RELATIVE ROLES OF TUBER- AND SOILBORNE INOCULUM IN VERTICILLIUM WILT OF POTATO AND QUANTIFICATION OF RESISTANCE IN MINT

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of JEREMIAH KAM SUNG DUNG find it satisfactory and recommend that it be accepted.

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Chair

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RELATIVE ROLES OF TUBER- AND SOILBORNE INOCULUM IN VERTICILLIUM WILT OF POTATO AND QUANTIFICATION OF RESISTANCE IN MINT

Abstract

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Inoculum of *Verticillium dahliae*, causal agent of Verticillium wilt (VW) of potato, exists as soilborne microsclerotia, however, it can also be found in vascular tissue of seed tubers. The effects of intratuber inoculum on VW symptoms were measured in the greenhouse and in fields with and without prior potato rotations. Vascular infection of seed tubers did not result in significant disease symptoms, stem colonization, microsclerotia production or progeny tuber infection in the greenhouse. The incidence of *V. dahliae* infection in seed lots was not related to differences in yield, symptom severity or progeny tuber infection in field experiments, however, disease incidence and severity were higher in common potato rotation fields compared to long rotation fields. Results indicate that efforts to reduce primary inoculum should focus on reducing pathogen populations in the soil.

Verticillium wilt is also a major constraint to mint (*Mentha*) production. The use of resistant cultivars is an important component of VW management and *Agrobacterium*-mediated transformation provides the opportunity to improve existing mint cultivars. Several *M. arvensis* and *M. longifolia* selections were evaluated for resistance to *V. dahliae* isolates from different hosts and vegetative compatibility groups (VCGs) in the greenhouse. Transgenic peppermint (*M.* x *piperita*) plants containing VW resistance-like sequences cloned from *M. longifolia* were also evaluated for resistance. *V. dahliae* isolates from peppermint caused significantly higher disease severity, yield reductions and plant mortality than isolates from other hosts, regardless of VCG. These data supports previous studies indicating host specificity in mint isolates of *V. dahliae* and suggest host origin may be a better indicator of isolate aggressiveness than VCG. Inoculations of *M. arvensis* and *M. longifolia* resulted in necrosis of the inoculated stems, however both species displayed the ability to recover from rhizomes. Both *M. arvensis* cultivars exhibited relatively low disease symptoms, aboveground stem colonization, yield reductions and plant mortality over repeated periods of cutback and regrowth. Transgenic peppermints containing VW resistance-like sequences did not display resistance to *V. dahliae*. Results indicate that the restriction of pathogen movement in aboveground stems and the ability to recover from infection may be important characteristics of VW resistance in mint.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

DEDICATION

This thesis is dedicated to my father, the late Albert K. S. Dung,

who taught me the importance of education, hard work

and living life to the fullest.

PREFACE

The chapters included in this thesis have been prepared for submission to professional journals. The manuscripts from Chapters I, III and IV will be submitted to Plant Disease. Chapter II will be submitted to American Journal of Potato Research. Citations within each chapter refer to the "Literature Cited" or "References" section of the chapter and follow the format used in their respective journals. Citations made in the "Introduction" section of this thesis are listed in the "Literature Cited" section at the end of the thesis.

INTRODUCTION

Verticillium dahliae Kleb., the primary causal agent of Verticillium wilt, is a soilborne, vascular wilt fungus with a worldwide distribution. The pathogen can infect hundreds of dicotyledonous species in various genera (1, 13, 74). Symptoms of *V. dahliae* infection depend upon the host but may include wilt, light brown vascular discoloration, unilateral chlorosis and necrosis, anthocyanescence, stunting, apical leaf curling, dieback and premature plant senescence (24, 31, 36, 74, 78).

The genus *Verticillium* was for a long time a heterogeneous form genus that included anamorphic fungi with verticillate, phialidic conidiophores and an unknown natural classification (89). Recent molecular analyses and subsequent revisions of the genus have resulted in the removal of the fungicolous, entomophagous and nematophagous *Verticillium* species, retaining only phytopathogens and saprophytes in *Verticillium s. str., viz*. the '*Nigrescentia'* (*Phyllachorales*); one exception is *V. fungicola*, causal agent of dry bubble on cultivated button mushrooms (*Agaricus bisporus*), which has not yet been removed from the genus despite molecular evidence suggesting it more closely related to the nonphytopathogenic species already removed from *Verticillium* (26, 66, 77, 89).

Two species in the genus *Verticillium* are known to cause Verticillium wilt, *V. albo-atrum* and *V. dahliae* (5). Morphologically, *V. dahliae* can be distinguished from *V. albo-atrum* by its shorter conidiophores (80-160 µm and 100-300 µm for *V. dahliae* and *V. albo-atrum*, respectively), which lack a darkened and swollen base. *V. dahliae* also possesses smaller conidia

(3-5.5 µm x 1.5-2 µm, as opposed to the conidia of *V. albo-atrum* which measure 3-12 µm x 2-3 µm) and forms microsclerotia as its sole resting structures rather than the melanized hyphae produced by *V. albo-atrum* (19, 35, 76). Some historical literature on *V. dahliae* often refer to the organism as a microsclerotial-form of *V. albo-atrum*, however *V. albo-atrum* is unable to grow at 30° C, is generally not pathogenic above 25° C and possesses a more limited host range than *V. dahliae* (19, 35, 76). The two can also be distinguished using nuclear DNA (ntDNA) and mitochondrial DNA (mtDNA) gene sequences, restriction fragment length polymorphisms (RFLP) and immunochemical techniques (5, 11, 22, 54, 66, 68). Isolates of *V. dahliae* with relatively longer conidia and approximately twice the DNA content comprise a significant proportion of isolates collected from cruciferous hosts and may be the result of interspecific hybridization (5). The species *V. longisporum* was proposed by Karapapa et al. (43) for these long-spored isolates of *V. dahliae* and is considered valid by some (22), however others prefer to use *V. dahliae* in reference to *Verticillium* isolates which produce microsclerotia as their sole resting structures (5). *V. nigrescens*, which produces chlamydospores as its resting structure, and *V. tricorpus*, which produces chlamydospores, microsclerotia and melanized hyphae (19, 35, 76) are considered weak plant pathogens and can be distinguished by ntDNA and mtDNA sequences and immunochemical methods (5, 22, 66, 68).

Despite its wide host range, *V. dahliae* isolates can vary in aggressiveness with respect to specific host species, with some isolates capable of causing more severe symptoms on certain hosts and possessing varying degrees of cross-pathogenicity to other plant species (7, 12, 68). Isolates can also be separated into vegetative compatibility groups (VCGs) based on their ability to undergo hyphal anastamosis with other isolates to form heterokaryons (13, 37). Correlations

between pathogenicity and VCG exist but are not found in all cases (7, 8, 45, 65). Random amplified polymorphic DNA analyses of isolates belonging to different VCGs have found a relatively high degree of homogeneity within and among VCGs regardless of host origin (7), however amplified fragment length polymorphism and ntDNA sequence analyses demonstrated diversity in European isolates belonging to VCG 2 suggesting it may be polyphyletic (15, 16). Correlations between molecular markers, such as RFLP and ntDNA sequences, with host origin exist in some cases but not in others (18, 63, 68).

Primary inoculum of *V. dahliae* consists of microsclerotia, which develop during plant senescence and can survive for long periods of time in field soils (29, 49, 74). Microsclerotia germinate in response to host root exudates (50) and hyphae invade the cortex and xylem, where the pathogen is systemically translocated through the host vascular system (24). Root colonization of resistant and nonhosts does occur, however it appears that the fungus is restricted from extensively colonizing the cortex and fails to reach the xylem (2, 3, 24, 83). A brief saprophytic stage occurs during host senescence and the pathogen produces microsclerotia and conidia in and on colonized tissue. Conidia are short-lived in comparison to microsclerotia and not thought to be important in the disease cycle (29). Microsclerotia production is most abundant on aerial plant parts (51) and colonized host debris can increase inoculum levels if incorporated into the soil.

Verticillium wilt management is focused on the reduction of primary inoculum through the use of disease-free planting stocks, rotations with nonsusceptible monocotyledonous crops and pre-plant fumigation (28, 52). Fumigation is expensive and the practice may be restricted in

coming years, while crop rotation is of only limited benefit due to the ability of *V. dahliae* to colonize roots of both host and nonhost crops and its long persistence in soil. The development and use of resistant cultivars is considered an important component of Verticillium wilt management, however the mechanisms of resistance are not completely understood and appear to differ depending on the host plant involved (24, 44, 72, 73, 85). Previous studies on Verticillium wilt resistance in potato, cotton and lettuce have suggested that host suppression of cortex colonization, stele penetration and xylem invasion are important components of resistant phenotypes (24, 30, 34, 83). Davis et al. (17) observed a correlation between limited vascular colonization and reduced microsclerotia production in resistant potato genotypes and demonstrated reductions in Verticillium wilt symptoms in susceptible cultivars planted after repeated croppings of resistant varieties.

Verticillium Wilt of Potato

Washington State is the second leading producer of potatoes (*Solanum tuberosum* L.) in the United States, with approximately 165,000 acres planted in 2007 and a production value of over $685 million (US) (59). Verticillium wilt of potato is a disease of major importance and concern in the Pacific Northwest. Previous literature often refers to Verticillium wilt as potato early dying, since symptoms of Verticillium wilt on potato are similar to the natural process of senescence but occur prematurely (67, 70). Verticillium wilt symptoms on potato include wilting, leaf curling, chlorosis and necrosis, with entire stems often dying prematurely but remaining upright. Symptoms progress upwards and are often unilateral both within a plant and

within individual leaves on a plant. Vascular discoloration in stems and tubers is often associated with Verticillium infection but is not diagnostic and may be due to physiological factors or other fungal pathogens such as *Fusarium* spp. (6, 36, 79). Variable effects of Verticillium wilt on yield reductions have been previously reported and range from 12% (41) to 50% (10), however reductions in potato yields are not necessarily correlated with the severity of aboveground symptoms (56, 71). Yield reductions and symptom severity were shown to be influenced by soil and environmental conditions (67) and can be especially severe during episodes of heat stress and high rates of evapotranspiration (21, 25, 62, 71).

Potatoes are a vegetatively propagated crop and *V. dahliae* can be found in the vascular system of commercial seed lots intended for production fields. The percentage of seed lots with *V. dahliae*-infected tubers can be as high as 44% in Israel (81) and 29% in Washington State (65), with the incidence of infection within seed lots typically < 5%. Most isolates collected from potatoes and potato fields in the Pacific Northwest belong to VCG 4A and were shown to be more aggressive than other VCGs (38, 64, 65). VCG 4A isolates from potato can also interact synergistically with the root lesion nematode (*Pratylenchus penetrans*) on potato (71).

The importance of *V. dahliae* in seed tubers on disease development, yield and quality was previously investigated. Robinson and Ayers (69) found that vascular infection of seed tubers by *V. albo-atrum* was not as important as soilborne inoculum**,** and Hoyman (33) concluded that *V. dahliae* infection of "Norgold Russet" seed tubers did not contribute to disease development, reduced yield or diminished quality. However, Verticillium wilt symptoms resulting from infected seed tubers can vary depending on the cultivar (69) and the effects of *V.*

dahliae infection in seed tubers is not known in "Russet Burbank", a major cultivar under production in the Pacific Northwest (57). In addition, research regarding the potential role of infected seed tubers in the production of new inoculum, i.e. microsclerotia, could not be found in the literature.

Verticillium Wilt of Mint

In addition to potatoes, the Pacific Northwest is also a leading producer of oil distilled from mint (*Mentha* spp.). Mint oil production in the United States is primarily focused on peppermint (*M*. x *piperita* "Black Mitcham") and two species of spearmint (*M*. x *gracilis*, or Scotch spearmint, and *M. spicata*, or native spearmint) (53). The shift of mint production in the early 1900's to the Pacific Northwest is primarily attributed to increased demand and decreased productivity in the Northeast and Midwest due to Verticillium wilt caused by *V. dahliae* (53, 60). Today the Pacific Northwest is known for its high mint yields and exceptional oil quality, with production in Washington State covering 35,700 acres with a value exceeding $58.8 million (US) in 2007 (58).

Verticillium wilt remains a major constraint to mint production in the United States and is the primary fungal disease affecting mint production in the Pacific Northwest. Symptoms of Verticillium wilt in mint can include wilt, chlorosis and/or anthocyanescence, bronzing and/or curling of the apical leaves, stunting, necrosis and premature senescence (31). Losses occur due to stand decline and reduced oil production, which can worsen over the lifetime of the perennial crop and make a field unsuitable for future mint production. Most isolates collected from mint in the Pacific Northwest were found to belong to a single VCG (2B) and are highly aggressive on

peppermint and Scotch spearmint, indicating the presence of a predominant pathotype (20, 27, 40). Isolates collected from peppermint were also shown to interact synergistically with *P. penetrans* on peppermint (23, 40).

Resistance is considered an important component of Verticillium wilt management in mint, however, since both peppermint and Scotch spearmint are sterile hybrids conventional breeding is not possible. Mint mutants resulting from treatment by irradiation has produced mixed results (32, 39, 55, 80) and characteristics such as oil composition, plant vigor and yield must be considered during cultivar development (48). The development of efficient *Agrobacterium tumefaciens*-mediated transformation of peppermint presents an opportunity to introduce resistance genes from other mint species into sterile mint hybrids while maintaining oil composition or other desirable traits (61, 84, 88). At least 18 species, 11 hybrids and numerous varieties of *Mentha* are described (82) and represent potential sources of Verticillium wilt resistance. *Mentha arvensis* (cornmint) is the most widely grown mint in the world and the menthol distilled from cornmint is used in cosmetics, foods and tobacco products (14). India is the leading producer of *M. arvensis* menthol, largely due to the integration of annual mint cropping systems with existing food production rotations, an efficient distilling infrastructure and the development of disease-resistant, high-yielding varieties (4, 46, 47, 75). The effects of powdery mildew (*Erysiphe cichoracearum*), rust (*Puccinia menthae*) and leaf spot (*Alternaria alternata*) on *M. arvensis* cultivars and genotypes were previously investigated (42), however the effects of Verticillium wilt on *M. arvensis* are not known. Another *Mentha* species, *M. longifolia* (horsemint), is a wild relative of cultivated mint with a broad geographic range and a high levels of intraspecific variation (82, 87). Verticillium wilt resistance-like sequences, similar

to the tomato *Ve* gene effective against *V. dahliae* race 1 (44, 73), were previously identified in *M. longifolia* using degenerate polymerase chain reaction primers and differences in Verticillium wilt response were demonstrated in USDA accessions of *M. longifolia* (86, 87).

Objectives

The objectives of the study described in Chapter I were to: (i) determine the role of vascular infection of "Russet Burbank" seed tubers in the development of Verticillium wilt symptoms, specifically chlorosis and necrosis; and (ii) quantify the incidence of vascular colonization, amount of microsclerotia formation in host debris and the frequency of progeny tuber infection to determine the potential contributions of seed tuber infection to future sources of inoculum. Since vascular infection of progeny tubers may occur via underground stolons, plants grown from apical and basal-end seed pieces derived from infected seed tubers were also compared. A field study, described in Chapter II, was conducted to quantify the effects of seed lot infection by *V. dahliae* on disease severity and yield characteristics in fields with and without potatoes in their recent cropping histories. Knowledge concerning the effects and importance of *V. dahliae*-infected seed tubers on the epidemiology and severity of Verticillium wilt will allow control efforts to focus on relevant sources of inoculum.

The objective of the research described in Chapter III was to evaluate *M. arvensis* and *M. longifolia* for resistance to Verticillium wilt using *V. dahliae* isolates from different VCGs and hosts. Knowledge regarding the responses of *M. arvensis* and *M. longifolia* to *V. dahliae* infection may aid in identifying sources of resistance for potential use in the development of

transgenic Verticillium wilt-resistant cultivars. In addition, the use of isolates with different VCG and host origin combinations may provide information useful in developing crop rotation strategies and determining the disease potential of a particular field. Chapter IV summarizes a study conducted to evaluate Verticillium wilt response in transgenic peppermint plants containing *Ve*-like sequences cloned from *M. longifolia* (86).

CONCLUSIONS

Relative role of seed tuber infection in the development of Verticillium wilt in potato

The results of these studies indicate that vascular infection of seed tubers by *V. dahliae* has a negligible effect on the development of Verticillium wilt symptoms in greenhouse-grown "Russet Burbank" potato plants. Vascular infection of seed tubers did not result in significant vascular colonization, microsclerotia production in host debris or increased incidence of progeny tuber infection in the greenhouse study. In addition, the incidence of seed tuber infection in certified seed lots by *V. dahliae* was not correlated to differences in yield, symptom severity or progeny tuber infection in field experiments, however, Verticillium wilt incidence and severity were higher in common potato rotation fields compared to long rotation fields, emphasizing the importance of soilborne inoculum. Results from both studies indicate that efforts to reduce primary inoculum should center on reducing pathogen populations in the soil and infected seed tubers should only be of concern when planting in fields not previously cropped to potato.

Evaluation of *Mentha arvensis, M. longifolia* and transgenic *M.* x *piperita* "Black Mitcham" for Verticillium wilt resistance

In these studies, *V. dahliae* isolates from peppermint were more aggressive on all mints tested than isolates from other hosts, including a VCG 2B isolate collected from spinach seed. These data support previous studies suggesting that *V. dahliae* isolates collected from mint are host-adapted (7, 20, 27). In addition, these results demonstrate the presence of pathogenic variation within VCGs and suggest that host origin may be a better indicator of isolate aggressiveness than VCG. The occurrence of *V. dahliae* populations belonging to numerous VCGs, each with subpopulations containing variation in host-adaptivity and cross-pathogenicity, may complicate detection and management efforts in the field.

Differences in disease severity among *Mentha* species were observed. Peppermint, the susceptible standard, showed high mortality, yield loss and aboveground stem colonization compared to the other mints tested while native spearmint, the resistant standard, showed no mortality, low yield loss and infrequent aboveground stem colonization. Root-dip inoculations of *M. arvensis* and *M. longifolia* often resulted in complete necrosis of the original stem, however both mints exhibited the ability to recover, sometimes asymptomatically, from underground rhizomes. Both *M. arvensis* cultivars exhibited relatively lower disease symptoms, yield reductions and mortality over time. The transgenic peppermint plants containing *mVe*1 and *mVe*2 sequences did not display increased resistance to Verticillium wilt in this study.

Results from this study are consistent with previous studies showing differences in pathogen isolation from stems of resistant and susceptible mint varieties (9, 31), indicating that

Verticillium wilt resistance in mint may involve the limitation of vascular and aboveground colonization by *V. dahliae*. The restriction of vascular invasion and aboveground colonization by *V. dahliae* may be a important facet of Verticillium wilt resistance and management in mint since microsclerotia, the primary form of inoculum, develop in host debris (74) and the perennial mint cropping systems of the Pacific Northwest present repeated opportunities for infested debris to become incorporated into field soils over time.

CHAPTER 1

Relative roles of tuber- and soilborne inoculum on Verticillium wilt in "Russet Burbank" potato

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ABSTRACT

Primary inoculum of *Verticillium dahliae*, causal agent of Verticillium wilt (VW) of potato, persists for years in soil as microsclerotia, however inoculum can also exist in the form of infected tubers used for seed. The relative impacts of soilborne and intratuber *V. dahliae* inoculum in the development of VW on potato were evaluated in the greenhouse. Naturally-infected and *V. dahliae*-free tubers were grown in *V. dahliae*-infested and noninfested potting mix. Area under the disease progress curve (AUDPC) was calculated from disease severity index ratings and aboveground stems and progeny tubers assayed for the presence of *V. dahliae*. The mean AUDPC did not differ for infected and noninfected tubers grown in noninfested soils. Plants grown in infested soils exhibited higher AUDPC than those grown in noninfested soil and mean AUDPC did not differ for plants arising from infected and noninfected tubers grown in infested soils. *V. dahliae* was successfully isolated from the vascular tissue of 94% of plants grown from infected and noninfected tubers in infested soils and 8% of plants arising from

infected tubers grown in noninfested soil. The mean microsclerotia colonization of stems was 48% of the total stem length for noninfected and for infected tubers in infested soil and 0.4% for the infected tuber/noninfested soil treatment. *V. dahliae* was recovered from 14% of progeny tubers from plants grown in infested soil and 0% of progeny tubers from plants grown in noninfested soil. Tuber infection did not significantly contribute to VW symptoms or inoculum production, indicating that efforts to reduce initial inoculum should focus on reducing pathogen populations in the soil.

