

HISTOLOGY OF SPOT BLOTCH INFECTION IN BARLEY, QTL MAPPING OF
RESISTANCE TO FUSARIUM HEAD BLIGHT, AND CHARACTERIZATION OF ROOT
ROT DISEASES IN WHEAT

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ABSTRACT

Three independent studies were conducted for spot blotch (*Bipolaris sorokiniana*), Fusarium head blight (FHB) (*Fusarium graminearum*), and root rot diseases (*Fusarium* species and *B. sorokiniana*). Histopathology of compatible and incompatible interactions between different pathotypes of *B. sorokiniana* and different genotypes of barley was examined with red fluorescent protein-tagged fungal isolates. The fungus penetrated the host cell wall and developed multicellular globular infection hyphae (IH) in the lumen of epidermal cells, but infected epidermal cells appeared to be alive till 16 hours post-inoculation (HPI). In the susceptible plants, the tip of IH was found to grow ahead of the dead tissue and invade the surrounding live mesophyll cells, whereas growth of IH in the resistant plants was restricted to the dead tissue after 20 HPI. The amount of H₂O₂ accumulation and the fungal biomass were also significantly higher in the susceptible hosts than in the resistant hosts.

To map resistance to FHB, two populations consisting 130 doubled haploid lines from the cross Grandin × PI277012 and 237 recombinant inbred lines from the cross Bobwhite × ND2710 were phenotyped and genotyped. QTL for Type I resistance were identified on chromosomes 1A, 2B, 4B, 5B and 6B in the GP population. These QTL explained 10.7-19 % of the total phenotypic variation. With the BN population, QTL for Type I resistance were identified on chromosomes 2A, 5A and 6B, explaining 6.2-13.7% of the total phenotypic variation.

To assess the prevalence, incidence and severity of wheat crown rot (CR) and common root rot (CRR) in ND, wheat root samples were collected from fields across the state in 2012, 2013, and 2014. Fungal isolations indicated that *B. sorokiniana* was most frequently recovered in all sampled years. Seedling tests on ten spring wheat lines showed that Glenn was the least susceptible while Steele-ND was the most susceptible to one *F. culmorum* isolate and one *B.*

sorokiniana isolate tested. Evaluation of 20 spring wheat genotypes for reaction to CRR at the adult plant stage showed that Freyr and RB07 were more resistant while Len and Briggs were more susceptible to CRR compared to other wheat genotypes evaluated.

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LITERATURE REVIEW

Hosts: Wheat and Barley

Wheat is an economically important crop around the world. North Dakota (ND) is a leading state for wheat production in the United States. According to a USDA report, ND produced 51.48% and 53.28% of the durum and spring wheat produced in the US, respectively, in the year of 2015 (<https://www.usda.gov/nass/PUBS/TODAYRPT/crop0816.pdf>). The genus *Triticum* encompasses species at three ploidy levels, including diploid ($2n = 14$; Einkorn wheat, *T. monococcum*), tetraploid ($2n = 28$; durum, pasta or Emmer wheat, *T. dicoccum*), and hexaploid ($2n = 42$; common or bread wheat, *T. aestivum*) (Feuillet et al., 2008). Common wheat has the AABBDD genomes and evolved through natural hybridization of domesticated tetraploid wheat (*Triticum turgidum*, AABB genomes) and diploid wild goat-grass (*Aegilops tauschii*, DD genomes) followed by polyploidization of the hybrid (Salamini et al., 2002). The genome size of hexaploid wheat is estimated to be 17 gigabases (Brenchley et al., 2012). Commercially, wheat is categorized into different classes such as hard red spring, hard red winter, soft red winter, white and durum wheat (FAO, 2011).

Barley (*Hordeum vulgare*) is another important cereal crop that is produced all over the world. It is also one of the major cereal crops in ND. According to the USDA, ND produced 31.36% of the total US barley crop in 2015 (<https://www.usda.gov/nass/PUBS/TODAYRPT/crop0816.pdf>). There are two types of barley based on spike morphology: two-row and six-row barley (Fischbech, 2002); and three types based on growth habit: winter, spring and facultative types (Poehlman, 1994). Barley is a diploid species with 14 chromosomes. The barley genome size is 5.1 gigabases (Gb) (International Barley Genome Sequencing Consortium, 2012.)

Wheat and barley production is impacted by several fungal diseases. Among them, spot blotch, Fusarium head blight (FHB or scab) and root rot are some of the important diseases that cause significant yield losses in these two crops.

Spot Blotch of Barley

Spot blotch is an important foliar disease of small grains. It is highly prevalent in the Upper Midwest region of the United States and Prairie Provinces of Canada (Mathre, 1997; Ghazvini and Tekauz, 2008). The disease causes significant yield loss ranging from 16 to 33 % (Clark, 1979; Fetch and Steffenson, 1999). Spot blotch is caused by the ascomycetous fungus *Bipolaris sorokiniana* [Sacc. in Sorok.] Shoem (Teleomorph: *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur), which is also the causal agent of common root rot and black point of wheat and barley (Wiese 1987). A typical symptom of spot blotch comprises circular to oval necrotic lesions surrounded by a chlorotic halo on the leaves (Fetch and Steffenson, 1999). In susceptible plants, the lesions can coalesce and collapse the whole leaves.

Four different pathotypes of *B. sorokiniana* have been identified based on their infection responses on three differential barley genotypes (Bowman, ND5883, and ND B112) (Valjavec-Gratian & Steffenson 1997b; Leng et al. 2016). Pathotype 0 (representative isolate: ND93-1) has low virulence on all three barley differentials; pathotype 1 (representative isolate: ND85F) has high virulence on ND5883 and low virulence on Bowman and ND B112; pathotype 2 (representative isolate: ND90Pr) has high virulence on Bowman and low virulence on ND5883 and ND B112 (Valjavec-Gratian & Steffenson 1997b). Pathotype 7 (representative isolate: ND4008) was recently identified and exhibits high virulence on all three barley differentials (Gayawli, 2010; Leng et al. 2016).

Spot blotch can be controlled by following an integrated approach which comprises crop rotation, reducing infected stubble in the field by burning them or by tillage, spraying fungicides like Triazoles, and growing resistant varieties (Friskop et al., 2016). Among these different measures, growing resistant barley varieties is considered as the most economical and ecological-friendly measure for controlling the disease (Mathre 1982; Wilcoxson et al. 1990).

Resistance to spot blotch in most of the six-rowed malting barley cultivars in the Upper Midwest region of the US was derived from NDB112 ('CIho 11531') (Wilcoxson et al. 1990). NDB112 was selected from the cross 'CIho 7117-77'/'Kindred' and its resistance has been effective for almost 50 years (Steffenson et al. 1996; Zhou and Steffenson, 2013; Leng et al., 2016). The resistance in NDB112 was overcome by the new pathotype 7 isolate ND4008, which was originally isolated from a root sample in 2008 in Langdon, ND (Gyawali, 2010). Leng et al. (2016) identified new sources of resistance against this new pathotype of spot blotch fungus from a barley core collection obtained from the USDA National Small Grains Collection in Aberdeen, ID. They found 40 barley accessions resistant to spot blotch at the seedling stage, and among them, 24 barley accessions also showed moderate to high levels of spot blotch resistance at the adult plant stage. Dominant genes for resistance to isolate ND4008 were identified in barley accessions PI235186, PI592275, and PI643242. Three quantitative trait loci (QTL), *Rcs- qtl-1H-11_10764*, *Rcs- qtl- 3H- 11_10565* and *Rcs- qtl- 7H- 11_20162* were reported to be associated with seedling and adult plant resistance to isolate ND85F (Zhou and Steffenson, 2013). Dominant genes conferring susceptibility to pathotype 1 (isolate ND85F) and pathotype 2 (isolate ND90Pr) were also identified in barley cv. Steptoe (Gazala et al., 2016) and Bowman (Valjavec-Gratian & Steffenson 1997a).

Among the genes identified to be involved in disease resistance and susceptibility in barley, *Mlo* is one of the most extensively studied genes in the barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) pathosystem (Büschges et al., 1979; Jørgensen, 1994; Stolzenburg et al., 1984). The mutation in the *Mlo* locus confers broad resistance to powdery mildew caused by the fungus *Blumeria graminis* f. sp. *hordei*. Interestingly, barley plants with the recessive *mlo* gene showed more susceptibility to the fungus *B. sorokiniana* (Kumar et al., 2001). *Mlo* was found to be associated with the mesophyll cell survival pathway (Kumar et al., 2001; Büschges et al., 1979). Barley genotypes with compromised *Mlo* pathways showed cell death of mesophyll cells when inoculated with *B. sorokiniana*. It is proven that *Mlo* is also associated with hydrogen peroxide (H₂O₂) accumulation mainly in the mesophyll cells.

B. sorokiniana is a hemibiotrophic fungus, which has a short biotrophic phase, followed by a necrotrophic phase (Kumar et al., 2002; Schäfer et al., 2004; Ibeagha et al., 2005). The biotrophic phase occurs in the early infection process and mainly in the epidermal cells where the fungus develops intracellular finger-like multicellular globular hyphae (Schäfer et al., 2004). On the other hand, the necrotrophic phase occurs in the late infection stage when the fungus grows inter- and intra- cellularly and kills massive amounts of leaf tissue. Toxins have been proposed to be involved in this process. Putative toxins have been isolated from fungal culture filtrates of *B. sorokiniana* (Gayed 1961; Pringle, 1979). Helminthosporol (Mayo et al., 1961), 9-hydroxyprehelminthosporol (Aldridge and Turner, 1970) and victoxinine (Pringle, 1979) are some of the toxins that have been isolated earlier from *B. sorokiniana*. However, these toxins are not host specific and do not produce the typical necrotic and chlorotic symptoms as that of spot blotch. Sorokinianin is another toxin isolated from fungal culture filtrates and identified as the condensed product from the sequesterpene and TCA pathways (Kumar et al., 2002). Recently,

Leng and Zhong (2012) showed that deletion of the gene for 4'-phosphopantetheinyl transferase (PPTase) in *B. sorokiniana* significantly reduced the virulence of the fungus. PPTase is required for activation of polyketide synthases (PKSs) and NRPSs. Further study indicated that two genes for nonribosomal peptide synthetases (NRPSs) are involved in high virulence of isolate ND90Pr (pathotype 2) on barley Bowman (Condon et al., 2013; Leng et al. unpublished data). These NRPSs are involved in biosynthesis of secondary metabolites, which serve as virulence factor on barley cv. Bowman (Condon et al., 2013). The toxins produced by these pathogens are important for pathogenesis by weakening or killing the host cells (Liljeroth et al., 1993).

Development of spot blotch in barley is a complex process. The spore of the fungus lands on the surface of barley leaves. The spore attaches on the surface and germinates by producing germ tubes on either or both polar cells (Kumar et al., 2002), and then produces appressoria-like structures (Schäfer et al., 2004). The fungus enters the epidermal cells by penetrating through the cell wall in between two epidermal cells. The fungus then develops primary infection hyphae in the lumen of the epidermal cells (Schäfer et al., 2004; Rodríguez-Decuadro et al., 2014). During fungal invasion, the host also responds to resist the fungal penetration by producing cell wall apposition (CWA) at the site of penetration. Also, the host expresses the hypersensitive reaction (HR) by producing defensive chemicals such as the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2), superoxides, nitric oxide etc. (Nanda et al., 2010). These biochemicals are involved in the production of phytoalexins with antimicrobial activities (Gadjev, and Gachev, 2008). ROS are also responsible for fortification of the host cell wall and signal transduction for activating genes involved in defensive activities in the attacked cells. ROS are suggested to be associated with salicylic acid (SA) in several HR systems, where SA suppresses or stimulates the programmed cell death (PCD) based on the level of ROS (Alvarez, 2000; Gadjev, and Gachev,

2008). This facilitates development of a boundary between dead and alive tissues. ROS are not only antimicrobial in nature, but also suicidal. Hence, the host produces enzymes like peroxiredoxin, glutathione peroxidase etc. and non-enzymatic anti-oxidants like ascorbate, glutathione, tocopherol and carotenoids to neutralize these ROS (Gadjev, and Gachev, 2008).

In the late infection stage, the fungus grows extensively and causes necrosis and chlorosis in leaf tissue of the susceptible host (Kumar et al., 2002; Schäfer et al., 2004; Ibeagha et al., 2005; Rodríguez-Decuadro et al., 2014). During this stage, the fungus lives as a necrotrophic pathogen, which releases toxins and kills the leaf tissues. Meanwhile, the host loses control over the production of H₂O₂ and phenolic compounds and triggers necrosis rather than the PCD (Kumar et al., 2001). Since H₂O₂ is antimicrobial, it is found that the fungus produces PKSs and NRPSs that are responsible for reducing H₂O₂ sensitivity (Leng and Zhong, 2012). However, Schäfer et al. (2004) proposed that H₂O₂ is linked with resistance rather than susceptibility. During early infection of barley by *B. sorokiniana*, H₂O₂ is associated with formation of CWAs and triggering HR during and after penetration in the epidermal cells. When the super-susceptible *albostrians* barley was inoculated with *B. sorokiniana*, there was no H₂O₂ accumulation in the infected mesophyll tissue (Schäfer et al., 2004). Therefore, H₂O₂ accumulation was proposed to be linked with resistance rather than the susceptibility. Yet, it is still unclear if the H₂O₂ accumulation is the cause or the consequence of fungal spread and toxin induced necrosis of the tissue because in the late infection stage the host produces a large amount of H₂O₂ to induce necrosis (Schäfer et al., 2004). They also suggested that late resistance response during the necrotrophic phase when fungal spread is restricted in mesophyll tissue is very important for limiting the disease outbreak compared to the early defense mechanism.

Fusarium Head Blight of Wheat

Fusarium head blight (FHB) or scab is an important disease of wheat and barley. The disease is caused by several *Fusarium* spp like *Fusarium culmorum*, *F. graminearum*, *F. pseudograminearum*, *F. avenaceum* (Xu and Nicholson, 2009). However, *F. graminearum* Schwabe (teleomorph: *Gibberella zae* (Schwein.) Petch) is the main fungal species that causes FHB in North America (O'Donnell et al., 2000; Puri and Zhong, 2010).

The typical symptom of the disease comprises bleached spikelets (Wiese, 1987; McMullen et al., 2008). As the disease progresses in a susceptible plant, the bleaching extends in all directions and causes the whole spike to collapse (Wiese, 1987). During warm and wet weather conditions, the infected tissue is covered with a light pink to salmon-orange colored fungal mass (McMullen et al., 2008; Trail, 2009). At the end of the growing season, under highly humid conditions, perithecia or sporodochia are formed on the outer surface of wheat spikes and peduncles (Bushnell et al. 2003). The infected grains usually exhibit pink discoloration and are shriveled. When these infected seeds are planted, seedling blight and poor crop stand can occur (Bai and Shaner, 1994).

FHB can cause large crop yield losses. During the 1990s, economic losses to FHB were estimated to be more than \$3 billion (McMullen et al., 1997). In addition to yield losses, FHB also affects the quality of the harvested crop due to contamination with mycotoxins such as deoxynivalenol (DON) and its derivatives, oestrogenic mycotoxin, aurofusarin, and zearalenone (Scott, 1990; Bai and Shaner, 1996; McMullen et al., 1997; Trail, 2009). These mycotoxins are highly toxic to humans and animals when consumed. In animals and livestock, the mycotoxins cause weight loss, diarrhea, hemorrhage of the alimentary canal and dermatitis. In humans, it causes nausea, vomiting, anorexia; alimentary toxic aleukia and akakabi toxicosis, as well as

neural disorders and immunosuppression (Nelson et al., 1993, Desjardins, 2006, Bennett and Klich, 2003). The U.S. Food and Drug Administration (FDA) has set the threshold for the allowable amount of DON in finished products of wheat for human consumption at 1 ppm and at 5-10 ppm for livestock and poultry (Aakre et al., 2005).

FHB is highly affected by environmental conditions. Moisture and temperature are vital for fungal growth, sporulation and disease development. The optimum temperature for ascospore production ranges from 15 to 20°C (Rossi et al., 2001), is 32 °C for conidiation (Xu et al., 2003), and 28-29 °C for disease infection (Rossi et al., 2001). When the soil moisture is above 30%, and in the presence of free moisture due to rainfall, perithecia of *F. graminearum* mature and release ascospores. Ascospores in the field are the primary source of inoculum (Xu et al., 2003). The large number of ascospores released in a conducive environment can cause FHB epidemics since none of the wheat varieties are completely resistant to FHB. Based on long-term field observations, a disease forecasting model was developed for farmers to predict disease development based on environmental conditions. Reducing the level of initial infection is an important measure to lower the impact of the disease in crop production.

FHB is a monocyclic disease, i.e. it has one disease cycle in one growing season. The airborne ascospores as primary source of inoculum land on the flowering heads of wheat, germinate, penetrates the host through stomata, and produces subcuticular hyphae that grows along the stomatal rows (Pritsch et al., 2000). The fungus then colonizes the glumes and parenchyma cells thereby causing discoloration of the infected spikelets. *F. graminearum* does not kill plant cells during initial infection, but do so when it grows intracellularly (Brown et al., 2010). Therefore, the fungus is considered as a hemibiotrophic pathogen. The fungus overwinters as mycelia or chlamydo spores on infected crop debris (Guenther and Trail, 2005). It

also produces perithecia as the sexual overwintering structure, which when exposed to conducive weather (warm and humid) release ascospores during next growing season. FHB is best controlled by an integrated approach, which combines use of fungicides and moderately resistant varieties. Growing resistant wheat varieties is the most reliable measure for controlling the disease since they help in reducing the disease severity and mycotoxin accumulation in the crop (Gilbert et al. 2000).

Resistance to FHB is a complicated system which is highly affected by genotype and genotype-by-environment (G x E) interactions (Campbell and Lipps 1998; Yang et al., 2005; Chu et al., 2011). It is also a quantitative trait that is governed by multiple major and minor genes. Resistance to FHB is categorized into five different types (Schroeder & Christensen, 1963; Mesterhazy et al. 1995). Type I is resistance to initial infection; type II is resistance to fungal spread from the infected spikelets along the rachis of a spike; type III is resistance to kernel infection; type IV involves tolerance; and type V is the resistance to mycotoxins. Of the different types of disease resistance, type I is more difficult to assess (reviewed in Buerstmayr et al., 2009). Type I resistance is measured as disease incidence (percentage of infected spikes in sprayed or naturally inoculated experimental units) or as disease severity (the percentage of infected spikelets per inoculated spike). Inoculation for type I resistance assessment is usually done by spraying plants with a spore suspension in order to mimic the natural way of disease infection. Type I resistance can also be studied by spreading *F. graminearum*-spoiled corn seeds evenly among plants on the soil surface. Inoculation by this method uses ascospores produced on the spoiled corn as the primary inoculum for infection.

Resistance to FHB is highly correlated with different morphological and developmental features of the plant like plant height (Mesterhazy, 1995; Paillard et al., 2004; Schmolke et al.,

2005; Draeger et al., 2007; Klahr et al., 2007), heading time (Miedaner et al., 2006; Klahr et al., 2007; Wilde et al., 2007), flower opening (Gilsinger et al. 2005), compactness of ears (Schmolke et al. 2005) and length of awns (Buerstmayr et al., 2012). There are several reports about the association of plant height with the level of FHB severity in wheat. Presence of height reducing genes *Rht-B1b* and *Rht-D1b* was associated with reduction in type I resistance (Gosman et al., 2008). Similarly, the presence of *Rht* alleles in short near isogenic lines showed increased FHB severity (Yan et al., 2011). However, when these near isogenic lines were physically elevated to the level where the spikes were at the same height, the differences in type I resistance among them disappeared, indicating that the association between these traits are mainly due to the microclimate created by plant height.

There are several sources of FHB resistance that are involved in reducing disease severity and mycotoxin accumulation (Gilbert et al. 2000). These sources of resistance are divided into three groups based on their types and origin (Gilbert et al. 2000; Bai et al. 2004). The first group comprises Asian spring wheat varieties including the Chinese cultivar ‘Sumai-3’ and ‘Wangshuibai’ (Derivative of ‘Ning’) (Lin et al. 2004; Lin et al. 2006), Japanese ‘Nobeoka Bozu’ (Mesterhazy, 1995), ‘Shinchunaga’ (Bai et al. 2001) and ‘Nyu Bai’ (Liu et al., 2003). The second group comprises Brazilian spring wheat cultivars ‘Frontana’ (Steiner et al., 2004) and ‘Encruzilhada’ (Bai et al 2004). The third group comprises winter wheat cultivars ‘Praag8’ and ‘Novokrumka’ (Snijders, 1990). Beside these, ‘Ernie’ (McKendry 1995), ‘Truman’ (McKendry et al., 2005) and ‘Goldfield’ (Gilsinger et al., 2005) are some cultivars from the United States that are moderately resistant to FHB and have been used for some U.S. breeding programs. Among these wheat cultivars, Sumai-3 is the most commonly used germplasm since it contains a major QTL, *Fhb1* (syn: *Qfhs.ndsu-3BS*), for FHB resistance. Waldron et al. in (1999) mapped

Fhb1 on chromosome 3BS from a population derived from the cross between ‘Sumai-3’ and ‘Stoa’. Since then, several studies have been conducted to verify this QTL (Anderson et al., 2001; Zhou et al., 2002). *Fhb1* confers a moderate level of resistance for FHB and explains 15 to 60% of phenotypic variation for FHB spread (Jin et al., 1997; Buerstmayr et al., 2002; Somers et al., 2003; Zhou et al., 2002; Yang et al., 2003; Anderson et al., 2011). *Fhb1* was also found in several other Chinese wheat cultivars like Ning894037 (Shen et al., 2003), Ning7840 (Bai et al., 1999, Zhou et al., 2002), Huapei57-2 (Bourdoncle et al., 2003). Recently, *Fhb1* has been cloned through map-based cloning and characterized using mutational analysis, gene silencing and transgenic expression (Rawat et al. 2016). The pore-forming toxin-like (*PFT*) gene was found at this locus associated with FHB resistance and presumably encodes a chimeric lecithin with two agglutinin domains and ETX/MTX2 toxin domain. This gene is proposed to confer FHB resistance by recognizing fungus-specific carbohydrates and causing toxicity to the fungus, i.e., arresting fungal growth by interacting with the fungal cell wall.

Unlike type II resistance, type I resistance is the resistance against the initial fungal colonization in the spike of the crop. QTL that confer type I resistance have been identified on chromosome 5A (Buerstmayr et al. 2003; Steiner et al. 2004 Yang et al., 2005; Chen et al., 2006; Lin et al. 2006;), 4B (Lin et al. 2006), 4A (Steed et al. 2005), 3BS (Yang et al., 2005; Chen et al., 2006; Lin et al., 2006), and 7B (Gilsinger et al., 2005). QTL *Qfhs.ifa-5A* (Buerstmayr et al., 2002; Buerstmayr et al., 2003; Buerstmayr et al., 2009; Schweiger et al., 2013) and *Fhb2* are located on chromosomes 5A and 6BS, respectively, and are considered as other important QTL besides *Fhb1*. *Qfhs.ifa-5A* is associated with type I resistance thereby inhibiting the rate of initial infection. Although it has some contribution to type II resistance, it is mainly associated with type I resistance. Depending on the resistance sources, *Qfhs.ndsu-3B* and *Qfhs.ifa-5A* together

explain 40-48% of phenotypic variance while *Fhb2* explains 21% of the phenotypic variance (Yang et al., 2003).

Although several QTL have been identified to be associated with FHB resistance, there is still need for identifying more DNA markers for marker-assisted selection (He et al., 2013). Recent success in cloning *Fhb1* is an important achievement for understanding the function of the gene associated resistance to FHB (Rawat et al., 2016). Its sequence can also be used for development of a perfect marker for marker-assisted selection of FHB resistance in breeding, gene pyramiding and genetic engineering. This will provide an ecological and economical solution for reducing the impact of FHB disease.

Crown rot (CR) and Common Root Rot (CRR) of Wheat

CR and CRR are important root diseases of wheat. CRR is mainly caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (S. Ito & Kuribayashi) Drechsler ex Dastur). This pathogen is also responsible for spot blotch. In CRR, the fungus infects the subcrown internode and causes fairly minor injury on plants to significant yield loss (Draper M.A. 2000).

Crown rot (CR), also called dryland foot rot, is a disease complex commonly caused by *Fusarium culmorum* (W.G. Sm.) Sacc., *F. pseudograminearum* (= *Gibberella coronicola*), and *F. graminearum* Schwabe (= *G. zae* (Schwein.) Petch) (Smiley and Patterson 1996; Paulitz 2006). CR damages the crown region and causes plants to mature early and spikes to become white with no or incomplete grain filling (Stack and McMullen, 1995). Whole plants or some tillers get stunted. These diseases can affect wheat plants of all growth stages and cause yield losses by reducing the number of standing crops, tillers and kernel weights.

CRR is a seed and soil borne disease. The pathogen *B. sorokiniana* overwinters as conidia and mycelia in soil and crop residues (Wiese, 1987). Onset for CRR is at the young seedling stage either by inoculum in soil or seed. During the process of infection, *B. sorokiniana* first forms appressoria, which penetrate the epidermal cells using infection pegs (Huang and Tinline, 1967). Once the fungus penetrates the host cells, it produces several branches. The fungus also produces the enlarged ball like structure at the tip of the infection peg in the cell lumen. The fungus infects the host by producing running hyphae and coarse, short celled hyphae. The running hyphae grows inter- and intra-cellularly in the epidermis, cortex, and occasionally in the stele, while the coarse, short-celled hyphae produces infection pegs that penetrate the neighboring cells. Some of the infected roots contain completely occluded xylem vessels. At the site of infection (outer layer of coleoptile or leaf sheath below the soil), necrotic lesions which range from light brown (in less susceptible genotypes) to dark brown lesion (in highly susceptible genotypes) can be observed. In severe cases, several small lesions coalesce into long necrotic lesion which may collapse the whole seedling (Wiese, 1987). In adult plants, these symptoms are seen in the seminal roots, crown root and subcrown internode (SCI). Among them, lesions in SCI are the most common symptom of CRR (Sallans and Tinline, 1965). When severely infected, the whole SCI and roots become dark in color. These necrotic lesions can sometimes extend up to the crown region giving crown rot symptoms along with stunted growth and reduced yield due to chaffy grains or empty spikes.

