MANAGEMENT OF FUSARIUM WILT IN SPINACH SEED CROPS IN THE MARITIME PACIFIC NORTHWEST USA

By

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MANAGEMENT OF FUSARIUM WILT IN SPINACH SEED CROPS IN THE MARITIME PACIFIC NORTHWEST USA

Abstract

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The maritime Pacific Northwest is the only region of the USA suitable for production of spinach seed, a cool-season, daylength-sensitive crop. However, the acidic soils of this region are highly conducive to spinach Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *spinaciae*. Rotations of 10 to 15 years between spinach seed crops are necessary to reduce the risk of losses to this disease. Raising soil pH with limestone partially suppresses spinach Fusarium wilt, but the suppressive effect is transitory, and the disease still limits seed crop acreage in the region. Experiments were conducted to: 1) assess the potential for annual applications of limestone for three years prior to a spinach seed crop to improve Fusarium wilt suppression compared to the level of suppression from a single limestone amendment, 2) develop a soil-based greenhouse bioassay to characterize the spinach Fusarium wilt risk of soil samples submitted from stakeholders' fields, and 3) explore the mechanism(s) of limestone-mediated Fusarium wilt suppression. Annual applications of limestone for each of three years prior to a spinach seed crop were superior to a single limestone application for suppressing Fusarium wilt and increasing seed yield. A soil bioassay to assess Fusarium wilt risk was developed in which

three spinach lines representing a range in Fusarium wilt susceptibility were used to test soil samples from growers' fields under consideration for spinach seed crops. In the four years that the soil bioassay has been offered as a risk assessment service, soil samples from 147 fields were submitted by stakeholders for evaluation. Follow-up visits to spinach seed crops planted in fields assessed with the soil bioassay validated the results. *In vitro* experiments demonstrated that deficiencies of iron, manganese, and zinc can reduce growth and sporulation of *F. oxysporum* f. sp. *spinaciae*. Furthermore, greenhouse experiments in naturally-infested field soil indicated that reduction in availability of these micronutrients in limestone-amended soils reduced Fusarium wilt inoculum potential. Together, these findings reveal relationships among soil properties and spinach Fusarium wilt development, increase the capacity for and profitability of USA spinach seed production, and will guide future research on soil-based management of this disease.

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CHAPTER ONE

INCREASING THE CAPACITY FOR SPINACH SEED PRODUCTION BY PROMOTING SOIL SUPPPRESION OF FUSARIUM WILT: A LITERATURE REVIEW

Spinach (*Spinacia oleracea* L.) is one of four members of the genus *Spinacia* (Chenopodiaceae) native to central and southwestern Asia, in what is now Iran (Rubatzky and Yamaguchi, 1997). Believed to have evolved from *Spinacia tetranda*, a wild relative that is still eaten in Anatolia, spinach was introduced to Europe in the 11th century and is now grown in temperate climates throughout the world. A low-growing, acaulescent, glabrous leafy green with a 20 to 25 cm diameter rosette, spinach is classified by leaf type as savoy, semi-savoy, or smooth-leaf (Compendium of Washington Agriculture, 2006; van der Vossen, 2004). Savoy types are dark-green and heavily crinkled; smooth leaf types have unwrinkled, hastate leaves that are typically used for processing; and semi-savoy types are intermediate, with slightly curly leaves that are easier to clean than full savoy types. Cultivars are also classified according to fruit type: var. *inermis* types have round, smooth seed and are more tolerant of warm temperatures; while var. *oleracea* have spiny seeds, a more branched and spreading growth habit, and are more cold-tolerant (van der Vossen, 2004). Flowers are produced in clusters of 6 to 20 on cymose inflorescences. The "seeds," which are one-seeded, indehiscent fruits, ripen unevenly. This presents a challenge for seed harvesting (Bewley et al, 2008).

The floral biology of spinach is complex (Sherry et al., 1993). While spinach is generally considered dioecious, individual plants can include extreme males with reduced, bract-like

leaves; vegetative males, which have all-male flowers but well-developed leafy inflorescences; monoecious, with male and female flowers on the same inflorescence; gynomonoecious, with complete and pistillate flowers; or gynoecious, with only pistillate flowers (Onodera et al., 2008). These floral variations are used by plant breeders and seed producers, but can also be problematic in the production of hybrid seed (Foss and Jones, 2005; Morelock and Correll, 2008). Spinach is wind-pollinated, and seed typically matures 60 to 70 days after flowering. Flowering in spinach, a long-daylength species, is highly photoperiodic (Metzger and Zeevaart, 1985). Because bolting is not desirable in spinach leaf crops, plants have been selected to require increasingly long daylengths (16+ hours) for induction of anthesis, which has resulted in all spinach seed production occurring in regions of high latitude. Optimal temperatures for spinach growth are from 15 to 20°C, but temperatures as low as 3°C are tolerated (van der Vossen, 2004).

The USA is the world's second largest producer of spinach (3%) following China (85%) (USDA-Economic Research Service). Consumption of spinach has increased steadily in the USA since the 1970s, driven primarily by the popularity of cello-packed, baby-leaf spinach and by increasing awareness of the nutritional benefits of this leafy green with high levels of vitamins C and A, iron, folic acid, magnesium, and the carotenoid lutein, which has been implicated in prevention of macular degeneration (Howard et al., 2002; Lucier, 2000). Fresh-market spinach now accounts for about three-quarters of total consumption in the USA (USDA-ERS). California, Arizona, New Jersey, and Texas are the largest spinach-producing states (Koike et al., 2011). The average farmgate value of spinach in 2004 to 2006 was $175 million (USDA ERS).

Spinach seed production. The dramatic expansion of the market for baby-leaf spinach has greatly increased the demand for spinach seed, as baby-leaf crops require planting up to 9

million seed/ha (LeStrange, 1996). There are few areas in the world where high-quality spinach seed can be produced (Metzger and Zeevart, 1985). Spinach seed production requires a mild climate, long daylength to initiate bolting, and dry conditions around the time of harvest to minimize seedborne pathogens. For this reason, the maritime Pacific Northwest and Denmark are currently the primary regions of spinach seed production for western markets. Up to 50% of the USA and 25% of the world supply of spinach seed is produced in western Washington and Oregon, on 1,200 to 1,600 ha annually at a value of $400 to 500/ha (Foss and Jones, 2005).

The primary limit to spinach seed production in the maritime Pacific Northwest is Fusarium wilt, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *spinaciae* (Foss and Jones, 2005). The highly-leached, acid soils of the Pacific Northwest are very conducive to this disease. The long-term survival of the pathogen in soils necessitates rotations of up to 15 years between spinach seed crops, and even then devastating losses to the disease can occur. When the disease was first reported in the Pacific Northwest in the 1960s, growers were able to avoid such losses by limiting spinach seed production to fields that had never been planted to spinach, but such ground is virtually non-existent at present in the Skagit Valley, the heart of the spinach seed production region in northwestern Washington. Partially resistant parent lines exist, but many widely-used parent lines are highly susceptible to the disease (Correll et al., 1994). Furthermore, spinach seed is grown on contract, so growers (and even seed company representatives of "third party" contracting seed companies) may not know the level of Fusarium wilt resistance of the parent lines they are provided. This complicates the selection of low-risk field sites for spinach seed crops. Because the disease is of minor importance for production spinach, breeding for resistance to Fusarium wilt has not been a high priority for many breeding programs (J. Schafer, spinach breeder, Schafer Agricultural Services, *personal communication*). With the additional

requirement to maintain isolation distances of up to 6.4 km between seed crops to prevent undesirable cross-pollination, finding ground suitable for spinach seed production represents a major limitation to the spinach seed production capacity in the Pacific Northwest and, therefore, in the USA.

Fusarium wilts: morphology and taxonomy. The genus *Fusarium* (division Ascomycotina), as described by Link in 1809, is currently subdivided into 70 species of filamentous fungi that are differentiated by the length and shape of macroconidia, the size and arrangement of microconidia, the presence or absence of chlamydospores, colony color on defined media, and most recently, by molecular genotyping based on sequence variation in the 28S ribosomal RNA subunit (Summerell et al., 2003; Leslie and Summerell, 2006). This classification is the culmination of efforts spanning centuries to make sense of the diversity within this genus, starting with Wollenweber and Reinking (1935), who described 65 species organized into 16 sections. Later reorganizations were undertaken by Snyder and Hansen (1940) and Nelson et al. (1983) to compress, expand, or otherwise modify the prevailing taxonomic structure. Identification of *Fusarium* species can be hindered by the high level of morphological plasticity and genetic variability both between and within species (Fourie et al., 2011).

Many species of *Fusarium* are found in soil and in association with plants, and are studied widely in agricultural systems for their roles as saprobes, plant pathogens, and antagonists (Windels, 1992). One of the most cosmopolitan and diverse groups within the genus is *F. oxysporum* Schlecht.:Fr. emend. Snyd & Hans. Distinguished from closely related species by the presence of microconidia born in false heads on relatively short monophialides, the presence of chlamydospores, and a characteristic sickle-shaped macroconidium with an attenuated apical cell, this species is further sub-categorized according to pathogenicity on a

wide range of cultivated crops (Armstrong and Armstrong, 1975; Burgess et al., 1989). Isolates capable of causing disease on a given plant host are termed formae speciales, and typically are indistinguishable morphologically from other isolates. More than 150 formae speciales have been identified, and are responsible for some of the world's most economically significant crop diseases, causing vascular wilts, yellows, corm and root rots, and damping-off (Agrios, 1997; Hawksworth et al., 1995). Within some formae speciales, pathogenic races have been distinguished based on differential virulence on host cultivars (Armstrong and Armstrong, 1975). *F. oxysporum* is an anamorphic species, propagating exclusively via clonal reproduction (Gordon and Martyn, 1997). The high level of phenotypic and genotypic diversity it exhibits is somewhat unusual for an asexual organism, but heterokaryosis, parasexual recombination, and a high mutation rate documented for *F. oxysporum* are most likely the sources of this variability (Buxton, 1956; Nelson et al., 1983).

Recent studies have suggested that *F. oxysporum* may be a complex of morphologically indistinguishable but phylogenetically distinct cryptic species (Baayen et al., 2000; O'Donnell et al., 2009). The lack of sexual reproduction means the biological species concept cannot be applied to *F. oxysporum*, leaving only morphological and phylogenetic approaches. The former is complicated by phenotypic plasticity, and the latter by the difficulty in differentiating between true species divergence and intra-specific diversity. It has also been determined that formae speciales in many cases do not represent monophyletic groupings of isolates, due to co-evolution of genetically diverse isolates with a host plant species and/or horizontal transfer of pathogenicity factors (Fourie et al., 2011). Such findings have stymied efforts to develop DNA-based diagnostic methods for host-specific Fusarium wilt pathogens (e.g., Okubara et al., 2013).

***Fusarium oxysporum*: life cycle, pathogenesis, and symptomology.** *F. oxysporum* persists in the soil in the form of long-lived chlamydospores or as mycelium colonizing non-host species (Gordon et al., 1989) or crop residues (Gordon and Okamoto, 1990). In the presence of a suitable host (i.e., nutrient source), the thick-walled chlamydospore germinates. Upon contact with an elongating root tip, the germ tube begins to colonize the surface of the root via expanding hyphae. Mycelia present in the soil can also colonize and invade the root epidermis. Chlamydospores need to be between 0.3 and 1.5 mm from the root in order to perceive the presence of plant leachates and germinate. Maximum germination occurs about 13 mm behind the root tip. Both pathogenic and non-pathogenic *F. oxysporum* have been shown to be strong colonizers of root surfaces and the root cortex (Beckman, 1987; Gordon and Martyn, 1997). Penetration of roots occurs mostly commonly through wounds in epidermal cells or at the junctures where lateral roots emerge, although the epidermis can be penetrated directly near the root tip where cell defenses are less well-developed. How many successful root penetrations are necessary for the onset of disease has been the subject of much investigation in Fusarium wilt research. The answer varies depending on the susceptibility and growth stage of the host, the inoculation method, the aggressiveness of the pathogen strain, and environmental conditions, including temperature, soil type and pH, matric potential, and soil fertility status (Baker, 1971; Hao et al., 2009).

After intercellular colonization of the cortex, hyphae invade the xylem through the pits and become established in the vasculature of the plant (Beckman, 1987; di Pietro et al., 2003). The xylem vessels of plant vascular tissue afford unique opportunities and challenges for the pathogen. On the one hand, this interconnected transport system is a conduit for the mycelium to move passively up to other parts of the plant, and the fungus is relatively protected from other

microorganisms once it reaches the vessels. On the other hand, the vessels are interrupted by perforation plates, which present a barrier to the movement of fungal hyphae. *F. oxysporum* circumvents this obstacle by producing microconidia, which are small enough to fit through the holes in the perforation plates, and which subsequently germinate, grow, and sporulate in the next vessel element (Beckman, 1987).

There are two theories as to what causes the wilting that results from a successful invasion of the vasculature by a pathogenic strain of *F. oxysporum* (Cook, 1981). One is that toxins produced by the fungus, primarily fusaric acid, cause plant cells to lose turgor pressure via disruption of cell membrane semi-permeability. The other is plugging of the vessels by tyloses, invaginations of parenchyma protoplasts through pits into the vessel lumen, and/or by polysaccharide gums of both plant and fungal origin. More evidence for the latter has been documented, although it is likely that toxins play a role in the pathogenesis of Fusarium wilts (Beckman, 1987). As a result of reduced water flow, a suite of symptoms ensues, beginning with slight mottling/interveinal chlorosis of young leaves, followed by loss of turgidity, epinasty, yellowing, and defoliation of leaves. Plants infected in the seedling stage may die soon after symptoms appear. Discoloration of the vascular stele and blackening of root tips are also diagnostic for some Fusarium wilts. In advanced stages of disease development as plant tissues begin to senesce, the pathogen moves out of the vascular tissue and colonizes dead and decaying tissue, often producing masses of sporodochia on crop residue. The sporodochia produce large numbers of macroconidia, which are eventually returned to the soil (Mace et al., 1981).

Spinach Fusarium wilt. Fusarium wilt of spinach was identified and described in 1923 in Idaho by Hungerford and Sherbakoff (Hungerford, 1923). It was designated a new species, *Fusarium spinaciae*, but later re-classified as *F. oxysporum* Schlecht. f. sp. *spinaciae* (Sherb.)

Snyd & Hans (Snyder and Hansen, 1940). The disease has been reported in spinach-producing regions throughout the world (Correll et al., 1994; Larsson and Gerhardson, 1992; Sumner et al., 1976). Because the pathogen seems to materialize wherever conditions are appropriate for disease expression, there has been much interest in potential sources and dissemination of inoculum. Bassi and Goode (1978) isolated *F. oxysporum* f. sp. *spinaciae* from commercial seed lots originating in the Pacific Northwest, and theorized that seedborne inoculum was most likely the source of pathogen dissemination to new regions of production. Wind-blown dust, irrigation water, and movement of infested soil on workers and equipment have all been documented as sources of *F. oxysporum* inoculum, but not specifically with the spinach-Fusarium wilt pathosystem (Beckman, 1987; Mace et al., 1981).

The challenge of Fusarium wilt management in spinach seed production must be viewed within the following framework: 1) it is difficult, if not impossible, to exclude the pathogen from seed production fields; 2) fumigation is not an economically viable management practice; and 3) many commonly-used parent lines have little to no field resistance to the disease, with growers having no option in regard to the inbred lines they are contracted to grow (Foss and Jones, 2005). This reality has led to the pursuit of alternative, sustainable approaches to Fusarium wilt management in spinach seed crops. du Toit et al. (2006) demonstrated the capacity for mustard biofumigant crops and seedmeal amendments to suppress Fusarium wilt, but these treatments have limited potential in the maritime Pacific Northwest because of the potential for cross-pollination with brassica vegetable seed crops and the limited availability and high cost of biofumigant seedmeals. The application of agricultural limestone to raise the pH and reduce conduciveness of soils to Fusarium wilt has been demonstrated to suppress the disease partially, especially when highly susceptible parent lines are used (du Toit et al., 2007, 2008, and 2011;

Gatch et al., 2011). However, elevation of soil pH following limestone amendment exacerbates another soilborne wilt disease of spinach seed crops, Verticillium wilt caused by *Verticillium dahliae* (du Toit et al., 2007, 2008, and 2011; Gatch et al., 2011). This disease is an emerging concern in spinach seed crops because the pathogen is readily seed-transmitted and because strains that infect spinach can infect some crops grown in rotation with production spinach in states such as California and Arizona, two major markets for Pacific Northwest spinach seed (Atallah et al. 2010; Bhat and Subbarao, 1999; du Toit et al., 2005). A better understanding of the dynamics of these wilt pathogens in soils is fundamental to the development of appropriate management practices.

Soil-host-pathogen interactions in Fusarium wilts. The documentation of soils in which the root systems of plants seem protected from soilborne plant pathogens is of much interest to researchers. Such "suppressive" soils have been found in the Châteaurenard region of France (Fravel et al., 2003), the Salinas Valley in CA (Sneh et al., 1984), and isolated field sites discovered in the course of working with soilborne pathogens (Alabouvette, 1999). These soils present opportunities for researchers to better understand and manipulate interactions between the soil biotic and abiotic environment, the pathogen, and the host plant (Haas and Defago, 2005). In the case of Fusarium wilts, growers and researchers have long noted the existence of soils that appear to be inhospitable to the diseases. According to Hornby (1983), prior to the turn of the 20th century cotton farmers were aware of soils that were mysteriously immune to "frenching," the term for Fusarium wilt of cotton. In the mid-1900s, when Fusarium wilt was ravaging the banana industry in Central America, Stotzky et al. (1961) found correlations between the presence of montmorillonite clay minerals and the degree of "resistance" of a soil to Fusarium wilt. Due to the successful use of commercial fumigants and the deployment of

resistance genes, the study of suppressive soils fell out of favor for a number of years, but decreasing efficacy and/or availability of these management options, combined with the advancement of molecular methods for characterizing suppressive soils, have resulted in a revisiting of this complex subject (e.g., Tousson, 1975). Studies of suppressive soils have become increasingly sophisticated, with multiple mechanisms elucidated and efforts made to manipulate soils for enhanced suppressiveness (Alabouvette et al., 2009).

A definition of soil suppression and overview of the various categories of suppressive mechanisms are critical to understanding the phenomenon. In order for a soil to be considered suppressive according to the definition provided by Baker and Cook (1971), disease incidence and severity must be low despite the presence of the pathogen, a conducive environment, and a susceptible host. The suppressive effect can be related to a reduction in inoculum concentration (numbers of propagative units) or reduction in the disease-causing potential of the pathogen population (Tousson, 1975). The mechanism(s) of suppression may be abiotic (e. g. soil mineralogy, pH, matric potential), biotic (antagonistic microbiota), or both (Hornby, 1983). Boundaries between these two categories are often blurred, as the perceived association of suppression with pH or mineralogy is often related to the effect of these abiotic factors on the soil microbiota. The biocontrol agents responsible for soil suppression are either directly or indirectly antagonistic to the wilt pathogen population, or both (Fravel, 2003). Direct antagonism includes parasitism, antibiosis, and competition, while indirect antagonism is exemplified by the induction of systemic resistance (ISR), a broad-spectrum activation of plant defenses such as that induced by non-pathogenic rhizobacteria (Baker and Cook, 1974; Kloepper 1992).

Suppressive soils can be divided further into those in which all Fusaria are suppressed, or those in which only pathogenic forms are suppressed (Tousson, 1975). The well-characterized

Fusarium-suppressive soils of the Châteaurenard region of France are an example of the latter (Fravel et al., 2003). These soils support large populations of non-pathogenic *F. oxysporum* and *F. solani*. Elimination of these non-pathogenic isolates by treatment of the soil at 55°C destroyed the suppressive mechanism, and re-introducing them restored suppressiveness, suggesting that these non-pathogenic Fusaria play a role in suppressive soils. Strains of non-pathogenic *F. oxysporum* have proven more effective in suppressing Fusarium wilts than other *Fusarium* species (Alabouvette, 2009). Studies with Fusarium wilts of celery, cucumber, watermelon, and other crops have also found non-pathogenic *F. oxysporum* to be key to the function of soil suppression (Larkin et al., 1996; Paulitz et al., 1987; Schneider, 1984). The term "non-pathogenic" refers only to the host plant in question; the non-pathogenic isolate that is antagonistic to the pathogen under consideration may be pathogenic on a different host, as was shown to be the case by Minerdi et al. (2008).

Both direct and indirect modes of action have been implicated in the suppression afforded by non-pathogenic *F. oxysporum* against Fusarium wilt pathogens. In one of the first studies on ISR against Fusarium wilt, Biles and Martyn (1989) inoculated watermelon with avirulent races of *F. oxysporum* f. sp. *niveum* and the non-pathogen *F. oxysporum* f. sp. *cucumerinum*, followed by inoculation with virulent races of *F. oxysporum* f. sp. *niveum*. Resistant responses and reduced wilt development resulted from both protective inoculations, although the level of resistance was higher with the avirulent *F. oxysporum* f. sp. *niveum*. Interestingly, symptoms of infection by *Colletotrichum lagenarium* were also reduced significantly following induction by *F. oxysporum* f. sp. *cucumerinum*, suggesting that the ISR in that case was non-specific. Benhamou and Garand (2001) carried out detailed cytological analysis of the resistance response elicited in pea roots colonized by non-pathogenic *F. oxysporum*. They described rapid and

extensive formation of cell wall appositions and other non-specific defense mechanisms typical of an ISR response that prevented hyphae from advancing beyond the outer cortex. Alabouvette et al. (2009) found that it was necessary for the protective strain to penetrate the plant root to induce ISR, as non-penetrating mutants did not elicit the resistance response. Split-root techniques, in which root systems are separated spatially and one portion is challenged with the pathogen while the other part of the root system is inoculated with a protective isolate, have been useful to prove that ISR is involved in the mitigation of Fusarium wilt symptoms by non-pathogenic formae speciales. In both split-root and whole plant bioassays, Kaur and Singh (2007) found that two non-pathogenic *F. oxysporum* isolates, one from India and one from the Châteaurenard region of France, effectively induced systemic resistance against a highly pathogenic strain of *F. oxysporum* f. sp. *ciceri*. Similar results have been reported for many other pathosystems, indicating that ISR is an important component of the biocontrol mechanisms operative in Fusarium wilt-suppressive soils.

With similar frequency, however, the occurrence of direct antagonism, particularly competition for nutrients or space in the soil environment or the rhizosphere has been noted. It is well-established that non-pathogenic *F. oxysporum* strains can colonize the surface of plant roots, and even invade and multiply within the root cortex (Beckman, 1987). In general, competition for glucose has been identified as the primary mechanism of the antagonism between pathogenic and non-pathogenic *F. oxysporum* (Fravel et al., 2003; Lemanceau et al., 1993). When glucose is not a limiting factor, suppression typically disappears, which provides evidence for nutrient competition as the mode of action. Harder to prove is the possibility that different strains of *F. oxysporum* compete for infection sites on the root surface and in the vessels of the host plant. Autofluorescent visualizations by Bolwerk et al. (2005) and Olivain and

Alabouvette (1999) tracked the early colonization behavior of pathogenic and nonpathogenic *F. oxysporum* in tomato roots. They found no evidence to dispute the hypothesis that spatial competition could play a role in the wilt suppression mediated by cross-inoculation (Olivain and Alabouvette, 1999). Ultimately, Alabouvette et al. (2006) concluded there is insufficient understanding of what constitutes an infection site for *F. oxysporum* to address the possibility of spatial competition between biocontrol and pathogenic strains.

While many studies have focused exclusively on the role of non-pathogenic *F. oxysporum* in Fusarium wilt suppression, the contribution of other microorganisms to suppression has also been demonstrated. These include *Bacillus* spp., *Trichoderma* spp., actinomycetes, *Penicillium oxalicum*, and *Pseudomonas* spp. (Cotxarrera et al., 2002; De Cal et al., 1997; Larkin et al., 1996). Of these, the *Pseudomonas* spp. collectively referred to as fluorescent pseudomonads, consistently have demonstrated high levels of protection against soilborne fungal pathogens, alone or in tandem with non-pathogenic *F. oxysporum* (Haas and Defago, 2005; Lemanceau et al., 1993; Park et al., 1988; Saman, 2009). The mechanisms of suppression associated with fluorescent pseudomonads are antibiosis, ISR, and more subtle interactions with the pathogen such as degradation of fungal toxins. The fluorescent pseudomonads produce Fe-chelating siderophores that further reduce the availability of this micronutrient in soils and, thus, inhibit growth of fungi such as *F. oxysporum* that do not have aggressive Fe uptake capability. Although not technically secondary metabolites, these bacterial siderophores are considered antibiotics because of their fungistatic properties (Haas and Defago, 2005). Other true antibiotics produced by fluorescent pseudomonads include phenazines, phloroglucinols, cyclic polypeptides, and hydrogen cyanide, but the modes of action of these

compounds and their effects on Fusarium wilt pathogens have not been investigated in great detail.

The induction of systemic resistance by fluorescent pseudomonads has been well-characterized (Duijff et al., 1998; Lemanceau et al., 1993). Leeman et al. (1995) and van Peer et al. (1991) performed experiments on radish and carnation, respectively, in which they were able to separate spatially biocontrol isolates of *P. fluorescens* from pathogenic *F. oxysporum* in the host plant from inoculation through harvest. Significant reductions in wilt symptoms were observed in the biocontrol-inoculated plants, and since antagonism was not a possibility due to the physical separation, ISR was implicated. Duijff et al. (1998) conducted a similar study with Fusarium wilt of tomato, but included a non-pathogenic *F. oxysporum* as well, and found this to be more effective than the fluorescent pseudomonad at suppressing Fusarium wilt. This result, or the finding that better control is achieved with multiple biocontrol agents, is not uncommon. This highlights the complexity of a suppressive soil and the challenge of isolating individual mechanisms or components of suppression (de Boer et al., 1999; Lemanceau et al., 1993).

Despite these complexities and the inconsistencies observed in performance of biocontrol agents derived from research on suppressive soils, there have been successful deployments of wilt-suppressive microorganisms in cropping systems. Strains of fluorescent pseudomonads have been commercialized as biopesticides, particularly for use in soil-less greenhouse production where conditions can be monitored carefully and controlled (Haas and Defago, 2005). One disadvantage of the fluorescent pseudomonads, however, is that they are not spore-formers like *Bacillus* spp., and therefore have a relatively short shelf-life in formulation. A non-pathogenic isolate of *F. oxysporum* has been registered as a pre-plant potting-mix amendment for greenhouse use (Alabouvette et al., 2006)

Effects of soil chemistry on wilt diseases. An extensive body of research demonstrates that many aspects of soil suppression are affected by abiotic characteristics of soils, particularly the levels and availabilities of macro- and micronutrients. The ways in which soil fertility status can condition host-pathogen interactions and the degree of soil suppressiveness have been categorized as: 1) alteration of pathogen growth, survival and/or aggressiveness; 2) alteration of plant growth and development; 3) alteration of plant resistance to the pathogen through suppression or promotion of specific resistance responses; and 4) alteration of the soil microbial community by stimulation or inhibition of microbes related to plant growth or pathogen antagonism (Duffy and Defago, 1997; Walters and Bingham, 2007).

Nitrogen (N) has been studied extensively in relation to plant disease (Datnoff, 2007). As a constituent of amino acids, proteins, phenolic defense compounds, and other cellular components and reactions, N is critical to the function of plants and their microbial associates (Huber and Watson, 1974). N status in plants affects growth rate, timing of maturity, cell size, and cell wall thickness. These factors all have implications for pathogens invading plant tissues, but just as critically, the form and availability of N can also affect vigor of the pathogen population. NH_4^+ (ammonium) and NO_3^- (nitrate) ions are the mineral forms of N available to plants and microbes in the soil, although microbes can also assimilate N in organic form in the soil and in the plant. The cycling that occurs between these two forms is shaped by the microbial processes of mineralization, conversion of biological N to ammonium, and nitrification, the oxidation of ammonium to nitrate (Havlin et al., 1999). While most plants and microbes are able to use either form, in many cases there is a preference for one over the other. Therefore, choice of N fertilizer can be important for optimizing plant heath or minimizing pathogen aggressiveness (Huber and Watson, 1974).

The form of N has proven more influential in development of vascular wilt diseases compared to overall N availability (Beckman, 1987). *F. oxysporum* has shown greater virulence when cultured on media supplied with ammonium N, and causing increased disease severity in field studies where ammonium fertilizers were used (Woltz and Jones, 1981). This response reflects, in part, the reduction in pH that occurs as a result of ammonium fertilization. The conversion of ammonium to nitrate generates H^+ ions, as does the exchange of ammonia for H^+ ions on the root surface. A lower soil pH increases the availability of micronutrients such as iron (Fe), manganese (Mn), and zinc (Zn), and applying these nutrients negates the disease-suppressing effect of raising the pH (Jones and Woltz, 1970). Soil-dwelling fungi are generally more subject to micronutrient deficiencies than plants, which are equipped with nutrient-mining root systems (Woltz and Jones, 1981). For soilborne pathogens, therefore, micronutrient deficiencies can be a limiting factor for maintenance of inoculum potential. This interaction between N form and micronutrient availability illustrates that the alteration of pathogen growth or pathogenicity can be indirect, in that the pathogen is not advantaged by the nutrient itself. The implications and practical applications of these effects for disease control must be evaluated within the context of crop nutrient requirements and responses to different fertilizer sources. The inherent complexity of any pathosystem poses challenges to the exploitation of mineral nutrition in plant health management.

Calcium (Ca) figures prominently in plant biochemistry and physiology, with roles in the structural integrity of cell walls, the maintenance of ionic selectivity in the plasma membrane, and the relay of environmental signals through intracellular signaling (White and Broadley, 2003). Ca is present in soil solutions or on cation exchange sites as the divalent cation Ca^{2+}, derived primarily from the dissolution of Ca carbonate and Ca sulfates (Rahman and Punja,

2007). While Ca deficiencies in soil are relatively rare, highly-weathered acid soils are prone to Ca deficiency as a result of cation leaching (Havlin et al., 1999). Adequate levels of Ca promote good soil tilth by stimulating growth of diverse bacterial populations that are involved in flocculation and aggregate formation (USDA NRCS).

Numerous studies have linked soil and plant Ca levels to disease development. Given the multiple and critical roles that Ca plays in plant cell function, it is no surprise that there are several mechanisms by which Ca can influence plant-pathogen relationships. For example, Ca in the cell wall binds to pectins in the middle lamella, filling spaces between the polygalacturonic acid chains that compose the pectins that might otherwise be sites for entry of degrading enzymes such as polygalacturonase, which is secreted by many fungal pathogens (Bateman and Lumsden, 1965). Spiegel et al. (1987) observed suppression of muskmelon Fusarium wilt with Ca salts, in particular $Ca(NO_3)_2$, and concluded that the effect of Ca was on the host and not on *F. oxysporum* f. sp. *melonis* because there was no effect of Ca concentration on dry weight of the pathogen *in vitro*. However, the authors measured pathogen growth, not production of pathogenesis-related enzyme production, and thus may have overlooked effect(s) of Ca on the pathogen. Corden (1965), for example, found that Ca directly inhibited the production of polygalacturonase by *F. oxysporum* in infected tomato plants and, thus, reduced aggressiveness of the pathogen. Ca plays a role in cellular signaling in response to environmental changes, and is involved in direct and indirect defense against pathogens. Normal cytosolic concentrations of Ca are low compared to apoplastic concentrations (Epstein and Bloom, 2005). Abiotic stresses such as flooding, chilling, heat, oxidation, hypoxia, and mechanical disturbance incite an influx of Ca ions into the cytosol, which signals downstream protective responses (Knight, 2000). These responses can include changes in membrane lipids, strategic accumulation of sugars,

proteins, and other metabolic products, and a host of other defense tactics. Plants deficient in Ca have a reduced capacity for self-protection against abiotic stresses; cells become damaged and leaky, providing nutrient sources for pathogens and/or chemical elicitors of pathogenesis (Wheeler and Hanchey, 1968).

Ca is involved in signaling that leads to both pathogen-targeted and non-specific alteration of a plant's resistance level. Callose deposition is a classic non-specific plant defense response known to be part of the reaction to invasion by vascular wilt pathogens (Agrios, 1997; Beckman, 1987). Callose is a polysaccharide that is deposited along the plasma membrane in response to physical or chemical stress, and its synthesis requires Ca ions (Kohle et al., 1985). A specific resistance mechanism is characterized by activation of defense compound synthesis by the presence of a specific pathogen elicitor. Phytoalexins (pathogen-inhibiting substances) can be induced by such elicitors (Agrios, 1997). Ca is part of the biochemical pathway that produces a specific phytoalexin (pathogen-inhibiting substance) in *Arabidopsis* plants that blocks the effect of fusaric acid, a toxin produced by *Fusarium* species (Bouzigarne et al., 2006). In alfalfa, Ca ions were found to mediate the production of a phytoalexin produced in response to a *Verticillium albo-atrum* elicitor (Smith et al., 1988). While these studies did not include field trials evaluating different soil Ca levels in relation to phytoalexin production and disease reduction, the results underlined the importance of Ca in specific as well as generic defense mechanisms.

A cation that can undergo several changes in oxidation state, Mn is involved in redox-based reactions that are central to photosynthesis and protection from free radicals in plant cells (Epstein and Bloom, 2005). Superoxide dismutases are a class of metalloenzymes (Cu, Fe, Zn, and Mn can all form these enzymes) that quenches free radicals in mitochondria and other plant

tissues (Bowler, 1992). Mn also participates in photosynthesis as part of an enzyme complex that donates electrons to photosystem II and, thus oxidized, can accept electrons from the oxidation of water (H_2O), resulting in the formation of oxygen (O_2) (Epstein and Bloom, 2005; Thompson et al., 2005). While Mn is perhaps most well-known for its role in these two enzyme systems, it is also an activator of enzymes involved in the synthesis of phenolic compounds that are building blocks for or initiators of plant defense responses, including formation of lignins, alkaloids, and flavonoids (Huber and McCay-Buis, 1993). This involvement in plant defense processes is important in discussion of Mn effects on plant disease.

Mn in soils exists in numerous forms: free, dissolved Mn^{2+}, Mn^{2+} bound to organic matter or exchangeable on the cation exchange capacity, and Mn^{3+} or Mn^{4+} oxides (Havlin et al., 1999; Schuman, 1991). Mn oxides, which tend to form at high pH and with the aid of bacterial and fungal members of the soil biota, are highly insoluble and unavailable to plants. As a result, Mn deficiencies are most commonly observed at higher soil pH. In water-logged soils that have low redox potential as a result of low O_2 levels, Mn^{2+} is the predominant form due to the use of Mn in oxidized states as an electron acceptor in microbial respiration. In poorly-drained, acid soils, Mn toxicities can occur. However, organic matter can form unavailable complexes with Mn to reduce availability, so acid soils that are high in organic matter, such as peat or muck soils, can have low Mn availability.

The formation of plant-unavailable Mn oxides through microbial processes can be a detriment to plant health and a component in the pathogenesis of take-all (*Gaeumannomyces graminis*), a fungal root disease of wheat that responds dramatically to soil fertility manipulations (Huber and McCay-Buis, 1993). The pathogen is able to oxidize Mn in the host rhizosphere, resulting in localized Mn deficiency. It is believed that the severity of take-all in

these Mn-deficient plants is due a weakened plant resistance response (Rengel et al., 1994). Mn deficiencies have also been studied with similar results in Fusarium crown and root rot of asparagus (Elmer, 1995). Suppression of Fusarium crown and root rot was associated with higher levels of Mn-reducing rhizobacteria, supporting the link between Mn availability and plant resistance (Elmer, 1995). One factor in the efficacy of the biocontrol fungus *Trichoderma harzianum* in boosting plant growth and health is presumed to be its ability to reduce and solubilize Mn in the rhizosphere, increasing availability of this micronutrient to plants (Altomare et al., 1999).

While Mn availability has been shown to be important for host plant resistance, Mn is also required by pathogens that attack plants. As mentioned for N, plants are better-equipped to mine nutrients from the soil with their far-reaching root systems, so for some diseases it can be advantageous to create conditions that reduce availability of certain micronutrients to the pathogens. Jones and Woltz (1970) found this to be the case with Fusarium wilt of tomato, which was less severe when high limestone amendment rates resulted in reduced availability of Zn and Mn. *In vitro* studies confirmed the importance of these micronutrients for growth and aggressiveness of the pathogen. This illustrates the need to assess the effect of nutrients for each pathogen and host plant, and weigh the benefits and drawbacks of fertility management practices.

Zn has the dubious distinction of causing the most frequent plant deficiencies of all the micronutrients (Cakmak, 2000). Like Mn, Zn is a divalent cation, but unlike Mn, it does not change valence and is thus not involved in soil redox cycles (Epstein and Bloom, 2005). Because Zn is readily complexed by organic matter, Zn concentrations in the soil solution are typically quite low. Availability of Zn is highly pH-dependent; soils with high pH and low aeration are

likely to be Zn deficient (Havlin et al., 1999). Zn has limited mobility in soils, a factor that can be manipulated in disease control. Zn is a component of numerous enzymes and is found in protein complexes referred to as zinc fingers that are involved in DNA transcription (Epstein and Bloom, 2005). Zn protects cells from ultraviolet light and free radicals, and participates in the formation of the plant hormones auxin and giberellin as well as other key metabolic processes (Duffy, 2007).

As for Mn, Jones and Woltz (1970, 1972) demonstrated with Fusarium wilt of tomato that liming soils decreased availability of Zn and, as a result, decreased aggressiveness of the pathogen. *In vitro* studies confirmed that Zn is essential for the growth, sporulation, and virulence of the pathogen (Woltz and Jones, 1968). Adding Zn to limed soils reversed the disease-reducing effects of liming (Jones and Woltz, 1970). Duffy and Defago (1997) found that Zn increased the number of microconidia and the total fungal biomass of *F. oxysporum* f. sp. *radicis-lycopersici* in culture. Because Zn is limiting for pathogens and the nutrient is immobile in soils, elevating soil pH with limestone, and banding or applying foliar Zn to supply the needs of the plant while minimizing pathogen access to Zn, could be a long-term approach to reducing *Fusarium* inoculum potential in the soil.

While adequate levels of soil Zn may boost pathogen growth and virulence, there is also evidence that Zn supports microbial populations that can have suppressive effects on some diseases. It has been demonstrated widely that certain strains and/or species of fluorescent pseudomonad bacteria in the soil microbial community help manage a range of fungal pathogens by colonizing and protecting plant roots and by inducing plant resistance (Handelsman and Stabb, 1996). Biocontrol activity and suppression of Fusarium crown and root rot of tomato by fluorescent pseudomonads has been correlated with soil Zn levels (Duffy and Defago, 1997).

Iron (Fe) plays a key role in many critical metabolic redox reactions in both microorganisms and plants. Many of the enzymes of the electron transport chains of respiration and photosynthesis contain Fe (Epstein and Bloom, 2005). Fe is also a constituent of the enzyme nitrogenase that facilitates N_2 fixation. Despite the magnitude of its role in biological systems and status as the fourth most abundant element in the lithosphere, the availability of Fe in soils is typically quite low (Havlin et al., 1999). The iron released by the dissolution of primary minerals is tied up in insoluble secondary minerals such as oxides and hydroxides, or is complexed by organic matter. What little free Fe is found in the soil solution occurs either as Fe^{3+} or Fe^{2+}, both of which can be absorbed by plants. The former is more common in aerobic (oxic) environments while the latter predominates in anaerobic (reduced) environments. Fe^{2+} is more soluble than Fe^{3+}, so Fe availability increases in waterlogged soils. Soil pH is also a major determinant of Fe availability; as pH decreases, Fe^{3+} concentration increases as the iron is released from $Fe(OH)_3$, the dominant secondary Fe mineral. Even in acidic soils, however, Fe is typically not available in sufficient concentrations to meet the needs of plants. To address this, plants have evolved a number of mechanisms to increase Fe availability in the rhizosphere. Plant roots release chelates, soluble organic compounds that complex with metals such as Fe (as well as Cu, Zn, and Mn) to transport these metals via diffusion pathways to the root surface, and release them for uptake by plants (reference). Citric acid and oxalic acid are examples of chelating agents released in root exudates. These acid chelates also lower the pH, which enhances release of Fe^{3+} from secondary minerals. Plant roots release H^+ ions as well, acidifying the rhizosphere and enhancing availability of several micronutrients.

Microorganisms in the soil environment are also subject to Fe deficiencies, and many have similar Fe acquisition strategies, such as the production of siderophores, which are similar

in structure and function to the chelates produced by plants (Crowley, D., 2006). Indeed, the Fe-chelating capacity of microbial siderophores is significantly greater than the chelating compounds produced by plants (Expert, 2007). A well-characterized group of siderophores are the pseudobactins produced by fluorescent pseudomonads. By reducing Fe availability to other microorganisms, these high-affinity Fe chelators play a role in suppression of soilborne pathogens (Duijff et al., 1994; Handelsman and Stabb, 1996; Scher and Baker, 1982). However, most fluorescent pseudomonads do not exhibit biocontrol activity, despite the production of siderophores, so Fe deprivation cannot be the sole mechanism underlying suppression (Haas and Defago, 2005).

The extensive work of Jones and Woltz (1981) demonstrates that various formae speciales of *F. oxysporum* have relatively high requirements for Fe as well as other micronutrients. Both growth and aggressiveness of the pathogen were enhanced in their experiments with increasing concentrations of Fe (Woltz and Jones, 1981), and the beneficial effects of liming on pathogen suppression were negated by adding chelates of Fe (Jones and Woltz, 1970). This could explain, in part, why Fusarium wilt severity is often associated with low pH soils, in which many micronutrients are maximally available. In comparison, *V. dahliae* does not appear to be as vulnerable to micronutrient deficiencies, since Verticillium wilts of some crops have been shown to be enhanced by high pH (Ochiai, 2008; Pegg and Brady, 2002). Studies indicate that this effect may be due to the reduction of Mn ions in acid soils, which have been shown to inhibit growth and microsclerotia production of *V. dahliae* (Shao and Foy, 1982). The opposite tendencies of these two pathogens in relation to soil pH confound efforts to manage both wilt pathogens simultaneously in crops such as spinach, which are susceptible to both Fusarium wilt and Verticillium wilt.

While soil pH appears to play a central role in Fusarium wilt and Verticillium wilt development, this is related primarily to the effect of pH on nutrient availability. A recent study by Caracuel et al. (2003) found that *F. oxysporum* contains a pH signaling transcription factor (PacC) that appears to regulate negatively two endopolygalacturonase genes linked to virulence. Loss of function mutants were significantly more virulent, while constitutively-expressed PacC mutants were slow to develop symptoms. The authors, as well as di Pietro et al. (2003), suggest that this phenomenon reflects the acidic interior of the host plant, but it is possible that the conduciveness of low pH soils to Fusarium wilts could be related to pH-mediated virulence gene expression.

While the complex relationships among mineral nutrients, plants, and pathogens makes it difficult to develop fertilization recommendations that apply to more than one crop, or to one crop in different production regions, nutrition can have profound effects on the development of wilt diseases. The mechanisms behind these effects offer unique opportunities for exploring the biology of host and pathogen.

Characterizing soil microbial communities. A limitation of many seminal studies examining the role of individual species or groups of microorganisms in suppressive soils is that, by isolating putative biocontrol strains and manipulating them *in vitro* or in sterilized soil environments, a major mediating component – the soil microbial community – is removed from the evaluation. There are reasons for doing this, however; the number of microorganisms in soil is large and many are unculturable, so finding new putatively suppressive organisms can be like looking for a needle in a haystack (Borneman and Becker, 2007). The tools used to identify microorganisms affiliated with Fusarium wilt suppression, and to track changes in microbial populations relative to shifts in degree of suppressiveness, have advanced greatly in the last few

decades. Historically, studies have relied upon culture-dependent techniques such as selective media for isolation and quantification of target microorganisms associated with a given soil or treatment. A classic example is a study by Scher and Baker (1982) in which they buried nylon screens colonized by *F. oxysporum* f. sp. *lini* in suppressive soil around the roots of flax plants, then retrieved the screens and placed them on a selective agar medium to isolate fluorescent pseudomonads. The bacterial isolates were able to render a conducive soil suppressive when added to the soil, unlike isolates obtained from a conducive soil. More advanced culture-dependent techniques are based on biochemical variability in soil microbial communities, such as community-level physiological profiling, and can be less biased and more comprehensive compared to morphology-based identification (Mazzola, 2004). While much has been learned from these methods, the vast majority of soil microorganisms (between 85 and 99.999%, depending on the study) elude culture-dependent methods (Amman et al., 1997; Liu et al., 1997). Typically, the researcher has in mind a group of organisms he/she is interested in tracking, and selects media or techniques to isolate only that group, thus precluding the possibility of identifying previously unknown but potentially important players in soil functions.

A fundamental shift was made possible with the advent of molecular techniques, particularly those using polymerase chain reactions (PCR), for amplification of target nucleic acids. PCR-based methods for characterizing soil microbial communities are culture-independent, so a much wider range of microorganisms can be detected (Clement et al., 1998). Some microorganisms, however, such as the spore-forming Gram positive bacteria, have demonstrated resistance to extraction and purification, so there can still be some level of bias to these approaches (Wintzingerode et al., 1997). A key component of molecular methods is the use of a marker system that provides insight into the phylogenetic composition of a microbial

community. The 16S and 18S small subunit ribosomal RNA (rRNA) or the associated genes on the ribosomal DNA, are commonly used for characterizing and tracking prokaryotic and eukaryotic populations, respectively, using culture-independent techniques (Sorensen et al., 2009).

The next step after extracting nucleic acids from a soil sample and generating PCR products based on a conserved region of the desired target is to use this mixture of products for microbial community fingerprinting. Three primary techniques have been used for rhizosphere microbial characterization: denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and single-strand conformation polymorphism (SSCP) (Garbeva et al., 2004; Sørensen et al., 2009). All three techniques rely upon separation of nucleic acid fragments and analysis of the resulting clusters of genetic elements that can be linked to groups of microbes. By studying differences in fingerprinting profiles, comparisons can be made between different soils or treatments, and associations investigated between function and diversity.

These techniques cannot resolve taxa on a very fine level, however, and cannot be used to calculate species richness and evenness indices (Yin et al., 2009). As a result, small differences or community components that could play a role in soil suppressiveness may be overlooked. With DGGE and SSCP methods, separated genetic elements can be recaptured and sequenced for comparison with database gene sequence accessions of known organisms, but this is not a high-throughput process (Sorensen et al., 2009). Recent innovations in sequencing technology, referred to collectively as massively parallel tag sequencing, have made sequence analyses sufficiently fast and affordable to allow a much more in-depth approach to microbial population structure surveys (Margulies et al., 2005; Sogin et al., 2006). These techniques are based not on

bacterial vectors or Sanger sequencing, but on the process referred to as pyrosequencing, a sequence-by-synthesis approach in which each nucleotide incorporated is detected when the pyrophosphate released is used to generate ATP, which is subsequently converted to light energy by the enzyme luciferase (Margulies et al., 2005). To use pyrosequencing for microbial community profiling, DNA must be extracted and amplified using primers for highly conserved regions flanking highly variable regions of bacterial or eukaryotic rRNA genes. A PCR amplicon library is thus generated for each sample. Each fragment in the library is bound to a bead, the beads are deposited into the wells of a picoliter reactor, and the fragments are sequenced (Margulies et al., 2005). Sequences are then used in BLAST queries, followed by identification of operational taxonomic units (OTUs) and calculation of species richness. The major advantage of this parallel sequencing strategy is that it allows minor components of the microbial community to be identified and tracked, and greatly enriches our understanding of phenomena such as soil suppression (Lin et al., 2009). Pyrosequencing has been used to characterize and compare microbial communities in diverse environments, from the human gastrointestinal tract to the phyllosphere of spinach leaves (Armougom and Raoult, 2009; Lopez-Velasco et al., 2011). In the context of soilborne pathogens, such microbial community analyses have facilitated the identification of previously uncharacterized antagonistic microorganisms and/or farming practices that cause shifts toward more suppressive soil microbial communities. However, evaluating the success of such shifts requires method(s) to assess soils for degree of suppressiveness to particular diseases.

Bioassays for soilborne disease risk prediction. The need for efficient and accurate assessment of inoculum potential of various soilborne pathogens in fields is an on-going challenge for growers, who must decide each season where to plant their crops, and for

researchers tasked with providing tools to aid in this decision-making process (Bloem et al., 2006).Various approaches, ranging from very basic to technologically intense, can be used for soil bioassays. The challenge is to take into account all of the contributing factors discussed above, such as the genetic susceptibility and growth stage of the host plant, relevance of the pathogen inoculation method, aggressiveness of pathogen strains, environmental conditions (temperature, soil type and pH, matric potential, soil fertility, cropping history), and the structure of the soil microbial community. Methods for quantifying the size of the pathogen population, whether through the use of selective media or DNA-based detection techniques, tell only part of the story.

An effective bioassay for Fusarium wilt risk of a given soil may necessitate developing a method in which the soil "speaks for itself," telling as much of the story as possible through visual observation of disease development in the soil under conditions highly favorable for the pathogen. Persson et al. (1999) accomplished this with an elegantly simple greenhouse bioassay for soil suppressiveness to Aphanomyces root rot of pea. They developed an oospore-based soil inoculation procedure and a root rot severity index, conducted a greenhouse bioassay with appropriate control treatments, and compared the bioassay results with disease assessments in field experiments. The greenhouse bioassay accurately detected differences in the capacity of different field soils to promote or suppress the pathogen. Other researchers have, in tandem with developing a bioassay method, examined the potential for predictive factors that can be readily measured to replace the need for a time- and resource-intensive soil bioassay. Borrero et al. (2004), for example, sought both to predict and better understand the tomato Fusarium wilt suppressiveness of various plant growth media. They developed a Fusarium wilt bioassay and conducted multiple regression analyses of various physical, chemical, and biological properties

to see whether some combination of these factors could predict wilt as accurately as the bioassay. Such an approach holds great promise for helping growers manage soilborne diseases, or better yet, evade them.

The work that follows was inspired by and conducted for the spinach seed growers of the maritime Pacific Northwest, who are intimately acquainted with soilborne diseases, and who remain committed to producing high quality spinach seed despite the exacting demands of the crop and the ever-present threat of Fusarium wilt. Many of the concepts and studies described in the foregoing review helped guide this project. The overall goal was to develop more effective methods for growers to manage Fusarium wilt in spinach seed crops in the highly conducive soils of the maritime Pacific Northwest, the only region of the USA suitable for spinach seed production. Towards this goal, three complementary studies were designed to address various aspects of spinach Fusarium wilt risk prediction and management. Chapter 1 describes a four-year, grower-cooperator field trial used to explore the possibility of coaxing a field site of a future spinach seed crop into a less wilt-conducive state through annual applications of limestone, compared to the current growers' practice of one limestone application in the spring of planting a spinach seed crop. Chapter 2 describes the development of a spinach Fusarium wilt soil bioassay similar to those described in this review, that was used to measure the risk of Fusarium wilt for soils submitted voluntarily by spinach seed stakeholders from 147 fields in northwestern Washington. The bioassay is being utilized now by growers to help determine when a field is of low enough risk for planting spinach seed crops with parent lines of a range in susceptibility to Fusarium wilt. Chapter 3 details *in vitro* and greenhouse trials that investigated the influence of soil abiotic factors, in particular micronutrient availability, on Fusarium wilt inoculum potential and some of the mechanisms of limestone-mediated suppression of spinach

Fusarium wilt. Together, the findings of these studies are increasing the carrying capacity of northwestern Washington farmland for spinach seed crops, and helping to preserve the viability of the spinach seed industry in the USA.

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CHAPTER TWO

EVALUATION OF ANNUAL APPLICATIONS OF LIMESTONE TO ENHANCE SUPPRESSION OF FUSARIUM WILT IN SPINACH SEED CROPS IN THE PACIFIC NORTHWEST USA

Introduction

Market demand for spinach (*Spinacia oleracea*) has increased sharply since the advent in the mid-1990s of fresh market, pre-packaged, baby and "teenage" leaf spinach, now the dominant spinach commodity in California and Arizona, the main fresh market spinach producing regions of the USA (Koike et al., 2011). Baby leaf spinach crops are short-season (40 days or less from planting to harvest), high-density plantings with populations of up to 9 million seed/ha, compared to 300,000/ha for processing crops. The dramatic increase in seed volume required to establish such high plant populations has led to downstream effects, most notably for the spinach seed industry. While spinach can be grown for fresh or processing markets in many different regions, spinach seed production requires long summer daylength to initiate bolting, mild temperatures, and low relative humidity during the period of seed set and maturation to ensure optimal seed quality (Metzger and Zeevart, 1985). The maritime Pacific Northwest (PNW) USA is one of the few regions in the world that meets these exacting requirements for spinach seed production (Foss and Jones, 2005). Together with the Willamette Valley of Oregon, Skagit, Whatcom, Snohomish, and Clallam counties in Washington produce up to 50% of the US spinach seed supply and up to 25% of the global supply, on 1,200 to 1,600 ha annually.

The primary limit to spinach seed production in the PNW is Fusarium wilt, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *spinaciae* (Foss and Jones, 2005). Fusarium wilts

affect most of the world's important crops (Beckman, 1987). The isolates that cause wilts are typically identified as host-specific formae speciales that can be very difficult to manage or eradicate once established in soils (Armstrong and Armstrong, 1981). The pathogen persists in the form of long-lived chlamydospores or as mycelium colonizing non-host species or crop residues (Gordon and Okamoto, 1990). Fungal hyphae penetrate roots through wounds in epidermal cells or at the junctures where lateral roots emerge, although the epidermis can be penetrated directly near the root tip where cell defenses are less well-developed (Beckman, 1987). Once established in the root cortex, hyphae ramify and may breach the endodermis to invade the vasculature of the root system. The reduced water flow that results from fungal proliferation, as well as plant defense responses in the form of gel production and tyloses, lead to classic wilt symptoms. A slight mottling/interveinal chlorosis of young leaves is followed by loss of turgidity, epinasty, yellowing, and defoliation. Belowground symptoms include discoloration of the vascular stele and, in spinach Fusarium wilt, blackening of root tips. In advanced stages of disease development, plant tissues senesce and the pathogen moves out of the vascular system to colonize dead and decaying tissue, often producing masses of macroconidia-bearing sporodochia on crop residue that are subsequently incorporated into the soil (Mace et al., 1981).

Rotations of at least 10 years are necessary to avoid economic losses to Fusarium wilt in PNW spinach seed crops (Foss and Jones, 2005; du Toit, 2004). When the disease was first reported in this region in the 1950s, growers were able to avoid such losses by limiting spinach seed production to "virgin" ground that had never been planted to spinach, but such ground is now virtually non-existent. Partially resistant parent lines do exist, but many parents used widely for seed crops are highly susceptible to the disease (du Toit, 2004). Furthermore, spinach seed is grown on a contractual basis (Foss and Jones, 2005), and growers as well as some seed company

representatives of "third party" companies that contract to produce seed for other companies, may not know the level of Fusarium wilt resistance of the parent lines they are provided. This complicates the selection of low-risk field sites. Because the disease is typically of minor importance for spinach food crops (Correll et al., 1994), breeding for resistance to Fusarium wilt has not been a high priority for many spinach breeding programs (J. Schafer, spinach breeder, Schafer Agricultural Services, *personal communication*). The consequences of the long rotation required between seed crops are significant for spinach seed growers, but affect the end users of spinach seed only in so much as they affect availability and thus, to some extent, the price of seed. With the additional requirement to maintain isolation distances of 1.0 to 6.4 km between adjacent, wind-pollinated spinach seed crops to prevent undesirable cross-pollination, the carrying capacity of farmland in the maritime PNW, the only region in the US where spinach seed can be grown on a commercial scale, has peaked. In contrast, spinach seed acreage in northern Europe, where Fusarium wilt is not a major concern, continues to expand, with Denmark now the top producer of spinach seed in the world (van Veldhuizen, 2011).

The variation in conduciveness of different soils to Fusarium wilts has been the subject of intense scrutiny (e.g., Scher and Baker, 1980; Tousson, 1975). In various regions of the world, the existence of soils that are in some way inhospitable to Fusarium wilts has prompted exploration of biotic and abiotic factors that may be implicated in the mechanism(s) of suppression. Early in the 19th century, researchers began to establish a connection between soil chemistry and Fusarium wilt severity in certain crops. In 1918, Edgerton found that soil application of limestone at the rate of 22.4 tons/ha significantly reduced Fusarium wilt incidence of tomato in both seed beds and in the field (Edgerton, 1918). Sherwood (1923) explored this response more thoroughly in greenhouse experiments with two soil types, a sandy loam and a silt

loam, sterilized and amended with the equivalent of 2.2, 4.5, 9.0, 17.9, and 26.9 t/ha, inoculated with a known isolate of *F. oxysporum* f. sp. *lycopersici*, and planted with a susceptible tomato cultivar. In both soils, a decrease in wilt was associated with an increase in pH. The author also performed experiments with culture media inoculated with the pathogen and adjusted to a range of pH levels using sodium hydroxide or hydrochloric acid, and found that the highest pathogen biomass production occurred between pH 2.8 and 5.0.

Similar research was conducted several decades later, when researchers in Florida devised a nutritional approach for management of several Fusarium wilts based on the suppressive effect of soil pH elevation on these diseases (Woltz and Jones, 1981). Experiments with watermelon (Everett and Blazquez, 1967; Jones and Woltz, 1975), cucumber (Jones and Woltz, 1975), tomato (Jones and Overman, 1971; Jones and Woltz, 1970; Jones and Woltz, 1972; Woltz and Jones, 1973), and chrysanthemum (Woltz and Engelhard, 1973) repeatedly demonstrated that alkalizing soil amendments, including calcium carbonate ($CaCO_3$), calcium oxide (CaO, also called burned lime), and calcium hydroxide ($Ca(OH)_2$), also called hydrated lime), applied at various rates reduced losses to Fusarium wilt. To address the possibility that the suppressive effects observed could be due to the increase in soil Ca levels with these amendments, steps were taken to equalize Ca levels regardless of limestone treatment (Jones and Overman, 1971), or to compare an alkalizing Ca compound such as $CaCO_3$ with a non-alkalizing Ca amendment such as calcium sulfate ($CaSO_4$) (Everett and Blazquez, 1967; Jones and Woltz, 1970). The results confirmed that increased soil pH, not Ca supply, was the factor most closely associated with Fusarium wilt suppression in these crops.

In addition to soil pH, the form of nitrogen (N) fertilizer also has been investigated as a factor influencing Fusarium wilt development, specifically the presence of ammonium (NH_4^+) or

nitrate (NO_3^-) as the nitrogenous ion. Both high pH and NO_3^--N reduced virulence of *F. oxysporum* f. sp. *vasinfectum* on cotton (Albert, 1946). For Fusarium wilts of tomato (Woltz and Jones, 1973), chrysanthemum (Woltz and Engelhard, 1973), cucumber and watermelon (Jones and Woltz, 1975), and celery (Schneider, 1985), use of NH_4^+- versus NO_3^--N fertilizers negated the suppressive effect of increased soil pH. Root uptake of the NO_3^- ion causes an increase in soil pH, while uptake of the NH_4^+ ion acidifies the surrounding soil (Havlin et al., 1999), so it is possible that the mechanism(s) underlying the suppressive effect of limestone application and NO_3^--N fertilization are similar. When *F. oxysporum* f. sp. *lycopersici* was cultured in a defined liquid medium with NH_4^+ or NO_3^- as the N source, spores harvested from NH_4^+ treatments were more virulent on inoculated tomato seedlings compared to spores cultured in NO_3^- media, suggesting that there could also be a direct effect of N source on fungal physiology (Woltz and Jones, 1973).

It is important to note that the results of a study on the effects of soil pH and N source on watermelon Fusarium wilt by Jones and Woltz (1975) could not be duplicated by Hopkins and Elstrom (1976). Similarly divergent results have been observed for the influence of soil pH on Fusarium wilts of banana (Peng et al., 1999; Stover, 1956), strawberry (Fang et al., 2011; Islas, 2012), and flax (Hoper et al., 1995). Furthermore, the alkaline soils of Arizona are conducive to Fusarium wilt of lettuce (Matheron and Koike, 2003). Clearly, generalities about relationships between soil pH and all Fusarium wilts are ill-advised, and caution is required when applying concepts of disease suppression to different regions and cropping systems. Part of the challenge in interpreting these studies and using the results to develop disease management strategies is that the mechanisms underlying the suppression achieved through manipulation of abiotic soil properties such as pH and N source are not well understood. The perceived association between

Fusarium wilt suppression and environmental factors is typically related, in part, to the effects of these factors on the soil microbiota, members of which may compete directly with or release toxins against the pathogen, or incite plant host defense responses (Baker and Cook, 1974).

From 2006 to 2008, studies were conducted in different fields in the Skagit Valley of northwestern Washington to determine whether the limestone-mediated suppression of Fusarium wilt observed in other crops could be deployed to manage spinach Fusarium wilt in this region (du Toit et al., 2007, 2008, and 2011). While the results varied with the level of Fusarium wilt resistance of the spinach parent lines evaluated and the specific location of each trial, agricultural limestone applied approximately two weeks prior to planting each spinach seed trial proved effective, with 4.48 tons/ha identified as an optimal rate for suppressing the disease and avoiding nutritional imbalances that can occur at higher application rates. However, the elevation of soil pH also increased the incidence of Verticillium wilt, caused by *Verticillium dahliae* (du Toit et al., 2007 and 2011). This disease is an emerging concern in spinach seed crops because it is readily seed-transmitted and because some strains that infect spinach can potentially infect crops grown in rotation with spinach in states such as California and Arizona, two major markets for PNW spinach seed (Atallah et al., 2011; Bhat and Subbarao, 1999; du Toit et al., 2005). In general, Verticillium wilts are enhanced by high soil pH and NO_3^-- rather than NH_4^+-N fertilizers (Lambert et al., 2005; Smiley, 1975), the opposite response of many Fusarium wilts.

The use of limestone amendments in spinach seed crops has intensified in response to these field studies in northwestern Washington (du Toit and Gatch, 2009). One limitation of this approach, however, is that the returns on the investment required for such applications are presumed to be short-lived, given that the soils in this region are typically well-buffered and may revert rapidly to an acidic, Fusarium-wilt conducive state well before the next spinach seed crop.

Most growers are reluctant to apply limestone more regularly, because some of the crops grown in rotation with spinach seed are either not valuable enough to recover the cost of regular limestone amendment (e.g., wheat) or do not require limestone for optimum yields. Also, the limited availability of land suitable for spinach seed production, due to the long rotation requirement and regional urban development pressure, necessitates convoluted land rental arrangements and annual negotiations between growers and seed companies, a reality which complicates long-term strategies for disease management (K. Johnson, president of the Puget Sound Seed Growers' Association, *personal communication*). However, the persistent nature and destructive potential of Fusarium wilt in these acid soils ultimately may demand the flexibility and patience of an approach such as annual applications of limestone for several years preceding a spinach seed crop, rather than one application the spring a spinach seed crop is planted. If such an approach proves effective, growers could employ shorter rotations and produce a greater percentage of their spinach seed crops on their own land, thereby increasing the returns on investment in this soil amendment.

In 2009, a four-year field trial was initiated in the Skagit Valley of Washington to evaluate the effects of the following on both Fusarium wilt and Verticillium wilt development in spinach seed production: 1) annual limestone applications for three years prior to a spinach seed crop compared to a single application the spring of planting, and 2) NO_3^-- versus NH_4^+-N fertilizers. Preliminary results have been published (Gatch et al., 2011).

Materials and Methods

Field site. A 0.8 ha field located on the farm of a grower-cooperator in Skagit County, WA was selected as the site of a four-year trial based on the following requirements: 1) a soil pH

typical of the acid soils of western Washington (pH <6.0); 2) an interval of approximately 5 years since the previous spinach seed crop to ensure adequate spinach Fusarium wilt pressure; and 3) a grower-cooperator willing to integrate the trial into his normal cropping system between spinach seed crops. The soil at the selected field site was classified as a Mount Vernon very fine sandy loam (USDA National Resources Conservation Service Soil Survey Staff), had been planted to a spinach seed crop by the grower-cooperator in 2005, and had a pH of 5.9 in March 2009 based on a commercial soil nutrient analysis (Soiltest Farm Consultants, Inc., Moses Lake, WA).