INTRODUCTION

Verticillium wilt of potato is a disease of major economic importance to potato (*Solanum tuberosum* L.) growing regions in North America. The primary causal agent of Verticillium wilt in the Pacific Northwest (PNW) is *Verticillium dahliae* Kleb., a soilborne fungal pathogen with an extensive host range and widespread distribution in temperate climates (35). Although no sexual stage is known to occur, genetic diversity does exist in the form of several distinct vegetative compatibility groups (VCG). Isolates from potatoes and PNW potato fields are predominantly VCG 4A and VCG 4B, and VCG 4A isolates have been found to be more aggressive on potato than other VCGs (18, 28, 29). Symptoms of Verticillium wilt of potato include leaf epinasty, wilting and chlorosis, which progress acropetally and often occur unilaterally. Entire stems eventually become necrotic and senesce prematurely, but remain upright. Vascular discoloration in stems and tubers is often associated with *V. dahliae* infection, but may also result from physiological factors or other pathogens and thus is not diagnostic (4,

17, 38). Reports on the effects of Verticillium wilt on yield are variable, ranging from 12% (20) to 30% or more (6), and reductions in potato yields are not necessarily correlated with aboveground symptoms (24, 36). Yield reductions and symptom expression may be influenced by soil and environmental conditions (30) and can be more pronounced during periods of heat stress and high rates of evapotranspiration (11, 14, 27, 36). Synergistic interactions between *Pratylenchus penetrans*, the root lesion nematode, and VCG 4A isolates of *V. dahliae* can increase disease severity, lower the disease thresholds of both pathogens and severely reduce yields (36).

A polyetic disease, primary inoculum of Verticillium wilt consists of microsclerotia, which form during plant senescence and can persist in soil for long periods of time (15, 21, 37). Reported disease thresholds for Verticillium wilt in potato range between 5-30 cfu/cm^3 of soil for *V. dahliae* alone and 2-13 cfu/cm^3 soil when *P. penetrans* is present (30). Microsclerotia are stimulated to germinate in response to host root exudates (22) and hyphae colonize the cortex and invade the xylem, where it produces is systemically translocated through the host vascular system (13). Root colonization of resistant and nonhosts has also been shown to occur, however the fungus appears to be prevented from extensively colonizing the cortex and fails to reach the xylem (1, 3, 13, 40). A short saprophytic phase occurs at host senescence, during which *V. dahliae* produces conidia and microsclerotia in colonized tissue. Conidia are short-lived and not thought to be significant in disease progress or development (15). Microsclerotia production is most abundant on aerial stems (23) and colonized host debris can increase inoculum levels if incorporated into the soil. Furthermore, infested soil carried on the surface of seed tubers has been shown to contribute to Verticillium wilt symptoms (33, 38).

In addition to soilborne inoculum, intratuber inoculum of *V. dahliae* can be found in the vascular system of certified seed tubers. A 1968-1969 survey detected *Verticillium albo-atrum* and *V. dahliae* in 39% of 244 certified seed lots, with incidence in lots typically between 1-2% and rarely greater than 5% (10). A more recent study of seed lots imported from northern Europe to Israel between 1995 and 1998 detected *V. dahliae* in 20-30% of seed lots at incidences < 5% and found up to 16% of seed lots with > 5% *V. dahliae* infection (39). Surveys of 224 seed lots intended for North American production fields in 1995 and 1996 detected *V. dahliae* in 29% of the lots and 3.6% of the seed tubers, of which 64% of isolates were VCG 4A, 33% were VCG 4B and 3% were VCG 4AB (29). The detection of *V. dahliae* in certified potato seed lots and prevalence of the more aggressive VCG 4A in both seed tubers and PNW fields (28) may have important implications in both seed and production systems as well as the epidemiology and distribution of the disease.

Despite the prevalence of *V. dahliae* in certified commercial seed lots, the contributions of intratuber inoculum in the development and epidemiology of Verticillium wilt are not fully understood. Robinson et al. (33) determined that vascular infection of seed tubers by *V. albo-atrum* did not cause symptomatic plants in several potato cultivars. A 1979 field study found no effect of intratuber infection by *V. dahliae* on plant growth, disease symptoms, yield or quality in the potato cultivar "Norgold Russet" (16). However, severity of wilt resulting from intratuber infection can vary among cultivars (33) and the effects of vascular infection by *V. dahliae* in seed tubers has not been evaluated in "Russet Burbank", the major cultivar under production in the PNW (25). The objectives of this study were to: (i) determine the relative roles of intratuber and soilborne inoculum in the development of Verticillium wilt symptoms in the potato cultivar

"Russet Burbank"; (ii) compare plants grown from apical and basal-end seed pieces cut from infected seed tubers, since vascular infection of progeny tubers may occur via underground stolons; (iii) quantify the incidence of vascular host colonization, total microsclerotial production on senescent stems and the incidence of infected progeny tubers to assess the potential contributions of seed tuber infection to overwintering inoculum.

MATERIALS AND METHODS

Seed tuber assays. Seventeen seed lots of potato cultivar "Russet Burbank", which is moderately susceptible to Verticillium wilt (41), were obtained in 2007 from Washington (WA), Idaho (ID), Montana (MT), and Alberta, Canada certified seed sources and assayed for natural *V. dahliae* infection. A total of ten WA, ID and MT certified seed lots were assayed in 2008. A random sample of thirty-five tubers from each lot were thoroughly scrubbed with a sponge under running distilled water and allowed to air dry. A ~15 mm round disk which included vascular tissue was aseptically cut from the stem-end of the tuber and plated onto either modified potato dextrose agar (19), NP-10 medium (5), or both. Plates were incubated at 23° C for 14 days. Positive identification of *V. dahliae* colonies was verified by sub-plating onto potato dextrose agar when necessary and eleven tubers were selected for use as naturally infected seed piece treatments. Eleven tubers were chosen as noninfected seed piece treatments based on negative results in the plate assays. Care was taken to use tubers in which other fungal pathogens (e.g. *Colletotrichum coccodes, Fusarium* spp.) were not detected.

Disease evaluation. Soil inoculum consisted of rye berries colonized with *V. dahliae* isolate 653 and was prepared as described by Atibalentja *et al* (2). Isolate 653 was isolated from potato and previously identified as VCG 4A and pathogenic on potato (9). Berries were ground in a mill (IKA MF10 Analytical Mill) and the ground inoculum was quantified via serial dilution. Infested soil treatments were prepared by adding ground inoculum to 5.0 L of Sunshine L2 greenhouse potting mix (Sun Gro Horticulture, British Columbia, Canada) to achieve a concentration of approximately 10 CFU/cm^3. Ground noninoculated rye was added to the noninfested soil treatments. Approximately 32 g of granular 16-16-16 N-P-K fertilizer (Agriliance Agronomy Co., St. Paul, MN) was added to each pot prior to planting the seed pieces. The 22 tubers selected from the assays previously described were cut aseptically crosswise and then lengthwise into four equal sized pieces (approximately 60 g) with at least two eyes each. Blocks consisted of four seed pieces derived from one infected tuber and four seed pieces derived from one disease-free tuber; both seed tubers were from the same lot. Apical and basal-end seed pieces were equally divided among seed and soil treatments. Pots (No. 2 nursery style, 6.6 liter; J. M. McConkey & Co., Inc., Sumner, WA) were arranged in the greenhouse as a randomized complete block (RCB) design with 11 replicates. The trial was performed once in 2007 and repeated in 2008.

Disease symptoms were assessed at 65 days after planting and approximately weekly thereafter until crop senescence (142 days for the first trial in 2007) and 133 days for the second trial in 2008). Plants were evaluated for total percentage of chlorosis and necrosis over the entire plant as well as on a 1-6 scale where 1= no symptoms, 2=slight chlorosis, 3= extensive chlorosis (≥50% of plant), 4 = extensive chlorosis and necrosis ≥25% of plant, 5 = extensive chlorosis and

necrosis ≥50% plant, and 6 = dead/nearly dead plant. Disease ratings and total chlorosis and necrosis over time were converted to area under disease progress curves (AUDPC), area under chlorosis progress curves (AUCPC) and area under necrosis progress curves (AUNPC), respectively, using the following equation:

$$\Sigma_{i}^{n-1} ((Y_{i} + Y_{i+1})/2)(t_{i+1} - t_{i})$$

where Y_i = cumulative disease severity at the i^{th} observation, t_i = time (days p.i.) at the i^{th} observation and n = number of observations.

Stem sampling and progeny tuber assays. A single aboveground stem from each plant was destructively sampled when plants appeared to be within a week of senescence (after the plant was >80% necrotic but before desiccation of the stem). A one cm section, taken 30 cm above the soil-line, was plated onto NP-10 medium and incubated for one week to detect vertical stem colonization of *V. dahliae.* The remaining stems were left to dry for 3 weeks in their containers and visually assayed for *V. dahliae* microsclerotial production using a dissecting microscope. Microsclerotial colonization was recorded as the percentage of stem colonization in relation to total stem length; all remaining stems were assayed and results combined to calculate the mean microsclerotial colonization per plant. Accuracy of the visual assay was confirmed by taking samples 1-2 cm above and below the transition between colonized and noncolonized tissue of 24 randomly selected stems and plating onto NP-10. A total of seven randomly selected progeny tubers from each plant were assayed for *V. dahliae* infection as described above.

Data analysis. Analysis of variance (ANOVA) was performed on AUDPC, AUCPC and AUNPC values using PROC MIXED in SAS (version 9.1; SAS Institute, Cary, NC). Data were

analyzed as a three-way factorial randomized complete block design, with treatments consisting of intratuber infection, soil infestation and seed piece origin (apical and basal-end treatments) and preplanned comparisons performed using Fisher's protected LSD.

RESULTS

Seed tuber assays. Assays of certified seed lots intended for Washington State production fields detected *V. dahliae* in 35% of lots with 2.0% of tubers infected in 2007 and in 70% of lots with 6.9% of tubers infected in 2008. Incidence of infected tubers within lots ranged from 0-11.4% in 2007 and 0-17.1% in 2008. *V. dahliae* was not detected in any of the four Canadian seed lots surveyed in 2007.

Disease evaluation. Mean AUDPC and AUNPC values were significantly ($P \leq 0.05$) higher in potato plants grown in infested soil than in noninfested soil in both trials (Table 1). Significant differences in AUDPC and AUNPC were not found ($P > 0.05$) between plants grown from infected and noninfected seed tubers in noninfested soil. AUCPC was significantly higher for the infected tuber treatment compared to the control treatment in the 2007 trial and mean AUCPC was significantly higher for both infested soil treatments in the 2008 trial, however these differences were not consistent over both years. Significant interactions or additive effects were not detected between intratuber infection and soilborne inoculum in either 2007 or 2008. Analysis of disease progress curves showed that necrosis and chlorosis began earlier in plants grown in infested soil compared to those grown in noninfested soil (Figs. 1 and 2). Differences in disease development were not found in plants grown from apical and basal-end seed pieces cut

from infected tubers, however mean AUNPC was significantly higher in plants grown from basal-end seed pieces during the 2007 trial ($P < 0.03$) and a significant seed piece x soil inoculum interaction was also detected ($P < 0.04$).

A number of experimental units were lost due to bacterial soft rot during the 2008 trial. Plants which failed to emerge were included in the ANOVA as missing data points. An entire block became heavily infested with aphids approximately 100 days after planting and was not included in the analysis, bringing the total number of experimental units down from 88 to 55. Despite the reduction in degrees of freedom, ANOVA results from the 2008 trial were consistent with those from the 2007 trial with regards to AUDPC, AUNPC and re-isolation data.

Stem sampling and progeny tuber assays. Vascular infection of seed tubers by *V. dahliae* did not significantly ($P > 0.05$) contribute to aboveground vascular colonization, progeny tuber infection or microsclerotia production in senescent stems compared to noninoculated controls (Table 2). Plants grown in infested soil exhibited significantly ($P \leq 0.05$) more vascular colonization by *V. dahliae* and produced significantly more *V. dahliae*-infected progeny tubers than infected tubers grown in noninfested soil. Mean microsclerotial colonization was significantly higher in plants grown in infested soil compared to plants grown from infected seed pieces in noninfested soil. Mean microsclerotia colonization of stems originating from infected tubers ranged from 0 to7% while stems obtained from plants grown in infested soil exhibited 0 to 91% mean colonization. *V. dahliae* was not detected in or on any stems or progeny tubers from control treatments. Based on the combined stem assays, incidence of aboveground stem infection in potato plants grown from infected tubers in noninfested soil was 21% in the 2007

trial and 13% in the 2008 trial and 100% for both infected and noninfected tubers grown in infested soil in both trials.

DISCUSSION

The role of soilborne microsclerotia as primary inoculum of *V. dahliae* has long been recognized, however, the pathogen can also be found in the vascular tissue of certified seed tubers. Several field experiments have demonstrated that, despite the presence of *Verticillium* spp. in certified seed lots (10, 29, 39), intratuber infection has little effect on Verticillium wilt symptoms or potato yields in various potato cultivars. These studies, however, have either used cultivars under limited current cultivation (16, 32), artificially inoculated tubers (38) or *V. albo-atrum* (33) and focused on the effects of tuber infection on aboveground symptoms, yield, quality and vascular discoloration of progeny tubers. In addition, the potential contribution of seed tuber infection to the formation of future inoculum, i.e. microsclerotia, was not previously quantified. The results of this study show that intratuber infection exhibited a negligible effect on the development of Verticillium wilt symptoms in the commonly grown but moderately susceptible potato cultivar "Russet Burbank". Seed piece infection did not significantly contribute to aboveground stem colonization of the plant or the formation of microsclerotia in debris.

Assays of certified seed lots detected higher levels of *V. dahliae* in 2008 compared to 2007. Incidence of *V. dahliae* among and within certified seed lots sampled in 2007 was comparable to a previous survey of North American seed lots (29). The higher incidence of *V.*

dahliae in 2008 may be due to the lack of seed sources from Canada, where the prevalent *Verticillium* species is *V. albo-atrum* (30, 31). Assays performed in 2007 did not detect *V. dahliae* in the four seed lots obtained from Canada, however *V. dahliae* has previously been reported in Canadian-grown seed tubers (29).

Previous studies of *V. dahliae* isolates collected from potatoes and PNW potato fields found that the majority of isolates to belong to VCG 4A and VCG 4B, with VCG 4A being highly aggressiveness on potato compared to other VCGs (18, 28, 29). Although the infected seed tubers used in this study were not tested for infection by the more aggressive VCG 4A, the results of this study are still of practical importance since naturally-infected tubers were used and the negligible effect of intratuber infection on necrosis and disease progression was definitive for both trials ($P > 0.73$). In addition, the density of soilborne inoculum used (10 cfu/cm^3 soil) was low, especially considering a recent study which found that 37% of PNW fields intended for potato production contained ≥ 10 cfu/g soil and 6% had inoculum densities > 30 cfu/g soil (28). Artificially inoculated tubers were not used since artificial inoculation does not simulate the natural infection process and it is difficult to obtain a sufficient number of tubers for study. Omer et al. (29) demonstrated that nearly two-thirds of infected seed tubers intended for Washington production contained isolates of the more aggressive VCG 4A and approximately one-third contained VCG 4B. It is reasonable to assume a roughly similar frequency of VCG distribution was present in seed tubers used in this study.

A second, unrepeated experiment was performed to provide additional confirmation of the results of the repeated trials. Tubers were taken from control and infested soil treatments in

the 2007 trial and assayed for vascular infection as described above. Infected tubers were taken from artificially inoculated soil and presumed to be infected with *V. dahliae* isolate 653 (VCG 4A). Plants were grown from uncut tubers and soil inoculum administered as described above. Treatments consisted of infected tubers grown in infested soil and noninfected tubers grown in infested and noninfested soil. Plants were arranged as a RCB in the greenhouse with eight replications of each treatment. Mean AUDPC and AUNPC values, vascular and microsclerotia colonization and progeny tuber infection were consistent with results obtained from the 2007 and 2008 trials using naturally-infected seed tubers obtained from commercial seed lots (data not shown).

Plants grown from *V. dahliae*-infected tubers and control plants exhibited similar development of chlorosis and necrosis over time in both trials, indicating that the senescence observed in plants grown from infected tubers was natural (Fig. 1). AUDPCs and AUNPCs of treatments grown in infested soil were similar regardless of intratuber infection and no interactive effects, either additive or synergistic, were detected between soilborne and intratuber inoculum ($P > 0.05$). Treatment comparisons of mean AUNPC and AUDPC were more consistent than mean AUCPC between trials, indicating that necrosis and/or disease ratings which incorporate both necrosis and chlorosis may provide a more consistent evaluation of Verticillium wilt symptoms. Comparisons of AUNPC values indicate that premature necrosis began between 95 and 105 days after planting in plants grown in infested soil, approximately one to two weeks earlier than plants grown in noninfested soils regardless of intratuber infection.

The importance of soilborne inoculum in Verticillium wilt of potato, both for disease development and long-term survival of the pathogen, has been recognized for quite some time (15, 21, 34, 37). Nitzan et al. (26) suggested that the distribution of soilborne *C. coccodes* inoculum in the root zone provides more potential points of infection. Although not significant, apical-end seed pieces planted in infested soil resulted in higher AUDPC and AUNPC values than basal-end seed pieces in both trials (data not shown); the likely presence of more eyes on apical-end seed pieces, which can sprout into infested soil and provide more opportunities for infection, provides one possible explanation. In addition to their distribution in the root zone, microsclerotia in soil are capable of repeated germination and can essentially function as several CFU over time, increasing their infection potential in comparison to conidia and reducing the number of propagules required to cause disease (12).

Since vascular colonization is thought to be required for symptom development (1, 37, 40) it is not completely understood why infection of *V. dahliae* in potato seed tubers did not result in significant Verticillium wilt symptoms. Vascular colonization of aboveground stems was only detected in a few plants grown from infected tubers, indicating that the pathogen does not readily translocate from tuber vascular tissue to aboveground vascular tissue. Pathogen populations in the vascular system of the tuber may be below the threshold required to initiate colonization of the growing stems and cause disease. The pathogen may also be compartmentalized in progeny tubers, either during infection, storage or growth, preventing complete colonization of the seed tuber and providing opportunities for sprouting eyes to escape infection. Prior research suggests that vascular infection of seed tubers by *V. dahliae* is often unilateral (38), indicating that if occlusion of the pathogen does occur may be during infection or

storage. Previous studies on potato have shown varietal differences in the progression and density of vascular colonization by *V. dahliae*, with Verticillium wilt-resistant plants showing less vascular colonization than susceptible ones (1, 3, 7).

The results of this study indicate that intratuber infection of seed tubers of potato cultivar "Russet Burbank" does not significantly contribute to symptoms, progeny tuber infection or inoculum production in plant debris, hence management strategies should focus on soilborne inoculum. The possibility exists that soilborne *V. dahliae* inoculum can be introduced into a field solely from infected seed pieces, which could be important if the fungus, or novel strains of the fungus, are introduced into soils not previously used to grow potatoes or where a management practice such as fumigation has been applied to reduce soilborne inoculum. Since the use of soil fumigants is both costly and subject to future restrictions, other methods of reducing *V. dahliae* propagules in field soils need to be utilized. The use of partial or completely resistant cultivars, which can restrict vascular colonization and subsequent microsclerotia formation by *V. dahliae*, has the potential to both reduce symptoms and limit the amount of inoculum in field soils (8). Molecular detection methods, such as quantitative polymerase chain reaction, can be utilized to help develop resistant cultivars and monitor pathogen populations in the soil (1, 3). A combination of control methods, including resistance, preplant monitoring, crop rotation, green manures, proper sanitation and other cultural practices will likely be necessary to sustainably manage potato production fields affected by Verticillium wilt in the future.

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LITERATURE CITED

1. Atallah, Z. K., Bae, J., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology 97:865-872.
2. Atibalentja, N., and Eastburn, D. M. 1997. Evaluation of inoculation methods for screening horseradish cultivars for resistance to *Verticillium dahliae*. Plant Disease 81:356-362.
3. Bae, J., Atallah, Z. K., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Colonization dynamics and spatial progression of *Verticillium dahliae* in individual stems of two potato cultivars with differing responses to potato early dying. Plant Disease 91:1137-1141.
4. Baribeau, B. 1952. Verticillium wilt and seed potato certification. American Journal of Potato Research 29:157-159.
5. Butterfield, E. J., and Devay, J. E. 1977. Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67:1073-1078.
6. Cappaert, M. R., Powelson, M. L., Christensen, N. W., and Crowe, F. J. 1992. Influence of irrigation on severity of potato early dying and associated yield loss. Phytopathology 82:1448-1453.

