apical and basal), oblong to oblong-clavate, slightly constricted, and form on pedicels up to 45 µ in length. Teliospores range in size from 30 to 45 x 13 to 17 µ with thin walls that are 1 to 2 µ at the sides and 4 to 9 µ at the apex (EPPO/CABI, 2004). Figure 1.9 illustrates teliospores of *Puccinia horiana* as drawn by Punithalingam (1968).

When conditions are optimal for germination (relative humidity (RH) > 96%, temperature 17 to 24°C), teliospores germinate without a period of dormancy, producing promycelia. Both apical and basal telial cells are capable of developing promycelia. However, promycelia usually arise from only apical cells. In vitro, the promycelium is a long, slender tube measuring 30 to 150 µ x 4 to 8 µ. (In contrast, developing promycelia in situ were short and thick, 33 x 8 µm in size). The promycelium usually forms septa that delineate 1 to 3 distal cells from which basidiospores develop (Kapooria and Zadoks, 1973).

Basidiospores at maturity can be oval, slightly curved, or broadly ellipsoid to fusiform in shape, measuring 7 to 14 x 5 to 9 µm, and are hyaline with a roughened wall. Initially, basidiospores have one or two nuclei, but become multinucleate with age (EPPO/CABI, 2004; Kapooria and Zadoks, 1973).

Basidiospores are disseminated 3 to 6 h after development by wind and/or water and are relatively short-lived. Under ideal conditions, basidiospores can be dispersed up to 700 m, but since ideal conditions rarely occur, the distance viable basidiospores travel is usually much shorter. It is likely that basidiospores remain viable for longer time periods at night than in the daytime. Teliospores are much longer-lived, and can survive for 8
weeks; however, shorter survival occurs if decomposition has started or if the teliospores are buried in soil (Coppock and Kreith, 1999). Upon landing on a susceptible host, basidiospores will germinate at RH > 70% and ≥ 5 h leaf wetness. The effect of temperature on spore germination is not clear, since germination has been reported to occur within the range of 6 to 36°C. Although germination does not occur at < 70% RH, spores can remain viable for an extended period at low RH (Water, 1981). Figure 1.10 illustrates a summary of the infection process of a *Puccinia* spp. into the plant epidermis layer. Teliospores and basidiospores also may be disseminated in and on asymptomatic, but CWR-infected, chrysanthemum cuttings (O'Keefe personal observations).

Critical parts of the disease cycle have not been reported. However, a film of water is likely necessary for resulting basidiospores to germinate. Kapooria and Zadoks (1973) summarized three patterns of basidiospore germination:

1. A germ tube of varying length that sharply tapers;

2. An appressorium-like vesicle which eventually develops an infection hypha;

and

3. A secondary basidiospore, although rare, can germinate by repetition.

Upon germination, basidiospores form a germ tube, and perhaps an appressorium, and penetration peg, which can penetrate leaves, flowers or stems, causing infection in as little as 2 h. Teliospores likely form a few days later (Baker, 1967; Firman and Martin, 1968; Kapooria and Zadoks, 1973; Zandvoort, et al., 1968). It is unknown if penetration occurs through the stomata or directly through the epidermis. Nevertheless, following penetration, initial colonization likely occurs via intercellular mycelia and intracellular haustoria. It is unknown if spermagonia are formed, but in other *Puccinia* spp.
“Anastomosis of vegetative mycelia at an early stage of basidiospore infection can occur (Lindfors, 1924; Walker, 1926)”. Thus, during karyogamy in the telial stage, rearrangement of chromosome can result in recombinant genotypes as reported by De Backer (2012) and Kohno et al. (1974, 1975) (Figure 1.11) However, many specific details of colonization were not reported.

Martin and Firman (1968) summarized four stages of CWR disease development as follows:

Stage 1. Development of germ tube and infection peg with no distinct difference between germ tube and appressorium;

Stage 2. Small fungal vesicle forms inside host epidermis;

Stage 3. Elongated fungal vesicle forms inside host epidermis; and

Stage 4: Septate fungal vesicle with branching hyphae forms.

The length of incubation period, defined as the period of time between penetration and appearance of first symptoms (Agrios, 2005), varies considerably among reports. Zandvoort et al. (1968) reported that the incubation period could be as short as 6 days at 17 to 21°C, increasing to 13 days at 10°C. They also reported the incubation period may be prolonged up to 56 days in the field if environmental conditions are not favorable for symptom development. Yamada (1956) reported a 10–day incubation period. Similarly, Stahl et al. (1964) determined the incubation period to be 12 days (at 15 to 20°C). However, Stark and Stach (1965) reported that the incubation period could vary from 10 days to 8 weeks.

Symptoms appear 7 to 10 days after penetration as pale green to yellow spots up
to 4 mm diameter on the adaxial leaf surface, presumably where air-borne basidiospores were deposited and initiated infection. The center of the spots becomes necrotic and sunken with age. Soon after the leaf spots appear, whitish/pink waxy telial clusters containing teliospores, form on the abaxial leaf surface, stems and flowers (Baker, 1967; Firman and Martin, 1968; Kapooria and Zadoks, 1973; Zandvoort, et al., 1968). Basidiospores form, and the life cycle repeats. However, in contrast, resistant chrysanthemum varieties may respond only with a hypersensitive reaction.

Infected, perennial chrysanthemums may overwinter, but the mechanism by which *P. horiana* overwinters is not fully understood. However, “CWR is known to overwinter in Europe where chrysanthemums overwinter (average minimum temperatures ranging from -10°F to 10°F” (Anon., 2010). In the United States, unconfirmed observations suggested that *P. horiana* may overwinter in Pennsylvania (Kim et al., 2011). In 2012, O’Keefe and Davis determined *P. horiana* was in fact able to overwinter in Pennsylvania in volunteer chrysanthemum plants (OKeefe and Davis, 2012).

**Detection of *Puccinia horiana***

Sensitive and specific detection protocols are critical for accurate diagnosis of fungal plant pathogens, including those that attack chrysanthemums. For quarantine pathogens, accurate pathogen diagnosis is essential in order to prevent ingress of new and economically important plant diseases such as CWR into the United States. Control measures also depend on accurate identification of the host plant. The first step in host identification is to properly identify the host genus, species, and variety, since not all chrysanthemum taxons are susceptible to *P. horiana*, and chrysanthemum varieties differ
greatly in morphological appearance.

Other rust fungi that attack chrysanthemum can cause symptoms that may be difficult to distinguish from CWR. For example, symptoms of basidiospore infection by the rust fungi *P. horiana* and *P. chrysanthemi* on chrysanthemums both appear as yellowish-green spots on the adaxial leaf surface, which are also similar to symptoms caused by insect feeding on chrysanthemum leaves. Morphological determination of the exact causal agent may be difficult without a microscope or other lab diagnostic techniques. For example, *P. horiana* is easily distinguished from *P. chrysanthemi* and several other rust species based on morphology of the sori and the teliospore (Punithalingam, 1968a,b). However, a major disadvantage of using morphology to detect *P. horiana*, is that fungal morphology cannot be used to accurately identify the pathogen within latent infections (Agrios 2005). That is, during the time period between infection and symptom development, colonization by rust fungi is primarily by intracellular mycelium. Identification of rust species using only mycelium is very difficult (Quilliam and Shattock, 2003). Another complication in developing diagnostic tests for *P. horiana* identification is the pathogen’s inability to grow on artificial media.

Although chlorophyll fluorescence image analysis, enzyme-linked immunosorbent assays (ELISA) and lateral flow devices (LFD) present possible options in *P. horiana* identification, their usefulness as routine diagnostics tool is limited (Alaei et al., 2009; De Backer, 2012). The development of polymerase chain reaction (PCR) protocols specific for *P. horiana* offers an expeditious as well as a definitive diagnostic tool. After DNA is extracted from an infected host, copies of the target DNA are amplified during a thermocyclic process involving repeated cycles of denaturation,
annealing of specific primers, and a final extension with a stable DNA polymerase (Mullis and Faloona, 1987). Minute amounts of the target DNA, as low as 10 fungal spores, can be detected using conventional PCR (Calderon et al., 2002a; Calderon et al., 2002b; Williams et al., 2001).

For conventional PCR, the PCR product is visualized on an agarose gel. In contrast, real time PCR (qPCR) uses fluorescent probes to detect the target DNA, where the amount of fluorescence detected is proportional to the amount of amplified PCR product. The number of PCR cycles required for an accurate level of exponentially increasing fluorescence, known as the cycle threshold value (CT value), is determined. The number of cycles needed to reach an accurate, diagnostic CT value is contingent on the initial amount of DNA extracted from the sample. The higher the initial target DNA value, the lower CT or fewer cycles are needed for qPCR detection. Relative amounts of initial samples required for detection can be determined based on results from standardized samples that have known concentrations of target DNA (Kubista et al., 2006; Schena et al., 2004).

Two types of fluorescent dyes are available. Fluorescent probes such as Taqman, which bind specifically to the amplified target, are labeled at the 5’ end with fluorophore and quenched at the 3’ end with a labeled fluorogenic quencher. Taqman probes hybridize the DNA target during the PCR cycle. The probe then degenerates, due to activity of the exonuclease of the DNA polymerase, and a fluorescent signal is produced from the quencher (Holland et al., 1991; Livak et al., 1995; Livak, 1999; Wittwer et al., 1997). The specificity of the hybridization with the target DNA limits false positive results due to primer dimers or unspecific amplification. The second type of fluorescent
dye is a non-specific DNA intercalating dye, such as SYBR green. The advantages of intercalating dyes are their ease of use and lower cost, as compared to the fluorescent probes. The disadvantages of intercalating dyes include the need for well-optimized reactions conditions in order to avoid formation of primer dimers and non-specific amplification, as well as very specific target primers since all of the DNA in the sample may be bound by such dyes. Assessment of the efficiency of the reaction requires analysis of a melting curve at the completion of the reaction (Mackay, 2004; Morrison et al., 1998).

Sugimura (2001) developed a primer set for *P. horiana*, but it was not species specific (Alaei et al., 2009b). Conventional PCR and real time qPCR protocols, which use *P. horiana* species-specific primers, have since been developed (Alaei et al., 2009b; Pedley, 2009). Both of these protocols are based on specific amplification of the ribosomal RNA gene cluster (rDNA) located in the internal transcribed spacer regions, ITS1 and ITS2, situated between the 18S, 5.8S and 28S subunits of the genes (White et al., 1990). The comparatively short, 500 to 800 bp length, of the ITS1–5.8–ITS2 is easily amplified by universal and rust-specific primer sets. This region also has a large amount of interspecific variation, but little to no intraspecific variation (De Backer, 2012; Lee and Taylor, 1992; White et al., 1990). Species-specific primers can be developed using available universal primers combined with knowledge of the interspecific variation region. The sensitivity of the protocol can be increased due to multiple copies of the rDNA ITS regions present in the genome allowing amplification of small or degraded amounts of target DNA (Borneman and Hartin, 2000; De Backer 2012; Gardes and Bruns, 1993).
Pedley (2009) developed *P. horiana* specific primers for conventional PCR and Taqman qPCR protocols. Using conventional PCR, the detection limit achieved was 1 ng of DNA. *Puccinia horiana* could be detected in symptomatic plant samples, but not accurately in asymptomatic plant samples. Pedley's qPCR protocol was determined to be 100 times more sensitive, allowing for some detection in asymptomatic samples. Alaei et al. (2009b) developed highly specific primers for *P. horiana* that had a detection limit of 10 fg target DNA in 10 ng DNA (0.001%) per real time qPCR reaction. As few as 10 target copies were detected using SYBR green dye. Alaei determined, based on the estimated size of the *P. graminis* genome, which has an expected 80 target copies, that he could theoretically detect less than one basidiospore/sample (Alaei et al., 2009b; De Backer, 2012).

Diagnostic PCR protocols necessitate adequate quantities of DNA or RNA, specific for *P. horiana*, to be present in the target organism. This is especially critical for accurate interception and identification of *P. horiana* in asymptomatic, but infected, chrysanthemum plants. Although conventional PCR could be used, real time qPCR offers more sensitive detection of lower target levels more quickly and at a reasonable cost.

**Pathotypes**

Recent research confirms that more than one pathotype of *P. horiana* exists (De Backer, 2012; Water, 1981). De Backer et al. (2012) reported “A study in which 6 Japanese isolates were inoculated on 40 cultivars involved the first systematic testing of multiple isolate in multiple hosts, and revealed differential interaction phenotype profiles
for several of the cultivars used (Yamaguchi, 1981).” They also determined that a minimum of 7 genes are involved in the pathosystem.

**Genotyping of *Puccina horiana***. Due to quarantine regulations and potential economic losses, rapid and accurate molecular diagnostics is an important aspect of pathogen control programs. Molecular techniques are available that allow detection and characterization of various pathogens, including their genetic diversity, as well as the ability to track and trace isolates. Genotyping of *P. horiana* isolates can allow the identification of new and highly virulent isolates in intercepted samples.

The genetic diversity of a population can be determined using dominant markers, based on Amplified Fragment Length Polymorphism (AFLP) or Random Amplification of Polymorphic DNA (RAPD). These two systems allow rapid screening of different genome loci without the need for sequence data. However, they are not suitable to develop markers for obligate biotrophs, such as *P. horiana*, since they don’t allow the amplification in a background of contaminating plant substances or microorganisms. In these cases co-dominant markers like Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphisms (SNPs), which are generally locus-specific, can be used. A SNP is a DNA sequence variation occurring when a single nucleotide, A, T, C, or G, in the genome differs between members of a biological species. SSRs, also known as microsatellites, are tandemly repeating sequences of 1-5 nucleotide base pairs of DNA (Turnpenny and Ellard, 2005).

SNPs are better suited for population genetic studies in distantly related isolates because, compared to SSR markers, SNPs exhibit less mutation (Brumfield et.al., 2003; Morin et al., 2004). However, more SNP markers are needed to determine the genetic
variation between isolates due to the biallelic nature of SNPs, while microsatellites have multiple alleles (Morin et al., 2004). *Puccinia horiana* species-specific SNP markers have been identified and an efficient SNP detection protocol using High Resolution Melting (HRM) technology has been developed. HRM allows the rapid identification of *P. horiana* isolates at a prudent cost (De Backer et al., 2012; Van Poucke et al., 2014). De Backer et al. (2012) identified 33 polymorphisms for *P. horiana* by sequencing 25 loci, representing 32 SNPs and 1 SSR. In 18 loci, a one single SNP was present, 4 loci had double SNPs (2 independent and 2 dependent), and 2 loci had triple SNPs (dependent). Using these markers, De Backer et al. (2012) determined 25 genotypes of *P. horiana* isolated from 45 samples in a worldwide collection. De Backer et al. (2012) determined that roughly 0.01% of the 2.35 x 10^6 nucleotide positions he initially analyzed were polymorphic, indicating that the occurrence of SNPs in *P. horiana* was considerably lower than in other fungi. He theorized that, as expected, the SNP markers he identified were stable. De Backer’s theory is supported by the clonal European isolates that were collected during 2003 to 2007. Except for SNP 431/1, all markers were also found in at least two isolates, implying they were not recently created (De Backer, 2012).

Based on a population analysis, the migration patterns of the pathogen and the exotic or endemic characteristics of the isolates collected from the different worldwide regions were analyzed. De Backer also observed clonal isolates within the geographic region where the pathogen is endemic. However, the genotypic diversity in each region was larger then expected. For example, in Europe, 12 genotypes were identified within 25 isolates, in Japan 3 genotypes were identified within 4 isolates and, in the United States, 3 genotypes were identified within 5 isolates. The marker profiles within an area did not
demonstrate a stepwise accumulation of mutations expected for organisms capable of asexual reproduction and certain haplotypes appeared to have recombinant SNP patterns in isolates originating from diverse geographic areas. These observations suggest recombination and migration of genotypes and that a parasexual cycle is present (DeBacker 2012). Based on reports by Kohno et al., (1974, 1975) recombination in P. horiana can be explained by heterokaryosis after anastomosis resulting in heterozygous vegetative mycelium; homozygous recombinant genotypes can be expected after somatic meiotic division during the telial stage (De Backer 2012, Kohno et al., 1974, 1975). Anastomosis has been observed in P. horiana (O’Keefe, personal observations) and most of the SNPS identified by De Backer were found in diverse groupings that also challenge theories of strictly clonal lineage.

De Backer (2012) determined that isolates from the United States grouped into two clusters belonging to three genotypes that do not directly relate as recombinants. He theorized that at least three introductions of P. horiana into the United States have occurred. He also suggested, that based on his results, that P. horiana may now be endemic in the United States or that introductions of the same isolate genotype(s) may have repeatedly occurred. De Backer also determined that all the isolates from the United States clustered with isolates of Asian origin.

De Backer (2012) determined that pathotype diversity was significantly greater than genotypic diversity. He explained this difference as being due to the interaction of at least seven resistance genes in chrysanthemums corresponding with avirulence genes in P. horiana (De Backer et al., 2011). De Backer reported distinct differences in pathotypes even within clonal isolates. He theorized that these differences could be due to avirulence
genes demonstrating independent selection pressure due to specific resistance genes in various chrysanthemum cultivars grown in different areas, or that a greater variation in pathotypes interactions could be due to epigenetic changes in disease resistance, as noted by Stokes et al. (2002). De Backer found a correlation between genotype and pathotype for some isolates including a unique pathotype group, containing highly virulent isolates. He theorized that these isolates contain few avirulence genes, including one corresponding to a frequently-used resistance gene (De Backer et al., 2011). Unfortunately, the location of avirulence genes on the *P. horiana* genome is unknown. Because recombination in *P. horiana* during asexual reproduction can increase the potential of new genotypes of greater virulence, co-existence of different genotypes within the same site should be avoided (De Backer, 2012).

