IDENTIFICATION OF EFFECTOR GENES FOR THE SPINACH FUSARIUM WILT PATHOGEN, *FUSARIUM OXYSPORUM* F. SP. *SPINACIAE*

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of ALEXANDER M. BATSON find it satisfactory and recommend that it be accepted.

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Abstract

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The maritime Pacific Northwest is the only region in the United States that is suitable for spinach seed production, where mild and dry summers with long day length are necessary for high yields of quality seed. Spinach Fusarium wilt caused by *Fusarium oxysporum* f. sp. *spinaciae* is the greatest biotic limitation to spinach seed production in this region. Management strategies for Fusarium wilt in spinach seed crops in this region are limited as they only partially or transiently suppress the pathogen. Little is known about what defines the causal agent of spinach Fusarium wilt as a pathogen of spinach genetically. However, host-specificity of other *F. oxysporum* ff. spp. is associated with unique combinations of effector genes, which are important for pathogenicity. In this study, *Fusarium* isolates were characterized phenotypically for pathogenicity to spinach and genotypically for a group of putative pathogenicity genes known as the *Secreted in Xylem* (*SIX*) genes. Thirty-nine isolates of *F. oxysporum* f. sp. *spinaciae* were identified and characterized into two pathogenicity groups based on wilt severity observed on three proprietary inbred spinach parent lines with differing levels of resistance to Fusarium wilt. *SIX8* and/or *SIX14* were detected in all *F. oxysporum* f. sp. *spinaciae* isolates identified;

however, the profile of *SIX* genes did not differentiate *F. oxysporum* f. sp. *spinaciae* from other *F. oxysporum* isolates that did not cause wilt of spinach. Illumina and PacBio platforms were used to sequence the genomes of seven *F. oxysporum* f. sp. *spinaciae* isolates and five spinach associated *F. oxysporum* isolates. Effector genes were predicted from the genome assemblies with a previously described software pipeline, which differentiated *F. oxysporum* f. sp. *spinaciae* isolates from non-pathogens of spinach. Furthermore, the two pathogenicity groups of *F. oxysporum* f. sp. *spinaciae* were differentiated based on predicted effector gene profiles. Further characterization of the predicted effector genes and other regions of the genome of *F. oxysporum* f. sp. *spinaciae* will aid in understanding mechanisms of pathogenicity, developing molecular tools for rapid detection and quantification of this pathogen, and breeding spinach cultivars and parent lines with increased resistance to Fusarium wilt.

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Dedication

For my loving parents, Elizabeth and Marcus.

CHAPTER ONE

LITERATURE REVIEW

1.1 *Spinacia oleracea* Linnaeus

1.1.1 Significance. Spinach (*Spinacia oleracea* L.) is a leafy green vegetable that is consumed across the globe for the leaves and petioles (Naeve 2015; Yamaguchi 1983). Spinach has been cultivated as a vegetable for over 1,300 years and is believed to have originated in what is now India or Afghanistan (Yamaguchi 1983). The first report of spinach cultivation in Europe dates to the 13th century, and spinach was later introduced to North America in the 16th to 17th century by European settlers (Morelock and Correll 2008).

The global production of spinach in 2014 was approximately 24.3 billion pounds [Food and Agriculture Organization of the United Nations Statistical-Database (FAOSTAT) 2014]. China, the United States, Japan, Turkey, and Indonesia are the top five spinach producing countries, which produced 91, 1.4, 1.1, 0.9, and 0.6% of the global supply of spinach in 2014, respectively (FAOSTAT 2014). Although China has the largest global share of spinach leaf production (FAOSTAT 2014), most of the production is of *Tetragonia* spp. (common name = New Zealand Spinach), another leafy vegetable. In the United States, fresh-market spinach consumption quintupled from 0.2 to 1.0 kg per capita from 1980 to 2007 [United States Department of Agriculture (USDA) Economic Research Service (ERS) 2007]. The total area of spinach production (fresh and processed markets) in the United States was approximately 2.1 x 10^5 ha [USDA National Agricultural Statistics (NASS) 2017], representing an approximately six-fold increase in domestic production from 5.4 x 10^7 kg in 2007 to 3.3 x 10^8 kg in 2017 (USDA ERS

2007; USDA NASS 2017). California leads in fresh market spinach production by land area and overall production with 69% of the area of production and 70% of total production by weight (USDA NASS 2017). California's spinach production occurs in roughly four areas of the state which include the southern desert valleys, the southern coast, the central coast, and the central San Joaquin Valley (Koike et al. 2011). Nearly 50% of the production of spinach occurs in the Salinas Valley where production of spinach occurs year-round (Koike et al. 2011). Although during winter months, a significant portion of baby leaf spinach production occurs in Yuma County, AZ due to the optimal climate for spinach production (Kerna et al. 2017). The next largest fresh-market spinach producing states are Arizona, Texas, and New Jersey with production by weight at 21, 4, and 4%, respectively.

The rapid increase in spinach consumption in the United States over the past 25 years is due, in part, to the convenience that pre-packaged and triple-washed baby leaf spinach presents to consumers, as well as a greater awareness of the health benefits conferred by the leafy vegetable (Boriss and Kreith 2006; University of the District of Columbia Center for Nutrition, Diet, and Health 2014; USDA ERS 2007). The health benefits of spinach include high levels of vitamin A, vitamin C, vitamin K1, folic acid, iron, calcium and additional minerals (Lucier et al. 2004). Furthermore, spinach is rich with carotenoids that are essential for vitamin A biosynthesis in humans and are antioxidants which aid in scavenging reactive oxygen species generated during cellular metabolism. The high carotenoid content of spinach has been documented to play a role in preventing macular degeneration (Johnson 2002).

1.1.2 Botany and physiology. Spinach is a diploid (2n = 12), annual, cool-season, and dicotyledonous species of vegetable that produces individual flowers that are wind pollinated (Koike et al. 2011; Welbaum 2005). Historically, *S. oleracea* belonged to the Chenopodiaceae

along with other species like table beet (*Beta vulgaris* var. *crassa*), swiss chard (*Beta vulgaris* var. *cicla*), sugar beet (*Beta vulgaris* var. *orientalis*), and orach (*Atriplex horensis*) (Yamaguchi 1983). However, the Angiosperm Phylogeny Group (APG) II system reclassified *S. oleracea* under family Amaranthaceae (subfamily Chenopodioideae) (Angiosperm Phylogeny Group (APG) II 2003). Progenitor species of *S. oleracea* are believed to be either *S. tetranda* or *S. turkestanica* (Xu et al. 2017).

Spinach seed germinates optimally between 5 and 30°C, and spinach seed can germinate at temperatures as low as 2°C (Koike et al. 2011). After germination, spinach plants develop leaves in a rosette, where new leaves radiate from the central stem on petioles (Koike et al. 2011; Welbaum 2005). Although spinach grows optimally at mild temperatures (i.e., 15 to 18°C), it is cold-tolerant, and mature plants can withstand temperatures as low as -9°C for several weeks during commercial production (Koike et al. 2011). Spinach leaves range from savoy to smooth; savoy type leaves are "crinkled" with deep ridges and a tough texture while flat leaf spinach is smooth and delicate with an absence of ridges (Navazio et al. 2007). Semi-savoy and flat-leafed spinach cultivars are commonly used for baby leaf and bunching spinach production, while savoy leaf types are favored for processing in the United States (LeStrange et al. 2006). This is driven by a combination of consumer preference and ease of cleaning/processing spinach leaves. For example, savoy spinach leaves are more difficult to clean, do not pack as efficiently in containers, and are typically more rigid, which make them a poor candidate for fresh-market spinach (LeStrange et al. 2006).

Spinach leaf production typically takes place in regions or times of the year with shorter day lengths (<14 hours) and cooler temperatures as longer day lengths and warmer temperatures induce the plant to switch from vegetative to reproductive growth (bolting) (Sherry et al. 1993).

However, a range from short- to long-day cultivars of spinach exist, which enables spinach growers to produce baby leaf spinach in regions and during times of the year when day lengths would normally induce bolting, which is undesirable because spinach leaves are not marketable post-bolting due to the bitterness of flavor (Koike et al. 2011; Mattson 2017). Spinach plants are typically dioecious with a 1:1 sex ratio, although plant breeding programs have developed lines of spinach plants that are primarily monoecious to facilitate hybrid seed production (Sherry et al. 1993). Spinach seed ranges from round to non-uniform and spikey, with spikey seed phenotypes are most likely derived from wild type *S. oleracea* (George, 2009). Round seeds are preferable when using precision planting equipment as seed with this shape are easier to plant compared to spikey seeds (LeStrange et al. 2006).

1.1.3 Spinach seed production. The increase in acreage, high seeding rate of baby leaf spinach crops ($\leq$ 9 million seed/ha) (Koike et al. 2011), and consumption of fresh-market spinach in the United States have increased the demand for a larger supply of high quality spinach seed. However, the production of spinach seed is limited to regions that have a long summer day length to induce flowering, as well as mild temperatures for this heat-sensitive species, and dry summers that minimize the risk of poor seed quality and infection of seed by fungal and bacterial pathogens (Foss and Jones 2005). The only region in the United States that fits these stringent climatic requirements for high quality spinach seed production is the maritime Pacific Northwest (PNW), which includes western Washington and western Oregon (Foss and Jones 2005). This region produces all the spinach seed that is grown in the United States, and up to 20% of the global market of spinach seed (Foss and Jones 2005). Washington State produces up to 75% of the United States' spinach seed production on 2,000 to 3,000 acres annually, with production concentrated in Skagit and Snohomish Counties (Foss and Jones 2005). The country that

produces the greatest amount of spinach seed (*S. oleracea* L.) for western hemisphere market types is Denmark (Navazio et al. 2007), followed by the United States. Other countries with suitable climates for spinach seed production include Chile, France, Germany, Italy, the Netherlands, and New Zealand.

In the maritime PNW, spinach seed crops are typically planted in spring between late March and mid-May and harvested any time from July to September depending on the day length sensitivity of the parent lines (Foss and Jones 2005). At least 90% of the acreage is hybrid seed production. Male and female inbred spinach lines are planted in the same field in rows that can range in ratios of females:males from 3:1 to 10:1. Individual plants that do not express true-varietal characteristics for these inbred lines, and any male plants that develop in the female rows are rogued to avoid the production of off-type hybrid seed or inbred seed, respectively. At harvest, the spinach plants bearing seed are cut, laid in windrows, dried for 10 to 14 days, threshed mechanically (the process of removing seed from the plants), and sent to a seed processing facility where the seed is cleaned to remove weed seed and other crop residues, and sized to marketable standards (Foss and Jones 2005). The seed produced by growers is subsequently analyzed for purity and viability (seed quality) and may be tested for certain seedborne pathogens before it is sold to fresh market or processing spinach growers. Since spinach seed is produced on contract by growers, seed companies distribute select inbred parent lines to the growers for hybrid spinach seed production (Foss and Jones 2005). To ensure the genetic purity of spinach seed crops, growers isolate spinach seed crops by distances up to 6.4 kilometers to restrict cross-pollination between other spinach seed crops that have different male (pollinator) inbred lines (Foss and Jones 2005). In western Washington, the locations for spinach seed production are finalized by growers and seed company field representatives at an annual

"pinning" ceremony at the Washington State University (WSU) Mount Vernon Northwestern Washington Research and Extension Center (NWREC) where the locations at which seed crops will be planted are marked on a map with pins to ensure minimum isolation standards are met (Foss and Jones, 2005). A similar seed crop mapping system is used in all other regions of spinach seed production to ensure genetic purity of the harvested seed.

The greatest biotic limitation to spinach seed production in the maritime PNW is spinach Fusarium wilt, which is caused by the soilborne fungus *Fusarium oxysporum* forma specialis (f. sp.) *spinaciae* (Foss and Jones 2005). The leached, acid soils in high rainfall regions of western Oregon and Washington are highly conducive to spinach Fusarium wilt, where the pathogen can survive in the soil >10 years, necessitating spinach seed crop rotations in excess of 10 to 15 years (du Toit and Ocamb 2008). Even then, crop rotations >15 years are sometimes still insufficient to avoid losses to this disease, with some crops incurring losses up to 100% if either of the parent lines in the hybrid seed crop is highly susceptible to Fusarium wilt. Historically, spinach seed growers have avoided fields with a risk to spinach Fusarium wilt in the maritime PNW by planting spinach seed crops on land that had not previously been planted with spinach; however, such virgin land for spinach seed production has become exceedingly rare after ~100 years of spinach seed production in this region (Biles 2018; Gatch 2013).

1.2 *Fusarium oxysporum*

1.2.1 History. There are over 80 known species of *Fusarium*, which are differentiated based on many characteristics such as the presence or absence of chlamydospores, the presence or absence of microconidia, the size and shape of macroconidia and microconidia, production of pigments in agar media and broth media, variation in specific DNA sequence(s), sexual

compatibility, etc. (Leslie and Summerell 2006). *Fusarium* spp. have a worldwide distribution, which are found in habitats that range from tropical rainforests to arid deserts and tundras (Kommedahl et al. 1988; Sangalang et al. 1995). *Fusarium* spp. are commonly found in association with plants as pathogens, commensalistic or mutualistic endophytes, or saprophytes (Beckman 1987). Numerous *Fusarium* spp. can cause plant diseases, with some of the more notable including *F. graminearum* (cause of Fusarium head blight in cereals), *F. solani* (root and crown rots), and *F. oxysporum* (vascular wilts and some bulb, crown, and root rots of many plant species) (Beckman 1987). Furthermore, several *Fusarium* spp. produce mycotoxins, the consumption of which can cause disease in humans and animals. For example, *F. graminearum* produces secondary metabolites classified as zearalenones and trichothecenes that can contaminate grains and feedstuff (Mirocha et al. 1976). These and other toxins such as diacetoxyscirpenol and deoxynivalenol (also known as vomitoxin) have been associated with consumption by cattle, dogs, and swine that resulted in bloody stools, hemorrhagic bowel syndrome, vomiting, and refusal of contaminated food (Mirocha et al. 1976).

The genus *Fusarium* was first described in 1809 by Johann H. F. Link, and in 1935 Wollenweber and Reinking (1935) published "Die Fusarien, ihre Beschreibung" (eng. The Fusaria, their description) which grouped the genus into 16 sections and 65 species (Snyder and Smith 1981). Snyder and Hansen (1940) later modified “Die Fusarien, ihre Beschreibung” because the Wollenweber and Reinking classification of *Fusarium* spp. relied on minute morphological differences among species, which in many cases, was cumbersome and complex even for adept mycologists. For example, under the Wollenweber and Reinking (1935) classification system, the subsections Orthocera, Constrictum, and Oxysporum (section Elegans) were differentiated by the presence or absence of sporodochia and dimensions of macroconidia.

However, the dimensions of macroconidia used to differentiate each subsection overlapped, which limited the ability to discriminate members of each subsection reliably. Furthermore, Wollenweber and Reinking (1935) did not standardize the growth medium used to differentiate *Fusarium* spp., which likely influenced morphological features that are critical for differentiation of species, such as the formation of sporodochia or macroconidial dimensions (Nelson 1991). Building off the monumental task of Wollenweber and Reinking (1935) to describe the Fusaria, Snyder and Hansen (1940) simplified the genus by condensing the number of *Fusarium* species from 65 to 9 based on similar morphologies and structural dimensions. Snyder and Hansen (1940) are termed "lumpers" in *Fusarium* systematics because they aimed to simplify morphological differentiation among *Fusarium* spp. by using broad morphological characteristics. One of the most notable amendments Snyder and Hansen (1940) made was condensing the section Elegans which contained 10 species from Wollenweber and Reinking's (1935) into one species, *F. oxysporum*.

1.2.2 F. oxysporum *as a plant pathogen: Fusarium wilts. F. oxysporum* is a facultative saprophyte comprised of a diverse group of isolates that can exploit various niches as saprophytes, plant mutualists (Alabouvette 1999), entomopathogens (Hasan and Vago 1972), opportunistic human pathogens in immunocompromised individuals (Nucci and Anaissie 2002), and, an economically important group of plant pathogens that induce wilts, rots, and damping-off of seedlings (Beckman 1987). Pathogenic strains of *F. oxysporum* were ranked the fifth most important fungal pathogen scientifically and economically by 495 international scientists (Dean et al. 2012). Plant pathogenic isolates of *F. oxysporum* can cause root and crown rots, bulb rots, and Fusarium wilts (Beckman 1987). Pathogenic isolates are subdivided into formae speciales (ff. spp.) based on host-specificity. Typically, each *F. oxysporum* f. sp. is specific to one host;

however, some formae speciales cause disease in a narrow range of closely related hosts. For example, some *F. oxysporum* isolates that cause wilts of plants in the Cucurbitaceae can cause disease of multiple cucurbit hosts (van Dam et al. 2016; Zhou and Everts 2007), and likewise for some isolates of *F. oxysporum* that can infect plant species in the Amaranthaceae (Armstrong and Armstrong 1976; Naiki and Kano 1977). Some *F. oxysporum* ff. spp. are further subdivided into races to distinguish the virulence of specific isolates against particular differential cultivars (Correll 1991).

1.2.3 Morphology of F. oxysporum. The species *F. oxysporum* is diverse and likely encompasses more than one species of morphologically indistinguishable (cryptic) fungi (Leslie and Summerell 2006). Colony morphology of this fungus ranges widely depending on the substrate on which the fungus is cultured: isolates may produce woolly to flat mycelia, which may range from a lack of pigmentation to deep violet or salmon-pink in color, and may be produced sparsely or in abundance (Leslie and Summerell 2006). Isolates of *F. oxysporum* have no known teleomorph and have only been observed to reproduce asexually through the production of two or three types of conidia: i) microconidia, ii) macroconidia, and iii) chlamydospores (Gordon 2017; Leslie and Summerell 2006). Microconidia produced by *F. oxysporum* are one to two-celled, hyaline, oval to kidney-shaped and are born on phialides produced through holoblastic conidiation (Elmer 2012; Leslie and Summerell 2006). The most characteristic and defining feature of the genus *Fusarium* is the fusiform-shaped macroconidium, which is a falcate phragmospore with tapered ends (Beckman 1987). *F. oxysporum* produces macroconidia that range from 3- to 4-septate, form on monophialides from branched conidiophores, and are occasionally clustered in sporodochia (Leslie and Summerell 2006). If sporodochia are produced, the color may differ among *F. oxysporum* isolates, but typically

sporodochia are pale orange (Leslie and Summerell 2006). Macroconidia are presumably too fragile to survive in soil for an extended period of time, although macroconidia can germinate and convert to chlamydospores in soil, or directly convert individual cells in each macroconidium to chlamydospores (Barran et al. 1977; Beckman 1987; Hsu and Lockwood 1973). Chlamydospores are durable resting structures that are thick-walled and form through the conversion of single cells within hyphae or conidia. Chlamydospores contain extensive nutrient reserves in the form of lipids relative to microconidia and macroconidia. The thick, chitinous cell wall and extensive nutrient reserves in chlamydospores likely aid in long term viability of these propagules (Beckman et al 1987).

Puhalla (1985) demonstrated that *F. oxysporum* isolates can be categorized further by vegetative compatibility group (VCG). Two *F. oxysporum* isolates that can form a stable dikaryon through anastomosis and plasmogamy are vegetatively compatible. Compatibility of hyphal fusion is determined by vegetative incompatibility (*vic*) genes, which are believed to be biallelic (Correll 1991). VCGs have been found to be associated with formae speciales or races, which suggests that a VCG is an indicator of host-specificity and race (Bosland and Williams 1986; Correll et al. 1986; Czislowski et al. 2017). However, VCG designation does not always correlate with host-specificity or races within a forma specialis, which can make their use as a predictor of host-specificity or race tenuous for some formae speciales (Elias and Schneider 1991; Fiely et al. 1995).

1.2.4 Disease cycle. Most Fusarium wilts are monocyclic diseases because the fungus typically is soilborne and, therefore, not disseminated until later in the growing season (Egel and Martyn 2007). For Fusarium wilt of banana and crown and root rot in tomato, caused by *F. oxysporum* ff. spp. *cubense* and *radicis-lycopersici*, respectively, overwintering conidia and

hyphae in the soil serve as the primary source of inoculum, and secondary sources of inoculum that can contribute to some Fusarium wilts include root-to-root contact between infected and healthy plants as well as dissemination of conidia aerially and by water splash (Ploetz 2015; Rekah et al. 2001). Although host-specificity typically differs among *F. oxysporum* ff. spp., the steps in the disease cycles are similar: i) fungal inoculum comes in contact with the host tissue (usually root hairs), ii) the fungus colonizes the outer root cortical tissues, iii) colonization of the roots is followed by spread of hyphae and conidia in the vasculature (xylem), iv) Fusarium wilt symptoms develop in the host plant, v) the fungus kills the host, and vi) the fungus produces chlamydospores and other conidia that may remain dormant in the soil or on crop residues until a new host or source of nutrients is available in the soil (Beckman 1987; Huang et al. 2012).

Overwintered inoculum of *F. oxysporum* germinate and grow toward roots in response to the secretion of root exudates (Beckman 1987; Nelson 1981, 1990). Research on the colonization *F. oxysporum* f. sp. *radicis-lycopersici* demonstrated that hyphae preferentially grow towards root hairs and, upon contact, grow along the root epidermis, forming a mantle-like mycelial mat (Lagopodi et al. 2002). The fungus then actively penetrates the root tissue, preferentially penetrating epidermal cell junctions. Furthermore, penetration is not mediated by appressoria or other specialized penetration-structures (Lagopodi et al. 2002). Post-penetration, the hyphae elongate through the apoplast of the root cortex and breach the casparian strip to reach the xylem (Beckman 1987). Hyphae enter the xylem via the pits of tracheid elements and, eventually, colonize the vessel elements. *F. oxysporum* spreads throughout the plant via elongation of hyphae from vessel element to vessel element, and through the production of microconidia. Upon detection of the pathogen, the host produces gels and gums, lignifies the infected tissues, and produces balloon-like structures known as tyloses in the xylem to limit the spread of the

pathogen (Baayen 1988; Beckman 1987; Charest et al. 1984; VanderMolen et al. 1977). Consequently, host-mediated occlusion of vessel elements limits transpiration, causing the host plant to wilt. The pathogen is then able to survive as a saprophyte on the dead host tissue, where the fungus produces conidia (Beckman 1987).

Chlamydospores are typically formed when the availability of carbohydrates decreases in dead host tissue or other substrates (Hsu and Lockwood 1973). Chlamydospores have been suggested to be more virulent per propagule than microconidia, according to Couteaudier and Alabouvette (1990). They found that Fusarium wilt symptoms in flax seedlings developed earlier and resulted in a greater disease incidence and severity in soils inoculated with chlamydospores compared to soils inoculated with an equivalent number of microconidia of *F. oxysporum* f. sp *lini*, the causal agent of Fusarium wilt of flax. In the same study, Couteadier and Alabouvette (1990) stained suspensions of either chlamydospores or microconidia with fluorescein diacetate (FDA), a stain that fluoresces when hydrolyzed by enzymes in cells, and found that chlamydospores exhibited a 100-fold greater enzymatic response than microconida. Greater enzymatic activity per propagule, as assessed by FDA, suggests that chlamydospores have a greater microbial "activity" than microconidia or are more primed to germinate in comparison to microconidia. This difference between chlamydospores and microconidia is further corroborated by work with *F. oxysporum* f. sp. *lycopersici* in tomato by De Cal et al. (1997).

1.3 Fusarium Wilt of Spinach.

1.3.1 F. oxysporum *f. sp.* spinaciae. Hungerford (1923) first reported Fusarium wilt of spinach in Idaho in 1923. Since then, spinach Fusarium wilt has been reported in other states in the United States (Armstrong and Armstrong 1976; Greathead et al. 1973), Canada (Reyes 1978;

Westerveld and Collins 2013), Japan (Naiki and Kano 1977), Italy (Sigillio et al. 2014), New Zealand (L. du Toit, *personal communication*), and Sweden (Larsson and Olofsson 1994). Spinach Fusarium wilt was first reported in the Skagit Valley of western Washington in the 1960s, where the disease is believed to have entered on infested stock seed. Although *F. oxysporum* f. sp. *spinaciae* is not as readily seed borne and seed transmitted in spinach as another wilt pathogen of spinach, *Verticillium dahliae*, *F. oxysporum* f. sp. *spinaciae* can be seedborne (Bassi et al. 1978; du Toit et al. 2005), which can aid in dissemination of the pathogen. The symptomology of Fusarium wilt in spinach includes damping-off, stunting, acropetal wilt of foliage, chlorosis, and leaf and root necrosis (Westerveld and Collins 2013). The fungus grows optimally on various agar media between 25 and 35°C, *F. oxysporum* f. sp. *spinaciae* isolates maintained at temperatures below 25°C grew at a third of the rate (Naiki and Morita 1983). Disease development on *F. oxysporum* f. sp. *spinaciae*-infected spinach plants is exacerbated at temperatures >25°C, and the rate of disease development decreases at temperatures <25°C (Naiki and Morita 1983).

Armstrong and Armstrong (1976) reported two races of *F. oxysporum* f. sp. *spinaciae*: race 1 included *F. oxysporum* f. sp. *spinacaie* isolates that induced symptoms of wilt on both spinach and beet, and race 2 included isolates that induced symptoms of wilt on beet only. Although useful for understanding host-specificity to certain members of the Amaranthaceae, the races of *F. oxysporum* f. sp. *spinaciae* reported by Armstrong and Armstrong (1976) are not true races since virulence was not described within a single host species. However, differential virulence of *F. oxysporum* f. sp. *spinaciae* on spinach has been reported. Fiely et al. (1995) demonstrated that *F. oxysporum* f. sp. *spinaciae* isolates obtained from spinach plants from the United States, Japan, and Sweden belonged to three VCGs: 0330, 0331, and 0332. Isolates of

VCG 0331 were not specific to *F. oxysporum* f. sp. *spinaciae* since two non-pathogenic *F. oxysporum* isolates were vegetatively compatible with *F. oxysporum* f. sp. *spinaciae* isolates. Furthermore, geographic origin of the *F. oxysporum* f. sp. *spinaciae* isolates was not associated with specific VCGs. When representative *F. oxysporum* f. sp. *spinaciae* isolates from each VCG were tested for pathogenicity on the spinach cultivar Grandstand, isolates from VCGs 0330 and 0332 induced more severe symptoms of Fusarium wilt than those from VCG 0331. The severity of disease did not differ significantly among plants inoculated with representative *F. oxysporum* f. sp. *spinaciae* isolates of VCGs 0330 and 0332.

Studies that aimed to understand the origins of host-specificity and relationship among strains of *F. oxysporum* f. sp. *spinaciae* have differing conclusions, which is likely due to differences in the genetic loci used to estimate phylogenetic relationships among *F. oxysporum* isolates as well as the specific samples of *F. oxysporum* f. sp. *spinaciae* isolates tested. Evidence presented by Baayen et al. (2000) suggested that *F. oxysporum* f. sp. *spinaciae* has a single evolutionary origin based on a multilocus phylogeny of the translation elongation factor 1α (*TEF-1α*) gene and the mitochondrial small subunit of ribosomal DNA (rDNA). However, more recent phylogenetic studies suggest that *F. oxysporum* f. sp. *spinaciae* is a polyphyletic forma specialis. Kwabe et al. (2007) demonstrated that 28 *F. oxysporum* f. sp. *spinaciae* isolates originating from Japan comprised four distinct clades based on the DNA sequences of the nuclear ribosomal intergenic spacer (IGS) of rDNA and the mating type allele *MAT1-2*. All *F. oxysporum* f. sp. *spinaciae* isolates were characterized as either VCG 0330 or 0331, and every isolate carried, *MAT1-2*. Furthermore, O'Donnell et al. (2009) demonstrated that *F. oxysporum* f. sp. *spinaciae* was divided into two phylogenetic clades based on the DNA sequence of *TEF-1α* and the IGS rDNA region of three *F. oxysporum* f. sp. *spinaciae* isolates. *F. oxysporum* f. sp.

spinaciae isolates from VCG 0330 and 0332 were grouped into the same phylogenetic clade, whereas the only *F. oxysporum* f. sp. *spinaciae* isolate from VCG 0331 was distinct phylogenetically from those of the other two VCGs (O'Donnell et al. 2009). More research is required to better understand the ancestry of host-specificity in *F. oxysporum* f. sp. *spinaciae*.

1.3.2 Management of spinach Fusarium wilt in the maritime PNW. Complete exclusion and/or eradication of spinach Fusarium wilt in seed production in the maritime PNW does not appear to be possible (Gatch 2013). Fumigation of infested soils with methyl bromide, chloropicrin, or other fumigants has been effective against other Fusarium wilts (Koike and Wilen 2009); however, fumigation is an expensive management practice that may not be economically viable for spinach seed growers. Several alternative, more environmentally-sound strategies have been developed to address the limitations of spinach seed production in the United States caused by spinach Fusarium wilt. A primary concern of spinach seed growers in this region is selecting land that has not been planted with spinach within the previous 10 years or longer, i.e., avoiding land that has a recent history of spinach or spinach seed production (du Toit and Ocamb 2008). Quantification of spinach Fusarium wilt risk of a field for spinach seed production has been offered as a service to growers annually using a soil bioassay carried out at the WSU Mount Vernon NWREC (Gatch and du Toit 2015). This bioassay has been instrumental for helping growers and seed companies make informed decisions on selecting fields with lower risk of Fusarium wilt to plant spinach seed crops, with >400 fields tested over the past 10 years (L. du Toit, *personal communication*). Although highly effective at assessing risk, the soil bioassay is labor-intensive and requires ~2 months to yield an estimate of risk. Okubara et al. (2013) attempted to develop a quantitative real-time PCR assay for the spinach Fusarium wilt pathogen, targeted at a guanine single nucleotide polymorphism (SNP) in the IGS

rDNA that was specific to a preliminary sample of *F. oxysporum* f. sp. *spinaciae* isolates tested. However, additional screening revealed that the assay failed to differentiate *F. oxysporum* f. sp. *spinaciae* isolates from a few isolates of *F. oxysporum* ff. spp. *lageneriae*, *lilii*, *melongenae,* and *raphani* as well as a larger number of *F. oxysporum* isolates that were obtained from spinach or soil in which spinach had been grown but were not pathogens of spinach. This real-time PCR assay was not effective at predicting the risk of Fusarium wilt of growers' soils, in contrast to the soil bioassay (Gatch and du Toit 2015).

In the event a field has some risk of spinach Fusarium wilt, growers have several options to reduce the impact of the disease if a spinach seed crop is planted; however, all economically-viable management practices for spinach Fusarium wilt only partially and/or transiently suppress the disease (du Toit et al. 2004, 2006, 2007; du Toit et al. 2014; Gatch et al. 2016). Historically, cultural practices to manage spinach Fusarium wilt aimed to avoid the disease completely or to modify soil conditions to make a less favorable environment for the disease. du Toit et al. (2004, 2006) demonstrated that growing a biofumigant crop of mixed *Brassica* spp. high in glucosinolates, or incorporating a and *Brassica* seed meal high in glucosinolates provided partial suppression of the disease. In both studies, biofumigation of soil with *Brassica* spp. or *Brassica* seed meal reduced the incidence of spinach Fusarium wilt and increased the overall yield of spinach seed relative to plots that were cover cropped with a non-biofumigant crop, wheat. Although biofumigation can be an effective management practice for suppression of *F. oxysporum* f. sp. *spinaciae*, it is not without downfalls. Some of the species used for *Brassica* cover crops can cross pollinate with some species of hybrid *Brassica* seed crops grown in the maritime PNW, so these cover crops can only be grown at a time of year that will not risk cross-pollination. Also, in some winters, the *Brassica* cover crops were killed by freezing conditions

resulting in very poor cover crop biomass and negligible benefit of biofumigation (L. du Toit, *personal communication*).

The application of composts to soil infested with *F. oxysporum* f. sp. *spinaciae* can also suppress spinach Fusarium wilt. du Toit et al. (2014) demonstrated that the application of a biosolids compost effectively reduced the incidence and severity of Fusarium wilt in a spinach seed crop, which translated to an increase in marketable yield of hybrid spinach seed. The marketable spinach seed yield of three inbred parent lines that differed in resistance to spinach Fusarium wilt (highly susceptible, moderately susceptible, and partially resistant) was increased by 20, 48, and 32%, respectively, relative to that of the same three parent lines grown in non-compost treated plots. The marketable seed yield of the three spinach inbred lines planted in soil treated with this compost also outperformed the yields of these same three inbreds in plots treated with the fungicide, Proline 480SC (prothioconazole).

Currently there is limited capacity to control soilborne inoculum of spinach Fusarium wilt with fungicides effectively. Seed treatments with active ingredients such as thiophanate methyl in Fungicide Resistance Action Committee (FRAC) Group 1, ergosterol biosynthesis inhibitors (FRAC Group 3), strobilurins (FRAC Group 11), and phenylpyrroles (FRAC Group 12) have been explored as options for seed treatments to control various *F. oxysporum* ff. spp. (Chase 2012; Garibaldi et al. 1997). Although effective at controlling seedborne inoculum, seed treatments only provide transient protection of the host since very little active ingredient can be applied to seed and the active ingredient typically degrades rapidly, so that the host is not protected for very long against soilborne inoculum of the target pathogens. Post-planting applications of prothioconazole (Proline 480SC) in spinach seed crops were evaluated by du Toit et al. (2014) and Gatch et al. (2016). Gatch et al. (2016) demonstrated that spinach seed crops

treated with prothioconazole had significantly less severe Fusarium wilt towards the end of the season. Spinach parent lines that differed in resistance to Fusarium wilt and that were treated with prothioconazole had marketable seed yields up to 18.4% greater compared to the non-treated control plots of the same parent line.

The environmental conditions that drive the progression and severity of Fusarium wilts tend to be acidic soils (low pH) and warm temperatures (generally 25 to 30°C) (Beckman 1987). Amelioration of soil pH to reduce severity of Fusarium wilt has been demonstrated in spinach seed crops by applying agricultural limestone (97% $CaCO_3$) to soils prior to planting (du Toit et al. 2007; du Toit et al. 2011; Gatch and du Toit 2017). The capacity for agricultural limestone to increase soil pH is mediated by the addition of calcium carbonate, which is a weak base. Gatch and du Toit (2017) demonstrated that applications of agricultural limestone at 2.24 and 4.48 tons/ha suppressed spinach Fusarium wilt in spinach plants when applied to a field infested severely with *F. oxysporum* f. sp. *spinaciae*. In one trial (du Toit et al. 2007), the application of limestone at 4.48 tons/ha increased the yield of hybrid seed by >100% (and as much as 350%) in comparison to plots without limestone amendment for three parent lines of that differed in susceptibility to Fusarium wilt. Furthermore, there was a cumulative suppressive effect of applying limestone over time. Annual application of limestone at 4.48 tons/ha for three years decreased severity of wilt midseason by an average of 20%, increased biomass of spinach plants by 33%, and increased marketable seed yield by 45% when compared to plots that received a single application of 4.48 tons/ha of limestone in one year only. Although the severity and incidence of spinach Fusarium wilt decreased among spinach plants in plots amended with limestone, the incidence of spinach plants with Verticillium wilt, caused by *V. dahliae*, increased (du Toit et al. 2007; Gatch and du Toit 2017). Verticillium wilt typically is favored when crops

are grown in basic soils, whereas the opposite is true for most Fusarium wilts (Beckman 1987; Lambert et al. 2005; Snyder and Smith 1981).

Jones and Woltz (1970) hypothesized that more basic soils (pH >7.0) decrease the availability of micronutrients that are essential for the fungus. The authors found that the addition of micronutrients such as iron (Fe), manganese (Mn), and zinc (Zn) to soil that had been treated with hydrated lime reversed the Fusarium wilt-suppression afforded by the lime application. Jones and Wolts (1970) ultimately postulated that the suppressive effect of liming on Fusarium wilt was likely mediated by the reduced availability of micronutrients vital for the fungus to cause disease. Gatch (2013) found that increasing the micronutrient concentration of Fe, Mn, and Zn in broth cultures of *F. oxysporum* f. sp. *spinaciae* increased fungal biomass and microconidial concentration significantly, and increased virulence of the inoculum on spinach.

The addition of limestone also increases calcium (Ca) concentrations in the soil. Ca is most commonly present as a divalent cation in plants which plays numerous roles in plant physiology such as intracellular signaling, cell division, membrane integrity, and more (Hepler and Wayne 1985). Ca has been demonstrated to play a role in suppressing various Fusarium wilts. For example, Keane and Sackston (1970) demonstrated that flax plants inoculated with *F. oxysporum* f. sp. *lini* had significantly reduced symptoms of wilt when grown in nutrient solutions with increased concentrations of Ca. When the concentration of Ca was increased 12-fold, Fusarium wilt severity ranged from 33 to 52% less than that observed on flax plants grown in Ca-limited hydroponic solutions (Keane and Sackston 1970). Edgington and Walker (1958) demonstrated similar findings with tomato Fusarium wilt. Both Edgington and Walker (1958) and Keane and Sackston (1970) hypothesized that increased Ca nutrition may reduce Fusarium

wilt by increasing the cross-connections of pectates in plant cells, which lowers the potential for the pectates to be hydrolyzed by fungal pectinases.

1.3.3 Fertilizer choice and Fusarium wilt. The nutrient composition of fertilizers has been demonstrated to affect the severity of Fusarium wilt significantly in some other cropping systems. In general, ammonia-based (NH_4^+) fertilizers have been shown to exacerbate the severity of Fusarium wilts, likely due to acidification of soil. Woltz and Englehard (1973) demonstrated that biweekly applications of ammonium nitrate (NH_4NO_3) to chrysanthemums in potting soil infested with the causal agent of Fusarium wilt of chrysanthemum, *F. oxysporum* f. sp. *chrysanthemi*, resulted in more severe wilt than when plants were fertilized with sodium nitrate ($NaNO_3$). Furthermore, when chrysanthemum plants were grown in soil amended with hydrated lime [$CaCO_3/Ca(OH)_2$] prior to inoculation with *F. oxysporum* f. sp. *chrsanthemi*, the severity of wilt was decreased ~10 to ~30%, depending on the fertilizer used. The least severe wilt was observed when chrysanthemum plants were grown in soil amended with hydrated lime and fertilized biweekly with $NaNO_3$. Furthermore, Woltz and Englehard (1973) demonstrated that, of the combinations of NH_4NO_3 or $NaNO_3$ and either $CaCO_3$ or $CaCO_3/Ca(OH)_2$ affected the final soil pH most dramatically, and resulted in a pH change that was inversely associated with disease severity. In general, incorporation of lime into potting soil increased the pH, and application of NH_4NO_3 decreased the pH. Overall, a more basic soil pH was associated with decreased severity of Fusarium wilt. Similar findings were observed with Fusarium wilt of tomato by Woltz and Jones (1973).

In a series of hydroponic growth experiments, Duffy and Défago (1999) demonstrated that the severity of Fusarium crown and root rot of tomato was positively correlated with the concentration of either ammonium sulfate [$(NH_4)_2SO_4$] or NH_4NO_3 in the growth medium.

However, the severity of Fusarium crown and root rot was inversely related to the concentration of ammonium chloride (NH_4Cl). Furthermore, fertilizers that contained calcium nitrate [$Ca(NO_3)_2$] were demonstrated to be suppressive of Fusarium wilt of chrysanthemum (Engelhard and Woltz 1973), cucumber (Jones and Woltz 1975), tomato (Jones and Woltz 1983), and crown and root rot of tomato (Duffy and Défago 1999) relative to other nitrates.

In the context of spinach seed production in the maritime PNW, avoiding further acidification of soil with the addition of ammonium fertilizers could help suppress Fusarium wilt. However, the application of fertilizers with nitrate as the principal N-source would be ineffective in this high rainfall region as nitrates readily leach in soils, and rains are very common in spring and early summer in the maritime PNW. The application of urea-based fertilizers could provide an effective alternative N-source given urea fertilizers cause less change in the soil pH.

1.3.4 Fusarium wilt suppressive soils. Although some soils have been demonstrated to be conducive to Fusarium wilts (e.g., acidic soils in the maritime PNW), other soils have been shown to be suppressive naturally to Fusarium wilts (Alabouvette 1986). Suppressive soils limit the incidence and severity of disease through abiotic and biotic factors, even in the presence of the pathogen and a susceptible host (Alabouvette 1986). The soils in the Châteaurenard region of France are suppressive to multiple Fusarium wilts and, in large part, the soils are believed to reduce severity of various wilts due to the large population of saprophytic Fusaria that have established in soils in that region. Other biological control agents have been demonstrated to suppress Fusarium wilts in other crops, such as fluorescent *Pseudomonas* spp. (van Peer et al. 1991), *Trichoderma* spp. (Larkin and Fravel 1998), chitinolytic bacteria such as *Paenibacillus* spp. and *Streptomyces* spp. (Singh et al. 1999), and other endophytic bacteria (Chen et al. 1995).

