GENOMICS AND MANAGEMENT OF FUSARIUM ROOT ROT OF FIELD PEAS

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Kishore Chittem

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Kishore Chittem

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Rubella S. Goswami
Co-Chair

Mohamed F. R. Khan
Co-Chair

Timothy L. Friesen

Kevin McPhee

Approved:

11-9-12
Date

Jack B. Rasmussen
Department Chair

ABSTRACT

Dry Pea or field pea (*Pisum sativum* L.) is an important cool season legume crop grown in the United States. Field peas are vulnerable to many diseases of which, soil borne diseases including wilt and root rot are of major economic importance and can cause significant reduction in yield. There is a dearth of satisfactory methods for control of root rot and no varieties with complete resistance to Fusarium root rot are currently available. Root rot disease was found to be prevalent in all the major pea growing counties of North Dakota surveyed in 2004, 2005, 2010 and 2011. *Fusarium* species were the most frequently isolated fungal species from the infected pea roots of which, *F. oxysporum* and *F. avenaceum* were the most common. 21 Field pea varieties were screened for resistance against *F. avenaceum* and *F. solani* f. sp. *pisi*, the *Fusarium* species traditionally associated with root rots of field pea in growth chamber experiments and field trials. Low levels of resistance were detected in a few cultivars but no variety was found to be completely resistant to any of the pathogens tested. Efficiency of precipitated calcium carbonate (PCC) in controlling *Fusarium* species most commonly associated with root rots was evaluated under *in vitro* and field conditions. Significant reduction in spore production, spore germination, and dry mycelial weight of *Fusarium* spp. were detected on PCC amended media in laboratory studies. In greenhouse and field experiments significant reduction in root rot disease severity was observed with PCC application compared to control. Fungal gene expression in artificially infected field pea roots and *F. graminearum* grown in culture was assessed using the Illumina mRNA-Seq technology. A total of 613 *F. graminearum* genes were found to be differentially expressed *in planta* on pea. Functional classes associated with amino acid metabolism, nitrogen metabolism, extracellular polysaccharide degradation, detoxification by degradation and defense related proteins were found to be significantly enriched in the up-regulated gene set as determined using FunCatDB. Expression of four up-

regulated genes was confirmed by RT-PCR to validate the inferences from the sequencing results.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

LITERATURE REVIEW

Introduction

Dry pea or field pea (*Pisum sativum* L.) is an important cool season legume crop grown in the United States (McPhee, 2003). Pea is believed to have possibly originated in southwestern Asia, i.e., northwestern India, Pakistan or adjacent areas of former USSR and Afghanistan (Meuhlbauer and Tullu, 1997). Central Asia, the Near East, Abyssinia and Mediterranean have been identified as centers of origins based on genetic diversity (Meuhlbauer and Tullu, 1997). Based on morphological similarities and cytological clues Zohary and Hopf (1973) identified that the Near East *humile* peas were the primary wild stock for cultivated peas. Pea is considered a native crop of Syria, Iraq, Iran, Turkey, Israel, Jordan, Ethiopia, and Lebanon and has been under cultivation in Europe for several thousand years (Nasiri et al., 2009). Based on the findings of carbonized remains of pea in archaeological sites, it can be concluded that pea plants were domesticated in the Near East arc, also referred as fertile crescent of Southwest Asia. Wild and primitive forms were found in ecologically diverse sites stretching from the Mediterranean to Afghanistan and into the highlands of Ethiopia (Kraft and Pfleger, 2001). Peas were discovered in Neolithic farming villages of the Near East which dates back to 7000-6000 B.C. Carbonized seeds were discovered in aceramic Jarmo, north Iraq, south Turkey, and Jericho. Peas are also found in Neolithic settlements in Europe (Zohary and Hopf, 1973). After domestication, pea was disseminated to other regions including Russia to the north, Europe to the west, the Indian subcontinent and China to the east. Pea was introduced into the Americas soon after Columbus discovered the country. Winter type pea was introduced from Austria in 1922 (Mehlbauer and Tullu, 1997).

Cultivation of pea in North Dakota began in the1990s and the area of production rapidly increased from 337,500 acres in 2003 to 756,000 acres in 2010. North Dakota, Montana and Washington are the leading dry edible pea producing states. North Dakota ranks first with an area of 430,000 acres planted to pea in 2010 and contributes more than 55 percent of value of production (NASS, 2010). Dry pea is susceptible to many root-rot pathogens including *Aphanomyces, Fusarium, Pythium,* and *Thielaviopsis*. Among these, Fusarium root rot is a serious disease present in all pea producing areas in the United States (Kraft and Pfleger 2001). Traditionally, *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyder & Hans was considered to be the primary causal agent of pea root rot. There is a dearth of satisfactory methods to control this root rot disease cultivars with complete resistance to Fusarium root rot are currently unavailable (Kraft and Pfleger, 2001). The importance of this crop to North Dakota and prevalence of the disease necessitates development of an integrated disease management program to reduce losses associated with Fusarium root rot in dry peas.

Fusarium root rot of field pea: Importance and causal agents

Fusarium root rot on pea was first reported in 1918 from Montana and in 1923 in Wisconsin, it was also reported in Europe around the same time (Kraft and Pfleger, 2001). Yield losses up to 30% were recorded in eastern Washington fields infested with *F. solani* f. sp. *pisi.* Twenty six percent increase in yield was observed when the soil population of *F. solani* f. sp. *pisi* was reduced by fumigation with chloropicrin (Kraft and Pfleger, 2001). Average yield losses of 35-37% due to root rot were reported in experimental plots in five Canadian provinces (Kraft and Pfleger, 2001).