**High Resolution Melting (HRM) Analysis.** High Resolution Melting (HRM) is a quantitative post-PCR analysis method used for identifying genetic variation in genomic sequences. HRM generates melting (dissociation) curves specific and sensitive enough to identify small sequence differences, including single-base variations. During HRM analysis, the target area is amplified by PCR in the presence of a saturating fluorescent dsDNA binding dye. After amplification, the PCR product is slowly melted while the fluorescence is measured and a dissociation curve is generated. The resulting profile indicates the amplicons present (Anon, 2013; Taylor et al., 2010; Van Poucke et al., 2014).

Benefits of HRM include that it is in many cases sensitive enough to detect a single base change between otherwise identical PCR fragments. In addition, HRM is nondestructive, allowing, if necessary, the resulting PCR product to be analyzed by gel electrophoresis or sequencing. Other benefits include the lower cost when compared to
fluorescent probe technologies, can accurately distinguish multiple SNPs located close to one another, and can accurately identify large number of SNPs in low target quantity (Anon, 2013; Taylor et al., 2013; Van Poucke et al., 2014). Drawbacks to HRM include that, for many SNPs, the melting temperature (Tm) difference between the two possible variants is very small resulting in potentially incorrect SNP assignments. Additionally, for *P. horiana* samples, it is difficult to acquire identical amounts of *P. horiana* DNA among samples for HRM diagnostics. Another drawback is that many positive controls are needed, since these are necessary for each SNP to be assigned to the correct SNP class (Anon, 2013; Taylor et al., 2013; Zhou et al., 2004).

As an alternative, HRM techniques using unlabeled probes have been developed. In general, SNP genotyping with HRM using unlabeled probes requires only one probe for each SNP and fewer controls (Van Poucke et al., 2014; Zhou, et al., 2004). An oligonucleotide is located at the 3’ end in order to block extension and, the Tm difference ($\Delta$Tm) is independent of the DNA concentration. The unlabeled probe is added to the master mix along with an asymmetric ratios of primers (Eranti et al., 2008; Van Poucke et al., 2014). The PCR is optimized to generate a large difference in melting temperature between a match and mismatch of the unlabeled probe. Short unlabeled probes of 20 to 30 bases can be designed to complement either of the two possible SNP variants. Greater differentiation and destabilization occurs when the mismatch is placed in the middle section of the probe. The differences in the melting temperature of the probes should be comparably different between the amplicon, the match, and the mismatch, allowing reliable SNP calling. The unlabeled probe, because it is smaller the amplicon, melts at a lower temperature, generating a peak to the left of the amplicon peak. And, when the
probe matches the SNP, due to stronger bonds, the probe dissociates at a higher temperature compared to a mismatch (Fig 1.12). With good primer and probe design, the Tm difference can be as much as 5-7°C (Anon, 2013; Van Poucke et al., 2014). Figure 1.12 illustrates a dissociation curve using unlabeled probe SNP mismatch, match and amplicon peaks (Van Poucke et al., 2014).

It is also possible to multiplex two unlabeled probes in order to identify two SNPs in one PCR amplicon (Erali et al., 2008; Poulson et al., 2007). In a multiplex run, there are four possible melting peaks, two are matches and two are mismatches. Provided the peaks do not overlap, it would be possible to assign the two SNPs correctly.

Van Poucke et al., (2014) developed an HRM protocol for *P. horiana* that results in generation of two peaks, one from the probe and one from the amplicon. The PCR is asymmetric with a forward primer concentration of 50 nM and a reverse primer concentration of 250 nM.

**Hosts.** The host range of CWR includes chrysanthemum species (*Chrysanthemum* spp.), as well as *Leucanthemella serotina* and *Nipponanthemum nipponicum*, which are close relatives in the Asteraceae family. In addition, Zeng et al., (2013) recently reported that *Ajania pacifica* and *A. shiwogiku* var *kinokuniense*, from Japan were also susceptible to CWR, as illustrated by artificial inoculations in greenhouse studies conducted in eastern China. Resistant chrysanthemum cultivars have been developed. Table 1.8 illustrates the CWR host range (USDA APHIS PPQ, 2012).

**Economic losses due to *P. horiana*.**

CWR is a serious disease that can cause complete loss of infected chrysanthemum crops. Currently *P. horiana* is a regulated pathogen that is not considered indigenous in
the United States. Many countries, including the United States, due to financial loss and difficulty of eradicating CWR, have established Phytosanitary Quarantines against CWR (USCFR, 2012). Plants, cuttings and cut flowers that are considered to be host of CWR are inspected upon entering the United States; if CWR is detected, the crop is eradicated. Eradication has economic ramifications, since concurrent outbreaks at multiple sites can result in large areas (e.g., counties) being quarantined. In such cases, growers and homeowners bear considerable eradication costs including lost revenue of commercial crops.

The earliest record of CWR interceptions occurring at ports in the United States is 1906 on chrysanthemums arriving from Japan (USDA ARS, personal communication). The first report of CWR identified at an inland location United States occurred in NJ and PA in 1977 (Coppock and Kreith, 1978; Peterson et al., 1978). In 2007, CWR was identified at 40 commercial and residential sites in CA, CT, ME, MD, NY, and PA. In 2008, 13 additional CWR sites were identified in CA, DE, MA, and MI. Positive CWR interceptions increased in 2009 when 16 locations were identified in NY and VA. California and MD reported 17 CWR positive locations in 2010. In a summary report in 2011, PA reported 139 CWR positive samples intercepted at 82 sites in 22 counties over 4 years (USDA APHIS NAPIS database). Chrysanthemum plants were eradicated at all of these positive locations, resulting in substantial financial loss. Figure 1.13 illustrates CWR interceptions in the United States and Canada since 1977.

In California, during 1990 to 1999, CWR was detected in California on plants arriving from Asia and Mexico. When CWR was detected during 1994 in California, the initial site was quarantined. A survey of the surrounding area identified an additional 70
positive sites with infected chrysanthemums. At the time, the cost to eradicate the interceptions was about $500 per site, or $32,000 total. Eight weeks later, during a follow-up survey, only a few sites remained infected. However, extension of the quarantine time resulted in the eradication cost increasing to approximately $7000 per site (Coppock and Kreith, 1999; USCFR 2012). The total eradication costs converted to 2014 values for $500, $32,000 and $7000 would be approximately $788, $50,500, and $11,000 respectively. During 1995, eradication of CWR was identified at commercial chrysanthemum growers in San Diego, and eradication cost was estimated at approximately $5000 per site. Although the infestation was quickly eradicated at most of the sites, one commercial grower experienced repeated site infestations that resulted in a loss of greater than $100,000, equal to approximately $153,000 in 2014 (Coppock and Kreith, 1999; USCFR 2012). Direct and indirect losses from CWR infestations intercepted in Santa Barbara County CA during 1992 to 1997 were estimated at $2 million dollars, greater then $3 million in 2014 dollar values. In 1997, the county reported the value of chrysanthemum production at greater than $10 million dollars, or over $14 million dollars in 2014 values (Coppock and Kreith, 1999; USCFR 2012).


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etNvIBFJoGrbGUHZQAsm%2FLec10CutflowerproductionofChrysanthemumand
Gladiolus.doc&ei=zuFHVPHcKLiPsQSrr4KgbQ&usg=AFQjCNQyF3m3DOEO RfywXZG0hxOVQ0Xt1Q


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Tables and Figures

TABLE 1.1. Current taxonomy of chrysanthemums.

<table>
<thead>
<tr>
<th>Classification of florist/hardy garden chrysanthemums</th>
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<tbody>
<tr>
<td><strong>Kingdom</strong></td>
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<tr>
<td><strong>Subkingdom</strong></td>
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<td><strong>Superdivision</strong></td>
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<td><strong>Division</strong></td>
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<td><strong>Subclass</strong></td>
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<td><strong>Order</strong></td>
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<td><strong>Family</strong></td>
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<td><strong>Genus</strong></td>
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<td><strong>Species</strong></td>
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TABLE 1.2 List of important dates and authorities involved in production of chrysanthemums in the western world.

<table>
<thead>
<tr>
<th>Dates and Authorities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1688. Breynius, Jakob (b. 37, d. 1797), (? as cultivated in Holland 1688-9).</td>
<td>Breynius, Amoenitates Exoticae.</td>
</tr>
<tr>
<td>1758. Linnaeus, Species Plantarum (C. sinense, white flowers, larg, and C. indicum, single white and double small yellow), rep. By Willd. 1764.</td>
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<tr>
<td>1784. Thunberg, Floris Japonica (as Matricaria).</td>
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<tr>
<td>1789. Introduced into France by Ramatuelle, and cultivated by M. Cels, then a celebrated nursery gardener of Paris, who in 1790 sent the “Old Purple” (Bot. Mag. t. 327) to Kew.</td>
<td></td>
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<tr>
<td>1790. Loureiro, Flora Cochinchinensis.</td>
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<tr>
<td>1790. Introduced into England by M. Cels from France, and first flowered in Colvill's Nursery, King's Road, Chelsea, in 1795.</td>
<td></td>
</tr>
<tr>
<td>1796. Curtis, Bot. Mag. t. 327. (The first large-flowered Chrysanthemum bloomed in England. The variety was called the “Old Purple,” and closely resembles the more modern variety known as “Dr. Sharpe” to-day.”)</td>
<td></td>
</tr>
<tr>
<td>1797. Willdenow, Species Plantarum (1797-1810).</td>
<td></td>
</tr>
<tr>
<td>1802. First “sports” appeared in England. The first was also the first white Chrysanthemum in English gardens, and called “The Changeable White,” or “Tasselled White.” It was a “sport” from the “Old or Tasselled Purple” as first introduced, and resembled it in all points save colour. (Haworth.) First “sport” white form “Old Purple,” and a pale pink “sport” from the “Changeable Buff.”</td>
<td></td>
</tr>
<tr>
<td>1824-6. Sabine, Hort. Trans. 1821-1826. p. 326, etc.; 1824, vol. vi. p.412; 1826, p. 322. (Twenty-seven introduced varieties from China, including “sports” appearing in English gardens, were cultivated at Chiswick up to 1824.) (Sabine.)</td>
<td></td>
</tr>
<tr>
<td>1825. First Chrysanthemum exhibition at Chiswick (700 pots).</td>
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<tr>
<td>1826. Frist French seedlings by M. Bernet.</td>
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</tr>
<tr>
<td>1830. First English seedlings by Mr. Wheeler, for which a Banksian Medal was awarded by the Royal Horticultural Society, December 4, 1832. First Chrysanthemum show held at Norwich.</td>
<td></td>
</tr>
<tr>
<td>1832. Forty-nine varieties of Chrysanthemums grown at Chiswich for the Royal Horticultural Society. First arrangement of varieties into sections suggested by Mr. Douglas Munro, F.L.S., and published in Hort. Trans. vol. i. (2nd series).</td>
<td></td>
</tr>
<tr>
<td>1845. Small-flowered or Pompon Chrysanthemum introduced by Mr. Fortune, viz. “Chusan Daisy” and Chinese “mini-mum.”</td>
<td></td>
</tr>
<tr>
<td>1846. First competitive Chrysanthemum show for cut blooms held in England, at Stoke Newington.</td>
<td></td>
</tr>
<tr>
<td>1862. Japanese Chrysanthemums introduced by Mr. Fortune (seven varieties).</td>
<td></td>
</tr>
<tr>
<td>1865. Salter, “The Chrysanthemum.”</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chrysanthemum variety - type</th>
<th>No. producers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Wholesale value (10&lt;sup&gt;5&lt;/sup&gt; dollars)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2011</td>
<td>2012</td>
</tr>
<tr>
<td>Hardy/garden</td>
<td>868</td>
<td>855</td>
</tr>
<tr>
<td>Indoor/potted patio/florist</td>
<td>174</td>
<td>170</td>
</tr>
<tr>
<td>Cut/pom-pom</td>
<td>25</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>a</sup> No. producers in 15 program states (CA, FL, HI, IL, MD, MI, NJ, NY, OH, OR, PA, SC, TX, WA)
<table>
<thead>
<tr>
<th>Rank</th>
<th>Company</th>
<th>Estimated million cuttings produced</th>
<th>Greenhouse square footage</th>
<th>Headquarters</th>
<th>Countries(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fides B.V.</td>
<td>800-850</td>
<td>7,535,880</td>
<td>Holland</td>
<td>five</td>
</tr>
<tr>
<td>2</td>
<td>Beekenkamp</td>
<td>635</td>
<td>8,611,128</td>
<td>Holland</td>
<td>six</td>
</tr>
<tr>
<td>3</td>
<td>Syngenta Flowers</td>
<td>550-600</td>
<td>n/a</td>
<td>Switzerland</td>
<td>seven</td>
</tr>
<tr>
<td>4</td>
<td>Selecta Klemm</td>
<td>230-250</td>
<td>3,659,730</td>
<td>Germany</td>
<td>three</td>
</tr>
<tr>
<td>5</td>
<td>Dummen</td>
<td>200-220</td>
<td>6,048,196</td>
<td>Germany</td>
<td>three</td>
</tr>
<tr>
<td>6</td>
<td>Oro Farms</td>
<td>200</td>
<td>6,100,000 (shade and field)</td>
<td>Guatemala</td>
<td>one</td>
</tr>
<tr>
<td>7</td>
<td>Ball FloraPlant</td>
<td>190-200</td>
<td>4,700,000</td>
<td>United States</td>
<td>four</td>
</tr>
<tr>
<td>8</td>
<td>ForemostCo</td>
<td>190-200</td>
<td>653,400 (plus 150 shade acres)</td>
<td>United States</td>
<td>nine</td>
</tr>
<tr>
<td>9</td>
<td>Cohen Nurseries</td>
<td>155</td>
<td>1,151,247</td>
<td>Israel</td>
<td>one</td>
</tr>
<tr>
<td>10</td>
<td>McGregor Plant Sales/Florexpo</td>
<td>120-135</td>
<td>4,305,564</td>
<td>Costa Rica</td>
<td>one</td>
</tr>
</tbody>
</table>