It has been hypothesized that large populations of "non-pathogenic" Fusaria or other biological control agents can outcompete pathogens for resources, asymptomatically colonize host root and cortical tissue, and/or activate induced systemic resistance (ISR) in the host (Aimé et al. 2013; Alabouvette et al. 2009; Fravel et al. 2003). Olivain and Alabouvette (1997) demonstrated that a non-pathogenic strain of *F. oxysporum* colonized the outer surface and cortical tissues of tomato seedlings but did not colonize the xylem. Furthermore, tomato plants inoculated with a non-pathogenic *F. oxysporum* isolate had induced defense reactions such as intercellular plugging and deposition of unidentified osmiophilic compounds (substances that take up the stain osmium tetraoxide), which may have blocked potential pathogens from infecting the host. Aimé et al. (2013) demonstrated that tomato plants inoculated with a non-pathogenic *F. oxysporum* isolate had reduced severity of disease when challenged by the causal agent of tomato Fusarium wilt. Furthermore, inoculation with the non-pathogenic *F. oxysporum* isolate induced the host plant to activate three genes implicated in generalized defense responses: extracellular chitinase (*CHI3*), beta-1,3-glucanase (*GLUA*), and a pathogenesis related (PR)-1 protein isoform (*PR-1a*). However, it remains unclear by what pathway or mechanism(s) the non-pathogenic *F. oxysporum* isolates induce defense responses in a host, nor is it well understood how asymptomatic colonizing fungi and bacteria evade the host defense responses they elicit.

1.3.5 Resistant spinach cultivars. In the context of spinach seed production in the maritime PNW, spinach parent lines with greater resistance to Fusarium wilt have been one of the best options for optimizing marketable seed yield when the pathogen is present in a field (Gatch and du Toit 2016). However, there currently are no known immune spinach cultivars, there is limited resistance to Fusarium wilt among parent lines that are available for spinach seed

producers, and seed companies sometimes may not be aware of the degree of susceptibility of a parent line to spinach Fusarium wilt. This limits growers' capacity to select lines with greater resistance to Fusarium wilt. Furthermore, spinach seed growers typically have no choice of selecting parent lines with Fusarium wilt resistance because spinach seed crops are grown on contract with the seed companies that develop the cultivars (Foss and Jones 2005). Fusarium wilt resistance in spinach has been a low priority for spinach breeders given the many other traits breeders have to consider for fresh- and processed-markets: superior agronomic traits (e.g., upright growth habit, increased tolerance to day length to avoid bolting, flavor, vitamin/mineral content, etc.) and resistance to other diseases and pests (Morelock and Correll 2008; Jay Schafer, Schafer Ag Services, *personal communication*). For example, downy mildew caused by *Peronospora effusa*, and white rust caused by *Albugo candida* are the main disease threat to baby leaf spinach producers in the United States and, thus, resistance to these diseases has been the focus of most spinach breeding programs (Correll et al. 1994; Morelock and Correll 2008).

O'Brien and Winters (1977) and Laguna (2000) demonstrated that spinach plant introduction (PI) accessions with partial resistance to Fusarium wilt exist in the USDA germplasm collection. Complete resistance to Fusarium wilt in spinach is not known. The Vegetable Seed Pathology program at the WSU Mount Vernon NWREC is in the process of evaluating >500 spinach germplasm accessions for resistance to Fusarium wilt, including PIs from the USDA National Plant Germplasm System (NPGS), advanced breeding lines from the University of Arkansas spinach breeding program, commercial cultivars, and spinach wild relatives of *S. turkestanica* and *S. tetrandra* (Gyawali et al. 2019). The goal is to identify spinach germplasm with greater resistance to Fusarium wilt that is available currently in commercial hybrids and identify molecular markers associated with Fusarium wilt resistance that can be

utilized in spinach breeding programs for developing cultivars with better resistance to Fusarium wilt. Both association and bi-parental mapping approaches are being employed to map the genetics of Fusarium wilt resistance in spinach.

1.4 *Fusarium oxysporum* pathogenicity and genetics

1.4.1 Host-pathogen interactions. The genetics of pathogenicity of *F. oxysporum* f. sp. *spinaciae* is poorly understood. Research efforts focused to better understand the genetic determinants of host-specificity in other *F. oxysporum* ff. spp. have resulted in the development of molecular tools that are being used to detect the pathogens rapidly (Doan et al. 2014; Lievens et al. 2009; van Dam et al. 2018), differentiate races of a forma specialis rapidly (Fraser-Smith et al. 2014; Lievens et al. 2009), as well as provide information to breeders to aid in the development of resistant cultivars (Schmidt et al. 2016).

1.4.2 The zigzag model of plant-pathogen interactions. One conceptual model that is used to understand how plant pathogens interact with host plants is the zigzag model described by Jones and Dangl (2006). Plants first respond to invasion by pathogens first by sensing microbial-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs), which are conserved chemical signatures produced by microbes and nematodes (e.g., chitin, ergosterol, lipopolysaccharides, flagellin, etc.) (Bittel and Robatzek 2007). For example, flagellin is a bacterial protein that polymerizes to form the flagellum, a structure that aids in movement (Agrios 2005). A group of 22 amino acids at the N-terminal domain of flagellin induces plant defense responses in *Arabidopsis thaliana*, potato, tobacco, and tomato, with flagellin detected by the *A. thaliana* receptor-like plant kinase, FLS2 (Felix et al. 1999; Hayashi et al. 2001). Receptor proteins produced by plant hosts detect epitopes of MAMPs/PAMPs. Typically, the

receptor proteins are transmembrane peptides with extracellular binding domains (Agrios 2005). Upon binding to MAMPs/PAMPs, plant receptor proteins activate defense genes through a signaling cascade that up regulates genes involved in host defense (Boller and Felix 2009; Boller and He 2009). Regulation of defense genes leads to several biochemical and structural changes of the host-cell: production of reactive oxygen species, production of plant hormones (salicylic acid, jasmonic acid, and ethylene), cross-linkage of cell wall proteins, production of phenolics and antimicrobial compounds, and programmed cell death (hypersensitive response) (Agrios 2005; Jones and Dangl 2006). Plant pathogens overcome and/or evade host-defenses by secreting effector proteins, which are small peptides that suppress host defenses and cellular function (Jones and Dangl 2006). Some effector proteins are also avirulence (Avr) factors that are detectable by host resistance (R) genes (Flor 1971), which result in complete resistance to a pathogen.

1.4.3 Genome structure of F. oxysporum. Whole genome sequencing of several closely related *Fusarium* spp. has revealed that *F. oxysporum* f. sp. *lycopersici*, the tomato wilt pathogen, has a genome size of 59.9 Mb, while *F. graminearum*, *F. solani*, and *F. verticillioides* have genomes of 36.2, 54, and 41.7 Mb, respectively (Coleman et al. 2009; Ma et al. 2010). The disparity in genome size among *F. oxysporum* f. sp. *lycopersici* and other *Fusarium* spp. is due to the presence of four lineage specific (LS) chromosomes that are separate from the "core" genome structure which is syntenic among *F. graminearum*, *F. solani*, and *F. verticillioides* (Ma et al. 2010). The core chromosomes are transmitted vertically and carry genes that are necessary to perform basal metabolic functions. The LS chromosomes typically contain large amounts of repetitive DNA and genes involved in pathogenicity (Ma et al. 2010; Schmidt et al. 2013). Pathogen-specific regions of the genome also have been found in other *F. oxysporum* ff. spp.

including *cepae* (Armitage et al. 2018), *cubense* (Guo et al. 2014), and legume-infecting *F. oxysporum* ff. spp. (Williams et al. 2016).

Ma et al. (2010) was the first to demonstrate that LS chromosomes can be transferred horizontally. The transfer of pathogenicity chromosome 14 from a *F. oxysporum* f. sp. *lycopersici* microconidial suspension converted a *F. oxysporum* isolate that was not pathogenic to tomato to become a pathogen of tomato. The conversion from non-pathogenic to pathogenic was mediated by the transfer of host-specific effector genes on chromosome 14 (Ma et al. 2010; Schmidt et al. 2013). The mechanism for LS chromosome transfer is still not understood fully in *F. oxysporum* (Schmidt et al. 2013); however, Mehrabi et al. (2011) suggested that horizontal chromosome transfer between two *F. oxysporum* isolates is mediated by hyphal fusion. Two *F. oxysporum* isolates that belong to the same VCG can form anastomosis tubes between each other and undergo plasmogamy. Although many formae speciales include isolates occupy multiple VCGs and carry similar accessory chromosomes and effector gene profiles (van Dam et al. 2016), which is contradictory since *F. oxysporum* isolates of differing VCGs are incompatible. Interestingly, Shahi et al. (2016) demonstrated that two vegetatively incompatible *F. oxysporum* isolates could form a stable heterokaryon when the isolates were co-cultivated in a carbon-limited medium. The isolates selected were *F. oxysporum* f. sp. *lycopersici* strain 4287 and the tomato non-pathogenic *F. oxysporum* strain 47, commonly referred to as *Fol4287* and *Fo47*, respectively. Although the incidence of heterokaryon formation between *Fol4287* and *Fo47* was limited (1 to 5% depending on growth medium), when heterokaryotic "offspring" were selected, genetic markers specific to *Fol4287* chromosome 14 were found, suggesting the transfer of DNA.

Horizontally transferrable chromosomes are not unique to *F. oxysporum*. Other fungal plant pathogens have been documented to carry LS chromosomes, including *Alternaria alternata* (Akagi et al. 2009), *Colletotrichum gloeosporioides* (Masel et al. 1990), and *Fusarium solani* (Coleman et al. 2009; Ma et al. 2010). Common characteristics among LS chromosomes are the enrichment of transposable elements, the presence of effector genes, and the absence of genes that are necessary for basal metabolism (Schmidt et al. 2013).

1.4.4 Secreted in Xylem *genes.* LS regions of the *F. oxysporum* f. sp. *lycopersici* genome have been found to carry a group of 14 effector genes that have been characterized through biochemical and bioinformatic means, called *Secreted in Xylem* (*SIX*) genes (Rep et al. 2004; Schmidt et al. 2013). The *SIX* genes encode small (<300 amino acids), cysteine rich proteins that were originally found in the xylem of Fusarium wilt-symptomatic tomato plants (Rep et al. 2002; Rep et al. 2004; Schmidt et al. 2013). Three of the 14 known *SIX* genes (*SIX1*, *SIX3*, and *SIX4*) are Avr factors in the *F. oxysporum* f. sp. *lycopersici*-tomato pathosystem (Houterman et al. 2008, 2009; Rep et al. 2004). In the *F. oxysporum* f. sp. *lycopersici*-tomato pathosystem, certain tomato cultivars are resistant to the pathogen monogenically, which is mediated by R genes that are called immunity, or *I,* genes (Takken and Rep 2010). The resistance genes *I, I-2*, and *I-3* have been introgressed into commercial tomato cultivars from closely related *Solanum* relatives, *S. pimpinellifolium* and *S. pennelli*. Consequently, the deployment of resistance genes in commercial tomato cultivars promoted the selection for *F. oxysporum* f. sp. *lycopersici* race 1, race 2 and race 3 (Takken and Rep 2010). Specifically, *SIX4*, *SIX3*, and *SIX1* are recognized by *I, I-2* and *I-3*, respectively. Deletion of *SIX4* in *F. oxysporum* f. sp. *lycopersici* race 1 breaks virulence to *I-2* and *I-3* tomato cultivars, suggesting that *SIX4* suppresses *I-2* and *I-3* mediated resistance, whereas the loss of *SIX4* allows *F. oxysporum* f. sp. *lycopersici* to evade *I*-mediated

defense (Houterman et al. 2008). Furthermore, *I-2* and *I-3* mediated resistance is suppressed by the protein Six4 even when the Avr protein Six3 is detected by the proteins I-2 and I-3. Houterman et al. (2009) demonstrated that a single point mutation in *SIX3* differentiates *F. oxysporum* f. sp. *lycopersici* race 2 from race 3. Two distinct and separate point mutations were found among the race 3 isolates: one population maintained a G to C transversion at base pair 134, which results in a R45 to H substitution, whereas another population maintained a G to C transversion at base pair 137, which results in a R46 to P substitution (Houterman et al. 2009). Both point mutations affected the translated peptide such that the peptide was not recognized by *I-2* tomato cultivars. Furthermore, deletion of *SIX1* (Rep 2005), *SIX3* (Houterman et al. 2009), *SIX5* (Ma et al. 2015), and *SIX6* (Gawehns et al. 2014) from isolates of *F. oxysporum* f. sp. *lycopersici* reduces virulence to tomato either partially or completely

Regions of the genome that contain effector genes are commonly enriched for transposable elements, which most likely maintain diversity and genomic plasticity (Ma et al. 2010; Schmidt et al. 2013). In *F. oxysporum*, sexual reproduction has not been observed. Thus, it is posited that the high transposable element content in the accessory chromosomes maintains variation. Many of the effector genes found in *F. oxysporum* ff. spp. are in the proximity of transposable elements, which are regions of DNA that can change positions in the genome through a variety of mechanisms (Feschotte and Pritham 2007). Eukaryotic transposable elements are divided into three classes: 1) double stranded DNA transposons that are excised from a location in the genome and are reinserted at another point in the genome, commonly referred to as "cut-and-paste" transposons; 2) *Helitron* transposons that move throughout the genome with a single-stranded DNA intermediate (similar to rolling-circle DNA replication); and 3) *Mavericks* which, as the name implies, have a different mechanism of transposition from the former classes,

although the exact mode of transposition is unknown (Feschotte and Pritham 2007). Transposable elements have been used in several studies to identify putative effector genes in *F. oxysporum* (Schmidt et al. 2013; van Dam et al. 2016). Effector genes have been found in association with transposable elements or highly repetitive regions of the genome of other pathogens as well, including *Magnoporthe oryzae* (Chuma et al. 2011), *Leptosphaeria maculans* (Rouxel et al. 2011), and *Phytophthora infestans* (Raffaele et al. 2010). The importance of transposable elements among fungal genomes includes that they may confer genome plasticity, by facilitating intrachromosomal recombination. For example, the process of selection of different races of *F. oxysporum* f. sp. *lycopersici* may be facilitated by recombination of transposable elements surrounding effector genes. Biju et al. (2017) proposed that *F. oxysporum* f. sp. *lycopersici* races 2 and 3 were generated through recombination of *Helitron* (*HelA* and *HelB*) transposons flanking the 5' and 3' region of the gene *SIX4*, which ultimately resulted in a deletion of the *SIX4* locus. Further analysis confirmed that *F. oxysporum* f. sp. *lycopersici* races 2 and 3 are comprised of 2 groups: each group contained a specific genomic deletion that surrounded the region of DNA proximal to *SIX4*.

1.4.5 SIX *genes and their association with* F. oxysporum *ff. spp.* The "effectorome," or the combination of effector genes of a given *F. oxysporum* f. sp., has been implicated as a determinant of host specificity (Lievens et al. 2009; van Dam et al. 2016). Interestingly, unique profiles of the *SIX* genes have been found in *F. oxysporum* ff. spp. other than *F. oxysporum* f. sp. *lycopersici*, including *canariensis*, *cepae*, *ciceris*, *conglutinans*, *cubense*, *fragariae*, *lini*, *melonis*, *niveum*, *palmarum*, *physali*, *pisi*, *radicis-cucumerinum*, *raphani*, *vasinfectum*, *zingiberri* (Czilowski et al. 2017; Laurence et al. 2015; Lievens et al. 2009; Meldrum et al. 2012; Simbaqueba 2017; Taylor et al. 2016; Thatcher et al. 2012; van Dam et al. 2016; Williams et al.

2016). Taylor et al. (2016) demonstrated that isolates of the causal agent of onion basal rot, *F. oxysporum* f. sp. *cepae,* had a *SIX* gene profile that was consistent among 18 of 21 highly virulent isolates, i.e., *SIX3, SIX5, SIX7, SIX9, SIX10, SIX12,* and *SIX14*. However, three *F. oxysporum* f. sp. *cepae* isolates that were less virulent on onion carried fewer or no *SIX* genes. It is still unclear what function *SIX* genes play in host-specificity, and how the protein products of *SIX* genes are involved in pathogenicity. However, it is known that some *SIX* genes contribute to virulence in other *F. oxysporum* ff. spp. as well. For example, Thatcher et al. (2012) demonstrated that *F. oxysporum* f. sp. *conglutinans,* the causal agent of Fusarium wilt of *Brassica* spp., carries *SIX4*. Deletion of *SIX4* in *F. oxysporum* f. sp. *conglutinans* reduced disease severity in *Arabadopsis* plants, and complementation of *SIX4*-deletion mutants restored virulence. Furthermore, Widinugraheni et al. (2018) found that deletion of *SIX1a* from *F. oxysporum* f. sp. *cubense* tropical race 4 significantly reduced virulence of the isolate on the banana cultivar Cavendish, and complementation of *SIX1a* restored virulence to *F. oxysporum* f. sp. *cubense.* Interestingly, several *F. oxysporum* ff. spp. share homologous *SIX* genes. Taylor et al. (2016) demonstrated that *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* ff. spp. *cepae, cubense*, *dianthi*, *lini*, and *narcissi* all carried *SIX7*, albeit with varying DNA sequences. Moreover, *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* ff. spp. *cepae*, *dianthi*, *freesia*, *lini*, *narcissi*, and *pisi* race 1 all carried the homolog *SIX12*. Overall, it is unclear whether the function of the *SIX* homologs are conserved amongst formae speciales.

F. oxysporum f. sp. *spinaciae* has not been the focal point of the extensive genetic studies involving *F. oxysporum* ff. spp. such as *cepae* (Taylor et al. 2016), *cubense* (Czislowski et al. 2017), *ciceris* and *pisi* (Williams et al. 2016), *lycopersici* (Rep et al. 2004; Lievens et al. 2009), and *melonis* (van Dam et al. 2016). However, the discovery of *SIX* genes in *F. oxysporum* f. sp.

lycopersici lead to several studies that attempted to characterize the *SIX* gene profile of many other *F. oxysporum* ff. spp. Lievens et al. (2009) assessed the *SIX* gene profile of 15 *F. oxysporum* ff. spp. and three other *Fusarium* spp., including *F. oxysporum* f. sp. *spinaciae*, for *SIX1* to *SIX7*. *SIX*1 to *SIX7* was not detected among the *F. oxysporum* f. sp. *spinaciae* isolates that were included in the sample of formae speciales. Covey et al. (2014) reported similar findings when attempting to detect *SIX1* and *SIX6* in each of three *F. oxysporum* f. sp. *spinaciae* isolates, as no *SIX* genes were detected. When Lievens et al. (2009) first reported the absence of *SIX1* to *SIX7* in *F. oxysporum* f. sp. *spinaciae*, the other seven *SIX* genes were not publicly known. It is unclear why Covey et al. (2014) decided to test for the presence of only *SIX1* and *SIX6* in three representative *F. oxysporum* f. sp. *spinaciae* isolates, given that all 14 *SIX* genes were known at the time that work was published.

1.4.6 Expression of SIX *genes.* The *SIX* genes are expressed selectively *in planta* by isolates of *F. oxysporum* (Michielse et al. 2009; Taylor et al. 2016) and are not required for colonization of their respective host (Michielse et al. 2009). *In planta* expression, specifically in the xylem, is likely in response to endogenously produced host signals such as MAMPs, PAMPs, and damage associated molecular patterns (DAMPs). However, it is unclear what specific signals trigger the expression of *SIX* genes. There are several regulatory elements implicated in the upregulation of *SIX1* in *F. oxysporum* f. sp. *lycopersici* (Michielse et al. 2009; van der Does et al. 2016). Interestingly, the transcription factors that upregulate *SIX1* are encoded in both the core and accessory chromosomes, suggesting that regulation of *SIX1*, is at least, controlled by regulatory elements that are transferred horizontally and vertically (van der Does et al. 2016). A transcription factor that is only found in the core region of *F. oxysporum* f. sp. *lycopersici*, *SIX Gene Expression 1* (*SGE1*), regulates at least *SIX1* to *SIX5* (Michielse et al. 2009). *F. oxysporum*

f. sp. *lycopersici* isolates with *SGE1* deletions did not express *SIX1* to *SIX5 in planta*. Furthermore, deletion of *SGE1* broke pathogenicity of *F. oxysporum* f. sp. *lycopersici* to susceptible tomato cultivars (Michielse et al. 2009). Deletion of *SGE1* may have pleiotropic effects on the fungus or may affect the regulation of other genes involved in pathogenicity. However, deletion of *SGE1* did not prevent *F. oxysporum* f. sp. *lycopersici* from infecting the host roots, suggesting that *SGE1* controls the expression of effectors and other genes involved in pathogenicity post-infection (Michielse et al. 2009).

1.4.7 SIX *genes in environmental* F. oxysporum *isolates.* Although the presence of *SIX* genes has been implicated in pathogenicity in multiple *F. oxysporum* ff. spp., the presence of *SIX* genes alone may be insufficient to determine host-range of a given isolate. Jelinski et al. (2017) obtained 74 *F. oxysporum* isolates from soil with a history of tomato Fusarium wilt and found that two of the 74 isolates carried a *SIX* gene profile that matched that of *F. oxysporum* f. sp. *lycopersici* race 2. The two isolates did not induce symptoms of wilt in tomato cultivars susceptible to *F. oxysporum* f. sp. *lycopersici* race 2. Jelinski et al. (2017) proposed several hypotheses to explain why isolates that had the core race 2 or 3 *SIX* profile were non-pathogenic on tomato: i) genetic regulatory elements, such as transcription factors, which are crucial for the expression of genes necessary for pathogenicity, may be absent or nonfunctional; ii) other genes necessary for pathogenicity may be absent, such as genes required for colonization or other *SIX* genes that were not assayed; or iii) the unsuccessful transfer of genetic material resulted in a non-functional effector suite.

Tesdall et al. (2017) demonstrated that endophytic *F. oxysporum* isolates collected from asymptomatic plants of banana cultivars susceptible to *F. oxysporum* f. sp. *cubense* tropical race 4, carried *SIX* gene profiles similar to that of pathogenic strains. Specifically, 11 and 15 of the

103 endophytic *F. oxysporum* strains had *SIX2* and *SIX9*, respectively. Both *SIX2* and *SIX9* are present in isolates of *F. oxysporum* f. sp. *cubense* tropical race 4. Of the 15 isolates that carried *SIX9*, 12 had a *SIX9* homolog that was distinct phylogenetically from that of *F. oxysporum* f. sp. *cubense* tropical race 4, while the other three endophytic strains with *SIX9* had an identical sequence match with the homolog from *F. oxysporum* f. sp. *cubense* tropical race 4 (Tesdall et al. 2017).

1.5 Research needs for spinach Fusarium wilt.

Fusarium wilt of spinach is a recalcitrant disease that is the most economically important biological limitation to spinach seed production in the maritime PNW (Foss and Jones 2005). The causal agent, *F. oxysporum* f. sp. *spinaciae*, is difficult to control as the fungus can survive in soils for >15 years in this region. Spinach seed growers have little recourse to control this disease since all economically viable management practices only partially and transiently suppress the disease. Furthermore, there is limited capacity for spinach seed growers to use resistant cultivars that would facilitate shorter rotation intervals than 10 to 15 years and, potentially, increase the carrying capacity for spinach seed crops in the United States.

Previous studies on the association of *SIX* genes with other *F. oxysporum* ff. spp. suggest that formae speciales can be differentiated by unique effector profiles (Lievens et al. 2009; Taylor et al. 2016; van Dam et al. 2016). Furthermore, the *SIX* genes that are shared among formae speciales differ based on DNA sequences, which could be useful for developing molecular markers for detection of the target pathogen(s). It is currently unknown whether *F. oxysporum* f. sp. *spinaciae* has a unique *SIX* gene profile that can be differentiated from those of other formae speciales.

Overall, research on the genetics behind host-specificity of *F. oxysporum* f. sp. *spinaciae* to spinach has been limited. By determining what differentiates this pathogen genetically from other *F. oxysporum* ff. spp. isolates, it may be possible to provide a framework for understanding host-specificity of this pathogen. This will help with the development of molecular tools to detect and quantify *F. oxysporum* f. sp. *spinaciae* rapidly, as well as provide valuable information for breeders to develop cultivars with greater resistance to spinach Fusarium wilt than is available currently to growers. The following objectives were the focus of this research:

1. Determine the *Secreted in Xylem* gene profile of *F. oxysporum* f. sp. *spinaciae*. This objective was addressed by the following:
    a. *F. oxysporum* isolates from a variety of sources were characterized for pathogenicity to spinach; and
    b. The *F. oxysporum* isolates also were characterized for presence or absence of the 14 known *SIX* genes.
2. Identify other genomic loci unique to *F. oxysporum* f. sp. *spinaciae* among the formae speciales of *F. oxysporum*. This was accomplished by:
    a. Sequencing and assembling the genomes of seven *F. oxysporum* f. sp. *spinaciae* isolates and five *F. oxysporum* isolates that were non-pathogenic on spinach but were associated with spinach, spinach seed, or soil in which spinach had been grown; and
    b. Identifying genetic features such as putative pathogenicity genes, housekeeping loci, and structural elements of the genome that differentiate *F. oxysporum* f. sp. *spinaciae* from other *F. oxysporum* isolates.

The goal of this project is to provide spinach seed growers and seed companies with tools and information to improve the ability to manage spinach Fusarium wilt. The outcomes of this research are identification of genetic loci that differentiate *F. oxysporum* f. sp. *spinaciae* from other *F. oxysporum* ff. spp. and non-pathogenic isolates of *F. oxysporum* to identify what functionally determines host-specificity of *F. oxysporum* f. sp. *spinaciae* to spinach. Ultimately, by understanding what defines *F. oxysporum* f. sp. *spinaciae* genetically as a pathogen of spinach, it will be possible to enhance management strategies for spinach Fusarium wilt.

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CHAPTER TWO

PATHOGENICITY AND THE *SECRETED IN XYLEM* GENE PROFILE OF THE CAUSAL AGENT OF SPINACH FUSARIUM WILT

2.1 Introduction

The global demand for spinach has increased dramatically since the mid-1980s when pre-packaged and triple-rinsed baby-leaf spinach became readily accessible to consumers [United States Department of Agriculture (USDA) Economic Research Service (ERS) 2007]. Consumption of fresh-market spinach in the United States quintupled from 0.2 to 1.0 kg/capita from 1980 to 2007, respectively. The increase in spinach consumption in the United States has demanded greater acreage of baby-leaf spinach crops, which are produced primarily in California, Arizona, Texas, New Jersey, and Florida [USDA National Agricultural Statistics Service (NASS) 2017]. Commercial production of baby-leaf spinach is intensive, with short cropping intervals of 18 to 45 days, seeding rates up to 9 million seed/ha and, often, multiple plantings in the same field in the same season (Koike et al. 2011). Consequently, the market demand for fresh and processed spinach necessitates a reliable source of high quality spinach seed. Although spinach is produced as a vegetable crop in many locations in the United States, spinach seed production is limited to the maritime Pacific Northwest (PNW) due to the stringent climatic requirements necessary to produce high quality seed (Foss and Jones 2005). Specifically, spinach seed can be produced only in locations around the globe that have a long summer day length necessary to trigger spinach plants to convert from vegetative to reproductive growth (bolting), mild temperatures for this heat-sensitive species, and low humidity or rainfall

for optimal seed quality and seed health. Spinach seed produced commercially in the maritime PNW region of the United States (western Washington and western Oregon) accounts for up to 20% of the global supply of spinach seed (Foss and Jones 2005).

Despite the optimal climatic conditions for spinach seed production in the maritime PNW, the acidic soils in this region are highly conducive to spinach Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *spinaciae* (Foss and Jones 2005). In these conducive soils, the pathogen is persistent, often surviving >10 years, which necessitates long spinach seed crop rotations (du Toit and Ocamb 2018). In some fields, crop rotations >15 years have been insufficient to avoid the disease, with crop losses up to 100% (Gatch 2013). Historically, spinach seed growers in the maritime PNW have avoided fields with a risk to spinach Fusarium wilt by planting spinach seed crops on land that had not previously been planted with spinach; however, land without a history of spinach or spinach seed production in this region is rare after ~100 years of spinach seed production (Gatch 2013).

Several management strategies are used to address Fusarium wilt as a limitation to spinach seed production in the maritime PNW. A soil bioassay for spinach Fusarium wilt is offered annually to seed growers by Dr. Lindsey du Toit's program at the Washington State University (WSU) Mount Vernon Northwestern Washington Research & Extension Center, to quantify the risk of Fusarium wilt using soil sampled from fields being considered for spinach seed production that spring (Gatch and du Toit 2015). This tool has been effective at helping growers and seed companies select fields with low risk of Fusarium wilt to plant spinach seed crops. In the event a field has some risk of Fusarium wilt, spinach seed growers have several practices they can implement to reduce the severity of the disease such as adding agricultural limestone to the soil prior to planting to increase soil pH and calcium, which reduce the

conduciveness of the soil to the disease (Gatch and du Toit 2017); application of composts or other amendments to fields to improve soil health (du Toit et al. 2014); biofumigation with a *Brassica* seed meal or *Brassica* cover crops (du Toit et al. 2006); and application of fungicides, particularly those with active ingredients in the Fungicide Resistance Action Committee (FRAC) Groups 1 or 3 (benzimidazoles or triazoles, respectively) as seed treatments or as soil treatments (du Toit et al. 2014). However, these economically viable management practices for spinach Fusarium wilt only partially and/or transiently suppress the disease in such naturally and highly conducive soils. Spinach cultivars are available that have partial resistance to Fusarium wilt (Laguna 2000; O'Brien and Winters 1977), but many cultivars are highly susceptible and spinach seed growers typically have no choice of the parent lines they are contracted by seed companies to plant. Therefore, to improve spinach seed yields in the maritime PNW, it is necessary to develop additional management strategies for Fusarium wilt.

The fungus *F. oxysporum* is a diverse species complex comprised of isolates that are non-pathogenic saprobes, biocontrol agents (Alabouvette et al. 2009), endophytes of plants (Gordon and Martyn 1997), human pathogens (Nucci and Anaissie 2002), and plant pathogens. Plant pathogenic isolates of *F. oxysporum* are grouped into >120 host-specific formae speciales, each of which cause disease on a single host or a narrow range of hosts that are closely related (Leslie and Summerell 2006). For example, some isolates of *F. oxysporum* f. sp. *spinaciae* were demonstrated to cause disease on spinach as well as beet and swiss chard, which are also in the Amaranthaceae (Armstrong and Armstrong 1976; Naiki and Kano 1977). Likewise, *F. oxysporum* ff. spp. *cucumerinum* and *niveum* were reported to cause wilts on multiple *Cucurbita* spp. (Cafri et al. 2005; Zhou and Everts 2007). Efforts to distinguish pathogenic *F. oxysporum* ff. spp. from non-pathogenic *F. oxysporum* isolates morphologically have fallen short as these

isolates typically are indistinguishable (Leslie and Summerell 2006). Furthermore, many *F. oxysporum* ff. spp. are polyphyletic and cannot be differentiated genotypically based on multilocus DNA sequence haplotypes or DNA sequence variation among housekeeping genes and non-coding loci (Baayen et al. 2000; O'Donnell et al. 2009). Pathogenicity tests have remained the standard for phenotypic identification of isolates of particular *F. oxysporum* ff. spp. (Leslie and Summerell 2006).

Recent advances in next generation sequencing and genomic studies of various *F. oxysporum* ff. spp. have improved our conceptual understanding of genetic similarities among formae speciales (Armitage et al. 2018; Ma et al. 2010; van Dam et al. 2016). Whole genome sequencing of several closely related *Fusarium* spp. revealed that *F. oxysporum* f. sp. *lycopersici*, the causal agent of Fusarium wilt of tomato, has a genome of 59.9 Mb, while *F. graminearum*, *F. solani*, and *F. verticillioides* have genomes of 36.2, 54.0, and 41.7 Mb, respectively (Coleman et al. 2009; Ma et al. 2010). The larger genome size of *F. oxysporum* f. sp. *lycopersici* compared to other *Fusarium* spp. is due, primarily, to the presence of four lineage specific chromosomes in *F. oxysporum* f. sp. *lycopersici* that are distinct from the core chromosomes. The core genome includes chromosomes that are syntenic among *F. graminearum*, *F. solani*, and *F. verticillioides* (Ma et al. 2010). The core chromosomes encode genes necessary to perform basal metabolic functions, while the lineage specific chromosomes are highly repetitive and encode genes required for pathogenicity and host-specificity to tomato (Ma et al. 2010; Schmidt et al. 2013). Furthermore, the transfer of one lineage specific chromosome, chromosome 14, from *F. oxysporum* f. sp. *lycopersici* to a non-pathogenic *F. oxysporum* isolate converted that recipient isolate to a pathogen of tomato (Ma et al. 2010).

The lineage specific chromosomes of *F. oxysporum* f. sp. *lycopersici* encode a group of 14 genes known as *Secreted in Xylem* (*SIX*) genes, which are translated into proteins that are secreted by *F. oxysporum* f. sp. *lycopersici* into the xylem of colonized tomato plants (Rep 2005; Rep et al. 2004; Schmidt et al. 2013). The proteins are relatively small (<300 amino acids) and rich in cysteine residues (Schmidt et al. 2013). Deletion of *SIX1* (Rep 2005), *SIX3* (Houterman et al. 2009), *SIX5* (Ma et al. 2015), and *SIX6* (Gawehns et al. 2014) from isolates of *F. oxysporum* f. sp. *lycopersici* reduced virulence to tomato either partially or completely, demonstrating their role in pathogenicity of the fungus to tomato. Furthermore, the three known races of *F. oxysporum* f. sp. *lycopersici* are differentiated by the genes *SIX1*, *SIX3*, and *SIX4*, each of which is an avirulence gene (also referred to as *Avr3*, *Avr2*, and *Avr1*, respectively). *SIX1*, *SIX3*, and *SIX4* encode proteins that are detected by proteins encoded by resistance genes in certain tomato cultivars, also known as immunity or *I* genes. The *I* genes that correspond to *SIX1*, *SIX3*, and *SIX4* in *F. oxysporum* f. sp. *lycopersici* were discovered among wild *Solanum* relatives of tomato (Gerdemann and Finley 1951; McGrath et al. 1987), and have been introgressed into commercial breeding lines of tomato to confer resistance to the three races of *F. oxysporum* f. sp. *lycopersici* (Catanzariti et al. 2015; Simons et al. 1998).

Subsequent to the discovery and characterization of the *SIX* genes in *F. oxysporum* f. sp. *lycopersici*, many other formae speciales have been demonstrated to have *SIX* genes, e.g., *canariensis*, *cepae*, *ciceris*, *conglutinans*, *cubense*, *fragariae*, *lini*, *melonis*, *niveum*, *palmarum*, *physali*, *pisi*, *radicis-cucumerinum*, *raphani*, *vasinfectum*, and *zingiberri* (Czilowski et al. 2017; Laurence et al. 2015; Lievens et al. 2009; Meldrum et al. 2012; Simbaqueba et al. 2018; Taylor et al. 2016; Thatcher et al. 2012; van Dam et al. 2016; Williams et al. 2016). Lievens et al. (2009) first demonstrated that the profile of *SIX* genes had the potential to differentiate among *F.*

oxysporum ff. spp. as well as distinguish isolates of these ff. spp. from non-pathogenic isolates. Furthermore, Taylor et al. (2016) demonstrated that *SIX3*, *SIX5*, *SIX7*, *SIX9*, *SIX10*, *SIX12*, and *SIX14* were present in highly virulent isolates of *F. oxysporum* f. sp. *cepae*, the causal agent of Fusarium basal rot in onion, while isolates with diminished virulence to onion had fewer *SIX* genes or none at all. The profiles of *SIX* genes present in *F. oxysporum* f. sp. *cepae* isolates differentiated the pathogenic isolates from non-pathogenic isolates of *F. oxysporum* associated with onion plants, and differentiated isolates of this forma specialis from those of eight other formae speciales evaluated in that study. Similarly, Czislowski et al. (2018) demonstrated that *SIX* gene profiles in the causal agent of Fusarium wilt of banana, *F. oxysporum* f. sp. *cubense*, were associated strongly with different races and vegetative compatibility groups (VCG) of the pathogen. The *SIX* genes also have been demonstrated to play a role in virulence of the causal agent of Fusarium wilt of banana (Widinugraheni et al. 2018), and for the causal agent of Fusarium wilt of *Brassica* spp., *F. oxysporum* f. sp. *conglutinans* (Thatcher et al. 2012).

Despite these recent advances in identifying common genetic markers and different profiles of these markers among formae speciales, Jelinski et al. (2017) demonstrated that the profile of *SIX* genes may not be a sufficient predictor of host specificity. They obtained *F. oxysporum* isolates from the soil of commercial tomato fields that were demonstrated not to be pathogens of tomato but carried partial or full profiles of the 14 known *SIX* genes. Two of 74 isolates they collected did not induce symptoms of Fusarium wilt in tomato cultivars susceptible to *F. oxysporum* f. sp. *lycopersici* race 2, but these two isolates had an identical *SIX* gene profile to *F. oxysporum* f. sp. *lycopersici* race 2. This indicated that the *SIX* genes alone do not determine pathogenicity to tomato.

As of 2018, there was a dearth of publicly available research on the genetics of *F. oxysporum* f. sp. *spinaciae*, nor was it known what *SIX* genes or other effector genes are associated with pathogenicity of *F. oxysporum* f. sp. *spinaciae* to spinach (Batson et al. 2018). Fiely et al. (1995) demonstrated three VCGs present among *F. oxysporum* f. sp. *spinaciae* isolates collected from a variety of geographic locations. Isolates in VCGs 0330 and 0332 induced greater severity of wilt on the spinach cultivar Grandstand than isolates in VCG 0331 (Fiely et al. 1995). Subsequently, *F. oxysporum* f. sp. *spinaciae* has been described as polyphyletic with respect to two loci, the *translation elongation factor-1α* (*TEF-1α*) and the intergenic spacer (IGS) region of ribosomal DNA (rDNA). Based on these two loci, representative isolates of *F. oxysporum* f. sp. *spinaciae* in VCGs 0330 and 0332 grouped together while an isolate belonging to VCG 0331 was in a separate clade (O'Donnell et al. 2009). A few studies have included *F. oxysporum* f. sp. *spinaciae* isolates in screens for *SIX* genes, but the *F. oxysporum* f. sp. *spinaciae* isolates were not the focal point of those investigations. For example, Lievens et al. (2009) included three *F. oxysporum* f. sp. *spinaciae* isolates in a genotypic screen for *SIX1* to *SIX7* in 255 isolates of 16 formae speciales and 15 non-pathogenic *F. oxysporum* isolates, none of which was detected in the three *F. oxysporum* f. sp. *spinaciae* isolates. At that time, only *SIX1* to *SIX7* were known. Later, Covey et al. (2014) screened *F. oxysporum* ff. spp. *betae* and *spinaciae* for *SIX1*, *SIX6*, and other loci that they used to genotype these two formae speciales. Neither *SIX1* nor *SIX6* was detected in the three *F. oxysporum* f. sp. *spinaciae* isolates evaluated. Thus, there was no publicly available record of the presence or association of *SIX* genes or other putative pathogenicity genes with the causal agent of spinach Fusarium wilt. By identifying effector genes associated with *F. oxysporum* f. sp. *spinaciae*, it should be possible to understand more about the mechanisms of host-pathogen interactions,

develop molecular tools to detect the pathogen rapidly and accurately, identify markers associated with resistance to the pathogen, and aid spinach breeders at breeding for resistance to Fusarium wilt.

The objectives of this study were to characterize isolates of *F. oxysporum* f. sp. *spinaciae* phenotypically and genotypically to determine if the causal agent of spinach Fusarium wilt has *SIX* genes, and if so, if there is a unique profile of *SIX* genes that differentiates isolates of this pathogen from those of other *F. oxysporum* ff. spp. and non-pathogenic isolates. The presence/absence profile of the 14 known *SIX* genes of *F. oxysporum* f. sp. *spinaciae* was compared to that of other *F. oxysporum* ff. spp. with known *SIX* gene profiles. Furthermore, *Fusarium* isolates found in association with spinach but that were not pathogenic to spinach were included in the genotypic and phenotypic comparisons to help identify genotypic characteristics unique to isolates of the spinach pathogen.

2.2 Materials and Methods

2.2.1 Phenotypic characterization of F. oxysporum *isolates.* Six pathogenicity tests were completed from April 2017 to May 2018 to evaluate a total of 69 *Fusarium* isolates (Table 2.1) from a larger collection of >700 *Fusarium* isolates in Dr. Lindsey du Toit's program, for pathogenicity to each of three spinach inbred lines that differ in susceptibility to Fusarium wilt. The number of treatment combinations of the parent line and isolate was prohibitively large to assess in a single pathogenicity test; thus, isolates were assessed for pathogenicity over six tests. Cultures of *Fusarium* were contributed to this work by Dr. Jim Correll (University of Arkansas), Dr. Frank Dugan (USDA ARS), Dr. Tom Gordon (University of California Davis), Dr. Linda Hanson (USDA ARS), Dr. Sierra Hartney (Sakata Seed America), and Dr. Howard Schwartz

(Colorado State University). Initially, the isolates were screened for pathogenicity to spinach by drenching the roots of each parent line with a spore suspension of the isolates as detailed below. However, the inoculation method was modified three times to improve the capacity to evaluate pathogenicity quantitatively. In pathogenicity test 1, the spore suspension was drenched over the root plugs of plants growing in 6-cell packs, with a root plug volume of 40 ml/cell and one plant per cell. For pathogenicity tests 2 to 5, the plants were grown in 6-cell packs that had a root plug volume of 120 ml/cell to reduce the issue of plants becoming root-bound in the small plugs used in the first pathogenicity test. For pathogenicity test 6, the inoculation method was changed to planting seed of each spinach line into potting medium that had been amended with the spore suspension. The modifications increased the uniformity of wilt development among replicate plants receiving the same treatment, and among repeats of the test. Confirmed pathogenic isolates of *F. oxysporum* ff. spp. *cepae* (pathogen of onion), *ciceris* (chickpea), and *pisi* (pea) were evaluated along with *Fusarium* isolates that were putative ff. spp. *betae* (beet), *cepae* (onion), and *phaseoli* (bean). Isolates designated as putative ff. spp. had not been verified for pathogenicity on the original host plants by the Vegetable Seed Pathology program at Washington State University. Pathogenicity test 1, completed in April 2017, as well as previous research with these isolates demonstrated that Fus187 was not a pathogen of spinach while Fus254 was a pathogen of spinach. The two isolates were selected as negative and positive *F. oxysporum* control treatments, respectively, for all subsequent pathogenicity tests. A water treatment was also included in each pathogenicity test as a negative control treatment to account for wilting symptoms that might result from factors not related to pathogenicity of the *Fusarium* isolates (e.g., moisture stress from plants becoming root-bound in the smaller 6-pack cells used in pathogenicity test 1).