The genus *Fusarium* is a well-known group of agriculturally important plant pathogens. *Fusarium graminearum, F. solani, F. oxysporum,* and *F. avenaceum* etc., are few examples of

the species that are serious pathogens of many crop plants. The diseases caused by *Fusarium* spp. include seedling rots, root rots, foot rots, wilt, and head blight. Some of these pathogens have the ability to produce mycotoxins and secondary metabolites during pathogenesis process. The ability to produce mycotoxins contributed agricultural importance and raised concerns about animal and human health. Several *Fusarium* species are known to be associated with foot and root rots of pea in the US, with *F. solani* f. sp. *pisi* being considered the most important. Other *Fusarium* species which have been isolated from peas affected with root rot are *F. avenaceum, F. culmorum, F. graminearum, F. sambucinum* var. *coeruleum, F. equiseti, F. poae, F. sporotrichioides* and *F. tabacinum* (Clarkson 1978). Fernandez, (2007) reported that *Fusarium avenaceum* was the most common and prevalent *Fusarium* spp. isolated from the discolored roots of lentils and pea grown in the eastern part of Saskatchewan, Canada. Presence of *F. equiseti, F. acuminatum, F.culmorum, F.oxysporum,* and *F.sporotrichioides* was also reported (Fernandez, 2007). In addition to pea, *F. avenaceum* has also been isolated from crown and root tissue of other hosts such as clovers, ryegrass, soybeans and potatoes. The highest populations of *F. avenaceum* were recovered in soil just prior to harvest of peas from Prince Edward Island, Canada (Fernandez, 2007). Preliminary surveys conducted in ND in the summer of 2004 in 47 fields in 10 counties suggest *Fusarium* spp. were the primary causal agent of root rot in this area (Gregoire and Bradley, 2005) among which *F. avenaceum* was suspected to be the most prevalent (Carl Bradley, personal communication).

F. avenaceum: History, distribution and importance

In 1822, Swedish botanist E.M Fries first described this species as *Fusisporium avenaceum.* It was first isolated from oats (*Avena sativa*) and got its epithet *avenaceum* from its host name. Later in 1886, P. A. Saccardo, famous Italian mycologist transferred the name

Fusisporium to the genus *Fusarium*. Teleomorph of *F. avenaceum* is *Gibberella avenacea* (Cook). The ascospore isolations from perithecia of *G. avenacea* collected from wheat stalks and from stems of *Peritridium* produced anamorphic colonies which were identified as *F. avenaceum* (Booth and Spooner, 1984). Production of perithecia in cultures has not yet been reported (Desjardins, 2006).

F. avenaceum is distributed worldwide. It is a soil borne pathogen, found predominantly in temperate regions where cold and wet conditions prevail. So far, the species has been reported to be present in 21 states in the US. Chiefly this species is regarded as a cereal root pathogen, but later reports have established its pathogenicity on other crops grown in rotation with cereals (Nalim et al., 2009, Uhlig et al., 2007). *F. avenaceum* is widely distributed in soil and soil debris, and the host range is very wide. It has been reported on 160 plant genera spread over 26 families (Booth, 1971, Desjardins, 2006, Maekinaitë, 2005). Root rots and ear rots of cereals, root rots of legumes, dry rot of potato tubers, seedling blights, wet core rot of apples, and stem and crown rot of lisianthus are some examples of diseases caused by *F. avenaceum* (Desjardins, 2006., El-Hamalawi and Stanghellini, 2005, Sørensen et al., 2009).

F. avenaceum has also been studied as a biological control agent for weed management. Hershenhorn et al., (1992) Winder (1997), and Oleskevich et al., (1998), evaluated the potential of *F. avenaceum* for biocontrol of spotted knap weed, marsh reed grass, and invasive *Rubus* spp. in Canada, respectively (Winder, 1999). Pouleur et al., (1992), found ice nucleation activity of *F. avenaceum* in a study with 20 fungal species. Maximum temperature of ice nucleation activity for *F. avenaceum* was -2.5^{o} C and was stable through a pH range of 1 to 13. The ice nuclei were cell free and stable at 60^{o} C. These ice nuclei have potential applications in biotechnology.

In addition to causing root rot, *F. avenaceum* is frequently found on cereal grains, where it causes seedling and head blights and produces mycotoxins. *F. avenaceum*, together with other *Fusarium* species, is associated with foot and root rot or crown rot and Fusarium head blight (FHB) diseases of cereals grown in Finland and Canada (Yli-Mattila, et al., 2002, Fernandez, 2007). Considerable storage losses due to *F. avenaceum* have been reported in potato. *F. avenaceum* is known to produce mycotoxins like moniliformin, beauvericin and enniatins (Yli-Mattila, et al., 2002).

