Helminthosporium solani: Biology, Occurrence, and Control

By

Chakradhar Mattupalli

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The dissertation is approved by the following members of the Final Oral Committee:
Amy O. Charkowski, Professor, Plant Pathology
Glen R. Stanosz, Professor, Plant Pathology
Douglas I. Rouse, Professor, Plant Pathology
James P. Kerns, Assistant Professor, Plant Pathology
Shelley H. Jansky, Associate Professor, Horticulture
ABSTRACT

Potatoes are one of the top five organic vegetables purchased for home consumption in the United States. Many biotic and abiotic factors limit organic potato production and to meet the increasing consumer demand, especially from the fresh market, there is a need to produce good quality blemish-free potatoes. This research shows that silver scurf (caused by Helminthosporium solani) and black dot (caused by Colletotrichum coccodes) are two prevalent diseases in organic potato production in Wisconsin, with high incidences across locations and cultivars. Seventy-five percent and 94% of asymptomatic tubers assessed were positive for H. solani and C. coccodes respectively. Because no cultivar has yet been identified that is completely resistant to silver scurf, we performed minituber inoculation assays and identified a diploid interspecific hybrid C287 that consistently supported little sporulation of H. solani, suggesting it has partial resistance to silver scurf. Although important, H. solani is an understudied pathogen with poor genomic information, and herein we report the draft genome of the fungus. The estimated genome size of H. solani is ~35 megabases. In silico genomic analysis suggests that the fungus possesses a large suite of genes encoding putative cell wall degrading enzymes.

We also explored alternate methods to control fungal pathogens of potato. We performed in vitro tests with tobacco phylloplanins (Nt-phylloplanins) using an inexpensive microfluidic approach to test for its inhibitory activity against plant and human fungal pathogens. Spores of Colletotrichum coccodes and Aspergillus fumigatus treated with Nt-phylloplanins did not germinate at the concentrations tested at 48 and 30 hours post treatment respectively. Inhibition of spore germination of the human pathogen A. fumigatus by Nt-phylloplanins also reveals a new venue for medicinal antifungal drug discovery that has not been explored previously.
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INTRODUCTION
Potato (*Solanum tuberosum* L.) is an annual herbaceous plant belonging to the family Solanaceae. The crop is well known to plant pathologists in particular due to the Irish potato famine caused by the late blight disease in the mid 1840’s. In terms of human consumption, potatoes are the third most important food crop in the world after rice and wheat. Potatoes are an excellent source of low fat carbohydrates, vitamins B and C, as well as iron, potassium and zinc. (CIP, 2013). Potatoes are consumed fresh, frozen, dehydrated, canned, or processed into forms such as potato chips and French fries. In developed countries, up to 60% of potatoes are consumed in a processed form (Kirkman, 2007). Potatoes are a critical crop in terms of food security; as an example China, which is the world’s largest consumer of potatoes, intends to meet 50% of its increased food demand in the next 20 years through increased potato production (CIP, 2013).

Potatoes are propagated commercially using “seed” tubers. However, individual cells, meristems, sprouts, true seeds, and stem cuttings can also be used as propagules (Struik and Wiersema, 1999). A seed tuber produces sprouts in its eyes, which develop into shoots, and produce roots from primordial on the sprouts. A potato plant is in fact a cluster of stems, originating from one seed tuber or seed piece (Struik, 2007). Potatoes have modified underground shoots called stolons, with elongated internodes, rudimentary leaves and hooked tips. Tubers are the swollen parts of stolons bearing axillary buds and leaves, evident as eyes on the tuber skin. The life cycle of a potato plant can be described through five growth stages: 1) sprout development (sprouts develop from tubers and emerge from the soil) 2) vegetative growth (leaves and stems develop from above-ground nodes, roots and stolons develop from below-ground nodes) 3) tuber initiation (tubers start forming at stolon tips) 4) tuber bulking (tubers
enlarge and develop as a strong sink organ), and 5) maturation (tuber growth ceases and aerial parts start to senesce) (Miller and Hopkins, 2008).

Tubers are the economically significant portion of the potato plant and because they develop in the soil, they are attacked by myriad pathogens that affect quality and yield. These pathogens may cause symptoms on the tuber that are either external, internal or both (Table 1). Fiers et al. (2012) recently reviewed various biotic, abiotic and management factors that influence the occurrence and development of soil-borne potato pathogens. Understanding how these pathogens attack the tuber requires some knowledge about the tuber periderm. In the first part of this review, a general overview of how tubers naturally protect themselves from soil micro-biota is provided. The second part of the review focuses on four pathogens that cause potato tuber-surface blemish diseases that are commonly observed in Wisconsin.

Native and Wound Periderm:

The potato tuber surface is protected by a specialized suberin-rich layer called the native periderm. This layer provides protection against water loss, mechanical damage, and pathogens (Lulai, 2001). Epidermis exists on the developing tubers for a very short period which is replaced by the native periderm and lenticels are formed below each stoma (Peterson and Barker, 1979). The native periderm is composed of three layers: phellem, phellogen, and phelloderm. The phellem is the outermost layer consisting of suberized cells and is often referred to as the skin of the potato tuber. The phellogen or the cork cambium is the middle layer and gives rise to phellem and phelloderm. The phelloderm is the innermost layer of the periderm consisting of parenchyma-like cells. Tubers with immature periderm are fragile and are susceptible to skinning
and mechanical damage during harvest and handling operations. The reason for this fragile state is because phellogen cells are meristematic and have thin cell walls, so they loosely hold the phellem to the phelloderm, allowing easy scuffing from the tuber (Lulai and Freeman, 2001). This wounding damage is overcome by killing the potato vines about 3 weeks before harvest which promotes good skin-set (Lulai and Orr, 1993). Histological and immunolabeling studies by Sabba and Lulai (2004) indicated that the phellogen walls are reinforced by pectin depositions during native periderm maturation providing resistance to skinning injuries.

Tubers that are cut or wounded lack protection from native periderm and as the wounded tuber begins to heal, it forms a wound periderm which is very similar in organization to the native periderm (Sabba and Lulai, 2002). Before the formation of wound periderm, a suberized closing layer may form. Suberin is a biopolymer composed of polyphenolic and polyaliphatic domains. The two domains are spatially separate. The polyphenolic domain which is embedded in the primary cell wall is cross-linked by glycerol to the polyaliphatic domain which is located between the primary cell wall and the plasma membrane (Bernards, 2002). During wound healing, differential deposition of these two domains play separate roles in conferring resistance to bacterial and fungal pathogens. Lulai and Corsini (1998) observed that the polyphenolic domain deposited within 2 to 3 days of wound healing provided resistance to the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (causal organism of bacterial soft rot) but not *Fusarium sambucinum* (causal organism of fungal dry rot). Resistance to *F. sambucinum* developed after 5 to 7 days of wound healing process which corresponded with the completion of deposition of the polyaliphatic domain. These results suggest that only complete suberin matrix
(suberin polyphenolic and aliphatic domains) can provide resistance to both fungal and bacterial infections.

The relative importance of potato diseases that cause external defects has been increasing as the fresh market becomes a more significant source of potato sales. Fresh market consumers demand washed potatoes with a good quality and healthy looking external appearance. Here we will focus on the following four pathogens that cause external tuber defects and are common in the state of Wisconsin: *Rhizoctonia solani*, *Streptomyces scabies*, *Helminthosporium solani*, and *Colletotrichum coccodes*.

**Rhizoctonia solani:**

The Rhizoctonia disease complex of potatoes consists of two phases: Rhizoctonia canker (infection of growing plants) and black scurf (infestation of tubers by sclerotia). This disease complex is present wherever potatoes are grown (Banville and Carling 2001) and is caused by the fungus, *Rhizoctonia solani* Kuhn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk). Isolates of *R. solani* are categorized into Anastomosis Groups (AGs) based on hyphal incompatibility; AG-3 is the most prevalent AG found on potatoes (Tsror, 2010). Symptoms of Rhizoctonia canker appear as reddish brown to black lesions leading to girdling of sprouts, stolons and roots. Poor crop stands and delayed emergence occur as the fungus kills the growing tip of the sprouts. Above ground symptoms may manifest in the form of aerial tubers in the axils of branches and petioles, leaf rolling, stunting, rosetting and purple pigmentation of leaves (Banville and Carling, 2001). Hymenia of the sexual stage of the fungus may be observed as a white to gray powdery mass on the lower stems near the soil surface (Carling et al. 1989). The other phase of the disease complex, black scurf, occurs on the tubers. The most obvious sign of
this disease is the presence of flat or raised, irregularly shaped brown to black sclerotia. These sclerotia are often referred as “the dirt that won’t wash off” as they adhere tightly to the tuber skin (Banville et al. 1996). On tubers that are harvested before skin set, the pathogen can also cause dark spots on tubers during storage (Buskila et al. 2011). Yield losses due to \( R. solani \) can be as high as 20 to 30 % (Banville 1989; Carling et al. 1989).

Disease cycle:

The primary sources of inoculum for this disease complex are mycelia and sclerotia infested seed tubers and soil. Roots and stolons are infected at any time during the growing season (Banville and Carling, 2001). After vine-killing and during tuber maturation, volatile tuber exudates increase the formation of sclerotia on the tubers (Dijst, 1990). Chand and Logan (1984) found an increase in the number of sclerotia per tuber on tubers harvested 4 weeks after vine-killing as compared to those that were harvested one week after vine-killing. Incidence and severity of black scurf have been shown to increase during storage (Chand and Logan, 1984; Spencer and Fox, 1979).

Host-pathogen interactions:

Histopathological studies revealed that \( R. solani \) mycelia invade the tuber periderm tissues inter-cellularly causing tissues surrounding the hyphae to collapse, arresting further growth of the pathogen (Schaal, 1939; Chand and Logan, 1984). Hyphae did not penetrate beyond the phellogen but in some cases extended to the cortical tissues (Chand and Logan, 1984; Schaal, 1939; Spencer and Fox, 1979). However, when native periderm on tubers was removed and the exposed areas of the tuber were artificially inoculated with \( R. solani \), a thicker and less organized closing layer with over suberized cell walls was formed. An increase in the expression
of suberin biosynthesis potato genes such as StKCS6, CYP86A33, and POP_A was also observed as a response to *R. solani* inoculation (Buskila et al. 2011).

Aoki et al. (1963) and Nishimura and Sabaki (1963) identified phytotoxic compounds such as phenylacetic acid and its hydroxyl derivatives (meta-hydroxyphenylacetic acid and para-hydroxyphenylacetic acid) produced from the culture filtrates of *R. solani*. Rioux et al. (2011) compared putative pathogenesis related genes expressed by *R. solani* AG1 and AG3 in infected rice leaves and potato sprouts respectively. They identified 12 genes similar between the two pathosystems which play key roles in the early processes of infection such as appresorium formation, cell wall degradation and toxin secretion. However, at present similar information related to the genes involved in the tuber infection process is lacking.

**Streptomyces spp.:**

Common scab of potatoes is caused by Gram-positive, filamentous bacteria belonging to the genus *Streptomyces*. Most of the 900 known species of *Streptomyces* are soil-inhabiting saprophytes, except a few species which are pathogens on plants (Bignell et al. 2010a). Loria et al. (2006) described various pathogenic species of *Streptomyces* that cause typical scab symptoms. However, the most common pathogenic species of *Streptomyces* that cause common scab of potato are: *Streptomyces scabies* (synonym *S. scabiei*), *Streptomyces turgidiscabies and Streptomyces acidiscabies*. *S. scabies* is the oldest characterized scab pathogen with a worldwide distribution while the other two *Streptomyces* species are newly emergent pathogens. *S. acidiscabies* causes acid scab whose symptoms are indistinguishable from common scab, but the pathogen occurs in soils with pH below 5.2 (Lambert and Loria, 1989). *S. turgidiscabies* causes distinctly erumpent lesions on potato tubers in Japan (Miyajima et al. 1998). All three
species are neither tissue nor host specific, causing scab lesions on various root and tuber crops. They also cause root and shoot stunting and root tissue necrosis of model plants such as tobacco and *Arabidopsis* (Bignell et al. 2010a). However, DNA homology studies suggest that these species are not closely related (Miyajima et al. 1998; Healy and Lambert, 1991). Common scab symptoms are mostly limited to the tubers, but the pathogen can also infect stolons (Loria, 2001). Initially, the lesions are circular or irregular and tan or brown-colored and enlarge as the tuber swells. Commercially, infection of the first four to five formed internodes is critical for scab infection as these early internodes expand more rapidly than the later-formed internodes, causing more scabby lesions (Lapwood et al. 1970). As the lesions mature, they become rough in texture, coalesce, grow up to 5 to 10 mm in diameter, and in severe cases cover most of the tuber surface. When scab lesions are superficial, the disease is called russet scab, and when they are sunken, it is called pitted scab. Likewise, if the lesions are raised, the disease is called erumpent scab. Lesion types are influenced by several factors such as the pathogen strain, cultivar, age of the tuber at the time of infection, and environmental factors (Loria, 2001). Common scab does not affect tuber yield but unsightly corky lesions impact the marketability of the tubers (van der Wolf and Boer, 2007).

*Disease cycle:*

Infected tubers and infested soil are the primary sources of inoculum for common scab (Loria, 2001). The pathogen gains entry into the tuber through recently formed unsuberized lenticels (Fellows, 1926). The early tuber development period (2 or 3 weeks after tuberization) is the critical stage for common scab infection and symptoms develop within seven days after exposure of the tuber to the pathogen (Lapwood and Hering, 1970; Khatri et al. 2011). Scab
symptoms are more severe on the first formed internodes of the tuber as compared to the later-formed internodes, because enlarging tubers are more susceptible to infection (Fellows, 1926; Lapwood and Hering, 1970). Initially the pathogen grows vegetatively, producing a branched, tangled network of filamentous cells, but as the tissues affected by scab mature the pathogen produces reproductive hyphae that transform into spore chains (Jones, 1931).