The spinach plants were rated weekly after inoculation using an ordinal scale of 0 to 5 (0 = no wilt symptoms observed, 1 = first pair of true leaves wilted, 2 = first and second pairs of true leaves wilted, 3 = first through third pairs of true leaves wilted, 4 = all above-ground tissue flaccid, 5 = plant dead; Fig. 2.1). The weekly ratings for all six inoculated seedlings/replication were then converted to a Fusarium wilt severity index (%) (see below). The area under the disease progress curve (AUDPC) was calculated using the trapezoidal method (Sparks et al. 2008) from 0 to 42 days after inoculation. The above-ground biomass of each spinach plant was harvested anywhere from 28 to 42 days after inoculation, depending on the test, by cutting each plant at the base of the stem. All plants for each replication of a treatment combination were dried in a paper bag at 65°C for 72 h, and the dried biomass measured.

2.2.1.1 Pathogenicity test 1. *Fusarium* isolates were cultured at room temperature (23 ± 1°C) under ambient light on half-strength potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) amended with 100 ppm chloramphenicol. A microconidial suspension of each isolate was prepared by inoculating Kerr's broth (Kerr 1963), a nutrient-limited broth that promotes production of microconidia. The Kerr's broth was prepared as follows: 60 g sucrose and 1 g of yeast extract were suspended in 1 liter deionized water to which 20 ml of each of the following stock solutions was added: 20 g $NaNO_3$/100 ml deionized water, 10 g KH_2PO_4/100 ml deionized water, 5 g KCl/100 ml deionized water, 5 g $MgSO_4 \bullet 7H_2O$/100 ml deionized water, and 0.183 g $FeSO_4 \bullet 7H_2O$/100 ml deionized water. The volume was increased to 1 liter using deionized water. A 50 ml aliquot of the broth was placed in a 125 ml Erlenmeyer flask for each isolate. The broth was autoclaved for 20 minutes (121°C and 124 kPa). Each flask of Kerr's broth was then inoculated with three agar plugs (each 1 mm^3) excised from the leading edge of hyphal growth of a 5-d-old culture on half-strength PDA. The inoculated broth was incubated at

room temperature under ambient light on a gyrotary shaker at 125 rpm for 7 d. Each microconidial suspension was then filtered through one layer of cheesecloth, followed by a second filtration through two layers of cheesecloth, the spore concentration quantified with a hemocytometer, and the suspension diluted to 1 x 10^5 spores/ml deionized water.

Spinach plants of three proprietary, female, inbred lines differing in resistance to Fusarium wilt (highly susceptible, moderately susceptible, and partially resistant) were used for the pathogenicity tests. Spinach seed of each parent line were planted in RediEarth Propagation Mix (SunGro Horticulture, Agawam, MA) in 72-cell flats (TLC Polyform, Inc., Salem, OR). Each 72-cell flat was then cut into rows of six cells. The root plugs in each of four replicate rows of six cells (= six seedlings/row) per parent line were drenched with the microconidial suspension of the appropriate *Fusarium* isolate or water (5 ml/seedling root plug) 12 days after planting (DAP). The experiment was maintained in a greenhouse with a 9 h day:15 h night cycle and a temperature cycle of 28°C by day:24°C by night for 28 days. The pathogenicity test was set up as a randomized complete block design (RCBD) with a 3 x 15 factorial design of three spinach lines and 15 inoculation treatments (14 fungal isolates and the water control treatment), and four replicate blocks. The plants were fertigated daily with water into which 20:20:20 fertilizer (N:P:K) (Plant Marvel, Chicago, IL) was injected at a ratio of 1:100 for a final concentration of 200 ppm N.

2.2.1.2 Pathogenicity tests 2 to 5. For each of pathogenicity tests 2 to 5, the inoculation protocol, disease ratings, and spinach biomass measurements were completed as described for pathogenicity test 1 with the modifications detailed below to increase uniformity of results among cells of the same replicate block. Spinach plants were grown in larger-sized 6-cell packs (5.7 cm x 5.7 cm x 7.6 cm per cell) (The HC Companies, Twinsburg, OH) with an approximate

volume of 120 ml/cell (triple the volume of the 6-cell-packs used in pathogenicity test 1). Accordingly, the amount of inoculum drenched over the root plugs of each spinach plant 12 DAP was tripled to 15 ml of 1 x 10^5 spores/ml (the final concentration of spores per volume of propagation mix was equivalent to that used in pathogenicity test 1). Inoculum was applied to each cell with a pipette for pathogenicity tests 2 and 3, and with an Omnispense Elite automatic dispenser (Wheaton Science Products, Millville, NJ) for pathogenicity tests 4 and 5 to increase the speed and uniformity of application.

2.2.1.3 Comparison of root drench and pre-plant soil inoculation methods. Due to issues with greater variability than desired in development of spinach Fusarium wilt symptoms across cells within replicate blocks and among blocks for individual *Fusarium* isolates tested, and relatively limited severity of disease for the positive control treatment in some cells for pathogenicity tests 1 to 5, an alternative inoculation method was evaluated in which the plant propagation mix was amended with the inoculum prior to planting spinach seed. A trial was set up to determine if the pre-plant soil inoculation method yielded more homogeneous disease severity for each inoculation treatment compared to the root drench inoculation method, and to compare wilt severity and spinach growth (dry biomass) using the two inoculation methods. Inoculum was prepared and enumerated as described for pathogenicity tests 2 to 5. For the root drench inoculation treatment, spinach plants of the highly susceptible parent line were inoculated as described for pathogenicity tests 2 to 5. For the pre-plant soil inoculation method, 270 ml of inoculum (1 x 10^5 spores/ml) were added to 2.2 liters of RediEarth for the same final concentration of 12,500 spores/ml propagation mix for both inoculation methods. The inoculated RediEarth was mixed vigorously in a 4-liter plastic bag for 2 min. A 120 ml aliquot of the inoculated mix was then placed in each cell of a two-by-three 6-cell planter pack (5.7 cm x 5.7

cm x 7.6 cm height per cell). Two seeds of the highly susceptible spinach parent line were sown into each cell at a depth of 1 cm and thinned to one plant/cell, if two plants emerged. Plants were rated for disease severity weekly as described previously. Since the plants inoculated using the root drench method were 12 days old at the time of inoculation, whereas seed was planted into infested soil for the pre-plant soil inoculation method, a standard comparison of dry spinach biomass was inappropriate to compare the two inoculation methods. Thus, a biomass ratio (ρ) was assessed for each inoculation method, where the dry biomass/plant (β) of the i^{th} inoculation method, j^{th} isolate, and k^{th} replicate block was divided by β of the i^{th} inoculation method, the control, and k^{th} replicate block.

$$\rho = \frac{\beta_{i,j,k}}{\beta_{i,control,k}}$$

2.2.1.4 Pathogenicity test 6. Microconidial suspensions of 12 *Fusarium* isolates were produced, harvested, and enumerated as described for pathogenicity tests 2 to 5, and applied using the pre-plant soil inoculation method (Table 2.1). For this test, the spinach plants had to be grown in two Percival Incubators (Series 101) (Percival Scientific Inc., Perry, IA) as the daylength in western Washington in June 2018, when this experiment was started, would have caused the plants to bolt in the greenhouse. Only the highly susceptible parent line was used in this pathogenicity test due to the limited area available in the growth chambers. The photoperiod in each incubator was set to 9 h by day and 15 h by night. For the first 10 days, the temperature was set to 22°C by day and 18°C by night. After 10 days, the temperature was raised to 28°C day and 24°C night. The experimental design for pathogenicity test 6 was the same as pathogenicity tests 1 to 5. Twelve isolates were tested for pathogenicity on the highly susceptible parent line, which included the positive control isolate Fus254 and the negative control isolate Fus187. The

plants for each block were placed on a different shelf of an incubator, with two blocks in each incubator. Severity of Fusarium wilt was rated weekly, and the plants harvested 39 DAP to measure dry spinach biomass as described previously.

2.2.1.5 Data analyses. The ordinal Fusarium wilt severity ratings were converted to a Fusarium severity index (Gatch 2013):

$$\text{FSI (Fusarium severity index)} = \frac{\sum(\text{Ordinal severity rating} * \text{number of seedlings})}{(\text{Total number of seedlings} * 5)} * 100\%$$

Correlations were calculated for weekly disease ratings, the AUDPC, and the biomass/plant with PROC CORR in SAS Version 9.4 (SAS Institute, Cary, NC). The AUDPC was selected to differentiate isolates of *F. oxysporum* f. sp. *spinaciae* from isolates not pathogenic to spinach, and to facilitate quantitative discrimination among isolates in terms of virulence to spinach (see justification in Results). Descriptive statistics were calculated in RStudio (RStudio Team, Boston, MA), and analyses of variance (ANOVAs) and treatment comparisons were calculated with PROC MIXED in SAS Version 9.4. The assumption of homogeneity of variances was assessed using PROC MIXED by the null-model likelihood ratio test by specifying the model to estimate treatment variances with a first order variance-covariance structure [type = un(1)] simultaneously. Determination of the distribution of residuals was assessed with PROC UNIVARIATE. If either assumption was violated, the data were transformed by log, log(x+1), square root, squareroot^{-1}, or angular (arcsin) transformation. The data were rank-transformed with PROC RANK if the transformations failed to meet the assumptions of normality of the data or homogeneity of variances. Means separation was estimated with Fishers' protected least significant difference (LSD) at $P < 0.05$. Groupings of the mean estimates were calculated with the macro pdmix800 (Saxton 1998).

2.2.2 Genetic characterization of F. oxysporum *isolates.* In total, 73 *Fusarium* isolates were characterized genetically by sequencing the housekeeping gene *TEF-1α* and testing for the presence or absence of the 14 known *SIX* genes using PCR with published primer pairs (Table 2.2). All 69 isolates that were characterized for pathogenicity to spinach were also characterized genetically. Four isolates were not tested for pathogenicity to spinach because only DNA was received for the three isolates of *F. oxysporum* f. sp. *lycopersici* (Fol1, Fol2, and Fol3), and the culture of isolate Fus396 became contaminated.

2.2.2.1 DNA extraction. The *Fusarium* isolates were each grown on half-strength PDA amended with 100 ppm chloramphenicol, from which two 3-mm^3 agar plugs were taken from the leading edge of fungal hyphae and placed in 75 mL potato dextrose broth (PDB) in a 125 mL Erlenmeyer flask. Each inoculated flask was shaken on an orbital shaking-platform at 125 rpm at room temperature for seven days under ambient light. After the mycelium had been vacuum-filtered and frozen at -80°C for 24 h, DNA was extracted from the mycelium with the FastDNA kit (MP Biomedicals, Santa Ana, CA). Minor modifications were made to the manufacturer's protocol for the FastDNA kit: approximately 200 mg mycelium was loaded into a satellite disruption tube containing 500 μm garnet and 600 mm ceramic bearings (OPS Diagnostics, Lebanon, NJ), and macerated with a MINIBEADBEATER (Biospec Products, Bartlesville, OK) for 2 min. The DNA-silica pellet was then washed twice with the SEWS-M buffer instead of once. The concentration of DNA was assessed on a Qubit Fluorometer (Invitrogen, Carlsbad, CA).

2.2.2.2 Distribution of SIX *genes.* A polymerase chain reaction (PCR) assay was used to amplify the *TEF-1α* in a total reaction volume of 20 μl with approximately 20 ng DNA and a final concentration of 0.5 μM of each primer (Table 2.2), AmpliTaq (Applied Biosystems, Foster

City, CA), and dNTPs (Applied Biosystems). A touchdown PCR protocol was used to amplify the partial *TEF-1α* region as follows: 1 cycle of 2 min at 94°C; 9 cycles of 30 s at 94°C, 30 s at 66°C and 1°C lower for each subsequent cycle, and 1.5 min at 72°C; 28 cycles of 30 s at 94°C, 30 s at 56°C, and 1.5 min at 72°C, followed by 1 cycle for 10 min at 72°C. Each of the *SIX* genes was amplified with the following thermocycler protocol: 1 cycle of 2 min at 94°C; 30 cycles of 45 s at 94°C, 30 s annealing (see Table 2.2 for specific annealing temperatures for the various *SIX* gene protocols), and 1 min at 72°C; followed by 1 cycle of 5 minutes at 72°C. All PCR amplifications were performed on a ThermoHybaid Express (Ashford, United Kingdom) thermocycler.

Two different primer sets were used to amplify each of *SIX9* and *SIX14* (Table 2.2). One primer set was designed to detect the homologs of *SIX9* and *SIX14* found in the genome *F. oxysporum* f. sp. *lycopersici* strain 4287 (FOL SIX9 and FOL SIX14), whereas the additional primer sets were designed specifically to detect *SIX9* and *SIX14* of *F. oxysporum* f. sp. *cepae* (FOC SIX9 and FOC SIX14) based on the sequence differences in the *SIX9* and *SIX14* homologs of *F. oxysporum* ff. spp. *cepae* and *lycopersici* (Taylor et al. 2016). The FOC SIX14 primer set was only used to amplify *SIX14* from DNA extracts of *F. oxysporum* f. sp. *cepae* isolates, whereas the FOL SIX14 primer set was used to detect the presence/absence of *SIX14* in all other isolates. Initially, the primer set FOL SIX14 nest was used to amplify DNA fragments generated from the reaction performed with the FOL SIX14 primer set. The PCR product generated with the latter was purified with ExoSAP-IT (Applied Biosystems), and the purified product was subjected to a PCR assay with the FOL SIX14 nest primer set. The presence of PCR products was confirmed by gel electrophoresis (1.5% agarose gel, 1X Tris-borate-ethylenediaminetetraacetic acid gel) using GelRed (Biotium, Fremont, CA) to visualize DNA

fragments according to the manufacturer's protocol. The DNA fragments were each purified with ExoSAP-IT (Applied Biosystems), and quantified with a Qubit Fluorometer. Purified DNA fragments were sequenced bidirectionally at Elim Biopharmaceuticals (Hayward, CA).

2.2.2.3 Phylogenetic analysis. The chromatograms obtained from bidirectional, dideoxy Sanger sequencing reactions for each of the amplified DNA fragments for each gene of interest were inspected manually using Chromas (Technelysium, South Brisbane, Australia) and aligned with MEGA 7.0 (Kumar et al. 2016) with the ClustalW alignment protocol. The resulting alignment of forward and reverse sequences generated from each DNA fragment were inspected manually for inconsistent and ambiguous base calls (Ns), and a consensus sequence was generated. Consensus sequences generated for *TEF-1α* and any of the 14 *SIX* genes detected were compared with publicly available sequences of these genes in GenBank using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). All *SIX* gene consensus sequences (MK906591 to MK906742) and *TEF-1α* consensus sequences (MK906743 to MK906815) were deposited in Genbank.

Maximum likelihood trees were estimated for the *TEF-1α* locus for all 73 isolates characterized genetically, and for the two *SIX* genes detected in those isolates that were pathogenic to spinach, i.e., *SIX8* and/or *SIX14* (see Results). Each tree was estimated using MEGA 7.0 with the models of evolution selected using the 'Model Test' function based on the lowest value of BIC. The models selected for *TEF-1α*, *SIX8*, and *SIX14* were the Kimura-2-parameter, Jukes-Cantor, and Kimura-2-parameter, respectively. Clade stability for each node was estimated with 1,000 bootstrap replicates (Felsenstein 1985).

2.3 Results

2.3.1 Pathogenicity tests.

2.3.1.1 Overall results of pathogenicity tests 1 to 6. There was a significant positive correlation between AUDPC and weekly wilt severity ratings in pathogenicity test 1 for ratings completed 14, 21, and 28 days after inoculation (DAI), with r = 0.834, 0.987, and 0.975 ($P \leq$ 0.0001), respectively. AUDPC ratings were not significantly correlated with wilt ratings 7 DAI (r = 0.044, P = 0.55). Similar relationships between wilt severity ratings and AUDPC were observed in subsequent pathogenicity tests.

Wilt symptoms were first observed approximately 14 DAI for pathogenicity tests 1 to 5 that were completed using the root drench inoculation method, and 14 DAP for pathogenicity test 6 completed using the pre-plant soil inoculation method. The positive control isolate, Fus254, induced the most severe wilt or the second-most severe wilt on the highly susceptible parent line of all the isolates evaluated in each pathogenicity test. The water control treatment and non-pathogenic isolate, Fus187, did not cause wilt symptoms on spinach plants in any of the pathogenicity tests, with one exception for test 4 in which Fus187 caused wilt symptoms on the partially resistant parent line (see below).

Over all six pathogenicity tests, 36 of the 69 *Fusarium* isolates inoculated onto spinach plants induced severe enough wilt symptoms that resulted in AUDPC values significantly greater ($P < 0.05$) than those of the two negative control treatments (water and Fus187) on at least one of the three parent lines evaluated, i.e., 36 isolates were identified as *F. oxysporum* f. sp. *spinaciae* (Fig. 2.2). However, an additional two isolates, Fus172 and Fus287, induced some wilt symptoms on at least one parent line, although the mean AUDPC value was not significantly

greater than that of the water and non-pathogenic isolate control treatments for each parent line. For the highly susceptible parent inoculated with Fus287, four plants developed symptoms of Fusarium wilt (each rated at least 1 out of 5 by 35 DAI) and one of those plants died after. For the moderately susceptible parent inoculated with Fus172, three plants developed symptoms of Fusarium wilt that were rated at least 2 out of 5 by 39 DAI, and two of those plants died. Therefore, these isolates were designated as *F. oxysporum* f. sp. *spinaciae*. Fus001 did not cause wilting on spinach in pathogenicity test 1, but was highly virulent on spinach in previous pathogenicity tests completed by the WSU Vegetable Seed Pathology program in 2001 to 2007. Therefore, Fus001 was designated as *F. oxysporum* f. sp. *spinaciae*. Of the 36 isolates that induced significant symptoms of wilt, 17 isolates induced the expected differences in severity of wilt (AUDPC) among the three parent lines, i.e., the highly susceptible inbred developed the most severe wilt followed by the moderately susceptible inbred and then the partially resistant inbred (Fig. 2.2). However, 19 *F. oxysporum* f. sp. *spinaciae* isolates induced similarly severe wilt symptoms (AUDPC values) on all three parent lines. The 17 isolates that induced the expected differences in wilt response among the three parent lines were defined as group 1 isolates, and the 19 isolates that induced a different response, i.e., the partially resistant inbred had more severe wilting, were characterized as group 2 isolates. The two isolates that induced very limited average disease severity (Fus172 and Fus287) could not be classified into either pathogenicity group because of inadequate average wilt severity.

None of the isolates of *F. oxysporum* ff. spp. *cepae*, *ciceris*, *pisi*, or the isolates that were provided as putative *F. oxysporum* ff. spp. *betae* or *phaseoli* induced wilting on spinach plants (Fig. 2.2 and Appendix Table 2.1). In addition, 11 *Fusarium* isolates that originated from spinach, spinach seed, or soil in which spinach had been grown did not induce symptoms of wilt

on any of the spinach parent lines, i.e., isolates Fus187, Fus191, and Fus192 in pathogenicity test 1; or on the highly susceptible parent line evaluated in pathogenicity test 6, i.e., Fus017, Fus023, Fus250, Fus259, Fus263, Fus265, Fus317, and Fus327 (Fig. 2.2; Appendix Table 2.1). These 11 isolates are referred to as non-pathogenic, spinach associated (NPS) isolates.

2.3.1.2 Pathogenicity test 1. The FSI at the final rating 28 DAI for the highly susceptible, moderately susceptible, and partially resistant parent lines averaged 25.9, 35.2, and 16.9%, respectively; and, the FSI ranged from 0 to 81.7, 0 to 96.7, and 0 to 73.3%, respectively. AUDPC values were significantly negatively correlated with spinach dry biomass ($r = -0.8205$, $P < 0.0001$). Similarly, weekly disease ratings 14, 21, and 28 DAI were significantly negatively correlated with spinach biomass ($-0.8191 \leq r \leq -0.6596$, $P < 0.0001$).

AUDPC values were ranked due to heterogeneity of variances and non-normal residuals of the raw data. Based on the ANOVAs for the ranked AUDPC and biomass data, both the spinach parent line and inoculation treatment main effects were significant at $P < 0.0001$, as was the parent-by-isolate interaction term (Table 2.3). Ten of the 14 *Fusarium* isolates induced symptoms of wilt (AUDPC) that were significantly greater from that of the control plants treated with water for at least one of the three spinach parent lines: Fus004, Fus057, Fus058, Fus059, Fus185, Fus193, Fus254, Fus267, Fus270, and Fus322 (Fig. 2.2 and Appendix Table 2.1). For the highly susceptible, moderately susceptible, and partially resistant parent lines, the AUDPC values of plants treated with eight ($P \leq 0.0066$), 10 ($P \leq 0.0002$), and seven ($P \leq 0.015$) isolates, respectively, were significantly greater than that of control plants treated with water. Similarly, the average biomass of the highly susceptible, moderately susceptible, and partially resistant parent lines inoculated with eight ($P \leq 0.0246$), eight ($P \leq 0.0115$), and six ($P \leq 0.0349$) *Fusarium* isolates was significantly less than the biomass of the same parent lines treated with

water (Fig 2.3 and Appendix Table 2.1). Fus001, Fus187 (negative control isolate), Fus191, and Fus192 did not induce significant symptoms of wilt on any of the spinach plants, nor did they cause a significant reduction in plant biomass relative to the control plants treated with water for each parent line (Figs. 2.2 and 2.3, and Appendix Table 2.1).

Differential virulence to each of the three spinach parent lines was observed for 9 of the 10 isolates identified as *F. oxysporum* f. sp. *spinaciae*, i.e., the AUDPC values differed significantly ($P < 0.05$) among parent lines for Fus004, Fus057, Fus059, Fus185, Fus193, Fus254, Fus267, Fus270, and Fus322 (Fig. 2.2), but not for isolate Fus058 ($0.1288 \leq P \leq 0.8363$) (Fig. 2.2). Isolates Fus057, Fus059, Fus193, Fus254, and Fus322 induced the expected differences in severity of wilt for phenotypic group 1 isolates, i.e., the highly susceptible and moderately susceptible parent lines had significantly greater AUDPC values (more severe wilt) than the partially resistant parent line (Fig. 2.2 and Appendix Table 2.1). However, for each of Fus004, Fus185, Fus267, and Fus270 demonstrated results typical of phenotypic group 2, i.e., the highly susceptible parent line had either similar or less severe wilt compared to the moderately susceptible and partially resistant parent lines.

2.3.1.3 Pathogenicity test 2. Ten of the 14 *F. oxysporum* isolates tested caused wilt symptoms on the three parent lines: Fus020, Fus021, Fus183, Fus254, Fus272, Fus287, Fus321, Fus323, Fus324, and Fus333 (Fig. 2.2 and Appendix Table 2.1). The FSI for the highly susceptible, moderately susceptible, and partially resistant parent lines at the last rating 35 DAI averaged 19.2, 13.1, and 5.8%, respectively; and ranged from 0 to 71.7, 0 to 73.3, and 0 to 66.6%, respectively. The ranked AUDPC values were significant for the main effects of parent line ($P < 0.0001$) and inoculation treatment ($P < 0.0001$), as well as the parent line-by-isolate interaction term ($P = 0.0295$) (Table 2.3). Wilt severity (AUDPC) and spinach biomass were

significantly negatively correlated (r = -0.654, $P < 0.0001$). Similarly, the weekly wilt ratings measured 15, 22, 28, and 35 DAI were significantly positively correlated with AUDPC values (r = 0.7009, 0.9733, 0.9883, and 0.9598, respectively at $P < 0.0001$) and, the weekly disease ratings were significantly negatively correlated with spinach biomass (r = -0.3301, -0.5042, -0.4996, and -0.4880, respectively at $P < 0.0001$). Wilt severity (AUDPC values) for the highly susceptible, moderately susceptible, and partially resistant parent lines averaged 245, 157, and 78, respectively; and ranged from 0 to 1,310, 0 to 1,004, and 0 to 1,063, respectively. Eight (Fus021, Fus183, Fus254, Fus272, Fus321, Fus323, Fus324, and Fus333), seven (Fus020, Fus183, Fus254, Fus272, Fus323, Fus324, and Fus333), and two (Fus020 and Fus324) *F. oxysporum* isolates induced more severe wilting compared to symptoms observed on plants treated with water for the highly susceptible, moderately susceptible, and partially resistant inbred lines, respectively (Fig. 2.2 and Appendix Table 2.1). Isolate Fus287 induced very limited wilting on the highly susceptible parent, with an average AUDPC value of 94, ranging from 0 to 288, which did not differ significantly from that of control plants treated with water ($P = 0.6345$). Spinach biomass was affected significantly by the main effects of parent line ($P < 0.0001$) and *Fusarium* isolate ($P < 0.0001$), as well as the parent-by-isolate interaction term ($P = 0.0157$) (Table 2.3). The biomass of the highly susceptible, moderately susceptible, and partially resistant parents averaged 0.32, 0.27, and 0.26 g, respectively; and ranged from 0.09 to 0.50, 0.10 to 0.44, and 0.08 to 0.38 g, respectively. The biomass of the highly susceptible parent line was reduced significantly by three isolates (P ranged from 0.0038 to 0.0335 for Fus183, Fus254, and Fus272), but none of the isolates reduced the biomass of the other two spinach parent lines compared to the biomass of the same parent lines treated with water or the non-pathogenic isolate Fus187 (Fig. 2.3 and Appendix Table 2.1).

Nine of the 14 *Fusarium* isolates evaluated in test 2 induced symptoms of wilt on at least one of the three spinach parent lines (Fig. 2.2 and Appendix Table 2.1). Five of these isolates (Fus021, Fus254, Fus272, Fus321, and Fus333) caused the expected differences in wilt severity among the spinach lines for phenotypic group 1 isolates, i.e., the most severe wilt was observed on the highly susceptible inbred followed by the moderately susceptible and partially resistant inbred lines (Fig. 2.2 and Appendix Table 2.1). However, similar AUDPC values were observed among all three parent lines inoculated with four isolates (Fus020, Fus183, Fus323, and Fus324) (Fig. 2.2 and Appendix Table 2.1). The putative isolates of *F. oxysporum* ff. spp. *betae*, *cepae*, and *phaseoli* did not induce significant symptoms of wilt on any of the spinach parent lines (Fig. 2.2 and Appendix Table 2.1).

2.3.1.4 Pathogenicity test 3. Less severe wilt developed on spinach plants in pathogenicity test 3 compared to the first two pathogenicity tests, so the plants were rated until 42 DAI before measuring biomass. Of the 13 *Fusarium* isolates tested, 11 induced symptoms of wilt (AUDPC values) that were significantly greater than that of the control plants treated with water for at least one of the three spinach parent lines (Fig. 2.2 and Appendix Table 2.1), including Fus159 to Fus168 and the positive control isolate, Fus254. The average AUDPC value for the highly susceptible, moderately susceptible, and partially resistant parent lines was significantly greater for plants treated with 11 ($P \leq 0.0182$), nine ($P \leq 0.0113$), and six ($P \leq 0.0163$) *F. oxysporum* isolates compared to that of control plants treated with water for each parent line (Fig. 2.2 and Appendix Table 2.1). The FSI 42 DAI on the highly susceptible, moderately susceptible, and partially resistant parent lines averaged 22.4, 24.1, and 12.3%, respectively; and ranged from 0 to 86.7, 0 to 86.7, and 0 to 84.0%, respectively. Ratings for plants inoculated with Fus442 were removed from the statistical analyses as this isolate did not

produce conidia or mycelium in liquid culture. Isolate Fus442 subsequently was identified as *F. equiseti* based on genetic (*TEF-1α*) and morphological features (see below). Wilt symptoms measured by the AUDPC values were significantly negatively correlated with spinach dry biomass ($r = -0.413$, $P < 0.0001$). Similarly, wilt ratings 15, 22, 28, 35, and 42 DAI were significantly negatively correlated with spinach dry biomass ($-0.4191 \leq r \leq -0.26254$, $P \leq 0.0006$); and significantly positively correlated with AUDPC values ($0.6085 \leq r \leq 0.9718$, $P < 0.0001$). For the ranked AUDPC values, both the main effects and the interaction term were significant in the ANOVA, the parent and isolate main effects at $P < 0.0001$, and the parent-by-isolate interaction term at $P = 0.0039$ (Table 2.3). For the highly susceptible, moderately susceptible, and partially resistant parent lines, the mean AUDPC values were 296, 268, and 188, respectively; and ranged from 0 to 1,123, 0 to 1,510, and 0 to 2,178, respectively.

For spinach biomass data, the main effects of parent and isolate ($P < 0.0001$) were significant, but not the interaction term ($P = 0.0751$) (Table 2.3). The mean spinach dry biomass of the highly susceptible, moderately susceptible, and partially resistant parent line was 1.04, 0.94, and 0.75 g, respectively; and ranged from 0.47 to 1.53, 0.38 to 1.49, and 0.18 to 1.12 g, respectively. Six of the *F. oxysporum* isolates significantly reduced the biomass of the three parent lines relative to that of the control plants (Fig. 2.3 and Appendix Table 2.1), i.e., Fus166, Fus168, Fus254 (the positive control isolate), Fus165, Fus167, and Fus162 ($P \leq 0.0294$). The biomass of plants inoculated with the other six *Fusarium* isolates (Fus159, Fus160, Fus161, Fus163, Fus164, and Fus440) and the negative control isolate Fus187 was not reduced for any of the three parent lines ($0.0879 \leq P \leq 0.7222$). The negative control isolate Fus187 and isolate Fus440 did not induce significant symptoms of wilt on any of the spinach lines, nor did they

cause a significant reduction in spinach biomass relative to that of the control plants treated with water for each parent line (Figs. 2.2 and 2.3, and Appendix Table 2.1).

Of the 11 *F. oxysporum* isolates that induced symptoms of wilt on at least one spinach parent line, significant differences in ranked AUDPC values were observed among the three parent lines for four of the isolates **(**Fus159, Fus161, Fus163, and Fus166) and the positive control isolate (Fus254) (Fig. 2.2 and Appendix Table 2.1). All of these isolates except Fus163 induced differences in severity of wilting expected among the three parent lines for phenotypic group 1 isolates, i.e., the highly susceptible and moderately susceptible parent lines had more severe wilt than the partially resistant parent line (Fig. 2.2 and Appendix Table 2.1). In contrast, the ranked AUDPC values did not differ significantly among the partially resistant parent and highly susceptible parent lines ($P = 0.8719$) inoculated with Fus163, although wilting was less severe on the moderately susceptible parent inoculated with Fus163 (Fig. 2.2 and Appendix Table 2.1). Similar wilt severity was observed among the three parent lines inoculated with Fus160, Fus162, Fus164, Fus165, Fus167, and Fus168 ($0.1024 \leq P \leq 0.9905$).

2.3.1.5 Pathogenicity test 4. For the 13 *Fusarium* isolates tested in pathogenicity test 4, the average FSI at the last rating 42 DAI of the highly susceptible, moderately susceptible, and partially resistant parent line was 14.6, 22.8, and 9.1%, respectively; and ranged from 0 to 63.3, 0 to 90.0, and 0 to 80.0%, respectively. The AUDPC values were significantly negatively correlated with spinach biomass ($r = -0.6126$, $p < 0.0001$). Similarly, severity ratings 15, 22, 28, 35, and 42 DAI were significantly negatively correlated with dry spinach biomass ($-0.6093 \leq r \leq -0.3782$, $P < 0.0001$); and the weekly severity ratings were significantly positively correlated with the AUDPC values ($0.6826 \leq r \leq 0.9774$, $P < 0.0001$). For the ranked AUDPC data, both main effects and the interaction term were significant at $P < 0.0001$ based on the ANOVA (Table

2.3). A single plant within one replicate block of the partially resistant parent line was rated a 2 out of 5 for wilt severity at the last rating 42 DAI with the negative control isolate Fus187. However, the other two parent lines demonstrated no wilting when inoculated with this negative control isolate or with water (Fig. 2.2 and Appendix Table 2.1).

Of the 13 *Fusarium* isolates tested, including the two control isolates, 10 induced symptoms of wilting on at least one spinach parent line (Fig. 2.2 and Appendix Table 2.1). The ranked AUDPC values of the highly susceptible, moderately susceptible, and partially resistant parent lines treated with nine ($P \leq 0.0225$), nine ($P \leq 0.0365$), and four ($P \leq 0.0127$) *F. oxysporum* isolates was significantly greater than that of plants of the same parent line treated with water. Isolates Fus172, Fus447, and Fus452 did not induce significant symptoms of wilt on any of the spinach plants or a significant reduction in spinach biomass relative to the control plants treated with water. However, four of the 24 plants of the moderately susceptible parent line treated with Fus172 developed symptoms of Fusarium wilt, three of which had died (rated 5) by 35 DAI. The plants that died were all in the same replicate block. Eight of the 10 *F. oxysporum* isolates that induced wilting of spinach plants caused significant differences in AUDPC values among the three parent lines: Fus169, Fus170, Fus173, Fus182, Fus184, Fus186, Fus188, and Fus189; as did the positive control isolate Fus254 and the negative control isolate Fus187. Only isolate Fus173 did not cause significant differences in wilt severity among spinach lines ($0.1060 \leq P \leq 0.8185$ for the three lines). Isolates Fus169, Fus182, Fus184, and Fus188 as well as the positive control isolate Fus254 caused reactions typical of phenotypic group 1, i.e., more severe wilt on the highly susceptible and moderately susceptible lines than the partially resistant line (Fig. 2.2 and Appendix Table 2.1). Isolate Fus189 only caused wilt symptoms on the moderately susceptible line, and Fus186 induced the least severe wilt on the highly

susceptible parent line (mean AUDPC ± standard error of 42 ± 27), which was significantly less than that of the partially resistant parent (265 ± 120, $P = 0.0294$) but not that of the moderately susceptible parent (171 ± 65, $P = 0.0602$) (Fig. 2.2 and Appendix Table 2.1). For isolate Fus170, the ranked AUDPC of the moderately susceptible parent line was almost significantly greater than that of the highly susceptible parent line ($P = 0.0585$) and was greater than that of the partially resistant parent line ($P = 0.0111$), but the highly susceptible and partially resistant parent lines did not differ significantly ($P = 0.5308$).

Spinach dry biomass was affected significantly by the main effects of parent and inoculation treatment, and by the parent-by-isolate interaction ($P < 0.0001$) (Table 2.3). Six of the 13 *Fusarium* isolates tested reduced spinach biomass relative to that of plants treated with water for at least one parent line (Fig. 2.3 and Appendix Table 2.1). Two (Fus182 and the positive control isolate, $P \leq 0.0261$), five (Fus167, Fus170, Fus173, Fus182, and the positive control isolate, $P \leq 0.0466$), and two (Fus173 and Fus186, $P \leq 0.0411$) *F. oxysporum* isolates resulted in significantly smaller plants (less biomass) than plants of the highly susceptible, moderately susceptible, and partially resistant lines treated with water, respectively.

2.3.1.6 Pathogenicity test 5. Fusarium wilt symptoms were not observed for any of the *Fusarium* test isolates inoculated on any of the spinach parent lines in pathogenicity test 5 except for the positive control isolate Fus254. This was expected because all other isolates tested were of *F. oxysporum* ff. spp. *betae*, *cepae*, *ciceris*, and *pisi* (Table 2.1). The average FSI at the last rating for the highly susceptible, moderately susceptible, and partially resistant parent line for the 15 inoculation treatments was 2.9, 2.1, and 0.2%, respectively; and ranged from 0 to 56.0, 0 to 48.3, and 0 to 10.0%, respectively. In contrast, the average FSI for the highly susceptible, moderately susceptible, and partially resistant parent lines inoculated with the positive control

isolate Fus254 was 42.3, 30.8, and 3.0%, respectively; and ranged from 11.7 to 56.0, 16.7 to 48.3, and 0 to 10.0%, respectively. The AUDPC data were not analyzed statistically for this pathogenicity test because only the positive control isolate induced symptoms of Fusarium wilt on spinach plants (Fig. 2.2). For spinach biomass data, the main effects of parent and isolate were significant ($P < 0.0001$), but there was not a significant parent-by-isolate interaction ($P = 0.5761$) (Table 2.3). There were significant differences in dry biomass per plant among the three parent lines, as the highly susceptible and moderately susceptible parents had similar biomass ($P = 0.8639$) but were larger than the partially resistant parent ($P < 0.0001$) as the partially resistant parent is a slower-growing inbred line than the other two parent lines in the absence of Fusarium wilt (Gatch 2013; Appendix Table 2.1). The biomass of the three parent lines ranged from 0.99 to 1.43 g/plant (Appendix Table 2.1), and the mean biomass of spinach plants inoculated with the positive control isolate was significantly less than that of all other treatments (0.99 g, $P < 0.0001$). No other differences were observed among the 13 *Fusarium* test isolates evaluated in test 5.

2.3.1.7 Pathogenicity test 6. Of the 12 *Fusarium* isolates tested for pathogenicity in test 6 on the highly susceptible spinach line in growth chambers, only the positive control isolate induced symptoms of wilt. The AUDPC values of individual experimental units ranged from 0 to 996. Similar to the data for pathogenicity test 5, the AUDPC values were not analyzed statistically because only the positive control isolate caused Fusarium wilt. The average (and range) in FSI for all treatments at the final rating was 9.8% (0 to 86.7%), and for the positive control isolate Fus254 was 75.0% (63.3 to 86.7%). For spinach biomass data, there was a significant isolate main effect ($P < 0.0056$), and the biomass ranged from 0.15 to 0.34 g/plant (Fig. 2.3 and Appendix Table 2.1) with significantly smaller plants that had been inoculated with

Fus254 compared to plants inoculated with all other isolates and water ($P < 0.0013$). No other differences were observed among the other 11 *Fusarium* isolates tested for pathogenicity (Appendix Table 2.1).

2.3.1.8 Soil amendment vs. drench inoculation. Differences in the uniformity and severity of Fusarium wilt were observed between the root drench and pre-plant soil amendment methods of inoculation (Appendix Fig. 2.1). Spinach plants planted in propagation mix inoculated prior to planting with *F. oxysporum* f. sp. *spinaciae* isolates Fus254 and Fus322 developed more uniform symptoms of Fusarium wilt across cells within each replicate and among replicate blocks compared to spinach plants that were inoculated by drenching the root plugs (Appendix Fig. 2.1). Overall, the variation in disease severity ratings, as measured by standard deviations, was less for spinach plants grown by planting seed into planting medium inoculated with Fus254 or Fus322 compared to plants that were drench-inoculated with the same isolates (Appendix Table 2.2). All spinach plants grown from seed sown into soil inoculated with one of the two *F. oxysporum* f. sp. *spinaciae* isolates developed consistent wilt symptoms and were dead by 28 DAP, whereas only 10 and 13 spinach plants had developed wilt symptoms by 28 days after drench-inoculation with Fus254 and Fus322, respectively (Appendix Table 2.2).

Symptoms of Fusarium wilt were first observed 14 DAI. There was a significant inoculation-by-isolate effect on the AUDPC values ($P = 0.0031$, Appendix Table 2.3). The greatest severity of wilt (AUDPC value) was observed for spinach plants grown from seed planted into soil inoculated with Fus254 and Fus322, followed by spinach plants that were drench-inoculated with Fus254 and Fus322. The least severe wilt was measured on plants in the water control treatment for both inoculation methods (Appendix Table 2.4). There was no significant difference between the mean AUDPC values of spinach plants that were drench

inoculated with isolates Fus254 or Fus322 (P = 0.5142). Similarly, there was no significant difference between the mean AUDPC values of spinach plants grown in soil that was amended with Fus254 or Fus322 (P = 0.8265).

There were significant inoculation method and isolate main effects on the biomass of spinach plants (P = 0.0021 and 0.0088, respectively), while the inoculation-by-isolate interaction was not significant (P = 0.4799). The average biomass of spinach plants for which the root plugs had been drenched with water or the seed sown in potting medium treated with water was 0.56 $\pm$ 0.03 g and 0.24 $\pm$ 0.02 g, respectively. As expected, the dry biomass of the drench-inoculated plants was greater than that of spinach plants seeded in potting medium because of the difference in age of the plants at the time of inoculation (12 vs. 0 DAP, respectively). There was a significant inoculation main effect on spinach biomass ratio (P = 0.0207), calculated to account for the difference in age of plants at the time of inoculation between the two inoculation methods, but there was not a significant isolate main effect (P = 0.4571) and there was not a significant interaction effect (P = 0.6360), i.e., results were the same for both isolates of *F. oxysporum* f. sp. *spinaciae*. The mean $\pm$ standard deviation of ρ for the drench-inoculation and pre-plant soil-amendment inoculation methods were 0.56 $\pm$ 0.22 and 0.10 $\pm$ 0.02, respectively (Table 2.4), i.e., on average, spinach seeded directly into potting medium infested with Fus254 or Fus322 had ~10% of the biomass compared to spinach plants grown in control soil inoculated with water, whereas spinach plants drench-inoculated over the root plugs with Fus254 or Fus322 had ~54% the biomass of control plants drench-inoculated with water.