Taxonomy

According to the tree of life web project (http://tolweb.org/Hypocreales/29328), the current classification, which is based on sequence data of the teleomorph *Gibberella avenacea*, is given below (Note: the tree of life page contains classification only up to family level):

Kingdom : Fungi

Phylum : Ascomycota

Sub-phylum : Pezizomycotina

Class ; Sordariomycetes

Sub-class : Hypocreomycetidae

Order : Hypocreales

Family : Nectriaceae

Genus : *Gibberella*

Species : *avenacea*

F. avenaceum has been referred with different synonyms in earlier literature. The common synonyms are:

F. roseum ‘Avenaceum’

F. roseum var. *avenaceum*

F. avenaceum ssp. *avenaceum* (Nelson et al., 1983, Leslie et al., 2006).

Fusarium avenaceum was placed in the section Arthosporiella by Booth (1971) along with *Fusarium semitectum, Fusarium camptoceras, Fusarium sporotrichioides*, and *Fusarium chlamydosporum* based on the presence of polyphialides and microconidia. Later, Nelson et al., (1983), changed it to section Roseum.

Morphology and colony characteristics

Morphology of the colony is highly variable. The growth rate on Potato Dextrose agar varies from slow to rapid. Mycelium is generally aerial, profuse, dense, and varies in color. The color varies from white to light yellow to grayish rose to tan to reddish brown (Leslie et al., 2006, Nelson et al., 1983). The sporodochia are pale orange to orange to brown in color and are formed in a central spore mass. The pigment formed in agar gives varying colors to the abaxial surface. The abaxial surface color varies from grayish rose to tan to carmine to red to burgundy (Leslie et al., 2006, Nelson et al., 1983). It may be confused with *F. graminearum* or *F. culmorum* when the under surface is carmine red, but can be distinguished based on macroconidial morphology. Sometimes, *F. avenaceum* may be confused with *F. acuminatum* in morphology. These two species can be distinguished by the presence or absence of chlamydospores. *F. avenaceum* does not produce chlamydospores, whereas *F. acuminatum* produces chlamydospores in culture (Leslie et al., 2006, Nelson et al., 1983).

Spore morphology

Macroconidia are slender, long, thin walled, and straight to slightly curved. Macroconidia have 3-5 septate. The apical cell is long and tapering. Sometimes the apical cell may be bent. The basal cell is generally notched, but foot-shaped cells are also produced by some isolates. Macro

conidia are more abundantly produced in sporodochia (Leslie et al., 2006). Macroconidial morphology of cultures grown on Carnation Leaf Agar is recommended for identifying the species. Size of macroconidia varies from 40-80 µ long X 3.5 – 4 µ wide (Booth, 1972). Microconidia are sparsely produced either on monophialidic or polyphialidic conidiogenous cells. Microconidia vary in size, 1 to 2 or 3 septate and fusoid in shape. Microconidia vary in their size ranging from 8 – 50 µ long X 3.5 – 4.5 µ wide (Booth, 1972). Chlamydospores are not known to be produced by *F. avenaceum*. This is the key character to distinguish *F. avenaceum* from *F. acuminatum*, when the colony morphology is the same. The perithecial stage is not found in cultures grown on artificial media. This stage is only produced on naturally infected host. Perithecia are deep purple in color, and may appear black. They may be present either singly or in groups. The perithecial shape varies from irregularly globose to pyriform. Paraphyses are absent, whereas periphyses can be seen. The asci are cylindrical to clavate, 8 spored, and measure 70 – 100 X 9 – 12 µ. The ascospores are colorless, fusoid, and septate. Spores are constricted at the septum and measure 13 -19 X 4 – 5µ.

Habit and habitat

Fusarium avenaceum survives as a saprophyte in soil in the absence of a host or as a pathogen on cereals, legumes, and other hosts. Being a soil inhabitant, *Fusarium avenaceum* exists in association with other soil borne fungi, including other *Fusarium* spp. and fungi belonging to other genera. Maekinaite (2005) studied the interaction of *F. avenaceum in vitro* with nine other root rot associated fungi and noticed the prevalence of fungistatic, trophic and mutual antagonisms among the interactions. Growth of *F. avenaceum* was greatly inhibited by *Aspergillus ochraceus, Chaetomium globosum* and *Rhizoctonia* spp. Forbes and Dickinson (1977) studied the behavior of *Fusarium avenaceum* in soil growth analysis plates and found that

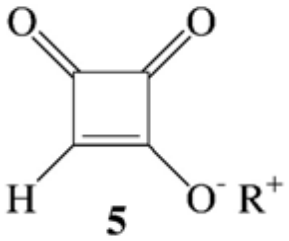
maximum growth takes place in soil of pF 2.55 and pH 5.8, incubated at 15^{o} C. Optimum temperature for survival of *Fusarium avenaceum* in soil is 20 – 30^{o} C under experimental conditions. During the winter, at natural soil temperatures survival rates for *Fusarium avenaceum* were 20% and 30% at 15-20 cm and 30 cm depths respectively (Kovacikova, 1993). Growth of *Fusarium avenaceum* was optimal on PDA at 15-28^{o} C than on Malt Agar (Winder, 1999). It has also been reported by Winder (1999), that juxtaposition of PDA and MA resulted in increased sporulation of *Fusarium avenaceum*. Sporulation levels were higher at 30^{o} C in 30 days old cultures. The authors also reported that higher day temperatures will result in abundant macroconidial proportion and less numbers of microconidia (Winder, 1999).

F. avenaceum **toxins**

Fusarium avenaceum is known to produce different mycotoxins like moniliformin, enniatins, and beavericin. However, *F. avenaceum* is not known to produce any trichothecenes. There were reports about trichothecene production by *F. avenaceum*, but it was not confirmed. This was possibly due to misidentification of a different species as *F. avenaceum*. So far, none of the *F. avenaceum* mycotoxins have been associated with any severe outbreaks of either human or animal toxicosis. Recent studies from Europe indicate that the toxic potential of *F. avenaceum* mycotoxins like enniatins and beavericin have been underestimated (Ivanova et al., 2006).