\(^a\) Number of countries in which chrysanthemum cuttings are grown.
<table>
<thead>
<tr>
<th>Insect</th>
<th>Symptoms</th>
<th>Management</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aphids</td>
<td>Chrysanthemum aphids, <em>Macrosiphoniella sanborni,</em> and other aphid species are pests on chrysanthemums. Chrysanthemum aphids are brown to black, while other species range from green to pink. Aphids feed by piercing plant tissue and sucking plant sap from new growth of shoots, leaves, buds, and flowers. Feeding can result in distorted growth, stunting and sometimes plant death. As aphids feed on plant sap, they excrete honeydew (a sugary material). The sooty mold fungus feeds on the honeydew, resulting in unsightly, dark fungal growth. In addition, chrysanthemum aphids can transmit various plant viruses.</td>
<td>Aphids can be removed from plants by applying a forceful spray of water to the plants every 2-3 days, especially to the undersides of leaves. Several naturally occurring enemies feed on aphids. As much as possible, these predators should be allowed to reduce aphid populations. Aphids are very difficult to control with insecticides. In addition, the use of insecticides kills the beneficial insects that kill aphids. If serious damage is occurring, spray with a pesticide.</td>
</tr>
<tr>
<td>Spider-mites</td>
<td>Two-spotted spider mites, <em>Tetranychus urtica</em>, and other mite species are pests of chrysanthemums. Mites tend to be a problem during hot, dry periods. Mites are extremely small and can barely be seen without a magnifying lens. They have piercing mouthparts with which they puncture plant tissue and suck plant sap. With a light infestation, leaves develop stipples (tiny yellow spots) and appear dusty. Early damage is often overlooked until damage is severe. With heavier infestations, symptoms include distorted leaves, and withered and discolored blooms. In addition, fine webbing can be seen on flower buds, between stems and on the undersides of leaves.</td>
<td>Consider destroying severely infested plants or portions of plants, as spider mites are often difficult to control. Spider mites can be removed by spraying plants forcefully with water 2-3 times. Insecticidal soap, if started early in the infestation, is effective at controlling spider mites. Spray with a pesticide. Read and follow all label directions and precautions.</td>
</tr>
<tr>
<td>Leafminers</td>
<td>Chrysanthemum leafminer, <em>Phytomyza syngenesiae</em>, is the larva (immature form) of small (about 1/8-inch) dark-colored flies. The adult female lays eggs on the undersurfaces of leaves. The larvae hatch and penetrate the surface to enter the leaf and live between the upper and lower surfaces of the leaves. As they move through the leaf feeding, they create winding trails that are pale green to brown in color. Dots of black waste products are visible in some of the trails. Severely infested leaves may dry up and droop downward along the stems.</td>
<td>Prune off and destroy infested leaves. Any leaves that fall to the ground should be picked up and destroyed. Remove and destroy any plant remains in the fall. Spray with a foliar systemic insecticide.</td>
</tr>
<tr>
<td>Disease and Pathogen/Cause</td>
<td>Symptoms</td>
<td>Management</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------</td>
<td>------------</td>
</tr>
</tbody>
</table>
| ASCOCHYTA RAY BLIGHT
*Ascochyta (Mycosphaerella)* | Flower development is retarded on one side of the bud. Petals exhibit a brown discoloration. Browning and blackening extends down the stem, causing the flower to droop. Brown to black irregularly shaped spots develop on leaves. | Avoid overhead irrigation. Apply a fungicide to protect healthy plants. |
| ALTERNARIA OR STEMPHYLIUM RAY SPECK
*Alternaria* or *Stemphylium* | Pin-point dead spots develop on petals. These spots may not enlarge. If enough spots are present, the entire flower dies. | Avoid overhead irrigation. Maintain greenhouse humidity below 98%. Apply a fungicide to protect healthy plants. |
| BACTERIAL BLIGHT
*Erwinia chrysanthemi* | Cuttings turn dark brown and collapse. Surviving cuttings may be infected but have no symptoms. Established plants wilt during the day when infected and recover at night. | Purchase culture-indexed cuttings that are free of the pathogen. Disinfect propagation beds between crops. Destroy infected cuttings. |
| BACTERIAL LEAF SPOT
*Pseudomonas cichorii* | Small dark brown to black spots on lower leaves enlarge and become irregular in shape. When infected leaves dry, the spots become brittle and crack. The disease often spreads up plants in one side of the pot, eventually to the flowers. | Do not plant infected cuttings. Avoid overhead irrigation. Water in a manner that keeps leaf surfaces dry at all times. Protect plants grown outdoors from splashing. |
| BOTRYTIS BLIGHT
*Botrytis cinerea* | Light brown spots form on lower petals. Browning spreads to other petals. Infected tissues become covered with dusty gray spores. | Maintain greenhouse humidity below 98% at all times. Apply a fungicide to protect healthy plants. |
| CHLOROTIC MOTTLE
Chrysanthemum chlorotic mottle viroid | Leaves, at first mottled, become completely yellow. Infected plants grown under low light conditions and when temperatures average less than 20°C (69°F) exhibit no symptoms. | Purchase virus-indexed plants that are free of the pathogen. Destroy infected plants and disinfect tools used to handle them. Do not handle healthy chrysanthemums after handling infected plants. |
| FUSARIUM WILT
*Fusarium oxysporum* | Symptoms vary with the cultivar infected. Yellowing of leaves, wilting, and discoloration of the vascular tissue develops up one side of the plant. | Management: Purchase culture-indexed cuttings free of the pathogen. Plant in pasteurized soil or soilless mix free of the pathogen. Maintain soil pH between 6.5 and 7.0. Use nitrate rather than ammonium forms of fertilizer. Apply a fungicide to protect healthy plants. |
<table>
<thead>
<tr>
<th>Disease and Pathogen/Cause</th>
<th>Symptoms</th>
<th>Management</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POWDERY MILDEW</strong>&lt;br&gt;Golovinomyces cichoracearum (formerly Erysiphe)**</td>
<td>Leaves have white, dry fungal growth on their surfaces.</td>
<td>Apply a fungicide to protect healthy plants.</td>
</tr>
<tr>
<td><strong>PYTHIUM ROOT AND STEM ROT</strong>&lt;br&gt;<em>Pythium</em></td>
<td>Stems turn dark brown to black at the soil line. Plants are stunted, wilt, and die.</td>
<td>Plant in pasteurized soil or soilless mix free of the pathogen. Apply a fungicide to protect healthy plants.</td>
</tr>
<tr>
<td><strong>RHIZOCTONIA STEM ROT</strong>&lt;br&gt;<em>Rhizoctonia solani</em></td>
<td>Young infected plants wilt during the day and recover at night. Reddish-brown dead areas develop at the soil line and girdle the plant.</td>
<td>Plant in pasteurized soil or a soilless mix free of the pathogen. Apply a fungicide to protect healthy plants.</td>
</tr>
<tr>
<td><strong>BROWN RUST</strong>&lt;br&gt;<em>Puccinia chrysanthemi</em></td>
<td>Dark brown masses of spores form in pustules on both leaf surfaces.</td>
<td>Remove and destroy infected leaves. Apply a fungicide.</td>
</tr>
<tr>
<td><strong>RUST, WHITE</strong>&lt;br&gt;<em>Puccinia horiana</em></td>
<td>Small, yellow to tan spots are observed on the upper surface of leaves. On the underside of the leaf below the spots, raised, pinkish to white to cream-tan areas develop in which spores of the fungus are produced.</td>
<td>Contact your state plant inspector and comply with regulations requiring the destruction of infected plants and fungicide treatment of remaining chrysanthemums.</td>
</tr>
<tr>
<td><strong>STUNT</strong>&lt;br&gt;Chrysanthemum stunt viroid</td>
<td>Symptoms vary with the cultivar infected. Young leaves are light green and very upright. Plants are stunted to half their normal height at maturity. Infected plants flower prematurely and flower size is reduced. Some cultivars exhibit small dead spots or flecks on the leaves.</td>
<td>Purchase virus-indexed plants that are free of the pathogen. Destroy infected plants and disinfect tools used to handle them. Do not handle healthy chrysanthemums after handling infected plants.</td>
</tr>
<tr>
<td><strong>VERTICILLIUM WILT</strong>&lt;br&gt;<em>Verticillium</em></td>
<td>The margins of lower leaves wilt and die. Or, the entire leaf dies. Symptoms proceed up one side of the plant.</td>
<td>Plant in pasteurized soil or soilless mix free of the pathogen.</td>
</tr>
</tbody>
</table>
Table 1.7. Taxonomic classification of *P. horiana* to species

<table>
<thead>
<tr>
<th>Classification of <em>Puccinia horiana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
</tr>
<tr>
<td>Phylum</td>
</tr>
<tr>
<td>Subphylum</td>
</tr>
<tr>
<td>Class</td>
</tr>
<tr>
<td>Subclass</td>
</tr>
<tr>
<td>Order</td>
</tr>
<tr>
<td>Family</td>
</tr>
<tr>
<td>Genus</td>
</tr>
<tr>
<td>Species</td>
</tr>
</tbody>
</table>
Table 1.8 Plant hosts of *Puccinia horiana*, causal agent of CWR (USDA-APHIS-PPQ, 2012).

<table>
<thead>
<tr>
<th>Accepted scientific name</th>
<th>Synonym(s)</th>
<th>Common name</th>
</tr>
</thead>
</table>
| *Chrysanthemum articum* L. | Arctanthermum arcticum (L.) Tzvelev   
Dendranthera arcticum (L.) Tzvelev | Arctic chrysanthemum Arctic daisy   |
| *Chrysanthemum boreale* (Makino) Makino | Chrysanthemum indicum L. var. boreale Makino 
Dendranthera boreale (Makino) Ling ex Kitam. |                                       |
| *Chrysanthemum indicum* L. | Dendranthera indicum (L.) Des Moul. |                                       |
| *Chrysanthemum japonense* Nakai | Dendranthera japonense (Nakai) Kitam. 
Dendranthera occidentali-japonense Kitam. | Nojigiku                            |
| *Chrysanthemum japonicum* Makino | Chrysanthemum makinoi Matsum. and Nakai 
Dendranthera japonicum (Makino) Kitam. | Ryuno-giku                          |
| *Chrysanthemum ×morifolium* Ramat. | Anthemis grandiflorum Ramat. 
Anthemis stipulacea Moench 
Chrysanthemum sinense Sabine ex Sweet 
Chrysanthemum stipulaceum (Moench) W. Wight Dendranthera ×grandiflorum (Ramat.) Kitam. 
Dendranthera ×morifolium (Ramat.) Tzvelev 
Matricaria morifolia Ramat. | Florist's chrysanthemum Chrysanthemum Mum |
| *Chrysanthemum pacificum* Nakai | Ajania pacifica (Nakai) K. Bremer & Humphries 
Dendranthera pacificum (Nakai) Kitam. | Iso-giku                            |
| *Chrysanthemum shiwogiku* Kitam | Ajania shiwogiku (Kitam.) K. Bremer & Humphries 
Dendranthera shiwogiku (Kitam.) Kitam. | Shio-giku                           |
| *Chrysanthemum yoshinaganthum* Makino ex Kitam | Dendranthera yoshinaganthum (Makino ex Kitam.) Kitam. |                                       |
| *Chrysanthemum zawadskii* Herbich subsp. Yezoense (Maek.) Y. N. Lee | Chrysanthemum arcticum subsp. maekawanum Kitam 
Chrysanthemum arcticum var. yezoense Maek. [basionym] 
Chrysanthemum yezoense Maek. [basionym], Dendranthera yezoense (F. Maek.) D. J. N. Hind, 
Leucanthemum yezoense (Maek.) Á. Löve & D. Löve |                                       |
| *Chrysanthemum zawadskii* Herbich subsp. Zawadskii | Chrysanthemum sibiricum Turcz. ex DC., nom. inval. Dendranthera zawadskii (Herbich) Tzvelev 
Dendranthera zawadskii var. zawadskii |                                       |
| *Leucanthemella serotina* (L.) Tzvelev | Chrysanthemum serotinum L. 
Chrysanthemum uliginosum (Waldst. & Kit. ex Willd.) Pers. 
Pyrus uliginosum (Waldst. & Kit. ex Willd.) |                                       |
| *Nipponanthemum nipponicum* (Franch. ex Maxim.) Kitam | Chrysanthemum nipponicum (Franch. ex Maxim.) Matsum. Leucanthemum nipponicum 
Franch. ex Maxim. | Nippon daisy Nippon-chrysanthemum |

Fig. 1.1. Wild chrysanthemum soup made from Ye Juhua chrysanthemums.
Fig. 1.2. SuZu the Shih tzu “Chrysanthemum” dog.
Fig. 1.3. Worldwide Chrysanthemum production, 2006
Fig. 1.4. Pom-pom and incurve type chrysanthemums.
Fig. 1.5. Distribution of chrysanthemum species studied in relation to chromosome number. The majority of diploid species occur in the Mediterranean region. A higher degree of polyplody is found in China and Japan. The European species are probably younger than those found in Asia. (Dowrick, 1952).
Fig. 1.6. Kinds and sequence of spores and spore-producing structures in rust fungi and nuclear condition of each (Agrios, 2005).
Fig. 1.7. Distribution map of *P. horiana*
Fig. 1.8. Life cycle of Puccinia horiana (DeBacker, 2012)
Fig. 1.9. Teliospores of Puccinia horiana (Punithalingam, 1968)
Fig. 1.10. Infection schematic diagram summary of infection process of a *Puccinia* spp (Morin et al., 1992).
Fig. 1.11. Schematic illustration of recombination in *Puccinia horiana* (A) “Tip-to-Toe” anastomosis as also described by Wang and McCallum (2009) between vegetative mycelium or germ tubes from two different genotypes (illustrated with black versus white nuclei) of *P. horiana* eventually results in heterozygous mycelium with two haploid nuclei. (B) During the telial stage karyogamy occurs with a rearrangement of the chromosomes resulting in a recombinant genotype (illustrated with gray nuclei). After the second somatic meiotic division in the promycelium, two identical haploid nuclei migrate in each of the two basidiospores, which give rise to homozygous recombinant mycelium (Kohno et al., 1974; Kohno et al., 1975). The nuclear status is indicated for each stage in the rectangular insets: c represent the number of chromosomes, n represents the ploidy number (De Backer, 2012).
Fig. 1.12. Dissociation Curve using unlabeled probe SNP mismatch, match and amplicon peaks (Van Poucke et al. 2014).
Fig. 1.13. CWR Interceptions in the United States and Canada since 1977
Chapter 2: Morphology of *Puccinia horiana*

**G. OKeefe** and **D.D. Davis.** Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, Pennsylvania, 16802

Corresponding author: D.D. Davis

E-mail: ddd2@psu.edu

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**ABSTRACT**


Chrysanthemum white rust (CWR), caused by *Puccinia horiana* Henn., is pathogenic on many *Chrysanthemum* spp. and close relatives within the family Asteraceae. CWR is economically important and infects florist chrysanthemum cultivars (*Chrysanthemum x morifolium*) throughout the world. Due to its worldwide importance and economic significance, many countries, including the United States, have established Phytosanitary Quarantines against CWR, and *P. horiana* is a regulated pathogen. Although CWR does not directly result in chrysanthemum death, growers with infected plants often experience severe economic loss due to quarantine regulations requiring eradication. The presence of overwintering systemic pathogens, such as *P. horiana*, within chrysanthemums has important economic and regulatory implications. This is
especially true in the case of systemically infected, but asymptomatic, chrysanthemums. We previously reported the presence of *P. horiana* in the roots and stems of asymptomatic chrysanthemum plants. However, we had not previously provided detailed descriptions of the morphology, intercellular colonization, and intracellular growth of *P. horiana* in chrysanthemums.

**INTRODUCTION**

Chrysanthemum white rust (CWR), caused by *Puccinia horiana* Henn., is an autoecious, microcyclic rust that is pathogenic on many *Chrysanthemum* spp. and close relatives within the family Asteraceae. CWR is economically important and infects florist chrysanthemum cultivars (*Chrysanthemum x morifolium*) throughout the world (Baker, 1967; Dickens, 1990; Firman and Martin, 1968; Horst and Nelson, 1997). Due to its worldwide importance and economic significance, many countries, including the United States, have established Phytosanitary Quarantines (USCFR, 2014) against CWR. Although CWR does not directly result in chrysanthemum death, growers with infected plants often experience severe economic loss due to quarantine regulations requiring eradication of chrysanthemum crops.

CWR is indigenous to Japan, where it was first reported in 1895 (Pedley, 2009). By the 1960s, CWR was found throughout Europe and later spread to Africa, Oceana, South America, and additional countries in Asia. In North America, CWR was reported in Mexico (EPPO/CABI) and in the United States within New Jersey and Pennsylvania (1977), Oregon and Washington (1990), and California (1991). Additional detections of
CWR were later reported in 22 Pennsylvania counties during 2004, and from 2006 to 2010 (Kim et al., 2011). Eradication was attempted at some Pennsylvania sites, but unconfirmed observations suggested that *P. horiana* might overwinter in volunteer chrysanthemum plants (Kim et al., 2011). This suggestion is important, since overwintering of *P. horiana* in the United States might imply that the fungus has become established in certain parts of the country. Establishment, in turn, might influence CWR federal quarantine regulations. And, since “CWR is known to overwinter in Europe where chrysanthemums overwinter (average minimum temperatures ranging from –10°F to 10°F)” (Anon, 2010), the possibility that *P. horiana* might overwinter in volunteer chrysanthemum plants in Pennsylvania prompted us to study this aspect of the disease cycle.

*Puccinia horiana* morphology was described in general terms by several authors in the 1960s and early 1970s (Firman and Martin, 1968; Kapooria and Zadoks, 1973; Punithalingam, 1968). *Puccinia horiana* produces only two spore stages, dikaryotic/diploid teliospores and haploid basidiospores. Aeciospores, urediospores, and pycnia are not produced. Since pycnia, which produce spermatia, are not produced, the process by which karyogamy occurs in *P. horiana* is unknown. We suggest that karyogamy results following anastomosis (Agrios 2005) of compatible *P. horiana* hyphae. This suggestion is supported in that multiple nuclei have been reported within *P. horiana* mycelium (Kapooria and Zadoks, 1973). However, anastomosis within infected chrysanthemum tissues has not been reported. Although not a major objective, microscopic observation of *P. horiana* anastomosis within infected chrysanthemum tissue is a secondary, although important, objective of this paper.
*Puccinia horiana* teliospores are two-celled and stalked, typical of the genus *Puccinia* (Agrios, 2005). Telia, often aggregated within clusters that are ca. 2 to 4 mm in diameter, can be distributed over an entire infected plant. Individual teliospores are fusiform, cylindrical, and oblong to oblong-clavate, seldom elliptical, with a smooth cell wall that is 1 to 2 µm wide enlarging to 3 to 10 µm at the spore apex. Teliospores are 32 to 45 µm long x 12 to 18 µm wide, and usually marginally constricted at the single septum, although occasionally biseptate. The hyaline pedicle is up to 45 µm long and persistent (Anon., 2004; Firman and Martin, 1968; Kapooria and Zadoks, 1973).

Kapooria and Zadoks (1973) illustrated the morphology and cytology of *P. horiana* promycelium and basidiospore formation, but presented only line drawings or images of low magnification. Teliospores can germinate from one or both cells, but usually from the apical cell where a long slender (e.g., in vitro on glass slides) promycelium forms and develops two septa. In contrast, developing promycelia on infected plant material (e.g., in vivo on infected leaves) are short and thick. In either case, after 12 h of germination, up to four reniform to round basidiospores are produced on the two distal cells, usually on sterigmata, which may be short, coiled, or curved. The first basidiospore is produced at the distal part of an apical promycelium cell. However, promycelia may occasionally branch and form basidiospores without visible sterigmata.

Under high RH and presence of free moisture, basidiospores can germinate in several ways. Kapooria and Zadoks (1973) described three patterns of basidiospore germination: i) a germ tube of varying length that sharply tapers; ii) an appressorium-like vesicle which eventually develops an infection hypha; and iii) a secondary basidiospore, although rare, can germinate by repetition.
With rust fungi in general, upon basidiospore germination the germ tube can penetrate the host either directly through the epidermis, or indirectly through host stomata. In either case, biotrophic fungi such as rusts generally release low levels of cell wall degrading enzymes at the host penetration site. Rust haustoria form soon after host cell penetration, which initiate further enzyme degradation of host cell walls. During early stages of colonization, these simple, filamentous, intracellular haustoria appear similar to intercellular hyphae (Larous and Losel, 1993). Cell wall degradation allows the rust haustoria to enter the cell through the cell wall, and initiate direct contact with the cell membrane and begin to withdraw nutrients.