CR is also a seed and soil borne disease. Fusarium species overwinters as mycelia, macroconidia, or thick walled chlamydospores (Cook, 1981; Paulitz, 2006). *F. culmorum* overwinters by producing viable chlamydospores, while *F. graminearum* overwinter as mycelia inside the infected non-decayed crop residues. CR affects the wheat crowns about 2-3 cm below

the soil surface (Cook, 1981; Wiese, 1991). The fungus infects the coleoptile by penetrating through the stomata and between epidermal cells (Malalasekera et al., 1973) and proliferating in the parenchymatous tissue (Pisi and Innocenti, 2001). Histological studies done by Beccari et al. (2011) with wheat seedlings inoculated with *F. culmorum* indicated that within 24 hours post inoculation (hpi), the fungus profusely colonizes the rhizodermal cell layer and first layer of cortex cells intra and inter-cellularly. At 48 hpi, the fungus proliferates by developing a dense mycelial network inside the lumen of cortical cells near the site of the infection, and by 120 hpi, the inoculated site starts producing conidiophores and conidia. A study of the infection of wheat by *F. graminearum* done by Stephens et al. (2008) showed that there are three phases in the infection process: (i) spore germination and development of a superficial hyphal matrix at the site of infection at 2 days post inoculation (dpi), (ii) infection and colonization of the inner adaxial epidermis of the first leaf sheath at 14 dpi, and (iii) colonization of vascular tissue and pith of the crown at 35 dpi.

CRR and CR can be controlled by integrated crop rotation, cultural practice, chemical treatment and growing resistant or tolerant crop cultivars. Crop rotation with non-cereal crops such as soybeans are found to be effective in controlling root rot and FHB due to *F. graminearum* (Burgess et al., 2001; Cook, 2010), and rotation with flax is known to reduce the amount of viable inoculum of *B. sorokiniana* (Conner et al., 1996). Cultural practices like burning the crop stubbles and inverting the soil and surface residue by harrowing during the fall season is highly recommended for disease control (Burgess et al., 2001). It is also important to maintain balanced soil fertility and moisture to support healthy growth of the crops (Moya, 2011). Excessive nitrogen promotes vegetative growth and induces excessive tillering that cannot be supported by the water stored in the soil, thereby causing the plant to experience water stress

which favors root and crown rot development (Papendick and Cook, 1974; Cook 1980; Burgess et al., 2001; Cook, 2010; Stein, 2010).

Seed treatment with fungicides along with the use of healthy seeds reduce the disease in seedlings (Stein 2010; Cook 2010). Chemicals like Difenoconazole + Mefenoxam (Dividend Extreme 7.73%: 1.87%) have been used to control the root rot diseases of wheat (<https://www.ag.ndsu.edu/extplantpath/publications-newsletters/fungicides/barley-oat-rye-wheat/view>). Biological control agents like *Chaetomium sp.*, *Idriella bolleyi*, and *Gliocladium roseum* are proven to antagonize *B. sorokiniana* (Knudsen et al., 1995). Similarly, CR caused by *F. culmorum* and *F. graminearum* could be controlled by suppressing its sporulation in wheat straw with the use of isolates of saprophytic fungi *Clonostachys rosea* isolated from necrotic tissue of other crops (Luongo et al., 2005).

Using resistant or tolerant cultivars is considered the most effective and efficient measure to reduce the impact of root rot diseases (Burgess et al., 2001; Cook, 2010). Leader, ND 722, AC Cadillac, HJ 98, Argent and Scholar are some of the wheat cultivars that are resistant to CRR (Tobias et al., 2009). Bailey et al. (1995) identified CRR resistance genes derived from *Aegilops ovata*, which are different than the genes that are usually present in most of the spring wheat cultivars. These resistance genes were transferred into wheat by crossing *Aegilops ovata* with *Triticum aestivum* using the Chinese Spring *ph1b* deletion to enhance genetic recombination.

CR resistance is very complicated and it is found to be either associated with or unrelated to Fusarium head blight (FHB) resistance (Smiley et al., 2003). Resistance to CR is a quantitative trait where multiple genes are associated with the disease resistance. Several quantitative trait loci (QTL) were reported to be associated with CR resistance. Bovill et al. (2006) reported QTL for seedling resistance on chromosomes 2B, 2D, and 5D in a population

derived from a cross between ‘W21MMT70’ (partial resistance) and ‘Mendos’ (susceptible). Another study conducted by Collard et al. (2006) reported QTL on chromosomes 1D and 1A. These QTL were suggested to be different from each other and considered suitable for gene pyramiding to provide durable resistance to CR in wheat (Bovill et al., 2006).

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CHAPTER 1: COMPARATIVE STUDY OF COMPATIBLE AND INCOMPATIBLE INTERACTIONS BETWEEN BARLEY AND THE SPOT BLOTCH FUNGUS

BIPOLARIS SOROKINIANA

Abstract

Spot blotch, caused by *Bipolaris sorokiniana*, is an important foliar disease of barley. However, cellular and molecular mechanisms of the host-pathogen interaction in this pathosystem are not well understood. The objective of this study was to investigate the histopathological events of compatible and incompatible interactions between different genotypes of barley and different pathotypes of *B. sorokiniana*. A hypersensitive response (HR) occurred in resistant barley genotypes at the time of appressoria formation. Significant differences were also observed between compatible and incompatible interactions after fungal penetration. The fungus developed multicellular globular infection hyphae (IH) in the epidermal cells after penetrating the cell wall, but infected epidermal cells were alive till 16 HPI, suggesting its biotrophic nature in the early infection stage. After 20 HPI, the fungus grew extensively in the susceptible hosts compared to the restrained growth in the resistant hosts. In susceptible plants, the tip of IH was found to grow ahead of the dead tissue. This constituted biotrophic invasion of the live tissue during which the fungus proliferated intracellularly in the mesophyll cells. As expected, the amount of H₂O₂ accumulation measured by the 3,3'-Diaminobenzidine (DAB) staining method and the fungal biomass were significantly higher in the susceptible hosts than in the resistant hosts.

Introduction

Spot blotch, caused by the ascomycetous fungus; *Bipolaris sorokiniana* [Sacc. in Sorok.] Shoem (Teleomorph: *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur), is an economically important disease of barley in the Upper Midwest of the United States and the Prairie Provinces

of Canada. In a conducive environment, spot blotch can cause yield loss of 16 to 33 % (Clark 1979, Fetch and Steffenson, 1999). The fungus is also a causative agent of common root rot and black point of wheat and barley (Wiese, 1977). Leaves infected with spot blotch show oval to circular necrotic lesions surrounded by chlorotic halos and lesion sizes range from pin point to almost 8mm x 3.2mm with a chlorotic margin of 0.5 to 1.0 mm wide (Fetch et al. 1999). Spot blotch can be controlled by spraying fungicides, however, growing resistant cultivars is considered as the most sustainable and effective approach to control the disease (Mehta and McNab, 1998).

Four different pathotypes (0, 1, 2 and 7) of *B. sorokiniana* have been identified based on their pattern of virulence on three barley differentials (ND5883, Bowman, and ND B112) (Valjavec-Gratian & Steffenson 1997b; Leng et al. 2016). Pathotype 0 (representative isolate ND93-1) has low virulence on all three barley differentials; pathotype 1 (representative isolate ND85F) has high virulence on ND5883 and low virulence on Bowman and ND B112; pathotype 2 (representative isolate ND90Pr) has high virulence on Bowman and low virulence on ND5883 and ND B112 (Valjavec-Gratian & Steffenson 1997b). Pathotype 7 (representative isolate ND4008) was recently identified and exhibits high virulence on all three barley differentials (Gayawli, 2010).

Since the first genetic study of spot blotch resistance was conducted by Wilcoxson et al. (1990), a number of genes or QTL for susceptibility/resistance to spot blotch have been identified (Steffenson et al. 1996; Bilgic et al. 2005; Bilgic et al. 2006; Bovill et al. 2010; Grewal et al. 2012; Haas et al. 2016; Berger et al. 2013; Roy et al. 2010; Zhou and Steffenson 2013; Leng et al. 2016; Wang et al. 2017). A dominant gene in barley cv. Bowman confers susceptibility to pathotype 2 isolate ND90Pr (Valjavec-Gratian et al., 1997a) and a

corresponding virulence gene (*VHv1*) in pathotype 2 has been identified (Zhong et al., 2002; Condon et al. 2013), suggesting an inverse gene for gene interaction as described by Friesen et al. (2010) and Liu et al. (2017) in other cereal pathosystems (tan spot and *Stagonospora nodorum* blotch). Three quantitative trait loci (QTL), *Rcs- qtl-1H- 11_10764*, *Rcs- qtl- 3H- 11_10565* and *Rcs- qtl- 7H- 11_20162*, have been reported to be the associated with seedling and adult plant resistance to isolate ND85F (Zhou and Steffenson, 2013). More recently, a single dominant gene conferring seedling resistance to isolate ND4008 has been identified in barley accessions PI235186, PI592275, and PI643242 (Leng et al., 2016), indicating that the genetic mechanism for resistance to this isolate may be different from those for resistance to the other pathotype isolates (ND90Pr and ND85F). Interestingly, 24 barley accessions exhibiting seedling resistance to ND4008 also showed seedling resistance to ND85F and ND90Pr (Leng et al., 2016).

B. sorokiniana is described as a hemibiotrophic fungus, which has a short biotrophic phase, followed by a necrotrophic phase during plant infection (Kumar et al., 2002; Schäfer et al., 2004; Ibeagha et al., 2005). The early (biotrophic) infection phase involves germination of conidia, development of appressoria, penetration of the cuticle and host cell wall and development of finger-like globular hyphae inside the invaded living epidermal cells (Schäfer et al., 2004; Rodríguez-Decuadro et al., 2014). The later (necrotrophic) infection phase is more destructive and includes release of toxins and inter- and intracellular proliferation of fungal hyphae in epidermal and mesophyll tissue of the plants. Upon fungal penetration, the host produces cell wall apposition (CWA) at the site of penetration and/or the pre-penetration hypersensitive reaction (pre-PHR) is triggered as the first line of defense, followed by post penetration HR (post-PHR) in invaded cells. Previous studies showed that the host resistance is based on its ability to counterattack the necrotrophic phase of *B. sorokiniana*, since no significant

differences in the biotrophic phase of the infection were observed between susceptible and resistant plants (Schäfer et al., 2004; Ibeagha et al., 2005).

The plant cells under pathogen attack often experience an oxidative burst and produces reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxides, nitric oxide etc. (Nanda et al., 2010). ROS are associated with HR, fortification of the host cell walls, as well as signal transduction for activation of the defense induced genes in the attacked cells. They are also involved in phytoalexin production and acts as an antimicrobial agent (Gadjev and Gachev, 2008). Although ROS are important for the plant to fight against the pathogen attack, they are equally toxic to the plant itself. ROS have been proposed to be associated with salicylic acid (SA) in several HR systems. SA stimulates or suppresses programmed cell death (PCD) based on the level of ROS, thereby contributing in developing a boundary between the dead and live tissues (Alvarez, 2000; Gadjev and Gachev, 2008). In order to maintain its signaling functions yet prevent toxicity, it is very important to balance the levels of H_2O_2 and other ROS. Therefore, the plant also employs an anti-oxidant system where several enzymes are deployed to neutralize these ROS. ROS like H_2O_2 can be neutralized by antioxidant enzymes such as peroxiredoxin, glutathione peroxidase, etc. and nonenzymatic anti-oxidants like ascorbate, glutathione, tocopherol and carotenoids (Gadjev and Gachev, 2008).

H_2O_2 is one of the extensively studied ROS in host-pathogen interactions (Gadjev and Gachev, 2008, Rodríguez-Decuadro et al., 2014). It is the most stable ROS, mobile due to high membrane permeability, and is also considered the most prominent signaling molecule. The presence of H_2O_2 in plants can be detected by using 3-3' Diaminobenzidine (DAB). In the presence of peroxidase, DAB interacts with H_2O_2 and polymerizes locally and instantly. H_2O_2 was reported to be accumulated during the early cellular response of the plant to pathogen attack

(Rodríguez-Decuadro et al., 2014). H₂O₂ accumulation is associated with both resistance and susceptibility in the host. Various studies showed that H₂O₂ accumulation is linked to HR and cell wall fortification as an early defense that arrests fungal growth upon penetration of the host by a biotrophic fungus like *B. graminis* f. sp. *hordei* (Hückelhoven et al., 1999; Hückelhoven et al., 2001; Kumar et al., 2002; Schäfer et al. 2004). With necrotrophic fungi, H₂O₂ accumulation helps to kill host cells which ultimately benefit fungal growth (Govrinet al., 2000; Schäfer et al. 2004). With a hemibiotrophic pathogen like *B. sorokiniana*, H₂O₂ accumulation has two contrasting functions. In the early biotrophic phase, H₂O₂ accumulation is associated with cell wall apposition, pre-PHR (pre-penetration hypersensitive response), post-PHR in epidermal cells and encapsulation of the fungus within the infected epidermal cell, all of which serve to restrict fungal penetration and growth (Schäfer et al. 2004). In the late stage of infection (necrotrophic phase), H₂O₂ accumulation is associated with the killing of a large number of epidermal and mesophyll cells (Rodríguez-Decuadro et al., 2014). Although H₂O₂ accumulation is presumably associated with the killing of host cells in the necrotrophic growth stage, it is ambiguous if H₂O₂ accumulation is the cause or the consequence of fungal spread (Schäfer et al. 2004).

All of the previous studies of the host-pathogen interaction in the barley-*B. sorokiniana* pathosystem have been conducted by using a single pathotype (Clay et al., 1997; Felle et al., 2007; Ibeagha et al., 2005; Kumar et al., 2001; Rodríguez-Decuadro et al., 2014; Schäfer et al. 2004). Till date, four different pathotypes of *B. sorokiniana* have been identified, based on their reaction on the barley differentials carrying different dominant susceptibility and resistance genes for spot blotch resistance (Valjavec-Gratian et al., 1997a; Zhou and Steffenson, 2013; Leng et al., 2016). We hypothesized that there exist differential interactions between these pathotypes and their corresponding resistant and susceptible barley genotypes. Thus, the

objective of this study was to characterize histopathological differences and similarities between compatible and incompatible interactions of barley with *Bipolaris sorokiniana* at multiple time points.

Materials and Methods

Pathogen Isolates and Hosts

Isolates ND93-1, ND85F, ND90Pr, and ND4008 belonging to pathotypes 0, 1, 2 and 7 of *C. sativus*, respectively, were used in the study. For fluorescence microscopy, the fungal isolates were transformed by using a vector (pCA56) containing a gene *mRFP1* expressing the red fluorescent protein (RFP) (Andrie et al., 2005). Fungal transformation was done by following the polyethylene glycol (PEG)-mediated transformation method as explained by Liu and Friesen (2012). The transformants that had high expression of RFP and exhibited the same phenotypes as their corresponding wild-types were chosen for the histopathological study (Figure 1.1). These RFP-expressing isolates were used to inoculate four barley genotypes (Bowman, ND5883, PI235186, and PI356746) (Table 1. 1). Bowman and ND5883 were used to observe the infection process of pathotypes 0, 1 and 2; whereas PI235186 and PI356746 were used to study the infection process of pathotype 7 (Table 1.1).

Table 1.1. Pathotypes of *B. sorokiniana* and barley cultivars/accessions used in the study

Pathotypes	Isolates	Resistant barley genotypes	Susceptible barley genotypes
0	ND93-1	Bowman ¹ and ND5883 ¹	--
1	ND85F	Bowman	ND5883
2	ND90Pr	ND5883	Bowman
7	ND4008	PI235186 ²	PI356746 ²

¹Barley genotypes used as differential lines for identifying different pathotypes of *B. sorokiniana* (Valjavec-Gratian and Steffenson, 1997b).

²Barley accessions obtained from the USDA National Small Grains collection, Aberdeen, ID.

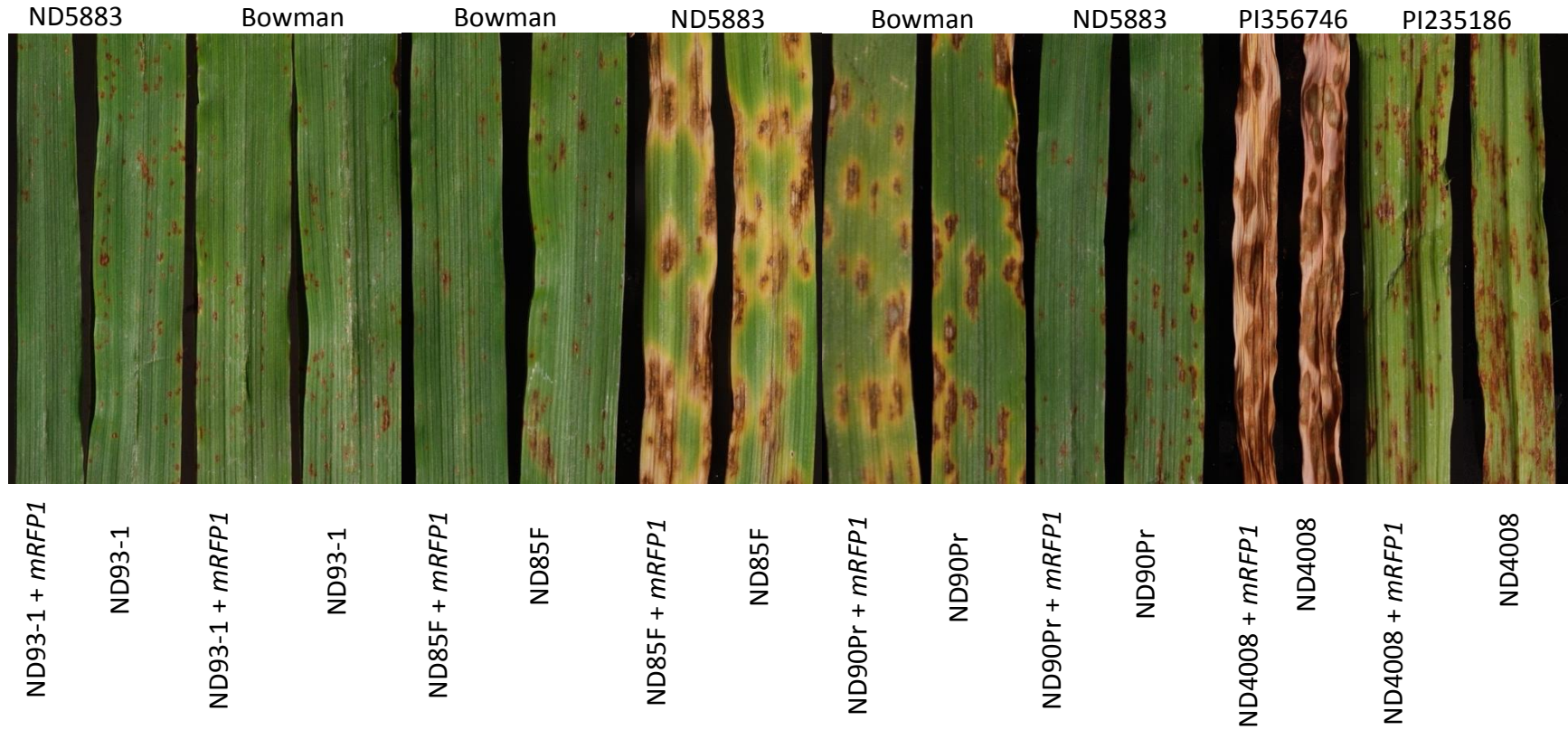


Figure 1.1. Disease symptoms of barley genotypes inoculated with different *B. sorokiniana* pathotypes. Plants were inoculated when second leaves were fully expanded. Wild types and red fluorescent protein (RFP)-expressing transformants (with + *mRFP1*) of isolates ND90Pr, ND85F and ND4008 were used to inoculate resistant and susceptible barley genotypes (Table 1.1). The barley genotypes are shown on the top, while the wild type isolates and their corresponding transformants are indicated at the bottom. The pictures were taken at 168 hours post inoculation (HPI). No differences in infection responses were observed between the wild type and transformant from the same isolate on the same barley genotype.

Planting, Inoculation and Sample Collection

Planting for this experiment was done as described by Leng et al. (2016). Three seeds of each barley genotype were planted in a D40 Deepots (6.4 cm diameter and 25.4 cm deep; Stuewe and Sons, Tangent, OR) filled with potting mix media (Pro-Mix LP15; Premier Tech Horticulture, Quakertown, PA) and slow-release fertilizer (Osmocote Plus 15-9-12 N-P-K plus minors; Everris Inc., Dublin, OH) was added. The experiment was conducted in a completely randomized design (CRD) with three replications (pots) per treatment. Plants were grown in a greenhouse at $23 \pm 2^\circ\text{C}$ with light duration of 14 hours for 12-14 days and inoculated when the second leaves were fully expanded.

The fungal isolates were grown on minimum media, and spores were harvested from the 7 day old cultures by adding distilled water, scraping the surface of the plate, and filtering the spores/mycelia mixture through one layer of miracloth (EMD Millipore Corporation, Temecula, CA). The spore suspension collected was quantified using a hemocytometer and brought to a final concentration of 5000 spores per ml. Tween-20 was added to the spore suspension at a ratio of 1 μl /ml. The prepared spore suspension was used to spray inoculate the 12-14 days old barley seedlings using a Preval Power Unit (Chicago Aerosol, Coal City, IL). Inoculated plants were kept in the dark in a misting chamber for 24 hours (with a misting cycle of 15 seconds every 6 minutes), and then returned to the greenhouse. Leaf samples were collected at 4, 8, 12, 16, 20, 24, 36, 48, 72, and 168 hours post inoculation (HPI) for fluorescence microscopy and DAB staining. For qPCR, leaf samples were collected at 12, 24, 36, 72 and 168 HPI.

Fluorescence Microscopy

The leaf samples collected at each of time points mentioned above were mounted on Fluoromount-G and examined under a Zeiss AxioImager M2 fluorescence microscope with Zeiss

ApoTome 2 module (Carl Zeiss AG, Oberkochen, Germany), using software AxioVision rel. 4.8. software. Different channels of fluorescent light were used to capture images of the plant and fungal tissues (Table 1.2).

Table 1.2. Fluorescent light channels used in this experiment

Light Channels	Tissue	Excitation (nm)	Beamsplitter (nm)	Emission (nm)
Blue (<i>DAPI</i>)	Plant	335-383	395	420-470
Green	Plant	450-490	495	500-550
Red (<i>DsRed</i>)	Fungus	538-562	570	570-640
Magenta (<i>Cy 5</i>)	Plant	625-655	660	665-715

Some of the leaf samples were also observed for pathogen structures inside the epidermal and mesophyll cells by using a Zeiss AxioObserver Z1, inverted microscope with LSM700 laser scanning unit, 405 solid state laser using red fluorescent light (excitation: 555nm and emission: 580nm) and green fluorescent light (excitation: 405nm and emission: 490nm).

3, 3'-Diaminobenzidine (DAB) Staining

The leaf samples collected at each of the time points were submerged in 1mg/ml aqueous DAB solution at pH 3.8 and incubated for four hours in dark at room temperature according to Tada et al. (2004). DAB stained leaves were then clarified and fixed by boiling in 95 % ethanol for 10 minutes at 75°C and stored in 50% glycerol at 4°C following the method of Thordal-Christensen et al. (1997). For each time point, three leaves were examined for the presence of DAB staining and/or presence of spores. The leaf samples were mounted on 50% glycerol. An Olympus BX51 microscope (Olympus, Center Valley, PA) and CCD camera (Dignosatic Instruments, Inc., Sterling Height, MI) was used to take photographs of the infected tissues. Each infection area was recorded using Image J software (<http://imagej.nih.gov/ij/>).

Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR, leaf tissue samples were collected at five time points for each interaction with three biological replicates for each time points. The genomic DNA was extracted and qPCR was performed to quantify the total fungal DNA. For each sample, three technical replicates were used. The standards were prepared using the genomic DNA of ND90Pr with five-fold dilution series with concentrations ranging from 50ng – 0.13pg. qPCR was done using a reaction volume of 20µl containing 10 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA), 80ng of genomic DNA and 0.5 µM of each primer (Cs-RT-Actin Forward Primer: GTATGGGCCAAAAGGACTCA and Cs-RT-Actin Reverse Primer: CACGCAGCTCGTTGTAGAAG). Amplification was done using initial denaturation at 95°C, for 5 min followed by 40 cycles of 15 sec of denaturation at 95°C and annealing/extension at 60°C for 1 min. Finally, a melt curve analysis was conducted between 60°C and 95°C with an increment of 0.5°C after every 5 sec. All the qPCR were performed in a Bio-RAD Laboratories C1000 thermocycler with CFX 96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The absolute quantity of total fungal DNA for each time point was calculated using their C_q values.

Results

Fungal Invasion

In all the interactions, spores started to germinate from both or either ends at 4 hours post-inoculation (HPI). The germ tubes branched and formed appressoria at the tip during 8 HPI (Figures 1.2, 1.3, 1.4 and 1.5). Sometimes, the germ tubes continued growing after the appressoria formation. At 12 HPI, the appressoria started to penetrate either in grooves between two cell walls or directly through the stomata.