Experimental and treatment designs. In 2009, three rates of agricultural limestone application, three proprietary female spinach inbred lines, and two N fertilizers were evaluated using a split-split plot, randomized complete block design with five replications. N fertilizer treatments were applied to main plots, spinach lines planted in split plots, and limestone amendment rates applied to split-split plots. The limestone treatments consisted of 0, 2.24, or 4.48 metric tons of agricultural limestone/ha (Imperial Ground limestone, Oregon Lime Score = 97, calcium carbonate equivalent (CCE) = 97%, 97% $CaCO_3$, and 38.8% Ca). These rates of limestone amendment were chosen based on the results of previous field trials that evaluated the potential for limestone amendments to suppress Fusarium wilt (du Toit et al., 2007, 2008, and 2011). The three spinach inbred lines were characterized by the seed companies that developed the lines as highly susceptible, moderately susceptible, and moderately resistant to spinach Fusarium wilt (referred to henceforth as the susceptible, moderate, and resistant inbreds, respectively), and were used with permission from these companies. Inbred lines were selected instead of publicly available, open-pollinated lines because of the lack of adequate genetic uniformity in open-pollinated spinach lines for traits such as susceptibility to Fusarium wilt. The

in-furrow, at-planting fertilizer treatments evaluated were monoammonium phosphate (11-52-0), which is typically used by spinach seed growers in western Washington (Jeff Schwab, Wilbur-Ellis Company, *personal communication*) and is an acidifying fertilizer (acidity score = 58); and calcium nitrate (15.5-0-0) as an alkalizing fertilizer, supplied at equivalent units of N. The addition of 67 kg/ha phosphorus with the NH_4^+-N fertilizer and 22 kg/ha calcium with the NO_3^--N fertilizer were potential confounding factors, but no attempt was made to apply these nutrients at equivalent rates because the soil tests did not indicate a deficiency of either nutrient.

In 2010 and 2011, the grower-cooperator planted potatoes and winter wheat, respectively, using typical production practices for northwestern Washington. The limestone treatments applied in the 2009 spinach trial were applied again in April 2010 to the identical plots as in 2009 and incorporated prior to planting the potato crop, and again in September 2011 after harvest of the winter wheat crop (Fig. 2.1). In 2012, the spinach seed crop trial was repeated in the same plots established in 2009, with several modifications. A major precipitation event (2.5 cm in one day) within two weeks of planting spinach in the 2009 season caused loss of the mobile NO_3^- ion, with subsequent N deficiency developing in the main plots treated with $CaNO_3$ fertilizer. This treatment was thus deemed inappropriate for the wet spring climate typical of the maritime PNW, and was not evaluated again in the 2012 trial. The plots that received no limestone in 2009 to 2011 were each divided in two in spring 2012, with half of each plot receiving no limestone and the other half receiving 4.48 t limestone/ha within one month of planting. The latter treatment was intended to simulate the common practice of limestone amendment for spinach seed crops in this region, i.e., to apply 4.48 t limestone/ha in the spring a spinach seed crop is planted. The design of the 2012 field trial thus became a randomized split

block, with three spinach inbred lines in the main plots and four limestone treatments in the split plots (Fig. 2.1).

Field planting and maintenance. *2009*. Following a winter wheat crop in 2008-2009, the field site was chiseled and plowed on 8 April 2009. On 22 April, cycloate (RoNeet , Helm Agro US, Memphis, TN) applied at 3 liters/ha and diazinon (Diazinon, MANA Inc., Raleigh, NC) applied at 4.7 liters/ha were broadcast and incorporated with a mulcher-packer for weed and insect control, respectively (Foss and Jones, 2005). Limestone was applied on 10 April at 0, 2.24, and 4.48 t/ha with a 1.8 m wide Gandy drop spreader (Gandy, Owatonna, MN), and rototilled 15 to 20 cm deep. Spinach seeds were planted on 22 April (1.3 cm deep) using a Monosem planter (Monosem, Edwardsville, KS), with 56 cm spacing between rows and 6.4 cm spacing within rows. Six 9-m long rows of the appropriate female line were planted in each split plot, with one row of a proprietary male line on each side of the six female rows. The male line was the same as that of the nearest commercial spinach seed crop in order to avoid violating the minimum required pollen isolation requirements established by the Western Washington Small Seed Advisory Committee (1998). Fertilizer treatments were applied in-furrow at planting to main plots: 11-52-0 monoammonium phosphate (308 kg/ha) or 15-5-0 calcium nitrate (114 kg/ha). Plots were hand-weeded regularly, and a fertilizer side-dressing (27-0-0) was applied at 200 kg/ha on 5 June with a single-shank applicator.

2010-2011. On 16 April 2010, limestone treatments were applied and incorporated into the same plots as described above for the 2009 trial. The grower-cooperator then planted the field site and surrounding area to a potato crop (cultivar 'Cascade') on 19 April. The potato crop was harvested on 9 September, and a winter wheat crop was planted in late September 2010. In mid-August 2011, the winter wheat crop was harvested by the grower, and the same limestone

treatments were applied and incorporated into identical plots on 8 September 2011, as described above.

2012. The field site was disked, chiseled, plowed, and mulched on 29 March and 6, 9, and 10 April 2012, respectively, to incorporate the wheat stubble and volunteer wheat plants. Limestone treatments were applied and incorporated on 11 April to plots in replications 1 to 3, and on 12 April to plots in replications 4 and 5, due to the onset of rain the afternoon of 11 April. Ro-Neet and Diazinon were broadcast and mulched on 23 April at the same rates as in 2009. Two weeks of rain delayed planting until 8 May. Monoammonium phosphate amended with boron (10-50-0-0.55B) was applied in-furrow at planting (373 kg/ha) to all plots. On 6 June, rows were cultivated for weed control, and on 13 June, plants were side-dressed with 27-0-0 (224 kg/ha). Plots were hand-weeded regularly through the season. On 21 June, a foliar application of chelated manganese (5%) and zinc (7%) was applied to plants in all plots (2.33 liters of each product/ha, Foli-Gro Zn and Mn, Wilbur Ellis, Walnut Creek, CA) to address the potential for micronutrient deficiencies in spinach plants growing in plots treated with limestone for multiple years. On 2 August, pyraclostrobin and boscalid (Pristine WG at 0.78 kg/ha, BASF Corp., Research Triangle Park, NC) was applied to plants to manage Stemphylium and Cladosporium leaf spots (du Toit et al., 2004).

To simplify references to these multi-year limestone treatments, the following designations are used in this chapter:

2009

0 tons limestone/ha = 0 t/ha

2.24 tons limestone/ha = 2.24 t/ha

4.48 tons limestone/ha = 4.48 t/ha

2010 to 2012

0 tons limestone/ha each year for 3 years = 0 t/ha/year

2.24 tons limestone/ha each year for 3 years = 2.24 t/ha/year

4.48 tons limestone/ha each year for 3 years = 4.48 t/ha/year

0 tons limestone/ha each year for 3 years and 4.48 tons/ha in 2012 = 0/4.48 t/ha

Soil chemical and microbial analyses. In 2009, soil samples (12 cores/plot, sampled 0-15 cm deep, then combined and mixed manually for each plot) were collected at three week intervals throughout the season. The primary objective of the soil sampling was to track changes in soil pH related to the limestone treatments. On 5 May, 18 June, and 8 and 28 July, soil samples were collected from split-split plots (limestone treatments). On 27 May, soil from split plots (fertilizer-by-limestone treatment combinations) was sampled for evaluation of the effect of N fertilizer treatments in addition to limestone treatments on soil pH. At the final, post-trial sampling on 18 August, soil was sampled from split-split plots for all treatment combinations. In 2010, the year a potato crop was grown in the field, soil samples were collected from split-split plots on 7 May (approximately 3 weeks after limestone application), 14 July (mid-season), and 5 September (just prior to potato harvest). In 2011, when wheat was grown in the site, soil samples were collected from split-split plots on 2 September (just prior to limestone application) and on 29 September (3 weeks after limestone application) to compare the soil pH before and after the limestone application applied that year. In 2012, samples were collected from split plots (limestone treatments) on 10 April (prior to limestone application), 9 May (3 weeks after planting), and 9 to 10 July (mid-season); and from inbred-by-limestone split plots on 24 August (after spinach seed harvest). Soil samples were collected using a 2-cm diameter soil probe from between rows of spinach plants, with the exception of the final sampling dates in 2009 and 2012, when soil cores were collected from between plants within spinach rows to assess the potential

influence of inbred parent line on nutrient and microbial variables measured. Except for soil samples collected prior to the limestone applications in 2011 and 2012, and main plot (inbred line) samples collected in 2012, soils were shipped overnight to Soiltest Farm Consultants, Inc. for analysis of the following properties: NO_3^--N, NH_4^+-N, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), pH, electrical conductivity (EC), organic matter (OM), sulfur (S), boron (B), zinc (Zn), manganese (Mn), copper (Cu), iron (Fe), cation exchange capacity (CEC), and buffer pH.

Subsamples of the soils collected on the first and last sampling dates in 2009, the three sampling dates in 2010, the second sampling date in 2011, and the three sampling dates in 2012 were air-dried, crushed with a marble rolling pin, and passed through a 1 mm aperture sieve. The subsamples were subsequently assayed to quantify colony forming units (CFU) of *F. oxysporum*/g air-dried soil. A 10 g subsample of each soil sample was added to 100 ml sterilized, 0.1% water agar (WA) in a 240-ml glass French square, agitated on a rotary shaker at 250 rpm for 10 min, and diluted serially to a 10^{-2} concentration. Aliquots (0.5 ml) of the 10^{-2} and 10^{-1} dilutions were each placed onto three replicate, 95-mm diameter plastic petri plates containing modified Komada's agar medium (Komada, 1975; Scott et al., 2010), and the aliquot distributed evenly over the plate using a sterilized glass rod. The plates were then incubated on a laboratory bench at room temperature (25 ± 2°C) in ambient light in a room with windows. Fungal colonies with fluffy, white to pale salmon-pink morphology typical of *F. oxysporum* growth on this semi-selective medium were counted 7 and 14 days after plating. *Verticillium dahliae* populations were also quantified for the first and last soil sampling dates of 2009 and 2012 by distributing a 1 g subsample on the surface of each of 10 95-mm diameter plates of NP-10 agar medium, a *Verticillium*-selective medium (Goud and Termorshuizen, 2003; Sorenson et al., 1991), using a

separate, sterilized, 29.6 ml glass salt shaker (Tablecrafts Products Company, Gurnee, IL) for each subsample. The plates were incubated in the dark at 26°C for 28 days, followed by enumeration of *V. dahliae*-type colonies using a dissecting microscope. The total number of colonies on all 10 plates was summed to calculate CFU *V. dahliae*/g soil.

Fusarium wilt and plant growth assessment. Plant stand and incidence of damped-off or wilted spinach plants were counted in a 3 m section of each of the two middle rows of each plot on 15 May, 4 and 24 June, and 14 July 2009; and on 29 May, 19 and 20 June, 9 and 10 July, 31 July, and 1 August 2012. For the latter two dates in 2012, wilt severity of each plant was also assessed using a 0 to 5 ordinal rating scale, with 0 representing a healthy, asymptomatic plant, and 5 representing a plant that had died due to wilt. The male inbred was included in these wilt assessments until the third rating in 2009 and the fourth rating in 2012, when senescence of the male line precluded further wilt ratings.

Isolations for plant pathogens were completed on 8 June 2009 and 29 May 2012 from a selection of wilted seedlings representing the four parent lines to assess the cause(s) of early season damping-off and wilt. Leaves were removed after washing the seedlings under running tap water, and the remaining tissue was cut into pieces approximately 1 cm long with a sterilized blade; surface-sterilized for 60, 90, or 120 s in 0.6% NaOCl; triple-rinsed in sterilized, distilled water; dried; and the pieces plated onto potato dextrose agar (Difco Laboratories, Detroit, MI) amended with chloramphenicol (cPDA) and WA amended with chloramphenicol (cWA). Fungi growing from these tissue pieces were transferred to new plates of cPDA and subsequently examined with a compound microscope to identify potential seedling pathogens.

On 29 June and 22 July 2009, and 11 July 2012, all plants, regardless of Fusarium wilt symptoms, were dug carefully with root systems attached from 1 m of row/parent line using a

hand trowel. The soil was shaken manually from the plants from each plot, and the plants were placed in a brown paper sack, dried at approximately 35°C for 10 to 12 days, and weighed. A subsample of the dried plant tissue was sent to Soiltest Farm Consultants, Inc. for plant nutrient analyses. Prior to drying the samples collected on 22 July 2009 and 11 July 2012, the root and crown of each plant were cut longitudinally and assessed for incidence of dark vascular discoloration typical of Fusarium wilt (Hungerford, 1923).

To evaluate potential effects of limestone treatments on yield and incidence of tuber diseases of the potato crop planted at the field site in 2010, permission was obtained from the grower-cooperator to harvest potato tubers from 3 m of row/split-split plot (limestone treatments) on 8 September. Potato yield was determined by measuring the weight of the total bulk yield from each 3 m of row, as well as the weight of potatoes in each of the following commercial weight classes: <57 g, 57 to 142 g, 143 to 255g, >255 g, and culls. Incidence of tuber diseases was also assessed for each split-split plot.

Seed yield and quality. Seeds were harvested by hand from plants in 3 m of the center four rows of each split-split plot on 8 August 2009, and from 13 to 23 August 2012. In 2009, whole plants were cut just above the soil line and, due to rainy conditions, placed in an air-drier set at 26 ± 3°C prior to hand-stripping the seeds from the plants on 8 to 20 August. In 2012, the weather in August was optimal for drying seed in the field, so plants were windrowed in the field onto sections of Reemay, a polyester spunbond fabric (Reemay, Inc., London, UK), for several days prior to hand-stripping the seed in the field. The 2012 harvest was staggered over the course of 10 days in order of the sequential maturation of seed from the parent lines, starting with the susceptible female inbred and ending with the resistant inbred. Seeds were cleaned and screened to marketable size (screen sizes 7 to 13) by first hand-sieving to break up seed clusters and

separate out larger chaff. Seeds were then passed through a Clipper air screen seed cleaner (M-2B, Ferrell-Ross, Oklahoma City, OK), an inclined belt separator, and a table-top Clipper.

A subsample of 100 seeds/plot was tested for germination using a blotter assay modified from the protocol of the Association of Official Seed Analysts (AOSA, 2008). For each of two sets of 50 seeds, the seeds were placed between two pieces of Steel blue blotter paper (25.4 cm x 38.1 cm, #38 regular weight; Anchor Paper Co., St. Paul, MN) that had been moistened with deionized water, rolled between wax paper (61.0 cm x 91.4 cm; Anchor Paper, Co.), the rolls placed upright in a plastic bag, the seeds cold-stratified at 40°C for 72 h to break seed dormancy, and the seeds incubated in a seed germinator (Stultz Scientific Engineering Corp., Springfield, IL) at 15°C in the dark. The number of germinated seeds for each of two rolls of 50 seeds/plot was assessed after 7, 14, and 21 days of incubation. At 21 days, the percentages of abnormal seedlings, non-germinated hard seed, and rotten seed were also determined (AOSA, 2008).

A freeze-blotter seed health assay for necrotrophic fungi, modified slightly from that described by du Toit et al. (2005), was carried out using an additional 100 seeds/plot. Seeds were surface-sterilized in 1.2% NaOCl for 60 s, and triple-rinsed in sterilized, deionized water to reduce infestations of common saprophytes such as *Alternaria* spp. that can interfere with detection of target fungal spinach pathogens. After drying overnight, the seeds were placed on damp, sterilized blotters (Anchor Paper Co.) in 10 cm x 10 cm, sterilized, acrylic boxes (Hoffman Manufacturing, Inc., Jefferson, OR) (32 to 34 seeds/box), imbibed in the dark for 25 h, frozen at -20°C for 25 h, and then incubated for 12 days at 24°C using a 12 h/12 h day/night cycle with near-UV and cool white fluorescent light by day. Seeds were examined 5, 9, and 14 days after plating using a dissecting microscope (8 to 100X magnification). In 2012, a modified protocol (du Toit, 2011) was used in which the substrate for the seeds consisted of an

approximately 0.5 cm deep layer of NP-10 agar medium poured into the same acrylic boxes, with no -20°C freezing step needed prior to incubation since the agar medium minimizes the issue of seed germination that the freeze-blotter method was designed to prevent. The NP-10 and freeze-blotter methods were demonstrated to be comparable for detection of fungi such as *V. dahliae* on spinach seed (du Toit, 2011).

Statistical analyses. Analyses of variance (ANOVAs) and means separation using Fisher's protected least significant difference (LSD) (P <0.05) were performed on all dependent variables using PROC GLM of SAS (Version 9.2, SAS Institute, Cary, NC). Data were transformed using logarithmic, square root, or arcsine square root as needed when assumptions of homogeneous variances and normally distributed residuals were not met, or were subjected to Friedman's non-parametric rank test if these transformations did not resolve violations of these assumptions.

Results

Spinach wilt and plant growth assessment. Symptoms of Fusarium wilt were observed in both the 2009 and 2012 spinach seed crop trials, with incidence of wilt increasing throughout each season from an incidence across all treatments of 2.0 ± 0.2% (mean ± standard error) on 15 May 2009 to 90.8 ± 1.6% on 14 July 2009, and from 2.1 ± 0.2% on 29 May 2012 to 100% on 31 July 2012. In 2009, the mean daily temperature and total rainfall for April, May, June, July, and August were 8.8°C and 4.3 cm, 12.0°C and 2.6 cm, 15.6°C and 0.4 cm, 17.9°C and 1.3 cm, and 16.5°C and 1.3 cm, respectively. The 2009 trial was irrigated (3.3 cm water) on 18 June. In 2012, the mean daily temperature and total rainfall for April, May, June, July, and August were 9.9°C

and 11.0 cm, 11.8°C and 6.1 cm, 13.4°C and 8.0 cm, 15.9°C and 3.3 cm, and 17.1°C and 0.03 cm, respectively.

In the 2009 trial, spinach plants growing in plots in the first replication had markedly less wilt compared to plants in the other replications, regardless of the treatment. Data collected from this replication were, therefore, not included in the statistical analyses, as the very low level of disease in these plots probably reflected cropping history irregularities (i.e., the portion of the field with the first block of treatments may not have been planted in the 2005 spinach seed crop as the plots were close to the edge of the field). In 2009, the main effect of limestone application rate on wilt incidence was not significant earlier in the season, but was significant by 24 June (Table 2.11), when plots amended with 4.48 t limestone/ha had fewer wilted plants (5.3 ± 0.9%) than plots with 2.24 (12.9 ± 3.3%) or 0 t/ha (33.6 ± 6.7%) when averaged across all spinach parents and both N treatments (Table 2.2, Fig 2.2A). By July 14, incidence of wilt averaged 89.1 to 92.0% for all limestone treatments, with no significant main effect of limestone treatments (Tables 2.1 and 2.2, Fig. 2.2A).

In 2012, the main effect of limestone amendment was not significant on 29 May, when wilt incidence was <2.1%, but was significant on 19 June and 9 July, at which time the effects varied depending on spinach inbred line (Table 2.3, and Figs. 2.2B, 2.3D, 2.3E, and 2.3F). On 19 June, there were significant differences in wilt incidence among limestone treatments for the susceptible female line only, with less wilt in the 0/4.48 t/ha and the 4.48 t/ha/year plots compared to the 0 and 2.24 t/ha/year plots (Table 2.4 and Fig. 2.3E). On 9 July, wilt incidence was so great for the susceptible female and male lines that no significant differences associated with limestone treatment were observed, while for the moderate and resistant female lines, the

4.48 t/ha/year plots had the least wilt, followed in order of increasing wilt by the plots with 2.24 t limestone/ha/year, 0/4.48 t/ha, and 0 t/ha/year plots; for the moderate female, wilt incidences associated with the latter two limestone treatments were not significantly different (Table 2.4 and Fig. 2.3F). Wilt severity, which was rated in addition to wilt incidence on 9 and 31 July in 2012, was greatest in the 0 t/ha/year and 0/4.48 t/ha plots on 9 July (2.58 ± 0.18 and 2.26 ± 0.17 out of a maximum of 5, respectively), and wilt in in plots with 2.24 t/ha/year (2.07 ± 0.18) was more severe than in the 4.48 t/ha/year plots (1.66 ± 0.22) (Table 2.4 and Fig. 2.4A). Wilt severity in the 0/4.48 t/ha plots on 9 July was not significantly different from wilt in the 0 or 2.24 t/ha/year plots, but was more severe than in the 4.48 t/ha/year plots. By 31 July, wilt severity was greater in the 0 and 0/4.48 t/ha plots compared to the 2.24 and 4.48 t/ha/year plots (3.63 ± 0.16 and 3.47 ± 0.16 vs. 3.27 ± 0.14 and 3.14 ± 0.15, respectively) (Table 2.4 and Fig. 2.4B).

In the 2009 trial, fungi identified as *F. oxysporum* were isolated from each of the 10 wilted seedlings collected/female line, and from 9 out of 10 seedlings of the male line (no other fungal seedling pathogens were detected). In the 2012 trial, two wilted seedlings collected from each of the susceptible and moderate female lines were infected with *F. oxysporum* isolates, and no other known spinach pathogens were isolated. Of two wilted seedlings collected from plots of the resistant female, one was infected with *F. oxysporum*, and the other with an unidentified species of *Rhizoctonia*. Four healthy seedlings were also examined, one from each inbred line, from each of which *F. oxysporum* isolates were obtained. In addition, a species of *Rhizoctonia* was isolated from a moderate female seedling. These *F. oxysporum* isolates were not tested for pathogenicity on spinach, so it was not determined whether they were *F. oxysporum* f. sp. *spinaciae* isolates.

The limestone treatments had a significant effect on incidence of vascular discoloration, spinach biomass production, and seed yield in both the 2009 and 2012 trials (Tables 2.1 and 2.3). In 2009, plants in the 0 and 2.24 t/ha plots had a greater incidence of vascular discoloration compared to 4.48 t/ha plots (98.3 ± 1.2 and 97.1 ± 2.1% vs. 91.0 ± 3.1%) (Table 2.2). Conversely, in 2012, fewer spinach plants in the 0 t/ha/year plots had vascular discoloration (70.7 ± 6.4%) compared to plants in the 0/4.48 t/ha plots (87.0 ± 3.5%), but not compared to the 2.24 or 4.48 t/ha/year plots (83.4 ± 3.7% and 77.4 ± 4.5%) (Table 2.4). On 29 June 2009, dry plant biomass was greater in the 4.48 t/ha plots (124.3 g/m row) compared to 2.24 t/ha plots (95.0 g/m row), which in turn produced more plant biomass than the 0 t/ha plots (60.6 g/m row) (Table 2.2 and Fig. 2.5A). When a second biomass assessment was conducted on 22 July 2009, spinach plants were significantly smaller in plots with no limestone (83.2 g/m row) vs. plots with 2.24 or 4.48 t limestone/ha (129.7 to 153.7 g/m row, respectively) (Table 2.2 and Fig. 2.5B). For the single date of biomass assessment conducted on 11 July in the 2012 trial, plots that received 4.48 t limestone/ha for four years yielded larger spinach plants (221.8 g/m row) than plots that received a single application of 4.48 t/ha just prior to planting the 2012 trial (167.0 g/m row), which in turn had larger plants than plots that received 0 t/ha/year throughout the four year trial (114.1 g/m row) (Table 2.4 and Fig. 2.5C).

In the 2009 trial, the 4.48 t/ha plots had greater marketable spinach seed yields (554 kg/ha) than the 0 ton/ha plots (206 kg/ha) (Table 2.2 and Fig. 2.6A). In the 2012 trial, seed yields were significantly different among all four limestone treatments, with 0 t/ha/year plots yielding the least seed (407 kg/ha), followed by the 0/4.48 t/ha plots (702 kg/ha), 2.24 t/ha/year plots (920 kg/ha), and 4.48 t/ha/year plots (1,018 kg/ha) (Table 2.4, Fig. 2.6D). For the susceptible female, seed yield in plots with 0 t/ha/year and 0/4.48 t/ha were not significantly different. There were no

significant effects of limestone treatment on potato yield or incidence of tuber diseases in 2010 however, no scab was observed on any of the tubers harvested for disease assessments (*data not shown*);.

The effects of spinach parent line susceptibility to Fusarium wilt on wilt development, spinach biomass production, vascular discoloration, and seed yield were significant in both the 2009 and 2012 trials, although not at each evaluation date in 2009 (Tables 2.1 and 2.3). In the 2009 trial, spinach inbred lines did not affect wilt incidence significantly at the first rating (15 May) when overall disease incidence was very low (<3%) (Table 2.1). However, by 4 June, the susceptible and resistant inbreds had significantly more damping-off or wilt (4.7 and 7.0%, respectively) than the male line only (0.6%), which probably reflected the presence of other damping-off/wilt pathogens like *Rhizoctonia*, this early in the season when the overall wilt incidence was still <10% (Table 2.2 and Fig. 2.3A). By 24 June, there was a notable but not significant trend toward decreasing wilt with increasing level of Fusarium wilt resistance in the spinach parent lines (Table 2.2 and Fig. 2.3B). By the final wilt assessment on 14 July, there were significant differences among the three female inbred lines, with the least wilt in plots with the resistant female (80.4%), and the greatest wilt incidence in plots with the susceptible female (99.5%) (Table 2.2 and Fig. 2.3C). In the 2012 trial, a significant difference was observed at the first rating date when wilt incidence averaged <3%, primarily because the male line had more wilt than the three female inbreds. More wilt was observed in plots of the susceptible female inbred and the male line by 19 June compared to the moderate and resistant females (24.3 and 29.6% vs. 10.7 and 9.4%, respectively) (Table 2.4 and Figs. 2.3D and 2.3E). By 9 July 2012, the susceptible female line and the male line had 98.3 and 99.3% wilt incidence, respectively,

whereas the moderate female averaged 84.6% wilt incidence, and the resistant female only 68.5% incidence of wilt (Table 2.4).

Because these trials were within pollination distance of a nearby commercial spinach seed field each year, it was necessary to plant seed of the male line planted in the closest commercial spinach seed crop. Consequently, the male line used in 2012 was not the same male used in the 2009 trial. The 2012 male line bolted and senesced later but was more susceptible to Fusarium wilt than the 2009 male line, as evidenced by wilt ratings and other variables measured (Tables 2.2 and 2.4). By 9 July 2012, wilt severity was greatest in the male line (2.98 out of a maximum of 5.00), followed by the susceptible, moderate, and resistant female inbreds in decreasing order of Fusarium wilt severity (2.62, 1.76, 1.22, respectively) (Table 2.4 and Fig. 2.4A). By the final 2012 wilt severity rating on 31 July, the male line had senesced and could no longer be rated, leaving the susceptible female with the most severe wilt (3.97), followed by the moderate (3.46) and the resistant (2.76) females, with significant differences among these female lines (Table 2.4 and Fig. 2.4B).

In the 2009 trial, the susceptible inbred had 100% incidence of vascular discoloration typical of Fusarium wilt on 22 July, which was greater than that of the moderate and resistant female lines (94.0 and 92.5%, respectively) (Table 2.2). Similarly, in the 2012 trial the susceptible female and male lines had greater incidences of vascular discoloration (96.2 and 95.1%) than the moderate and resistant females (69.4 and 57.7%), the latter having the lowest incidence of vascular discoloration of all the lines (Table 2.4). On 29 June 2009, the male and susceptible female lines had significantly more biomass than the moderate female (113.9 and 97.6 vs. 89.9 g/m row), which in turn had more biomass than the resistant female (71.7 g/m row), reflecting the genetically-predetermined smaller phenotype of the resistant female inbred

compared to the other inbreds (Table 2.2 and Fig. 2.5A). By the second biomass assessment on 22 July 2009, however, there were no significant differences in biomass among inbreds, suggesting that the reduced level of wilt in the resistant female had, by then, compensated for the size differential (Table 2.2 and Fig. 2.5B). In the 2012 trial, the susceptible and moderate inbreds had greater biomass than the resistant and male lines (183.1 and 185.5 vs. 161.8 and 166.6 g/m row), regardless of limestone application rate (Table 2.4 and Fig. 2.5C). Despite the smaller size of the resistant female line, the moderate and resistant inbreds had 62 and 50% greater marketable seed yields, respectively, than the susceptible inbred in the 2009 trial (Table 2.2 and Fig 2.6A). In the 2012 trial, differences in yield were even more pronounced, with the resistant inbred yielding 171% more seed than the susceptible inbred, and 55% more than the moderate female inbred (Table 2.4), despite the smaller frame of the resistant inbred (Table 2.4 and Fig. 2.6D).

Rainfall (2.5 cm) 10 days after planting in the 2009 trial caused severe leaching of nitrate in the plots fertilized in-furrow with NO_3^--N. Unlike NH_4^+ cations, NO_3^- anions are not retained by the CEC of soils, and are susceptible to leaching (Havlin et al., 1999). As a result, spinach plants in the plots fertilized with NO_3^--N expressed severe symptoms of N deficiency, including chlorosis and stunting, throughout the 2009 trial, culminating with NH_4^+-N fertilized plots out-yielding NO_3^--N plots by 83% (Table 2.2). However, early in the 2009 season (4 June), plants of the moderate female line had significantly less wilt in NO_3^--fertilized plots compared to NH_4^+-fertilized plots, with no significant differences among the other inbreds. This accounts for the significant inbred line-by-N form interaction in the ANOVA (Table 2.1). Similarly, the moderate female had more vascular discoloration in plots fertilized with NH_4^+-N vs. NO_3^--N, while fertilizer did not affect vascular discoloration of the other two female lines (Table 2.2). By 14

July, plants in plots fertilized with NO_3^--N had significantly more wilt than plants in plots fertilized with NH_4^+-N for the susceptible and resistant lines, and there was no significant effect of fertilizer on wilt incidence for the moderate parent. Spinach biomass on 29 June was greater in plots fertilized with NH_4^+-N vs. NO_3^--N for the susceptible female and male lines but not for the moderate or resistant female lines, reflecting the significant interaction of inbred line and N form in Table 2.1 ($P = 0.0143$).

In addition to the significant interactions of N-form with spinach inbred line described above, the effects of N treatment were influenced by limestone treatments for two of the field variables measured in 2009 (Table 2.1). In plots with 0 and 2.24 t limestone/ha, NO_3^--N fertilizer significantly increased wilt incidence compared to NH_4^+-N on 24 June, but the fertilizers had no effect in plots with 4.48 t limestone/ha (Table 2.2), reflecting the significant limestone-by-N form interaction on this date (Table 2.1). Similarly, spinach biomass on 29 June was not affected by N-fertilizer in plots with 4.48 t limestone/ha, but biomass was greater in plots fertilized with NH_4^+-N vs. NO_3^--N in plots with 0 or 2.24 t limestone/ha.

Seed germination and health assays. There were no significant effects of limestone application rate on seed germination or incidence of necrotrophic fungi found on seed harvested in the 2009 trial (Tables 2.5 and 2.6). However, in the 2012 trial, the four years of annual limestone treatments resulted in significant effects on the incidence of seedborne *V. dahliae* and *Alternaria* spp. (Table 2.7). Seed harvested from plots that received 4.48 t limestone/ha for each of four years had significantly more *V. dahliae* (11.5%) compared to seed harvested from plots receiving 2.24 t/ha/year and the 0/4.48 t/ha plots (5.6 and 3.2%), which in turn had significantly more *V. dahliae* than seed from the 0 t/ha/year plots (0.9%) (Table 2.8 and Fig. 2.6E). Fewer

seeds from the 4.48 t/ha/year plots were infested with *Alternaria* spp. (33%) compared to seed from the 0 t/ha/year (45.5%) and 0/4.48 t/ha plots (46.1%) (Table 2.7).

The main effect of spinach inbred line susceptibility on the incidence of rotten seed and incidence of seed with *Fusarium* spp. and *Stemphylium botryosum* was significant in the 2009 trial (Table 2.5). More of the seed harvested from plots with the susceptible female were rotten (20.0%) compared to seed of the moderate and resistant female lines (8.3 and 7.4%, respectively), and more of the susceptible female seed was infested with *Fusarium* spp. than the moderate or resistant female seed (4.2 vs. 0.9 and 0.7%, respectively) (Table 2.6 and Fig. 2.6C). Seed of the moderate female had a greater incidence of *S. botryosum* (7.4%) compared to seed of the susceptible (3.2%) and resistant females (3.3%). In the 2012 trial, spinach parent line significantly affected the incidence of germinated, non-germinated, and rotten seed, as well the percentage of seed on which *Fusarium* spp., *V. dahliae*, and *Alternaria* spp. were detected (Table 2.7). The percentage of rotten seed harvested from plots of the susceptible female (32.6%) was greater than that of seed harvested from plots of the moderate female (20.5%), which was greater than that of seed harvested from plots of the resistant female (6.3%) (Table 2.8). The percentage of viable (germinated) seed was greater for seed harvested from plots with the resistant and moderate females (67.0% and 61.5%, respectively) compared to seed from plots with the susceptible female (50.8%). However, the incidence of non-germinated, but not rotten (dormant), seed was greater for seed harvested from plots of the resistant female (26.2%) compared to the susceptible and moderate females (15.7 and 17.2%, respectively). Seed of the susceptible female sustained a greater level of infection with *Fusarium* spp. (10.6%) compared to seed of the moderate female (2.1%), which in turn had more seed infected with *Fusarium* spp. than seed of the resistant female (0.4%) (Table 2.8 and Fig. 2.6F). The reverse was observed in the 2012 trial

for *V. dahliae* seed infection, with more seed infected with this fungal pathogen for the resistant and moderate female lines (5.7 and 7.8%, respectively) compared to seed of the susceptible female (2.5%) (Table 2.8 and Fig. 2.6E). A significant limestone-by-inbred interaction (P = 0.0136) for *V. dahliae* seed infection (Table 2.7) was manifested by the lack of significant differentiation among plots amended with 0/4.48, 2.24, and 4.48 t limestone/ha for the susceptible inbred compared to the moderate and resistant inbred lines, the seed of which was increasingly infected with *V. dahliae* with increasing limestone rate (Table 2.8). Less seed of the susceptible female was infested with *Alternaria* spp. than seed of the moderate and resistant females, which did not differ significantly (Table 2.8).

In 2009, the effects of fertilizer N-form were significant for the incidence of germinated, non-germinated, and rotten seed, but not for the incidence of any of the seed-associated necrotrophic fungi (Tables 2.5 and 2.6). Seeds harvested from plots fertilized with NO_3^--N were of poorer quality than seeds harvested from plots fertilized with NH_4^+-N, as demonstrated by a lower germination rate, as well as greater incidences of rotten seeds and non-germinated (dormant) seeds (Table 2.6).

Plant nutrient analyses. The effect of limestone application rate on plant nutrient analyses was significant for P, Ca, Mg, S, Zn, and Mn in the 2009 trial; however, the effects on Mg and Mn were also influenced by the N-form of fertilizer, and the effect of limestone on N was influenced by both N-form of fertilizer and inbred line (significant three-way interaction) (Table 2.9). Plants in plots with 2.24 and 4.48 t limestone/ha had more Ca (1.27 and 1.37%, respectively) than plants in 0 t/ha plots (1.07%) (Table 2.10). Conversely, spinach levels of Zn and Mn were greater in the 0 t/ha plots (117.2 and 178.2 mg/kg, respectively) compared to the 4.48 t/ha plots (97.1 and 33.8 mg/kg, respectively), and compared to the 2.24 t/ha plots for Mn

(143.0 mg/kg). Spinach plants had more Mg but less P in 0 t/ha plots compared to 2.24 and 4.48 t/ha plots (Table 2.10). Spinach inbred line significantly affected all plant nutrient levels in the 2009 trial, although the effects on N, P, Ca, Mg, and S varied with N-form, with greater levels in the NH_4^+-N than the NO_3^--N plots (Tables 2.9 and 2.10). Plants of the susceptible female inbred had less N, Ca, Mg, S, B, and Zn compared to plants of the moderate and resistant inbreds (Table 2.10). The male line had less N, P, and Fe compared to the female lines. The moderate female line had more P and Mn compared to the other lines (Table 2.10). N-form affected spinach levels of N, P, Mg, S, B, and Zn, although the effect varied with inbred line, and the effect on Mg also varied among limestone treatments. Spinach plants in plots fertilized with NH_4^+-N had significantly more N, P, and S, but less Mg, B, and Zn compared to plants in plots fertilized with NO_3^--N. For the male line, Ca was more concentrated in NO_3^--N fertilized plots compared to NH_4^+-N fertilized plots (Table 2.10)

In the 2012 trial, there were significant main effects of limestone application rates on spinach plant levels of Mg, B, Zn, and Mn (Table 2.11), with the most concentrated levels of these nutrients in plants growing in 0 t/ha/year plots and the least concentrated in the 2.24 to 4.48 t/ha/year plots (Table 2.12). The concentration of Zn decreased by 40.8% and the concentration of Mn decreased by 66.1% in plants growing in the 4.48 t/ha/year plots compared to the 0 t/ha/year plots. The main effects of limestone treatment and inbred line on spinach Ca levels were not significant, but the interaction term was significant ($P = 0.0056$) (Table 2.11 and 2.12). Spinach Ca was less concentrated in plants growing in 0 t/ha/year plots compared to 2.24 and 4.48 t/ha/year plots, but only for the susceptible and resistant female inbreds. The resistant inbred had more concentrated N (3.37%) compared to the other inbreds (3.11, 3.08, and 3.08% for the susceptible, moderate, and male lines, respectively), but this was the sole main effect of inbred

line on plant nutrient levels, which contrasted sharply with the 2009 trial in which the effects of inbred lines on spinach nutrient levels were significant for every nutrient tested.

Soil populations of *F. oxysporum* and *V. dahliae*. In the 2009 trial, limestone application rate did not significantly affect the soil population of *F. oxysporum* or *V. dahliae* detected at the beginning or the end of the season (Table 2.13), although the *F. oxysporum* population more than doubled by the end of the season across all limestone rates, from 1,951 to 4,989 CFU/g soil. There was, however, a significant effect of spinach inbred line on soilborne *F. oxysporum* detected at the end of the 2009 trial (Table 2.13), with a smaller *F. oxysporum* population detected in soil from plots planted with the resistant female compared to plots with the susceptible and moderate inbreds (3,802 vs. 4,874 and 5,339 CFU/g soil, respectively) (Tables 2.13 and 2.14). There was no effect of inbred line on the soilborne *V. dahliae* population, but soil in plots fertilized with NO_3^--N had more *V. dahliae* (47.9 CFU/g soil) at the end of the 2009 season than soil from plots fertilized with NH_4^+-N (25.3 CFU/g soil) (Table 2.14).

Soil samples collected on 7 May, 14 July, and 5 September 2010 (when the field was planted to a potato crop) did not reveal significant effects of the rates of limestone amendment on the soilborne *F. oxysporum* population detected (*data not shown*). However, there were significant effects of both limestone treatment and spinach inbred lines on soilborne populations of *F. oxysporum* and *V. dahliae* by the end of the 2012 trial (Table 2.15). At the start of the 2012 trial, the *F. oxysporum* population had dropped back to levels similar to and, in fact, slightly less than those observed at the start of the 2009 season (2,089 CFU/g soil at the beginning of the 2009 trial vs. 1,210 CFU/g soil at the beginning of the 2012 trial), while the *V. dahliae* population was greater across all treatments at the beginning of the 2012 trial (average of 64.2 CFU/g soil) than at the end of the 2009 season (average of 36.5 CFU/g soil). Significant effects

of both spinach inbred and limestone rates on both of these soil fungus populations were detected in the 2012 trial (Table 2.15). Limestone application rate did not influence soilborne *F. oxysporum* populations significantly at the early and mid-season soil samplings in 2012, but by the end of the season, the 0/4.48 t/ha plots (but not the 0 t/ha/year plots) had a larger population of this fungus than the 2.24 and 4.48 t/ha/year plots (6,027 vs. 5,147 and 4,822 CFU/g soil, respectively) (Table 2.16). Limestone rates and spinach inbreds also affected soilborne *V. dahliae* populations, with the plots planted to the resistant female and those amended with 4.48 t limestone/ha/year having the largest *V. dahliae* populations (Table 2.16). Even at the beginning of the trial, the 0 t/ha/year plots had smaller soilborne *V. dahliae* populations compared to the 2.24 and 4.48 t/ha/year plots (45.8 vs. 72.8 and 81.2 CFU/g soil, respectively). By the end of the 2012 season, the 4.48 t/ha/year plots had 103.1 CFU *V. dahliae*/g soil, which was significantly greater than 62.1 CFU/g soil in the 2.24 t/ha/year plots, which was greater than 47.8 CFU/g soil in the 0/4.48 t/ha plots, which in turn was greater than 32.6 CFU/g soil in the 0 t/ha/year plots (Table 2.16).

Soil nutrient analyses. In the 2009 trial, soil nutrient analyses for soil samples collected periodically through the season revealed significant effects of limestone treatments on soil chemical and physical properties. A pattern of decreasing soil NO_3^--N levels with increasing limestone application rate was observed from 8 July to 19 August (Table 2.17). By 8 July, soil in plots with 4.48 t limestone/ha had less NO_3^--N (9.3 mg/kg) than plots with 0 t/ha (15.7 mg/kg), and by the final sampling date on 19 August, soil NO_3^--N in both the 0 t/ha plots (64.0 mg/kg) and the 2.24 t/ha plots (51.0 mg/kg) was greater compared to soil from the 4.48 t/ha plots (33.3 mg/kg). Changes in soil NH_4^+-N levels associated with limestone treatments were less evident, with a greater concentration of this cation in the 4.48 t/ha plots (1.55 mg/kg) compared to the 0

t/ha plots (1.23 mg/kg) only at the earliest (5 May) sampling date. There were no effects of limestone treatment on soil P or B, and none on soil K and Mg until the final sampling date (19 August), when soil K was less in the 4.48 t limestone/ha plots compared to the 0 and 2.24 t/ha plots (226.3 vs. 247.5 and 246.2 mg/kg, respectively). Similarly, soil Mg was more concentrated in the 0 and 2.24 t/ha plots (0.683 and 0.679 meq/100 g soil, respectively) compared to the 4.48 t/ha plots (0.633 meq/100 g). The effect of limestone application rate on soil Ca was significant throughout the season, with more Ca in soil from the 4.48 t/ha plots than the 2.24 t/ha plots, and more Ca in soil from the 2.24 t/ha plots than the 0 t/ha plots; by the end of the trial, soil Ca was 3.52, 4.88, and 5.83 meq/100 g in the 0, 2.24, and 4.48 t/ha plots, respectively (Table 2.17). The reverse was observed with soil S, which was lower on 27 May, 8 July, and 19 August in plots with 4.48 t limestone/ha compared to plots with 0 t/ha (Table 2.17). Increasing the rate of limestone application decreased soil Mn at all six sampling dates, and decreased soil Zn for four of the six sampling dates. On 8 and 28 July, soil Fe was less concentrated in plots amended with 4.48 t limestone/ha (41.5 and 38.0 mg/kg, respectively) than in plots with 2.24 (57.0 and 58.3 mg/kg, respectively) or 0 t/ha (63.0 and 57.3 mg/kg, respectively). There were significant effects of limestone treatments on soil pH in the 2009 trial, and soil pH decreased gradually throughout the season for all limestone treatments (Table 2.17 and Fig. 2.7). Plots treated with 4.48 t limestone/ha had a higher pH than plots with 2.24 t/ha, and plots with 2.24 t/ha had a higher pH than plots with 0 t/ha, with the exception of 28 July, when soil pH did not differ significantly between the 2.24 and 4.48 t/ha plots. There were no significant effects of limestone treatment on soil OM or CEC in the 2009 trial (*data not shown*).

The following soil nutrients were affected by N-form of fertilizer used in the 2009 trial, although not at every sampling date: NH_4^+-N, P, K, S, B, Mn, Zn, and Fe (Table 2.17). On 27

May, soil in plots fertilized with NO_3^--N had much less NH_4^+-N (2.21 mg/kg) than plots fertilized with NH_4^+-N (9.13 mg/kg). On both 27 May and 19 August, the two sampling dates when the effects of spinach inbred lines on soil nutrient levels were measured, soil P and S were lower, and B higher in plots fertilized with NO_3^--N compared to plots fertilized with NH_4^+-N. The effect of N-form of fertilizer on soil K was only significant at the 27 May sampling, when soil from NO_3^--N -fertilized plots had less K (247.7 mg/kg) than soil in NH_4^+-N-fertilized plots (267.3 mg/kg). Soil Mn and Zn were lower in NO_3^--N plots compared to NH_4^+-N plots on 18 August, whereas on 27 May, soil Fe was higher in NO_3^--N plots compared to NH_4^+-N plots. Plots fertilized with NO_3^--N had a higher soil pH (6.25) than plots fertilized with NH_4^+-N (6.05), but only at the 27 May sampling.

In the 2012 field trial, limestone treatments significantly affected soil levels of NO_3^- and NH_4^+-N, P, Ca, S, Mn, Zn, and Fe, although not at all three sampling dates (Table 2.18). On 9 May, soil from plots amended with 2.24 and 4.48 t limestone/ha/year had more NO_3^--N (5.84 and 5.60 mg/kg, respectively) compared to soil from 0/4.48 t/ha and 0 t/ha/year plots (4.84 and 4.04 mg/kg, respectively). This pattern reversed over the course of the season, so that at the mid- and end-of-season sampling dates (9 July and 18 August, respectively), the 0 t/ha/year plots had more soil nitrate (15.12 and 17.4 mg/kg, respectively) than plots to which any limestone had been applied (6.89 to 12.26 mg/kg). On 9 July, soil from the 0 t/ha/year plots had less NH_4^+-N (0.76 mg/kg) than plots to which any limestone had been applied (1.66 to 2.09 mg/kg). On the final sampling date, the 4.48 t/ha/year plots had more soil P (302 mg/kg) compared to plots with the other limestone treatments (215 to 256 mg/kg). At the start and middle of the season, soil Ca increased with increasing limestone application rate, but by the end of the season these differences were not significant, i.e, at the end of this four-year trial, there were no significant

effects of limestone application on soil Ca status. Soil K, Mg, and B were not affected significantly by limestone application rates, but soil S was lower in plots amended with 4.48 t limestone/ha/year compared to 0 t/ha/year and 0/4.48 t/ha plots at the mid-season sampling, and in all limestone-amended plots compared to non-amended plots at the end of the season (Table 2.18). Soil Mn, Zn, and Fe were lower in all limestone-amended plots than in non-amended plots. At the start of the season, the 0 t/ha/year plots had more available Mn in the soil (3.29 mg/kg) compared to any of the limestone-amended plots (1.31 to 1.18 mg/kg), which were not statistically different. By the end of the season, the effects of limestone treatments on soil Mn were even more distinct, with the highest Mn level detected in 0 t/ha/year plots (3.51 mg/kg), followed in order by 0/4.48 t/ha (1.17 mg/kg), 2.24 t/ha/year (0.99 mg/kg), and 4.48 t/ha/year plots (0.84 mg/kg). Similar effects on soil Zn and Fe were observed, with available levels of these micronutrients lowest in 4.48 t/ha/year plots and highest in 0 t/ha/year plots throughout the season, with varying degrees of differentiation among the remaining limestone treatments for each sampling date (Table 2.18). There was no effect of limestone treatments on soil OM in 2012 (*data not shown*). On 9 July, the 0/4.48 t/ha plots had a lower CEC compared to 2.24 t/ha plots. Soil pH increased slightly from 9 May to 9 July 2012, and then decreased by 24 August for all limestone treatments (Table 2.18 and Fig. 2.7). However, the pH was higher at both the start and end of the 2012 season compared to the 2009 season in plots that had received 2.24 and 4.48 t limestone/ha/year for four years (pH of 6.77 and 7.18, respectively, on 9 May 2012) compared to the pH on 24 August 2012 for plots that had been amended with a single application of the comparable rate of limestone at the start of the 2009 trial (pH of 5.84 and 6.28, respectively) (Fig. 2.7). Furthermore, plots that received a single application of 4.48 t limestone/ha just prior to planting the 2012 spinach trial had a lower pH than plots amended with 2.24 or 4.48 t/ha/year for

four years, indicating that a shift in soil pH had occurred following annual applications of limestone over four years compared to a single application.

Discussion

The results of this four-year field trial demonstrate that the suppression of spinach Fusarium wilt achieved with a single limestone application at 2.24 or 4.48 t/ha in the spring of planting a spinach seed crop in the highly conducive soils of the maritime PNW, can be augmented by annual applications of limestone at similar rates for three seasons preceding a spinach seed crop. While exceptions exist, acid soils are generally thought to be conducive to Fusarium wilts of various crops. This may explain why spinach seed production in Denmark, and fresh market and processing spinach production in southern Texas, where soils are naturally alkaline, are not limited by this disease (L. J. du Toit, *personal communication*). The hypothesis that a three-to-four-year approach to limestone-mediated Fusarium wilt management could enhance wilt suppression was based, in part, on observations that soils in the Skagit Valley typically revert rapidly to an acid state within the same season following a single limestone application (du Toit et al., 2007, 2008, and 2011).

Research on Fusarium wilts of other crops indicates that in vitro growth and aggressiveness of Fusarium wilt pathogens can be enhanced under conditions of low pH (Jones et al., 1989). *F. oxysporum* formae speciales may not be particularly competitive saprophytes in the soil microbial community (Garrett, 1970). However, these pathogens have evolved successful survival and pathogenicity strategies based on the formation of dormant, persistent chlamydospores which germinate in the presence of host plants, as well the ability to infect and proliferate on the roots of non-host crops (Armstrong and Armstrong, 1981; Beckman, 1987). In

the highly-leached, low pH environment typical of maritime PNW soils, *F. oxysporum* f. sp. *spinaciae* is able to invade the vascular system of spinach plants, even when the rotation out of spinach seed crops approaches 20 years (L. J. du Toit, *personal communication*).

The objective of this research was to determine whether a field in the maritime PNW designated for a spinach seed crop can be modified over the course of three years into a less favorable environment for this pathogen, as a result of annual soil applications of limestone for three years prior to planting the spinach seed crop. To be effective, this approach must be compatible with the needs of other crops grown in rotation with spinach seed crops in the Skagit Valley, which is one of the reasons the trial was conducted in the field of a grower-cooperator who carried out his planned rotation in the years between the 2009 and 2012 spinach seed crop trials. The results of this research indicate that such applications can stabilize soil pH at levels closer to neutral, thereby reducing inoculum potential of the pathogen population. Findings from the 2012 spinach trial showed a compound beneficial effect of annual limestone applications for four years prior to a spinach seed crop compared to one application made just before planting a spinach seed crop, which is the typical practice in the maritime PNW. While the study did not include treatments with two-year or three-year annual limestone applications to compare with the four-year annual treatments and the 0/4.48 t/ha treatment, and was limited to just one field site in this region of fairly diverse soil types, the annual application of 2.24 or 4.48 t/ha/year resulted in an increase in soil pH, less Fusarium wilt, and greater marketable seed yield compared to plots amended just once with 4.48 t limestone/ha and non-amended soil. In previous field trials evaluating a single limestone application just prior to planting a spinach seed crop for spinach Fusarium wilt suppression, 4.48 t limestone/ha applied within two weeks of planting elevated soil pH to close to 7.0 from a pH <6.0 (du Toit et al., 2007 and 2008; Gatch et al., 2011). In the

2012 trial in this study, soil in plots that received 4.48 t limestone/ha for four years surpassed pH 7.0, and remained >7.0 through the end of the season. This seemingly stable shift in soil pH was accompanied by an average 20% reduction in wilt incidence mid-season, 33% increase in spinach plant biomass, and 45% increase in spinach seed yield compared to plots amended once with the equivalent rate of limestone, the spring of planting. These results are consistent with the numerous studies conducted in the 1970s in Florida on the use of limestone amendments to suppress Fusarium wilts of tomato, watermelon, chrysanthemum, and other crops (Jones et al., 1989), although those studies did not evaluate sequential, annual applications of limestone.

As observed in previous field trials in the Skagit Valley of Washington, the degree of wilt suppression achieved with limestone application was influenced significantly by the susceptibility to Fusarium wilt of the spinach parent lines evaluated. In some years, the benefits of limestone-mediated suppression of Fusarium wilt have been more evident for the susceptible line than the partially resistant line, as appeared to be the case in the 2009 trial in this study. In the 2012 trial, however, the partially resistant line had the greatest increase in seed yield and decline in incidence and severity of wilt symptoms with increasing rate of limestone application. It is likely that nuances of temperature and precipitation in a given season affect the interactions among *F. oxysporum* f. sp. *spinaciae*, spinach parent line, and limestone application. In 2009, the wet, cool spring followed by a hot, dry summer created ideal conditions for Fusarium wilt development. The disease advanced rapidly, making wilt incidence assessment after the middle of July ineffectual for differentiating treatment effects on disease development. Wilt was slower to progress and yields were greater in the 2012 season, which was cooler and milder than in 2009. This may have enhanced the observed benefit of annual limestone applications on spinach seed production and Fusarium wilt suppression in the 2012 trial. Nevertheless, incidence of wilt

reached 100% toward the end of the season in both 2009 and 2012, and plots not treated annually with limestone, or amended with a single application the spring of planting, clearly had more severe wilt compared to plots with annual applications at either 2.24 or 4.48 t/ha for four years by the final disease rating in 2012.

The 2012 field trial was arranged in the same plots, with the same treatments (except for the NO_3^--N vs. NH_4^+-N fertilizers) as the 2009 trial. As evidenced by the 2009 results, spinach inbred line susceptibility to Fusarium wilt influenced the amount of soilborne *F. oxysporum* detected at the end of the season, which is an indirect indicator that there may have been similar effects on the population of *F. oxysporum* f. sp. *spinaciae*. Quantification of the pathogen population would have required greenhouse pathogenicity testing of an adequate subsample of *F. oxysporum* isolates obtained from the soil dilution plating process, to distinguish strains of *F. oxysporum* f. sp. *spinaciae*, other formae speciales, and non-pathogenic *F. oxysporum*, a task that was not feasible for a trial of this size. While there was no significant effect of limestone application rate or form of N fertilizer on the *F. oxysporum* population detected at the end of the 2009 season, there was a slight but significant decrease in *F. oxysporum* population by the end of the 2009 trial in plots planted with the resistant female compared to the susceptible and moderate female lines. This may have resulted in lower disease pressure in the same plots planted to the resistant female in 2012 compared to plots planted with the moderate and susceptible inbreds, if the reduction in *F. oxysporum* population detected in 2009 corresponded to a reduction in Fusarium wilt inoculum potential. However, the lack of significant limestone treatment effects on the *F. oxysporum* population in the 2009 trial suggests that differences among these treatments in 2012 were not associated significantly with historical effects of the 2009 treatments on the pathogen population. This potential confounding could have been avoided if the four-year

trial was set up in a field with a 5 to 6 year rotation out of a spinach seed crop, the limestone treatments applied annually prior to each rotational crop, and then a spinach seed crop trial planted to evaluate the effects of the limestone treatments and spinach inbreds with different levels of Fusarium wilt susceptibility.

Soil chemical properties and plant nutrient status were assessed in both the 2009 and 2012 trials to characterize chemical shifts in both soil and plant tissue as a result of limestone treatments. Because plant genotype can influence the uptake and accumulation of mineral elements (Clark, 1983), the effect of spinach parent line was also evaluated in these nutrient analyses. Raising soil pH can reduce the availability of certain soil nutrients, particularly Fe, Mn, and Zn, through the formation of insoluble metal hydroxides (Havlin et al., 1999). In Denmark, where spinach seed crops are produced on soils with a pH ranging from 7.5 to 8.0, foliar sprays of Mn and Zn are necessary to avoid deficiencies of these micronutrients (H. van Veldhuizen, *personal communication*). In the 2009 trial, soil Mn levels were reduced significantly with increasing rates of limestone amendment and, at some of the sampling dates, fell below the 1.5 mg/kg level that is considered sufficient for most crops (Marx et al., 1996). In 2012, only soils from plots that had received no limestone amendments all four years of the trial had Mn concentrations that met or exceeded this level. Soil Zn was also reduced with limestone amendments in both trials, and by the 2012 trial was slightly below the recommended sufficiency of 1.0 mg/kg both mid-season and at the end of the season.

These soil micronutrient deficiencies associated with limestone application were expected. One theory about the mechanism of Fusarium wilt suppression with limestone application is that raising the soil pH limits availability of these so-called “acid micronutrients” for the pathogen, thereby limiting the growth and virulence of the pathogen (Jones et al., 1989;

Woltz and Jones, 1968). Regardless of whether starving the pathogen of these micronutrients contributes to limestone-based suppression, starving the plant of these essential elements is an undesirable side effect. Therefore, as a precaution against the development of spinach plant deficiencies, a foliar Mn and Zn application was made to the entire trial in 2012 at the rosette stage of growth. Plants in plots amended with 4.48 t limestone/ha had significantly lower Zn and Mn in both trials compared to plants in plots that were not amended with limestone. In the 2012 trial, annual applications of limestone at 4.48 t/ha/year resulted in lower plant Zn and Mn levels compared to plants growing in plots amended with the same amount of limestone once, just prior to planting the 2012 spinach seed crop. Plant Zn and Mn levels did not fall below the sufficiency range in any of the plots in 2012 (Olsen's Agricultural Laboratory, 2013). Soil Fe, but not plant Fe, was reduced in plots with 4.48 t limestone/ha/year. Further evaluation of annual limestone applications in different fields would be valuable to determine whether prophylactic micronutrient foliar sprays are critical to the successful use of annual limestone amendments as a management practice by spinach seed growers.

Another theory regarding the mechanism(s) of Fusarium wilt suppression achieved with soil limestone application is that the Ca provided by the limestone is beneficial for plant defense responses, since Ca fortifies cell wall structure by cross-linking pectins and is involved in cellular defense signaling (Marschner, 1995). While some studies have shown that pH elevation, not Ca, is implicated in limestone-mediated Fusarium wilt suppression (Jones and Woltz, 1969), others provide evidence for the involvement of Ca in reducing Fusarium wilt development (Corden, 1965). It was important to track soil and plant Ca levels in this study to assess whether results were confounded by a Ca deficiency in non-limestone-amended soils. A soil Ca level of 5 meq/100 g soil and a spinach plant level of 0.6% are considered adequate for normal plant

growth (Marx et al., 1996; Olsen Laboratory, 2013). In 2009, soil Ca was below this in plots amended with 0 and 2.24 t limestone/ha, while in 2012, only plots that were not amended with limestone were below this level, and only at the first two sampling dates. Furthermore, spinach Ca levels were above the sufficiency range in both trials. The fact that in the 2012 trial there were no significant differences in plant Ca, or in soil Ca by the end of the trial, associated with limestone treatments, suggests that Ca probably did not play a critical role in the mechanism(s) of limestone-mediated spinach Fusarium wilt suppression.

Any potential disease management strategy designed for spinach seed crops in the Skagit Valley of Washington State must be vetted for possible negative consequences as well as potential benefits to crops grown in rotation with spinach seed, the most valuable of which is fresh-market potatoes (McMoran, 2011). Although some studies have indicated that elevation of soil pH through limestone applications can increase the incidence of potato scab, a disease caused by *Streptomyces scabies* that results in cosmetic tuber damage that can lead to significant economic losses (Lambert and Manzer, 1991; Odland and Allbritten, 1950), other factors, most notably soil moisture, affect scab risk (Lapwood and Adams, 1975). To address the concern of some potato growers that an increase in the use of limestone in spinach seed crops may enhance potato scab, potatoes were harvested from a 3 m section of row of each plot in the four-year limestone trial just prior to harvest of the 2010 potato crop that was part of the grower-cooperator's rotational plan for the field. No significant effects on potato yield or tuber disease incidence were observed among the plots with different limestone treatments.

Also important for grower adoption of annual applications of limestone in spinach seed production are analyses demonstrating the economic viability of this longer-term approach to Fusarium wilt management. A minimum estimated increase in a typical spinach seed crop that

yields about 1,350 kg/ha, to justify the cost of applying 2.24 or 4.48 t limestone/ha, is 52 and 91 kg seed/ha, respectively, based on current expenses for the purchase, application, and incorporation of these two rates of agricultural limestone (J. Schafer and P. Klein, *personal communication*). This represents a yield increase of 4 and 7%, respectively. In the 2009 trial, soil amendment with 2.24 t limestone/ha approximately doubled spinach seed yield, and 4.48 t/ha increased yields by 169% compared to yields from plots with no limestone amendment, so the minimum required increases in seed yield were far exceeded. To cover expenses for annual limestone applications of 4.48 t/ha for three or four years prior to a spinach seed crop, including the year of planting the seed crop, a yield increase of 44% would be necessary. In the 2012 trial, annual applications of 4.48 t limestone/ha for four years compared to 0 t/ha and 4.48 t/ha applied once just prior to the seed crop, increased spinach seed yields by 150 and 45% respectively. A 45% increase represents a substantial yield gain, and it may be worth conducting further research to determine whether similar seed yield increases can be achieved with three, or even two, limestone applications prior to planting a spinach seed crop. Any economic analysis should also take into account potential economic benefits of annual limestone applications to crops grown in rotation with spinach, such as brassica or beet seed crops and the various small fruit crops grown in the Skagit Valley. For example, limestone is routinely applied to brassica crops to help suppress clubroot caused by *Plasmodiophora brassicae*, and to raspberry crops to help suppress Phytophthora root rot caused by *Phytophthora rubi* (Pacific Northwest Plant Disease Management Handbook, 2013).

The increased incidence of *V. dahliae*-infected seed harvested from plots amended with 4.48 t limestone/ha is an issue that needs to be addressed in the development of a limestone-based Fusarium wilt management strategy. Verticillium wilts of many crops have been shown to

be favored by alkaline soils and/or NO_3^--N fertilization, the opposite response of some Fusarium wilts to these conditions (Dutta, 1981; Elmer and Ferrandino, 1994; Jones and Woltz, 1972). The 11.5% incidence of *V. dahliae* on seed harvested from plots amended with 4.48 t limestone/ha/year in the 2012 trial would cause a commercial spinach seed lot to be rejected for export to Mexico, which has a phytosanitary requirement of <10% incidence of *V. dahliae* on spinach seed (International Phytosanitary Certificate, 2003). Most commercial spinach seed lots, regardless of origin, sustain some level of *V. dahliae* infection (du Toit et al., 2005). However, the recent scrutiny of spinach seed as one of several possible sources of *V. dahliae* strains found in California fields where lettuce is grown in rotation with spinach and other crops susceptible to Verticillium wilt, and where Verticillium wilt has emerged as a major disease of lettuce (Atallah et al., 2010), underlines the importance of taking steps to reduce *V. dahliae* incidence on spinach seed or develop means of treating infected seed lots to eradicate the pathogen and/or prevent seed transmission. Spinach seed treatments have been identified that reduce *V. dahliae* incidence on seeds from 64% to <10% (du Toit et al., 2010; du Toit and Correll, 2012), which indicates that contending with seedborne *V. dahliae* in harvested spinach seed will prove easier than managing Fusarium wilt in spinach seed crops since there are currently no effective and economically viable chemical seed, foliar, or soil treatments for this disease.

A crop that requires an 8 to 15 year rotation to avoid significant losses to a disease, and cannot be relocated to new regions of production because of unique and stringent climatic and geographic requirements, is clearly one that is both valued by stakeholders and worthy of extraordinary measures to protect. Until Fusarium wilt management practices are developed that are both effective and economically viable, the USA acreage suitable for spinach seed production will continue to be restricted severely as a result of the risk posed by this disease. This study

demonstrates the potential for enhanced suppression of Fusarium wilt with annual soil applications of limestone for several years, rendering the soils of the maritime PNW less hospitable to this persistent pathogen.