Moniliformin

Moniliformin is a highly toxic mycotoxin produced by *Fusarium avenaceum* and other *Fusarium* species like *F. moniliforme, F. acuminatum,* and *F. subglutinans* (Filek and Linder, 1996). Moniliformin is the sodium or potassium salt of 3-hydroxycyclobut-3-ene-1,2-dione (Filek and Linder, 1996). The structure of moniliformin is shown below.

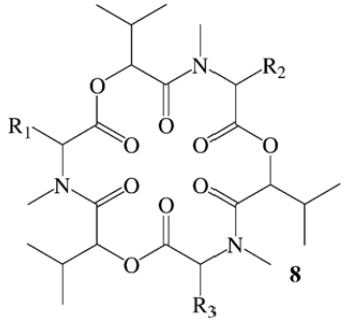


Structure of moniliformin (Uhlig et al., 2007)

Moniliformin has been detected as a natural grain contaminant in corn, triticale, wheat, rye, and oats from different parts of the world (Abramson et al., 2002). It causes myocardial degeneration and necrosis in experimental animals and is an inhibitor of thiamine pyrophosphate depending enzymes such as pyruvate dehydrogenase, ketoglutarate dehydrogenase and pyruvate decarboxylase (Uhlig et al., 2007).

Enniatins and beavericin

Enniatins and beavericins are secondary metabolites produced by *Fusarium avenaceum*. Chemically they are cyclohexadepsipeptides (Kamyar et al., 2006). These are inhibitors of acyl coenzyme A cholesterol acyltransferase. Apart from *Fusarium spp.* enniatins are also known to be produced by *Alternaria, Halosarpheia,* and *Verticillium* (Ivanova et al., 2006). Enniatins are known for their antibiotic, antifungal activities and phytotoxic effects associated with wilt and necrosis (Ivanova et al., 2006, Uhlig et al., 2007).



Structure of enniatins (Uhlig et al., 2007)

Enniatins are synthesized by the enniatin synthetase. Herrmann et al., (1996) developed enniatin non-producing strains by disrupting the enniatin synthetase gene. The virulence of enniatin non-producing *F. avenaceum* strains on potato tubers was significantly reduced. This indicates that enniatin contributes to the virulence of *F. avenaceum.* Kulik et al., (2007) developed primer sets based on the enniatin synthetase gene, *esyn1*, for detection of enniatin producing *Fusarium* species. In a cytotoxicity assay of enniatins produced by *F. avenaceum*, the cytotoxicity of enniatins was found to be comparable with that of deoxynivalenol indicating that that enniatins may have an underestimated toxic potential (Ivanova et al., 2006).

Phylogeny of *F. avenaceum*

Kristensen et al., (2005) studied the phylogenetic relationships between 17 different species of *Fusarium* based on partial translation elongation factor α (tef1-α) sequences. *F. avenaceum* formed a major monophyletic group together with three presumed distantly related taxa *F. flocciferum, F. torulosum* and *F. tricinctum.* All four species in this group are known moniliformin producers. This study reported the formation of two monophyletic groups in congruence with their toxin profiles i.e., trichothecene producers and moniliformin producers. Combined analysis of β- tubulin, IGS, and ITS sequences showed that *F. avenaceum/F. arthrosporioides/ F. tricinctum* species complex can be divided into seven clusters (Yli-Mattila et al., 2002). The same authors also reported that two main European *F. avenaceum* groups often morphologically indistinguishable can be distinguished in the tree based on β-tubulin from each other. Phylogenetic analysis based on a multilocus approach showed that *F. avenaceum* isolates form an exclusive group with strong bootstrap values (Nalim et al., 2009).

Disease screening methods and available sources of resistance

Integration of different disease management strategies, including the use of resistant cultivars is often recommended for controlling soil borne pathogens. Use of resistant cultivars is a safe, economical, and effective method for crop disease management (Infantino et al., 2006). Various methods have been used to screen the pea germplasm for resistance to root rot under artificially inoculated conditions. In the most commonly used method, seeds are inoculated by soaking in 50- 60 ml of conidial suspension at room temperature overnight and then planted in plastic trays (Infantino et al., 2006). Ondrej et al., (2008) recommended a modification to the above method of inoculation since the overnight soaking of non-primed pea seed (that had not been soaked prior to inoculation) in the inoculum did not result in sufficient infection. Successful infection was observed only on fully primed seed (24hr soaking). In another approach, the sand cornmeal layer method described for screening dry bean against root rot by Bilgi et al., (2008) was successfully adopted by Mathew et al., (2008) for evaluating root rot severity. As with differences in the methods, different scales have also been used for evaluation of pea root rot resistance. A 0-5 scale where 0: no symptoms, 1: slight hypocotyl lesions; 2: lesions coalescing around epi- and hypocotyl; 3: lesions starting to spread into the root system, with root tips starting to be infected; 4: epicotyl, hypocotyl and root system almost completely infected and only slight amount of white uninfected tissue left; 5: completely infected root was widely used. (Infantino et al., 2006, Ondrej et al., 2008). However, Hwang et al., (1995) assessed root rot severity on a scale of 0-4, where 0: healthy; 1: 1-10% discoloration; 2: 11-25%; 3: 26-50% and 4: 51-100%.