In later phases of colonization, rust mycelium proceeds in the host mesophyll or vascular system via intercellular mycelium and form the more classic intracellular branched, lobed haustoria. Finally, rust colonization may spread systemically throughout the entire host plant, progressing from leaves to stem to roots (Larous and Losel, 1993). However, the above description of the colonization process is for rusts in general, not for *P. horiana.* There have been few published papers specifically describing the *P. horiana* infection/colonization process in detail, or describing and illustrating, in detail, the internal *P. horiana* structures within infected chrysanthemum host tissue. The most useful observations are those of Firman and Martin (1968), who generally described the following four stages of the infection process of *P. horiana* on a sensitive chrysanthemum variety: i) development of germ tube and infection peg with no distinct difference between a germ tube and an appressorium; ii) formation of small fungal vesicles inside the host epidermis; iii), formation of elongated fungal vesicles inside the host epidermis; and iv) formation of septate fungal vesicles with branching hyphae.
As colonization progresses, CWR signs and symptoms develop on susceptible chrysanthemum plants. Infected plants initially exhibit yellow chlorotic spots on the adaxial leaf surface, where air-borne P. horiana basidiospores deposit, germinate, and initiate infection. Later, telial pustules form on various portions of the infected plant, including leaves, stems, and flowers. However, we also suggest that colonization may proceed in some chrysanthemum varieties without production of visible symptoms or signs. That is, the P. horiana mycelium may systemically colonize the host vascular system, perhaps from the leaves to the roots, but colonization remains asymptomatic.

Although the above general descriptions of P. horiana fungal structures and the infection process have been reported, illustrations of these structures on or within infected chrysanthemum plants using modern techniques have not been presented. Nor has it been proven that P. horiana can overwinter in central Pennsylvania. Thus, the main objectives of this study were to: (i) determine the ability of P. horiana to overwinter in Pennsylvania and (ii) illustrate morphological details of P. horiana within infected chrysanthemum leaves. As stated above, a secondary objective was to determine if anastomosis occurred, and if so, to illustrate the phenomenon.

**MATERIALS AND METHODS**

**Study sites.** The study area consisted of six outdoor garden locations in University Park, Pennsylvania, where CWR had been detected on perennial chrysanthemums in 2010 as well as one homeowner site in adjacent State College, where
CWR had been identified in 2011. At all seven study sites, eradication of infected chrysanthemums was attempted as described below.

**Evaluating ability of P. horiana to overwinter in Pennsylvania.** During October 2010, chrysanthemum plants with symptoms of CWR were observed at the six University Park sites. Upon detection of CWR, the six sites were quarantined and eradication attempted as per the USDA APHIS eradication protocol for CWR (USDA-APHIS-PPQ, 2012). Putatively eradicated sites were surveyed approximately every 2 weeks for presence of volunteer chrysanthemum plants. If present, volunteer plants were examined for morphological signs and symptoms of *P. horiana*. In July 2011, volunteer chrysanthemum plants, with putative CWR pustules were identified at two of the University Park sites. Symptomatic samples were collected at these two sites and examined with a Nikon model SMZ 1500 dissecting scope for morphological signs of *P. horiana*. Putative *P. horiana* teliospores were observed and examined further with a Nikon model 80i compound microscope to achieve better detail.

Based on these initial observations, eradication of CWR at the two University Park infested sites, as well as the newly identified homeowner site in State College, was attempted. The three putatively eradicated sites were then surveyed approximately every 2 weeks throughout the fall and winter of 2011 to 2012. During February 2012, asymptomatic volunteer chrysanthemum plants arising from root pieces were observed at all three of the sites. Two asymptomatic volunteer plants, arising from root pieces, were collected from each site. Each sample was washed with tap water to remove excess soil, surface sterilized with 10% bleach, and divided into two subsamples. One subsample from each site was divided into crown and root portions and approximately 100 mg of
each portion excised for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit. The DNA extractions were stored at -20°C prior to molecular screening. Molecular analysis was performed on the DNA extractions using primers ITS 5 and Rust1, followed by conventional PCR and real time qPCR, per published protocol (Pedley, 2009). Samples positive for *P. horiana* were then sequenced following Pedley's 2009 protocol.

The remaining two subsamples from each site were transplanted into sterilized potting soil and placed in a clean controlled environment chamber at maintained 18°C and 85% relative humidity (RH). After 6 weeks, six actively growing chrysanthemum plants were transferred to a second clean controlled environment chamber set at 17°C and 90 to 100% RH. Plants were examined weekly for CWR signs and symptoms. Plants that exhibited developing telia and teliospores were examined morphologically. If telia and teliospores resembled those of *P. horiana*, they were subjected to molecular analyses as described above.

**Determine morphological details of *P. horiana***. Fresh symptomatic and asymptomatic chrysanthemum leaf and stem samples from CWR-positive plants were collected for morphological imaging using a Nikon SMZ 1500 dissecting microscope. If putative *P. horiana* structures were observed, they were further examined using a Nikon 80i compound microscope as follows. Fresh samples of telia, promycelia, and intercellular *P. horiana* structures were free-hand sectioned and mounted in either 0.05% lactoglycerol cotton blue or 0.05% lactoglycerol. Sections were viewed at 10, 40, 60, and 100x. Microscopic images were captured with a Nikon DS-Fi1 digital camera, and edited for clarity when necessary with Helicon Focus 4.4 software (Helicon Soft Ltd.) as well as Adobe Photoshop (Adobe Systems). Morphology of *P. horiana* teliospore development,
promycelium formation, basidiospore formation and germination, as well as systemic infection and colonization were described and photographed.

Prior to examination, basidiospores were collected in sterile distilled water drops on acid-washed glass slides. Collection was achieved by attaching CWR infected leaves, which contained germinating telia, to the underside of a petri plate cover. The cover was placed over the slide, which was resting in the bottom of a petri plate containing distilled water to maintain elevated humidity. Samples were incubated in the dark at 18°C until examination. Slides containing basidiospores were examined after 2, 4, 6, 8, and 24 h of incubation. Basidiospore dimensions were measured and presence of germination recorded.

Intact telia with teliospores, which were attached to host leaves and/or stems, were free-hand sectioned at 2, 4, 6, 8 and 24 h after collection. Telia and teliospores were removed from telial sori using a fine needle, mounted on glass slides and examined. Mycelia and associated structures were also teased from the samples and mounted. Telia were examined mainly for telia and teliospore morphology, teliospore germination, promycelium development, and basidiospore formation. Mycelia were examined mainly to describe hyphal morphology, as well as to document the presence of haustoria and anastomosis.

Additional fresh symptomatic and asymptomatic samples from plants positive for *P. horiana* were prepared as per The Pennsylvania State University Huck Institute modified protocols for Scanning Electron Microscopy (SEM) (Dykstra and Reuss, 2003; Hall and Hawes, 1991). Samples containing telial sori were free-hand sectioned through the sori prior to fixation. Samples consisting of asymptomatic stems were also sectioned
prior to fixation. Specimens were fixed overnight at 4°C in 2.8% (v/v) glutaraldehyde in 0.1M HEPES buffer (pH 7.2) and 0.02% (v/v) Triton X-100 at RT for 2 to 4 hours. Samples were washed 3 times in buffer for 15 min each and fixed in 1% OsO4 in 0.1 HEPES buffer (pH 7.2) with 0.02% Triton X-100 in a dark container at 4°C for 24 h. Samples were then washed 3 times in the same buffer for 15 min each prior to dehydration in a % graded acetone series (20, 30, 40, 50, 60, 70, 80, 90 and 3 x 100%) for 15 min each. Each sample was critical point dried in a Bal-Tec CPD030 dryer, mounted to an aluminum stub with conductive tabs sputter-coated with 10 nm gold palladium (Au/Pd), and examined under either a JSM5400 Scanning Electron Microscope, a FEI Quanta 200 Environmental Scanning Electron Microscope or a Zeiss Merlin Compact SEM.

Additional fresh symptomatic samples from plants positive for *P. horiana* were prepared as per the Pennsylvania State University Huck Institute modified protocol (Owen and Makaroff, 1995). Symptomatic stem samples were sectioned into 5 mm pieces prior to fixation and fixed overnight at 4°C in 2.8% (v/v) glutaraldehyde in 0.1M HEPES buffer (pH 7.2) and 0.02% (v/v) Triton X-100 at RT for 2 to 4 h. Samples were washed 3 times in buffer for 15 min each and fixed in 1% OsO4 in 0.1 HEPES buffer (pH 7.2) with 0.02% Triton X-100 in a dark container at 4°C for 24 h. Samples were washed 3 times in the same buffer for 15 min each prior to dehydration in a % graded acetone series (20, 30, 40, 50, 60, 70, 80, 90 and 3 x 100%) for 15 min each. Each sample was infiltrated with acetone and Spurr’s resin at 3:1 for 2 to 4 h, 1:1 overnight and 3:1 for 10 to 12 h. Samples were then infiltrated with 100% Spurr’s resin overnight, then again for 10 to 12 h and overnight prior to embedding and polymerization in Spurr’s at 60°C for 2
days. Embedded sample were thin-sectioned with a microtome, mounted on glass slides and examined with an Olympus BX41 compound microscope. Images were captured with cellSens software (Olympus Corp of the Americas) and edited for clarification as needed.

RESULTS

Overwintering of P. horiana in Pennsylvania. *Puccinia horiana* telial sori (Fig. 2.1) were identified morphologically in July 2011 on the abaxial leaf surface volunteer chrysanthemum plants at two of the originally infested University Park sites. The adaxial leaf surface also exhibited chlorotic spots characteristic of CWR (but not restricted to this disease). These two sites had been putatively eradicated from CWR the previous year. During October 2012, CWR symptoms on the adaxial leaf surfaces, as well as telial sori on the abaxial surface, were confirmed morphologically on volunteer chrysanthemums at the putatively eradicated State College site.

In addition, *P. horiana* was detected molecularly in plant roots from one site and in the plant crowns from two sites. On 6 April 2012, CWR symptoms and signs were confirmed morphologically on two plants sampled from one site. On 19 April 2012, CWR signs and symptoms were confirmed morphologically and by molecular analysis on leaves of plant samples removed from volunteer plants at one University Park site. DNA extractions were sequenced and shared a 100% maximum identity to a known *P. horiana* accession (EU816920.1) in GenBank.
Teliospore and basidiospore formation and germination, and colonization of stem and leaf tissue, were observed within and on symptomatic as well as asymptomatic *P. horiana* infected host plants using compound microscopy and SEM (Fig. 2.2 – Fig. 2.9). Telial clusters averaged 1 to 5 mm diameter, were compact and pinkish to dark in color. Single teliospores were borne on pedicles arising from a basal layer of sporogenous cells, had smooth outer cell walls 1 to 2 µm thick, were oblong or clavate, and were 28 to 43 x 8 to 18 µm. Pedicels were persistent, hyaline and varied in length up to 40 µm. Teliospores were commonly observed on the plant surface, but were occasionally observed developing intracellularly within the chrysanthemum stem host cells. Promycelia formed most commonly from the apical cell, and occasionally from the basal cell or from both cells (Fig 2.2D, Fig. 2.8A and B). The promycelium was long and tubular, 65 x 5 µm and occasionally greater than 155 µm in length. Septa were observed within the promycelium and basidiospores formed on the distal cell. Sterigmata were not always visible, but when present, usually had an attenuated pointed/conical tube shape (Fig. 2.8B).

Basidiospores were oval to kidney shaped, 13 x 6 µm, with a roughened wall, and germinated on the leaf epidermis. Basidiospores did not become swollen and produced a single germ tube that emerged laterally from the spore (Fig. 2.2A and B, Fig. 2.5M). Secondary basidiospores, formed by budding, were observed.

A mucilaginous exudate formed at the site of attachment and penetration of the leaf epidermis. Appressoria and stomatal penetration were not observed. Germ tubes of various lengths developed from basidiospores and gave rise to hyphae. As colonization progressed, intercellular mycelium with intracellular haustoria and hyphae, developed
Mycelium was observed adhering to mesophyll cells and was found in association with tracheid cells in the xylem (Fig. 2.5N). M-haustoria, initiated by basidiospore-induced infections, were observed using both compound microscopy and SEM. Most haustoria were delimited by septa (Fig. 2.2B and Fig. 2.3E). During colonization, a mucilaginous exudate similar to that observed adjacent to the site of penetration, formed around the plant cell tissue being colonized by \textit{P. horiana}.

Masses of vermiform branching hyphae with septa (Fig. 2.3E and F, Fig. 2.4K and L), typical of M-haustoria, were also observed within both symptomatic and asymptomatic host stem tissue (Fig. 2.4 L). Infectious mycelia invaded the leaf spongy parenchyma cell layer, later forming telia primordial. Telia eventually produced pedicels that erupted through the epidermis of both stem and leaf tissue (Fig. 2.2A and C, Fig. 2.3 G and H, Fig. 2.4I and J, Fig. 2.5O). Colonizing mycelia occasionally followed vessel elements in older infected specimens and anastomosis was observed between adjacent hyphal strands (Fig. 2.4K, Fig. 2.7).

**DISCUSSION**

Although we previously reported the presence of \textit{P. horiana} in the roots and stems of asymptomatic chrysanthemum plants, we had not provided detailed descriptions of the morphology, intercellular colonization, and intracellular growth of \textit{P. horiana} in chrysanthemums until this investigation. Images produced by compound microscopy and
SEM confirmed that the morphology of *P. horiana* teliospore sori, germinating teliospores, and germinating basidiospores (Fig 2.6) were generally as previously described (Firman and Martin, 1968; Kapooria and Zadoks, 1973; Punithalingam, 1968). However, this paper provides additional, detailed illustrations regarding *P. horiana* colonization of chrysanthemum stems and leaves. Also, anastomosis between two adjacent strands of *P. horiana* hyphae is illustrated for the first time.

**Morphological details of *P. horiana***. Our images revealed that *P. horiana* telia formed on chrysanthemum leaves, stems and flowers in an unorganized pattern that coalesced at high levels of infection. Although telia were mostly hypophyllous on leaves, epiphyllous telia were also observed. Similar to other *Puccinia* spp., *P. horiana* telia originated subepidermally and were erumpent when not sheathed by the host epidermis (Cummings and Hiratsuka, 1984; Mendgen, 1984).

As described by Punithalingam (1968), most *P. horiana* teliospores were 2-celled, but occasionally 1- or 3-celled. We observed that teliospores germinated without a period of dormancy, as reported by others (Firman and Martin, 1968; Kapooria and Zadoks, 1973). Promycelium typically formed from the apical cell, but basal cells also formed promycelium and, in some cases, promycelium developed from both cells as reported by others (Firman and Martin, 1968; Kapooria and Zadoks, 1973). *Puccinia horiana* promycelium was usually long and tubular, but was often branched as described by Firman and Martin (1968) and Kapooria and Zadoks (1973). The length and shape of the promycelium within an infected plant was somewhat different from a promycelium formed in vitro in water on glass slides, but not as dramatically different as that described in line drawings by Kapooria and Zadoks (1973). Sterigmata were not always visible, but
when observed, usually had attenuated pointed/conical tubes, similar to those described by Kapooria and Zadoks (1973). Figure 2.8 illustrates the typical shape of *P. horiana* promycelium that we observed.

Figure 2.8 also illustrates *P. horiana* basidiospore formation. The number of basidiospores formed on each promycelium was difficult to ascertain, but appeared to vary from one to four. De Backer (2012) and Kohno et al. (1974) reported that *P. horiana* normally produces two basidiospores per promycelium, whereas Kim (personal communication) stated that the most common number was four, implying that the number of *P. horiana* basidiospores per promycelium may vary with strain or environmental factors. We observed *P. horiana* basidiospore germ tubes of various lengths, that gave rise to hypha or to secondary basidiospores formed by repetition as described by Kapooria and Zadoks (1973).

**Colonization by *P. horiana***. We did not observe stomatal penetration. Therefore, we hypothesize that germ tubes arising from *P. horiana* basidiospores penetrate the host epidermis directly. In addition, basidiospores germinated without noticeable germ tube swelling or appressorium formation. Although many rust fungi produce appressoria on basidiospore germ tubes immediately prior to infection (Littlefield and Heath, 1979), Waterhouse (1921) determined that appressoria were not required for host penetration in many rust fungi. Likewise, Larous and Losel (1993) and Plowright (1889) demonstrated that basidiospores of some rust fungi were able to directly penetrate the host epidermis and initiate perennial mycelium in the stem, provided the host tissue was not old. Morin et al. (2004) also observed direct penetration of basidiospore germ tubes into host epidermal cells without the formation of an appressorium. These latter references support
our observations that appressoria are likely not present during *P. horiana* infections of chrysanthemums.

Following penetration, subsequent colonization by *P. horiana* within systemically infected chrysanthemum plants was at times asymptomatic, and the outward appearance of colonized, yet asymptomatic, plants was similar to that of non-infected host plants. This observation may explain why latent infections are at times reported years after an infected plant was obtained. Although symptoms such as swelling and elongation of shoots due to hypertrophy or hyperplasia are associated with monokaryotic phases of rust life cycles and are absent in dikaryotic phases (Larous and Lösell, 1993), minor growth alterations could easily go un-noticed in cuttings and perennial plants of chrysanthemums infected perennial mycelium of *P. horiana*.

In general, biotrophic fungi often release cell wall degrading enzymes in sufficient quantities for localized reactions at the entry site of host cell walls (Larous and Losel, 1993; Morin et al., 2004). We observed a mucilaginous exudate surrounding teliospores, as well as at the site of attachment and penetration of the leaf epidermis and internal cell walls. Gold and Mendgen (1984) determined mucilaginous exudates anchor the penetrating mycelium to the host epidermis, seal the penetration site, protect the fungus from adverse conditions, and provide enzymes necessary for cell wall degradation. Bonde et al. (2013) also reported that *P. horiana* colonization among tracheid cells of chrysanthemums appeared to be enzymatic. Larous and Losel (1993) reported that cell wall degrading enzyme activity is necessary for mycelium to grow intercellularly within the host tissue. Similar to observations in other rusts (Larous and
Losel, 1993), *P. horiana* colonizing mycelia occasionally followed vascular tissue in older host tissue.