Host-pathogen interactions:

After *S. scabies* gains entry into the tuber through immature lenticels, the meristem is stimulated to form closely packed elongated cells which later collapse and turn brown. Cell proliferation, expansion, and subsequent browning are attributable to thaxtomin, the toxin produced by the pathogen (Lawrence et al. 1990). Gradually the meristematic activity ceases and the last-formed cells suberize to form a wound cork which separates infected cells from the healthy tissue below. The pathogen can further infect the healthy cells by growing through incompletely suberized cells, leading to the formation of a second barrier of wound cork. These barriers are formed as long as the tuber grows actively resulting in severe scab lesions (Jones, 1931). Khatri et al. (2011) studied pathogen-induced changes in the tuber periderm at 10 days after tuber initiation in a hydroponic or a non-destructive pot culture system. They observed a significant increase in phellem thickness, number of phellem cell layers and phellem suberin content, and these changes were greater in the scab-resistant cultivar Russet Burbank than in the susceptible cultivar Desiree. These results suggest that potato cultivars respond differentially to scab infection by reinforcing their periderm structure.

Lawrence et al. (1990) were the first to establish the role of a toxin in *S. scabies* infection. By artificially inoculating aspetically grown minitubers with either *S. scabies* or purified extracts
obtained from scab lesions, they were able to reproduce common scab symptoms. They later identified the phytotoxic agents as thaxtomin (in honor of Roland Thaxter who first described the causal agent of common scab) A and B which are produced only by pathogenic *Streptomyces* species. Thaxtomin A is cyclic dipeptides (2, 5-diketopiperazines) derived from 4-nitrotryptophan and phenylalanine. The presence of a 4-nitroindole moiety plays a key role in thaxtomin phytotoxicity (King and Calhoun, 2009). Inhibition of cellulose biosynthesis has been identified as the primary mode of action of thaxtomin A (Bignell et al. 2010a). So far, 11 members of the thaxtomin family have been characterized, 10 of which are produced by *S. scabies* (King and Calhoun, 2009). Similarly, another class of phytotoxins called concanamycins A and B were shown to be produced by *S. scabies*, but not by *S. acidiscabies*. Their role as a virulence factor is yet to be determined (Natsume et al. 1998).

Another virulence factor that was accidentally discovered while screening a cosmid library is *nec1* (Bukhalid and Loria 1997). When this gene was expressed in the non-pathogen *Streptomyces lividans*, it caused necrosis of potato tuber disks and produced scab-like symptoms on immature potato tubers. The *nec1* gene is structurally conserved among *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies*, indicating that this gene may have been horizontally transferred among these species (Bukhalid et al. 1998; Bukhalid et al. 2002). Although *nec1* is not directly involved in the biosynthesis of thaxtomin A, it is physically linked to thaxtomin A biosynthesis genes (Bukhalid et al. 1998). Kers et al. (2005) described a large pathogenicity island (discrete cluster of pathogenicity and virulence genes acquired by horizontal gene transfer) of 325-660 kb in size conserved among the three pathogenic species of *Streptomyces* described earlier. One of the genes identified in this pathogenicity island *tomA*, encodes a homologue of
tomatinase enzyme, which can detoxify anti-microbial saponins secreted by plants. Although Seipke and Loria (2008) showed that *tomA* is not important for pathogenicity in potatoes, conservation of this gene among *S. scabies*, *S. acidiscabies*, *S. turgidiscabies* suggests a role in plant-microbe interactions.

Coronofacic acid is a component of the phytotoxin coronatine, produced by plant pathogens such as *Pseudomonas syringae* and *Pectobacterium atrosepticum*. Bignell et al. (2010b) performed mutational studies and identified a secondary metabolite cluster that is predicted to synthesize a compound similar to coronafacic acid. It is interesting to note that this biosynthetic cluster is present only in *S. scabies* but not *S. acidiscabies* or *S. turgidiscabies*. Genomic analysis of *S. scabies* 87-22 revealed the presence of other virulence factors such as expansin-like proteins, cutinases and auxin biosynthetic genes. These results warrant further investigations for their role and mechanism of action during host-pathogen interactions (Bignell et al. 2010a).

**Helminthosporium solani:**

Silver scurf, caused by the imperfect fungus, *Helminthosporium solani* Durieu & Mont. is a potato disease that affects tuber appearance. The disease is known to occur in various parts of the world (Errampalli et al. 2001; Loria and Secor 2001). Symptoms are observed only on the tubers and are localized to the periderm. At harvest, diseased tubers have grey lesions on the periderm that appear silvery when moistened. The silvery appearance of the tubers is attributed to air pockets that develop due to cellulolytic activity of the pathogen (Heiny and McIntyre, 1983). The outer cell layers of severely diseased tubers may slough off and *H. solani* infection increases the permeability of the periderm to water vapor, resulting in tuber shrinkage and
weight loss (Burke, 1938; Jellis and Taylor, 1974). In severely diseased tubers loss of pigmentation occurs, especially on red-skinned cultivars reducing their fresh market value (Jellis and Taylor, 1977). The disease affects the processing market as chips made from severely diseased tubers have unacceptable black burnt edges (Holley and Kawchuck, 1996). Field studies indicate that *H. solani* does not affect emergence and growth of the potato plants or tuber yields (Mooi, 1968; Read and Hide, 1984; Cunha and Rizzo, 2004). Silver scurf has been gaining economic importance in the past three decades due to the quality standards demanded by both processing and fresh market consumers as well as the emergence of *H. solani* isolates resistant to the broad-spectrum thiabendazole fungicides that are applied at postharvest by conventional potato growers (Errampalli et al. 2001).

*Disease cycle:*

*H. solani* has a very narrow host range comprising only tuber-bearing *Solanum* species and the weed species *Solanum elaeagnifolium* (Burke, 1938; Kamara and Huguelet, 1972; Sethuraman et al. 1997; Rodriguez et al. 1995). The disease spreads both in the field and during storage. Diseased seed tubers (Jellis and Taylor, 1977) and infested soil (Merida and Loria, 1994) act as the primary source of inoculum in the field while diseased seed tubers act as the initial source of inoculum in storage (Rodriguez et al. 1996). After planting diseased seed tubers, expansion of lesions occurs rapidly within 4 to 5 weeks so that the entire tuber surface is affected (Jellis and Taylor, 1977). The mechanism of pathogen transmission from a diseased seed tuber to its progeny tubers is unknown as the pathogen is not known to infect either stems or stolons (Burke, 1938). Jellis and Taylor (1977) recovered viable conidia of *H. solani* from soil surrounding progeny tubers at 14 weeks after planting and observed that the first infection sites
on progeny tubers occur at the stolon end of the tubers. They attributed the increase in disease in the field and during storage to the expansion of lesions on the diseased tubers as well as to the spread of the pathogen from diseased tubers to healthy tubers. In studying *H. solani* conidial production in storage, Rodriguez et al. (1996) found that conidia were produced throughout the storage season, with production levels ranging from 0 to 12,000 conidia per day at 4°C and from 0 to 24,000 conidia per day at 10°C. *H. solani* colonized and sporulated in-vitro on senescent leaf tissue of alfalfa, sorghum, rye, oats, corn and wheat suggesting the pathogen may be able to survive saprophytically in soil (Merida and Loria, 1994). The pathogen can also survive in dry soil of potato stores for a period of five months (Carnegie et al. 1996) and conidia may serve as over-wintering structures for the pathogen (Kamara and Huguelet, 1972).

*Host-pathogen interactions:*

Histological studies by Hunger and McIntyre (1979) showed that the hyphae of *H. solani* are localized to the periderm and that conidiophores arise from beneath the periderm. Fewer cell layers were observed in infected (4 to 6 layers) compared to healthy (6 to 8 layers) periderm (Hunger and McIntyre, 1979). Conidial germination occurred at 6 and 16 hours after inoculation on tubers of Dark Red Norland (Martinez et al. 2004) and Norchip (Heiny and McIntyre, 1983), respectively. Likewise, penetration by germ tubes into the periderm also differed between these cultivars. For Dark Red Norland, germ tube penetration occurred 6 to 9 hours after inoculation, whereas in Norchip germ tube penetration did not occur until five days after inoculation. Appresoria-like structures and suberin deposits in cortical cell layers were noted by Heiny and McIntyre (1983), but not by Martinez et al. (2004). Hyphae grow intra- or inter-cellularly and move from one cell to another by direct cell wall penetration (Martinez et al. 2004). Martinez et
al. (2004) observed necrosis in both colonized and neighboring cells and suggested that *H. solani* may be a necrotroph.

**Colletotrichum coccodes:**

*Colletotrichum coccodes* (Wallr.) causes a tuber blemish disease called black dot of potato, which has a world-wide distribution (Lees and Hilton, 2003). The presence of small black sclerotia on tubers, roots, and above- and below-ground stems gives the disease its common name. Diseased tubers may have gray-brown blemishes on the periderm, or less clearly delimited discolored areas on which sclerotia may or may not be present (Jellis and Taylor, 1974). The fungus has been shown to cause dark brown, deep sunken lesions following artificial inoculation of mini-tubers stored at 5-15°C for 5 months (Glais and Andrivon, 2004). Diseased roots have a stringy appearance when pulled out of the soil due to cortical tissue desiccation. The pathogen infects the cortical tissues of the stem; after drying, amethyst discoloration on the inside of the vascular cylinder is observed. Stolons may be attacked at any stage of plant growth and the lesions cause severing, thus leaving a part of the stolon adhering to the stem end of the tuber (Dickson, 1926). Diseased foliage bears dark brown to black lesions similar to those caused by *Alternaria solani*, but the lesions of *C. coccodes* lack concentric rings (Johnson and Miliczky, 1993). The disease also causes a reduction in yield, in some cases even with very little symptom expression (Johnson, 1994; Mohan et al. 1992; Barkdoll and Davis, 1992). Also, *C. coccodes* is known to interact with other pathogens like *Verticillium dahliae*, causing increased disease symptoms and more extensive fungal colonization in some potato cultivars (Tsror and Hazanovsky, 2001). Fresh weight losses of 5 to 10 % were observed by Hunger and McIntyre (1979) when tubers naturally infected with *C. coccodes* were stored for 18 weeks.
**Disease cycle:**

Hosts of *C. coccodes* include vegetables such as tomato, pepper and squash in the Solanaceae and Cucurbitaceae families (Dillard, 1992). Isolates of the pathogen collected from potato were capable of producing typical anthracnose lesions on ripe tomato fruit and vice-versa (Illman et al. 1959, Chesters and Hornby, 1965). The fungus can also colonize roots of winter cherry, marrow and symptomless hosts such as chrysanthemum, white mustard, cress, cabbage and lettuce (Chesters and Hornby, 1965). Also, *C. coccodes* colonizes the stems of rotation crops such as yellow mustard, soybean, spring canola, alfalfa, oats, as well as the stems of a number of weeds, including eastern black night shade, velvetleaf, giant foxtail and Timothy grass (Nitzan et al. 2006a).

Black dot is a soil-borne and tuber-borne disease. Sclerotia are the over-wintering structures. Greenhouse studies by Blakeman and Hornby (1966) found 53% of *C. coccodes* sclerotia remained viable and no conidia remained viable after burying them in soil for 83 and 3 weeks respectively. Tuber-borne inoculum may be present as sclerotia on the tuber surface or as hyphae in the vascular bundles (Tsror et al. 1999). Contaminated seed act as an important source in introducing the disease to non-infested soils (Barkdoll and Davis, 1992). However, once infested, soil-borne inoculum has a higher disease causing potential than tuber-borne inoculum (Nitzan et al. 2008). Nitzan et al. (2008) conducted greenhouse studies and observed a nonlinear trend in disease severity (sclerotial density on the roots and the length of the stem with visible sclerotia) with increasing soilborne inoculum concentration. Aerial infection of potato foliage can occur in certain regions where sandstorms and sprinkler irrigation are common. Johnson and
Miliczky (1993) obtained infections by sandblasting potato foliage followed by inoculation with a conidial suspension of *C. coccodes*. In this type of dispersal, blowing sand creates wounds on the foliage and sprinkler irrigation aids in the dissemination of conidia and sclerotia.

Infection of below and above ground parts occurs early in the growing season (Davis and Johnson, 2001). But, the pathogen has a latent period evident by restricted colonization in the plant stems. As the plant enters the tuber bulking phase and starts to senesce, *C. coccodes* colonization is activated and sclerotia develop on above and below-ground plant parts (Nitzan et al. 2006b). However, Ingram and Johnson (2010) did not observe such a colonization pattern on potato roots. Glais-Varlet et al. (2004) observed an increase in the development of black dot symptoms upon artificial inoculation of minitubers stored at 5 to 15°C. These results suggest that the disease may be able to spread on tubers during the storage period.

*Host-pathogen interactions:*

Not much literature is available for *C. coccodes* interactions with potato tubers. However, considerable work was done in tomato on which the fungus causes anthracnose disease. The extent to which these results can be extrapolated to potato is debatable owing to the differences in the environment and the plant part affected. Reasons for slow progress on this subject may be due to the under-estimation of the disease as the symptoms appear late in the cropping season, typically coinciding with natural senescence. In addition, symptoms on stems and stolons resemble those of Rhizoctonia canker, symptoms on leaves are similar to Verticillum wilt or early blight and symptoms on tubers are similar to silver scurf (Davis and Johnson, 2001).
Conclusion:

Potato growers must manage tuber surface defect diseases, but have few non-pesticide based methods for this problem. All four diseases discussed in this review are soil- or tuber-borne and are monocyclic, so control involves reducing the amount of initial inoculum. One way to achieve this goal is by the use of certified seed potatoes. One challenge is to determine the permissible level of surface defects in a certified seed tuber, which is especially difficult when considering latent pathogens like *C. coccodes* (Glais-Varlet et al. 2004). The percentage of surface defects that are permissible on certified seed tubers varies among states, with 5% surface defects permitted in some states like Wisconsin. However, in chapter 1, we show visual assessment of tubers at harvest for silver scurf and black dot may be misleading because a very high percentage of asymptomatic tubers are already infected with *H. solani* and *C. coccodes*.