Currently, there are no cultivars available with complete resistance to root rot. Germplasm with partial resistance has been developed and commercial cultivars have been

released with tolerance to root rot caused by *F. solani* f. sp. *pisi* (Kraft and Pfleger 2001). However, there is no information available on resistance to *F. avenaceum.* The genetics of resistance to Fusarium root rot in pea is quantitatively inherited (Infantino et al., 2006). Comparison of disease resistance data for Aphanomyces root rot and Fusarium root rot showed a weak, but significant and positive correlation (Infantino et al., 2006). Three F_8-derived breeding lines W6 26740, W6 26743 and W6 26745, believed to be unique in combining high levels of resistance to Fusarium root rot with acceptable agronomic traits have been developed by Coyne et al., (2008). The parentage of these breeding lines is a cross between Dark Skin Perfection and 90-2131. These lines can be used as parental lines to develop green pea cultivars with improved resistance to Fusarium root rot. In a study conducted on the *Pisum* core collection obtained from USDA Western Regional Plant Introduction Station, (USDA WRPIS, Pullman) 44 plant introduction lines with a disease severity rating of 2.5 or less among 387 accessions screened were identified as being partially resistant to root rot (Infantino et al., 2006). Only a few accessions from the above 44 PIs retained high levels of resistance under field conditions (Infantino et al., 2006). Hwang et al., (1995) observed that all twenty cultivars tested in a study under field conditions in Alberta, Canada were susceptible to moderately susceptible. Ondrej et al., (2008) identified a higher level of resistance in the accessions LPKE 36, Herold, Kamelot and Gotik among the 19 selected pea accessions. Five of the 184 accessions tested at Semo Smrzic, Czech Republic had high tolerance to root rot (Ondrej et al., 2008). Cultivars 'Lifter' and 'Franklin', with a high degree of tolerance to Fusarium root rot caused by *F. solani* f. sp. *solani,* have been registered (Infantino et al., 2006).

Current methods of root rot management in field peas and precipitated calcium carbonate as a soil amendment

Tillage practices and seed treatment are two important strategies for management of root rot in field peas. Pea root rot incidence and severity increases with soil compaction (Burke et al., 1970). Fludioxonil, pyraclostrobin, and trifloxystrobin are labeled for seed treatment for controlling seed borne and soil borne fungal (*Fusarium* and *Rhizoctonia*) diseases in ND (McMullen and Markell, 2010). However, seed treatments often fail to provide satisfactory control of the disease (Samuel Markell, personal communication).

Root rot diseases caused by similar pathogens affect a wide range of crops including those often grown in rotation with dry peas. Therefore, disease control measures which gave positive results in other crops hold the potential to have similar effects on pathogens attacking peas. Precipitated Calcium Carbonate (PPC) also known as spent lime or waste lime, a byproduct of the sugar industry is used as a soil pH amendment in Europe. Previous reports suggest that PPC has been effective in reducing root rot and/or increases yield in sugar beet grown in North Dakota and Minnesota (Windels et al., 2004). PPC when applied at 3t/ac or 10 t/ac reduced the *Aphanomyces* root rot of sugar beet from 93-100% to 62% compared to non-treated plots (Windels et al., 2004). A reduction in *Aphanomyces* soil index values was recorded in sugar beet as a result of soil application of PPC (Windels et al., 2004). Lime has also been used to control club root of cabbage in the US (Campbell and Greathead, 1989). In addition to its effect on soil pH, it also contains nitrogen, phosphorus, potassium, and other inorganic and organic nutrients that fertilize crops and alters physical properties of soil, e.g., improving water drainage, which results in less root disease.

Virulence factors identified in *Fusarium* – pea interaction

Little information is available about the pathogenicity/ virulence factors in *F. avenaceum.* Hermann et al., (1996), through disruption of the enniatin synthase gene *Esyn1*, showed that enniatin production contributes to virulence of *F. avenaceum* on potato tubers. In studies conducted on other *Fusarium* spp. infecting pea, pisatin demethylase (PDA) of *Nectria haematococca* (teleomorph of *F. solani*), was shown to be a host-specific virulence factor on pea (Wasmann and VanEtten, 1996). Han et al., (2001), identified the gene encoding pisatin demthylase (*PDA1*) which is clustered with three other genes involved in pea pathogenicity (*PEP* genes). In a recent study involving field pea and *Nectria haematococca,* Coleman et al., (2011) reported that an ABC transporter (*NhABC1*) and a cytochrome P450 gene are virulence factors. Cytochrome P450 acts in detoxification of the phytoalexin 'pisatin' produced by pea, and the ABC transporter *NhABC1* confers tolerance to pisatin.