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Table 2.1. Probability values from the analyses of variance (ANOVAs) for the fixed effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on spinach wilt incidence, vascular discoloration, dried plant biomass, and seed yield in a 2009 spinach seed crop field trial in Skagit Co., WA

	Wilt incidence (%)[b]				Vascular discoloration	Dried plant biomass (g/m row)[d]		Marketable seed yield
ANOVA factor[a]	**15 May**	**4 June**	**24 June**	**14 July**	**(%)[c]**	**29 June**	**22 July**	**(kg/ha)[e]**
Limestone rate	0.8232	0.1099	0.0381*	0.5556	0.0082*	0.0024*	0.0165*	0.0320*
Spinach inbred line	0.5409	0.0014*	0.1596	<0.0001*	0.0251*	0.0002*	0.1659	0.0332*
Nitrogen (N) form	0.2101	0.1073	0.0344*	0.3393	0.0707	0.0528	0.0903	0.0095*
Limestone-by-inbred	0.1818	0.4583	0.8024	0.1939	0.2084	0.4130	0.9344	0.5854
Limestone-by-N form	0.1005	0.5249	0.0191*	0.0684	0.9351	0.0146*	0.6208	0.8731
Inbred line-by-N form	0.2427	0.0187*	0.1010	0.0046*	0.0429*	0.0143*	0.0525	0.1242
Limestone-by-inbred-by-N form	0.1315	0.5192	0.1832	0.3629	0.1178	0.2563	0.5796	0.1294
R^2	0.9375	0.9018	0.9681	0.9133	0.8509	0.9259	0.8916	0.9471
CV	64.89	38.37	23.84	38.00	39.23	34.81	42.12	43.60
Transformation	-	Rank	Rank	Rank	Rank	Rank	Rank	-

[a] Experimental design was a completely randomized, split-split block with nitrogen (N) fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] Incidence of wilt was measured by determining the presence or absence of wilt symptoms for each plant in 3 m of each of two rows/plot on the dates indicated, and calculating the percentage of plants with wilt symptoms.

[c] Vascular discoloration was measured by determining the presence or absence of dark internal vascular discoloration in the root tissue of plants harvested from 1 m of row in each plot on 22 July, and calculating the percentage of plants with vascular discoloration.

[d] Dried plant biomass (g/m row) was measured by harvesting, drying at 35°C, and weighing the tissue of whole plants from 1 m of row/plot.

[e] Marketable seed yield (kg/ha) was determined by harvesting seed from all plants in 1 m of the center four rows/plot, followed by drying, cleaning, and sizing the seed (sizes 7 to 13).

Table 2.2. Effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on wilt incidence, vascular discoloration, dried plant biomass, and seed yield in a 2009 spinach seed crop field trial in Skagit Co., WA

	Wilt incidence (%)[b]				Vascular discoloration	Dried plant biomass (g/m row)[d]		Marketable seed yield
Factor[a]	**15 May**	**4 June**	**24 June**	**14 July**	**(%)[c]**	**29 June**	**22 July**	**(kg/ha)[e]**
N-form								
Ammonium (NH_4)	2.2	4.6	8.3 b	88.8	96.7	112.7	144.7	506 a
Nitrate (NO_3)	1.9	3.4	26.3 a	92.9	94.2	73.9	99.8	276 b
LSD	NS	NS	Rank	NS	NS	NS	NS	123
Spinach inbred line								
Susceptible	2.4	4.7 ab	24.4	99.5 a	100.0 a	97.6 a	105.6	291 b
Moderate	2.2	3.6 b	18.2	92.6 b	94.0 b	89.9 b	124.3	458 a
Resistant	1.6	7.0 a	9.3	80.4 c	92.5 b	71.7 c	127.6	423 a
Male	2.0	0.6 c	-	-	-	113.9 a	131.4	-
LSD	NS	Rank	NS	Rank	Rank	Rank	NS	121
Limestone (t/ha)								
0	1.9	5.4	33.6 a	92.0	98.3 a	60.6 c	83.2 b	206 b
2.24	2.4	3.5	12.9 ab	89.1	97.1 a	95.0 b	129.7 a	413 ab
4.48	1.8	3.1	5.3 b	91.4	91.0 b	124.3 a	153.7 a	554 a
LSD	NS	NS	Rank	NS	Rank	Rank	Rank	238
Inbred-by-N form								
Susceptible								
NH_4	-	5.1	-	99.0 b	100.0	123.1 a	-	-
NO_3	-	4.3	-	100.0 a	100.0	72.2 b	-	-
LSD	-	NS	-	Rank	NS	39.7	-	-
Moderate								
NH_4	-	5.3 a	-	95.1	99.2 a	102.9	-	-
NO_3	-	2.0 b	-	90.0	88.9 b	76.8	-	-
LSD	-	Rank	-	NS	Rank	NS	-	-
Resistant								
NH_4	-	7.4	-	72.3 b	91.1	82.6	-	-
NO_3	-	6.5	-	88.5 a	93.8	60.9	-	-
LSD	-	NS	-	Rank	NS	NS	-	-

Factor[a]	Wilt incidence (%)[b]				Vascular discoloration (%)[c]	Dried plant biomass (g/m row)[d]		Marketable seed yield (kg/ha)[e]
	15 May	4 June	24 June	14 July		29 June	22 July	
Male								
NH_4	-	0.6	-	-	-	142.3 a	-	-
NO_3	-	1.7	-	-	-	85.5 b	-	-
LSD	-	NS	-	-	-	42.6	-	-
Limestone (t/ha)-by-N form								
0								
NH_4	-	-	13.4 b	-	-	98.1 a	-	-
NO_3	-	-	53.8 a	-	-	23.1 b	-	-
LSD	-	-	Rank	-	-	Rank	-	-
2.24								
NH_4	-	-	6.4 b	-	-	112.3 a	-	-
NO_3	-	-	19.5 a	-	-	77.7 b	-	-
LSD	-	-	Rank	-	-	Rank	-	-
4.48								
NH_4	-	-	4.9	-	-	127.8	-	-
NO_3	-	-	5.7	-	-	120.7	-	-
LSD	-	-	NS	-	-	NS	-	-

[a] Each value is the mean of four replications. Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. Raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant differences.

[b] Incidence of wilt was measured by determining the presence or absence of wilt symptoms for each plant in 3 m of each of two rows/plot on the dates indicated, and calculating the percentage of plants with wilt symptoms.

[c] Vascular discoloration was measured by determining the presence or absence of dark internal vascular discoloration in the root tissue of plants harvested from 1 m of row in each plot on 22 July, and calculating the percentage of plants with root vascular discoloration.

[d] Dried plant biomass (g/m row) was measured by harvesting, drying at 35°C, and weighing the tissue of whole plants from 1 m of row/plot.

[e] Marketable seed yield (kg/ha) was determined by harvesting seed from all plants in 1 m of the center four rows/plot, followed by drying, cleaning, and sizing the seed (sizes 7 to 13).

Table 2.3. Analyses of variance (ANOVAs) for the fixed effects of limestone application rate and spinach inbred line susceptibility on incidence and severity of spinach wilt, vascular discoloration, dried spinach plant weight, and seed yield in a 2012 spinach seed crop field trial in Skagit Co., WA

	Wilt incidence (%)[b]			Wilt severity (0 to 5)[c]		Vascular discoloration (%)[d]	Dried plant biomass (g/m row)[e]	Marketable seed yield (kg/ha)[f]
ANOVA factor[a]	**29 May**	**19 June**	**9 July**	**9 July**	**31 July**		**11 July**	
Limestone rate	0.2225	0.1534	0.0001*	0.0003*	0.0004*	0.0367*	<0.0001*	<0.0001*
Spinach inbred line	0.0133*	0.0028*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0130*	<0.0001*
Limestone-by-inbred	0.3685	0.0233*	0.0002*	0.8600	0.1258	0.3762	0.6135	0.0015*
R^2	0.7611	0.9570	0.9481	0.9452	0.9759	0.8859	0.9151	0.9685
CV	71.75	6.98	16.96	18.93	13.04	26.98	12.24	14.90
Transformation	–	Log	Rank	Rank	Rank	Rank	–	Rank

[a] Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to the split plots.

* = significant at $P \leq 0.05$. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to log or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA.

[b] Incidence of wilt measured by determining the presence or absence of wilt symptoms for each plant in 3 m of each of two rows/plot, and calculating the percentage of plants with wilt symptoms.

[c] Severity of wilt measured by rating wilt symptoms on a 0 to 5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt) for each plant in 3 m of two rows/plot.

[d] Vascular discoloration measured by determining the presence or absence of dark internal vascular discoloration in the root tissue of plants harvested from 1 m of row in each plot on 22 July, and calculating the percentage of plants with vascular discoloration.

[e] Dried plant biomass (g/m row) was obtained by harvesting, drying at 35°C,and weighing the combined tissue of whole spinach plants from 1 m of row/plot.

[f] Marketable seed yield (kg/ha) determined by harvesting seed from all spinach plants from 1 m of each of four rows/plot, followed by drying, cleaning, and sizing the seed (sizes 7 to 13).

Table 2.4. Effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on incidence and severity of spinach wilt, vascular discoloration, dried spinach plant biomass, and seed yield in a 2012 spinach seed crop field trial in Skagit Co., WA

	Wilt incidence (%)[b]			Wilt severity (0 to 5)[c]		Vascular discoloration	Biomass (g/m row)	Marketable seed yield
Factor[a]	**29 May**	**19 June**	**9 July**	**9 July**	**31 July**	**(%)[d]**	**11 July[e]**	**(kg/ha)[f]**
Spinach inbred line								
Susceptible	1.3 b	24.3 a	98.3 a	2.62 b	3.97 a	96.2 a	183.1 a	418 c
Moderate	1.8 b	10.7 b	84.6 b	1.76 c	3.46 b	69.4 b	185.5 a	731 b
Resistant	2.0 b	9.4 b	68.5 c	1.22 d	2.70 c	57.7 c	161.8 b	1,136 a
Male	3.1 a	29.6 a	99.3 a	2.98 a	-	95.1 a	166.6 b	-
LSD	1.0	Log	Rank	Rank	Rank	Rank	15.5	Rank
Limestone (t/ha)								
0	1.8	23.9	96.0 a	2.58 a	3.63 a	70.7 b	114.1 c	407 d
0/4.48	1.2	16.7	92.8 ab	2.26 ab	3.47 a	87.0 a	167.0 b	702 c
2.24	2.9	20.0	87.7 b	2.07 b	3.27 b	83.5 ab	194.2 ab	920 b
4.48	2.4	13.5	74.1 c	1.66 c	3.14 b	77.4 ab	221.8 a	1,018 a
LSD	NS	NS	Rank	Rank	Rank	Rank	31.2	Rank
Inbred-by-limestone (t/ha)								
Susceptible								
0	-	35.5 a	99.8	-	-	-	-	238 b
0/4.48	-	16.5 b	100.0	-	-	-	-	340 b
2.24	-	29.3 a	99.8	-	-	-	-	515 a
4.48	-	15.8 b	93.5	-	-	-	-	579 a
LSD	-	Sq root	NS	-	-	-	-	147
Moderate								
0	-	15.0	94.6 a	-	-	-	-	350 c
0/4.48	-	10.9	92.8 a	-	-	-	-	693 b
2.24	-	10.0	84.8 b	-	-	-	-	879 a
4.48	-	7.0	66.3 c	-	-	-	-	1,003 a
LSD	-	NS	Rank	-	-	-	-	168
Resistant								

Factor[a]	Wilt incidence (%)[b]			Wilt severity (0 to 5)[c]		Vascular discoloration (%)[d]	Biomass (g/m row)	Marketable seed yield (kg/ha)[f]
	29 May	19 June	9 July	9 July	31 July		11 July[e]	
0	-	7.6	89.7 a	-		-	-	632 c
0/4.48	-	13.7	78.4 b	-		-	-	1,074 b
2.24	-	10.3	66.4 c	-		-	-	1,366 a
4.48	-	6.0	39.5 d	-		-	-	1,473 a
LSD	-	NS	Rank	-		-	-	209
Male								
0	-	37.2	100.0	-		-	-	-
0/4.48	-	25.6	100.0	-		-	-	-
2.24	-	30.6	100.0	-		-	-	-
4.48	-	25.1	97.3	-		-	-	-
LSD	-	NS	NS	-		-	-	-

[a] Each value is the mean of five replications. Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to the split plots. Raw data were subjected to log, square root (sq. root), or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] Incidence of wilt measured by determining the presence or absence of wilt symptoms for each plant in 3 m of each of two rows/plot, and calculating the percentage of plants with wilt symptoms.

[c] Severity of wilt measured by rating wilt symptoms on a 0 to 5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt) for each plant in 3 m of two rows/plot.

[d] Vascular discoloration measured by determining the presence or absence of dark internal vascular discoloration in the root tissue of plants harvested from 1 m of row in each plot on 22 July, and calculating the percentage of plants with vascular discoloration.

[e] Dried plant biomass (g/m row) was obtained by harvesting, drying at 35°C,and weighing the combined tissue of whole spinach plants from 1 m of row/plot.

[f] Marketable seed yield (kg/ha) determined by harvesting seed from all spinach plants from 1 m of each of four rows/plot, followed by drying, cleaning, and sizing the seed (sizes 7 to 13).

Table 2.5. Probability values from the analyses of variance (ANOVAs) for the fixed effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on spinach seed germination and health assays in a 2009 spinach seed crop field trial in Skagit Co., WA

	Seed germination assay[b]			**Freeze-blotter seed health assay[c]**			
ANOVA factor[a]	**Germinated seed (%)**	**Non-germinated seed (%)**	**Rotten seed (%)**	***Fusarium* spp. (%)**	***Verticillium dahliae* (%)**	***Stemphylium botryosum* (%)**	***Alternaria* spp. (%)**
Limestone rate	0.3530	0.0847	0.2701	0.1222	0.0713	0.2377	0.2673
Spinach inbred line	0.1634	0.2425	0.0022*	0.0185*	0.1705	0.0118*	0.0721
Nitrogen form	0.0126*	0.0106*	0.0044*	0.1599	0.0663	0.1035	0.6159
Limestone-by-inbred	0.0869	0.5974	0.1178	0.6353	0.1684	0.2805	0.6931
Limestone-by-N form	0.4067	0.7007	0.8316	0.7853	0.9307	0.1390	0.3046
Inbred-by-N form	0.3209	0.1131	0.4695	0.7036	0.4620	0.7942	0.2940
Limestone-by-inbred-by-N form	0.0735	0.7437	0.2901	0.3124	0.0831	0.6205	0.3020
R^2	0.9886	0.9876	0.9856	0.9348	0.9573	0.8852	0.8648
CV	18.0	7.01	7.66	35.35	30.77	42.23	19.15
Transformation	Rank	Log	Log	Rank	NS	Arcsine	NS

[a] Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to arcsine, log, or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] A subsample of 100 seeds/plot was tested for germination using a blotter assay modified from the protocol of the Association of Official Seed Analysts (2008), as described in the main text.

[c] A freeze-blotter seed health assay for necrotrophic fungi was carried out on 100 seeds/plot as described by du Toit (2011) and in the main text.

Table 2.6. Effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on seed germination and health assays in a 2009 spinach seed crop field trial in Skagit Co., WA

	Seed germination assay[b]			Freeze-blotter seed health assay[c]			
Factor[a]	**Germinated seed (%)**	**Non-germinated seed (%)**	**Rotten seed (%)**	***Fusarium* spp. (%)**	***Verticillium dahliae* (%)**	***Stemphylium botryosum* (%)**	***Alternaria* spp. (%)**
N form							
Ammonium (NH_4)	83.2 a	5.3 b	7.4 a	0.9	3.3	4.9	20.8
Nitrate (NO_3)	58.9 b	16.4 a	16.6 b	2.9	9.1	4.4	21.3
LSD	Rank	Log	Log	NS	NS	NS	NS
Spinach inbred line							
Susceptible	64.8	10.8	20.0 a	4.2 a	1.8	3.2 b	17.2
Moderate	75.3	9.0	8.3 b	0.9 b	7.9	7.4 a	23.6
Resistant	75.1	11.7	7.4 b	0.7 b	7.9	3.3 b	21.7
LSD	NS	NS	NS	Rank	NS	Arcsine	NS
Limestone (t/ha)							
0	69.8	10.2	13.1	3.4	2.2	5.5	20.4
2.24	68.6	12.1	13.7	1.7	6.3	4.6	20.0
4.48	77.0	9.0	8.6	0.6	9.1	3.9	22.6
LSD	NS	NS	NS	NS	NS	NS	NS

[a] Each value is the mean of four replications. Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. Raw data were subjected to log, arcsine, or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] A subsample of 100 seeds/plot was tested for germination using a blotter assay modified from the protocol of the Association of Official Seed Analysts (2008), as described in the main text.

[c] A freeze-blotter seed health assay for necrotrophic fungi was carried out on 100 seeds/plot as described by du Toit (2011) and in the main text.

Table 2.7. Probability values from the analyses of variance (ANOVAs) for the fixed effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on spinach seed quality and incidence of seedborne fungi in a 2012 spinach seed crop field trial in Skagit Co., WA

	Seed germination assay[b]			**NP-10 agar seed health assay[c]**			
ANOVA factor[a]	**Germinated (%)**	**Non-germinated (%)**	**Rotten (%)**	***Fusarium* spp. (%)**	***Verticillium dahliae* (%)**	***Stemphylium botryosum* (%)**	***Alternaria* spp. (%)**
Limestone rate	0.8809	0.9908	0.7998	0.1626	<0.0001*	0.0555	0.0059*
Spinach inbred line	0.0134*	0.0188*	<0.0001*	<0.0001*	0.0071*	0.1195	0.0014*
Limestone-by-inbred	0.8683	0.8246	0.9134	0.1647	0.0136*	0.1020	0.7170
R^2	0.9110	0.8581	0.8941	0.9440	0.9325	0.7973	0.8629
CV	12.20	26.90	27.24	30.83	27.85	37.71	14.97
Transformation	-	-	Rank	Arcsine	Arcsine	Rank	-

[a] Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to split plots. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to arcsine or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] A subsample of 100 seeds/plot was tested for germination using a blotter assay modified from the protocol of the Association of Official Seed Analysts (2008), as described in the main text.

[c] An NP-10 agar seed health assay for necrotrophic fungi was carried out on 100 seeds/plot as described by du Toit (2011) and in the main text.

Table 2.8. Effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on spinach seed quality and incidence of seedborne fungi in a 2012 spinach seed crop field trial in Skagit Co., WA

	Seed germination assay[b]			NP-10 agar seed health assay[c]			
Factor[a]	**Germinated seed (%)**	**Non-germinated seed (%)**	**Rotten seed (%)**	***Fusarium* spp. (%)**	***Verticillium dahliae* (%)**	***Stemphylium botryosum* (%)**	***Alternaria* spp. (%)**
Spinach inbred line							
Susceptible	50.8 b	15.7 b	32.6 a	10.6 a	2.5 b	67.3	33.6 b
Moderate	61.5 a	17.2 b	20.5 b	2.1 b	7.8 a	74.4	47.8 a
Resistant	67.0 a	26.2 a	6.3 c	0.4 c	5.7 a	68.8	42.3 a
LSD	9.66	7.05	Rank	Arcsine	Arcsin	NS	5.7
Limestone (t/ha)							
0	57.3	19.5	22.2	5.7	0.9 c	62.5	45.5 a
0/4.48	61.9	19.1	18.5	5.2	3.2 b	77.9	46.1 a
2.24	59.2	20.3	19.8	3.3	5.6 b	72.3	39.9 ab
4.48	60.7	19.9	18.7	3.1	11.5 a	68.0	33.3 b
LSD	NS	NS	NS	NS	Arcsine	NS	7.0
Inbred-by-limestone (t/ha)							
Susceptible							
0	-	-	-	-	0.0 b	-	-
0/4.48	-	-	-	-	2.4 a	-	-
2.24	-	-	-	-	3.4 a	-	-
4.48	-	-	-	-	4.2 a	-	-
LSD	-	-	-	-	Arcsine	-	-
Moderate							
0	-	-	-	-	1.2 c	-	-
0/4.48	-	-	-	-	3.0 c	-	-
2.24	-	-	-	-	8.6 b	-	-
4.48	-	-	-	-	18.2 a	-	-
LSD	-	-	-	-	Arcsine	-	-
Resistant							
0	-	-	-	-	1.4 c	-	-

Factor[a]	Seed germination assay[b]			NP-10 agar seed health assay[c]			
	Germinated seed (%)	Non-germinated seed (%)	Rotten seed (%)	*Fusarium* spp. (%)	*Verticillium dahliae* (%)	*Stemphylium botryosum* (%)	*Alternaria* spp. (%)
0/4.48	-	-	-	-	4.2 b	-	-
2.24	-	-	-	-	4.8 b	-	-
4.48	-	-	-	-	12.2 a	-	-
LSD	-	-	-	-	Arcsine	-	-

[a] Each value is the mean of five replications. Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to the split plots. Raw data were subjected to arcsine or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] A subsample of 100 seeds/plot was tested for germination using a blotter assay modified from the protocol of the Association of Official Seed Analysts (2008), as described in the main text.

[c] An NP-10 agar seed health assay for necrotrophic fungi was carried out on 100 seeds/plot, as described by du Toit (2011) and in the main text.

Table 2.9. Probability values from the analyses of variance (ANOVAs) for the fixed effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on spinach plant nutrient analyses in a 2009 spinach seed crop field trial in Skagit Co., WA

	Plant nutrient analyses (29 June)[b]								
ANOVA factor[a]	**N (%)**	**P (%)**	**Ca (%)**	**Mg (%)**	**S (%)**	**B (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Fe (mg/kg)**
Limestone rate	0.4337	0.0004*	0.0028*	0.0025*	0.0019*	0.1676	0.0211*	0.0457*	0.6527
Spinach inbred line	<0.0001*	<0.0001*	0.0002*	0.0004*	0.0002*	0.0096*	<0.0001*	0.0005*	0.0004*
N form	0.0380*	0.0042*	0.0988	0.0283*	0.0106*	0.0033*	0.0006*	0.1496	0.2162
Limestone-by-inbred	0.0819	0.1554	0.2343	0.0637	0.2877	0.1710	0.0010*	0.1124	0.2914
Limestone-by-N form	0.6544	0.6861	0.5069	0.0219*	0.2019	0.1178	0.4568	0.0167*	0.0718
Inbred-by-N form	0.0044*	0.0109*	0.0075*	0.0496*	0.0046*	0.2324	0.2404	0.4115	0.1719
Limestone-by-inbred-by-N form	0.0179*	0.2950	0.4744	0.2673	0.4533	0.0828	0.1342	0.3862	0.2252
R^2	0.9595	0.9745	0.9456	0.9301	0.9572	0.9390	0.9634	0.9061	0.8739
CV	5.54	3.75	10.00	33.76	7.97	31.58	2.49	39.18	45.42
Transformation	Arcsine	Sq root	-	Rank	Arcsine	Rank	Log	Rank	Rank

[a] Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to arcsine, square root (sq root), log, or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] Plant nutrient analyses were carried out on whole plants harvested from 1 m of row/plot on 29 June, dried at 35°C, and sent to Soiltest Farm Consultants, Inc. in Moses Lake, WA for testing.

Table 2.10. Effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on spinach plant nutrient analyses in a 2009 spinach seed crop field trial in Skagit Co., WA

	Plant nutrient analyses (29 June)[b]								
Factor[a]	**N (%)**	**P (%)**	**Ca (%)**	**Mg (%)**	**S (%)**	**B (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Fe (mg/kg)**
N form									
NH_4	3.18 a	0.54 a	1.19	0.54 b	0.24 a	23.5 b	93.2 b	155.1	3,728
NO_3	2.81 b	0.41 b	1.29	0.70 a	0.16 b	30.8 a	120.8 a	148.2	5,064
LSD	Arcsine	Sq root	NS	Rank	Arcsine	Rank	Log	NS	NS
Spinach inbred line									
Susceptible	2.57 c	0.47 b	1.07 c	0.56 b	0.17 b	23.7 b	80.3 c	147.9 bc	5,068 a
Moderate	3.14 b	0.52 a	1.22 b	0.65 a	0.22 a	28.8 a	122.8 a	166.5 a	5,063 a
Resistant	3.73 a	0.47 b	1.37 a	0.71 a	0.23 a	30.6 a	98.8 b	153.8 b	4,086 a
Male	2.53 d	0.43 c	1.28 b	0.59 b	0.17 b	25.6 b	126.3 a	138.4 c	3,367 b
LSD	Arcsine	Sq root	0.09	Rank	Arcsine	Rank	Log	Rank	Rank
Limestone (t/ha)									
0	2.96	0.43 b	1.07 b	0.75 a	0.17 c	29.3	117.2 a	178.2 a	6,021
2.24	3.09	0.48 a	1.27 a	0.62 a	0.20 b	27.6	106.8 ab	143.0 b	3,699
4.48	2.94	0.52 a	1.37 a	0.51 b	0.22 a	24.5	97.1 b	33.8 b	3,468
LSD	NS	Sq root	0.13	Rank	Arcsine	NS	Log	Rank	NS
Inbred-by-limestone (t/ha)									
Susceptible									
0	-	-	-	-	-	-	86.5	-	-
2.24	-	-	-	-	-	-	78.1	-	-
4.48	-	-	-	-	-	-	76.1	-	-
LSD	-	-	-	-	-	-	NS	-	-
Moderate									
0	-	-	-	-	-	-	128.3	-	-
2.24	-	-	-	-	-	-	123.6	-	-
4.48	-	-	-	-	-	-	116.4	-	-
LSD	-	-	-	-	-	-	NS	-	-
Resistant									
0	-	-	-	-	-	-	120.9 a	-	-
2.24	-	-	-	-	-	-	93.6 b	-	-

Factor[a]	Plant nutrient analyses (29 June)[b]								
	N (%)	P (%)	Ca (%)	Mg (%)	S (%)	B (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Fe (mg/kg)
4.48	-	-	-	-	-	-	81.9 b	-	-
LSD	-	-	-	-	-	-	Sq root	-	-
Male									
0	-	-	-	-	-	-	133.1	-	-
2.24	-	-	-	-	-	-	133.9	-	-
4.48	-	-	-	-	-	-	112.0	-	-
LSD	-	-	-	-	-	-	NS	-	-
Inbred-by-N form									
Susceptible									
NH_4	2.95 a	0.54 a	1.07	0.49	0.21 a	-	-	-	-
NO_3	2.20 b	0.39 b	1.07	0.62	0.13 b	-	-	-	-
LSD	Rank	0.06	NS	NS	Arcsine	-	-	-	-
Moderate									
NH_4	3.33 a	0.60 a	1.16	0.58	0.27 a	-	-	-	-
NO_3	2.96 b	0.44 b	1.28	0.72	0.18 b	-	-	-	-
LSD	Rank	0.05	NS	NS	Arcsine	-	-	-	-
Resistant									
NH_4	3.93 a	0.54 a	1.34	0.62	0.29 a	-	-	-	-
NO_3	3.54 b	0.41 b	1.40	0.79	0.17 b	-	-	-	-
LSD	Rank	0.04	NS	NS	Arcsine	-	-	-	-
Male									
NH_4	2.51 a	0.47 a	1.17 b	0.49 b	0.18	-	-	-	-
NO_3	2.55 b	0.39 b	1.40 a	0.68 a	0.16	-	-	-	-
LSD	Rank	0.03	0.19	Rank	NS	-	-	-	-
Limestone (t/ha)-by-N form									
0									
NH_4	-	-	-	0.58 a	-	-	-	168.0	-
NO_3	-	-	-	0.91 b	-	-	-	188.4	-
LSD	-	-	-	Rank	-	-	-	NS	-
2.24									
NH_4	-	-	-	0.55 b	-	-	-	151.6	-

	Plant nutrient analyses (29 June)[b]								
Factor[a]	**N (%)**	**P (%)**	**Ca (%)**	**Mg (%)**	**S (%)**	**B (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Fe (mg/kg)**
NO_3	-	-	-	0.69 a	-	-	-	134.3	-
LSD	-	-	-	Rank	-	-	-	NS	-
4.48									
NH_4	-	-	-	0.50	-	-	-	145.7 a	-
NO_3	-	-	-	0.52	-	-	-	121.8 b	-
LSD	-	-	-	NS	-	-	-	Rank	-

[a] Each value is the mean of four replications. Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. Raw data were subjected to log, arcsine, square root (sq. rot), or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each main factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] Plant nutrient analyses were carried out on whole plants harvested from 1 m of row/plot on 29 June, dried at 35°C, and sent to Soiltest Farm Consultants, Inc., in Moses Lake, WA for testing.

Table 2.11. Probability values from analyses of variance (ANOVAs) for the fixed effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on spinach plant nutrient analyses in a 2012 spinach seed crop field trial in Skagit Co., WA

	Plant nutrient analyses (11 July)[b]								
ANOVA factor[a]	**N (%)**	**P (%)**	**Ca (%)**	**Mg (%)**	**S (%)**	**B (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Fe (mg/kg)**
Limestone rate	0.2328	0.8781	0.0657	0.0096*	0.1104	0.0105*	0.0033*	0.0012*	0.6022
Spinach inbred line	0.0149*	0.5484	0.4050	0.1321	0.1387	0.1232	0.1588	0.1443	0.1636
Limestone-by-inbred	0.5537	0.4009	0.0056*	0.5520	0.2251	0.1508	0.6544	0.6501	0.8758
R^2	0.7795	0.7022	0.8494	0.7080	0.6807	0.7992	0.7851	0.8388	0.6868
CV	37.94	12.03	12.22	16.03	11.96	36.18	37.44	16.57	33.38
Transformation	Rank	-	-	-	-	Rank	Rank	-	-

[a] Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to split plots. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = when necessary, raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] Plant nutrient analyses were carried out on whole plants harvested from 1 m of row/plot on 11 July, dried at 35°C, and sent to Soiltest Farm Consultants, Inc. in Moses Lake, WA.

Table 2.12. Effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on spinach plant tissue nutrient analyses in a 2012 spinach seed crop field trial in Skagit Co., WA

	Plant nutrient analyses (11 July)[b]								
Factor[a]	**N (%)**	**P (%)**	**Ca (%)**	**Mg (%)**	**S (%)**	**B (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Fe (mg/kg)**
Spinach inbred line									
Susceptible	3.11 b	0.60	1.17	0.37	0.23	71.8	63.0	88.4	1,211
Moderate	3.08 b	0.59	1.19	0.39	0.23	59.8	75.7	97.9	1,582
Resistant	3.37 a	0.62	1.22	0.43	0.25	85.1	71.8	103.3	1,649
Male	3.08 b	0.60	1.27	0.40	0.22	83.4	70.1	95.1	1,274
LSD	Rank	NS	NS	NS	NS	NS	NS	NS	NS
Limestone (t/ha)									
0	3.34	0.60	1.03	0.46 a	0.23	102.7 a	83.5 a	124.6 a	1,393
0/4.48	3.17	0.60	1.21	0.39 b	0.23	79.6 ab	70.3 b	97.3 b	1,362
2.24	3.08	0.63	1.29	0.38 b	0.24	63.0 bc	67.6 bc	87.8 bc	1,536
4.48	3.04	0.60	1.31	0.36 b	0.23	54.9 c	59.3 c	75.0 c	1,425
LSD	NS	NS	NS	0.053	NS	Rank	Rank	20.1	NS
Limestone (t/ha)-by-inbred									
Susceptible									
0	-		0.93 b	-	-	-	-	-	-
0/4.48	-		1.15 ab	-	-	-	-	-	-
2.24	-		1.31 a	-	-	-	-	-	-
4.48	-		1.27 a	-	-	-	-	-	-
LSD	-		0.24	-	-	-	-	-	-
Moderate									
0	-		1.18	-	-	-	-	-	-
0/4.48	-		1.01	-	-	-	-	-	-
2.24	-		1.34	-	-	-	-	-	-
4.48	-		1.22	-	-	-	-	-	-
LSD	-		NS	-	-	-	-	-	-
Resistant									
0	-		0.95 b	-	-	-	-	-	-
0/4.48	-		1.39 a	-	-	-	-	-	-

	Plant nutrient analyses (11 July)[b]								
Factor[a]	**N (%)**	**P (%)**	**Ca (%)**	**Mg (%)**	**S (%)**	**B (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Fe (mg/kg)**
2.24	-		1.21 a	-	-	-	-	-	-
4.48	-		1.32 a	-	-	-	-	-	-
LSD	-		0.19	-	-	-	-	-	-
Male									
0	-		1.07	-	-	-	-	-	-
0/4.48	-		1.29	-	-	-	-	-	-
2.24	-		1.31	-	-	-	-	-	-
4.48	-		1.43	-	-	-	-	-	-
LSD	-		NS	-	-	-	-	-	-

[a] Each value is the mean of five replications. Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to the split plots. Raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each main factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] Plant nutrient analyses were carried out on whole plants harvested from 1 m of row/plot on 11 July, dried at 35°C, and sent to Soiltest Farm Consultants, Inc. in Moses Lake, WA.

Table 2.13. Probability values from the analyses of variances (ANOVAs) for the fixed effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on soil populations of *Fusarium oxysporum* and *Verticillium dahliae* in a 2009 spinach seed crop field trial in Skagit Co., WA

	***Fusarium oxysporum* (CFU/g soil)[b]**		***Verticillium dahliae* (CFU/g soil)[c]**	
Factor[a]	**5 May**	**19 August**	**5 May**	**19 August**
Limestone rate	0.3910	0.3237	0.1478	0.2859
Spinach inbred line	-	0.0425*	-	0.3654
N form	-	0.3172	-	0.0460*
Limestone-by-inbred	-	0.6178	-	0.2621
Limestone-by-N form	-	0.2204	-	0.5034
Inbred-by-N form	-	0.2373	-	0.6742
Limestone-by-inbred-by-N form	-	0.8579	-	0.3255
R^2	0.5202	0.9529	0.5220	0.8977
CV	23.27	29.00	22.83	68.47
Transformation	-	Rank	-	-

[a] Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. R^2 = coefficient of determination. CV = coefficient of variance. Rank = raw data subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$. For soil sampling, 12 cores (2.5 cm x 15 cm depth) were obtained from each plot, mixed by hand, and a subsample dried and passed through a 1-mm aperture sieve.

[b] Population of *F. oxysporum* (including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae*) was quantified by dilution plating soil on Komada's agar medium (Komada, 1975) (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after plating.

[c] Population of *V. dahliae* was quantified by distributing 1 g soil/sample on the surface of each of 10 plates of NP-10 agar medium (Sorensen et al., 1991) using a sterilized glass salt shaker for each sample. Plates were incubated in the dark at 26°C for 28 days, followed by enumeration of *V. dahliae*-type colonies using a dissecting microscope.

Table 2.14. Effects of limestone application rate, spinach inbred line susceptibility to Fusarium wilt, and nitrate vs. ammonium fertilizers on soil populations of *Fusarium oxysporum* and *Verticillium dahliae* in a 2009 spinach seed crop field trial in Skagit Co., WA

	***Fusarium oxysporum* (CFU/g soil)[b]**		***Verticillium dahliae* (CFU/g soil)[c]**	
Factor[a]	**5 May**	**19 August**	**5 May**	**19 August**
N form				
Ammonium (NH_4)	-	5,328	-	25.3 b
Nitrate (NO_3)	-	4,016	-	47.9 a
LSD	-	NS	-	21.78
Spinach inbred line				
Susceptible	-	4,874 a	-	31.0
Moderate	-	5,339 a	-	34.5
Resistant	-	3,802 b	-	44.3
LSD	-	Rank		NS
Limestone (t/ha)				
0	1,780	4,721	21.3	34.6
2.24	2,283	4,875	28.0	29.7
4.48	2,183	4,420	31.0	45.7
LSD	NS	NS	NS	NS

[a] Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. Rank = raw data subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$. For soil sampling, 12 cores (2.5 cm x 15 cm depth) were obtained from each plot, mixed by hand, and a subsample dried and passed through a 1-mm aperture sieve. Means followed by the same letter for each main factor are not significantly different based on Fisher's least protected significant difference ($P \leq 0.05$). NS = no significant difference.

[b] Population of *F. oxysporum* (including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae*) was quantified by dilution plating soil on Komada's agar medium (Komada, 1975) (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after plating.

[c] Population of *V. dahliae* was quantified by distributing 1 g soil/sample on the surface of each of 10 plates of NP-10 agar medium (Sorensen et al., 1991) using a sterilized glass salt shaker for each sample. Plates were incubated in the dark at 26°C for 28 days, followed by enumeration of *V. dahliae*-type colonies using a dissecting microscope.

Table 2.15. Probability values from analyses of variance (ANOVAs) for the fixed effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on soil populations of *Fusarium oxysporum* and *Verticillium dahliae* in a 2012 spinach seed crop field trial in Skagit Co., WA

	***Fusarium oxysporum* (CFU/g soil)[b]**			***Verticillium dahliae* (CFU/g soil)[c]**	
Factor[a]	**9 May**	**9 July**	**24 August**	**9 May**	**24 August**
Limestone rate	0.8639	0.8241	0.0374*	0.0018*	<0.0001*
Spinach inbred line	-	-	0.0079*	-	0.0149*
Limestone-by-inbred	-	-	0.0529	-	0.7470
R^2	0.3899	0.1160	0.8643	0.8178	0.8165
CV	42.00	34.49	15.28	18.02	9.43
Transformation	-	-	-	-	Log

[a] Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to split plots. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data were subjected to log transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$. For soil sampling, 12 cores (2.5 cm x 15 cm depth) were collected from each plot, mixed by hand, and a subsample dried and passed through a 1-mm aperture sieve.

[b] Population of *F. oxysporum* (including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae*) was quantified by dilution plating soil on Komada's agar medium (Komada, 1975) (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after plating.

[c] Population of *V. dahliae* was quantified by distributing 1 g soil/sample on the surface of each of 10 plates of NP-10 agar medium (Sorensen et al., 1991) using a sterilized glass salt shaker for each sample. Plates were incubated in the dark at 26°C for 28 days, followed by enumeration of *V. dahliae*-type colonies using a dissecting microscope.

Table 2.16. Effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on soil populations of *Fusarium oxysporum* and *Verticillium dahliae* in a 2012 spinach seed crop field trial in Skagit Co., WA

	***Fusarium oxysporum* (CFU/g soil)[b]**			***Verticillium dahliae* (CFU/g soil)[c]**	
Factor[a]	**9 May**	**9 July**	**24 August**	**9 May**	**24 August**
Spinach inbred line					
Susceptible	-	-	6,323 a	-	53.7 b
Moderate	-	-	5,245 b	-	55.5 b
Resistant	-	-	4,388 b	-	75.0 a
Male	-	-	1,031	-	Log
LSD					
Limestone (t/ha)					
0	1,080	2,280	5,280 ab	45.8 c	32.6 d
0/4.48	1,347	1,893	6,027 a	57.0 bc	47.8 c
2.24	1,173	1,947	5,147 b	72.8 ab	62.1 b
4.48	1,240	2,007	4,822 b	81.2 a	103.1 a
LSD	NS	NS	796	15.9	Log

[a] Each value is the mean of five replications. Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to the split plots. Split plots were sampled in the beginning of the season (and mid-season for *F. oxysporum*), while all plots were sampled at the end of the season. Log = raw data subjected to log transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference. For soil sampling, 12 cores (2.5 cm x 15 cm depth) were collected from each plot, mixed by hand, and a subsample dried and passed through a 1-mm aperture sieve.

[b] Population of *F. oxysporum* (including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae*) was quantified by dilution plating soil on Komada's agar medium (Komada, 1975) (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after plating.

[c] Population of *V. dahliae* was quantified by distributing 1 g soil/sample on the surface of each of 10 plates of NP-10 agar medium (Sorensen et al., 1991) using a sterilized glass salt shaker for each sample. Plates were incubated in the dark at 26°C for 28 days, followed by enumeration of *V. dahliae*-type colonies using a dissecting microscope.

Table 2.17. Effects of limestone application rates and nitrate vs. ammonium fertilizers on soil chemical properties in a 2009 spinach seed crop field trial in Skagit Co., WA

	Soil sampling date					
Factor[a]	**5 May**	**27 May**	**18 June**	**8 July**	**28 July**	**19 August**
				NO_3 (mg/kg)[b]		
N form						
NH_4	-	33.5	-	-	-	44.2
NO_3	-	32.3	-	-	-	54.6
LSD	-	NS	-	-	-	NS
Limestone (t/ha)						
0	5.4	29.7	10.3	15.7 a	27.7 a	64.0 a
2.24	6.1	30.2	9.2	12.9 ab	20.3 b	51.0 a
4.48	6.2	38.7	9.0	9.3 b	13.4 b	33.3 b
LSD	NS	NS	NS	3.8	7.0	Rank
				NH_4 (mg/kg)		
N form						
NH_4	-	9.13 a	-	-	-	3.18
NO_3	-	2.21 b	-	-	-	2.81
LSD	-	Rank	-	-	-	NS
Limestone (t/ha)						
0	1.23 b	9.90	2.00	1.33	2.55	2.96
2.24	1.45 ab	3.88	2.03	1.50	3.43	3.09
4.48	1.55 a	3.24	2.10	1.15	1.63	2.94
LSD	0.30	NS	NS	NS	NS	NS
				P (mg/kg)		
N form						
NH_4	-	230.3 a	-	-	-	312.5 a
NO_3	-	138.1 b	-	-	-	280.4 b
LSD	-	73.1	-	-	-	20.7
Limestone (t/ha)						
0	107.0	175.0	285.3	319.0	255.5	303.6
2.24	105.5	174.1	300.8	320.5	251.0	295.0
4.48	107.8	196.0	292.8	309.0	241.3	290.8
LSD	NS	NS	NS	NS	NS	NS
				K (mg/kg)		
N form						
NH_4	-	267.3 a	-	-	-	241.4
NO_3	-	247.7 b	-	-	-	238.5
LSD		Rank				NS
Limestone (t/ha)						
0	263.5	263.9	271.8	295.5	280.3	247.5 a
2.24	269.0	266.9	273.0	281.5	263.3	246.2 a
4.48	255.0	241.8	261.3	268.8	254.3	226.3 b
LSD	NS	NS	NS	NS	NS	14.3

	5 May	27 May	18 June	8 July	28 July	19 August
				Ca (meq/100 g)		
N form						
NH_4	-	4.78	-	-	-	4.66
NO_3	-	4.82	-	-	-	4.82
LSD		NS				NS
Limestone (t/ha)						
0	3.30 c	3.46 c	3.73 c	3.58 c	3.43 c	3.52 c
2.24	4.58 b	4.95 b	4.83 b	4.73 b	4.78 b	4.88 b
4.48	5.60 a	5.98 a	5.70 a	5.80 a	5.68 a	5.83 a
LSD	0.62	0.49	0.74	0.31	0.62	0.38
				Mg (meq/100 g)		
N form						
NH_4	-	0.583	-	-	-	0.669
NO_3	-	0.600	-	-	-	0.661
LSD		NS				NS
Limestone (t/ha)						
0	0.550	0.575	0.650	0.650	0.625	0.679 a
2.24	0.575	0.613	0.675	0.675	0.600	0.683 a
4.48	0.575	0.588	0.650	0.650	0.600	0.633 b
LSD	NS	NS	NS	NS	NS	Rank
				S (mg/kg)		
N form						
NH_4	-	14.4 a	-	-	-	10.5 a
NO_3	-	8.8 b	-	-	-	8.4 b
LSD		Rank				Rank
Limestone (t/A)						
0	5.5	12.4 a	5.8	7.0 a	6.3	10.3 a
2.24	4.8	11.8 ab	7.3	7.0 a	6.3	9.6 ab
4.48	5.8	10.6 b	6.0	4.5 b	4.5	8.4 b
LSD	NS	Rank	NS	Sq. root	NS	Rank
				B (mg/kg)		
N form						
NH_4	-	0.187 b	-	-	-	0.231 b
NO_3	-	0.238 a	-	-	-	0.259 a
LSD		0.042				Rank
Limestone (t/ha)						
0	0.160	0.213	0.203	0.175	0.145	0.245
2.24	0.123	0.210	0.238	0.175	0.178	0.258
4.48	0.138	0.215	0.210	0.140	0.125	0.232
LSD	NS	NS	NS	NS	NS	NS

	5 May	27 May	18 June	8 July	28 July	19 August
				Mn (mg/kg)		
N form						
NH_4	-	2.29	-	-	-	3.62 a
NO_3	-	2.10	-	-	-	2.98 b
LSD		NS				Rank
Limestone (t/ha)						
0	1.75 a	3.15 a	2.58 a	2.70 a	3.03 a	4.92 a
2.24	0.90 b	1.88 b	1.95 ab	1.55 b	1.85 b	2.91 b
4.48	0.80 c	1.56 b	1.43 b	1.13 c	1.18 c	2.05 c
LSD	Rank	0.44	0.84	0.38	0.51	Rank
				Zn (mg/kg)		
N form						
NH_4	-	2.25	-	-	-	2.13 a
NO_3	-	2.03	-	-	-	1.95 b
LSD		NS				Rank
Limestone (t/ha)						
0	2.15	2.63 a	2.35	2.35 a	2.08 a	2.48 a
2.24	1.75	2.10 b	2.05	1.88 b	1.95 a	2.03 b
4.48	1.60	1.70 b	1.68	1.33 c	1.18 b	1.62 c
LSD	NS	0.50	NS	0.38	0.42	Rank
				Fe (mg/kg)		
N form						
NH_4	-	67.3 b	-	-	-	73.9
NO_3	-	81.7 a	-	-	-	76.9
LSD		Rank				NS
Limestone (t/ha)						
0	61.5	80.0	68.0	63.0 a	57.3 a	83.6
2.24	54.5	72.9	71.0	57.0 a	58.3 a	77.9
4.48	56.8	70.6	61.0	41.5 b	38.0 b	64.9
LSD	NS	NS	NS	7.04	12.53	NS
				pH		
N form						
NH_4	-	6.05 b	-	-	-	5.76
NO_3	-	6.25 a	-	-	-	5.79
LSD	-	0.16	-	-	-	NS
Limestone (t/ha)						
0	5.85 c	5.68 c	5.68 c	5.68 c	5.43 b	5.22 c
2.24	6.40 b	6.20 b	6.13 b	6.12 b	6.30 a	5.84 b
4.48	6.60 a	6.59 a	6.48 a	6.63 a	6.55 a	6.28 a
LSD	0.16	0.19	0.26	0.13	0.42	0.14

[a] Each value is the mean of four replications. Experimental design was a completely randomized, split-split block with nitrogen fertilizer treatments (ammonium vs. nitrate-N) applied to the main plots, spinach inbred lines to the split plots, and limestone application rates to the split-split plots. Raw data were subjected to square root (sq. root) or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] For each soil sampling date, 12 cores (2.5 cm x 15 cm depth) were collected from each plot, mixed by hand, and a subsample sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA) for nutrient analyses.

Table 2.18. Effects of limestone application rate on soil chemical properties in a 2012 spinach seed crop field trial in Skagit Co., WA

Limestone application rate (t/ha)[a]	9 May	9 July	24 Aug
		NO_3 (mg/kg)	
0	4.04 c	15.12 a	17.40 a
0/4.48	4.84 b	8.58 b	12.26 b
2.24	5.60 a	9.50 b	12.03 b
4.48	5.84 a	6.89 b	10.27 b
LSD	0.47	Sq. root	2.71
		NH_4 (mg/kg)	
0	2.84	0.76 b	2.58
0/4.48	2.46	1.66 a	3.46
2.24	2.34	2.05 a	3.32
4.48	2.26	2.09 a	3.18
LSD	NS	Rank	NS
		P (mg/kg)	
0	294	280	215 b
0/4.48	270	288	253 b
2.24	277	293	256 b
4.48	280	284	302 a
LSD	NS	NS	Rank
		K (mg/kg)	
0	251	278	192
0/4.48	254	267	244
2.24	260	270	187
4.48	239	259	203
LSD	NS	NS	NS
		Ca (meq/100 g)	
0	3.27 d	3.11 d	5.40
0/4.48	5.09 c	5.20 c	6.42
2.24	6.22 b	5.84 b	6.43
4.48	8.17 a	8.27 a	5.32
LSD	0.62	0.50	NS
		Mg (meq/100 g)	
0	0.670	0.572	0.654
0/4.48	0.660	0.572	0.762
2.24	0.664	0.560	0.602
4.48	0.628	0.536	0.774
LSD	NS	NS	NS
		S (mg/kg)	
0	7.28	6.89 a	9.38 a
0/4.48	6.50	7.06 a	7.05 b
2.24	6.06	5.66 b	7.15 b
4.48	6.38	4.80 b	6.79 b
LSD	NS	Rank	Rank

Limestone application rate (t/ha)[a]	9 May	9 July	24 Aug
		B (mg/kg)	
0	0.120	0.116	0.750
0/4.48	0.098	0.086	0.892
2.24	0.126	0.128	0.818
4.48	0.158	0.108	1.170
LSD	NS	NS	NS
		Mn (mg/kg)	
0	3.29 a	2.45 a	3.51 a
0/4.48	1.31 b	1.36 b	1.17 b
2.24	1.30 b	1.11 bc	0.99 c
4.48	1.18 b	0.93 c	0.84 d
LSD	Rank	0.40	Rank
		Zn (mg/kg)	
0	2.17 a	2.19 a	2.35 a
0/4.48	1.48 b	1.23 b	1.39 b
2.24	1.33 bc	1.09 bc	1.28 b
4.48	1.06 c	0.81 c	0.98 c
LSD	0.39	Rank	Log
		Fe (mg/kg)	
0	96.0 a	80.6 a	74.2 a
0/4.48	72.2 b	65.2 b	55.2 b
2.24	68.8 b	55.6 bc	48.4 c
4.48	64.4 b	44.2 c	39.6 d
LSD	21.6 b	12.6	Rank
		pH	
0	5.80 d	5.68 d	5.42 d
0/4.48	6.44 c	6.58 c	6.26 c
2.24	6.77 b	6.81 b	6.51 b
4.48	7.18 a	7.33 a	7.08 a
LSD	0.15	0.17	0.12
		CEC (meq/100 g)	
0	8.90	8.11 ab	8.83
0/4.48	9.34	7.61 b	8.38
2.24	9.88	8.33 a	8.72
4.48	9.47	8.27 ab	8.44
LSD	NS	0.68	NS

[a] Each value is the mean of five replications. Experimental design was a completely randomized, split block with spinach inbred lines planted in the main plots and limestone amendment rates applied to the split plots. When necessary, raw data were subjected to log, square root (sq. root), or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each main factor are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference. Main plots (inbred line) were not sampled for soil nutrient analyses in the 2012 trial.

[b] For each soil sampling date, 12 cores (2.5 cm x 15 cm depth) were collected from each plot, mixed by hand, and a subsample sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA) for nutrient analyses.

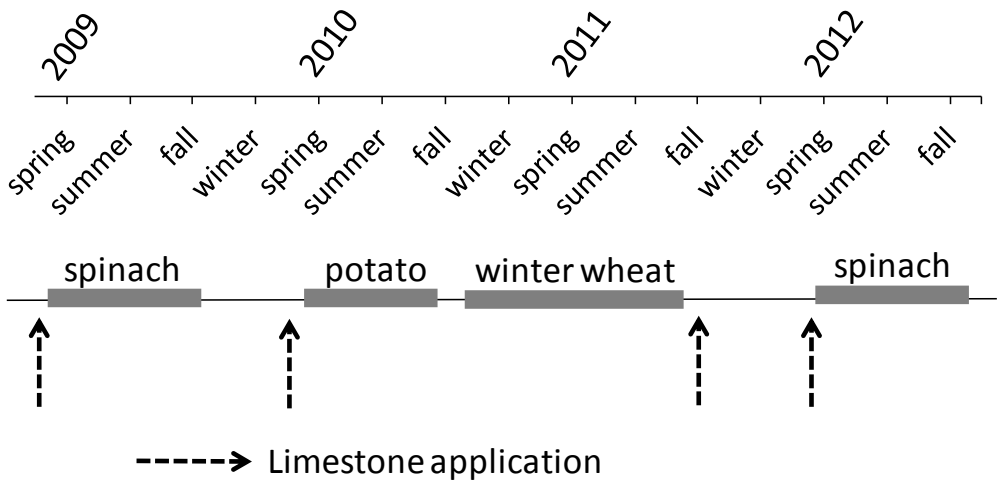

Fig. 2.1. Timeline of agricultural limestone applications in a field trial evaluating three annual applications vs. one application of limestone to soil for suppression of Fusarium wilt in spinach seed crops in Skagit Co., WA. The trial was located in a grower-cooperator's field. Following a spinach seed crop trial in 2009, the grower planted a typical rotation of potatoes (2010) followed by winter wheat (2011). In 2012, the spinach seed crop field trial was repeated in the same plots and with the same limestone application rates and spinach inbred lines as in the 2009 trial, to assess wilt severity. Limestone application rates of 0, 2.24, and 4.48 t/ha were applied to the same plots each season in 2009 to 2011. In the 2012 spinach seed crop trial, a fourth limestone treatment was added in which the control plots that had received no limestone from 2009 to 2011 were divided in half, with one half again receiving no limestone and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop.

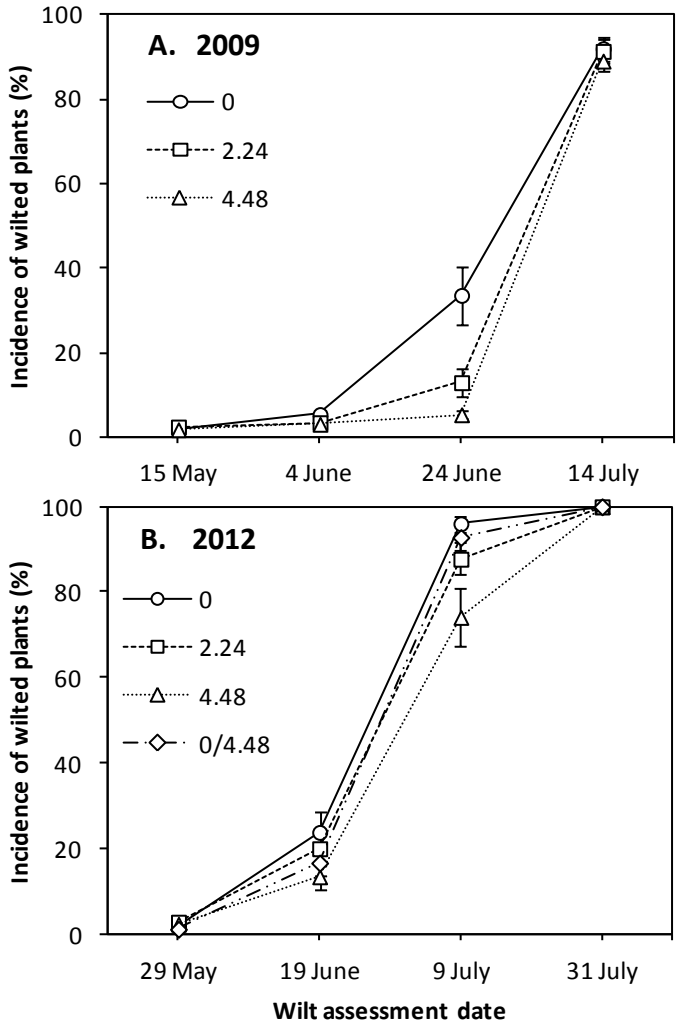

Fig. 2.2. Influence of agricultural limestone application rate on incidence of Fusarium wilt in 2009 (**A**) and 2012 (**B**) spinach seed crop field trials. Wilt incidence was measured as the percentage of plants with wilt symptoms in 3 m of the center two rows/plot, averaged over four (2009) or five (2012) replications. For 2009, each data point is the mean and standard error of 32 (15 May and 4 June) or 24 (24 June and 14 July) observations. In 2012, each data point for each date is the mean and standard error of 20 observations. Limestone amendment rates were 0, 2.24, and 4.48 t/ha/year from 2009 to 2011. In the 2012 spinach seed crop trial, a fourth limestone treatment was added in which the control plots that had received no limestone from 2009 to 2011 were divided in half, with one half again receiving no limestone and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop (the 0/4.48 treatment).

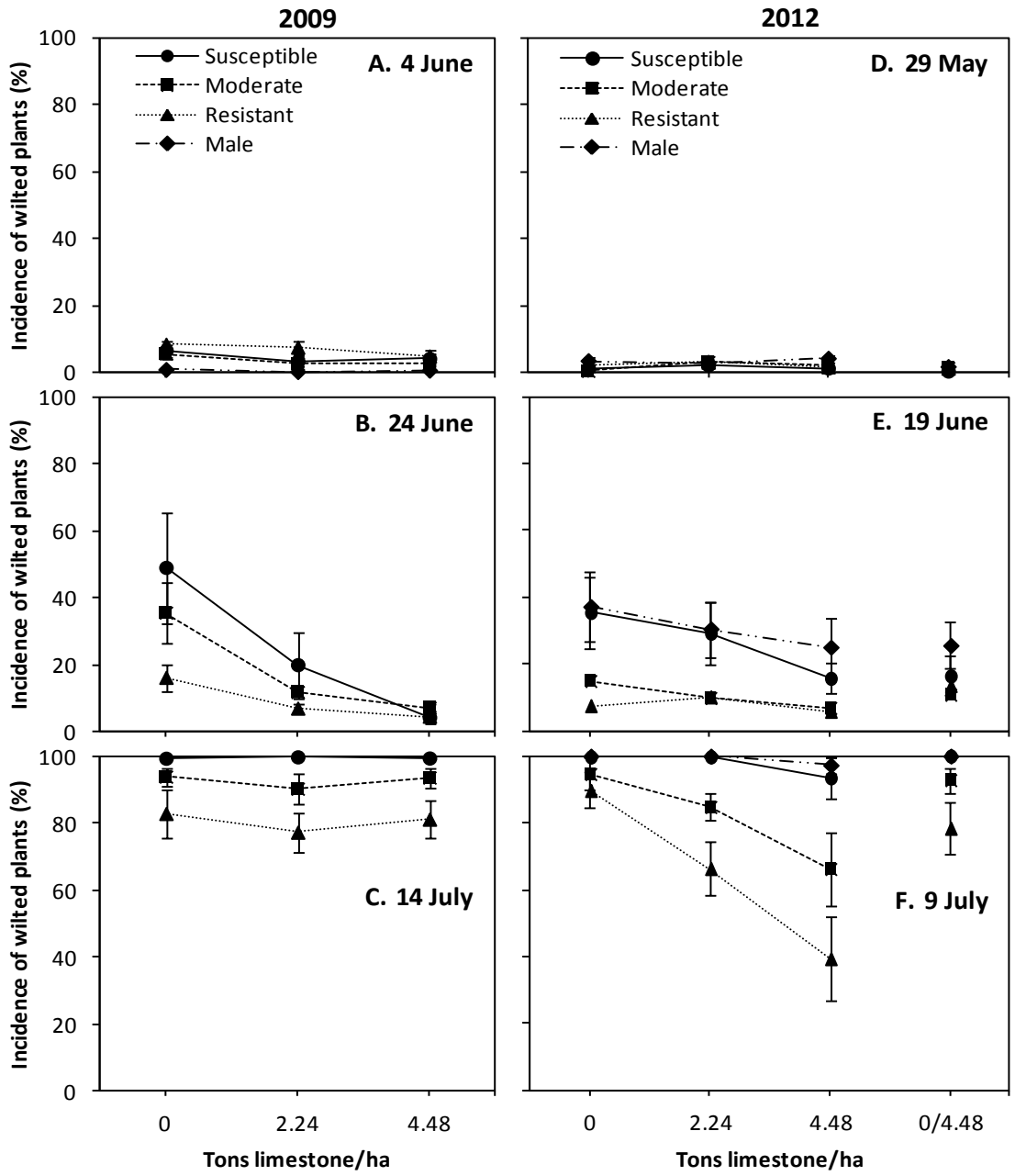

Fig. 2.3. Influence of limestone application rate and susceptibility of spinach inbreds to Fusarium wilt on the incidence of wilt in 2009 (**A**, **B**, and **C**) and 2012 (**D**, **E**, and **F**) spinach seed crop field trials. Wilt incidence was measured as the percentage of plants with wilt symptoms in 3 m of the center two rows/plot, averaged over four (2009) or five (2012) replications. Each data point is the mean and standard error of 8 (2009) or 5 (2012) observations. Limestone amendment rates were 0, 2.24, and 4.48 t/ha/year from 2009 to 2011. In the 2012 spinach seed crop trial (**D**, **E**, and **F**), a fourth limestone treatment was added in which the control plots that had received no limestone from 2009 to 2011 were divided in half,

with one half again receiving no limestone and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop (the 0/4.48 treatment).

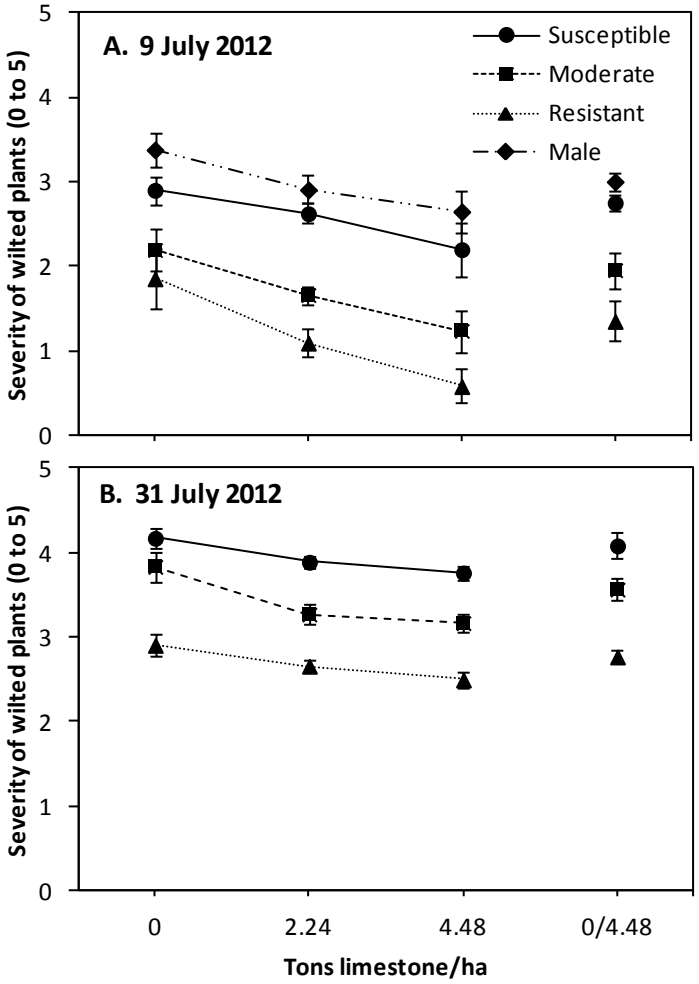

Fig. 2.4. Influence of limestone application rate and susceptibility of spinach inbred lines to Fusarium wilt on the severity of wilt in a spinach seed crop trial on 9 (**A**) and 31 (**B**) July 2012. Wilt severity of each plant in 3 m of the center two rows/plot was measured using a 0 to 5 ordinal rating scale, with 0 = healthy plant, and 5 = plant dead due to wilt. Each data point is the mean and standard error of five replicate plots. The male spinach line had senesced by 31 July and was, therefore, excluded from wilt rating. Limestone application rates of 0, 2.24, and 4.48 t/ha/year were applied to the same plots each season in from 2009 to 2011. In 2012, a fourth limestone treatment was added in which the control plots that had received no limestone from 2009 to 2011 were divided in half, with one half again receiving no limestone and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop (the 0/4.48 treatment).