2.3.2 Genotypic characterization of isolates based on TEF-1α sequences. Phylogenetic analysis of part of the *TEF-1α* gene indicated that the 39 *F. oxysporum* f. sp. *spinaciae* isolates were monophyletic as the sequences of the *TEF-1α* locus were 100% identical for all 39 isolates

(Fig. 2.4). One NPS isolate, Fus263, grouped with the 39 *F. oxysporum* f. sp. *spinaciae* isolates but was different from all other *F. oxysporum* f. sp. *spinaciae* isolates at three bases. Furthermore, the NPS isolates were separated into four clades (Fig. 2.4). Isolates of *F. oxysporum* ff. spp. *pisi* and *lycopersici* were polyphyletic, while isolates of *F. oxysporum* ff. spp. *cepae* and *ciceris* were monophyletic (Fig. 2.4). Several isolates received for this study that were identified as the submitter as putative isolates of *F. oxysporum* ff. spp. *betae* and *phaseoli* had a relatively low identity to *F. oxysporum* when compared with isolates in GenBank (96 to 98% identity), although all variable nucleotides in the *TEF-1α* sequences of these isolates were in non-coding regions. Fus442 had a *TEF-1α* sequence that was ~99% identical to GenBank accessions of *F. equiseti*, and produced macroconidia diagnostic of *F. equiseti* when grown on half-strength PDA and on carnation leaf agar (Appendix Fig. 2.2). Furthermore, Fus442 did not produce microconidia in Kerr's broth nor on half-strength PDA. The absence of microconidia production is an attribute *F. equiseti* (Leslie and Summerell 2006).

2.3.3 SIX *gene characterization of* Fusarium *isolates.* The *SIX* gene profiles of isolates of *F. oxysporum* ff. spp. *cepae* and *lycopersici* included in this study matched previously published profiles for both *cepae* (Taylor et al. 2016) and *lycopersici* (Schmidt et al. 2013) (Table 2.4). Similarly, the *SIX* profiles for isolates of *F. oxysporum* ff. spp. *ciceris* and *pisi* were similar to published profiles (Taylor et al. 2016; Williams et al. 2016) (Table 2.4). For the 39 *F. oxysporum* f. sp. *spinaciae* isolates identified by pathogenicity testing, only *SIX8* and/or *SIX14* were detected out of all 14 *SIX* genes, with the exception of *SIX11* which was detected in a single isolate, Fus004 (Table 2.4). Overall, the three profiles of *SIX* genes detected among the 39 *F. oxysporum* f. sp. *spinaciae* isolates (*SIX8* alone, *SIX14* alone, or *SIX8* plus *SIX14*) did not differentiate *F. oxysporum* f. sp. *spinaciae* uniquely from those of other *F. oxysporum* ff. spp. or from NPS

isolates of *F. oxysporum* (Table 2.4). *SIX8* only, *SIX14* only, or *SIX8* and *SIX14* were detected in four, 18, and 17 *F. oxysporum* f. sp. *spinaciae* isolates, respectively. For the 11 NPS isolates tested, *SIX14* was detected in two isolates (Fus187 and Fus327), but none of the other *SIX* genes was detected (Table 2.4). The *F. oxysporum* f. sp. *spinaciae* isolates carried homologs of *SIX8* and *SIX14* that had unique DNA sequences relative to the *SIX8* and *SIX14* sequences amplified from other isolates (Figs. 2.5 and 2.6). The *SIX8* DNA sequences of *F. oxysporum* f. sp. *spinaciae* isolates were ~91% identical to that of *F. oxysporum* f. sp. *lycopersici* isolates. The *SIX14* DNA sequences of *F. oxysporum* f. sp. *spinaciae* isolates were ~92% identical to the *F. oxysporum* f. sp. *lycopersici* isolates, including exons and an intron. The two exon sequences of *SIX14* in the *F. oxysporum* f. sp. *spinaciae* isolates and the *F. oxysporum* f. sp. *lycopersici* isolates were ~93% identical.

The coding sequences of *SIX8* and *SIX14* were clearly differentiated among isolates of the various formae speciales of *F. oxysporum* evaluated in this study (Figs. 2.5 and 2.6). Furthermore, little to no sequence variation was observed among *F. oxysporum* isolates of the same forma specialis. For example, there was no sequence variation in *SIX8* for *F. oxysporum* ff. spp. *ciceris*, *lycopersici*, *pisi*, and *spinaciae.* The isolates identified as putative *F. oxysporum* f. sp. *betae* (Fus440, Fus441, and Fus452) were separated into two clades based on the *SIX8* sequences. Similarly, the phylogenetic tree for *SIX14* sequences differentiated isolates of *F. oxysporum* ff. spp. *ciceris* from those of *pisi*, *cepae*, and *lycopersici*, as well as from isolates of *spinaciae*. The putative *F. oxysporum* f. sp. *betae* isolates Fus439, Fus440, and Fus441 as well as *F. equiseti* isolate Fus442 were grouped together (Fig. 2.6).

SIX14 was detected in all 17 of the *F. oxysporum* f. sp. *spinaciae* isolates that had been identified as belonging to group 1 in the pathogenicity test, i.e., isolates that caused more severe

symptoms on the highly susceptible spinach line than the partially resistant line. *SIX8* was detected in only one of these 17 isolates of *F. oxysporum* f. sp. *spinaciae* in pathogenicity group 1 (Fus333). In contrast, *SIX8* was detected in all 19 isolates of *F. oxysporum* f. sp. *spinaciae* placed in phenotypic group 2, whereas *SIX14* was detected in only 10 of these 20 isolates. *SIX8* and *SIX14* were detected in two *F. oxysporum* isolates that were putative f. sp. *betae* (Fus440 and Fus441). Another three isolates provided for this study by Dr. Linda Hanson (USDA ARS, Lansing, MI) that previously had been characterized as putative wilt pathogens of beet and spinach, i.e., Fus439, Fus441, and Fus452, did not have a consistent profile of *SIX* genes: *SIX3*, *SIX5*, and *SIX14* were detected in Fus439; *SIX8* and *SIX14* were detected in Fus441; and *SIX8* was detected in Fus452 (Table 2.4). However, the *F. oxysporum* isolates identified by Dr. Hanson as putative pathogens of sugar beet and of both sugar beet and spinach did not induce symptoms of wilt on any of the three spinach parent lines tested in this study (Fig. 2.2 and Table 2.4). *SIX14* was detected in the NPS isolate Fus187, with 100% identical DNA sequence to that of the *SIX14* homolog of *F. oxysporum* f. sp. *cepae* isolates Fus125 and Fus127.

Interestingly, the DNA sequence of the *SIX14* homolog amplified with the primer set FOC SIX14 (Table 2.2) did not align with the *SIX14* sequences amplified with the FOL SIX14 primer set. Thus, the *SIX14* homolog amplified with the FOC SIX14 primer set was not included in the phylogenetic comparison (Fig. 2.6). *SIX14* was noticeably more abundant (25 to 33% more concentrated DNA) when amplified from *F. oxysporum* f. sp. *spinaciae* isolates in pathogenicity group 1 compared to isolates in group 2 (Fig. 2.7) (*data not shown*).

Initially, *SIX14* was detected in one NPS isolate using the FOL SIX14 primer set. However, when the FOL SIX14 nest primer set was used with DNA amplified from the FOL SIX14 primers, four NPS isolates (Fus187, Fus191, Fus192, and Fus327) were detected to carry

SIX14. However, when the reaction was repeated independently with both primer sets, *SIX14* was not detected in three of these NPS isolates (Fus191, Fus192, and Fus327). Furthermore, for isolate Fus324, DNA sequences produced with two independent assays using the FOL SIX14 nest primer set produced different results. One DNA fragment had a sequence 100% identical to that of the *F. oxysporum* f. sp. *spinaciae SIX14* homolog, while the other DNA fragment was 100% identical to the *F. oxysporum* f. sp. *lycopersici SIX14* homolog (*data not shown*). As a result, sequences generated using the FOL SIX14 nest primer set were not used to determine the presence or absence of *SIX14* or for comparison of *SIX14* sequences among the fungal isolates.

2.4 Discussion

This study demonstrated the presence of two *SIX* genes, *SIX8* and/or *SIX14*, in each of 39 isolates of the causal agent of spinach Fusarium wilt, *F. oxysporum* f. sp. *spinaciae*, that originated from different geographic locations and years. However, a single profile of these two *SIX* genes was not consistent among all *F. oxysporum* f. sp. *spinaciae* isolates, nor did the presence or absence of *SIX8* and/or *SIX14* differentiate *F. oxysporum* f. sp. *spinaciae* isolates from other *F. oxysporum* isolates characterized in this study that were not pathogens of spinach. Two primary differential reactions of three spinach inbred lines to the 39 isolates of *F. oxysporum* f. sp. *spinaciae* were demonstrated with pathogenicity tests. The isolates were placed into two pathogenicity groups based on the severity of wilt symptoms induced on the three inbred lines. Seventeen of the 39 isolates caused the expected responses of the most severe wilt symptoms on the spinach inbred that previously had been characterized as highly susceptible to Fusarium wilt, less severe symptoms on the moderately susceptible inbred line, and the least severe symptoms on the partially resistant inbred line (Gatch and du Toit 2015). Surprisingly, 19

of the 39 isolates characterized as *F. oxysporum* f. sp. *spinaciae* induced more severe symptoms on the partially resistant or moderately susceptible parent line than on the highly susceptible line. A remainder of three isolates that were previously identified as *F. oxysporum* f. sp. *spinaciae* could not be characterized into a group due to the low severity of wilt they induced on spinach plants. This suggests that resistance to Fusarium wilt in spinach may not be driven exclusively by quantitative resistance loci in the host and/or quantitative effector genes in the pathogen. This is corroborated by Fiely et al. (1995) who characterized 53 *F. oxysporum* f. sp. *spinaciae* isolates for VCG and for virulence on the spinach cultivar Grandstand. The severity of wilt induced on this cultivar differed between two groups of *F. oxysporum* f. sp. *spinaciae* isolates, and the two groups also were associated with specific VCGs. *F. oxysporum* f. sp. *spinaciae* isolates that induced more severe wilt belonged to VCGs 0330 and 0332 (22 and 13 isolates, respectively), whereas isolates that induced less severe wilt belonged to VCG 0331 (18 isolates).

The objectives of this work were to identify whether any of the 14 known *SIX* genes are present in isolates of *F. oxysporum* f. sp. *spinaciae* isolates and, if so, if there is a unique *SIX* gene profile that differentiates isolates of *F. oxysporum* f. sp. *spinaciae* from isolates of other *F. oxysporum* ff. spp. and non-pathogenic *F. oxysporum* isolates. These objectives were inspired by the preponderance of evidence of the association of unique *SIX* gene profiles with other *F. oxysporum* ff. spp., e.g., the Fusarium wilt pathogens of banana (Czislowski et al. 2018; Meldrum et al. 2012), legumes (Williams et al. 2016), onion (Sasaki et al. 2015; Taylor et al. 2016), tomato (Lievens et al. 2009; Schmidt et al. 2013), and other crops. The *SIX* genes are known to play a functional role in virulence of some *F. oxysporum* ff. spp., since the deletion of specific *SIX* genes led to a reduction or loss of virulence in some *F. oxysporum* ff. spp. (Rep et

al. 2004; Thatcher et al. 2012; Widinugraheni et al. 2018). However, the functions of the 14 *SIX* genes that have been detected remain unknown.

It is unclear whether the *SIX8* and/or *SIX14* homologs identified in isolates of the spinach pathogen in this study play a role in determining host-specificity of *F. oxysporum* f. sp. *spinaciae* to spinach, particularly as *SIX14* also was detected in some NPS isolates. To our knowledge, there has not been previous research demonstrating the presence of *SIX* genes or other putative effector genes in *F. oxysporum* f. sp. *spinaciae*. However, *F. oxysporum* f. sp. *spinaciae* isolates have only been included in a limited number of studies that aimed to characterize *F. oxysporum* ff. spp. genotypically based on the presence or absence of *SIX1* to *SIX7* (Covey et al. 2014; Lievens et al. 2009), and other molecular markers (Covey et al. 2014). The DNA sequences of *SIX8* and *SIX14* amplified from the NPS isolates and other *F. oxysporum* ff. spp. in this study were distinct from those of *F. oxysporum* f. sp. *spinaciae*, which suggests that *SIX8* and/or *SIX14* may play a role in inducing Fusarium wilt symptoms on spinach. Furthermore, isolates Fus440, Fus441, and Fus452, which were described as putative isolates of *F. oxysporum* f. sp. *betae* by the researcher who provided these isolates, had *SIX* gene profiles that could not be differentiated from those of the *F. oxysporum* f. sp. *spinaciae* isolates in this study. Armstrong and Armstrong (1976) and Naiki and Kano (1977) demonstrated that some isolates of *F. oxysporum* f. sp. *spinaciae* can cause Fusarium wilt on beet and, to a more limited degree, on Swiss chard, which is not too unexpected given these hosts are in the same family as spinach, Amaranthaceae. Interestingly, the limited number of *F. oxysporum* f. sp. *betae* isolates evaluated in this study were not able to cause wilt on any of the three spinach inbred lines used, which corroborated previous work by Armstrong and Armstrong (1976). A more diverse set of isolates of this forma specialis should be examined to confirm this. The *SIX8* gene was detected in all 19 *F. oxysporum*

f. sp. *spinaciae* isolates characterized as belonging to group 2 in this study, i.e., isolates that caused more severe wilt on the partially resistant spinach inbred than the highly susceptible inbred. However, *SIX8* also was detected in one *F. oxysporum* f. sp. *spinaciae* isolate classified in group 1 (Fus333). *SIX14* was detected in all 17 isolates characterized in group 1, and in 16 of the 19 isolates placed in group 2. However, for six of these 16 isolates in group 2, amplification of *SIX14* was very limited, to the point that the concentration of DNA amplified was insufficient for sequencing.

Similar to previous work by Taylor et al. (2016) and van Dam et al. (2016), the DNA sequences of *SIX8* and *SIX14* detected in this study distinguished among *F. oxysporum* formae speciales unambiguously, whereas DNA sequence variation in the core housekeeping gene *TEF-1α* did not differentiate the formae speciales. Incongruencies between effector gene genealogies and housekeeping gene phylogenies have been described for this species (Czislowski et al. 2018; Laurence et al. 2015; Ma et al. 2010; van Dam et al. 2016). The dominant hypothesis for addressing these incongruencies has been horizontal transfer of accessory chromosomes, which has also been demonstrated experimentally (Ma et al. 2010; Shahi et al. 2016).

A limitation of placing the *F. oxysporum* f. sp. *spinaciae* isolates into one of two pathogenicity groups is the failure to describe the quantitative differences in virulence observed among *F. oxysporum* f. sp. *spinaciae* isolates within each group. These quantitative differences may be valuable for seeking genetic loci which confer different degrees of virulence on spinach. For example, in pathogenicity test 1, Fus267 induced more severe wilt on the partially resistant and moderately susceptible parent lines than on the highly susceptible parent line, and Fus058 induced even greater severity of wilt on the partially resistant parent compared to Fus267. Despite this difference in virulence, there was no difference in the *SIX* gene profiles of Fus267

and Fus058, which suggests that other genetic loci in *F. oxysporum* f. sp. *spinaciae* are likely associated with the ability to cause Fusarium wilt of spinach. *F. oxysporum* f. sp. *spinaciae* isolates with different levels of virulence to spinach will be invaluable to assess the impacts of specific pathogen effector genes at causing spinach Fusarium wilt.

For the *SIX14* sequence data collected from 27 *F. oxysporum* f. sp. *spinaciae* isolates in this study, an ambiguous peak was present in the bidirectional reads of 25 isolates, i.e., the presence of a guanine and a cytosine peak at base pair 64 in the first exon. Competing peaks in chromatogram data can indicate the presence of a heterozygotic single nucleotide polymorphism in a diploid or polyploid organism and indicates there could be multiple copies of *SIX14* in *F. oxysporum* f. sp. *spinaciae* isolates. However, it is unclear whether the sequence difference detected reflects duplicate copies of *SIX14* in some isolates or variation that occurred during the amplification process or the sequencing process. Finding multiple copies of effector genes would not be novel since the genome assembly of *F. oxysporum* f. sp. *lycopersici* isolate 4287 revealed a partial duplication event in *SIX1*, at least eight copies of *SIX8*, two copies of *SIX13*, and a partial duplication event for *SIX14* (Schmidt et al. 2013; Williams et al. 2016). Determining whether *F. oxysporum* f. sp. *spinaciae* has multiple copies of *SIX14* and/or other putative effector genes is addressed in Chapter 3 of this thesis, on sequencing the whole genomes of isolates of *F. oxysporum* f. sp. *spinaciae*.

All 39 *F. oxysporum* f. sp. *spinaciae* isolates examined in this study were monophyletic for the *TEF-1α* sequence. This corroborated the findings of Baayen et al. (2000) who described 89 *F. oxysporum* isolates comprising eight formae speciales (including three *F. oxysporum* f. sp. *spinaciae* isolates) using sequences of the *TEF-1α* gene and mitochondrial small subunit (SSU) ribosomal RNA genes. However, O'Donnell et al. (2009) expanded on this work by including

850 *F. oxysporum* isolates comprising at least 68 *F. oxysporum* ff. spp. and other *F. oxysporum* isolates collected from environmental substrates, and demonstrated that the three isolates *F. oxysporum* f. sp. *spinaciae* were polyphyletic for the IGS rDNA sequence but not the *TEF-1α* sequence. It is unclear whether the two groups of *F. oxysporum* f. sp. *spinaciae* identified in this study based on spinach parent line pathogenicity tests are associated with the two phylogenetic lineages for the spinach Fusarium wilt pathogen described by O'Donnell et al. (2009). It will be valuable to examine multiple genetic loci of *F. oxysporum* f. sp. *spinaciae* to yield a greater understanding of the genetic relationships among *F. oxysporum* f. sp. *spinaciae* isolates and spinach cultivars differing in resistance to Fusarium wilt.

Several limitations in this study influenced the capacity to characterize *Fusarium* isolates phenotypically and genotypically. The variability in severity of Fusarium wilt observed among some replicate blocks in pathogenicity tests 1 to 5 limited to some degree the power of the tests for differentiating levels of virulence among isolates of *F. oxysporum* f. sp. *spinaciae* quantitatively. Three isolates previously identified as *F. oxysporum* f. sp. *spinaciae* based on pathogenicity tests (Fus001, Fus172, and Fus287), did not induce significant wilt symptoms in this study. However, the lack of wilt symptoms also could reflect a loss of virulence of the isolates in storage, as has been observed with other isolates of this pathogen in the WSU Vegetable Seed Pathology program. The methods used to characterize the *Fusarium* isolates phenotypically were modified over the six pathogenicity tests in this study in an effort to reduce variability among replicate blocks of the same treatment. This confounded to some degree the ability to compare isolates among tests, although inclusion of positive and negative inoculation treatments enabled standardization of the spinach plant responses to the isolates across tests. Changing the method of inoculation from a root plug-drench to sowing spinach seed into

inoculated potting medium reduced variability in symptoms of Fusarium wilt across replicate blocks of the same treatment.

Detection of the *SIX* genes in this study was based on accurate scoring of the presence or absence of a DNA product on an agarose gel. Weak amplifications of the *SIX* genes occurred for some of the PCR assays, e.g., *SIX14* did not amplify as readily for *F. oxysporum* f. sp. *spinaciae* group 2 isolates as it did with group 1 isolates. This could have been due to a variety of factors such as poor binding of PCR primers to genomic DNA, or lack of an optimized PCR assay for this locus. The inability to amplify *SIX1* to *SIX7*, and *SIX9* to *SIX13* in any of the 39 *F. oxysporum* f. sp. *spinaciae* isolates does not prove that those genes or loci were not present in these isolates, but that each locus was not detected using the published PCR protocols for each of these *SIX* genes.

Prior to this study, there had been no published research describing the presence of *SIX8* and/or *SIX14* in isolates of *F. oxysporum* f. sp. *spinaciae*. However, several *Fusarium* isolates tested in this study that did not induce symptoms of wilt on spinach plants also had a profile of *SIX* genes that matched the three permutations of *SIX8* and/or *SIX14* present in the *F. oxysporum* f. sp. *spinaciae* isolates. Therefore, it will be important to determine what other putative effector genes are associated with *F. oxysporum* f. sp. *spinaciae.* van Dam et al. (2016) demonstrated the capacity to predict effector genes associated with *miniature impalas* (*mimps*), which are repetitive transposable elements associated with the promoter regions of *SIX* genes (Schmidt et al. 2013). Leveraging this biological phenomenon detected in *F. oxysporum* f. sp. *lycopersici* enabled van Dam et al. (2016) to determine that *F. oxysporum* ff. spp. *cucumerinum*, *niveum*, *melonis*, *radicis-cucumerinum*, and *lycopersici* have unique profiles of predicted effector genes which may be important for host-specificity. Exploration of the effector suite of *F. oxysporum* f.

sp. *spinaciae* will be necessary to determine the genetic basis of pathogenicity of this fungus to spinach.

Due to the stringent climatic conditions required to raise high quality spinach seed crops, the production of spinach seed is restricted to the maritime PNW region of the United States, where the need to enhance management strategies for Fusarium wilt is of paramount concern for sustaining spinach seed production in the United States. Insights from this work ultimately are expected to aid in the development of molecular tools that can be used to detect, differentiate, and quantify this pathogen rapidly from plants, soil, and seed, and contribute to understanding the molecular and genetic basis of *F. oxysporum* f. sp. *spinaciae* interactions with spinach. This should provide invaluable information to breeders for identifying molecular markers for resistance to different isolates of the spinach Fusarium wilt pathogen, and developing spinach breeding lines and cultivars with enhanced resistance to Fusarium wilt.

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Table 2.1. *Fusarium* isolates characterized phenotypically in this study for pathogenicity to spinach and genotypically for the presence of 14 *Secreted in Xylem* genes.

Isolate code	*Fusarium* species and forma specialis[a]	Origin of isolate					
		Substrate of origin	Country	State	City or county	Year isolated	Source
Fus001	*F. oxysporum*	Spinach seed	USA	WA	Skagit Co.	2000	L. du Toit[b]
Fus004	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2000	L. du Toit[b]
Fus017	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2007	L. du Toit[b]
Fus020	*F. oxysporum*	Spinach seed crop	USA	WA	Skagit Co.	2007	L. du Toit[b]
Fus021	*F. oxysporum*	Spinach seed crop	USA	WA	Skagit Co.	2007	L. du Toit[b]
Fus023	*F. oxysporum*	Spinach seed crop	USA	WA	Skagit Co.	2007	L. du Toit[b]
Fus057	*F. oxysporum*	Spinach seed crop	USA	WA	Skagit Co.	2006	L. du Toit[b]
Fus058	*F. oxysporum*	Spinach seed crop	USA	WA	Skagit Co.	2006	L. du Toit[b]
Fus059	*F. oxysporum*	Spinach seed crop	USA	WA	Skagit Co.	2006	L. du Toit[b]
Fus062	*F. oxysporum* f. sp. *cepae*	Onion	USA	CO	Brighton	1982	H. Schwartz[c]
Fus125	*F. oxysporum* f. sp. *cepae*	Onion	USA	CO	Brighton	1982	H. Schwartz[c]
Fus127	*F. oxysporum* f. sp. *cepae*	Onion	USA	CO	West Slope	1983	H. Schwartz[c]
Fus129	*F. oxysporum* f. sp. *cepae*	Onion	USA	CO	West Slope	-	H. Schwartz[c]
Fus159	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	WA	-	-	Fiely et al. (1995)
Fus160	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	AR	-	-	Fiely et al. (1995)
Fus161	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	CA	-	-	Fiely et al. (1995)
Fus162	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	WA	-	-	Fiely et al. (1995)
Fus163	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	AR	-	-	Fiely et al. (1995)
Fus164	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	AR	-	-	Fiely et al. (1995)
Fus165	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	AR	-	-	Fiely et al. (1995)
Fus166	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	AR	-	-	Fiely et al. (1995)
Fus167	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	AR	-	-	Fiely et al. (1995)
Fus168	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	TN	-	-	Fiely et al. (1995)
Fus169	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	TN	-	-	Fiely et al. (1995)

Table 2.1. Continued.

Isolate code	*Fusarium* species	Origin of isolate					
		Substrate of origin	Country	State	City or county	Year isolated	Source
Fus170	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	OK	-	-	Fiely et al. (1995)
Fus172	*F. oxysporum* f. sp. *spinaciae*	Spinach	Japan	-	-	-	Fiely et al. (1995)
Fus173	*F. oxysporum* f. sp. *spinaciae*	Spinach	Japan	-	-	-	Fiely et al. (1995)
Fus182	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus183	*F. oxysporum*	Spinach	USA	WA	-	2008	L. du Toit[b]
Fus184	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus185	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus186	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus187	*F. oxysporum* (NPS)[i]	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus188	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus189	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus191	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus192	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus193	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2008	L. du Toit[b]
Fus250	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2009	L. du Toit[b]
Fus254	*F. oxysporum* f. sp. *spinaciae*	Spinach	USA	WA/ OR	-	2009	L. du Toit[b]
Fus259	*F. oxysporum*	Soil	USA	WA	Skagit Co.	2009	L. du Toit[b]
Fus263	*F. oxysporum*	Spinach seed	USA	WA	Skagit Co.	2009	L. du Toit[b]
Fus265	*F. oxysporum*	Soil	USA	WA	Skagit Co.	2009	L. du Toit[b]
Fus267	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2009	L. du Toit[b]
Fus270	*F. oxysporum*	Spinach	USA	WA	Skagit Co.	2009	L. du Toit[b]
Fus272	*F. oxysporum*	Spinach seed	USA	WA	-	2009	L. du Toit[b]
Fus287	*F. oxysporum*	Spinach	Italy	-	Battipagua	2012	J. de Visser[d]
Fus317	*F. proliferatum*	Soil	USA	WA	Skagit Co.	2010	L. du Toit[b]
Fus321	*F. oxysporum*	Spinach	USA	CA	Porterville	2011	L. du Toit[b]
Fus322	*F. oxysporum*	Spinach	USA	CA	-	2011	L. du Toit[b]
Fus323	*F. oxysporum*	Spinach	USA	CA	Porterville	2011	L. du Toit[b]
Fus324	*F. oxysporum*	Spinach	USA	-	-	2011	L. du Toit[b]
Fus327	*F. oxysporum*	Spinach	USA	-	-	-	S. Hartney[e]
Fus333	*F. oxysporum*	Spinach	USA	-	-	-	S. Hartney[e]
Fus393	*F. oxysporum* f. sp. *ciceris* race 1	Chickpea	India	-	-	-	F. Dugan[f]
Fus394	*F. oxysporum* f. sp. *ciceris* race 2	Chickpea	India	-	-	-	F. Dugan[f]
Fus395	*F. oxysporum* f. sp. *ciceris* race 3	Chickpea	India	-	-	-	F. Dugan[f]
Fus396	*F. oxysporum* f. sp. *ciceris* race 4	Chickpea	-	-	-	-	F. Dugan[f]

Table 2.1. Continued.

Isolate code	*Fusarium* species	Origin of isolate					
		Substrate of origin	Country	State	City or county	Year isolated	Source
Fus400	*F. oxysporum* f. sp. *pisi* race 1	Pea	-	-	-	-	F. Dugan[f]
Fus401	*F. oxysporum* f. sp. *pisi* race 1	Pea	-	-	-	-	F. Dugan[f]
Fus402	*F. oxysporum* f. sp. *pisi* race 2	Pea	-	-	-	-	F. Dugan[f]
Fus435	*Fusarium* sp. (putative pathogen of bean)	Bean	USA	NE	Mitchell	1998	L. Hanson[g]
Fus436	*F. oxysporum* (putative pathogen of onion)	Sugarbeet	USA	MN	Renville	2005	L. Hanson[g]
Fus439	*F. oxysporum* (putative pathogen of beet and spinach)	Sugarbeet Stem	USA	OR	-	2001	L. Hanson[g]
Fus440	*F. oxysporum* (putative pathogen of beet)	Sugarbeet Leaf	USA	OR	-	2001	L. Hanson[g]
Fus441	*F. oxysporum* (putative pathogen of beet and spinach)	Sugarbeet	USA	OR	-	1995	L. Hanson[g]
Fus442	*F. equiseti*	Sugarbeet	USA	MN	Crookston	2004	L. Hanson[g]
Fus447	*F. oxysporum* (putative pathogen of beet)	Sugarbeet	USA	MN	-	2002	L. Hanson[g]
Fus448	*F. oxysporum* (putative pathogen of beet)	Sugarbeet	USA	WY	Worland	2002	L. Hanson[g]
Fus452	*F. oxysporum* (putative pathogen of beet and spinach)	Sugarbeet mid root	USA	CO	Fort Morgan	2002	L. Hanson[g]
Fol1	*F. oxysporum* f. sp. *lycopersici* race 1	-	-	-	-	-	T. Gordon[h]
Fol2	*F. oxysporum* f. sp. *lycopersici* race 2	-	-	-	-	-	T. Gordon[h]
Fol3	*F. oxysporum* f. sp. *lycopersici* race 3	-	-	-	-	-	T. Gordon[h]

[a] Forma specialis designation is determined by previous pathogenicity tests performed by the individual cited as a source.

[b] Washington State University, Mount Vernon, WA.

[c] Colorado State University, Fort Collins, CO.

[d] Pop Vriend Seeds, Andijk, Netherlands.

[e] Sakata Seed America, Burlington, WA.

[f] United States Department of Agriculture (USDA) Agricultural Research Service (ARS), Pullman, WA.

[g] USDA ARS, Lansing, MI.

[h] University of California, Davis, CA.

[i] NPS = Isolate of *F. oxysporum* that was not a pathogen of spinach but was isolated from spinach seed, spinach plant, or soil in which spinach had been grown.

Table 2.2. Primer pairs used to characterize *Fusarium* isolates for the presence of the *Secreted in Xylem* (*SIX*) genes and for amplification and sequencing of the *translation elongation factor-1α* (*TEF-1α*) gene.

Gene	Primer set	Primer name	DNA sequence (5' - 3')	Expected product size	Annealing temperature (°C)	Source
SIX1	SIX1	P12-F2B	GTATCCCTCCGGATTTTGAGC	992	59	van der Does et al. (2008)
		P12-R1	AATAGAGCCTGCAAAGCATG			Rep et al. (2004)
SIX2	SIX2	SIX2-F2	CAACGCCGTTTGAATAAGCA	749	59	van der Does et al. (2008)
		SIX2-R2	TCTATCCGCTTTCTTCTCTC			
SIX3	SIX3	SIX3-F1	CCAGCCAGAAGGCCAGTTT	608	59	van der Does et al. (2008)
		SIX3-R2	GGCAATTAACCACTCTGCC			
SIX4	SIX4	SIX4-F1	TCAGGCTTCACTTAGCATAC	967	59	Lievens et al. (2009)
		SIX4-R1	GCCGACCGAAAAACCCTAA			
SIX5	SIX5	SIX5-F1	ACACGCTCTACTACTCTTCA	667	59	Lievens et al. (2009)
		SIX5-R1	GAAAACCTCAACGCGGCAAA			
SIX6	SIX6	SIX6-F1	CTCTCCTGAACCATCAACTT	793	59	Lievens et al. (2009)
		SIX6-R1	CAAGACCAGGTGTAGGCATT			
SIX7	SIX7	SIX7-F1	CATCTTTTCGCCGACTTGGT	862	59	Lievens et al. (2009)
		SIX7-R1	CTTAGCACCCTTGAGTAACT			
SIX8	SIX8	SIX8-F	TCGCCTGCATAACAGGTGCCG	250	59	Meldrum et al. (2012)
		SIX8-R	TTGTGTAGAAACTGGACAGTCGATGC			
SIX9	FOL SIX9[a]	-	GGGTGGACCATATCACGATGTTCG	458	69	Taylor et al. (2016)
		-	GAATACCTGAGTGGAGTTGTGTCTTG			
SIX9	FOC SIX9[a]	-	GGCCCAGCCCTAGTCTAACTCC	347	67	Taylor et al. (2016)
		-	AACTTAACATGCTGGCCGTCAATCG			
SIX10	SIX10	-	GTTAGCAACTGCGAGACACTAGAA	636	65	Taylor et al. (2016)
		-	AGCAACTTCCTTCCTCTTACTAGC			
SIX11	SIX11	-	ATTCCGGCTTCGGGTCTCGTTTAC	559	61	Taylor et al. (2016)
		-	GAGAGCCTTTTTGGTTGATTGTAT			
SIX12	SIX12	-	CTAACGAAGTGAAAAGAAGTCCTC	449	61	Taylor et al. (2016)
		-	GCCTCGCTGGCAAGTATTTGTT			

Table 2.2. Continued.

Gene	Primer set	Primer name	DNA sequence (5' - 3')	Expected product size	Annealing temperature (°C)	Source
SIX13	SIX13	-	CCTTCATCATCGACAGTACAACG	1027	61	Taylor et al. (2016)
		-	ATCAAACCCGTAACTCAGCTCC			
SIX14	FOL SIX14[a]	-	ATAAAGTGCGACTGGACTTCTGCC	422	67	Taylor et al. (2016)
		-	ACCCCCATCCACATTCCTAAGCGA			
SIX14	FOL SIX14 nest[b]	-	GATCCCAATGGGGGCTGTGT	232	59	Taylor et al. (2016)
		-	GCTGGTGGCTAGAATCTCTTTGGA			
SIX14	FOC SIX14[a]	-	ACAACACCGCGACGCTAAAAAT	438	61	Taylor et al. (2016)
		-	GCACACTCAGTGCGACAAGTTC			
TEF-1α	TEF-1α	526F	GTCGTYGTYATYGGHCAYGT	1041	Touchdown protocol (66 to 56°C)	Rehner and Buckley (2005)
		1567R	ACHGTRCCRATACCACCRATCTT			

[a] Primer sets preceded by FOC were designed by Taylor et al. (2016) against the *SIX* gene homolog of *F. oxysporum* f. sp. *cepae*. Primer sets preceded by FOL were designed by Taylor et al. (2016) for the *SIX* gene homolog of *F. oxysporum* f. sp. *lycopersici*.

[b] The primer set FOL SIX14 nest was designed by Taylor et al. (2016) to amplify 232 bp within the DNA fragment amplified with the primer set FOL SIX14.

Table 2.3. Probability values from the analyses of variance (ANOVAs) for the fixed effects of spinach parent (inbred) line and *Fusarium* isolate on area under the disease progress curve (AUDPC) and dry biomass/spinach plant for six pathogenicity tests completed from April 2017 to June 2018.

		ANOVA factor[a]			
Test	Response factor	Parent	Isolate	Parent-by-isolate	Transformation
1	AUDPC[b]	<0.0001	<0.0001	<0.0001	Rank
	Biomass/plant[c]	<0.0001	<0.0001	<0.0001	-
2	AUDPC[b]	<0.0001	<0.0001	0.0295	Rank
	Biomass/plant[c]	<0.0001	<0.0001	0.0157	-
3	AUDPC[b]	<0.0001	<0.0001	0.0039	Rank
	Biomass/plant[c]	<0.0001	<0.0001	0.0081	-
4	AUDPC[b]	<0.0001	<0.0001	<0.0001	Rank
	Biomass/plant[c]	<0.0001	<0.0001	<0.0001	-
5	AUDPC[b]	-	-	-	-
	Biomass/plant[c]	<0.0001	<0.0001	0.5761	-
6	AUDPC[b]	-	-	-	-
	Biomass/plant[c]	-	0.0056	-	-

[a] The experimental design for each pathogenicity test was a completely randomized block with four replications. Sixty-nine *Fusarium oxysporum* isolates were assessed for pathogenicity in six independent pathogenicity tests rather than one large pathogenicity test due to the amount of space and time required to assess 69 isolates for pathogenicity to three different spinach inbred lines simultaneously. Pathogenicity tests 1 to 5 were each completed in a greenhouse as a factorial treatment design (three spinach parent lines-by-isolate of *Fusarium*), whereas pathogenicity test 6 was completed in growth chambers with the highly susceptible spinach parent only. Rank indicates a rank transformation of the response factor (AUDPC or biomass) was calculated for ANOVA to address heterogeneous variances of the original data.

[b] Mean AUDPC value for six plants (subsamples) from each of four replications. Ranks were compared using Fisher's protected least significant difference ($P < 0.05$).

[c] Mean dry biomass/plant for six spinach plants evaluated in each of four replications per treatment combination.

Table 2.4. *Secreted in Xylem* (*SIX*) gene profile assessed for 73 *Fusarium* isolates used in this study.

Isolate code	*Fusarium* species designation	Pathogenicity group[a]	Origin	Reaction from PCR assays with specific *SIX* gene primer sets[b]														
				1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14-FOL*	*14-FOC*[c]
Fol1	*F. oxysporum* f. sp. *lycopersici* (race 1)	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NT
Fol2	*F. oxysporum* f. sp. *lycopersici* (race 2)	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	NT
Fol3	*F. oxysporum* f. sp. *lycopersici* (race 3)	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	NT
Fus021	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus057	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus059	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus159	*F. oxysporum* f. sp. *spinaciae*	1	WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus161	*F. oxysporum* f. sp. *spinaciae*	1	CA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus166	*F. oxysporum* f. sp. *spinaciae*	1	AR	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus169	*F. oxysporum* f. sp. *spinaciae*	1	TN	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus182	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus184	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus188	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus189	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus193	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus254	*F. oxysporum* f. sp. *spinaciae*	1	WA/OR	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus272	*F. oxysporum* f. sp. *spinaciae*	1	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus321	*F. oxysporum* f. sp. *spinaciae*	1	CA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus322	*F. oxysporum* f. sp. *spinaciae*	1	CA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus333	*F. oxysporum* f. sp. *spinaciae*	1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT
Fus004	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	+	-	-	-	NT
Fus020	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT
Fus058	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus160	*F. oxysporum* f. sp. *spinaciae*	2	AR	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT
Fus162	*F. oxysporum* f. sp. *spinaciae*	2	WA	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus163	*F. oxysporum* f. sp. *spinaciae*	2	AR	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus164	*F. oxysporum* f. sp. *spinaciae*	2	AR	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT

Table 2.4. Continued.

Isolate code	*Fusarium* species designation	Pathogenicity group[a]	Origin	Reaction from PCR assays with specific *SIX* gene primer sets[b]														
				1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14-FOL*	*14-FOC*[c]
Fus165	*F. oxysporum* f. sp. *spinaciae*	2	AR	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT
Fus167	*F. oxysporum* f. sp. *spinaciae*	2	AR	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus168	*F. oxysporum* f. sp. *spinaciae*	2	TN	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus170	*F. oxysporum* f. sp. *spinaciae*	2	OK	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus173	*F. oxysporum* f. sp. *spinaciae*	2	Japan	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus183	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	-	NT
Fus185	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus186	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus267	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus270	*F. oxysporum* f. sp. *spinaciae*	2	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT
Fus323	*F. oxysporum* f. sp. *spinaciae*	2	CA	-	-	-	-	-	-	-	+	-	-	-	-	-	-	NT
Fus324	*F. oxysporum* f. sp. *spinaciae*	2	-	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)[d]	NT
Fus001	*F. oxysporum* f. sp. *spinaciae*	?	Skagit Co., WA	-	-	-	-	-	-	-	+	-	-	-	-	-	-	NT
Fus172	*F. oxysporum* f. sp. *spinaciae*	?	Japan	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus287	*F. oxysporum* f. sp. *spinaciae*	?	Battipagua, Italy	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus393	*F. oxysporum* f. sp. *ciceris*	-	India	-	-	-	-	-	-	-	+	-	-	+	-	-	+	NT
Fus394	*F. oxysporum* f. sp. *ciceris*	-	India	-	-	-	-	-	-	-	+	-	-	+	-	-	+	NT
Fus395	*F. oxysporum* f. sp. *ciceris*	-	India	-	-	-	-	-	-	-	+	-	-	+	-	+	+	NT
Fus396	*F. oxysporum* f. sp. *ciceris*	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	NT
Fus400	*F. oxysporum* f. sp. *pisi* race 1	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	NT
Fus401	*F. oxysporum* f. sp. *pisi* race 2	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	NT
Fus402	*F. oxysporum* f. sp. *pisi* race 5	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	NT
Fus062	*F. oxysporum* f. sp. *cepae*	-	Brighton, CO	-	-	+	-	+	-	+	-	+	+	-	+	-	-	+
Fus125	*F. oxysporum* f. sp. *cepae*	-	Brighton, CO	-	-	+	-	+	-	+	-	+	+	-	+	-	+	+
Fus127	*F. oxysporum* f. sp. *cepae*	-	West Slope, CO	-	-	+	-	+	-	+	-	+	+	-	+	-	+	+
Fus129	*F. oxysporum* f. sp. *cepae*	-	West Slope, CO	-	-	+	-	+	-	+	-	+	+	-	+	-	-	+
Fus442	*F. equiseti*	-	Crookston, MN	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus435	*Fusarium* sp. (putative pathogen of bean)	-	Mitchell, NE	-	-	(+)[d]	-	-	-	-	-	-	-	-	-	-	-	NT

Table 2.4. Continued.