*Puccinia horiana* colonization in chrysanthemum was systemic with intercellular mycelium and intracellular haustoria, similar to that described by Bonde et al. (2013). We observed hyphae and haustoria within both symptomatic and asymptomatic host tissue. Similar to other rust fungi, some haustoria and many hyphae had septa (Chen et al., 1996). Littlefield and Heath (1979) presented the terms monokaryotic (M-Haustoria) for unspecialized intracellular hyphae produced after basidiospore infection, and dikaryotic (D-haustoria) for true haustoria originating from aeciospore or urediniospore infections. We observed only M-haustoria, as expected since *P. horiana* is a monokaryotic rust (Larous and Losel, 1993; Quilliam and Shattock, 2003). Presence of M-haustoria is also related to a microcyclic lifecycle, where telia develop after basidiospore germ tube infection with or without pycnia being produced, but without production of aeciospores or urediniospores (Quilliam and Shattock, 2003).

**Anastomosis in *P. horiana***. Anastomosis in *P. horiana* had not been previously reported (De Backer, 2012). This paper provides the first observation that anastomosis (Fig. 2.4K) occurs within chrysanthemums infected with *P. horiana*. Although the sequence of events prior to and during anastomosis are poorly understood (Wang and McCallum, 2009), documentation of anastomosis is important in plant pathogenic fungi. Increased virulence in *Puccinia* spp. can occur via somatic hybridization due to anastomosis, which is required in the parasexual cycle for many *Puccinia* spp. (Wang and McCallum, 2009). In this paper, we present the first cytological evidence demonstrating anastomosis in *P. horiana*, indicating that asexual fusion between different *P. horiana*
pathotypes and/or genotypes may occur. Previously, it was believed that *P. horiana* reproduced only clonally. Our observations support those of De Backer et al., (2012) and Ono (2002) that new, possibly more virulent pathotypes and/or genotypes may arise via anastomosis.

**Overwintering of *P. horiana* in Pennsylvania.** Systemic rust infections that overwinter are common in coniferous trees infected with rust fungi such as the stem-infecting Cronartium rusts (Buller, 1950). Also, Puccinia rusts that attack biennial thistles can overwinter systemically in the roots, in addition to overwintering as teliospores within infested plant debris (Berner et al., 2012). Systemic infections of herbaceous host plants with rust fungi capable of overwintered are less documented (Larous and Losel, 1993). In chrysanthemums infected with CWR, the mechanism by which *P. horiana* overwinters is not understood. Although no overwintering data were presented, it was reported that “CWR is known to overwinter in Europe where chrysanthemums overwinter (average minimum temperatures ranging from -10°F to 10°F” (Anon., 2010). In the United States, unconfirmed observations suggested that *P. horiana* may overwinter within infected chrysanthemums (Kim et al., 2011), a hypothesis confirmed by OKeefe and Davis (2012), who reported *P. horiana* mycelium in the roots of infected, overwintered chrysanthemum plants in the field. In support of this latter finding, Bonde et al. (2013) placed chrysanthemum plants infected with *P. horiana* in a controlled environment chamber “…simulating fall, winter, and spring temperatures in northeastern U.S.” After the simulated winter, systemic rust infections were observed in the crown, root, and newly formed stems. However, the temperature regimes, and whether constant or fluctuating, were not reported and thus these findings cannot be related to the field
situation. The presence of overwintering systemic pathogens, such as *P. horiana*, within chrysanthemums has important economic and regulatory implications, especially in the case of systemically infected, but asymptomatic, chrysanthemums. To our knowledge, this is the first confirmed report of *P. horiana* overwintering outdoors in Pennsylvania. A brief description of this dissertation study has been published (OKeeffe and Davis, 2012).
LITERATURE CITED


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chrysanthemums. Plant Dis. 93:1252-1258


Fig. 2.1. Overwintered volunteer chrysanthemum plant with *P. horiana* telial sori.
Fig. 2.2. (A) Germinating *P. horiana* basidiospores and germ tubes on chrysanthemum leaf epidermis; (B) Intracellular *P. horiana* hypha in symptomatic chrysanthemum stem. (C) Intracellular *P. horiana* teliospores in symptomatic chrysanthemum stem; (D) *P. horiana* teliospore with promycelium developing from the basal cell. Teliospore is intracellular within a symptomatic chrysanthemum stem.
Fig. 2.3. (E) Intracellular *P. horiana* hypha growing in a symptomatic chrysanthemum stem; (F) Intracellular *P. horiana* hypha growing within an asymptomatic chrysanthemum stem; (G) Germinating *P. horiana* teliospores erupting through symptomatic chrysanthemum leaf epidermis; (H) Closer view of germinating *P. horiana* teliospores erupting through symptomatic chrysanthemum leaf epidermis.
Fig. 2.4. (I) Germinating *P. horiana* teliospores erupting through symptomatic chrysanthemum leaf epidermis germ tube emanating from teliospores; (J) Germinating *P. horiana* teliospore with initial germ tube; (K) Anastomosis occurring between hyphal strands of *P. horiana*; (L) Intracellular *P. horiana* mycelia with M haustoria in symptomatic chrysanthemum leaf tissue as viewed under compound microscope.
Fig. 2.5. (M) Basidiospores (a) germinating on promycelium (b) developing from teliospores; (N) Intracellular mycelium in association with symptomatic chrysanthemum mesophyll cells; (O) *P. horiana* telia sori containing germinating basidiospores (a), conical shaped promycelium (b), teliospore pedicles (c) and teliospores (d); (P) Intracellular *P. horiana* mycelium in symptomatic chrysanthemum mesophyll cells (a, b) and xylem (c).
Fig. 2.6. Germinating *P. horiana* basidiospores with elongating germ tubes.
Fig. 2.7. *Puccinia horiana* mycelium within xylem in chrysanthemum stem stained blue with lacto-phenol and cotton blue.
Fig. 2.8. A. Promycelium developing from both apical and basal cells of a *P. horiana* teliospore; B. Conical shaped sterigmata and *P. horiana* basidiospores at tips of promycelium.
Fig 2.9. (Q) Thin section of *P. horiana* sori with basidiospores; (R) Thin section of *P. horiana* sori with basidiospores and germinating teliospores.
Chapter 3: Detection of Chrysanthemum White Rust

G. OKeefe, K. Van Poucke, K. Heungens, and D.D. Davis. First and fourth authors: Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, Pennsylvania, 16802; Second and third authors: Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit, Burg. Van Gansberghelaan 96 bus 2, 9820 Merelbeke, Belgium.

Corresponding author: D.D. Davis
E-mail: ddd2@psu.edu
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ABSTRACT


Chrysanthemum white rust (CWR), caused by *Puccinia horiana*, is economically important and can infect many *Chrysanthemum* species, as well as closely related plants. *Puccinia horiana* can cause serious damage on chrysanthemums in commercial greenhouses as well as at homeowner sites. Many countries, including the United States, have established Phytosanitary Quarantines based on loss due to CWR infections and difficulty in eradicating CWR-infected plants. Early detection and identification of *P. horiana* leading to management of CWR would significantly reduce economic loss, as well as prevent ingress of new introductions and/or strains into the United States. Unfortunately, sensitive and specific diagnosis protocols that are critical for accurate identification of *P. horiana* have not been developed. Currently, identification of *P. horiana* is based on visible morphology (e.g., signs and symptoms on the host plant). However, visible morphology cannot be used to identify *P. horiana* within latent infections. In this paper, we suggest use of molecular techniques, especially PCR, for
accurate identification of \textit{P. horiana} within asymptomatic, yet infected, chrysanthemum plants. Early, sensitive, and accurate molecular diagnosis of \textit{P. horiana} in chrysanthemum plants could greatly reduce loss for wholesale and retail horticultural businesses, as well as homeowners, due to CWR.

\textbf{INTRODUCTION}

Chrysanthemum white rust (CWR), caused by the autoecious, microcyclic rust fungus \textit{Puccinia horiana} Henn., is a disease on many \textit{Chrysanthemum} spp. and is economically important due to its ability to infect florist chrysanthemum cultivars (\textit{Chrysanthemum} \textit{$\times$} \textit{morifolium}) throughout the world (Baker, 1967; Dickens, 1990; Firman and Martin, 1968; Horst and Nelson, 1997). Many countries, including the United States, have established Phytosanitary Quarantines based on the cost of lost chrysanthemum crops and difficulty in eradicating CWR-infected plants (USCFR, 2014).

\textit{Puccinia horiana} can cause serious damage to chrysanthemums in commercial greenhouses as well as at homeowner sites (Anon, 2010), resulting in unprecedented economic losses. In 2007, CWR was identified at 40 commercial and residential sites in CA, CT, MD, NY, PA, and ME. In 2008, an additional 13 sites with CWR were identified in CA, DE, MA, and MI. Positive interceptions increased in 2009 when 16 positive locations were identified in NY and VA. In 2010, CA and MD reported 17 positive locations. In 2011, PA reported that 139 CWR positive samples had been intercepted at 82 sites in 22 counties during 4 years. Due to federal regulation of \textit{P. horiana}, chrysanthemum plants were eradicated at all of these positive locations, resulting in substantial crop and financial loss. For example, during 1990 to 1999, CWR
was detected at 64 infested sites in CA on plants shipped from Asia and Mexico. At that time, the cost to eradicate the interceptions was approximately $500 per site, or $32,000 total. Due to an extension of the quarantine, the eradication cost increased to approximately $7,000 per site. The total eradication costs converted to 2014 values for $500, $32,000 and $7,000 would be approximately $788, $50,500, and $11,000 respectively. During 1995, eradication costs of CWR for commercial chrysanthemum growers in San Diego, CA, was estimated at approximately $5,000 per site, which equals > $7,000 dollars in 2014 dollars. Direct and indirect losses from CWR infestations intercepted in Santa Barbara County CA, between 1992 to 1997 were estimated at $2 million, which is > $3 million in 2014 values. In 1997, Santa Barbara County reported the value of chrysanthemum production to be greater than $10 million, or > $14 million dollars in 2014 values (Coppock, and Kreith, 1999). However, in spite of the numerous interceptions of CWR in the United States, the causal pathogen *P. horiana*, is not considered to be established in this country (USCFR, 2012).

The above examples of dollar loss clearly illustrate that early detection of CWR leading to management of the disease would significantly reduce economic losses for both commercial and homeowner chrysanthemum growers. However, sensitive and specific diagnosis protocols are critical for accurate identification of CWR. In addition, for regulated quarantine pathogens such as *P. horiana*, early identification is also essential to prevent ingress of new introductions, including possibly new strains, into the United States. Unfortunately, identification of critical fungal pathogens, such as *P. horiana*, is often based on morphology (e.g., visible signs and symptoms on the host plant). However, visible morphology cannot be used to accurately identify pathogens
hidden within latent infections (Agrios, 2005). In this paper, we suggest use of molecular techniques, especially PCR, for accurate interception and identification of *P. horiana* within asymptomatic chrysanthemum plants. Diagnostic PCR protocols require sufficient sensitivity to detect minute quantities of DNA or RNA in the target organism. Conventional PCR and real time qPCR protocols, which use *P. horiana* species-specific primers, have been developed (Alaei et al., 2009; Pedley, 2009). Although conventional PCR can be used, real time qPCR currently offers more sensitive detection of low target levels of *P. horiana* quickly and at lower cost.

The main objectives of this study were to (i) determine the economic impact of CWR based on incidence and severity of *P. horiana* on selected varieties at one location in central Pennsylvania, and (ii) develop a molecular screening technique sensitive enough to detect latent, hidden infections of CWR.

**MATERIALS AND METHODS**

**Study sites.** All study (survey) sites were located on the campus of The Pennsylvania State University (PSU), University Park, PA where chrysanthemum cuttings and plantings had exhibited visible CWR symptoms in past years. In this paper, selected varieties of chrysanthemums were evaluated for presence or absence of CWR in 2012 and 2013.

**Determining the incidence and severity of CWR on 10 chrysanthemum varieties during fall 2012.** From 1 October to 25 November 2012, approximately 100 chrysanthemum plants of each of ten varieties were evaluated weekly for presence or
absence of visible symptoms of CWR. The chrysanthemums were growing either in planters or in the ground at various locations across the PSU campus. Each plant was rated morphologically for CWR disease severity (none, low, medium or high), and location of visible signs and symptoms on leaves, stems, flowers and/or buds were recorded. Infected plants were eradicated as discovered per USDA regulations (USCFR, 2012). However, prior to eradication, symptomatic and asymptomatic cuttings were removed from a sample of infected chrysanthemum plants for molecular analyses as follows.

Approximately 100 mg of symptomatic leaves were excised for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit. The DNA extractions were stored at -20°C prior to molecular analyses. On 14 November 2013, 150 asymptomatic cuttings were collected from each of two varieties exhibiting the lowest disease severity rating. A random number generator was used to designate 3 reps of 42 plants/variety. Cuttings were potted in Sun Gro Metro-Mix 600 growing medium (Sun Gro Horticulture Canada Ltd, Bellevue, WA) and rooted in a controlled environment maintained at approximately 40% RH, 17°C, and a 16-h photoperiod. These environmental conditions are optimal for chrysanthemum growth, but not for CWR symptom development (Pertuit, 1995), and were selected to suppress symptom development until rooted plants became established.

One chrysanthemum variety was maintained under these conditions for 2 weeks and the second for 2 months. After the initial establishment period, the cuttings were placed in a controlled environment at approximately 100% RH, 17°C and a 16-h photoperiod, environmental conditions conducive for development of CWR signs and symptoms (Anon, 2010). Plants were surveyed weekly and disease incidence and severity
recorded for a maximum of 1 month. In order to prevent secondary infections, symptomatic plants were removed from the growth chamber as symptoms developed. As per USDA CWR regulations and eradication protocol, all chrysanthemum plants within an CWR-infected of the variety were removed and eradicated (USCFR, 2012; APHIS PPQ, 2012). Due to the high level of CWR incidence and severity observed during 2012, this study was expanded to include concurrent morphological and molecular research in 2013.

**Determining the incidence and severity of CWR on 17 chrysanthemum varieties in 2013.** On 11 June 2013, commercially purchased chrysanthemum cuttings arrived at the PSU nursery, were rooted and potted in Sun Gro Metro-Mix 600, numbered, and placed outside in nursery beds under an automatic watering and fertilizing system. Seventeen varieties consisting of 102 cuttings each were included in this study. Three of the 17 varieties were symptomatic with CWR during the 2012-growing season, but all cuttings were asymptomatic for CWR at time of arrival in 2013. The potted plants within the nursery were evaluated at biweekly intervals from June to early September. Campus plants were surveyed weekly from mid-September until November, the end of the growing season. This survey design resulted in the potted plants being evaluated 7 times in the nursery and 10 times after being planted on campus.

Potted plants in the nursery that had visible CWR signs and symptoms were sampled and were examined with a Nikon model SMZ 1500 dissecting scope for *P. horiana*. If present, teliospores were further examined with a Nikon model 80i compound microscope. All chrysanthemums, including both symptomatic and asymptomatic plants, within a CWR-positive variety were removed and eradicated (USCFR, 2012; APHIS
PPQ, 2012). However, prior to eradication, symptomatic and asymptomatic cuttings were collected from two of the CWR-positive varieties. Approximately 100 mg of each positive sample was excised for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit and stored at -20°C until used for molecular screening, which was performed on the DNA extraction using the protocol of Alaei et al. (2009) as described in detail later.

On 29 August 2013, prior to eradication, 50 asymptomatic cuttings were collected from one variety of potted chrysanthemum plants at the PSU nursery that had visible CWR symptoms. Cuttings were rooted in rock wool and maintained in a controlled environment chamber set at 100%RH, 17°C and a 16-h photoperiod, conditions optimal for CWR disease development (Anon, 2010). Cuttings were evaluated weekly, at which time disease incidence and severity was recorded for a maximum of 2 months. To prevent secondary infections, symptomatic plants were removed from the growth chamber as telial pustules developed and eradicated, resulting in the evaluation period ranging from 1 week to 2 months.

On 31 October 2013, prior to disposal, based on morphology, 50 asymptomatic cuttings were collected from a second CWR-positive variety of chrysanthemums that had been planted on campus. This variety had also been found to be CWR-positive in 2012. Cuttings were rooted in rock wool and maintained in a controlled environment chamber under environmental conditions optimal for CWR disease development (Anon 2010). The rooted cuttings were surveyed weekly, and disease incidence and severity recorded for a maximum of 2 months. In order to prevent secondary infections, symptomatic plants were removed from the growth chamber as telial pustules developed and eradicated.
Development of molecular screening protocol. To develop a molecular technique for screening asymptomatic CWR samples, the protocols and primer sets reported by Pedley (2009) and Alaei et al. (2009) were evaluated for sensitivity to *P. horiana* (Table 3.1). In addition, a slightly modified protocol was developed using primers of Alaei et al. (2009). The modified PCR protocol was performed using the Alaei et al. primers in 25µl reactions containing 10µM of each primer and SensiMix SYBR No-ROX PCR Master Mix (Applied Biosystems, Inc). Reactions were conducted using a Smart Cycler II (Cepheid, Inc. Sunnyvale, CA) with the initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62°C for 15 s, and 72°C for 15 s acquired at the end of the step. Approximately 100 mg of CWR-positive samples from two symptomatic, from two asymptomatic but CWR-positive plants, a single sample of *P. chrysanthemi*, and a single sample collected from a healthy chrysanthemum plant were excised for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit and stored at -20°C. The nucleic acid concentration of the samples was determined using a Nanodrop 2000 (Thermo Scientific, Inc.) and adjusted to approximately 20 ng/µl. The extracted DNA and a Negative Template Control (NTC) were compared using the three protocols. The threshold cycle (Ct) value was recorded as the cycle at which a fluorescence signal intercepted the threshold set at 30. Each sample was analyzed in triplicate using three protocols. The Ct values from each protocol were then evaluated and compared.