7. Davis, J. R., Pavek, J. J., and Corsini, D. L. 1983. A sensitive method for quantifying *Verticillium dahliae* colonization in plant tissue and evaluating resistance among potato genotypes. Phytopathology 73:1009-1014.

8. Davis, J. R., Pavek, J. J., Corsini, D. L., Sorensen, L. H., Schneider, A. T., Everson, D. O., Westerman, D. T., and Huisman, O. C. 1994. Influence of continuous cropping of several potato clones on the epidemiology of Verticillium wilt of potato. Phytopathology 84:207-214.

9. Douhan, L. I., and Johnson, D. A. 2001. Vegetative compatibility and pathogenicity of *Verticillium dahliae* from spearmint and peppermint. Plant Disease 85:297-302.

10. Easton, G. D., Nagle, M. E., and Bailey, D. L. 1972. *Verticillium albo-atrum* carried by certified seed potatoes into Washington and control by chemicals. Amer Potato J 49:397-402.

11. Ewing, E. 1981. Heat stress and the tuberization stimulus. American Journal of Potato Research 58:31-49.

12. Farley, J. D., Wilhelm, S., and Snyder, W. C. 1971. Repeated germination and sporulation of microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 61:260-264.

13. Fradin, E. F., and Thomma, B. P. H. J. 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7:71-86.

14. Francl, L. J., Madden, L. V., Rowe, R. C., and Riedel, R. M. 1990. Correlation of growing season environmental variables and the effect of early dying on potato yield. Phytopathology 80:425-432.

15. Green, R. J. 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.

16. Hoyman, W. G. 1974. Consequence of planting Norgold Russet seed infected with *Verticillium albo-atrum*. American Potato Journal 51:22-25.

17. Isaac, I., and Harrison, J. A. C. 1968. The symptoms and causal agents of early-dying disease (Verticillium wilt) of potatoes. Annals of Applied Biology 61:231-244.

18. Joaquim, T. R., and Rowe, R. C. 1991. Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. Phytopathology 81:552-558.

19. Johnson, D. A., Rowe, R. C., and Cummings, T. F. 1997. Incidence of *Colletotrichum coccodes* in certified potato seed tubers planted in Washington State. Plant Disease 81:1199-1202.

20. Johnson, K. B., Radcliffe, E. B., and Teng, P. S. 1986. Effects of interacting populations of *Alternaria solani*, *Verticillium dahliae*, and the potato leafhopper (*Empoasca fabae*) on potato yield. Phytopathology 76:1046-1052.

21. Menzies, J. D., and Griebel, G. E. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.

22. Mol, L. 1995. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. European Journal of Plant Pathology 101:679-685.

23. Mol, L., and Scholte, K. 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. Potato Research 38:143-150.

24. Nachmias, A., and Krikun, J. 1984. Transmission of *Verticillium dahliae* in potato tubers. Phytopathology 74:535-537.

25. National Agricultural Statistics Service. Crop Production. 2008. United States Department of Agriculture,, Washington D.C.

26. Nitzan, N., Cummings, T. F., and Johnson, D. A. 2008. Disease potential of soil- and tuberborne inocula of *Colletotrichum coccodes* and black dot severity on potato. Plant Disease 92:1497-1502.

27. Nnodu, E., and Harrison, M. 1979. The relationship between *Verticillium albo-atrum* inoculum density and potato yield. American Journal of Potato Research 56:11-25.

28. Omer, M. A., Johnson, D. A., Douhan, L. I., Hamm, P. B., and Rowe, R. C. 2008. Detection, quantification, and vegetative compatibility of *Verticillium dahliae* in potato and mint production soils in the Columbia Basin of Oregon and Washington. Plant Disease 92:1127-1131.

29. Omer, M. A., Johnson, D. A., and Rowe, R. C. 2000. Recovery of *Verticillium dahliae* from North American certified seed potatoes and characterization of strains by vegetative compatibility and aggressiveness. American Journal of Potato Research 77:325-331.

30. Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. Annual Review of Phytopathology 31:111-126.

31. Powelson, M. L., and Rowe, R. C. 2008. Managing diseases caused by seedborne and soilborne fungi and fungus-like pathogens. Pages 183-195 in: Potato Health Management, D. A. Johnson, ed. APS Press, St. Paul.

32. Robinson, D. B., and Ayers, G. W. 1953. The control of Verticillium wilt of potatoes by seed treatment. Canadian Journal of Agricultural Science 33:147-152.

33. Robinson, D. B., and Ayers, G. W. 1961. Verticillium wilt of potato in relation to vascular infection of the tuber. Canadian Journal of Plant Science 41:703-708.

34. Rowe, R. C. 1985. Potato early dying - a serious threat to the potato industry. American Potato Journal 62:157-161.

35. Rowe, R. C., Davis, J. R., Powelson, M. L., and Rouse, D. I. 1987. Potato early dying: causal agents and management strategies. Plant Disease 71:482-489.

36. Rowe, R. C., Riedel, R. M., and Martin, M. J. 1985. Synergistic interactions between *Verticillium dahliae* and *Pratylenchus penetrans* in potato early dying disease. Phytopathology 75:412-418.

37. Schnathorst, W. C. 1981. Life cycle and epidemiology of *Verticillium*. Pages 81-111 in: Fungal Wilt Diseases of Plants, M. E. Mace, A. A. Bell and C. H. Beckman, eds. Academic, New York.

38. Thanassoulopoulos, C. C., and Hooker, W. J. 1968. Factors influencing infection of field grown potato by *Verticillium albo-atrum*. American Journal of Potato Research 45:203-216.

39. Tsror (Lahkim), L., Aharon, M., and Erlich, O. 1999. Survey of bacterial and fungal seedborne diseases in imported and domestic potato seed tubers. Phytoparasitica 27:215-226.

40. Vallad, G. E., and Subbarao, K. V. 2008. Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. Phytopathology 98:871-885.

41. Whitworth, J. L., and Davidson, R. D. 2008. Quality seed: seed improvement, cultivar and seed lot selection, and certification. Pages 31-41 in: Potato Health Management, D. A. Johnson, ed. APS Press, St. Paul.

TABLE 1. Mean AUDPC, AUCPC and AUNPC values for potato cultivar "Russet Burbank" grown from *V. dahliae*-infected and noninfected seed tubers in the presence and absence of soilborne inoculum.

Inoculum Source(s)		AUDPC		AUCPC		AUNPC	
Soil	**Tuber**	**2007 Trial[a]**	**2008 Trial**	**2007 Trial**	**2008 Trial**	**2007 Trial**	**2008 Trial**
None	**None**	217 a	169 a	1165 a	814 a	1749 a	1385 a
None	**Infected**	219 a	169 a	1544 b	832 ab	1781 a	1475 a
Infested	**None**	274 b	216 b	1372 b	1048 c	3022 b	1975 b
Infested	**Infected**	272 b	216 b	1529 b	992 bc	2905 b	1916 b

[a] Treatment means compared with Fischer's protected least significant difference; values with the same letter indicate no significant difference within the trial ($P > 0.05$).

TABLE 2. Vascular colonization, microsclerotia production and progeny tuber infection in "Russet Burbank" potato plants grown from infected and noninfected seed tubers in the presence and absence of soilborne inoculum.

Inoculum Source(s)		Stem Colonization (% isolated at 30 cm above soil-line)		Microsclerotia Colonization (% total length)		Infected Progeny Tubers (% isolated)	
Soil	**Tuber**	**2007 Trial[a]**	**2008 Trial**	**2007 Trial**	**2008 Trial**	**2007 Trial**	**2008 Trial**
None	**None**	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
None	**Infected**	8.3 a	6.7 a	0.5 a	0.3 a	0.0 a	0.0 a
Infested	**None**	95.8 b	92.3 b	52.5 b	46.1 b	14.3 b	15.4 b
Infested	**Infected**	95.8 b	92.9 b	47.8 b	41.8 c	13.7 b	13.3 b

[a] Treatment means compared with Fischer's protected least significant difference; values with the same letter indicate no significant difference within the trial ($P > 0.05$).

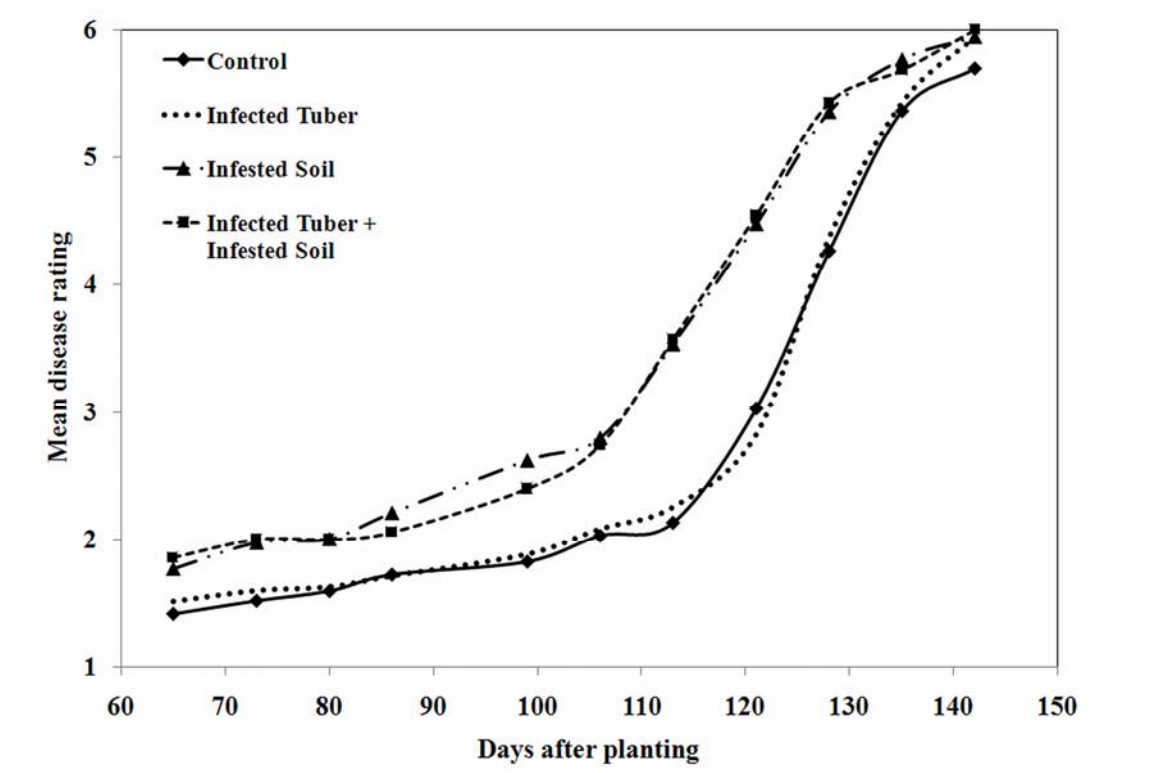

Fig 1. Disease progress curves for potato plants grown from *V. dahliae*-infected and noninfected tubers in infested and noninfested soil. Data combined from both years.

CHAPTER 2

The Effect of Vascular Infection in Potato Seed Lots by *Verticillium dahliae* on Disease Severity and Yield

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Dung, J. 2009. The Effect of Vascular Infection in Potato Seed Lots by *Verticillium dahliae* on Disease Severity and Yield.
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Abstract *Verticillium dahliae*, causal agent of Verticillium wilt, can be found in the vascular tissue of potato seed tubers. The effects of *V. dahliae* infection in certified seed lots on Verticillium wilt symptoms and yield were compared over two seasons in fields with and without prior histories of potato crops (common and long rotation, respectively). The number of stems colonized by *V. dahliae* at vine kill differed significantly among seed lots in both field types during the first season ($P < 0.05$). Yield, progeny tuber number and incidence of *V. dahliae*-infected progeny tubers differed significantly in three of four fields ($P \leq 0.05$) and crop canopy necrosis differed significantly among seed lots grown in one field. The amount of *V. dahliae* infection in seed lots was not related to differences in yield or symptom severity, however, the higher incidence and severity of Verticillium wilt in common potato rotation fields compared to long rotation fields emphasize the importance of soilborne inoculum in the development of the disease in potato.

Introduction

Verticillium wilt of potato (*Solanum tuberosum* L.), caused by the fungus *Verticillium dahliae* Kleb., is a disease of significant importance in temperate potato growing regions. The pathogen has a broad host range and worldwide distribution (Rowe et al., 1987). Symptoms of Verticillium wilt of potato include unilateral wilting, chlorosis and necrosis which progress acropetally. Entire stems and plants senesce prematurely, with stems often remaining upright. Reports on yield reductions due to Verticillium wilt are variable, ranging from 12% (Johnson et al., 1986) to 30-50% (Powelson and Rowe, 1993). Symptoms and yield reductions are affected by soil and environmental conditions (Powelson and Rowe, 1993) and can be more severe during times of relatively high temperatures and high levels of evapotranspiration (Ewing, 1981; Francl et al., 1990; Nnodu and Harrison, 1979; Rowe et al., 1985).

Primary inoculum of *V. dahliae* consists of microsclerotia which can remain viable for long periods of time (Green, 1969; Menzies and Griebel, 1967; Schnathorst, 1981) and are stimulated to germinate in response to host root exudates (Mol, 1995). The pathogen invades the xylem of susceptible hosts where it is translocated systemically (Fradin and Thomma, 2006). Microsclerotia are formed primarily in colonized aerial stems during plant senescence (Mol and Scholte, 1995) and can increase inoculum levels if infested debris is plowed into the soil. Moreover, infested soil carried on the surface of seed tubers has been shown to contribute to the development of Verticillium wilt in potato (Robinson and Ayers, 1961; Thanassoulopoulos and Hooker, 1968).

In addition to soilborne microsclerotia, *V. dahliae* inoculum can be found in the vascular system of certified seed tubers. The percentage of seed lots with *V. dahliae*-infected tubers can be as high as 44% in Israel (Tsror (Lahkim) et al., 1999) and 29% in Washington State (Omer et al., 2000), with incidence of infection within seed lots typically < 5%. Robinson and Ayers (1961) found that vascular infection of seed tubers by *V. albo-atrum* was not as important as external and soilborne inoculum**,** and Hoyman (1974) concluded that *V. dahliae* infection of "Norgold Russet" seed tubers did not contribute to disease development or reduced yield or quality. However, wilt severity can vary among cultivars (Robinson and Ayers, 1961) and the importance of *V. dahliae* infection in seed tubers of the widely grown cultivar "Russet Burbank" is not known. The objective of this study was to quantify the effects of seed tuber infection on disease severity and yield using certified seed lots with varying degrees of *V. dahliae* infection grown in fields with and without potatoes in their recent cropping histories.

Materials and Methods

Seed tubers of potato cultivar "Russet Burbank" were obtained from grower and distributor storages in the spring of 2007 and 2008. Seed tubers were from certified seed lots intended for Washington State production fields. Approximately 45 kg (about 225 tubers) of seed tubers were collected from ten seed lots each year and were Generation 3 in accordance with the Washington limited generation scale for seed potatoes. Seed lots were assayed to determine the incidence of intratuber infection by *V. dahliae*. Thirty-five seed tubers from each seed lot were removed from 4 C storage and warmed at 23 C for 12 hours. The seed tubers were washed with

soapy water and gently scrubbed with a sponge, rinsed with distilled water and air-dried. A 15 mm diameter disk, centered on the site of stolon attachment and consisting of periderm, cortex and vascular tissue, was aseptically cut from the basal end of each tuber and plated onto either modified potato dextrose agar (Johnson et al., 1997) or NP-10 medium (Butterfield and Devay, 1977). Plates were incubated at 23 C in the dark for 14 days and evaluated for the presence of *V. dahliae* growing on the media. The identification of *V. dahliae* colonies was verified by subculturing onto potato dextrose agar when necessary. The incidence of *V. dahliae* infection in each seed lot was calculated based on the number of infected seed tubers found in the assays. Six seed lots were selected to provide a range of *V. dahliae* incidence (Tables 1 & 2). Tubers from the six seed lots were removed from 4 C storage, cut into 56- to 113-g (2- to 4-oz) seed pieces and stored at 10 C and 95% relative humidity until planting.

The study was performed in 2007 and repeated in 2008 in fields located in the Columbia Basin of Washington State. The common rotation fields were under five-year potato rotations and were located at the WSU Othello Research Station (Shano Silt Loam, 46° 47' 14.46N, 119° 02' 38.60W). The long rotation field in 2007 (Chedahap Fine Sandy Loam, 46° 41' 18.92N, 118° 51' 17.09W) was never planted to potatoes while the long rotation field in 2008 had not been planted to potatoes in over 30 years (Shano Silt Loam, 46° 45' 39.48N; 118° 51' 04.84W). Common rotation fields were irrigated with an overhead, linear system. Plots located in long rotation fields were situated within commercial potato fields and irrigated using center-pivot systems. Fields were planted on May 3, 2007 and April 24, 2008. Each plot contained seed pieces from one of the six seed lots selected. Plots were arranged in a completely randomized design in 2007 and a randomized complete block design in 2008 and were replicated four times

both years. Seed pieces were planted at a depth of 20 cm (8 in) and spaced approximately 25 cm (10 in) apart. Plots contained three rows, 4.6 m in length (15 ft) and spaced 86 cm (34 in) apart, providing a population of approximately 45,588 plants ha^{-1} (18,449 plants $acre^{-1}$). The center row was used for yield evaluation and an adjacent row was used to assess symptom severity. All three rows were used to evaluate canopy necrosis.

The insecticide thiamethoxam (Platinum 8 oz $acre^{-1}$) was applied in furrow at a rate of 140 g a.i. ha^{-1}. Fungicides or seed treatments were not used at planting. Both fields were otherwise managed according to standard practices used in the Columbia Basin, except the irrigation system was inoperative for 10 days in the long rotation field during the last week of June and first week of July 2007.

Disease and Yield Assessment

Total crop canopy necrosis was assessed in the common rotation field at 119 days after planting (DAP) and in the long rotation field at 125 DAP in 2007 (Tables 1 & 2). Crop canopy necrosis was assessed at 137 DAP for both fields in 2008. Colonization of aboveground stems by *V. dahliae* was assessed in a sample of three intact plants that were removed from each replication at 109 DAP in the common rotation field and 125 DAP in the long rotation field in 2007. Plant colonization was assessed in both fields at 109 DAP in 2008. The sampled plants were dug and petioles were removed using pruning shears. Plants were placed in coolers for transportation and stored overnight at 4 C. The following day, plants were rinsed in tap water, surface sterilized in a 0.5% NaOCl solution for 5 minutes and rinsed in distilled water. Aboveground stem

colonization by *V. dahliae* was assessed by aseptically plating three 5 mm cross-sections taken at 2-, 6- and 12-cm up the stem onto NP-10 medium. Plates were incubated at 23 C for 14 days and evaluated for the presence of *V. dahliae.* Colonization at the three heights was recorded as a binomial response, with 1 = presence of *V. dahliae* and 0 = absence of *V. dahliae*. A colonization severity score was assigned to each subsample (stem) based on maximum *V. dahliae* colonization height above the soil line at 2-, 6-, and 12-cm using the following formula: $[(1* x_{2cm}) + (2 * x_{6cm})+ (3* x_{12cm})]$ where x_y = the binomial response at stem height y. The values for each cross-section were then totaled within each subsample and the mean value of three subsamples was calculated for each replication.

Vines were mechanically flailed prior to harvest and the number of stems exhibiting *V. dahliae* microsclerotia was recorded in each plot. Plots were dug at 125 DAP in the common rotation field and at 131 DAP in the long rotation field in 2007. Both fields were dug at 146 DAP in 2008. Yields were taken from the center row of each plot. The tubers from each replication were mechanically counted and weighed and total yields ha^{-1} calculated. Progeny tubers were assessed for intratuber infection by *V. dahliae* as described above using 14 tubers randomly selected from each replication.

Data analysis

Analysis of variance (ANOVA) was performed on data for canopy necrosis, number of stems showing *V. dahliae* colonization prior to harvest, stem colonization severity, percentage of progeny tubers infected, total yield and progeny tuber quantity using PROC GLM in SAS

(version 9.1; SAS Institute, Cary, NC). Comparisons were made between seed lots field using Fisher's protected least significant difference.