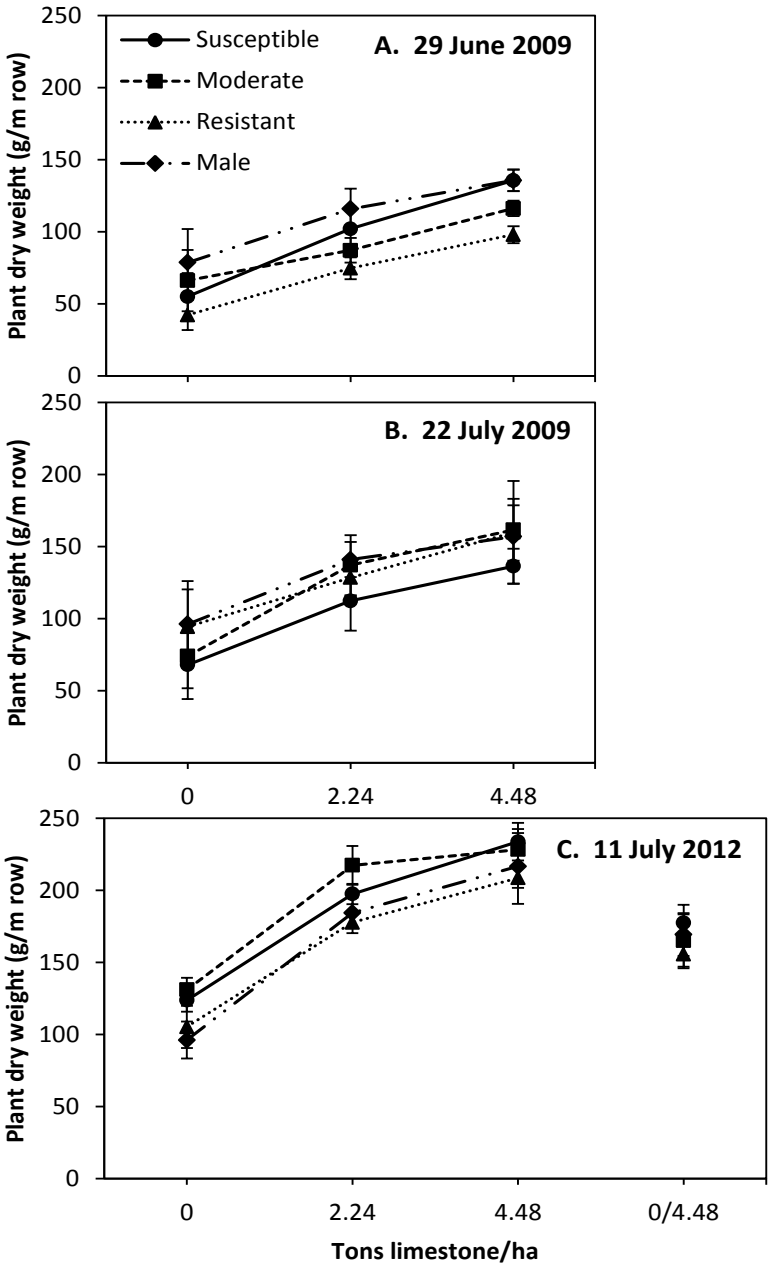

Fig. 2.5. Influence of limestone application rate and susceptibility of spinach inbred lines to Fusarium wilt on dried spinach plant biomass in a spinach seed crop trial in 2009 (**A** and **B**) and 2012 (**C**). Biomass was measured by harvesting, drying, and weighing plants in 1 m of row/plot. In 2009, biomass was measured on 29 June (**A**) and 22 July (**B**). In 2012, biomass was measured on 11 July (**C**). Each data point represents the mean and standard error of eight (2009) or five replicate plots (2012). Limestone amendment rates were 0, 2.24, and 4.48 t/ha from 2009 to 2011. In the 2012 spinach seed crop trial, a fourth limestone treatment was added in which the control plots that had received no limestone from 2009

to 2011 were divided in half, with one half again receiving no limestone and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop (the 0/4.48 treatment).

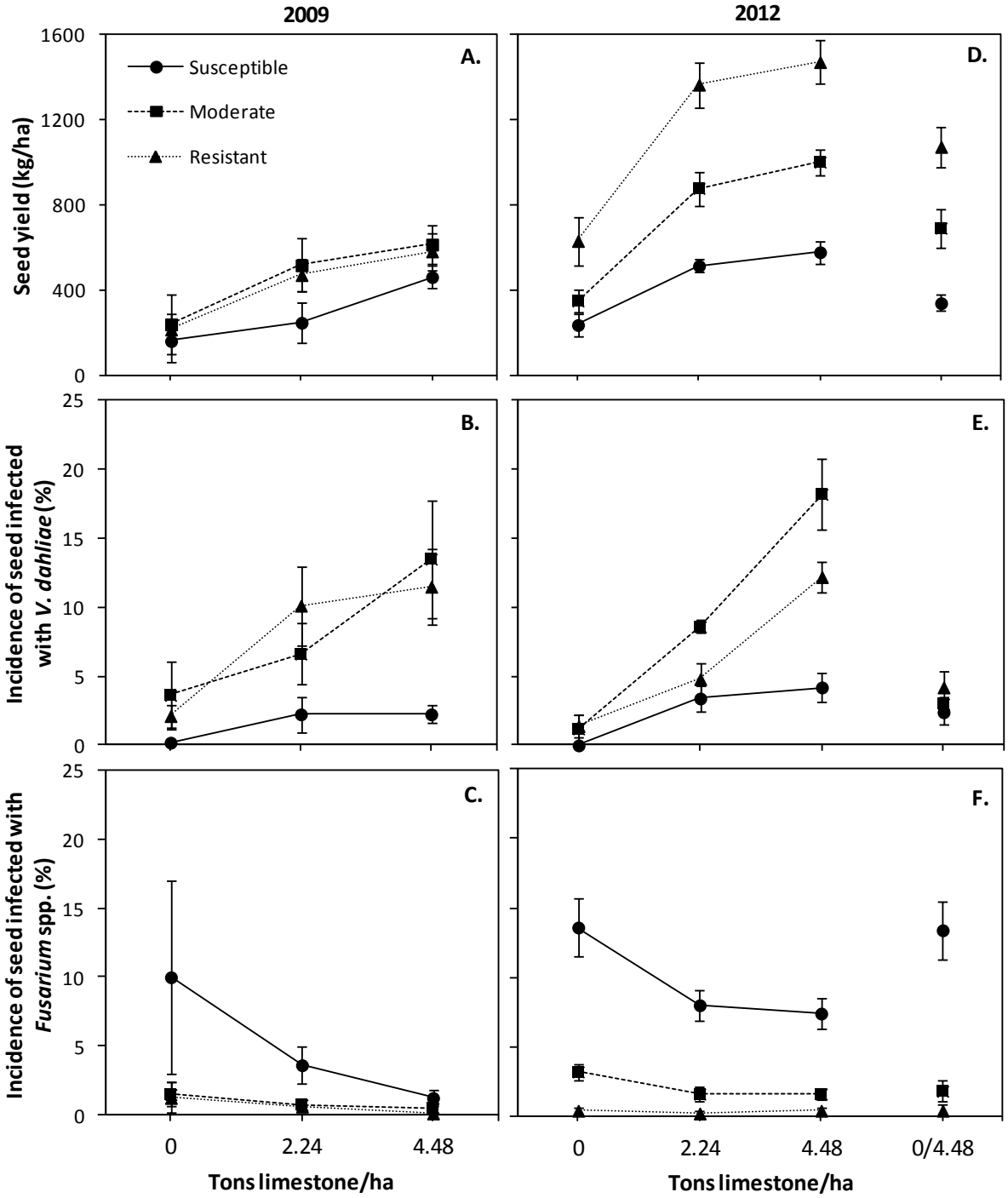

Fig. 2.6. Influence of limestone application rate and susceptibility of three female spinach inbred lines to Fusarium wilt on marketable spinach seed yield (**A** and **D**), incidence of seed infected with *Verticillium dahliae* (**B** and **E**), and incidence of seed infected with *Fusarium* spp. (**C** and **F)** in spinach seed crop

trials in 2009 (**A**, **B**, and **C**) and 2012 (**D**, **E**, and **F)**. Each data point represents the mean and standard error of 8 (2009) or 5 (2012) plots. Limestone application rates of 0, 2.24, and 4.48 t/ha were applied to the same plots each season from 2009 to 2011. In the 2012 spinach seed crop trial, a fourth limestone treatment was added in which the control plots that had received no limestone from 2009 to 2011 were divided in half, with one half again receiving no limestone and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop (the 0/4.48 treatment).

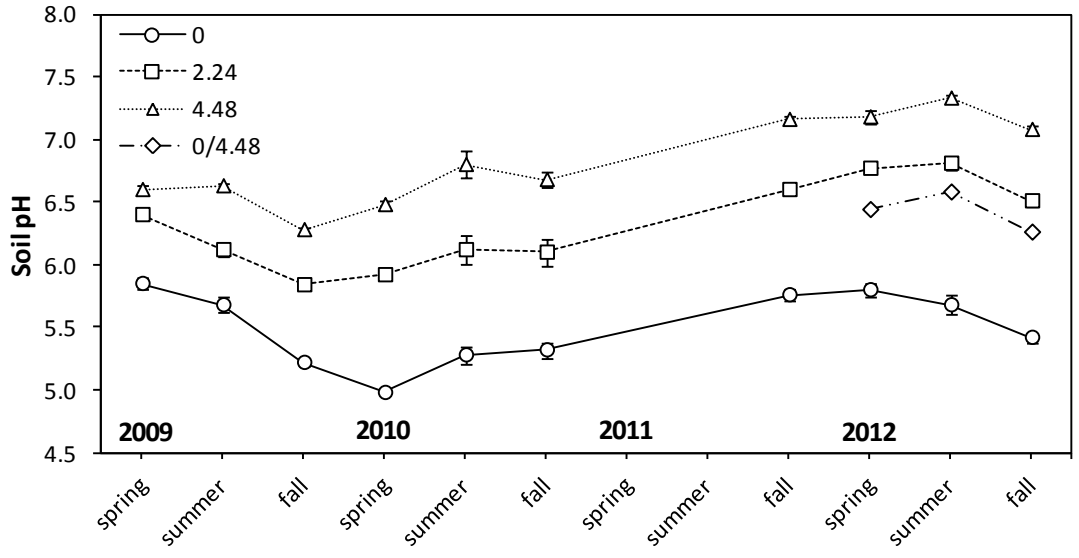

Fig. 2.7. Effect of limestone application rate on soil pH in a field trial evaluating three annual applications vs. a single application of limestone to soil for suppression of Fusarium wilt in spinach seed crops in Skagit Co., WA. Each data point represents the mean and standard error of five replicate plots, with the exception of fall 2009, when the effect of limestone rate was averaged across three spinach inbred lines and two N-treatments for 30 plots/mean. For soil sampling, 12 cores (2.5 cm x 15 cm depth) were collected from each plot, mixed by hand, and a subsample dried and passed through a 1-mm aperture sieve. Limestone amendment rates were 0, 2.24, and 4.48 t/ha/year from 2009 to 2011. In the 2012 spinach seed crop trial, a fourth limestone treatment was added in which the control plots that had received no limestone from 2009 to 2011 were divided in half, with one half again receiving 0 t limestone/ha and the other half amended with 4.48 t limestone/ha prior to planting the spinach crop (the 0/4.48 treatment).

CHAPTER THREE

DEVELOPMENT OF A SOIL BIOASSAY FOR FUSARIUM WILT RISK PREDICTION IN SPINACH SEED PRODUCTION, AND IDENTIFICATION OF SOIL PROPERTIES ASSOCIATED WITH SPINACH FUSARIUM WILT INOCULUM POTENTIAL

Introduction

Production of high quality spinach seed requires a mild climate for this cool-season crop, long summer daylength to trigger uniform bolting, and dry conditions during seed set and maturation (Metzger and Zeevart, 1985). The maritime Pacific Northwest (PNW) of the United States of America (USA) is one of the few regions in the world that meets these requirements, and is the source of up to 50% of the USA and 25% of the world spinach seed supply (Foss and Jones, 2005). However, the highly-leached, acid soils of the PNW are very conducive to spinach Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *spinaciae* (Foss and Jones, 2005). This disease can cause severe economic losses in a spinach seed crop, with symptoms including wilting and chlorosis of leaves, stunting, internal vascular discoloration and necrosis of roots, early senescence, and reduced seed yield (Correll et al., 1994). As with many of the Fusarium wilts that affect that world's food crops, the spinach-specific strains of the pathogen persist in the soil for an extended period once introduced to a field, forcing growers to wait up to 15 years between spinach seed crops (Foss and Jones, 2005). Partial resistance to the disease has been identified, but many of the most commonly used parent lines have little to no resistance (Correll et al., 1994). Because growers contract with seed companies to produce hybrid spinach seed, they typically have no choice and/or knowledge of the resistance to Fusarium wilt of the proprietary lines they are contracted to grow. Furthermore, pollen isolation distances between

seed crops of this cross-pollinating, dioecious species must be maintained, further complicating the placement of spinach seed crops.

The only means currently used for predicting the spinach Fusarium wilt risk of a field is knowledge of the cropping history and the patience to observe the recommended 10 to 15 year rotation interval between spinach seed crops (Foss and Jones, 2005). Rotation interval is not always a reliable predictor of Fusarium wilt risk, however. There have been documented cases of near-total yield loss due to this disease in fields that had not had a spinach seed crop for 18 or more years, while occasionally fields considered high-risk for Fusarium wilt based on rotation interval have developed only mild wilt symptoms (L. J. du Toit, *personal communication*). Because spinach seed is one of the more lucrative crops in the maritime PNW (McMoran, 2011), and availability of fields that meet the rotation requirement are increasingly scarce, growers and seed companies need a more accurate tool for determining the earliest that a field may be "safe" for planting a spinach seed crop. To do this requires a method for estimating the Fusarium wilt inoculum potential of a field under consideration prior to planting a spinach seed crop.

Inoculum potential, as defined by Garrett (1970), is the energy available for substrate (host) colonization at the point of infection, which in turn is a function of pathogen density and vigor, physico-chemical and microbial properties of the soil, and environmental variables such as temperature and precipitation. Baker (1971) proposed that inoculum potential combined with host susceptibility (disease potential) determines the level of disease risk. One approach to measuring inoculum potential is to estimate the size of the pathogen population through direct measurement. Dilution plating of soils onto a selective medium is a common method for pathogen quantification (van Bruggen and Grunwald, 1996). This method does not work well for *F. oxysporum* f. sp. *spinaciae*, however, because colonies of the pathogen are morphologically

indistinguishable from those of other formae speciales and non-pathogenic *F. oxysporum* isolates when plated onto *F. oxysporum*-selective Komada's agar medium (Komada, 1975). Differentiating *spinaciae* isolates from other *F. oxysporum* colonies identified on Komada's agar medium requires time-consuming pathogenicity tests (Armstrong and Armstrong, 1981). To circumvent such testing, a real-time PCR assay was developed by Okubara et al. (2013) in an attempt to detect and quantify *F. oxysporum* f. sp. *spinaciae* in soils via extraction and amplification of DNA using pathogen-specific primers. However, the correlation between quantity of purported pathogen DNA obtained from soil test samples and the development of spinach Fusarium wilt in those soils in greenhouse assays was low. The low predictive value of the real-time PCR assay for soil Fusarium wilt risk was hypothesized to be due to the very limited amount of soil that could be used practically for DNA extraction (up to 0.8 g samples), variation in virulence among *F. oxysporum* f. sp. *spinaciae* isolates (E. W. Gatch and L. J. du Toit, *unpublished data*), and/or the high level of cross-reaction of the assay with non-pathogenic *F. oxysporum* isolates present on spinach plants or in soil in which spinach seed crops had been grown. Regardless, it is likely that any method of assessing spinach Fusarium wilt inoculum potential of soils that relies strictly on quantitative estimation of the pathogen population, ignoring the influence of the soil environment and host susceptibility on disease development, will fall short as a risk-prediction tool. These limitations of soil pathogen DNA quantification methods have been encountered by researchers working with Fusarium wilt risk prediction in other crops (e.g., Jiménez-Fernández et al., 2011).

Another approach to predicting soilborne disease risk is to collect soil from the field(s) of interest and conduct a bioassay or grow-out test of each soil in pots planted with known cultivars, in controlled conditions conducive to disease development. At appropriate intervals,

disease severity is evaluated and the data used to calculate a risk index for each soil tested (van Bruggen and Grunwald, 1996). This "natural inoculum potential" method can be very effective because it takes into account the influence of the soil environment, and has been used to estimate inoculum potential of soils for pea root rot caused by *Aphanomyces euteiches* and *Fusarium solani* f. sp. *pisi* in both the US and Europe (Malvick et al., 1994; Oyarzun et al., 1994; Reiling et al., 1996). With one or at most two indicator cultivars used, and no mention of cultivar susceptibility to these pathogens, these studies did not attempt to account for the contribution of host plant disease potential to bioassay outcomes, perhaps because the role of host susceptibility in the development of these root rots is not as important as it is for diseases like spinach Fusarium wilt (McGee et al., 2012; Oyarzun, 1993).

In the process of developing methods to understand and predict soilborne disease risk, many researchers have observed associations between different soil types or properties and disease severity, leading to a large body of literature on the characteristics of soils suppressive to certain diseases (Alabouvette et al., 2009; Hoper and Alabouvette, 1996; Mazzola, 2004). Soil physical and chemical properties, including macro- and micronutrient availability, pH, cation exchange capacity (CEC), electrical conductivity (EC), organic matter (OM), bulk density, texture analysis, and clay mineralogy; and biological properties such as microbial mass, activity, and diversity, have been examined in relation to soil receptivity to plant diseases. Many studies have sought to determine whether some of these associations might be strong enough to use in lieu of more time-consuming bioassays to predict risk or identify soils suppressive to disease despite the presence of pathogen inoculum (Janvier et al., 2007). For example, Stotzky and Martin (1963) found that soils with smectite clay, which has a higher CEC compared to other clay types, were suppressive to Fusarium wilt of banana, while soils without smectite clay were

conducive. Lazarovits et al. (2007) analyzed relationships between potato common scab and various soil edaphic factors such as organic matter, soil pH, mineral nutrients, and CEC to identify potential scab predictor variables. In a study examining the relationship between various soil factors and occurrence of Aphanomyces root rot in sugar beet fields in southern Sweden, Olsson et al. (2011) identified high soil calcium (Ca) concentration as reliably predictive of this soilborne disease. One challenge with these sorts of studies is that relationships identified may be specific to a given region and not applicable to others, as was the case with the potato scab research (Lazarovits et al., 2007).

The objectives of this study were to: 1) develop a greenhouse soil bioassay integrating the various factors contributing to inoculum and disease (host) potential that will serve as an effective Fusarium wilt risk-prediction tool for spinach seed growers; and 2) evaluate abiotic and biotic soil characteristics in relation to spinach Fusarium wilt severity measured with the soil bioassay to identify correlative relationships that could aid in risk prediction. For the soil bioassay-based diagnostic service to meet the needs of spinach seed producers and stakeholders, the following were important considerations: 1) each soil sample submitted must be collected in such a manner to represent adequately the field in question, with a sufficient final volume to accommodate the designated pot size and number of replications employed in the bioassay; 2) spinach parent lines exhibiting a range in Fusarium wilt susceptibility representative of what is planted in commercial spinach seed fields in the region should be used for Fusarium wilt risk index calculation; 3) the service must be completed and results communicated to stakeholders prior to the date each spring when growers and seed companies finalize the placement of spinach seed crops for that season to avoid unwanted cross-pollination; 4) to complete the bioassay in the two-month period between soil submission and delivery of results, the bioassay must be

conducted under conditions conducive to rapid development of Fusarium wilt, i.e., high temperature and adequate soil moisture; 5) bioassay results should be compared to spinach seed crop assessments to determine how well the bioassay predicts actual Fusarium wilt risk observed in growers' fields; and 6) the service should be offered annually, since the Fusarium wilt risk of a particular field will change with time, crop rotations, and farming practices.

Materials and Methods

Fusarium wilt soil bioassay development: Stage 1. Preliminary experiments were conducted in fall 2009 and spring 2010 to identify some of the basic parameters for successful execution of a spinach Fusarium wilt soil bioassay in January and February each year in a controlled greenhouse environment. Observational trials were conducted in July and August 2009 to determine how to grow spinach plants successfully in pure field soil compared to soil amended with vermiculite or other potting mix, and to identify an appropriate container size and shape for the bioassay. Unadulterated field soil was determined to be suitable for growing spinach for up to 2 months in 12 cm diameter x 12 cm tall pots (Anderson Die, Portland, OR), as long as overhead watering was done carefully (gently) to avoid compaction.

A randomized complete block (RCB) experiment with four replications was subsequently designed to evaluate three factors for development of the soil bioassay: 1) soil Fusarium wilt risk, using soil sampled from each of two fields considered high risk and low risk for spinach Fusarium wilt based on spinach seed crop rotation interval; 2) a partial soil pasteurization treatment or no pasteurization to generate further differences in Fusarium wilt risk within each of these two soil samples; and 3) three proprietary spinach parent lines previously characterized as highly susceptible (S), moderately susceptible (M), and moderately resistant (R) to Fusarium wilt

(du Toit et al., 2007). The objective was to determine whether the three parent lines grown in pots filled with each of the four soil-by-pasteurization treatments could adequately differentiate the Fusarium wilt risk level of these treatments in a controlled greenhouse environment, and in a timely fashion.

Soil preparation. Soil was collected on 28 September 2009 from each of two fields: a high risk field that had been in spinach seed production two months prior to the start of the experiment, and a low risk field that had not had a spinach crop for approximately 12 years. The high risk soil was classified as a Mount Vernon very fine sandy loam, and the low risk soil as a Sedro Woolley silt loam (Soil Survey Staff of the United States Department of Agriculture (USDA) National Resources Conservation Service (NRCS)). A portion of the soil collected from each site was heated for 1.5 h at approximately 65°C in a 200 liter Pro-Grow SS-30 model electric soil sterilizer (Pro-Grow Supply Corp., Brookfield, WI). The soil sterilizer was half-filled for each treatment, such that the metal rods from which the heat emanated were covered entirely. Each soil sample was mixed thoroughly by hand in a large plastic bin after heating and prior to planting to minimize the effect of variation in sections of the field sampled as well as variation in temperature within the soil sterilizer. The high risk soil dried into clods prior to planting, so the soil was passed through a 4.7 mm-aperture sieve to achieve an appropriate tilth before hand-mixing. The low risk soil required only a raking to remove crop debris prior to heating and/or mixing.

Greenhouse conditions and pest management. The bioassay was conducted in a greenhouse set at 23 to 28°C with supplemental lighting provided on a 10 h light/14 h dark daily schedule. Plants were watered as needed with a fertigation system (General Purpose 20-20-20 fertilizer, Plant Marvel, Chicago, IL, injected with the irrigation water at a 1:100 ratio for

applying 200 ppm nitrogen). Seeds were treated with thiram (Thiram 42-S, Bayer CropScience, Research Triangle Park, NC) and mefenoxam (Apron XL LS, Syngenta, Basel, Switzerland) at 521 ml and 20 ml/100 kg seed, respectively, for control of damping-off and seedling pathogens other than *Fusarium*, e.g., *Rhizoctonia* and *Pythium*, respectively.

Planting, disease ratings, and spinach biomass measurement. On 3 October 2009, pots (15 cm diameter and 11.3 cm deep, Anderson Die) were filled with soil and sown with four seeds of the appropriate spinach parent line at a depth of approximately 1 cm. On 12 October, seeds were re-sown to replace those that did not germinate (7 seeds out of a total of 192 planted). This was not ideal, however, since Fusarium wilt development is affected by plant growth stage and the re-planted seedlings were 9 days less mature than the rest of the plants. Fusarium wilt symptoms were rated at the onset of symptoms 32 days after planting (DAP), and again 39, 44, and 51 DAP. A 0-to-5 ordinal wilt rating scale was devised: 0 = no visible symptoms; 1 = a few flaccid older leaves with a dull green cast; 2 to 4 = progressive increase in the percentage of leaves wilting and chlorotic; and 5 = dead plant due to wilt. The number of plants in each of these categories was recorded for each pot at each rating date. At 51 DAP, aboveground spinach biomass was measured by cutting all the plants in each pot at the soil line, combining the plants from each pot into a paper bag, drying the plants at approximately 35°C for 3 to 7 days, and weighing the dried plants.

Soil microbial evaluation. A subsample of each soil-by-pasteurization treatment combination was air-dried in the greenhouse, crushed, and passed through a 1 mm-aperture sieve. To quantify colony forming units (CFU) of *F. oxysporum*/g air-dried soil, 10 g of each subsample was added to 100 ml sterilized, 0.1% water agar (WA) in a 240 ml glass French square, agitated for 10 min on a rotary shaker at 250 rpm, and diluted serially to a 10^{-2}

concentration. An aliquot (0.5 ml) of each of the 10^{-1} and 10^{-2} dilutions was distributed across the surface of each of three replicate plates of modified Komada's agar medium (Komada, 1975; Scott et al., 2010) using a sterilized glass rod. The plates were incubated on a laboratory bench at room temperature (25 ± 2°C) in ambient light in a room with windows. Colonies typical of *F. oxysporum* on this semi-selective medium (dense, fluffy, white to pale-pink, aerial mycelium) were enumerated 7 and 14 DAP. The soil dilution and enumeration procedure was repeated three times, and the average count converted to CFU/g soil.

Soil bioassay development: Stage 2. To refine further the protocol developed in stage 1, a second experiment was conducted with several modifications. The number of replications (pots) of each treatment combination was increased from four to five. Soil from an additional field that had an eight-year rotation out of spinach seed production was included to approximate a moderate risk soil (the soil classification was a Skagit silt loam). An additional soil heat treatment was added, consisting of 1.5 h at 65°C followed by 2 h at 80°C, to further reduce the spinach Fusarium wilt pathogen population. Two negative control soil treatments were included: 1) the high risk soil autoclaved twice at 121°C and 1.1 kg/cm^2 for 30 min with 24 h between autoclavings, and 2) soil collected from an orchard in Skagit Co, WA that had never been planted to a spinach seed crop. These negative control treatments were not subjected to the two heat treatments. To maintain the three-way factorial structure of the treatments for statistical analyses, the negative control treatments were excluded from the analyses of variance (ANOVA) and used, instead, as a baseline for comparison with the ANOVA results for the primary treatments.

Soil preparation. The soil for each treatment was sieved prior to planting (and pasteurization, when applicable) with a 6.3 mm-aperture sieve to remove larger pieces of organic

matter, rocks, etc. Soils were stored in a cooler at 4°C when not being processed, and were hand-mixed prior to planting as described previously.

Greenhouse conditions and pest management. This stage 2 experiment was conducted under the same conditions and in the same greenhouse as the first preliminary bioassay, from 9 December 2009 until 8 January 2010, at which point the pots were moved to another greenhouse set at 24 to 27°C during the day and 21 to 24°C at night, with supplemental lighting provided on a day/night cycle of 10 h/14 h. Seeds were treated with Thiram 42S and Apron XL LS as described previously. Bifenthrin (Talstar P granular insecticide, FMC Corp., Philadelphia, PA) was applied to the soil surface in each pot (0.11 kg/m^2) for control of cranefly (*Tipula paludosa*) larvae, and watered into the soil. *Bacillus thuringiensis* subspecies *israelensis* (Gnatrol WDG, Valent BioSciences, Libertyville, IL) was applied four times during the bioassay at a rate of 2 g/liter for control of fungus gnat (Mycetophilidae and Sciaridae) larvae. A regular rotation of insecticides for thrips (*Thrips* spp. and/or *Frankliniella* spp.) control was implemented in this and subsequent bioassays, including imidacloprid (Provado 1.6F, Bayer CropScience, applied at 45 ml/ha), *Beauveria bassiana* (Botanigard 22WP, Laverlam International, Butte, MT, applied at 2.4 g/liter), spinosad (Success 480SC, Dow AgroSciences, Indianapolis, IN, applied at 50 ml/1,000 liters), and acephate (Orthene 97, AmVac Chemical Co., Newport Beach, CA, applied at 1.2 g/liter).

Planting and disease rating. The bioassay was planted on 12 December 2009. Prior to filling the pots with soil, the six holes in the bottom of each pot were covered with flat-bottomed marbles (1.25 cm diameter, Panaceae Products, Columbus, OH) to prevent loss of soil through the holes when watering. Eight seeds/pot (increased from four seeds in the first bioassay) were sown approximately 1 cm deep in the same pot size used previously, and thinned to four

seeds/pot. The number of emerged seedlings/pot was recorded at 7 and 14 DAP. Fusarium wilt symptoms were rated 28, 35, and 42 days DAP using the 0-to-5 scale developed for the first bioassay. Aboveground spinach dry biomass was measured. A wilted seedling was collected on 6 January from a pot of every treatment combination in which wilting seedlings were observed (along with an asymptomatic seedling collected on 25 January), surface-sterilized in 1.2% NaOCl, triple-rinsed in sterilized, deionized water, dried, cut into pieces, and plated onto potato dextrose agar (Difco Laboratories, Detroit, MI) amended with chloramphenicol (cPDA) and chloramphenicol-amended water agar (cWA). Transfers were made from candidate *F. oxysporum* colonies growing out from the tissue pieces, and identified as *F. oxysporum* isolates by examination with a compound microscope. A total of 15 isolates were then single-spored and transferred to plates of cPDA with four 1.5 cm-diameter disks of sterilized filter paper (413, VWR Scientific, West Chester, PA) on the surface of the agar medium. When the disks were fully colonized by the mycelium, the disks were removed from the agar, placed in sterilized coin envelopes (Westvaco Envelope Division, Springfield, MA), dried in a laminar flood hood overnight, and stored at -20°C in an air-tight tissue culture box containing anhydrous calcium sulfate (DrieRite, Mallinckrodt Baker, Inc., Phillipsburg, NJ).

Soil chemical and microbial evaluation. A soil nutrient analysis of nitrate (NO_3^-)- and ammonium (NH_4^+)-nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), zinc (Zn), manganese (Mn), copper (Cu), iron (Fe), pH, CEC, EC, OM, and buffer pH, was performed on a sample of soil for each soil source-by-heat treatment combination at Soiltest Farm Consultants, Inc. (Moses Lake, WA). The methods used for these analyses are detailed in Table 3.1.

Three separate subsamples of soil (approximately 50 g) were collected prior to planting, dried, and sieved for enumeration of *F. oxysporum* (as described for the first bioassay) and *Verticillium dahliae*. To assay soil for *V. dahliae,* a sterilized glass salt shaker with a metal screw cap was used to sprinkle 1 g of the dried, crushed, and sieved soil of each sample onto each of 10 95 mm-diameter plates of NP-10 agar medium, a semi-selective medium for *Verticillium* (Goud and Termorshuizen, 2003; Sorenson et al., 1991). After 28 days of incubation in the dark at 26°C, colonies typical of *V. dahliae* were enumerated using a dissecting microscope, and the number of microsclerotia/g soil was calculated. At the end of the bioassay, the soil from each pot of three of the five replicate pots/treatment was mixed, dried, crushed, sieved, and stored for a post-assay enumeration of *F. oxysporum*.

The bioassay was repeated in the spring of 2010 with several modifications. Instead of using the orchard soil as a negative control treatment, a soil sample collected from a field in Skagit Co. that had been fumigated with methyl bromide:chloropicrin (57:43% at 3,274 liters/ha) several weeks prior to the trial was used as a negative control treatment. The eight seeds sown in each pot were not thinned to four seedlings as the pot size could accommodate eight plants adequately for the duration of the bioassay. When rating the plants for disease, each plant was rated individually at weekly intervals so that the progression of wilt was followed for each plant. This repeat bioassay was planted on 30 April 2010, the plants rated for Fusarium wilt 21, 28, and 35 DAP, and plants harvested for biomass 38 DAP.

2010 grower soil bioassay. The protocol developed in the preliminary bioassays was used to evaluate the Fusarium wilt risk of soil samples submitted by growers and seed company representatives from fields in northwestern Washington being considered by these stakeholders for planting spinach seed crops in 2010. The same three spinach parent lines were planted in

each soil sample. Three control soils were included: a positive control soil consisting of the same high risk soil as the preliminary bioassays, an intermediate risk soil developed by subjecting the high risk soil to a heat treatment of approximately 65°C for 1.5 h followed by 80°C for 2 h in the electric soil sterilizer, and a negative control soil developed by autoclaving the high risk soil for 30 min on each of two consecutive days. The treatment design was a two-way factorial of three spinach parent lines and 29 soil samples (26 grower soil samples and 3 control soils), and the experimental design was an RCB with five replications.

Soil preparation. Following a request for soils sent by email to spinach seed growers and seed company representatives in November 2009, 26 soil samples from fields in Skagit and Snohomish Counties were submitted from 21 December 2009 to 6 January 2010. Two 19-liter buckets of soil were requested for each field to be assayed. Participants were instructed to sample fields as thoroughly as possible to reflect Fusarium risk accurately. Samples were assigned a number (1 to 26) when received, and stored in a shed that maintained a consistently cool temperature through the winter. The two buckets of soil for each sample were mixed and spread out on a tarp on a greenhouse bench, and dried until the moisture content enabled the soil to be sieved through a 6.3 mm aperture sieve. For clay soils that had a tendency to form clods with air-drying, a piece of PVC pipe was used as a rolling pin to break the larger clumps into smaller pieces. After sieving, each soil was returned to the appropriate buckets and placed in cool storage. A subsample of each soil was sent to Soiltest Farm Consultants for nutrient analysis, as described for the preliminary bioassays.

Greenhouse conditions and pest management. The greenhouse was set to a 10 h light/14 h dark daily schedule with supplemental lighting during the day. The temperature was set at 22 to 25°C during the day and 19 to 22°C during the night. Seeds of the three spinach parent lines

were treated with Thiram 42-S and Apron XL LS as described above. Insect management was carried out as described above.

Planting and disease rating. On 14 January 2010, 15 cm-diameter pots were filled with the appropriate soil, and planted on 15 January with eight seeds of the appropriate spinach parent line. The total number of emerged seedlings was recorded as described above, and severity of Fusarium wilt symptoms was rated 21, 28, 35, and 42 DAP using the same scale developed for the preliminary bioassays. Each of the eight seedlings/pot was rated and tracked separately. A sample of wilted seedlings was collected on 25 January (one seedling) and 1 February (nine seedlings) from each of the three inbred lines, surface-sterilized for 30 s in 1.2% NaOCl, triple-rinsed in sterilized, deionized water, dried on sterilized paper towels, cut aseptically into pieces, and plated onto cPDA and cWA to isolate potential plant pathogens. Nine isolates of *F. oxysporum* collected from the seedlings were then single-spored and stored on colonized filter disks at -20°C, as described above. Aboveground dried spinach biomass was measured as described previously, with plants harvested from 56 to 59 DAP (completed daily by replication). A subsample (approximately 50 g) of each soil was collected, dried, and sieved for enumeration of *F. oxysporum* and *V. dahlie* as described for the preliminary bioassays, with three replications of triplicate dilution plating/soil sample.

Statistical analyses. Each disease rating was converted to a disease index for each plot (pot) using the weighted average formula below, and then analyzed using ANOVA:

$$\text{Fusarium wilt index} = \frac{\sum(\text{severity rating} \times \text{number of seedlings within that rating})}{(\text{total number of emerged seedlings}) \times 5}$$

Rank transformation of disease index data was performed prior to ANOVA due to non-homogeneous variances associated with the low risk soils. This is a permitted, although not

preferred, method for dealing with ordinal scale data in factorial experiments (Shah and Madden, 2004). Soils and spinach parent lines were considered fixed effects, and replications random effects in the ANOVAs, which were calculated for each dependent variable using SAS Version 9.2 (SAS Institute, Cary, NC).

Dissemination and validation of results. On 13 February 2010, an open house was held for growers and seed company representatives to observe the results of the soil bioassay prior to the annual early March meeting when seed company representatives finalize decisions about the locations of seed crops (referred to as the seed crop "pinning" meeting) to ensure minimum pollen isolation standards are met. The pots were arranged by treatment (soil source-by-spinach parent combination) to allow stakeholders to view all five replications together for each soil. Visits to 14 of the 26 fields evaluated in the bioassay that ended up being planted to a spinach seed crop were made in the summer of 2010 to determine whether the Fusarium wilt severity observed in each of these 14 fields corresponded with that observed in the bioassay. A transect of each field was walked, and the incidence and severity of Fusarium wilt was assessed visually (no numerical disease ratings were assigned).

Corroboration of field visits. In fall 2010, a follow-up bioassay was performed to confirm or clarify some potential discrepancies observed in the summer of 2010 in two of the 14 spinach seed fields that had been evaluated in the bioassay. Four soil samples were collected in mid-July, two from a field where the location of the 2010 spinach seed crop overlapped with a spinach seed crop planted just three years prior in that field, compared to the rest of the field that had been planted to a spinach seed crop at least 14 years previously. In the overlapped section, symptoms of Fusarium wilt were very severe. Soil was sampled between the rows of spinach, avoiding roots as much as possible, in each of these two sections. The second pair of soil

samples was from a field that appeared to be relatively high risk for Fusarium wilt in the bioassay, yet the spinach seed crop planted showed no symptoms of Fusarium wilt. After discussing the observations for that field with the grower, he reported that, after seeing the results of the soil bioassay, he relocated the spinach seed crop to another part of the field that he expected to have less inoculum potential. To test whether the bioassay could differentiate the level of risk between these two areas of the field, soil was collected from where the 2010 seed crop was located and showed no Fusarium wilt symptoms, and from the area where the soil sample for the winter bioassay had been collected. The bioassay was performed for these four soil samples plus two control soil treatments, a positive control treatment consisting of the same high risk soil used previously, and a negative control treatment consisting of the fumigated soil used in the stage 2 preliminary bioassay. The bioassay was set up on 20 September 2010, and spinach plants rated for Fusarium wilt symptoms 11, 18, and 25 DAP. Plants were harvested for aboveground dried biomass on 29 October 2011, 39 DAP.

2011 grower soil bioassay. A second soil bioassay was carried out in the winter of 2010/2011, beginning with a request for soil samples sent to stakeholders in November 2010. Soil samples from 42 fields in Skagit and Clallam Counties were submitted. Each soil sample was mixed and processed with a gas-operated soil shredder (Royer Model 10, Oshkosh, WI) that greatly reduced the time required to achieve the proper tilth for planting. Pots were filled and planted on 14 January 2011. A subsample of each soil was collected prior to planting for nutrient analysis, as described above. The same high risk soil used in the 2010 bioassay was used as a positive control treatment. A steam-operated soil pasteurizer (Patzek, *unpublished report*) was used to make the no risk and intermediate risk control soils. For the negative control soil, a sample of the high risk soil was steamed at 56°C for 1 h; for the intermediate risk control soil,

the high risk soil was steamed at 40°C for 1 h. Greenhouse conditions and pest management were as described for the 2010 grower soil bioassay, with the exception that from 25 January until 7 February 2011, the temperature was lowered to 20 to 23°C/day and 18 to 21°C/night to help establish seedlings during a series of warm, sunny days that kept the greenhouse too hot. On 26 January, isolations from seven wilted seedlings, each from a different soil, were performed as described above, and similarly from nine seedlings on 7 February. Plants were rated for Fusarium wilt 21, 28, and 35 DAP, and harvested for aboveground biomass on 2 to 3 March, 47 to 48 DAP (completed daily by replication), a week earlier than the 2010 soil bioassay as this proved to be an adequate duration for differentiating risk levels. A subsample of each soil (approximately 50 g) was collected, dried, and sieved for enumeration of *F. oxysporum* and *V. dahliae* as described for the preliminary bioassays, with one replication of triplicate dilution plating performed/soil sample. An open house was held the week of 21 February for growers and seed company representatives to observe the Fusarium wilt risk in their respective soil samples. Follow-up visits to 11 of the 42 fields evaluated in the bioassay that were planted to spinach seed crops in 2011 were made during the summer of 2011 to determine whether the Fusarium wilt severity observed in each seed crop corresponded to that predicted by the bioassay.

2012 grower soil bioassay. A solicitation for field soil samples to be submitted for the 2012 soil bioassay was sent to stakeholders in November 2011. The initial funding for this project from the USDA Western Region Integrated Pest Management (WRIPM) program and the Western Sustainable Agriculture and Research Education (WSARE) program covered expenses for the first two years. A fee of $150/soil sample was instituted for the 2012 grower soil bioassay. Even with this fee, soil samples were received from 39 fields for evaluation. The soils were processed in December 2011, and the bioassay set up on 11 January 2012 using the same

protocol as the 2011 grower soil bioassay. Fusarium wilt symptoms were rated 21, 28, and 35 DAP, and plant biomass was harvested and dried on 24 February, 44 DAP. The greenhouse lighting regime and pest management practices were the same as for the previous bioassays. The temperature regime was 22 to 25°C/day and 24 to 26°C/night until 25 January, when the temperature was increased to 24 to 26°C/day and 22 to 25°C/night. Isolations from 11 wilted seedlings were carried out on 25 January, and from an additional three seedlings on 6 February.

In addition to the soil samples submitted by growers and seed company representatives, soil samples were collected from a long-term limestone-Fusarium wilt field trial (Gatch et al., 2011; see Chapter 2) to serve as validation control soils for the bioassay. That spinach seed crop trial was conducted in 2009 in a grower-cooperator's field in Skagit Co. that had been planted to a spinach seed crop in 2005 and, therefore, had a very high risk of Fusarium wilt in 2009. The effects of three rates of limestone application (0, 2.24, and 4.48 t/ha) and the same three spinach parent lines used in the bioassay were evaluated in the field trial, as described in Chapter 2. From 2009 to 2012, the grower-cooperator planted a typical wheat/potato rotation. Each year, the same limestone treatments were applied to the same plots, and in spring 2012 the spinach seed crop trial was planted in the same plots as the 2009 trial, as described in Chapter 2. The objective of that trial was, in part, to determine whether annual applications of limestone for three years prior to a spinach seed crop could enhance Fusarium wilt suppression compared to a single application of 4.48 tons limestone/ha the spring that the seed crop was planted. As a result of these annual limestone treatments, spinach plots in this trial demonstrated a wide range in Fusarium wilt severity in 2012 (see Chapter 2). Soil samples were collected in December 2011 from plots in three of the five replications of the three limestone treatments. The nine soil samples were processed and planted in the bioassay along with the 39 growers' soils. Spinach plants grown in

soil from the limestone-amended field plots developed very light green foliage compared to the control soils, so the foliage of the resistant and moderate parent lines (no seedlings of the susceptible inbred survived in soil from the non-limed plots due to severe Fusarium wilt) in each of three replicate plots/limestone treatment was harvested on 24 February, when the spinach was harvested for the rest of the bioassay. The foliage from the moderate and resistant inbred lines in the limestone field trial soils was dried at 35°C and sent to Soiltest Farm Consultants, Inc. for nutrient analysis to determine the potential cause of chlorosis.

Another opportunity for validation of the bioassay was made possible by a field production manager of one of the participating seed companies, who offered to plant approximately 1 m of row of each of the three spinach parent lines used in the bioassay in each of 10 fields that were included in the 2012 bioassay and ended up being planted to spinach seed crops in 2012. Two of the 10 fields had a high risk of Fusarium wilt based on the soil bioassay. Prior to 2012, assessing the predictive value of the soil bioassay for fields with a high Fusarium wilt risk scale had not been possible because fields that appeared to be of high risk in the bioassay were not planted with spinach seed crops by the growers because of the high value of these crops. The validation test rows of the three spinach parent lines planted in 10 spinach seed crops in fields tested in the bioassay allowed for direct validation, even though the test rows of the three female lines were not replicated and were very short. The plants in these test plots were assessed visually for Fusarium wilt severity on 27 July using the 0-to-5 rating scale.

2013 grower soil bioassay. In the 2013 grower soil bioassay, 40 soil samples were received from growers and seed company representatives. Soil processing, planting of the three spinach inbred lines, disease and soil microbial assessments, soil nutrient analyses, and spinach biomass harvest were performed as described for the 2010 to 2012 soil bioassays. The bioassay

was planted on 11 January 2013 and harvested on 20 February, 37 DAP. A fee of $200/soil sample was charged, and only four replicate pots/soil-parent line combination were planted to reduce the amount of soil each grower needed to submit. Submitters were given the option of a limestone treatment (4.48 t/ha equivalent rate) applied to their soil sample(s) prior to planting the bioassay, since growers seldom plant a spinach seed crop in northwestern Washington without amending the soil with up to 4.48 t limestone/ha prior to planting. A second set of control soils (high Fusarium wilt risk, medium risk, and low risk, created using the protocols of the 2012 bioassay) was included that were each amended with the equivalent of 4.5 t limestone/ha to compare to the non-limed control soils. Isolations from seven wilted seedlings were performed on 23 January 2013, as described for previous bioassays.

Correlation and regression analyses. Simple Pearson's correlation analyses were carried out to discern relationships among the various soil properties measured and the severity of Fusarium wilt assessed in the bioassay. The nature of these relationships was explored further using stepwise multiple regression with PROC REG in SAS Version 9.2 to identify significant regression models that explained the variability in disease severity. Soil samples from fields that had no prior history of spinach seed production, along with the control soil treatments and soils with poor spinach growth due to herbicide carryover effects, were excluded from the regression analyses, reducing the total number of soil samples assayed and included in the regression analyses to 121.

Results

Soil bioassay development. *First preliminary soil bioassay.* There were significant effects of Fusarium wilt risk level of the soil, Fusarium wilt susceptibility of the spinach inbred

line, and soil heat treatment on wilt development, although not at every rating date for the latter (Table 3.2). Fusarium wilt severity ratings increased from 32 to 51 DAP (Table 3.3). Plants growing in the high risk soil had greater Fusarium wilt severity at each rating and, thus, greater AUDPC and reduced biomass compared to plants in the low risk soil that had not had a spinach seed crop for 12 years (Table 3.3, and Fig. 3.1A and 3.1B). The AUDPC of high risk soils was nearly seven times larger than that of low risk soils (Table 3.3). The susceptible spinach line had greater Fusarium wilt severity ratings than the other two lines, starting at 40 DAP and continuing through 51 DAP, with an AUDPC approximately twice that of the moderate and resistant inbreds (Table 3.3 and Fig 3.1A). At 40, 44, and 51 DAP, this effect was observed only in the heat-treated soil (Table 3.3, and Fig. 3.1A and 3.1B). There was no overall significant difference between wilt severity of the moderate and resistant inbred lines, but in the non-heated soil, AUDPC was smallest for the resistant inbred (5.7) compared to the moderate inbred (7.6), which in turn had a smaller AUDPC than the susceptible inbred (11.3) (Tables 3.2 and 3.3). There was no significant effect of the Fusarium wilt susceptibility of the spinach inbred lines on spinach biomass (Table 3.2).

Heat treatment of the soils reduced Fusarium wilt severity starting at the second rating, 40 DAP, and continuing to 51 DAP, although the effect of heat treatment was not as significant as the main effects of soil Fusarium wilt risk level or spinach inbred line (Tables 3.2 and 3.3, Fig. 3.1A). Furthermore, at 40 and 51 DAP, a significant effect of soil heat treatment on Fusarium wilt severity was observed only in the high risk soil (Tables 3.2 and 3.3). Soil heat treatment increased spinach plant biomass only in the high risk soil (1.50 vs. 0.74 g/pot for heated and non-heated soils, respectively) (Table 3.3 and Fig. 3.1B). The population of *F. oxysporum* detected in

the heated, low risk soil; the non-heated, low risk soil; heated, high risk soil; and non-heated, high risk soil was 267; 5,200; 3,867; and 9,133 CFU/g soil, respectively.

Second preliminary bioassay. In the second preliminary soil bioassay, there were again significant main effects of soil Fusarium wilt risk level, Fusarium wilt susceptibility of spinach inbred lines, and soil heat treatment on Fusarium wilt severity ratings, AUDPC, and spinach plant biomass, except for soil heat treatment main effect on wilt severity 42 DAP (Table 3.4). There was a significant interaction between spinach inbred line and soil Fusarium wilt risk level for the 28 and 42 DAP severity ratings, AUDPC, and plant biomass (Table 3.4). Wilt developed more rapidly compared to the first preliminary bioassay, with the high risk soil displaying near maximum wilt severity (0.92 out of 1.00) at the first rating (28 DAP) compared to the first preliminary bioassay, in which wilt severity was 0.16 out of 1.00 at the first rating (32 DAP) (Tables 3.3 and 3.5). Colonies with morphology typical of *F. oxysporum* were isolated from every seedling collected, including the single asymptomatic seedling, although 5 of the 24 seedlings assayed were also infected with *Rhizoctonia* spp. (*data not shown*).

Spinach plants in the high risk soil had greater Fusarium wilt severity than spinach plants in the medium risk soil, which in turn had greater Fusarium wilt severity than plants in the low risk soil (Table 3.5 and Fig. 3.1C). This was true at each of the three weekly severity ratings and for AUDPC (AUDPC of 26.4, 9.1, and 1.5 for the high, medium, and low risk soils, respectively). Plants in the high risk soil were smaller (biomass of 0.03 g/pot) compared to the medium and low risk soils (1.27 and 1.24 g/pot, respectively) (Table 3.5. and Fig. 3.1D). Spinach plants in the two negative control soils, one autoclaved and one from an orchard with no history of spinach seed production, had a mean biomass of 1.64 and 1.90 g/pot, respectively. The susceptible and moderate spinach lines had similar Fusarium wilt severity ratings that were

greater compared to that of the resistant line throughout the trial, with AUDPC values of 13.8 and 13.3 vs. 9.9, respectively (Table 3.5). This effect varied with soil Fusarium wilt risk level, however (Table 3.4). In the low risk soil, there was no difference in Fusarium wilt severity among spinach lines, while in the medium and, to a lesser extent, the high risk soils, the susceptible and moderate inbreds had significantly greater wilt severity compared to the resistant line (Table 3.5 and Fig. 3.1C). Wilt was so severe in the high risk soil that by 35 and 42 DAP there was no significant differentiation in wilt severity among the three inbred lines. Conversely, in the low risk soil, there was negligible wilt, regardless of inbred line (Fig. 3.1C). A similar interaction between spinach line and soil risk level was observed for spinach biomass/pot and biomass/plant (Tables 3.4 and 3.5, and Fig. 3.1D). In the medium risk soil, the resistant inbred had greater biomass than the susceptible inbred, which had greater biomass than the moderate inbred (1.55, 1.26, and 0.98 g/pot, respectively). In the low risk soil, there was no significant effect of inbred line on biomass, while in the high risk soil, the resistant inbred had more biomass than the susceptible inbred but not significantly more biomass than the moderate inbred (0.06 vs. 0.00 and 0.02 g/pot, respectively) (Table 3.5).

The high heat soil treatment reduced Fusarium wilt severity at 28 and 35 DAP, but not at 42 DAP, reduced AUDPC, and increased spinach biomass compared to non-treated soil, but the effects were smaller than the effects of soil risk level and spinach parent line (Tables 3.4 and 3.5, and Fig. 3.1C and 3.1D). The AUDPC of soils exposed to the high and low heat treatments was 11.4 and 11.9, respectively, compared to 13.7 for non-treated soil. Biomass of plants in soils treated with high or low heat was greater (0.92 and 0.88 g/pot, respectively) compared to that of plants from non-treated soils (0.73 g/pot) (Table 3.5). The high and low heat treatments did not result in significantly different Fusarium wilt severity ratings or plant biomass. The heat

treatments reduced *F. oxysporum* populations in each soil; 1,978 to 622, 2,022 to 355, and 4,556 to 2,133 CFU/g in non-treated vs. high heat treated soil of the low, medium, and high risk soils, respectively, but these reductions were no longer detected at the end of the trial (Table 3.6).

Third preliminary bioassay. Results of the third preliminary bioassay were similar to those of the second preliminary bioassay, with significant main effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on weekly Fusarium wilt severity ratings, AUDPC, and spinach biomass, except for the soil heat treatment main effect on severity of wilt rated 20 DAP (Table 3.7). Overall, Fusarium wilt severity was less in the third preliminary bioassay compared to the first and second bioassays (Fig 3.1E vs. 3.1A and 3.1C, respectively). Nevertheless, beginning at 28 DAP, plants in the high risk soil had greater Fusarium wilt severity compared to plants in the medium risk soil, which had greater wilt severity than plants in the low risk soil, with final AUDPC values of 5.4, 1.5, and 0.5, respectively (Table 3.8). The spinach biomass in the negative control soil collected from a fumigated field was 9.83 g/pot (*data not shown*), compared to 5.38, 8.00, and 2.99 g/pot in the low, medium, and high risk soils, respectively (Table 3.8). A possible reason the medium risk soil produced larger plants than the low risk soil (Table 3.8 and Fig. 3.1F) may be found in results of soil nutrient analyses (Table 3.6), i.e., the low risk soil had lower levels of most nutrients, less OM, a lower CEC, and was even in the deficiency range for Ca and B compared to the medium risk soil (Marx et al., 1996). The pots in these trials were watered daily with fertigated water intended to prevent nutrient deficiencies, but in the low risk soil, the fertility program may not have been sufficient to overcome nutrient deficiencies, particularly when combined with the poor nutrient retention of this relatively low OM soil.

Beginning with the wilt severity rating 28 DAP, the susceptible inbred line had more severe wilt than the moderate line, which had more severe wilt than the resistant line, but the effect of inbred line was again conditioned by the level of Fusarium wilt risk of the soil, as estimated by the number of years since the soil had previously been planted to a spinach seed crop (Tables 3.7 and 3.8). In the low risk soil, the moderate and resistant inbred lines did not differ significantly in Fusarium wilt severity or spinach biomass, while in the medium and high risk soils, the moderate inbred had more severe Fusarium wilt than the resistant inbred, and the susceptible inbred had more severe wilt than the moderate inbred (Table 3.8 and Fig.3.1E).

The effect of soil heat treatment varied with soil risk level for wilt severity 20 DAP, AUDPC, and spinach biomass, as well as with inbred line for wilt severity measured 20 DAP (Table 3.7). There were no significant effects of soil heat treatments on Fusarium wilt severity in the low and medium risk soils, but in the high risk soil, the high and low heat treatments reduced Fusarium wilt severity compared to non-treated soil (AUDPC of high heat, low heat, and non-treated soils was 3.9, 3.6, and 8.7, respectively, in the high risk soil) (Table 3.8). Both the high and low heat treatments resulted in significantly greater spinach biomass (3.40 and 3.79 g/pot, respectively) compared to non-heated, high risk soil (1.78 g/pot), while in the medium risk soil, only the high heat treatment significantly reduced biomass compared to the low heat treatment and non-heated soil (8.97 vs. 8.02 and 7.00 g/pot, respectively) (Table 3.8 and Fig. 3.1F). In the low risk soil, there were no significant effects of soil heat treatments on any of the variables measured (Table 3.8).

2010 to 2013 grower soil bioassays: Fusarium wilt and plant growth assessments. Data for each of the bioassay soil samples are arranged in Figs. 3.2 to 3.9 in order of increasing Fusarium wilt severity based on the mean Fusarium wilt index for each soil sample averaged

over all three inbred lines on the final rating date. Control soils (high, medium, and low Fusarium wilt risk) are shown at the far right in each figure.

2010 grower soil bioassay. A total of 26 soil samples were submitted by eight stakeholders (growers and seed company representatives) for the first grower soil Fusarium wilt bioassay, with 25 soils from Skagit Co. and one from Snohomish Co., WA. Plants in four of the growers' soils exhibited abnormal growth characterized by stunting and chlorosis, which was attributed to a soil pH too low (4.8) to foster normal growth of spinach (Beattie, 1937), and probable herbicide carryover for the other three soils (T. W. Miller, Extension Weed Scientist, Washington State University, *personal communication*). Fusarium wilt could not be assessed for plants growing in these soils, so they were excluded from the evaluations. A species of *Pythium* was isolated from a wilting seedling of the moderately susceptible inbred line plated onto agar media on 25 January 2010. Of the nine wilting seedlings collected on 1 February, seven (four seedlings of the susceptible inbred, two of the moderate inbred, and one of the resistant inbred) were infected with *F. oxysporum*, one seedling of the resistant inbred was infected with a *Pythium* sp., and one seedling of the resistant line was not infected with any known spinach pathogen.

Symptoms of Fusarium wilt were observed in the bioassay, with severity affected significantly by soil sample and spinach inbred line susceptibility to the disease, as well as the interaction of these factors (Table 3.9). By the final rating, 42 DAP, the severity of Fusarium wilt averaged across inbred lines was >0 for every soil (Fig. 3.2A). A wide range in apparent Fusarium wilt risk was observed among the 26 submitted soil samples, despite the fact that the samples were all from fields under consideration for planting a spinach seed crop in the 2010 season. The Fusarium wilt severity index measured 42 DAP ranged from 0.01 to 0.95, with a

mean of 0.49 (Fig 3.2A). Fusarium wilt severity for each inbred line and soil sample increased over the course of the 42 days of the bioassay, although in five soil samples the final Fusarium wilt index remained <0.2 (soils 13, 7, 25, 16, and 14; Table 3.10 and Fig. 3.2A).

Overall, the susceptible inbred line had significantly more severe Fusarium wilt than the moderate line, which in turn had more severe Fusarium wilt than the resistant line at each rating and for AUDPC (AUDPC of 16.5, 9.6, and 6.1, respectively) (Table 3.10), but these effects varied depending on the soil sample (Fig 3.3A). For example, at 35 DAP, the moderate and resistant inbred lines in soil 1 did not differ significantly in Fusarium wilt severity index (0.17 and 0.15, respectively), but both had less severe wilt severity than the susceptible inbred (0.76) (Fig. 3.3A). Soil 27, the high risk control soil, had such severe wilt for all three inbred lines that there were no significant differences in severity ratings among the inbreds. In contrast, the Fusarium wilt inoculum potential of soils 7 and 13 was so low that mild wilt symptoms were observed only for the susceptible inbred line. In nine soil samples (soils 2, 5, 6, 9, 10, 11, 18, 19, and 23), spinach biomass was reduced to ≤0.3 g/pot, highlighting the potential for severe losses to Fusarium wilt when a susceptible inbred is planted in a high risk soil (Fig. 3.3A)

Spinach biomass (g/plant and g/pot) was significantly affected by soil sample and spinach inbred line, as well as the interaction of these two factors (Table 3.9). The resistant and moderate inbred lines had greater biomass overall compared to the susceptible line (3.19 and 3.35 vs. 2.70 g/pot, respectively) (Table 3.10), but this effect was also dependent on the soil sample (Fig. 3.3B). In soils with a low Fusarium wilt risk index, the resistant line typically had smaller plants (less biomass) than the moderate and susceptible inbred lines (e.g., soils 7, 13, 14, and 25 in Fig. 3.3B). As Fusarium wilt severity increased, this pattern reversed, such that the resistant inbred line tended to produce plants with greater biomass than the moderate and susceptible inbred lines

(e.g., soils 2, 5, 6, and 23 in Fig. 3.3B). Despite the influence of spinach inbred line, the mean Fusarium wilt severity index 28 DAP of all three inbred lines was negatively correlated with spinach biomass (-0.8863, P <0.0001, Table 3.11), as evidenced by the opposing trends for wilt severity index and biomass (Fig. 3.2 and 3.3).

Fusarium wilt development and plant growth in the control soils designated as high (soil 27), intermediate (soil 28), and low risk (soil 29) for Fusarium wilt was, overall, as expected, although the medium risk soil developed a greater level of Fusarium wilt than anticipated (Fig. 3.2A and 3.3A). The heat treatment used to generate this soil treatment was meant to create a level of Fusarium wilt that corresponded to roughly half the Fusarium wilt risk in the high risk control soil (Fig 3.2A). By the end of the bioassay, the medium and high risk soils did not differ significantly in Fusarium wilt severity index when averaged across inbred lines. However, the resistant inbred grown in the medium risk control soil had significantly less wilt than in the high risk soil (0.79 vs. 0.98) (Fig. 3.3A). The low risk, autoclaved control soil had a very low level of Fusarium wilt by 42 DAP, indicating that the autoclaving treatment did not completely eliminate the pathogen, but reduced the level to negligible, as symptoms were only observed on the final rating (42 DAP) (Fig. 3.2A). More spinach biomass (larger plants) was produced in this low risk control soil than in the medium and high risk control soils (2.95 vs. 0.30 and 0.07 g/pot, respectively) (Fig. 3.2B). However, plants in four of the growers' soil samples produced approximately twice as much biomass compared to the low risk control soil (Fig 3.2B).

2011 grower soil bioassay. The 42 soil samples received for the 2011 Fusarium wilt soil bioassay were submitted by 10 stakeholders from fields in Skagit Co. (35 samples) and Clallam Co. (6 samples), as well as British Columbia, Canada (1 sample). Of the seven wilted seedlings collected for isolations on 26 January 2011, two were infected with *Rhizoctonia* spp. (one

resistant inbred seedling and one moderate inbred seedling), one was infected with both *Rhizoctonia* and *F. oxysporum* (a moderate inbred seedling), three had no fungi isolated (two moderate inbred seedlings and one resistant inbred seedling), and one was infected with an unidentified fungus (a susceptible inbred seedling). The four seedlings collected from soil 5 on 3 February 2011 were all infected with *F. oxysporum*, and one of these seedlings was also infected with a *Rhizoctonia* sp. A seedling of the susceptible line in the medium risk control soil (soil 46) collected on 3 February was infected with *F. oxysporum*. Of the four spinach seedlings from soil 24 plated on 3 February, one was infected with *F. oxysporum*, two with both *F. oxysporum* and a *Rhizoctonia* sp., and one with an unidentified fungus. Nine other wilted seedlings selected at random on 3 February and plated were infected with *F. oxysporum* (one seedling of each of the susceptible, moderate and resistant inbred lines), *Rhizoctonia* spp. (three seedlings of the susceptible inbred line), or unidentified fungi (two seedlings of the moderate line). These isolations, which were more extensive than those conducted in the 2010 bioassay, confirmed what appeared to be a problem with Rhizoctonia seedling blight in the first few weeks of the bioassay.

The overall mean Fusarium wilt severity index at each rating was less in the 2011 bioassay compared to the soils evaluated in the 2010 bioassay (Table 3.10). Of the 42 soils submitted, 7 (soils 15, 20, 28, 30, 31, 34, and 40) had no apparent Fusarium wilt risk in the 2011 bioassay based on the absence of Fusarium wilt symptoms at every rating (Fig. 3.4A and Fig. 3.5A). Five soils (soil 15, 27, 29, 30, and 32, all of which were collected from fields in Clallam Co.) were reported by the submitters as never previously having been planted to a spinach seed crop, and yet three of these samples (27, 29, and 32) had very low severity ratings. Overall, mean Fusarium wilt severity increased from 0.03 at 21 DAP to 0.21 at the final rating (35 DAP) in the

2011 trial, compared to a mean Fusarium wilt severity of 0.44 at 35 DAP in the 2010 bioassay (Table 3.10). As in the 2010 bioassay, a wide range in apparent Fusarium wilt risk was observed among soil samples, despite the fact that these samples were all from fields under consideration for planting spinach seed crops in 2011. The mean Fusarium wilt severity index of the three inbred lines at the end of the bioassay, 35 DAP, ranged from 0.00 to 0.80, with a mean of 0.21 (Fig 3.4A). However, in some soil samples, plants of the susceptible inbred line had wilt indices >0.90 (Fig. 3.5A).

The main effects of soil sample and spinach parent line, and the interaction of these two factors, were highly significant for Fusarium wilt severity at each rating and for AUDPC (Table 3.9). Although the main effect of spinach inbred line on spinach biomass production was not significant, the interaction between inbred line and soil sample was highly significant (Table 3.9). The coefficients of determination (R^2) were slightly smaller, and the coefficients of variation (CV) slightly greater (except for severity of wilt 21 DAP), for the 2011 soil bioassay compared to the 2010 bioassay, indicating there was slightly more unexplained variability in the 2011 bioassay data; however, an R^2 >0.7 for all variables except wilt severity measured 21 DAP suggests that the 2011 bioassay data was quite robust. The highly significant interaction between soil sample and spinach parent line is evident in Fig. 3.5A, with soil 24 exhibiting significant differentiation in Fusarium wilt severity in accordance with the susceptibility of the inbred lines (Fusarium wilt index of 0.93, 0.66, and 0.12 for the susceptible, moderate, and resistant spinach inbreds, respectively), while soils such as 19 and 23 had wilt severity index values that deviated from this pattern (0.88, 0.35, and 0.67 wilt index for the susceptible, moderate, and resistant inbreds, respectively, for soil 19; and 0.59, 0.13, and 0.16, respectively, for soil 23).

Efforts to create a medium risk soil (soil 46) using steam-pasteurization equipment were unsuccessful. Plants in the medium risk soil displayed even more severe Fusarium wilt (Fig. 3.4A) and produced less biomass (Fig. 3.4B) than plants in the high risk soil (soil 45), when the reverse was expected. However, the steam pasteurizer was superior to autoclaving for reducing Fusarium wilt inoculum potential in the low risk control soil (soil 47) without adversely affecting plant growth, as no wilt symptoms were observed for any of the inbred lines in this soil throughout the bioassay, and plant biomass was in the top 15% of the 45 soils assayed (Fig. 3.4A and 3.4B).