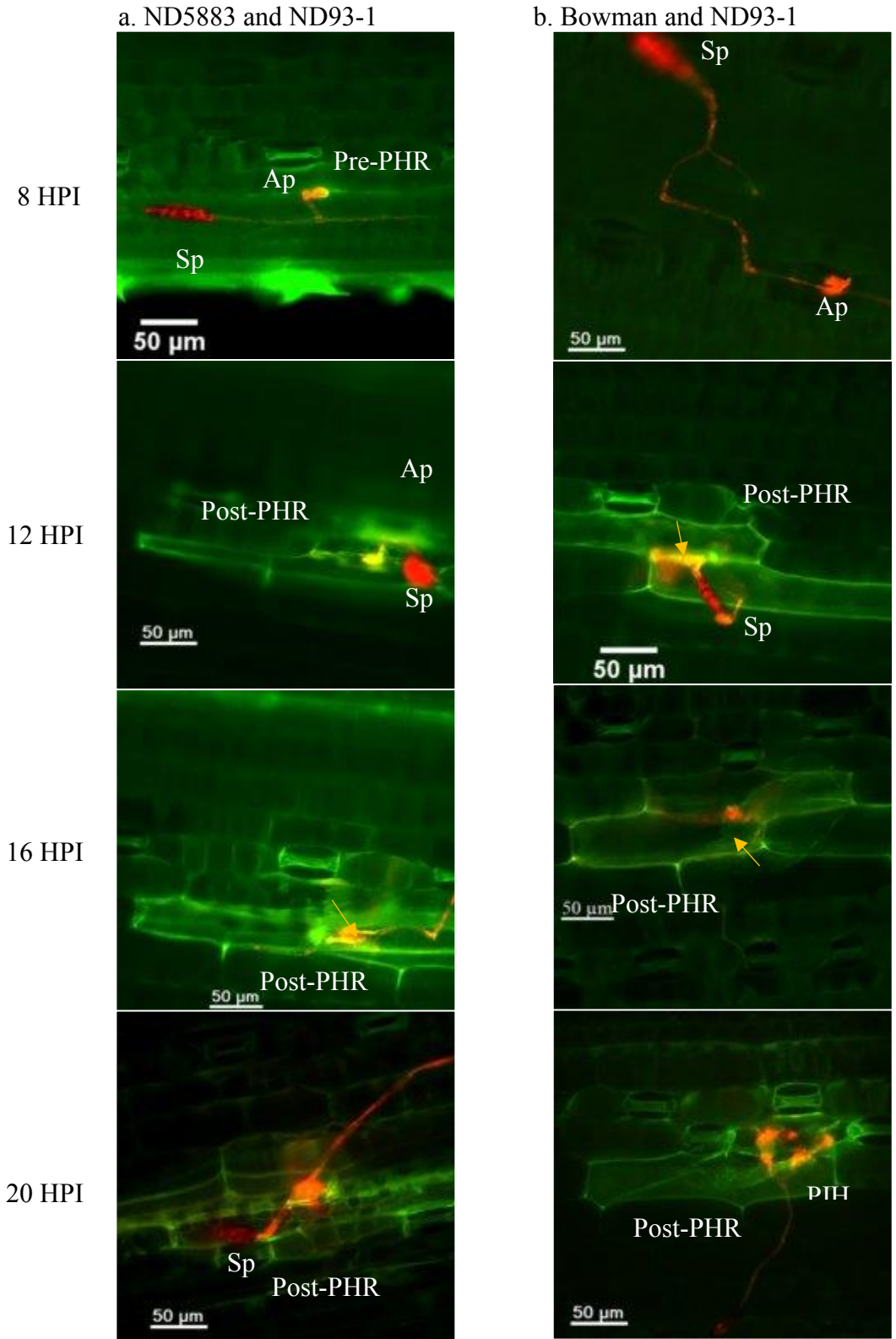


Figure 1.2. Histopathology of incompatible interactions between isolate ND93-1 (pathotype 0) and barley genotypes ND5883 (a) and Bowman (b).

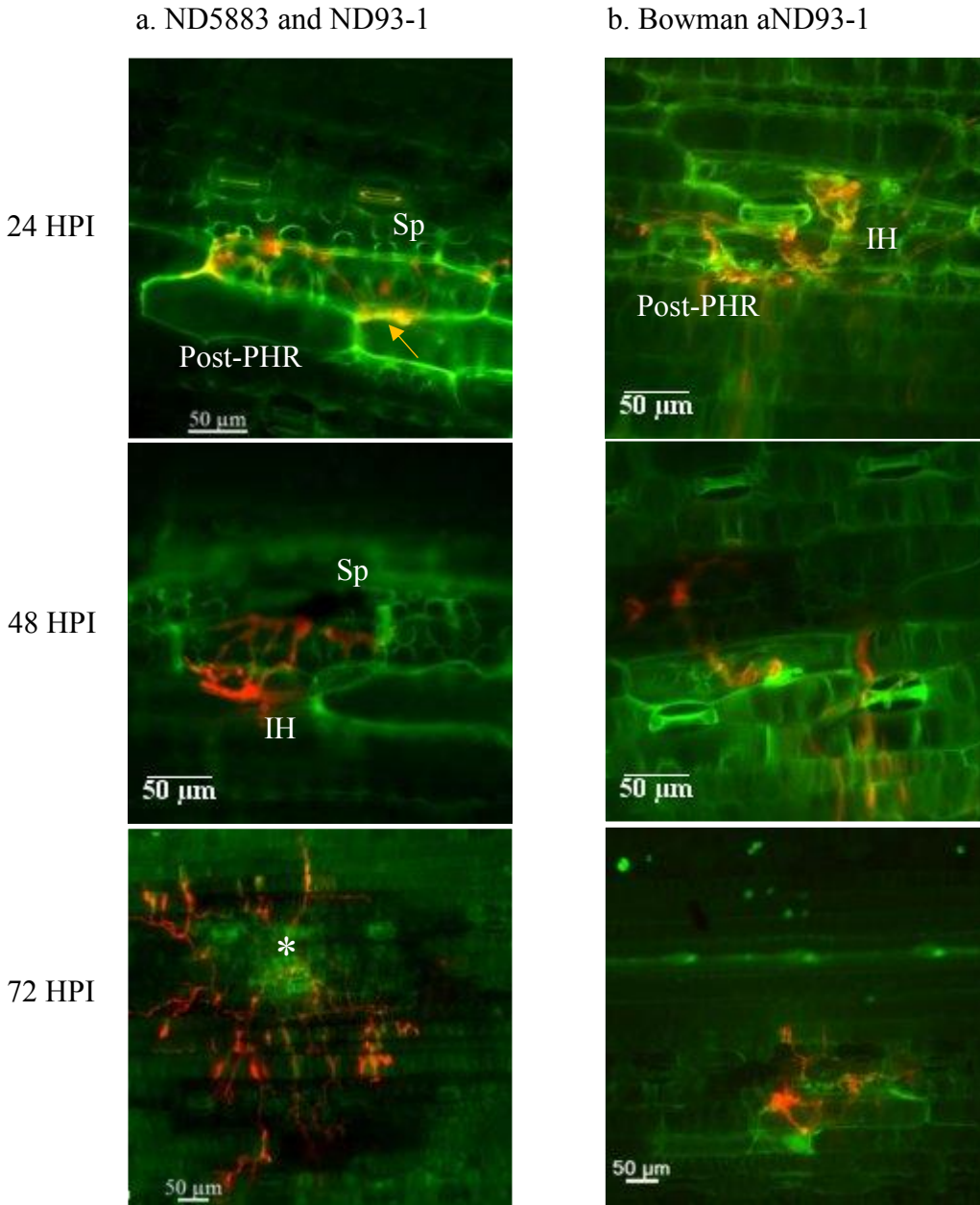


Figure 1.2. Histopathology of incompatible interactions between isolate ND93-1 (pathotype 0) and barley genotypes ND5883 (a) and Bowman (b) (Continued). The fungal spores (Sp) germinated, formed appressoria (Ap) at the tip of the germ tube (Gt), and triggered a pre-penetration hypersensitive response (Pre-PHR) at 8 hours post-inoculation (HPI). The fungus started to penetrate the epidermal cells (Epi) and triggered a post-penetration hypersensitive response (Post-PHR) at 12 HPI. The fungus developed primary infection hyphae (PIH) in the epidermal cells, and the host post-PHR was observed at the site of penetration at 16 HPI. In both barley genotypes, autofluorescence around infection sites increased from 16 HPI onwards and the infection hypha (IH) grew to the neighboring cells. However, the fungal proliferation was constrained to the dead tissues. *Yellow arrows show the site of penetration. White asterisks represent dead plant cells due to the necrosis.

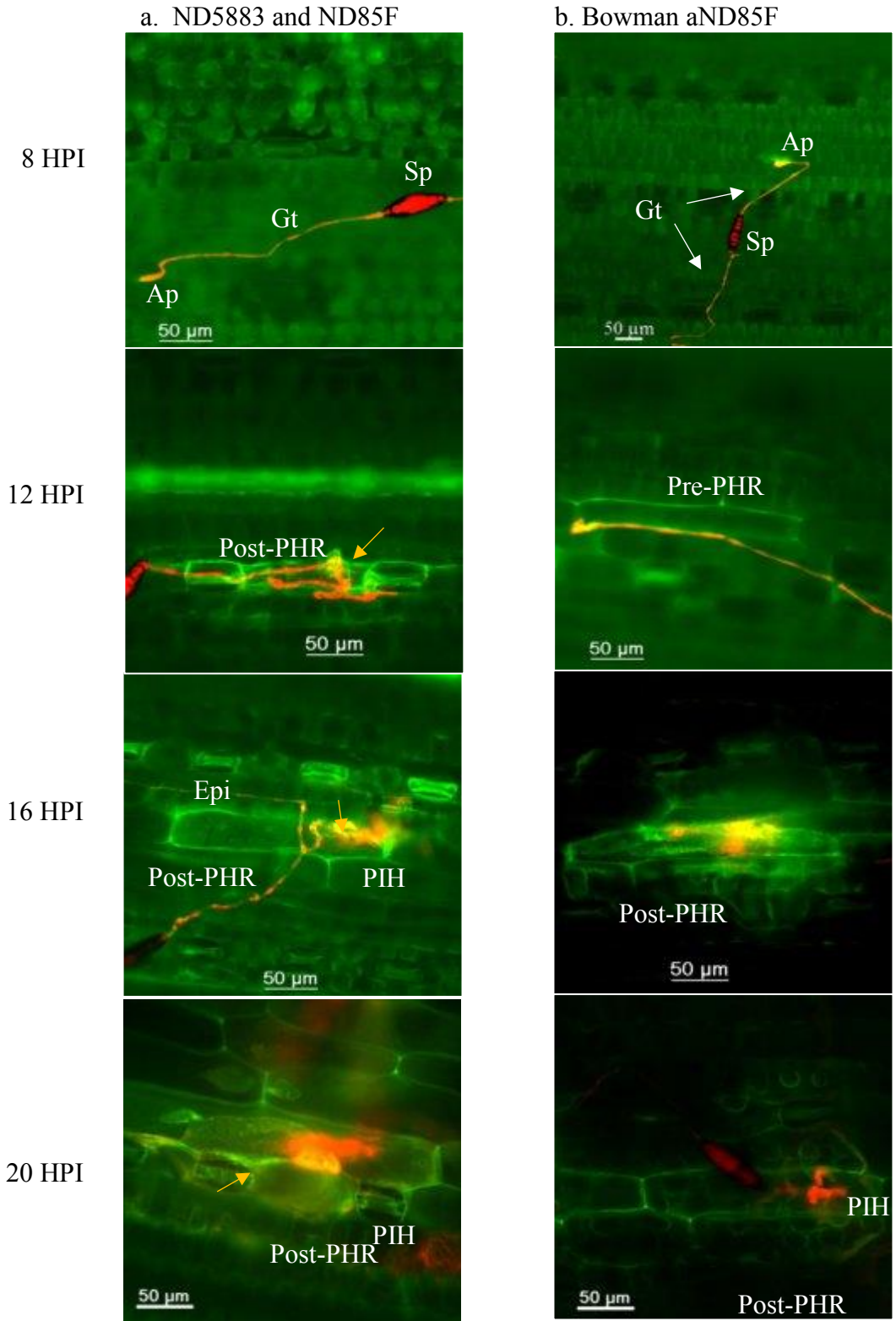


Figure 1.3. Histopathology of the interactions between isolate ND85F (pathotype 1) and two barley genotypes (ND5883 and Bowman).

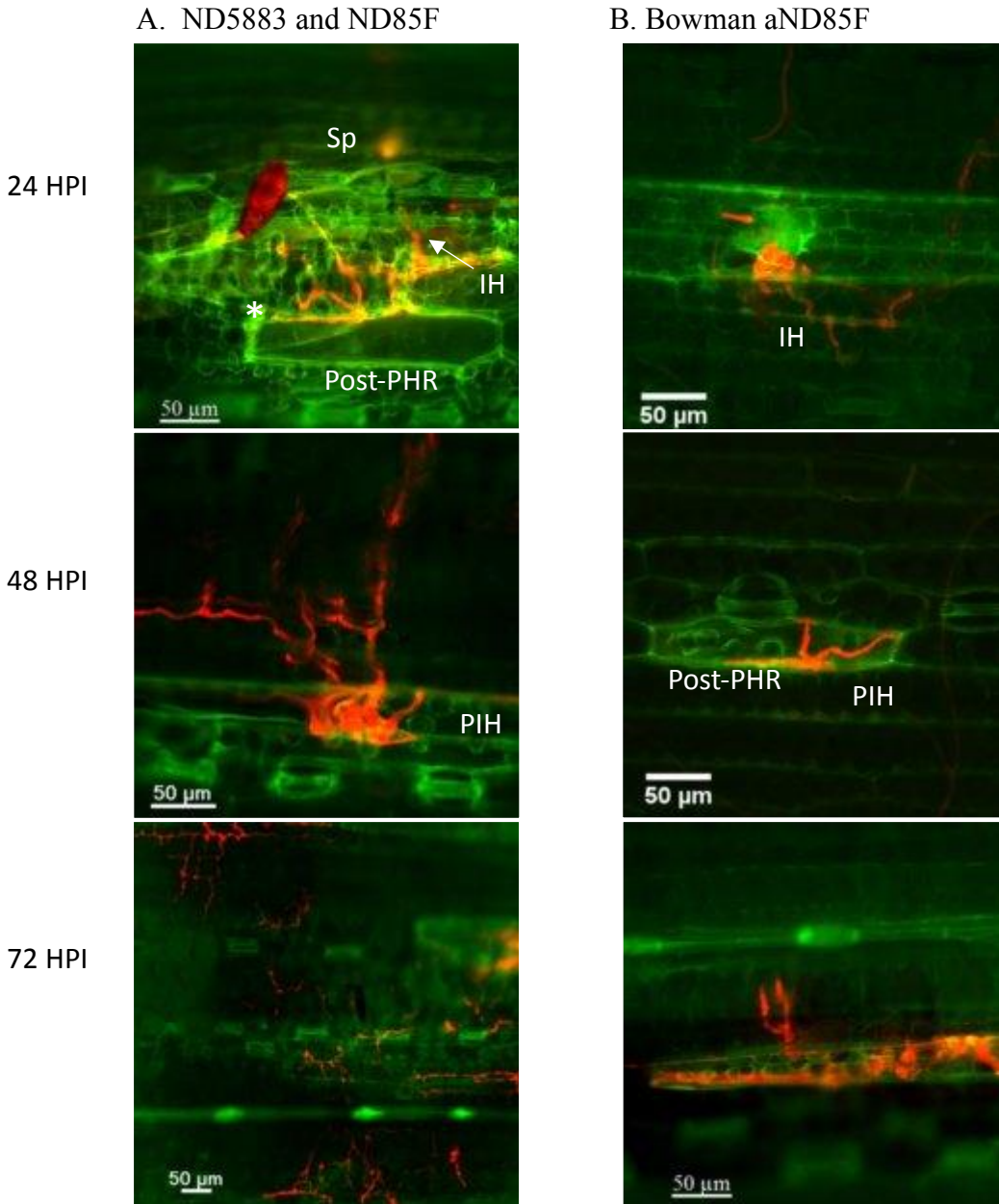


Figure 1.3. Histopathology of the interactions between isolate ND85F (pathotype 1) and two barley genotypes (ND5883 and Bowman) (Continued). (a) Compatible interaction between ND85F and ND5883 and (b) incompatible interaction between ND85F and Bowman. Spore (Sp) germination and appressoria (Ap) formation at the tip of the germtube (Gt) were observed at 8 HPI. The fungus started to penetrate the host cell wall in between the two epidermal cells at 12 HPI and triggered pre-penetration hypersensitive response (pre-PHR) in Bowman while post-penetration hypersensitive response (post-PHR) was observed in the penetrated epidermal cells of ND5883 at this time point. The autofluorescence of the penetrated host cells increased at 16 HPI. The autofluorescent cells started to collapse at 20 HPI indicating cell death. The fungus proliferated profusely in ND5883 whereas its growth was constrained in Bowman. *Yellow arrows show the site of penetration. White asterisks represent dead plant cells due to the necrosis.

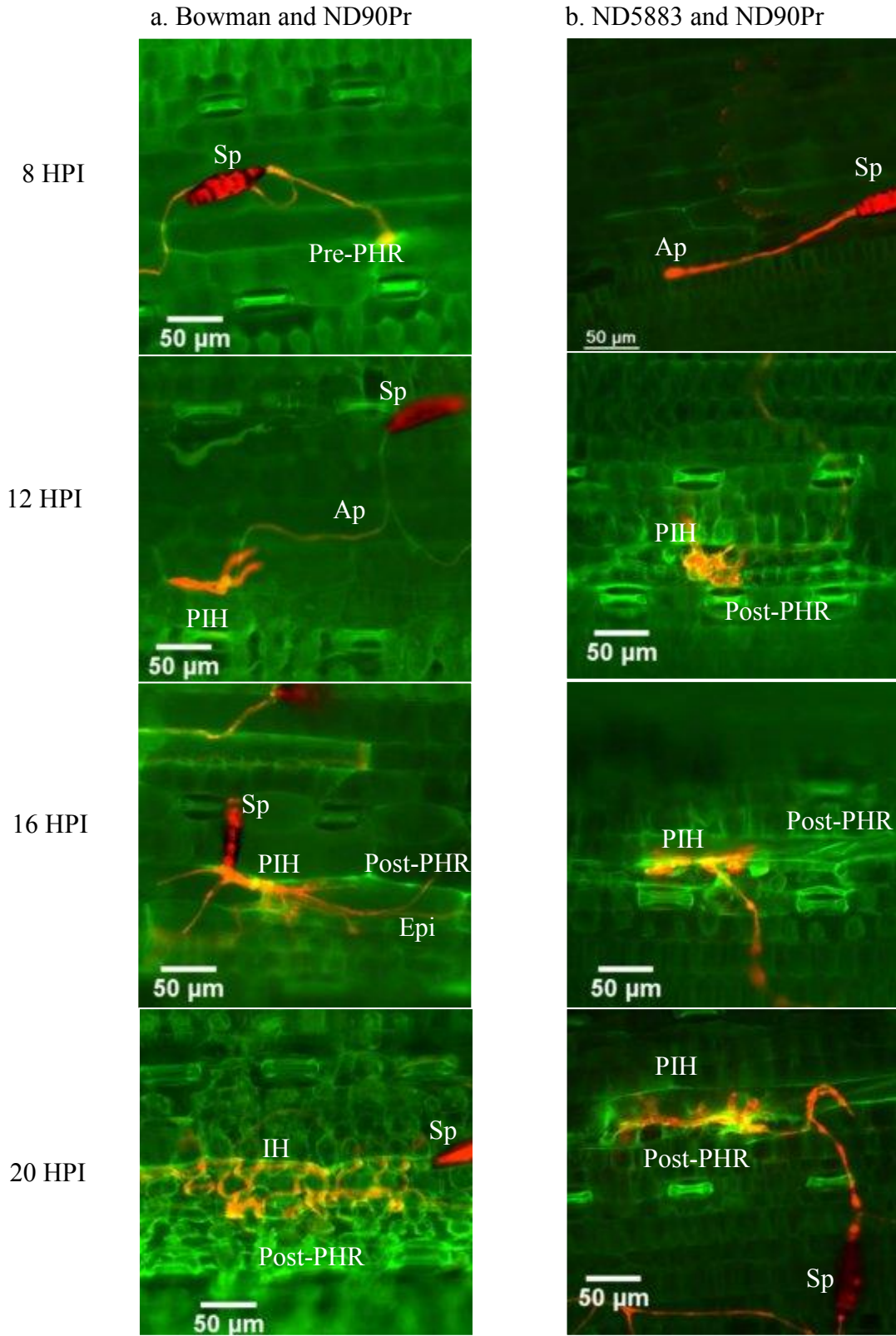


Figure 1.4. Histopathology of the interaction between isolate ND90Pr (pathotype 2) and two barley genotypes (Bowman and ND5883).

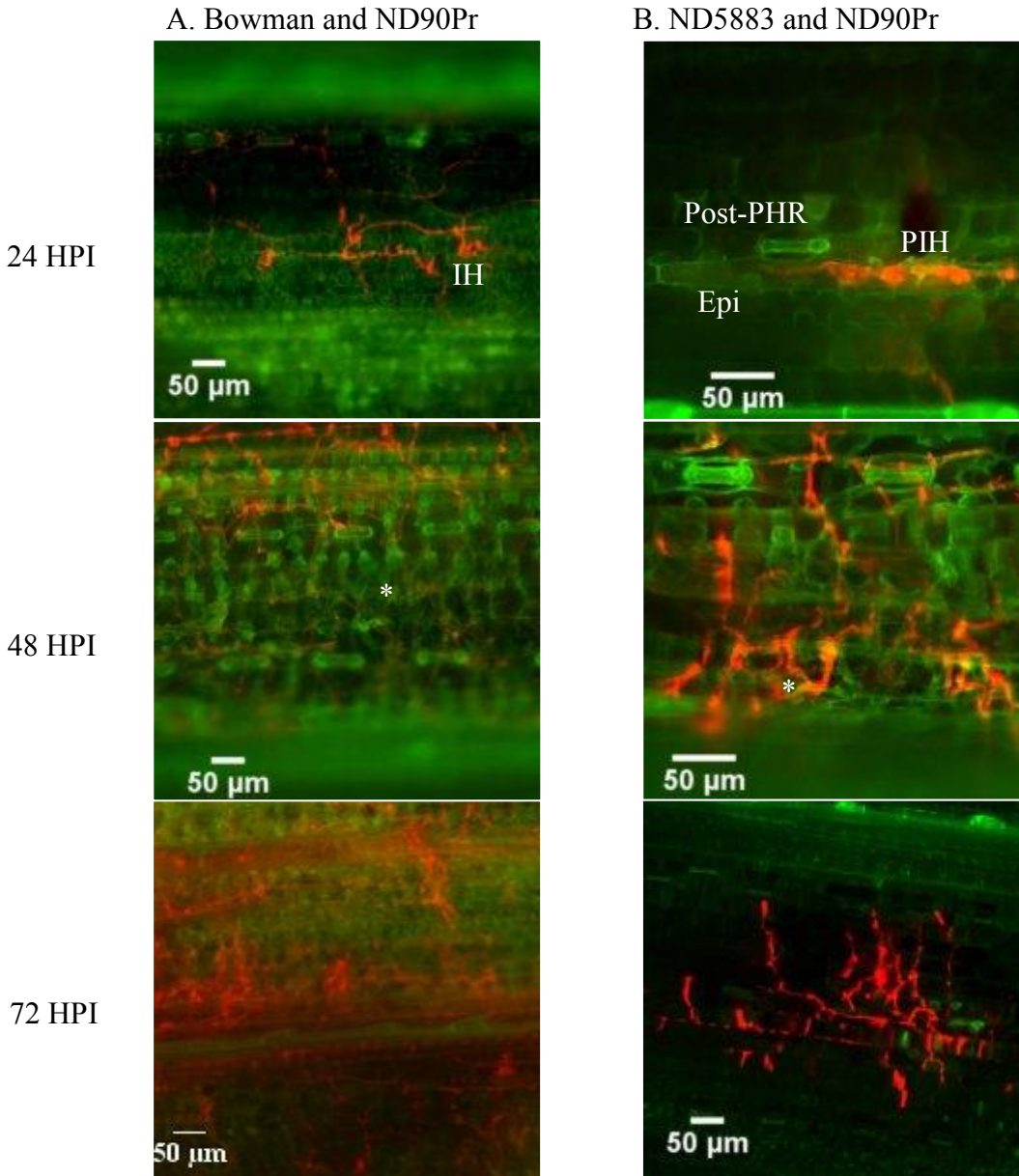


Figure 1.4. Histopathology of the interaction between isolate ND90Pr (pathotype 2) and two barley genotypes (Bowman and ND5883) (Continued). (a) Compatible interaction between ND90Pr and Bowman; (b) incompatible interaction between ND90Pr and ND5883. Spore (Sp) germination and appressoria (Ap) formation were observed at 8 HPI and pre-penetration hypersensitive response (pre-PHR) beneath the appressoria was also observed in Bowman at this time point. The fungus started to penetrate the host cell through the groove between the epidermal cell walls and developed the primary infection hyphae (PIH) at 12 HPI and triggered post-penetration hypersensitive response (post-PHR) in ND5883 as indicated by the presence of increased intensity of autofluorescence around the penetrated host cells. In the susceptible barley host Bowman, the fungus proliferated quickly by developing intracellular and intercellular infection hyphae (IH) after 20 HPI, but in the resistant barley line ND5883, the fungal growth was restricted to the dead tissues. *Yellow arrows show the site of penetration. White asterisks represent dead plant cells due to the necrosis.

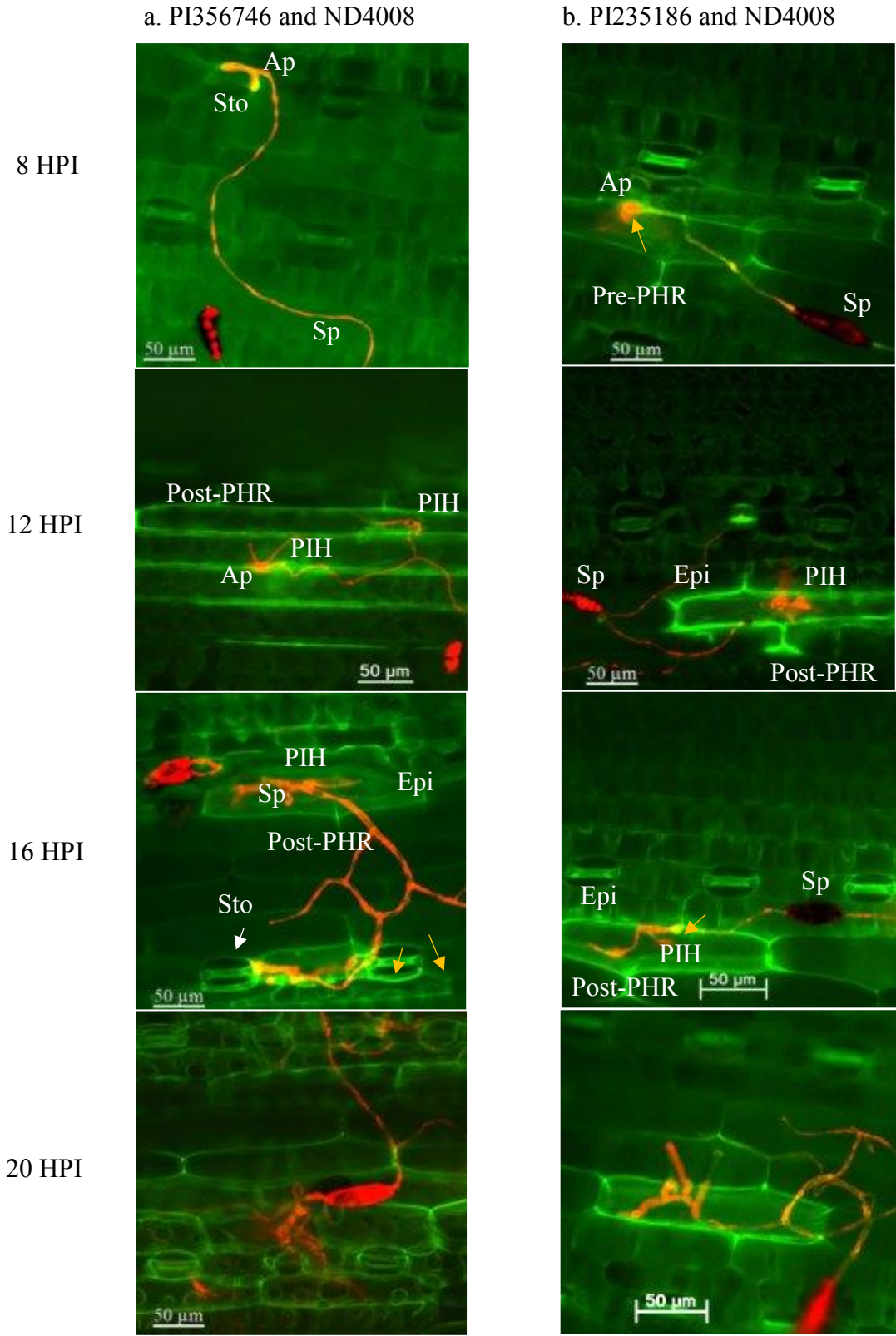


Figure 1.5. Histopathology of the interaction between isolate ND4008 (pathotype 7) and two barley genotypes (PI356747 and PI235186).