Relationships between seed lot infection and dependent variables such as disease severity and yield were assessed by performing linear regression analysis using PROC REG in SAS (version 9.1; SAS Institute, Carey NC). The percentage of seed tubers infected with *V. dahliae* was log transformed and relationships individually assessed relative to total yield, progeny tuber quantity and incidence of progeny tuber infection. These specific analyses were chosen because of significant differences among seed lots found in three of four fields. The number of plants with *V. dahliae*-colonized stems at vine kill was only significantly different among seed lots in two of four fields but was also subjected to simple linear regression.

Results

Verticillium wilt severity and yield characteristics varied among all four fields but were not consistently correlated with the incidence of infected seed in seed lots (Tables 1 & 2). Total crop canopy necrosis varied significantly among seed lots only in the common rotation field in 2007 ($P < 0.05$). The number of plants with stems colonized with *V. dahliae* microsclerotia at vine kill was significantly different among seed lots in 2007 in both field types ($P < 0.05$), however no significant differences in aboveground colonization severity were found ($P > 0.05$). Incidence of *V. dahliae*-infection in progeny tubers was significantly different among seed lots in the 2008 common rotation field and in both long rotation fields. Correlations were not observed between seed lot and progeny tuber infection. Significant differences in yield were found among seed lots

in long rotation fields during both years and in the common rotation field in 2007 ($P \leq 0.05$) but the differences were not correlated with the percentage of seed lot infected with *V. dahliae* ($P > 0.1$). Significant differences in the number of progeny tubers were observed in three of four fields ($P \leq 0.05$) but differences were not correlated with incidence of *V. dahliae* infection in seed lots. Exploratory regression analysis did not identify any relationships between significant differences in yield or disease characteristics such as total crop canopy necrosis or the incidence of infected stems at vine kill.

Discussion

The percentage of *V. dahliae*-infected seed tubers in a seed lot was not associated with disease symptoms, canopy necrosis or yield in the popular potato cultivar "Russet Burbank". Data from this study are consistent with previous studies on other cultivars which concluded that vascular infection of seed tubers was not as important as soilborne inoculum in the development Verticillium wilt (Hoyman, 1974; Robinson and Ayers, 1961; Thanassoulopoulos and Hooker, 1968). Previous studies report variable effects of Verticillium wilt on yield (Nachmias and Krikun, 1984; Powelson and Rowe, 1993; Rowe et al., 1985). Correlations between Verticillium wilt severity and yield reductions were not found in this study and the significant differences in yield observed were likely due to some other causes such as physiological characteristics, microbiological processes or a combination of factors. Although seed lot infection by *V. dahliae* was not related to disease development or reductions in yield, infected seed pieces themselves may become a source of inoculum for future potato crops.

Although a lack of repetition prohibits formal statistical comparisons between the common and long rotation fields, differences in disease severity were evident (Tables 1 & 2). Canopy necrosis was greater in the common rotation fields compared to long rotation fields. Common rotation fields also contained more infected stems at vine kill, higher disease severity and a greater incidence of *V. dahliae* infection in progeny tubers. In addition, all but one of the seed lots planted in long rotation fields produced higher yields compared to the same seed lot planted in common rotation fields during the same year. The role and importance of soilborne inoculum in the development of Verticillium wilt is well-documented and, although soilborne inoculum was not quantified, the higher incidence and severity of the disease in common rotation soils could likely be attributed to soilborne inoculum. The amount of soilborne inoculum required to incite Verticillium wilt in potato is relatively low, ranging between 5-30 cfu cm^{-3} of soil (Powelson and Rowe, 1993), and inoculum residing in soil may have overshadowed any minor effects of vascular infection of seed tubers. Additionally, the incidence of *V. dahliae* infection within certified seed lots is generally less than 5% and may not be high enough to significantly contribute to disease in the field.

The pathogen was isolated from aboveground stems of plants grown in long rotation fields, one of which was considered virgin potato ground while the other had not grown a potato crop in over 30 years. Although it remains undetermined whether the source of *V. dahliae* inoculum was undetected infected seed, microsclerotia in soil or both, it is known that soilborne microsclerotia are effective sources of inoculum capable of long-term survival (Green, 1969; Schnathorst, 1981) and repeated germination (Farley et al., 1971). The pathogen can colonize

the roots of resistant and nonhost plants (Eynck et al., 2007; Fradin and Thomma, 2006; Vallad and Subbarao, 2008) which may serve as asymptomatic green bridges and prolong its survival.

The results obtained from this study indicate that vascular infection of seed tubers by *V. dahliae* is not related to differences in disease severity or yield. Trends were not observed which could explain the differences in yield or disease symptoms observed among seed lots, however seed lots grown in field soils under five-year potato rotations generally exhibited greater Verticillium wilt severity and produced lower yields than seed lots grown in long rotation fields. The reasons for yield differences among seed lots within a field are not known, but possible explanations include slight genetic differences between clones, the geographic origin or physiological age of the seed lot or a combination of factors (Fennell and de Jong, 1996; Love et al., 1992; Miller et al., 1995). The differences in yield observed among the seed lots emphasizes the importance of considering the recommended best practices and guidelines for potato seed lot selection (Bohl et al., 2003; Secor and Johnson, 2008) when choosing and handling seed lots for production.

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References

Bohl, W.H., N. Olsen, S.L. Love, and P. Nolte. 2003. Seed and planting management. In Potato Production Systems, (eds.) J. C. Stark and S. L. Love, 91-113.

Butterfield, E.J., and J.E. Devay. 1977. Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67:1073-1078.

Ewing, E. 1981. Heat stress and the tuberization stimulus. American Journal of Potato Research 58:31-49.

Eynck, C., B. Koopmann, G. Grunewaldt-Stoecker, P. Karlovsky, and A. von Tiedemann. 2007. Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. European Journal of Plant Pathology 118:259-274.

Farley, J.D., S. Wilhelm, and W.C. Snyder. 1971. Repeated germination and sporulation of microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 61:260-264.

Fennell, J., and R. de Jong. 1996. Effect of seed production location on the performance of Russet Burbank clones in southern Australia. American Journal of Potato Research 73:569-575.

Fradin, E.F., and B.P.H.J. Thomma. 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7:71-86.

Francl, L.J., L.V. Madden, R.C. Rowe, and R.M. Riedel. 1990. Correlation of growing season environmental variables and the effect of early dying on potato yield. Phytopathology 80:425-432.

Green, R.J. 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.

Hoyman, W.G. 1974. Consequence of planting Norgold Russet seed infected with *Verticillium albo-atrum*. American Potato Journal 51:22-25.

Johnson, D.A., R.C. Rowe, and T.F. Cummings. 1997. Incidence of *Colletotrichum coccodes* in certified potato seed tubers planted in Washington State. Plant Disease 81:1199-1202.

Johnson, K.B., E.B. Radcliffe, and P.S. Teng. 1986. Effects of interacting populations of *Alternaria solani*, *Verticillium dahliae*, and the potato leafhopper (*Empoasca fabae*) on potato yield. Phytopathology 76:1046-1052.

Love, S., A. Thompson, T. Baker, and D. Corsini. 1992. Comparison of Russet Burbank clones from various geographical regions of the United States and Canada. American Journal of Potato Research 69:299-307.

Menzies, J.D., and G.E. Griebel. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.

Miller, J., D. Smallwood, J. Miller, and G. Fernandez. 1995. Norgold Russet and Norgold Russet strain M — additional evidence for genetic dissimilarity. American Journal of Potato Research 72:273-286.

Mol, L. 1995. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. European Journal of Plant Pathology 101:679-685.

Mol, L., and K. Scholte. 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. Potato Research 38:143-150.

Nachmias, A., and J. Krikun. 1984. Transmission of *Verticillium dahliae* in potato tubers. Phytopathology 74:535-537.

Nnodu, E., and M. Harrison. 1979. The relationship between *Verticillium albo-atrum* inoculum density and potato yield. American Journal of Potato Research 56:11-25.

Omer, M.A., D.A. Johnson, and R.C. Rowe. 2000. Recovery of *Verticillium dahliae* from North American certified seed potatoes and characterization of strains by vegetative compatibility and aggressiveness. American Journal of Potato Research 77:325-331.

Powelson, M.L., and R.C. Rowe. 1993. Biology and management of early dying of potatoes. Annual Review of Phytopathology 31:111-126.

Robinson, D.B., and G.W. Ayers. 1961. Verticillium wilt of potato in relation to vascular infection of the tuber. Canadian Journal of Plant Science 41:703-708.

Rowe, R.C., R.M. Riedel, and M.J. Martin. 1985. Synergistic interactions between *Verticillium dahliae* and *Pratylenchus penetrans* in potato early dying disease. Phytopathology 75:412-418.

Rowe, R.C., J.R. Davis, M.L. Powelson, and D.I. Rouse. 1987. Potato early dying: causal agents and management strategies. Plant Disease 71:482-489.

Schnathorst, W.C. 1981. Life cycle and epidemiology of *Verticillium*. In Fungal Wilt Diseases of Plants, (eds.) M. E. Mace, A. A. Bell and C. H. Beckman, 81-111. Academic, New York.

Secor, G.A., and S.B. Johnson. 2008. Seed tuber health before and during planting. In Potato Health Management, 2nd ed., (ed.) D. A. Johnson, 43-53. APS Press, St. Paul, Minn.

Thanassoulopoulos, C.C., and W.J. Hooker. 1968. Factors influencing infection of field grown potato by *Verticillium albo-atrum*. American Journal of Potato Research 45:203-216.

Tsror (Lahkim), L., M. Aharon, and O. Erlich. 1999. Survey of bacterial and fungal seedborne diseases in imported and domestic potato seed tubers. Phytoparasitica 27:215-226.

Vallad, G.E., and K.V. Subbarao. 2008. Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. Phytopathology 98:871-885.

Table 1. Disease severity and yield characteristics of seed lots with and without *V. dahliae*-infected tubers planted in fields under five-year potato rotations (common rotation).

	Seed Lot	Seed infected per lot (%)	Mean(%) Canopy Necrosis	*V. dahliae*-colonized stems per plot at vine kill	Aboveground Stem Severity	Incidence of Infected Progeny Tubers	Progeny Tuber Quantity	Yield (mt ton ha^{-1})
2007	A	0	47	4.3 bc	5.8	0.24	175 abc	87.4 a
	B	0	45	4.0 bc	6.0	0.39	184 ab	83.4 a
	C	0	48	3.8 c	6.0	0.23	158 c	74.2 b
	D	3	45	4.5 abc	6.0	0.34	179 abc	72.2 b
	E	3	60	7.3 a	6.0	0.25	193 a	74.9 b
	F	11	60	6.8 ab	6.0	0.22	169 bc	78.7 ab
	Regression with seeds lots		*NS*	$P < 0.05$ $R^2 = 0.67$	*NS*	*NS*	*NS*	*NS*
2008	U	0	85 b	8	5.7	0.34 ab	128 b	60.3
	V	0	89 ab	10	5.6	0.27 b	155 a	75.1
	W	0	84 b	10	5.3	0.48 a	153 a	70.8
	X	3	95 a	9	6.0	0.36 ab	125 b	60.1
	Y	11	91 ab	9	6.0	0.41 ab	128 b	61.4
	Z	14	90 ab	8	6.0	0.29 b	130 b	67.7
	Regression with seeds lots		*NS*	*NS*	$P < 0.03$ $R^2 = 0.74$	*NS*	*NS*	*NS*

[a] Treatment means compared with Fischer's protected least significant difference; values with the same letter indicate no significant difference within the trial ($P > 0.05$).
[b] NS = not significant
[c] DAP = days after planting

Table 2. Disease severity and yield characteristics of seed lots with and without *V. dahliae*-infected tubers planted in fields which had not grown potatoes in 30 years (long rotation).

	Seed Lot	Seed infected per lot (%)	Mean(%) Canopy Necrosis	*V. dahliae*-colonized stems per plot at vine kill	Aboveground Stem Severity	Incidence of Infected Progeny Tubers	Progeny Tuber Quantity	Yield (mt ton ha^{-1})
2007	A	0	11	3.3 b	1.5	0.14 a	206 ab	79.6 ab
	B	0	10	2.5 b	1.5	0.14 a	166 b	63.0 b
	C	0	11	1.8 a	2.3	0.07 ab	190 ab	76.2 ab
	D	3	15	3.5 b	2.3	0.00 b	205 ab	85.0 a
	E	3	13	4.0 b	1.1	0.05 b	194 ab	78.2 ab
	F	11	11	3.3 b	1.6	0.09 ab	221 a	85.4 a
	Regression with Seeds Lots[b]		*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
2008	U	0	10	0	5.0	0.01 c	181	91.2 ab
	V	0	5	0.3	4.5	0.11 a	172	97.5 a
	W	0	6	0	4.4	0.00 c	169	85.2 b
	X	3	7	0.3	3.7	0.09 ab	145	83.4 b
	Y	11	9	0.5	4.2	0.04 bc	145	86.3 b
	Z	14	9	0.5	5.3	0.11 a	147	83.4 b
	Regression with Seeds Lots		*NS*	$P < 0.02$ $R^2 = 0.80$	*NS*	*NS*	$P < 0.02$ $R^2 = 0.80$	*NS*

[a] Treatment means compared with Fischer's protected least significant difference; values with the same letter indicate no significant difference within the trial ($P > 0.05$).
[b] NS = not significant
[c] DAP = days after planting

CHAPTER 3

Evaluation of Verticillium Wilt resistance in *Mentha arvensis* and *M. longifolia* using *Verticillium dahliae* isolates from various hosts and vegetative compatibility groups

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ABSTRACT

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is a major constraint to mint (*Mentha*) production in the United States and resistant cultivar development is considered an important component of Verticillium wilt management. Two *M. arvensis* cultivars and four *M. longifolia* genotypes were evaluated for resistance to *V. dahliae* isolates obtained from different hosts and belonging to different vegetative compatibility groups (VCGs) in separate greenhouse experiments. Isolates of *V. dahliae* obtained from peppermint caused significantly higher disease severity, yield reduction and plant mortality than isolates obtained from other hosts regardless of VCG, demonstrating variation in aggressiveness on mint among and within VCGs. Disease severity, plant mortality and pathogen isolation frequency in aboveground stems were consistently higher and yields consistently lower in peppermint (*M.* x *piperita* "Black Mitcham"), the susceptible standard, compared to the resistant standard native spearmint (*M. spicata*). Root-dip inoculations of *M. arvensis* and *M. longifolia* genotypes with isolates of *V.*

dahliae obtained from peppermint resulted in AUDPC values similar to or greater than peppermint, however, *M. arvensis* and *M. longifolia* plants displayed the ability to recover from infection by developing new growth from rhizomes. Both *M. arvensis* cultivars exhibited significantly lower mean disease severity ratings than peppermint and were not significantly different from native spearmint following each cutback and regrowth period. The restriction of pathogen movement in vascular or aboveground tissue and ability to recover from infection may be important components of *V. dahliae* resistance in mint.

INTRODUCTION

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is a major disease affecting mint (*Mentha* L.) production in the United States. Symptoms of Verticillium wilt in mint can include anthocyanescence, bronzing and/or curling of the apical leaves, chlorosis, stunting, wilt, necrosis and premature senescence (19). Losses occur due to decreased oil production and stand reduction, which can worsen over the lifetime of the perennial crop. Fields of peppermint (*M.* x *piperita* L.) and Scotch spearmint (*M.* x *gracilis* Sole) can be severely damaged by Verticillium wilt while another commercially grown species, native spearmint (*M. spicata* L.), is more resistant (5).

The host range of *V. dahliae* includes hundreds of dicotyledonous species in various genera (1, 9, 39). Despite its broad host range, isolates of *V. dahliae* exhibit varying degrees of host specificity, with some displaying increased aggressiveness and/or cross-pathogenicity on certain host species (6, 8). In addition, isolates can be separated into vegetative compatibility

groups (VCGs) based on their ability to undergo hyphal anastamosis with other isolates (9, 22). Most isolates collected from mint belong to VCG 2B and are highly aggressive on mint, indicating the presence of a predominant mint pathotype (11, 15, 24). *V. dahliae* isolates collected from mint can also interact synergistically with the root lesion nematode *Pratylenchus penetrans* (13, 24).

Initial inoculum of *V. dahliae* consists primarily of soilborne microsclerotia, which form in senescing plants and can persist in soils for years (17, 31, 39). Microsclerotia germinate in response to plant root exudates (32) and hyphae colonize the root surface and cortex. Verticillium wilt symptoms occur when the pathogen penetrates the stele and invades the xylem, where it is systemically translocated through the host vascular system (14). The pathogen is capable of colonizing the roots of resistant and nonsusceptible hosts but it appears the fungus is restricted from extensively colonizing the cortex and is unable to infiltrate the xylem (2, 3, 14, 44). Microsclerotia are produced at host senescence and colonized plant debris can contribute to future inoculum levels if incorporated into soil. Conidia can also be produced during host senescence but are shorter-lived than microsclerotia and not thought to be significant in the disease cycle (17, 44). Primary inoculum may also be present in the form of infected rhizomes used for planting (29).

Verticillium wilt is managed primarily through the use of disease-free planting stock, rotations with nonsusceptible monocotyledonous crops and preplant fumigation (16, 33). Environmental and economical concerns may limit the future use of chemical controls and crop rotation is of only limited benefit, largely due to the ability of *V. dahliae* to colonize and survive

on numerous hosts and nonhosts and its ability to persist in soils. The development of resistant cultivars offers a promising tool to manage Verticillium wilt, however, both peppermint and Scotch spearmint are sterile hybrids and conventional breeding is not an option. Mint mutants derived from irradiation treatment has produced mixed results (20, 23, 34, 41) and other characteristics such as plant vigor, oil composition and yield are of critical importance during cultivar development (30). Recent advances in *Agrobacterium tumefaciens*-mediated transformation of peppermint offers the opportunity to introduce Verticillium wilt resistance genes from other *Mentha* species into sterile mint hybrids without altering desirable oil composition or quality characteristics (35, 45, 48).

The genus *Mentha* includes at least 18 species, 11 hybrids and numerous varieties (42), all of which are potential sources of Verticillium wilt resistance. *M. arvensis* L. is the most widely grown mint in the world and menthol derived from *M. arvensis* is used in a variety of cosmetics, foods and tobacco products (10). India is now the leading producer of *M. arvensis* menthol, mostly due to the development and integration of an annual mint cropping system with existing food production systems, an efficient distilling infrastructure and the development of high-yielding, disease-resistant cultivars (4, 27, 28, 40). A prior study investigated the impacts of powdery mildew (*Erysiphe cichoracearum*), rust (*Puccinia menthae*) and leaf spot (*Alternaria alternata*) on *M. arvensis* cultivars and genotypes (25), however the potential effects of *V. dahliae* infection of *M. arvensis* are not known. Another *Mentha* species, *M. longifolia* (L.) L., is a wild relative of cultivated mint with a wide geographic range and a relatively high degree of intraspecific variation (42, 47). Verticillium wilt resistance-like sequences, similar to the tomato *Ve* gene effective against *V. dahliae* race 1 (26, 38), were previously identified in *M. longifolia*

using degenerate polymerase chain reaction primers and differences in Verticillium wilt response were demonstrated in USDA accessions of *M. longifolia* (46, 47). The objectives of this greenhouse study were to: (i) determine the aggressiveness of *V. dahliae* isolates from various hosts and VCGs on *M.* x *piperita* "Black Mitcham" and *M. spicata*; (ii) evaluate the aggressiveness of the same *V. dahliae* isolates on *M. arvensis* cultivars "Paraguayan" and "Shivalik", *M. longifolia* accessions CMEN 584 and CMEN 585 (previously described as susceptible and resistant to Verticillium wilt, respectively), a single progeny from a cross of the two *M. longifolia* accessions (F_1) and a single progeny from an F_1 self-cross (F_2); and (iii) measure the progression of Verticillium wilt symptoms and its effects on stem colonization and yield in the above mint genotypes over successive croppings.

MATERIALS AND METHODS

Plant materials and *V. dahliae* isolates. Isolates of *V. dahliae* used in this study were obtained from peppermint and other hosts in Washington State (Table 1). The identities of isolates were confirmed as *V. dahliae* using polymerase chain reaction and species-specific primers based on the *β*-tubulin 2 gene as described by Atallah et al. (2) Disease assays on *M. arvensis* cultivars were conducted independently of *M. longifolia* assays in separate greenhouse trials and both experiments were repeated once. The first experiment consisted of two Mint Industry Research Council lines of *M. arvensis,* "Paraguayan" and "Shivalik", while the second experiment consisted of *M. longifolia* USDA accessions CMEN584 (PI 557769) and CMEN585 (PI 557767), a single progeny from a CMEN584 X CMEN585 cross (designated "F_1") and a single

progeny from an F_1 self-cross (designated "F_2"). *Mentha arvensis* cultivars were obtained from Summit Laboratories, Inc. (Fort Collins, CO) and *M. longifolia* genotypes were obtained from Kelly Vining (University of New Hampshire, Durham, NH). All experiments consisted of four repetitions and included *M.* x *piperita* "Black Mitcham" and *M. x spicata* as susceptible and resistant standards, respectively. Mint plants were vegetatively propagated by treating 7-10 cm apical cuttings with Root-Tone (0.20% 1-Naphthaleneacetamide and 4.04% tetramethylthiuram-disulfide; Black Leaf Products, Louisville, KY) and rooted in flats filled with Sunshine LC1 peat-based media (SunGro, Bellevue, WA) for four to six weeks.