Starting on 25 June 2013, eight random leaf samples were collected from each of the 17 varieties of potted chrysanthemum plants from the PSU nursery. Approximately 100 mg of asymptomatic leaves sampled from each of the eight chrysanthemum samples
were excised for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit, and stored at -20°C prior to molecular screening. The nucleic acid concentration of the samples was determined using a Nanodrop 2000 (Thermo Scientific, Inc.) and nucleic acid concentration adjusted to approximately 20 ng/µl. Extracted DNA was screened using only the modified protocol of Alaei et al. as described above. The threshold cycle (Ct) value was recorded and each sample was run in duplicate.

Asymptomatic samples were analyzed with two sets of primers and/or independently analyzed by collaborators at the Instituut voor Landbouw- en Visserijonderzoek (ILVO), Merelbeke, Belgium. Samples analyzed at ILVO were performed in an ABI 7900 (Applied Biosystems) consisting of 25µl reactions containing 2µl DNA template and either10 µM of each primer and SensiMixSYBR PCR Master Mix (Applied Biosystems) or 10 µM of each primer and Takara Master Mix (Takara). The initial denaturing step was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. Samples that resulted in a Ct value between 13 to 40 were considered putatively positive for CWR. Putatively positive plants were tagged at the nursery for (additional) morphological screening and DNA extracted prior to eradication. In addition, samples were collected from putatively positive plants that did not develop CWR signs in the nursery. These samples were included within the 50 asymptomatic plants placed in controlled environmental chamber for the incidence and severity study, as described above.

RESULTS
Determining the incidence and severity of CWR on 10 chrysanthemum varieties growing on the PSU campus in fall 2012. Chrysanthemum varieties exhibiting visible CWR symptoms were sampled at PSU on three different dates in 2012. On 24 October 2012, symptomatic samples were collected from one variety out-planted on PSU campus. On 1 November 2012, samples exhibiting visible symptoms of CWR were collected from two symptomatic chrysanthemum varieties out-planted on PSU campus. And finally, on 16 November 2012, symptomatic samples were collected from three chrysanthemum varieties out-planted on the PSU campus, as well as from symptomatic potted plants growing within the PSU nursery. Microscopic examination of samples confirmed CWR on or in some plants of all symptomatic varieties. Per USDA CWR regulations and eradication protocol, all chrysanthemum plants representing each infected variety were removed and eradicated (USCFR, 2012; USDA APHIS, 2012). However, prior to eradication, ca. 100 mg of symptomatic tissue was excised from each variety for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit. Samples were stored at -20°C for molecular analysis at a later date.

Five of the 10 chrysanthemum varieties evaluated on PSU campus were judged to be CWR positive, based on visible signs and symptoms, and were rated for relative disease severity using 0 = no disease, 1 = light infection level, 2 = medium infection level, and 3 = high level of infection. The mean frequency level of CWR on infected plants ranged from 0.16 to 2.61 (Table 3.2). Within symptomatic varieties, most plants were rated as a symptom level of 1, a light level of infection. However, Variety 2 was rated mostly at a level of 2, a medium level, for frequency (Fig. 3.1). All plants representing the five symptomatic varieties of chrysanthemums were eradicated.
Determining the incidence and severity of CWR on 17 chrysanthemum varieties in 2013. On 20 August and 26 August 2013, symptomatic leaf tissue samples were collected from two potted chrysanthemum varieties in the PSU nursery that exhibited visible CWR symptoms. Microscopic examination confirmed CWR in or on both varieties. The remaining 15 varieties were asymptomatic. Symptom severity on the two symptomatic varieties was rated using the 1 to 3 criteria. All asymptomatic plants within in each variety were rated at a level of 0, and symptomatic plants were rated as 1, 2, or 3. The least symptomatic variety exhibited CWR infection on only one of 102 plants evaluated, with a mean severity rating of 0.01. The second variety exhibited more severe symptoms on 25 of 102 plants, with a mean severity rating of 0.25. All chrysanthemum plants representing both symptomatic varieties were eradicated, but prior to eradication, ca. 100 mg of symptomatic leaf was excised and stored at -20°C.

Development of molecular screening protocol. Three different protocols, using two different primer sets, were evaluated and compared in three replications to determine the most sensitive qPCR protocol for detecting *P. horiana* in chrysanthemum. Protocols included those reported by Pedley (2009), Alaei et al. (2009), and our modified versions of their protocols (Table 3.3). Sample types included healthy plant tissue (no *P. horniana*), symptomatic plant tissue known to contain *P. horniana*, asymptomatic but CWR-positive plant tissue, and a negative template control (NTC). Results demonstrated that the modified protocols of Aleai et al. (columns 4 and 5 in Table 3.3), were most sensitive. The qPCR values for both the healthy plants and NTC were >40 using any of the protocols or primer sets evaluated, indicating lack of detectable *P. horiana* DNA.
On 25 June 2013 eight randomly selected asymptomatic leaf samples were collected from eight plants each of the three varieties of potted chrysanthemums from the PSU nursery that had been identified as positive for CWR in 2012. Based on the results shown in Table 3.4, molecular analysis was performed on the DNA extractions using the modified Alaei protocols with primers Ph236F/Ph264R and Ph263F/Ph264R. Four of the eight asymptomatic samples from one variety tested “positive” (based on Ct values between 13 and <40) for *P. horiana* at both the PSU lab and at the ILVO, Belgium lab. Three of the eight asymptomatic samples of the second variety and two of the eight asymptomatic samples from the third variety also tested positive for *P. horiana* at the PSU lab. Plants that tested positive by qPCR were tagged at the nursery for additional morphological screening, and DNA extracted prior to eradication. All plants that tested positive by qPCR eventually developed CWR symptoms either at the nursery, or in later controlled environment chamber studies. All plants within CWR-positive varieties were eradicated.

Following testing of the three varieties that had been identified as positive for CWR, all other asymptomatic samples, collected between 29 June 2013 and 5 August 2013, from the remaining 14 varieties were tested at the PSU lab using the modified Alaei protocols with primers Ph236F/Ph264R and Ph263F/Ph264R. All samples proved negative for *P. horiana*.

Plant samples were collected from putative positive plants that did not develop CWR signs in the nursery and were included within the 50 asymptomatic plants placed in controlled environmental chamber as part of the 2013 incidence and severity study. Table 3.4 illustrates the chrysanthemum varieties and number of plants that screened putative
positive for *P. horiana* prior to symptom development, i.e. the plants developed yellow leaf spots and/or telial sori.

**DISSCUSION**

**Determining the incidence and severity of CWR chrysanthemum varieties growing on the PSU campus.** *Puccinia horiana* can cause serious damage to chrysanthemums in commercial greenhouses as well as at homeowner sites (Anon., 2010), resulting in unprecedented economic losses. As stated earlier, the United States and many other countries have established Phytosanitary Quarantines due to the seriousness of CWR (USCFR, 2012) and thus *P. horiana* is considered to be a regulated pathogen. Because of the United States federal regulation of *P. horiana*, five varieties of chrysanthemums that tested positive for CWR on the PSU campus in 2012 were eradicated. These five varieties contained 582 plants resulting in a 2012 financial loss of approximately $3,550, not including the cost for eradication of the plants. In contrast, only two chrysanthemum varieties, consisting of 204 plants, tested positive for CWR on campus in 2013 and needed to be eradicated, resulting in a $1,400 approximate loss, not including the eradication costs.

Thus, the dollar loss on campus due to CWR during 2013 was approximately 40% that of 2012, or reduced approximately by half. Only 204 chrysanthemum plants within two varieties were lost in 2013, as opposed to 582 within five varieties in 2012. The reduced dollar and crop loss in 2013 was likely due to two factors. First, we had identified several CWR-sensitive varieties of chrysanthemum in 2012. Some of these sensitive varieties were deliberately not purchased in 2013 because the campus
landscaping personnel did not, of course, want CWR-sensitive chrysanthemum varieties on campus. Although not used in our loss calculations, some of the CWR-sensitive varieties that were not ordered in 2013 may also represent an aesthetic loss. That is, some CWR-sensitive varieties that were not purchased in 2013 may have had desirable characteristics, including form and color, which would have fit well into the campus-landscaping scheme. Unfortunately, they may have been replaced in 2013 by less aesthetically pleasing chrysanthemum varieties or even entirely different plant species.

Secondly, in 2013 we detected CWR-sensitive chrysanthemum varieties on campus earlier in the growing season than in 2012. In 2012 we had surveyed for CWR only on chrysanthemums planted out on campus, not on potted plants in the nursery prior to outplanting. In 2012, we did not detect CWR on campus until the fall, on 24 October. Based on these 2012 observations, we began our 2013 surveys on potted chrysanthemum plants while they were still in the nursery and had not yet been planted on campus. This more intensive survey in 2013 resulted in our detecting CWR much earlier, on 20 August. The 2-month earlier detection in 2013, as compared to 2012, resulted in considerable cost savings in that the CWR-sensitive varieties were still in pots, as well as being grouped by variety in the nursery, and thus could be eradicated more easily and cost-effectively than if they had been established on campus in the ground or in planters.

One of our more interesting findings was how quickly severe CWR infections occurred. During 2012, the most sensitive variety had a mean severity index of 2.61, and was therefore not purchased in 2013. The second-most sensitive variety in 2012 had a 1.08 severity rating, but was purchased again in 2013. In 2013, this second variety was identified as CWR-positive on 20 August. At that time, only one symptomatic plant with
telia was identified and *P. horiana* confirmed. However, 2 days later, on 22 August 2013, telial clusters of *P. horiana* were observed on an additional 17 plants. That is, *P. horiana* telial clusters increased from being present on only one plant, to 18 out of 102 plants within 2 days, a very rapid increase in disease incidence. In addition to incidence, the severity rating of this variety in 2012 was 1.08, while in 2013, due to the earlier interception, the severity rating was only 0.03.

The weather during October 2012 on the PSU campus was cooler and wetter than in August 2013, which was hot and dry. The average temperature during October 2012 was 12.0°C and the average rainfall was 127 mm. In comparison, the average temperature during August 2013 was 20.5°C and the average rainfall was 1.04 mm. Based on these environmental conditions, higher CWR severity and incidence ratings 2012 were to be expected during October 2012. However, the CWR disease was more severe in 2013 for unknown reasons. In fact, the number of positive plants during 2013 went from one to 18 in 2 hot dry days, an observation that was enlightening and demonstrated the ability of *P. horiana* to quickly produce symptoms on otherwise asymptomatic plants.

In 2010 and 2013, we visited three non-commercial (“hobbyist”) chrysanthemum growers, who grew prize-winning show chrysanthemums. We observed CWR in chrysanthemum varieties at all three locations. Two of the hobbyists had three varieties with mild CWR symptoms. However, the third hobbyist, who grew twice as many plants as the other two hobbyists, had CWR symptoms within all chrysanthemum varieties. Unfortunately, the third grower grew many irreplaceable heirloom varieties that are no longer available. All plants representing symptomatic varieties were eradicated. Not only
were these situations devastating to the hobbyists financially, but also emotionally, especially in the case where heirloom varieties could not be replaced.

A molecular screening protocol (see next section) might have allowed even earlier detection in 2013 of CWR within infected, but asymptomatic cuttings, plants, and varieties at PSU. A molecular screening protocol also might have prevented the hobbyists a financial and emotional loss.

**Molecular screening protocol.** Currently, best management practices for growers to maintain chrysanthemum crops free of CWR include routine scouting for visible CWR signs and symptoms, microscopic confirmation of teliospores, and the use of prophylactic fungicides. Since *P. horiana* can grow systemically yet asymptotically within infected chrysanthemum plants, initial stages of infection and colonization of the pathogen can occur without visual detection (Bonde et al., 2013; OKeefe and Davis, 2012). Accurate and early detection of *P. horiana* can limit the spread, ensure clean stock, and reduce costs associated with eradication.

Development of diagnostic protocols that allow accurate and early detection of plant diseases is critical for preventing introduction of pathogens and minimizing losses within various pathosystems (Pedley, 2009; Schaad et al., 2003). Visual diagnostic confirmations, which are slow and require diagnostic expertise, are the only option for identifying some pathogens. However, plant samples that contain pathogens, but yet are asymptomatic, can go undetected. Sensitive molecular diagnostics capable of identifying pathogens during the asymptomatic stages have been developed for many pathogens, but not for *P. horiana* (Alaei et al., 2009b; Schaad et al., 2003). The purpose of this phase of
the research was to develop a real-time molecular protocol for screening asymptomatic, yet *P. horiana* positive, chrysanthemum plants.

Alaei et al. (2009b) developed qPCR primers and probes sensitive enough to detect as few as eight *P. horiana* basidiospores, with mean Ct values of approximately 33.5 (0.1 pg). Although they developed the qPCR protocol for an ABI 7900 (Applied Biosystems), which used either SYBR Green I PCR Master Mix (Applied Biosystems) or a Taqman probe, we modified their protocol by using SensiMix SYBR No-ROX PCR Master Mix (Applied Biosystems, Inc) and by optimizing the annealing temperatures for each of the two primer sets with a Cepheid Smart Cycler II. Alaei et al. (2009b) also recommended primer sets Ph263F/Ph264R and Ph234F/Ph259R. However, we determined that primer set Ph234F/Ph229R was more sensitive and accurate when used in combination with Ph263F/Ph264R for screening asymptomatic yet *P. horiana*-positive chrysanthemum plants. Using this modified molecular protocol, three asymptomatic chrysanthemum varieties tested positive for *P. horiana* in 2013. All three later developed macroscopic CWR symptoms and microscopic *P. horiana* teliospores, whereas all chrysanthemum varieties that tested negative for *P. horiana* did not develop CWR signs and symptoms.

The federal regulations state that, for a chrysanthemum plant to be considered positive for *P. horiana*, it must exhibit visible teliospores on or in the infected plant (Pedley, 2009; USCFR, 2014). Thus, chrysanthemum plants that are determined to be positive for *P. horiana* via molecular screening do not legally have to be eradicated, if teliospores or telia sori are not visible. If the PSU nursery manager waited for development of telia and teliospores on plants known to contain *P. horiana* by using
molecular methods, the infected chrysanthemums might have been out-planted on campus. This delay in identification of CWR would increase the financial costs, since eradication would now involve digging and removing established plants on campus. Sensitive and accurate molecular screening of chrysanthemum plants such as demonstrated by our research, employed prior to dispersal of asymptomatic yet *P. horiana*-positive chrysanthemum plants, could greatly reduce cost of production for wholesale and retail horticultural businesses, as well as homeowners.
Literature Cited


   §Title 7. Part 319.37-2. USDA, APHIS, PPQ.
TABLE 3.1. PCR primers, probes and nucleotide sequences, location and reference.

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5' to 3')</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph-F2</td>
<td>CCCCTTTTTTATTATATAACACAAG</td>
<td>ITS1</td>
<td>Pedley (2009)</td>
</tr>
<tr>
<td>Ph-R1</td>
<td>CAAAAATTATTTTGAGAGGG</td>
<td>ITS2</td>
<td>Pedley (2009)</td>
</tr>
<tr>
<td>FAM-Probe</td>
<td>FAM-CCAAAAGGTACACCTGGTTTGA GTGCA-TAMRA</td>
<td>ITS2</td>
<td>Pedley (2009)</td>
</tr>
<tr>
<td>Ph229R</td>
<td>TTTTCAAAAACAAAAGCTGCAAGT</td>
<td>ITS2</td>
<td>Alaei (2009b)</td>
</tr>
<tr>
<td>Ph234F</td>
<td>CCCTCTAAAAATAATTTTTGTTAATTA</td>
<td>ITS2</td>
<td>Alaei (2009b)</td>
</tr>
<tr>
<td>Ph263F</td>
<td>ACCCTTTAAAAATATATACCACCAACTAT</td>
<td>ITS1</td>
<td>Alaei (2009b)</td>
</tr>
<tr>
<td>Ph264R</td>
<td>CTTGTGTTATATAATAAAAAGGGGTAAAA6-FAM-</td>
<td>ITS1</td>
<td>Alaei (2009b)</td>
</tr>
<tr>
<td>FAM-Probe</td>
<td>ACTTGGTTGCATGAATT-MGB</td>
<td></td>
<td>Alaei (2009b)</td>
</tr>
</tbody>
</table>
TABLE 3.2. Frequency of CWR incidence among 10 chrysanthemum varieties in the field during 2012.

<table>
<thead>
<tr>
<th>Plant variety</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plants</td>
<td>72</td>
<td>95</td>
<td>102</td>
<td>23</td>
<td>6</td>
<td>38</td>
<td>29</td>
<td>7</td>
<td>32</td>
<td>67</td>
</tr>
<tr>
<td># CWR pos</td>
<td>70</td>
<td>94</td>
<td>84</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td># CWR neg</td>
<td>2</td>
<td>1</td>
<td>18</td>
<td>15</td>
<td>6</td>
<td>38</td>
<td>25</td>
<td>7</td>
<td>32</td>
<td>67</td>
</tr>
<tr>
<td>% Positive</td>
<td>97</td>
<td>99</td>
<td>82</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE 3.3. Mean real-time Ct values obtained using protocols of Pedley (2009), Alaei et al., (2009b) and a protocol we modified.