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CHAPTER ONE. IDENTIFICATION AND CHARACTERIZATION OF *FUSARIUM* SPP. ASSOCIATED WITH ROOT ROTS OF FIELD PEA IN NORTH DAKOTA

Abstract

Root rots are a major concern in dry pea production in North Dakota. However, it is unclear which pathogens are involved in causing disease. This report brings together findings from surveys conducted over four years (2004, 2005, 2008 and 2009). The initial studies (2004 and 2005) were mainly aimed at establishing the importance of root rot and providing a broad idea about the most prevalent root rot pathogen. The later studies (2008 and 2009) involved a thorough evaluation of not only disease incidence and severity but also included isolation and characterization of *Fusarium* species found to be associated with the disease. Average disease incidence and severity ranged between 0 to 50% and 0 to 5.79% in 2004 and from 0 to 25% and 0 to 3.41% in 2005 respectively. Root rot incidence and severity in 2008 varied between 0 to 60% and 4.0 to 16.5%, and higher disease incidence (20-100%) and disease severity (3.50 to 53.75%) were observed during the year 2009. *Fusarium* species were the most frequently isolated fungal species from the infected pea roots of which, *F. oxysporum* and *F. avenaceum* were most common but many other species, including *F. graminearum, F. culmorum* and *F. sporotrichiodes* were also recovered. Pathogenicity tests showed that all the *Fusarium* species isolated from the symptomatic roots were capable causing disease. Among the nine *Fusarium* species tested, *F. avenaceum* was the most aggressive in causing root rot, followed by *F. culmorum* and *F. graminearum. F. sporotrichioides* was found to be as aggressive as the *F. solani* isolates. Differences in aggressiveness existed among the *F. avenaceum*. The prevalence of *F. avenaceum* on dry peas, and its ability to cause severe root rot emphasizes the possibility of this pathogen to emerge as a potential risk under the current cropping practices for pulse crops.

Introduction

Dry field pea (*Pisum sativum* L.) is an important cool season legume crop grown in the north central plain states of the United States. North Dakota produces over 50% of the US crop with 430,000 acres planted in 2010 (United States Department of Agriculture National Agricultural Statistics Service [USDA-NASS]). Soil borne diseases, including wilts and root rots are of major economic importance and can cause significant reduction in yield (13, 14). Root rots in dry pea can be caused by many pathogens including *Alternaria alternata, Aphanomyces euteiches*, *Fusarium oxysporum* f. sp. *pisi*, *F. solani* f. sp. *pisi*, *Mycosphaerella pinodes, Pythium* spp., *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* often referred to as the Pea root rot complex (4, 26,19). Among these, Fusarium root rots are thought to cause the most serious diseases in the United States (16). Fusarium root rot on pea was first reported in 1918 from Minnesota and in 1923 from Wisconsin (15). Yield losses to Fusarium root rots are significant. In eastern Washington plots infested with *F. solani* f. sp. *pisi* 30% yield losses were recorded, and a 26% increase in yield was observed when the population of *F. solani* f. sp. *pisi* was reduced in soil by fumigation with chloropicrin (16). From experimental plots in five Canadian provinces, average yield losses of 35-37% to Fusarium root rot were reported (16).

Fusarium solani (Mart.) Sacc. f. sp. pisi (Jones) Snyder & Hans has been thought to be the primary causal agent of the root rot complex. However, several other *Fusarium* species have also been associated with root rots in field peas, including *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. sambucinum* var. *coeruleum, F. equiseti, F. poae, F. sporotrichioides* and *F. tabacinum* (4). Fernandez (6) and Fernandez et al., (7) reported that *F. avenaceum* was the most prevalent *Fusarium* spp. isolated from the discolored roots of lentils and field peas grown in the eastern part of Saskatchewan, Canada. In addition to peas, *F. avenaceum* has also been isolated

from crown and root tissue of other hosts such as clovers, ryegrass, soybeans and potatoes. In recent years, root rots appear to be developing as a major constraint in field pea production in North Dakota and preliminary reports by Gregoire et al., (10) suggested that *Fusarium* species were the predominant pathogens associated with this disease in the state. Therefore, the objectives of this study were (i) to assess the importance of this disease in the major field pea production areas of the state, (ii) to assess the major pathogens associated with root rots in this region, and (iii) to characterize pathogenicity and aggressiveness of associated pathogens.

Materials and methods

Field surveys and disease rating

Surveys were conducted in major dry pea growing counties of North Dakota in 2004, 2005, 2008 and 2009 to assess the prevalence of pea root rot disease. Selected fields were located at least 3 miles apart. Twenty plants were sampled from each field during the years 2004 and 2005. In 2008 and 2009, 10 plants were collected from each field surveyed following a 'W' pattern, placed in Ziploc bags and stored in coolers until brought to laboratory. The roots were washed under running tap water and assessed for incidence and severity of root rot. Root rot severity was measured as the percentage of root length covered by lesions (length of lesions/total root length X 100). Random samples submitted by growers and crop consultants from the major pea production areas of ND in 2007 were also included in the study.

Pathogen isolation and identification

Roots were washed under running tap water and tissue with lesions on tap root or hypocotyl showing dark brownish discoloration were excised. Excised lesions from five of the roots collected were surface sterilized and other five were not surface sterilized. Surface

sterilization involved a soaking in 0.5% NaOCl (10% solution of commercial bleach containing 5.25% a.i.) and 70% ethanol for one minute each, followed by two rinses in sterile distilled water. The samples were subsequently blotted dry on sterile paper towels in a laminar flow hood. Dried root segments were plated on half strength potato dextrose agar (PDA) containing streptomycin and penicillin, 250 ppm each, and incubated for 3-5 days at 20-25^{o} C under 12 hours of alternating light and dark conditions. Subsequently pure cultures were established through single spore isolation. Presumptive identification of the single spore isolates was done based on colony characteristics including colony morphology, color on PDA, and spore morphology. *Fusarium* isolates were identified to species level using the keys described in Booth (2), and Leslie and Summerell (17). Morphological identification of representative isolates was confirmed by PCR amplification and sequencing translation elongation factor alpha 1 (TEF-1 α) region followed by comparison to Fusarium sequencing available at GenBank and the Fusarium ID database (8). In 2007, in addition to isolation and identification of Fusarium species according to the procedures mentioned above, a DNA hybridization array based on TEF-1α sequences was used for identifying different *Fusarium* species associated with field pea root rot (24). DNA was extracted from infected roots of samples obtained from selected root rot infected fields using the Qiagen DNAeasy kit. Confirmation of identification was also conducted independently by the Fungal Identification service at AgCanada during 2007-2008.