Conidial suspensions of each *V. dahliae* isolate were prepared by inoculating 125 ml of Czapeks-Dox broth (MP Biomedicals, Solon, OH) with plugs taken from single-spore isolates grown on potato dextrose agar (PDA). Liquid cultures were incubated on a 150 rpm shaker at 22-23° C in the dark for 5-7 days. Conidia were strained through four layers of cheesecloth to remove mycelia. Conidia concentrations were quantified with a hemacytometer and adjusted to 1×10^6 conidia/ml by adding sterile distilled water (sdH_2O). Rooted cuttings were uplifted and potting media gently rinsed from the roots prior to inoculation. Rooted cuttings were soaked for five minutes in 100 ml of conidial suspension, with control treatments consisting of a five minute soak in sdH_2O. Plants were transplanted into 10 cm square pots (J. M. McConkey & Co., Inc., Puyallup, WA,) filled with Sunshine LC1 media and arranged in a randomized complete block design. Natural light was supplemented to achieve a photoperiod of at least 15 hours when necessary.

Disease severity, stem assays and yield measurements. Verticillium wilt symptoms were assessed approximately four weeks postinoculation (p.i.) and weekly thereafter using the following disease severity index (DSI): 0 = no visible symptoms, 1 = mild chlorosis <10% of plant, 2 = distinct chlorosis 10-20% of plant, 3 = asymmetrical apical growth, chlorosis 20-40% of plant and/or stunting (<80% height of control plants), 4 = chlorosis ≥40% of plant and/or severe stunting (<60% height of control plants), 5 = necrosis ≥40% of plant and 6 = dead/nearly dead plant. At eight weeks p.i. plants were cut at 1-2 cm above the soil line and allowed to regrow for an additional eight weeks (16 weeks p.i.), at which point disease ratings were recorded and plants cut back again. An exception to this was the first *M. arvensis* trial, during which the plants entered a six week dormant period following cutback, most likely due to insufficient photoperiod length. In this case symptoms were assessed following eight weeks of regrowth after bud break (22 p.i.). Symptoms were assessed a final time at 24 weeks p.i. (30 weeks p.i. for the first *M. arvensis* trial).

Stem assays were conducted after DSI assessments at 8-, 16- and 24 weeks p.i. (8-, 22- and 30 weeks p.i. for the first *M. arvensis* trial). Two 4 cm basal sections were taken from symptomatic stems of each plant, surface-sterilized in 0.5% NaOCl for 3 min and plated onto Whatman filter paper moistened with sdH_2O. Plates were incubated for 5 days in the dark and checked for *V. dahliae* conidiophores and microsclerotia formation. Identification was performed by microscopic examination of conidiophores, conidia and microsclerotia and verified by subplating onto PDA and when necessary. Following regrowth ratings and stem assays at 16- and 24 weeks p.i. (22 and 30 weeks p.i. in the case of the first *M. arvensis* trial), the remaining

stems were cut to 1-2 cm above the soil line, dried for two weeks and masses recorded. Yields were converted to yield ratios using the following formula:

$$\text{Yield ratio}_{n(x)} = \frac{\text{yield}_{n(x)}}{\text{mean yield}_{control(x)}}$$

where n(x) = individual yield observation of mint species (x), control(x) = the mean yield of mint species (x) control and yield ratios < 1 indicating reduced yield compared to the mean yield of control treatments.

Data analysis. Areas under the disease progress curve (AUDPC) were calculated for ratings taken during the first eight weeks using the following formula:

$$\Sigma_{i}^{n-1} ((Y_{i} + Y_{i+1})/2)(t_{i+1} - t_{i})$$

where Y_i = cumulative disease severity at the i^{th} observation, t_i = time (days p.i.) at the i^{th} observation and n = number of observations. Analysis of variance (ANOVA) was performed using PROC GLM in SAS (version 9.1; SAS Institute, Cary, NC). AUDPC data, regrowth ratings and yield ratios were analyzed separately and comparisons were made between *V. dahliae* isolates and *Mentha* species using Tukey's honest significant difference test.

RESULTS

Aggressiveness of *V. dahliae* isolates. Observable differences in resistance and aggressiveness were evident among mint cultivars and *V. dahliae* isolates approximately three to four weeks

following root-dip inoculation. Inoculations with *V. dahliae* isolates obtained from peppermint often resulted in complete wilt, necrosis and senescence of the original, inoculated cutting by the conclusion of the first eight-week growth period. The exception was *M. x spicata*, which exhibited only mild chlorosis and crescent leaf symptoms and only on rare occasion. Isolates obtained from hosts other than peppermint were able to cause mild symptoms in all mints, including slight to moderate chlorosis and occasional stunting or asymmetric apical growth, however symptoms were not as severe as those caused by peppermint isolates and necrosis or plant mortality due to Verticillium wilt was not observed. Isolates obtained from peppermint produced significantly higher regrowth ratings at 16- and 24 weeks postinoculation than isolates obtained from hosts other than peppermint in all trials (Table 2). The mean disease severity rating of plants inoculated with *V. dahliae* isolates from hosts other than peppermint remained below 2.0 at the end of all three growth periods in each experiment, with symptoms following cutback generally limited to varying levels of chlorosis.

DSI of peppermint isolates. Root-dip inoculations of *M.* x *piperita* "Black Mitcham", *M. arvensis* and *M. longifolia* with *V. dahliae* isolates obtained from peppermint resulted in significantly higher ($P \leq 0.0001$) AUDPC values than inoculations with isolates obtained from other hosts (Table 3). Subsequent comparisons between *Mentha* species were restricted to plants inoculated with *V. dahliae* isolates obtained from peppermint based on their increased aggressiveness relative to isolates obtained from other hosts. *M. arvensis* "Shivalik" exhibited significantly higher AUDPC values than "Paraguayan" and *M.* x *piperita* "Black Mitcham" in both *M. arvensis* trials. AUDPC values of *M. longifolia* CMEN584 were significantly higher than those for CMEN585 in both trials. *M. longifolia* CMEN585 exhibited significantly lower

AUDPC values than *M*. x *piperita* "Black Mitcham" and the other *M. longifolia* genotypes in the second trial. Inoculations of *M. x spicata*, the resistant standard, resulted in significantly lower AUDPC values than all mints in both experiments except the first *M. arvensis* trial, in which it was not significantly different than *M. arvensis* "Paraguayan". AUDPC values for all other mint genotypes were similar to or significantly higher than *M*. x *piperita* "Black Mitcham", the susceptible standard, at eight weeks p.i.

M. x *piperita* "Black Mitcham" exhibited the highest mean DSI ratings at 16- and 24 weeks p.i. in both experiments (Table 2). Both *M. arvensis* cultivars had significantly lower mean DSI ratings than *M*. x *piperita* "Black Mitcham", the susceptible standard, and were not significantly different from *M. spicata*, the resistant standard, at 16- and 24 weeks p.i. in both trials. At 16 weeks p.i., all *M. longifolia* genotypes had significantly higher mean DSI ratings than *M. spicata* with the exception of F_2 and CMEN585 in the first and second trials, respectively. *M. longifolia* CMEN584 had the highest rating out of all *M. longifolia* genotypes. At 24 weeks p.i., DSI ratings for *M. longifolia* F_1 and F_2 were not significantly different than *M. spicata* in the first trial while *M. longifolia* CMEN585 and F_1 were not significantly different than the resistant standard in the second trial.

There was a notable difference in Verticillium wilt progression between mint species inoculated with *V. dahliae* isolates obtained from peppermint. Mean disease severity ratings for *M*. x *piperita* "Black Mitcham" ranged between 4.0 and 6.0 at the conclusion of all three growth periods (Table 2). Conversely, the average disease severity ratings for *M. x spicata* and *M. arvensis* cultivars remained below 1.8 following the first cutback and asymptomatic stems were

frequently produced from underground rhizomes. Symptoms following cutback were generally limited to various levels of chlorosis and stunting. Recovery following cutback was not as dramatic in *M. longifolia* genotypes compared to *M. arvensis*, however there was a general decrease in mean ratings over time and asymptomatic stems were frequently produced. Early mortality caused by Verticillium wilt only occurred in plants inoculated with *V. dahliae* isolates from peppermint and differences in plant survival were observed between *Mentha* species inoculated with peppermint isolates (Table 4).

Yield ratio and pathogen isolation from aboveground stems. Yield ratios at 16 weeks p.i. were significantly lower in plants inoculated with mint isolates in both experiments (Figures 1 and 2). Mean yield ratios were lowest for *M*. x *piperita* "Black Mitcham" in both *M. arvensis* trials and the difference was significant in the first trial. Yield ratios for *M. arvensis* cultivars were not significantly different than *M. spicata* in the first trial, but yield ratios for "Shivalik" were significantly lower than *M. spicata* and "Paraguayan" in the second trial. *M. spicata* plants inoculated with *V. dahliae* isolates obtained from peppermint resulted in significantly higher mean yield ratios than *M.* x *piperita* "Black Mitcham" peppermint and *M. longifolia* CMEN584 in both trials. At 24 weeks p.i., yield ratios were significantly lower in plants inoculated with *V. dahliae* isolates obtained from peppermint in both *M. longifolia* trials and one *M. arvensis* trial (Figures 3 and 4). Yield ratios for *M. x spicata* were significantly higher than *M.* x *piperita* "Black Mitcham" in both experiments. Yield ratios for "Paraguayan" were significantly higher than *M*. x *piperita* "Black Mitcham" in both *M. arvensis* trials, while "Shivalik" was not significantly different than *M*. x *piperita* "Black Mitcham". Yield ratios for F_1 and F_2 were not significantly different than *M. spicata*, which exhibited the highest yield ratio at 24 weeks in

both trials. Assays of aboveground stems resulted in the isolation of the pathogen from all mint genotypes inoculated with *V. dahliae* isolates obtained from peppermint, with isolation frequencies between 8 and 96% (Table 5). Isolates of *V. dahliae* obtained from other hosts were isolated from aboveground stems at low frequencies and were not recovered at all from any *M. longifolia* genotypes or *M. arvensis* "Shivalik".

DISCUSSION

Previous studies demonstrated variation in pathogen-host interactions among *V. dahliae* isolates and the presence of host-adapted populations capable of causing more severe symptoms on certain hosts, with some isolates exhibiting cross-pathogenicity to other host species (6, 37). Correlations between pathogenicity and VCG have been found to exist in some cases (36) and most *V. dahliae* isolates collected from peppermint belong to VCG 2B and exhibit increased aggressiveness on peppermint (11). In this study, root-dip inoculations of *M.* x *piperita* "Black Mitcham", *M. arvensis* and *M. longifolia* with *V. dahliae* isolates from peppermint resulted in significantly higher disease severity than isolates obtained from other hosts, including a VCG 2B isolate collected from spinach seed. These results support previous studies suggesting that *V. dahliae* isolates collected from mint are host-adapted (6, 11, 15) and indicate that knowledge of both host origin and VCG are important in predicting the aggressiveness of a certain isolate on a particular host species. The presence of *V. dahliae* populations of different VCGs, each possibly containing subpopulations with varying degrees of aggressiveness and cross-pathogenicity, may complicate efforts to manage and quantify the pathogen in field soils.

In addition to variation in isolate aggressiveness, differences in disease severity among *Mentha* species were evident. Disease severity, plant mortality and pathogen isolation frequencies were consistently higher and yield ratio consistently lower in the susceptible standard, *M*. x *piperita* "Black Mitcham", compared to the resistant standard *M. spicata*. Although root-dip inoculations of *M. arvensis* and *M. longifolia* genotypes with peppermint isolates of *V. dahliae* resulted in AUDPC values similar to or higher than *M*. x *piperita* "Black Mitcham" at 8 weeks p.i., *M. arvensis* and *M. longifolia* plants exhibited a range of symptoms following cutback, ranging from 0 (no symptoms) to 6 (dead plant) while 96% of *M*. x *piperita* "Black Mitcham" plants exhibited moderate to severe symptoms (DSI $\geq$ 4) at the completion of all trials. The reasons for the observed variation in disease severity and plant mortality among *M. arvensis* and *M. longifolia* clones are not known, however, the rhizomatous and stoloniferous nature of the mint plants tested may have allowed the plants to recover from initial infection or provided a source of variation. Results in the presence of soilborne inoculum may differ, however, since multiple opportunities for new infection events would exist over space and time.

Yields were significantly reduced only in *M*. x *piperita* "Black Mitcham" plants inoculated with *V. dahliae* isolates obtained from peppermint, most likely due to its high susceptibility and rate of mortality compared to the resistant standard *M. spicata*. Both *M. arvensis* cultivars exhibited relatively high yield ratios and low mortality rates compared to the standards. Among *M. longifolia* genotypes, *M. longifolia* CMEN584 generally exhibited the lowest yield ratios and *M. longifolia* CMEN585 the highest. Differences in frequencies of pathogen isolations from stems were also evident among cultivars. The pathogen was isolated from nearly 100% of stems collected from the susceptible standard *M*. x *piperita* "Black

Mitcham" and less than 10% of stems collected from the resistant standard *M. x spicata*. Isolations were relatively low (< 15%) in both *M. arvensis* cultivars tested as well as *M. longifolia* CMEN585. Among *M. longifolia* genotypes, the pathogen was isolated in highest numbers from *M. longifolia* CMEN584 and in lowest numbers from *M. longifolia* CMEN585. Results from this study are consistent with a previous assessment by Vining et al. (49) identifying CMEN584 and CMEN585 as relatively susceptible and resistant USDA *M. longifolia* accessions, respectively. Interestingly, although CMEN585 consistently had lower DSI ratings and pathogen isolation rates and higher yield ratios than *M. longifolia* CMEN584, it also exhibited one of the highest mortality rates among all mints evaluated.

Another study by Vining et al. (46) identified nucleotide sequences in *M. longifolia* CMEN585 sharing a 57% predicted amino acid identity with the dominant *Ve* gene, which confers *V. dahliae* race 1 resistance in tomato. Although the genotype tested from the F_1 generation of a CMEN584 x CMEN585 cross had a lower mortality rate than CMEN585, it did not perform as well as CMEN585 with regards to disease severity, yield ratio or pathogen isolation frequency from aboveground stems. Single-gene, race-specific resistance to *V. dahliae* has only been identified in tomato and lettuce (38, 43) and results from experiments using peppermint plants transformed with *Ve*-like sequences cloned from *M. longifolia* CMEN585 suggest other genes may be involved (12).

Previous studies on Verticillium wilt resistance in lettuce, potato and other crops have suggested that host suppression of cortex colonization, stele penetration and xylem invasion are important components of resistant phenotypes (14, 18, 21, 44). Data from this study are

consistent with prior studies demonstrating differences in pathogen isolation from stems of resistant and susceptible mint species (7, 19), suggesting that resistance in mint may involve the prevention of vascular and/or aboveground colonization by the pathogen. This may be especially important in mint considering the perennial, rhizomatous and stoloniferous nature of the crop, which may allow the plant to escape from pathogen foci found in soil. The restriction of aboveground colonization by *V. dahliae* may also be a significant component of Verticillium wilt management in perennial mint cropping systems since repeated harvests present multiple opportunities for infested debris to become incorporated into field soils.

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LITERATURE CITED

1. Agrios, G. N. 1997. Plant Pathology. Academic Press, San Diego.
2. Atallah, Z. K., Bae, J., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology 97:865-872.
3. Bae, J., Atallah, Z. K., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Colonization dynamics and spatial progression of *Verticillium dahliae* in individual stems

of two potato cultivars with differing responses to potato early dying. Plant Disease 91:1137-1141.

4. Bahl, J. R., Bansal, R. P., and Kumar, S. 2002. Direct planting of the short-duration summer crop using cold stored suckers of menthol mint *Mentha arvensis.* Journal of Horticultural Science & Biotechnology 77:217-219.

5. Berry, S. Z., and Thomas, C. A. 1961. Influence of soil temperature, isolates, and method of inoculation on resistance of mint to Verticillium wilt. Phytopathology 51:169-174.

6. Bhat, R. G., and Subbarao, K. V. 1999. Host range specificity in *Verticillium dahliae.* Phytopathology 89:1218-1225.

7. Brandt, W. H., Lacy, M. L., and Horner, C. E. 1984. Distribution of *Verticillium* in stems of resistant and susceptible species of mint. Phytopathology 74:587-591.

8. Chang, R. J., and Eastburn, D. M. 1994. Host range of *Verticillium dahliae* from horseradish and pathogenicity of strains. Plant Disease 78:503-506.

9. Chen, W. 1994. Vegetative compatibility groups of *Verticillium dahliae* from ornamental woody plants. Phytopathology 84:214-219.

10. Clark, G. S. 2007. Aroma chemical profile: menthol. Perfumer and Flavorist 32:38-47.

11. Douhan, L. I., and Johnson, D. A. 2001. Vegetative compatibility and pathogenicity of *Verticillium dahliae* from spearmint and peppermint. Plant Disease 85:297-302.

12. Dung, J. 2009. Evaluation of Verticillium Wilt resistance in transgenic *Mentha* x *piperita* "Black Mitcham" expressing *Ve*-like sequences from *M. longifolia*. M.S. thesis. Washington State University, Pullman, WA.

13. Faulkner, L. R., and Skotland, C. B. 1965. Interactions of *Verticillium dahliae* and *Pratylenchus minyus* in Verticillium wilt of peppermint. Phytopathology 55:583-586.

14. Fradin, E. F., and Thomma, B. P. H. J. 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7:71-86.

15. Green, R. J. 1951. Studies on the host range of the *Verticillium* that causes wilt of *Mentha piperita* L. Science 113:207-208.

16. Green, R. J. 1967. Control of Verticillium wilt of peppermint by crop rotation sequences. Plant Dis Rep 51:441-453.

17. Green, R. J. 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.

18. Griffiths, D. A. 1971. The development of lignitubers in roots after infection by *Verticillium dahliae* Kleb. Can J Microbiol 17:441-444.

19. Horner, C. E. 1954. Pathogenicity of *Verticillium* isolates to peppermint. Phytopathology 44:239-242.

20. Horner, C. E., and Melouk, H. A. 1977. Screening, selection, and evaluation of irradiation-induced mutants of spearmint for resistance to Verticillium wilt. Int. Atomic Energy Agency - SM:214/20:253-262.

21. Huisman, O. C. 1988. Seasonal colonization of roots of field-grown cotton by *Verticillium dahliae* and *V. tricorpus*. Phytopathology 78:708-716.

22. Joaquim, T. R., and Rowe, R. C. 1990. Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology 80:1160-1166.

23. Johnson, D. A., and Cummings, T. F. 2000. Evaluation of mint mutants, hybrids, and fertile clones for resistance to *Verticillium dahliae.* Plant Disease 84:235-238.

24. Johnson, D. A., and Santo, G. S. 2001. Development of wilt in mint in response to infection by two pathotypes of *Verticillium dahliae* and co-infection by *Pratylenchus penetrans.* Plant Disease 85:1189-1192.

25. Kalra, A., Singh, H. B., Patra, N. K., Pandey, R., Shukla, R. S., and Kumar, S. 2001. The effect of leaf spot, rust and powdery mildew on yield components of nine Japanese mint (*Mentha arvensis*) genotypes. The Journal of Horticultural Science & Biotechnology 76:546-548.

26. Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R., and Prufer, D. 2001. Tomato *Ve* disease resistance genes encode cell surface-like receptors. Proceedings of the National Academy of Sciences of the United States of America 98:6511-6515.

27. Kumar, S., Bahl, J. R., Bansal, R. P., Gupta, A. K., Singh, V., and Sharma, S. 2002. High economic returns from companion and relay cropping of bread wheat and menthol mint in the winter-summer season in north Indian plains. Industrial Crops and Products 15:103-114.