Isolate code	*Fusarium* species designation	Pathogenicity group[a]	Origin	Reaction from PCR assays with specific *SIX* gene primer sets[b]														
				1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14-FOL*	*14-FOC*[c]
Fus440	*F. oxysporum* (putative pathogen of beet)	-	OR	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus447	*F. oxysporum* (putative pathogen of beet)	-	MN	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)[d]	NT
Fus448	*F. oxysporum* (putative pathogen of beet)	-	Worland, WY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus439	*F. oxysporum* (putative pathogen of beet and spinach)	-	OR	-	-	+	-	+	-	-	-	-	-	-	-	-	+	NT
Fus441	*F. oxysporum* (putative pathogen of beet and spinach)	-	OR	-	-	-	-	-	-	-	+	-	-	-	-	-	+	NT
Fus452	*F. oxysporum* (putative pathogen of beet and spinach)	-	Fort Morgan, CO	-	-	-	-	-	-	-	+	-	-	-	-	-	-	NT
Fus436	*F. oxysporum* (putative pathogen of onion)	-	Renville, MN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus017	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus023	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus187	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	NT
Fus191	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus192	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus250	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus259	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus263	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus265	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus317	*F. proliferatum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
Fus327	*F. oxysporum* (NPS)	-	Skagit Co., WA	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)[d]	NT

[a] *Fusarium* isolates that induced the most severe wilt on the highly susceptible and moderately susceptible spinach parent lines relative to a partially resistant parent line were categorized in group 1. *Fusarium* isolates that induced a different response in severity of Fusarium wilt on the three parent lines were categorized into group 2. '-' = not applicable. '?' = pathogenicity group could not be determined due to limited and/or inconsistent disease development on the three parent lines. Refer to Table 2.1 for details of the isolates.

[b] Primer sets described in Table 2.2. '+' indicates the presence of a DNA fragment of the expected size. '-' indicates the absence of a DNA fragment of the expected size. '(+)' indicates the DNA fragment produced by the PCR assay was too low in concentration for sequencing. Genbank accession numbers for the sequenced DNA fragments of the *SIX* genes: MK906591-MK906742.

[c] The primer sets SIX14 FOL and SIX14 FOC (Taylor et al. 2016) were used to detect the presence of *SIX14*. The DNA fragment amplified from *F. oxysporum* f. sp. *cepae* isolates Fus062, Fus125, Fus127, and Fus129 had little sequence identity to *SIX14* genes amplified from other *Fusarium* isolates. 'NT' = sample not tested with the corresponding primer set.

[d] DNA fragment was not sequenced.

Fig. 2.1. Ordinal wilt rating scale used for spinach plants inoculated with isolates of *Fusarium oxysporum* f. sp. *spinaciae*. Numbers above plants represent wilt severity ratings.

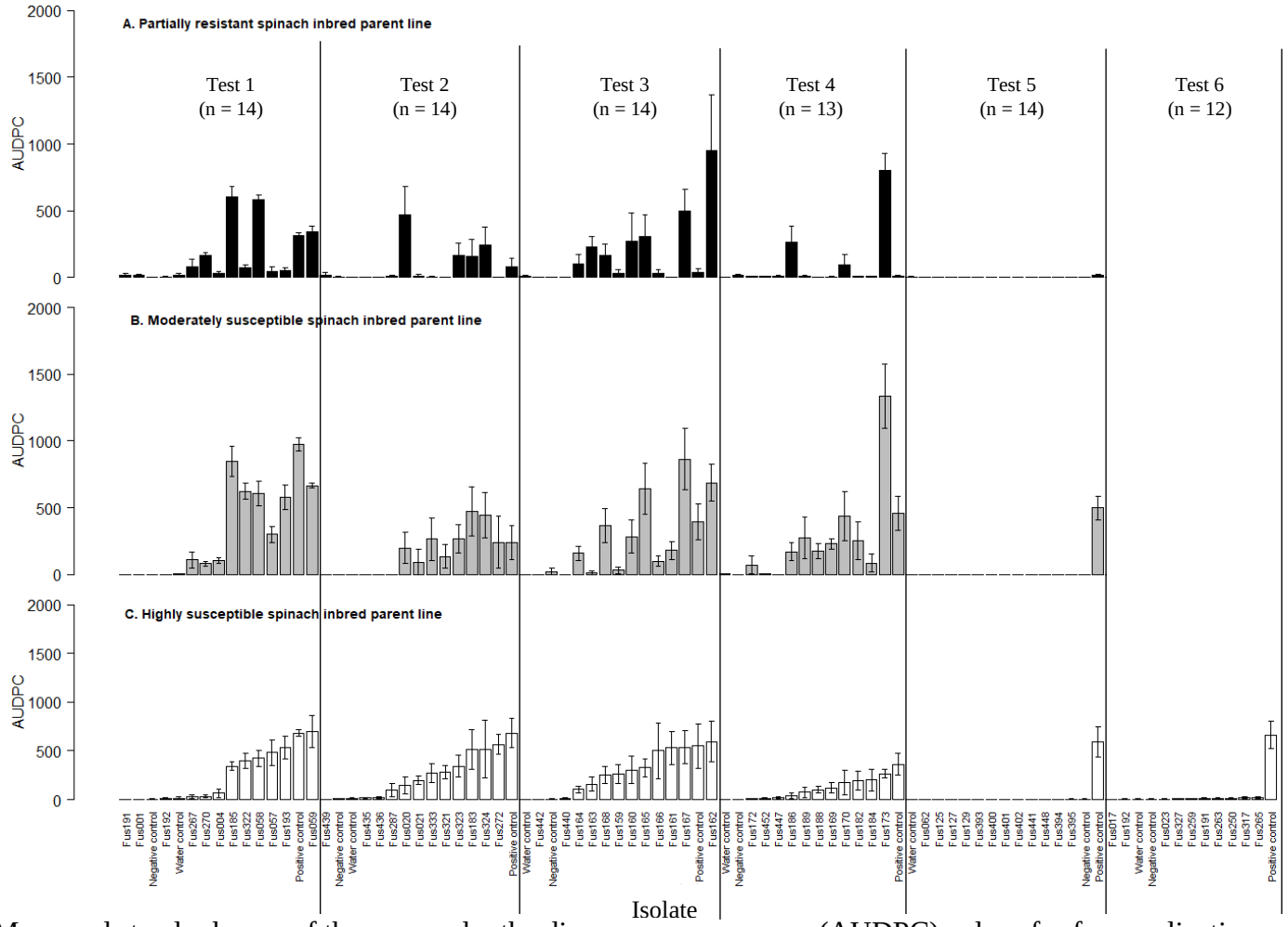

Fig. 2.2. Mean and standard error of the area under the disease progress curve (AUDPC) values for four replications of each spinach parent-line-by-inoculation treatment combination for 69 *Fusarium* isolates characterized phenotypically on A) a partially resistant spinach inbred line, B) a moderately susceptible spinach inbred line, and C) a highly susceptible spinach inbred line. The isolates were screened over six pathogenicity tests. n = number of *Fusarium* isolates evaluated in that test, including the positive control isolate (Fus254) of *F. oxysporum* f. sp. *spinaciae* and the negative control isolate (Fus187). Refer to the main text for details of the inoculations. Each test was carried out as a randomized complete block design. AUDPC was calculated based on weekly ratings of severity of wilt symptoms.

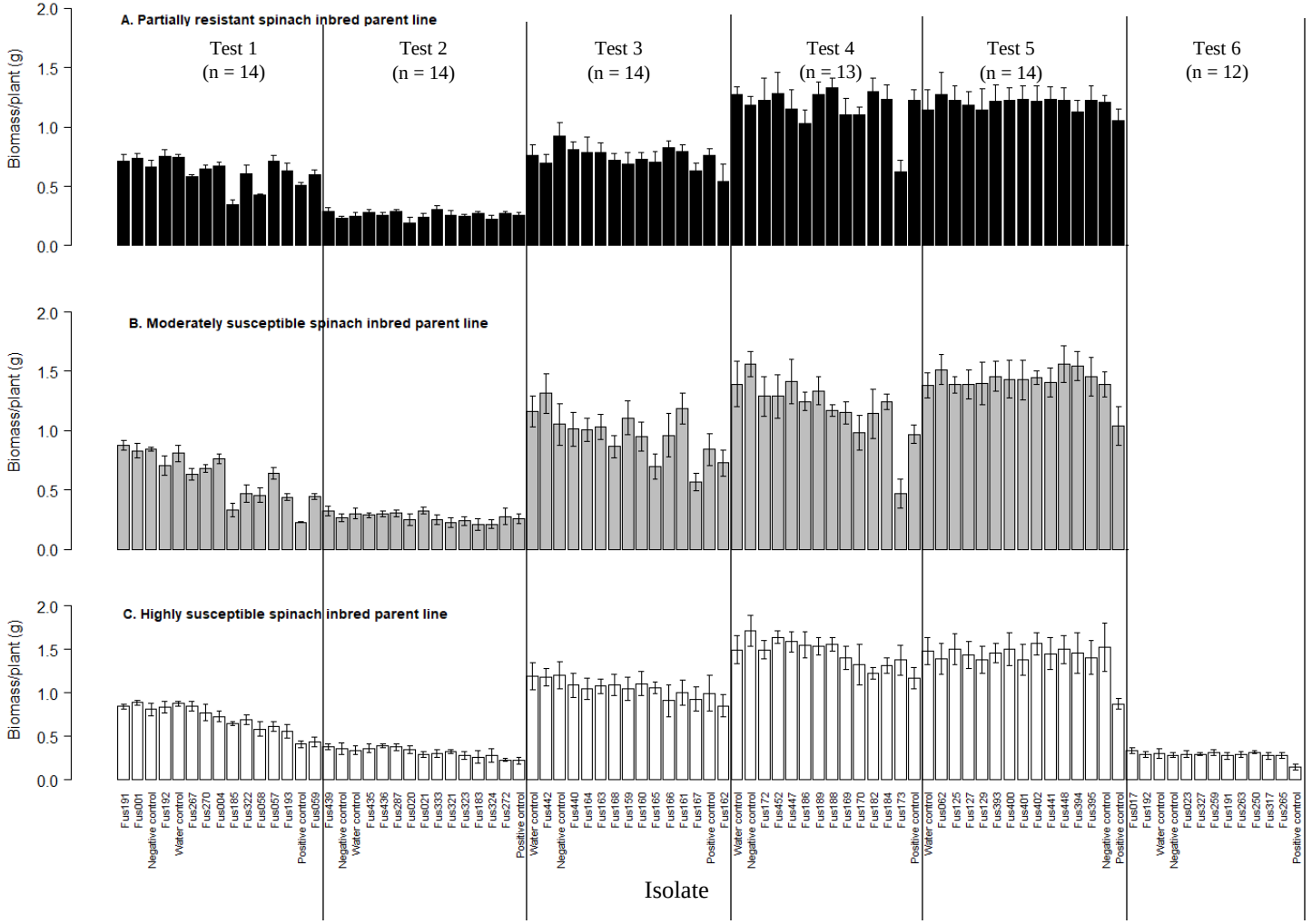

Fig. 2.3. Mean and standard error of biomass/plant (g) for four replications of each spinach parent-line-by-inoculation treatment combination for 69 *Fusarium* isolates characterized phenotypically on A) a partially resistant inbred line, B) a moderately susceptible inbred line, and C) a highly susceptible spinach inbred line. The isolates were screened over six pathogenicity tests. n = number of *Fusarium* isolates tested, including the positive control isolate of *F. oxysporum* f. sp. *spinaciae* (Fus254) and the negative control isolate (Fus187). Refer to the main text for details of the inoculations. Each test was carried out as a randomized complete block design.

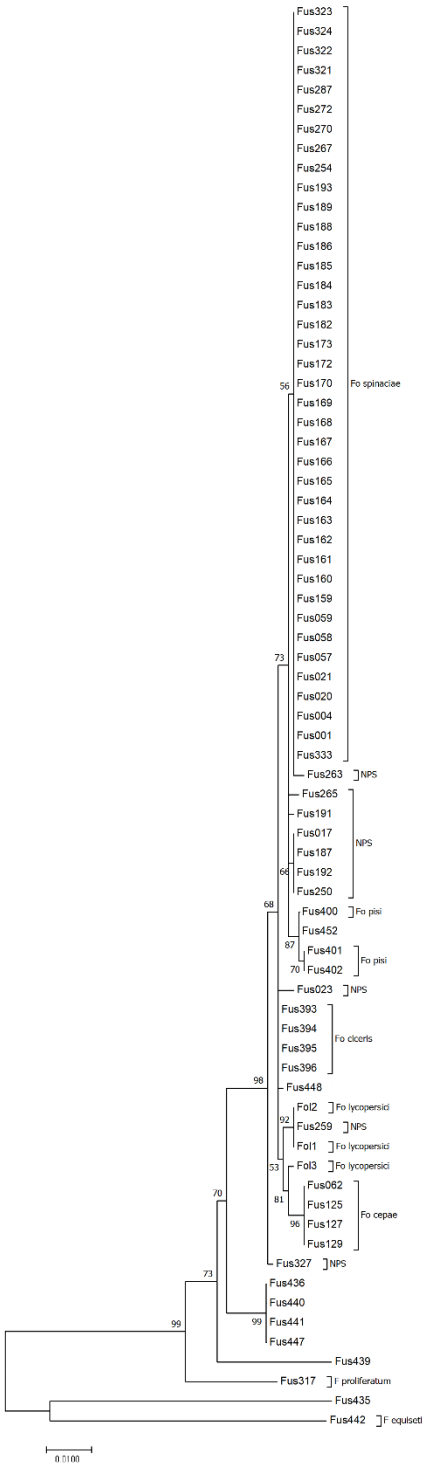

Fig. 2.4. Unrooted maximum likelihood phylogeny estimated from the partial coding region of the *translation elongation factor-1α* (Genbank accession numbers MK906743 to MK906815) gene amplified from *Fusarium* isolates. 'Fo' = *F. oxysporum*, 'NPS' = non-pathogenic, spinach associated isolates. The number at each node represents the percentage of trees with the node out of 1,000 bootstrap replicates. The scale bar indicates the average number of substitutions per site. Refer to Table 2.1 for details of the isolates.

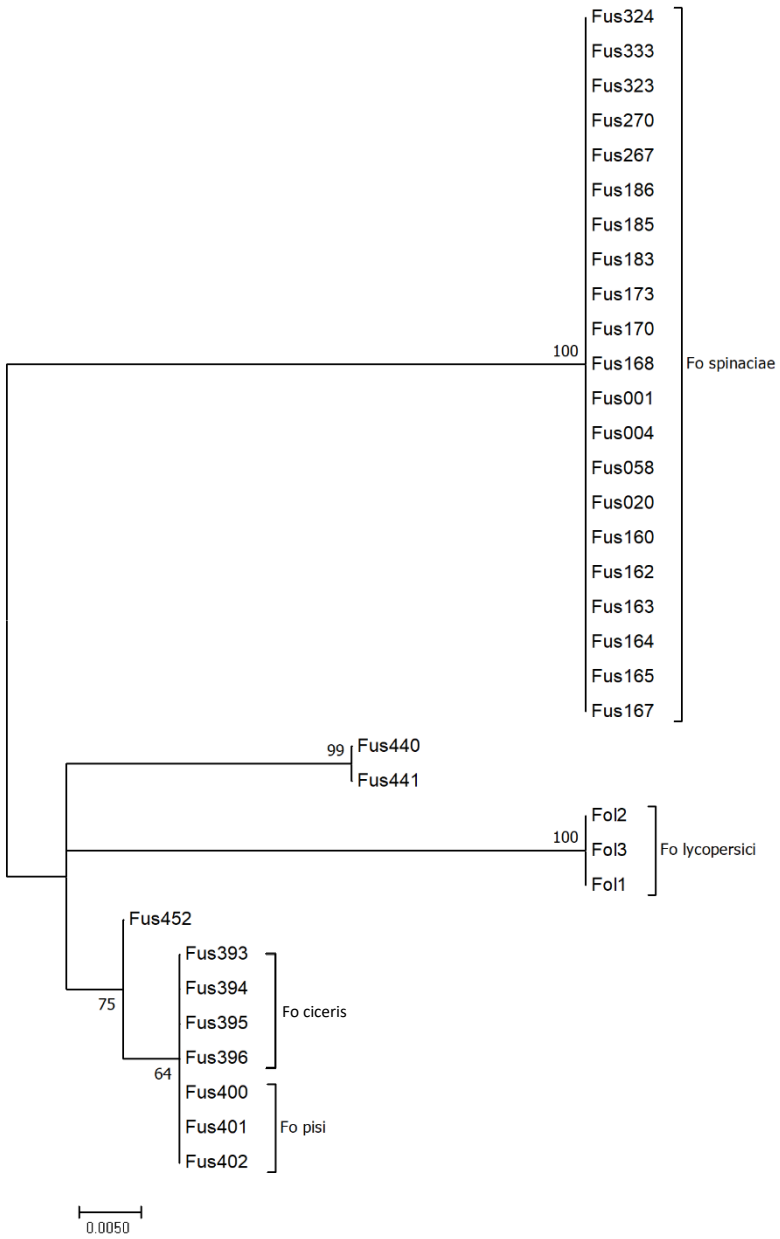

Fig. 2.5. Unrooted maximum likelihood phylogenetic tree estimated from coding region of the putative effector gene *Secreted in Xylem 8* (*SIX8*) amplified from *Fusarium* isolates. 'Fo' = *F. oxysporum*, 'NPS' = non-pathogenic, spinach associated isolates. The number at each node represents the percentage of trees with the node out of 1,000 bootstrap replicates. The scale bar indicates the average number of substitutions per site. Refer to Table 2.1 for details of the isolates.

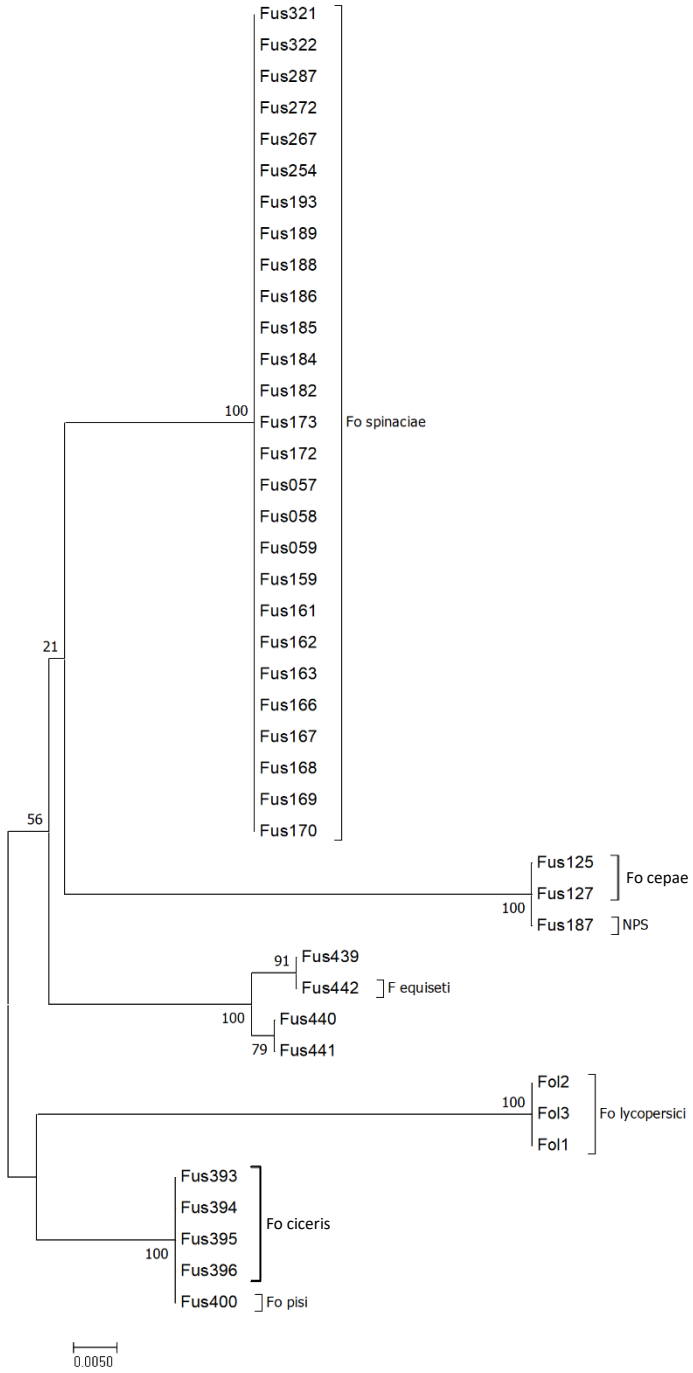

Fig. 2.6. Unrooted maximum likelihood phylogeny estimated from the putative effector gene *Secreted in Xylem 14* (*SIX14*) amplified from *Fusarium* isolates. 'Fo' = *F. oxysporum*, 'NPS' = non-pathogenic, spinach associated isolates. The number at each node represents the percentage of trees with the node out of 1,000 bootstrap replicates. The scale bar indicates the average number of substitutions per site. Refer to Table 2.1 for details of the isolates.

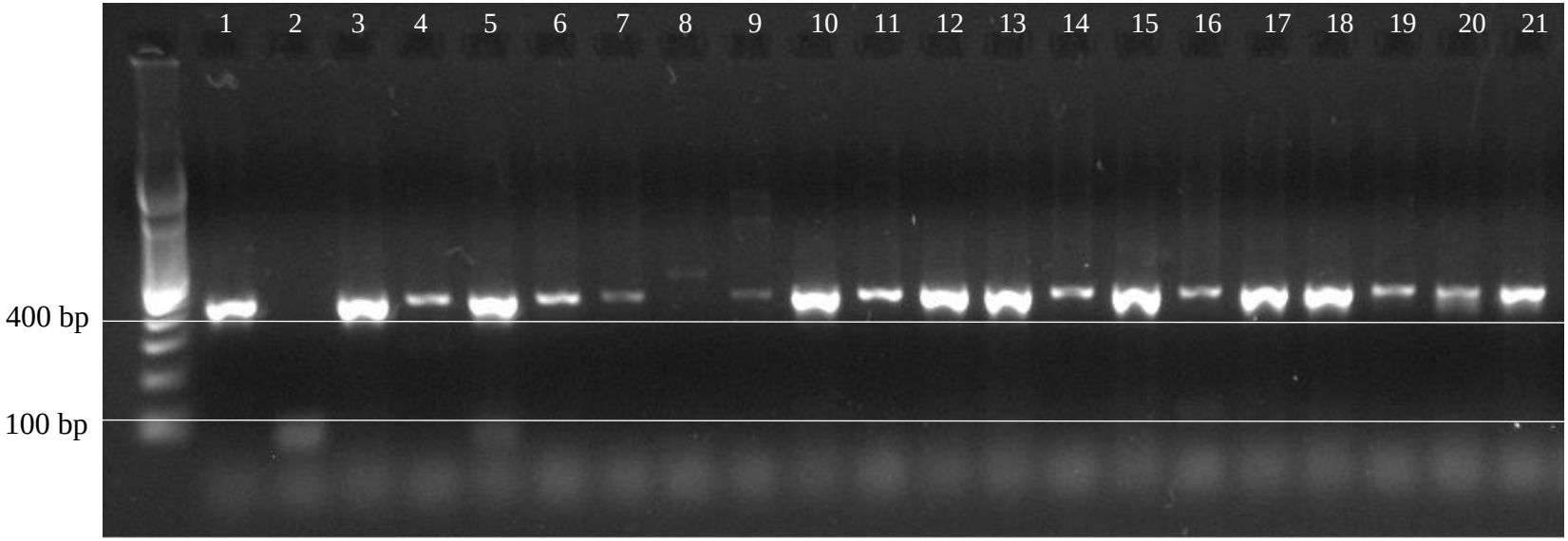

Fig. 2.7. *Secreted in Xylem 14* (*SIX14*) DNA sequence amplified from genomic DNA extracted from a selection of *Fusarium oxysporum* isolates. From lanes 1 to 22: ladder, Fol1, negative control (water), Fus159 (1), Fus160 (2), Fus161 (1), Fus162 (2), Fus163 (2), Fus164 (2), Fus165 (2), Fus166 (1), Fus167 (2), Fus168 (2), Fus169 (1), Fus170 (2), Fus172 (ND), Fus173 (2), Fus182 (1), Fus184 (1), Fus186 (2), Fus188 (1), and Fus189 (2). Refer to Tables 2.1 and 2.4 for details. The number in parentheses after each isolate indicates the pathogenicity group to which the isolate of *F. oxysporum* f. sp. *spinaciae* was categorized, as detailed in the main text and in Table 2.4. 'ND' indicates the pathogenicity group for that isolate was not determined.

CHAPTER THREE

PRELIMINARY GENOME ANALYSIS AND IDENTIFICATION OF PUTATIVE PATHOGENICITY GENES IN THE CAUSAL AGENT OF SPINACH FUSARIUM WILT

3.1 Introduction

The only region of the United States that is suitable for production of high quality spinach seed is the maritime Pacific Northwest (western Washington and Oregon), because of the requirement for long summer day length, mild summer temperatures, and low summer rainfall for optimal spinach seed health and quality (Foss and Jones 2005). This region produces nearly 100% of the spinach seed grown in the United States and up to 20% of the global supply of spinach seed of cultivars suitable for western markets (Foss and Jones 2005). The greatest biotic limitation to spinach seed production in this region is Fusarium wilt, caused by the soilborne fungal pathogen *Fusarium oxysporum* f. sp. *spinaciae* (Foss and Jones 2005; Gatch 2013). In the acid soils of this region, which are highly conducive to spinach Fusarium wilt, the pathogen is persistent, typically surviving for >10 years, which necessitates long spinach seed crop rotations (du Toit and Ocamb 2018). In some cases, crop rotations >15 years have been insufficient to avoid losses as great as 100% (Gatch 2013).

Several management strategies are used for spinach Fusarium wilt in seed crops in the maritime PNW. A quantitative soil bioassay which assesses the risk of spinach Fusarium wilt is offered annually to seed growers by the Vegetable Seed Pathology program at the Washington State University (WSU) Mount Vernon Northwestern Washington Research & Extension Center

(NWREC) by testing soil sampled from fields the growers are considering planting to spinach seed crops that spring (Gatch and du Toit 2015). This tool has been effective at helping growers and seed companies avoid fields with greater risk of Fusarium wilt to plant spinach seed crops. Even with this soil bioassay, seed growers use several practices to reduce the severity of the disease, such as adding agricultural limestone (97% $CaCO_3$) to the soil prior to planting to raise the soil pH and calcium levels (Gatch and du Toit 2017); using other soil amendments such as composts to increase soil health and spinach vigor (du Toit et al. 2014); biofumigation with *Brassica* seedmeal or *Brassica* cover crops (du Toit et al. 2006); and application of fungicides, particularly those in the Fungicide Resistance Action Committee (FRAC) Groups 1 and 3 (benzimidazoles and triazoles, respectively), as seed treatments or as soil applications (du Toit et al. 2014). However, these management practices only partially and/or transiently suppress the disease in such highly conducive soils. Although some spinach cultivars have partial resistance to Fusarium wilt (Laguna 2000; O'Brien and Winters 1977), many cultivars are highly susceptible. Furthermore, spinach seed is grown on contract with seed companies, and the parent lines that growers are contracted to plant may be highly susceptible to Fusarium wilt (Foss and Jones 2005; Gatch 2013). Thus, to improve spinach seed yields in the maritime PNW, additional management strategies are needed for Fusarium wilt, including the ability to develop parent lines with greater resistance, and the ability to detect and differentiate isolates of the pathogen rapidly and quantitatively for effective diagnoses and risk assessment.

F. oxysporum is a species complex since the species is composed of a genetically heterogeneous group of cryptic individuals, which includes isolates that are saprophytes, plant mutualists (Alabouvette 1999), entomopathogens (Hasan and Vago 1972), opportunistic human pathogens (Nucci and Anaissie 2002), and plant pathogens that induce wilts, rots, and damping-

off (Beckman 1987). As a plant pathogen, >140 host specific formae speciales (ff. spp.) are known, each of which cause a wilt disease of a single plant species or several closely related species (Edel-Hermann and Lecomte 2019). Efforts to differentiate *F. oxysporum* ff. spp. by sequencing barcoding loci (i.e., housekeeping genes and non-coding loci), and differentiation through multilocus DNA haplotyping have demonstrated that host-specificity is largely polyphyletic (Baayen et al. 2000; O'Donnell et al. 2009; van Dam et al. 2016). However, exceptions to this exist, such as *F. oxysporum* ff. spp. *cepae* and *ciceris*, which appear to have one evolutionary origin (Armitage et al. 2018; Jiménez-Gasco 2002). More recently, whole genome sequencing of the causal agent of tomato Fusarium wilt, *F. oxysporum* f. sp. *lycopersici*, revealed that the genome of this pathogen is composed of a core set of 11 chromosomes as well as four lineage-specific chromosomes (Ma et al. 2010). The core genome encodes genes that are essential for basal metabolism of this fungus, while the lineage-specific chromosomes are typically repeat-rich and encode effector genes that are necessary for pathogenicity (Ma et al. 2010).

The lineage-specific chromosome 14 was demonstrated to be transferred horizontally from *F. oxysporum* f. sp. *lycopersici* to a non-pathogenic strain of tomato, *F. oxysporum* strain Fo47, which transformed Fo47 into a pathogen of tomato (Ma et al. 2010). The transfer of pathogenicity from one isolate to another is determined by effector genes. One such group of effector genes is the *Secreted in Xylem* (*SIX*) genes. The 14 known *SIX* genes encode relatively small proteins (<300 amino acids) that are rich in cysteine residues (Rep 2005; Rep et al. 2004; Schmidt et al. 2013). Thirteen of the *SIX* genes were found on chromosome 14 of *F. oxysporum* f. sp. *lycopersici*, and one *SIX* gene was found in a lineage-specific region of chromosome 6 of *F. oxysporum* f. sp. *lycopersici* (Schmidt et al. 2013). Knockout experiments demonstrated that

SIX1 (Rep 2005), *SIX3* (Houterman et al. 2009), *SIX5* (Ma et al. 2015), and *SIX6* (Gawehns et al. 2014) reduced the virulence of *F. oxysporum* f. sp. *lycopersici* either partially or completely. Although it is known that some of the *SIX* genes are necessary for pathogenicity in the causal agent of tomato Fusarium wilt, the functions of the Six proteins remain unknown.

Analysis of the genomic locations of *SIX1* to *SIX7* on lineage-specific chromosome 14 of *F. oxysporum* f. sp. *lycopersici* revealed that these effector genes were associated with transposable elements: a miniature inverted-repeat transposable element (MITE) in the 5' promoter region called a *miniature Impala* (*mimp*), and mFot5 found downstream of the open reading frames of *SIX1*, *SIX2*, *SIX3*, *SIX5*, and *SIX7* (Schmidt et al. 2013). The association of *mimps* in the promoter region of each of the *SIX* genes facilitated the discovery of *SIX9* to *SIX14* (Schmidt et al. 2013) and, subsequently, *mimps* were used as markers to discover other putative effector genes in other *F. oxysporum* ff. spp. (Armitage et al. 2018; Schmidt et al. 2016; van Dam et al. 2016). The presence or absence of *SIX* genes and *mimp*-predicted effector genes can be used to differentiate some *F. oxysporum* ff. spp. and even among races of some specific formae speciales (Czislowski et al. 2018; Lievens et al. 2009; Taylor et al. 2016; van Dam et al. 2016), which is promising for the development of molecular tools that can discriminate formae speciales. Van Dam et al. (2016) demonstrated that *F. oxysporum* ff. spp. *cucumerinum*, *niveum*, *melonis*, *radicis-cucumerinum*, and *lycopersici* were differentiated by candidate effector genes predicted by association with a *mimp* in the 5' region of the start codon. Interestingly, *F. oxysporum* ff. spp. that cause disease of Cucurbitaceae genera and species, i.e., *cucumerinum*, *melonis*, *niveum*, and *radicis-cucumerinum*, had similar predicted effector gene profiles, suggesting that the repertoire of effector genes present in isolates of a forma specialis is associated with host-specificity.

Little is known about what drives host-specificity to spinach genetically in *F. oxysporum* f. sp. *spinaciae*. Previous work that aimed to characterize *F. oxysporum* f. sp. *spinaciae* isolates genetically focused on determination of vegetative compatibility groups (VCG) (Fiely et al. 1995), and DNA sequence variation among housekeeping genes and non-coding loci (Baayen et al. 2000; Kwabe et al. 2009; O'Donnell et al. 2009). Fiely et al. (1995) demonstrated that *F. oxysporum* f. sp. *spinaciae* isolates that induced more severe wilt symptoms on the spinach cultivar Grandstand were all in VCGs 0330 and 0332, and isolates that were less virulent were in VCG 0331. They described two groups of *F. oxysporum* f. sp. *spinaciae* isolates based on disease severity. Similarly, Chapter 2 of this thesis grouped 36 isolates of *F. oxysporum* f. sp. *spinaciae* into two phenotypic groups based on which of three spinach inbred lines developed more severe symptoms of wilt. However, very few studies have focused on determining genetic characteristics associated with host-specificity of *F. oxysporum* f. sp. *spinaciae*. Previous studies that profiled *F. oxysporum* ff. spp. for the presence of *SIX* genes only included three isolates of *F. oxysporum* f. sp. *spinaciae*, and only screened for the presence of *SIX1* to *SIX7*, none of which was detected in isolates of the spinach Fusarium wilt pathogen (Covey et al. 2014; Lievens et al. 2009). In Chapter 2 of this thesis, *SIX8* and/or *SIX14* were demonstrated to be present in 39 isolates of *F. oxysporum* f. sp. *spinaciae* examined. However, the presence of these *SIX* genes in *F. oxysporum* f. sp. *spinaciae* did not differentiate these isolates from other *F. oxysporum* isolates found in association with spinach but which did not cause wilt symptoms on spinach. Moreover, functional characterization or prediction of putative effector genes other than the *SIX* genes in *F. oxysporum* f. sp. *spinaciae* has not been documented publicly.

The objectives of this study were to identify genomic factors that differentiate isolates of *F. oxysporum* f. sp. *spinaciae* from *F. oxysporum* isolates not pathogenic on spinach, particularly

non-pathogenic isolates that originate from environments in which spinach is grown. Understanding the genetic underpinnings of host-specificity of *F. oxysporum* f. sp. *spinaciae* should make it possible to identify regions of the genome suitable for development of molecular markers that can be used to detect and quantify the pathogen more rapidly than tedious pathogenicity testing, which is currently the only means of identifying and

differentiating isolates of this pathogen from isolates not pathogenic on spinach. Furthermore, this understanding is expected to provide information for spinach breeders to develop parent lines and cultivars with greater levels of resistance to Fusarium wilt than is available currently.

3.2 Materials and Methods

3.2.1 Phenotypic characterization.

3.2.1.1 Isolate selection. In Chapter two, details are described for 39 isolates identified as *F. oxysporum* f. sp. *spinaciae* as they induced wilt symptoms on spinach plants, and 11 *Fusarium* isolates that did not induce wilt symptoms on spinach. Seven of the *F. oxysporum* f. sp. *spinaciae* isolates and five of the isolates that were not pathogens of spinach but were obtained from spinach plants, soil in which spinach was grown, or spinach seed (NPS isolates) were selected for repeat pathogenicity testing and whole genome sequencing. Selection was based on the locations in which the isolates were originally collected, the years of isolation, and the pathogenicity groups described in Chapter 2 (Table 3.1). All of the NPS isolates were collected in Washington State and were selected for whole genome sequencing based on the year of isolation. Each isolate was tested for pathogenicity on each of two proprietary inbred spinach lines, one highly susceptible and the other partially resistant to Fusarium wilt (Gatch and du Toit

2015). The pathogenicity test was designed as a 2-by-13 factorial treatment design of two spinach lines and 13 inoculation treatments (12 isolates plus a water control treatment), laid out as a randomized complete block design with four replicate blocks. The trial was repeated.

3.2.1.2 Inoculation. The 12 isolates were grown at room temperature in ambient light on half-strength potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) amended with 100 ppm chloramphenicol. A microconidial suspension of each *F. oxysporum* isolate was generated by placing three colonized, 1 mm^3 agar cubes from the leading edge of hyphal growth of a 5-day-old PDA colony into 200 mL of sterilized Kerr's Broth [28.6 g sucrose, 0.48 g yeast extract, 1.9 g $NaNO_3$, 0.95 g KH_2PO_4, 0.48 g KCl, 0.48 g $MgSO_4 \bullet 7H_2O$, 0.02 g $FeSO_4 \bullet 7H_2O$, per liter deionized water (Kerr 1963)]. The inoculated Kerr's broth was shaken at 125 RPM on a gyrorotary shaker at room temperature with ambient light for seven days. Each microconidial suspension was then filtered through one layer of autoclave-sterilized cheesecloth, followed by a second filtration through two layers of sterilized cheesecloth to remove mycelium. The spore concentration of each microconidial suspension was assessed using a hemocytometer, and diluted in water to 9.93 x 10^5 spores/ml with sterilized, deionized water in a final volume of 200 ml. The 200 ml of inoculum were mixed into 5,300 ml of RediEarth propagation mix (SunGro Horticulture, Agawam, MA) by spraying the spore suspension over the propagation mix as the propagation mix was tumbled in a Gustafson Batch Seed Treater to achieve a final concentration of 3.75 x 10^4 microconidia/ml propagation mix. The inoculum or water (negative control treatment) was applied to the propagation mix over 5 min, and an additional 100 ml of deionized water was applied to the substrate to wash any microconidia remaining in the spray bottle onto the propagation mix. The inoculated propagation mix was separated into eight 6-cell packs (each cell 5.7 cm x 5.7 cm x 7.62 cm tall) (The HC Companies, Twinsburg, OH). Two seeds of the

appropriate spinach parent line were sown into each cell at a depth of approximately 1 cm. The spray bottle, seed treater, and benchtops were sanitized with 70% isopropyl alcohol and triple-rinsed with deionized water between inoculation treatments. The plants were maintained for 39 days in a growth chamber, as detailed below. The repeat trial (trial 2) was set up in a greenhouse and maintained for 35 days because of accelerated development of wilt symptoms compared to trial 1 in a growth chamber.

3.2.1.3 Maintenance of plants. In trial 1, the spinach plants were maintained in a growth chamber (Percival, Perry, IA) with a photoperiod of 9 h day:15 h night and temperature set at 22°C by day and 18°C by night. After 10 days, the spinach plants were thinned to one plant per cell (6 plants/experimental unit), and the growth chamber temperature was increased to 28°C by day and 24°C by night to increase transpirational demand and, therefore, enhance development of Fusarium wilt. The plants were fertigated daily with water supplemented with 20:20:20 (N:P:K) fertilizer (Plant Marvel, Chicago, IL) injected at a ratio of 1:100 for a final rate of 200 ppm N. Trial 2 was completed in a greenhouse between 28 September 2018 and 2 November 2018 when the daylength was short enough to prevent bolting of the spinach plants. The plants were supplemented with incandescent lighting on a 9 h day:15 h night schedule, and a temperature of 22°C by day and 18°C by night. After 10 days, the spinach plants were thinned to one plant per cell (6 plants per experimental unit) and the greenhouse temperature was increased to 28°C by day and 24°C by night.

3.2.1.4 Disease assessment. The spinach plants were rated for wilt symptoms weekly using an ordinal scale of 0 to 5 (0 = no wilt symptoms, 1 = first pair of true leaves wilted, 2 = first and second pairs of true leaves wilted, 3 = first through third pairs of true leaves wilted, 4 = all aboveground tissue flaccid, and 5 = plant dead; Fig. 2.1). The weekly ratings for all six

inoculated seedlings per replication were then converted to a Fusarium wilt severity index (%) as described by Gatch (2013):

$$\text{FSI (Fusarium severity index)} = \frac{\sum(\text{Ordinal severity rating} * \text{number of seedlings})}{(\text{Total number of seedlings} * 5)} * 100\%$$

The area under the disease progress curve (AUDPC) was calculated using the trapezoidal method (Sparks et al. 2008) from time points 0 to 39 (trial 1) or 35 (trial 2) days after planting. The aboveground biomass of each spinach plant was harvested 39 (trial 1) or 35 (trial 2) days after planting by cutting each plant at the base of the stem. All replicate plants per treatment were dried in a paper bag at 65°C for 72 h, and the dried biomass measured.

3.2.1.5 Statistical analyses. Statistical analyses were performed in SAS Version 9.4 (SAS Institute, Cary, NC) with PROC MIXED. PROC CORR was used to calculate Pearson's correlation coefficient for weekly disease ratings, the AUDPC, and biomass/plant. Normality of errors of the response variables were determined with PROC UNIVARIATE. Heterogeneity of variances was assessed by modeling variances with a first-order variance-covariance structure using the parameter 'type = un(1)' in the repeated statement. Log, square root, or arcsin transformations were used to transform response data when parametric assumptions of normally distributed residuals and/or homogeneous variances were not met. If the transformed response data did not meet parametric assumptions for analysis of variance (ANOVA), the response variables were rank transformed, and statistical analyses performed on the ranked data. Means separations were calculated with Fisher's protected least significant difference (LSD) at $P < 0.05$, and means were grouped with pdmix800 (Saxton 1998).