Pathogenicity tests

To determine the pathogenicity of the various *Fusarium* spp. recovered from symptomatic dry pea roots, pathogenicity tests were conducted using a root rot susceptible field pea cultivar DS Admiral following the sand cornmeal inoculum layer method previously described for (1). The experiment was laid out in a completely randomized block design with 4

replications where one cup was considered a replication. The experiment was conducted in a greenhouse with day and night temperatures of 21^{o} C and 18^{o} C, respectively and the experiment was repeated two times. Two isolates of each of the nine *Fusarium* species isolated from dry pea roots, namely, *F. acuminatum, F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. oxysporum, F. redolens, F. solani, and F. sporotrichioides* were selected randomly and grown on half strength PDA for 10 days at 23^{o} C with a 12 h photoperiod. The inoculum was prepared by growing each isolate on pre-sterilized (at 15lbs for 45 min) sand-cornmeal mixture (45g sand, 5g cornmeal and 10ml distilled water) for 7-10 days under conditions mentioned above. Three isolates of *F.solani* f. sp. *pisi* were used as positive control (11). Three pre-germinated seeds were planted in each cup. The plants were rated for root rot severity 10 d after inoculation. Disease severity was calculated as the percentage of root length covered by lesions (length of lesions/total root length X 100).

Aggressiveness of F. avenaceum isolates

Variation in aggressiveness of seventeen randomly chosen *F. avenaceum* isolates representing different years of collection and counties was determined in growth chamber experiment with alternating cycles of 14 h light, 8 h darkness with day and night temperatures of 21^{o} C and 18^{o} C respectively. The experiment was laid out in a completely random design (CRD) with four replications per isolate where one cup representing a replication. The experiment was repeated three times. As in the case of the pathogenicity tests, the susceptible field pea cultivar DS Admiral and the sand cornmeal inoculum layer method were used in this study and disease rating was also conducted in a similar manner. *F.solani* f. sp. *pisi* was used as positive control, whereas sterilized sand cornmeal mixture served as negative control.

Analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc. Cary, NC). Homogeneity of variance between experiments was tested using LEVENE's test. Similar experiments were combined and analyzed using all replications using PROC ANOVA. Mean separation was performed using Fisher's protected least significant difference (LSD) test.

Results

Field surveys, isolation and identification of pathogens

Plant samples were collected from 47 and 41 fields representing 10 counties in 2004, and 2005 respectively. In 2008 and 2009, plant samples were collected from 77 growers' fields spread over 11 counties and from 38 fields located in 7 counties respectively. Root rot disease was found to be present in all the counties surveyed during the four years (Table 1.1). Average disease incidence ranged between 0 to 50% and 0 to 25% during 2004, and 2005 respectively. Root rot severity ranged between 0 to 5.79% in 2004 and 0 to 3.41% in 2005. In 2008, root samples were collected from 77 fields. The overall disease incidence varied between 0 to 60% and disease severity ranged from 4.0 to 16.5% (Table 1.1). Higher disease incidence (20-100%) and disease severity (3.50 to 53.75%) were observed during the year 2009.

Random isolations from the infected pea root samples from 2004 and 2005 surveys showed that *Fusarium* spp was the most commonly isolated pathogen from root rot infected dry pea (10) and *F. avenaceum* appeared to be predominant among the *Fusarium* spp isolated (data not shown). In 2007, 5 diseased root samples were obtained from root rot infected fields. These samples were primarily used for plating and DNA array hybridization conducted to assess the major pathogens prevalent in the state. Hybridization to a DNA array based on the TEF-1α

region revealed the diversity of *Fusarium* species associated with field pea root rot in ND. Eleven different species of *Fusarium* were detected using the DNA array (Figure 1.1).This was further confirmed by isolations of these species from the infected roots. *F. solani, F. oxysporum, F. avenaceum, F. redolens, F. graminearum,* and *F. acuminatum* were isolated from the affected roots and their identity was confirmed by morphological characteristics and TEF-1 α sequence comparisons (8). Various *Fusarium* species isolated constituted 93% of the fungal pathogens identified from symptomatic pea root samples in 2007 (Figure 1.1). *Didymella pinodes* (5%) and *Rhizoctonia solani* (2%) were the other fungal pathogens isolated from pea roots. Pathogen isolation was conducted from a subset of (39) the total fields surveyed. *F. avenaceum*, the most prevalent species, was found in 71.8% (Table 1.1) of the fields. *F. oxysporum* was the next most frequently isolated *Fusarium* species from the infected roots and it was isolated from 66.67% of the fields. *F. acuminatum, F. solani, F. redolens, F. sporotrichioides, F. equiseti, F. culmorum,* and *F. graminearum* were also isolated from infected roots, with varying prevalence (Figure 1.2). Similar to the 2008, *Fusarium* species were the most frequently isolated fungal species from the infected pea roots. *F. oxysporum* and *F. avenaceum* were isolated from almost 90% of the fields surveyed in 2009 (94.4 and 89.5% respectively, Figure 1.2). Other *Fusarium* species prevalent in 2009 were *F. acuminatum* (57.9%), *F. redolens* (57.9 %), *F. equiseti* (36.8%), *F. solani* (28.9%), *F. culmorum* (23.6%) and *F. sporotrichioides* (18.4 %) of the field surveyed respectively. Apart from the *Fusarium* species, another root rot pathogen associated with root rot, *R. solani,* was also present in 2.56 % and 15.8% of the fields surveyed in the state during 2008 and 2009 respectively (19).