Plants in six of the growers' soils exhibited abnormal growth characterized by stunting and chlorosis, which was attributed to probable herbicide carryover (T. W. Miller, *personal communication*). Because these symptoms interfered with Fusarium wilt assessment, ratings for plants in these soils were delayed until the final rating (35 DAP). Based on this single wilt assessment, three of these soils (soils 5, 24, and 41) were determined to be medium or high risk for Fusarium wilt, and three were low risk (soils 20, 37, and 42) (Fig. 3.4A). The plant biomass harvested from these three low-risk, herbicide-contaminated soils all had less biomass than predicted based on the soil Fusarium wilt risk alone. These soils, as well as two others in which plants did not grow as vigorously as expected for unknown reasons (soils 11 and 30), or soils in which there was very poor germination (soil 44), may account for the lower negative correlation between Fusarium wilt severity index and spinach biomass in the 2011 soil bioassay (-0.5085, P <0.0001) compared to the 2010 bioassay (-0.8863, P <0.0001) (Table 3.11).

A similar relationship to the one observed between spinach inbred line, soil Fusarium wilt severity index, and spinach biomass for the 22 growers' soils in the 2010 bioassay was also observed for the 40 growers' soils in the 2011 bioassay (Fig. 3.5). In soils with low Fusarium

wilt severity index, the susceptible inbred line tended to have greater plant biomass production compared to the resistant line, while in soils with high Fusarium wilt severity index, the resistant inbred line generally produced greater biomass compared to the susceptible line. In both bioassays, some soils presented exceptions to this pattern (e.g., soils 20 and 30 in the 2011 bioassay). However, the average spinach biomass/pot for soils with lower Fusarium wilt indices, i.e., the first 20 soils in Fig. 3.5B, was 3.11, 2.75, and 2.59 for the susceptible, moderate, and resistant parent lines, respectively; and the average spinach biomass/pot for soils with high Fusarium wilt risk index, i.e., the next 20 soils, was 1.13, 2.03, and 2.09 for the susceptible, moderate, and resistant inbred lines, respectively.

2012 grower soil bioassay. The 39 soils submitted by nine stakeholders for inclusion in the 2012 Fusarium wilt soil bioassay were all from Skagit County, WA. For two of the soil samples, seedlings developed severe chlorosis and stunting after emergence and died soon thereafter, presumably due to herbicide carryover in the soil based on symptoms (T. W. Miller, *personal communication*). These soils could not be included in Fusarium wilt assessments. Of the 11 wilted seedlings collected on 25 January 2012, four were infected with *F. oxysporum* (three seedlings of the susceptible inbred and one of the moderate inbred), five with *Rhizoctonia* spp. (one seedling of the susceptible inbred, one of the moderate inbred, and three of the resistant inbred), and two (both of the susceptible line) with unidentified fungi. Three additional seedlings collected on 6 February were all infected with *F. oxysporum* (one susceptible, one moderate, and one resistant inbred seedling).

There were highly significant main effects of soil sample and spinach inbred line, and a highly significant interaction between these two factors for all dependent variables (Table 3.9). The mean Fusarium wilt severity indices averaged over all soils and inbred lines were greater at

each rating date in the 2012 bioassay compared to the 2011 bioassay, but less than those of the 2010 bioassay (Table 3.10). As with the previous bioassays, Fusarium wilt severity increased over the 35 day duration of the 2012 soil bioassay (Table 3.10, Fig. 3.6A). The protocol used to create a medium risk control soil (soil 50) in the 2012 soil bioassay was more successful than the methods used in the previous bioassays. Mixing the high risk soil (soil 51) with pasteurized soil (soil 49) in a 1:9 ratio resulted in a soil Fusarium wilt severity index that was greater than that of the pasteurized, low risk soil and less than that of the high risk soil (Fig. 3.6A).

Overall, the susceptible inbred line had more severe Fusarium wilt than the moderate inbred line, which had more severe Fusarium wilt than the resistant inbred line for individual ratings 21, 28, and 35 DAP, and for AUDPC (AUDPC of 6.6, 3.2, and 1.4, respectively) (Table 3.10), but the reactions of the three inbred lines were highly influenced by individual soil samples (Fig. 3.7A). In soil 6, for example, there was a low and statistically similar Fusarium wilt severity index for all three inbred lines (Fig. 3.7A). In soils 3 and 29, however, the susceptible and moderate inbreds did not differ significantly in Fusarium wilt severity, but wilt severity for these two inbreds was greater than that of the resistant parent (Fig. 3.7A).

A wide range in Fusarium wilt severity was observed among the 39 growers' soil samples (Fig. 3.6A). Nineteen soils appeared low to very low risk for Fusarium wilt (mean Fusarium wilt index averaged across inbred lines was <0.2), and 18 soils appeared moderate to high risk (mean Fusarium wilt index ≥0.4) (Fig. 3.7A). However, for some of the soils that appeared to be of moderate to high risk based on the mean indices, the soil-by-inbred line data provide a better assessment of the potential for losses to Fusarium wilt. In soils 7, 13, 14, 20, 22, 29, and 30, for example, Fusarium wilt could be minimal if a resistant inbred line were deployed, while severe losses could be incurred if a susceptible inbred were planted. For soils 1, 2, 10, 16, and 26, even

an inbred known to have some resistance to Fusarium wilt could be risky, given the moderate severity of the disease observed in the bioassay with the resistant inbred (Fig. 3.7A).

Mean spinach biomass (g/plant and g/pot) averaged over all soil samples was significantly greater for the moderate and resistant inbred lines compared to the susceptible line (2.01 and 1.90 vs. 1.59 g/pot, respectively) (Table 3.10). The negative correlation between spinach plant biomass and Fusarium wilt severity was larger in the 2012 bioassay compared to the 2011 bioassay, as evidenced by the more consistent decrease in spinach biomass with increasing Fusarium wilt severity (Figs. 3.6A and 3.6B), and by a Pearson's correlation coefficient of r = -0.8489 (P <0.0001) compared to -0.5085 (P <0.0001) in the 2011 bioassay for Fusarium wilt severity index (35 DAP) and spinach plant biomass (Table 3.11). A pattern similar to that observed in the 2010 and 2011 bioassays was apparent in the 2012 soil bioassay, with the susceptible inbred line producing bigger plants in low risk soils compared to the resistant line, and the reverse in high risk soils (Fig. 3.7B).

2013 grower soil bioassay. A total of 40 soils submitted by nine stakeholders was evaluated in the 2013 Fusarium wilt soil bioassay. Overall, Fusarium wilt severity increased from 0.16 at 21 DAP to 0.33 at 28 DAP (Table 3.10). This final Fusarium wilt severity index was similar to the 35 DAP rating in the 2012 bioassay (0.32), and greater than the 35 DAP rating in the 2011 bioassay (0.21) (Table 3.10). The Fusarium wilt severity of plants in each soil increased during the bioassay. However, as in previous bioassays, there was a wide range in wilt severity among the soils, with a minimum of 0.01 (soil 17) and maximum of 0.81 (soil 37), and with more than half of the soil samples displaying a mean Fusarium wilt index <0.5 by the final rating (Fig. 3.8A). Two of the wilted seedlings collected for isolation and identification of spinach pathogens were infected with *F. oxysporum* (one moderate inbred seedling and one resistant

inbred seedling), one seedling of the susceptible inbred was infected with a *Rhizoctonia* sp., and four were infected with both *F. oxysporum* and *Rhizoctonia* spp. (all seedlings of the resistant inbred line). Spinach plants in six soil samples (3, 5, 7, 9, 17, and 18) had negligible Fusarium wilt symptoms (severity index <0.1) (Fig. 3.8A). The correlation between spinach biomass production and Fusarium wilt severity was significantly negative ($r = -0.8125$, $P \leq 0.0001$).

There were highly significant main effects of soil samples and spinach inbred lines, and a highly significant interaction between these two factors for all dependent variables in the 2013 bioassay (Table 3.9). Fusarium wilt was significantly more severe in the susceptible inbred line compared to the moderate inbred, and more severe in the moderate inbred than in the resistant inbred, at each rating date and for the AUDPC (AUDPC was 5.3, 3.1, and 1.9 for the susceptible, moderate, and resistant inbred lines, respectively) (Table 3.10). Spinach biomass (g/plant and g/pot) was significantly greater for the resistant inbred compared to the moderate inbred, and for the moderate inbred compared to the susceptible inbred (Table 3.10). This degree of significant differentiation among all three inbred lines was not observed in the previous three grower soil bioassays, in which the moderate and resistant inbreds did not differ significantly in biomass but had significantly more biomass than the susceptible inbred (2010 and 2012), or did not differ significantly at all (2011) (Table 3.10).

Figs. 3.9A and 3.9B illustrate the significant inbred line-by-soil interaction for Fusarium wilt severity ratings 28 DAP, and for spinach biomass production in the 2013 soil bioassay. In soils 12 and 23, for example, there was little differentiation among the three inbred lines, while in soils 31 and 36, the susceptible inbred had significantly more severe Fusarium wilt than the moderate inbred, which had significantly more severe Fusarium wilt than the resistant inbred (Fig. 3.9A). A pattern similar to that observed in previous soil bioassays was observed in the

2013 soil bioassay, with the susceptible inbred line producing bigger plants in low risk soils compared to the resistant line, and the reverse in high risk soils (Fig. 3.9B).

The limestone amendment included in the 2013 bioassay suppressed Fusarium wilt in the medium and high risk control soils (soils 45 and 46, respectively, in Figs. 3.8 and 3.9) compared to the non-limed, medium and high risk soils (soils 42 and 43, respectively). The medium and high risk soils, with or without limestone amendment, did not differ significantly in Fusarium wilt severity index at 28 DAP (Fig. 3.8A). Spinach plants growing in the pasteurized, low risk control soil, with or without limestone (soils 44 and 41, respectively), should have had negligible Fusarium wilt symptoms. Although this was the case for the non-limed, low risk control soil (soil 41), wilt was observed in the limestone-amended, low risk control soil (soil 44, with a Fusarium wilt index of 0.31), possibly as a result of incomplete pasteurization for that soil sample.

Bioassay validation. *2010.* Not all fields that were sampled and evaluated in the soil bioassays were planted to a spinach seed crop that season, although the bioassay samples submitted were all from fields under consideration by growers for planting a spinach seed crop that season. Visual evaluations of spinach seed crops planted in 2010 were conducted for 15 of the fields from which soil samples were evaluated in the 2010 soil bioassay. Observations of Fusarium wilt severity generally substantiated the results of the bioassay. For one soil, the field appeared to be high risk in the soil bioassay but very little Fusarium wilt was observed in the spinach seed crop planted in that field in 2010. However, the grower revealed that the planting site had been moved, based on results of the soil bioassay, to a lower risk portion of the field that had been rotated out of spinach seed crops longer than the original section of the field that was sampled for the bioassay. A subsequent greenhouse bioassay completed in August 2010 with soil collected from both parts of this field confirmed what was observed in the growers' spinach seed

crop in 2010, alleviating the concern that the results of the bioassay had overestimated Fusarium wilt risk for this field (*data not shown*).

2011. Cold and wet spring conditions in 2011 severely restricted planting of spinach seed crops throughout northwestern Washington, thus reducing the number of fields available to visit for validation of the 2011 soil bioassay results. Nonetheless, visual observations were completed for 11 fields evaluated in the 2011 bioassay that were planted to spinach seed crops in 2011. Six of these fields (soils 12, 28, 31, 34, 30, and 11) had almost no Fusarium wilt in the bioassay (severity index <0.1, Fig. 3.4A), and the absence of Fusarium wilt symptoms in the corresponding spinach seed crops validated these predictions. Three of the soils (soils 9, 10, and 33) had low risk for Fusarium wilt in the 2011 soil bioassay (mean Fusarium wilt index <0.2), and two (soils 36 and 13) had a low to medium risk (mean Fusarium wilt index between 0.2 and 0.5) (Fig. 3.4A). The incidence and severity of Fusarium wilt in these fields, based on visual assessments of the proprietary inbred lines of unknown Fusarium wilt susceptibility planted by the growers in those fields, corroborated the bioassay results (*data not shown*).

The test plots in the field corresponding to soil 37 in the 2011 bioassay did not have any symptoms of Fusarium wilt in any of the three inbred lines. This, together with the absence of wilt symptoms in the surrounding inbred lines of the grower's seed crop, confirmed the results of the bioassay showing low Fusarium wilt risk for this field (Fig. 3.4A and 3.5A). Additional test plots planned for the 2011 season were not planted due to weather-related delays or cancellations of some spinach seed crops by the growers or seed companies.

2012. Of the test plots that were planted in 10 growers' spinach seed crops in the 2012 season, two (soils 18 and 22) were severely damaged by flooding where the test rows were planted, and could not be assessed for Fusarium wilt development. Fusarium wilt ratings in the

remaining eight test plots for each of the three inbred lines were comparable to those of the corresponding soils in the 2012 bioassay (Fig. 3.10). Plants in soils 12, 15, 19, and 28 had very mild Fusarium wilt symptoms in both the bioassay (Fig. 3.10B) and the field test plots (Fig. 3.10A), as well as in the rest of each field that was planted by the growers with inbred lines of unknown susceptibility to Fusarium wilt. Spinach plants growing in soils 13, 20, and 21 had moderate to severe Fusarium wilt in the 2012 bioassay, depending on the inbred line, and similarly in the field test plots (Fig. 3.10) and each commercial spinach seed crop. In the 2012 bioassay, soil 26 had a moderate risk of Fusarium wilt (Fig. 3.10B), and yet little wilt was observed in the corresponding field test plots and in the inbred lines planted by the grower (Fig. 3.10A). This discrepancy may be associated with the use of limestone in the field by the grower, but not in the soil bioassay.

The bioassay results for soil samples collected in November 2011 from a four-year, limestone-spinach Fusarium wilt field trial were similar to those of the corresponding plots in the 2012 field trial (Tables 3.12 and 3.13; Fig. 3.11). In the greenhouse soil bioassay, there were highly significant main effects (P <0.0001) of both limestone application rate and spinach inbred line on Fusarium wilt severity measured 28 and 35 DAP, and on spinach biomass (Table 3.12). In the subsequent 2012 spinach field trial, the effects of limestone rate on spinach biomass and Fusarium wilt severity on 9 July were also significant (Tables 3.12 and 3.13), but less so compared to the bioassay, and limestone rate did not affect Fusarium wilt severity significantly on 31 July in the field trial (Tables 3.12 and 3.13). Spinach inbred lines affected Fusarium wilt severity on 9 and 31 July in the field trial, with the most severe wilt in the susceptible inbred, followed by the moderate and resistant inbreds, but had no significant effect on spinach biomass production (Tables 3.12 and 3.13).

In the 2012 greenhouse soil bioassay for the limestone-amended soils, Fusarium wilt severity was greatest in soil from plots with no limestone, followed in decreasing order by the soil amended with 2.24 and 4.48 t/ha at both 28 and 35 DAP (Table 3.13, Fig. 3.11A and 3.11C). In the field, however, the 2.24 and 4.48 t/ha plots did not differ significantly in wilt severity on either 9 or 31 July, although both had less wilt compared to the 0 t/ha plots (Table 3.13, Fig. 3.11B and 3.11D). In both the greenhouse bioassay and the field, spinach plants in soil amended with 4.48 t limestone/ha had significantly greater biomass compared to spinach plants in plots with 2.24 t limestone/ha, which in turn had greater plant biomass compared to plants in plots with no limestone amendment (Table 3.13). In the greenhouse bioassay, the susceptible inbred had more severe wilt and smaller plant biomass compared to the moderate inbred, which, in turn, had more severe wilt and smaller biomass compared to the resistant inbred (Table 3.13). The same trends were observed in the field, although the effect of inbred line on plant biomass was not significant (Tables 3.12 and 3.13).

Spinach plants grown in soils collected from field plots that had received 2.24 and 4.48 t limestone/ha annually for three years developed a chlorotic appearance during the bioassay. Plants in soils sampled from the 0 t/ha plots has significantly higher levels of Zn, Mn, Cu, and Fe, and lower N and B compared to plants in soil amended with 2.24 or 4.48 t limestone/ha/year, although none of the tissue nutrient levels were in the deficiency range for any of the limestone treatments (Table 3.14) (Maynard and Hochmuth, 1997).

Association of soil properties with Fusarium wilt inoculum potential. The means and standard errors for the soil nutrient analyses are provided in Table 3.15 for each year the grower soil bioassay was conducted. These 147 soils are representative of agricultural soils in northwestern Washington. The soil nutrient analyses indicated that maritime Pacific Northwest

soils tend to be moderately acid (mean pH of 5.70 to 6.15), with adequate to high levels of most macro- and micronutrients (Marx et al., 1996). Furthermore, the average buffer pH was >6.4 for soils submitted each year, which suggests that the soils collectively are not highly buffered and, therefore, could be amenable to long-term pH manipulation using limestone amendments.

Sixteen soil properties correlated significantly with Fusarium wilt severity measured 28 DAP in the greenhouse soil bioassays, although the number of significant correlations varied among the three spinach inbred lines (Table 3.16). The susceptible inbred had the greatest number of significant correlations between Fusarium wilt severity and soil properties (13), followed by the moderate inbred (10) and the resistant inbred (8) (Table 3.16). Two macronutrients, NH_4^+-N and K, were positively correlated with Fusarium wilt severity, NH_4^+-N for all three inbred lines and K for the susceptible and resistant inbreds. Ca and B, which contribute to cell wall integrity and plant defense (Marschner, 2012), were also positively correlated with Fusarium wilt severity, but only for the moderate and susceptible inbreds for B, and for the susceptible inbred alone for Ca. Three of the acid micronutrients (Fe, Mn, and Cu) were positively correlated with Fusarium wilt severity: Fe for all three inbreds, Mn for the resistant inbred, and Cu for the susceptible inbred (Table 3.16). Soil pH was negatively correlated with Fusarium wilt severity only for the susceptible inbred, while SMP buffer pH was negatively correlated with Fusarium wilt severity for the susceptible and moderate inbreds (Table 3.16). The soil EC was positively correlated with Fusarium wilt severity for the susceptible and moderate inbreds, as was the population of *F. oxysporum* estimated by dilution plating. The percentages of sand and clay were negatively and positively correlated, respectively, with Fusarium wilt severity for all three inbreds, whereas the percentage of silt was negatively correlated with wilt severity for the moderate inbred only. The number of years since the

previous spinach seed crop (rotation interval) was negatively correlated with Fusarium wilt severity for all three inbreds (Table 3.16). Variables that did not correlate significantly with Fusarium wilt severity included NO_3-N, P, Mg, S, Na, Zn, CEC, and the *V. dahliae* population (*data not shown*).

Relationships between soil properties and soil Fusarium wilt severity ratings, analyzed using a separate stepwise forward-selection regression analysis for each inbred line, revealed a six-variable model for the susceptible inbred in which spinach rotation interval, NH_4^+-N, soil pH, buffer pH, and percent sand and clay explained the maximum variability associated with Fusarium severity wilt in the 2010 to 2013 soil bioassays. For the moderate inbred, the best model included rotation interval, *V. dahliae* population, NH_4^+-N, soil pH, and clay as the predictor variables. For the resistant inbred, the best model included rotation interval, NH_4^+-N, K, and soil pH. The following equations best described the predicted Fusarium wilt severity at 28 DAP, Y:

Susceptible inbred: $Y = -0.4556 – 0.0172(\text{rotation}) + 0.0854(NH_4^+\text{-N}) – 0.3875(\text{soil pH}) + 0.4037(\text{buffer pH}) + 0.0056(\text{sand}) + 0.0245(\text{clay})$ ($R^2 = 0.3396$ at $P < 0.0001$)

Moderate inbred: $Y = 1.0777 – 0.0136(\text{rotation}) + 0.0005(V.\ dahliae) + 0.0563(NH_4^+\text{-N}) – 0.1528(\text{soil pH}) + 0.0045(\text{clay})$ ($R^2 = 0.3213$ at $P < 0.0001$)

Resistant inbred: $Y = 0.6161 – 0.0094(\text{rotation}) + 0.0498(NH_4^+\text{-N}) + 0.0003(\text{K}) – 0.0947(\text{soil pH})$ ($R^2 = 0.2415$ at $P < 0.0001$)

One of the complicating issues with such multiple regression analyses is the multicollinearity of many of the soil properties measured (Table 3.17). Although this does not violate the assumptions for multiple regression, it can make the interpretation of partial regression coefficients difficult (Freund and Littell, 1991). For example, soil CEC was positively

correlated with the levels of many soil nutrients in these soils. The percentage of sand, which was negatively correlated with CEC, was thus negatively correlated with the levels of many soil nutrients. Levels of Fe and Zn, two of the acid micronutrients that are more available in low pH soils, did correlate positively with pH of the soils evaluated in the bioassays (Table 3.17). However, Mn, another of the acid micronutrients, did not correlate significantly with any other soil property (Table 3.17).

Discussion

Spinach seed growers and seed company stakeholders have for years sought a way to predict the spinach Fusarium wilt risk of fields in the maritime Pacific Northwest, where conducive soils and the persistent nature of *F. oxysporum* f. sp. *spinaciae* can lead to devastating and unexpected yield losses in spinach seed crops despite the routine practice of 10 to 15 year rotations between spinach seed crops (Foss and Jones, 2005). Given the recent success of molecular methods for detection and quantification of some soilborne pathogens (Ophel-Keller et al., 2008), this approach was pursued for spinach Fusarium wilt risk assessment of soils by Okubara et al. (2013). However, a limited correlation between quantity of the purported DNA detected with the Taq-man real-time PCR assay for the spinach pathogen and Fusarium wilt severity across a diversity of growers' fields suggested that, for this pathosystem, inoculum density may not be the most valuable predictive factor. Use of that molecular assay was also confounded by a relatively high level of cross-reaction of the assay with non-pathogenic strains of *F. oxysporum* obtained from spinach seed, plants, and soil sampled from northwestern Washington. Soil edaphic properties are known to influence disease progression in Fusarium wilts of other crops (e.g., Hoper and Alabouvette, 1996). In this study, a greenhouse soil

bioassay for evaluation of spinach Fusarium wilt inoculum potential (risk) was developed, deployed, and to a limited extent, validated, over the course of four years in northwestern Washington, the primary region of spinach seed production in the USA (Foss and Jones, 2005). In addition to developing an effective soil risk prediction tool, a secondary objective was to identify soil chemical and physical properties that are associated most closely with soil conduciveness to spinach Fusarium wilt, as a potential aid in the selection of fields suitable for spinach seed crops.

Soil bioassays conducted in controlled greenhouse environments have been used successfully for screening host germplasm for resistance to various soilborne diseases, including Fusarium wilts (Subramanian et al., 2006). Often such bioassays use a range of inoculum concentrations in sterilized soil or other growing media to assess host resistance (e.g., Becerra Lopez-Lavalle et al., 2012). When the soil, not the host plant, is evaluated for conduciveness to disease, similar standardized methods can be used in which the soil(s) under consideration are inoculated with increasing concentrations of the pathogen (Alabouvette et al., 2005). Growing a susceptible host plant at different infestation levels allows a disease progress curve to be calculated for each soil and compared to those of other soils. This method is effective only when there is no initial pathogen inoculum in the soil, which can be achieved by selecting sampling sites that have no history of the disease in question or by pasteurizing or sterilizing the soil to eliminate the pathogen. In the case of spinach Fusarium wilt, the former would be very difficult in the spinach seed-producing areas of northwestern Washington, since fields that have never had a spinach seed crop and are thus guaranteed to be free of the Fusarium wilt pathogen, are increasingly rare in this primary region of spinach seed production. However, the latter is

undesirable because soil sterilization precludes assessment of the contribution of the soil microbial community to disease development or suppression (Lockwood, 1964).

The soil bioassay developed in this study evolved from these considerations and the unique challenges of working with a wind-pollinated seed crop, the placement of which is highly regulated to maintain genetic purity of the harvested seed, in a region with limited agricultural acreage. To begin bioassay development, two soil samples, one collected three months after the completion of a spinach seed crop field trial in which severe Fusarium wilt occurred (Gatch et al., 2011), and one from a field that had not had a spinach seed crop for 12 years, were used for a preliminary greenhouse soil bioassay. Results of this bioassay demonstrated that spinach plants grown in naturally-infested soils in pots in a greenhouse environment can develop Fusarium wilt in less than two months, and that a high risk soil can be distinguished from a low risk soil based on severity of Fusarium wilt symptoms, with supporting evidence provided by reduced spinach biomass in the high vs. low risk soils. While the susceptible spinach inbred line had greater wilt severity compared to the moderate and resistant inbred lines, the moderate and resistant lines did not differ significantly for wilt severity (except in the low risk soil, and only for cumulative wilt severity represented by the AUDPC), and there were no significant differences in final spinach biomass among the three inbred lines. Increasing the number of replications from four to five in the second preliminary bioassay improved statistical differentiation of the moderate and resistant lines for wilt severity or biomass production.

The preliminary bioassay protocol proved sensitive enough to detect a difference in Fusarium wilt inoculum potential, and accompanying spinach growth, between a high risk soil that was heat-treated to reduce the pathogen population and the non-treated, high risk soil. Although significant, the reduction in Fusarium wilt severity achieved with the soil heat

treatment was slight, with a severity index of 0.40 for treated soil vs. 0.52 for non-treated soil. The electric soil sterilizer did not expose the soil uniformly to 65°C, and Fusarium wilt inoculum potential was only slightly reduced by the heat treatment. To address this, an additional treatment was evaluated in the second preliminary trial, consisting of a higher temperature and longer exposure of the moist soil to heat.

The amount of time (51 days) required for completion of the first preliminary bioassay was not optimal. Final decisions regarding the placement of spinach seed crops in the Skagit Valley of Washington State are made at an annual seed-crop mapping (pinning) meeting held in early March to ensure minimum pollen isolation distances are maintained. Results of the bioassay need to be available to stakeholders several weeks prior to this meeting to assist in the final field selection process for spinach seed crops. After consultation with stakeholders, soil samples were delivered from target field sites each year in December, to minimize the length of time soils were stored prior to the bioassay, while accommodating the schedules of the growers and seed company representatives sampling fields. Several weeks were needed to process the ~38 liters of soil received for each sample prior to planting. With these bounds for completion of the bioassay, the maximum duration of the soil bioassay was five to six weeks. In the first preliminary bioassay, Fusarium wilt symptoms were slower to develop than anticipated. However, it was subsequently determined that the original greenhouse used for this bioassay often maintained temperatures lower than the desired 23 to 28°C. Temperature is important in the development of many Fusarium wilts (Baker and Cook, 1974), so the second preliminary bioassay was completed in a greenhouse with superior temperature regulation. The rate of Fusarium wilt development was faster in this second bioassay than in the first bioassay, and by

28 DAP, Fusarium wilt symptoms were evident in all but the low risk soil. This enabled the first wilt rating to be done 21 DAP and a final rating by 35 DAP.

In the second preliminary bioassay, the addition of a soil sample from a field with an eight year spinach seed crop rotation, along with a second, higher-temperature soil heat treatment, created additional levels of Fusarium wilt potential with which to test sensitivity of the bioassay. Based on Fusarium wilt severity ratings, the eight-year soil was intermediate in risk and differentiated significantly from the high risk, 0-year rotation soil as well as the low risk, 12-year rotation soil. Spinach plants in this medium risk soil produced more biomass than plants in the high risk soil, but not significantly less biomass than plants in the low risk soil. In the second preliminary bioassay, the susceptible and moderate inbreds did not differ significantly in Fusarium wilt severity, but both had greater wilt severity compared to the resistant inbred in the medium risk soil. In the high risk soil, this same pattern was observed at the first rating date, 28 DAP. For AUDPC ratings, the susceptible inbred alone had significantly more severe wilt than the resistant inbred. These findings suggested that, for soils with low Fusarium wilt risk, fine gradations in risk level are difficult to detect with this soil bioassay, as expected.

The influence of spinach inbred line on plant biomass production in the second preliminary bioassay, as well as the manner in which soil Fusarium wilt risk level modified this effect, revealed genetic differences among the inbred lines. In the absence of Fusarium wilt, the resistant inbred produced plants with a smaller, more compact frame and darker-green leaves compared to the moderate and susceptible inbred lines. This explains why, in the low risk soil, there was no significant difference in spinach biomass production among inbred lines. In the medium risk soil, Fusarium wilt had a negative impact on spinach biomass. The smaller phenotype of the resistant line was counteracted by the capacity of this inbred to withstand

Fusarium wilt pressure, as opposed to the susceptible line, which was stunted severely by the disease despite a genetic potential for larger size. This shift was further delineated in the high risk soil, in which the resistant inbred line had significantly more biomass and less severe wilt than the susceptible line.

In the third preliminary bioassay, with further minor modifications to the protocol, similar results were obtained for the effects of soil Fusarium wilt risk level, spinach inbred line, and soil heat treatment on Fusarium wilt severity and spinach biomass production. The susceptible, moderate, and resistant lines differed significantly for Fusarium wilt severity, unlike the first two bioassays, but only in the medium and high risk soils. Increasing the number of spinach plants/pot from four to eight also improved the statistical power of the bioassay for differentiating different levels of Fusarium wilt risk.

The bioassay developed in these preliminary trials was deployed as a risk prediction service for spinach seed stakeholders, starting in the winter of 2009-2010, and has been carried out at the Washington State University-Northwestern Washington Research and Extension Center (WSU-NWREC) each year since. It is expected that the service will be transferred to a private soil testing agency in the future, but operation of the bioassay has continued at this facility each winter. Soil samples from 147 growers' fields in northwestern Washington were submitted for Fusarium wilt risk assessment from 2010 to 2013. All of these fields were being considered for a spinach seed crop the season following the assay, yet displayed a very wide range in risk of Fusarium wilt. Including three inbred lines in the assay with a range of Fusarium wilt susceptibility reflective of the range in susceptibility seed growers might encounter, was invaluable for the risk characterization process. The bioassay demonstrated that it may, in some fields, be safe to plant a spinach seed crop in a field with medium Fusarium wilt risk detected in

the bioassay, as long as inbred lines with moderate levels of resistance to Fusarium wilt are planted. In other fields, even a partially resistant line may not hold up against the inoculum potential of the soils. Both scenarios are possible with soils of similar Fusarium wilt risk levels when the risk is averaged across inbreds, but the disparate responses of the three individual inbred lines in some soils gave a more accurate assessment of risk. This relates to the dilemma that growers often face of not knowing the susceptibility to Fusarium wilt of the inbred lines they are contracted to grow for a given spinach seed crop, regardless of what they may know about the Fusarium wilt risk of a potential field site as a result of the soil bioassay. To help address this situation, a separate screening service has been initiated at the WSU-NWREC to determine the susceptibility of spinach inbred lines submitted voluntarily for testing by seed companies. This is carried out concurrently with the soil bioassay each winter (*data not shown*).

The growth of spinach plants in the autoclaved soil used in the preliminary bioassay and 2010 grower soil bioassay as a negative control treatment was mediocre, despite the near-absence of wilt symptoms in that soil treatment. Autoclaving alters soil chemical and physical properties in a manner that can be detrimental to plant growth (Wolf and Skipper, 1994). A significant increase in extractable Mn due to release of this micronutrient from the soil organic fraction is a common side effect of autoclaving, as is the loss of soil structure, which is maintained, in part, by microbial activity (Wolf and Skipper, 1994). The steam pasteurization treatment (56°C for 1 h) also caused shifts in soil chemistry in two of the control soils in the 2011 grower soil bioassay, but the effects were not as severe as autoclaving, and plant growth in the negative control soil was comparable to that of other low risk soils submitted by stakeholders.

Creating an intermediate risk soil proved challenging. In the 2011 bioassay, the 40°C soil treatment was intended to reduce the pathogen population partially, but may have diminished populations of other soil microbes more than the pathogen population, minimizing microbial competition and enhancing Fusarium wilt severity compared to the non-heated, high risk control soil. In the 2012 grower soil bioassay, a mixture of 1:9 parts raw, high risk soil:the same soil pasteurized, effectively diminished Fusarium wilt potential. The exact ratio of high risk:pasteurized soil blending may need to be adjusted each year the bioassay is offered, depending on the Fusarium wilt inoculum potential of the high risk soil used. Despite these difficulties with formulating a range of suitable control soils to compare to grower-submitted soils for Fusarium wilt development, the bioassays have provided valuable information about soil Fusarium risk level for stakeholders. Growers have continued to submit soil samples annually, even with implementation of a $200 fee/field sample. Growers and seed company representatives have reported changes in their decisions regarding the location of spinach seed crops, primarily by avoiding fields that were high risk for Fusarium wilt in the soil bioassay. It is yet unclear if stakeholders are willing to risk a shorter rotation interval than they would otherwise employ based on the soil bioassay demonstrating a field to be of lower risk for Fusarium wilt than expected. Further validation by stakeholders comparing Fusarium wilt severity in the bioassay with disease development in their fields is expected to enhance stakeholder confidence in the bioassay results.

In similar studies on the development of bioassays for soil disease prediction in other cropping systems, validation can be a relatively straightforward process (e.g., Persson et al., 1999). Researchers can designate fields to evaluate, sample and assay those fields, and then plant the bioassay indicator cultivar(s) in a field trial following the bioassay to compare results

directly. However, this is not possible with spinach seed crops in northwestern Washington. The placement of spinach seed crops is regulated to ensure pollen isolation between fields, with distance of 0.8 to 8.0 km depending on how closely the parent lines are related genetically. This limits the number of fields in the maritime Pacific Northwest that can be planted to spinach seed crops. Furthermore, growers and seed companies are unwilling to risk economic losses in high-value spinach seed crops by placing a crop in a field that, by virtue of the rotation interval or bioassay assessment, appears to have a high risk of Fusarium wilt. These impediments have limited the opportunities to compare the Fusarium wilt soil bioassay results directly with Fusarium wilt severity in growers' fields that represent the spectrum of Fusarium wilt risk.

Each year that the soil bioassay was completed, growers were notified of the necessity of thorough field sampling, the influence of spinach inbred line susceptibility on the risk of Fusarium wilt, and the limited validation efforts that had been completed in growers' fields. For example, with the visual assessments of commercial spinach seed crops that had been sampled and evaluated in the bioassay, the inbred lines that the growers planted in those fields were not the same as the inbred lines used in the bioassay, and the levels of susceptibility of the inbred lines planted in growers' fields typically were not known. Caution was thus required in drawing definitive conclusions based on comparisons between the soil bioassay and the seed crop field results.

In the 2012 field season, two opportunities arose to provide further validation of the bioassay. A representative of one of the participating seed companies offered to plant non-replicated test strips of the three bioassay spinach inbred lines in each of 10 fields that were evaluated in the 2012 soil bioassay, including two high-risk field sites. Overall, comparisons of the bioassay results and Fusarium wilt severity in these fields indicated that the bioassay was a

reliable predictor of wilt severity. For one field (soil 26), however, the bioassay predicted moderate to high Fusarium wilt risk, but very little wilt was observed in the grower's seed crop in that field. Most growers amend their fields in spring with 2.24 to 4.48 t limestone/ha within a few days or weeks of planting spinach seed crops, which has been shown to suppress spinach Fusarium wilt (du Toit et al., 2007; du Toit et al., 2008; du Toit et al., 2011; Gatch et al., 2011). In the first several years of offering the bioassay to test growers' fields, most stakeholders did not want limestone added to the soils they submitted because they wanted to see the "worst-case" scenario for Fusarium wilt risk in their field(s). Therefore, the severity of Fusarium wilt in the growers' fields was likely to be mitigated compared to the soil bioassay results. This may explain the results for soil 26 in the 2012 soil bioassay, compared to the very low severity of wilt observed in the grower's spinach seed crop planted in that field in 2012. Whether this false positive bioassay result was an anomaly or reflected the use of limestone by the grower, partial resistance of the parent lines planted by the grower, or inadequate field sampling, the potential exists for over-estimating risk of Fusarium wilt with the soil bioassay. As of the 2013 soil bioassay, limestone amendment has been offered as an option to the soil bioassay protocol that individual growers can request to reflect more accurately the widespread use of limestone pre-planting.

The four-year (2009 to 2012) limestone-mediated Fusarium wilt spinach seed field trial (Chapter 2) provided another opportunity for validation of the bioassay. Disease development in the 2012 field trial generally mirrored that of the 2012 bioassay for soil sampled from three replicate plots of the limestone treatments (0, 2.24, and 4.48 t limestone/ha/year), but was less severe overall in the field than in the bioassay. Differences in Fusarium wilt severity among limestone treatments and among the three inbred lines were more pronounced in the bioassay,

reflecting the highly conducive conditions in the greenhouse bioassay used to induce rapid development of Fusarium wilt. Also, the field plots were treated with an additional limestone amendment in spring 2012 prior to planting the 2012 field trial, compared to the soils sampled in December 2011 for the bioassay. The decline in Fusarium wilt severity observed with increasing rate of limestone amendment in the field trial was associated with highly significant increases in seed yield (see Chapter 2). Although the bioassay was terminated 5 weeks after planting, the Fusarium wilt severity ratings in the bioassay aligned more closely to seed yield than to disease severity ratings in the field.

Correlation and multiple regression analyses revealed significant relationships among various soil properties and spinach Fusarium wilt severity, but none that could replace the bioassay adequately as a Fusarium wilt risk prediction tool. The significance of the correlation between a given soil variable and Fusarium wilt severity (risk) of that soil was influenced significantly by susceptibility of the inbred line. A greater number of soil variables (n = 13) was significantly correlated with wilt development for the susceptible inbred line compared to the moderate and resistant inbred lines (n = 10 and 8, respectively). For example, soil pH was significantly negatively correlated with Fusarium wilt severity only for the susceptible inbred line. *F. oxysporum* CFU/g soil correlated positively with wilt severity for the susceptible and moderate inbred lines, but not for the resistant line. From a risk management perspective, this is desirable because risk prediction is most critical when growers plant susceptible inbred lines. Unexpectedly, the percentages of sand and clay in the soil texture analysis were negatively and positively correlated with Fusarium wilt severity, respectively. Studies on Fusarium wilts of other crops have identified coarse-textured soils as a risk factor for those wilt diseases (Baker and Cook, 1974; Hoper and Alabouvette, 1996), although exceptions have been noted (e.g.,

Scher and Baker, 1980). Of the acid micronutrients (Fe, Mn, and Zn) for which soil availability has been implicated in Fusarium wilt development in other studies (Jones and Woltz, 1970; Woltz and Jones, 1981), only Fe was correlated consistently with Fusarium wilt severity in this study for all three inbred lines. This may indicate that availability of these micronutrients is not as closely linked to Fusarium wilt development as demonstrated in other studies, or that other factors such as spinach seed crop rotation interval may have masked the relationship.

The negative correlation between the rotation interval between spinach seed crops and Fusarium wilt severity was one of the strongest predictive relationships identified, with correlation coefficients ranging from -0.2988 in 2011 to -0.4732 in 2010, but still left a non-trivial portion of the variability in Fusarium wilt severity among the 121 soils unexplained. For almost every soil assayed, the rotation out of spinach was ≥10 years, and yet many fields demonstrated a high risk of Fusarium wilt. This underlines the challenges spinach seed growers face in the PNW in identifying fields suitable for a spinach seed crop.

The assessment of relationships between soil properties and Fusarium wilt calculated using stepwise multiple regression yielded models that further revealed the nature of some of these significant correlations. Based on the regression equations, for example, if a grower wanted to plant a field that had an eight year rotation out of spinach seed crops, and keep Fusarium wilt severity index <0.2 when planting susceptible spinach inbreds, the grower should select a field with ≤15% clay and low buffering capacity (high CEC), and raise the pH to 7.2 via limestone amendment. Using the equations, one can estimate the rotation interval required to bring the Fusarium wilt risk of a field to 0 for each inbred line, assuming typical values for other soil properties in the equations: 37, 25, and 21 years for a susceptible, moderate, and resistant inbred line, respectively. These estimations based on the regression analyses are of limited value,

however, because even the best-fitting regression models explained no more than 31% of the variation in wilt severity observed among the 121 soils assayed. Nonetheless, the results are a source of hypotheses regarding the role of some these factors in determining spinach Fusarium wilt risk. Furthermore, accumulating additional results from several more years of operating the soil bioassay could improve the predictive value of these models. Ideally, there should be 10 observations for every independent variable to optimize the power and validity of regression analyses (Stevens, 2002).

Identifying significant relationships among independent soil variables and soil Fusarium wilt risk does not indicate causality (James and McCulloch, 1990). Common sense is required to assess the equations generated to determine which of the significant predictor variables may be influencing disease development. The identification of potential predictor variables can lead to new questions and hypotheses about the ecology of the spinach Fusarium wilt pathosystem. For example, the strong correlation between NH_4^+-N and Fusarium wilt severity in the correlation and regression analyses justifies exploration of the potential for choice of N-fertilizer to mitigate or exacerbate Fusarium wilt severity, as demonstrated for other Fusarium wilts (e.g., Woltz and Engelhard, 1973). Similarly, it would be intriguing to explore the significant correlations between soil texture and spinach Fusarium wilt risk, as texture of soils can be characterized readily. No single practice or piece of information will eliminate risk of this disease in maritime PNW fields, and management of spinach Fusarium wilt will continue to depend on a combination of risk assessment and soil pH adjustment. The multivariate approach in this study provided an introductory foray into the complex subterranean realm of a spinach seed crop, adding to the results of the soil Fusarium wilt bioassay and enhancing our understanding of factors that shape Fusarium wilt risk.

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Table 3.1. Methods used for nutrient analyses of soil samples[a] submitted by spinach seed crop stakeholders in northwestern Washington for evaluation in a spinach Fusarium wilt soil bioassay

Variable (unit of measure)	Method used for measurement[b]
P (mg/kg)	Bray P1
K (mg/kg), Ca, and Mg (meq/100 g soil)	Ammonium acetate extraction
S, B, Fe, Mn, Zn, Cu (mg/kg)	Diethylene triamine pentaacetic acid-sorbitol extraction + inductively coupled plasma spectroscopy
Nitrate-N (mg/kg)	Cadmium reduction
Ammonium-N (mg/kg)	KCl extraction
pH	1:1 soil:water
Buffer pH	Shoemaker, McLean, and Pratt
Organic matter (%)	Walkley-Black wet oxidation
Cation exchange capacity (meq/100 g soil)	Sodium acetate saturation
Electrical conductivity (mmhos/cm)	Saturated paste extract

[a] Soil samples were collected by spinach seed stakeholders (growers and seed company representatives) from commercial fields located in northwestern Washington. A subsample of the approximately 38-liter sample for each field submitted for the bioassay was collected after manual processing and mixing of the sample, and sent to a commercial soil testing lab (Soiltest Farm Consultants, Inc., Moses Lake, WA) for analysis.

[b] Protocols described by Gavlak et al. (2007).

Table 3.2. Probability values from the analyses of variance (ANOVAs) for the fixed effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on Fusarium wilt ratings 32, 40, 44, and 51 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass in the first preliminary greenhouse Fusarium wilt bioassay

	Fusarium wilt severity (0-to-5)					Spinach
ANOVA factor[a]	**32 DAP[b]**	**40 DAP**	**44 DAP**	**51 DAP**	**AUDPC**	**biomass[c]**
Soil Fusarium wilt risk	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Spinach inbred line	0.0058*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.6073
Heat treatment	0.7252	0.0175*	0.0007*	0.0026*	0.0153*	0.1932
Soil-by-inbred interaction	0.0058*	0.1067	0.1848	0.1042	0.3498	0.2748
Soil-by-heat treatment	0.7252	0.0074*	0.0706	0.0395*	0.0493*	0.0028*
Inbred-by-heat treatment	0.0644	0.0776	0.0965	0.0376*	0.0300*	0.0093*
Soil-by-inbred-by-heat	0.0644	0.8473	0.8346	0.6961	0.5592	0.7433
R^2	0.80	0.85	0.8834	0.8876	0.9053	0.8664
CV	25.05	24.09	21.77	21.41	10.85	23.40
Transformation	Rank	Rank	Rank	Rank	Sq root	Rank

[a] Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12 and 0 year rotations out of a spinach seed crop, characterized as low and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C, or not heated. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to square root (sq root) or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] For each of four replications/treatment combination, four spinach plants in one pot were rated for severity of Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0 to 1 index, with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g).

Table 3.3. Effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on Fusarium wilt ratings 32, 40, 44, and 51 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass in the first preliminary greenhouse bioassay

	Fusarium wilt severity (0-to-5)					Spinach biomass
Factor[a]	**32 DAP[b]**	**40 DAP**	**44 DAP**	**51 DAP**	**AUDPC**	**(g)[c]**
Soil Fusarium wilt risk						
High risk	0.16 a	0.52 a	0.66 a	0.74 a	12.5 a	1.12 b
Low risk	0.00 b	0.09 b	0.12 b	0.17 b	1.8 b	3.00 a
LSD	Rank	Rank	Rank	Rank	Sq root	Rank
Spinach inbred line						
Susceptible	0.15 a	0.47 a	0.59 a	0.65 a	11.6 a	2.05
Moderate	0.03 b	0.25 b	0.31 b	0.39 b	5.2 b	2.18
Resistant	0.05 ab	0.22 b	0.30 b	0.36 b	5.3 b	1.95
LSD	Rank	Rank	Rank	Rank	Sq root	NS
Soil heat treatment						
Heat-treated	0.08	0.26 b	0.32 b	0.40 b	6.3 b	2.20
Non-treated	0.08	0.36 a	0.47 a	0.52 a	8.2 a	1.93
LSD	NS	Rank	Rank	Rank	Sq root	NS
Soil risk-by-heat treatment						
High risk						
Heat-treated	0.15	0.42 b	0.53	0.64 b	10.7	1.50 a
Non-treated	0.17	0.62 a	0.79	0.84 a	14.3	0.74 b
LSD	NS	0.16	0.18	0.17	NS	Rank
Low risk						
Heat-treated	0.00	0.08	0.10	0.20	1.5	2.89
Non-treated	0.00	0.09	0.15	0.15	2.1	3.12
LSD	NS	NS	NS	NS	NS	NS
Heat treatment-by-inbred line						
Heat-treated						
Susceptible	0.16	0.48 a	0.56 a	0.67 a	11.9 a	2.13
Moderate	0.01	0.16 b	0.18 b	0.24 b	2.9 b	2.52
Resistant	0.06	0.17 b	0.25 b	0.33 b	4.8 b	1.94
LSD	NS	Rank	Rank	Rank	Rank	NS
Non-treated						
Susceptible	0.15	0.46	0.61	0.63	11.3 a	1.97
Moderate	0.06	0.34	0.45	0.54	7.6 b	1.85
Resistant	0.04	0.26	0.34	0.39	5.7 c	1.97
LSD	NS	NS	NS	NS	Rank	NS

[a] Each value is the mean of four replications. Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12 and 0 year rotations out of a spinach seed crop, characterized as low risk and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C, or not heated. When necessary, raw data were subjected to square root (sq root) or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] For each of four replications/treatment combination, four spinach plants in one pot were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g).

Table 3.4. Probability values from the analyses of variance (ANOVAs) for the fixed effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on Fusarium wilt ratings 28, 35, and 42 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass per plant and per pot in a second preliminary greenhouse bioassay

	Fusarium wilt severity (0-to-5)				Spinach biomass	
ANOVA factor[a]	**28 DAP[b]**	**35 DAP**	**42 DAP**	**AUDPC**	**g/plant[c]**	**g/pot**
Soil Fusarium wilt risk	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Spinach inbred line	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0071*	0.0016*
Soil heat treatment	0.0209*	0.0240*	0.0551	0.0247*	0.0027*	0.0004*
Soil-by-inbred	0.0055*	0.0085*	0.0003*	0.0197*	0.0037*	0.0020*
Soil-by-heat treatment	0.3360	0.9354	0.9234	0.6456	0.2777	0.1531
Inbred-by-heat treatment	0.7133	0.9139	0.9014	0.8640	0.6050	0.8048
Soil-by-inbred-by-heat trt	0.7374	0.5088	0.6364	0.6197	0.4392	0.3216
R^2	0.8678	0.8644	0.8761	0.8646	0.7946	0.8056
CV	21.91	22.79	21.72	23.01	28.45	27.68
Transformation	Rank	Rank	Rank	Rank	Rank	Rank

[a] Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12, 8, and 0 year rotations out of a spinach seed crop, characterized as low, medium, and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C followed by 2 hr at 80°C (high heat), for 1.5 h at 65°C (low heat), or not heated (non-treated). R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] For each of five replications/treatment combination, four spinach plants in one pot were rated for severity of Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, weighing the dried plants from each pot (g/plot), and dividing by the number of plants/pot to calculate biomass/plant (g/plant).

Table 3.5. Effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on Fusarium wilt ratings 28, 35, and 42 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass per plant and per pot in a second preliminary greenhouse bioassay

	Fusarium wilt severity (0-to-5)				Spinach biomass	
Factor	**28 DAP**	**35 DAP**	**42 DAP**	**AUDPC**	**g/plant**	**g/pot**
Soil Fusarium wilt risk						
High risk	0.92 a	0.97 a	0.99 a	26.4 a	0.10 b	0.03 b
Medium risk	0.24 b	0.41 b	0.57 b	9.1 b	0.35 a	1.27 a
Low risk	0.02 c	0.10 c	0.14 c	1.5 c	0.32 a	1.24 a
LSD	Rank	Rank	Rank	Rank	Rank	Rank
Spinach inbred line						
Susceptible	0.44 a	0.55 a	0.64 a	13.8 a	0.24 a	0.85 a
Moderate	0.43 a	0.53 a	0.61 a	13.3 a	0.20 b	0.73 b
Resistant	0.31 b	0.40 b	0.46 b	9.9 b	0.25 a	0.95 a
LSD	Rank	Rank	Rank	Rank	Rank	Rank
Soil heat treatment						
High heat	0.37 b	0.45 b	0.52	11.4 b	0.25 a	0.92 a
Low heat	0.37 b	0.49 ab	0.58	11.9 ab	0.23 a	0.88 a
Non-treated	0.45 a	0.54 a	0.61	13.7 a	0.20 b	0.73 b
LSD	Rank	Rank	NS	Rank	Rank	Rank
Soil risk-by-inbred line						
Low risk						
Susceptible	0.05	0.13	0.19	2.4	0.33	1.29
Moderate	0.00	0.10	0.14	1.2	0.31	1.13
Resistant	0.01	0.06	0.09	1.0	0.31	1.23
LSD	NS	NS	NS	NS	NS	NS
Medium risk						
Susceptible	0.32 a	0.52 a	0.72 a	11.7 a	0.38 a	1.26 b
Moderate	0.32 a	0.51 a	0.69 a	11.5 a	0.27 b	0.98 c
Resistant	0.09 b	0.21 b	0.30 b	4.1 b	0.41 a	1.55 a
LSD	Rank	Rank	Rank	Rank	Rank	Rank
High risk						
Susceptible	0.97 a	0.99	1.00	27.4 a	0.00 b	0.00 b
Moderate	0.96 a	0.99	1.00	27.2 ab	0.01 ab	0.02 ab
Resistant	0.83 b	0.94	0.98	24.5 b	0.02 a	0.06 a
LSD	Rank	NS	NS	Rank	Rank	Rank

[a] Each value is the mean of five replications. Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12, 8, and 0 year rotations out of a spinach seed crop, characterized as low, medium, and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C followed by 2 h at 80°C (high heat), for 1.5 h at 65°C (low heat), or not heated (non-treated). When necessary, raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] For each of five replications/treatment combination, four spinach plants in one pot were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, weighing the dried plants from each pot (g/plot), and dividing by the number of plants/pot to calculate biomass/plant (g/plant).

Table 3.6. Properties of soils evaluated in the second and third preliminary soil bioassays, and the first grower soil bioassay in 2010, for determining the risk of spinach Fusarium wilt

	Low risk soil (12 year rotation)			Medium risk soil (8 year rotation)			High risk soil (0 year rotation)			Negative control soils				Inter-	
Variable[a]	**Non-treated**	**Low heat**	**High heat**	**Non-treated**	**Low heat**	**High heat**	**Non-treated**	**Low heat**	**High heat**	**Auto-claved**	**Orch-ard**	**Fumi-gated**	**Steam-past.**	**mediate control**	**Positive control**
pH	6.3	6.3	6.3	6.8	6.6	6.6	5.7	5.8	5.8	5.5	6.3	6.4	5.6	5.5	5.4
Buffer pH	6.9	6.9	6.9	6.9	6.8	6.8	6.6	6.6	6.5	6.5	6.6	6.6	6.4	6.4	6.5
NO_3 (mg/kg)	3.6	3.7	3.6	3.9	5.5	5.0	5.6	4.9	4.3	4.2	10.1	8	9.9	15.0	25.9
NH_4 (mg/kg)	1.3	1.1	5.2	1.8	3.9	6.2	1.4	1.3	6.3	6.0	2.6	.	8.3	3.2	2.2
P (mg/kg)	80	81	82	192	192	192	273	273	309	208	142	102	132	338	292
K (mg/kg)	227	227	238	401	412	416	259	276	271	275	404	275	378	358	476
Ca (meq/100 g)	3.8	3.6	3.8	10.0	9.1	9.3	3.6	3.6	3.6	4.1	8.3	127	3.4	3.4	2.7
Mg (meq/100 g)	0.7	0.7	0.7	1.5	1.4	1.4	0.6	0.6	0.6	0.7	1.6	132	0.7	0.7	0.6
S (mg/kg)	6	5	9	12	16	16	4	6	5	14	10	3	15	13	15
B (mg/kg)	0.24	0.24	0.32	0.71	0.49	0.49	0.08	0.10	0.12	0.36	0.43	0.60	0.32	0.28	0.26
Fe (mg/kg)	73	72	71	84	84	79	35	46	42	53	116	62	88	88	63
Mn (mg/kg)	1.2	1.1	3.3	2.6	3.0	3.5	1.2	1.8	3.5	54.7	2.6	2.0	14.3	4.0	3.8
Zn (mg/kg)	0.8	0.8	0.9	1.6	1.4	1.2	1.2	1.6	1.5	2.1	5.1	4.5	4.0	3.7	2.2
Cu (mg/kg)	3.9	3.8	3.8	6.9	6.3	5.4	1.2	1.5	1.3	1.5	12.5	3.3	2.2	2.2	2.0
CEC (meq/100 g)[b]	11.6	8.4	10.8	19.7	17.2	16.6	10.4	9.8	9.7	10.3	15.6	9.0	10.9	11.9	9.8
OM (%)	2.0	1.9	2.0	3.0	3.4	3.2	2.7	2.7	2.8	3.0	4.0	2.3	3.2	3.2	2.8
EC (mmhos/cm)	0.29	0.26	0.29	0.47	0.47	0.44	0.29	0.21	0.23	0.55	0.60	0.20	0.12	0.22	0.43
F. oxysporum pre-trial (CFU/g)[c]	1,978	1,400	622	2,022	1,044	355	4,556	3,200	2,133	0	711	133	0	733	2,556
F. oxysporum post-trial (CFU/g)[c]	8,215	6,896	9,281	4,570	7,052	6,193	11,422	11,214	12,014	0	4,786	363	-	-	-

[a] Soils were collected in fall 2009 from fields in Skagit County, WA. The low risk soil was from a field that had not had a spinach seed crop for 12 years, the medium risk soil from a field that had not had a spinach seed crop in 8 years, and the high risk soil from a field that had a spinach seed crop planted the year prior to the bioassay. The negative control soils represent different approaches used to achieve soils free of the spinach Fusarium wilt pathogen, *Fusarium oxysporum* f. sp. *spinaciae*. The autoclaved soil consisted of the high risk soil autoclaved twice at 121°C and 1.1 kg/cm^2 for 30 min with 24 h between autoclavings. The orchard soil was collected from a fruit orchard that had never had a spinach seed crop. The fumigated soil was collected from a research field trial that had been fumigated with methyl bromide:chloropicrin (57:43% at 3,274 liters/ha). The steam pasteurized soil (steam-past.) consisted of the high risk soil subjected to steam pasteurization at 60°C for 1 h. The intermediate control soil used in the first grower soil bioassay in 2010 was made by heating the high risk soil with an electric sterilizer (SS-30, Pro-Grow Supply Corp.) at 65°C for 1.5 h and at 80° for 2 h. A subsample of each soil (following heat treatment, if applicable) was collected and sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA) for nutrient analysis.

[b] CEC = cation exchange capacity, EC = electrical conductivity, and OM = organic matter.

[c] Population of *F. oxysporum* (including possible non-pathogenic isolates and other formae speciales which cannot be distinguished morphologically from strains of the spinach pathogen) quantified in soils before and after the second preliminary bioassay by dilution plating on Komada's agar medium (1975). Fungal colonies with fluffy, white to pale salmon-pink morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after plating.

Table 3.7. Probability values from the analyses of variance (ANOVAs) for the fixed effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on Fusarium wilt ratings 20, 28, and 35 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass per plant and per pot in the third preliminary greenhouse bioassay

	Fusarium wilt severity (0-to-5)				Spinach biomass	
ANOVA factor[a]	**20 DAP[b]**	**28 DAP**	**35 DAP**	**AUDPC**	**g/plant[c]**	**g/pot**
Soil Fusarium wilt risk	<0.0001*	<0.0001	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Spinach inbred line	<0.0001*	<0.0001	<0.0001*	<0.0001*	<0.0001*	0.2486
Soil heat treatment	0.3015	0.0033*	0.0002*	0.0014*	<0.0001*	<0.0001*
Soil-by-inbred line	0.1853	0.0027*	<0.0001*	<0.0001*	0.0005*	0.0002*
Soil-by-heat treatment	0.0062*	0.1322	0.0543	0.0145*	0.0005*	0.0037*
Inbred-by-heat treatment	0.0310*	0.8071	0.9042	0.8625	0.8835	0.3267
Soil-by-inbred-by-heat	0.2552	0.6555	0.2694	0.3505	0.6723	0.3558
R^2	0.5509	0.7024	0.7722	0.7719	0.8546	0.8610
CV	31.07	32.26	29.24	29.30	11.60	18.82
Transformation	Rank	Rank	Rank	Rank	Rank	-

[a] Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12, 8, and 0 year rotations out of a spinach seed crop, characterized as low, medium, and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C followed by 2 h at 80°C (high heat), for 1.5 h at 65°C (low heat), or not heated (non-treated). R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] For each of five replications per treatment combination, up to eight spinach plants in one pot were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, weighing the dried plants from each pot (g/plot), and dividing the weight by the number of plants/pot to calculate biomass/plant (g/plant).

Table 3.8. Effects of soil Fusarium wilt risk level, spinach inbred line susceptibility to Fusarium wilt, and soil heat treatment on Fusarium wilt ratings 20, 28, and 35 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass per plant and per pot in the third preliminary greenhouse bioassay

	Fusarium wilt severity (0-to-5)				Spinach biomass	
Factor[a]	**20 DAP**[b]	**28 DAP**	**35 DAP**	**AUDPC**	**g/plant**[c]	**g/pot**
Soil Fusarium risk level						
High risk	0.09 a	0.28 a	0.57 a	5.4 a	0.29 c	2.99 c
Medium risk	0.01 b	0.09 b	0.20 b	1.5 b	0.81 a	8.00 a
Low risk	0.01 b	0.03 c	0.06 c	0.5 c	0.53 b	5.38 b
LSD	Rank	Rank	Rank	Rank	Rank	0.43
Spinach inbred line						
Susceptible	0.07 a	0.24 a	0.47 a	4.3 a	0.49 b	5.46
Moderate	0.04 b	0.12 b	0.24 b	2.2 b	0.51 b	5.27
Resistant	0.01 b	0.04 c	0.13 c	0.9 c	0.64 a	5.63
LSD	Rank	Rank	Rank	Rank	Rank	NS
Soil heat treatment						
High heat	0.02	0.10 b	0.21 b	1.8 b	0.62 a	6.05 a
Low heat	0.02	0.10 b	0.26 b	2.0 b	0.56 a	5.69 a
Non-treated	0.07	0.20 a	0.37 a	3.8 a	0.45 b	4.62 b
LSD	NS	Rank	Rank	Rank	Rank	0.43
Soil risk-by-inbred line						
Low risk						
Susceptible	0.02	0.08 a	0.13 a	1.3 a	0.92 a	6.17 a
Moderate	0.00	0.00 b	0.00 b	0.0 b	0.76 ab	5.10 b
Resistant	0.00	0.01 b	0.05 b	0.2 b	0.69 b	4.86 b
LSD	NS	Rank	Rank	Rank	Rank	0.92
Medium risk						
Susceptible	0.03 a	0.18 a	0.41 a	3.1 a	1.25 a	7.73
Moderate	0.01 ab	0.09 b	0.19 b	1.5 b	1.11 b	7.82
Resistant	0.00 b	0.00 c	0.01 c	0.0 c	1.21 ab	8.43
LSD	Rank	Rank	Rank	Rank	Rank	NS
High risk						
Susceptible	0.15	0.46 a	0.86 a	8.5 a	0.34 b	2.47
Moderate	0.10	0.26 b	0.52 b	5.1 b	0.42 ab	2.89
Resistant	0.03	0.12 c	0.33 c	2.5 c	0.51 a	3.60
LSD	NS	Rank	Rank	Rank	Rank	NS
Soil risk-by-heat treatment						
Low risk						
High heat	0.01	0.03	0.04	0.5	0.90	5.78
Low heat	0.01	0.04	0.07	0.6	0.74	5.26
Non-treated	0.00	0.03	0.07	0.4	0.72	5.09
LSD	NS	NS	NS	NS	NS	NS
Medium risk						
High heat	0.00	0.06	0.11	0.9	1.38 a	8.97 a
Low heat	0.02	0.08	0.21	1.6	1.18 b	8.02 b
Non-treated	0.01	0.13	0.29	2.1	1.00 c	7.00 b
LSD	NS	NS	NS	NS	Rank	Rank

High risk						
High heat	0.05 b	0.21 b	0.48 b	3.9 b	0.46 a	3.40 a
Low heat	0.04 b	0.18 b	0.49 b	3.6 b	0.55 a	3.79 a
Non-treated	0.20 a	0.45 a	0.75 a	8.7 a	0.26 b	1.78 b
LSD	Rank	Rank	Rank	Rank	Rank	Rank

[a] Each value is the mean of five replications. Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12, 8, and 0 year rotations out of a spinach seed crop, characterized as low, medium, and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C followed by 2 h at 80°C (high heat), for 1.5 h at 65°C (low heat), or not heated (non-treated). When necessary, raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[b] For each of five replications per treatment combination, up to eight spinach plants in one pot were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting the plants in each pot at the soil line, drying the plants at 35°C, weighing the dried plants from each pot (g/plot), and dividing the weight by the number of plants/pot to calculate biomass/plant (g/plant).

Table 3.9. Probability values from the analyses of variance (ANOVAs) for the fixed effects of soil sample and spinach inbred line susceptibility to Fusarium wilt on Fusarium wilt severity ratings 21, 28, 35, and 42 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass per plant and per pot in the 2010 to 2013 grower soil Fusarium wilt bioassays

	Fusarium wilt severity (0-to-5)					Spinach biomass	
ANOVA factor[a]	**21 DAP[b]**	**28 DAP**	**35 DAP**	**42 DAP**	**AUDPC**	**g/plant[c]**	**g/pot**
2010							
Soil	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Spinach inbred	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Soil-by-inbred	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
R^2	0.8044	0.8810	0.8999	0.8967	0.8982	0.8633	0.8633
CV	27.17	21.76	20.13	20.49	20.48	23.71	23.71
Transformation	Rank	Rank	Rank	Rank	Rank	Rank	Rank
2011							
Soil	<0.0001	<0.0001	<0.0001	-[d]	<0.0001	<0.0001	<0.0001
Spinach inbred	<0.0001	<0.0001	<0.0001	-	<0.0001	0.2483	0.2353
Soil-by-inbred	<0.0001	<0.0001	<0.0001	-	<0.0001	<0.0001	<0.0001
R^2	0.5719	0.7930	0.8294	-	0.8278	0.7144	0.8103
CV	26.84	24.40	24.13	-	23.88	34.44	28.07
Transformation	Rank	Rank	Rank	-	Rank	Rank	Rank
2012							
Soil	<0.0001	<0.0001	<0.0001	-	<0.0001	<0.0001	<0.0001
Spinach inbred	<0.0001	<0.0001	<0.0001	-	<0.0001	<0.0001	<0.0001
Soil-by-inbred	<0.0001	<0.0001	<0.0001	-	<0.0001	<0.0001	<0.0001
R^2	0.7193	0.8662	0.9025	-	0.8893	0.8846	0.8513
CV	23.29	22.25	19.57	-	20.85	21.91	24.85
Transformation	Rank	Rank	Rank	-	Rank	Rank	Rank
2013							
Soil	<0.0001	<0.0001	-	-	<0.0001	<0.0001	<0.0001
Spinach inbred	<0.0001	<0.0001	-	-	<0.0001	<0.0001	<0.0001
Soil-by-inbred	<0.0001	<0.0001	-	-	<0.0001	<0.0001	<0.0001
R^2	0.6749	0.8167	-	-	0.7580	0.7822	0.7810
CV	35.96	28.13	-	-	32.33	31.00	31.08
Transformation	Rank	Rank	-	-	Rank	Rank	Rank

[a] Experimental design each year was a randomized complete block with two factors: 1) soil samples submitted by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female spinach inbred lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] For each replication, up to 8 plants/pot were each rated for severity of Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt development (all plants dead).