<table>
<thead>
<tr>
<th>Primer set:</th>
<th>Pedley protocol</th>
<th>Alaei’s protocol 1</th>
<th>Alaei’s protocol 2</th>
<th>Modified protocol 1</th>
<th>Modified protocol 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample:</td>
<td>Ph-F1/Ph-F2/</td>
<td>Ph234F/Ph229R</td>
<td>Ph263F/Ph264R</td>
<td>Ph234F/Ph229R</td>
<td>Ph263F/Ph264R</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>29.12± 0.85</td>
<td>26.60±0.91</td>
<td>25.02±1.05</td>
<td>25.37±0.59</td>
<td>23.7±0.80</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>39.41±0.73</td>
<td>34.30±2.08</td>
<td>34.11±1.79</td>
<td>31.25±1.19</td>
<td>33.52±1.92</td>
</tr>
<tr>
<td>Healthy plant</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NTC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Threshold cycle.
b Chrysanthemum samples with *P. horiana* teliospores present.
c Chrysanthemum samples positive for CWR but no symptoms present.
d Chrysanthemum Samples with no CWR present.
e Negative template control
TABLE 3.4. Chrysanthemum plants from three varieties putatively rated positive with molecular assay during 2013.

<table>
<thead>
<tr>
<th>Plant #</th>
<th>PSU 229F/234R</th>
<th>PSU 263F/264R</th>
<th>ILVO 263F/264R</th>
<th>ILVO 263F/264R</th>
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<tbody>
<tr>
<td></td>
<td>SensiMix-SCII&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SensiMix-SCII&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sensi-Mix/ABI&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Takara/ABI&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>for 3</td>
<td>13&lt; Ctd ≤40</td>
<td>13&lt; Ctd ≤40</td>
<td>13&lt; Ctd ≤40</td>
<td>13&lt; Ctd ≤40</td>
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<tr>
<td>varieties&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two plants in variety 1, 4 plants in variety 2, and 3 plants in variety 3.

<sup>b</sup> Cepheid Smart Cycler II.

<sup>c</sup> ABI 7900.

<sup>d</sup> Threshold value.
Fig. 3.1. Frequency of CWR among 10 chrysanthemum varieties, as rated from 0-3.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Rated &quot;0&quot;</th>
<th>Rated &quot;1&quot;</th>
<th>Rated &quot;2&quot;</th>
<th>Rated &quot;3&quot;</th>
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<tr>
<td>1</td>
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Chapter 4: Genotyping and Analysis of 61 \textit{P. horiana} Isolates from the United States

G. OKeefe, K. Van Poucke, M. De Backer, K. Heungens, K. Pedley, M. Mangano, G. McMackin and D.D. Davis. First and seventh authors: Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, Pennsylvania, 16802; Second, third and fourth authors: Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit, Burg. Van Gansberghelaan 96 bus 2, 9820 Merelbeke, Belgium. Fifth author: United States Department of Agriculture-Agricultural Research Service, Foreign Disease-Weed Science Research Unit, 1301 Ditto Ave., Ft. Detrick, MD 21702, sixth and seventh authors: New Jersey Department of Agriculture, Division of Plant Industry, Trenton, New Jersey.

Corresponding author: D.D. Davis

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Abstract


Chrysanthemum White Rust (CWR), caused by \textit{Puccinia horiana} Henn., is an autoecious, microcyclic rust that is pathogenic on many chrysanthemum species.

Chrysanthemum White Rust is economically important due to its ability to infect florist chrysanthemum (\textit{Chrysanthemum \times morifolium}) cultivars used as cut flowers and potted plants throughout the world. \textit{Puccinia horiana} can cause serious damage to chrysanthemums in commercial greenhouses as well as at homeowner sites. \textit{Puccinia horiana} has been found in most chrysanthemum-producing regions. Many countries, including the U.S., have established Phytosanitary Quarantines due to the cost of lost mum crops and the difficulty of eradicating CWR. Recent research confirms there is more than one genotype of \textit{P. horiana}. SNP and SSR markers have been developed for HRM techniques to identify and analyze genotypes of \textit{P. horiana} isolates. The objectives
of this study were to genotype *P. horiana* isolates collected throughout the United States and analyze and compare the genotypes of the United States isolates with worldwide isolates previously collected and genotyped.

**Introduction**

Chrysanthemum White Rust (CWR), caused by *Puccinia horiana* Henn., is an autoecious, microcyclic rust that is pathogenic on many chrysanthemum species. Chrysanthemum White Rust is economically important due to its ability to infect florist chrysanthemum (*Chrysanthemum × morifolium*) cultivars used as cut flowers and potted plants throughout the world (Baker, 1967; Dickens, 1990; Firman and Martin, 1968; Horst and Nelson, 1997). *Puccinia horiana* can cause serious damage to chrysanthemums in commercial greenhouses as well as at homeowner sites (Anon, 2010). *Puccinia horiana* has been found in most chrysanthemum-producing regions (Eppo/CABI, 2004; Punithalingam, 1968). Many countries, including the U.S., have established Phytosanitary Quarantines due to the cost of lost mum crops and the difficulty of eradicating CWR. (US C.F.R., 2012).

If the moisture and temperature are optimal, symptoms appear on leaves 7 to 10 days post-infection (dpi) as yellow chloric spots. *Puccinia horiana* Henn., has two known spore stages, basidial and telial. Teliospores grow *in situ* within pustules (sori). Teliospores are not normally disseminated except in wind-driven debris. When conditions are optimal (RH > 96%, temperature 17 – 24 C), teliospores germinate without a period of dormancy, producing promycelia. If environmental conditions remain
optimal for at least 3 hours, up to four basidiospores, the infective mobile propagule, form on each promycelium. Basidiospores are disseminated 3 - 6 hours after development by wind and/or water. Upon landing on a susceptible host, spore germination will take place if the RH > 70% and there are approximately 5 hours of leaf wetness. Teliospores and basidiospores may likely be spread in and on asymptotic plant cuttings. Puccina horiana is a self-fertilizing rust, therefore the basidiospores are genetically uniform unless anastomosis or mutation followed by recombination of various genotypes ensues.

Recent research confirms there is more than one pathotype of *P. horiana* (De Backer, 2012; Dickens, 1968; Water, 1981). In 2011, De Backer et al., demonstrated the complexity of the *P. horiana* pathosystem by testing 22 isolated collected from a broad geographic area on 36 chrysanthemum cultivars. They determined that, based on Flor’s gene-for-gene theory, a minimum of 7 avirulence genes are involved in the *P. horiana* pathosystem. In 2012, De Backer et al. reported “…a study in which 6 Japanese isolates were inoculated on 40 cultivars involved the first systematic testing of multiple isolate in multiple hosts, and revealed differential interaction phenotype profiles for several of the cultivars used (Yamaguchi, 1981).” In order to understand the pattern and changes within the *P. horiana* isolate population, De Backer et al., (2012) developed a genotyping protocol for *P. horiana* based on single-nucleotide polymorphisms (SNPs) and simple-sequence repeats (SSRs). In his research, De Backer included 5 *P. horiana* isolates collected from 5 locations in the United States, California, Massachusetts, Connecticut, Maryland and Pennsylvania. These isolates were unique because *P. horiana* is not considered endemic in the United States. The five isolated include three non-recombinant
genotypes, indicating a minimum of three separate introductions of the pathogen into the United States. Although collected in different states over three years, two sets of two isolates each had the same multilocus genotype. De Backer hypothesized that, based on the genotype information of the 5 isolates, that survival and spread of the pathogen within the United States is possible and that *Puccinia horiana* may now in fact be endemic. This is supported by the reports of overwintering of *P. horiana* on infected chrysanthemum plants in Pennsylvania (OKeefe and Davis, 2012; Kim et al., 2011). However, “…genotyping of more USA isolates and isolated from the possible geographic origins of imported plant material will provide further evidence of one of these hypotheses…” (De Backer, 2012).

**Genotyping of *P. horiana***. Due to quarantine regulations and potential economic losses, rapid and accurate molecular diagnostics is an important aspect of pathogen control programs. Molecular techniques are available that allow detection and characterization of various pathogens, including their genetic diversity, as well as the ability to track and trace isolates. Genotyping of *Puccinia horiana* isolates can allow the identification of new and highly virulent isolates in intercepted samples.

The genetic diversity of a population can be determined using dominant markers, based on Amplified Fragment Length Polymorphism (AFLP) or Random Amplification of Polymorphic DNA (RAPD). These two systems allow rapid screening of different genome loci without the need for sequence data. However, they are not suitable to develop markers for obligate biotrophs, such as *P. horiana*, since they don’t allow the amplification in a background of contaminating plant substances or microorganisms. In these cases co-dominant markers like Simple Sequence Repeat (SSR) and Single
Nucleotide Polymorphisms (SNPs), which are generally locus-specific, can be used. A SNP is a DNA sequence variation occurring when a single nucleotide, A, T, C, or G, in the genome differs between members of a biological species. SSRs, also known as microsatellites, are tandemly repeating sequences of 1-5 nucleotide base pairs of DNA (Turnpenny & Ellard, 2005).

SNPs are better suited for population genetic studies in distantly related isolates because, compared to SSR markers, SNPs exhibit less mutation (Brumfield et al., 2003; Morin et al., 2004). However, more SNP markers are needed to determine the genetic variation between isolates due to the biallelic nature of SNPs, while microsatellites have multiple alleles (Morin et al., 2004). For P. horiana species-specific SNP markers are identified and an efficient SNP detection protocol using High Resolution Melting (HRM) technology has been developed. This allows the rapid identification of P. horiana isolates at a prudent cost (De Backer et al., 2012; Van Poucke et al., 2014). De Backer et al. (2012) identified 33 polymorphisms for P. horiana by sequencing 25 loci, representing 32 SNPs and 1 SSR. In 18 loci a one single SNP was present, 4 loci had double SNPs (2 independent and 2 dependent) and 2 loci had triple SNPs (dependent). Using these markers, De Backer et al. (2012) determined 25 genotypes of P. horiana isolated from 45 samples in a worldwide collection. De Backer et al. (2012) determined that roughly 0.01% of the 2.35x10^6 nucleotide positions he initially analyzed were polymorphic, indicating that the occurrence of SNPs in P. horiana was considerably lower than in other fungi. He theorized that, as expected, the SNP markers he identified are stable, as supported by the clonal European isolates collected between 2003 – 2007. Except for SNP 431/1, all
markers were also found in at least two isolates, implying they were not recently created (De Backer, 2012).

Based on a population analysis, the migration patterns of the pathogen and the exotic or endemic characteristics of the isolates collected from the different worldwide regions were analyzed. De Backer also observed clonal isolates within the geographic region where the pathogen is endemic. However, the genotypic diversity in each region was larger than expected. For example, in Europe, 12 genotypes were identified within 25 isolates, in Japan 3 genotypes were identified within 4 isolates and, in the United States, 3 genotypes were identified within 5 isolates. The marker profiles within an area did not demonstrate a stepwise accumulation of mutations expected for organisms capable of asexual reproduction and certain haplotypes appeared to have recombinant SNP patterns in isolates originating from diverse geographic areas. These observations suggest recombination and migration of genotypes and that a parasexual cycle is present (DeBacker 2012). Based on reports by Kohno et al., (1974, 1975) recombination in *P. horiana* can be explained by heterokaryosis after anastomosis resulting in heterozygous vegetative mycelium and homozygous recombinant genotypes can be expected after somatic meiotic division during the telial stage (De Backer 2012, Kohno et al., 1974, 1975). As noted previously in this dissertation, anastomosis has been observed in *P. horiana* and most of the SNPS identified by De Backer were found in diverse groupings that also challenge theories of strictly clonal lineage.

De Backer (2012) determined that isolates from the United States grouped into two clusters belonging to 3 genotypes that do not directly relate as recombinants. He theorized that at least 3 introductions of *P. horiana* into the United States have occurred.
He also suggested, that based on his results, that *P. horiana* may now be endemic in the United States or that introductions of the same isolate genotype(s) may have repeatedly occurred. De Backer also determined that all the isolates from the United States clustered with isolates of Asian origin.

De Backer (2012) determined that pathotype diversity was significantly greater than genotypic diversity. He explained this difference as being due to the interaction of at least 7 resistance genes in chrysanthemums corresponding with avirulence genes in *P. horiana* (De Backer et al., 2012). De Backer reported distinct differences in pathotypes even within clonal isolates. He theorized that these differences could be due to avirulence genes demonstrating independent selection pressure due to specific resistance genes in various chrysanthemum cultivars grown in different areas, or that a greater variation in pathotypes interactions could be due to epigenetic changes in disease resistance, as noted by Stokes et al. (2002). De Backer found a correlation between genotype and pathotype for some isolates including a unique pathotype group, containing highly virulent isolates. He theorized that these isolates contain few avirulence genes, including one corresponding to a frequently-used resistance gene (De Backer et al., 2011). Unfortunately, the location of avirulence genes on the *P. horiana* genome is unknown. Because recombination in *P. horiana* during asexual reproduction can increase the potential of new genotypes of greater virulence, co-existence of different genotypes within the same site should be avoided (De Backer, 2012).

**High Resolution Melting (HRM) Analysis.**

High Resolution Melting (HRM) is a quantitative post-PCR analysis method used for identifying genetic variation in genomic sequences. HRM generates melting (dissociation)
curves specific and sensitive enough to identify small sequence differences, including
single-base variations. During HRM analysis, the target area is amplified by PCR in the
presence of a saturating fluorescent dsDNA binding dye. After amplification, the PCR
product is slowly melted while the fluorescence is measured and a dissociation curve is
generated. The resulting profile indicates the amplicons present (Anon., 2013; Taylor et
al., 2010; Van Poucke et al., 2014).

Benefits of HRM include that it is in many cases sensitive enough to detect a
single base change between otherwise identical PCR fragments. In addition, HRM is
nondestructive allowing, if necessary, for the resulting PCR product to be analyzed by gel
electrophoresis or sequencing. Other benefits include the lower cost when compared to
fluorescent probe technologies, can accurately distinguish multiple SNPs located close to
one another, and can accurately identify large number of SNPs in low target quantity
(Anon., 2013; Taylor et al., 2010; Van Poucke et al., 2014). Drawbacks to HRM include
that, for many SNPs, the melting temperature (Tm) difference between the two possible
variants is very small resulting in potential incorrect SNP assignment. Additionally, for *P.
horiana* samples, it is difficult to acquire identical amounts of *P. horiana* DNA among
samples for HRM diagnostics. Another drawback is that many positive controls are
needed, since these are necessary for each SNP to be assigned to the correct SNP class
(Anon., 2013; Taylor et al., 2010; Zhou et al., 2004).

As an alternative, HRM techniques using unlabeled probes have been developed.
In general, SNP genotyping with HRM using unlabeled probes requires only one probe
for each SNP and fewer controls (Van Poucke et al., 2014; Zhou, et al., 2004). An
oligonucleotide is located at the 3’ end in order to block extension and, the Tm difference
(ΔTm) is independent of the DNA concentration. The unlabeled probe is added to the master mix along with an asymmetric ratios of primers (Erali et.al., 2008; Van Poucke et.al., 2014). The PCR is optimized to generate a large difference in melting temperature between a match and mismatch of the unlabeled probe. Short unlabeled probes of 20-30 bases can be designed to complement either of the two possible SNP variants. Greater differentiation and destabilization occurs when the mismatch is placed in the middle section of the probe. The differences in the melting temperature of the probes should be comparably different between the amplicon, the match, and the mismatch, allowing reliable SNP calling. The unlabeled probe, because it is smaller the amplicon, melts at a lower temperature, generating a peak to the left of the amplicon peak. And, when the probe matches the SNP, due to stronger bonds, the probe dissociates at a higher temperature compared to a mismatch (Fig 4.1). With good primer and probe design, the Tm difference can be as much as 5-7°C (Anon., 2013; Van Poucke et al., 2014). Figure 4.1 illustrates a dissociation curve using unlabeled probe SNP mismatch, match and amplicon peaks (Van Poucke et al., 2014).

The main objectives of this study were to (i) genotype *P. horiana* isolates collected through out the United States and (ii) analyze and compare the genotypes of the United States isolates with world wide isolates previously collected and genotyped.

**Materials and Methods**

*Puccinia horiana* isolates. Thirty-eight *P. horiana* isolates were collected from chrysanthemum plants determined positive for CWR between 2007 and 2013 (Table 4.1). Isolates were selected based on submissions to the USDA APHIS Eastern Region Plant
Pathology Lab. The isolates were submitted from Connecticut, Maryland, Pennsylvania, Virginia and West Virginia. An additional isolate was collected from the New Jersey Department of Agriculture, Division of Plant Industry, Plant Diagnostic Lab plant disease herbarium. This sample was a 1978 historical herbarium sample collected from one of the original 14 New Jersey sites where CWR had been intercepted (Petersen et al., 1978). An additional 17 *P. horiana* DNA extractions, collected in California, were submitted for genotyping from the USDA-ARS, Foreign Disease-Weed Science Research Unit, Fort Detrick, Maryland. The data for the remaining 45 isolates were collected by De Backer et al. (2013).

For fresh samples and dried herbarium samples collected between 2007 and 2010, approximately 100 mg of symptomatic leaves were excised for DNA extraction using a Qiagen DNeasy Plant Mini extraction kit. The DNA extractions were stored at -20°C prior to molecular screening and genotyping. The nucleic acid concentration of the samples were determined using a Epoch Microplate Spectrophotometer (BioTek, Inc.)