As an alternative, growers could adopt new methods of producing seed tubers such as seed potato sprout technology. This technology facilitates production of seed tubers by planting detached sprouts, thus minimizing the risk of soil-borne pathogen movement (Souza-Dias et al. 2008). Reducing soil inoculum using fumigants is an option, but one that is limited due to environmental concerns. Future efforts should focus on breeding for new cultivars resistant to multiple diseases, a solution that is eco-friendly and has long-term viability. Although cultivated potatoes are tetraploid, which complicates breeding efforts, the use of wild *Solanum* species in potato breeding programs may be valuable as the progeny tubers following crosses with wild relatives have long dormancy with good tuber size and set (Jansky and Peloquin 2006). For instance, by crossing [H551 × *S. chacoense*] with [US-730 × (*S. berthaultii* × *S. tarijense*)], a diploid interspecific clone C545 was obtained, which exhibited improved resistance to a number
of important diseases including soft rot, common scab, pitted scab, early dying disease and early blight (Jansky and Rouse 2003). Further in chapter 1, we show that these diploid inter-specific clones exhibit partial resistance to other tuber diseases such as silver scurf.

Several cultural and chemical control methods are available for the management of the tuber blemish diseases discussed here, although their effectiveness is limited (Errampalli et al. 2001; Lees and Hilton, 2003). As an addition to the existing disease management options, we explored the possibility of utilizing leaf surface proteins that are naturally produced by tobacco by testing for their inhibitory effects on fungal pathogens such as C. coccodes. The results of this project are discussed in chapter 2.

This review demonstrates the dearth of information about the molecular basis of host-pathogen interactions for the fungal pathogens discussed here (Table 2). With the advent of high throughput sequencing techniques, the cost of obtaining genome data has dropped considerably. This has led to the description of several fungal genomes and provided insights into the pathogen biology, survival and infection strategies (Ellwood et al. 2010; de Wit et al. 2012; Islam et al. 2012; Manning et al. 2013). A next step to advance our understanding of fungal diseases of potato is to sequence the genomes of fungi that infect tuber surfaces and to perform comparative genomics to study factors involved in host-pathogen interactions. Based on what we know about fungal pathogenicity, plant cell wall degrading enzymes and fungal toxins are likely candidates for virulence factors important for infection of potato tubers. Because numerous fungi are present in soil, but only these three species (H. solani, C. coccodes, and R. solani) commonly infect potato tuber surfaces in Wisconsin, we anticipate that evidence of convergent evolution will be found once the molecular determinants of pathogenicity have been identified for these fungi.
chapter 3, we address questions related to *H. solani*’s biology by generating the draft genome of the fungus.
Literature Cited:


Table 1: Bacterial, fungal and oomycete pathogens affecting potato tubers

<table>
<thead>
<tr>
<th>Disease</th>
<th>Causal agent</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial ring rot</td>
<td><em>Clavibacter michiganensis</em> subsp. <em>sepedonicus</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Brown rot</td>
<td><em>Ralstonia solanacearum</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Soft rot</td>
<td><em>Pectobacterium</em> spp., <em>Clostridium</em> spp.</td>
<td>External and internal</td>
</tr>
<tr>
<td>Common scab</td>
<td><em>Streptomyces</em> spp.</td>
<td>External</td>
</tr>
<tr>
<td>Black dot</td>
<td><em>Colletotrichum coccodes</em></td>
<td>External</td>
</tr>
<tr>
<td>Black pit</td>
<td><em>Alternaria alternata</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Charcoal rot</td>
<td><em>Macrophomina phaseolina</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Early blight</td>
<td><em>Alternaria solani</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Fusarium dry rot</td>
<td><em>Fusarium</em> spp.</td>
<td>External and internal</td>
</tr>
<tr>
<td>Gangrene</td>
<td><em>Phoma foveata</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Gray mold</td>
<td><em>Botrytis cinerea</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Late blight</td>
<td><em>Phytophthora infestans</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Leak</td>
<td><em>Pythium</em> spp.</td>
<td>External and internal</td>
</tr>
<tr>
<td>Pink rot</td>
<td><em>Phytophthora erythroseptica</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Powdery scab</td>
<td><em>Spongospora subterranea</em> f. sp. <em>subterranea</em></td>
<td>External</td>
</tr>
<tr>
<td>Black scurf</td>
<td><em>Rhizoctonia solani</em></td>
<td>External</td>
</tr>
<tr>
<td>Rosellinia black rot</td>
<td><em>Rosellinia</em> spp.</td>
<td>External and internal</td>
</tr>
<tr>
<td>Silver scurf</td>
<td><em>Helminthosporium solani</em></td>
<td>External</td>
</tr>
<tr>
<td>Skin spot</td>
<td><em>Polyscytalum pustulans</em></td>
<td>External</td>
</tr>
<tr>
<td>Stem rot</td>
<td><em>Sclerotium rolfsii</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Thecaphora smut</td>
<td><em>Angiosorus solani</em></td>
<td>External and internal</td>
</tr>
<tr>
<td>Verticillium wilt</td>
<td><em>Verticillium</em> spp.</td>
<td>Internal</td>
</tr>
<tr>
<td>Wart</td>
<td><em>Synchytrium endobioticum</em></td>
<td>External</td>
</tr>
<tr>
<td>White mold</td>
<td><em>Sclerotinia sclerotiorum</em></td>
<td>External and internal</td>
</tr>
</tbody>
</table>
Table 2: Comparison of four potato tuber surface blemish diseases that are common in Wisconsin

<table>
<thead>
<tr>
<th></th>
<th>Common Scab</th>
<th>Black Scurf</th>
<th>Black Dot</th>
<th>Silver Scurf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Causal agent</td>
<td><em>Streptomyces</em> spp.</td>
<td><em>Rhizoctonia solani</em></td>
<td><em>Colletotrichum coccodes</em></td>
<td><em>Helminthosporium solani</em></td>
</tr>
<tr>
<td>Source of inoculum</td>
<td>Infected tubers, Infested soil</td>
<td>Infected tubers, Infested soil</td>
<td>Infected tubers, Infested soil</td>
<td>Infected tubers, Infested soil</td>
</tr>
<tr>
<td>Primary inoculum</td>
<td>Spores</td>
<td>Mycelia and sclerotia</td>
<td>Conidia</td>
<td>Conidia</td>
</tr>
<tr>
<td>Invasion</td>
<td>Filamentous cell growth restricted by formation of wound cork</td>
<td>Hyphae grow intercellularly and localized to the periderm, but may sometimes extend to cortex</td>
<td>Systemic and hyphae colonize cortical and vascular bundle cells</td>
<td>Hyphae grow intra- and intercellularly and localized to the periderm</td>
</tr>
<tr>
<td>Symptom/sign development</td>
<td>Tuber</td>
<td>Tuber</td>
<td>Tuber, stolons, roots, above-ground stems</td>
<td>Tuber</td>
</tr>
<tr>
<td>Tuber symptoms/signs</td>
<td>Corky lesions that may be raised or pitted or superficial</td>
<td>Irregularly shaped brown to black sclerotia adhering to the periderm</td>
<td>Grey-brown blemishes that are less clearly delimited and with/without sclerotia</td>
<td>Grey lesions on the periderm that appear silvery when moistened</td>
</tr>
<tr>
<td>Overwintering structures</td>
<td>Spores</td>
<td>Mycelia and sclerotia</td>
<td>Sclerotia</td>
<td>Conidia</td>
</tr>
<tr>
<td>Active disease spread in storage</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Pathogenicity and virulence factors during tuber-pathogen interactions</td>
<td>Thaxtomin, nec1, concanamycins</td>
<td>Not known</td>
<td>Not known</td>
<td>Not known</td>
</tr>
</tbody>
</table>
CHAPTER 1

Evaluating incidence of *Helminthosporium solani* and *Colletotrichum coccodes* on asymptomatic organic potatoes and screening potato lines for resistance to silver scurf

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Abstract

Silver scurf, caused by *Helminthosporium solani*, and black dot, caused by *Colletotrichum coccodes*, cause tuber blemishes on potato (*Solanum tuberosum* L.) which affect processing and fresh market trade. Tubers from ten cultivars were collected at harvest from three organic farms in Wisconsin and categorized as symptomatic or asymptomatic based on visual symptoms of silver scurf and black dot and/or signs of *H. solani* and *C. coccodes*. Tuber incubation and PCR assays were performed on asymptomatic tubers to detect *H. solani* and *C. coccodes*. Tuber incubation and PCR assays were in slight to fair agreement (kappa coefficient < 0.4) for detecting both pathogens. Most asymptomatic tubers tested were positive by one or both assays for *H. solani* (75%) or *C. coccodes* (94%). Minituber inoculation assays were also performed to screen potato lines for resistance to silver scurf. Of the 14 lines tested, a diploid interspecific hybrid, C287, had consistently low sporulation, suggesting it has partial resistance to silver scurf. Since the majority of tubers harvested are already infected with one or both pathogens further research should focus on organically acceptable management practices that may inhibit disease development in field and in storage.
Introduction

Potato (*Solanum tuberosum* L.) is the third most important food crop in the world for human consumption (CIP 2012) and one of the top five organic vegetables purchased for home consumption in the U.S. (Stevens-Garmon et al. 2007). Although organic potatoes comprise less than one percent of total potato sales, Greenway et al. (2011) forecast a 19% annual growth rate in U.S. per capita organic potato consumption from 2008 to 2013. Many biotic and abiotic factors limit organic potato production. In order to meet the increasing consumer demand, especially from the fresh market, there is a need to produce good quality blemish-free organic potatoes.

Silver scurf, caused by the fungus *Helminthosporium solani* Durieu & Mont. is a little-studied disease, particularly in organic potato production. At harvest, diseased tubers have grey lesions on the periderm that appear silvery when moist. Under favorable storage conditions, fungal sporulation makes tubers appear black and sooty. At later stages, the outer cell layers of tubers slough off leading to tuber shrinkage and weight loss (Burke 1938; Jellis and Taylor 1974). Silver scurf has been gaining economic importance in the past three decades due to the quality standards demanded by processing and fresh market consumers as well as the emergence of *H. solani* isolates resistant to the broad-spectrum thiabendazole and thiophanate-methyl fungicides (Errampalli et al. 2001; Geary et al. 2007). Initial infection of tubers by *H. solani* occurs in the field either from the seed tuber (Jellis and Taylor 1977) or soil (Merida and Loria 1994). Disease spread occurs during storage, as air flow in storages aids in the dissemination of fungal spores (Rodriguez et al. 1996).
Colletotrichum coccodes (Wallr.) causes black dot, and the presence of small black sclerotia on tubers, roots, and above- and below-ground stems gives the disease its common name. Infected tubers may have gray-brown blemishes on the periderm, or less clearly delimited discolored areas on which sclerotia may or may not be present (Jellis and Taylor 1974). The pathogen can cause dark brown, deep sunken lesions on minitubers stored at 5 to 15 C for five months (Glais and Andrivon 2004). Fresh weight losses of 5 to 10% occurred when tubers naturally infected with C. coccodes were stored for 18 weeks (Hunger and McIntyre 1979). The pathogen also infects roots, making them appear stringy when pulled out of the soil. Stolons may also be attacked at any stage of tuber growth and the lesions can sever the stolon, leaving a part of the stolon adhering to the tuber at harvest (Davis and Johnson, 2001). Foliar infections manifest as dark brown to black lesions similar to those caused by Alternaria solani, but lacking concentric rings (Johnson and Miliczky, 1993). Soil, infected seed tubers and air-borne inoculum serve as sources of inoculum for this disease (Lees and Hilton 2003). The disease may be overlooked as the symptoms appear late in the cropping season, coinciding with natural senescence. In addition, symptoms on stems and stolons are similar to those of Rhizoctonia canker, and on leaves similar to those of Verticillium wilt or early blight (Davis and Johnson, 2001).

Tuber symptoms of silver scurf and black dot are frequently confused owing to the similarity of blemishes caused on the periderm, and because they may be present on the same tuber (Jellis and Taylor, 1974). Depending on environmental conditions, both diseases can increase during storage. Rodriguez et al. (1996) collected air-borne conidia of H. solani ranging
from 0 to 12,000 conidia per day in storages at 4 C, and from 0 to 24,000 conidia per day in storages at 10 C during one year of the study. They detected *H. solani* conidial production throughout the storage season and also showed that greenhouse grown minitubers placed in the storages could be infected by *H. solani* conidia. Although such extensive work has not been done with black dot spread during storage, Mawson (1999) showed that preventing water condensation during storage led to increased levels of black dot and decreased levels of silver scurf on tubers. Latent infections by *C. coccodes* and *H. solani* have been documented (Glais-Varlet et al. 2004; Tsror et al. 1999; Jellis and Taylor 1974) and may contribute to disease increase during storage. To avoid continued underestimation of these diseases based on visual symptoms alone, the frequency of latent infections on asymptomatic tubers, and the potential for disease spread from such tubers, should be better understood. This information will help to determine whether future efforts to manage these diseases in organic farming should focus on reduction of inoculum spread or disease development in storage.

Organic potato production is unique from conventional production both in terms of agronomic and plant protection practices. Fludioxonil, fludioxonil plus quintozene, and azoxystrobin can reduce severity of silver scurf (Geary et al. 2007). However, these chemicals cannot be used in organic potato production. Seed tuber treatments with salts acceptable in organic production such as sodium carbonate and sorbic acid were not effective in controlling silver scurf (Geary et al. 2007). In addition, to date, no potato cultivars with complete resistance to silver scurf have been identified (Merida et al. 1994; Secor 1994; Thomas et al. 2005). Since organic potato growers have few control strategies for silver scurf, we hypothesize that a high percentage of organically grown potatoes, even if asymptomatic, are infected with *H. solani*
and/or *C. coccodes*. With the availability of interspecific *Solanum* hybrids that have resistance to multiple potato diseases (Jansky and Rouse 2003), resistance to silver scurf may be present in potato germplasm suitable for breeding. Our objectives were to investigate the abundance of asymptomatic tubers that harbor *H. solani* or *C. coccodes* in tubers harvested from organic potato farms, and to identify potato germplasm that has resistance to silver scurf.