[c] Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, weighing the dried plants from each pot (g/plot), and dividing the total weight by the number of plants/pot to calculate biomass/plant (g/plant).

[d] - = No rating was done for that interval, as the bioassay was terminated prior to that many DAP.

Table 3.10. Effects of spinach inbred line susceptibility on Fusarium wilt ratings 21, 28, 35, and 42 days after planting (DAP), the area under the disease progress curve (AUDPC), and aboveground spinach biomass per plant and per pot measured in the 2010 to 2013 grower soil Fusarium wilt bioassays

Year and spinach	Fusarium wilt severity (0-to-5)					Spinach biomass	
inbred line[a]	21 DAP[b]	28 DAP	35 DAP	42 DAP	AUDPC	g/plant[c]	g/pot
2010	0.24	0.35	0.44	0.49	10.7	0.385	3.08
Susceptible	0.40 a	0.55 a	0.66 a	0.71 a	16.5 a	0.337 b	2.70 b
Moderate	0.19 b	0.31 b	0.42 b	0.49 b	9.6 b	0.419 a	3.35 a
Resistant	0.14 c	0.20 c	0.26 c	0.29 c	6.1 c	0.399 a	3.19 a
LSD	Rank	Rank	Rank	Rank	Rank	Rank	Rank
2011	0.03	0.12	0.21	-	1.7	0.433	2.41
Susceptible	0.05 a	0.21 a	0.34 a	-	3.1 a	0.454	2.35
Moderate	0.01 b	0.08 b	0.17 b	-	1.2 b	0.413	2.48
Resistant	0.01 b	0.06 c	0.11 c	-	0.9 c	0.432	2.42
LSD	Rank	Rank	Rank	-	Rank	NS	NS
2012	0.07	0.23	0.32	-	3.7	0.255	1.83
Susceptible	0.15 a	0.39 a	0.48 a	-	6.6 a	0.231 b	1.59 b
Moderate	0.04 b	0.21 b	0.33 b	-	3.2 b	0.264 a	1.90 a
Resistant	0.02 c	0.10 c	0.14 c	-	1.4 c	0.271 a	2.01 a
LSD	Rank	Rank	Rank	-	Rank	Rank	Rank
2013	0.16	0.33	-	-	3.4	0.169	0.90
Susceptible	0.26 a	0.48 a	-	-	5.3 a	0.141 c	0.75 c
Moderate	0.14 b	0.31 b	-	-	3.1 b	0.172 b	0.76 b
Resistant	0.08 c	0.20 c	-	-	1.9 c	0.195 a	1.18 a
LSD	Rank	Rank	-	-	Rank	Rank	Rank

[a] Each value is the mean of five replications. Experimental design each year was a randomized complete block with two factors: 1) soil samples submitted by growers or seed company representatives from fields in northwestern Washington (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. The means for soils are not presented due to space constraints. R^2 = coefficient of determination. CV = coefficient of variance. When necessary, raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. * = significant at $P \leq 0.05$.

[b] For each replication, up to eight plants/pot were rated for severity of Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt development.

[c] Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, weighing the dried plants from each pot (g/plot), and dividing the weight by the number of plants/pot to calculate biomass/plant (g/plant).

Table 3.11. Pearson's correlation coefficients for spinach biomass/plant, number of years since a field was last planted to a spinach seed crop (rotation interval), and soil pH of the field with Fusarium wilt severity at 28 days after planting (DAP) in Fusarium wilt soil bioassays completed for growers' fields in each of 2010, 2011, 2012, and 2013

	Fusarium wilt severity (28 DAP)[b]			
Variables[c]	**2010 (n = 22)**	**2011 (n = 36)**	**2012 (n = 37)**	**2013 (n = 40)**
Spinach biomass/plant	-0.8863[a]	-0.5085	-0.8489	-0.8125
	(*P* <0.0001)*	(*P*<0.0001)*	(*P* <0.0001)*	(*P* <0.0001)*
Spinach rotation interval	-0.4732	-0.2988	-0.3353	-0.3020
	(*P* = 0.0015)*	(*P* = 0.0001)*	(*P* = 0.0003)*	(*P* = 0.0176)*
Soil pH	-0.2995	-0.1551	-0.2339	-0.1965
	(*P* = 0.0828)	(*P* = 0.0950)	(*P* = 0.0135)	(*P* = 0.0314)

[a] Experimental design each year was a randomized complete block with two factors: 1) soil samples submitted by growers or seed company representatives (two 19-liter buckets/sample); and 2) female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each value in the table is Pearsons correlation coefficient for that pair of variables, with the probability of no significant correlation (*P*) in parentheses. * = significant at $P \leq 0.05$.

[b] For each of four (2013) or five (2010 to 2012) replications/treatment combination, up to eight spinach plants/pot were rated for severity of Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = plant dead due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity.

[c] Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, weighing the dried plants from each pot, and dividing the total weight by the number of plants/pot (g/plant). Rotation interval = the number of years since the field from which the soil sample was collected had previously been planted with a spinach seed crop. pH = soil pH of each soil sample submitted, measured at the start of the bioassay.

Table 3.12. Probability values from the analyses of variance (ANOVAs) for the fixed effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on spinach wilt severity measured 28 and 35 days after planting (DAP), area under the disease progress curve (AUDPC), and dried spinach plant biomass in a 2012 greenhouse soil bioassay and subsequent spinach seed crop field trial planted in Skagit Co., WA in 2012

Trial and ANOVA factors[a]	Fusarium wilt severity		Spinach biomass[c]
	28 DAP[b]	35 DAP	
Greenhouse soil bioassay			
Limestone rate	<0.0001*	<0.0001*	<0.0001*
Spinach inbred	<0.0001*	<0.0001*	<0.0001*
Limestone-by-inbred	0.4883	0.2122	0.3049
R^2	0.753	0.771	0.8141
CV	30.9	28.53	17.70
Transformation	-	Rank	Arcsine
Spinach seed crop field trial	9 July	31 July	Biomass
Limestone rate	0.0204*	0.0830	0.0007*
Spinach inbred	<0.0001*	0.0020*	0.1488
Limestone-by-inbred	0.0835	0.1948	0.9397
R^2	0.9716	0.9837	0.8881
CV	10.26	3.98	14.64
Transformation	-	-	-

[a] Soil samples were collected in December 2011from three of five replicate plots of a four-year limestone/Fusarium wilt spinach seed crop field trial (see Chapter 2) to serve as validation for the greenhouse Fusarium wilt soil bioassay. The samples were processed and planted in the bioassay along with 37 growers' soil samples. The Fusarium wilt ratings and spinach biomass measurements in the bioassay were compared subsequently to wilt ratings and spinach biomass production for these same plots in a 2012 spinach seed crop field trial to determine how well the bioassay predicted Fusarium wilt risk for the plots sampled. Experimental design for this validation trial carried out in the 2012 greenhouse soil bioassay was a randomized complete block with two factors and three replications. The factors were: 1) limestone applications of 0, 2.24, or 4.48 t/ha for each of three years (2009 to 2011); and 2) female spinach inbred lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. The experimental design of the field trial was a two-factor, completely randomized, split block with the same spinach inbred lines used in the bioassay applied to main plots, and the limestone application rates applied to split plots (see Chapter 2 for details). R^2 = coefficient of determination. CV = coefficient of variance. Transformation = when necessary, raw data were subjected to arcsine or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA.

[b] In the greenhouse soil bioassay, up to 8 plants in a pot for each replication were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index with 1 representing maximum Fusarium wilt development. Fusarium wilt severity was assessed in the field trial for each plant in 3 m of each of two rows on the dates indicated, using a similar rating scale and severity index.

[c] Dried, aboveground spinach biomass was measured in the greenhouse soil bioassay by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot). In the field trial, biomass was measured by harvesting, drying at 35°C, and weighing whole plants sampled from 1 m of row/plot.

Table 3.13. Effects of limestone application rate and spinach inbred line susceptibility to Fusarium wilt on Fusarium wilt severity measured 28 and 35 days after planting (DAP), area under the disease progress curve (AUDPC), and dried plant biomass in a 2012 greenhouse soil bioassay, and in a subsequent spinach seed crop field trial planted in Skagit Co., WA in 2012

	Fusarium wilt severity		Spinach biomass
Trial and factor[a]	**28 DAP[b]**	**35 DAP**	**(g/pot)[c]**
Bioassay			
Limestone rate (t/ha)			
0	0.76 a	0.91 a	0.25 c
2.24	0.50 b	0.66 b	0.92 b
4.48	0.24 c	0.44 c	1.19 a
LSD	0.08	Rank	Arcsine
Spinach inbred line			
Susceptible	0.67 a	0.84 a	0.57 c
Moderate	0.47 b	0.69 b	0.77 b
Resistant	0.36 c	0.48 c	1.04 a
LSD	0.08	Rank	Arcsine
Spinach seed crop field trial	**9 July**	**31 July**	**Biomass (g/m of row)**
Limestone rate (t/ha)			
0	0.54 a	0.74	114.89 c
2.24	0.42 b	0.67	185.19 b
4.48	0.34 b	0.65	216.79 a
LSD	0.10	NS	28.93
Spinach inbred line			
Susceptible	0.52 a	0.82 a	180.09
Moderate	0.39 b	0.71 b	172.11
Resistant	0.26 c	0.55 c	153.61
LSD	0.05	0.09	NS

[a] Soil samples were collected in December 2011from three of five replicate plots of a separate, four-year limestone/Fusarium wilt spinach seed crop field trial to serve as validation for the greenhouse Fusarium wilt soil bioassay. The samples were processed and planted in the bioassay along with 39 growers' soil samples. The Fusarium wilt ratings and spinach biomass measurements were compared subsequently to wilt ratings and spinach biomass production for these same plots in a 2012 spinach seed crop field trial, to determine how well the bioassay predicted Fusarium wilt risk for the plots sampled. Experimental design for this validation trial carried out in the 2012 greenhouse soil bioassay was a randomized complete block with three replications of two factors: 1) limestone applications of 0, 2.24, or 4.48 t/ha for each of three years (2009 to 2011); and 2) female spinach inbred lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. The experimental design of the field trial was a two-factor, completely randomized, split block with the same spinach inbred lines used in the bioassay applied to main plots, and the limestone application rates applied to split plots (see Chapter 2 for details). When necessary, raw data were subjected to arcsine or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA.

[b] In the greenhouse soil bioassay, up to 8 plants/pot for each replication were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt development. Fusarium wilt severity was assessed in the field trial for each plant in 3 m of each of two rows on the dates indicated, using a similar rating scale and severity index. Means followed by the same letter for each factor or interaction are not significantly different based on Fisher's protected least significant difference (LSD) ($P \leq 0.05$). NS = no significant difference.

[c] Dried, aboveground spinach biomass was measured in the greenhouse soil bioassay by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot). In the field trial, biomass was measured by harvesting, drying at 35°C, and weighing whole plants sampled from 1 m of row/plot.

Table 3.14. Mean results of tissue nutrient analyses for spinach plants grown in soil collected from plots of a limestone spinach seed crop field trial (see Chapter 2) and evaluated in the 2012 greenhouse Fusarium wilt soil bioassay

	Plant tissue nutrient levels						
Limestone rate (t/ha/year)[a]	**Zn (mg/kg)**	**Mn (mg/kg)**	**Cu (mg/kg)**	**Fe (mg/kg)**	**N (%)**	**Ca (%)**	**B (mg/kg)**
0	148 a	246 a	19 a	7,189 a	4.48 c	1.93	46 b
2.24	97 b	99 b	16 b	2,850 b	5.75 b	2.07	64 a
4.48	76 c	73 b	15 b	1,252 b	6.55 a	2.10	70 a
LSD	12	41	2	2,462	0.63	NS	13

[a] Soil samples were collected in November 2011 from plots that were part of a four-year limestone/Fusarium wilt spinach seed crop field trial located in Skagit County, WA, in which limestone applications of 0, 2.24, or 4.48 t/ha were applied to the same plots each year from 2009 to 2012 in a grower cooperator's field (details described in Chapter 2). Soil samples were collected from plots representing three replications of each of the limestone treatments, and evaluated in the 2012 greenhouse soil bioassay along with 39 field soil samples submitted by spinach seed growers and stakeholders. A spinach seed trial was then planted in 2012 in this field with the same three female spinach parent lines used in the bioassay, and wilt development and plant growth were evaluated throughout the season as described in Chapter 2. The Fusarium wilt severity ratings for the soils in the bioassay were compared to results of the field trial to assess how well the bioassay predicted field levels of wilt. Tissue samples were collected at the end of the trial and sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA) to determine whether the limestone treatments may have caused micronutrient deficiencies that may have resulted in symptoms of chlorosis in spinach plants growing in the limestone-amended soils in the bioassay.

Table 3.15. Means and standard errors (SE) of soil nutrient analyses for soils submitted by growers to test in the Fusarium wilt soil bioassays in 2010 to 2013

	2010 (n = 26)		2011 (n = 42)		2012 (n = 39)		2013 (n = 40)	
Soil property[a]	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
pH	5.70	0.06	6.10	0.06	6.15	0.06	6.27	0.07
Buffer pH	6.45	0.03	6.47	0.04	6.41	0.04	6.37	0.04
NO_3 (mg/kg)	12.1	1.1	11.3	0.9	14.3	1.5	7.3	0.7
NH_4 (mg/kg)	2.0	0.1	2.1	0.1	2.4	0.1	4.6	0.2
P (mg/kg)	186	11	164	11	165	11	162	10
K (mg/kg)	329	18	281	15	319	20	276	18
Ca (meq/100 g)	7.9	0.4	7.1	0.4	8.0	0.4	7.8	0.4
Mg (meq/100 g)	1.4	0.1	1.3	0.13	1.2	0.08	1.2	0.1
S (mg/kg)	21	2	29	4	39	4	31	4
B (mg/kg)	0.85	0.04	0.56	0.06	0.78	0.09	0.82	0.09
Fe (mg/kg)	172	10	170	13	237	19	204	15
Mn (mg/kg)	2.51	0.24	2.20	0.20	1.64	0.14	4.05	0.35
Zn (mg/kg)	2.28	0.20	2.18	0.19	3.16	0.47	2.36	0.35
Cu (mg/kg)	5.60	0.44	4.53	0.31	5.08	0.43	5.10	0.34
CEC (meq/100 g)[b]	16.2	0.54	16.9	0.58	15.4	0.5	14.9	0.6
OM (%)	4.71	0.24	4.00	0.18	4.97	0.26	3.67	0.20
EC (mmhos/cm)	0.58	0.04	0.21	0.01	0.31	0.02	0.27	0.03

[a] Soil samples were collected by spinach seed stakeholders (growers and seed company representatives) in commercial fields located in northwestern Washington. A subsample of the approximately 38 liters of soil submitted for each field for the bioassay was collected after processing the soil sample. The subsample was sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA) for analysis of: pH, buffer pH, nitrate-nitrogen (NO_3^-), ammonium-nitrogen (NH_4^+), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), iron, (Fe), manganese (Mn), zinc (Zn), copper (Cu), cation exchange capacity (CEC), organic matter (OM), and electrical conductivity (EC).

Table 3.16. Pearson's correlation coefficients between soil properties and spinach Fusarium wilt severity ratings measured 28 days after planting in Fusarium wilt soil bioassays conducted from 2010 to 2013 using soil sampled from 121 growers' fields in northwestern Washington[a]

	Spinach inbred line susceptibility to Fusarium wilt		
Soil property[b]	**Susceptible**	**Moderate**	**Resistant**
NH_4-N	0.1844	0.2765	0.2700
	(*P* = 0.0428)*	(*P* = 0.0021)*	(*P* = 0.0022)*
K	0.1846	0.1769	0.1975
	(*P* = 0.0427)*	(*P* = 0.0522)	(*P* = 0.0299)*
Ca	0.2019	0.1589	0.0800
	(*P* = 0.0264)*	(*P* = 0.0818)	(*P* = 0.3832)
B	0.3098	0.2618	0.1112
	(*P* = 0.0005)*	(0.0037)*	(*P* = 0.2247)
Fe	0.3128	0.3066	0.2228
	(*P* = 0.0005)*	(*P* = 0.0006)*	(*P* = 0.0141)*
Mn	0.1664	0.1313	0.2164
	(*P* = 0.0681)	(*P* = 0.1511)	(*P* = 0.0172)*
Cu	0.2048	0.1229	0.1127
	(*P* = 0.0242)*	(*P* = 0.1793)	(*P* = 0.2184)
pH	-0.1949	-0.1572	-0.1385
	(*P* = 0.0322)*	(*P* = 0.0852)	(*P* = 0.1298)
Buffer pH	-0.1944	-0.2024	-0.1750
	(*P* = 0.0326)*	(*P* = 0.0260)*	(*P* = 0.0549)
EC	0.2986	0.24	0.1255
	(*P* = 0.0009)*	(*P* = 0.0075)*	(*P* = 0.1702)
Fusarium oxysporum[c]	0.2156	0.19	0.1451
	(*P* = 0.0175)*	(*P* = 0.0408)*	(*P* = 0.1124)
Sand (%)	-0.2436	-0.2603	-0.1800
	(*P* = 0.0071)*	(*P* = 0.0039)*	(*P* = 0.0483)*
Clay (%)	0.3478	0.3259	0.2113
	(*P*<0.0001)*	(*P* = 0.0003)*	(*P* = 0.0200)*
Silt (%)	0.1650	0.2025	0.1487
	(*P* = 0.0705)	(*P* = 0.0259)*	(*P* = 0.1035)
Rotation interval[d]	-0.3068	-0.3138	-0.2700
	(*P* = 0.0007)*	(*P* = 0.0005)*	(*P* = 0.0029)*
Total significant correlations	13	10	8

[a] Experimental design in each bioassay was a randomized complete block with two factors: 1) soil samples submitted by growers or seed company representatives (one or two 19-liter buckets/sample); and 2) female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. For each of five replications/treatment combination, up to eight spinach plants/ pot were rated for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Data were combined for four soil bioassays (2010 to 2013). Soils that had never had a spinach seed crop, soils with phytotoxic herbicide carryover effects that precluded Fusarium wilt assessments, and the control soils each year were not included in the correlations analyses, bringing the total number of soils included in the correlation analyses to 121. * = significant at $P \leq 0.05$.

[b] Soil samples were collected by spinach seed stakeholders (growers and seed company representatives) from commercial fields located in northwestern Washington. A subsample of the approximately 38-liter soil sample for each field submitted for each field was collected after processing and mixing the sample, and sent to Soiltest Farm

Consultants, Inc. (Moses Lake, WA) for analysis. Only those soil properties which correlated significantly with Fusarium wilt severity ratings for one or more of the spinach inbred lines are included in this table: ammonium-nitrogen (NH_4^+), potassium (K), calcium (Ca), boron (B), iron (Fe), manganese (Mn), copper (Cu), pH, buffer pH, and electrical conductivity (EC)

[c] The soil population of *Fusarium oxysporum* (including possible non-pathogenic isolates and other formae speciales which cannot be distinguished morphologically from strains of the spinach pathogen, *F. oxysporum* f. sp. *spinaciae*) was quantified by dilution plating a sample of each soil onto Komada's agar medium (Komada, 1975). Fungal colonies with fluffy, white to pale salmon-pink morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after plating. Rotation = number of years since the field was planted with a spinach seed crop.

[d] Rotation interval = number of years since the field was planted with a spinach seed crop.

Table 3.17. Pearson's correlation coefficients among properties of soil samples collected from 147 fields under consideration for spinach seed crops in northwestern Washington, and evaluated in greenhouse soil Fusarium wilt bioassays from 2010 to 2013

Soil property[a]	NO_3	NH_4	P	K	Ca	Mg	S	B	Fe	Mn	Zn	Cu	pH	Buffer pH	CEC	EC	OM	Sand	Clay	Silt
NO_3-N	-	-0.06	-0.02	0.17	0.12	0.12	-0.10	0.06	0.12	0.03	**0.39**	-0.14	**0.18**	0.00	-0.12	0.07	**0.25**	0.15	-0.05	0.08
NH_4-N	-0.06	-	**-0.20**	0.00	**0.31**	0.14	**0.36**	**0.27**	**0.29**	0.07	0.18	-0.09	0.17	**0.26**	**0.39**	**-0.35**	**0.47**	**-0.22**	0.02	0.13
P	-0.02	**-0.20**	-	**-0.19**	0.00	**0.27**	-0.09	0.07	0.03	-0.11	**0.18**	**0.39**	-0.12	-0.01	**0.19**	**0.36**	-0.08	-0.03	**-0.56**	**0.43**
K	0.17	0.00	**-0.19**	-	0.15	**-0.37**	-0.11	-0.15	**-0.28**	0.14	0.07	-0.17	**-0.21**	**-0.26**	**-0.29**	**-0.22**	-0.05	-0.05	**0.25**	**-0.30**
Ca	0.12	**0.31**	0.00	0.13	-	**0.29**	**0.29**	**0.26**	**0.43**	0.17	**0.48**	0.05	0.08	**0.39**	**0.42**	-0.04	**0.26**	-0.09	-0.04	**0.22**
Mg	0.12	0.13	**0.27**	**-0.37**	**0.29**	-	**0.36**	**0.23**	**0.35**	-0.08	**0.40**	0.05	0.17	**0.63**	**0.44**	**0.39**	**0.27**	0.12	-0.32	**0.50**
S	-0.10	**0.36**	-0.09	-0.11	**0.29**	**0.36**	-	**0.26**	**0.29**	0.06	0.11	-0.03	-0.05	**0.54**	**0.55**	**-0.33**	0.23	**-0.60**	-0.12	**0.31**
B	0.06	**0.27**	0.07	-0.15	**0.26**	**0.23**	**0.26**	-	**0.43**	0.07	**0.45**	0.16	0.11	**0.35**	**0.59**	-0.15	**0.34**	**-0.31**	**-0.34**	**0.39**
Fe	0.12	**0.29**	0.03	**-0.28**	**0.43**	**0.34**	**0.29**	**0.43**	-	0.02	**0.41**	0.17	**0.28**	**0.48**	**0.53**	-0.10	**0.39**	-0.15	-0.13	**0.29**
Mn	0.03	0.07	-0.11	0.14	0.17	-0.07	0.06	-0.07	0.02	-	0.10	0.04	0.03	-0.01	0.12	-0.14	0.01	-0.10	0.00	**0.04**
Zn	**0.39**	0.18	**0.18**	0.07	**0.48**	**0.40**	0.11	**0.45**	**0.41**	0.10	-	0.13	**0.21**	**0.35**	**0.40**	-0.02	**0.44**	-0.04	**-0.25**	**0.33**
Cu	-0.14	-0.09	**0.39**	-0.17	0.05	0.05	-0.03	0.16	0.17	0.04	0.13	-	0.04	**0.20**	**0.21**	-0.15	0.07	**-0.30**	**-0.20**	0.15
pH	**0.18**	0.17	-0.11	**-0.21**	0.08	0.17	-0.05	0.11	**0.28**	0.03	**0.21**	0.04	-	**0.22**	0.03	0.17	**0.21**	**0.23**	0.14	-0.08
SMP pH	0.00	**0.26**	-0.01	**-0.26**	**0.39**	**0.63**	**0.54**	**0.35**	**0.48**	-0.13	**0.35**	**0.20**	**0.22**	-	**0.58**	-0.10	**0.23**	**-0.34**	0.02	**0.24**
CEC	-0.11	**0.39**	**0.19**	**-0.29**	**0.42**	**0.44**	**0.55**	**0.59**	**0.53**	0.12	**0.40**	**0.21**	0.03	**0.58**	-	**-0.23**	**0.21**	**-0.51**	**-0.36**	**0.52**
EC	0.07	**-0.35**	**0.36**	**-0.22**	-0.05	**0.39**	**-0.33**	-0.15	-0.10	-0.14	-0.02	-0.15	0.17	-0.10	**-0.23**	-	**-0.31**	**0.71**	**-0.29**	**0.20**
OM	**0.25**	**0.47**	-0.08	-0.05	**0.26**	**0.27**	**0.23**	**0.34**	**0.39**	0.01	**0.44**	0.07	**0.21**	**0.23**	0.21	**-0.31**	-	-0.02	0.00	0.16
Sand	0.15	**-0.22**	-0.03	-0.04	-0.08	0.12	**-0.60**	**-0.31**	**-0.15**	-0.10	-0.04	**-0.30**	**0.23**	**-0.34**	**-0.51**	**0.71**	-0.02	-	0.15	**-0.21**
Clay	-0.05	0.02	**-0.56**	**0.25**	-0.04	**-0.32**	-0.12	**0.34**	-0.13	0.00	**-0.25**	**-0.20**	0.14	0.02	-0.36	**-0.29**	0.00	0.15	-	**-0.92**
Silt	0.08	0.13	**0.43**	**-0.30**	**0.22**	**0.50**	**0.31**	**0.39**	**0.29**	0.04	**0.33**	0.15	-0.08	**0.24**	**0.52**	**0.20**	0.16	**-0.21**	**-0.92**	-

[a] Soil samples were collected by spinach seed stakeholders (growers and seed company representatives) from commercial fields located in northwestern Washington. A subsample of the approximately 38-liter soil sample submitted for each field for the bioassay was collected after processing and mixing the sample, and sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA) for analysis of: pH, buffer pH, nitrate-nitrogen (NO_3), ammonium-nitrogen (NH_4^+), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), iron, (Fe), manganese (Mn), zinc (Zn), copper (Cu), cation exchange capacity (CEC), organic matter (OM), and electrical conductivity (EC). Soil samples were submitted from 147 fields for evaluation in the soil bioassay from 2010 to 2013. Numbers in bold font represent significant correlation coefficients ($P \leq 0.05$).

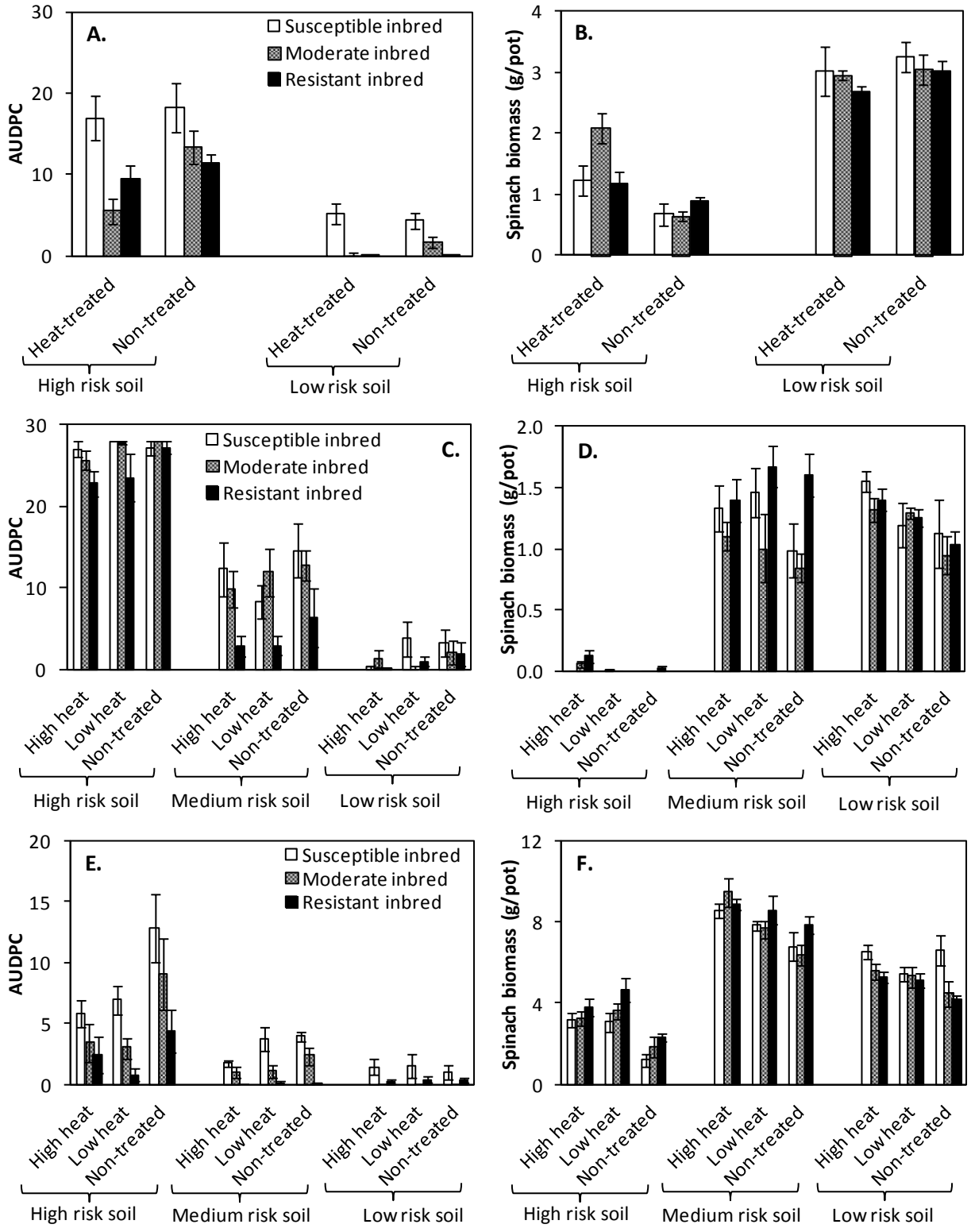
A.
Susceptible inbred
Moderate inbred
Resistant inbred
AUDPC
30
20
10
0
Heat-treated
Non-treated
Heat-treated
Non-treated
High risk soil
Low risk soil
B.
Spinach biomass (g/pot)
4
3
2
1
0
Heat-treated
Non-treated
Heat-treated
Non-treated
High risk soil
Low risk soil
C.
Susceptible inbred
Moderate inbred
Resistant inbred
AUDPC
30
20
10
0
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High risk soil
Medium risk soil
Low risk soil
D.
Spinach biomass (g/pot)
2.0
1.5
1.0
0.5
0.0
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High risk soil
Medium risk soil
Low risk soil
E.
Susceptible inbred
Moderate inbred
Resistant inbred
AUDPC
20
15
10
5
0
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High risk soil
Medium risk soil
Low risk soil
F.
Spinach biomass (g/pot)
12
8
4
0
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High heat
Low heat
Non-treated
High risk soil
Medium risk soil
Low risk soil

Fig. 3.1. Effects of soil Fusarium wilt risk level, soil heat treatment, and spinach inbred line on area under the disease progress curve (AUDPC) for weekly severity ratings and spinach biomass (g/pot) in the first (**A** and **B**), second (**C** and **D**), and third (**E** and **F**) preliminary bioassays for assessing the risk of Fusarium wilt. Experimental design was a randomized complete block with three factors: 1) soil collected from fields with 12, 8 (second and third bioassays), and 0 year rotations out of a spinach seed crop, characterized as low, medium, and high risk for Fusarium wilt, respectively; 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt; and 3) soil heated for 1.5 h in an electric soil sterilizer at 65°C followed by 2 h at 80°C (high heat) (second and third bioassays), for 1.5 h at 65°C (low heat), or not heated (non-treated). Each data point is the mean ± standard error of five replicate plots, with four (**A** or **B**) or eight (**C** to **F**) seedlings/pot.

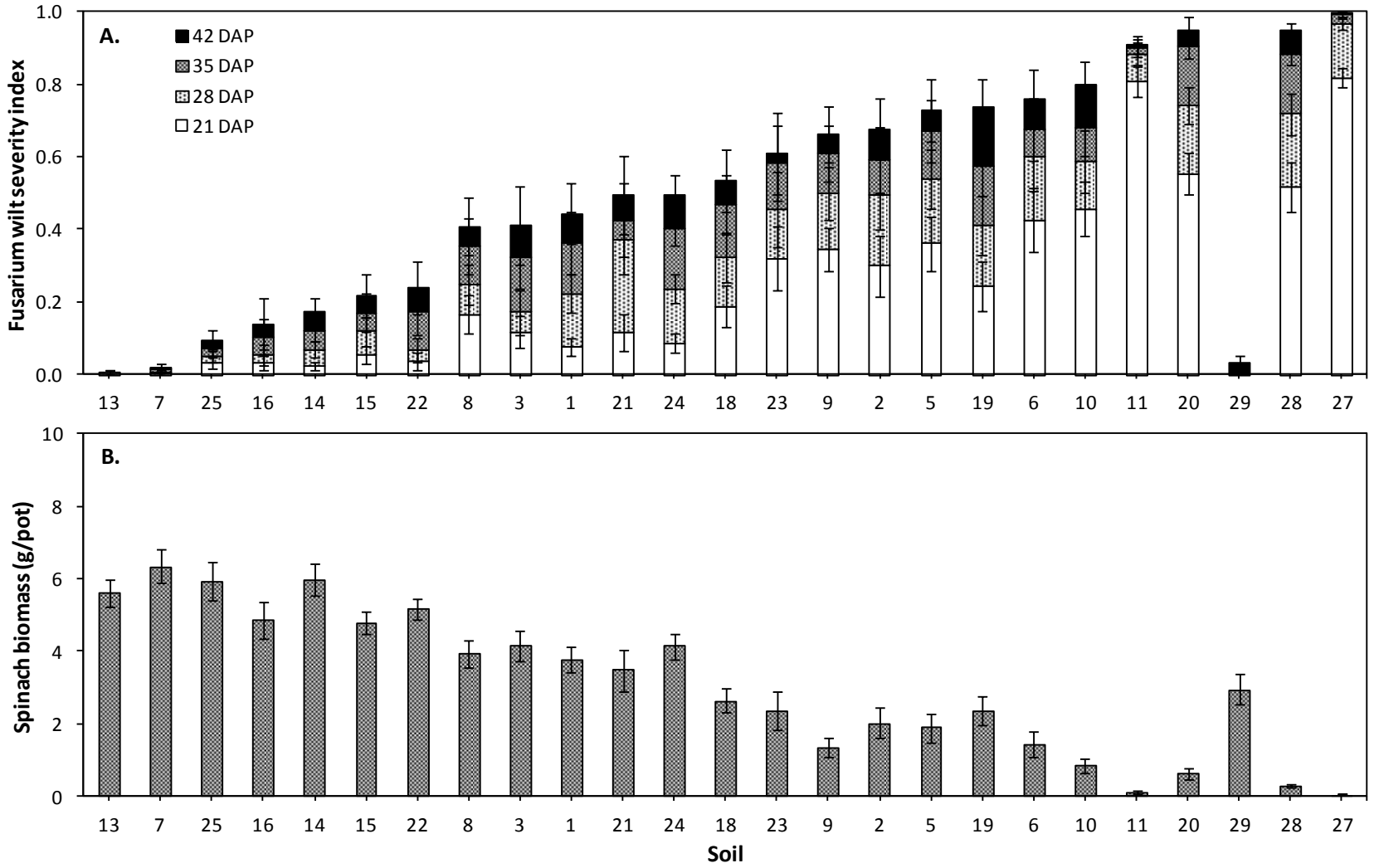

Fig. 3.2. Effects of 22 grower field soil samples (soils 1 to 22) and three control soils (soils 27 to 29, representing high, medium, and low risk of spinach Fusarium wilt, respectively) on spinach Fusarium wilt severity index (0-to-1 scale, with 1 = maximum wilt) measured 21, 28, 35, and 42 days after planting (DAP) (**A**), and aboveground spinach biomass (g/pot) measured 56 to 59 DAP (**B**)

in the 2010 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a randomized complete block with five replications of two factors: 1) soil samples submitted from fields in northwestern Washington by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of 15 pots (five replicate pots of each of three spinach inbred lines), with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

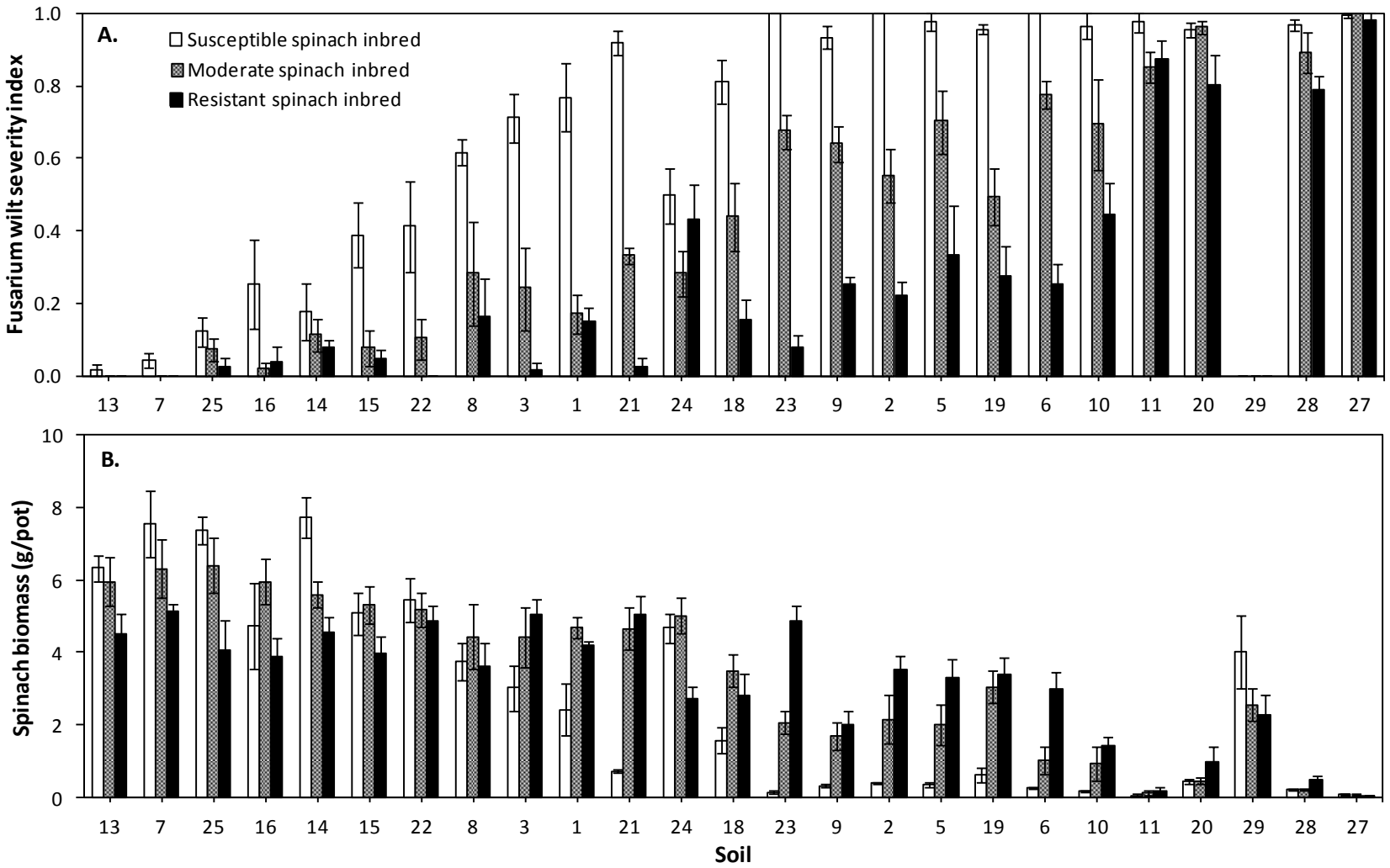

Fig. 3.3. Effects of 22 grower field soil samples (soils 1 to 22) and three control soils (soils 27 to 29, representing high, medium, and low risk of spinach Fusarium wilt, respectively), on Fusarium wilt severity index (0-to-1 scale with 1 = maximum Fusarium wilt) 35 days after planting (**A**), and on aboveground spinach biomass (g/pot) (**B**) in the 2010 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a randomized complete block with

five replications of two factors: 1) soil samples submitted from fields in northwestern Washington by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of five replicate pots, with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant, and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line 56 to 59 days after planting, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

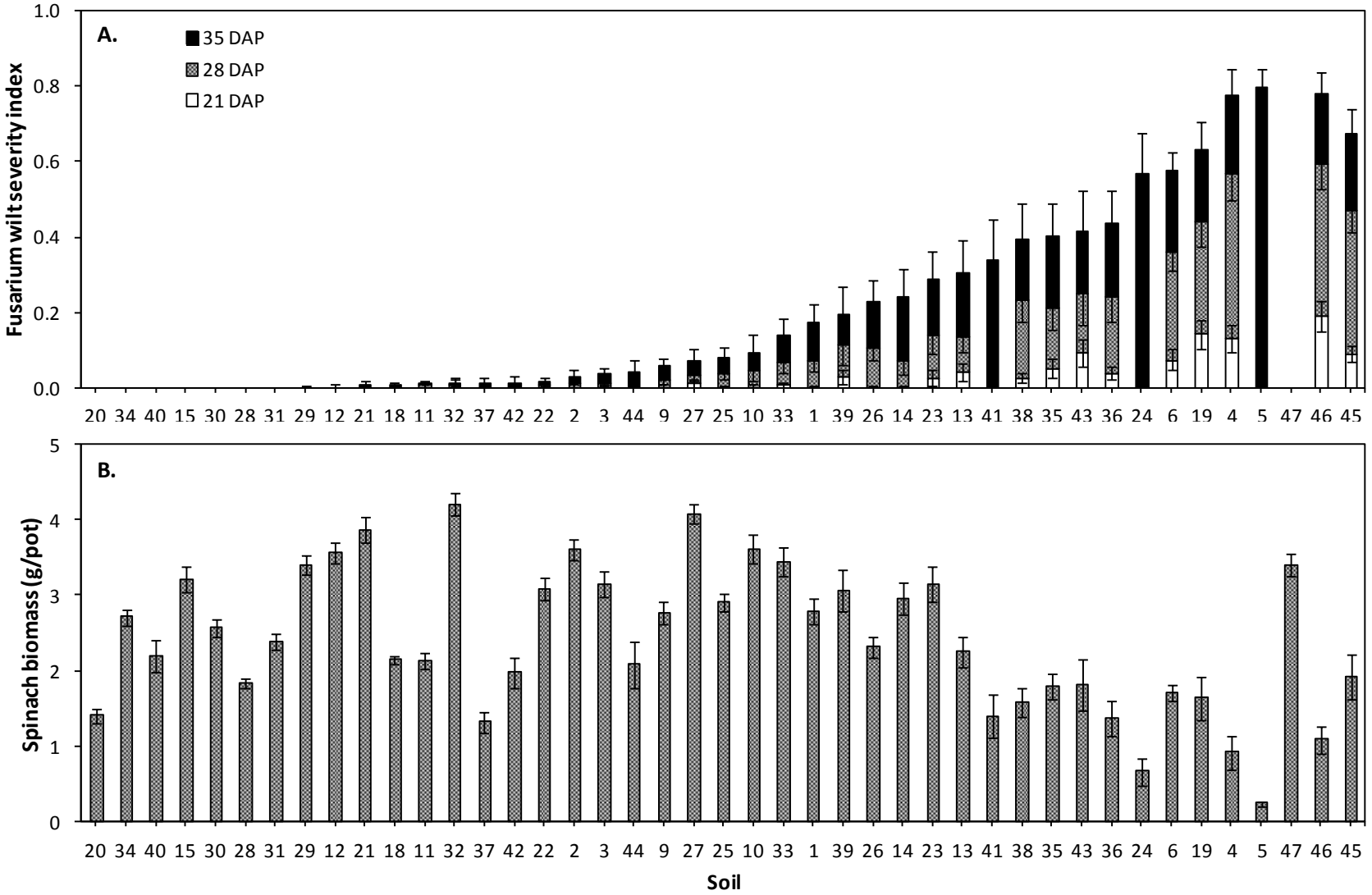

Fig. 3.4. Effects of 40 grower field soil samples (soils 1 to 40) and three control soils (soils 45, 46, and 47, representing high, medium, and low risk, respectively) on Fusarium wilt severity index (0-to-1 scale, with 1 = maximum wilt) measured 21, 28, and

35 days after planting (DAP) (**A**), and aboveground spinach biomass (g/pot) measured 47 to 48 DAP (**B**) in the 2011 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a randomized complete block with five replications of two factors: 1) soil samples submitted from fields in northwestern Washington by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of 15 pots (five replicate plots of each of three spinach inbred lines), with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot). Spinach plants in soils 5, 20, 24, 37, 41, and 42 showed symptoms of herbicide carryover toxicity that confounded Fusarium wilt ratings until the final rating 35 DAP (**A**).

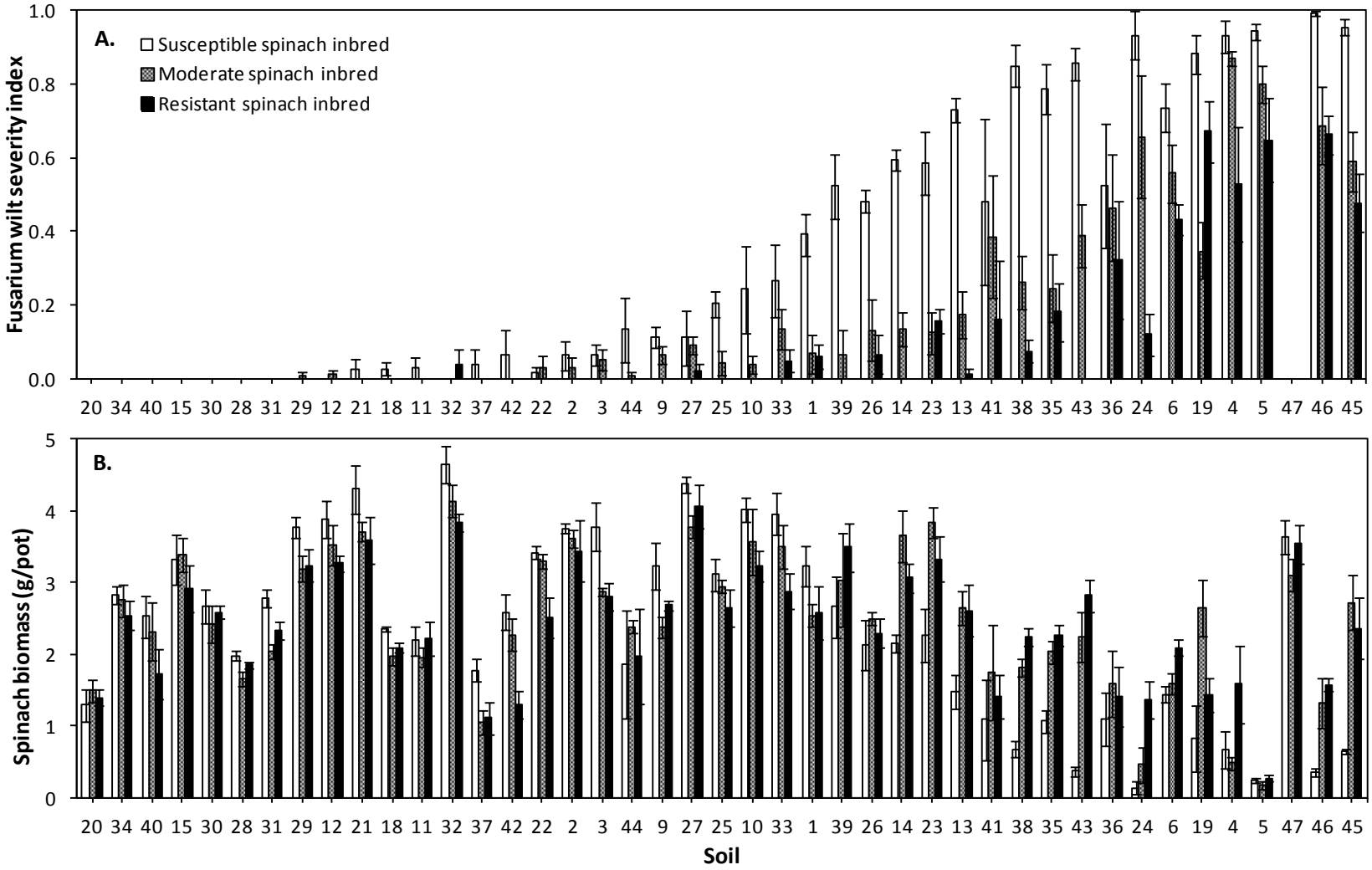

Fig. 3.5. Effects of 40 grower field soil samples (soils 1 to 40) and three control soils (soils 45, 46, and 47, representing high, medium, and low risk, respectively), as well as spinach inbred line, on Fusarium wilt severity index (0 to 1 scale with 1 = maximum Fusarium wilt) 35 days after planting (**A**), and on aboveground spinach biomass (g/pot) (**B**) in the 2011 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a

randomized complete block with five replications of two factors: 1) soil samples submitted by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of five replicate pots, with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line 47 to 48 days after planting, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

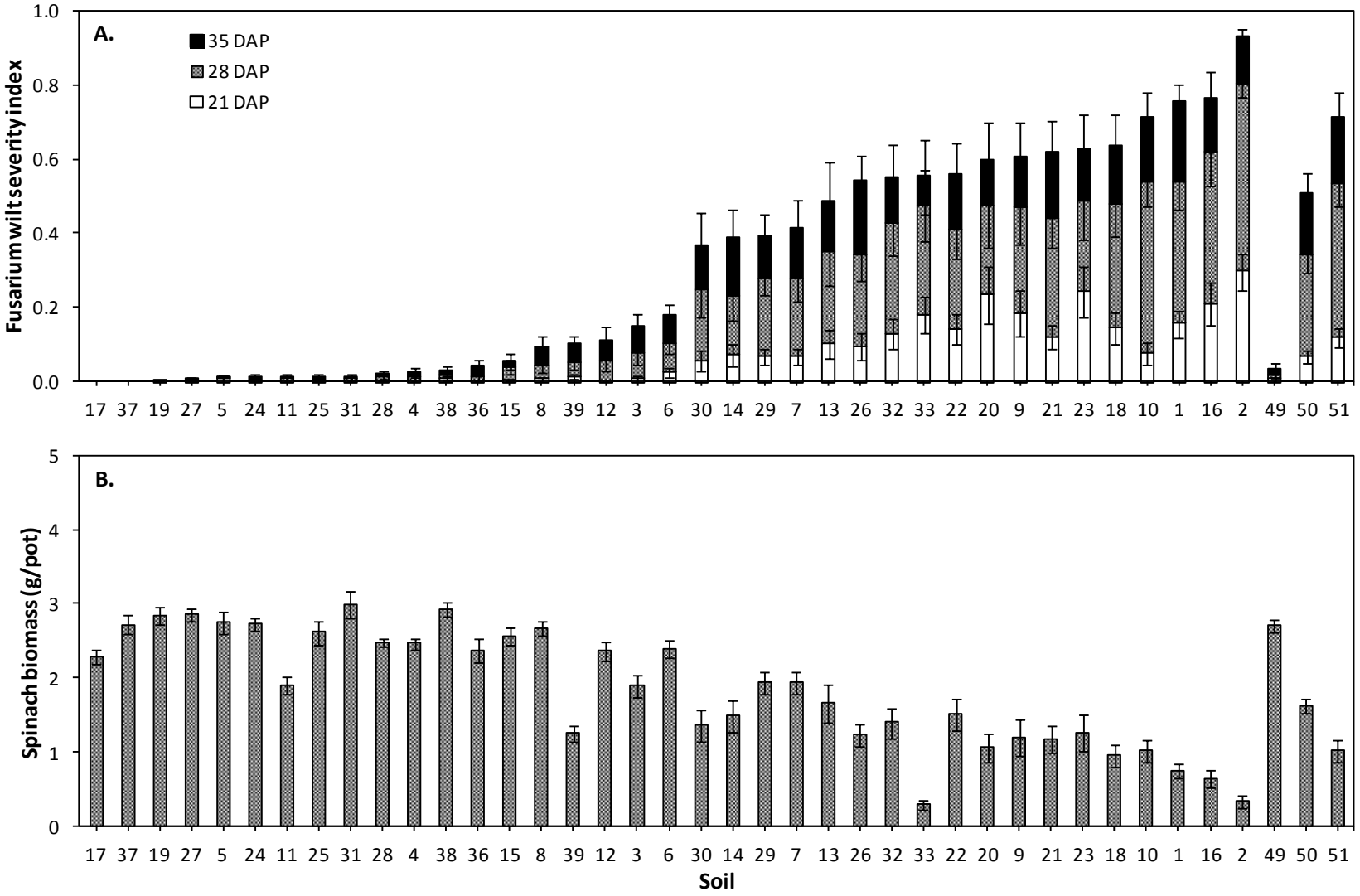

Fig. 3.6. Effects of 37 grower field soil samples (soils 1 to 37) and three control soils (soils 49, 50, and 51, representing low, medium, and high risk of spinach Fusarium wilt, respectively) on Fusarium wilt severity index (0-to-1 scale, with 1 = maximum

wilt) measured 21, 28, and 35 days after planting (DAP) (**A**), and aboveground spinach biomass (g/pot) measured 47 to 48 DAP (**B**) in the 2012 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a randomized complete block with five replications of two factors: 1) soil samples submitted from fields in northwestern Washington by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of 15 pots (five replicate pots of each of three spinach inbred lines), with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

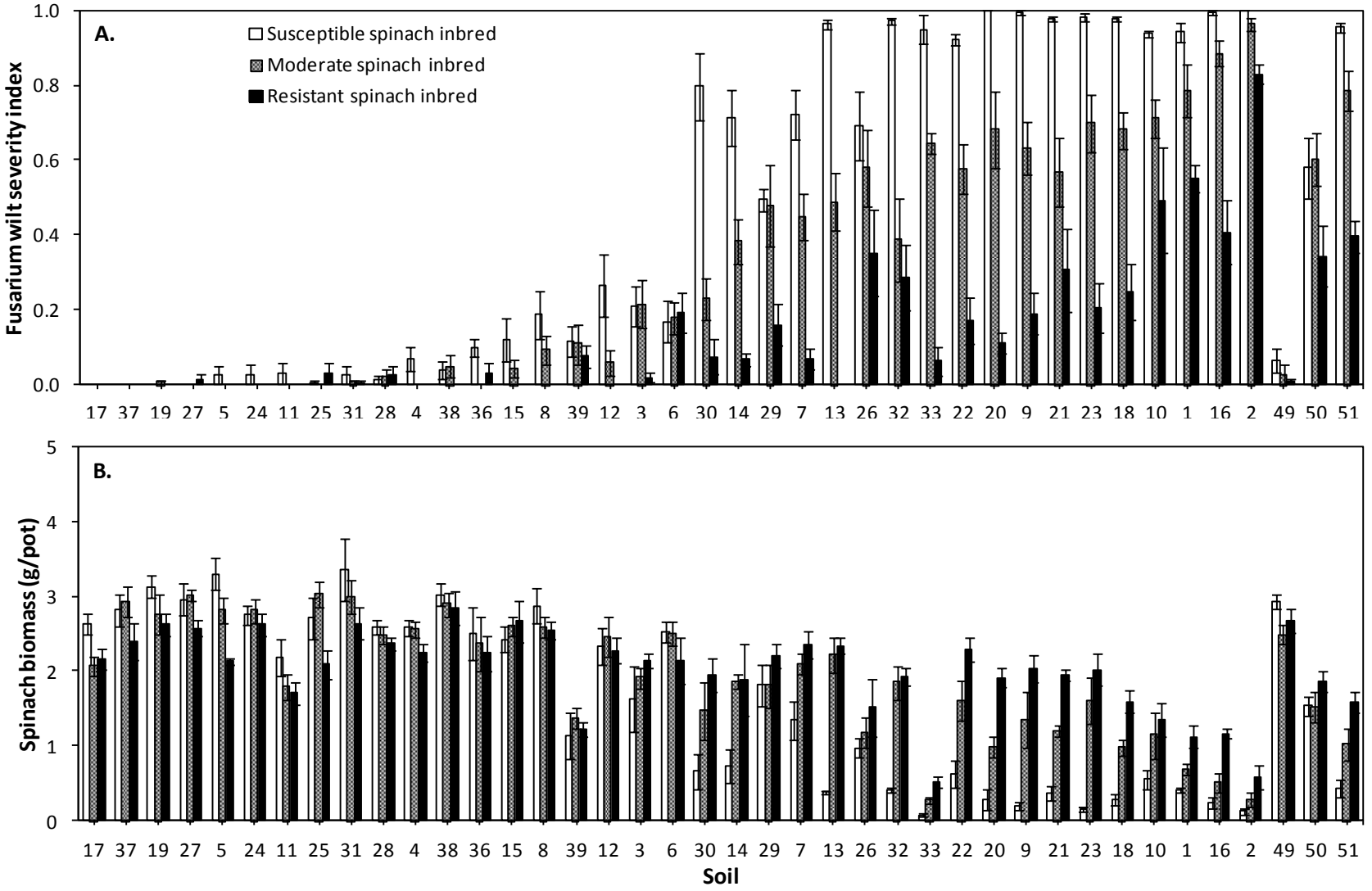

Fig. 3.7. Effects of 37 grower field soil samples (soils 1 to 37) and three control soils (soils 49, 50, and 51, representing low, medium, and high risk of spinach Fusarium wilt, respectively), as well as spinach inbred lines, on Fusarium wilt severity index (0-

to-1 scale with 1 = maximum Fusarium wilt) measured 35 days after planting (**A**), and on aboveground spinach biomass (g/pot) (**B**) in the 2012 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a randomized complete block with five replications of two factors: 1) soil samples submitted from fields in northwestern Washington by growers or seed company representatives (two 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of five replicate pots, with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line 47 to 48 days after planting, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

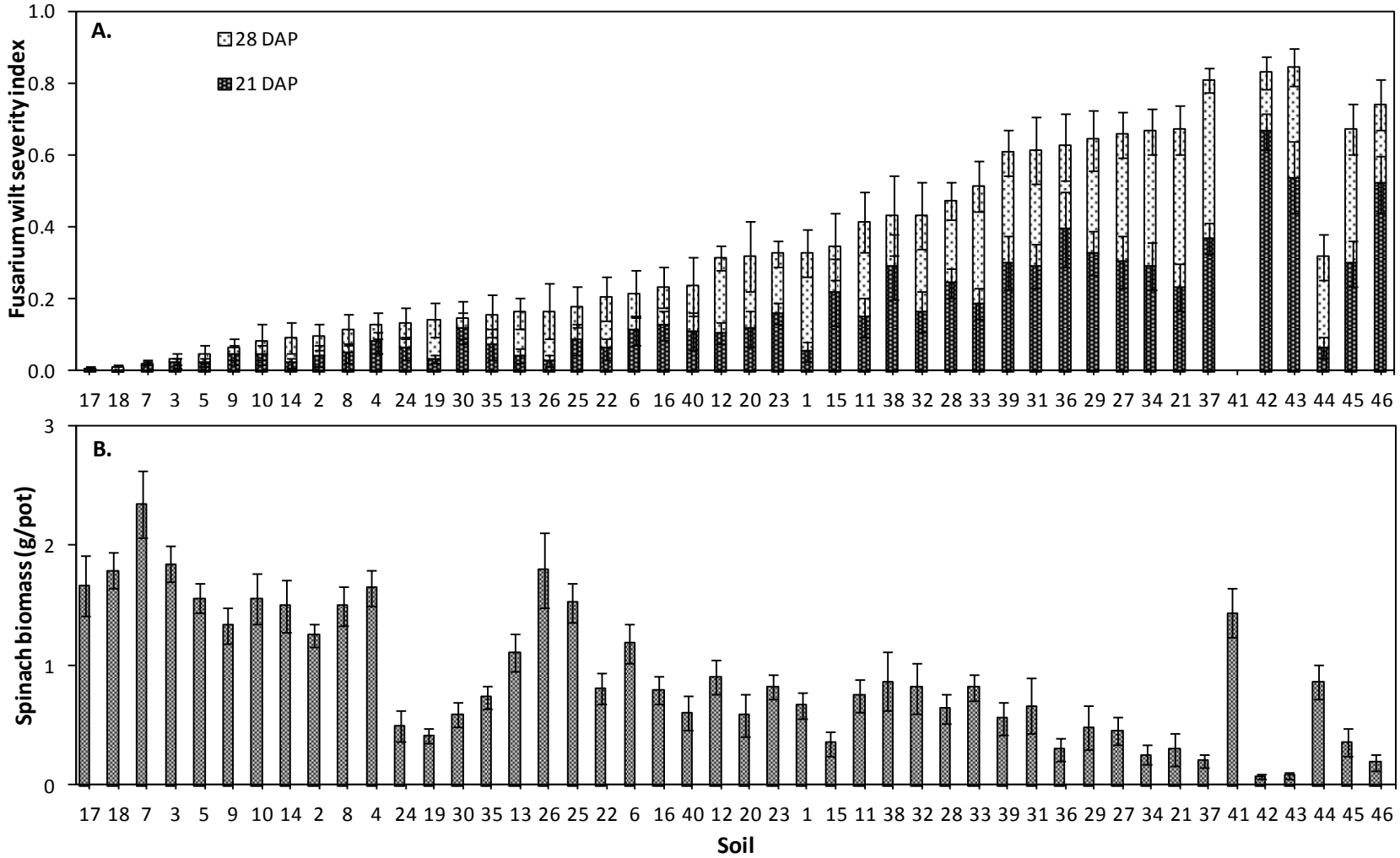

Fig. 3.8. Effects of 40 grower field soil samples (soils 1 to 40) and six control soils (soils 41, 42, and 43, representing low, medium, and high risk of spinach Fusarium wilt, respectively; and soils 44, 45, and 46, representing these same three control soils amended with the equivalent of 4.48 t limestone/ha) on Fusarium wilt severity index (0-to-1 scale, with 1 = maximum wilt) measured 21 and 28 days after planting (DAP) (**A**) and aboveground spinach biomass (g/pot) measured 37 DAP (**B**) in the 2013 grower soil

Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a randomized complete block with four replications of two factors: 1) soil samples submitted by growers or seed company representatives (one 19-liter bucket/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of 12 pots (four replicate pots of three spinach inbred lines), with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to the 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

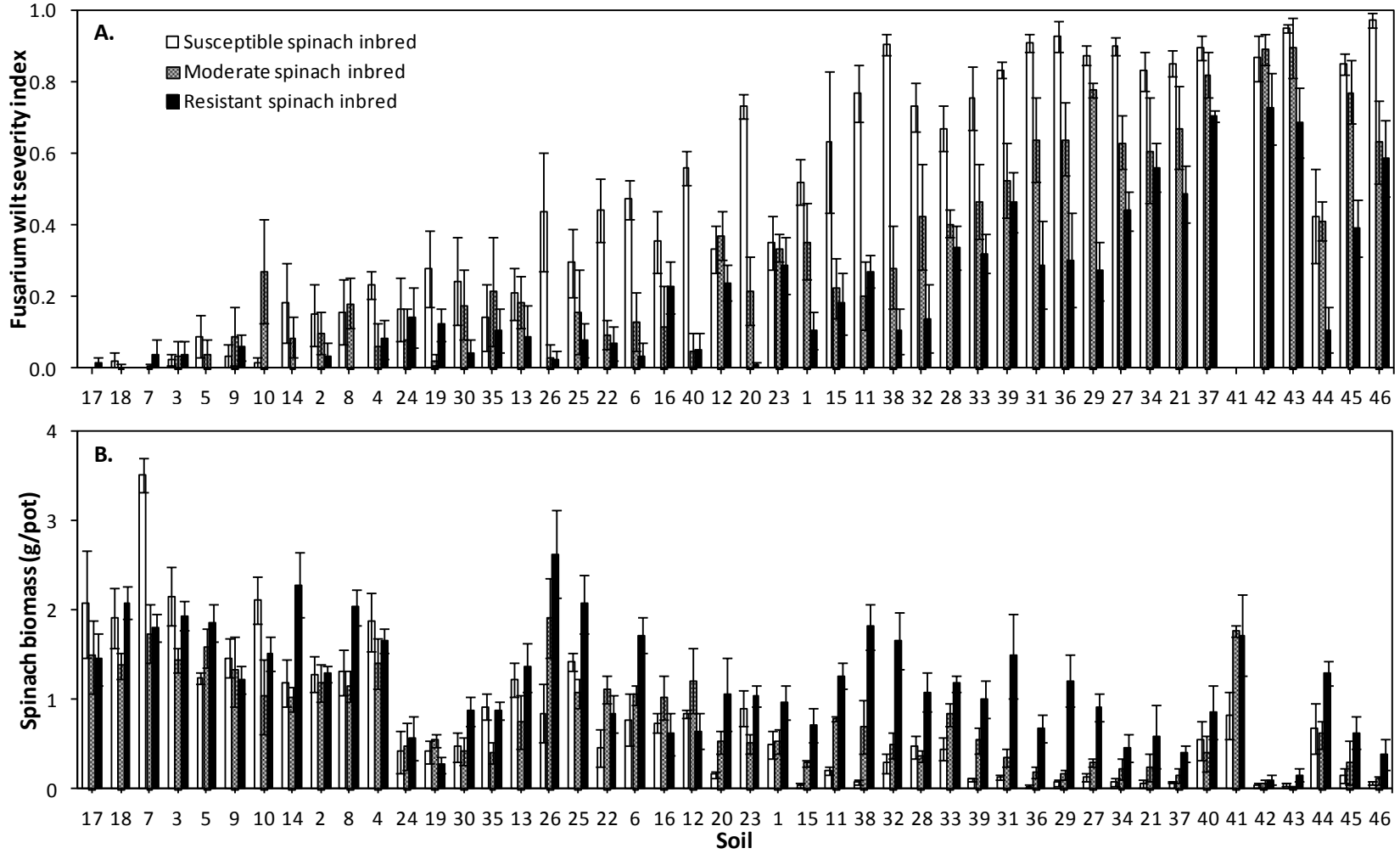

Fig. 3.9. Effects of 40 grower field soil samples (soils 1 to 40) and six control soils (soils 41, 42, and 43, representing low, medium, and high risk of spinach Fusarium wilt, respectively; and soils 44, 45, and 46, representing these same three control soils amended with the equivalent of 4.48 t limestone/ha) as well as spinach inbred line, on Fusarium wilt severity index (0-to-1 scale with 1 = maximum Fusarium wilt) measured 28 days after planting (**A**), and on aboveground spinach biomass (g/pot) (**B**) in the 2013 grower soil Fusarium wilt bioassay. Details of the bioassay and wilt ratings are described in the main text. Experimental design was a

randomized complete block with four replications of two factors: 1) soil samples submitted from fields in northwestern Washington by growers or seed company representatives (one 19-liter buckets/sample); and 2) three female inbred spinach lines characterized as highly susceptible, moderately susceptible, or moderately resistant to spinach Fusarium wilt. Each data point is the mean and standard error of four replicate pots, with up to eight spinach plants evaluated/pot for Fusarium wilt symptoms based on a 0-to-5 rating scale, with 0 = healthy plant and 5 = dead plant due to Fusarium wilt. Ratings were subsequently converted to a 0-to-1 index. Dried, aboveground spinach biomass was measured by harvesting plants at the soil line 37 days after planting, drying the plants at 35°C, and weighing the dried plants from each pot (g/plot).