28. Kumar, S., Bahl, J. R., Shukla, P., Singh, A., Ram, G., Bansal, R. P., and Sharma, S. 1999. Screening of genotypes of menthol mint *Mentha arvensis* for high yields of

herbage and essential oil under late cropping conditions of the sub-tropical Indo-Gangetic plains. The Journal of Horticultural Science & Biotechnology 74:680-684.

29. Lacy, M. L., and Horner, C. E. 1966. Behavior of *Verticillium dahliae* in the rhizosphere and on roots of plants susceptible, resistant, and immune to wilt. Phytopathology 56:427-430.

30. Lawrence, B. M. 2007. The composition of commercially important mints. Pages 217-323 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton, FL.

31. Menzies, J. D., and Griebel, G. E. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.

32. Mol, L. 1995. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. European Journal of Plant Pathology 101:679-685.

33. Morrell, A., Schreiber, A., and Lundy, R. 1997. Pests, pest control, and fertilizer use in Washington mint. Washington State University Cooperative Extension, MISC0188 Pullman, WA.

34. Murray, M. J., and Todd, W. A. 1972. Registration of Todd's Mitcham peppermint. Crop Science 12:128.

35. Niu, X., Li, X., Veronese, P., Bressan, R. A., Weller, S. C., and Hasegawa, P. M. 2000. Factors affecting *Agrobacterium tumefaciens*-mediated transformation of peppermint. Plant Cell Reports 19:304-310.

36. Omer, M. A., Johnson, D. A., and Rowe, R. C. 2000. Recovery of *Verticillium dahliae* from North American certified seed potatoes and characterization of strains by vegetative compatibility and aggressiveness. American Journal of Potato Research 77:325-331.

37. Qin, Q. M., Vallad, G. E., Wu, B. M., and Subbarao, K. V. 2006. Phylogenetic analyses of phytopathogenic isolates of *Verticillium* spp. Phytopathology 96:582-592.

38. Schaible, L., Cannon, O. S., and Waddoups, B. 1951. Inheritance of resistance to Verticillium wilt in a tomato cross. Phytopathology 41:986-990.

39. Schnathorst, W. C. 1981. Life cycle and epidemiology of *Verticillium*. Pages 81-111 in: Fungal Wilt Diseases of Plants, M. E. Mace, A. A. Bell and C. H. Beckman, eds. Academic, New York.

40. Singh, A. K., Srivastava, R. K., Kalra, A., Bansal, R. P., Tomar, V. K. S., Bahl, J. R., and Kumar, S. 2003. New practices in the cultivation of the mint, *Mentha arvensis* in the Indo-Gangetic plains. Experimental Agriculture 39:199-207.

41. Todd, W. A., Green, R. J., and Horner, C. E. 1977. Registration of Murray Mitcham peppermint. Crop Science 17:188.

42. Tucker, A. O., and Naczi, R. F. C. 2007. *Mentha*: An overview of its classification and relationships. Pages 1-39 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton, FL.

43. Vallad, G. E., Qin, Q. M., Grube, R., Hayes, R. J., and Subbarao, K. V. 2006. Characterization of Race-Specific Interactions Among Isolates of Verticillium dahliae Pathogenic on Lettuce. Phytopathology 96:1380-1387.

44. Vallad, G. E., and Subbarao, K. V. 2008. Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. Phytopathology 98:871-885.

45. Veronese, P., Li, X., Niu, X., Weller, S. C., Bressan, R. A., and Hasegawa, P. M. 2001. Bioengineering mint crop improvement. Plant Cell, Tissue and Organ Culture 64:133-144.

46. Vining, K. J., Zhang, Q., Smith, C. A., and Davis, T. M. 2007. Identification of resistance gene analogs and Verticillium wilt resistance-like sequences in *Mentha longifolia*. Journal of the American Society for Horticultural Science 132:541-550.

47. Vining, K. J., Zhang, Q., Tucker, A. O., Smith, C., and Davis, T. M. 2005. *Mentha longifolia* (L.) L.: a model species for mint genetic research. HortScience 40:1225-1229.

48. Wildung, M. R., and Croteau, R. B. 2005. Genetic engineering of peppermint for improved essential oil composition and yield. Transgenic Research 14:365-372.

TABLE 1. List of *Verticillium dahliae* isolates used in the study along with their vegetative compatibility group designation and host origin.

Isolate[a]	VCG	Host origin
109	2B	Peppermint
111	2B	Peppermint
695	2B	Spinach seed
601	4A	Cherry
653[b]	4A	Potato
240	4B	Potato

[a] All isolates obtained from Dennis Johnson (Department of Plant Pathology, Washington State University, Pullman, WA) except isolate 695 obtained from Lindsey DuToit (Northwestern Washington Research and Extension Center, Washington State University, Mount Vernon, WA).
[b] Isolate Vd653 not used in *M. arvensis* experiment.

TABLE 2. Mean disease severity index ratings of different mint genotypes at 8-, 16- and 24 weeks following root-dip inoculation with *V. dahliae* isolates from mint and other hosts.[a]

	Mint isolates[b]			Isolates from other hosts[c]		
Mint selection	**8wk**	**16wk**	**24wk**	**8wk**	**16wk**	**24wk**
***M.* x *piperita* "Black Mitcham"**	4.7	5.2	5.1	0.9	0.4	0.3
M. *arvensis* "Paraguayan"	3.8	1.5	1.7	1.4	0.5	1.0
***M. arvensis* "Shivalik"**	5.6	1.3	1.8	0.7	0.4	0.6
***M. longifolia* CMEN584[d]**	5.5	4.4	3.9	0.9	0.1	0.6
***M. longifolia* CMEN585[d]**	4.0	3.3	3.3	0.8	0.3	0.5
584 x 585 (F_1)	5.6	3.7	2.6	0.8	0.4	0.3
F_1 x F_1 self-cross (F_2)	5.5	3.6	3.0	1.0	0.3	0.3
***M. spicata* (Native spearmint)**	2.0	0.6	0.8	0.7	0.4	0.3

[a] Native spearmint (*M. spicata*) and peppermint (*M.* x *piperita* "Black Mitcham") were used as resistant and susceptible standards, respectively. Data combined from all experiments.
[b] Mean ratings combined for *V. dahliae* isolates Vd109 and Vd111 (VCG 2B from peppermint).
[c] Mean ratings combined for *V. dahliae* isolates Vd695 (VCG 2B from spinach), Vd601 (VCG 4A from cherry), Vd653 (VCG 4A from potato) and Vd240 (VCG 4B from potato). Isolate Vd653 was not used in the *M. arvensis* experiments.
[d] *M. longifolia* USDA accessions CMEN584 (PI 557769) and CMEN585 (PI 557767).

TABLE 3. Mean AUDPC for *M. arvensis* cultivars "Paraguayan" and "Shivalik" and *M. longifolia* accessions CMEN584, CMEN585, a CMEN584 x CMEN585 cross (F_1) and an F_1 x F_1 self-cross (F_2)[a].

	VCG 2B			VCG 4A		VCG 4B
	Peppermint		Spinach	Cherry	Potato	Potato
Mint selection	**Vd109**	**Vd111**	**Vd695**	**Vd601**	**Vd653[b]**	**Vd240**
***M.* x *piperita* "Black Mitcham"**	84	77	14	11	17	16
***M. arvensis* "Paraguayan"**	62	76	20	23	NT	25
***M. arvensis* "Shivalik"**	98	99	14	9	NT	19
***M. longifolia* CMEN584[c]**	111	106	13	17	14	12
***M. longifolia* CMEN585[c]**	74	76	13	14	13	11
584 x 585 (F_1)	109	100	9	12	9	17
F_1 x F_1 self cross (F_2)	91	100	12	12	9	19
***M. spicata* (Native spearmint)**	36	36	16	8	13	11

[a] Plants were inoculated with *V. dahliae* isolates from different hosts and VCGs. Native spearmint (*M. spicata*) and peppermint (*M.* x *piperita* "Black Mitcham") were used as resistant and susceptible standards, respectively. Data combined from all experiments.
[b] Isolate Vd653 not included in *M. arvensis* experiments.
[c] *M. longifolia* USDA accessions CMEN584 (PI 557769) and CMEN585 (PI 557767).

TABLE 4. Mortality rates of mint genotypes inoculated with *V. dahliae* isolates from peppermint[a].

Mint selections	Mortality (%)
***Mentha* x *piperita* "Black Mitcham"**	34
***M. arvensis* "Paraguayan"**	6
***M. arvensis* "Shivalik"**	6
***M. longifolia* CMEN 584[b]**	25
***M. longifolia* CMEN 585[b]**	44
CMEN 584 x CMEN 585 (F_1)	19
F_1 x F_1 (F_2)	44
***M. spicata* (Native spearmint)**	0

[a] Data combined for *V. dahliae* isolates Vd109 and Vd111(both isolates were VCG 2B from peppermint) from all experiments.
[b] *M. longifolia* USDA accessions CMEN584 (PI 557769) and CMEN585 (PI 557767)

TABLE 5. Incidence of *V. dahliae* recovery from aboveground stems.[a]

	Pathogen Isolation (%)	
	Mint isolates[b]	**Isolates from other hosts[c]**
***Mentha* x *piperita* "Black Mitcham"**	96.8	0.9[e]
***M. arvensis* "Paraguayan"**	14.4	0.7[e]
***M. arvensis* "Shivalik"**	13.3	0
***M. longifolia* CMEN 584[b]**	72.5	0
***M. longifolia* CMEN 585[b]**	14.3	0
CMEN 584 x CMEN 585 (F_1)	48.8	0
F_1 x F_1 (F_2)	40.3	0
***M. spicata* (Native spearmint)**	8.3	0.3[e]

[a] Native spearmint (*M. spicata*) and peppermint (*M.* x *piperita* "Black Mitcham") were used as resistant and susceptible standards, respectively. Data combined from all experiments.
[b] Mean ratings combined for *V. dahliae* isolates Vd109 and Vd111 (VCG 2B from peppermint).
[c] Mean ratings combined for *V. dahliae* isolates Vd695 (VCG 2B from spinach), Vd601 (VCG 4A from cherry), Vd653 (VCG 4A from potato) and Vd240 (VCG 4B from potato). Isolate Vd653 was not used in the *M. arvensis* experiments.
[d] *M. longifolia* USDA accessions CMEN584 (PI 557769) and CMEN585 (PI 557767)
[e] Isolate Vd695 was recovered once each from "Black Mitcham" and "Paraguayan and isolate Vd240 was recovered once each from "Black Mitcham" and *M. spicata*.

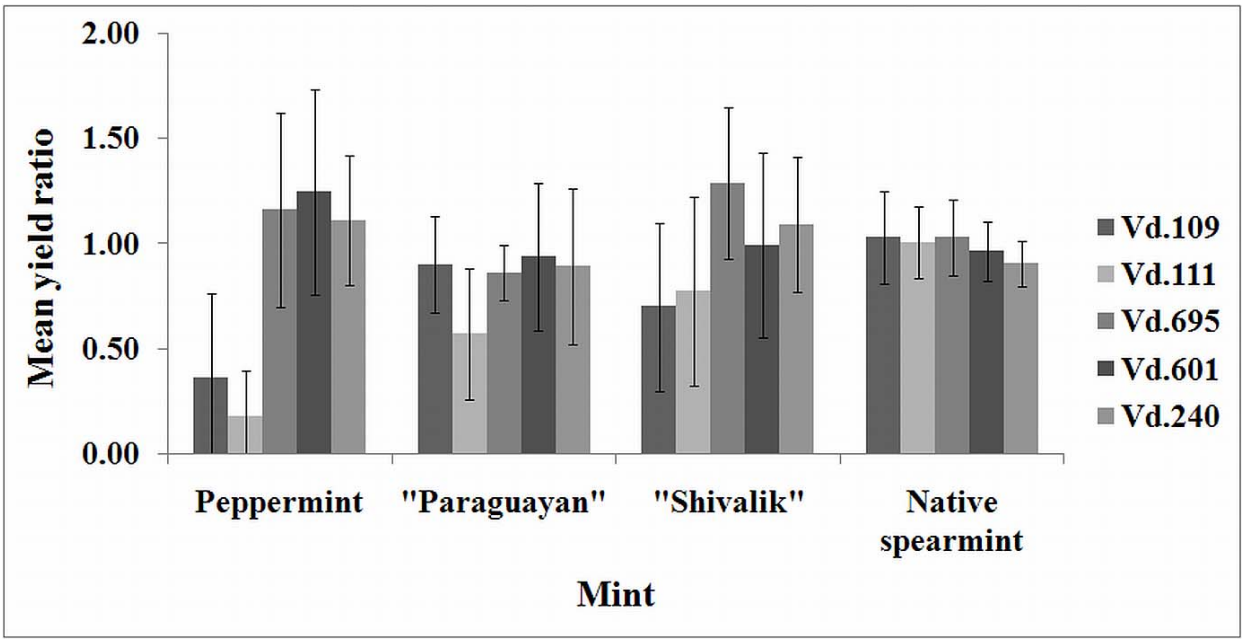

Fig 1. Combined mean yield ratios from both *M. arvensis* experiments following cutback and 8 weeks of regrowth. Isolates Vd.109 and Vd.111 were from peppermint (VCG 2B), Vd.695 was from spinach seed (VCG 2B), Vd.601 was from cherry (VCG 4A) and Vd.240 was from potato (VCG 4B). Error bars indicate standard deviation.

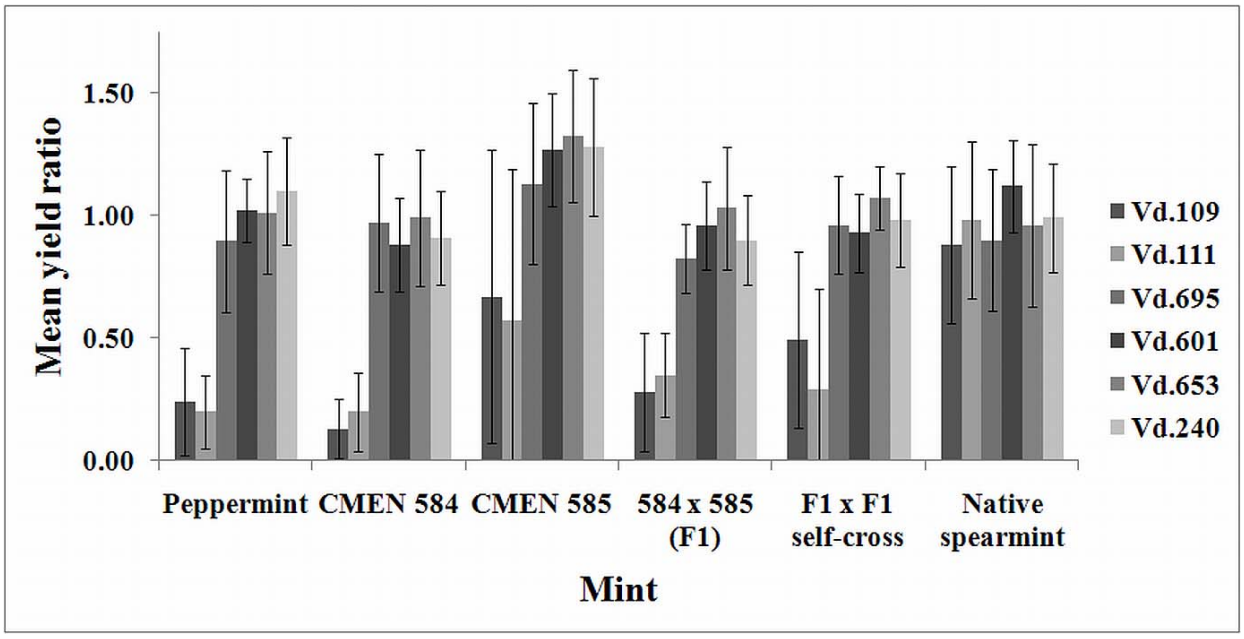

Fig 2. Combined mean yield ratios from both *M. longifolia* experiments following cutback and 8 weeks of regrowth. Isolates Vd.109 and Vd.111 were from peppermint (VCG 2B), Vd.695 was from spinach seed (VCG 2B), Vd.601 was from cherry (VCG 4A) and Vd.653 and Vd.240 were from potato (VCG 4A and VCG 4B, respectively). Error bars indicate standard deviation.

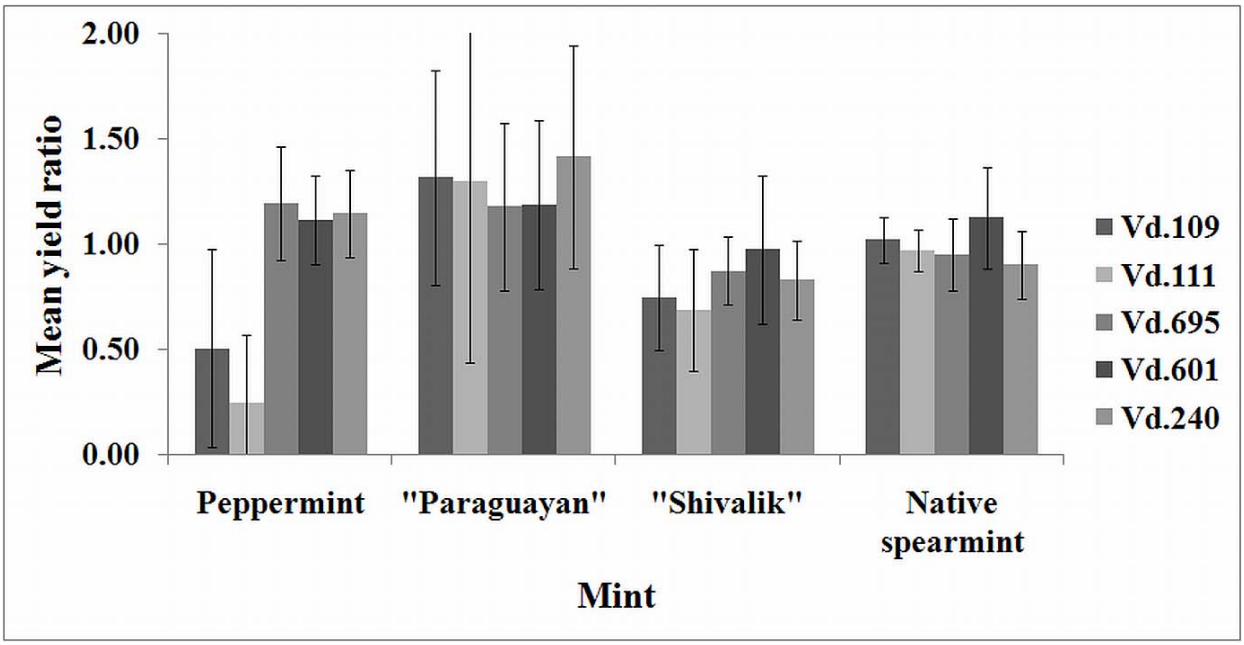

Fig 3. Combined mean yield ratios from both *M. arvensis* experiments following two successions of cutback and regrowth (24 weeks p.i.). Isolates Vd.109 and Vd.111 were from peppermint (VCG 2B), Vd.695 was from spinach seed (VCG 2B), Vd.601 was from cherry (VCG 4A) and Vd.240 was from potato (VCG 4B). Error bars indicate standard deviation.