**Materials and Methods**

*Tuber samples*

Field trials were conducted during the 2011 growing season in conjunction with the on-farm organic variety trials at three locations in Wisconsin: Antigo (A), Rosholt (B), and Cottage Grove (C). At locations A and B, the experimental design was a randomized complete block design with four replications at each location. The plots were hand planted and consisted of two rows with 20 plants per row. Plots measured 1.8 m wide by 6.1 m long with 0.9 m row spacing. At location C, a single replicate was planted, and cultivar location within the block was randomized. The plots were hand planted, and consisted of single rows with 20 plants per row. Plots measured 1.8 m wide by 6.1 m long with 0.9 m row spacing. Soil type, previous crop history, and planting and harvest dates for each location are presented in Table 1. There were 31 cultivars planted at each location, including yellow, red, russet, and specialty cultivars. Of these cultivars, 10 were selected that included multiple market classes and maturity groups, and are commonly grown by conventional and/or organic farmers in Wisconsin (Table 2). Potatoes were grown at these locations by farmers following organic management practices. The cultivars planted had four different seed sources (Table 2) and all seed was grown under conventional
management. Yield samples were harvested from measured lengths of each plot: 6.1 m lengths for each plot row at location A, 3.05 m lengths of each plot row at location B, and 6.1 m lengths of the single plot row at location C (data not shown). After harvest, the tubers from each plot were bulked and thirty tubers of each cultivar were arbitrarily selected from one replicate plot at each location. Sampled tubers were processed within a period of one week from the sampling date, during which time the tubers were stored at 4°C. Each tuber was handled individually and washed thoroughly under running tap water to remove soil deposits and then grouped as either symptomatic or asymptomatic based on visual scoring. Tubers were considered to be asymptomatic if they showed no signs of either *H. solani* or *C. coccodes* or symptoms of black dot or silver scurf. Ten asymptomatic tubers were selected per cultivar per location for PCR and tuber incubation assays, although in some cases there were fewer than ten asymptomatic tubers available (Table 3). At location C there were fewer than ten asymptomatic tubers for some cultivars. Hence, tubers that showed only signs of *C. coccodes* (sclerotia) but that lacked symptoms of either disease and signs of *H. solani* were grouped as asymptomatic for silver scurf but symptomatic for black dot (Table 3).

*Tuber incubation assay*

A humid chamber was made by placing a sterile paper towel in a 450 g deli container and dispensing 10 ml of sterile distilled water onto the towel. A small rectangular portion on the side of the container was cut out and the hole was sealed with Micropore 3M tape to facilitate aeration. Each symptomatic (up to 15 tubers per cultivar per location) or asymptomatic tuber was then placed in an individual deli container, and incubated in the dark at 24°C for a period of three
weeks. Asymptomatic tubers were number coded so that the PCR and tuber incubation assays were always conducted on the same tuber. At the end of the incubation period, the entire tuber surface was examined for the presence of conidiophores and conidia of *H. solani* and sclerotia of *C. coccodes*.

**PCR assay**

A one centimeter diameter circle of periderm was arbitrarily collected from each asymptomatic tuber using a cork borer, excised and chopped into small pieces with a sterile blade and used for DNA extraction. DNA was extracted from this tissue using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer’s protocol except that the homogenization of the sample was carried out in a mini beadbeater (Biospec Products, Bartlesville, OK) for two minutes. To detect *H. solani*, two sets of primer pairs (First-round: Hs1F1/Hs2R1; Nested: Hs1NF1/Hs2NR1) developed by Cullen et al. (2001) were used to amplify ITS regions. Sequences designed by Cullen et al. (2002) were used to amplify *C. coccodes* (First-round: Cc1F1/Cc2R1; Nested: Cc1NF1/Cc2NR1). Each PCR reaction consisted of one µl of undiluted potato DNA extract, 0.3 µM of each primer, and 1x PCR Master Mix (Promega Corporation, Madison, WI) in 25 µl of total volume. One µl of the first-round PCR product was used for nested PCR. Amplification of *H. solani* was carried out with cycling parameters slightly modified from Cullen et al. (2001): 95 C for 150 s, followed by 30 cycles of 95 C for 45 s, annealing at 66 C (first-round) and 60 C (nested) for 60 s, extension at 72 C for 90 s, and a final elongation at 72 C for 5 min. All reactions were run in a Techne TC-512 thermocycler. *H. solani* or *C. coccodes* DNA was used as the positive control and the negative control was nuclease free
water (Promega Corporation, Madison, WI). PCR products were analyzed by electrophoresis on 2% agarose gel in 1X TAE buffer, stained with SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA) and visualized under a UV transilluminator (Universal Hood II: Bio-Rad, Hercules, CA). An asymptomatic tuber was considered positive for *H. solani* or *C. coccodes* if the pathogen was detected by either PCR or the tuber incubation assay.

**Minituber inoculation assay**

Fourteen lines of minitubers were screened in two separate experiments (Table 4). Minitubers grown in a nutrient film technique system were washed, surface disinfested with calcium hypochlorite (0.5% active chlorine) for five minutes and rinsed twice in sterile water. B-AC-16A isolate of *H. solani* recovered from an infected potato was grown on V8 medium containing 177 ml V8 juice, 20 g agar and 3 g CaCO₃ per liter (Goth and Webb 1983). Cultures were incubated in the dark at 24 C and a conidial suspension was prepared by scraping a four-week old colony in a small volume of sterile water. The conidial suspension concentration was determined with a hemacytometer and adjusted to the required concentration (10⁵ spores per milliliter) by dilution with 0.1% water agar. Lines C287, Dark Red Norland, Early Epicure and Epicure Banana were screened in both experiments. A minimum of ten minitubers per line were arbitrarily selected and inoculated with *H. solani* conidial suspension until wet using an aspirator. Up to three inoculated minitubers of the same line were placed together in individual humid chambers, which were designed as described earlier for the tuber incubation assay. Three minitubers per line included as negative controls were sprayed with 0.1% water agar. After one month, each minituber was placed in a tube containing 15ml of deionized water plus 10 µl of
10% Tween20 and washed for five minutes on a laboratory shaker to extract conidia. The minitubers were then removed from the suspension and weighed. To standardize across the samples, the volume in each tube was adjusted to 20 ml with deionized water. A 10 µl portion of this suspension was filtered through a membrane filter (Munck and Stanosz 2009) and conidia of *H. solani* were counted with the aid of a dissecting microscope. The mean number of conidia obtained from three such readings was adjusted for the total volume of water in which the minituber was washed and divided by tuber weight to estimate the number of conidia extracted per gram of minituber.

*Statistical analyses*

Tubers asymptomatic for silver scurf and black dot at harvest (Table 2), and the asymptomatic tubers positive for *H. solani* and *C. coccodes* after performing tuber incubation and PCR assays (Table 3) were binary responses. Hence, Fisher’s exact tests were performed to compare the proportion of asymptomatic tubers among cultivars within a location and also to compare among locations within a cultivar. PCR and tuber incubation assays were considered as two ratings assessing an asymptomatic tuber, hence the inter-rating agreement between the assays was measured using the kappa coefficient. The kappa coefficient lies between -1 and 1, where values less than zero indicate less agreement than expected by chance and values greater than zero indicate more agreement than expected by chance (Cohen 1960). Interpretation of the kappa coefficient was made using an arbitrary benchmark scale (Landis and Koch 1977). For minituber inoculation assays, significant differences were noted between the two experiments. Hence, a linear model was fit treating the lines that were common in both experiments as separate lines.
Comparisons among lines were made using ANOVA, with means separated when necessary using Fisher’s LSD. All statistical analyses were performed with R software version 2.14.1. Statistical significance was set at $P < 0.05$.

**Results**

*Disease incidence at harvest*

Ten potato cultivars were assayed for the incidence of black dot and/or silver scurf after harvest. At each location, significant differences were observed among cultivars for the number of asymptomatic tubers for silver scurf and/or black dot at harvest ($P < 0.05$). However, none of the cultivars assayed were resistant to these diseases (Table 2). Barring three cultivars (Purple Majesty, Freedom Russet and Dark Red Norland) from location A, and three cultivars (Freedom Russet, Keuka Gold and Satina) from location B, more than 50% of the sampled tubers for each cultivar were symptomatic for silver scurf and/or black dot. Significant differences ($P < 0.05$) for the number of asymptomatic tubers at harvest were found among locations except for Adirondack Blue and Adirondack Red (Table 2). The cultivar Freedom Russet was an extreme example of this variability. All assayed Freedom Russet tubers from location B were asymptomatic, while only 30% from location C appeared healthy.

*Tuber incubation and PCR assays*

Asymptomatic tubers were assayed for *H. solani* and *C. coccodes* by tuber incubation and PCR assays to determine if these tubers carried either pathogen (Table 3). Tubers were considered infected if they were positive for the pathogen by either assay. The majority of asymptomatic
tubers assayed from these fields were infected with one or both of the pathogens: 75% of the asymptomatic tubers assayed were positive for \textit{H. solani} and 94% were positive for \textit{C. coccodes} (Fig. 1). The proportion of asymptomatic tubers that were positive for \textit{H. solani} following tuber incubation and PCR assays (Table 3), differed significantly among cultivars at each of the three locations \((P < 0.05)\). Only Adirondack Blue, Superior and Yukon Gold showed significant differences among locations for incidence of \textit{H. solani} on asymptomatic tubers. It is interesting to note that tubers of Freedom Russet from location B showed no lesions at harvest (Table 1), yet all tubers were infected with \textit{H. solani} (Table 3). No significant differences were found among locations or cultivars for \textit{C. coccodes} (Table 3).

For both pathogens, among most of the cultivars and locations tested, there was slight to fair agreement between the tuber incubation and PCR assays (Table 5). A moderate agreement for \textit{H. solani} was observed with Dark Red Norland and Yukon Gold. Location B had a substantial agreement between the two \textit{H. solani} assays. Tuber incubation and PCR assays did not agree in 37% and 26% of the asymptomatic tubers assessed for \textit{H. solani} and \textit{C. coccodes} respectively (Fig. 1). In samples where the two assays did not match, the tuber incubation assay resulted in more positives for \textit{H. solani} (29%) than \textit{C. coccodes} (8%), while the PCR assay detected more positives for \textit{C. coccodes} (18%) than \textit{H. solani} (8%).

The majority of the tubers that were assayed were infected with both \textit{H. solani} and \textit{C. coccodes}. Both pathogens occurred on the same tuber in 69% of symptomatic and 65% of asymptomatic tubers assessed (Fig. 2). In many cases, conidiophores of \textit{H. solani} and sclerotia of \textit{C. coccodes} were observed in close proximity when the tuber periderm was viewed with the aid
of a dissecting microscope (Fig. 3). Co-occurrence of these two fungi was reported earlier (Nitzan et al. 2005), but there is essentially no information available on how these fungi may interact on tuber surfaces.

Minituber inoculation assays

In the minituber inoculation assays, significant differences were observed among lines for *H. solani* sporulation (Table 4). C287 consistently had the lowest conidial count per gram of minituber, and Peruvian Blue had the highest level of sporulation. Epicure Banana from one experiment had low sporulation that was not significantly different from C287, but it produced higher number of conidia (24806 ± 3764) per gram of minituber in the other experiment, suggesting variability either within the line or the experiments. However, since C287 supported a low level of sporulation in both experiments, it appeared to have partial resistance to silver scurf.

Discussion

A high incidence of silver scurf and black dot was found across locations and cultivars with 75% and 94% of asymptomatic tubers assessed positive for *H. solani* and *C. coccodes* respectively. These results, coupled with the difficulty of distinguishing between the symptoms of silver scurf and black dot, especially on blue-skinned cultivars, indicate that visual assessment of the tubers at harvest for silver scurf and black dot symptoms is not predictive of pathogen incidence. Since high incidences of both fungi were observed on asymptomatic tubers, the tuber infections might have occurred shortly before harvest, or, alternatively infections may remain latent in developing tubers. Consistent with these results, others have reported tuber latent infections by *C. coccodes* (Glais-Varlet et al. 2004 and Tsror et al. 1999). Likewise, Jellis and
Taylor (1974) did not observe symptoms of silver scurf until three to five weeks after infection. Our results have direct implications for seed potato production. Black dot (Johnson et al. 1997) and silver scurf (Geary and Johnson 2006) incidence increases with each year that potatoes are replanted. Thus, organic farmers would likely face a very high incidence of both silver scurf and black dot if they replant the tubers they harvested.

Since initial information about the presence of either pathogen on asymptomatic tubers prior to our assays is unobtainable, this limited our ability to judge whether the tuber incubation or PCR assay is more accurate. The fast, but relatively expensive PCR assay was in slight to fair agreement with the slower, but inexpensive tuber incubation assay. The periderm samples for the PCR assay were taken arbitrarily from each tuber and it is unlikely that these pathogens are distributed evenly over the tuber surface at the time of harvest. Indeed, previous work showed that *C. coccodes* is isolated at greater frequency from tuber stem ends than from either lateral sections or bud ends (Johnson et al. 1997). These pathogens may also be present on the tuber but lack sclerotia or fruiting bodies after incubation, which would result in low agreement between the assays. Both scenarios likely occurred in this study since *H. solani* was detected more frequently by the tuber incubation assay, suggesting that infected regions of some tubers were not assayed, and *C. coccodes* was detected more frequently by PCR, suggesting that sclerotia did not form consistently in our tuber incubation assay.

No cultivar has yet been identified that is completely resistant to silver scurf (Merida et al. 1994; Secor 1994; Thomas et al. 2005). However, partial resistance to silver scurf was reported in wild *Solanum* species such as *S. demissum, S. chacoense, S. acaule, S. stoloniferum,*
*S. oxycarpum* and *S. hondelmannii* (Rodriguez et al. 1995). Previous studies indicate that tuber assays are likely to give a more accurate assessment of cultivar resistance to silver scurf than field tests, since disease severity increases with the length of time that tubers remain exposed to the pathogen in the field and this time period varies between early- and late-maturing cultivars (Merida et al. 1994). In this study, minitubers were used instead of field-grown potatoes, because when surface disinfested field-grown tubers were inoculated and incubated, the majority of the samples were lost due to tuber decay pathogens. Minitubers grown by the nutrient film technique remain nearly pathogen free, which minimized tuber decay.