3.2.2 Genomic characterization.

3.2.2.1 DNA extraction. The 12 isolates selected for whole genome sequencing were grown on half-strength PDA amended with 100 ppm chloramphenicol. For each isolate, two 3-mm^3 colonized agar plugs were taken from the leading edge of hyphal growth and placed in 75 mL of sterilized potato dextrose broth in a 125 mL Erlenmeyer flask. Each inoculated flask was shaken on an orbital shaking-platform at 125 RPM at approximately 24°C for 7 days under ambient light. The fungal mycelium was then harvested from each isolate in a laminar flow hood by vacuum-filtration through size 1 Whatman filter paper (Whatman PLC, Maidstone, United Kingdom) in a Büchner funnel. The mycelium from each isolate was stored in a sterilized 1.7 mL Eppendorf tube at -80°C until further use. DNA was extracted from approximately 100 mg of frozen mycelium from each isolate with the Purelink Total Genomic DNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Genomic DNA concentration and integrity were determined with the Qubit fluorometer (Invitrogen, Carlsbad, CA) and gel electrophoresis, respectively.

3.2.2.2 Genome sequencing. Library preparation and sequencing were completed by the Molecular Research DNA Lab (Shallowater, TX). Eleven isolates were sequenced with the Illumina HiSeq platform (2 x 250 bp, ~10 million reads per paired end). The DNA samples were prepared with the KAPA HyperPlus Kits (Roche, Basel, Switzerland) following the manufacturer's protocol. In addition, genomic DNA isolated from the twelfth isolate of *F. oxysporum* f. sp. *spinaciae*, Fus254, was sequenced with the Pacific Biosciences Sequel. Isolate Fus254 was selected for long read sequencing to generate a more complete genome assembly. This isolate was chosen for the PacBio platform since it had been characterized extensively for pathogenicity of spinach as a positive control treatment in the pathogenicity tests described in

Chapter 2. The library for the sample was prepared using SMRTbell Template Prep Kit (Pacific Biosciences, Menlo Park, CA) following the manufacturer's protocol. The DNA sample of *F. oxysporum* f. sp. *spinaciae* isolate Fus254 was sheared to a size of approximately 7.2 Kbp using the Covaris G-tube (Covaris Inc., Woburn, MA), and 1.0 µg of the sample was used to enter the SMRTbell Template Prep protocol. The library pool was then sequenced on two SMRT Cells using a 10-hour movie time on the PacBio Sequel (Pacific Biosciences).

Additionally, three genome assemblies of *F. oxysporum* f. sp. *spinaciae* were provided by Drs. Martijn Rep and Mara de Sain at the University of Amsterdam, Amsterdam, Netherlands for this study. The genome assemblies of these isolates were labeled Fospi001, Fospi002, and Fospi003 and are representative of *F. oxysporum* f. sp. *spinaciae* isolates deposited in the USDA Agricultural Research Service (ARS) culture collection Northern Regional Research Laboratory (NRRL) (Fospi001 = NRRL26874, Fospi002 = NRRL26875, and Fospi003 = NRRL26876). These isolates also, circumstantially, turned out to be the isolates Fus165, Fus166, and Fus167, respectively, that were tested for pathogenicity on spinach as detailed in Chapter 2 (Appendix Table 2.1, Figs. 2.2 and 2.3).

3.2.2.3 Raw read data and genome assembly. The sequence reads of the 12 isolates used for this part of the study were deposited in the sequence read archive (SRA) under the BioProject number PRJNA540981. BioSample numbers for the 12 isolates sequenced can be found in Table 3.1. Illumina reads were quality-trimmed using Fastq-MCF Version 1.04.676 (https://expressionanalysis.github.io/ea-utils) with a quality threshold of 20, and Illumina adapters removed. The trimmed reads were assembled with CLC-workbench Version 8.0 (Qiagen) with the following parameters: insert size of the sequenced fragments was set to a range of 1 to 1,000 bp (default), de Bruijn word and bubble size were set to automatic, and minimum

contig length was set to 500. *De novo* assembly of the PacBio reads of isolate Fus254 was accomplished using the SMRT Analysis Hierarchical Genome Assembly Process (HGAP) Version 3.0. HGAP consists of three primary processes: preassembly, assembly, and consensus polishing. The expected genome size was estimated to be 50 Mbp based on the genome size of other *F. oxysporum* ff. spp. that have been sequenced (Ma et al. 2010; van Dam et al. 2016). The single pass reads were mapped against the seed reads, which averaged 13.1 Kb. Assembly statistics were calculated with QUAST (Gurevich et al. 2013). Additionally, Benchmarking Universal Single-Copy Orthologs (BUSCO) was used as an estimate of genome completeness (Simão et al. 2015). BUSCO assesses the presence of single copy orthologs that are conserved evolutionarily among phylogenetic lineages. Ultimately, the analysis determines the number of orthologs that are either present in single copies, fragmented, duplicated, or missing completely. The ortholog database selected to estimate genome completeness was Sordariomyceta *obd9*. To get an estimate of coverage, and regions of the genome that were not sequenced with shorter read technology (Illumina), the trimmed paired-end Illumina reads generated from the isolates that were sequenced for this study (Table 3.1) were mapped to the assembly of Fus254 with Bowtie 2 (Langmead and Salzberg 2012). The depth of coverage of the reads across the assembly of Fus254 was visualized with the open-source plotting tool gnuplot. Additionally, a rough estimate of depth of coverage for each Illumina assembly was estimated with the Lander/Waterman approach by multiplying the average read length of paired end sequences (L) by the number of reads (n), and dividing by the assembly size (G), resulting in average coverage (C) (Lander and Waterman 1988).

3.2.2.4 Phylogenetic analyses of housekeeping genes. The housekeeping genes *translation elongation factor 1α* (*TEF-1α*), *RNA polymerase binding protein 1* (*RPB1*), and β-

tubulin (β-*TUB*) were extracted from the genome assemblies of all *F. oxysporum* f. sp. *spinaciae* isolates, NPS isolates, and 105 publicly accessible genomes of 24 *F. oxysporum* ff. spp. from Genbank (Appendix Table 3.1). These loci were selected for describing intraspecies variation among individual isolates within the *F. oxysporum* species complex (Geiser et al. 2004; Laurence et al. 2014). Each locus was detected among the 120 genome assemblies by submitting a query with the BLASTn command-line tool, the coordinates for each locus in each assembly were extracted, and the sequences were aligned with Clustal Omega using default parameters. The alignments were imported into MEGA 7.0 (Kumar et al. 2016), and maximum likelihood trees were estimated for *TEF-1α, RPB1*, β-*TUB*, and the concatenated sequences of all three loci. Models of evolution were selected based on the lowest Bayesian Information Criterion value in the MEGA 7.0 native 'Model Test' function. Clade stability was estimated with 100 bootstrap replicates.

3.2.2.5 Effector gene prediction. The pipeline described by van Dam et al. (2016) to search for putative effector genes associated with *mimps* was used to predict effector genes associated with *mimps* among the 12 *F. oxysporum* f. sp. *spinaciae* and NPS genome assemblies generated in this study. The pipeline identified *mimps* in each genome assembly, and identified open reading frames (ORFs) 2,500 bp downstream of the *mimp* by gene prediction with AUGUSTUS 3.1 (Stanke 2004) and by detecting codons that encode a methionine or stop signal. The predicted genes were screened with SignalP 4.1 (Petersen et al. 2011) for the presence of a secretion signal at the N-terminus of the predicted amino acid. The pipeline then predicted ORFs with a secretion signal as noted above, that were then pooled so that redundant records could be removed. The list of curated effectors was searched with the Basic Local Alignment Search Tool (BLASTn) among all query genome assemblies, and the presence/absence plot generated was

clustered hierarchically by effector profile (y-axis) and distribution of predicted effectors throughout the assemblies (x-axis). The 14 known *SIX* genes were also searched for in the genome assemblies of all 12 *F. oxysporum* f. sp. *spinaciae* and NPS isolates used in this study (Table 3.4). The DNA sequences of *SIX1* to *SIX14* acquired, as detailed in Chapter 2, from *F. oxysporum* f. sp. *lycopersici* race 1 (isolate Fol1, Table 2.1) were used as a query in each genome assembly.

3.3 Results

3.3.1 Phenotypic characterization overview. Overall, the results of the two pathogenicity trials were congruent (Tables 3.2 and 3.3), with similar trends in the severity of wilt that each isolate of *F. oxysporum* f. sp. *spinaciae* induced on the two spinach parent lines. Similar to the results in Chapter 2, two main groups of *F. oxysporum* f. sp. *spinaciae* isolates were identified as characterized by the severity of wilt induced on the highly susceptible vs. partially resistant parent lines (Appendix Fig. 3.1; Table 3.3). Isolates identified as belonging to *F. oxysporum* f. sp. *spinaciae* pathogenicity group 1 induced the expected response, i.e., the highly susceptible parent line developed more severe wilt than the partially resistant parent line. However, isolates classified as belonging to pathogenicity group 2 of *F. oxysporum* f. sp. *spinaciae* induced either a similar severity of wilt on both parents or more severe wilt on the partially resistant parent relative to the highly susceptible parent (Table 3.3). Although weekly ratings of spinach Fusarium wilt were made, the AUDPC was used as an estimate of wilt severity over the duration of both trials to quantify differences in severity of Fusarium wilt symptoms among treatments. As expected, the AUDPC was positively correlated with all wilt severity ratings in trial 1 (0.3163

≤ r ≤ 0.9920 for the first to last weekly ratings, respectively, P <0.0011) and trial 2 (0.5920 ≤ r ≤ 0.9941, P < 0.0001).

3.3.1.1 Growth chamber pathogenicity test (trial 1). Symptoms of Fusarium wilt were first apparent 14 days after planting (DAP). There were significant differences in the fixed effects of spinach parent line and isolate (P < 0.0001), and the parent-by-isolate interaction term (P < 0.0001) for the response variable AUDPC (Table 3.2). Disease severity based on the AUDPC ranged from 0 to 1,847 and 0 to 1,195 for the highly susceptible and partially resistant spinach parent lines, respectively. For the 13 inoculation treatments on the highly susceptible spinach line, 10 isolates induced an AUDPC that was significantly greater than the AUDPC measured for plants that received the water control treatment: Fus001, Fus057, Fus059, Fus167, Fus173, Fus191, Fus250, Fus254, Fus259, and Fus322 (P ≤ 0.0077 for all comparisons with the control treatment). The low AUDPC values for the highly susceptible parent treated with isolates Fus191, Fus250, and Fus259 probably reflected low wilt ratings early in the trial in one to two replicate blocks that appeared to be associated with overwatering the plants in the growth chamber. The mean ± standard error of the final FSI of spinach plants of the highly susceptible parent line inoculated with Fus191, Fus250, and Fus259 was 0.0 ± 0.0, 4.33 ± 4.16, and 6.67 ± 13.33%, respectively. In contrast, the average FSI of the highly susceptible parent grown in substrate inoculated with Fus254 was 71.3 ± 5.7% by 39 DAP. For the partially resistant parent line, seven of 12 isolates resulted in a mean AUDPC that differed significantly from that of the same parent treated with water: Fus001, Fus057, Fus059, Fus167, Fus173, Fus254, and Fus322 (P < 0.0001).

The dry biomass of each spinach plant ranged from 0.05 to 1.09 g and 0.12 to 0.66 g for the highly susceptible and partially resistant parents, respectively. The biomass of spinach plants

of the highly susceptible parent grown in substrate inoculated with six of the 12 *F. oxysporum* isolates (Fus057, Fus059, Fus167, Fus173, Fus254, and Fus322) was significantly less than the biomass of the same parent treated with water ($P \leq 0.0246$). In comparison, the dry biomass of the partially resistant parent line was reduced significantly when grown in substrate inoculated with the following six of the 12 *F. oxysporum* isolates: Fus001, Fus057, Fus059, Fus167, Fus173, and Fus254 ($P \leq 0.0253$). The dry biomass/spinach plant of the partially resistant parent grown in soil inoculated with Fus017, Fus187, Fus191, Fus250, Fus259, and Fus322 was similar to the biomass of that parent treated with water ($0.2315 \leq P \leq 0.9189$). Furthermore, mean AUDPC values and the dry biomass/plant were negatively correlated (Pearson's correlation coefficient r = -0.802, $P < 0.0001$), indicating that increased disease severity as measured by AUDPC was correlated with a decrease in spinach biomass

Differential virulence of the *F. oxysporum* f. sp. *spinaciae* isolates was observed between the two spinach parent lines. The mean AUDPC of the highly susceptible parent was greater than that of the partially resistant parent in soil infested with isolates Fus057, Fus059, Fus254, and Fus322 ($P \leq 0.0001$). The AUDPC values of the highly susceptible parent were ~4 to 7 times greater than the AUDPC values of the partially resistant parent inoculated with these four isolates. However, the AUDPC of the partially resistant parent was either greater or comparable to that of the highly susceptible parent when inoculated with isolates Fus001, Fus167, and Fus173 ($0.0144 \leq P \leq 0.0315$).The AUDPC values of the partially resistant parent were ~2 to 3 times greater than the AUDPC values of the highly susceptible parent when inoculated with these three isolates.

3.3.1.2 Pathogenicity test (trial 2). Similar responses in virulence or avirulence to spinach were observed in trial 2 compared to trial 1. There were significant differences in AUDPC values

among the fixed effects of spinach parent line and isolate ($P < 0.0001$), and the parent-by-isolate interaction term ($P < 0.0001$) (Table 3.2). Among the fungal isolates used to inoculate the highly susceptible parent line, the AUDPC values ranged from 0 to 1,832, compared with 0 to 1,400 for the partially resistant parent line. Overall, seven of the 12 *F. oxysporum* isolates resulted in AUDPC values that were greater than that of the water control treatment on both parent lines (Table 3.3). For the highly susceptible parent, AUDPC values for the following seven isolates differed from that of the water control treatment: Fus001, Fus057, Fus059, Fus167, Fus173, Fus254, and Fus322 ($P < 0.0001$). Symptoms of wilt were not observed on plants of the highly susceptible parent line inoculated with isolates Fus017, Fus187, Fus191, Fus250, and Fus259. The average FSI of the highly susceptible parent line at the final rating (35 DAP) in soil inoculated with Fus017, Fus187, Fus191, Fus250, or Fus259 was 0 to 0.83%, for which the ranked AUDPC ranged from 0 to 2.91. The AUDPC values of the partially resistant parent line inoculated with isolates Fus001, Fus057, Fus059, Fus167, Fus173, Fus254, and Fus322 was significantly greater than that of the water control treatment ($P < 0.0001$). However, the AUDPC value was not different from that of the control treatment when the partially resistant parent was inoculated with five of the 12 isolates, Fus017, Fus187, Fus191, Fus250, or Fus259 ($P \geq 0.1301$).

For the ranked dry biomass/spinach plant, significant differences were observed for the fixed effects of spinach parent ($P = 0.0233$) and isolate ($P < 0.0001$), and the parent-by-isolate interaction term ($P < 0.0001$) (Table 3.2). The average dry biomass/plant ranged from 0.02 to 0.57 g and 0.04 to 0.38 g for the highly susceptible and partially resistant parent lines, respectively. Plants of the highly susceptible parent inoculated with Fus001, Fus057, Fus059, Fus167, Fus173, Fus254, or Fus322 had significantly less biomass than that of the plants treated with water ($P < 0.0001$), while plants inoculated with Fus017, Fus0187, Fus191, Fus250, or

Fus259 had a similar biomass to the plants treated with water ($0.2733 \leq P \leq 0.8546$). Plants of the partially resistant parent line inoculated with isolates Fus001, Fus057, Fus059, Fus167, Fus173, or Fus254 had less average biomass than the same parent treated with water ($P \leq 0.0004$), in contrast to the biomass of the partially resistant parent inoculated with Fus017, Fus0187, Fus191, Fus250, Fus259, or Fus322 which was similar to that of the same parent line treated with water ($0.1222 \leq P \leq 0.9854$). AUDPC values and the dry biomass/plant of each experimental unit were negatively correlated (Pearson's correlation coefficient r = -0.8778, $P < 0.0001$), i.e., increased disease severity measured by the AUDPC value was negatively correlated with spinach biomass at the end of the trial.

Differential virulence was observed among the seven *F. oxysporum* f. sp. *spinaciae* isolates with respect to the two spinach parent lines (Table 3.3). The AUDPC values did not differ significantly between the highly susceptible and partially resistant parent lines in soil inoculated with Fus001 ($P = 0.7473$), Fus167 ($P = 0.6521$), or Fus173 ($P = 0.1528$) (Table 3.3). However, the mean AUDPC value differed between the two parent lines planted in potting medium infested with four of the seven *F. oxysporum* f. sp. *spinaciae* isolates: Fus057 ($P < 0.0001$), Fus059 ($P < 0.0001$), Fus254 ($P < 0.0001$), and Fus322 ($P = 0.0021$). Similar to trial 1, isolates Fus057, Fus059, Fus254, and Fus322 induced a greater wilt severity on the highly susceptible parent line in both trials while isolates Fus001, Fus167, and Fus173 induced a similar or greater severity of wilt on the partially resistant inbred.

3.3.2 Genomic characterization.

3.3.2.1 Genome assemblies. For the 11 genome assemblies generated from Illumina sequencing, the number of contigs per assembly ranged from 1,144 to 4,525, and the total

assembly size ranged from 47.2 to 55.7 Mbp (Table 3.4). The average Lander/Waterman coverage, after trimming the 11 Illumina assemblies generated, ranged from 59 to 74X. Overall, the Illumina-generated assemblies of the six *F. oxysporum* f. sp. *spinaciae* assemblies were more fragmented than the genome assemblies of the five NPS isolates. The six Illumina-generated *F. oxysporum* f. sp. *spinaciae* assemblies ranged from 3,849 to 4,525 bp per assembly with an assembly size of 55.1 to 55.7 Mbp, whereas the five NPS assemblies ranged from 1,144 to 2,148 contigs with an assembly size of 47.2 to 51.0 Mbp (Table 3.4). The Fus254 (PacBio) assembly was 56.2 Mbp over 279 contigs.

Based on the estimate of genome completeness with the tool BUSCO for each genome assembly, which assesses the presence of evolutionarily conserved orthologues in each assembly relative to a lineage-specific data set of 3,725 orthologous genes from the Sordariomyceta *obd9* dataset, the percentage of complete BUSCOs ranged from 97.3 to 99.1% (3,625 to 3,691) (Table 3.4). BUSCOs that were either fragmented (incomplete orthologous genes) or completely missing from the assemblies ranged from 0.9 to 2.5% (35 to 93 orthologs) of all orthologs missing from the assemblies.

After re-mapping the trimmed Illumina reads of the 11 isolates to the PacBio assembly of *F. oxysporum* f. sp. *spinaciae* isolate Fus254, several regions of these assemblies appeared to be unique to *F. oxysporum* f. sp. *spinaciae* as they were not present in the NPS read data (Appendix Fig. 3.2). However, exact amount of sequence unique to all *F. oxysporum* f. sp. *spinaciae* isolates remains to be determined, as this method was only used to demonstrate that there are unique genomic regions of the collective genomes of the *F. oxysporum* f. sp. *spinaciae* isolates compared to the NPS isolates.

3.3.2.2 Comparison of housekeeping genes among isolates of F. oxysporum. An unrooted maximum likelihood phylogenetic tree inferred from a concatenated alignment of the housekeeping genes β-*TUB*, *RPB1*, and *TEF-1α* demonstrated that *F. oxysporum* f. sp. *spinaciae* groups 1 and 2 were differentiated into two phylogenetic clusters (Fig. 3.1). Isolates from the two groups were differentiated by 17 polymorphic sites in the concatenated alignment of these three genes. Isolates of *F. oxysporum* f. sp. *nicotianae* clustered with *F. oxysporum* f. sp. *spinaciae* group 1 isolates while *F. oxysporum* f. sp. *spinaciae* group 2 isolates clustered with isolates of *F. oxysporum* ff. spp. *cucumerinum* and *vasinfectum*. The NPS isolates grouped into two clusters separate from the two groups of *F. oxysporum* f. sp. *spinaciae* isolates (Fig. 3.1).

Two of the three individual gene trees showed roughly similar topologies to the concatenated tree with respect to the relationship of *F. oxysporum* f. sp. *spinaciae* groups 1 and 2 (Appendix Figs. 3.3, 3.4, and 3.5). *F. oxysporum* f. sp. *spinaciae* groups 1 and 2 isolates were discriminated into two clades for *RPB1* and β-*TUB* (Appendix Figs. 3.3 and 3.5). However, all the *F. oxysporum* f. sp. *spinaciae* isolates shared identical DNA sequences at the *TEF-1α* locus, which is congruent with results reported in Chapter 2 (Appendix Fig. 3.4). The placement of the NPS isolates was variable among each of these three gene trees with respect to their relationship to the isolates of *F. oxysporum* f. sp. *spinaciae* (Appendix Figs. 3.3, 3.4, and 3.5). The Maximum Likelihood analysis of the alignment of *RPB1* showed bootstrap support of 87 and 86% for *F. oxysporum* f. sp. *spinaciae* groups 1 and 2, respectively. However, both clades were not comprised of *F. oxysporum* f. sp. *spinaciae* isolates exclusively. *F. oxysporum* f. sp. *nicotianae* and two NPS isolates were in the same clade as the group 1 isolates of *F. oxysporum* f. sp. *spinaciae*, while isolates of *F. oxysporum* ff. spp. *cucumerinum*, *lagenariae*, *lilii*, *momordicae*, *niveum*, and *vasinfectum* were in the same clade as the group 2 isolates of *F. oxysporum* f. sp.

spinaciae (Appendix Fig. 3.5). Similarly, the two *F. oxysporum* f. sp. *spinaciae* pathogenicity groups were discriminated at the β-*TUB* locus into two groups (Appendix Fig. 3.3). The tree inferred from the β-*TUB* alignment demonstrated bootstrap support of groups 1 and 2 isolates of *F. oxysporum* f. sp. *spinaciae* of 63 and 99%, respectively (Appendix Fig. 3.3).

3.3.2.3 Identification of effector genes in F. oxysporum *f. sp.* spinaciae. Distinct profiles of predicted effector genes differentiated the *F. oxysporum* f. sp. *spinaciae* isolates from the NPS isolates. Among the 10 *F. oxysporum* f. sp. *spinaciae* isolates (nine unique *F. oxysporum* f. sp. *spinaciae* isolates because it turned out that the assemblies of Fus167 and Fospi003 were generated from the same isolate) and five NPS isolates, 48 putative effector genes were identified (Fig. 3.2). Of these 48 predicted effector genes, 19 were common to all 15 genomes and 14 were only found in the *F. oxysporum* f. sp. *spinaciae* isolates. The 15 genomes were clustered hierarchically into three groups based on predicted effector profiles (Fig. 3.2). The *F. oxysporum* f. sp. *spinaciae* isolates were differentiated into two groups based on effector profiles: Fus001, Fus167, Fus173, Fospi001, and Fospi003 grouped separately from Fus057, Fus059, Fus254, Fus322, and Fospi002. In contrast, NPS isolates Fus017, Fus187, Fus191, Fus250, and Fus259 clustered into a single group (Fig. 3.2).

3.2.2.4 Detection of SIX *genes.* Of the 14 known *SIX* gene homologs in isolates of the tomato Fusarium wilt pathogen, *SIX4*, *SIX8*, *SIX9*, and *SIX14* were detected in the 10 genomes sequenced for isolates of *F. oxysporum* f. sp. *spinaciae* and the five genomes of NPS isolates (Table 3.5). Partial matches of *SIX4* and *SIX9* were found among all the *F. oxysporum* f. sp. *spinaciae* group 1 and group 2 assemblies, respectively. All group 2 isolate assemblies had a complete copy of *SIX8*, whereas a *SIX8* homolog of low identity (~86%) was detected in the

genome assemblies of all group 1 isolates (Table 3.5). *SIX14* was detected among the *F. oxysporum* f. sp. *spinaciae* group 1 assemblies and one NPS isolate, Fus187. Unexpectedly, *SIX14* was not detected in the genome assemblies of the *F. oxysporum* f. sp. *spinaciae* group 2 isolates Fus167 and Fus173, although this gene was detected in these isolates using the PCR assay (Chapter 2 and Table 3.5).

SIX14 was detected on three different contigs of the PacBio assembly of isolate Fus254, a group 1 isolate. Two of the copies of *SIX14* were identical, and one had an insertion of a cytosine in the first exon of the gene. A conceptual translation of the three copies of *SIX14* indicated that the cytosine insertion shifted the reading frame downstream to a non-synonymous G22R substitution, which ultimately led to a conceptually truncated peptide (Fig. 3.3).

3.4 Discussion

Based on the genomes of six *F. oxysporum* f. sp. *spinaciae* isolates and five NPS isolates sequenced with the Illumina platform, a seventh *F. oxysporum* f. sp. *spinaciae* isolate with long-read sequences using PacBio, and three genome assemblies of *F. oxysporum* f. sp. *spinaciae* provided by collaborators at the University of Amsterdam that were duplicate isolates of three isolates tested previously for pathogenicity to spinach and the presence of *SIX* genes (Fus165, Fus166, and Fus167; Chapter 2), the two pathogenicity groups into which the *F. oxysporum* f. sp. *spinaciae* isolates were differentiated based on the severity of wilt induced on two spinach inbred lines were supported by both phylogenetic lineage and the predicted effector gene profiles. Moreover, the predicted effector profiles of the NPS isolates were differentiated from those of the two groups of isolates of *F. oxysporum* f. sp. *spinaciae*. The two phenotypic groups identified

in this study for seven isolates of *F. oxysporum* f. sp. *spinaciae* corroborated the pathogenicity test results described in Chapter 2, as well as the pathogenicity test results described by Fiely et al. (1995). Fiely et al. (1995) demonstrated that *F. oxysporum* f. sp. *spinaciae* isolates of VCGs 0330 and 0332 induced more severe Fusarium wilt on the spinach cultivar Grandstand compared to isolates belonging to VCG 0331. In the first pathogenicity trial in this study with seven isolates of the spinach pathogen, three isolates in group 2 (Fus001, Fus167, and Fus173) induced more severe wilt symptoms on the partially resistant parent line than on the highly susceptible parent line, as had been observed in previous pathogenicity tests (Chapter 2). However, in the second trial, the severity of wilt was similar among both parents inoculated with the group 2 isolates. As for all Fusarium wilts, the severity of spinach Fusarium wilt is increased by transpirational stress (Beckman 1987). Differences in virulence observed between the two trials likely reflect differences in environmental conditions in the trials. Trial one was completed in a growth chamber in order to avoid bolting (conversion from vegetative to reproductive growth) of the spinach plants under the longer daylength at the time of year this trial was completed. Airflow was more limited and the humidity (although not quantified) was greater in the growth chamber trial compared to conditions in the greenhouse during trial two. The greater relative humidity likely reduced transpiration of the spinach plants (Beckman 1987; Gates 1968), thereby influencing the rate of development of wilt symptoms.

Previous phylogenetic analyses of *F. oxysporum* suggested that this species is comprised of several cryptic phylospecies, and that most *F. oxysporum* ff. spp. are polyphyletic (Aoki et al. 2014; Baayen et al. 2000; O'Donnell et al. 2009; van Dam et al. 2016). In this study, isolates of *F. oxysporum* f. sp. *spinaciae* phenotypic groups 1 and 2 also were differentiated into two clusters based on a concatenated phylogenetic tree of the β-*TUB, RPB1,* and *TEF-1α* genes.

These lineages were not specific to *F. oxysporum* f. sp. *spinaciae* when compared to the same gene sequences of other *F. oxysporum* ff. spp. The individual gene tree for *TEF-1α* indicated that *F. oxysporum* f. sp. *spinaciae* groups 1 and 2 were not differentiated based on DNA sequence variation, in contrast to the individual trees for β-*TUB* and *RPB1*, which may be explained by low resolution of this formae speciales at the *TEF-1α* locus. Further elucidation of the phylogenetic relationship between these two groups of isolates of *F. oxysporum* f. sp. *spinaciae*, using a combination of housekeeping genes and effector genes, will be helpful for understanding how and when host-specificity to spinach evolved.

Using PCR assays for detection of the 14 known *SIX* genes, two putative effector genes, *SIX8* and/or *SIX14*, were detected in all 39 isolates of *F. oxysporum* f. sp. *spinaciae* that were characterized as detailed in Chapter 2. When the genome assemblies of 10 isolates of *F. oxysporum* f. sp. *spinaciae* were searched for the 14 known *SIX* genes, *SIX14* was only detected among the pathogenicity group 1 isolates, in contrast to the PCR assay-based results, i.e., *SIX14* was not found in the raw Illumina reads of the group 2 isolates. This suggests that either *SIX14* is not present in the genomes of pathogenicity group 2 isolates, and some results reported in Chapter 2 might be erroneous or, more likely, *SIX14* is located in a region of the genome of pathogenicity group 2 isolates that was not covered in the genome sequencing effort. When the Illumina reads generated from group 2 isolates were mapped to the assembly of Fus254, the depth of coverage was highly variable among the Fus254 contigs that encode *SIX14*. In contrast, the read depth was less variable for the Illumina reads from *F. oxysporum* f. sp. *spinaciae* group 1 isolates (Fus057, Fus059, Fus322, and Fospi002), indicating more uniform coverage along contigs on which *SIX14* is located.

The three copies of *SIX14* found in the PacBio assembly of Fus254 were differentiated into two sequence variants which coded conceptually distinct amino acids. One of these variants translated into an amino acid that resulted in a premature stop codon. The presence of multiple copies of *SIX* genes and other putative effector genes has been well documented among *F. oxysporum* ff. spp. (Armitage et al. 2018; Schmidt et al. 2013; Williams et al. 2016). *F. oxysporum* f. sp. *lycopersici* was found to have eight copies of *SIX8* (Williams et al. 2016), and *F. oxysporum* f. sp. *cepae* has multiple copies of both *SIX3* and *SIX9* (Armitage et al. 2018). The mechanism for multiplication of effector genes is unknown, but hypotheses concerning the involvement of transposon-mediated multiplication or loss of effector genes have been suggested. For example, Biju et al. (2017) proposed that race 2 isolates of *F. oxysporum* f. sp. *lycopersici* were generated through recombination of *Helitron* (*HelA* and *HelB*) transposons flanking the 5' and 3' region of *SIX4*, which ultimately resulted in a deletion of the *SIX4* locus. *SIX4* encodes a protein associated with an avirulence gene in *Immunity* (*I*)-1 cultivars of tomato. The *I-1* tomato cultivars recognize Six4 produced by the pathogen and are fully resistant to Fusarium wilt. The loss of the *SIX4* gene in *F. oxysporum* f. sp. *lycopersici* race 1 isolates resulted in a breakdown of this resistance (Houterman et al. 2008).

Effector genes of *F. oxysporum* are associated with repeat-dense regions of the genome (Armitage et al. 2018; Rep 2005; Schmidt et al. 2013; van Dam et al. 2016). Similarly, other fungal and oomycete pathogens have effector genes located in genomic regions that are densely populated with transposable elements and repetitive elements, and that are AT-rich regions (Chuma et al. 2011; Haas et al. 2009; Raffaele et al. 2010; Rouxel et al. 2011). Transposable elements in the genome of *F. oxysporum* ff. spp. *cepae* and *lycopersici* are most densely concentrated in the lineage-specific chromosomes (Armitage et al. 2018; Ma et al. 2010). The

evolutionary significance of transposable elements is that they contribute to genomic plasticity and, perhaps, contribute to genetic diversity in an otherwise asexual fungus. The accessory and core regions of the genomes of both groups 1 and 2 isolates of *F. oxysporum* f. sp. *spinaciae* have not been defined yet, but this will be an important step to aid in identification of lineage-specific and pathogen-specific regions of the genome that are unique to *F. oxysporum* f. sp. *spinaciae*. Armitage et al. (2018) were able to identify lineage-specific regions of the genome of isolates of *F. oxysporum* f. sp. *cepae* by comparison with the genome assemblies of non-pathogenic, onion-associated isolates of *F. oxysporum*.

It is not yet known whether the effector genes predicted among the *F. oxysporum* f. sp. *spinaciae* isolates in this study are expressed during infection of spinach plants, or whether these genes play a functional role in host-specificity to spinach. Van Dam et al. (2016) demonstrated that ~60% of the putative effector genes identified among five *F. oxysporum* ff. spp. using this pipeline were expressed *in planta* 10 days after inoculation. This suggests that effector genes may play a role in pathogenicity or host-specificity. *Mimps* are strongly associated with the promoter region of all the *SIX* genes in *F. oxysporum* f. sp. *lycopersici*, and the presence of *mimps* has been useful for *de novo* identification of putative effector genes in other *F. oxysporum* ff. spp. (Schmidt et al. 2013; Schmidt et al. 2016; van Dam et al. 2016; van Dam et al. 2017). The putative effector genes predicted in this study were limited to those downstream of a *mimp*. However, it remains to be determined if the association of *mimps* with effector genes in the genome of *F. oxysporum* f. sp. *spinaciae* is indicative of effector genes that are necessary for host-specificity to spinach.

These results represent a starting point for identifying what determines host-specificity in *F. oxysporum* f. sp. *spinaciae* to spinach genetically and functionally. The results provide insight

into potentially unique loci associated with *F. oxysporum* f. sp. *spinaciae*. The effector genes identified need to be characterized in order to facilitate identification of lineage-specific regions of the genome of *F. oxysporum* f. sp. *spinaciae* that will enable identification of markers for spinach-specific effector genes, and corresponding host resistance loci that potentially could be utilized in spinach breeding programs. For example, identification and functional characterization of the effector genes *SIX1*, *SIX3*, and *SIX4* in *F. oxysporum* f. sp. *lycopersici* demonstrated that the products of these genes are also avirulence factors that are recognized by cultivars of tomato with corresponding resistance genes (Houterman et al. 2008, 2009; Rep et al. 2004). Similarly, *AVRFOM2* in *F. oxysporum* f. sp. *melonis* race 2 is recognized by resistance gene *Fom-2*, which is present in some muskmelon germplasm (Schmidt et al. 2016). By characterizing effector genes identified in this study, it should be possible to understand better how the spinach Fusarium wilt pathogen interacts with spinach.

This study complements other efforts in the Vegetable Seed Pathology program at the WSU Mount Vernon NWREC to evaluate >500 spinach germplasm accessions for relative susceptibility or resistance to Fusarium wilt. Among these accessions are plant introduction (PI) lines from the USDA National Plant Germplasm System (NPGS), advanced breeding lines from the University of Arkansas spinach breeding program, commercially available cultivars, and collections of wild spinach relatives *Spinacia turkestanica* and *S. tetrandra* (Gyawali et al. 2019). The goal of that work is to identify spinach germplasm with greater resistance to Fusarium wilt, and identify molecular markers associated with Fusarium wilt resistance that can be employed in breeding programs for developing improved resistance to Fusarium wilt. Ultimately, developing spinach parent lines with resistance to Fusarium wilt will aid in increasing the carrying capacity of spinach seed production in the maritime PNW, the only

region in the United States that is suitable to produce seed crops of this temperature- and daylength-sensitive species.

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Table 3.1. List of *Fusarium oxysporum* isolates associated with spinach for which the whole genomes were sequenced in an attempt to identify putative effector genes that define the spinach Fusarium wilt pathogen, *F. oxysporum* f. sp. *spinaciae*.

			Origin			
Isolate	BioSample number[a]	*Fusarium* species identification	Country	State	Substrate of origin	Year isolated
Fus001	SAMN11520591	*F. oxysporum* f. sp. *spinaciae*	USA	Washington	Spinach Seed	2000
Fus057	SAMN11520593	*F. oxysporum* f. sp. *spinaciae*	USA	Washington	Spinach Seed Crop	2006
Fus059	SAMN11520594	*F. oxysporum* f. sp. *spinaciae*	USA	Washington	Spinach Seed Crop	2006
Fus167	SAMN11520595	*F. oxysporum* f. sp. *spinaciae*	USA	Arkansas	Spinach	1991/92[b]
Fus173	SAMN11520596	*F. oxysporum* f. sp. *spinaciae*	Japan	-	Spinach	1991/92[b]
Fus254	SAMN11520602	*F. oxysporum* f. sp. *spinaciae*	USA	WA/OR	Spinach	2009
Fus322	SAMN11520601	*F. oxysporum* f. sp. *spinaciae*	USA	CA	Spinach	2011
Fus017	SAMN11520592	*F. oxysporum*	USA	WA	Spinach seed crop	2007
Fus187	SAMN11520597	*F. oxysporum*	USA	WA	Spinach	2008
Fus191	SAMN11520598	*F. oxysporum*	USA	WA	Spinach	2008
Fus250	SAMN11520599	*F. oxysporum*	USA	Washington	Spinach	2009
Fus259	SAMN11520600	*F. oxysporum*	USA	Washington	Soil	2009

[a] Raw Illumina and PacBio read data were deposited into the sequence read archive under the BioProject number PRJNA540981, with the associated BioSample number.

[b] Fus167 and Fus173 are described in Fiely et al. (1995) as MF42 or SP6, respectively. These isolates were collected in either 1991 or 1992.

Table 3.2. Probability values from the analyses of variance (ANOVAs) for the fixed effects of spinach parent line, *Fusarium oxysporum* isolate, and parent-by-isolate interaction for area under the disease progress curve (AUDPC) and biomass/plant in two trials completed in 2018.

	Trial 1[b]		Trial 2[c]	
ANOVA factor[a]	AUDPC[d]	Biomass/plant (g)[e]	AUDPC[d]	Biomass/plant (g)[e]
Parent	<0.0001	0.6772	<0.0001	0.0233
Isolate	<0.0001	<0.0001	<0.0001	<0.0001
Parent-by-isolate	<0.0001	<0.0001	<0.0001	<0.0001
Transformation	Rank	Log	Rank	Rank

[a] Each trial was set up as a completely randomized block design with four replicate blocks. Raw data were either log or rank transformed due to heterogeneity of variances and/or non-normal residuals. Refer to the main text for details of the spinach parent lines and inoculation treatments.

[b] The spinach plants in trial 1 were maintained in a growth chamber (Percival, Perry, IA) as described in the main text.

[c] The spinach plants in trial 2 were maintained in a greenhouse as described in the main text.

[d] Mean AUDPC value for six plants (subsamples) from each of four replications. Ranks were compared using Fisher's protected least significant difference ($P < 0.05$).

[e] Mean dry biomass/plant for six spinach plants evaluated in each of four replications per treatment combination.

Table 3.3. Effects of spinach parent line-by-isolate interaction based on the analyses of variance (ANOVAs) for area under the disease progress curve (AUDPC) and biomass/spinach plant in two trials completed in 2018 to identify isolates of the spinach Fusarium wilt pathogen, *F. oxysporum f. sp. spinaciae*.

ANOVA factor[a]		Trial 1		Trial 2	
Spinach parent (inbred) line	Isolate	AUDPC	Biomass/plant (g)	AUDPC	Biomass/plant (g)
Partially resistant	Water (control)	2.08 ij	0.55 bcd	0.00 g	0.30 bc
	Fus001	851.46 bc	0.23 hi	1066.92 b	0.08 gh
	Fus167	628.92 cde	0.30 gh	1222.96 b	0.05 hi
	Fus173	1154.79 ab	0.15 j	953.75 bc	0.11 gh
	Fus057	242.71 f	0.40 ef	281.75 e	0.21 ef
	Fus059	395.83 ef	0.29 h	699.71 cd	0.13 fg
	Fus254	192.71 f	0.40 ef	660.33 cde	0.12 g
	Fus322	201.46 f	0.47 def	88.96 f	0.27 cd
	Fus017	7.71 hij	0.55 bcd	7.29 g	0.29 c
	Fus187	6.25 ghij	0.46 def	14.58 g	0.31 bc
	Fus191	1.04 j	0.53 cd	87.5 g	0.29 c
	Fus250	0.00 j	0.52 cde	5.83 g	0.31 bc
	Fus259	0.00 j	0.51 cde	0.00 g	0.27 cd
Highly susceptible	Water (control)	0.00 j	0.64 abc	0.00 g	0.40 a
	Fus001	430.21 def	0.58 bcd	1165.79 b	0.09 gh
	Fus167	230.42 f	0.46 def	1163.75 b	0.10 gh
	Fus173	708.33 cd	0.40 fg	697.96 cd	0.23 de
	Fus057	1681.25 a	0.07 l	1684.67 a	0.03 i
	Fus059	1556.46 a	0.10 k	1751.46 a	0.03 i
	Fus254	848.38 bc	0.31 gh	1705.08 a	0.03 i
	Fus322	1210.21 ab	0.20 ij	548.92 de	0.22 de
	Fus017	4.79 ghij	0.83 a	0.00 g	0.40 a
	Fus187	1.04 j	0.78 a	0.00 g	0.46 a
	Fus191	10.83 gh	0.74 a	0.00 g	0.42 a
	Fus250	26.50 g	0.65 abc	0.00 g	0.39 a
	Fus259	59.38 ghi	0.72 ab	2.92 g	0.38 ab
Transformation		Rank	Log	Rank	Rank

[a] Each value is the mean of four replications. The experimental design in both trials was a completely randomized block. Raw data were either log or rank transformed due to heterogeneity of variances and/or non-normal residuals. Values that share at least one letter are not different statistically based on Fisher's protected least significant difference (LSD) ($P < 0.05$).

[b] The spinach plants in trial 1 were maintained in a growth chamber (Percival, Perry, IA) as described in the main text.

[c] The spinach plants in trial 2 were maintained in a greenhouse as described in the main text.

[d] Mean AUDPC value for six plants (subsamples) from each of four replications. Ranks were compared using Fisher's protected least significant difference ($P < 0.05$).