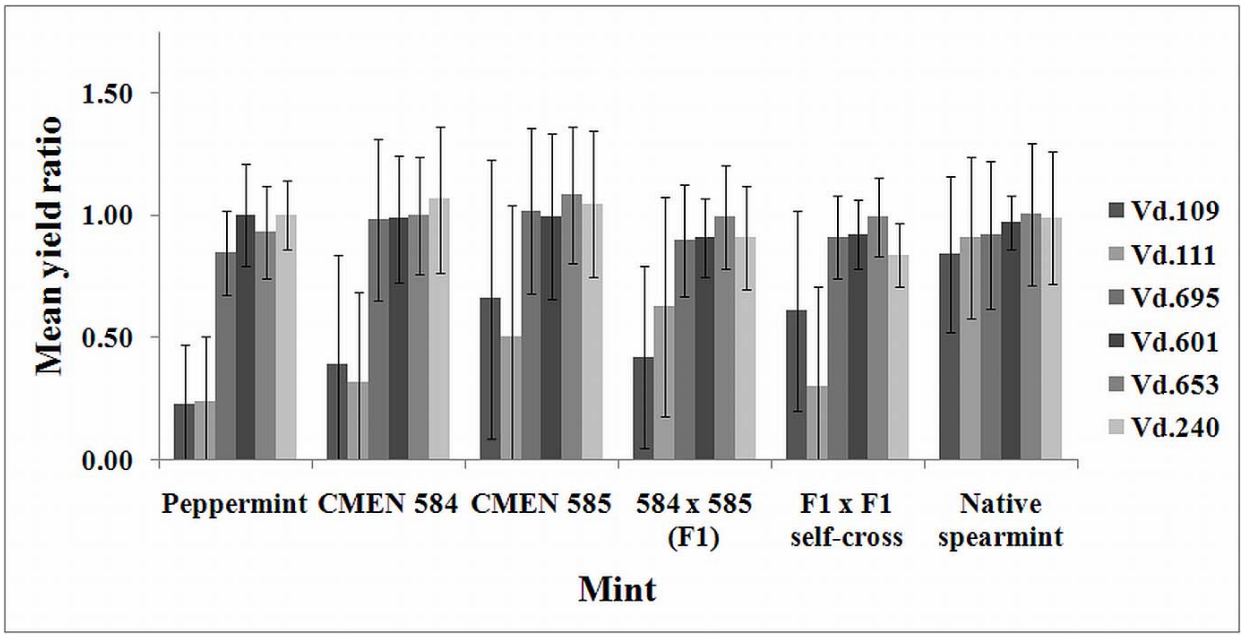

Fig 4. Combined mean yield ratios from both *M. longifolia* experiments following two successions of cutback and regrowth (24 weeks p.i.). Isolates Vd.109 and Vd.111 were from peppermint (VCG 2B), Vd.695 was from spinach seed (VCG 2B), Vd.601 was from cherry (VCG 4A) and Vd.240 was from potato (VCG 4B). Error bars indicate standard deviation.

CHAPTER 4

Evaluation of Verticillium Wilt resistance in transgenic *Mentha* x *piperita* "Black Mitcham" containing *Ve*-like sequences from *M. longifolia*

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ABSTRACT

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is the primary fungal disease affecting mint (*Mentha*) production in the Pacific Northwest. The use of resistant cultivars is considered an important facet of Verticillium wilt management and the development of *Agrobacterium*-mediated transformation provides opportunities to improve existing mint cultivars, some of which are sterile hybrids. A total of 67 transgenic peppermint (*M*. x *piperita* "Black Mitcham") plants containing *M. longifolia* sequences similar to the Verticillium resistance (*Ve*) gene found in tomato were inoculated with a conidial suspension of *V. dahliae* and evaluated for Verticillium wilt resistance in the greenhouse. Sixty-three transformants exhibited AUDPC values similar to wild-type *M*. x *piperita* "Black Mitcham" controls at 8 weeks postinoculation (p.i.), while four transformants exhibited significantly higher ($P < 0.05$) AUDPC values than controls. Significant differences in disease severity were not found between transformants and wild-type controls at 16 weeks p.i. following cutback and 8 weeks of regrowth

($P > 0.20$). Transformation of *M.* x *piperita* "Black Mitcham" with the *Ve*-like sequences found in *M. longifolia* did not provide detectable levels of Verticillium wilt resistance, however, genetic transformation remains a promising approach to develop Verticillium wilt-resistant mint cultivars.

INTRODUCTION

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is an important fungal disease affecting mint (*Mentha* L.) production in the United States. Symptoms of Verticillium wilt in mint can include chlorosis, anthocyanescence, bronzing and/or curling of the apical leaves, stunting, wilt, necrosis and plant mortality (13). Losses due to stand reduction and decreased oil production can worsen over time in a perennial mint crop (18). The disease is difficult to control due to its wide host range (1, 5, 33) and persistence in field soils (12, 24).

Despite the diverse host range of *V. dahliae*, a degree of host specificity has been documented and some isolates are highly aggressiveness on certain hosts (3, 4). Isolates of *V. dahliae* from mint are collectively more aggressive on mint and can interact synergistically with the root lesion nematode (*Pratylenchus penetrans*) (9, 17), indicating the presence of a mint-adapted pathotype (10, 17). In addition, *V. dahliae* isolates can be separated into vegetative compatibility groups (VCGs) based on their ability to undergo hyphal anastamosis with other isolates (5, 15) and most isolates collected from mint belong to VCG 2B (7).

Verticillium wilt is managed primarily through the use of disease-free planting stock, rotations with resistant or nonhost crops and preplant fumigation (11, 25), however fumigant treatment is costly and their use may soon be restricted. Crop rotation is of only limited benefit, largely due to the ability of *V. dahliae* to colonize and survive on numerous host and nonhost plants and survive for years as microsclerotia in soil (12, 24). The use of resistant cultivars is considered an effective approach to manage Verticillium wilt, however two of the most important cultivars grown in the United States, peppermint (*M.* x *piperita* L.) and Scotch spearmint (*M*. x *gracilis* Sole), are quite susceptible. Another commercially grown species, native spearmint (*M*. *spicata* L.), is more resistant (2), but peppermint and Scotch spearmint are sterile hybrids and cultivar improvement through conventional breeding is not possible (35). Mutants generated by irradiation treatment of mint rhizomes has produced mixed results (14, 16, 26, 34) and other desirable crop characteristics such as plant vigor, oil composition and yield must be considered during cultivar development (22).

The genus *Mentha* is comprised of at least 18 species, 11 hybrids and numerous varieties and subspecies (35), all of which are potential sources of Verticillium wilt resistance. *M. longifolia* (L.) L. is a wild relative of cultivated mint with a wide geographic range and a relatively high degree of intraspecific variation (35, 39). Variability in Verticillium wilt resistance is documented in *M. longifolia* and Verticillium wilt resistance-like sequences, similar to the *Ve* gene found in tomato (*Solanum lycopersicum* L.) (20, 32), have been cloned and sequenced in several *M. longifolia* accessions (38, 39). The tomato *Ve* gene has provided fairly durable resistance against race 1 isolates of *V*. *dahliae* and is found in most commercial tomato cultivars grown today. Recent research demonstrated that the *Ve* locus in tomato actually consist

of two closely-linked genes, *Ve*1 and *Ve*2, which encode for cell surface glycoproteins (20, 30). Both genes independently convey resistance to *V. albo-atrum* race 1 when transferred to potato (*S. tuberosum* L.) (20). The development of *Agrobacterium tumefaciens*-mediated transformation of peppermint provides an effective means to introduce disease resistance genes into cultivated mints while at the same time conserving oil composition and other desirable characteristics (27, 36, 40). The objective of this study was to evaluate Verticillium wilt response in transgenic peppermint plants containing *Ve*-like sequences cloned from *M. longifolia* (38).

MATERIALS AND METHODS

Vector construct and *Agrobacterium*-mediated transformation. Transgenic peppermint plants (*M.* x *piperita* "Black Mitcham") containing *mVe*1 and *mVe*2 sequences from *M. longifolia* (38) were generated in the laboratory of Rodney Croteau (Institute of Biological Chemistry, Washington State University, Pullman, WA) using the following procedure: the NOS promoter sequence of plasmid pBI121 was amplified using polymerase chain reaction (PCR) primers which placed a *Bam*HI and a *Pst*I restriction site upstream and downstream, respectively, of the promoter. The NOS fragment from pBI121 was ligated into *Bam*HI and *Pst*I-digested pCambia 1380 to generate plasmid pCNOS. Plasmid pCNOS contained a NOS terminator and hygromycin selection marker coupled with a 35S CaMV promoter. PCR amplification of *M. longifolia mVe*1 and *mVe*2 sequences using primers containing a *Pst*I restriction site was performed and the product ligated into *PstI*-digested pCNOS. Insert orientation was determined

by PCR amplification and inserts were sequenced to ensure that no unintentional mutations occurred during PCR. Confirmed plasmids were electroporated into competent cells of the hypervirulent *Agrobacterium tumefaciens* strain EHA105 and greenhouse-grown "Black Mitcham" peppermint were transformed as described by Niu et al. (28) with the following exceptions: instead of sterile plants, greenhouse tissue was surface-sterilized with 15% household bleach for 15 minutes, tobacco feeder cells were omitted during co-cultivation and coconut milk was not used in the selection media for the initial six weeks in order to reduce bacterial overgrowth. Resulting plantlets were transferred to soil and propagated in the greenhouse.

Verticillium wilt resistance screening. Separate experiments were conducted in the greenhouse on two lots of transformed plants with four replicates of each plant. Both experiments included two nontransformed, wild-type peppermint (*M*. x *piperita* "Black Mitcham") controls, one obtained from the laboratory of R. Croteau (IBC) and one from Dennis Johnson (Department of Plant Pathology, Washington State University, Pullman, WA). Plants were vegetatively propagated by rooting 7-10 cm apical cuttings in Sunshine LC1 peat-based media (SunGro, Bellevue, WA) for four to six weeks.

Liquid cultures of *V. dahliae* isolate Vd109 were prepared by inoculating 125 ml of Czapeks-Dox broth (MP Biomedicals, Solon, OH) with plugs taken from single-spore isolates grown on potato dextrose agar (PDA). Isolate Vd109 (VCG 2B; Grant County, WA) was found to be highly pathogenic to *M*. x *piperita* "Black Mitcham" in previous studies (7, 16). Broth cultures were incubated on a 150 rpm shaker at 22-23° C in the dark for 5-7 days. Conidia were

strained through four layers of cheesecloth to remove mycelia and the six cultures were combined. Conidia concentration was quantified using a hemacytometer and adjusted to 1 x 10^6 conidia/ml with sterile distilled water (sdH_2O). Rooted cuttings were uplifted and potting media gently rinsed from the roots prior to inoculation. Cuttings were soaked for five minutes in 100 ml of conidial suspension, with a control inoculation of each transformant consisting of a five minute soak in sdH_2O. Plants were transplanted into 10 cm square pots (J. M. McConkey & Co., Inc., Puyallup, WA) filled with Sunshine LC1 media and arranged in a randomized complete block design in the greenhouse. Natural light was supplemented to achieve a photoperiod of at least 15 hours when necessary.

Disease severity assessment and stem assays. Verticillium wilt symptoms were assessed approximately four weeks postinoculation (p.i.) and weekly thereafter using the following disease severity index (DSI): 0 = no visible symptoms, 1 = mild chlorosis <10% of plant, 2 = distinct chlorosis 10-20% of plant, 3 = asymmetrical apical growth, chlorosis 20-40% of plant and/or stunting (<80% height of control plants), 4 = chlorosis ≥40% of plant and/or severe stunting (<60% height of control plants), 5 = necrosis ≥40% of plant and 6 = dead/nearly dead plant. At eight weeks p.i. plants were cut to 1-2 cm above the soil line and DSI recorded after eight weeks of regrowth (16 weeks p.i.). Stem assays were conducted after DSI assessments at 8- and 16 weeks to determine the incidence of pathogen colonization in aboveground stems. Two 4 cm basal sections were taken from all replications of 12 randomly selected transformants and both nontransformed controls. Stem sections were surface-sterilized in 0.5% NaOCl for 3 min and plated onto Whatman filter paper moistened with sdH_2O. Stems were incubated for 5

days in the dark and checked for the production *V. dahliae* conidiophores and microsclerotia on stems and filter paper.

Data Analysis. Areas under the disease progress curve (AUDPC) were calculated for DSI ratings taken during the first eight weeks using the following formula:

$$\Sigma_{i}^{n-1} ((Y_{i} + Y_{i+1})/2)(t_{i+1} - t_{i})$$

where Y_i = cumulative disease severity at the i^{th} observation, t_i = time (days p.i.) at the i^{th} observation and n = number of observations. Analysis of variance (ANOVA) was performed using PROC MIXED in SAS (version 9.1; SAS Institute, Cary, NC). AUDPC data and regrowth ratings were analyzed separately and comparisons were made against nontransformed wild-type controls using Dunnett's test.

RESULTS

A total of 67 transformant *M.* x *piperita* "Black Mitcham" plants containing Ve-like sequences were evaluated for resistance against an isolate of *V. dahliae* previously shown to be aggressive on "Black Mitcham" peppermint. Significant differences in AUDPC values were not found between transformants and wild-type controls in the first experiment ($P > 0.16$; Table 1). In the second experiment, four transformants exhibited significantly higher ($P < 0.05$) AUDPC values than nontransformed controls. Significant differences ($P > 0.20$) in ratings following cutback and regrowth were not found at 16 weeks p.i. and the pathogen was isolated in high

frequencies (≥ 88%) from the aboveground stems of all transformant and control treatments at 8- and 16 weeks p.i.

DISCUSSION

Vining et al. (38) identified nucleotide sequences in *M. longifolia* accessions sharing approximately 57% predicted amino acid identity with the dominant *Ve* gene which confers resistance in tomato to *V. dahliae* race 1. The transgenic *M.* x *piperita* "Black Mitcham" plants containing *mVe*1 and *mVe*2 genes did not exhibit increased resistance to Verticillium wilt compared to nontransformed controls in this study. Sequences similar to *mVe*1 and *mVe*2 were found in both resistant and susceptible accessions of *M. longifolia* as well as the susceptible *M.* x *piperita* "Black Mitcham", indicating that it may not be a major factor in the variations in Verticillium wilt response observed in different USDA accessions of *M. longifolia*. Results from inoculations of resistant and susceptible *M. longifolia* accessions and single progeny from their cross and subsequent self-cross also suggest other mechanisms may be involved in the variations in Verticillium wilt response observed in *M. longifolia* (8). Since gene expression was not measured, the possibility exists that expression was insufficient or lacking in the transgenic peppermint plants.

Research investigating the chromosomal location of the *Ve* locus yielded conflicting results, prompting some workers to suggest the possibility that multiple loci are involved (6, 19, 29). Another gene associated with Verticillium wilt resistance, *VET1*, was identified in *Arabidopsis thaliana* (37) and QTLs for resistance to *V. longisporum* were identified in *Brassica*

napus (31). These reports, in addition to the variations in aggressiveness and cross-pathogenicity observed among populations of *V. dahliae,* indicate a variety of genetic mechanisms may be involved in interactions between *V. dahliae* and different hosts. The development of *A. tumefaciens*-mediated transformation offers enormous potential for the improvement of vegetatively propagated crops and was previously used to introduce herbicide resistance (23) and alter oil biosynthesis pathways (21, 40) in mint. Preliminary data by Veronese et al. (36) demonstrated lower disease severity and yield reductions in transgenic peppermint which produced a tobacco PR protein. Genetic transformation remains a promising approach to managing Verticillium wilt in peppermint and the pyramiding of multiple genes, including PR proteins, *R* genes and QTLs, may now be possible given the development of *A. tumefaciens*-mediated transformation of peppermint (40).

ACKNOWLEDGEMENTS

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LITERATURE CITED

1. Agrios, G. N. 1997. Plant Pathology. Academic Press, San Diego.
2. Berry, S. Z., and Thomas, C. A. 1961. Influence of soil temperature, isolates, and method of inoculation on resistance of mint to Verticillium wilt. Phytopathology 51:169-174.

3. Bhat, R. G., and Subbarao, K. V. 1999. Host range specificity in *Verticillium dahliae.* Phytopathology 89:1218-1225.

4. Chang, R. J., and Eastburn, D. M. 1994. Host range of *Verticillium dahliae* from horseradish and pathogenicity of strains. Plant Disease 78:503-506.

5. Chen, W. 1994. Vegetative compatibility groups of *Verticillium dahliae* from ornamental woody plants. Phytopathology 84:214-219.

6. Diwan, N., Fluhr, R., Eshed, Y., Zamir, D., and Tanksley, S. D. 1999. Mapping of *Ve* in tomato: a gene conferring resistance to the broad-spectrum pathogen, *Verticillium dahliae* race 1. Theoretical and Applied Genetics 98:315-319.

7. Douhan, L. I., and Johnson, D. A. 2001. Vegetative compatibility and pathogenicity of *Verticillium dahliae* from spearmint and peppermint. Plant Disease 85:297-302.

8. Dung, J. 2009. Evaluation of Verticillium Wilt resistance in *Mentha arvensis* and *M. longifolia* using *V. dahliae* isolates from various hosts and vegetative compatibility groups. M.S. thesis. Washington State University, Pullman.

9. Faulkner, L. R., and Skotland, C. B. 1965. Interactions of *Verticillium dahliae* and *Pratylenchus minyus* in Verticillium wilt of peppermint. Phytopathology 55:583-586.

10. Green, R. J. 1951. Studies on the host range of the *Verticillium* that causes wilt of *Mentha piperita* L. Science 113:207-208.

11. Green, R. J. 1967. Control of Verticillium wilt of peppermint by crop rotation sequences. Plant Dis Rep 51:441-453.

12. Green, R. J. 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.

13. Horner, C. E. 1954. Pathogenicity of *Verticillium* isolates to peppermint. Phytopathology 44:239-242.

14. Horner, C. E., and Melouk, H. A. 1977. Screening, selection, and evaluation of irradiation-induced mutants of spearmint for resistance to Verticillium wilt. Int. Atomic Energy Agency - SM:214/20:253-262.

15. Joaquim, T. R., and Rowe, R. C. 1990. Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology 80:1160-1166.

16. Johnson, D. A., and Cummings, T. F. 2000. Evaluation of mint mutants, hybrids, and fertile clones for resistance to *Verticillium dahliae.* Plant Disease 84:235-238.

17. Johnson, D. A., and Santo, G. S. 2001. Development of wilt in mint in response to infection by two pathotypes of *Verticillium dahliae* and co-infection by *Pratylenchus penetrans.* Plant Disease 85:1189-1192.

18. Johnson, D. A., Zhang, H., and Alldredge, J. R. 2006. Spatial pattern of Verticillium wilt in commercial mint fields. Plant Disease 90:789-797.

19. Juvik, J. A., Bolkan, H., and Tanksley, S. D. 1991. The *Ve* gene for race 1 *Verticillium* resistance is on chromosome 7. Tomato Genetics Cooperative 41:23-25.

20. Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R., and Prufer, D. 2001. Tomato *Ve* disease resistance genes encode cell surface-like receptors. Proceedings of the National Academy of Sciences of the United States of America 98:6511-6515.

21. Krasnyanski, S., May, R. A., Loskutov, A., Ball, T. M., and Sink, K. C. 1999. Transformation of the limonene synthase gene into peppermint (*Mentha piperita* L.) and preliminary studies on the essential oil profiles of single transgenic plants. Theoretical and Applied Genetics 99:676-682.

22. Lawrence, B. M. 2007. The composition of commercially important mints. Pages 217-323 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton, FL.

23. Li, X., Gong, Z., Koiwa, H., Niu, X., Espartero, J., Zhu, X., Veronese, P., Ruggiero, B., Bressan, R. A., Weller, S. C., and Hasegawa, P. M. 2001. *Bar*-expressing peppermint (*Mentha* x *piperita* L. var. Black Mitcham) plants are highly resistant to the glufosinate herbicide Liberty. Molecular Breeding 8:109-118.

24. Menzies, J. D., and Griebel, G. E. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.

25. Morrell, A., Schreiber, A., and Lundy, R. 1997. Pests, pest control, and fertilizer use in Washington mint. Washington State University Cooperative Extension, MISC0188 Pullman, WA.

26. Murray, M. J., and Todd, W. A. 1972. Registration of Todd's Mitcham peppermint. Crop Science 12:128.

27. Niu, X., Li, X., Veronese, P., Bressan, R. A., Weller, S. C., and Hasegawa, P. M. 2000. Factors affecting *Agrobacterium tumefaciens*-mediated transformation of peppermint. Plant Cell Reports 19:304-310.

28. Niu, X., Lin, K., Hasegawa, P. M., Bressan, R. A., and Weller, S. C. 1998. Transgenic peppermint (*Mentha* x *piperita* L.) plants obtained by cocultivation with *Agrobacterium tumefaciens.* Plant Cell Reports 17:165-171.

29. Rick, C. M., Martin, F. M., and Gentile, A. 1959. Linkage of Verticillium resistance (*Ve*). Tomato Genetics Cooperative 9:44.

30. Ruthardt, N., Kawchuk, L. M., Fischer, R., and Emans, N. 2007. Tomato protein of the resistance gene *Ve2* to Verticillium wilt [*Verticillium* spp.] is located in the endoplasmic reticulum. Canadian Journal of Plant Pathology 29:3-8.

31. Rygulla, W., Snowdon, R. J., Friedt, W., Happstadius, I., Cheung, W. Y., and Chen, D. 2008. Identification of quantitative trait loci for resistance against *Verticillium longisporum* in oilseed rape (*Brassica napus*). Phytopathology 98:215-221.

32. Schaible, L., Cannon, O. S., and Waddoups, B. 1951. Inheritance of resistance to Verticillium wilt in a tomato cross. Phytopathology 41:986-990.