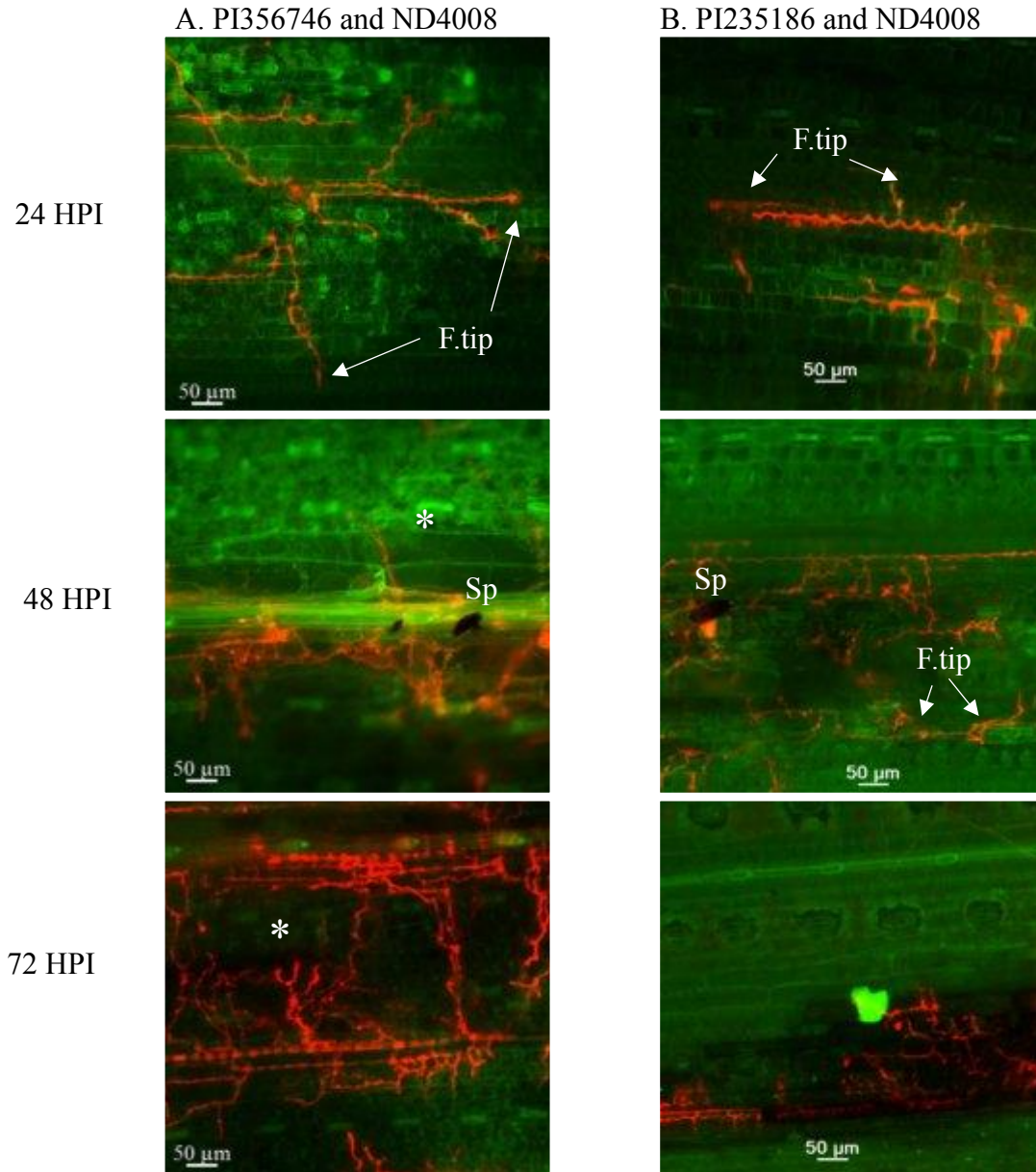


Figure 1.5. Histopathology of the interaction between isolate ND4008 (pathotype 7) and two barley genotypes (PI356747 and PI235186) (Continued). (a) Compatible interaction between and PI356746, and (b) incompatible interaction between ND4008 and PI235186. Spore (Sp) germination and appressoria (Ap) formation started at 8 HPI either over the stomata or in between two epidermal cells and pre-penetration hypersensitive response (pre-PHR) was observed at the sites of appressoria formation in both barley genotypes. The fungus penetrated and developed primary infection hyphae (PIH) in the epidermal cell at 12 HPI and triggered pre-penetration hypersensitive response (post-PHR). The autofluorescence intensity of the infected cells increased substantially after 16 HPI and cell death was observed at 24 HPI. The fungal growth was widespread in the susceptible barley line PI356746 while restrained fungal growth was observed in the resistant barley line PI235186 at 72 HPI. *Yellow arrows show the site of penetration. White asterisks represent dead plant cells due to the necrosis.

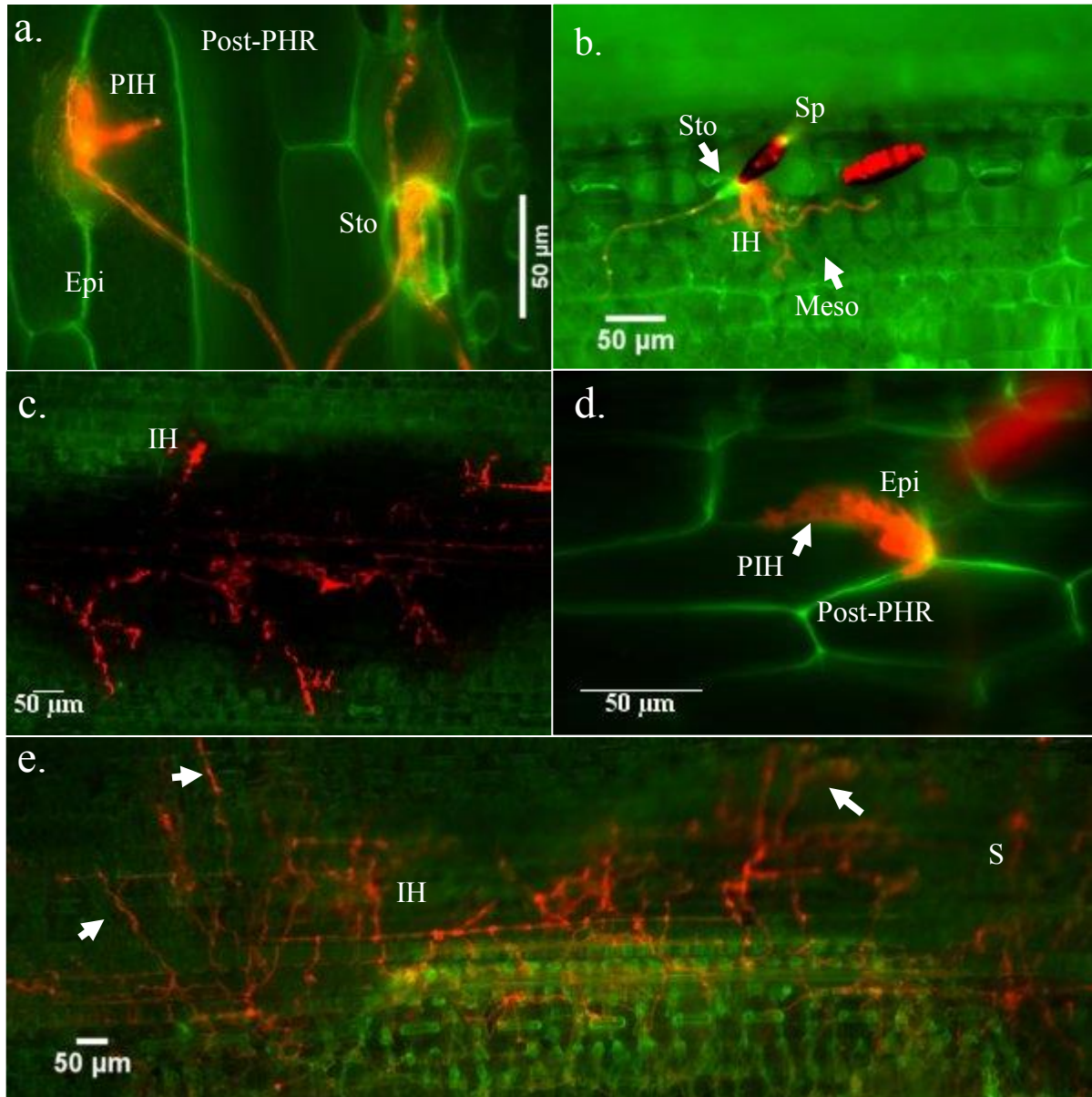


Figure 1.6. Fungal growth in barley plants. (a) Isolate ND4008 penetrated PI235186 leaf tissue through the stomata or through the groove in between two epidermal cells and triggered a post-penetration hypersensitive response (post-PHR) at the site of penetration at 48 hours post inoculation (HPI). (b) Isolate ND90Pr reached to mesophyll cells early at 8 HPI when it penetrated through stomata of Bowman. (c) The infection hyphae (IH) of ND90Pr in the resistant barley line ND5883 were restricted to the dead tissue (represented by the dark area) at 168 HPI. (d) Isolate ND4008 developed globular primary infection hyphae (PIH) in the epidermal cells of PI356746 at 16 HPI. (e) Isolate ND90Pr grew extensively in the susceptible barley cv. Bowman at 48 HPI; the tips of the IH (shown by white arrows) grew ahead of the autofluorescent (dead) tissues and invaded living cells.

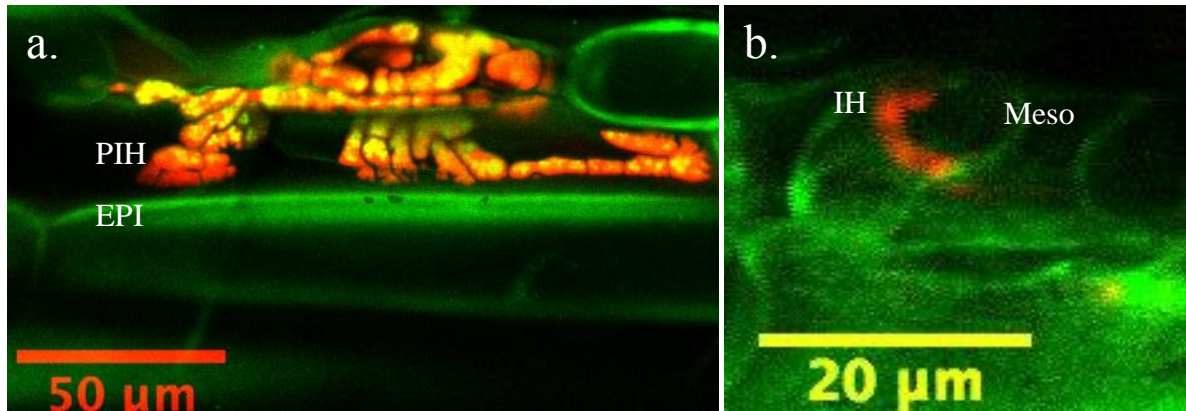


Figure 1.7. Confocal microscopy of *B. sorokiniana* infection in barley leaves. (a) The primary infection hypha (PIH) of isolate ND4008 was restricted in an epidermal cell (Epi) of a resistant host PI235186 at 20 hours post inoculation (HPI). (b) Infection hypha (IH) of isolate ND4008 was constrained inside a mesophyll cell of PI235186 at 20 HPI.

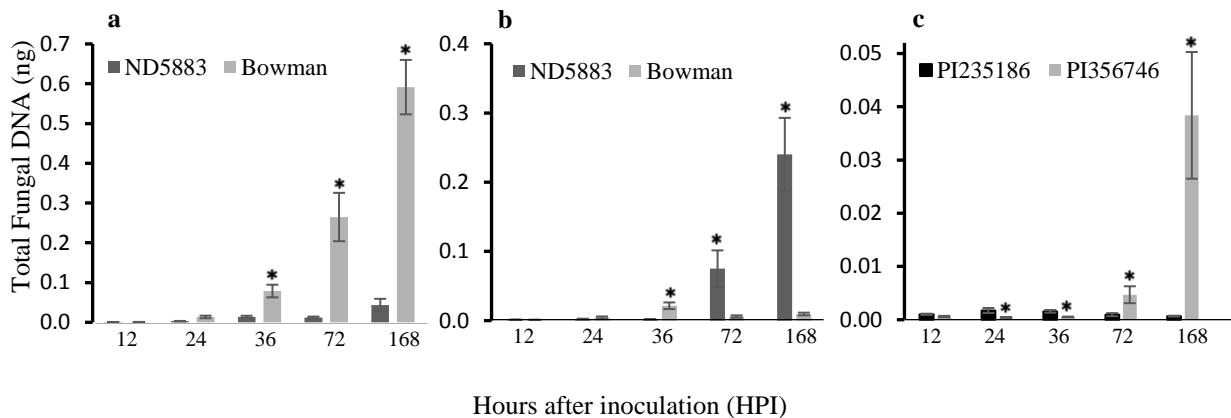


Figure 1.8. Quantification of fungal biomass using qPCR. Genomic DNA for qPCR assay was extracted from inoculated leaf samples collected at 5 time points for the three different interactions: (a) DNA quantification of isolate ND90Pr during infection of barley genotypes ND5883 (resistant) and Bowman (susceptible), (b) DNA quantification of Isolate ND85F during infection of barley genotypes ND5883 (susceptible) and Bowman (resistant), and (c) DNA quantification of isolate ND4008 during infection of barley genotypes PI235186 (resistant) and PI356746 (susceptible). The total fungal DNA in nano gram (ng) was calculated using cq values. Bars on the graph represent mean \pm standard error (S.E.). An asterisk indicates a significant difference in fungal mass accumulation between the resistant and susceptible barley genotypes in two-tailed Student's t-test at $p < 0.05$.

During penetration, the fungus appeared to push the epidermal cell walls apart and develop a penetration peg through the space. Penetration through the juncture of epidermal cell walls led to the development of primary infection hyphae (PIH) in the epidermal cells (Figure

1.6a) while penetration through the stomata led to fungal growth directly into the mesophyll cells (Figure 1.6b). The PIH developed in the epidermal cells in both compatible and incompatible interactions at 12 to 16 HPI, but the fungal growth was much faster in the susceptible host than in the resistant host.

Significant differences in the growth of the fungus were observed among the different interactions after 16 HPI. The fungus started to grow intra- and inter-cellular to the neighboring epidermal and mesophyll cells in both resistant and susceptible hosts, but the PIH were constricted within a few infected cells in the resistant hosts (Figure 1.7a). As expected, fungal growth to the neighboring cells was more profound in susceptible than in resistant hosts. In the compatible interactions, the fungus grew extensively and killed massive amounts of leaf tissue, whereas in the incompatible interactions the fungal growth was restricted (Figure 1.2, 1.3, 1.4, 1.5, 1.6 c and 1.6 e). Tips of infection hyphae were found to grow ahead of the leaf tissue with HR or dead cells in susceptible barley plants (Figure 1.6e). Some of the mesophyll cells were also penetrated during the late infections (Figure 1.7b). The qPCR results showed that the fungal biomass increased in susceptible hosts as the infection progressed with time, whereas it did not increase significantly over time in the resistant host (Figure 1.8). Especially, at 168 HPI the fungal biomass was found to be significantly more in the susceptible host as compared to the fungal biomass in the resistant host.

Host Responses

In resistant barley genotypes, the plant cells responded to fungal invasion (all pathotypes) as early as 8 HPI when the fungus formed appressoria (Figures 1.2, 1.3, and 1.5), but, for the interactions with pathotype 2, the susceptible genotype, Bowman, showed the early responses to the fungal invasion (Figure 1.4).

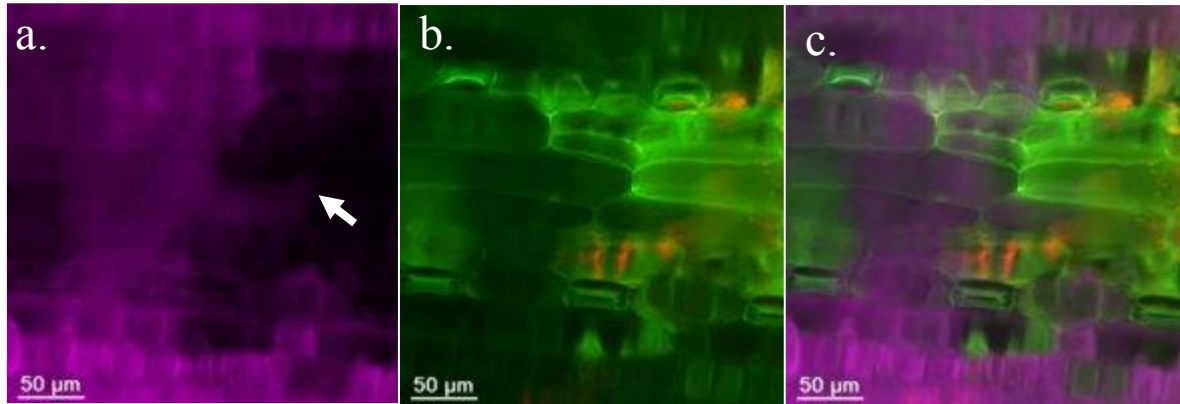


Figure 1.9. Chlorophyll loss during early infection of spot blotch in barley cv. Bowman. (a) The plant tissue with chlorophylls showed the magenta color using *Cy 5* channel. The dark patches (white arrow) indicate the plant tissue with chlorophylls degraded. (b) The leaf tissue (visualized with green autofluorescence using *Green* channel) and fungal hyphae of isolate ND90Pr (visualized with red fluorescence using *DsRed* channel) (c) Merged images of a and b. The green autofluorescence coincided with the loss of chlorophylls in the infected leaf tissue.

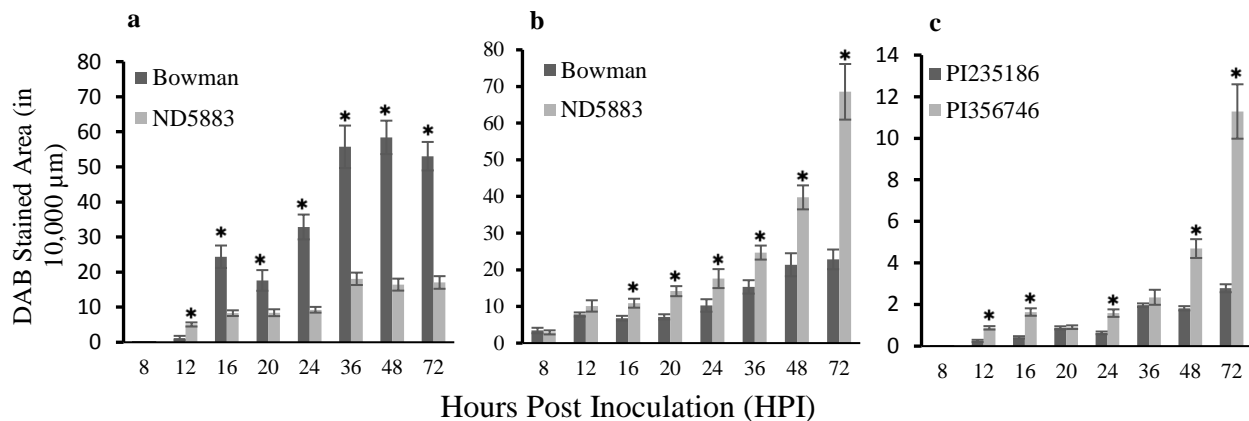


Figure 1.10. Quantitative assay of H_2O_2 accumulation at the infection sites using DAB staining. Leave samples were collected at 8 time points for the three interactions: (a) DAB staining area in barley genotypes ND5883 (resistant) and Bowman (susceptible) after inoculation with isolate ND90Pr, (b) DAB staining area in barley genotypes ND5883 (susceptible) and Bowman (resistant) after inoculation with isolate ND85F, and (c), DAB staining area in barley genotypes PI235186 (resistant) and PI356746 (susceptible) after inoculation with isolate ND4008. Bright-field microscopy was used to take pictures of the infected sites and the DAB stained areas were measured using ImageJ software. H_2O_2 accumulation is represented as the DAB stained area in μm . Bars on the graph represent mean \pm S.E. An asterisk indicates significant difference in H_2O_2 accumulation between resistant and susceptible barley genotypes in a two-tailed Student's t-test at $p < 0.05$.

Green autofluorescence increased slightly at the site of appressoria formation and pre-PHR was observed. In all interactions, the barley host failed to resist the fungal penetration through stomata, leading to early access to the mesophyll tissue (Figure 1.6 b). This might be due to the fact that the stomata are the natural opening of the leaves. Beside these, the magenta autofluorescence (visible at maximum 675-680) was lost around the host-pathogen interaction site (Figure 1.9). When the fungus penetrated the host through the stomata or in between the two epidermal cells at 12 to 16 HPI, post-PHR was triggered in the host. By 12 HPI, no significant differences in the intensity of green autofluorescence at the fungal penetration sites (the epidermal cells) were observed between the compatible and incompatible interactions (data not shown). The autofluorescence intensity during the early infection phase was comparatively lower compared to the intensity during late infection stage. After 16 HPI, the green fluorescent intensity of the infected cells increased accompanied with irregularity in the outline of the host plasma membrane, which was indicative of cell death. The pre-PHR and post-PHR of epidermal cells of resistant genotypes were not efficient to completely restrict fungal growth, but the pathogen only continued to grow to a few mesophyll and epidermal cells and was restricted to the dead tissues (dark areas) by 72 HPI (Figures 1.2, 1.3, 1.4, 1.5, 1.6 c and 1.6e). In contrast, the fungus grew profusely in the susceptible host tissue. The infection hyphae grew intra- and inter-cellular in all possible directions. During late infection, the fungus killed the host tissues and collapsed the mesophyll and epidermal cells as represented by collapsed cells. DAB staining indicated that H₂O₂ accumulation occurred during early infection in both resistant and susceptible hosts. In the interactions involving ND85F, H₂O₂ accumulation started from 8 HPI (during appressorium formation), while in the interactions with ND90Pr and ND4008, H₂O₂ accumulation started from 12 HPI (during penetration) (Figure 1.10).

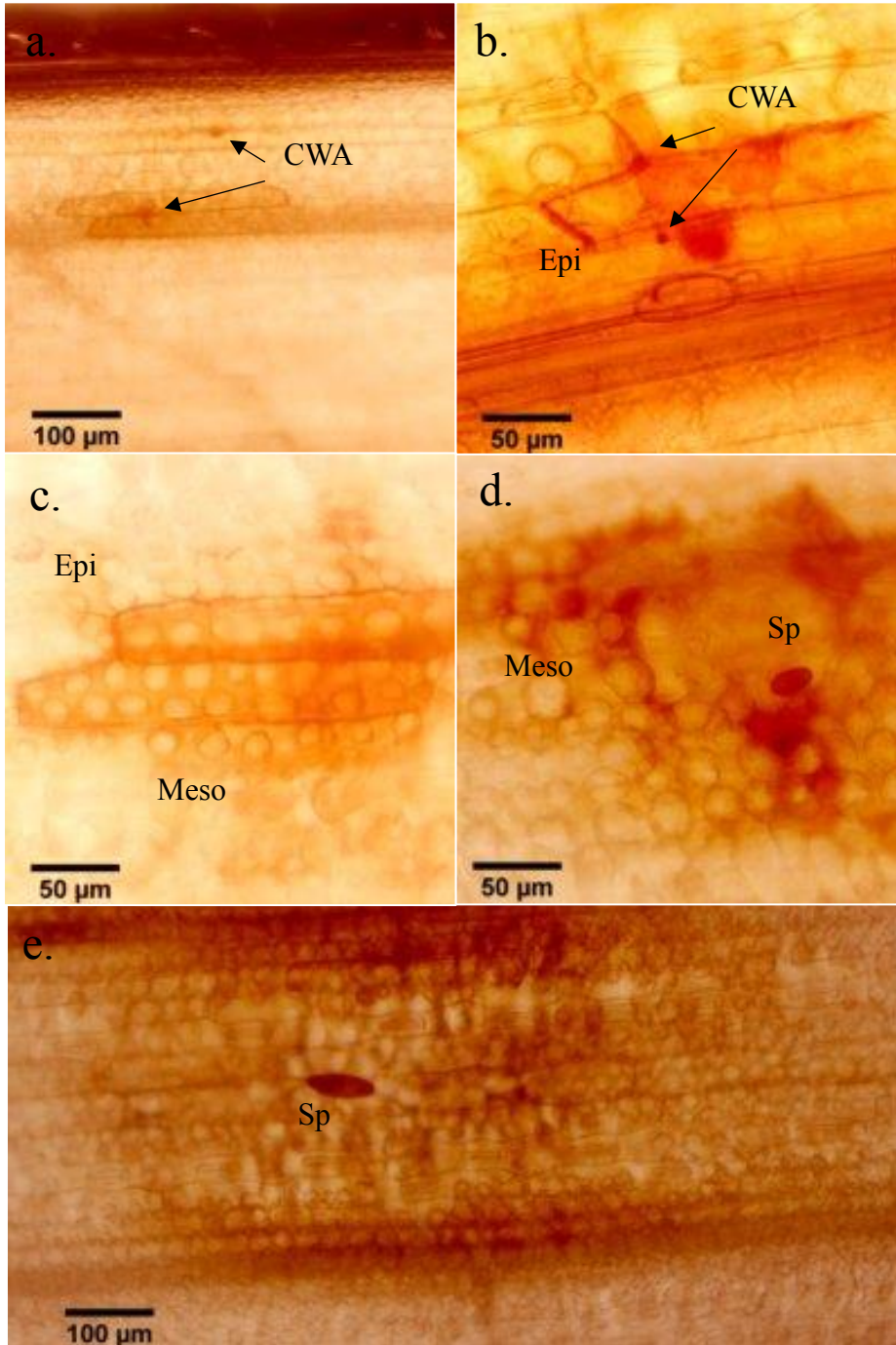


Figure 1.11. Histochemical study of the spot blotch disease on barley leaves. (a and b) Cell wall apposition (CWA), during fungal penetration (black arrows), along with the hypersensitive response (HR) in epidermal cells (Epi) of barley line PI356746 (a) and cv. Bowman (b) at 16 hours post inoculation (HPI) with isolate ND4008 and ND85F, respectively. (c and d) Hypersensitive response (HR) in epidermal and mesophyll cells (meso) of barley line PI356746 at 12 HPI with isolate ND4008. (e) Fungal spread in the host is shown by DAB stained (brown) area at 48 HPI.