The herbarium sample from New Jersey was examined under a compound microscope for telia sori. Approximately 50 telia sori were excised from the leaf using a Harr’s Uni-Core (Harr’s Inc.) 1.20mm plant corer (Figure 4.2). Using a modified protocol of Kistler’s (2012) protocol for extraction of ancient DNA from plants, DNA was extracted as follows: CTAB buffer containing 2 g Hexadecyl trimethyl-ammonium bromide (CTAB), 10 ml 1 M Tris pH 8, 4 ml 0.5 M Ethylenediaminetetra Acetic acid Disodium salt (EDTA) pH 8, 28 ml 5 M NaCl, 40 ml H2O and 1 g Polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) (PVP, Mw 40,000). The pH was adjusted to 5 using HCL and the solution was brought up to 100 ml with H2O. The extraction buffer was
made by adding CTAB buffer to 1 mM PVP (40 mg/mL) and β-mercaptoethanol to 0.5% (v/v, 5µL/mL). The sample was placed in a 1.5 mL tube, ground with a sterile pellet pestle, Next, 500µL of extraction buffer was added to the tube, gently stirred and heated for 30 m at 55°C. 500µL of chloroform was then added to the solution and gently mixed followed by centrifuging for 7 m at 14,000 rpm. The aqueous phase was then transferred to a new tub, while the bottom layer and any debris was left behind. Based on the amount of the aqueous phase, 0.08 volumes of cold ammonium acetate and 0.54 volumes of cold isopropanol were added to the tube and the tube was gently inverted approximately 25 times. The tube was then incubated on ice for 30 m followed by centrifuging for 3 m at 14,000 rpm. The supernatant was carefully discarded and 700µL of 70% ethanol was added to the sample. The tube was gently inverted 10 times, then centrifuged for 1 m at 14,000 rpm. The supernatant was carefully discarded and 700µL of 95% ethanol was added to the pellet prior to centrifuging for 1 m at 14,000 rpm. The supernatant was discarded and the tube was inverted on a paper towel for 3 m to eliminate any remaining moisture. The tubes were then placed right side up overnight until thoroughly dried. Prior to molecular analysis, the sample was rehydrated overnight at room temperature with 50µL TE buffer. The nucleic acid concentration of the sample was determined using a Nanodrop 2000 (Thermo Scientific, Inc.)

**Genotyping using SNP markers.** The SNP and SSR markers developed by De Backer (2012), then modified by Van Poucke (2014) for HRM analysis, were used to genotype the 57 *P. horiana* isolates collected in the United States during this study. Fragment specific primer sequences used in the HRM PCR reactions are listed in Table
4.2. The results from the 57 US isolates were then combined with the data previously analyzed by De Backer et al. (2012).

The PCR reactions for the 57 *P. horiana* isolates collected in the United States were performed in a 7900HT Fast Real-Time PCR System and contained 1x MeltDoctor HRM Master Mix (Applied Biosystems, CA), 50 nM forward primer, 250 nM reverse primer and 250 nM unlabeled probe in a 20µl reaction volume. The PCR program consisted of an enzyme activation step at 94°C for 10 min, followed by 50 cycles of 15 sec at 94°C, 30 sec at 53°C and 30 sec at 72°C. After the PCR a melting analysis was performed consisting of an initial denaturation step at 94°C for 15 sec and a annealing step at 45°C for 1 min, followed by melting of the dsDNA by slowly heating the reaction product from 45°C to 90°C at a ramp rate of 5% (corresponding to a temperature increase of 6.98°C/min). The average SNP melt temperature and Δ melt are listed in Table 4.3.

**SNP data analysis.** As per the De Backer et al. (2012) study, the SNP and SSR data results were converted to binary data by assigning a value of “0” to the most-frequent allele among all isolates and a value of “1” to the least-frequent allele. Similarity matrices were calculated using the simple matching coefficient from the binary data (De Backer et al., 2012; Grower, 1971). Using Bionumerics software (Applied Maths, Belgium), cluster analysis was performed using the un-weighted pair group method utilizing arithmetic averages (UPGMA). Due to low number of markers and isolates, low bootstrap values were expected, so the cophenetic correlation coefficients of the original matrix of genetic similarities and the dendrogram-derived similarities were calculated for each cluster in order to verify the agreement of both matrices (De Backer et al., 2013; Farris, 1969). Neighbor joining, maximum–likelihood methods and the resulting
dendrogram were compared and evaluated. Using the Bayesian approach preformed with STRUCTURE v2.3.4 (De Backer et al., 2013; Falush, 2003; Pritchard, 2000), using the diploid data as input, the genetic structure in the collection was analyzed. The admixture model along with the correlated allele frequencies and with no prior origin information for the isolates, were used to determine the approximate number of genetic clusters (K). Simulations were run with a burn-in of 50,000 iterations of the Markov chain and the K ranging from 1 to 15, followed by 500,000 iterations runs. For each model, using 20 parallel Markov chains, the In likelihood \[ L(K) \] was determined. Based on the mean L(K)>20 runs for each K(44) and the second-order rate of change of the likelihood function with respect to \( K(\Delta K) \), the most likely value of K was resolved (De Backer et al., 2013; Evanno et al., 2005).

**Recombination and pathotypes correlation analysis.** Each genotype was analyzed to determine if it could be a recombinant of any other genotypes within the collections. A genotype was considered a punitive recombinant if it contained all of the alleles shared by potential parental isolates and a combination of alleles different between the potential parental isolates.

**RESULTS**

Table 4.4 shows the HRM results from 32 single-nucleotide polymorphism (SNPs) and one simple-sequence repeat for 57 *P. horiana* isolates collected in the United States between 1978 and 2013. The dendrogram in Figure 4.3.A illustrates the cluster relationship of the United States isolates, based on the Gower simple matching similarity coefficients and the UPGMA method. The numbers within the tree represent the
cophenetic correlation coefficients for the cluster nodes. Similar clustering was observed using maximum-likelihood and neighbor-joining methods.

The Bayesian analysis of population structure determined an optimal K value of six for the 61 US isolates when analyzed alone and an optimal K value of five when the 101 worldwide isolates were analyzed (Figure 4.3 C and Figure 4.5 C). The rendered tree for the United States isolates (Figure 4.4) reveals six populations containing one or two genotypes. Figure 4.3 B represents the allele for each SNP of the 61 isolates and Figure 4.3 C illustrates the mean probability of assignment of each the isolates to each of six populations (K=6) as determined with STRUCTURE software using 20 parallel Markov chains.

Combining our results with those of De Backer et al. (2012) revealed that the 61 P. horiana United States isolates collected from eight states (CA, CT, MA, MD, NJ, PA, VA and WV) grouped into six genotypes. Figure 4.8 illustrates the distribution of the 61 isolates and six genotypes within eight states.

Forty-nine of the isolates matched three previously described genotypes (De Backer et al., 2013), while seven of the isolates comprised three new genotypes. The denodrogram (Figure 4.5 A), comparing the United States isolates, shows eight populations containing up to six genotypes each. The dendrogram is based on the UPGMA method and simple matching similarity coefficients with the cophenetic correlation coefficients (CCC) for the cluster nodes. For the United States set of isolates, the CCC was >98%, while for the worldwide set of isolates, the CCC was >85% (De Backer et al., 2013), indicating that the dendrograms present a good representation of the similarity matrix.
Figure 4.5 B represents the allele for each SNP of the 101 worldwide isolates and Figure 4.5 C illustrates the mean probability of assignment of each the isolates to each of five populations (K=5) as determined with STRUCTURE software using 20 parallel Markov chains. Figure 4.6 illustrates a rendered tree showing the relationship of the 101 worldwide isolates of *P. horiana*. The United States isolates are highlighted in yellow.

Twenty of the 31 United States Penmar genotype isolates of *P. horiana* were collected from seven varieties of chrysanthemums grown on PSU campus during a 4-year time period. These isolates include five isolates collected from one variety grown at one site where *P. horiana* overwintered in volunteer chrysanthemum plants. The genotype of the five isolates collected from overwintering chrysanthemum plants did not change during the 4-year time period. The remaining 15 isolates were collected from six other chrysanthemum varieties. All of the chrysanthemum varieties grown on the PSU campus originated from cuttings of one source.

The genotype Penmar was identified in isolates from four states (MD, PA, VA and WV). This isolate had been identified previously in only two states (MD and PA) (De Backer et al., 2013), The newly identified genotype, Conn (Con), clustered closely with the Conmas isolates, while Pencon, one of the newly identified genotypes, closely clustered with isolate LK1 from Sri Lanka. The newly identified genotype for the NJ 1978 isolate clustered with isolates from Belgium, Columbia, Great Britain and Poland. The Japanese isolates JP1 and JP2 were distinctly different from the rest of the isolates.

**DISCUSSION**
Genotyping data analysis. As previously noted by De Backer (2012), all the SNPs analyzed in this study were homozygous. This homozygosity is explained by the *P. horiana* microcyclic life cycle where basidia produce only two basidiospores each of which receives two nuclei (De Backer et al., 2013; Kohno et al., 1974; Kohno et al., 1975). The nuclei are identical since they are derived from the second meiotic division. The pustules and hyphae contain identical nuclei derived from each basidiospore, therefore the homozygous state of the markers occurs in single pustule isolates. A heterozygous state can only occur in a marker if anastomosis with another genotype or mitotic mutation has occurred. Although rare, anastomosis has been reported (O'Keefe and Davis, 2014). Also, even if anastomosis or mutation has occurred, the nuclear cycle will restore the homozygous state of the markers in the basidiospores (De Backer et al., 2013).

This study was comprised of 101 isolates including 61 collected within the United States. A total of six multilocus genotypes were identified within the United States and 28 were identified for the worldwide collection, an increase of three genotypes as compared to those identified by De Backer et al. (2013). For the worldwide collection, eight multilocus genotypes are represented by more than one isolate, including the United States Penmar genotype consisting of 31 isolates and the United States Cal genotype consisting of 13 isolates (Figures 4.3 - 4.6). The remaining isolates described by De Backer et al. (2013) clustered as previously reported.

Within the United States isolates, we determined there are four main genotypes in the United States, including the one newly discovered in this study, CONN (Con). This newly identified fourth genotype is not a recombinant of any two of the other three
United States genotypes since there are three inconsistent markers. It was also determined that the other United States genotypes are not recombinants of each other. This indicates that at least four introductions likely have become established in the United States.

Many of the United States genotypes cluster geographically in the upper East Coast (Connecticut and Massachusetts), lower East Coast (Pennsylvania, Maryland, West Virginia and Virginia) and California. The Conmas genotype, identified in California, was also found in Connecticut and Massachusetts. This clustering pattern indicates mainly local establishment and spread. However, because the Conmas genotype was identified in California and the upper East Coast, this indicates later and longer distance migration within the United States or an additional international introduction. The Conmas genotype also could have spread from California to the East Coast within asymptomatic cuttings (O’Keefe and Davis, 2014).

The Conn (Con) isolate is interesting because it is a new genotype, but is only represented by one isolate. Except for SNPs 003-1/2 and 595, it has the same profile as the Conmas genotype. The Con isolate may represent another international introduction, or it may be a recombinant of the Conmas genotype and the Penmar genotype. This genotype does not represent a recombinant of any other combination of United States genotypes, and therefore suggests at least one local recombination event occurring within the United States. Recombination in P. horiana can be explained by heterokaryosis after anastomosis of mycelium or germ tubes, which results in heterozygous vegetative mycelium. (Figure 4.7) Homozygous recombinant genotypes occurring after somatic meiotic division during the telial stage, have been observed (Kohno et al., 1974; 1975).
As mentioned by De Backer et al. (2013), most of the SNPs identified occur in various combinations, which cannot be explained by asexual propagation of clonal lines.

The NJ 1978 isolate appears to be a single introduction. Although the isolate was reported on chrysanthemum plants shipped directly from Japan to New Jersey during 1978, this isolate clustered closely with those from Belgium, Columbia, Great Britain and Poland. Based on this study, this report appears unlikely and leads us to question the possibility that the report of origin of the chrysanthemum plants was wrong. Based on the research of De Backer et al. (2013), the Colombian isolates are probably introductions from Europe, so, it is likely that the NJ 1978 isolate has a link with the European isolates while other United States isolates appear to have a link with Asian isolates. It appears that this isolate was successfully eradicated since other findings of this genotype have not occurred while the United States isolates with an Asian link have not been eradicated.

Groups of the United States isolates share the same genotype and were often observed with a geographic region. Based on the groupings of the genotypes, some isolates may be clonal. However, as we have only analyzed a limited number of markers, it is possible this hypothesis might change if more markers or the complete genome were analyzed. The exception being the United States genotype Conmas, which was identified in isolates collected in California, Connecticut and Massachusetts. While clonal isolates collected in other worldwide locations represent relatively recent local dispersal, the Conmas genotype appears to be a later introduction and a longer distance migration within the United States or an additional international introduction.

Until the study by De Backer et al. (2013), *P. horiana* was not considered endemic in the United States. This study indicates that five or six separate introductions
into the United States have occurred. The 61 United States isolates were collected from seven states. Supported by confirmation of *P. horiana* overwintering in the United States (O'Keefe and Davis 2012), this study indicates that *P. horiana* is now endemic within the United States.
LITERATURE CITED


Table 4.1 Isolate information code, isolate name, collection location, collection year and source.

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Source: ¹Pennsylvania Department of Agriculture, ²Virginia Department of Agriculture and Consumer Services, ³Connecticut Department of Agriculture, ⁴Maryland Department of Agriculture, ⁵data furnished by De Backer et al., (2012).
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Table 4.2. List of primers and probes used for HRM analysis of *P. horiana* isolates.
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TABLE 4.4. HRM results using 32 single-nucleotide polymorphisms (SNPs) and one simple-sequence repeat (SSR) for 55 US isolates.

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*Penmar = 31 isolates, Pencon = 5 isolates, Conn = 1 isolate, NJ 1978 = 1 isolate, Conmas = 5 isolates, Cal = 13 isolates.
TABLE 4.X. Comparison of HRM results using 32 single-nucleotide polymorphisms (SNPs) and one simple-sequence repeat (SSR), for 55 US isolates.

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<sup>a</sup>Conmas = 2 isolates, Cal = 1 isolate, Penmar = 2 isolates. <sup>b</sup>Penmar = 31 isolates, Pencon = 5 isolates, Conn = 1 isolate, NJ 1978 = 1 isolate, Conmas = 5 isolates, Cal = 13 isolates.
Fig. 4.1. Dissociation Curve using unlabeled probe SNP mismatch, match and amplicon peaks (Van Poucke et.al. 2014).
Figure 4.4. Rendered tree illustrating relationship of 61 United States isolates of *P. horiana*.
Fig. 4.5. A. Dendrogram showing cluster relationship of 101 Puccinia horiaria isolates based on the Gower similarity measure and paired group algorithm method using 32 SNPs and 1 SSR. B. Alleles for the 32 SNPs and 1 SSR for each isolate. C. Mean probability for each isolate assigned to 5 populations (K=5) based on 20 Markov chains determined with STRUCTURE program analysis.
Fig. 4.6. Rendered tree illustrating the relationship of 101 worldwide isolates of P. horians. The United States isolates are highlighted in yellow.
Figure 4.7 A. Anastomosis occurring between hyphal strands of *P. horiana*; B. Schematic illustration of recombination in *Puccinia horiana* (A) “Tip-to-Toe” anastomosis as also described by Wang and McCallum (2009) between vegetative mycelium or germ tubes from two different genotypes (illustrated with black versus white nuclei) of *P. horiana* eventually results in heterozygous mycelium with two haploid nuclei. (B) During the telial stage karyogamy occurs with a rearrangement of the chromosomes resulting in a recombinant genotype (illustrated with gray nuclei). After the second somatic meiotic division in the promycelium, two identical haploid nuclei migrate in each of the two basidiospores, which give rise to homozygous recombinant mycelium (Kohno et al., 1974; Kohno et al., 1975). The nuclear status is indicated for each stage in the rectangular insets: c represent the number of chromosomes, n represents the ploidy number (De Backer, 2012).
Figure 4.8. Distribution of 61 *P. horiana* isolates and 6 genotypes within 8 U.S. states.
VITA
Grace O’Keefe

Education

• Ph. D. Plant Pathology, The Pennsylvania State University, December 2014
• M.S. Plant Pathology, Rutgers University, The State University of New Jersey, May 1991
• B.S. Plant Science, Cook College, Rutgers University, The State University of New Jersey, May 1977

Research Experience

• Doctoral Research under Dr. Donald D. Davis, Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, 2010 - 2014
• Masters Research under Dr. Joseph L. Peterson, Rutgers University, The State University of New Jersey, 1986 -1991

Employment

• Plant Pathologist, USDA-APHIS-PPQ, 2006 – present
• State Plant Pathologist, Virginia Department of Agriculture and Consumer Services, Richmond, VA, 2003 – 2006
• Assistant State Horticulturist, Maine Department of Agriculture, Augusta ME, 1994 – 2003
• Senior Scientist, Georgia Department of Agriculture, Tifton, GA, 1990 – 1994
• Plant Pathologist, Horticulturist and Horticulturist Trainee, New Jersey Department of Agriculture, 1989 – 1990

Awards and Grants

• Gamma Sigma Delta, The Honor Society of Agriculture, 2014
• Leonard J. Francl Endowment, PPEM, 2014
• PPEM Travel Grants, 2013, 2014
• Henry W. Popp Award (Outstanding graduate student in the PPEM Dept), 2012

Publications