[e] Mean dry biomass/plant for six spinach plants evaluated in each of four replications per treatment combination.

Table 3.4. Genome assembly statistics of isolates of *Fusarium oxysporum* sequenced to identify putative effectors that define the spinach Fusarium wilt pathogen, *F. oxysporum* f. sp. *spinaciae*.

Isolate	ID[a]	Platform[b]	No. of contigs	Largest contig	Total length (bp)	GC (%)[c]	N50 (bp)[d]	L50[d]	N's per 100 kbp[e]	% Sordariomycete genes (BUSCO)[f]
Fus057	*Fos* (1)	Illumina HiSeq	4,492	815,478	55,133,722	47.59	90,308	143	10.77	97.6
Fus059	*Fos* (1)	Illumina HiSeq	4,525	790,793	55,114,739	47.59	90,581	152	10.31	97.8
Fus254	*Fos* (1)	PacBio Sequel	279	1,713,855	56,261,220	48.37	625,464	28	0	98.8
Fus322	*Fos* (1)	Illumina HiSeq	4,269	944,781	55,110,552	47.60	82,330	147	11.01	97.8
Fospi002 (Fus166)[g]	*Fos* (1)	Illumina	3,330	1,680,321	54,711,642	47.60	129,734	78	300.7	99.0
Fus001	*Fos* (2)	Illumina HiSeq	4,052	873,215	55,133,078	47.45	118,130	110	12.63	97.3
Fus167	*Fos* (2)	Illumina HiSeq	4,403	679,154	55,665,772	47.47	120,794	122	11.6	97.7
Fus173	*Fos* (2)	Illumina HiSeq	3,849	933,858	55,271,922	47.45	135,099	96	10.06	97.3
Fospi001 (Fus165)[g]	*Fos* (2)	Illumina	3,117	1,611,665	55,662,373	47.43	219,224	53	272.25	99.1
Fospi003 (Fus167)[g]	*Fos* (2)	Illumina	2,983	1,611,824	55,562,735	47.47	296,361	51	289.91	99.0
Fus017	NPS	Illumina HiSeq	2,148	932,278	50,973,513	47.65	209,173	68	7.67	98.2
Fus187	NPS	Illumina HiSeq	1,861	1,090,389	48,789,438	47.71	233,677	60	7.48	96.6
Fus191	NPS	Illumina HiSeq	1,144	868,096	47,238,916	47.55	243,532	59	6.67	97.6
Fus250	NPS	Illumina HiSeq	1,609	1,988,080	49,644,441	47.70	208,491	63	5.43	97.4
Fus259	NPS	Illumina HiSeq	1,389	1,648,444	48,835,076	47.54	272,410	51	5.87	97.7

[a] *Fos* (n) = *Fusarium oxysporum* f. sp. *spinaciae* (pathogenicity group); NPS = *F. oxysporum* isolate that is not a pathogen of spinach but was associated with spinach plants, spinach seed, or soil in which spinach was grown.

[b] The next generation sequencing platform that was used to generate read data for each isolate.

[c] The percentage of guanine and cytosine residues that comprise each genome assembly.

[d] Conceptually, the N50 of a genome assembly is assessed by ordering contigs from largest to smallest, and the N50 is the length (in base pairs) of the contig, along with all larger contigs, which account for 50% of the assembly length. Similar to N50, L50 represents the n^{th} contig for which all contigs of equal or greater length account for 50% of the assembly length.

[e] Number of unidentified base pairs (N's) per 100,000 base pairs in a genome assembly.

[f] The percentage of single copy orthologs present in a genome assembly, as assessed by the program BUSCO (Simão et al. 2015).

[g] The genome assemblies of Fospi001, Fospi002, and Fospi003 were provided by Drs. Martijn Rep and Mara de Sain at the University of Amsterdam, Amsterdam, the Netherlands. The original isolates are from the United States Department of Agriculture Agricultural Research Service culture collection at the Northern Regional Research Laboratory (NRRL). These isolates were originally described in Fiely et al. (1995). Fospi001, Fospi002, and Fospi003 were each tested for pathogenicity on spinach as described in Chapter 2, where they are identified as Fus165, Fus166, and Fus167, respectively.

Table 3.5. Comparison of the presence or absence profiles of 14 *Secreted in Xylem* (*SIX*) genes in the genome assemblies of *Fusarium oxysporum* f. sp. *spinaciae* (*Fos*) isolates and *F. oxysporum* isolates that were not pathogenic on spinach but were isolated in association with spinach (NPS isolates).

		Method used to search for *SIX* genes[a]																									
		BLASTn[b] search														PCR amplification[c]											
		SIX genes														*SIX* genes											
Isolate	ID[d]	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Fus057	*Fos* (1)	-	-	-	+[e]	-	-	-	+[e]	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Fus059	*Fos* (1)	-	-	-	+[e]	-	-	-	+[e]	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Fospi002 (Fus166)	*Fos* (1)	-	-	-	+[e]	-	-	-	+[e]	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Fus254	*Fos* (1)	-	-	-	+[e]	-	-	-	+[e]	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Fus322	*Fos* (1)	-	-	-	+[e]	-	-	-	+[e]	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Fus001	*Fos* (2)	-	-	-	-	-	-	-	+	+[e]	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Fospi001 (Fus165)	*Fos* (2)	-	-	-	-	-	-	-	+	+[e]	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	(+)
Fus167	*Fos* (2)	-	-	-	-	-	-	-	+	+[e]	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Fospi003 (Fus167)	*Fos* (2)	-	-	-	-	-	-	-	+	+[e]	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Fus173	*Fos* (2)	-	-	-	-	-	-	-	+	+[e]	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Fus017	NPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fus187	NPS	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Fus191	NPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fus250	NPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fus259	NPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] '+' = presence of *SIX* gene; '-' = absence of *SIX* gene.

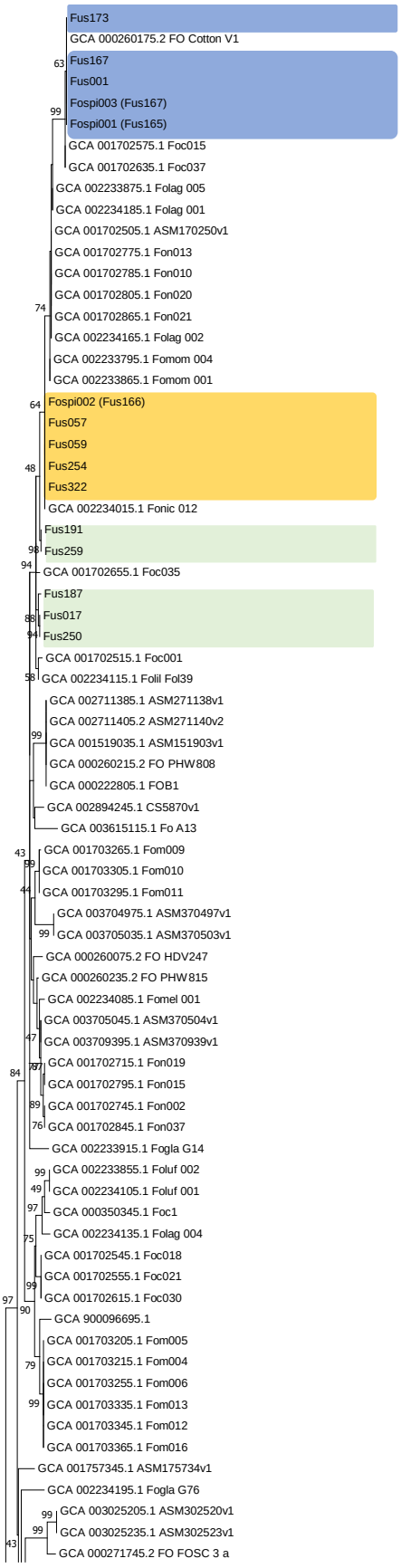
[b] BLASTn = Basic Local Alignment Search Tool (nucleic acids). All 14 *SIX* genes were submitted as a query against the genome assemblies of the isolates in the table.

[c] The presence or absence of the *SIX* genes as detected by polymerase chain reaction (PCR) assays (detailed in Table 2.5) is noted for comparison. (+) = weak amplification of a DNA fragment in the PCR assay.

[d] *Fos* (n) = *Fusarium oxysporum* f. sp. *spinaciae* (pathogenicity group); NPS = *F. oxysporum* isolate that is not a pathogen of spinach but was associated with spinach plants, spinach seed, or soil in which spinach was grown.

[e] Partial hit or identity <90%.

TEF-1α + *RPB1* + β*TUB*
(9,518 positions)
TN93 + Γ + I



***Fos* group 2**

***Fos* group 1**

NPS

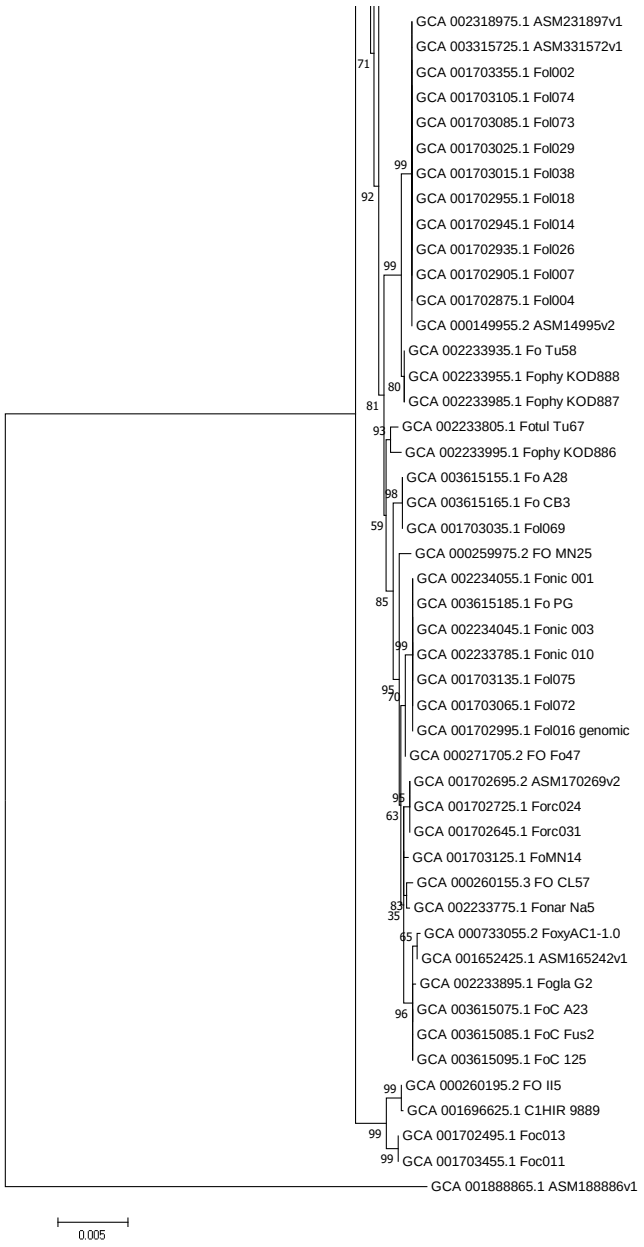


Fig. 3.1. Unrooted maximum likelihood tree based on the concatenated DNA sequences of *translation elongation factor 1α* (*TEF-1α*)*, RNA polymerase binding protein 1* (*RPB1*)*,* and β *tubulin* (β-*TUB*) (9,518 nt total) extracted from 120 publicly available genome sequences of *Fusarium oxysporum*. The tree was estimated with the Tamura 3-parameter model, and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Nodes with bootstrap support <33 are not shown for clarity. The scale bar indicates the average number of nucleotide substitutions per site.

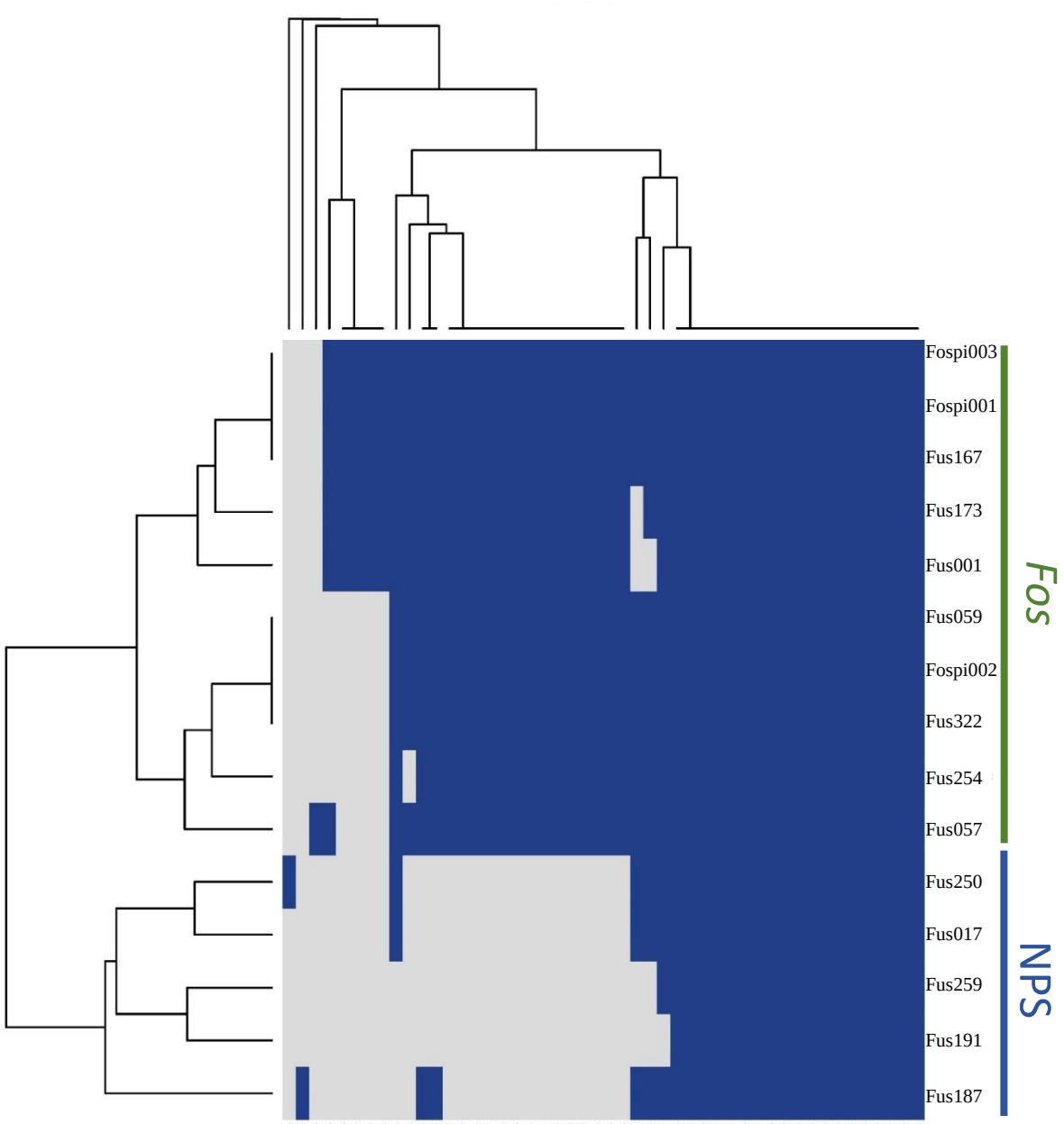


Fig. 3.2. Presence or absence plot of putative effector genes found in *Fusarium oxysporum* f. sp. *spinaciae* (*Fos*) isolates in comparison with non-pathogenic isolates of *F. oxysporum* associated with spinach (NPS). Solid blue boxes indicate the presence of an open reading frame (ORF) while shaded gray boxes indicate the absence of an ORF in a genome assembly. The genome assemblies (rows) are clustered by the similarity of putative effector gene distribution (left, y-axis), and the putative effector genes (columns) are clustered by their presence or absence within each assembly (top, x-axis).

Fig. 3.3. Conceptual amino acid translation of three copies of the *Secreted in Xylem 14* (*SIX14*) gene extracted from the PacBio assembly of *Fusarium oxysporum* f. sp. *spinaciae* isolate Fus254. Fus254_SIX14a_1, Fus254_SIX14a_2, and Fus254_SIX14b were found on contigs 091, 140, and 180, respectively. Asterisks (*) above the amino acid alignment indicate identical amino acids at that position for all three copies of the gene. Asterisks (*) in place of an amino acid residue and surrounded by a gray box indicate a predicted stop codon.

APPENDIX

Appendix Table 2.1. Means separation of the area under the disease progress curve (AUDPC) for severity of Fusarium wilt and biomass/plant for spinach plants inoculated with microconidial suspensions of *Fusarium* isolates.

	ANOVA factor[a]			
Pathogenicity test	Parent	Isolate[c]	AUDPC[b]	Biomass/plant (g)
1	Highly susceptible	Control	13.13 efg	0.88 a
		Fus187	4.38 fg	0.81 abc
		Fus254	681.04 a	0.41 h
		Fus001	1.46 fg	0.89 a
		Fus004	64.17 d	0.73 bcd
		Fus057	482.71 abc	0.61 def
		Fus058	422.92 bc	0.58 ef
		Fus059	700.00 ab	0.44 gh
		Fus185	344.17 c	0.64 def
		Fus191	0.00 g	0.84 ab
		Fus192	8.75 fg	0.84 ab
		Fus193	532.29 abc	0.56 fg
		Fus267	27.71 def	0.85 ab
		Fus270	33.54 de	0.77 abc
		Fus322	401.04 c	0.69 cde
	Moderately susceptible	Control	4.38 e	0.81 abc
		Fus187	0 e	0.84 a
		Fus254	975.62 a	0.23 h
		Fus001	0.00 e	0.83 ab
		Fus004	102.08 cd	0.76 abcd
		Fus057	300.42 c	0.64 de
		Fus058	606.67 b	0.46 fg
		Fus059	665.00 ab	0.44 fg
		Fus185	845.83 ab	0.33 gh
		Fus191	0.00 e	0.88 a
		Fus192	0.00 e	0.71 bcde
		Fus193	580.42 b	0.44 fg
		Fus267	110.83 d	0.63 e
		Fus270	83.13 d	0.68 cde
		Fus322	622.71 ab	0.47 f
	Partially resistant	Control	18.96 fghi	0.75 a
		Fus187	0 i	0.67 abcd
		Fus254	312.08 c	0.51 ef
		Fus001	16.04 fgh	0.74 ab
		Fus004	30.63 fgh	0.67 abcd
		Fus057	48.13 efg	0.71 abc
		Fus058	583.33 ab	0.43 fg
		Fus059	339.79 bc	0.60 cde
		Fus185	602.29 a	0.35 g
		Fus191	17.5 ghi	0.71 abc
		Fus192	4.38 hi	0.76 a

Appendix Table 2.1. Continued.

Pathogenicity test	ANOVA factor[a] Parent	Isolate[c]	AUDPC[b]	Biomass/plant (g)
	Partially resistant	Fus193	53.96 ef	0.63 abcd
		Fus267	84.58 de	0.58 de
		Fus270	164.79 cd	0.64 abcd
		Fus322	74.38 de	0.61 bcde
2	Highly susceptible	Control	15.22 de	0.34 abcd
		Fus187	8.54 de	0.36 abc
		Fus254	680.63 a	0.22 g
		Fus020	145.42 bcd	0.35 abcd
		Fus021	196.25 abc	0.29 cdefg
		Fus183	516.46 ab	0.26 efg
		Fus272	566.46 ab	0.23 fg
		Fus287	93.96 cde	0.38 ab
		Fus321	281.88 ab	0.32 abcde
		Fus323	343.13 ab	0.28 defg
		Fus324	518.75 ab	0.28 defg
		Fus333	270.63 ab	0.30 bcdef
		Fus435	16.67 cde	0.36 abc
		Fus436	21.04 cde	0.39 a
		Fus439	2.92 e	0.38 a
	Moderately susceptible	Control	0.00 d	0.30 ab
		Fus187	0.00 d	0.27 abc
		Fus254	236.04 abc	0.26 abc
		Fus020	200.21 abc	0.25 bc
		Fus021	93.75 cd	0.33 a
		Fus183	473.96 a	0.21 c
		Fus272	242.71 abc	0.28 abc
		Fus287	0.00 d	0.31 ab
		Fus321	135.83 bcd	0.23 bc
		Fus323	265.63 ab	0.24 bc
		Fus324	446.67 a	0.21 c
		Fus333	266.88 abc	0.25 bc
		Fus435	0.00 d	0.29 ab
		Fus436	0.00 d	0.30 ab
		Fus439	0.00 d	0.33 a
	Partially resistant	Control	0.00 e	0.25 ab
		Fus187	4.17 cde	0.23 ab
		Fus254	81.25 bcde	0.26 ab
		Fus020	472.71 a	0.19 b
		Fus021	13.96 cde	0.24 ab
		Fus183	161.46 bcd	0.27 a
		Fus272	0.00 e	0.27 a
		Fus287	8.33 cde	0.29 a
		Fus321	1.46 de	0.26 ab
		Fus323	163.96 abc	0.24 ab
		Fus324	241.25 ab	0.23 ab

Appendix Table 2.1. Continued.

Pathogenicity test	ANOVA factor[a] Parent	Isolate[c]	AUDPC[b]	Biomass/plant (g)
	Partially resistant	Fus333	5.63 cde	0.30 a
		Fus435	0.00 e	0.28 a
		Fus436	2.92 cde	0.26 ab
		Fus439	18.13 cde	0.29 a
3	Highly susceptible	Control	1.46 c	1.19
		Fus187	5.83 c	1.20
		Fus254	552.08 a	1.00
		Fus159	263.54 ab	1.05
		Fus160	303.96 ab	1.11
		Fus161	529.79 a	1.00
		Fus162	592.71 a	0.85
		Fus163	159.79 ab	1.08
		Fus164	104.58 b	1.04
		Fus165	326.67 ab	1.06
		Fus166	503.13 ab	0.91
		Fus167	535.21 a	0.93
		Fus168	253.33 ab	1.09
		Fus440	8.75 c	1.09
		Fus442	1.46	1.18
	Moderately susceptible	Control	0 e	1.16
		Fus187	23.33 de	1.05
		Fus254	394.60 abc	0.84
		Fus159	30.63 de	1.11
		Fus160	284.58 bc	0.95
		Fus161	179.81 bc	1.19
		Fus162	686.56 a	0.73
		Fus163	14.58 e	1.03
		Fus164	158.98 c	1.01
		Fus165	641.44 ab	0.7
		Fus166	100.04 cd	0.96
		Fus167	865.25 a	0.57
		Fus168	366.88 abc	0.87
		Fus440	0 e	1.01
		Fus442	0	1.31
	Partially resistant	Control	7.08 e	0.76
		Fus187	0.00 e	0.92
		Fus254	35.63 de	0.76
		Fus159	30.00 e	0.69
		Fus160	271.46 bcd	0.73
		Fus161	0.00 e	0.79
		Fus162	954.25 a	0.54
		Fus163	228.25 bc	0.78
		Fus164	104.79 cde	0.79
		Fus165	306.96 abc	0.71

Appendix Table 2.1. Continued.

Pathogenicity test	ANOVA factor[a] Parent	Isolate[c]	AUDPC[b]	Biomass/plant (g)
	Partially resistant	Fus440	0.00 e	0.81
		Fus442	0	0.69
4	Highly susceptible	Control	2.92 e	1.50 abcd
		Fus187	2.92 e	1.71 a
		Fus254	362.50 a	1.17 e
		Fus169	120.46 ab	1.40 bcde
		Fus170	172.92 abc	1.32 cde
		Fus172	8.75 de	1.50 abcd
		Fus173	265.63 a	1.38 bcde
		Fus182	194.17 ab	1.23 e
		Fus184	201.04 ab	1.31 de
		Fus186	42.08 bcd	1.55 abc
		Fus188	101.67 ab	1.56 abc
		Fus189	76.46 bcd	1.54 abcd
		Fus447	17.50 de	1.59 ab
		Fus452	12.25 cde	1.64 a
	Moderately susceptible	Control	4.38 ef	1.39 abc
		Fus187	0.00 f	1.56 a
		Fus254	457.08 ab	0.97 e
		Fus169	229.42 ab	1.15 de
		Fus170	438.13 ab	0.98 e
		Fus172	71.46 de	1.29 bcd
		Fus173	1335.83 a	0.47 f
		Fus182	256.25 bc	1.14 de
		Fus184	84.58 cd	1.24 bcd
		Fus186	170.83 bc	1.25 bcd
		Fus188	176.46 bc	1.17 cde
		Fus189	276.04 bc	1.33 abcd
		Fus447	0.00 f	1.41 ab
		Fus452	2.92 ef	1.29 bcd
	Partially resistant	Control	0.00 d	1.28 a
		Fus187	14.58 bc	1.18 ab
		Fus254	10.21 bcd	1.22 ab
		Fus169	4.38 cd	1.10 ab
		Fus170	94.79 b	1.10 ab
		Fus172	7.08 cd	1.23 ab
		Fus173	799.79 a	0.62 c
		Fus182	8.75 bcd	1.30 a
		Fus184	7.29 bcd	1.23 ab
		Fus186	265.21 a	1.03 b
		Fus188	1.46 d	1.33 a
		Fus189	10.21 bcd	1.27 a
		Fus447	8.13 cd	1.15 ab
		Fus452	7.29 cd	1.29 a

Appendix Table 2.1. Continued.

Pathogenicity test	ANOVA factor[a] Parent	Isolate[c]	AUDPC[b]	Biomass/plant (g)
5	Highly susceptible	-	40.04	1.42 a
	Moderately susceptible	-	33.26	1.42 a
	Partially resistant	-	1.66	1.20 b
	-	Control	2.17	1.33 a
	-	Fus187	1.08	1.38 a
	-	Fus254	369.13	0.99 b
	-	Fus062	0	1.39 a
	-	Fus125	0	1.37 a
	-	Fus127	0	1.34 a
	-	Fus129	0	1.31 a
	-	Fus393	0	1.38 a
	-	Fus394	1.39	1.38 a
	-	Fus395	1.08	1.36 a
	-	Fus400	0	1.39 a
	-	Fus401	0	1.35 a
	-	Fus402	0	1.41 a
	-	Fus441	0	1.36 a
	-	Fus448	0	1.43 a
6		Control	5.00	0.30 a
		Fus187	5.00	0.28 a
		Fus254	663.42	0.15 b
		Fus017	0.00	0.34 a
		Fus023	5.83	0.29 a
		Fus191	10.67	0.28 a
	Highly susceptible	Fus192	3.33	0.29 a
		Fus250	12.00	0.32 a
		Fus259	8.33	0.32 a
		Fus263	10.83	0.29 a
		Fus265	19.17	0.28 a
		Fus317	18.33	0.28 a
		Fus327	7.33	0.29 a

[a] Means were compared only for significant ANOVA effects ($P < 0.05$) for each pathogenicity test, unless there was a significant interaction. Values with the same letters are not statistically different based on Fishers protected least significant difference ($P < 0.05$). All letter comparisons are grouped by the main effect of 'Parent' in pathogenicity tests 1 to 4 for clarity. Letters are not displayed for biomass/plant for pathogenicity test 4, or for the AUDPC data for pathogenicity test 5. See the main text for clarification.

[b] Means comparisons are based on ranked data, but the unranked means are displayed.

[c] Isolates were tested for pathogenicity on spinach in six pathogenicity tests. Refer to Table 2.1 for a description of each isolate.

Appendix Table 2.2. Variation in severity and incidence of Fusarium wilt based on the mean standard deviation and count of wilted plants of a highly susceptible spinach parent line inoculated with two *Fusarium oxysporum* f. sp. *spinaciae* isolates, Fus254 and Fus322.

		Average standard deviation of Fusarium wilt severity within experimental units[a]				
Inoculation method	Isolate	14 DAI	21 DAI	28 DAI	Plants with a disease severity > 0[b]	Total number of plants[c]
Root-drench	Control	0.00	0.15	0.07	1	15
	Fus254	1.69	2.38	2.37	10	17
	Fus322	0.47	1.35	1.61	13	16
		14 DAP	21 DAP	28 DAP		
Soil-amendment	Control	0.00	0.00	0.00	0	18
	Fus254	1.01	0.46	0.00	17	17
	Fus322	1.08	0.48	0.00	18	18

[a] The standard deviation of Fusarium wilt severity per experimental unit was calculated based on disease severity at the rating intervals shown in terms of days after inoculation (DAI) or days after planting (DAP). No statistical analyses were calculated as these descriptive values are indicative of the variation observed in disease severity within replicates. Only the highly susceptible spinach parent line was tested.

[b] Plants with a disease severity rating > 0 at 28 DAI or DAP.

[c] The total number of plants evaluated for three replicate blocks of each treatment combination. Although enough seed was planted for 18 plants per treatment combination, seed germination was not 100%.

Appendix Table 2.3. Analyses of variance (ANOVAs) for the fixed effects of inoculation method, *Fusarium oxysporum* isolate, and inoculation-by-isolate interaction on area under the disease progress curve for spinach Fusarium wilt severity index and dry spinach biomass.

ANOVA factor[a]	AUDPC	Biomass/plant (g)	ρ [b]
Inoculation method	0.0022	0.0021	0.0207
Isolate	<0.0001	0.0088	0.4751
Inoculation-by-isolate	0.0031	0.4799	0.6360
Transformation[c]	Rank	-	-

[a] Each value represents the probability (*P*-value) associated with inoculation method, isolate, and inoculation-by-isolate interaction effects. Values are significant at $P < 0.05$.

[b] The ratio of biomass/plant (g) for inoculated plants compared to that of the control plants treated with water. Variances were modeled with the repeated statement and a type = un(1) variance-covariance structure to compensate for heterogeneous variances among treatments.

[c] When the assumptions of homogeneity of variances and normality of residuals were not met, the data were subjected to a non-parametric rank transformation.

Appendix Table 2.4. Means separation of area under the disease progress curve (AUDPC) values for severity of Fusarium wilt and biomass/plant for spinach plants treated with two isolates of *Fusarium oxysporum* f. sp. *spinaciae* using two methods of inoculation.

ANOVA factor[a]	Treatment	AUDPC[b]	Biomass/plant (g)	ρ [c]
Inoculation method	Root-drench	-	0.39 a	0.56 a
	Soil-amendment	-	0.094 b	0.10 b
Isolate	Control	-	0.40 a	-
	Fus254	-	0.15 b	-
	Fus322	-	0.19 b	-
Inoculation-by-isolate	Root-drench			
	Control	11.67 c	-	-
	Fus254	545.22 b	-	-
	Fus322	455.39 b	-	-
	Soil-amendment			
	Control	0 c	-	-
	Fus254	1113.78 a	-	-
	Fus322	1092.78 a	-	-

[a] Means were only compared for significant ANOVA effects ($P < 0.05$), unless there was a significant interaction. Values with the same letters are not statistically different based on Fishers protected least significant difference ($P < 0.05$).

[b] Means comparisons are based on ranked data, but the unranked means are displayed.

[c] The ratio of biomass/plant (g) to that of the water control treatment. Variances were modeled with the repeated statement, and a type = un(1) variance-covariance structure to compensate for heterogeneous variances among treatments.

Appendix Table 3.1. List of publicly available *Fusarium oxysporum* genome assemblies used in this study for phylogenetic analyses.

Accession number[a]	Assembly name	Strain description
GCA000149955.2	ASM14995v2	*Fusarium oxysporum* f. sp. *lycopersici* 4287
GCA000222805.1	FOB1	*F. oxysporum* Fo5176
GCA000259975.2	FO_MN25_V1	*F. oxysporum* f. sp. *lycopersici* MN25
GCA000260075.2	FO_HDV247_V1	*F. oxysporum* f. sp. *pisi* HDV247
GCA000260155.3	FO_CL57_V1	*F. oxysporum* f. sp. *radicis-lycopersici* 26381
GCA000260175.2	FO_Cotton_V1	*F. oxysporum* f. sp. *vasinfectum* 25433
GCA000260195.2	FO_II5_V1	*F. oxysporum* f. sp. *cubense* tropical race 4 54006
GCA000260215.2	FO_PHW808_V1	*F. oxysporum* f. sp. *conglutinans* race 2 54008
GCA000260235.2	FO_PHW815_V1	*F. oxysporum* f. sp. *raphani* 54005
GCA000271705.2	FO_Fo47_V1	*F. oxysporum* Fo47
GCA000271745.2	FO_FOSC_3_a_V1	*F. oxysporum* FOSC 3-a
GCA000350345.1	Foc1_1.0	*F. oxysporum* f. sp. *cubense* race 1
GCA000733055.2	FoxyAC1-1.0	*F. oxysporum*
GCA001519035.1	ASM151903v1	*F. oxysporum* f. sp. *conglutinans*
GCA001652425.1	ASM165242v1	*F. oxysporum* f. sp. *medicaginis*
GCA001696625.1	C1HIR_9889	*F. oxysporum* f. sp. *cubense*
GCA001702495.1	Foc013	*F. oxysporum* f. sp. *cucumerinum*
GCA001702505.1	ASM170250v1	*F. oxysporum* f. sp. *niveum*
GCA001702515.1	Foc001	*F. oxysporum* f. sp. *cucumerinum*
GCA001702545.1	Foc018	*F. oxysporum* f. sp. *cucumerinum*
GCA001702555.1	Foc021	*F. oxysporum* f. sp. *cucumerinum*
GCA001702575.1	Foc015	*F. oxysporum* f. sp. *cucumerinum*
GCA001702615.1	Foc030	*F. oxysporum* f. sp. *cucumerinum*
GCA001702635.1	Foc037	*F. oxysporum* f. sp. *cucumerinum*
GCA001702645.1	Forc031	*F. oxysporum* f. sp. *radicis-cucumerinum*
GCA001702655.1	Foc035	*F. oxysporum* f. sp. *cucumerinum*
GCA001702695.2	ASM170269v2	*F. oxysporum* f. sp. *radicis-cucumerinum*
GCA001702715.1	Fon019	*F. oxysporum* f. sp. *niveum*
GCA001702725.1	Forc024	*F. oxysporum* f. sp. *radicis-cucumerinum*
GCA001702745.1	Fon002	*F. oxysporum* f. sp. *niveum*
GCA001702775.1	Fon013	*F. oxysporum* f. sp. *niveum*
GCA001702785.1	Fon010	*F. oxysporum* f. sp. *niveum*
GCA001702795.1	Fon015	*F. oxysporum* f. sp. *niveum*
GCA001702805.1	Fon020	*F. oxysporum* f. sp. *niveum*
GCA001702845.1	Fon037	*F. oxysporum* f. sp. *niveum*
GCA001702865.1	Fon021	*F. oxysporum* f. sp. *niveum*
GCA001702875.1	Fol004	*F. oxysporum* f. sp. *lycopersici*
GCA001702905.1	Fol007	*F. oxysporum* f. sp. *lycopersici*

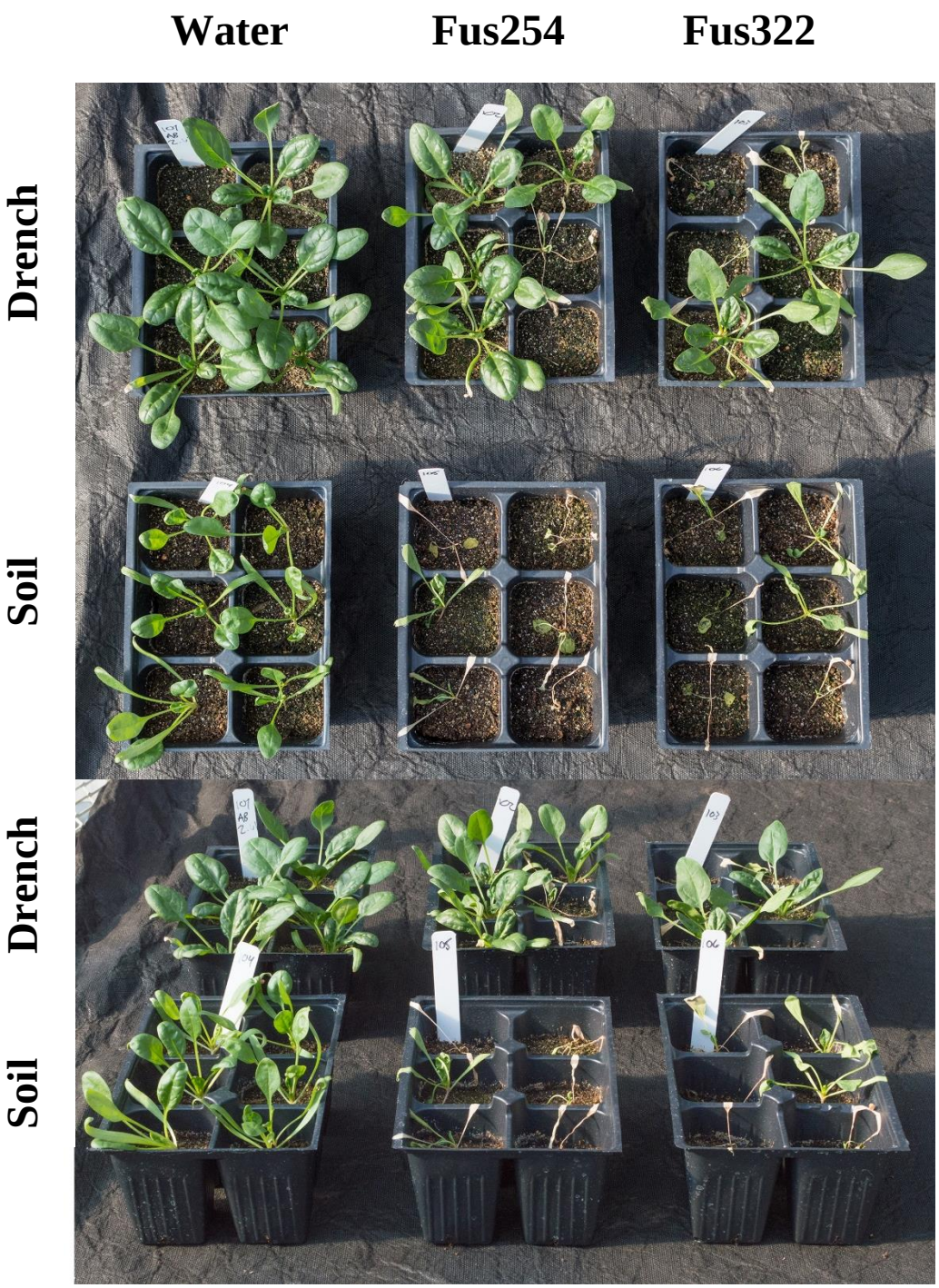
Appendix Table 3.1. Continued.