At 12 HPI, a significant difference was observed between resistant and susceptible hosts infected with ND90Pr and ND4008, while the difference was not observed until 16 HPI in ND85F inoculated hosts. The DAB stained area in the barley leaves increased with time for all interactions with the susceptible host showing a more substantial increase compared to the resistant host. Pathotype 0 (ND93-1) did not show a significant difference between ND5883 and Bowman for the DAB stained area (data not shown). The histochemical study also showed cell wall apposition (CWA) at 16 HPI in some infection sites, which could be part of an early defense response (Figures 1.11 a and b). However, CWA was not observed frequently during the early fungal invasion.

Discussion

B. sorokiniana is considered a hemibiotrophic fungus, which has a short and early biotrophic phase followed by a late necrotrophic phase (Kumar et al. 2002). During the early infection stage, the fungal spore germinates from polar cells (germination of either or both polar cells) and produces appressoria on the stomata or in between epidermal cells. Hence, the cues for appressoria formation and penetration are the grooves between the epidermal cells and the stomatal openings.

During this early infection process, the grooves between the epidermal cells of plants were widened by fungal penetration. Similar results were reported by Clay et al. (1997) who indicated that the barley cell walls were displaced, without any damage, from their original position during penetration by *B. sorokiniana*. From the infected epidermal cells, PIH branched and invaded further neighboring epidermal and mesophyll cells.

During late infection, the fungus becomes necrotrophic along with the continued biotrophic infection of new and healthy cells in susceptible barley genotypes where the infection

hyphae grow inter- and intra-cellular in the epidermal and the mesophyll tissue and proliferate ahead of highly autofluorescent (dead) cells. This indicates that the fungus needs new plant tissue for biotrophic invasion (Kankanala et al., 2007). It appears that the fungus continues to infect new cells with the biotrophic strategy in the susceptible plants, and grows for short period as a biotrophic fungus and then switches to a necrotrophic phase. In contrast, in the incompatible interactions the growth of infection hyphae was restricted in the dead tissue and rarely expanded to the surrounding living tissue.

In response to the fungal infection, the host also exhibited various defense mechanisms at different stages. Green autofluorescence was observed as the first host response when the fungus formed appressoria on epidermal cells. The green autofluorescence was higher at the site of appressorium formation compared to the unaffected leaf tissue. The increased green autofluorescence was reported to be associated with the accumulation of phenolic compounds like tannin and flavonoids since these compounds show autofluorescence under the emission wavelength of 500-550 nm (green-yellow region) (García-Plazaola et al., 2015). However, the plasma-membranes of the autofluorescent cells were smooth and the intensity of autofluorescence was rather low during fungal penetration, suggesting that these cells were still alive. After 20 HPI, the intensity of the green autofluorescence of the infected cells increased and the cells with strong autofluorescence had irregular plasma-membranes, suggesting they were dead cells. Koga et al. (1988) found that when barley leaves were inoculated with the powdery mildew fungus, the infected cells showed some autofluorescence during the early infection stage but those cells were alive because they were able to plasmolyze and take up neutral red. In the later stage, the infected cells showed increased autofluorescence, indicating they were dead cells that failed to plasmolyze and take up neutral red (Koga et al., 1988).

Our result showed that ND90Pr (pathotype 2) differed from the other pathotype isolates during the early infection process. When infected by isolate ND90Pr, the susceptible barley cultivar Bowman showed stronger pre-PHR and post-PHR than the resistant barley line ND5883), whereas no obvious differences were observed in the interactions of resistant and susceptible barley genotypes with the other fungal isolates in the early infection process. Recent study indicated that two nonribosomal protein synthetases (NRPS) encoding genes (NPS) (IDs: 115356 and 140513) are required in isolate ND90Pr for high virulence on Bowman (susceptible host) and these two genes are highly upregulated at 12 HPI (Condon et al., 2013). It is possible that a secondary metabolite toxin produced by the NRPSs in the fungus interacts with the sensitivity gene in Bowman and triggers a HR in the host.

H₂O₂ accumulation was also observed in the host cells during appressoria formation. At the time of basal defense, the host cells produce H₂O₂ and phenolic compounds. H₂O₂ is associated with fortifying the cell wall, signaling for the activation of genes associated with the defense response and for production of antimicrobial agents like phytoalexins (Thordal-Christensen et al., 1997). The histochemical study showed that there was some difference in the amount of H₂O₂ accumulation in the resistant and susceptible hosts during the interaction with different pathotypes of *B. sorokiniana*. During interaction with pathotype 2 (ND90Pr) and pathotype 7 (ND4008), significant differences in H₂O₂ accumulation between the resistant and susceptible hosts were observed at 12 HPI. However, in the interaction with ND85F, the H₂O₂ accumulation was significantly different between the resistant and susceptible genotypes at 16 HPI. The evidence from the fluorescence microscopy and the DAB staining supports the hypothesis that resistant and susceptible hosts react differently to the different pathotype isolates.

Although early defense by the host was not sufficient to control fungal growth, the susceptible and resistant plants had different responses to the fungal infection during the late infection stage. The area and intensity of the autofluorescence, the DAB stained area, and the fungal biomass of the infected leaf tissue increased substantially in the susceptible host, but they were restricted in the resistant host. The host cells during late infection were shrunken and the cell organelles were collapsed, indicative of the complete collapse of the host cells. Ibeagha et al. (2005) showed that after successful penetration and colonization of the epidermal cells, the fungus spreads to the mesophyll cells. If the host is able to block the invasion of the mesophyll cells and reduce the spread, then it is considered a successful defense mechanism. Thus, late resistance to fungal spread is more effective than the early defense response.

During the late infection phase, the fungus hijacked the host defense mechanism to produce a large amount of H₂O₂. The susceptible host leaves had significantly larger DAB stained areas than those of the resistant host, and the intensity of DAB staining was high in the dead host cells (Figure 1.7). Therefore, the necrotrophic phase of the fungus induced the production of H₂O₂ and phenolic compounds and the host cell lost control over its production and triggered necrosis rather than programmed cell death (Kumar et al., 2001). These biochemicals are responsible for killing the host cell itself and allow the fungus to thrive on it. H₂O₂ is toxic to both the host and the pathogen. Leng and Zhong (2012) found that *PPT1* in ND90Pr is involved in the activation of polyketide synthases (PKSs) and NRPSs, which are involved in reducing sensitivity to H₂O₂. Therefore, it is possible that the fungus produces these kinds of biochemicals that either detoxify the dead leaf tissue or helps to reduce the sensitivity to H₂O₂.

Conclusion

Histological comparisons of compatible and incompatible interactions between different pathotypes of *B. sorokiniana* and different barley genotypes indicate that the resistant and susceptible hosts respond similarly to all the pathotypes except pathotype 2 during early pathogenesis. The fungus grows profusely in the susceptible host while its growth is restricted in the resistant host. The pre-PHR and post-PHR are not efficient enough to stop the fungal growth in both compatible and incompatible interactions. However, resistance during the late infection stage is more successful in inhibiting fungal proliferation. The information gained from this study helps us to understand the strategies used by the fungus to invade and colonize the host and the strategies used by the host to resist and suppress the fungal invasion. These findings will aid further unraveling of the different aspects of this plant-pathogen interaction and the development of durable strategies for management of the spot blotch disease in barley.

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CHAPTER 2: IDENTIFICATION OF QTL FOR TYPE I RESISTANCE TO FUSARIUM HEAD BLIGHT IN TWO SPRING WHEAT MAPPING POPULATIONS

Abstract

Fusarium head blight (FHB), mainly caused by *Fusarium graminearum*, is an important disease of wheat and other small grains in North America. Use of resistance is one of the most important components in management of the disease. Among different types of FHB resistance, type II resistance (resistance to disease spread) is the most studied, while type I resistance (resistance to initial infection) has not been well characterized. This project was focused on mapping QTL associated with Type I resistance to FHB in spring wheat using 130 doubled haploid (DH) lines from the cross Grandin × PI277012 (GP) and 237 recombinant inbred lines (RILs) from the cross Bobwhite × ND2710 (BN). The GP population was previously genotyped with SSR markers and the 9K SNP chips while the BN population was genotyped with the 90K SNP chips. The two populations were further evaluated for type I resistance by spay inoculation in the field and greenhouse. For the GP population, QTL analysis using composite interval mapping (CIM) identified three QTL on chromosomes 1A, 4B and 6B, respectively, under field environments, and two QTL on chromosomes 2B and 5B, respectively, under greenhouse conditions. These QTL explained 10.7-19% of the total phenotypic variation. For the BN population tested under field conditions, three QTL were detected on chromosomes 2A, 5A and 6B, respectively, whereas one QTL was detected on chromosome 5A under greenhouse conditions. These QTL explained 6.2-13.7% of the total phenotypic variation. The QTL identified in this study mapped to genomic regions with previously reported QTL for FHB resistance, except for one QTL (*Qfhb.ndwp-5A.2*) on chromosome 5A which explained 6.6% of

the phenotypic variation. The markers associated with the QTL for type I resistance will be useful for selection and pyramiding of different FHB resistance loci in breeding programs.

Introduction

Fusarium head blight (FHB) or scab is an economically important fungal disease of wheat and barley caused by several *Fusarium* spp. In North America, FHB is predominantly caused by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zae* (Schwein.) Petch) (O'Donnell et al., 2000; and Puri and Zhong, 2010). This disease can cause significant yield losses and reduce the quality of grains due to contamination with mycotoxins like deoxynivalenol (DON) and nivalenol. Upon consumption by humans and animals, contaminated grains can be detrimental to health (Nelson et al., 1993). FHB is highly favored by warm and humid weather conditions in conjunction with production practices such as minimum tillage, inadequate crop rotation and cultivation of susceptible cultivars (Dill-Macky and Jones, 2000; Lu et al, 2013). Fungicides can be used to control FHB, but they add cost, lack consistent efficacy, and have negative impact on the environment (Parry et al., 1995). Therefore, growing resistant cultivars is considered the most effective and ecologically friendly measure to control the disease.

Resistance to FHB is a complex trait governed by quantitative trait loci (QTL) and is categorized into different types (Schroeder & Christensen, 1963; Mesterhazy et al. 1995), including Type I (resistance to initial infection), Type II (resistance to fungal spread from the infected spikelets along the rachis), Type III (resistance to kernel infection), Type IV (tolerance) and Type V (resistance to toxins). Among the different types of resistance, type I and type II resistances are widely recognized and considered as the key components contributing to field resistance to FHB (Yan et al., 2011). However, type II resistance is considered to be more stable

than type I resistance (Bai and Shaner, 1994; Bai and Shaner, 2004). Type I resistance is assessed mainly by spray inoculation, while Type II resistance is evaluated mainly by point inoculation (reviewed in Buerstmayr et al., 2009).

Wheat genotypes with varying levels of FHB resistance have been reported from different parts of the world and genomic regions governing FHB resistance have been identified in many of the genotypes (reviewed in Buerstmayr et al., 2009). However, fewer QTL have been identified for Type I resistance than for Type II resistance. QTL associated with Type I resistance have been reported in Sumai-3 (Xu et al., 2001), Frontana (Steiner et al., 2004) and Goldfield (Gilsinger et al., 2005). Sumai-3 contains three types of resistance: type I (Buerstmayr et al. 2003), type II (Waldron et al. 1999; Anderson et al. 2001; Zhou et al. 2002), and type III resistance (Lemmens et al. 2005). *Fhb1* (*syn: Qfhs.ndsu-3BS*) is one of the most important QTL mapped on 3BS (Waldron et al. 1999; Anderson et al. 2001; Buerstmayr et al., 2002; Buerstmayr et al., 2003; Cuthbert et al., 2006; Liu et al. 2006; Schweiger et al., 2013). It is derived from the highly resistant cultivar ‘Sumai-3’ and mainly provides resistance against disease spread (type II resistance) explaining up to 60% of the phenotypic variation. Recently, *Fhb1* has been cloned and identified as the pore-forming toxin-like (*PFT*) gene that encodes a chimeric lectin with agglutinin domains and confers resistance against the pathogen presumably by interacting with the fungal cell wall (Rawat et al. 2016). *Qfhs.ifa-5A* (Buerstmayr et al., 2002; Buerstmayr et al., 2003; Buerstmayr et al., 2009; Schweiger et al., 2013) and *Fhb2* are other important QTL located on wheat chromosomes 5A and 6BS, respectively. *Qfhs.ifa-5A* is associated with type I resistance thereby inhibiting the rate of initial infection. Although it has some contribution to type II resistance, it is mainly associated with type I resistance. Depending on the environment, *Qfhs.ndwp-3B* and *Qfhs.ifa-5A* together explain 40-48% of phenotypic variance (Buerstmayr et

al., 2003) while *Fhb2* explains 21% of the phenotypic variance (Yang et al., 2003). Frontana is another source of moderate type I resistance to FHB presumably due to morphological features like hard glumes and narrow flower opening (Gilsinger et al., 2005). However, no major QTL have been reported in 'Frontana' derived populations. Goldfield is a winter wheat and the QTL identified on 2B was associated with narrow flower opening. Despite the identification of multiple FHB resistance QTL, complete resistance to FHB has not been found in wheat (Yu et al., 2008).

The correlations between the FHB severity and the other traits like, flowering or heading time and plant height have been reported in previous studies (Gervais et al., 2003, Paillard et al., 2004, Somers et al., 2003). For example, the presence of semi dwarfing genes, *Rht-B1b* and *Rht-D1b* (derived from Japanese cultivar 'Norin 10') exhibited little or no negative impact on type I resistance in wheat under moderate disease pressure while it had negative effect on FHB severity under high disease pressure (Gosman et al., 2009).

PI277012 is a hexaploid wheat that has a high level of FHB resistance comparable to Sumai-3 but it does not have *Fhb1* from Sumai-3 (Chu et al. 2011). Using a doubled haploid population from a cross between PI277012 and Grandin, two QTL (*Qfhb.rwg-5A.1* and *Qfhb.rwg-5A.2*) for FHB resistance II have been identified in PI277012 (Chu et al., 2011). *Qfhb.rwg-5A.1* on 5AS explains up to 20% phenotypic variation, while *Qfhb.rwg-5A.2* on 5AL is a unique QTL that explains 20-32% of FHB phenotypic variation. ND2710 is a FHB-resistant spring wheat line developed by NDSU spring wheat breeding program. It was selected from the progeny of the cross between Grandin and ND2603, a wheat line derived from the cross between Sumai-3 and Wheaton (Frohberg et al. 2004). QTL analysis detected two QTL for FHB resistance in ND2710, which mapped to the same regions as *Fhb1* and *Fhb2* on chromosome

3BS and 6B, respectively (Zhao et al. 2014). However, it is not known if QTL for Type I resistance in PI277012 and ND2710 are the same as those QTL reported in these two wheat genotypes. The objectives of this study were to i) identify and map the QTL associated with type I resistance to FHB in PI277012 and ND2710, respectively, ii) investigate effects of plant height and flowering time on type I resistance.

Materials and Methods

Plants Materials

Two different mapping populations were used in this study, including a recombinant inbred population (F₉ generation) from the cross between ND2710 (PI633976) and Bobwhite (PI520554) (BN) and a doubled haploid (DH) population from the cross between Grandin (PI531005) and PI277012 (GP). ND2710 is a FHB resistant spring wheat line released in 1998 by North Dakota Agriculture Experiment Station (NDAES) (Frohberg et al. 2004). Bobwhite is the susceptible parent. This population consisted of 237 recombinant inbred lines (RILs). The doubled haploid population from the cross between Grandin and PI277012 (GP) was developed by Dr. Steven Xu, USDA-ARS, Northern Crop Science Laboratory (Chu et al., 2011). Grandin is a spring wheat cultivar used as a FHB susceptible parent. This population consisted of 130 DH lines.

Inoculum Preparation

Two 3ADON isolates and two 15 ADON isolates of *Fusarium graminearum* were used in this study. Each isolate was grown on moong bean agar for four days and 12 to 15 plugs (5 mm) with mycelia and spores were transferred to 500 ml of carboxymethyl cellulose (CMC) media (Cappellini and Peterson, 1965). After growth for 7 days at room temperature under constant light conditions and by shaking (150 rpm) in an incubation shaker, spores were

harvested by filtration of the cultures through a layer of miracloth and diluted to the concentration of 50,000 spores/mL. Tween 20 was added at a concentration of 1mL/L of the spore suspension. The spore suspensions from the four isolates were mixed in equal volumes for spray inoculations in the greenhouse and field experiments.

Greenhouse Experiment

Greenhouse experiment was conducted only once for both populations in the spring of 2016. A completely randomized design was used for each experiment. Three replications of each line were planted in three different pots with three seeds per pot. Seeds were planted in 6-inch clay pots filled with potting mix media (Pro-Mix LP15; Premier Tech Horticulture, Quakertown, PA) and supplemented with slow-release fertilizer (Osmocote Plus 15-9-12 N-P-K plus minors; Everris Inc., Dublin, OH) after planting. A temperature of 68-70 °F with day light duration of 14 hours per day was maintained from planting to the plant heading stage. When more than 50% of the spikes in a pot were flowering, the spikes were inoculated by spraying spore suspension as prepared above on all sides until they were covered by the fine drops of the inoculum. Inoculation was done on two consecutive days using a spray bottle containing 50,000 spores/mL. The inoculated plants were kept in a misting room and misted for one minute each hour over three days at a temperature 72-75 °F. After inoculation, the plants were returned to the greenhouse room with the same conditions as before inoculation except for the temperature being raised to 72-75 °F.

Field Experiment

Field experiments were conducted at Fargo in the summers of 2015 and 2016. The wheat lines were planted in hills with 5-8 seeds per hill. The field experiments were conducted in a randomized complete block design (RCBD) with three blocks. Similar to the greenhouse

experiment, inoculation was done when approximately 50% of the spikes were flowering in each hill. They were inoculated by spraying inoculum on all sides of the spikes until they were covered with fine droplets of inoculum. Inoculations were done using a Roundup Professional 4-Gallon S-2 Backpack USA Sprayer on two consecutive days. To provide enough moisture, inoculated plants were over-head misted overnight with a misting cycle of 2 minutes in every 2 hours.

Phenotype Assessments

The percentage of infected spikelets (PIS) was used to assess type I resistance in this study. PIS was recorded 10 days post inoculation by counting infected spikelets per inoculated spike. Plant height was measured from the base of the plant to the tip of the tillers and the number of days from planting to flowering was also recorded for each wheat line at the time of inoculation. The data for plant height was collected in the 2015 field experiment and the 2016 greenhouse experiment, while the flowering data were collected only in the field experiments.

Statistical Analysis

All the statistical analyses for this project were conducted using different SAS procedures and their commands in SAS 9.4 (SAS 2011). Shapiro-Wilk normality tests for the distribution of the residuals of PIS, plant height and days to flowering in each experiment were conducted using PROC UNIVARIATE. Homogeneity of variance among 2015 and 2016 field data were verified by the Levene's test, and if homogenous, data from the two years were combined to obtain means for QTL analysis. The correlations between the experiments for each trait and between the traits in each year were estimated with Spearman rank-correlation coefficients (r). Broad sense heritability for each trait was calculated using the restricted maximum likelihood (REML) method in SAS (Holland et al., 2003). Heritability coefficients for single year data were

estimated using the equation $H = \delta^2_G / (\delta^2_G + \delta^2_E / r)$, while for multiple years the equation used was $H = \delta^2_G / (\delta^2_G + \delta^2_{GXY} / y + \delta^2_E / yr)$, where δ^2_G = genotypic variation, δ^2_{GXY} = genotype by year interaction variance, δ^2_E = residual variance, y = number of years, r = number of replications.

Genotyping, Linkage Mapping and QTL Analysis

The BN population was genotyped by Monsanto using the wheat 90k Illumina iSelect assay (Wang et al. 2014), and the GP population was genotyped using the 9K Illumina iSelect assay (Cavanagh et al. 2013) along with 319 SSR markers previously used by Chu et al. (2011) for mapping QTL for FHB resistance in the population. A total of 761 and 765 polymorphic and non-co-segregating markers were identified and used for linkage analysis and QTL mapping analysis in the BN and GP populations, respectively.

The linkage maps for both populations were developed in MapDisto (Lorieux 2012) using a Kosambi mapping function (Kosambi, 1943) with a LOD threshold of 5.0. A total of 28 linkage groups spanning 1462.05 cM of genetic distance and 26 linkage groups spanning 3368.86 cM of genetic distance were obtained for the BN and GP populations, respectively. For the BN population, the linkage groups were anchored to individual wheat chromosomes using the consensus map developed with the 90K iselect assay (Wang et al., 2014). For the GP population, the linkage groups were previously anchored with SSR markers (Chu et al. 2011). These linkage maps along with the phenotypic data were used for QTL analyses with the software Qgene 4.0 (Joehanes and Nelson 2008). Composite interval mapping (CIM) was used to identify significant loci associated with FHB PIS, plant height and days to flowering. Cofactors were selected using the forward selection method with maximum number of cofactors set at auto (0). A permutation test with 1000 permutations was used to compute the LOD threshold for an experiment-wise significance level of 0.05 for each trait in each population.

Results

BN Population

The BN population showed a continuous distribution for the three traits studied (Figure 2.1). Transgressive segregates were observed for all three traits in this population in all field and greenhouse experiments. ND2710 was consistently more resistant to FHB, taller and flowered earlier compared to the susceptible parent Bobwhite (Table 2.1). The average PIS of ND2710 were 6.69%, 8.99% and 34.35% in the 2015 field experiment, 2016 field experiment and 2016 greenhouse experiment, respectively. The mean PIS of Bobwhite were 26.61%, 34.27% and 77.17% in the 2015 field experiment, 2016 field experiment and 2016 greenhouse experiment, respectively. Moderate (for PIS and days to flowering) to high (for height) heritabilities were calculated for the BN population (Table 2.1).

Table 2.1. Heritability of percentages of infected spikelets (PIS), plant height, and days to flowering in the Bobwhite × ND2710 (BN) population

Population	Trait	Experiments	Parents		Population			Heritability ^a
			Bobwhite	ND2710	Mean	Min	Max	
Bobwhite × ND2710 (BN)	PIS	2015 Field	26.61	6.69	25.85	0	100	0.69 ^b
		2016 Field	34.27	8.99	14.91	0	100	0.75
		Overall Mean	30.04	7.53	20.42	0	100	0.42 ^c
	Plant Height	2016 Greenhouse	77.17	34.35	42.39	1.78	84.47	0.72
		2015 Field	29.17	39.45	31.89	22.5	42.67	0.88
	Days to Flowering	2016 Greenhouse	24	38.67	27.90	42	16	0.8
		2015 Field	70	65.56	65.20	48	88	0.79
		2016 Field	62	57.64	61.10	50	78	0.42
		Overall Mean	66	61.60	63.17	48	88	0.478

^a Broad sense heritability was calculated using the restricted maximum likelihood (REML) method in SAS (Holland et al. 2003)

^b Heritability coefficient for single year data was estimated using equation $H = \delta^2_G / (\delta^2_G + \delta^2_E/r)$

^c Heritability coefficient for multiple year data was estimated using equation $H = \delta^2_G / (\delta^2_G + \delta^2_{GXY}/y + \delta^2_E/yr)$, where, δ^2_G = genotypic variation, δ^2_{GXY} = genotype by year interaction variance, δ^2_E = residual variance, y = number of years, r = number of replications.