33. Schnathorst, W. C. 1981. Life cycle and epidemiology of *Verticillium*. Pages 81-111 in: Fungal Wilt Diseases of Plants, M. E. Mace, A. A. Bell and C. H. Beckman, eds. Academic, New York.

34. Todd, W. A., Green, R. J., and Horner, C. E. 1977. Registration of Murray Mitcham peppermint. Crop Science 17:188.

35. Tucker, A. O., and Naczi, R. F. C. 2007. *Mentha*: An overview of its classification and relationships. Pages 1-39 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton, FL.

36. Veronese, P., Li, X., Niu, X., Weller, S. C., Bressan, R. A., and Hasegawa, P. M. 2001. Bioengineering mint crop improvement. Plant Cell, Tissue and Organ Culture 64:133-144.

37. Veronese, P., Narasimhan, M. L., Stevenson, R. A., Zhu, J. K., Weller, S. C., Subbarao, K. V., and Bressan, R. A. 2003. Identification of a locus controlling *Verticillium* disease symptom response in *Arabidopsis thaliana*. Plant Journal 35:574-587.

38. Vining, K. J., Zhang, Q., Smith, C. A., and Davis, T. M. 2007. Identification of resistance gene analogs and Verticillium wilt resistance-like sequences in *Mentha longifolia*. Journal of the American Society for Horticultural Science 132:541-550.

39. Vining, K. J., Zhang, Q., Tucker, A. O., Smith, C., and Davis, T. M. 2005. *Mentha longifolia* (L.) L.: a model species for mint genetic research. HortScience 40:1225-1229.

40. Wildung, M. R., and Croteau, R. B. 2005. Genetic engineering of peppermint for improved essential oil composition and yield. Transgenic Research 14:365-372.

TABLE 1. Mean AUDPC and ratings of transformant *Mentha* x *piperita* "Black Mitcham" containing *mVe*1 and *mVe*2 Verticillium wilt resistance-like sequences cloned from *M. longifolia*. Nontransformed wild-type peppermint plants from two sources were used as controls.

Transformant	Mean AUDPC[a]	Mean Rating[a]	Transformant	Mean AUDPC[a]	Mean Rating[a]
***mVe*1**			***mVe1***		
1.1	104 a	4.75	20	130 a	5.75
1.2	108 a	5.25	21	131 a	5.50
1.3	115 a	4.75	22	126 a	5.50
1.4	94 a	4.50	23	127 a	5.75
2.1	105 a	5.50	24	122 a	5.50
2.2	108 a	5.00	25	129 a	5.25
2.3	102 a	4.50	26	114 a	5.50
2.4	105 a	4.50			
2.5	118 a	4.88	***mVe2***		
3.1	94 a	4.75	1.1	109 a	5.00
3.2	96 a	5.00	2	131 a	5.50
3.3	114 a	4.75	3.1	119 a	5.00
3.4	110 a	5.25	4	127 a	5.50
4.1	117 a	5.00	5	128 a	5.25
4.2	115 a	5.50	6	140 b	5.75
4.3	102 a	4.25	7.1	109 a	4.75
4.4	120 a	5.00	8	123 a	5.75
5.1	101 a	5.00	9	131 a	5.50
5.2	103 a	5.00	10	118 a	5.50
5.3	105 a	4.50	11	130 a	5.25
6.1	124 a	4.63	12	133 a	5.75
6.2	99 a	4.75	14	141 b	5.75
7.1	103 a	4.25	15	123 a	5.75
7.2	99 a	5.00	16	123 a	5.75
7.3	91 a	4.25	17	134 a	5.75
8.1	101 a	4.75	18	126 a	5.75
9.1	103 a	4.75	19	129 a	5.75
10.1	111 a	4.25	20	124 a	5.75
11.1	108 a	5.25	21	132 a	5.50
12	132 a	5.75	22	135 a	5.75
13	123 a	5.50	23	132 a	5.50
14	131 a	5.75	24	137 b	5.75
15	138 b	5.50			
16	127 a	5.50	**Wild-type controls**		
17	123 a	5.25			
18	129 a	5.50	IBC	114 a	5.25
19	130 a	5.50	Plant Path.	113 a	5.38

[a] AUDPC values calculated from disease severity ratings taken weekly from four to eight weeks postinoculation. Plants were then cut back to soil level, allowed to regrow for 8 weeks and disease ratings assessed again at 16 weeks postinoculation. Comparisons between transformants and controls were performed using Dunnett's test.

LITERATURE CITED

1. Agrios, G. N. 1997. Plant Pathology. Academic Press, San Diego.
2. Atallah, Z. K., Bae, J., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology 97:865-872.
3. Bae, J., Atallah, Z. K., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Colonization dynamics and spatial progression of *Verticillium dahliae* in individual stems of two potato cultivars with differing responses to potato early dying. Plant Disease 91:1137-1141.
4. Bahl, J. R., Bansal, R. P., and Kumar, S. 2002. Direct planting of the short-duration summer crop using cold stored suckers of menthol mint *Mentha arvensis.* Journal of Horticultural Science & Biotechnology 77:217-219.
5. Barbara, D. J., and Clewes, E. 2003. Plant pathogenic *Verticillium* species: how many of them are there? Molecular Plant Pathology 4:297-305.
6. Baribeau, B. 1952. Verticillium wilt and seed potato certification. American Journal of Potato Research 29:157-159.
7. Bhat, R. G., and Subbarao, K. V. 1999. Host range specificity in *Verticillium dahliae.* Phytopathology 89:1218-1225.
8. Botseas, D. D., and Rowe, R. C. 1994. Development of potato early dying in response to infection by two pathotypes of *Verticillium dahliae* and co-infection by *Pratylenchus penetrans*. Phytopathology 84:275-282.

9. Brandt, W. H., Lacy, M. L., and Horner, C. E. 1984. Distribution of *Verticillium* in stems of resistant and susceptible species of mint. Phytopathology 74:587-591.

10. Cappaert, M. R., Powelson, M. L., Christensen, N. W., and Crowe, F. J. 1992. Influence of irrigation on severity of potato early dying and associated yield loss. Phytopathology 82:1448-1453.

11. Carder, J. H., and Barbara, D. J. 1991. Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticillium*. Mycological Research 95:935-942.

12. Chang, R. J., and Eastburn, D. M. 1994. Host range of *Verticillium dahliae* from horseradish and pathogenicity of strains. Plant Disease 78:503-506.

13. Chen, W. 1994. Vegetative compatibility groups of *Verticillium dahliae* from ornamental woody plants. Phytopathology 84:214-219.

14. Clark, G. S. 2007. Aroma chemical profile: menthol. Perfumer and Flavorist 32:38-47.

15. Collado-Romero, M., Mercado-Blanco, J., Olivares-Garcia, C., and Jimenez-Diaz, R. M. 2008. Phylogenetic analysis of *Verticillium dahliae* vegetative compatibility groups. Phytopathology 98:1019-1028.

16. Collado-Romero, M., Mercado-Blanco, J., Olivares-Garcia, C., Valverde-Corredor, A., and Jimenez-Diaz, R. M. 2006. Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathology 96:485-495.

17. Davis, J. R., Pavek, J. J., Corsini, D. L., Sorensen, L. H., Schneider, A. T., Everson, D. O., Westerman, D. T., and Huisman, O. C. 1994. Influence of continuous cropping of several potato clones on the epidemiology of Verticillium wilt of potato. Phytopathology 84:207-214.

18. Dobinson, K. F., Patterson, N. A., White, G. J., and Grant, S. 1998. DNA fingerprinting and vegetative compatibility analysis indicate multiple origins for *Verticillium dahliae* race 2 tomato isolates from Ontario, Canada. Mycological Research 102:1089-1095.

19. Domsch, K. H., Gams, W., and Anderson, T.-H. 1980. Compendium of soil fungi. Academic Press, London; New York.

20. Douhan, L. I., and Johnson, D. A. 2001. Vegetative compatibility and pathogenicity of *Verticillium dahliae* from spearmint and peppermint. Plant Disease 85:297-302.

21. Ewing, E. 1981. Heat stress and the tuberization stimulus. American Journal of Potato Research 58:31-49.

22. Fahleson, J., Hu, Q., and Dixelius, C. 2004. Phylogenetic analysis of *Verticillium* species based on nuclear and mitochondrial sequences. Archives of Microbiology 181:435-442.

23. Faulkner, L. R., and Skotland, C. B. 1965. Interactions of *Verticillium dahliae* and *Pratylenchus minyus* in Verticillium wilt of peppermint. Phytopathology 55:583-586.

24. Fradin, E. F., and Thomma, B. P. H. J. 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7:71-86.

25. Francl, L. J., Madden, L. V., Rowe, R. C., and Riedel, R. M. 1990. Correlation of growing season environmental variables and the effect of early dying on potato yield. Phytopathology 80:425-432.

26. Gams, W., and Zare, R. 2002. New generic concepts in *Verticillium* Sect. *Prostrata*. Mycological Research 106:130-131.

27. Green, R. J. 1951. Studies on the host range of the *Verticillium* that causes wilt of *Mentha piperita* L. Science 113:207-208.

28. Green, R. J. 1967. Control of Verticillium wilt of peppermint by crop rotation sequences. Plant Dis Rep 51:441-453.

29. Green, R. J. 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.

30. Griffiths, D. A. 1971. The development of lignitubers in roots after infection by *Verticillium dahliae* Kleb. Can J Microbiol 17:441-444.

31. Horner, C. E. 1954. Pathogenicity of *Verticillium* isolates to peppermint. Phytopathology 44:239-242.

32. Horner, C. E., and Melouk, H. A. 1977. Screening, selection, and evaluation of irradiation-induced mutants of spearmint for resistance to Verticillium wilt. Int. Atomic Energy Agency - SM:214/20:253-262.

33. Hoyman, W. G. 1974. Consequence of planting Norgold Russet seed infected with *Verticillium albo-atrum*. American Potato Journal 51:22-25.

34. Huisman, O. C. 1988. Seasonal colonization of roots of field-grown cotton by *Verticillium dahliae* and *V. tricorpus*. Phytopathology 78:708-716.

35. Isaac, I. 1967. Speciation in *Verticillium*. Annual Review of Phytopathology 5:201-222.

36. Isaac, I., and Harrison, J. A. C. 1968. The symptoms and causal agents of early-dying disease (Verticillium wilt) of potatoes. Annals of Applied Biology 61:231-244.

37. Joaquim, T. R., and Rowe, R. C. 1990. Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology 80:1160-1166.

38. Joaquim, T. R., and Rowe, R. C. 1991. Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. Phytopathology 81:552-558.

39. Johnson, D. A., and Cummings, T. F. 2000. Evaluation of mint mutants, hybrids, and fertile clones for resistance to *Verticillium dahliae.* Plant Disease 84:235-238.

40. Johnson, D. A., and Santo, G. S. 2001. Development of wilt in mint in response to infection by two pathotypes of *Verticillium dahliae* and co-infection by *Pratylenchus penetrans.* Plant Disease 85:1189-1192.

41. Johnson, K. B., Radcliffe, E. B., and Teng, P. S. 1986. Effects of interacting populations of *Alternaria solani*, *Verticillium dahliae*, and the potato leafhopper (*Empoasca fabae*) on potato yield. Phytopathology 76:1046-1052.

42. Kalra, A., Singh, H. B., Patra, N. K., Pandey, R., Shukla, R. S., and Kumar, S. 2001. The effect of leaf spot, rust and powdery mildew on yield components of nine Japanese mint (*Mentha arvensis*) genotypes. The Journal of Horticultural Science & Biotechnology 76:546-548.

43. Karapapa, V. K., and Typas, M. A. 2001. Molecular characterization of the host-adapted pathogen *Verticillium longisporum* on the basis of a Group-I intron found in the nuclear SSU-rRNA gene. Current Microbiology 42:217-224.

44. Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R., and Prufer, D. 2001. Tomato *Ve* disease resistance genes encode cell surface-like receptors. Proceedings of the National Academy of Sciences of the United States of America 98:6511-6515.

45. Korolev, N., Perez-Artes, E., Bejarano-Alcazar, J., Rodriguez-Jurado, D., Katan, J., Katan, T., and Jimenez-Diaz, R. M. 2001. Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. European Journal of Plant Pathology 107:443-456.

46. Kumar, S., Bahl, J. R., Bansal, R. P., Gupta, A. K., Singh, V., and Sharma, S. 2002. High economic returns from companion and relay cropping of bread wheat and menthol mint in the winter-summer season in north Indian plains. Industrial Crops and Products 15:103-114.

47. Kumar, S., Bahl, J. R., Shukla, P., Singh, A., Ram, G., Bansal, R. P., and Sharma, S. 1999. Screening of genotypes of menthol mint *Mentha arvensis* for high yields of herbage and essential oil under late cropping conditions of the sub-tropical Indo-Gangetic plains. The Journal of Horticultural Science & Biotechnology 74:680-684.

48. Lawrence, B. M. 2007. The composition of commercially important mints. Pages 217-323 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton, FL.

49. Menzies, J. D., and Griebel, G. E. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.

50. Mol, L. 1995. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. European Journal of Plant Pathology 101:679-685.

51. Mol, L., and Scholte, K. 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. Potato Research 38:143-150.

52. Morrell, A., Schreiber, A., and Lundy, R. 1997. Pests, pest control, and fertilizer use in Washington mint. Report No. MISC0188. Washington State University Cooperative Extension, Pullman, WA.

53. Morris, M. A. 2007. Commercial mint species grown in the United States. Pages 87-136 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton.

54. Morton, A., Carder, J. H., and Barbara, D. J. 1995. Sequences of the internal transcribed spacers of the ribosomal RNA genes and relationships between isolates of *Verticillium alboatrum* and *V. dahliae*. Plant Pathology 44:183-190.

55. Murray, M. J., and Todd, W. A. 1972. Registration of Todd's Mitcham peppermint. Crop Science 12:128.

56. Nachmias, A., and Krikun, J. 1984. Transmission of *Verticillium dahliae* in potato tubers. Phytopathology 74:535-537.

57. National Agricultural Statistics Service. Crop Production. 2008. United States Department of Agriculture,, Washington D.C.

58. National Agricultural Statistics Service. Mint Release. 2008. United States Department of Agriculture,, Washington, D.C.

59. National Agricultural Statistics Service. Potato Release. 2008. United States Department of Agriculture,, Washington, D.C.

60. Nelson, R. 1950. Verticillium wilt of peppermint. Michigan State College of Agriculture, East Lansing, MI.

61. Niu, X., Li, X., Veronese, P., Bressan, R. A., Weller, S. C., and Hasegawa, P. M. 2000. Factors affecting *Agrobacterium tumefaciens*-mediated transformation of peppermint. Plant Cell Reports 19:304-310.

62. Nnodu, E., and Harrison, M. 1979. The relationship between *Verticillium albo-atrum* inoculum density and potato yield. American Journal of Potato Research 56:11-25.

63. Okoli, C. A., Carder, J. H., and Barbara, D. J. 1994. Restriction fragment length polymorphisms (RFLPs) and the relationships of some host-adapted isolates of *Verticillium dahliae*. Plant Pathology 43:33-40.

64. Omer, M. A., Johnson, D. A., Douhan, L. I., Hamm, P. B., and Rowe, R. C. 2008. Detection, quantification, and vegetative compatibility of *Verticillium dahliae* in potato and mint production soils in the Columbia Basin of Oregon and Washington. Plant Disease 92:1127-1131.

65. Omer, M. A., Johnson, D. A., and Rowe, R. C. 2000. Recovery of *Verticillium dahliae* from North American certified seed potatoes and characterization of strains by vegetative compatibility and aggressiveness. American Journal of Potato Research 77:325-331.

66. Pantou, M. P., Strunnikova, O. K., Shakhnazarova, V. Y., Vishnevskaya, N. A., Papalouka, V. G., and Typas, M. A. 2005. Molecular and immunochemical phylogeny of *Verticillium* species. Mycological Research 109:889-902.

67. Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. Annual Review of Phytopathology 31:111-126.

68. Qin, Q. M., Vallad, G. E., Wu, B. M., and Subbarao, K. V. 2006. Phylogenetic analyses of phytopathogenic isolates of *Verticillium* spp. Phytopathology 96:582-592.

69. Robinson, D. B., and Ayers, G. W. 1961. Verticillium wilt of potato in relation to vascular infection of the tuber. Canadian Journal of Plant Science 41:703-708.

70. Rowe, R. C. 1985. Potato early dying - a serious threat to the potato industry. American Potato Journal 62:157-161.

71. Rowe, R. C., Riedel, R. M., and Martin, M. J. 1985. Synergistic interactions between *Verticillium dahliae* and *Pratylenchus penetrans* in potato early dying disease. Phytopathology 75:412-418.

72. Rygulla, W., Snowdon, R. J., Friedt, W., Happstadius, I., Cheung, W. Y., and Chen, D. 2008. Identification of quantitative trait loci for resistance against *Verticillium longisporum* in oilseed rape (*Brassica napus*). Phytopathology 98:215-221.

73. Schaible, L., Cannon, O. S., and Waddoups, B. 1951. Inheritance of resistance to Verticillium wilt in a tomato cross. Phytopathology 41:986-990.

74. Schnathorst, W. C. 1981. Life cycle and epidemiology of *Verticillium*. Pages 81-111 in: Fungal Wilt Diseases of Plants, M. E. Mace, A. A. Bell and C. H. Beckman, eds. Academic, New York.

75. Singh, A. K., Srivastava, R. K., Kalra, A., Bansal, R. P., Tomar, V. K. S., Bahl, J. R., and Kumar, S. 2003. New practices in the cultivation of the mint, *Mentha arvensis* in the Indo-Gangetic plains. Experimental Agriculture 39:199-207.

76. Smith, H. C. 1965. The morphology of *Verticillium albo-atrum*, *V. dahliae*, and *V. tricorpus*. New Zeal J Agr Res 8:450-478.

77. Sung, G. H., Spatafora, J. W., Zare, R., Hodge, K. T., and Gams, W. 2001. A revision of *Verticillium* sect. *Prostrata*. II. Phylogenetic analyses of SSU and LSU nuclear rDNA sequences from anamorphs and teleomorphs of the *Clavicipitaceae*. Nova Hedwigia:311-328.

78. Sunseri, M., and Johnson, D. A. 2001. Verticillium wilt. Washington State University, Cooperative Extension,, Pullman, WA.

79. Thanassoulopoulos, C. C., and Hooker, W. J. 1968. Factors influencing infection of field grown potato by *Verticillium albo-atrum*. American Journal of Potato Research 45:203-216.

80. Todd, W. A., Green, R. J., and Horner, C. E. 1977. Registration of Murray Mitcham peppermint. Crop Science 17:188.

81. Tsror (Lahkim), L., Aharon, M., and Erlich, O. 1999. Survey of bacterial and fungal seedborne diseases in imported and domestic potato seed tubers. Phytoparasitica 27:215-226.

82. Tucker, A. O., and Naczi, R. F. C. 2007. *Mentha*: An overview of its classification and relationships. Pages 1-39 in: Mint: The genus *Mentha*, B. M. Lawrence, ed. CRC Press, Boca Raton, FL.

83. Vallad, G. E., and Subbarao, K. V. 2008. Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. Phytopathology 98:871-885.

84. Veronese, P., Li, X., Niu, X., Weller, S. C., Bressan, R. A., and Hasegawa, P. M. 2001. Bioengineering mint crop improvement. Plant Cell, Tissue and Organ Culture 64:133-144.

85. Veronese, P., Narasimhan, M. L., Stevenson, R. A., Zhu, J. K., Weller, S. C., Subbarao, K. V., and Bressan, R. A. 2003. Identification of a locus controlling *Verticillium* disease symptom response in *Arabidopsis thaliana*. Plant Journal 35:574-587.

86. Vining, K. J., Zhang, Q., Smith, C. A., and Davis, T. M. 2007. Identification of resistance gene analogs and Verticillium wilt resistance-like sequences in *Mentha longifolia*. Journal of the American Society for Horticultural Science 132:541-550.

87. Vining, K. J., Zhang, Q., Tucker, A. O., Smith, C., and Davis, T. M. 2005. *Mentha longifolia* (L.) L.: a model species for mint genetic research. HortScience 40:1225-1229.

88. Wildung, M. R., and Croteau, R. B. 2005. Genetic engineering of peppermint for improved essential oil composition and yield. Transgenic Research 14:365-372.

89. Zare, R., Gams, W., and Schroers, H. J. 2004. The type species of *Verticillium* is not congeneric with the plant-pathogenic species placed in *Verticillium* and it is not the anamorph of '*Nectria' inventa*. Mycological Research 108:576-582.