Screening for resistance was performed based on the ability of *H. solani* to sporulate on the tuber surface, a measure of resistance for silver scurf used previously by Merida et al. (1994) and Rodriguez et al. (1995). Of the 14 lines examined, only C287 had consistently low spore counts in both minituber inoculation experiments. C287, a diploid ($2n = 2x = 24$) interspecific hybrid line has wild *Solanum* species in its parentage [Female parent: (US-W730 × (*S. berthaultii* × *S. tarijense*)) and male parent: (US-W730 × *S. tarijense*)]. C287 is resistant to Verticillium wilt and other surface blemish diseases such as common scab and black scurf (Jansky and Rouse, 2003). The results suggest that C287 may also have partial resistance to silver scurf, which may be usefully transferred to commercial cultivars as has been done for common scab (Murphy et al. 1995) and Verticillium wilt (Frost et al. 2006) resistance.

The high prevalence of *H. solani* and *C. coccodes* on organic potatoes and the lack of tuber resistance in currently available cultivars suggest that future research should also focus on preventing disease development during the growing season and in storage. Field data indicate a
complex relationship between pathogen incidence, disease expression, cultivar, and location. Although the percentage of asymptomatic tubers (Table 2) and the frequency of *H. solani* infections (Table 3) in asymptomatic tubers differed significantly between some cultivars and locations, no clear trends were seen when comparing cultivars and locations. Further research into the influence of soil characteristics and management choices on disease development during the growing season is needed. For organic growers with operational flexibility and a ready market, harvesting and marketing immediately at plant maturity may be an adequate control for silver scurf, since disease severity increases if tubers remain in the field (Merida et al 1994).
References


Table 1. Soil type, previous crop, and planting and harvest dates for each sampling location

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Location A</th>
<th>Location B</th>
<th>Location C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil type</td>
<td>Langlade silt loam</td>
<td>Sandy loam</td>
<td>Dodge silt loam</td>
</tr>
<tr>
<td>Planting date</td>
<td>30 May 2011</td>
<td>20 May 2011</td>
<td>16 May 2011</td>
</tr>
<tr>
<td>Harvest date</td>
<td>11 October 2011</td>
<td>20 September 2011</td>
<td>17 October 2011</td>
</tr>
<tr>
<td>Previous crop</td>
<td>Oats and Clover</td>
<td>Alfalfa and Rye</td>
<td>Popcorn</td>
</tr>
</tbody>
</table>

*Sampling locations A, B and C were farms located in Antigo, Rosholt, and Cottage Grove, WI respectively.*
Table 2. Percentage of potato tubers asymptomatic for both silver scurf and black dot after harvest from three locations in Wisconsin.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Seed source</th>
<th>Seed certification</th>
<th>Maturity group</th>
<th>Location A</th>
<th>Location B</th>
<th>Location C</th>
<th>P-value^z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Market class: Blue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adirondack Blue</td>
<td>D</td>
<td>Certified</td>
<td>Medium</td>
<td>33.3 (30)</td>
<td>50.0 (30)</td>
<td>26.7 (30)</td>
<td>0.155</td>
</tr>
<tr>
<td>Purple Majesty</td>
<td>R</td>
<td>Certified</td>
<td>Medium</td>
<td>82.1 (28)</td>
<td>31.0 (29)</td>
<td>10.0 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Market class: Red</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adirondack Red</td>
<td>D</td>
<td>Certified</td>
<td>Early to Medium</td>
<td>26.7 (30)</td>
<td>50.0 (30)</td>
<td>26.7 (30)</td>
<td>0.089</td>
</tr>
<tr>
<td>Chieftain</td>
<td>C</td>
<td>Certified</td>
<td>Medium</td>
<td>20.7 (29)</td>
<td>40.0 (30)</td>
<td>0.0 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dark Red Norland</td>
<td>L</td>
<td>Foundation</td>
<td>Early</td>
<td>60.0 (30)</td>
<td>0.0 (30)</td>
<td>10.0 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Market class: Russet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freedom Russet</td>
<td>L</td>
<td>Foundation</td>
<td>Medium to late</td>
<td>65.4 (26)</td>
<td>100.0 (30)</td>
<td>30.0 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Market class: Yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keuka Gold</td>
<td>D</td>
<td>Certified</td>
<td>Medium to late</td>
<td>46.7 (30)</td>
<td>63.3 (30)</td>
<td>10.0 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Satina</td>
<td>C</td>
<td>Certified</td>
<td>Medium early</td>
<td>30.4 (24)</td>
<td>53.3 (30)</td>
<td>23.3 (30)</td>
<td>0.047</td>
</tr>
<tr>
<td>Superior</td>
<td>L</td>
<td>Foundation</td>
<td>Early</td>
<td>21.4 (28)</td>
<td>46.7 (30)</td>
<td>0.0 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yukon Gold</td>
<td>L</td>
<td>Foundation</td>
<td>Early</td>
<td>26.7 (30)</td>
<td>36.7 (30)</td>
<td>10.0 (30)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

^z Sampling locations A, B and C were organic farms located in Antigo, Rosholt, and Cottage Grove, WI respectively. Numbers in parentheses indicate sample size.

^y Seed source: D= David Gallenberg farm, WI; C = Childstock farm, NY; L= Lelah Starks farm, WI; R= Rocky Farms, CO.

^z Statistical analysis was carried out in R (Version 2.14.1) using Fisher’s exact test at 5% significance level.
Table 3. Percentage of asymptomatic tubers positive for *Helminthosporium solani* and *Colletotrichum coccodes* after performing PCR and tuber incubation assays

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Location</th>
<th>H. solani</th>
<th>C. coccodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>ADB</td>
<td>80.0 (10)</td>
<td>10.0 (10)</td>
<td>50.0 (10)</td>
</tr>
<tr>
<td>PMJ</td>
<td>60.0 (10)</td>
<td>33.3 (9)</td>
<td>85.7 (7)</td>
</tr>
<tr>
<td>ADR</td>
<td>75.0 (8)</td>
<td>100.0 (10)</td>
<td>100.0 (8)</td>
</tr>
<tr>
<td>CFT</td>
<td>83.3 (6)</td>
<td>100.0 (10)</td>
<td>100.0 (8)</td>
</tr>
<tr>
<td>DRN</td>
<td>30.0 (10)</td>
<td>NA</td>
<td>66.7 (9)</td>
</tr>
<tr>
<td>FDR</td>
<td>100.0 (10)</td>
<td>100.0 (10)</td>
<td>100.0 (9)</td>
</tr>
<tr>
<td>KKG</td>
<td>90.0 (10)</td>
<td>80.0 (10)</td>
<td>100.0 (10)</td>
</tr>
<tr>
<td>STN</td>
<td>100.0 (7)</td>
<td>60.0 (10)</td>
<td>90.0 (10)</td>
</tr>
<tr>
<td>SUP</td>
<td>83.3 (6)</td>
<td>0.0 (10)</td>
<td>90.0 (10)</td>
</tr>
<tr>
<td>YKG</td>
<td>100.0 (8)</td>
<td>30.0 (10)</td>
<td>100.0 (10)</td>
</tr>
</tbody>
</table>

\(^x\) Cultivar: ADB= Adirondack Blue, PMJ = Purple Majesty, ADR = Adirondack Red, CFT = Chieftain, DRN = Dark Red Norland, FDR= Freedom Russet, KKG = Keuka Gold, STN = Satina, SUP = Superior and YKG = Yukon Gold.

\(^y\) Sampling locations A, B and C were organic farms located in Antigo, Rosholt, and Cottage Grove, WI respectively. Numbers in parentheses indicate sample size.

\(^z\) Statistical analysis was carried out using Fisher’s exact test at 5% significance level.
Table 4. Spore production on fourteen potato minituber lines inoculated with *Helminthosporium solani*  

<table>
<thead>
<tr>
<th>Line</th>
<th>Number of conidia extracted per gram of minituber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peruvian Blue</td>
<td>49283(^{x}) ± 6437.7 a</td>
</tr>
<tr>
<td>Epicure Banana-A(^{y})</td>
<td>24806 ± 3763.9 b</td>
</tr>
<tr>
<td>Peanut</td>
<td>23846 ± 6363.6 bc</td>
</tr>
<tr>
<td>Early Epicure-A</td>
<td>24307 ± 5815.1 bcd</td>
</tr>
<tr>
<td>Dark Red Norland-A</td>
<td>17793 ± 3845.4 bcd</td>
</tr>
<tr>
<td>Nosebag</td>
<td>12744 ± 2950.9 bcde</td>
</tr>
<tr>
<td>Superior</td>
<td>10957 ± 1854.3 cdef</td>
</tr>
<tr>
<td>Early Epicure-B(^{z})</td>
<td>11717 ± 1882.1 def</td>
</tr>
<tr>
<td>Dark Red Norland-B</td>
<td>11892 ± 2456.1 def</td>
</tr>
<tr>
<td>White Cobbler</td>
<td>9920 ± 2633.9 ef</td>
</tr>
<tr>
<td>Scotia Blue</td>
<td>9728 ± 2278.9 ef</td>
</tr>
<tr>
<td>Trina</td>
<td>8514 ± 1596.4 ef</td>
</tr>
<tr>
<td>Pike</td>
<td>7021 ± 1372.3 ef</td>
</tr>
<tr>
<td>Round Blue Andean</td>
<td>10387 ± 6066.8 f</td>
</tr>
<tr>
<td>Tundra</td>
<td>3775 ± 1235.0 g</td>
</tr>
<tr>
<td>Epicure Banana-B</td>
<td>2360 ± 410.9 gh</td>
</tr>
<tr>
<td>C287-B</td>
<td>2031 ± 403.7 hi</td>
</tr>
<tr>
<td>C287-A</td>
<td>1216 ± 292.5 i</td>
</tr>
</tbody>
</table>

\(^{x}\) Means (±SE) followed by the same lowercase letters are not significantly different within a column (\(\alpha = 0.05\)).

\(^{y}\) Lines followed by the letter A are sporulation data from experiment 1

\(^{z}\) Lines followed by the letter B are sporulation data from experiment 2
Table 5. Kappa coefficient values showing agreement between tuber incubation and PCR assays for detecting *Helminthosporium solani* and *Colletotrichum coccodes* from asymptomatic tubers

| Cultivar / Location<sup>z</sup> |  
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                 |  
| **Kappa coefficient<sup>z</sup>** |  
| **H. solani** | **C. coccodes** | **H. solani** | **C. coccodes** | **H. solani** | **C. coccodes** | **H. solani** | **C. coccodes** | **H. solani** | **C. coccodes** |
| Cultivar:                       |  
| Adirondack Blue                 | 0.11 | 0.24 | 0.11 | 0.24 | 0.11 | 0.24 | 0.11 | 0.24 | 0.11 | 0.24 |
| Purple Majesty                  | 0.21 | 0.0  | 0.21 | 0.0  | 0.21 | 0.0  | 0.21 | 0.0  | 0.21 | 0.0  |
| Adirondack Red                  | 0.16 | 0.19 | 0.16 | 0.19 | 0.16 | 0.19 | 0.16 | 0.19 | 0.16 | 0.19 |
| Chieftain                       | 0.04 | 0.0  | 0.04 | 0.0  | 0.04 | 0.0  | 0.04 | 0.0  | 0.04 | 0.0  |
| Dark Red Norland                | 0.55 | 0.0  | 0.55 | 0.0  | 0.55 | 0.0  | 0.55 | 0.0  | 0.55 | 0.0  |
| Freedom Russet                  | 0.0  | 0.23 | 0.0  | 0.23 | 0.0  | 0.23 | 0.0  | 0.23 | 0.0  | 0.23 |
| Keuka Gold                      | 0.09 | 0.30 | 0.09 | 0.30 | 0.09 | 0.30 | 0.09 | 0.30 | 0.09 | 0.30 |
| Satina                          | 0.17 | 0.01 | 0.17 | 0.01 | 0.17 | 0.01 | 0.17 | 0.01 | 0.17 | 0.01 |
| Superior                        | 0.27 | 0.0  | 0.27 | 0.0  | 0.27 | 0.0  | 0.27 | 0.0  | 0.27 | 0.0  |
| Yukon Gold                      | 0.41 | -0.07| 0.41 | -0.07| 0.41 | -0.07| 0.41 | -0.07| 0.41 | -0.07|
| Location:                       |  
| A                               | 0.24 | 0.25 | 0.24 | 0.25 | 0.24 | 0.25 | 0.24 | 0.25 | 0.24 | 0.25 |
| B                               | 0.62 | 0.09 | 0.62 | 0.09 | 0.62 | 0.09 | 0.62 | 0.09 | 0.62 | 0.09 |
| C                               | 0.03 | 0.30 | 0.03 | 0.30 | 0.03 | 0.30 | 0.03 | 0.30 | 0.03 | 0.30 |

<sup>z</sup> Kappa coefficients (Landis and Koch 1977): 0.81 to 1.00 = almost perfect agreement, 0.61 to 0.80 = substantial agreement, 0.41 to 0.60 = moderate agreement, 0.21 to 0.40 = fair agreement, 0.00 to 0.20 = slight agreement, <0.00 = poor agreement.