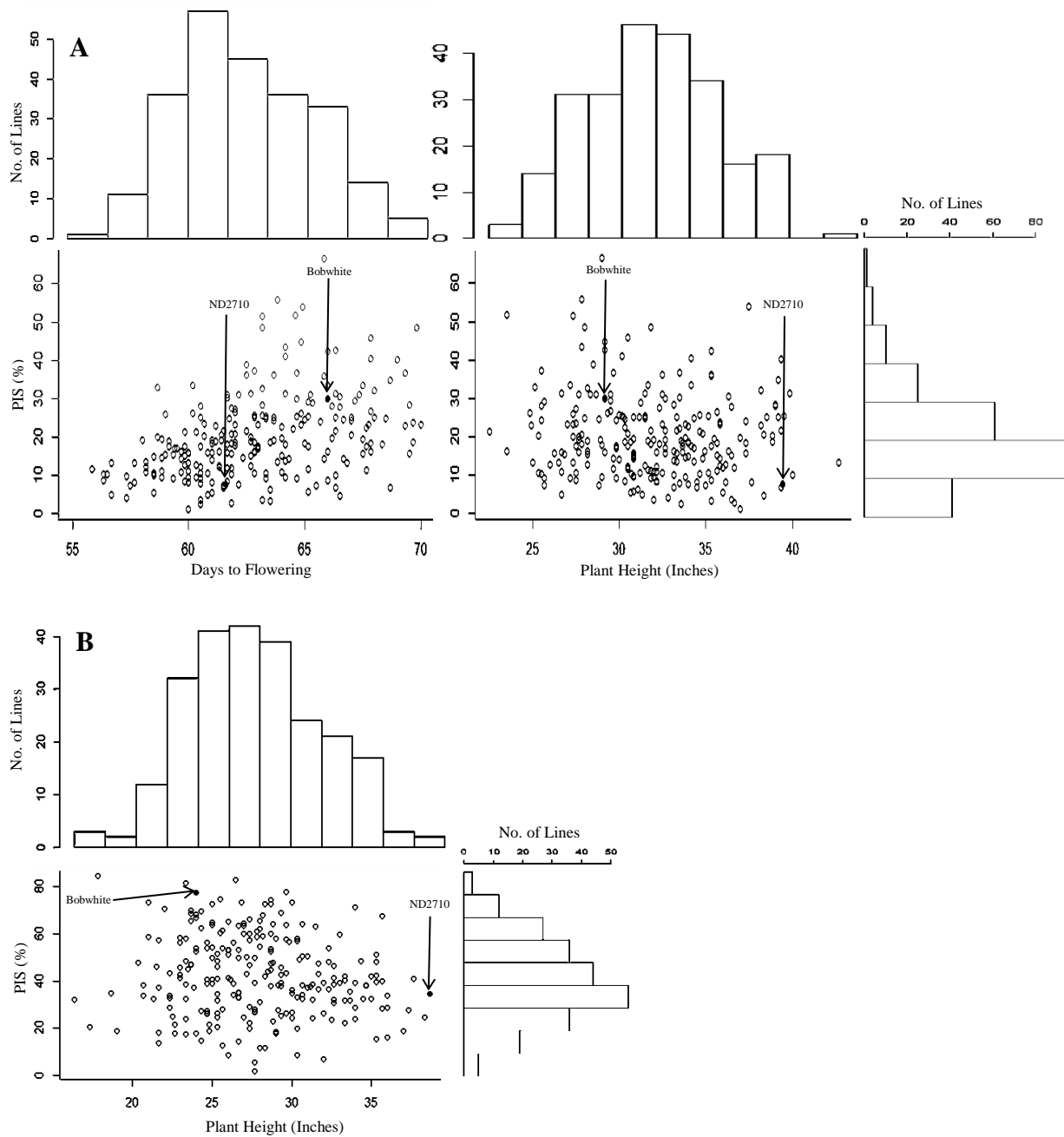


Figure 2.1. Scatterplot and histogram of traits in the BN population. (A) Averages of the 2015 and 2016 field data were used to generate the histograms and scatter plots of percentages of infected spikelets (PIS) and days to flowering. For plant height the 2015 field data were used. (B) 2016 Green house data were used to generate the histogram and scatter plot for plant height. Arrows show the resistant parent ND2710 and the susceptible parent Bobwhite.

Table 2.2. Spearman correlation coefficients between percentages of infected spikelets (PIS), plant height and days to flowering of the BN population grown in different environments

	2015 Field				2016 Field				2016 Green House	
	Plant Height		Days to Flowering		Plant Height ^a		Days to Flowering		Plant Height	
	r	p	r	p	r	p	r	p	r	p
PIS	-0.22	<.01	0.09	0.19	-0.02	0.771	0.64	<.01	-0.13	0.05
Plant Height			0.29	<.01			0.17	<.01		

^a Plant heights were not measured in the 2016 field trial. The data from 2015 were used to compute the correlations, r=spearman correlation coefficient, p=Level of significance. p<0.05 indicates a significant correlation

Table 2.3. Spearman correlation coefficients between different experiments for the percentage of infected spikelets (PIS), plant height, and days to flowering of the BN population

	PIS				Plant Height		Days to Flowering	
	2015 Field		2016 Field		2015 Field		2016 Field	
	r	p	r	p	r	p	r	p
2015 Field			0.40	<.01			0.43	<.01
2016 Greenhouse	0.23	<.01	-0.16	0.02	0.71	<.01	NA	NA

r=spearman correlation coefficient; p=Level of significance. p<0.05 indicates a significant correlation

The PIS for the population ranged from 0 - 100% in the field experiments and 1.78 - 84.47% in the greenhouse. Plant heights ranged from 22.5 – 42.67 inches in the field and 16 – 42 inches in the greenhouse. The days to flowering ranged from 48 - 88 days in 2015 the field experiment and 50 – 78 days in the 2016 field experiment. Spearman rank correlation coefficients (r) showed a significant (p < 0.05) and negative correlation (r = -0.22) between PIS and plant height only in the 2015 field experiment (Table 2.2). The PIS was positively correlated with the days to flowering with the correlation coefficient of 0.64 for the 2016 field experiments only. Significant correlation was also observed between the different experiments for the traits (Table 2.3). A significant positive correlation was observed between the two field experiments for PIS and days to flowering.

Homogeneity tests showed that the results for all the traits from the field experiments involving the BN population were adequately similar, and thus QTL analysis was conducted using the data from each experiment separately as well as the combined data. Three QTL for type I resistance were identified in ND2710 in the 2015 field experiment and they were designated as *Qfhb.ndwp-2A*, *Qfhb.ndwp-5A.1* and *Qfhb.ndwp-6B*, respectively (Table 2.4). *Qfhb.ndwp-2A* peaked at marker *IWB7310* with a LOD value of 6.257. It was localized between markers *IWB64705* and *IWB34883* on chromosome 2A and explained 11.7% of the total phenotypic variation. *Qfhb.ndwp-5A.1* peaked at the marker *IWB52454* on chromosome 5A, and explained 5.5% of the phenotypic variation. *Qfhb.ndwp-6B* was located between markers *IWA5722* and *IWB28183* with the peak closer to marker *IWB2587* and explained 6.2% of the phenotypic variation. QTL analysis using the combined data of the two years detected the same two QTL, *Qfhb.ndwp-2A* and *Qfhb.ndwp-5A.1*, which explained 13.7% and 7.7% of the total variation, respectively. In addition, a different QTL was identified on chromosome 5A in the 2016 greenhouse experiment, which was designated as *Qfhb.ndwp-5A.2*. It peaked at marker *IWB52862* with a LOD value of 3.44 and explained 6.6% of the phenotypic variation. No QTL for type I resistance were found with the data from the 2016 field experiment (Table 2.4). The QTL for plant height, designated as *QHt.ndwp-4B*, was detected in the same region on chromosome 4B in both the 2015 field experiment and the 2016 greenhouse experiment (Table 2.4). It peaked at marker *IWA6850*, was flanked by *IWB89610* and *IWB53624*, and respectively explained 10.6 and 18.1 % of the phenotypic variation in the 2015 field experiment and the 2016 greenhouse experiment. The QTL for days to flowering were not consistent among the two years of field experiments.

Table 2.4. Significant QTL for type I resistance to FHB identified in the BN population grown in different environments

Trait	Environment	QTL	Chr ^b	Flanking Markers	Closest Marker	Lod ^c	Add ^d	PV % ^e
Type I Resistance	2015 Field	<i>Qfhb.ndwp-2A</i>	2A	IWB64705- IWB34883	IWB7310	6.26	-4.05	11.7
		<i>Qfhb.ndwp-5A.1</i>	5A	IWA7061- IWB11019	IWB52454	4.45	-3.39	8.5
		<i>Qfhb.ndwp-6B</i>	6B	IWA5722- IWB28183	IWB2587	3.25	-2.83	6.2
	2016 Field Combined data ^a	<i>Qfhb.ndwp-2A</i>	2A	IWB64705- IWB7315	IWB7310	7.41	-3.59	13.7
		<i>Qfhb.ndwp-5A.1</i>	5A	IWA7061- IWB11019	IWB52454	4.01	-2.61	7.7
		<i>Qfhb.ndwp-5A.2</i>	5A	IWB36131- IWA5612	IWB52862	3.44	-4.12	6.6
Plant Height	2015 Field	<i>QHt.ndwp-4B</i>	4B	IWA6850 - IWB53624	IWA6850	5.7	3.93	10.6
	2016 Greenhouse	<i>QHt.ndwp-4B</i>	4B	IWA6850 - IWB53624	IWA6850	10.07	2.29	18.1
Days to Flowering	2015 Field	<i>QFlo.ndwp-4A</i>	4A	IWB10456 - IWB56312	IWB60255	4.5	-0.96	8.6
		<i>QFlo.ndwp-7B</i>	7B	IWB73515 - IWB11138	IWA4306	3.8	0.93	7.3
	2016 Field	<i>QFlo.ndwp-4B</i>	4B	IWB71718 - IWB7783	IWB30432	4.86	1.48	9.2
		<i>QFlo.ndwp-7A</i>	7A	IWB33919 - IWB8825	IWB59817	5.1	-1.38	9.7
	Combined data	<i>QFlo.ndwp-7A</i>	7A	IWB33919 - IWB8825	IWB59817	3.48	-0.89	6.7

^a The averaged data from the 2015 and 2016 Field experiments were used for QTL analysis.

^b Chr = Chromosome.

^c Significant LOD values that are above $\alpha_{0.05}$. The $\alpha_{0.05}$ values were obtained using the 1000 permutation test in Qgene.

^d Negative values indicate QTL contributed by the resistant parent ND2710 while positive values represent QTL contributed by the susceptible parent Bobwhite.

^e Phenotypic variation explained by the QTL.

In the 2015 field experiment, two QTL were detected, one (*QFlo.ndwp-4A*) on chromosome 4A and the other (*QFlo.ndwp-7B*) on chromosome 7B, which explained 8.6 and 7.3 % of phenotypic variation, respectively (Table 2.4). In the 2016 field experiment, QTL were detected on 4B (*QFlo.ndwp-4B*) and 7A (*QFlo.ndwp-7A*) that explained 9.2 and 9.7% of the phenotypic variation, respectively (Table 2.4).

GP Population

The GP population also showed continuous distribution and transgressive segregation for all traits studied in the experiments (Figure 2.2). Variation in mean PIS of the two parents was observed in the different experiments (Table 2.5). The FHB susceptible parent Grandin showed higher PIS compared to the FHB resistant parent PI277012. Mean PIS in Grandin was respectively 15.8%, 2.5% and 46.3% more than in PI277012 in the 2015 field experiment, the 2016 field experiment, and the 2016 greenhouse experiments. PI277012 was respectively 13.2 and 10 inches taller than Grandin in the 2015 field experiment and the 2016 greenhouse experiment.

The PIS for the GP population ranged from 0 to 94.4% in the 2015 field experiment and from 0 to 100% in both the 2016 field and 2016 greenhouse experiments. The heights ranged from 20.87 to 33.84 inches and from 17 to 44 inches in the 2015 field and 2016 greenhouse experiments, respectively. The days to flowering ranged from 54 to 77 and 53 to 86 days in the 2015 field and 2016 field experiments, respectively. As in the BN population, the GP population also showed moderate to high heritability for the different traits studied (Table 2.5). Like the BN population, a negative correlation was observed between plant height and PIS in the GP population (Table 2.6). Significant correlation between plant height and flowering date was observed only in the 2016 field experiments. Likewise, positive and significant correlations for each trait was observed between experiments conducted in different environmental conditions (Table 2.7). *Qfhb.ndwp-1A* and *Qfhb.ndwp-6B.1* were Grandin-derived and located on chromosomes 1A and 6B, respectively, while *Qfhb.ndwp-4B.1* was contributed by PI277012 and located on chromosome 4B.

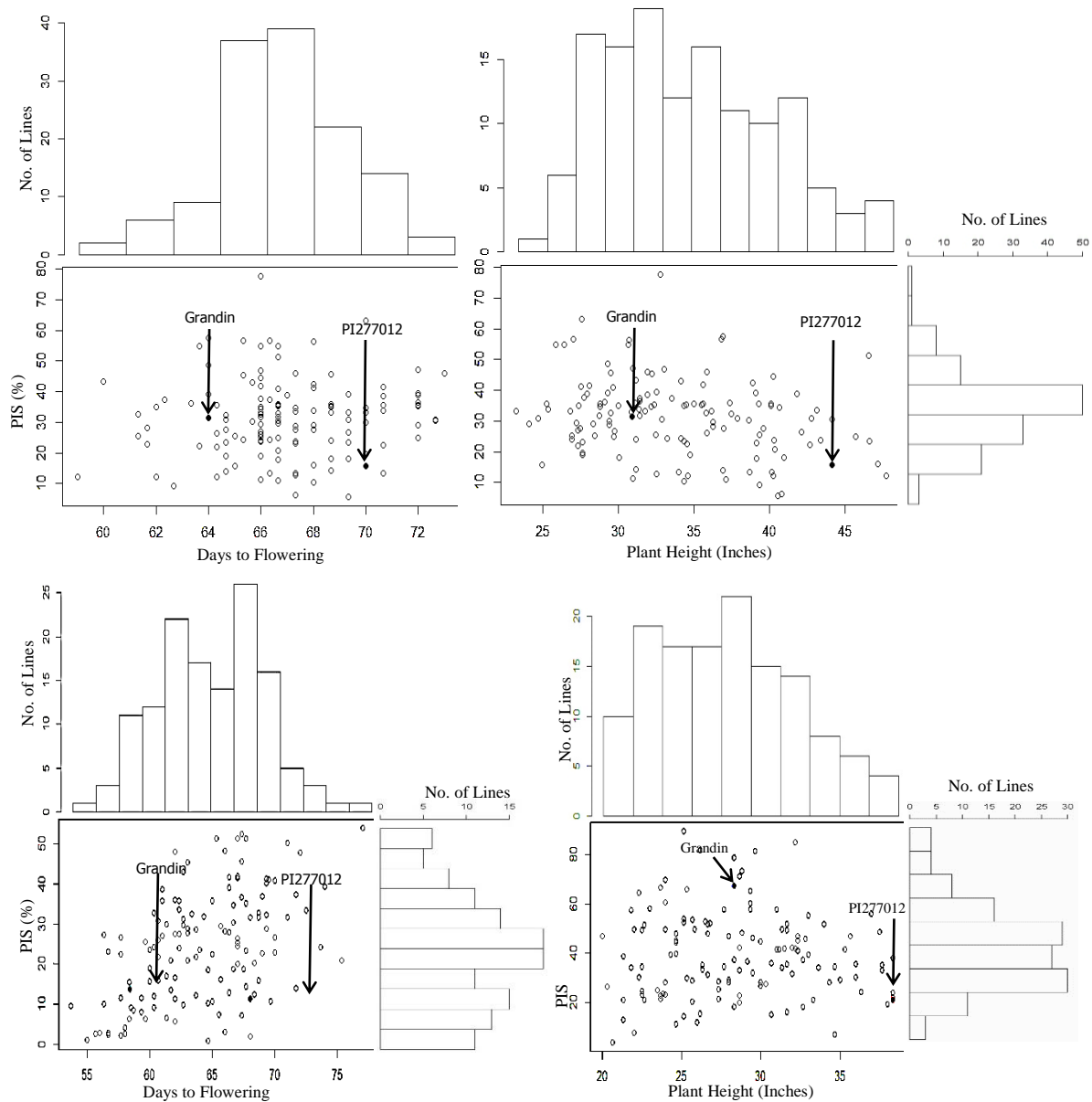


Figure 2.2. Scatterplots and histograms of traits in the GP population. (A) Percentage of infected spikelets (PIS), days to flowering and plant height data from the 2015 field trial. (B) PIS and days to flowering data from the 2016 field trial, and (C) PIS and plant height data from the 2016 greenhouse trial. The arrows show resistant parent PI277012 and susceptible parent Grandin.

Table 2.5. Heritability of percentage infected spikelets (PIS), plant height, and days to flowering in the GP population

Population	Trait	Experiments	Parents		Population			Heritability ^a
			Grandin	PI277012	Mean	Min	Max	
Grandin × PI277012 (GP)	PIS	2015 Field	31.43	15.64	31.08	0	94.44	0.6 ^b
		2016 Field	13.78	11.32	24.53	0	100	0.64
		2016 Greenhouse	67.37	21.07	31.48	0	100	0.75
	Height	2015 Field	30.88	44.14	57.09	20.8 7	33.84	0.33
		2016 Greenhouse	28.33	38.33	28.54	17	44	0.25
	Days to Flowering	2015 Field	64	70	64.19	53.6 7	77	0.10
2016 Field		58.41	68	64.05	53	86	0.12	

^a Broad sense heritability was calculated using the restricted maximum likelihood (REML) method in SAS (Holland et al. 2003)

^b Heritability coefficients for single year data were estimated using equation $H = \delta^2_G / (\delta^2_G + \delta^2_E/r)$ where, δ^2_G = genotypic variation, δ^2_E = residual variance, r = number of replications.

Table 2.6. Spearman correlation coefficients between PIS, plant height and days to flowering for the GP population grown in different environments

	2015 Field		2016 Field				2016 Greenhouse			
	Plant Height		Days to Flowering		Plant Height ^a		Days to Flowering		Plant Height	
	r	p	r	p	r	p	r	p	r	p
PIS	-0.26	<.01	0.07	0.45	-0.29	<.01	0.47	<.01	<.01	0.99
Plant Height			-0.03	0.75			<.01	<.01		

^a Plant heights were not measured in 2016 field. The data from 2015 were used to compute the correlations. r=spearman correlation coefficient; p = Level of significance. p<0.05 indicates a significant correlation

Table 2.7. Spearman correlation coefficients between different experiment for percentages of infected spikelets (PIS), plant heights, and days to flowering in the GP population

	PIS				Plant Height		Days to Flowering	
	2015 Field		2016 Field		2015 Field		2016 Field	
	r	p	r	p	r	p	r	p
2015 Field			0.17	0.05			0.44	<.01
2016 Green House	0.22	0.01	0.03	0.77	0.88	<.01	NA	NA

r=spearman correlation coefficient; p = Level of significance. p<0.05 indicates a significant correlation

Table 2.8. Significant QTL for type I resistance to FHB in the GP population grown in different environments

Trait	Environment	QTL	Chr. ^a	Flanking Marker	Closest Marker	Lod ^b	Add ^c	PV % ^d
Type I Resistance	2015 Field	<i>Qfhb.ndwp-1A</i>	1A	SNP6553- barc269	SNP4291	3.71	7.66	12.4
		<i>Qfhb.ndwp-4B.1</i>	4B	gwm375- barc20	wmc419.1	3.74	-3.67	12.5
		<i>Qfhb.ndwp-6B.1</i>	6B	SNP1901 - SNP2479	SNP2492	3.16	3.31	10.7
	2016 Field	<i>Qfhb.ndwp-4B.2</i>	4B	Rht-B1- gwm375	SNP2194	4.83	-4.32	15.8
		<i>Qfhb.ndwp-6B.2</i>	6B	SNP3352- SNP2975	SNP2305	4.13	-3.94	13.7
	2016 Greenhouse	<i>Qfhb.ndwp-2B</i>	2B	SNP2440- barc183	SNP1359	3.29	-4.75	11.1
		<i>Qfhb.ndwp-5B</i>	5B	gwm604- SNP47	SNP2500	5.91	-6.57	19
Plant Height	2015 Field	<i>QHt.ndwp-4B</i>	4B	Rht-B1 - SNP5863	SNP2194	22.44	4.74	55.1
	2016 Greenhouse	<i>QHt.ndwp-4B</i>	4B	Rht-B1 - SNP5863	SNP2194	33.19	3.52	69.4
Days to Flowering	2015 Field	<i>QFlo.ndwp-2B</i>	2B	wmc25.2 - SNP1359	SNP2440	15.05	1.44	41.6
		<i>QFlo.ndwp-3A</i>	3A	SNP2291 - barc67.2	SNP720	6.52	-2.15	20.8
		<i>QFlo.ndwp-5B</i>	5B	SNP47 - SNP8395	wmc75	4.04	0.67	13.4
		<i>QFlo.ndwp-7B</i>	7B	SNP8007 - wmc606	SNP783	9.17	1.06	27.9
		<i>QFlo.ndwp-2B</i>	2B	wmc25.2 - SNP1359	SNP2440	8.30	2.49	25.6
	2016 Field	<i>QFlo.ndwp-3D</i>	3D	SNP4209 - SNP2727	SNP2293	4.20	2.03	13.9

^a Chr = Chromosome.

^b Significant LOD values that are above $\alpha_{0.05}$. The $\alpha_{0.05}$ values were obtained using 1000 permutation test in Qgene.

^c Negative values indicate the QTL contributed by the resistant parent ND2710 while positive values represent the QTL contributed by the susceptible parent Bobwhite.

^d Phenotypic variation explained by the QTL.

QTL analysis identified three QTL for type I resistance in the 2015 field experiment (Table 2.8). *Qfhb.ndwp-1A* was located within the marker interval *SNP6553* to *barc269*, with peak near marker *SNP429* and explained 12.4% of the phenotypic variation. *Qfhb.ndwp-6B.1* peaked at marker *SNP2492* explaining 10.7% of the phenotypic variation. *Qfhb.ndwp-4B.1* was mapped between markers *gwm375* and *barc20* with the peak close to *wmc419.1* (LOD = 3.7) and

explained 12.5% of the phenotypic variation. In the 2016 field experiment, two PI277012-derived QTL viz. *Qfhb.ndwp-4B.2* and *Qfhb.ndwp-6B.2* were identified on chromosomes 4B and 6B, respectively. *Qfhb.ndwp-4B.2* occurred between *Rht-B1* and *gwm375* with the peak near *SNP2194* and explained 15.8 % of the phenotypic variation. *Qfhb.ndwp-6B.2* occurred between *SNP3352* and *SNP2975* with the peak near marker *SNP2305* with LOD of 4.1 and explained 13.7% of the phenotypic variation. In the 2016 greenhouse experiment, two PI277012-derived QTL were identified viz. *Qfhb.ndwp-2B* and *Qfhb.ndwp-5B* on chromosomes 2B and 5B, respectively. *Qfhb.ndwp-2B* was located between *SNP2440* and *barc183* with peak near *SNP1359* (LOD = 3.3) and explained 11.1% of the phenotypic variation. *Qfhb.ndwp-5B* mapped between *gwm604* and *SNP47* with peak close to *SNP2500* (LOD = 5.9) and explained 19 % of the phenotypic variation.

A major QTL for plant height was found on chromosome 4B (*QHt.ndwp-4B*) in the GP population (Table 2.8). As expected, the QTL associated with height is closer to marker *Rht-B1*, located in the same region as *Qfhb.ndwp-4B.1* and explained almost 55 – 69 % of the phenotypic variation. Similar to the results in the BN population, QTL for days to flowering in the GP population were different in the 2015 and 2016 field experiments, except for the QTL identified on chromosome 2B (*QFlo.ndwp-2B*), which was identified in both years of the experiments (Table 2.8). In the 2015 field experiment, four QTL were detected on chromosome 2B (*QFlo.ndwp-2B*), 3A (*QFlo.ndwp-3A*), 5B (*QFlo.ndwp-5B*), and 7B (*QFlo.ndwp-7B*), with peaks at SNP 2440, SNP 720, *wmc75* and *SNP783*, and explained 41.6%, 20.8%, 13.4%, and 27.9% of the phenotypic variances, respectively. In the 2016 field experiment, two new QTL were detected, which peaked at *SNP2440* and *SNP2293* on chromosomes 2B (*QFlo.ndwp-2B*), and 3D (*QFlo.ndwp-3D*), respectively. *QFlo.ndwp-2B* and *QFlo.ndwp-3D* explained 25.6% and 13.9%

of the phenotypic variance, respectively.

Discussion

In this study, both the GP and BN populations showed a continuous distribution for PIS, plant height and days to flowering, suggesting that these traits are quantitative. Transgressive segregation was observed in both populations, suggesting the favorable genes for these traits are present in both of the parents in each population (Buerstmayr et al., 2015). With the phenotypic and genotypic data, QTL for FHB Type I resistance, plant height, and days to flowering were detected in the two different mapping populations (BN and GP) under different environmental conditions.

QTL for Type I Resistance

Three QTL associated with type I resistance in ND2710 were identified on chromosomes 2A (*Qfhb.ndwp-2A*), 5A (*Qfhb.ndwp-5A.1* and *Qfhb.ndwp-5A.2*) and 6B (*Qfhb.ndwp-6B*), respectively. The FHB resistance of ND2701 is presumably derived from Sumai-3 because Sumai-3 was used as the resistant parent in the pedigree (Frohberg et al. 2004). A recent study confirmed that ND2710 carries *Fhb1* on 3B and *Fhb2* on 6B (Zhao et al. 2014). Previous studies indicated that some QTL are responsible for either type I or type II resistance under different environmental conditions (Steiner et al. 2004; Lin et al. 2006), while other QTL contribute both types of FHB resistance (Yu et al. 2008). In this study, no QTL for type I resistance were identified in the *Fhb1* region on 3BS, indicating that *Fhb1* was not significant for type I resistance under the conditions used in the study. This result is consistent with those of Lin et al. (2006) but differs from those of Yu et al. (2000). One QTL for type I resistance was identified on 6B, but it is not known if it is localized in the same region as *Fhb2* because the markers used for QTL mapping in this population are different from the markers used in the previous studies.

Peterson et al. (2016) identified QTL *Qfhb.nc-2A* associated with disease severity and DON accumulation in the same region harboring *Qfhb.ndwp-2A*. It is likely that *Qfhb.nc-2A* and *Qfhb.ndwp-2A* are not the same QTL but closely linked to each other since significant QTL for FHB resistance has not been detected in the study of Zhao et al. (2014). The QTL (*Qfhb.ndwp-5A.1* and *Qfhb.ndwp-5A.2*) on chromosome 5A were identified in the 2015 field and 2016 greenhouse experiments, respectively. Chu et al. (2011) reported two QTL for FHB resistance on 5A, designated as *Qfhb.rwg-5A.1* and *Qfhb.rwg-5A.2* in the GP population. Since the study by Chu et al. (2011) was done using SSRs only, their result could not be related to the present study. However, a recent study using 9K SNP and other markers identified the same two QTL associated with FHB Type II resistance in PI277012 (Zhao et al., 2015). Based on the consensus maps for 9K and 90K SNPs, *Qfhb.ndwp-5A.1* mapped in the same region as *Qfhb.rwg-5A.1*, but *Qfhb.ndwp-5A.2* is located in a different region from *Qfhb.rwg-5A.2* and may represent a unique QTL for type I resistance.