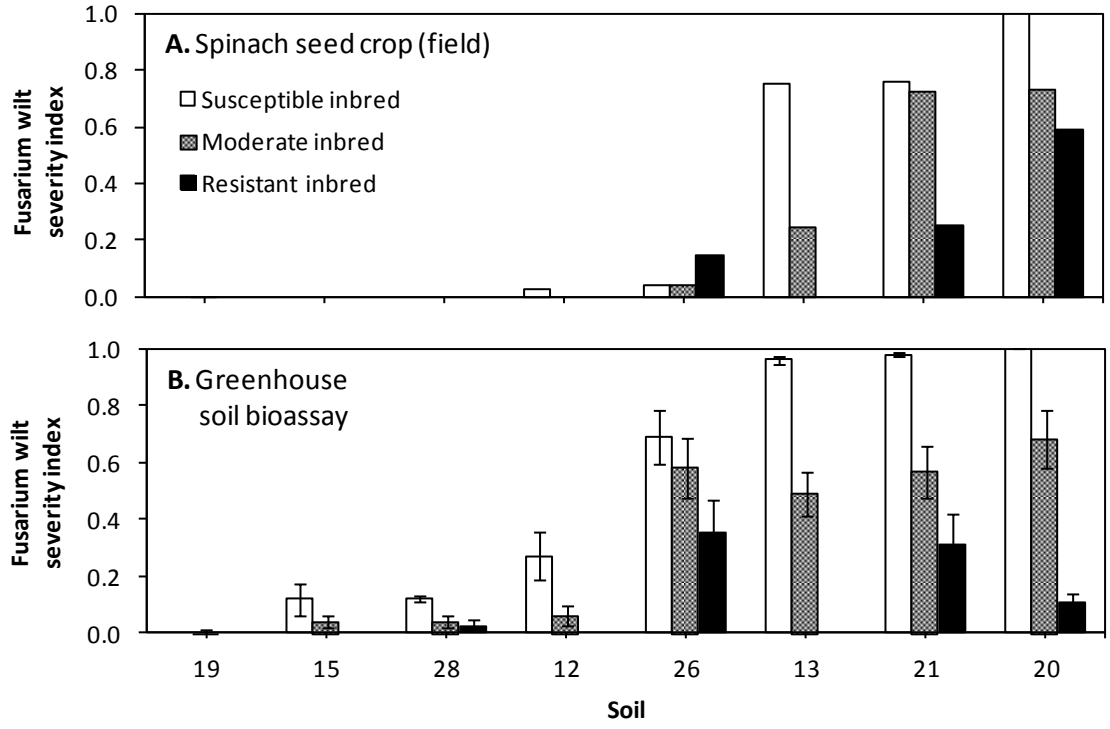

Fig. 3.10. Results of eight fields (represented by soils 12, 13, 15, 19, 20, 21, 26, and 28) planted with spinach seed crops in 2012 that had been evaluated in the 2012 greenhouse soil bioassay for spinach Fusarium wilt risk. Approximately 1 m of row was planted in each crop with each of three female spinach inbred lines characterized as highly susceptible, moderately susceptible, and moderately resistant to Fusarium wilt, the same three spinach inbred lines used in the bioassay. The test plots were planted alongside the proprietary male and female lines in each field by the participating grower and seed company, rated for Fusarium wilt severity on 27 July 2012 using the same 0-to-5 rating scale (converted to a 0-to-1 index) as the bioassay (**A**), and the ratings compared to the greenhouse bioassay results for soils sampled from these fields the previous winter (**B**). The plants in the test plots were incorporated into the soil prior to seed set to avoid contamination of the commercial seed crop. Each data point in the greenhouse soil bioassay (**B**) represents the mean ± standard error of five replicate pots of soil, each planted with eight seed of the appropriate spinach inbred line. The data points for the field trial (**A**) each represent an average of the wilt ratings for all the plants of that inbred line in 1 m of row.

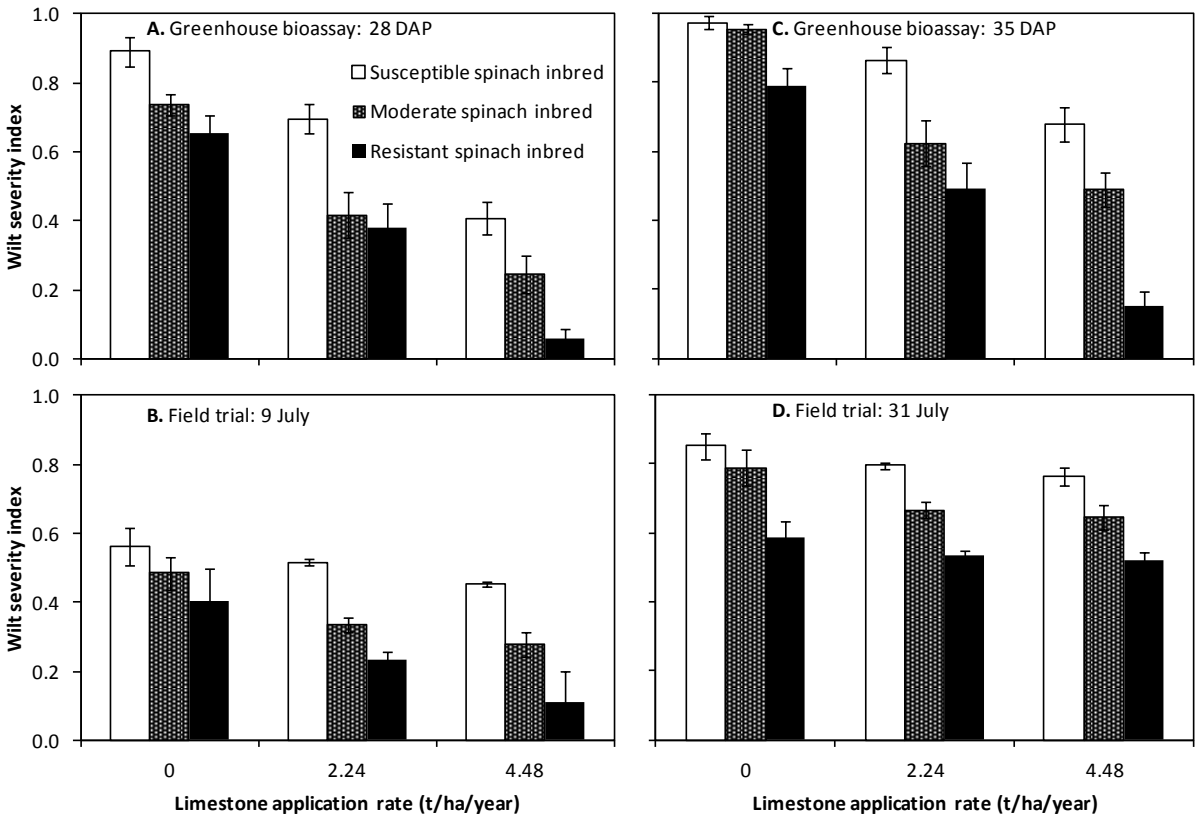

Fig. 3.11. Comparison of Fusarium wilt severity ratings from a greenhouse soil bioassay (**A** and **B**) for assessing Fusarium wilt risk with results of a 2012 spinach seed crop field trial (**C** and **D**). Soil samples were collected in November 2011 from plots that were part of a four-year limestone/Fusarium wilt field trial located in Skagit County, WA, in which limestone was applied at 0, 2.24, or 4.48 t/ha to the same plots each year from 2009 to 2012 in a grower cooperator's field (see Chapter 2). Soils were collected from plots of three replications of each of the limestone treatments, and evaluated in the 2012 greenhouse Fusarium wilt soil bioassay, along with soil samples submitted by spinach seed growers and stakeholders from 37 other fields. A spinach seed crop trial was planted in this site again in spring 2012, with the same three female spinach inbred lines used in the bioassay (highly susceptible, moderately susceptible, and moderately resistant to Fusarium wilt), and wilt development and spinach growth were evaluated through the season as described in detail in Chapter 2. The Fusarium wilt severity ratings for these soil samples in the bioassay (**A** and **C**) were compared to results from the 2012 field trial (**B** and **D**), to assess how well the bioassay predicted levels of Fusarium wilt in the field. Each data point represents the mean ± standard error of spinach Fusarium wilt severity (0-to-1 scale, where 1 = all plants dead from Fusarium wilt) of 15 pots (five replicate pots of each of three limestone rates) in the greenhouse soil bioassay (**A** and **C**), and mean spinach Fusarium wilt

severity (0-to-1 scale) for spinach plants growing in three replicate plots in the 2012 field trial (**B** and **D**).

CHAPTER FOUR

EFFECTS OF SELECT MICRONUTRIENTS ON *FUSARIUM OXYSPORUM* F. SP. *SPINACIAE* AND LIMESTONE-MEDIATED SUPPRESSION OF FUSARIUM WILT

Introduction

Mineral nutrition can influence the outcome of plant-pathogen interactions profoundly (Graham, 1983; Walters and Bingham, 2007). A plant that has access to adequate levels of essential macro and micronutrients in bioavailable forms in the soil is better-equipped to off-set the effects of pathogen invasion, by replacing damaged tissue, accelerating or delaying plant maturation, and deploying physiological and/or biochemical resistance mechanisms (Marschner, 1995). Nutrient deficiencies can compromise the capacity of a plant to outgrow or defend against microbial onslaught, resulting in heightened susceptibility to disease and reduced survival, growth, and/or reproduction (Colhoun, 1973). Equally important to understanding the dynamics of plant pathogenesis are the nutritional requirements of the pathogen, which must be met for full expression of genetic virulence (Tousson et al., 1960; Weinhold et al., 1969). Plants and their pathogenic associates can differ in the kinds and amounts of minerals identified as essential (Garraway and Evans, 1984; Gerloff, 1963; Steinberg, 1950a). Calcium (Ca), for example, is essential for plant growth, cell wall integrity, membrane selectivity, and cellular signaling pathways (Marschner, 1995), but has not been shown to be required by many fungi, including wilt pathogens within the *Fusarium oxysporum* complex (Steinberg, 1950a and 1950b; Youatt, 1993). Conversely, strains of *F. oxysporum* have been shown to require comparatively high levels of iron (Fe), manganese (Mn), and zinc (Zn), and are highly sensitive to deficiencies of these trace elements (Steinberg, 1950b; Woltz and Jones, 1981). This sensitivity may reflect, in

part, the limited volume of soil that can be explored by microbes (Woltz and Jones, 1973). Plants generally have an advantage over microbial pathogens in the relative ease of obtaining access to soil mineral nutrients, with root systems that can expand into new territory when faced with zones of nutrient depletion, compared to the relatively limited mobility of soil microbes. These differences present potential opportunities for the management of certain plant diseases through manipulation of soil fertility to the detriment of the pathogen but not the plant host (Woltz and Jones, 1981).

In the 1960s, researchers in Florida, building upon earlier work by Edgerton (1918), Scott (1923), Sherwood (1923), and Albert (1946), determined that raising soil pH through application of agricultural limestone ($CaCO_3$) suppressed Fusarium wilts of various crops, caused by formae speciales of *F. oxysporum* (Jones et al., 1989; Jones and Overman, 1971; Jones and Woltz, 1970; Jones and Woltz, 1975; Woltz and Engelhard, 1973; Woltz and Jones, 1981). Application of gypsum ($CaSO_4$), which does not affect soil pH, did not reduce wilt symptoms to the same extent (Jones and Woltz, 1967). Furthermore, a reduction in tomato Fusarium wilt symptoms was observed in studies using NaOH to adjust soil pH, rather than a Ca-based compound (Scott, 1923). Together, these results suggest that an increase in soil pH, more so than added Ca, is critical to the underlying mechanism(s) of limestone-mediated suppression of Fusarium wilt.

Hypotheses about the nature of such mechanisms requires an understanding of how the availability of some essential micronutrients is affected by shifts in soil chemistry, as well as the roles of these elements in plant and microbial physiology (Rice, 2007; Woltz and Jones, 1981). As soil pH increases, Fe, Mn, and Zn precipitate out of the soil solution in the form of oxides or hydroxides, becoming unavailable to both plant roots and the soil microbial community (Brady and Weil, 1999). These transition metals are required in micro-amounts by all known living

organisms (Epstein and Bloom, 2005; Pais, 1983). Fe has the widest range of roles of these micronutrients (Marschner, 1995). One of the most common but least accessible elements found in soils, Fe is required for chlorophyll synthesis as well as the function of redox enzymes in photosynthetic and respiratory electron transport chains, lipoxygenases, and superoxide dismutases, with the latter two classes of enzymes involved in the function and integrity of cell membranes (Marschner, 1995). Studies on *F. oxysporum* have linked Fe availability to the production of mycotoxins and pectin methyl esterases, both of which are considered virulence factors in wilt development (Sadasivan, 1965). Numerous studies have examined the role of Fe deficiency on stimulation of antagonistic populations of fluorescent pseudomonads, which compete vigorously with other soil microbes for Fe through the production of Fe-chelating siderophores, and thus have a competitive advantage (and superior Fusarium wilt biocontrol activity) in low-Fe environments (e.g., Scher and Baker, 1980; Simeoni et al., 1987).

Mn is a critical component of the redox reactions leading to oxidation of water in Photosytem II, and several enzymes in the biosynthetic pathway of lignin, a key compound in plant structural defense against pathogens (Graham, 1983; Marschner, 1995). Mn is also a cofactor for a superoxide dismutase, an enzyme that deactivates cell membrane-destroying free radicals. Zn occurs in a number of enzymes in both plants and microbes, including carbonic anhydrase and a superoxide dismutase. Zn is a component of proteins called zinc fingers that help regulate DNA transcription (Epstein and Bloom, 2005). Zn is required for the synthesis of indole acetic acid, a hormone which is, in turn, required for the formation of tyloses in xylem cells under attack by Fusarium wilts (Beckman, 1987). In *F. oxysporum* f. sp. *vasinfectum* (referred to in the cited study as *F. vasinfectum*), Zn has been implicated in the production of

fusaric acid, and is required for normal growth and sporulation of the pathogen (Kalyanasundarum and Sarawathi-Devi, 1955).

Various strategies are employed by both plants and microbes to solubilize these essential trace elements in high pH soils, including the excretion of protons, organic acids, CO_2, reductases, and various types of chelators, but deficiencies may still occur (Epstein and Bloom, 2005; Rengel, 2008). Woltz and Jones (1968), and Jones and Woltz (1967, 1970) identified an association between decreased availability of Fe, Mn, and Zn in limestone-amended soils and reduced growth, sporulation, and virulence of the tomato Fusarium wilt pathogen. These results laid the groundwork for the development of an economical and environmentally-sound management program for Fusarium wilts based on starving the pathogen population of these micronutrients, and in doing so, reducing the inoculum potential of the pathogen (Woltz and Jones, 1981). However, not all Fusarium wilts are affected by soil pH in the same way (Hoper et al., 1995; Islas, 2012; Matheron and Koike, 2003; Peng et al., 1999; Stover, 1956) and under alkaline conditions, the host plant may also be vulnerable to micronutrient deficiencies (du Toit et al., 2007).

The need for a program to manage Fusarium wilt of spinach seed crops in the maritime Pacific Northwest (PNW) region of the USA is dire, as growers currently must use rotations of up to 15 years between spinach seed crops to reduce the risk of losses to this disease (Foss and Jones, 2005). Market demand for spinach seed has increased sharply since the advent in the mid-1990s of fresh market, pre-packaged, baby and "teenage" leaf spinach, now the dominant spinach commodity in California and Arizona, the main fresh market spinach producing regions of the USA (Koike et al., 2011). Baby leaf spinach crops are short-season (40 days or less from planting to harvest), high-density plantings with populations of up to 1.6 million seed/ha,

compared to 90,000 seed/ha for processing crops. The narrow corridor that comprises the maritime PNW is the sole region of the USA suitable for spinach seed production, which requires moderate summer temperatures, long daylengths to initiate bolting, and dry conditions during seed maturation for optimal seed quality (Metzger and Zeevart, 1985). Together with the Willamette Valley of Oregon, Skagit, Whatcom, Snohomish, and Clallam Counties in Washington produce up to 50% of the US spinach seed supply and up to 25% of the global supply, on 1,200 to 1,600 ha annually (Foss and Jones, 2005).

Application of 4.48 t $CaCO_3$/ha prior to planting a spinach seed crop has proven effective for partial suppression of spinach Fusarium wilt in acid soils typical of the maritime PNW (du Toit et al., 2007, 2008, and 2011), and has been widely adopted by spinach seed growers in the PNW (see Chapter 2). However, a rotation interval of 5 to 8 years, which would approximately double available acreage for spinach seed production significantly, remains elusive. Investigating the mechanism(s) behind limestone-mediated suppression of spinach Fusarium wilt, and attempting to unravel the complex biological and chemical dynamics of the spinach rhizosphere could help to optimize the use of limestone to manage this disease. Such studies could also lead to alternative management strategies such as strategic placement, application timing, and/or formulation of fertilizer. Based on previous studies identifying Fe, Mn, and Zn as potential factors in the development of Fusarium wilt (Woltz and Jones, 1971; du Toit et al., 2007, 2008), the objectives of this study were to: 1) evaluate the effects of a range of Fe, Mn, and Zn concentrations on biomass production and conidiation of the spinach Fusarium wilt pathogen *in vitro*, and 2) determine whether the disease suppression induced by limestone amendment of naturally-infested, acid soils could be negated by soil micronutrient amendment. The results may

lead to a greater understanding of the influence of soil micronutrient status on spinach Fusarium wilt development.

Materials and Methods

***In vitro* assays**. Experiments were conducted to assess the effects of Fe, Mn, and Zn concentration on *in vitro* growth and spore production of *F. oxysporum* f. sp. *spinaciae*. Fos001, a single-spore isolate from Skagit Co., WA that was determined previously to be pathogenic on spinach, was cultured in a defined liquid minimal medium with a range of concentrations of each of these micronutrients using a batch culture system. Separate experiments were performed for each micronutrient and both fungal biomass and spore production. Each experiment was conducted twice. For each experiment, a one-way, completely randomized design was used with four replications of each micronutrient concentration. The liquid minimal medium consisted of 1 g NH_4NO_3 (G. Mallinckrodt & Co., St. Louis, MO), 0.4 g KH_2PO_4 (Sigma-Aldrich, St. Louis, MO), 0.2 g KCl (Sigma-Aldrich), 0.2 g $MgSO_4$ (Sigma-Aldrich), 0.01 g $FeSO_4 \cdot 7H_2O$ (J. T. Baker Chemical Co., Phillipsburg, NJ), 0.0088 g $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich), 0.006 g $MnSO_4 \cdot H_2O$ (Sigma-Aldrich), and 20 g sucrose (Macron Chemicals, Phillipsburg, NJ) per liter deionized water. For adding Mn and Zn, autoclaved stock solutions of $MnSO_4 \cdot H_2O$ and $ZnSO_4 \cdot 7H_2O$, respectively, were prepared and added separately to the autoclaved and cooled main solution. For Fe, a stock solution of $FeSO_4 \cdot 7H_2O$ was prepared aseptically in a laminar flow hood in sterilized, deionized water, and the solution was not autoclaved prior to adding to the main solution. These steps were taken to avoid precipitation of these elements and, in the case of Fe, oxidation from the ferrous (soluble) to ferric (insoluble) state.

For each micronutrient evaluated, the basal medium was prepared without that micronutrient. This basal medium was then divided into seven equal volumes in separate autoclaved flasks, and a four-fold range of micronutrient concentration added, from 0 to 2 mg/liter, was established by adding 0.5 ml of the appropriate micronutrient stock solution to each flask. This range was chosen based on preliminary experiments showing that, in the defined nutrient medium used in this study, biomass production and sporulation of *F. oxysporum* f. sp. *spinaciae* Fos001 stabilized at concentrations <2 mg/liter for each of the three micronutrients evaluated. Following pH measurement (SympHony Electrode SB70P, VWR International, Radnor, PA) of each of these solutions, 50 ml aliquots were transferred into 100 ml, autoclaved glass flasks, and each flask was inoculated with an agar plug obtained with a size 2 cork borer (Humboldt Manufacturing, Schiller Park, IL) from the leading edge of a 5- to 7 day old culture of Fos001 grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) amended with chloramphenicol (cPDA) at 100 mg/liter. After 7 days of incubation on a rotary shaker at 125 rpm (2100 platform shaker, Innova, Fountain Valley, CA), the filtrate was removed by vacuum from each flask using a porcelain Buchner funnel (CoorsTek, Golden, CO) and Whatman filter paper #1 (7 cm diameter, GE Healthcare, Kent, UK). The filtrate pH was measured for the combined four replications of each treatment following separation of the mycelial mat. The mycelial mat remaining on the filter paper was then rinsed with deionized water, dried in an oven at 65°C for 4 h, and weighed. The average weight of filter paper alone dried under the same conditions was subtracted from the total weight of filter paper with the mycelial mat to obtain a net measurement of dried fungal biomass.

In separate experiments using the media preparation and incubation protocols described above, spore production by *F. oxysporum* f. sp. *spinaciae* Fos001 and the percentage of

germinated spores were measured by filtering the contents of each flask through four layers of cheesecloth after a 7-day incubation, and enumerating microconidia/ml using a hemocytometer. A spore was considered germinated when there was visual evidence of a germ tube emerging from the conidium. Germinated and non-germinated microconidia were recorded to obtain an estimate of the percentage of germinated spores at the time of harvest.

Greenhouse experiments. Soil sampled from a field in Skagit Co., WA that had previously been characterized as high risk for spinach Fusarium wilt (see Chapter 3) was selected for greenhouse experiments to evaluate the effects of limestone application and Fe, Mn, and Zn concentrations on spinach Fusarium wilt development. Each micronutrient was the focus of a separate experiment, and each experiment was arranged as a randomized complete block experimental design with a three-way factorial treatment design and five replications of each treatment combination. The factors were: 1) steam-pasteurization of the soil or no pasteurization, to evaluate the effects of micronutrients and limestone in the presence and absence of Fusarium wilt; 2) soil limestone amendment at equivalent rates of 0 or 4.48 t/ha; and 3) a soil drench application of 0, 19, or 190 mg of each micronutrient in a chelated formulation/liter soil (w/v). The Mn and Fe experiments were conducted twice, and the Zn experiment was conducted three times. In the repeat trials for Mn and Zn, an additional micronutrient concentration of 1.9 mg/liter was added because soil solution concentration of Mn is commonly between 0.5 and 1.0 mg/liter (Havlin et al., 1999). For the Fe trials, all four concentrations were evaluated in both trials. These micronutrient rates were chosen based on similar work by Jones and Woltz (1970), who found significant differences in the effects of these application rates on tomato Fusarium wilt development.

Soil preparation. The field from which the soil was collected had been planted to a spinach seed crop in 2009, and was classified as a Mount Vernon very fine sandy loam (Soil Survey Staff, USDA National Resources Conservation Service). Fifteen 19-liter buckets of soil were collected in mid-November 2011, and the soil passed through a 6.3 mm-aperture sieve to remove debris from a winter wheat crop harvested in fall of 2011, and to break up large soil clods. For the pasteurization treatment, soil was either steam-pasteurized at 56°C for 1 h with a custom-built soil steamer (Patzek, *unpublished report*) or left non-pasteurized. Agricultural limestone (Imperial Ground limestone, Oregon Lime Score = 97, calcium carbonate equivalent (CCE) = 97%) was mixed with pasteurized or non-pasteurized soil in an 85-liter cement mixer at rates equivalent to 0 or 4.48 t/ha at a 15 cm depth of incorporation (w/v) in 19 liter batches. Soil with no limestone amendment was mixed in the cement mixer also. After limestone amendment, soil for each treatment was stored in clean buckets in a greenhouse set at 22 to 26°C to facilitate reaction of the limestone in the soil. After two weeks of storage, a subsample of approximately 200 cm^3 from each pasteurization-by-limestone soil treatment combination was collected, air-dried, crushed with a marble rolling pin, and passed through a 1 mm-aperture sieve. The pH of each subsample was measured using a 1:1 soil:deionized water protocol. A subsample of 40 g soil was mixed with 40 ml deionized water, and the suspension stirred for 30 s, followed by a 3 min interval and another 30-s stirring, for a total of five stir/wait cycles. After the soil settled and a supernatant had formed, pH was measured. Soil pH was also measured at the end of each trial for each pasteurization-limestone-micronutrient treatment combination. Similarly, samples of each soil treatment combination were collected upon completion of each trial (with the exception of the second Fe trial and third Zn trial), and sent to a commercial soil laboratory (Soiltest Farm Consultants Inc., Moses Lake, WA) for analysis of available Fe, Mn, and Zn.

Planting, greenhouse conditions, and pest management. Plastic pots (12.5 cm diameter and 12.5 cm deep, Anderson Die, Portland, OR) with drainage holes were each filled with the appropriate soil treatment after each drainage hole was plugged with a piece of expanded polyether foam plug to prevent drainage of soil from the pots. Seven seeds/pot of a proprietary spinach inbred line determined previously to be moderately susceptible to Fusarium wilt (see Chapters 2 and 3) were sown at a depth of approximately 1 cm. Prior to planting, seeds were treated with thiram (Thiram 42-S, Bayer CropScience, Research Triangle Park, NC) and mefenoxam (Apron XL LS, Syngenta Crop Protection, Basel, Switzerland) at 521 ml and 20 ml/100 kg seed, respectively, for control of *Rhizoctonia* and *Pythium* damping-off and seedling pathogens, respectively. Bifenthrin (Talstar P granular insecticide, FMC Corp., Philadelphia, PA) was applied to the soil surface in each pot and watered into the soil for control of cranefly (*Tipula paludosa*) larvae (0.11 kg/m^2). Two days after planting, the appropriate micronutrient soil drench was applied to the appropriate pots. For each pot, the micronutrient solution was applied in two 50-ml volumes, with 10 to 15 min between applying each aliquot to prevent pooling of water and resulting soil compaction. This was done to ensure that the drench treatment wetted the soil just to the point that water was starting to drip out of the bottom of the pot. The micronutrients used were in chelated form (Versatile-IDS Chelated Micronutrients, Wilbur-Ellis, San Francisco, CA), with ethylenediaminetetracetic acid (EDTA) and iminosuccinic acid (IDS) as the chelating agents, and a guaranteed analysis of 4% Fe, 9% Zn, and 5% Mn. Use of these products was recommended by an agronomist (Jeff Schwab, Wilbur Ellis, Burlington, WA; *personal communication*) for these soil experiments instead of the sulfate forms of the micronutrients used for the *in vitro* experiments, because of the stability of the chelated formulations across a wide range of pH values (3 to 12).

Experiments were conducted in a greenhouse set at 24 to 26°C by day and 22 to 25°C by night, with supplemental lighting provided on a 10 h light/14 h dark schedule. Plants were watered as needed with a modified Hoagland's solution (Hoagland and Arnon, 1950) that did not include the three specific micronutrients evaluated. A weekly rotation of insecticides was applied for control of thrips (*Thrips* spp. and/or *Frankliniella* spp.): imidacloprid (Provado 1.6F, Bayer CropScience) at the equivalent rate of 45 ml/ha, *Beauveria bassiana* (Botanigard 22WP, Laverlam International, Butte, MT) at 2.4 g/liter, spinosad (Success 480SC, Dow AgroSciences, Indianapolis, IN) at 50 ml/1,000 liters, and acephate (Orthene 97, AmVac Chemical Co., Newport Beach, CA) at 1.2 g/liter.

Disease ratings and biomass measurement. Fusarium wilt symptoms were rated at the onset of symptoms 21 days after planting (DAP), and again 28 and 34 DAP. A 0-to-5 ordinal rating scale was used, with 0 = no visible symptoms; 1 = a few, flaccid older leaves with a dull green cast; 2 to 4 = progressive increase in leaf wilting and chlorosis; and 5 = dead plant due to Fusarium wilt. The number of plants in each of these categories was recorded for each pot at each rating date. After the 35 DAP rating, aboveground spinach biomass was measured by cutting all the plants in each pot at the soil line, combining the plants in a paper sack, drying the plants at approximately 35°C, and weighing the dried plant material. Isolations for plant pathogens were completed 10 to 14 DAP from seven seedlings to assess the cause(s) of early season damping-off and wilt. Two seedlings were collected for each of the three micronutrient trials, one from a non-pasteurized soil with symptoms of Fusarium wilt, and one from a pasteurized soil with symptoms of phytotoxicity. A third seedling was collected from the Fe experiment that was growing in a pasteurized soil and had symptoms of Fusarium wilt. Leaves were removed from the sampled seedlings, and the remaining tissue surface-sterilized for 30 s in

0.6% NaOCl; triple-rinsed in sterilized, deionized water; dried; cut aseptically into pieces approximately 3 mm long; and the pieces plated onto cPDA and water agar (Bacto agar, Difco Laboratories) amended with chloramphenicol (cWA). Fungi growing from these tissue pieces were transferred to new plates of cPDA and subsequently examined with a compound microscope to identify potential seedling pathogens.

Soil microbial evaluation. The population of *F. oxysporum* in each pasteurization-by-limestone soil treatment combination at the time of planting, and each soil pasteurization-by-limestone-by-micronutrient concentration treatment combination at the end of each trial (with the exception of the second Fe trial and third Zn trial), was quantified using a soil dilution series. A 10 g subsample of dried, crushed, and sieved soil was added to 100 ml 0.1% WA in a 240-ml glass French square, agitated for 10 min on a rotary shaker at 250 rpm, and diluted serially to a 10^{-1} concentration. Aliquots (0.5 ml) of the 10^{-1} dilution were plated onto each of three replicate petri plates of Komada's agar medium, (Komada, 1975; Scott et al., 2010). The plates were then incubated on a laboratory bench at room temperature (25 ± 2°C) in ambient light in a room with windows. Fungal colonies with fluffy, white to pale salmon-pink morphology typical of *F. oxysporum* growth on this semi-selective medium were counted 7 and 14 DAP.

Statistical analyses. Analyses of variance (ANOVAs) and means separation using Fisher's protected least significant difference (LSD) (P <0.05) were performed on all dependent variables using PROC GLM of SAS Version 9.2 (SAS Institute, Cary, NC). Disease ratings were first converted to a Fusarium wilt severity index for each plot (pot) using a weighted average formula, and then analyzed using ANOVA:

$$\text{Fusarium wilt index} = \frac{\sum(\text{category} \times \#\ \text{seedlings within that category})}{(\text{total \# of emerged seedlings}) \times 5}$$

Area under the disease progress curve (AUDPC) was calculated using the Fusarium wilt index data from each rating date. Rank transformation of Fusarium wilt index data was performed prior to ANOVA due to non-homogeneous variances resulting from the absence of wilt in the pasteurized soil treatments. This is an allowed, although not preferred, method for dealing with ordinal scale data in factorial experiments (Shah and Madden, 2004). Biomass measurements were subjected to logarithmic, square root, or arcsine square root transformations when assumptions of homogeneous variances and/or normally distributed residuals were not met, or were subjected to Friedman's non-parametric rank test if these transformations did not resolve violations of the ANOVA assumptions.

Results

In vitro experiments. *Mn assays.* The effect of Mn concentration on *in vitro* biomass production by *F. oxysporum* f. sp. *spinaciae* was highly significant ($P \leq 0.0001$) for both trials (Table 4.1). Increasing the concentration of Mn from 0 to 2.0000 mg/liter nutrient medium significantly increased fungal biomass production (Fig. 4.1A and 4.1D). In the first Mn trial, the nutrient medium with no Mn added and 0.0005 mg Mn/liter produced the least fungal biomass (Fig. 4.1A). With the next highest Mn concentration (0.0020 mg/liter), biomass increased by almost 50% compared to the control treatment. No further consistent increase or decrease in fungal biomass was observed from 0.0020 to 2.0000 mg Mn/liter. Similar results were observed in the second Mn trial, with no Mn added and 0.0005 mg Mn/liter resulting in significantly reduced biomass (60 and 41%, respectively) compared to ≥0.0020 mg/liter (Fig. 4.1D). There was no pattern detected in the fluctuation in fungal biomass measured between 0.0020 and 2.0000 mg Mn/liter in the second Mn trial.

The relatively large coefficient of variance for conidial production by *F. oxysporum* f. sp. *spinaciae* in response to different concentrations of Mn *in vitro* (CV = 28.73 and 27.71 for trials 1 and 2, respectively; Table 4.1), as well as the relatively large standard errors for mean sporulation data (Fig. 4.1B and 4.1E), indicated that the sporulation data were more variable than the biomass data. The number of conidia produced/ml spanned four orders of magnitude across the range of Mn concentrations tested in the first trial, from 19 x 10^3 conidia/ml at the lowest Mn concentration tested to 13 x 10^6 conidia/ml at 2.0000 mg Mn/liter (Fig. 4.1B). There was no significant difference in sporulation at the three lowest Mn concentrations (0 to 0.0020 mg/ml), but there was a significant increase in spore production from 0.0020 to 0.0080 mg Mn/liter, and no further significant increase or decrease from 0.0080 to 2.0000 mg Mn/liter (Fig. 4.1B). Similar results were observed in the second Mn trial (Fig. 4.1E). The effects of increasing Mn concentration on the percentage of germinated conidia were too variable to discern significant differences in the first Mn trial (Table 4.1 and Fig. 4.1C), but in the second Mn trial, the two lowest Mn concentrations resulted in no conidial germination compared to 3.4 to 9.3% germination at ≥0.0020 mg Mn/liter (Fig. 4.1F).

Mycelia harvested from the flasks containing no added Mn or 0.0005 mg Mn/liter had an abnormal appearance with shortened, highly branched hyphae compared to the hyphal morphology of mycelium from flasks containing higher Mn concentrations (Fig. 4.2A and 4.2B). In addition, distinct differences in degree of pigmentation of the mycelial mats and the liquid filtrate were observed at the end of the 7-day incubation period in association with increasing Mn concentration (Fig. 4.2C and 4.2E). Pigmentation was typically light or nearly absent in medium with the lowest micronutrient concentrations, and became increasingly darker pinkish-red and eventually dark purple at the highest concentrations.

Zn assays. The effect of Zn concentration on fungal biomass was also highly significant ($P \leq 0.0001$) for both trials (Table 4.1), with increasing concentration from 0 to 2.0000 mg Zn/liter nutrient medium significantly increasing fungal biomass production (Fig. 4.1A and 4.1D). In the first Zn trial, biomass increased across the range of Zn concentrations tested, leveling off only after reaching 0.5000 mg Zn/liter (Fig. 4.1A). The suppression of fungal biomass production was very severe in the medium with no Zn added, with approximately 90% reduction in biomass compared to biomass in the medium with 2.0000 mg Zn/liter treatment. This dramatic restriction of biomass production at very low Zn concentrations was also observed in the second Zn trial, in which the fungal biomass in the control flasks was reduced by 88% compared to flasks with ≥0.1250 mg Zn added/liter (Fig. 4.1D). Biomass production plateaued at 0.1250 mg Zn/liter in the second trial (Fig. 4.1D). Spore production also was reduced significantly in both Zn trials at the lower Zn concentrations (Fig. 4.1B and 4.1E). In both Zn trials, Zn concentrations ≥0.1250 mg/liter were required to restore spore production to $>10^7$ conidia/ml, and did not differ significantly above this concentration, whereas spore production reached $>10^7$ conidia/ml at only 0.0080 mg Mn/liter. In the first Zn trial, conidial germination data was highly variable (CV = 44.33) (Table 4.1) and did not reveal significant effects of Zn concentration on the percentage of germinated conidia (Fig. 4.1C). In the second Zn trial, while the germination data were also highly variable (CV = 31.81) (Table 4.1), there was an abrupt decline in the percentage of germinated spores when the Zn concentration was increased from 0.0300 to 0.1250 mg/liter, and remained low at the higher concentrations (0.5000 to 2.0000 mg Zn/liter) (Fig. 4.1F). As observed in the Mn trials, pigment production by *F. oxysporum* f. sp. *spinaciae* increased with increasing Zn concentration (Fig. 4.2C to 4.2E).

Fe assays. The effect of Fe concentration on production of biomass by *F. oxysporum* f. sp. *spinaciae* was highly significant ($P \leq 0.0001$) in both trials (Table 4.1), with increasing Fe concentration from 0 to 2.0000 mg/liter nutrient medium significantly increasing fungal biomass (Fig. 4.1A and 4.1D). Biomass production was least at the two lowest Fe concentrations (0 and 0.0020 mg/liter), and reached a statistical maximum at 0.0080 mg Fe/liter in both trials (Fig. 4.1A and Fig. 4.1D). Spore production also was affected significantly by increasing Fe concentration (Table 4.1). Fewer conidia were produced between 0 and 0.0800 mg Fe/liter than at ≥0.0300 mg Fe/liter in the first trial, and at ≥0.1250 mg/liter in the second trial (Fig. 4.1B and 4.1E). The reduction in sporulation at lower concentrations of Fe was less dramatic compared to that at low concentrations of Mn and Zn (Fig. 4.1B and 4.1E). In both Fe trials, there was no significant effect of Fe concentration on the percentage of germinated spores (Table 4.1, and Fig. 4.1C and 4.1F). Increasing the Fe concentration in the liquid medium increased pigment production by *F. oxysporum* f. sp. *spinaciae*, as observed for Mn and Zn (Fig. 4.2C to 4.2E).

Greenhouse experiments. For each greenhouse trial, the results of disease assessments for pasteurized soils were not included in the analyses because the incidence of Fusarium wilt in these soils was negligible (*data not shown*). In each trial, the 190 mg/liter soil drench, regardless of the specific micronutrient evaluated, resulted in symptoms of toxicity on the spinach plants, which confounded evaluation of Fusarium wilt severity. Results for that maximum micronutrient rate were, therefore, interpreted with caution.

Mn assays. The effect of limestone soil amendment on Fusarium wilt severity was highly significant at each rating date and, therefore, for AUDPC values in both Mn trials ($P \leq 0.0001$) (Table 4.2). Limestone amendment at the equivalent rate of 4.48 t/ha significantly reduced Fusarium wilt severity (Fig. 4.3A and 4.3D). The mean AUDPC value for plants in soil not

amended with limestone was more than two and three time as large as that of plants in soil amended with limestone in Trials 1 and 2, respectively (Fig. 4.3B and Fig. 4.3E). In the first Mn trial, the main effect of soil Mn concentration was significant for AUDPC, but not for individual weekly severity ratings (Table 4.2). The AUDPC values of plants growing in soil treated with 190.0 mg Mn/liter (13.9) was significantly greater than that of plants in the 0 and 19.0 mg Mn/liter treatments (12.0 and 12.2, respectively). In the second Mn trial, the effect of Mn concentration on severity of Fusarium wilt was significant at each weekly rating and for the AUDPC (Table 4.2). At 21 DAP, plants in soil treated with 1.9 mg Mn/liter had less severe wilt than plants in soil with no Mn added or 190.0 mg Mn/liter, but not compared to the 19.0 mg Mn/liter treatment (Fig. 4.3D). At 28 and 35 DAP ratings and for the AUDPC, the greatest severity of Fusarium wilt was observed for plants in the 0 or 190.0 mg Mn/liter treatments (Fig. 4.3D and 4.3E).

There was a significant interaction between limestone and Mn treatments on severity of Fusarium wilt at 28 DAP and for the AUDPC in the Trial 1, but no significant interaction on any of the wilt ratings in Trial 2 (Table 4.2). In soil amended with limestone in Trial 1, plants growing with no Mn added to the soil had less severe Fusarium wilt compared to plants in soil treated with 19.0 and 190.0 mg Mn/liter at the 28 DAP rating (Fig. 4.3A) and for the AUDPC (Fig. 4.3B). In soils that were not amended with limestone, Fusarium wilt severity approached 100% by 28 DAP in Trial 1, regardless of Mn treatment (Fig. 4.3A).

Limestone significantly affected spinach biomass production in both Mn trials, but only in the non-pasteurized soils (Table 4.3). Plants in non-pasteurized, limestone-amended soil produced 3.7 and 3.5 times the biomass of plants in non-pasteurized soil not amended with limestone in the Trials 1 and 2, respectively (Fig. 4.2C and 4.2F). In Trial 1, Mn concentration

did not affect spinach biomass production significantly, although there was a trend toward reduced biomass with increasing Mn concentration that was just short of significance for the non-pasteurized soil (P = 0.0550) (Table 4.2 and Fig. 4.3C). In Trial 2, the effect of Mn concentration on spinach biomass was affected by the limestone amendment (Table 4.3). In soils amended with limestone, plant biomass did not differ significantly among the 0, 1.9, and 19.0 mg Mn/liter treatments, but was reduced significantly in soil drenched with 190.0 mg Mn/liter in both pasteurized and non-pasteurized soil (Fig. 4.3F). In non-pasteurized soil not amended with limestone, plants in soil drenched with 1.9 mg Mn/liter had the greatest biomass (0.86 g/pot), followed by plants in the 19.0, 0, and 190.0 mg Mn/liter treatments (0.42, 0.35, and 0.23 g/pot respectively); the latter two treatments did not differ significantly, nor did the 0 vs. 19.0 mg Mn/liter treatments (Fig. 4.3F). In pasteurized soil not amended with limestone, plants in soil with no Mn added had greater biomass than plants in soil treated with 19.0 mg/liter, which in turn had greater biomass than plants in soil treated with 190.0 mg Mn/liter (2.25, 1.80, and 0.71 g/pot, respectively).

Non-pasteurized soil amended with 4.48 t limestone/ha had a higher pre-planting pH than non-pasteurized soil not amended with limestone (6.48 vs. 5.67, respectively) (Table 4.3). In non-pasteurized soil not amended with limestone, there was a decrease in post-trial soil pH with increasing concentration of Mn amendment (Table 4.3). In both pasteurized and non-pasteurized soil, Mn levels were higher in soils with no limestone amendment compared to soils amended with limestone (Table 4.3). Soil Mn availability increased with increasing concentration of Mn added to the soil, but not always by the expected 10-fold increase corresponding to the rates of Mn added. In Trial 1, *F. oxysporum* was not detected in the pre-trial or post-trial pasteurized soil, with one minor exception of 267 CFU/g soil detected in the non-limed control soil with no Mn

added (Table 4.3). In Trial 2, *F. oxysporum* was not detected in the pre-trial, pasteurized soil, but *F. oxysporum* was detected in the post-trial samples of some treatments, ranging from 44 to 1,022 CFU/g soil (Table 4.3). In non-pasteurized soil, the *F. oxysporum* population was larger in the post-trial soil samples than the pre-trial soil samples. Limestone-amended, non-pasteurized soils had slightly smaller post-trial *F. oxysporum* populations overall compared to non-limed, non-pasteurized soils, except for non-limed soil drenched with 190.0 mg Mn/liter (Table 4.3). The post-trial *F. oxysporum* population in non-pasteurized, non-limed soil was reduced in soil treated with 190.0 mg Mn/liter compared to that of soil treated with lower concentrations of Mn. Of the two seedlings collected for pathogen isolations in Trial 2, the seedling from the pasteurized soil with symptoms of phytotoxicity was not infected with any fungi, and the seedling from non-pasteurized soil with symptoms of Fusarium wilt was infected with *F. oxysporum.*

Zn assays. Three trials were conducted for Zn assessment due to inconsistent results obtained in the second trial. In all three trials, the effect of limestone on Fusarium wilt severity was highly significant ($P \leq 0.0001$) (Table 4.4), with wilt severity in non-limed soil approaching the maximum (1.00) at 35 DAP vs. severity ratings of 0.65, 0.63, and 0.47 in soils amended with limestone in Trials 1, 2, and 3, respectively (Fig. 4.4). In Trial 1, the effect of Zn concentration on Fusarium wilt severity was significant at each of the weekly ratings and for the AUDPC (Table 4.4). In Trial 2, there was no significant main effect of Zn concentration on Fusarium wilt severity ratings or AUDPC. In Trial 3, there were significant effects of Zn at each weekly rating and for AUDPC (Table 4.4).

At 21 DAP and for AUDPC in Trial 1, the high rate of Zn (190.0 mg/liter) resulted in significantly more severe Fusarium wilt compared to the 19.0 and 0 Zn/liter treatments (Fig.

4.4A and 4.4B). At 28 and 35 DAP, there was more severe wilt for plants in the 190.0 mg Zn/liter treatment compared to the control treatment, but not compared to the 19.0 mg Zn/liter treatment (Fig. 4.4A). The effect of Zn concentration on the 35 DAP rating was observed only in soil amended with limestone. In soil with no limestone, near-maximum Fusarium wilt severity (1.00) was observed, regardless of Zn concentration (*data not shown*).

While there was no significant main effect of Zn concentration on Fusarium wilt severity in Trial 2, there was an interaction between limestone and Zn at the 35 DAP rating (Table 4.4). In soil with no limestone amendment on this rating date, Fusarium wilt severity was near maximum regardless of Zn level. In contrast, in soil amended with limestone, plants in soil with no added Zn, 1.9, and 190.0 mg Zn/liter all had greater Fusarium wilt severity than plants in soil treated with 19.0 mg Zn/liter (*data not shown*). In Trial 3, there was a significant increase in Fusarium wilt severity in pots treated with 19.0 mg Zn/liter compared to 0 mg/liter at the 28 and 35 DAP ratings, as well as for AUDPC (Fig. 4.4D and 4.4E, data not shown for 35 DAP). Fusarium wilt severity of plants in soil treated with the highest Zn rate (190.0 mg/liter) was greater than that of all other Zn concentrations at each weekly rating.

The effects of Zn concentration on spinach biomass production were significant in both pasteurized and non-pasteurized soil in each of the three Zn trials (Table 4.4). In Trial 1, plants in soil treated with no Zn and 19.0 mg Zn/liter had significantly greater biomass (0.51 and 0.38 g/pot, respectively) than plants in soil treated with 190.0 mg Zn/liter (0.20 g/pot). In Trial 2, biomass of plants in non-pasteurized soil was greater when the soil was treated with 0, 1.9, and 19.0 mg Zn/liter (0.71, 0.61, and 0.74 g/pot, respectively) compared to plants in soils drenched with 190.0 mg Zn/liter (0.49 g/pot) (*data not shown*). In pasteurized soil, plants in soil treated with 0 Zn or 1.9 mg Zn/liter had greater biomass than plants in soils treated with 19.0 mg

Zn/liter, which in turn had greater biomass than plants in soil treated with 190.0 mg Zn/liter (*data not shown*). In Trial 3, biomass of plants in pasteurized soil treated with 0, 1.9, and 19.0 mg Zn/liter was greater than that of plants in soil treated with 190.0 mg/liter (Fig. 4.3F). In non-pasteurized soil, the effect of Zn concentration depended on the limestone amendment (Table 4.4). Plants in limestone-amended soils not treated with Zn had greater biomass (2.32 g/pot) than plants in soil with the 1.9 mg Zn/liter treatment (2.21 g/pot), which had greater biomass than plants in soil treated with 19.0 mg Zn/liter (1.93 g/pot), which in turn had greater biomass than plants in soil treated with 190.0 mg Zn/liter (0.06 g/pot) (Fig. 4.3F). In non-limed, non-pasteurized soil, there were no significant differences in spinach biomass among Zn treatments, despite a trend of decreasing biomass with increasing Zn concentration.

Soil amended with 4.48 t limestone/ha had a higher pH than soil not amended with limestone (Table 4.5). In non-pasteurized soil (with and without limestone), there was a trend of decreasing soil pH with increasing concentration of Zn amendment. In both pasteurized and non-pasteurized soil, available Zn was greater in soil with no limestone compared to soil amended with limestone, except for soil drenched with 190.0 mg Zn/liter. Soil Zn increased with increasing concentration of Zn treatment (Table 4.5). In Trial 1, *F. oxysporum* was not detected in the pre- or post-trial, pasteurized soil. In Trial 2, *F. oxysporum* also was not detected in the pre-trial, pasteurized soil, but some post-trial, pasteurized soils did have *F. oxysporum* (Table 4.5). In non-pasteurized soil, the *F. oxysporum* population was larger in the post-trial soil samples than the pre-trial soil samples (Table 4.5). A trend toward decreasing *F. oxysporum* populations was observed with increasing rate of Zn soil treatment in the non-pasteurized soil, regardless of whether the soil was limed. Of the two seedlings collected for pathogen isolations in Trial 2, the seedling from the pasteurized soil with symptoms of phytotoxicity was not

infected with any fungi, and the seedling from non-pasteurized soil with symptoms of Fusarium wilt was infected with *F. oxysporu*m and a *Rhizoctonia* sp.

Fe assays. In both Fe trials, limestone amendment significantly reduced Fusarium wilt severity at each weekly rating (Table 4.9, Fig. 4.5A and 4.5D). The AUDPC value for soil not amended with limestone was approximately three and two times that of soil amended with limestone in Trials 1 and 2, respectively (Fig. 4.5B and 4.5E). Fe concentration did not significantly affect Fusarium wilt severity ratings or AUDPC values in Trial 1 (Table 4.6). At the highest Fe concentration in Trial 1, toxicity symptoms were so severe that some plants could not be rated for Fusarium wilt, resulting in missing data (Fig. 4.5A and 4.5B). Overall, results of both Fe trials showed a higher degree of variability, indicated by a relatively large CV (32.52 to 36.33 in Trial 1, and 31.78 to 33.11 in Trial 2; Table 4.6) and relatively low coefficients of determination (R^2 = 0.6258 to 0.7069 in Trial 1, and 0.6879 to 0.7054 in Trial 2: Table 4.6). This suggested that a significant portion of the variability observed in wilt severity ratings could not be explained by the ANOVA models. Despite this variability, there was a significant main effect of Fe concentration at 28 and 35 DAP in the second Fe trial (Table 4.6). At 28 DAP, plants in soil treated with 190.0 mg Fe/liter had greater Fusarium wilt severity (0.85) compared to all lower Fe concentrations (0.66 to 0.73). At 35 DAP, Fusarium wilt severity of plants in soil treated with 190.0 mg Fe/liter (0.92) was greater than that of plants in soil treated with 19.0 and 1.9 mg Fe/liter (0.83 and 0.80, respectively), but not compared to soil with no added Fe (0.90).

Limestone amendment of soil significantly increased spinach biomass production in the non-pasteurized soil, with spinach biomass from limestone-amended soil approximately five times that of non-amended soil (Table 4.6, Fig. 4.5C and 4.5F). In pasteurized soil, there was no significant effect of limestone amendment on spinach biomass in the first Fe trial (Fig. 4.5C), but

biomass increased by 37% in limed vs. non-limed soil in the second trial (Fig. 4.5F). There was a significant effect of Fe concentration on spinach biomass production in both non-pasteurized and pasteurized soils (Table 4.6). In the first Fe trial, biomass of spinach in soils with no Fe added (1.11 g/pot) was greater than that of spinach in soils treated with 19.0 and 190.0 mg Fe/liter (0.87 and 0.26 g/pot, respectively), but not compared to 1.9 mg/liter (0.95 g/pot). Spinach in non-pasteurized soil treated with 190.0 mg Fe/liter was one-quarter the size of spinach in soil with no added Fe (Fig. 4.5C). In the second Fe trial, biomass of spinach in non-pasteurized soil did not differ significantly among the 0, 1.9, and 19.0 mg Fe/liter treatments, but biomass of plants in soil treated with 190.0 mg Fe/liter was significantly less than that of plants grown in soil treated with lower rates of Fe (Fig. 4.5F). The effect of Fe treatment on plant biomass in the pasteurized soil depended on limestone amendment (Table 4.6). In soil with no limestone, there was no significant difference in spinach biomass among the 0, 1.9, and 19.0 mg Fe/liter treatments, but a significant and severe reduction in biomass was associated with the 190.0 mg Fe/liter treatment (Fig. 4.5F). In soil amended with limestone, spinach biomass was greatest in soil treated with 0 and 1.9 mg Fe/liter, followed by soil with 19.0 mg/liter, and least in soil with 190.0 mg Fe/liter. However, the reduction in biomass at the highest Fe concentration in the pasteurized soil was not as severe as the biomass reduction observed in non-pasteurized soil with the highest Fe concentration (Fig. 4.5F).

Non-pasteurized soil amended with 4.48 t limestone/ha had a higher pre-trial pH than non-pasteurized, non-limed soil (6.81 vs. 5.71, respectively for Fe Trial 1) (Table 4.7). In non-pasteurized soil not amended with limestone, there was a trend of decreasing soil pH with increasing concentration of Fe amendment, except for soil drenched with 19.0 mg Fe/liter. Within each pasteurization treatment, Fe levels were lower in limed vs. non-limed soil (Table

4.7). Soil Fe availability generally increased with increasing concentration of Fe from 0 to 19.0, and from 19.0 to 190.0 mg/liter, but not by the 10-fold increase corresponding to each rate of Fe drench tested. In Trial 1, *F. oxysporum* was not detected in the pre-trial, pasteurized soil. *F. oxysporum* was detected in all of the pre-trial, non-pasteurized soil samples (1,222 to 1,644 CFU/g soil for non-limed and limed soil, respectively). Limestone-amended, non-pasteurized soils had smaller post-trial *F. oxysporum* populations compared to non-limed, non-pasteurized soil, except for soil treated with 190.0 mg Fe/liter (Table 4.7). Of the three seedlings collected for pathogen isolations in Trial 2, the seedling from non-pasteurized soil with symptoms of Fusarium wilt was infected with *F. oxysporu*m. Of the two seedlings collected from pasteurized soil, the seedling that had symptoms of phytotoxicity was not infected with any fungi, and the seedling that had symptoms of Fusarium wilt was infected with *F. oxysporum*.

Discussion

Results of the *in vitro* experiments in this study indicate that there are minimum levels of Fe, Mn, and Zn required by the spinach Fusarium wilt pathogen for normal growth and sporulation. The findings of these experiments were similar to those reported by Woltz and Jones (1971) and others working on fungal micronutrient nutrition (Foster, 1939). For example, Woltz and Jones (1971) found that Mn deficiency reduced mycelial growth of the tomato Fusarium wilt pathogen, but to a lesser extent than Fe and Zn, which supports the results of this study. The lowest concentration of added Mn that they evaluated, 0.06 mg/liter, restored normal growth of the tomato pathogen. In this study, it was necessary to dilute the Mn concentration of the basal medium to 0.0005 mg/liter to identify a concentration >0 that suppressed growth of isolate Fos001 of *F. oxysporum* f. sp. *spinaciae*. This illustrates the truly trace amounts of these

elements required by some organisms. In this study and in the work of Woltz and Jones (1971), restriction of mycelial growth by deficient levels of Zn and Fe was most severe with the control treatment of no added micronutrient, and the restriction did not disappear until adding 0.2500 mg/liter of each micronutrient. Similarly, in this and the work of Woltz and Jones, the greatest reduction in mycelial growth was observed with Zn deficiency compared to Fe and Mn deficiencies. Other similarities to the results of Woltz and Jones (1971) were also observed in this study with the effects of the three micronutrients on fungal sporulation. A greater reduction in conidial production was observed with Zn and Mn deficiency compared to Fe deficiency. Furthermore, sporulation levels were restored at lower concentrations of Mn and Fe compared to Zn, corroborating the findings of Bhatnagar and Prasad (1968).

The precise micronutrient concentrations at which growth of fungal cultures is restored in such *in vitro* experiments can vary. Kalyanasundaram and Saraswathi-Devi (1955), for example, noted suppression in fungal biomass production of the cotton Fusarium wilt pathogen (*F. oxysporum* f. sp. *vasinfectum*) at Zn concentrations <0.008 mg/liter of basal liquid medium, which was lower than the results of this study. Steinberg (1950b) identified optimal Fe, Zn, and Mn concentrations of 0.4, 0.4, and 0.2 ppm (mg/liter), respectively, for growth of *F. oxysporum* f. sp. *lycopersici*. These concentrations are higher, particularly for Fe and Mn, compared to the minimum concentrations identified in this spinach study for restoring growth of an isolate of *F. oxysporum* f. sp. *spinaciae*. Such discrepancies could reflect actual divergence in nutritional requirements of the isolates or formae speciales evaluated, and/or differences in formulation, preparation, or component purity of basal nutrient media, incubation conditions, and/or experiment durations. In many of the early seminal studies on fungal nutrition, great care was taken to purify chemical ingredients, glassware, water, and inoculum of heavy metal impurities

through procedures such as autoclaving stock solutions with calcium carbonate to precipitate contaminants, distilling and recrystallizing inorganic salts, and successive transfers of inoculum in liquid media deficient in the micronutrient in question to use up residual reserves (Steinberg, 1950b; Thind and Madan, 1977). Such impurities are the reason most synthetic media used to culture filamentous fungi do not include the heavy metal elements explicitly; historically, these nutrients were supplied in sufficient minute amounts in other media components, leading to confusion about whether or not they were, in fact, required for growth (Foster, 1939). Apart from the use of laboratory grade chemicals, deionized water, and an acid-rinse of glassware provided by an industrial dishwasher, additional purification measures were not pursued in this study, in part because preliminary experiments showed that existing impurities were not of sufficient scale to mask the effects of the selected range of micronutrient concentrations on fungal growth parameters.

A more relevant issue biologically is the relationship of these *in vitro* results to spinach rhizosphere ecology in the presence of the Fusarium wilt pathogen. The *in vitro* experiments were designed to remove as many confounding variables as possible for predicting pathogen growth, with the exception of the concentration in a liquid medium of the specific micronutrients of interest. In reality, numerous factors influence availability of Fe, Mn, and Zn in soil, especially in the rhizosphere where micronutrients interact with root exudates and microbial affiliates. The processes governing nutrient availability in the soil include dissolution from primary or secondary minerals and precipitation back into mineral form; adsorption/desorption from the soil cation exchange; immobilization/mineralization by soil biota; and uptake or release by plant roots (Havlin et al., 1999). Soil nutrient analyses are based on various techniques for extracting nutrients, such as use of the chelating agent DTPA for detecting and quantifying

heavy metal elements. The results are intended to estimate not just the concentration of the nutrient in the soil solution, which can fluctuate rapidly as a result of the abovementioned processes, but how much of the nutrient is available to plant roots, which can access some of the more labile pools of nutrients by manipulating the chemistry of the rhizosphere (Kabata-Pendias, 2001). Extensive correlation studies between extraction techniques and the nutritional status of plants have determined which methods are most accurate for assessing soil nutrient availability (Sims and Johnson, 1991).

However, what constitutes availability for plants may be different than for soil microbes (Woltz and Jones, 1981). For most plants, values >1.0 and 1.5 ppm of Zn and Mn, respectively, obtained via DTPA extraction are considered sufficient. Recommended levels of Fe typically are not provided in soil nutrient analyses because existing extraction protocols do not identify Fe-deficient soils reliably (Hansen et al., 2006; Marx et al., 1996). There is no equivalent to the soil nutrient analysis for soil microbes, but if soil test results are used to approximate bioavailability of nutrients for microbial as well as plant growth, then most soils, including the soil used in the greenhouse experiments in this study, supply more than the <0.5 mg/liter required in the *in vitro* trials to restore growth of the spinach Fusarium wilt pathogen. However, results of soil nutrient analyses do not represent the concentrations of nutrients typically found in the soil pore water. The analyses take into account the capacity of root systems to solubilize nutrients, and the capacity of soils to replace ions taken up by roots with reserves on the cation exchange (Brady and Weil, 1999). Typical soil solution concentrations range from 0.002 to 0.070 mg/liter for Zn, and 0.01 to 1.00 mg/liter for Mn (Havlin et al., 1999). These concentrations fall within the ranges used in the *in vitro* experiments in this study, which suggests it might be possible for isolates of *F. oxysporum* to encounter micronutrient deficiencies in the rhizosphere of spinach grown in the

maritime Pacific Northwest, especially when facing competition from plants and other microbes for these sparingly soluble trace elements.

The pigments produced by *F. oxysporum* f. sp. *spinaciae* in increasing amounts with increasing Fe, Mn, or Zn concentration during the *in vitro* trials were most likely rubrofusarins, secondary metabolites derived from napthoquinones that may play a role in pathogenesis (Baker and Tatum, 1983; Wolf, 1955), although this was not verified in this study. The pattern of increasing pigmentation observed with increaseing Fe, Mn, or Zn concentration was similar to that reported by Woltz and Jones (1968), who rated pigmentation of *F. oxysporum* f. sp. *lycopersici* cultures on a scale of 0-to-5 (0 = colorless, 1 = light pink, and 5 = dark purple). They found that high *in vitro* concentrations of Mn, Zn, and Fe in a liquid medium led to increasingly dark red to purple pigmentation by the tomato Fusarium wilt pathogen. According to Garraway (1984), fungi that are sensitive to Mn deficiency typically exhibit abnormal growth, with shortened, highly branched hyphae. This supports the observations in this study of abnormal hypahl morphology in cultures of *F. oxysporum* f. sp. *spinaciae* at the lower concentrations of Mn tested.