<sup>z</sup> Sampling locations A, B and C were organic farms located in Antigo, Rosholt, and Cottage Grove, WI respectively.
Figure 1. Percentage of asymptomatic tubers positive or negative for *Helminthosporium solani* and *Colletotrichum coccodes* by tuber incubation (T) and PCR (P) assays. (T0P0) Negative for both tuber incubation and PCR assays. (T1P1) Positive for both tuber incubation and PCR assays. (T1P0) Positive for tuber incubation and negative for PCR assay. (T0P1) Negative for tuber incubation and positive for PCR assay.
Figure 2. Percentage of tubers positive or negative for *Helminthosporium solani* (Hs) and *Colletotrichum coccodes* (Cc) by either tuber incubation or PCR assay for asymptomatic tubers and by tuber incubation assay for symptomatic tubers. (Hs1Cc0) Positive for *H. solani* and negative for *C. coccodes*. (Hs0Cc1) Negative for *H. solani* and positive for *C. coccodes*. (Hs0Cc0) Negative for both *H. solani* and *C. coccodes*. (Hs1Cc1) Positive for both *H. solani* and *C. coccodes*. 

![Graph showing the percentage of tubers positive or negative for *Helminthosporium solani* (Hs) and *Colletotrichum coccodes* (Cc)]
Figure 3. Conidia and conidiophores of *Helminthosporium solani* (left side) and sclerotia of *Colletotrichum coccodes* (right side) observed in close proximity on a potato tuber periderm under a dissecting microscope.
CHAPTER 2

A microfluidic assay for identifying differential responses of plant and human fungal pathogens to tobacco phylloplanins

The structure of this chapter has been formatted with submission to PeerJ journal in mind:

Mattupalli C, Spraker JE, Berthier E, Charkowski AO, Keller NP, Shepherd RW. A microfluidic assay for identifying differential responses of plant and human fungal pathogens to tobacco phylloplanins. PeerJ.

Some of the data published in this chapter was done in Dr. Keller and Dr. Shepherd lab.

Berthier E fabricated the microfluidic plates and Spraker JE was responsible for the Aspergillus assays.
Abstract

Phylloplanins are defensive glycoproteins secreted onto leaf surfaces by trichome bearing plants such as tobacco. They are of interest because of their anti-microbial properties, but like other natural product bioactives, the assessment and screening of phylloplanin biological activity is impeded by limited availabilities of active compounds. Here we report an inexpensive microfluidic approach that requires only a few microliters of Nicotiana tabacum (tobacco) phylloplanins (Nt-phylloplanins) to assess spore germination inhibition of plant and human fungal pathogens. Spores of Colletotrichum coccodes and Aspergillus fumigatus treated with Nt-phylloplanins did not germinate at 48 and 30 hours post treatment respectively. Nt-phylloplanins transiently inhibited spore germination of Fusarium sambucinum, but had no detectable activity against Alternaria alternata or Verticillium albo-atrum, demonstrating differential sensitivity of fungi to Nt-phylloplanins. Inhibition of spore germination of the human pathogen A. fumigatus by Nt-phylloplanins also reveals a new venue for medicinal antifungal drug discovery that has not been explored previously.
Introduction

The phyllosphere or microorganismal habitat of leaf surfaces is a complex and inhospitable habitat exposed to continuous fluctuations in physical environment (Hirano and Upper, 2000). It is inhabited by diverse genera of microbes including potential pathogens, that must also endure the array of preformed and induced plant defences of the leaf surface or phylloplane (Lindow and Brandl, 2003; Shepherd and Wagner, 2012). While many well-studied plant defenses and defensive processes are triggered by pathogens as they access the plant interior or penetrate the epidermal cell wall (Chisholm et al. 2006; Bent and Mackey 2007; Underwood and Somerville 2008), it is evident that sizable fortifications are also present at the outermost layers of the air-plant interface, and consist mainly of secreted plant-produced biochemicals that act directly against pathogens to hinder or obstruct their progress (Kennedy et al. 1992; Karamanoli and Lindow 2006; Shepherd and Wagner 2007).

The leaf surface is composed of a pavement of epidermal cells interspersed with stomata, trichomes and other leaf surface structures (Hirano and Upper, 2000). Trichomes are epidermal protuberances developing outwards on the surface of various plant organs (Werker, 2000) and can be broadly classified as simple/non-glandular and glandular secreting types. Glandular secreting trichomes are present in about 30% of vascular plants and secrete secondary metabolites such as terpenoids and phenylpropanoids that can provide disease and pest resistance (Kelsey et al. 1984; Wagner et al. 2004). Shepherd et al. (2005) discovered that plants produce and secrete to their leaf surfaces antifungal plant proteins termed phylloplanins. Native phylloplanins of Nicotiana tabacum (Nt-phylloplanin) can be collected by gently agitating undamaged leaves in water, lyophilizing the resulting solution and resuspending the lyophilized
product in sterile water. With SDS-PAGE, Nt-phyloplanin is visible as four bands with molecular masses of 16 kDa, 19 kDa, 21 kDa, and 25 kDa. The four bands all have the same N-terminal amino acid sequence and are believed to arise from glycosyl additions to a single 13 kDa core polypeptide encoded by the phylloplanin gene (Accession AY705384). Homologues of this gene have been reported to exist and be expressed only in N. tabacum trichome glands (Harada et al. 2010).

Analyses of the phylloplanin promoter using reporter genes indicate that it directs expression solely in short glandular secreting trichomes, thus revealing a highly specialized mechanism for localization and delivery of proteins to leaf surfaces (Shepherd et al., 2005). Short glandular trichomes are believed to continually secrete phylloplanins onto the leaf surface, although factors such as leaf age might influence secretion. After leaf surface proteins are removed, their levels are renewed within one week (Kroumova et al. 2007). Native Nt-phyloplanins have inhibitory activity against spore germination and hyphal growth of the basidiomycete Rhizoctonia solani, and the ascomycete Pyricularia oryzae (King et al. 2011). A recombinant 13 kDa Nt-phyloplanin polypeptide produced in Escherichia coli inhibited spore germination of Peronospora tabacina, causal agent of blue mold disease in tobacco (Shepherd et al. 2005).

Mechanized methods for harvesting phylloplane proteins on a large scale are not yet available. This impeded our ability to screen for their antimicrobial properties and led us to explore for techniques that are compatible with low volume of reagents. Microfluidic platforms are reputed for temporal and spatial control of microenvironments concurrently allowing assays to be performed in micro-channels using smaller volumes of samples (Paguirigan and Beebe, 2008). To date, these microsystems have been successfully used for applications in drug discovery, cell
culturing and characterization of fungal secondary metabolites (Dittrich and Manz, 2006; Gao et al. 2011; Berthier et al. 2013). Here, we developed a simple microfluidic channel array that enabled the in vitro study of antifungal effects of Nt-phylloplanin on plant and, for the first time, human pathogens. We verified the hypothesis that Nt-phylloplanins have inhibitory effects on a subset of fungi that have not been studied earlier and suggest that these molecules be considered for development in medicinal venues.

**Materials and Methods:**

*Nt-phylloplanin collection:* Tobacco (*Nicotiana tabacum* TI 1068) plants were grown in 15.2-centimeter diameter plastic pots in a greenhouse. MetroMix (Sun Gro Horticulture, BC, Canada) was used as potting soil and a slow releasing fertilizer (Osmocote) was applied at the rate of 7.5 g per each pot. Nt-phylloplanins were collected by washing fully expanded leaves (undetached from the plant) in 200 ml of distilled water with gentle agitation for 15 seconds. Leaves with visible damage were not washed to avoid contamination from leaf sap. The wash solution was then passed through a Miracloth (EMDMillipore, MA, USA) to filter debris and lyophilized to dryness. The lyophilized powder was resuspended in 1 ml of sterile Milli-Q water, and centrifuged at 12,000 × g for 5 minutes at 24 °C. The supernatant was filtered using a PVDF membrane syringe filter (13 mm / 0.22 μm; Fisher Scientific, PA, USA) and stored at 4°C until further use. Proteins were separated from the lyophilized product by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN Tetra cell electrophoresis system (Bio-Rad, Hercules, CA) and visualized with Coomassie Brilliant Blue. Nt-phylloplanin concentration of 1 mg mL⁻¹ was used in this study and the protein concentration was estimated using BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL).
**Proteinase treatment:** Proteinase K immobilized on Eupergit C beads (200mg, Sigma-Aldrich, MO, USA) was placed into mini-spin filters and 500 µL of leaf water wash samples were added to these filters. The filters were then placed in a 1.5 mL microcentrifuge tube and incubated at 37°C overnight. Flow-through was collected by centrifuging the tubes at 5000 g for 10 minutes which was again incubated at 95°C for 30 minutes, followed by a second centrifugation at 5000g for 5 minutes to pellet any precipitate.

**Fungal cultures:** *Alternaria solani* (Asol11), *Verticillium albo-atrum* (AC186), *Fusarium sambucinum* (PD1), *Colletotrichum coccodes* (DRN-MK), *Aspergillus fumigatus* (Af293) were the fungal pathogens used for *in vitro* testing of phylloplanin activity. *A. fumigatus* was grown on glucose minimal media (GMM) agar at 37°C for 3 days. *A. fumigatus* spore suspensions were made by applying 10 ml of sterile 0.01% Tween-20 solution to the plate and subsequently agitating conidiophores with a cell spreader. *A. solani* was grown on clarified V8 media, *F. sambucinum* on quarter strength PDA, *C. coccodes* and *V. albo-atrum* on full strength PDA. Spore suspensions were prepared by scraping 10 to 15 day old colonies into a small volume of sterile water. Spore suspensions were quantified using a haemocytometer and appropriate dilutions were made with sterile ddH₂O.

**Microfluidic channel fabrication:** Microfluidic channel assays were fabricated using a soft-lithography method (Fig. 1). The mold was fabricated from a 15 cm diameter silicon wafer upon which was spun 2 layers of SU8 epoxy photoresist, the first was set to a thickness of 150 µm and the second to 500 µm. Masks for patterning the designs in the photoresist were drawn on Adobe Illustrator (Adobe, USA) and printed at Imagesetter Inc (Madison, WI, USA). The soft-lithography process was performed by mixing polydimethylsiloxane (PDMS) in a ratio of 1:10,
degassing the mix in a vacuum desiccator, and pouring onto the mold. Following the application of PDMS, a transparent sheet, a rectangle of silicone rubber, a rectangle of rigid polyester, and 4 kg of weights were placed on the mold. The molds were baked at 80°C for 4 hours after which the PDMS was removed from the mold and bonded via to a glass microscope slide following an oxygen plasma surface activation treatment (FEMTO, Diener, Germany).

**Spore Germination Assays:** The spore germination assay was conducted in microfluidic channels prepared according to the described method (Fig. 1). Each micro-channel was loaded with 20 µl or 15 µl (*A. fumigatus*) total fluid volume, containing 10 µl or 13.5 µl (*A. fumigatus*) of phylloplanin solution and 10 µl or 1.5 µl (*A. fumigatus*) spore suspension, premixed in microcentrifuge tubes. Final spore concentration in each micro-channel was $4 \times 10^2$ for *A. solani*, $1.5 \times 10^3$ for *A. fumigatus*, $2 \times 10^3$ for *C. coccodes* and *F. sambucinum*, and $2 \times 10^4$ for *V. albo-atrum*. Controls of proteinase treated phylloplanin solution were used and loaded in equal volume to the treatment channels. Channels were incubated at room temperature in a moist chamber to prevent evaporation from the channels. Germination rates were quantified microscopically at 6, 12, 24, 30 or 48 hours post inoculation by counting 200 spores per treatment. A spore was considered germinated if the length of the germ tube exceeds half the length of the spore or multiple germ tubes developed. Each treatment had three biological replicates.

**Results and Discussion:**

Due to the large volumes of water used for collecting phylloplanins from leaf aerial surfaces, the samples first needed to be lyophilized to yield a concentrated product highly enriched in protein
but very limited in volume and quantities. This aspect of sample recoverability was a significant bottleneck in our ability to perform a large number of assays. Here, we developed a microfluidic channel array enabling the efficient study of phylloplanins requiring only microliters of reagents (Fig. 1). The accessible microscale assay leveraged simple pipette-based pumping methods (Berthier and Beebe, 2007) allowing simple fabrication and use of the channels. The channels positioned on a microscope slide allowed straightforward use and imaging at known locations and devoid of typical meniscus effects that occur in microwell plates. Using this microscale array, we performed tens of assays simultaneously with low volumes of Nt-phylloplanins, and demonstrated that this assay will be broadly useful in further large-scale screening of natural product bioactives against microorganisms.

Earlier studies indicated the antimicrobial effects of Nt-phylloplanins (Shepherd et al. 2005; Kroumova et al. 2007; King et al. 2011) against an oomycete and two fungal pathogens. Here, we show that the antimicrobial activity of Nt-phylloplanins is specific to some fungal species, suggesting that fungal resistance mechanisms exist. Differential responses to antimicrobial proteins by pathogens, which are likely driven by host-pathogen co-evolution, is not uncommon (Segura et al. 1998; Segura et al. 1999; Van Damme et al. 1999; Vigers et al. 1992). For instance, a 30 kDa protein isolated from leaves of *Engelmannia pinnatifida* strongly inhibited the growth of plant pathogens such as *Fusarium oxysporum, Alternaria solani, Gaeumannomyces graminis*, but not the human pathogen, *Candida albicans* (Huynh et al. 1996).

The inhibition of spore germination by tobacco leaf wash samples was tested *in vitro* against four fungal plant pathogens (*Alternaria solani, Verticillium albo-atrum, Fusarium sambucinum, Colletotrichum coccodes*), and a human pathogen (*Aspergillus fumigatus*). Nt-phylloplanins
strongly inhibited *C. coccodes* and *A. fumigatus*, transiently inhibited *Fusarium sambucinum*, and were inactive against *A. solani* and *V. albo-atrum*, thereby indicating a spectrum of pathogen sensitivity (Table 1). *C. coccodes* spores treated with tobacco leaf wash sample did not germinate at 48 hours post inoculation when treated with 1 mg mL$^{-1}$ Nt-phylloplanins (Fig. 2). The LD$_{50}$ for Nt-phylloplanins against *C. coccodes* spores was approximately 0.1 mg mL$^{-1}$ (Fig. 3) *C. coccodes* spores failed to germinate when they were treated with tobacco leaf wash sample overnight and washed twice with sterile water, thus the effect of Nt-phylloplanins was irreversible (Fig. 2).