Multiple QTL for type I resistance were found in the GP population derived from the cross of PI277012 and Grandin. They were mapped on chromosomes 1A (*Qfhb.ndwp-1A*), 4B (*Qfhb.ndwp-4B.1* and *Qfhb.ndwp-4B.2*) and 6B (*Qfhb.ndwp-6B.1*). Chu et al. (2011) reported a QTL *QHt.rwg-4B* associated with fusarium damaged kernel count in a region containing *Rht-B1* on the short arm of chromosome 4B. The present study identified QTL *Qfhb.ndwp-4B.2* associated type I resistance in the 2016 field experiment of the GP population. This QTL may be due to the effect of plant height as previous studies indicated a close relationship between plant height and FHB resistance (Rosman et al. 2009; Yan et al. 2011). The QTL *Qfhb.ndwp-2B* identified in the 2016 greenhouse experiment was mapped in the same region as a QTL identified by Chu et al. (2011) for days to heading. It might be possible that *Qfhb.ndwp-2B* is

associated with both disease incidence and days to heading. Lu et al. (2013) also reported a QTL associated with FHB resistance (after point inoculation) on chromosome 2B. The QTL *Qfhb.ndwp-2B* identified in the 2016 field experiment might be the same as the QTL identified by Lu et al. (2013), since they are located at the same distance from marker *brac35*. *Qfhb.ndwp-6B.2* was identified in the 2016 field experiment and mapped to the same region containing *Fhb2* (Cuthbert et al., 2007).

Qfhb.ndwp-5B was identified as a QTL for Type I resistance only in the 2016 greenhouse experiment. This QTL mapped to the same region as the minor QTL reported by Lu et al. (2013) which was associated with FHB severity in grain spawn and spray inoculated experiments, but not in their point inoculated experiment.

Heritability of Traits

The heritability of type I resistance based on PIS for both populations was moderate as expected, as it is evident from most of the previous studies that FHB disease establishment and development are highly influenced by environmental factors (reviewed in Buerstmayr et al., 2009). Higher heritability in the greenhouse (under controlled environment) compared to the field conditions corroborates the strong effect of $G \times E$ interaction on FHB resistance. However, heritability of plant height was higher in both field and greenhouse experiments for the BN population (Table 2.1) than for the GP population (Table 2.5). Despite high heritability, only one QTL for plant height was identified in the BN population, which explained 10.6% of the phenotypic variance in the 2015 field experiment (Table 2.4). This may be due to the presence of multiple minor alleles that contribute to height but could not reach a significant LOD threshold (Buerstmayr et al., 2015). In contrast, the GP population had intermediate heritability for plant height (Table 2.5), but a QTL (*QHt.ndwp-4B*) with a strong peak explaining a very high

percentage of phenotypic variance was identified (Table 2.8). *Qht.ndwp-4B* mapped to the same region as the semi-dwarfing gene *RhtB1b* (Hedden, 2003), suggesting that they are the same gene. *RhtB1b* is present in Grandin and previous identified by Chu et al. (2011) as a major QTL in the GP population. The lower heritability for this trait suggests the gene to be strongly influenced by the environment.

The heritability for days to flowering in the BN was high (Table 2.1) but QTL identified explained a small percentage of the phenotypic variance (Table 2.4). This result suggests the presence of multiple QTL with minor effect, which were not identified with the given data. Interestingly in GP population, the heritability for days to flowering was very low but QTL were identified on six different chromosomes with phenotypic variation ranging from 13.4 to 41.6 % (Table 2.4). The heritability for both height and days to flowering in GP population were low, suggesting these traits are more affected by the given environments in the GP population than in the BN population.

Associations between Traits

A significant negative correlation was calculated between height and PIS. This result is consistent with all the previous studies (Draeger et al. 2007, Gosman et al. 2009, Chu et al. 2011, Yan et al. 2011, Buerstmayr et al., 2015). Shorter plants might have shown higher PIS due to the microclimate created by tall plant (Yan et al, 2011).

Despite the significant negative correlation between height and PIS, QTL for disease resistance in the BN population was not found on chromosome 4B, suggesting that the FHB resistance from ND2710 is not associated with *Qht.ndwp-4B*. However, in the GP population, QTL for type I resistance were found on chromosome 4B in the field experiments of both years, though the chromosomal regions were different. In the 2016 field experiment, the disease

resistance QTL *Qfhb.ndwp-4B.2* occurred in the same region as the height reducing QTL *QHt.ndwp-4B*, but *Qfhb.ndwp-4B.1* resided distal to *QHt.ndwp-4B*. This suggests that some QTL for plant height could affect type I resistance or type II resistance as has also been found in previous reports (Yan et al., 2011). Unlike plant height, days to flowering showed a positive correlation with PIS. However, although multiple QTL for days to flowering were detected in both the BN and GP populations, none of them were mapped to the same regions where QTL for FHB resistance were identified, suggesting that the QTL for days to flowering were not associated with FHB resistance. The correlation may be due to some unidentified minor gene acting in background that impacts both of these traits.

Conclusion

A number of QTL associated with type I resistance to FHB were identified in this study. However, no QTL for type I resistance were found on chromosome 3B where *Fhb1* is located in the BN population where ND2710, the resistant parent, has *Fhb1* derived from Sumai-3. In both populations, QTL were identified on chromosome 6B. However, comparison of the QTL identified in the BN and GP populations could not be made since the markers used for QTL mapping were different. The QTL expression was inconsistent among the different trials due to highly variable environmental conditions. Therefore, multiple trials at multiple locations should be conducted in order to identify the QTL truly associated with reducing the disease incidence. The DNA markers associated with the QTL identified in the study could be used for marker-assisted selection to develop the cultivars with a higher level of FHB resistance.

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CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF FUNGAL SPECIES ASSOCIATED WITH ROOT ROT OF WHEAT IN NORTH DAKOTA

Abstract

Wheat crown rot (CR) and common root rot (CRR) are mainly caused by *Fusarium* species and *Bipolaris sorokiniana*, respectively. The diseases can cause average yield losses of 15-35% in a year. However, in North Dakota (ND), few studies have been conducted on these diseases in recent years. To assess the prevalence and severity of these diseases in ND, we collected wheat root samples from fields across the state in 2012, 2013, and 2014. The collected root samples were scored for CRR severity, and then fungi associated with the CRR and CR symptoms were isolated and identified. Higher incidence and severity of CRR were observed in 2012 (warm and dry year) than in 2013 and 2014. *B. sorokiniana* was more frequently isolated from both infected crown and sub crown internodes (SCI) than other fungal species in all sampled years. Although *F. oxysporum*, *F. solani* and *F. redolens* were isolated from the root rot samples, they were not pathogenic on the two spring wheat genotypes tested. One *F. culmorum* isolate and one *B. sorokiniana* isolate were used to evaluate seedling reactions of ten spring wheat lines for seedling rot, and the result showed that Glenn was the most resistant while Steele-ND was the most susceptible to the infection of these two isolates. Twenty different spring genotypes were also tested for reactions to CRR using one isolate of *B. sorokiniana* for inoculation and it was found that Freyr and RB07 were more resistant while Len and Briggs were more susceptible to this isolate compared to other wheat genotypes evaluated. This study provides useful information on fungal species causing the root rot disease complex of wheat in ND and resistant/susceptible reactions of some spring wheat varieties to representative pathogenic fungal isolates.

Introduction

Common root rot (CRR) and crown rot (CR) are among the most important and common seed and soil borne diseases of wheat and barley in many regions of North America including the Upper Midwest region (Harding 1978; Stack 1992; Moya-Elizondo et al. 2011). In spring wheat, CRR and CR can cause yield loss up to 15% and 35%, respectively (Machacek, 1943; Smiley et al., 2005). CRR is mainly caused by the fungal pathogen *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (S. Ito & Kuribayashi) Drechsler ex Dastur), which is also the causal agent of spot blotch on leaves of wheat and barley (Arabi et al., 2006). In contrast to CRR, CR is a disease complex caused by several pathogenic fungi (Paulitz et al. 2002). *Fusarium culmorum* (W.G. Sm.) Sacc., *F. pseudograminearum* (= *Gibberella coronicola*), and *F. graminearum* Schwabe (= *G. zae* (Schwein.) Petch) are the most common pathogens associated with this disease complex (Smiley and Patterson 1996; Paulitz 2006). These fungi also cause Fusarium head blight in wheat and barley (Cook et al., 2010). Some of the *Pythium* and *Rhizoctonia* species were also found to be associated with root rot of spring wheat (Cook et al., 1980). CRR and CR can affect both seedlings and adult plants. The most common symptoms of CRR are necrotic lesions in the sub-crown internode (SCI) that merges as plants mature, while plants with CR show reddish-brown lesions on the root and crown tissue (Stack and McMullen, 1999).

Ashley et al. (1997) showed that the CRR pathogen, *B. sorokiniana*, was found throughout the 5-leaf stage of diseased plants while *Fusarium*, *Pythium* and *Rhizoctonia* were detected at the soft dough stage. The presence of *Rhizoctonia* and *Pythium* was found to be favored in zero tillage farming compared to wheat grown in other tillage systems (Cook et al., 1980). No-till farming has gained more popularity over tilled farming in recent years. It

conserves soil moisture, reduces labor and fuel cost and also helps in reducing soil erosion (Dyer et al. 2009; Hogg et al. 2010). CRR and CR are favored by relatively high soil moisture and thus no-till farming increases the severity of CRR (Wildermuth et al., 1997) and CR (Papendick and Cook, 1974; Smiley et al., 1996; Burgess et al., 2001). Besides, conservation tillage increases inoculum pressures, cropping intensity, crown depths and early season moisture, which not only increase the disease severity (Papendick and Cook, 1974; Smiley et al., 1996; Burgess et al., 2001; Dyer et al., 2009) but also cause changes in the pathogens populations associated with root rot diseases (Cook 1980; Swan et al., 2000; Wildermuth et al., 2001; Dyer et al., 2009). Owing to the fact that the dynamics of root rot pathogens changes with cropping systems and weather patterns, disease surveys have been conducted in several regions of the United States, including the Pacific Northwest (Cook, 1968; Smiley and Patterson, 1996), Texas Panhandle (Specht and Rush, 1988), southeastern Idaho (Strausbaugh et al., 2004), upper coastal plain area of Mississippi (Gonzalez and Trevathan, 2000), and Canadian Prairies (Hall and Sutton, 1998; Fernandez and Jefferson 2004; Fernandez et al. 2007a, b; 2009), Montana (Moya et al., 2011). However, very few surveys have been conducted in North Dakota (ND) in the past 20 years (Ashley et al. 1997; Stack and McMullen 1999), although the cropping systems and the weather patterns have changed in recent years (www.ndsu.edu/ndsco/resources/growingseason summary). New surveys are required in ND to understand the population dynamics of root pathogens and to design an effective management strategy for root rot diseases. Therefore, the objectives of this study were to survey CRR and CR in North Dakota, identify the pathogens associated with them, and screen spring wheat genotypes for resistance to these diseases.

Materials and Methods

Surveys of Wheat Root Diseases in North Dakota

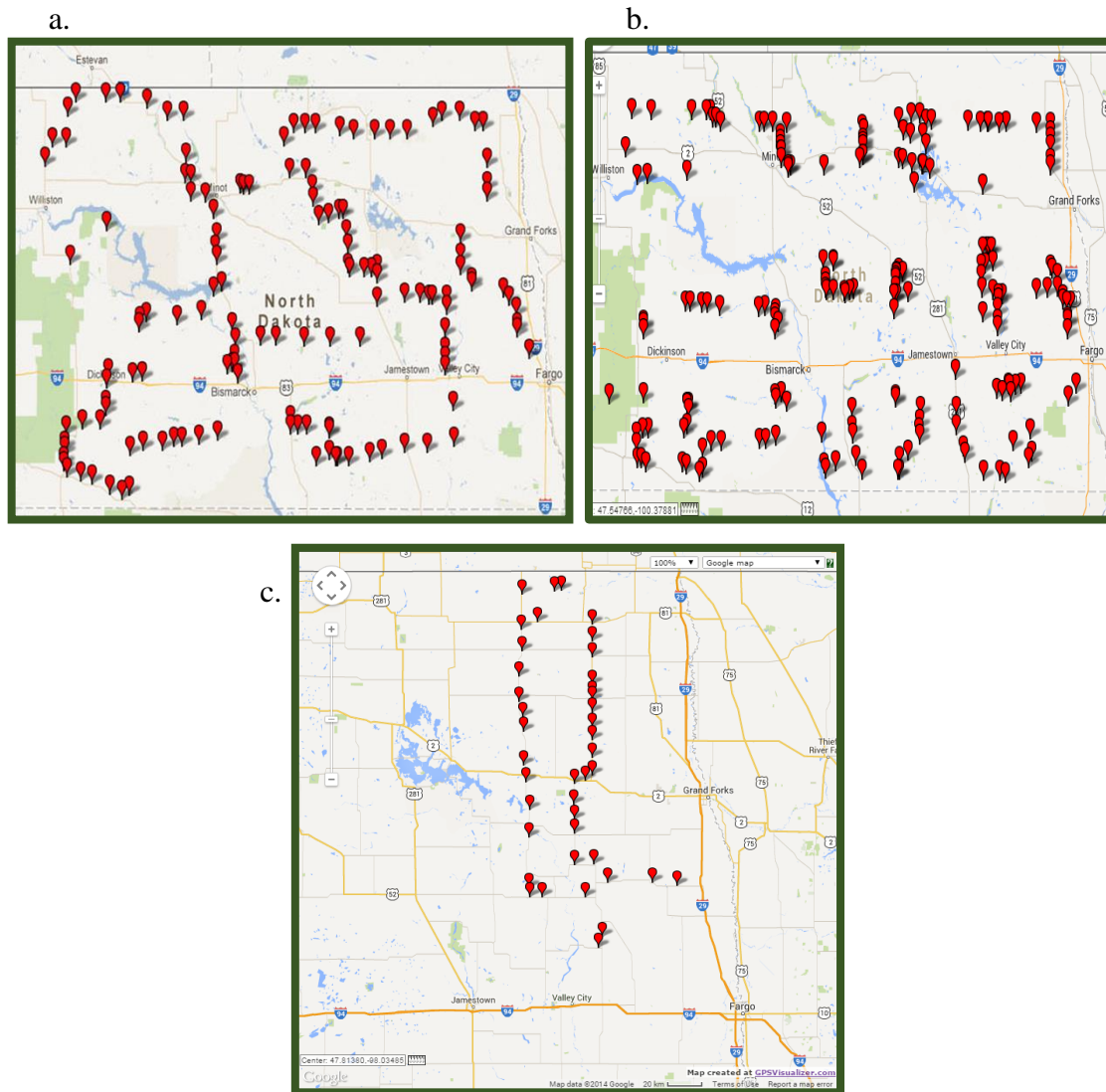


Figure 3.1. Map layout of wheat fields sampled in (a) 2012, (b) 2013, and (c) 2014 for CRR and CR in North Dakota. (a) In 2012, 140 fields were sampled across 46 counties. (b) In 2013, 206 fields sampled across 42 counties. (c) In 2014, 41 fields were sampled across 11 counties. Red balloons represent the fields that were sampled, marked by GPS co-ordinates and visualized by using www.gpsvisualizer.com.

The surveys were conducted by collecting samples from different wheat growing fields during the wheat growing seasons of 2012, 2013 and 2014. The samples were collected from 140

fields across 46 ND counties in 2012, 206 fields across 42 counties of ND in 2013 and 41 fields across 11 counties in 2014 (Figure 3.1). Eight to ten wheat plants within the flowering to dough stages were arbitrarily selected and uprooted from each field. Sampled fields were at least 5 miles apart and were marked by GPS co-ordinates. The GPS co-ordinates were uploaded in www.gpsvisualizer.com to obtain the maps of the sampled fields. Collected root samples were washed thoroughly with tap water and air dried. CRR severity of the root sample was rated by using the disease rating scale formulated by Ledingham et al. (1973). The samples were stored in a refrigerator at 4 °C until use.

Identification of Fungi Associated with the Wheat Root Rot Diseases

Four infected crown and SCI tissues from each of the collected samples were excised, surface sterilized in 0.5% NaOCl for 2 minutes, rinsed three times using sterilized distilled water, and plated on water agar. After three days of incubation, hyphal tips of fungal growth were transferred onto fresh PDA. *Bipolaris sorokiniana* was identified based on the morphology of mycelia and spores on PDA. For *Fusarium* species, isolates were grown on carnation leaf agar, and morphological features such as growth pattern (slow or fast), pigmentation on the media and the shape and size of macroconidia and microconidia as described by Leslie and Summerell (2006) were used for identification. The identity of isolates was then confirmed by sequencing the translation elongation factor alpha 1 (TEF-1 α) gene (Knutsen et al. 2004). The primer pair ef1 (forward primer; 5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (reverse primer; 5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3' (O'Donnell et al., 1998c) was used for PCR, and primer ef22 (5'-AGGAACCCTTACCGAGCTC- 3') was used for gene sequencing according to the method of Geiser et al. (2004). The internal transcribed spacer (ITS) of the rDNA gene was amplified by PCR with primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-

TCCTCCGCTTATTGATATGC-3') (Fujita et al., 2001) for fungal isolates in which the TEF-1 α gene was not amplified by ef1 and ef2. The obtained gene sequences were blasted against the Fusarium ID database (Geiser et al., 2004) and US National Center for Biology Information (NCBI) <http://www.ncbi.nlm.nih.gov/Entrez/>) for comparison of sequences of *Fusarium*. The fungal isolates were stored in 30% glycerol solution and kept in -80°C.

Pathogenicity Test

The different fungal species isolated from wheat samples were evaluated for pathogenicity by the sand-cornmeal inoculum layer method described by Bilgi et al. (2008) with slight modification. To prepare the inoculum, three different isolates from each fungal species were grown on PDA under a light and dark cycle of 13 and 11 hours, respectively, at room temperature. Then, five 5-mm plugs from 7 days old cultures of each isolate were used to inoculate a pre-sterilized (at 121 °C, 1.05 kg/cm² (15 psi) for 45 min) sand-cornmeal mixture consisting of 90g and 10g cornmeal and 20ml water in a 250 ml conical flask. The inoculated mixture was incubated for 12 days with hand shaking daily in order to let the fungus grow throughout the mixture. Two spring wheat genotypes, ND652 and Alsen, were used for the pathogenicity tests because they are moderately resistant to root rot of wheat (Stack and McMullen, 1999; Mergoum et al. 2005; Mitter et al., 2006 and Tobias et al. 2009). Seeds of each wheat genotype were surface sterilized in 0.5% NaOCl for 2 minutes and rinsed with sterilized distilled water for three times, and planted on the bed of inoculum sandwiched by layers of autoclaved vermiculite (Figure 3.2a). Plants were grown in humidity chambers employing light/dark cycles of 13/11 hours, respectively, with the humidity on for 15 seconds, every 6 minutes. Three cups per treatment and 3 seeds per cup were arranged in a completely

randomized design (CRD). Presence/absence of disease symptoms on roots of the plants was recorded at 10 days after planting. The experiment was repeated twice.

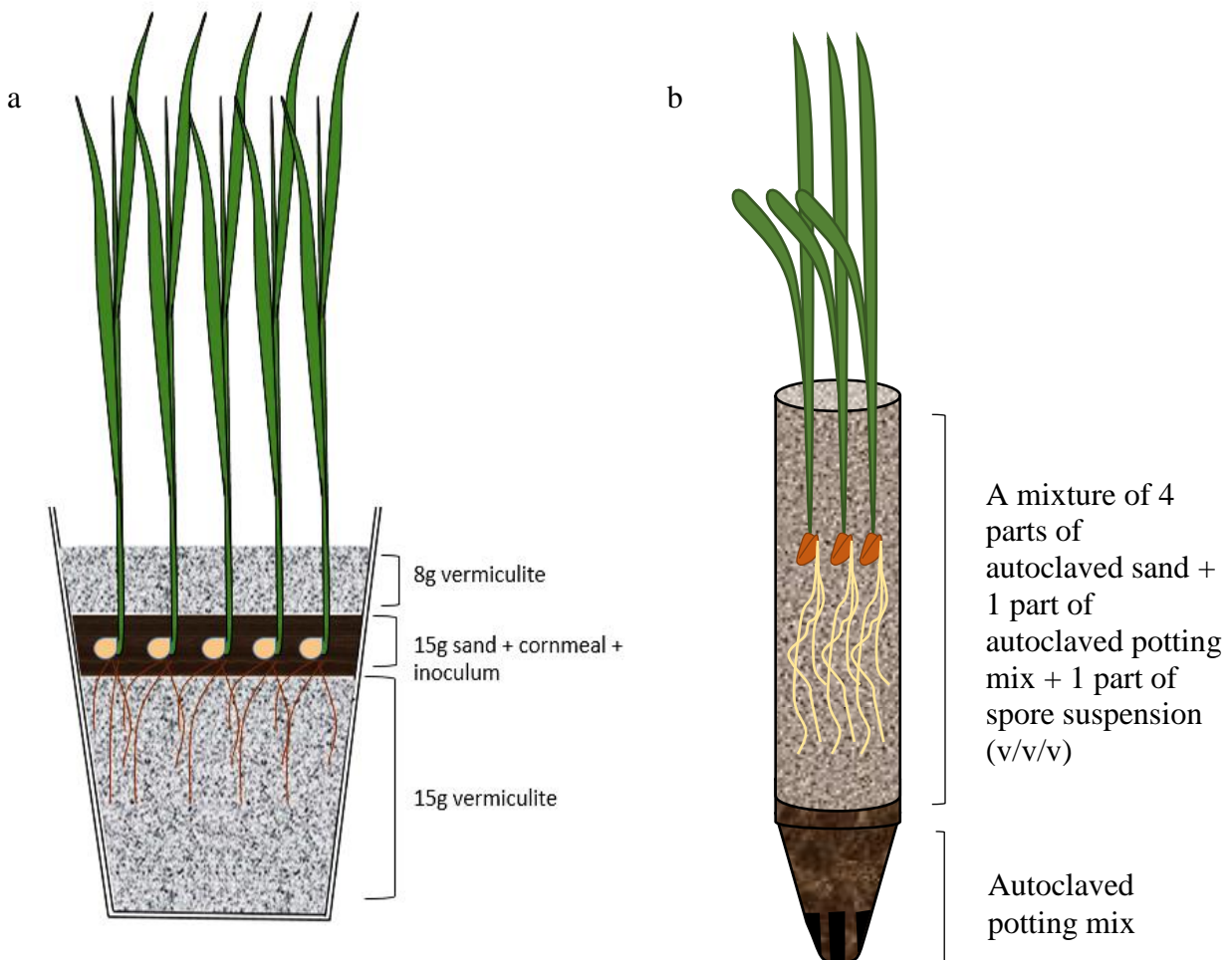


Figure 3.2. Diagrams of a pot and a cone filled with layers of inoculum and potting material for root rot experiments of wheat. (a) The sand-cornmeal inoculum layer method for pathogenicity tests of fungal isolates and for screening root rot resistance at the seedling stage in spring wheat; (b) Soil, sand and inoculum mixture for screening common root rot resistance at the adult plant stage.

Evaluation of Seedling Reactions to Root Rot Pathogens in Spring Wheat

Ten spring wheat genotypes (Table 3.1) were evaluated for reactions to the two pathogenic fungi *F. culmorum* and *B. sorokiniana*. One randomly picked isolate from each fungal species was used. Inoculum preparation and planting were done as described above for

Table 3.1. Different spring wheat genotypes used in the study

Genotypes	Pathogenicity test	Seedling test	Adult plant screening
Alsen	✓	✓	✓
Amidon	✗	✓	✓
Barlow	✗	✗	✓
Briggs	✗	✓	✓
Choteau	✗	✓	✓
Dapps	✗	✗	✓
Dulair	✗	✗	✓
Faller	✗	✓	✓
Freyr	✗	✗	✓
Glenn	✗	✓	✓
Len	✗	✓	✓
ND652	✓	✓	✓
Parshall	✗	✗	✓
Prosper	✗	✗	✓
RB07	✗	✗	✓
Reeder	✗	✓	✓
Rollag	✗	✗	✓
Steele-ND	✗	✓	✓
Velva	✗	✗	✓
Wheaton	✗	✗	✓

✓: Used in the experiment

✗: Not used in the experiment

the pathogenicity tests. Three cups (reps) with 5 seeds per cup were used for each treatment.

Disease severity and seedling height were recorded. The experiment was repeated twice.

Disease rating was done by using the severity scale that was modified by van Leur (1991), and Arabi and Jawhar (2002). The hypocotyls of the seedlings were examined for the presence of necrotic lesions and the level of severity assessed based on a 0-5 scale, where 0 = no lesions; 1 = small light brown lesions (1-10% tissue discoloration); 2 = light brown lesions (11-25% tissue discoloration); 3 = light brown to black lesions (26-40% tissue discoloration); 4 = black lesions (41-70% tissue discoloration); and 5 = black lesions (71-100% tissue discoloration). The rotten seeds that failed to germinate were scored as 5.

Screening Adult Plants for Reactions to CRR

One of the isolates from *B. sorokiniana* was used to evaluate reactions of 20 different varieties of spring wheat (Table 3.1) to CRR at the adult plant stage. A mixture of four parts of autoclaved sand, one part of autoclaved potting mix (Pro-Mix LP15; Premier Tech Horticulture, Quakertown, PA), and one part of spore suspension (v/v/v) were used as inoculum mixture for the experiment. A spore suspension was prepared by harvesting the spores from a 7 day old culture of the isolate using distilled water and adjusting it to a concentration of 1000 spores/ml. Water was used as control. D 40 Deepots (6.4 cm in diameter and 25.4 cm in depth) (Stuewe and Sons, Tangent, OR) were used for planting this experiment. Pots were filled with inoculum mixture till two thirds of the cone and three surface sterilized seeds were planted and covered with the inoculum (Figure 3.2b). In order to prevent running off of inoculum mixture through the drain holes, the bottom of the cones were first filled with potting mix. Plants were kept in the humidity chamber for 5 days (until the majority of seeds germinated) under 13 hours of light and misting for 15 seconds every 6 minutes. Seedlings were then moved to a greenhouse and a slow-release fertilizer (Osmocote Plus 15-9-12 N-P-K plus minors; Everris Inc., Dublin, OH) was applied. Plants were grown for 7 weeks before lesions in SCIs were observed and severity scores were recorded. The 0-5 scale described by van Leur (1991), and Arabi and Jawhar (2002) and explained above was once again used to rate the disease severity. The experiment layout was a Completely Randomized Design (CRD) with 3 replications (pots) per treatment and 3 plants per replication. The experiment was repeated once.