Accession number	Assembly name	Strain description
GCA001702935.1	Fol026	*F. oxysporum* f. sp. *lycopersici*
GCA001702945.1	Fol014	*F. oxysporum* f. sp. *lycopersici*
GCA001702955.1	Fol018	*F. oxysporum* f. sp. *lycopersici*
GCA001702995.1	Fol016	*F. oxysporum* f. sp. *lycopersici*
GCA001703015.1	Fol038	*F. oxysporum* f. sp. *lycopersici*
GCA001703025.1	Fol029	*F. oxysporum* f. sp. *lycopersici*
GCA001703035.1	Fol069	*F. oxysporum* f. sp. *lycopersici*
GCA001703065.1	Fol072	*F. oxysporum* f. sp. *lycopersici*
GCA001703085.1	Fol073	*F. oxysporum* f. sp. *lycopersici*
GCA001703105.1	Fol074	*F. oxysporum* f. sp. *lycopersici*
GCA001703125.1	FoMN14	*F. oxysporum*
GCA001703135.1	Fol075	*F. oxysporum* f. sp. *lycopersici*
GCA001703205.1	Fom005	*F. oxysporum* f. sp. *melonis*
GCA001703215.1	Fom004	*F. oxysporum* f. sp. *melonis*
GCA001703255.1	Fom006	*F. oxysporum* f. sp. *melonis*
GCA001703265.1	Fom009	*F. oxysporum* f. sp. *melonis*
GCA001703295.1	Fom011	*F. oxysporum* f. sp. *melonis*
GCA001703305.1	Fom010	*F. oxysporum* f. sp. *melonis*
GCA001703335.1	Fom013	*F. oxysporum* f. sp. *melonis*
GCA001703345.1	Fom012	*F. oxysporum* f. sp. *melonis*
GCA001703355.1	Fol002	*F. oxysporum* f. sp. *lycopersici*
GCA001703365.1	Fom016	*F. oxysporum* f. sp. *melonis*
GCA001703455.1	Foc011	*F. oxysporum* f. sp. *cucumerinum*
GCA001757345.1	ASM175734v1	*F. oxysporum* f. sp. *ciceris*
GCA001888865.1	ASM188886v1	*F. oxysporum* f. sp. *melongenae*
GCA002233775.1	Fonar_Na5	*F. oxysporum* f. sp. *narcissi*
GCA002233785.1	Fonic_010	*F. oxysporum* f. sp. *nicotianae*
GCA002233795.1	Fomom_004	*F. oxysporum* f. sp. *momordicae*
GCA002233805.1	Fotul_Tu67	*F. oxysporum* f. sp. *tulipae*
GCA002233855.1	Foluf_002	*F. oxysporum* f. sp. *luffae*
GCA002233865.1	Fomom_001	*F. oxysporum* f. sp. *momordicae*
GCA002233875.1	Folag_005	*F. oxysporum* f. sp. *lagenariae*
GCA002233895.1	Fogla_G2	*F. oxysporum* f. sp. *gladioli*
GCA002233915.1	Fogla_G14	*F. oxysporum* f. sp. *gladioli*
GCA002233935.1	Fo_Tu58	*F. oxysporum* f. sp. *tulipae*
GCA002233955.1	Fophy_KOD888	*F. oxysporum* f. sp. *physali*
GCA002233985.1	Fophy_KOD887	*F. oxysporum* f. sp. *physali*
GCA002233995.1	Fophy_KOD886	*F. oxysporum* f. sp. *physali*
GCA002234015.1	Fonic_012	*F. oxysporum* f. sp. *nicotianae*

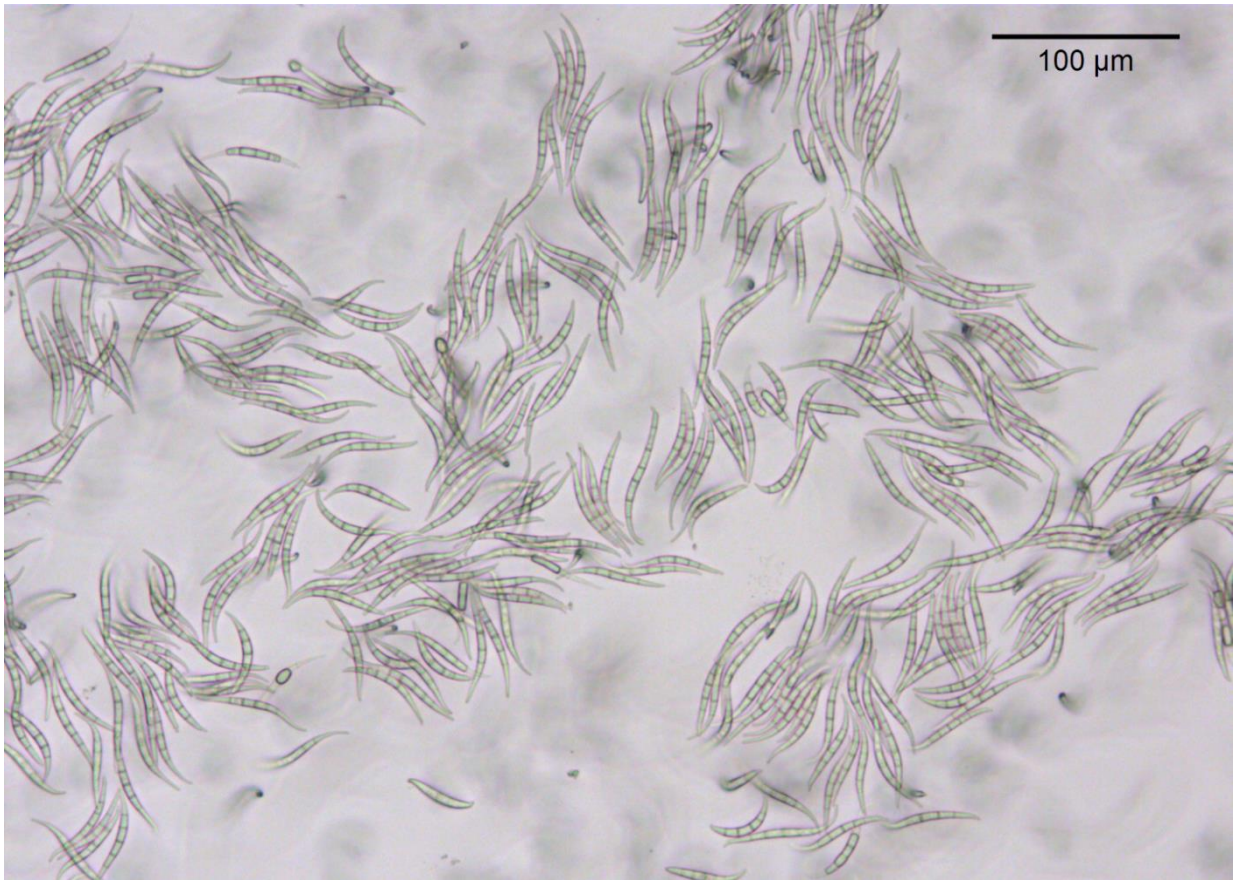
Appendix Table 3.1. Continued.

Accession number	Assembly name	Strain description
GCA002234045.1	Fonic_003	*F. oxysporum* f. sp. *nicotianae*
GCA002234055.1	Fonic_001	*F. oxysporum* f. sp. *nicotianae*
GCA002234085.1	Fomel_001	*F. oxysporum* f. sp. *melongenae*
GCA002234105.1	Foluf_001	*F. oxysporum* f. sp. *luffae*
GCA002234115.1	Folil_Fol39	*F. oxysporum* f. sp. *lilii*
GCA002234135.1	Folag_004	*F. oxysporum* f. sp. *lagenariae*
GCA002234165.1	Folag_002	*F. oxysporum* f. sp. *lagenariae*
GCA002234185.1	Folag_001	*F. oxysporum* f. sp. *lagenariae*
GCA002234195.1	Fogla_G76	*F. oxysporum* f. sp. *gladioli*
GCA002318975.1	ASM231897v1	*F. oxysporum* f. sp. *melonis 26406*
GCA002711385.1	ASM271138v1	*F. oxysporum* f. sp. *conglutinans*
GCA002711405.2	ASM271140v2	*F. oxysporum* f. sp. *conglutinans*
GCA002894245.1	CS5870v1	*F. oxysporum* CS5870
GCA003025205.1	ASM302520v1	*F. oxysporum*
GCA003025235.1	ASM302523v1	*F. oxysporum*
GCA003315725.1	ASM331572v1	*F. oxysporum* f. sp. *lycopersici* 4287
GCA003615075.1	FoC_A23_v1	*F. oxysporum* f. sp. *cepae*
GCA003615085.1	FoC_Fus2_v1	*F. oxysporum* f. sp. *cepae*
GCA003615095.1	FoC_125_v1	*F. oxysporum* f. sp. *cepae*
GCA003615115.1	Fo_A13_v1	*F. oxysporum*
GCA003615155.1	Fo_A28_v1	*F. oxysporum*
GCA003615165.1	Fo_CB3_v1	*F. oxysporum*
GCA003615185.1	Fo_PG_v1	*F. oxysporum*
GCA003704975.1	ASM370497v1	*F. oxysporum*
GCA003705035.1	ASM370503v1	*F. oxysporum*
GCA003705045.1	ASM370504v1	*F. oxysporum*
GCA003709395.1	ASM370939v1	*F. oxysporum*
GCA900096695.1	-	*F. oxysporum*

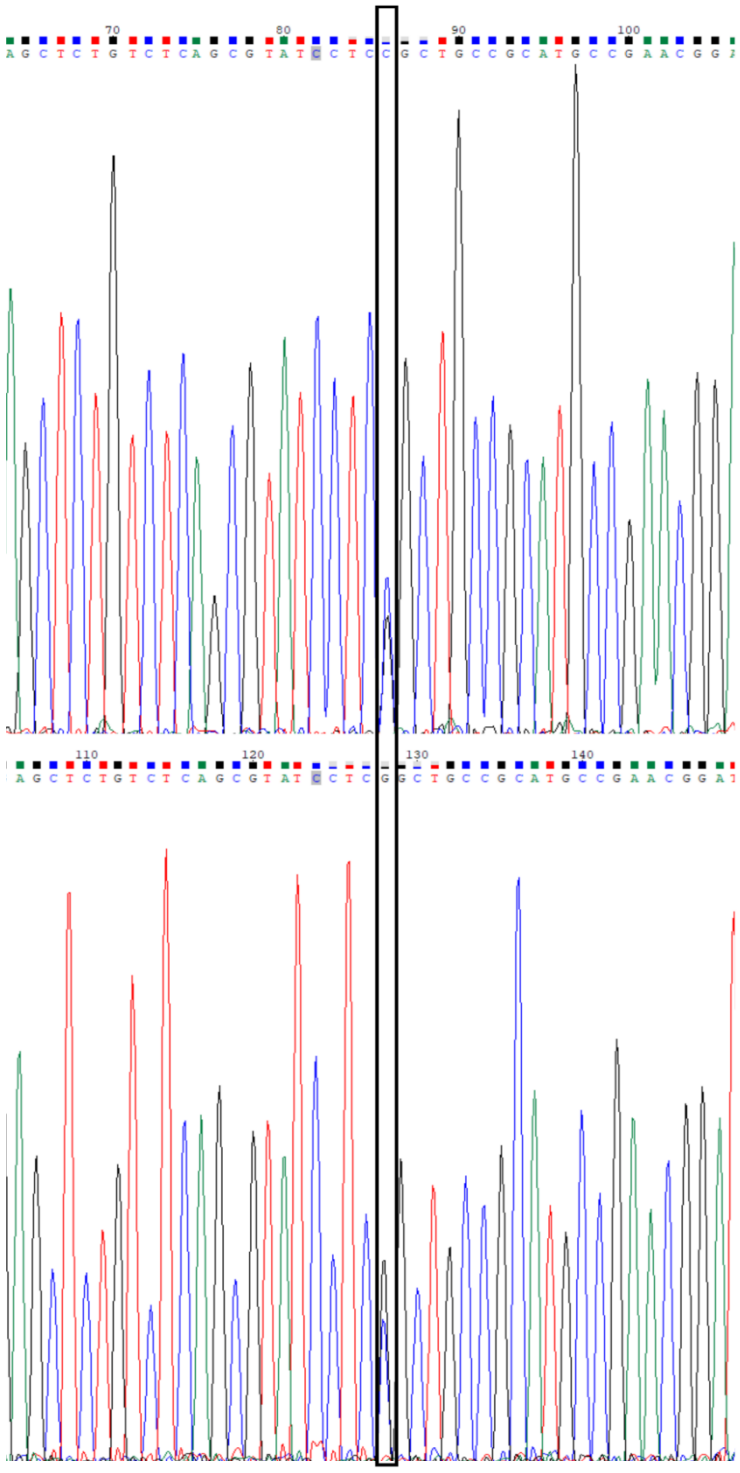
[a] GenBank accession number of the genome assembly.



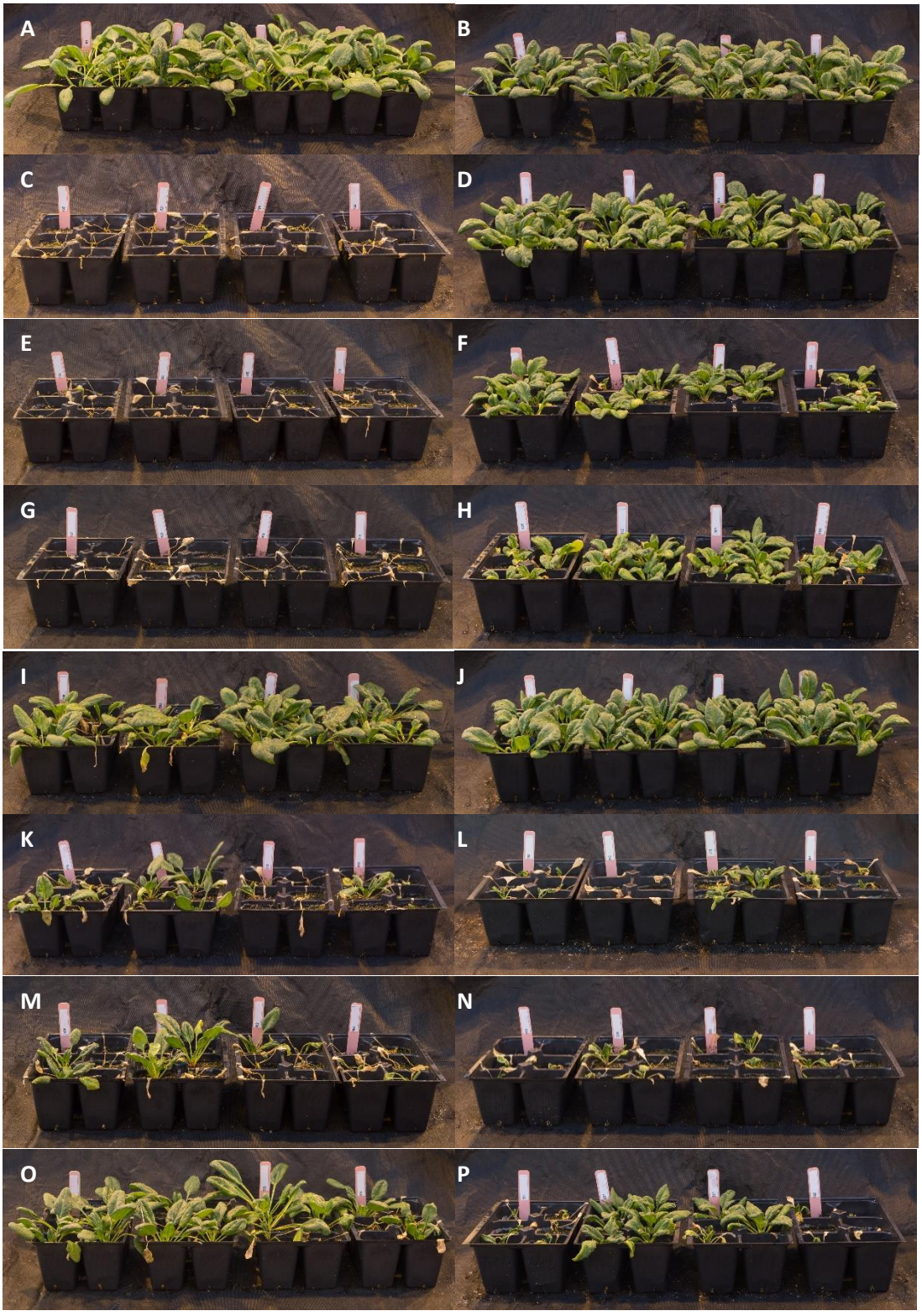
Appendix Fig. 2.1. Aerial (top) and profile (lower) photos of one replicate block of a spinach inbred line highly susceptible to Fusarium wilt, 21 days after inoculation or planting. The plants were either drench-inoculated over the root plugs or the seed was sown in infested soil with one of three inoculation treatments: water (negative control treatment), isolate Fus254 of *F. oxysporum* f. sp. *spinaciae*, or isolate Fus322 of *F. oxysporum* f. sp. *spinaciae*.

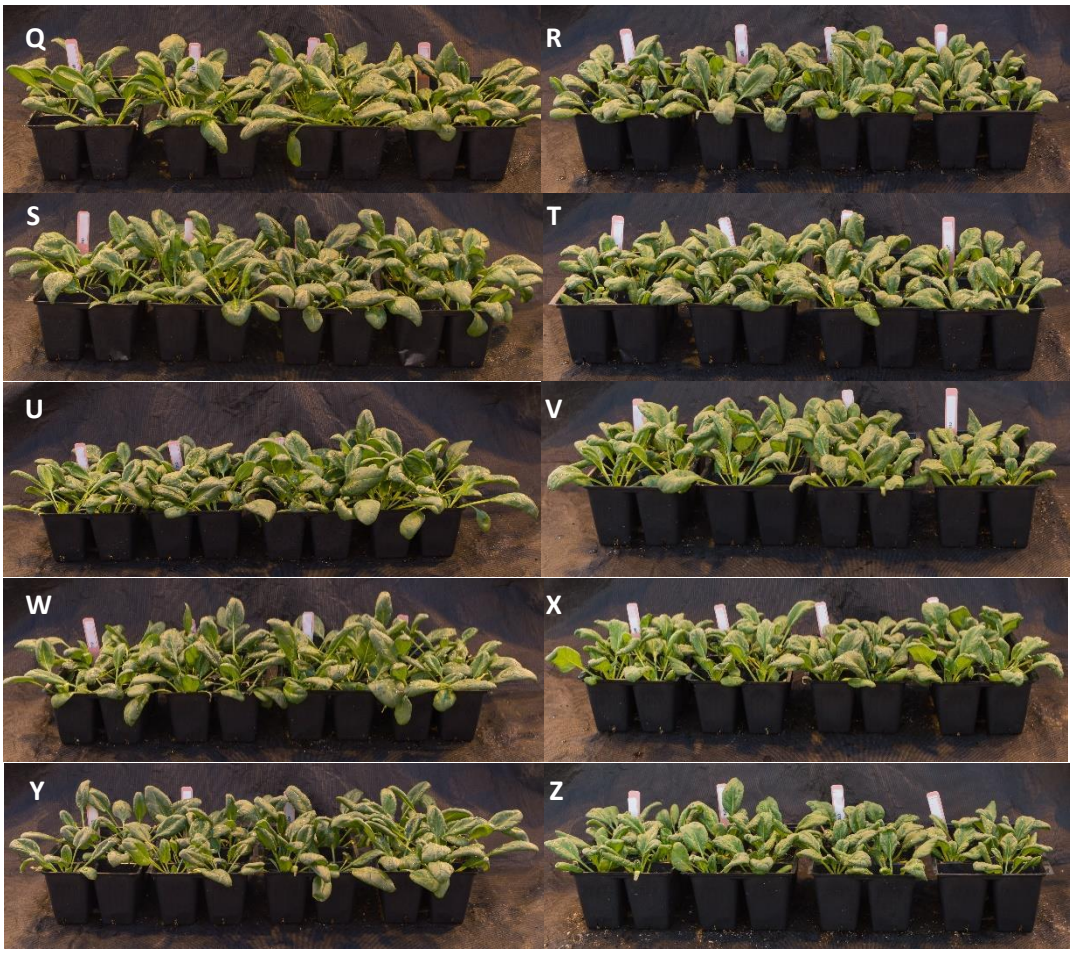


Appendix Fig. 2.2. Macroconidia of isolate Fus442, identified as *Fusarium equiseti*. The macroconidia were sampled from sporodochial stroma growing on the surface of a carnation leaf on carnation leaf agar.

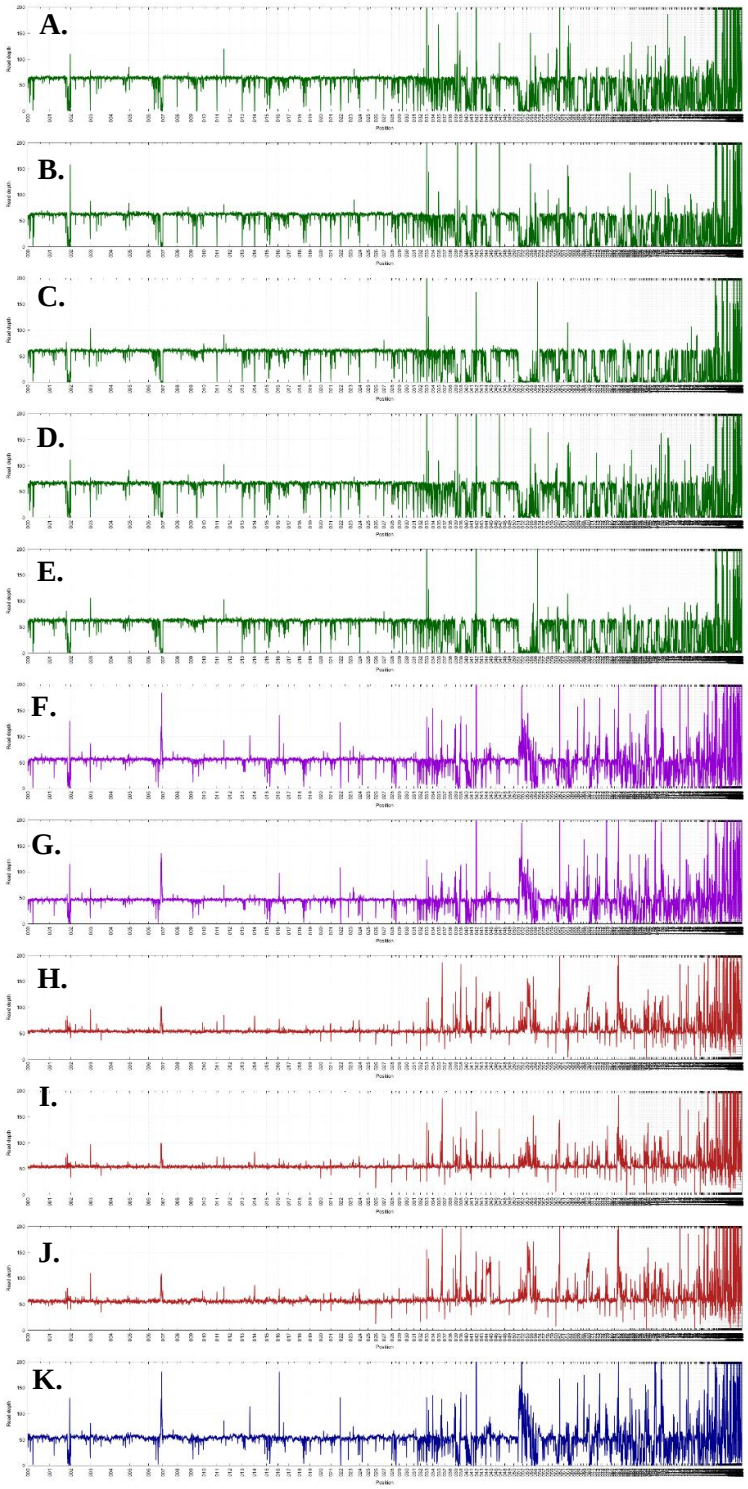


Appendix Fig. 2.3. Stacked and aligned chromatograms of the *Secreted in Xylem 14* (*SIX14*) gene from isolate Fus322 of *Fusarium oxysporum* f. sp. *spinaciae*. Top = forward read of *SIX14*, bottom = reverse complement of the reverse sequencing read of *SIX14*. The black box includes a base call for which the forward read called a C while a G was called at a similar relative intensity. The opposite was true for the reverse read.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

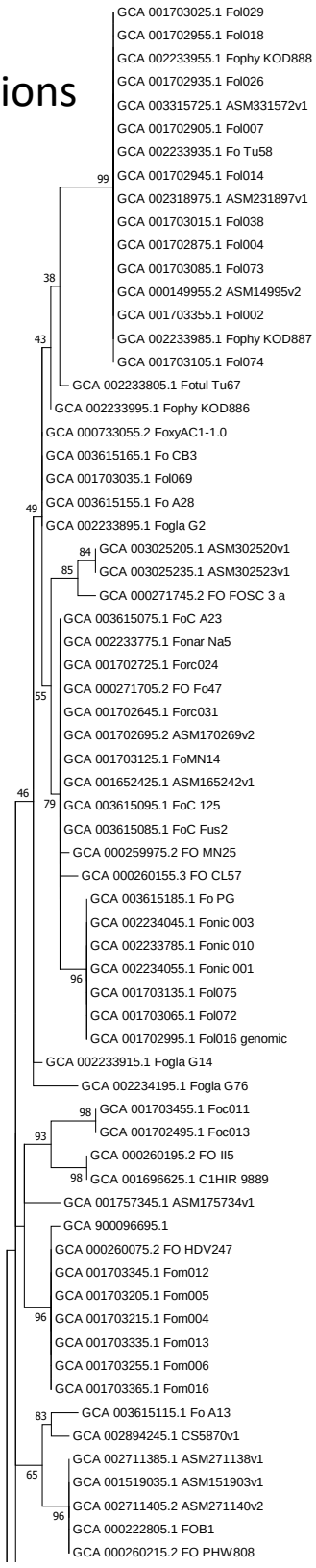


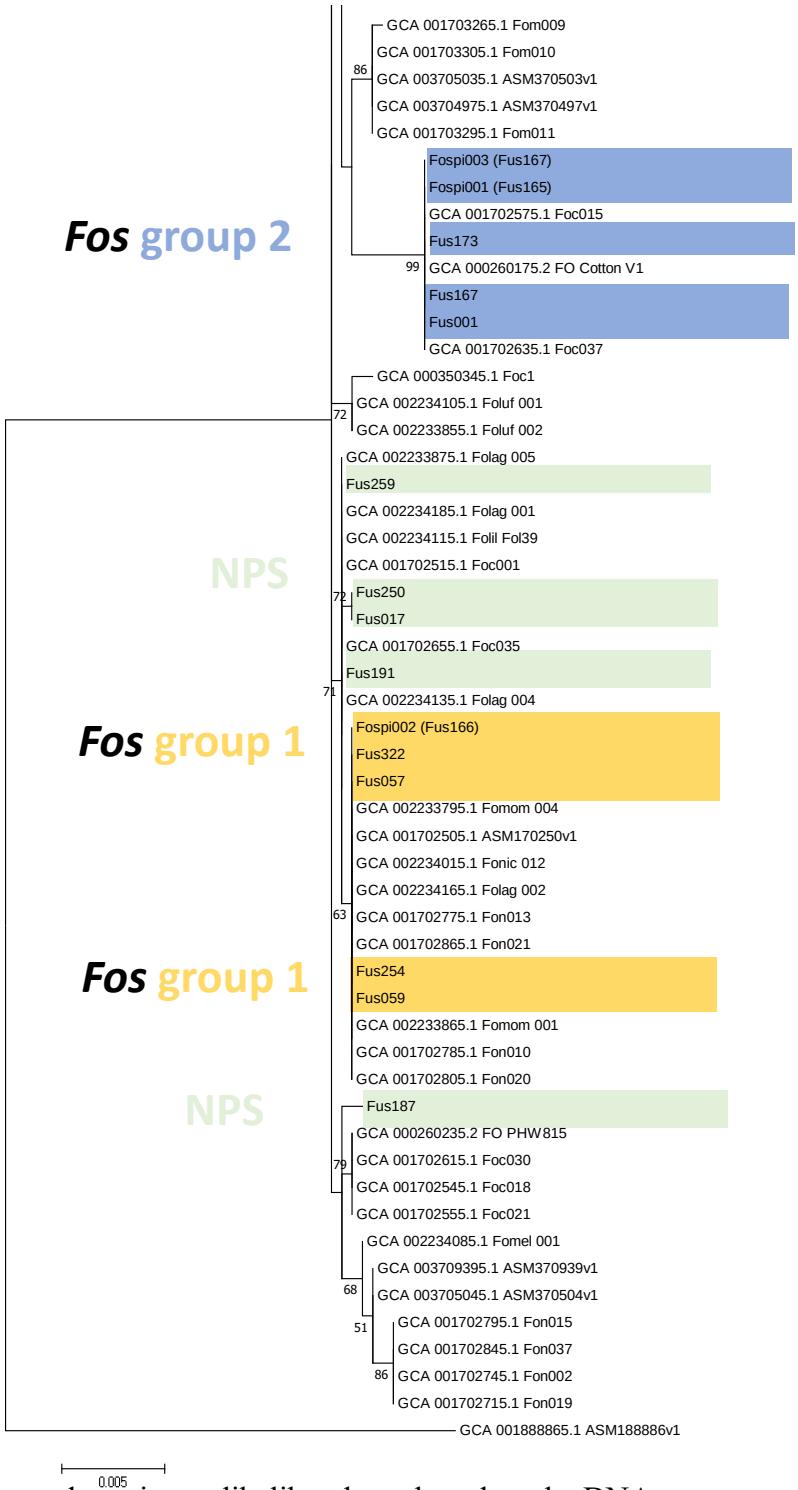
Appendix Fig. 3.1. Treatment combinations of two proprietary spinach inbred lines planted into potting mix inoculated with one of 12 *Fusarium oxysporum* isolates or water (control treatment). The photos are of spinach plants tested in pathogenicity trial 2. The plants were photographed 35 days after planting. A, C, E, G, I, K, M, O, Q, S, U, W, and Y represent spinach plants of the highly susceptible inbred in one of 13 inoculated soils. B, D, F, H, J, L, N, P, R, T, V, X, and Z represent plants of the partially resistant inbred in one of 13 inoculated soils. The soil was inoculated with the following *F. oxysporum* isolates (or water) prior to sowing spinach seed: A and B, water control treatment; C and D, Fus057; E and F, Fus059; G and H, Fus254; I and J, Fus322; K and L, Fus001; M and N, Fus167; O and P, Fus173; Q and R, Fus017; S and T, Fus187; U and V, Fus191; W and X, Fus250; Y and Z, Fus259. Refer to Table 3.1 for details of the isolates.



Appendix Fig. 3.2. Depth of Illumina reads mapped to the Fus254 PacBio assembly for: A) Fus017, B) Fus187, C) Fus191, D) Fus250, E) Fus259, F) Fus001, G) Fus167, H) Fus057, I) Fus059, J) Fus322, and K) Fus173. Plots with green lines represent read depths for *Fusarium oxysporum* isolates not pathogenic to spinach, and plots with purple, blue, or red lines are for *F. oxysporum* f. sp. *spinaciae* isolates. Refer to Table 3.1 for details of the isolates.

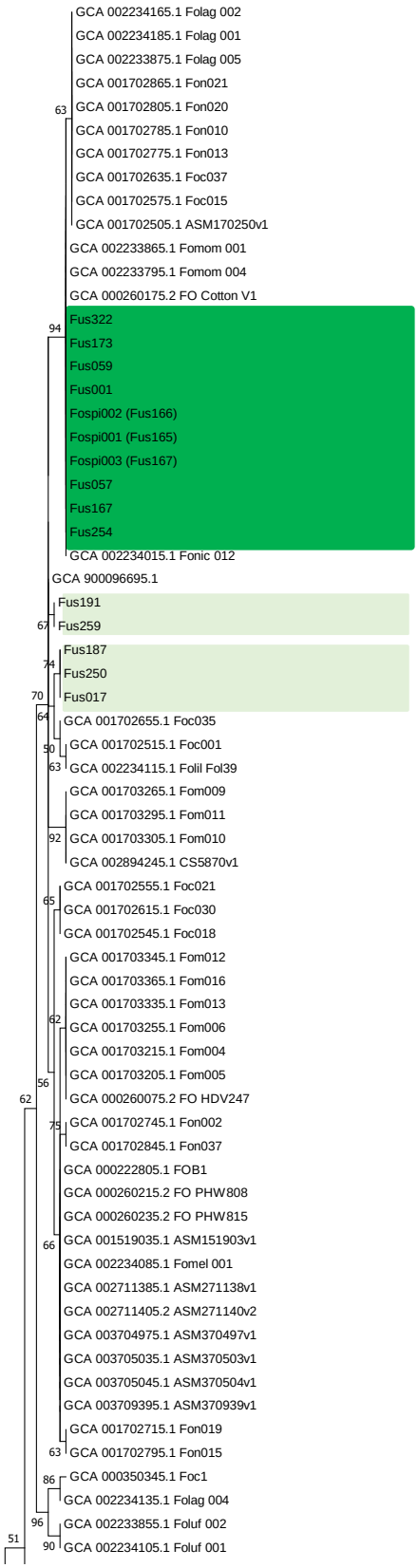
Locus: β-*TUB*
Number of positions: 2040 positions
Model: K2P + Γ

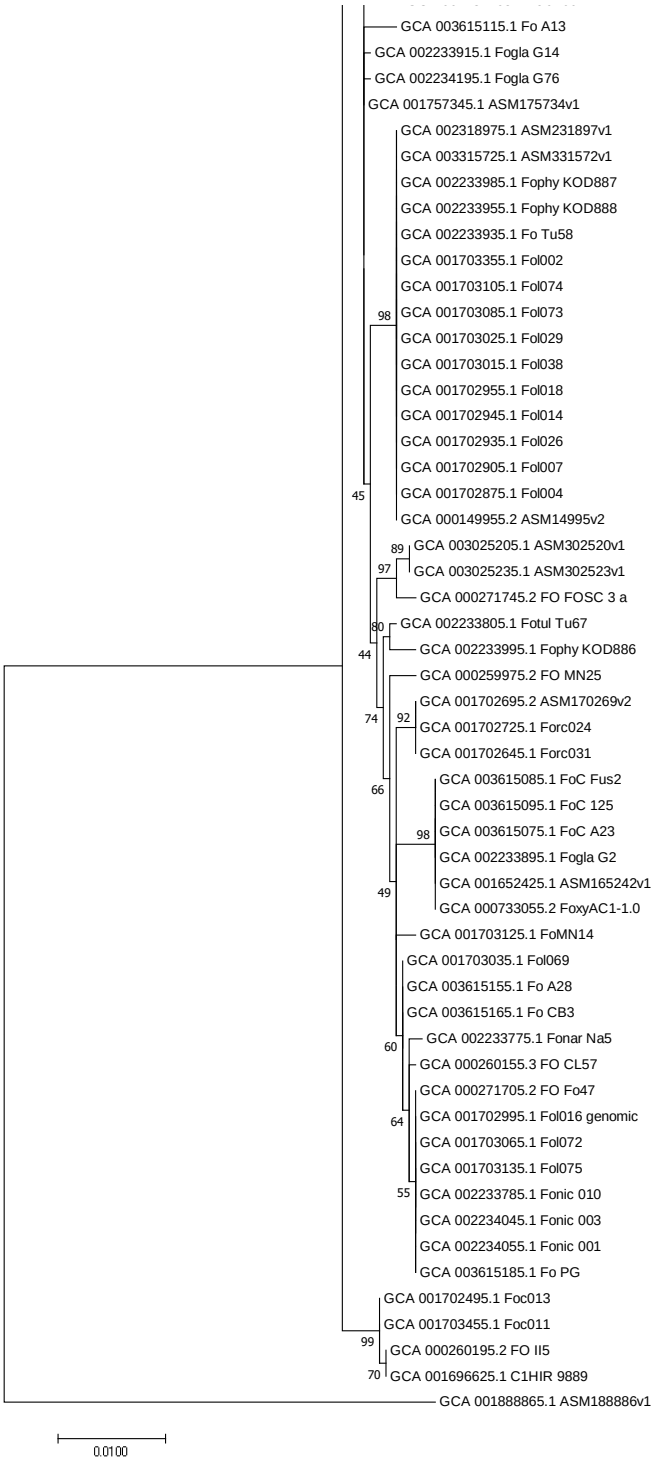




Appendix Fig. 3.3. Unrooted maximum likelihood tree based on the DNA sequence of the β *tubulin* (β-*TUB*) gene (2040 nt total) extracted from 120 publicly available *Fusarium oxysporum* genome sequences. The tree was estimated with the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches, and nodes with bootstrap support <33 are not shown for clarity. The scale bar indicates the average number of nucleotide substitutions per site.

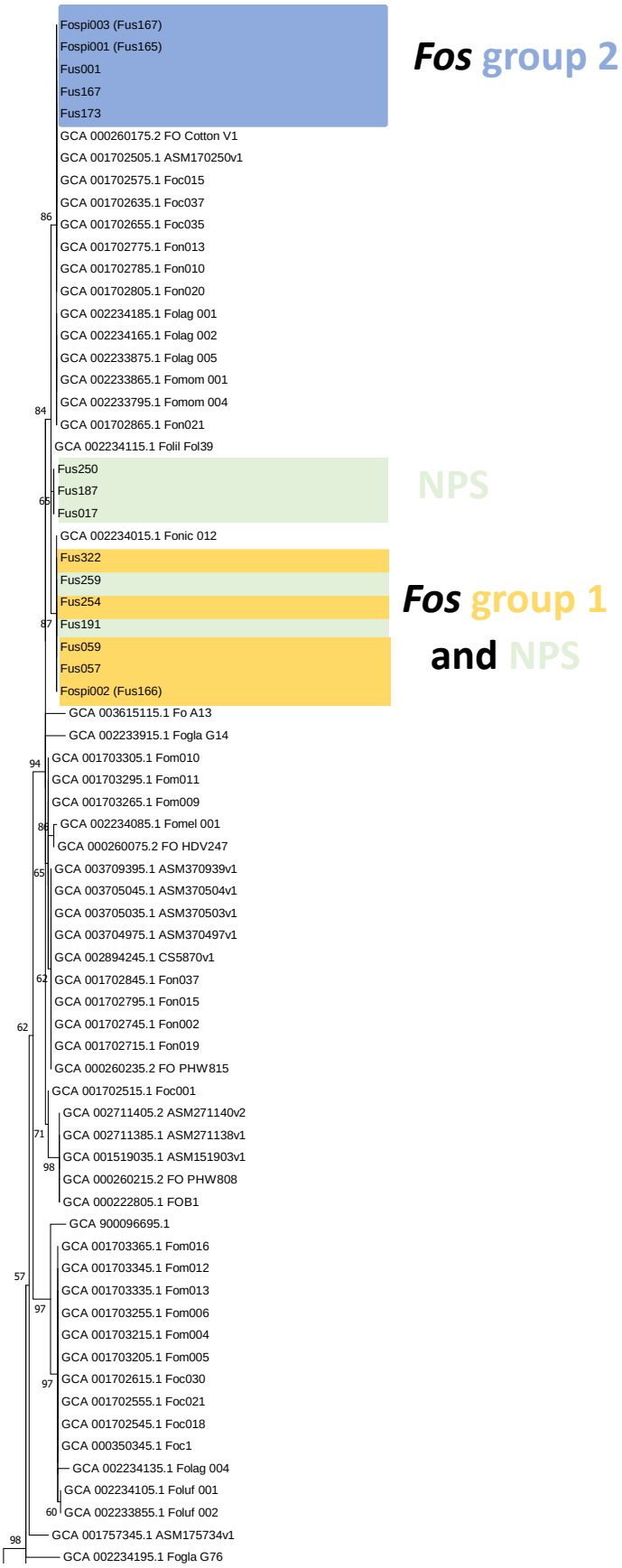
Locus: *TEF-1α*
Positions: 1671 positions
Model: HKY93 + Γ

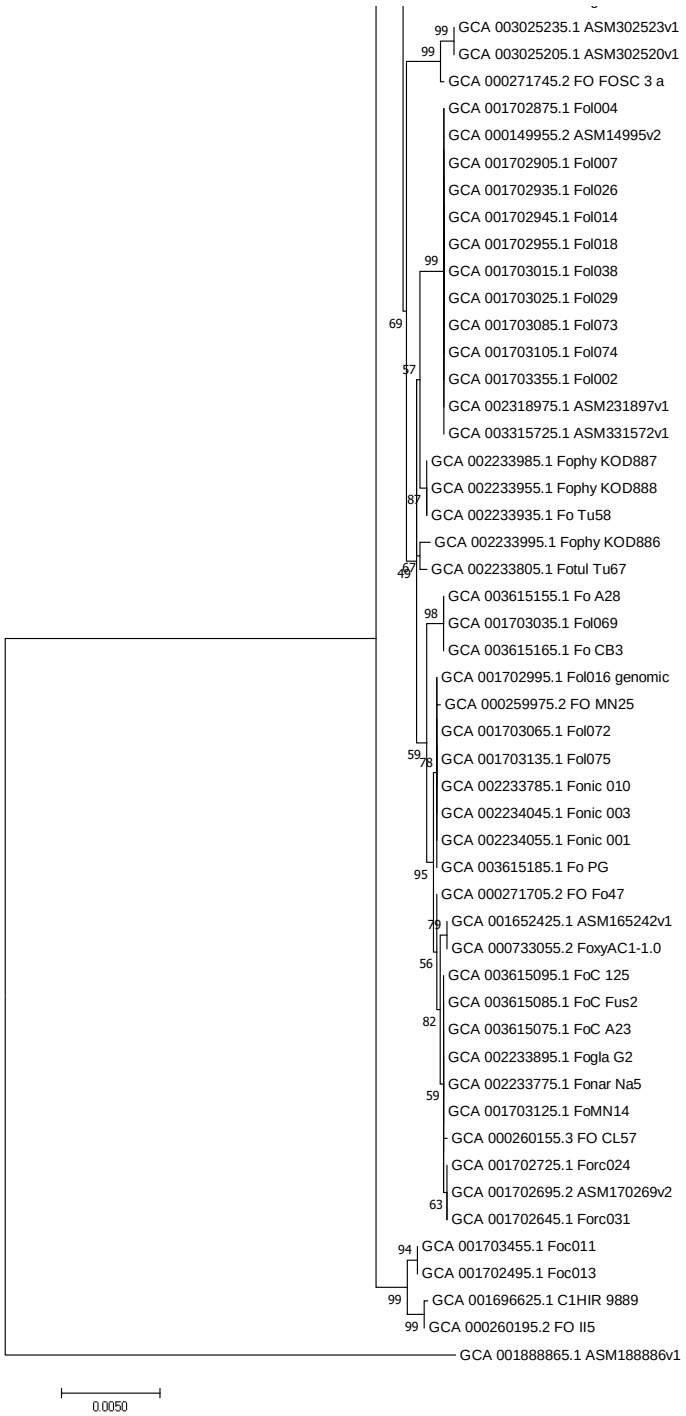




Appendix Fig. 3.4. Unrooted maximum likelihood tree based on the DNA sequence of the *translation elongation factor 1α* (*TEF-1α*) gene (1671 nt total) extracted from 120 publicly available *Fusarium oxysporum* genome sequences. The tree shown was estimated with the Hasegawa-Kishino-Yano model. The percentage of trees in which the associated taxa clustered together is shown next to the branches, and nodes with bootstrap support <33 are not shown for clarity. The scale bar indicates the average number of nucleotide substitutions per site.

Locus: *RPB1*
Positions: 5805
Model: TN93





Appendix Fig. 3.5. Unrooted maximum likelihood tree based on the DNA sequence of the *RNA polymerase binding protein 1* (*RPB1*) gene (5805 nt total) extracted from 120 publicly available *Fusarium oxysporum* genome sequences. The tree shown was estimated with the Tamura-Nei 3-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches, and nodes with bootstrap support <33 are not shown for clarity. The scale bar indicates the average number of nucleotide substitutions per site.