Few of the *A. fumigatus* spores germinated when incubated with tobacco leaf wash samples at the Nt-phylloplanin concentrations tested, and this effect persisted for at least 30 hours post inoculation (Fig. 4). The inhibition of *A. fumigatus* (Fig. 4) and *C. coccodes* (Fig. 2) spore germination was eliminated when proteins were digested with proteinase-K, suggesting that Nt-phylloplanins were necessary for inhibition.

Unlike *C. coccodes* and *A. fumigatus*, *F. sambucinum* spore germination was only transiently inhibited by Nt-phylloplanins. *F. sambucinum* spores germinated in negative controls at 12 hours post inoculation (hpi), but not when treated with Nt-phylloplanins. However, this inhibitory effect started to degrade at 16 hpi and by 24 hpi no inhibitory effect was evident and extensive hyphal growth occurred (Fig. 5).

The mechanism of action of Nt-phylloplanins remains unknown. Since, Nt-phylloplanins inhibit *C. coccodes* and *A. fumigatus* (this work), *Rhizoctonia solani* and *Pyricularia oryzae* (King et al. 2011), but only transiently inhibit *F. sambucinum* and are ineffective against *Alternaria solani*.
and, *Verticillium albo-astrum*, this suggests that host-pathogen co-evolution may select for Nt-phylloplanin resistance in some fungi. Knowledge of fungal resistance mechanisms and the ease of acquisition of resistance to phylloplanins would aid in decisions on whether to develop these antimicrobial proteins as a natural product for use in plant/human protection.

*Aspergillus fumigatus* is the most common *Aspergillus* species to cause human infections (Dagenais and Keller, 2009) and it causes a spectrum of diseases such as allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis, depending on the host’s immune status (Latge, 1999; Dagenais and Keller, 2009; Zaas and Alexander, 2009). Currently there are four classes of antifungal agents with activity against *Aspergillus*: the polyenes such as amphotericin B and nystatin; the triazoles such as itraconazole, voriconazole and posaconazole; the echinocandins such as caspofungin and micafungin; and the allylamines such as terbinafine (Chamilos and Kontoyiannis, 2005). However, treatments involving amphotericin B and triazoles may cause nephric and hepatic toxicity (Wingard et al., 1999; Tan et al., 2006). Lipid-based formulations of amphotericin B have reduced the incidence of nephrotoxicity, but they do so at a significantly increased drug acquisition cost (Pound et al., 2011). Also, in some countries such as UK, up to 15% of *A. fumigatus* strains have been found to be resistant to various azoles (Mayr and Lass-Florl, 2011). This highlights the ongoing requirement for new anti-fungal agents for this pathogen (Denning and Hope, 2010) and antimicrobial proteins are now being explored as potential therapeutics. Over 1000 antimicrobial peptides are now known to be produced by different classes of organisms, of which about 80 are plant defensins or plant defense peptides produced by 50 plant species (AMSDatabase). To date, a majority of the antimicrobial peptides utilized in drug development are of animal origin (Gordon et al., 2005).
Our results suggest that examination of plant antimicrobial proteins, such as Nt-phylloplanins, may prove fruitful for development of novel treatments for *A. fumigatus*. 
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signaling and iron acquisition in epiphytic bacteria by leaf surface compounds. Appl


Table 1: Comparison of the effects of Nt-phylloplanins on fungal spore germination

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Fungal family</th>
<th>Disease</th>
<th>Host</th>
<th>Inhibition of spore germination by Nt-phylloplanins</th>
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<td>Invasive aspergilosis</td>
<td>Humans</td>
<td>Yes</td>
</tr>
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Figure 1: Schematic representation of microfluidic device fabrication process.

(1) A multilayered mold is fabricated using photolithography methods from a light-sensitive negative photoresist SU8. Each layer of SU8 is spun on top of the previous one and patterned by exposure to UV light. (2) Using soft-lithography, PDMS polymer is cured on the mold to create a replicate of the channel array. (3) The PDMS layer is bonded to glass using an oxygen-plasma surface activation method. (4) The result is a platform containing an array of microscale channels on a microscope slide.
Figure 2. Inhibition of *Colletotrichum coccodes* spore germination after treatment with Nt-phyllloplanins in tobacco leaf wash

*C. coccodes* spores were subjected to various treatments: water control, proteinase-K treated Nt-phyllloplanins, Nt-phyllloplanins (1 mg mL\(^{-1}\)), treatment with Nt-phyllloplanins overnight followed by washing twice in sterile distilled water, and incubated in microfluidic channels (2 × 10\(^3\) spores per channel). Each point represents an average of three biological replicates with 200 spores assessed per each treatment. The data was arcsine transformed prior to being analyzed using ANOVA, with means separated using Fisher’s LSD in R 2.15.3. Treatments with the same lowercase letters are not significantly different at \( P < 0.05 \). Error bars represent standard error of the mean.
Figure 3: Inhibition curve of *Colletotrichum coccodes* spore germination by Nt-phylloplanins in tobacco leaf wash

*C. coccodes* spores were treated with tobacco leaf wash containing various concentrations of Nt-phylloplanins and incubated for a period of 12 hours. Each data point represents an average value from three biological replicates.
Figure 4. Inhibition of *Aspergillus fumigatus* spore germination after treatment with Nt-phyllloplanins in tobacco leaf wash

Spores of *A. fumigatus* were subjected to various treatments: GMM control, proteinase-K treated Nt-phyllloplanins, Nt-phyllloplanins (1 mg mL\(^{-1}\)), and incubated in microfluidic channels (1.5 \(\times\) 10\(^3\) spores per channel). Each point represents an average of three biological replicates with 200 spores assessed per each treatment. The data was arcsine transformed prior to being analyzed using ANOVA, with means separated using Fisher’s LSD in R 2.15.3. Treatments with the same lowercase letters are not significantly different at \(P < 0.05\). Error bars represent standard error of the mean.
Figure 5. Transient inhibition of *Fusarium sambucinum* spore germination by Nt-phylloplanins in tobacco leaf wash

A) *F. sambucinum* spores germinated in water controls at 12 hours post inoculation (hpi). B) Non-germination of Nt-phylloplanin treated *F. sambucinum* spores at 12 hpi. C) Extensive hyphal growth observed after treating *F. sambucinum* spores with Nt-phylloplanins at 24 hpi. Bar = 20 µm.
CHAPTER 3

A draft genome sequence reveals *Helminthosporium solani*’s arsenal for cell wall degradation
ABSTRACT

*Helminthosporium solani*, is a fungal pathogen belonging to the family Massarinaceae. It causes blemishes on potato tubers, affecting processing and fresh market trade. Despite its world-wide distribution, little is known about the biology of *H. solani*. Here we report the generation of a draft genome sequence of *H. solani* with an estimated genome size of ~35 megabases, which is also the first reference genome within the family Massarinaceae. We identified a large suite of genes in the *H. solani* genome that encode putative cell wall degrading enzymes. Based on comparison with other known genomes, we speculate that *H. solani* is a hemi-biotroph or necrotroph. The presence of a large number of genes in the glycoside hydrolase (GH) 10 and 43 families, which aid in the hydrolysis of glucoronoarabinoxylan, also suggests that *H. solani* may be able to survive on grass hosts or debris and indicates the need to re-examine the life cycle and host range of this pathogen. The *H. solani* draft genome will be a valuable resource for phylogenetic and pathogenicity studies on this pathogen.
INTRODUCTION

The fungus *Helminthosporium solani* causes silver scurf disease of potatoes and has a worldwide distribution (Errampalli et al. 2001). Potato tubers with silver scurf have grey lesions which are localized to the periderm and appear silvery when moistened. Under favorable storage conditions, fungal sporulation makes the tubers appear black and sooty. Loss of pigmentation occurs in severely diseased tubers, especially on red-skinned cultivars (Jellis and Taylor, 1977), reducing their fresh market value. Silver scurf also increases the permeability of the tuber skin to water vapor, resulting in tuber shrinkage and weight loss (Burke, 1938; Jellis and Taylor, 1974). Silver scurf negatively affects the processing market as chips made from severely infected tubers have unacceptable black burnt edges (Holley and Kawchuck, 1996). Although silver scurf does not affect tuber yields (Read and Hide, 1984; Cunha and Rizzo, 2004), the disease has been gaining economic importance in the past three decades due to the quality standards demanded by processing and fresh market consumers as well as the emergence of *H. solani* isolates resistant to the broad-spectrum thiabendazole fungicides and thiophanate-methyl fungicides used to control this disease (Errampalli et al. 2001; Geary et al. 2007).

Silver scurf spreads both in the field and during storage. Diseased seed tubers (Jellis and Taylor, 1977) and infested soil (Merida and Loria, 1994) act as the primary source of inoculum. *H. solani* has a very narrow host range comprised of tuber-bearing *Solanum* species and the weed species *Solanum elaeagnifolium* (Burke, 1938; Kamara and Huguelet, 1972; Sethuraman et al. 1997; Rodriguez et al. 1995). *H. solani* is an anamorphic fungus that can colonize senescent leaf tissues of alfalfa, sorghum, rye, oats, corn, and wheat *in vitro*, and can also survive in dry soil of potato stores for a period of five months (Merida and Loria, 1994; Carnegie et al. 1996). There
are no known specialized over-wintering structures, however conidia may serve this purpose (Kamara and Huguelet, 1972). Other aspects of the disease cycle such as pathogen spread from mother tuber to daughter tubers and its overwintering phases are poorly understood. Furthermore, genomic information for *H. solani* is scarce, as evidenced by the presence of only 36 nucleotide sequences of beta-tubulin and ribosomal genes in the National Center for Biotechnology Information (NCBI). There is no information available regarding *H. solani* genes required for virulence or pathogenesis. In this sense, *H. solani* is an understudied pathogen.

*Helminthosporium solani* is an ascomycete belonging to the order Pleosporales, the largest order in the class Dothideomycetes (Kirk et al. 2008). Analyses of ribosomal DNA and ITS sequences reveal that the closest relatives of *H. solani* is the wood saprophyte *Helminthosporium velutinum* and *Saccharicola bicolor*, causal agent of leaf scorch disease of sugarcane (Olivier et al. 2000). Currently *H. solani* is placed in the family Massarinaceae (Eriksson and Hawksworth, 2003). With the exception of *H. solani* and *S. bicolor*, the majority of the members of Massarinaceae are saprophytes (Liew et al. 2002; Zhang et al. 2009). Wood saprophytes possess multiple cell wall degrading enzymes capable of hydrolyzing cellulose and hemicelluloses (Bucher et al. 2004; Simonis et al. 2008; Sin et al. 2002). In a recent study, a large scale screening for hydrolytic activity of pathogenic and non-pathogenic fungi has also revealed that plant pathogens are promising sources for cell wall degrading enzymes (King et al. 2011). With the advent of high throughput sequencing techniques, the cost of sequencing has dropped considerably. This has led to the description of several fungal genomes with genes predicted to encode a number of carbohydrate-active enzymes (Hane et al. 2007; Ellwood et al. 2010; de Wit et al. 2012; Islam et al. 2012; Manning et al. 2013). Based on *H. solani*’s phylogeny and its growth in association with
plants, we hypothesized that *H. solani* encodes genes involved in cell wall degradation. The purpose of this study was to generate a draft genome of *H. solani* to gain insights into its biology and identify candidate virulence factors that are important for colonization of potato tubers.

**Materials and Methods:**

*Helminthosporium solani* genomic DNA isolation

The *H. solani* isolate B-AC-16A was recovered from a diseased potato tuber. Pure culture of *H. solani* was black in color when grown on V8 medium, conidiophores were septate and unbranched, and conidia were dark brown obclavate (7-8 × 18-64 µm) with up to 8 septa (Goth and Webb, 1983; Loria and Secor, 2001, Rivera-Varas et al. 2007). The single spore isolate of *H. solani* was grown in minimal medium (Olivier and Loria, 1998) for 2 weeks in the dark at 24°C on a rotary shaker. The fungal mycelia were then separated from the medium and placed between paper towels to squeeze out the remaining medium. The mycelia were then lyophilized overnight and DNA was extracted using phenol-chloroform-isoamyl alcohol (Bok and Keller 2012).

Sequencing of the *H. solani* genome and sequence assembly

*H. solani* genomic DNA was used for paired-end pyrosequencing on a full plate Roche (454) Genome Sequencer FLX+ with Titanium chemistry. *H. solani* genomic DNA was also sequenced in one lane of a 1X 100 bp flow cell on an Illumina HiSeq2000. We obtained approximately 1 million reads from the 454 sequencer and approximately 175 million reads from the Hiseq2000. The genome was assembled from a combination of 454 and Illumina data using the Roche GS De Novo Assembler (Newbler) Version 2.6 (Marquilies et al. 2005) The following parameters
were used in the assembly- seed step 12, seed length 16, seed count 1, min overlap length 40, minimum overlap identity 90, alignment identity score 2, alignment difference score –3. We used all of the 454 data in the assembly. The first 25 million reads from the Illumina data were used in the assembly to represent 50 X coverage of the genome assuming a genome size of 50 Mb.

**Retrieval and gene modeling for cell wall degrading enzymes**

Following genome assembly we retained all contigs larger than 100 bp and predicted genes using the self-training gene prediction program GenMark-ES Version 2.3c (Borodovsky and Lomsadze 2011). The search for cell wall degradation-associated genes in the *H. solani* genome was also performed with complete protein sequences from other ascomycetes such as *Leptosphaeria maculans* and *Cochliobolus heterostrophus*. The sequences were used as query in a stand-alone tblastn search with *H. solani* genome as the database (Tao, 2010). Preliminary annotations of proteins sequences obtained from translation of the genes predicted by GeneMark were obtained using InterProScan Version 4.8 (Zdobnov and Apweiler 2001) including membership in protein families and Gene Ontology (Ashburner et al. 2000) categories.