Data Analysis

All disease scores were converted to their corresponding percentage and mid values of the range were used for analysis. Levene's homogeneity of variance test was applied to the

repeated experiments. Tests showing no significant interaction at $P < 0.05$ were considered homogenous and thus combined for further analysis using general linear model procedure (PROC GLM) in SAS (SAS Institute Inc., Cary, NC). Treatment means were compared using Fisher's protected least significant difference (LSD) at $P = 0.05$.

Results

Field Surveys, Isolation and Identification of Fungi Associated with Root Diseases

In 2012, 146 fields were sampled across 44 counties with the mean CRR severity and incidence ranging from 25 to 88% and from 63.64 to 100%, respectively. There were 204 fields sampled across 41 counties in 2013. The mean CRR severity and incidence ranged from 4.19 to 82%, and 4.4 to 100%, respectively. In 2014, 41 fields were sampled across 11 counties with the mean CRR severity and incidence ranging from 0 to 58% and 0 to 100%, respectively. For the three years of surveys, mean disease severity and incidence were highest in 2012 in all the counties (Table 3.2).

Bipolaris sorokiniana, *Fusarium acuminatum*, *F. culmorum*, *F. graminearum*, *F. equiseti*, *F. pseudograminearum*, *F. oxysporum*, *F. redolens*, *F. sporotrachoides* and *F. solani* were isolated from the infected crown and SCI tissues. Other microorganisms like *F. poae*, *Pythium spp*, and some unknown fungal species were also recovered, but they were categorized as other species in the study due to their very slow growth. The frequencies of isolated fungal species varied among the three years (Figure 3.3). For CRR samples, *B. sorokiniana* was the most frequently isolated fungal species in all three years (19.90%, 19.83% and 34.78% in 2012, 2013 and 2014, respectively) (Figure 3.3a).

Table 3.2. Number of fields, mean root rot severity (%) and incidence (%) observed during surveys of wheat fields conducted in 2012, 2013 and 2014 in North Dakota

county	2012			2013			2014		
	# of fields	Severity (%)	Incidence (%)	# of fields	Severity (%)	Incidence (%)	# of fields	Severity (%)	Incidence (%)
Adams	4	46.43	95.65	-	-	-	-	-	-
Barnes	5	35.55	89.47	9	35.76	52.30	-	-	-
Benson	6	71.92	100.00	7	29.64	57.50	-	-	-
Billings	1	61.25	100.00	-	-	-	-	-	-
Bottineau	3	52.41	96.72	-	-	-	-	-	-
Bowman	6	69.37	100.00	8	21.59	28.30	-	-	-
Burleigh	1	47.10	90.00	6	23.05	43.90	-	-	-
Burke	3	43.86	88.57	-	-	-	-	-	-
Cass	3	60.58	100.00	8	32.43	63.40	-	-	-
Cavalier	5	72.01	94.37	3	4.19	4.40	3	42.83	100.00
Dickey	-	-	-	3	36.89	45.50	-	-	-
Divide	2	67.16	100.00	-	-	-	-	-	-
Dunn	3	65.05	91.49	3	66.34	100.00	-	-	-
Eddy	3	66.72	91.49	-	-	-	-	-	-
Emmons	5	58.27	89.47	8	46.83	70.80	1	58.00	100.00
Foster	3	64.08	93.10	-	-	-	-	-	-
Golden Valley	-	-	-	1	82.00	100.00	-	-	-
Grand Forks	1	57.16	95.74	-	-	-	2	only cr	-
Grant	4	44.42	86.36	5	33.83	71.40	1	32.71	100.00
Griggs	4	69.13	94.44	11	28.02	48.30	3	43.82	69.87
Hettinger	4	57.43	93.75	11	46.05	60.80	-	-	-
Kidder	2	55.50	79.49	4	46.85	76.70	-	-	-
LaMoure	4	58.14	77.27	2	5.69	25.00	-	-	-
Logan	3	26.00	63.64	5	34.77	58.10	-	-	-
McHenry	5	65.51	97.06	4	39.77	79.40	-	-	-
McIntosh	4	50.08	88.24	5	29.58	36.40	-	-	-
McKenzie	2	53.63	73.68	2	27.45	38.50	-	-	-
McLean	7	55.17	91.49	-	-	-	-	-	-
Mercer	2	78.71	100.00	-	-	-	-	-	-
Morton	6	48.25	92.59	6	34.18	46.80	-	-	-
Mountrail	-	-	-	3	41.10	58.30	-	-	-
Nelson	3	25.01	72.22	1	24.80	100.00	9	47.76	92.31
Oliver	2	75.42	100.00	5	36.99	50.00	-	-	-

Table 3.2. Number of fields, mean root rot severity (%) and incidence (%) observed during surveys of wheat fields conducted in 2012, 2013 and 2014 in North Dakota (continued)

county	2012			2013			2014		
	# of fields	Severity (%)	Incidence (%)	# of fields	Severity (%)	Incidence (%)	# of fields	Severity (%)	Incidence (%)
Pembina	3	72.95	100.00	-	-	-	1	0.00	0.00
Perkins	-	-	-	1	69.11	100.00	-	-	-
Pierce	3	57.94	95.83	10	46.20	62.50	-	-	-
Ramsey	-	-	-	1	19.81	60.00	4	28.09	95.45
Renville	2	33.21	83.33	4	64.93	100.00	-	-	-
Richland	-	-	-	5	80.14	42.80	-	-	-
Rolette	2	88	100.00	3	8.71	12.50	-	-	-
Sargent	-	-	-	4	16.73	30.00	-	-	-
Sheridan	-	-	-	5	12.95	30.80	-	-	-
Sioux	-	-	-	1	81.93	100.00	-	-	-
Slope	3	65.33	100.00	-	-	-	-	-	-
Stark	5	68.01	100.00	4	21.97	48.00	-	-	-
Steele	2	32.99	66.67	-	-	-	6	11.42	30.56
Stutsman	1	88.00	100.00	3	11.08	22.70	-	-	-
Towner	2	56.89	93.33	5	19.96	28.20	-	-	-
Traill	3	60.08	95.65	7	28.44	34.60	2	22.24	75.00
Walsh	2	68.08	100.00	10	22.04	38.60	9	23.14	57.64
Ward	6	69.85	100.00	11	36.29	57.90	-	-	-
Wells	3	54.37	95.24	7	43.23	84.10	-	-	-
Williams	3	30.93	93.33	3	28.43	59.10	-	-	-
Total	146			204			41		

- Indicates the county was not surveyed or the samples were not collected

Among *Fusarium* species isolated from CRR samples, *F. acuminatum* was more frequent in 2012 (10.68%) and 2013 (12.4%), but in 2014 more isolates of *F. redolens* (17.39%) were recovered. Also, *F. oxysporum* was isolated more frequently in 2012 than in 2013 and 2014. For CR samples, *B. sorokiniana* was again more frequently isolated than any other fungal species in all the years (30.07%, 17.98%, and 34.61% in 2012, 2013 and 2014, respectively) (Figure 3.3b).

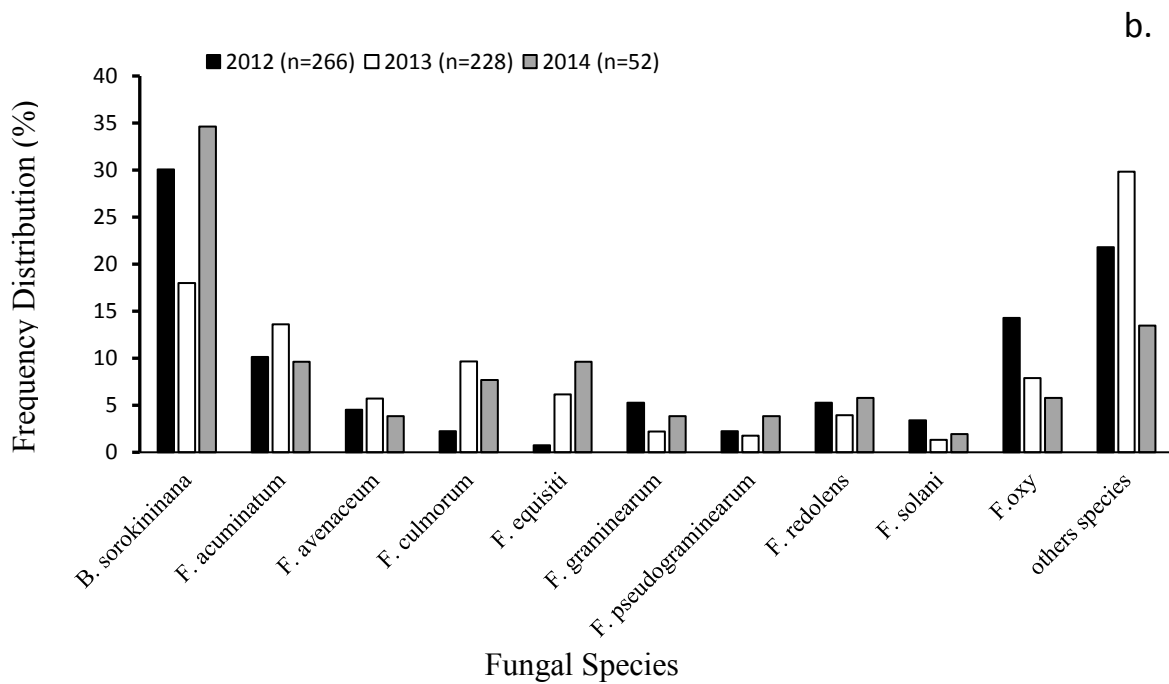
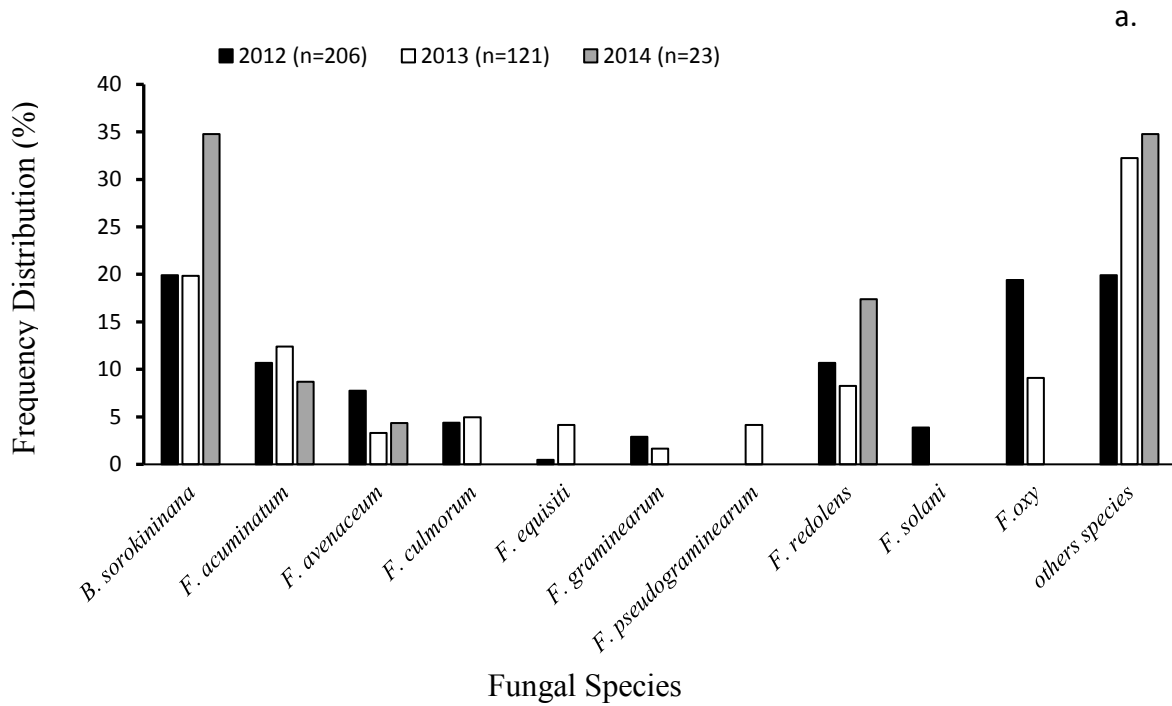


Figure 3.3. Percentage frequency distribution of different fungal species in wheat fields of North Dakota in years 2012, 2013, and 2013, respectively collected from (a) infected sub crown internodes (CRR) and (b) infected crowns (CR). The number in parenthesis represents the total number of fungal isolated from the respective crown or SCIs samples in each.

Table 3.3. Pathogenicity test of *Fusarium* species in spring wheat

Species	Isolates	1st repeat of the experiment		2nd repeat of the experiment	
		Host		Host	
		Alsen	ND652	Alsen	ND652
<i>F. equiseti</i>	13126-S1	+	+	+	+
	13147-C-1	+	+	+	+
	13037-C-2"	+	+	+	+
<i>F. avenaceum</i>	13063-C-1	-	+	-	-
	13046-C-3	+	+	+	+
	14028-C-1	+	+	+	+
<i>F. acuminatum</i>	14029-C-3	+	+	+	+
	13094-C-3	-	+	+	+
	13188-C-3	+	+	+	X
<i>F. graminearum</i>	13092-C-3	+	+	+	+
	13061-C-2	+	+	+	+
	13093-C-1	+	+	+	+
<i>F. pseudograminearum</i>	13093-C-3	+	+	+	+
	14013-C-2	X	X	X	X
	13098-C-3	+	X	+	X
<i>F. culmorum</i>	13197-C-2	X	+	X	+
	14022_C-4	X	+	X	+
	13133-C-1	X	+	X	+
<i>F. solani</i>	81-S-4	-	-		
	12-C-2	-	-		
<i>F. oxysporum</i>	92-C-3	-	-		
<i>F. redolens</i>	72-C-1	-	-		

+ indicates the presence of infection in hypocotyls of seedlings

- indicates the absence of infection in hypocotyls of seedlings

X indicates the complete failure of seed germination

Among different *Fusarium* species isolated from the CR samples in 2012, *F. oxysporum* (14.29%) was more frequently isolated. However, in 2013 and 2014, the number of *F. oxysporum* isolates was much less compared to 2012. In 2003 and 2014, *F. acuminatum* was the most frequently isolated *Fusarium* species (13.60% and 9.62% in 2013 and 2014, respectively).

Pathogenicity Tests

The pathogenicity tests showed that all of the isolates of *F. equiseti*, *F. avenaceum*, *F. acuminatum*, *F. graminearum*, *F. pseudograminearum* and *F. culmorum* were pathogenic to both spring wheat genotypes, Alsen and ND652 (Table 3.3). The isolates of *F. pseudograminearum* used in this test were highly virulent on both Alsen and ND652. All the seeds inoculated with the *F. pseudograminearum* isolates were rotten and failed to germinate. The cups treated with these isolates were completely colonized by the mycelia. The isolates of *F. culmorum* killed all seeds of Alsen before germination, while the isolates of *F. oxysporum*, *F. solani* and *F. redolens* were non-pathogenic on Alsen and ND652.

Seedling Reactions of Spring Wheat Genotypes to B. sorokiniana

The Levene's test for homogeneity showed that variance for disease severity in the 10 wheat varieties inoculated with *B. sorokiniana* (isolate: 120-C-3) was not significant at $P = 0.66$ between the two experiments conducted. Therefore, the data from the two repeats of experiments were combined for further analysis. The tested genotypes showed significant treatment \times genotype interaction (i.e. each inoculated wheat genotype was significantly different from their corresponding non-inoculated genotypes for the disease severity). Amidon was comparatively less susceptible with the disease severity of 60.38%, while Steele-ND was the most susceptible among the genotypes tested, with the disease severity of 84% (Figure 3.4a).

Seedling Reactions of Spring Wheat Genotypes to F. culmorum

The data from the two repeats of the experiments were combined for analysis since they were homogeneous with $P = 0.90$. Differences among the 10 spring wheat genotypes tested were significant with $P = <0.0001$ (Figure 3.4b).

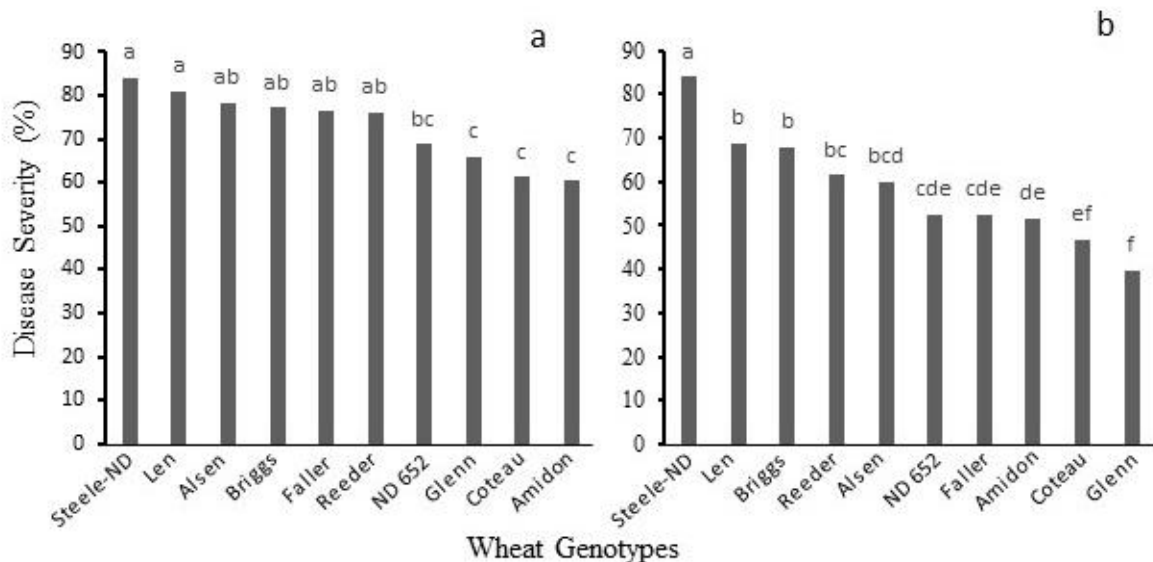


Figure 3.4. Seedling reactions of spring wheat genotypes to (a) *Bipolaris sorokiniana* and (b) *Fusarium culmorum*. Disease severity is represented as the mean percentage of root and hypocotyl rot of seedlings when inoculated with *B. sorokiniana* or *F. culmorum*. Same letters above the bars indicate no significant difference ($P=0.05$) in mean root and hypocotyl rot severity between different wheat genotypes.

Steele-ND was found to be the most susceptible wheat variety when inoculated with *F. culmorum* (isolate: 122-S-2) with a disease severity of 84%. Glenn was comparatively less susceptible with a disease severity of 39.79%.

Screening of Spring Wheat Genotypes for CRR Resistance at the Adult Plant Stage

The homogeneity test indicated that disease severity variances were not significantly ($P = 0.396$) different between the two experiments, and thus the data were combined for analysis. There were significant differences in the level of disease severity among the 20 wheat genotypes with $P < 0.0001$ (Figure 3.5). Len (73.43%) and Briggs (67.56%) were more susceptible, while RB07 (7.36%) and Freyr (9.63%) were more resistant. None of the wheat genotypes were immune to the isolate (120-C-3) of *B. sorokiniana*.

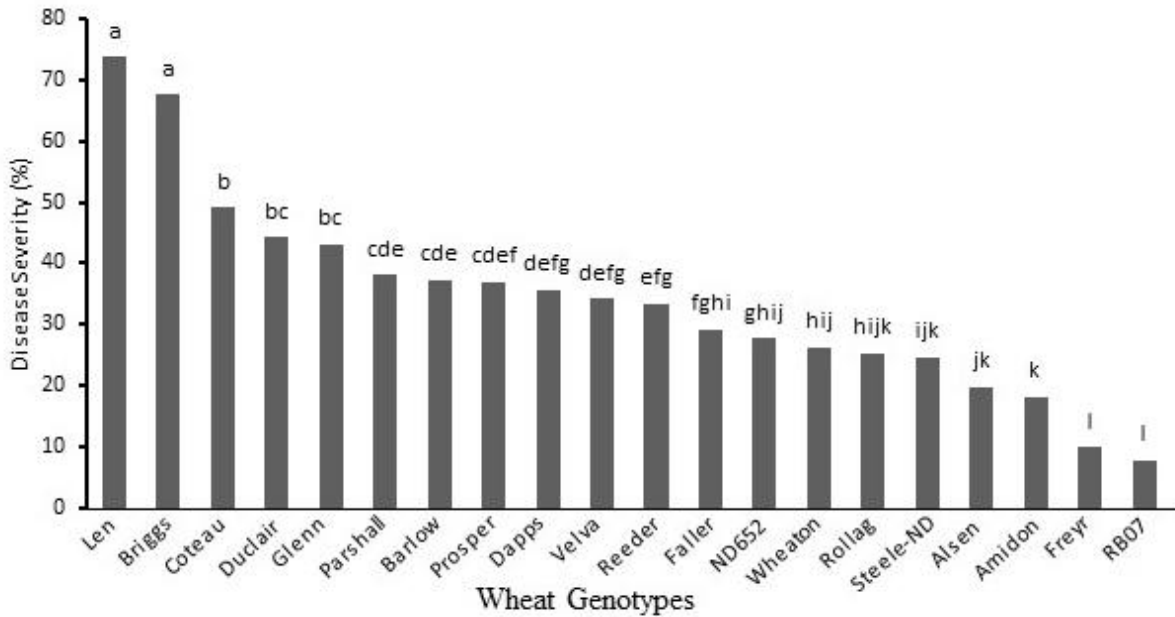


Figure 3.5. Reactions of spring wheat genotypes to common root rot (CRR) at the adult plant stage. Disease severity is represented as the mean percentage of CRR severity when inoculated with *Bipolaris sorokiniana*. Same letters above the bars indicate no significant difference ($P=0.05$) between different wheat genotypes in CRR severity in the sub-crown internodes.

Discussion

The field surveys in 2012, 2013 and 2014 showed that the root rot diseases were prevalent in wheat fields of North Dakota. However, the disease incidence and severity varied among these three years. Disease incidence and severity in the farmer's fields were higher in 2012 compared to 2013, which could be due to the contrasting weather patterns between the two years. According to the Growing Season Weather Summary for North Dakota, 2012 was the 13th driest growing season since 1895 and characterized as dry and warm when compared to the 30-year average from 1981. In contrast, 2013 is the 8th wettest growing season since 1895 and was characterized as a wet and cold growing season when compared to the 30-year average from 1981. CRR is highly favored by dry and warm weather (Grey and Mathre, 1984), which is likely the main reason for its higher incidence and severity in 2012. This also explains the reason for

the high frequency of *B. sorokiniana* isolated from both crown and the SCI tissues. Although the growing season of 2013 was wet, the weather was cooler. Therefore, the disease severity and incidence was comparatively lower in 2013 compared to 2012. In 2014, fewer fields were sampled so it is difficult to draw a conclusion about the disease scenario.

The fungal isolation and identification showed that *Fusarium* species were more prevalent than *B. sorokiniana* in all three years. However, *B. sorokiniana* was more prevalent than any other fungal species. This indicates that *B. sorokiniana* is one of the major root rot causing fungal pathogens of wheat. This result is consistent with the study of Ashley et al., (1997), which reported *B. sorokiniana* as one of the most commonly isolated root pathogen. The prevalence of different *Fusarium* species varied among the tissues collected in different years. This might be due to the differences in the weather, tillage and cropping pattern in different years. The no-till cropping system is gaining in popularity in North Dakota (Dyer et al. 2009; Hogg et al. 2010), and it changes the level of moisture in the soil. Soil moisture is an important factor for the dynamics of the microflora population and thus can influence the population dynamics of root pathogens (Cook 1980; Swan et al. 2000; Wildermuth et al. 2001; Dyer et al. 2009).

Among the different *Fusarium* species tested for pathogenicity on Alsen and ND652, *F. equiseti*, *F. avenaceum*, *F. acuminatum*, *F. graminearum*, *F. pseudograminearum*, and *F. culmorum* were able to cause disease in the seedlings. These fungal isolates also markedly affected germination of the seeds. Among them, *F. pseudograminearum* and *F. culmorum* isolates had the most severe effect on seed germination. Considering the fact that these fungal species can affect the germination of seeds, it is highly recommended to use fungicide-treated seeds for planting to protect seeds and seedlings from rotting. Besides, *F. oxysporum*, *F.*

redolens and *F. solani* were found non-pathogenic on the two wheat genotypes. These fungi might have been isolated from the infected root rot samples as secondary invaders or saprophytes living in the dead tissues. This is also supported by the fact that when the outer layer of the root rot samples was removed for fungal isolation in 2013 and 2014, the frequency of *F. oxysporum* isolates was reduced to almost half. Hence, to isolate the fungal species that are truly associated with the root rot disease, it is very important to remove the secondary invaders living on the outer layer of the dead tissue before fungal isolation.

Screening adult plants for CRR caused by *B. sorokiniana* and seedling rot caused by *B. sorokiniana* and *F. culmorum* showed that there were significant differences in the levels of disease severity among the genotypes tested. Len and Briggs were highly susceptible to CRR while Freyr and RB07 were resistant to CRR. Previous studies also showed that Len was the most susceptible wheat variety while ND652 was more resistant to CRR when compared to different wheat, durum and barley genotypes (Stack and McMullen in 1995; Mergoum et al. 2005; Tobias et al. 2009). Len was found to be highly susceptible to seedling rot when inoculated with *F. culmorum* and *B. sorokiniana* indicating that the Len is highly affected by root rot from the seedling to the adult plant stages. Amidon showed a comparatively lower level of root rot disease in both the seedling and adult plant stages. This suggests that Amidon is highly tolerant to root rot disease and can be used as one source of root rot resistance for wheat breeding programs.

The levels of disease severity are highly affected by the environment, cropping system and the crop rotation. However, choosing resistant or tolerant wheat genotypes plays an important role in preventing yield loss due to root rot diseases. This study highlights the prevalence of different root rot pathogens in wheat growing fields of ND and emphasizes the

need of similar studies to account for the changing pathogen composition associated with root diseases. The disease assay showed that widely grown wheat varieties might be at risk from root pathogens, if effective disease management is not developed to control the disease. The results also showed significant differences in reactions to the root rot pathogens tested among the wheat genotypes and thus it is important to screen more germplasm to identify effective resistance sources for breeding programs to develop wheat varieties with improved resistance to root rot.

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