The greenhouse experiments in this study were conducted to determine whether suppression of spinach Fusarium wilt afforded by amendment of soils with limestone could be negated, in whole or in part, by adding Fe, Mn, or Zn in chelated form to the soil as a drench. The hypothesis addressed was whether the mechanism(s) underlying the partial reduction in Fusarium wilt severity achieved with soil limestone amendment is (are) related to depriving the pathogen of these micronutrients. In similar experiments, Jones and Woltz (1970) established a link between induced deficiencies of these acid micronutrients via soil pH elevation, and reduced inoculum potential of the tomato Fusarium wilt pathogen. Sandy Florida soils, with or without

limestone amendment, were treated with a range of micronutrient concentrations in various combinations. The micronutrients were supplied as lignosulfonates (non-phytotoxic complexing agents), and foliar micronutrient sprays were applied to tomato plants to avoid plant deficiencies in non-treated control soils. They did not note any plant toxicity symptoms, even at the highest rate of micronutrient amendment (400 mg/kg soil). The addition of Mn and Zn, or Fe and Zn to soils appeared to reverse the suppressive effect of limestone amendment on tomato Fusarium wilt. Similar results were achieved by Duffy and Defago (1999), who noted an increase in severity of tomato crown and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* as the solution concentration of Fe, Mn, and Zn was increased from 0 to 100 mg/liter in a hydroponic production system.

The results of the greenhouse experiments in this study present a more complex picture of the interactions between soil micronutrient availability, the spinach Fusarium wilt pathogen, and the host plant compared to other studies. In the first Mn trial, treatment of soil with 19.0 mg Mn/liter soil enhanced Fusarium wilt severity only in soil amended with limestone. However, the suppressive effect of limestone on the disease was not negated completely by the addition of Mn. In soils not amended with limestone, wilt reached maximum severity by 28 DAP, regardless of the micronutrient treatment. Adding 19.0 mg Mn/liter did not reduce plant biomass significantly in either the pasteurized or non-pasteurized soil, so it is unlikely that the increase in disease severity was due to host plant resistance being compromised by Mn toxicity. Amendment with limestone did reduce the availability of Mn at all rates of Mn applied compared to non-limed soil, reflecting the precipitation of Mn in the form of insoluble oxides and hydroxides at higher pH. The post-trial *F. oxysporum* population was reduced in soil treated with 190.0 mg Mn/liter compared to lower concentrations in non-pasteurized, non-limed soil in which more of the added

micronutrient was available. This could reflect toxic effects of the element on the pathogen, despite apparent enhancement of disease potential at the highest Mn concentration. Mn in soil solution can, under certain conditions, produce reactive oxygen species that can be damaging to soil microbes, and an excess of any of these three micronutrients can cause an imbalance in the uptake of other micronutrients (Momma et al., 2011).

In the second Mn trial, unlike the first trial, a significant reduction in Fusarium wilt severity occurred when soil was drenched with 1.9 and 19.0 mg Mn/liter soil, suggesting that Mn level in the control soil was in the deficiency range for spinach, enhancing susceptibility to Fusarium wilt. Mn, as stated previously, is involved in plant defense responses, particularly those depending on the phenol biosynthesis pathway. For diseases such as rice blast (*Magnaporthe grisea*) and take-all of wheat (*Gaeumannomyces graminis* var. *tritici*), Mn deficiency is linked to enhanced disease severity (Graham, 1983). However, the theory of Mn-deficient spinach plants accounting for the greater Fusarium wilt severity observed in soil with no added Mn in Trial 2 is difficult to reconcile with the fact that Mn levels in the post-trial soils that received no Mn were above the plant sufficiency level of 1.5 mg Mn/kg (Marx et al., 1995).

In the first Zn trial, the effects of limestone amendment and Zn concentration were similar to those of the first Mn trial, with an increase in Fusarium wilt severity observed with increasing concentration of added Zn. However, the disease increase from 0 to 19.0 mg Zn/liter, the increment of most biological significance in that trial, was not significant statistically, perhaps due to very severe Fusarium wilt in the soil not amended with limestone. In the second Zn trial, there were fewer significant effects of the Zn treatments on Fusarium wilt severity and spinach biomass except for the phytotoxic highest rate of Zn drench. In the third Zn trial carried out to assess disparate results of the first two trials, drenching soil with 19.0 mg Zn/liter

significantly enhanced Fusarium wilt severity, as observed in the first Zn trial. There was no difference in spinach biomass among pasteurized soils drenched with 0, 1.9, and 19.0 mg Zn/liter, but a decrease in spinach biomass occurred from 0 to 1.9 and 19.0 mg Zn/liter treatments in the non-pasteurized soils. This suggested that the negative effect of Zn on growth of spinach could have been due to enhanced Fusarium wilt inoculum potential with the added Zn. As in the Mn trials, there were slight differences in the effects of limestone on Fusarium wilt severity between Trials 1 and 3. In the first Zn trial, it was in the non-pasteurized, limestone-amended soil that addition of Zn at 19.0 or 190 mg/liter soil exacerbated Fusarium wilt. In the third trial, increased Fusarium wilt severity with increasing Zn concentration was observed only in the non-limed soil. It is not evident why this occurred. In Trial 1 for both Zn and Mn, the inoculum potential in the non-limed, highly conducive soil for Fusarium wilt was so great that any potential effect of increasing micronutrient concentration was overwhelmed by the severity of wilt at all micronutrient drench rates. However, in the limed soil, Fusarium wilt severity was reduced to the point that comparatively smaller effects of micronutrient concentrations manifested. In Trial 2 for Mn and Trial 3 for Zn, Fusarium wilt severity was not as great in the non-limed soils compared to Trial 1 of each micronutrient, which may have revealed the wilt-enhancing effects of increasing micronutrient concentration.

The first Fe trial lacked significant effects of Fe concentrations on Fusarium wilt severity, although a trend toward increased wilt severity at ≥1.9 mg Fe/liter soil was observed in non-limed soils. A corresponding reduction in plant biomass with 1.9 mg Fe/liter non-limed soils, but not in limed or pasteurized soils, suggested that Fe could be enhancing pathogen virulence at this low level. However, the more severe wilt observed in soil drenched with 19.0 and, especially, 190.0 mg Fe/liter was confounded with phytotoxic effects of the two highest Fe drench rates. In

the second Fe trial, a significant increase in wilt severity was again found at the highest, phytotoxic level of Fe drench. The results also suggested that drenching soil with 1.9 mg Fe/liter soil may reduce Fusarium wilt severity, but severity of wilt increased with a further increase in soil drench rate to 19.0 mg Fe/liter in limed soil.

The results of these experiments indicate that, while Fe, Mn, and Zn may play a role in Fusarium wilt development, manipulating soil chemistry effectively to deprive the pathogen, but not the plant, of these trace elements can be difficult, given the complexity of forces governing micronutrient availability in the rhizosphere. The potential increase in resistance of spinach plants to Fusarium wilt with very low concentrations of micronutrient amendment, observed in some iterations of the trials, is similar to effects reported on Fusarium wilts of other crops. Fernández-Falcón et al. (2004) noted a reduction in Fusarium wilt infection of banana rhizomes with increasing concentration of Zn amendment, and attributed this to the role of Zn in production indole acetic acid (IAA) and the formation of tyloses, which block pathogen advance in the xylem. Zn amendment enhanced biocontrol activity of *Pseudomonas fluorescens* against Fusarium wilt of chickpea, presumably by decreasing fusaric acid production by the pathogen, *F. oxysporum* f. sp. *ciceri* (Saikia et al., 2009). Kalyanasundaram and Saraswathi-Devi (1955) determined that Zn is required for production of fusaric acids by *F. oxysporum*, but that fusaric acid production is repressed at high Zn concentrations. Fusaric acid has been shown to suppress production by fluorescent pseudomonads of diacetylphloroglucinol (DAPG), one of the compounds implicated in the biocontrol activity of this group of soil antagonists (Voisard et al., 1994). Contrasting results were reported in studies of Fusarium crown and root rot of tomato, which was exacerbated by Zn amendment (Duffy and Défago, 1997); and in another study, by Fe, Mn, and Zn amendment (Duffy and Défago, 1999), with no evidence of micronutrient-

mediated enhancement of plant resistance. These studies illustrate the intricacy of the interactions among nutrients, plant pathogens, the broader soil microbial community, and host plants, and the difficulty of evaluating the effects of any single factor on Fusarium wilt development in soil-based experiments.

It is thus noteworthy, given the nearly impenetrable tangle of competing influences in the soil environment, that significant increases in spinach Fusarium wilt severity were observed with increasing micronutrient amendment in this study. The findings of this study support the hypothesis that a reduction in micronutrient availability is a contributing factor in the suppression of spinach Fusarium wilt achieved with soil limestone amendment. However, as some of the variability observed in repeat trials indicated, there are other factors involved, such as the effects of these micronutrients on host plant defenses and on other soilborne microbes interacting with the pathogen and host. While the *in vitro* experiments confirmed that there are minimum Fe, Mn, and Zn requirements for normal growth and sporulation of *F. oxysporum* f. sp. *spinaciae*, attempts to evaluate subsequent effects of these *in vitro* micronutrient treatments on virulence of the pathogen on spinach were less successful. Experiments were attempted in which conidia produced under different micronutrient concentrations were harvested and used to inoculate spinach seedlings in a peat-based potting mix. Results were inconclusive (*data not shown*), perhaps because the residual effects of nutrient deprivation on inoculum potential were quickly reversed by the micro-availability of these nutrients in the potting mix to which the inoculum was added. Further experiments in more controlled and defined substrates (rock-wool, for example), might allow the effects of micronutrient availability on pathogen virulence to be explored in the absence of confounding factors.

There is sufficient evidence that micronutrients can exacerbate spinach Fusarium wilt to consider practical steps that can be taken by spinach seed growers, e.g., foliar applications of micronutrients when soil nutrient analyses indicate potential crop deficiencies, or finding alternatives to commonly-used fertilizers such as mono-ammonium phosphate which are known to contain Zn impurities (U.S. Environmental Protection Agency, 1999). Taking steps to reduce access of the spinach Fusarium wilt pathogen to these micronutrients in spinach seed crops will not eradicate the disease, but given the intractable nature of the pathogen and the conduciveness of acid, maritime PNW soils to this disease, all available tools and knowledge should be deployed to erode inoculum potential.

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Table 4.1. Probability values from the analyses of variance (ANOVAs) for the effects of manganese (Mn), zinc (Zn), and iron (Fe) concentration in a liquid medium on biomass production, sporulation, and spore germination of *Fusarium oxysporum* f. sp. *spinaciae* evaluated *in vitro*

Micronutrient, trial, and ANOVA factor[a]	Fungal biomass (g)[b]	Conidial production (conidia/ml)[c]	Conidial germination (%)[d]
Mn			
Trial 1			
Concentration	<0.0001	<0.0001	0.3419
R^2	0.7948	0.8021	0.2663
CV	7.54	28.73	54.77
Transformation	-	Rank	-
Trial 2			
Concentration	<0.0001	<0.0001	<0.0001
R^2	0.8176	0.8139	0.7146
CV	7.25	27.71	33.91
Transformation	-	Rank	Rank
Zn			
Trial 1			
Concentration	<0.0001	<0.0001	0.5175
R^2	0.9430	0.8408	0.2034
CV	13.51	7.29	44.33
Transformation	Rank	Log	-
Trial 2			
Concentration	<0.0001	0.0002	0.0028
R^2	0.8694	0.8175	0.7202
CV	0.3590	26.16	31.81
Transformation	Sq. root	Rank	Rank
Fe			
Trial 1			
Concentration	<0.0001	0.0017	0.0524
R^2	0.8700	0.6209	0.4348
CV	14.89	35.87	28.82
Transformation	-	Rank	-
Trial 2			
Concentration	<0.0001	0.0008	0.2816
R^2	0.7281	0.6367	0.2777
CV	29.63	46.05	37.60
Transformation	Rank	-	-

[a] Each experiment was a completely randomized design with micronutrient (Mn, Zn, or Fe) concentration of a liquid basal medium inoculated with an isolate of *F. oxysporum* f. sp. *spinaciae*. Seven micronutrient concentrations from 0 to 2 mg/liter were evaluated using a four-fold dilution series. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = raw data subjected to log, square root (sq. root), or non-parametric (rank) transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA. - = no transformation needed.

[b] Biomass of the pathogen was measured after 7 days of incubation by removing the liquid from the inoculated medium using a vacuum pump and Buchner funnel, drying the fungal biomass on filter paper at 65°C for approximately 4 h, weighing the filter paper and biomass, and subtracting the weight of the filter paper.

[c] Spore production was measured after 7 days of incubation by filtering the liquid medium through four layers of cheesecloth to remove mycelium, and counting the number of microconidia/ml of the filtrate using a hemocytometer.

[d] Spore germination (% germinated spores/ml) was determined by counting the number of germinated conidia, and dividing by the total number of conidia observed on the hemocytometer.

Table 4.2. Probability values from the analyses of variance (ANOVAs) for the effects of limestone and manganese (Mn) amendment of soil naturally infested with *Fusarium oxysporum* f. sp. *spinaciae* on spinach Fusarium wilt severity and dried plant biomass in pasteurized and non-pasteurized soils in greenhouse experiments

	Non-pasteurized soil[b]					Pasteurized soil
Trial and ANOVA factor[a]	**21 DAP[c]**	**28 DAP**	**35 DAP**	**AUDPC**	**Spinach biomass (g/pot)[d]**	**Spinach biomass (g/pot)**
Trial 1						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.1582
Mn concentration	0.1957	0.2493	0.3321	0.0067*	0.0550	0.1240
Limestone-by-Mn	0.3251	0.0373*	0.2719	0.0167*	0.4960	0.9767
R^2	0.7591	0.8754	0.8238	0.8629	0.8646	0.3895
CV	29.00	20.60	23.30	22.13	16.70	10.85
Transformation	Rank	Rank	Rank	Rank	Arcsine	Arcsine
Trial 2						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.8320
Mn concentration	0.0284*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Limestone-by-Mn	0.3185	0.2458	0.1671	0.1880	0.0305*	0.0155*
R^2	0.8493	0.8876	0.9156	0.8933	0.9154	0.7255
CV	23.46	20.40	17.62	19.88	18.11	32.66
Transformation	Rank	Rank	Rank	Rank	Rank	Rank

[a] Each experiment was a randomized complete block design with two factors: 1) amendment of soils with the equivalent of 0 or 4.48 t limestone/ha, and 2) a soil drench of 0, 19, or 190 mg Mn/liter soil (Trial 1), or 0, 1.9, 19.0, or 190/0 mg Mn/liter (Trial 2). R^2 = coefficient of determination. CV = coefficient of variance. Transformation = when necessary, raw data were subjected to arcsine or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA.

[b] Each limestone-by-Mn treatment combination was evaluated in both pasteurized (56°C for 1 h) and non-pasteurized soil to compare the effects of these treatments with and without Fusarium wilt. Plants in the pasteurized soil had negligible Fusarium wilt symptoms, so only the biomass data are included in this table for plants grown in the pasteurized soil.

[c] For each of five replications/treatment combination, up to seven spinach plants/pot were rated for severity of Fusarium wilt 21, 28, and 35 days after planting (DAP) using a 0-to-5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt). Ratings were converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Area under the disease progress curve (AUDPC) was calculated based on the results of these ratings.

[d] Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the plants (g/pot).

Table 4.3. Effects of soil pasteurization, limestone amendment, and manganese (Mn) concentration on soil pH, available Mn, and *Fusarium oxysporum* population in greenhouse experiments evaluating the role of Mn in limestone-mediated suppression of spinach Fusarium wilt

	Trial 1			Trial 2		
Factor[a]	**Soil pH[b]**	**Soil Mn (mg/kg)[c]**	***F. oxysporum* (CFU/g soil)[d]**	**Soil pH[b]**	**Soil Mn (mg/kg)[c]**	***F. oxysporum* (CFU/g)[d]**
Pasteurized soil						
4.48 t limestone/ha	Pre-trial: 6.86		Pre-trial: 0	Pre-trial: 6.92 ± 0.12		Pre-trial: 0
Mn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	6.66	2.7	0	6.90 ± 0.04	3.6 ± 0.8	1,022 ± 989
1.9	-	-	-	6.91 ± 0.06	4.1 ± 0.7	89 ± 89
19.0	6.92	5.4	0	6.97 ± 0.04	5.6 ± 0.9	0
190.0	6.94	74.9	0	6.75 ± 0.05	70.2 ± 4.8	222 ± 135
0 t limestone/ha	Pre-trial: 5.44		Pre-trial: 0	Pre-trial: 5.94 ± 0.16		Pre-trial: 0
Mn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	5.89	9.3	267	5.45 ± 0.08	8.1 ± 1.4	0
1.9	-	-	-	5.48 ± 0.07	7.7 ± 2.0	44 ± 44
19.0	5.68	18.8	0	5.51 ± 0.04	11.1 ± 1.5	333 ± 193
190.0	5.73	178.3	0	5.40 ± 0.10	93.2 ± 14.0	867 ± 867
Non-pasteurized soil						
4.48 t limestone/ha	Pre-trial: 6.48		Pre-trial: 1,800	Pre-trial: 6.81 ± 0.03		Pre-trial: 1644 ± 88
Mn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	6.62	1.5	3,200	6.95 ± 0.07	3.0 ± 0.7	3,422 ± 829
1.9	-	-	-	6.89 ± 0.07	3.5 ± 1.0	3,622 ± 311
19.0	6.88	3.1	2,533	6.87 ± 0.08	4.6 ± 1.5	3,289 ± 693
190.0	6.84	51.1	3,067	6.64 ± 0.03	53.9 ± 10.7	3,844 ± 386
0 t limestone/ha	Pre-trial: 5.67		Pre-trial: 2733	Pre-trial: 5.71 ± 0.03		Pre-trial: 1222 ± 222
Mn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	5.34	3.0	5,800	5.33 ± 0.03	8.9 ± 2.1	4,645 ± 712
1.9	-	-	-	5.46 ± 0.06	9.4 ± 2.0	5,200 ± 677
19.0	5.17	8.5	3,533	5.15 ± 0.04	18.5 ± 3.9	4,067 ± 335
190.0	5.04	154.2	2,600	4.93 ± 0.06	93.2 ± 14.0	3,444 ± 225

[a] Greenhouse experiments were conducted to assess the influence of a range of Mn concentrations on limestone-mediated Fusarium wilt suppression. Each Mn-by-limestone treatment combination was evaluated in both pasteurized and non-pasteurized soil naturally infested with the spinach Fusarium wilt pathogen, *F. oxysporum* f. sp. *spinaciae*, so that the effects of limestone and micronutrient amendment on plant growth could be assessed independently of Fusarium wilt development. Each experiment was set up as a randomized complete block design with five replications and two factors: 1) amendment of soil with the equivalent of 0 or 4.48 t limestone/ha, and 2) a soil drench of 0, 19, or 190 mg Mn/liter soil (Trial 1) or 0, 1.9, 19.0, or 190.0 mg Mn/liter soil (Trial 2). Numbers for Trial 2 represent the mean ± standard error of three replications. Numbers without standard errors represent single, non-replicated assessments.

[b] Soil pH was measured using a 1:1 soil:deionized water protocol on soil samples collected just prior to planting (pre-trial) or after completion of the trial (post-trial).

[c] Soil samples were collected upon completion of the trial and sent to Soiltest Farm Consultants, Inc. in Moses Lake, WA for determination of Mn availability (mg/kg soil).

[d] Population of *F. oxysporum*, including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae* on Komada's agar medium (Komada, 1975), was quantified for soil samples collected just prior to and at the completion of each experiment, by dilution plating soil on the agar medium (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after planting.

Table 4.4. Probability values from the analyses of variance (ANOVAs) for the effects of limestone and zinc (Zn) amendment of soil naturally infested with *Fusarium oxysporum* f. sp. *spinaciae* on spinach Fusarium wilt severity and dried plant biomass in pasteurized and non-pasteurized soils in greenhouse experiments

	Non-pasteurized soil[b]					Pasteurized soil
Trial and ANOVA factor[a]	**21 DAP[c]**	**28 DAP**	**35 DAP**	**AUDPC**	**Spinach biomass (g/pot)[d]**	**Spinach biomass (g/pot)**
Trial 1						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.5438
Zn concentration	0.0022*	0.0350*	0.0032*	0.0010*	<0.0001*	0.0001*
Limestone-by-Zn	0.6883	0.3969	0.0231*	0.5075	0.0894	0.9370
R^2	0.8127	0.7938	0.8974	0.8263	0.7792	0.6263
CV	25.71	26.11	17.84	24.91	27.92	25.88
Transformation	Rank	Rank	Rank	Rank	Rank	-
Trial 2						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001	<0.0001*	<0.0001*
Zn concentration	0.3838	0.1846	0.3698	0.5643	0.0003*	<0.0001*
Limestone-by-Zn	0.6211	0.2783	0.0175*	0.1668	0.3400	0.0815
R^2	0.6821	0.8138	0.8388	0.8120	0.8857	0.9039
CV	34.63	26.40	23.97	26.74	20.57	2.56
Transformation	Rank	Rank	Rank	Rank	Rank	Log
Trial 3						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0051*
Zn concentration	0.0026*	<0.0001*	0.0006*	0.0005*	<0.0001*	0.0001*
Limestone-by-Zn	0.4231	0.1544	0.1473	0.2444	<0.0001*	0.2207
R^2	0.7846	0.8265	0.8418	0.8417	0.8800	0.7054
CV	28.28	25.44	23.61	24.54	21.08	17.02
Transformation	Rank	Rank	Rank	Rank	Rank	-

[a] Each experiment was a randomized complete block design with two factors: 1) amendment of soils with the equivalent of 0 or 4.48 t/ha limestone, and 2) a soil drench of 0, 19, or 190 mg Zn/liter soil (Trial 1) or 0, 1.9, 19.0, or 190.0 mg Zn/liter (Trials 2 and 3). R^2 = coefficient of determination. CV = coefficient of variance. Transformation = when necessary, raw data were subjected to log or non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA.

[b] Each limestone-by-Zn treatment combination was evaluated in both pasteurized (56°C for 1 h) and non-pasteurized soil to compare the effects of these treatments with and without Fusarium wilt. Plants in the pasteurized soil had negligible Fusarium wilt symptoms, so only the biomass data are included in this table for plants grown in the pasteurized soil.

[c] For each of five replications/treatment combination, up to seven spinach plants/pot were rated for severity of Fusarium wilt 21, 28, and 35 days after planting (DAP) using a 0-to-5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt). Ratings were converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Area under the disease progress curve (AUDPC) was calculated based on the results of these ratings.

[d] Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the plants (g/pot).

Table 4.5. Effects of soil pasteurization, limestone amendment, and zinc (Zn) concentration on soil pH, available Zn, and *Fusarium oxysporum* population in greenhouse experiments evaluating the role of Zn in limestone-mediated suppression of spinach Fusarium wilt

	Trial 1			Trial 3		
Factor[a]	**Soil pH**[b]	**Soil Zn (mg/kg)**[c]	***F. oxysporum* (CFU/g soil)**[d]	**Soil pH**[b]	**Soil Zn (mg/kg)**[c]	***F. oxysporum* (CFU/g soil)**[d]
Pasteurized soil						
4.48 t limestone/ha	Pre-trial:		Pre-trial:	Pre-trial:		Pre-trial:
	6.86		0	6.92 ± 0.12		0
Zn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	6.56	2.2	0	7.06 ± 0.19	1.2 ± 0.1	0
1.9	-	-		6.97 ± 0.04	1.6 ± 0.1	0
19.0	6.71	15.7	0	6.99 ± 0.10	8.0 ± 0.3	133 ± 133
190.0	6.42	131.3	0	6.75 ± 0.11	51.9 ± 0.0	4,356 ± 1,927
0 t limestone/ha	Pre-trial:		Pre-trial:	Pre-trial:		Pre-trial:
	5.44		0	5.94 ± 0.16		0
Zn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	5.96	5.6	0	5.57 ± 0.10	3.1 ± 0.2	178 ± 146
1.9	-	-	-	5.66 ± 0.14	3.9 ± 0.4	1,200 ± 1,200
19.0	5.56	23.1	0	5.56 ± 0.17	13.0 ± 2.6	4,645 ± 4,611
190.0	5.66	130.5	0	5.52 ± 0.11	51.8 ± 0.1	2,200 ± 1,572
Non-pasteurized soil						
4.48 t limestone/ha	Pre-trial:		Pre-trial:	Pre-trial:		Pre-trial:
	6.48		1,800	6.81 ± 0.03		1,644 ± 88
Zn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	6.95	1.6	2,267	6.95 ± 0.03	1.4 ± 0.3	4,577 ± 878
1.9	-	-	-	6.83 ± 0.08	1.5 ± 0.2	3,689 ± 880
19.0	6.98	18.1	2,200	6.73 ± 0.08	9.4 ± 1.3	3,200 ± 575
190.0	6.51	131.1	4,467	6.28 ± 0.09	51.8 ± 0.1	2,467 ± 102
0 t limestone/ha	Pre-trial:		Pre-trial:	Pre-trial:		Pre-trial:
	5.67		2,733	5.71 ± 0.03		1,222 ± 222
Zn (mg/liter)	Post-trial:		Post-trial:	Post-trial:		Post-trial:
0	5.49	4.4	4,800	5.17 ± 0.06	3.1 ± 0.1	4,533 ± 804
1.9	-	-	-	5.28 ± 0.17	3.8 ± 0.5	3,867 ± 306
19.0	5.18	32.8	5,333	5.09 ± 0.05	15.4 ± 0.4	3,667 ± 529
190.0	4.68	129.7	3,867	4.57 ± 0.06	51.7 ± 0.0	2,335 ± 546

[a] Greenhouse experiments were conducted to assess the influence of a range of Zn soil concentrations on limestone-mediated Fusarium wilt suppression. Each Zn-by-limestone treatment combination was evaluated in both pasteurized and non-pasteurized soil naturally infested with the spinach Fusarium wilt pathogen, *F. oxysporum* f. sp. *spinaciae*, so that the effects of limestone and micronutrient amendment on plant growth could be assessed independently of Fusarium wilt development. Each experiment was set up as a randomized complete block design with five replications and two factors: 1) amendment of soils with the equivalent of 0 or 4.48 t limestone/ha, and 2) a soil drench of 0, 19, or 190 mg Zn/l soil (Trial 1) or 0, 1.9, 19.0, or 190.0 mg Zn/liter soil (Trial 2). Numbers for trial 3 represent the mean ± standard error of three replications. Numbers without standard errors represent single, non-replicated assessments.

[b] Soil pH was measured using a 1:1 soil:deionized water protocol on soil samples collected just prior to planting (pre-trial) or after completion of the trial (post-trial).

[c] Soil samples were collected upon completion of the trial and sent to Soiltest Farm Consultants, Inc. in Moses Lake, WA for determination of Zn availability (mg/kg soil).

[d] Population of *F. oxysporum*, including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae* on Komada's agar medium (Komada, 1975), was quantified for soil samples collected just prior to and at the completion of each experiment, by dilution plating soil on the agar medium (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after planting.

Table 4.6. Probability values from the analyses of variance (ANOVAs) for the effects of limestone and iron (Fe) amendment of soils naturally infested with *Fusarium oxysporum* f. sp. *spinaciae* on spinach Fusarium wilt severity and dried plant biomass in pasteurized and non-pasteurized soil in greenhouse experiments

	Non-pasteurized soil[b]					Pasteurized soil
Trial and ANOVA factor[a]	**21 DAP[c]**	**28 DAP**	**35 DAP**	**AUDPC**	**Spinach biomass (g/pot)[d]**	**Spinach biomass (g/pot)**
Trial 1						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.1319
Fe concentration	0.7466	0.6178	0.2757	0.5855	<0.0001*	<0.0001*
Limestone-by-Fe	0.9390	0.5045	0.4597	0.7389	0.1880	0.5194
R^2	0.6258	0.6559	0.7069	0.6457	0.8660	0.8004
CV	36.33	35.36	32.52	35.94	22.22	23.19
Transformation	Rank	Rank	Rank	Rank	Rank	-
Trial 2						
Limestone	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0014*
Fe concentration	0.4579	0.0263*	0.0314*	0.0995	0.0111*	<0.0001*
Limestone-by-Fe	0.1446	0.1095	0.0692	0.7215	0.3616	<0.0001*
R^2	0.7054	0.7167	0.6879	0.7053	0.6076	0.7974
CV	33.11	32.09	31.78	33.20	38.12	28.35
Transformation	Rank	Rank	Rank	Rank	Rank	-

[a] Each experiment was a randomized complete block design with two factors: 1) amendment of soils with the equivalent of 0 or 4.48 t limestone/ha limestone, and 2) a soil drench of 0, 19, or 190 mg Fe/liter soil. R^2 = coefficient of determination. CV = coefficient of variance. Transformation = when necessary, raw data were subjected to non-parametric rank transformation due to heterogeneous variances and/or non-normal distribution of residuals in the ANOVA.

[b] Each limestone-by-Fe treatment combination was evaluated in both pasteurized (56°C for 1 h) and non-pasteurized soils to compare the effects of these treatments with and without Fusarium wilt. Plants in the pasteurized soil had negligible Fusarium wilt symptoms, so only the biomass data are included in this table for plants grown in the pasteurized soil.

[c] For each of five replications/treatment combination, up to seven spinach plants/pot were rated for severity of Fusarium wilt 21, 28, and 35 days after planting (DAP) using a 0-to-5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt). Ratings were converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Area under the disease progress curve (AUDPC) was calculated based on the results of these ratings.

[d] Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the plants (g/pot).

Table 4.7. Effects of soil pasteurization, limestone amendment, and iron (Fe) concentration on soil pH, available Fe, and *Fusarium oxysporum* population in a greenhouse experiment evaluating the role of Fe in limestone-mediated suppression of spinach Fusarium wilt

	Trial 1		
Factor[a]	**Soil pH**[b]	**Soil Fe (mg/kg)**[c]	***F. oxysporum* (CFU/g soil)**[d]
Pasteurized soil			
4.48 t limestone/ha	Pre-trial: 6.92 0.12		Pre-trial: 0
Fe (mg/liter)	Post-trial:		Post-trial:
0	7.01 ± 0.02	41.7 ± 1.8	22 ± 22
1.9	7.01 ± 0.08	40.7 ± 1.5	0
19.0	6.96 ± 0.06	57.3 ± 8.3	0
190.0	6.99 ± 0.06	168.0 ± 7.2	267 ± 267
0 t limestone/ha	Pre-trial: 5.94 ± 0.16		Pre-trial: 0
Fe (mg/liter)	Post-trial:		Post-trial:
0	5.62 ± 0.09	69.0 ± 3.5	2,800 ± 1,408
1.9	5.42 ± 0.10	67.7 ± 4.7	1,756 ± 1,288
19.0	5.45 ± 0.06	82.0 ± 3.5	1,845 ± 1,237
190.0	5.65 ± 0.04	190.3 ± 15.6	2,534 ± 353
Non-pasteurized soil			
4.48 t limestone/ha	Pre-trial: 6.81 ± 0.03		Pre-trial: 1,644 ± 88
Fe (mg/liter)	Post-trial:		Post-trial:
0	6.86 ± 0.11	40.7 ± 0.9	2,222 ± 336
1.9	6.77 ± 0.08	37.3 ± 8.0	2,089 ± 118
19.0	6.76 ± 0.02	48.3 ± 1.2	2,289 ± 370
190.0	6.42 ± 0.17	190.3 ± 7.9	3,689 ± 525
0 t limestone/ha	Pre-trial: 5.71 ± 0.03		Pre-trial: 1222 ± 222
Fe (mg/liter)	Post-trial:		Post-trial:
0	4.98 ± 0.12	73.0 ± 2.3	3,689 ± 541
1.9	4.80 ± 0.09	66.3 ± 2.7	2,933 ± 270
19.0	5.07 ± 0.09	77.3 ± 1.7	2,867 ± 214
190.0	4.49 ± 0.02	241.0 ± 14.7	3,645 ± 647

[a] Greenhouse experiments were conducted to assess the influence of a range of Fe soil concentrations on limestone-mediated Fusarium wilt suppression. Each Fe-by-limestone treatment combination was evaluated in both pasteurized and non-pasteurized soil naturally infested with the spinach Fusarium wilt pathogen, *F. oxysporum* f. sp. *spinacia*e, so that the effects of limestone and micronutrient amendment on plant growth could be assessed independently of Fusarium wilt development. The experiment was a randomized complete block design with five replications and two factors: 1) amendment of soils with the equivalent of 0 or 4.48 t limestone/ha, and 2) a soil drench of 0, 1.9, 19.0, or 190.0 mg Fe/liter soil. Numbers represent the mean ± standard error of three replications.

[b] Soil pH was measured using a 1:1 soil:deionized water protocol on soil samples collected just prior to planting (pre-trial) or after completion of the trial (post-trial).

[c] Soil samples were collected upon completion of the trial and sent to Soiltest Farm Consultants, Inc. in Moses Lake, WA for determination of Fe availability (mg/kg soil).

[d] Population of *F. oxysporum* (including strains of the spinach pathogen, non-pathogenic isolates, and other formae speciales which cannot be distinguished morphologically from *F. oxysporum* f. sp. *spinaciae*) was quantified on

soil samples collected just prior to and at the completion of the experiment, by dilution plating soil on Komada's agar medium (Komada, 1975) (three replicate plates/dilution). Fungal colonies with fluffy, white to pale salmon-pink mycelial morphology typical of *F. oxysporum* growth on this medium were counted 7 and 14 days after planting.

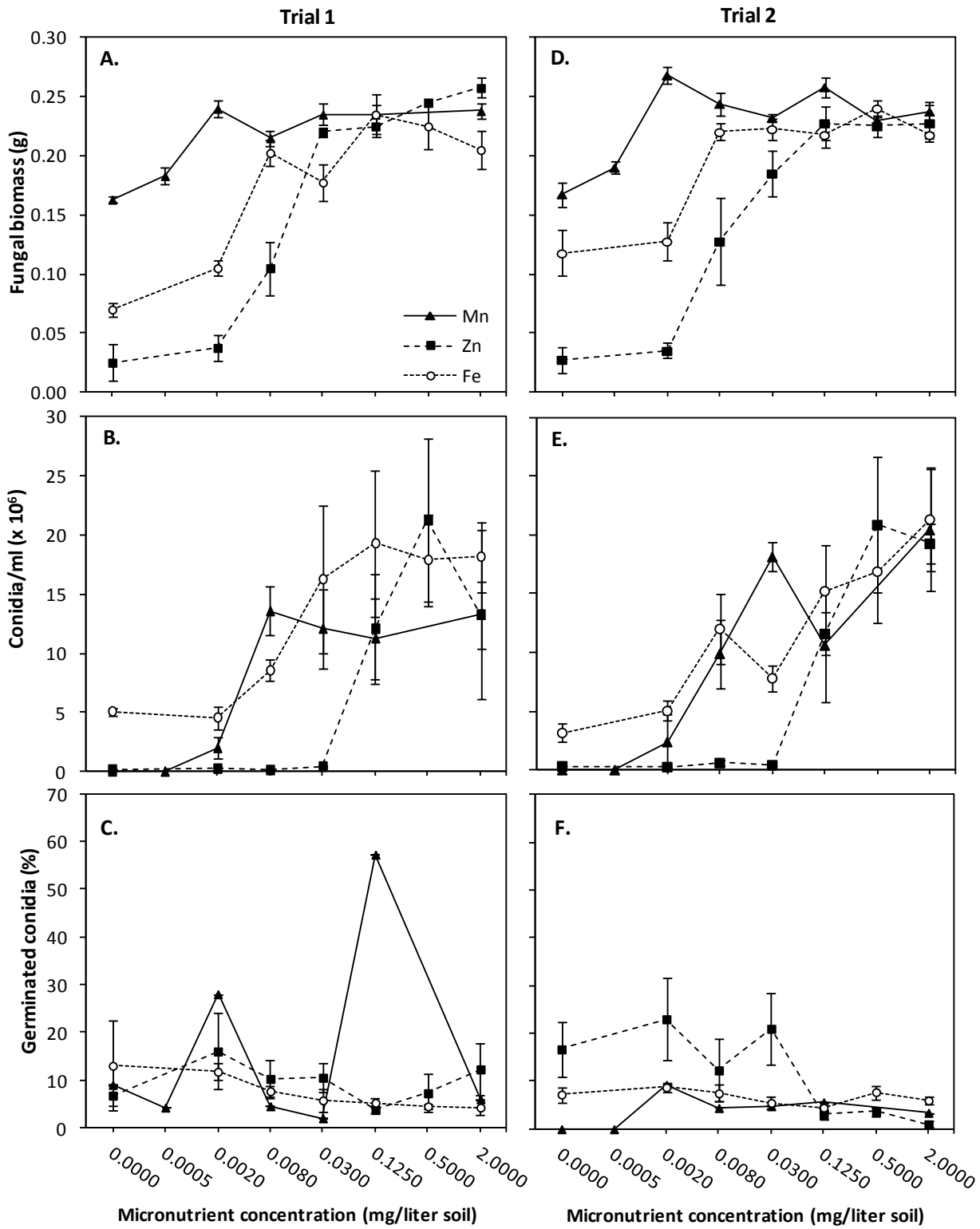

Fig. 4.1. Effects of a range of manganese (Mn), zinc (Zn), and iron (Fe) concentrations in a liquid medium on biomass production and sporulation of *Fusarium oxysporum* f. sp. *spinaciae*

evaluated *in vitro* in two trials (**A** to **C** = Trial 1, **D** to **F** = Trial 2). Each experiment was a completely randomized design with micronutrient (Mn, Zn, or Fe) concentration of a liquid medium inoculated with an isolate of *F. oxysporum* f. sp. *spinaciae*. Seven micronutrient concentrations from 0 to 2 mg/liter, obtained with a four-fold dilution series, were evaluated. Because a low concentration of Mn appeared to be required for normal growth of the fungus, an additional four-fold dilution of Mn (0.0005 mg Mn/liter soil) was evaluated that was not evaluated for Zn and Fe. Conversely, a higher concentration (0.5000 mg Mn/liter) was not evaluated for Mn due to space limitations. Biomass of the pathogen was measured after 7 days of incubation by removing liquid from the inoculated medium, and drying the remaining fungal biomass (**A** and **D**). Spore production was measured by filtering the liquid medium to remove mycelium, and counting the number of microconidia/ml (**B** and **E**). Conidial germination (% germinated spores/ml) (**C** and **F**) was determined by counting the number of germinated conidia, and dividing by the total number of conidia observed on the hemocytometer. Each data point is the mean ± standard error of four replications of that micronutrient concentration.

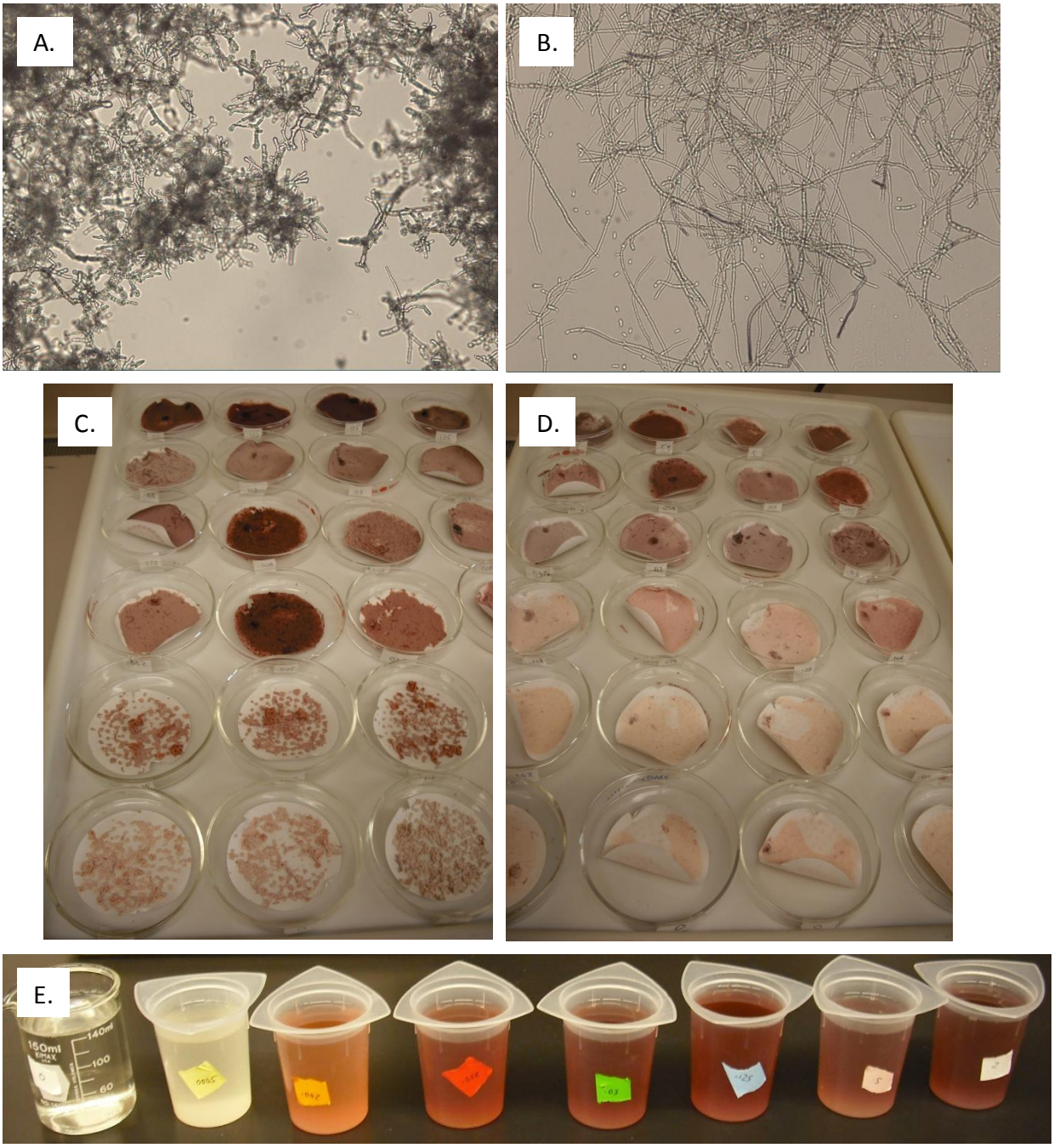

Fig. 4.2. Effects of a range micronutrient concentrations in a liquid medium on hyphal morphology and pigmentation of mycelium and culture filtrates of *Fusarium oxysporum* f. sp. *spinaciae*. Abnormal hyphae of the fungus were observed with no added Mn in the liquid medium (**A**), whereas normal hyphal growth occurred in media amended with ≥0.125 mg Mn/liter (**B**). Variation in mycelial pigmentation was associated with increasing Mn (**C**) and Fe (**D**) concentration, from no added Mn or Fe at the lower end of each photo to 0.125 mg Mn/liter and 0.5 mg Fe/liter at the top of each photo. Similar variation in pigmentation of the culture filtrates was observed with increasing Mn concentration (**E**) (0 to 2 mg Mn/liter from left to

right). Similar patterns of pigmentation of fungal mycelium and filtrates were observed with increasing concentration of each of the three micronutrients (Fe, Mn, and Zn).

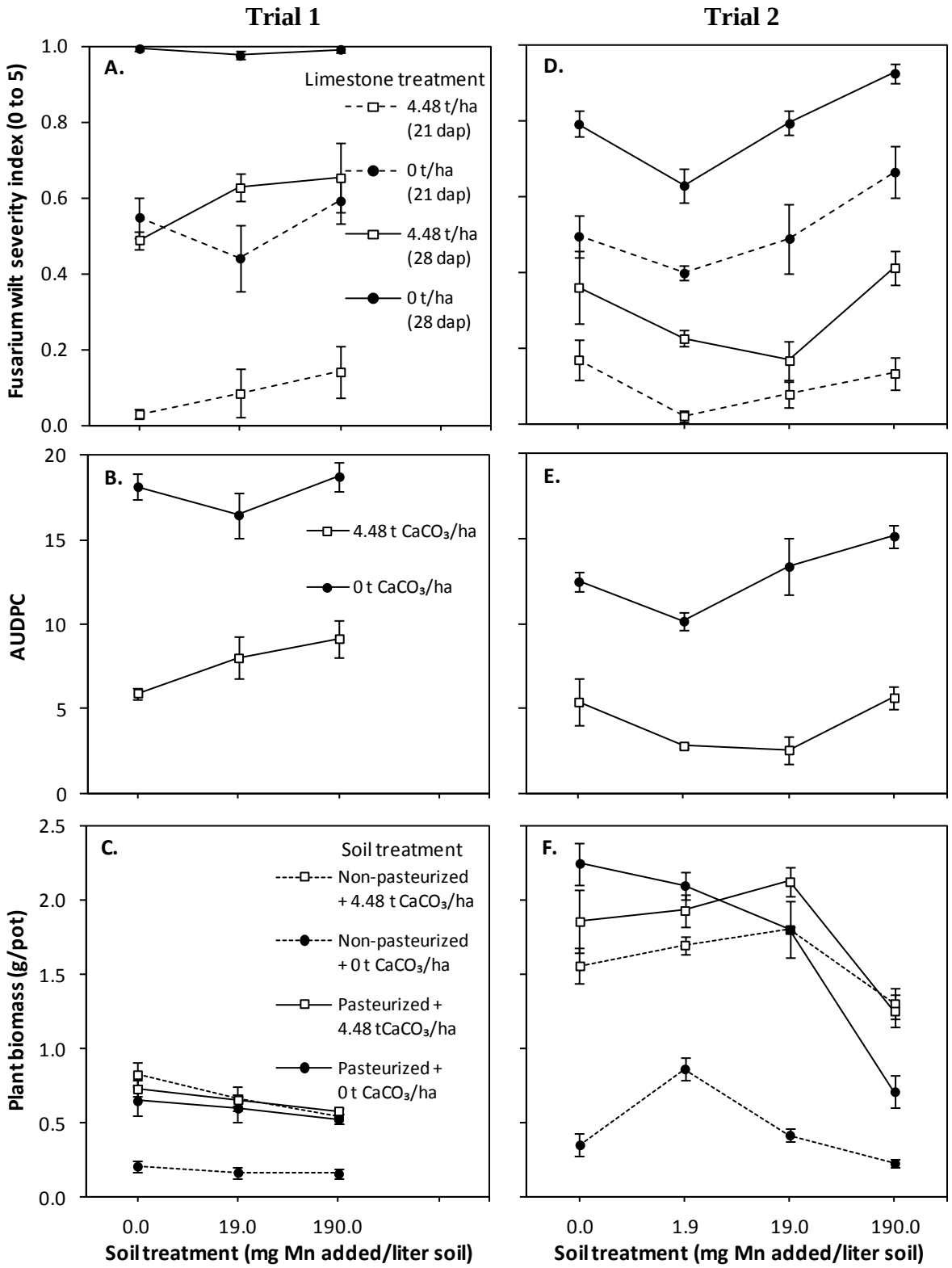

Fig. 4.3. Effects of limestone and manganese (Mn) amendment of soil naturally infested with *Fusarium oxysporum* f. sp. *spinaciae* on spinach Fusarium wilt severity and dried plant biomass

in pasteurized and non-pasteurized soil in a repeated greenhouse experiment (**A** to **C** = Trial 1, **D** to **F** = Trial 2). Each experiment was a randomized complete block design with two factors: 1) amendment of soil with the equivalent of 0 or 4.48 t limestone ($CaCO_3$)/ha, and 2) a soil drench of 0, 19, or 190 mg Mn/liter soil. In the repeat experiments, an additional level of 1.9 mg Mn/liter of soil was added. For each of five replications/treatment combination, up to seven spinach plants/pot were rated for severity of Fusarium wilt 21, 28, and 35 days after planting (DAP) using a 0-to-5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt) (**A** and **D**). Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Area under the disease progress curve (AUDPC) was calculated based on these ratings (**B** and **E**). Each limestone-by-Mn treatment combination was evaluated in both pasteurized (56°C for 1 h) and non-pasteurized soil to compare the effects of these treatments with and without Fusarium wilt. Plants in the pasteurized soil had negligible Fusarium wilt symptoms and, thus, were not included in the disease severity or AUDPC figures. Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the plants (g/pot) (**C** and **F**). Each data point is the mean ± standard error of five replications.

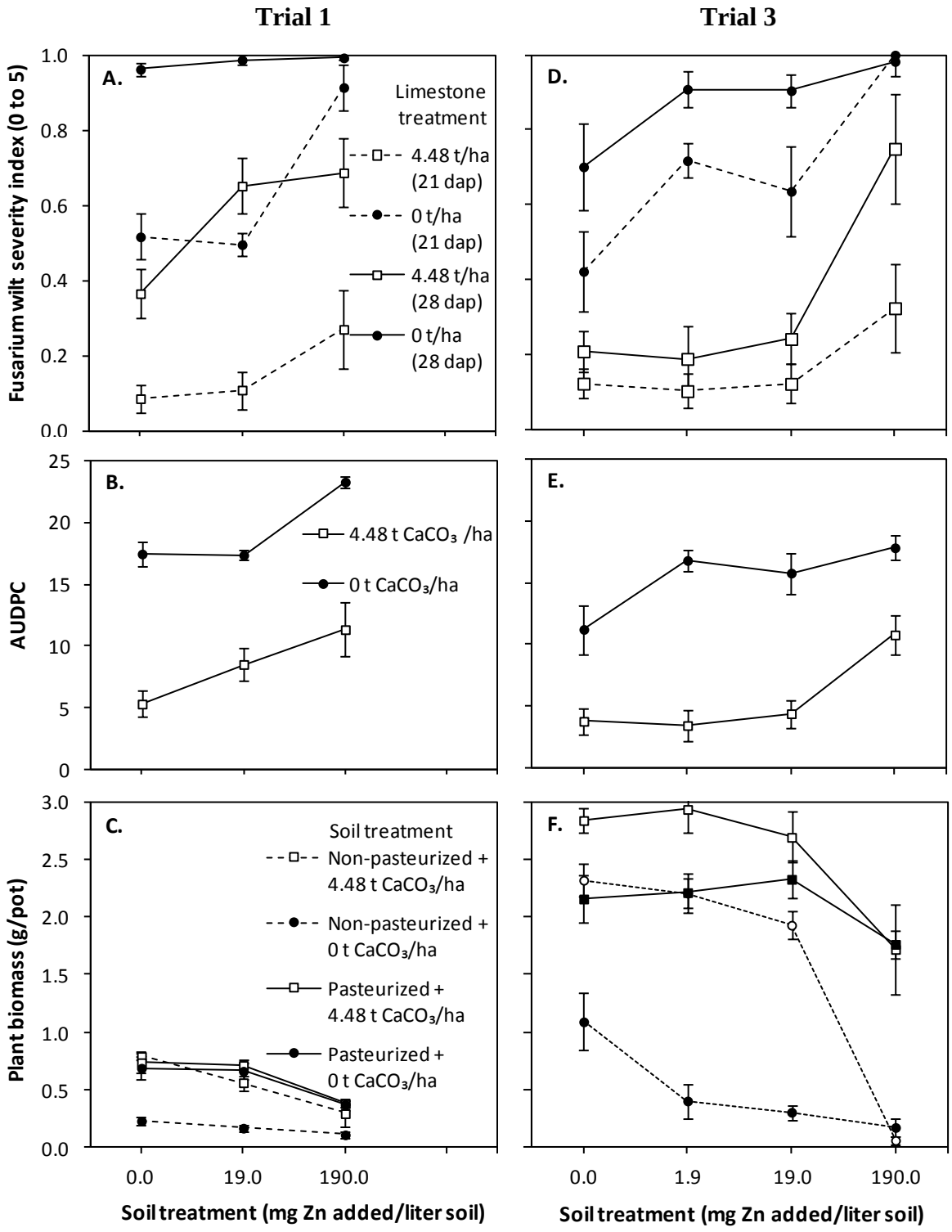

Fig. 4.4. Effects of limestone and zinc (Zn) amendment of soil naturally infested with *Fusarium oxysporum* f. sp. *spinaciae* on spinach Fusarium wilt severity and dried plant biomass in pasteurized and non-pasteurized soil in a repeated greenhouse experiment (**A** to **C** = Trial 1, **D** to

F = Trial 3). Three trials were conducted due to inconsistent results achieved in the first two trials. Results of the first and third trials are shown, since there were few significant effects in the second trial. Each experiment was a randomized complete block design with two factors: 1) amendment of soil with the equivalent of 0 or 4.48 t limestone ($CaCO_3$)/ha, and 2) a soil drench of 0, 19, or 190 mg Zn/liter soil. In the second and third trials, an additional level of 1.9 mg Zn/liter soil was added. For each of five replications/treatment combination, up to seven spinach plants/pot were rated for severity of Fusarium wilt 21, 28, and 35 days after planting (DAP) using a 0-to-5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt) (**A** and **D**). Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Area under the disease progress curve (AUDPC) was calculated based on these ratings (**B** and **E**). Each limestone-by-Zn treatment combination was evaluated in both pasteurized (56°C for 1 h) and non-pasteurized soil to compare the effects of these treatments with and without Fusarium wilt. Plants in the pasteurized soil had negligible Fusarium wilt symptoms and, thus, were not included in the disease severity or AUDPC figures. Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the plants (g/pot) (**C** and **F**). Each data point is the mean ± standard error of five replications.

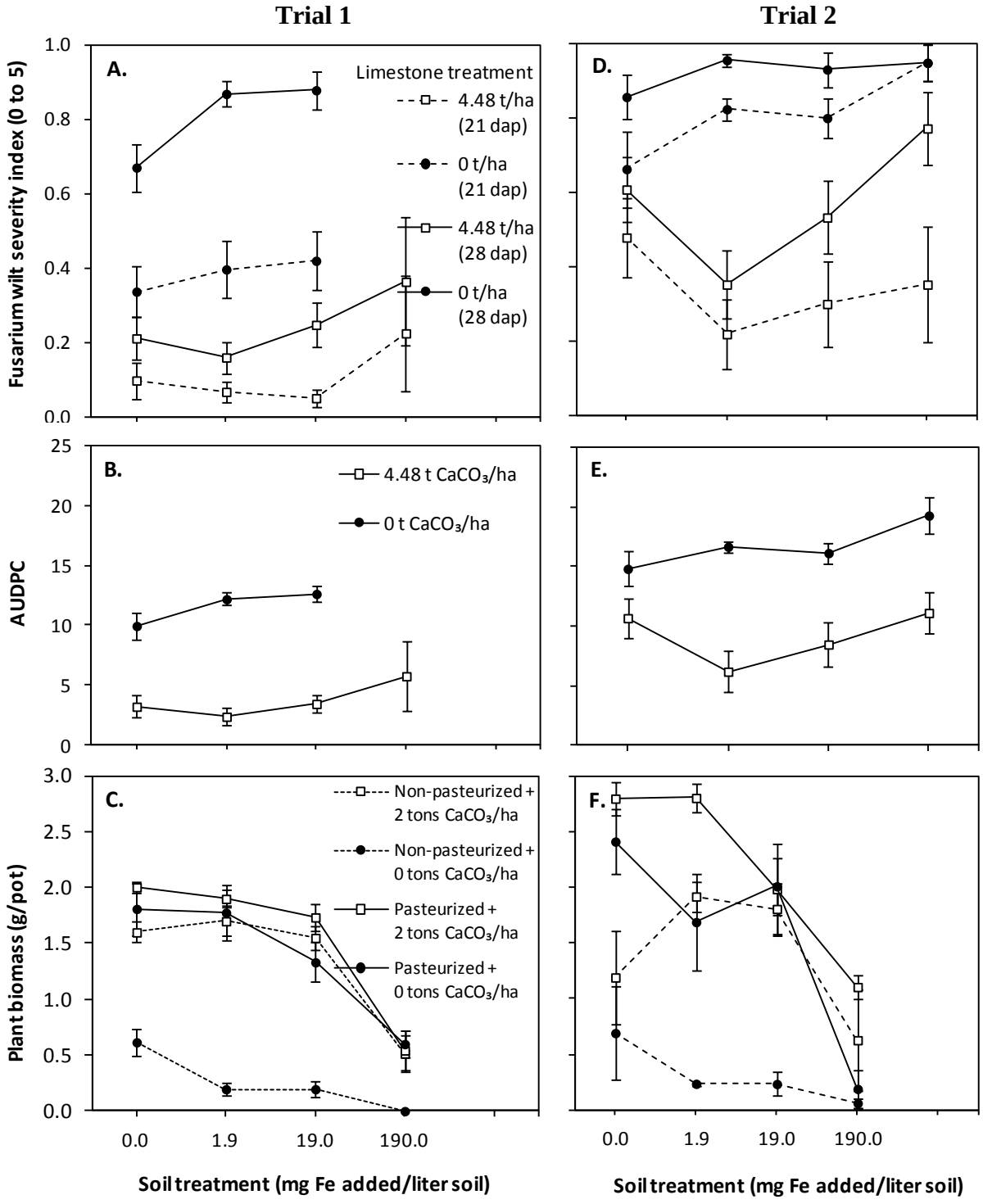

Fig. 4.5. Effects of limestone and iron (Fe) amendment of soil naturally infested with *Fusarium oxysporum* f. sp. *spinaciae* on spinach Fusarium wilt severity and dried plant biomass in pasteurized and non-pasteurized soil in a repeated greenhouse experiment (**A** to **C** = Trial 1, **D** to **F** = Trial 2). Each experiment was a randomized complete block design with two factors: 1)

amendment of soil with the equivalent of 0 or 4.48 t limestone ($CaCO_3$)/ha, and 2) a soil drench of 0, 1.9, 19, or 190 mg Fe/liter soil. For each of five replications/treatment combination, up to seven spinach plants/pot were rated for severity of Fusarium wilt 21, 28, and 35 days after planting (DAP) using a 0-to-5 ordinal scale (0 = healthy plant, 5 = plant dead due to wilt) (**A** and **D**). Ratings were subsequently converted to a 0-to-1 index, with 1 representing maximum Fusarium wilt severity. Area under the disease progress curve (AUDPC) was calculated based on these ratings (**B** and **E**). Each limestone-by-Fe treatment combination was evaluated in both pasteurized (56°C for 1 h) and non-pasteurized soil to compare the effects of these treatments with and without Fusarium wilt. Plants in the pasteurized soil had negligible Fusarium wilt symptoms and, thus, were not included in the disease severity or AUDPC figures. Dried, aboveground spinach biomass was measured by harvesting all plants in a pot at the soil line, drying the plants at 35°C, and weighing the plants (g/pot (**C** and **F**). Each data point is the mean ± standard error of five replications.

CHAPTER FIVE

CONCLUSIONS

When I started this Ph.D. project, early forays into the literature on Fusarium wilts led me to believe that I was going to be looking for ways to render the soils of the maritime Pacific Northwest suppressive to Fusarium wilt of spinach. As I spent time in spinach seed fields becoming familiar with the disease and its effects, and listened to the experiences of growers, spinach seed company representatives, and my advisor, it became clear to me that my goal would be more rightly characterized as trying to make the soils of this region less conducive, as opposed to more suppressive, to spinach Fusarium wilt. The difference is one of semantics, but was important to clarifying what was possible, given the persistence of the pathogen in these soils and the challenges associated with achieving a durable shift in an environment as complex as the plant rhizosphere.

The results presented in Chapter 2 on the four-year limestone trial demonstrate the potential for achieving such a shift. In previous field trials conducted in the region, a single application of limestone in the spring just prior to planting a spinach seed crop raised soil pH and partially suppressed Fusarium wilt. Typically, however, by the end of the season, the soil pH in these trials had reverted to an acidic state close to the pre-liming pH level, depending on the amount of limestone applied. Furthermore, growers still observed spinach seed crop rotations of 10 to 15 years despite intensifying the use of limestone as a Fusarium wilt management tool. The need remained for improvement in spinach Fusarium wilt risk prediction and mitigation. The objective of the four-year field trial established in 2009 was to determine whether the use of

limestone could be optimized through annual applications for three years prior to a spinach seed crop, compared to just one pre-plant application. Conclusive evidence of enhanced spinach Fusarium wilt suppression with a longer-term approach to limestone amendment was observed in the form of reduced severity of wilt symptoms and up to 45% increase in seed yields. Spinach plants growing in 2012 in plots that had been amended with limestone at 4.48 t/ha each year from 2009 to the spring of 2012 had an appearance and seed yields comparable to a typical commercial seed crop with a mild case of Fusarium wilt. This is remarkable given that the rotation interval for this seed crop trial was just three years. By the end of the 2012 season, the soil pH in plots amended with 4.48 t limestone/ha had been elevated to and remained above 7, suggesting that the buffering capacity of the soil had been overwhelmed by the repeated limestone applications.

Annual applications of limestone were also associated with an increase in the incidence of seed infected with *Verticillium dahliae*, which can be a cause for concern due to the wide host range of this wilt pathogen and existing phytosanitary restrictions for some countries on levels of *V. dahliae*-infected spinach seed. However, most spinach seed lots, regardless of origin, have been found to carry *V. dahliae,* and seed transmission of the pathogen can be reduced greatly or eliminated with fungicide seed treatments. No effective fungicide treatment is currently available for spinach Fusarium wilt, a disease that can reduce seed yields and seed quality very significantly. Thus, the possibility of higher levels of *V. dahliae* seed infection as a result of increased use of limestone applications should not be considered reason to abandon a potential tool for improved spinach Fusarium wilt management.

Stakeholders have been receptive to the concept of a longer-term approach to Fusarium wilt, and encouraged by the results of the limestone field trials. However, whether growers will

adopt annual applications of limestone amendment for several years preceding the planting of a spinach seed crop depends on economical and logistical factors. The soil bioassay developed and implemented in this project for evaluating each winter the Fusarium wilt risk associated with specific fields, has been integrated rapidly into spinach seed production practices of northwestern Washington. One participating seed company went so far as to mandate testing in the bioassay of any field being considered for a spinach seed crop by grower-contractees for that company. Decisions about placement of spinach seed crops have been influenced by results of the soil bioassay, mostly to avoid fields that were thought to be safe based on the rotation interval but that appeared to be of high risk in the bioassay. With time, however, it is expected that stakeholders may consider planting a field with a shorter rotation than currently practiced, if the bioassay results indicate the field is low risk for Fusarium wilt. It is also hoped that growers and seed production managers for seed companies will be able to make more informed decisions about appropriate inbred lines to plant in a field based on the level of risk indicated by the bioassay.

A third facet of this research project was to explore the mechanism(s) of limestone-mediated suppression of spinach Fusarium wilt, in particular the hypothesis that raising the soil pH deprives the pathogen of sufficient levels of iron, manganese, and/or zinc, as demonstrated by studies on Fusarium wilts of some other crops (cited in Chapters 2 to 4). With a reductionist, *in vitro* approach, this component of the study established that there are mimimum required levels of these micronutrients for growth and sporulation of the spinach Fusarium wilt pathogen. Greenhouse trials confirmed the potential role of this pH-driven micronutrient deprivation in Fusarium wilt suppresion, although the results were somewhat variable, reflecting the complexity of the soil substrate compared to the synthetic liquid medium used for the *in vitro*

trials. The contribution of the broader soil microbial community to limestone-mediated Fusarium wilt suppresion was not explored in this study, but is an important aspect of disease suppression that remains to be investigated. To this end, soil samples from each of the limestone treatments in the four-year field trial were collected and subjected to 454 pyrosequencing to characterize microbial communities associated with the various levels of Fusarium wilt observed in each plot. Completion of that analysis will add to our understanding of the processes within limestone-amended soils that result in less conducive conditions for spinach Fusarium wilt. With this knowledge, in combination with more effective deployment of limestone and improved Fusarium wilt risk prediction for growers' fields using the soil bioassay, there exists the very real opportunity to reduce the spinach seed crop rotation interval by 50%. This will, in turn, double the acreage suitable for spinach seed production in the Pacific Northwest and, therefore, in the USA.