**Phylogenetic analyses:**

Phylogenetic analyses of 16 putative cell wall degrading genes were conducted using MEGA version 5.05 (Tamura et al. 2011). The amino acid sequences were aligned using Clustal W with default parameters (Thompson et al. 1994). A neighbor-joining tree was constructed for each sequence group and a bootstrap support for internal branches was estimated from 1000 pseudo-replicates.
RESULTS AND DISCUSSION

*Helminthosporium solani* is an understudied pathogen with few genomic resources. Here, we report a draft genome sequence of *H. solani*, which is also the first reference genome within the family Massarinaceae. The *H. solani* genome assembly consisted of 35.0 Mb in 9,248 contigs, with about 34 Mb in contigs bigger than 500 bp (2939 contigs) that are contained in 486 scaffolds. The N50 scaffold size is 168,899 bp with an N50 contig size of 34,146 bp. The assembly contained 874,415 Roche 454 reads and 19,976,474 Illumina reads.

We searched the *H. solani* contig sequences with the complete complement of predicted protein sequences from the genomes of two closely related Ascomycete species *Leptosphaeria maculans* and *Cochiobolus heterostrophus* C5 using tblastn (Altschul et al. 1990) with a threshold evalue of 0.00001. The 36.36 Mb *C. heterostrophus* genome encodes 13,336 predicted proteins and has 10,159 putative homologs in the *H. solani* genome. The 44.89 Mb *L. maculans* genome encodes 12,469 predicted proteins and has 8,459 putative homologs in the *H. solani* genome. The average percentage identity between *H. solani* proteins and their homologs in either genome is about 60%. De novo gene prediction using GeneMark-ES revealed 14,862 predicted protein-coding genes from the *H. solani* contig sequences. Approximately two-thirds of these predicted proteins have some matching entry from the InterProScan searches.

*H. solani* genome sequence provided insight into several putative virulence genes, including genes homologous to plant cell wall degrading enzymes, non-ribosomal peptide synthetases, and polyketide synthetases. However, we focused our analysis on the plant cell wall degrading
enzymes because of the large base of knowledge about the enzyme function and enzyme content of plant-associated microbes.

*H. solani encodes numerous plant cell wall degrading enzymes:*

The complex polysaccharides of plant cell walls are hydrolyzed by a large variety of carbohydrate-active enzymes (CAZymes) which are classified into families such as glycoside hydrolases (GH), polysaccharide lyases, carbohydrate esterases and carbohydrate-binding modules (Cantarel et al. 2008). Because the lifestyle of a fungus is generally well correlated with CAZyme content, we compared the number of genes predicted to encode cellulolytic and hemicellulolytic enzymes in the genome of *H. solani* to a subset of ascomycetes that included a saprotroph (*Trichoderma reesei*), necrotrophs (*Fusarium graminearum, Sclerotinia sclerotiorum, Botrytis cinerea*), a hemibiotroph (*Magnaporthe grisea*) and a biotroph (*Blumeria graminis*). We observed that *H. solani* has at least 71 putative genes encoding GH enzymes, which was comparable to genomes of hemibiotrophs and necrotrophs (Table 1 and 2). For instance, biotrophs generally possess fewer cell wall degrading enzymes than necrotrophs and hemibiotrophs to evade plant defenses (deWit et al. 2012; Amselem et al. 2011; Goodwin et al. 2011). Based on GH enzyme content, *H. solani* likely has a hemibiotrophic or necrotrophic lifestyle, which is in accordance with earlier histological studies where necrosis was observed in both *H. solani* colonized and neighboring potato tuber cells (Martinez et al. 2004).

*No evidence of horizontal gene transfer among putative cell wall degrading genes:*

*H. solani* belongs to a family that consists mainly of saprophytes, suggesting that acquisition of novel genes or gene duplication resulted in *H. solani* gaining the ability to colonize potato tuber
surfaces. Because it is a tuber- and soil-borne pathogen and because soil is a complex environment hosting diverse organisms, there are likely to be ample sources for horizontal gene transfer for *H. solani* (Wenzl et al. 2005; Gorfer et al. 2011). Hence, we constructed phylogenetic trees, a primary method for identification of horizontal gene transfer event, with a subset of 16 *H. solani* plant cell wall degrading gene homologs (Richards et al. 2011). However, all of the GH enzyme gene sequence trees were congruent with the *H. solani* phylogeny (data not shown).

**Hemicellulase enzyme content suggests that *H. solani* can degrade monocot plant cell walls:**

Hemicelluloses are the second most abundant group of polysaccharides in the plant cell wall (van den Brink and de Vries 2011). Xyloglucans and arabinoxylans constitute two major families of hemicelluloses whose composition varies among plants. For instance, xyloglucans are predominant in dicots and nongraminaceous monocots whereas arabinoxylans are primarily present in monocot grasses in the family Poaceae (Albersheim et al. 2011). *H. solani* is a pathogen on potato, which is a dicot plant. For this reason, we were surprised by the presence of a single gene likely involved in xyloglucan degradation (family GH16) which was in contrast to other fungi compared in this study and another eighteen fungal species in the class *Dothideomycetes* (Ohm et al. 2012). This suggests that *H. solani* does not extensively digest dicot hemicelluloses, which may explain, in part, its narrow host range. But the fact that *H. solani* is well adapted to potato suggests that the pathogen may possess a different strategy. A caveat to this analysis is that the gene number is not predictive of enzyme expression or activity, as evidenced with *T. reesei* which produces highly efficient enzymes with a small number of genes (Martinez et al. 2008).
Glucuronoarabinoxylan, composed of a xylose backbone with arabinose and glucuronic acid side chains is the major hemicellulose component of grass cell walls (van den Brink and de Vries 2011; Vogel 2008). Degradation of glucuronoarabionxylan is aided by endoxylanases (GH10) and arabinofuranosidases (GH43). *Fusarium graminearum* and *Magnaporthe grisea*, which are predominantly pathogens on grass hosts possesses 15 and 16 genes respectively that encode GH10 and GH43 enzymes. It is interesting to note that *H. solani* possesses 13 GH10 and GH43 homologs, which is comparable with grass host pathogens. Although earlier *in vitro* studies showed *H. solani*’s ability to colonize senescent grass leaf tissues (Merida and Loria 1994), our genomic data suggest that *H. solani* may have a greater ability to survive on monocots than dicots, which indicates the need to re-examine the life cycle and host range of *H. solani*. Further studies should also focus on developing a transformation method for *H. solani* which would allow functional analyses of candidate genes associated with cell wall degradation and elucidate their role during pathogenesis.
Literature Cited:


reveals transduplication and the impact of repeat elements on pathogenicity and population divergence. G3 (Bethesda) 3: 41-63.


Table 1: Comparison of the number of cellulose degrading genes among ascomycete fungi

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Lifestyle</th>
<th>Glycoside Hydrolase (GH) family</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GH 1</td>
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<tr>
<td><em>Trichoderma reesei</em> a</td>
<td>Saprotroph</td>
<td>2</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em> a</td>
<td>Necrotroph</td>
<td>5</td>
</tr>
<tr>
<td><em>Sclerotinia sclerotiorum</em> a</td>
<td>Necrotroph</td>
<td>1</td>
</tr>
<tr>
<td><em>Fusarium graminearum</em> a</td>
<td>Necrotroph</td>
<td>3</td>
</tr>
<tr>
<td><em>Magnaporthe grisea</em> a</td>
<td>Hemi-biotroph</td>
<td>2</td>
</tr>
<tr>
<td><em>Helminthosporium solani</em> b</td>
<td>?</td>
<td>3</td>
</tr>
<tr>
<td><em>Blumeria graminis f.sp. hordei</em> a</td>
<td>Biotroph</td>
<td>0</td>
</tr>
</tbody>
</table>

a Gibson et al. (2011)

b Data from this study
Table 2: Comparison of the number of hemicelluloses degrading genes among ascomycete fungi

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Lifestyle</th>
<th>GH 10</th>
<th>GH 11</th>
<th>GH 16</th>
<th>GH 43</th>
<th>GH 51</th>
<th>GH 54</th>
<th>GH 62</th>
<th>GH 81</th>
<th>Total</th>
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<td>3</td>
<td>12</td>
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<td>2</td>
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<td>2</td>
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<tr>
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<td>2</td>
<td>11</td>
<td>4</td>
<td>3</td>
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<td>1</td>
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<tr>
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<td>0</td>
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<tr>
<td><em>Fusarium graminearum</em> a</td>
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<td>1</td>
<td>1</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

a Gibson et al. (2011)
b Data from this study
SYNTHESIS
Silver scurf (causal agent: *Helminthosporium solani*) and black dot (causal agent: *Colletotrichum coccodes*) are two potato surface blemish diseases that are frequently misidentified by potato growers and whose importance in organic farming is poorly understood. Organic potato production is unique from conventional production in terms of both agronomic and plant protection practices. Synthetic pesticides are not permitted in organic farming which leaves the crop vulnerable to various diseases. Very little on-farm research has been done to learn about the importance of these diseases in organic potato farming. This dissertation is the first to demonstrate that both *H. solani* and *C. coccodes* are highly prevalent in organic potato farms in Wisconsin. This research also suggests that the visual assessment of tubers at harvest for silver scurf and black dot may be misleading. Possible future work should focus on organic control measures to prevent disease development during the growing season and in storage. With the expanding fresh market for organic potatoes demanding blemish free potatoes, we believe there is an immediate need for research on tuber surface defects.

In our experiments, we also observed *H. solani* and *C. coccodes* together on about 65% of the asymptomatic and asymptomatic tubers assessed. Despite having different lifestyles, co-occurrence of both pathogens in such a high frequency raises interesting ecological questions. At one extreme, *H. solani* has a known host range limited to tuber-bearing *Solanum* species, some saprophytic ability, and no known special over-wintering structures. At the other extreme, *C. coccodes* has a broad host range, the ability to survive in crop debris, produce sclerotia, and colonize the same host tissue as *H. solani*. Generally speaking, two species sharing a single niche cannot co-exist for a long period of time, which raises questions about the nutrients that the pathogens sequester from the tuber, the time of infection and the growth patterns affecting their
reproductive potential. We have conducted preliminary experiments looking at carbon and nitrogen utilization patterns of these pathogens, but more experiments need to be performed to dissect this association and explore plausible interactions between these two pathogens.

As all current potato cultivars are susceptible to silver scurf, we screened potato cultivars and lines for resistance to silver scurf. Mature tuber incubation assays performed to screen for resistance were unsuccessful because 90% of the tubers were lost before the end of the assay due to tuber decay pathogens. As an alternative to mature tubers, we explored the utilization of hydroponically grown minitubers, which minimized tuber losses to decay pathogens. This work demonstrated that minitubers can be successfully employed to screen for resistance to silver scurf. This dissertation has identified a diploid inter-specific breeding line (C287) with consistently low *H. solani* sporulation. C287 is known to have resistance to other significant potato diseases such as verticillium wilt, common scab, and black scurf and findings from this study suggest that C287 possesses partial resistance to silver scurf as well. Further work should be focused on field and storage trials to evaluate silver scurf resistance of C287 at multiple locations.

*H. solani* is a little studied pathogen with many unanswered questions regarding its biology and lifecycle. For instance, little information is available regarding the genes involved in the infection process, overwintering phase of the pathogen, and virulence factors. In order to address some of these questions, we generated a draft genome sequence of *H. solani*, which is the first reference genome in the family Massarinaceae and will be made available for public research. This research led to the identification of a large suite of genes in the *H. solani* genome that encode putative cell wall degrading enzymes. Based on comparison with other known genomes,
we speculate that *H. solani* is a hemi-biotroph or necrotroph. Also, the presence of a large number of genes in Glycoside Hydrolase (GH) 10 and 43 families, which aid in the hydrolysis of glucoronoarabinoxylan, a major component of grass hemicelluloses, suggests that *H. solani* may be able to survive on grass hosts and indicates the need to re-examine the host range of this pathogen.

We believe draft genomic data will shed light on previously unknown aspects of the biology of *H. solani*. *In silico* genomic analysis indicates *H. solani* may be a promising target for identifying novel cell wall degrading enzymes for use in the biofuel industry. To this end, future research should focus on performing enzymatic assays to obtain more detailed characterization of the secretome of *H. solani*. It would also be useful to develop a new primer set for detecting *H. solani* because the current nested primer set is time and cost prohibitive for use in diagnostics. Analysis of the genome of *H. solani* also revealed several genes involved in secondary metabolite production. Although our attempts to transform *H. solani* by biolistic transfection and *Agrobacterium*-mediated transformation were unsuccessful, we strongly believe further work on the development of a transformation procedure that would aid in performing functional genomics.

Finally, we explored the antifungal properties of tobacco phylloplane (leaf surface) proteins to elucidate their potential effects on pathogen development, and determine if they can be utilized in post-harvest disease control. Due to the large volumes of water used for collecting phylloplanins from leaf aerial surfaces, the samples first needed to be lyophilized to yield a concentrated product highly enriched in protein. This aspect of sample recovery limited the volume of phylloplanins and was a significant bottleneck in our ability to perform large numbers
of assays. For this reason, we decided to use a microfluidic approach that has been successfully used earlier for applications in drug discovery, cell culturing, and characterization of fungal secondary metabolites. Using microfluidic channels, we were able to perform tens of assays simultaneously with low volumes of phylloplanins, and demonstrated that this assay will be broadly useful in further large-scale screening of natural product bioactives against microorganisms.

Tobacco phylloplanins strongly inhibited spore germination of *Colletotrichum coccodes* and *Aspergillus fumigatus*, transiently inhibited *Fusarium sambucinum* spore germination, and were inactive against *Alternaria solani* and *Verticillium albo-atrum*, indicating a spectrum of pathogen sensitivity that was not previously known. Most importantly, inhibition of spore germination of the human pathogen *A. fumigatus* by tobacco phylloplanins reveals a new venue for medicinal antifungal drug discovery that has not been explored previously. Future work should focus on mass scale production of tobacco phylloplanins by expressing them in a heterologus system and testing for inhibitory effects on additional species of *Aspergillus* that are human pathogens. Lastly because the mechanism of action of phylloplanins remains unknown, further research efforts should be directed at exploring the targets of phylloplanins.