Pathogenicity tests

Levene's test for homogeneity of variance suggested that the two individual experiments were homogenous and could be combined, so combined data from the two experiments was used for analysis. Pathogenicity tests showed that all the *Fusarium* species isolated from the symptomatic roots were capable causing root rot. The lesions on roots varied from dark brown to black, and rotting of tissue was observed. Significant differences in the severity of root rot symptoms were observed between different *Fusarium* species (Table 1.2). Disease severity varied from 6.5 % to 60.3 %. Among the 9 *Fusarium* species tested, *F. avenaceum* caused most severe root rot, followed by *F. culmorum* and *F. graminearum. F. sporotrichioides* was found to be as aggressive as the *F. solani* isolates. *F. equiseti, F. oxysporum* and *F. redolens* were found to be weakly pathogenic on field pea roots.

Aggressiveness of F. avenaceum isolates

Tests for homogeneity of variance showed that only two of the three experiments could be combined, and hence only the two experiments with homogeneous variance were combined for analysis. Significant variation in aggressiveness among the *F. avenaceum* isolates was observed (Table 1.3). Disease severity varied from 6.25 to 88.7 %. Three *F. avenaceum* isolates (FA0601, Pea41, and FPSM60) were found to be were more aggressive than *F.solani* f.sp.*pisi*, resulting in rotting of more than 85% of root length. Four isolates were as aggressive as *F. solani* f. sp. *pisi*, and ten isolates were found to be less aggressive than *F. solani* f. sp. *pisi.*

Discussion

In this study, *F. avenaceum* was found to be one of the two most common *Fusarium* spp. from (Figure 1.1) recovered from symptomatic dry pea roots. In agreement with the findings

from Saskatchewan mentioned earlier (6) and recent reports from Alberta, Canada where 80% of the Fusarium isolates from field pea root rot samples were *F. avenaceum* (5). In a disease survey conducted during 1993 and 1994, *F. avenaceum* was isolated from 41-75% of the pea fields in Sweden (23), where frequency of isolation of this pathogen was higher than expected. These findings are in contrast with earlier reports where *F. solani* f. sp. *pisi* was the major root rot pathogen of this crop (16). In ND, rotation of field pea with cereals and canola is a common practice. *F. avenaceum* is frequently found on cereal grains, where it causes seedling and head blights and produces mycotoxins like moniliformin, beauvericin and enniatins (27).). *F. avenaceum*, together with other *Fusarium* species is associated with foot and root rot or crown rot/ Fusarium head blight (FHB) diseases of cereals (6, 27) and seedling blight in canola resulting in poor stand establishment (3). As suggested by Feng et al., (10), lack of host specificity and rotation with other host crops could be attributed for the higher frequency of isolation of *F. avenaceum* from dry pea.

Persson et al., (23) pointed out the importance of pathogenicity tests in connection with disease surveys based on deviations from normally observed symptoms for *Fusarium* species. In this study, *F. solani*, known as a root rot pathogen was able to cause wilting, whereas *F. oxysporum* infection resulted in root rot in contrast to the normal wilting symptoms. Our pathogenicity studies with representative isolates from each species showed that *F. avenaceum* causes most severe symptoms compared to all the other *Fusarium* spp (Table 1.2) which is similar to findings reported by Persson et al., (23). We also found significant variation in root rot severity among *F. avenaceum* isolates from our aggressiveness studies using 17 isolates (Table 1.3). Similar differences in severity of symptoms among 75 *F. avenaceum* isolates were observed by Feng et al., (5) who grouped their isolates into three severity classes. We could classify the

17 isolates into highly aggressive (3), aggressive (4), and less aggressive (10) classes compared to the control *F. solani* f. sp. *pisi* used in this study.

F. oxysporum (Figure 1.2) was also frequently isolated from infected pea roots. This species is ubiquitous in nature and is considered to be primarily associated with wilts in various crops (9). However, there are reports from Europe and Canada that have established *F. oxysporum* as a causal agent of pea root rot along with other *Fusarium* spp. isolated from field pea roots (13, 23, 25). In contrast to reports from a study conducted in Southern Scandinavia (23) where *F. oxysporum* was found to cause severe root rot in a pathogenicity tests, we found this species to be a weak pathogen causing less severe symptoms than all the other *Fusarium* spp. evaluated. This could be attributed to variation in aggressiveness among *F. oxysporum* isolates as observed previously (25) with the isolates from field peas being significantly less aggressive than the other *Fusarium* spp. isolated (Table 1.2). Moreover, none of the field pea plants evaluated in this study showed symptoms of wilt and isolations were strictly conducted from roots. This leads us to hypothesize these isolates are part of the soil population and are unlikely to be a major contributor to root rots.

Pathogenicity assays using representative isolates proved that all the *Fusarium* spp. isolated from the surveys are capable of causing root rot to varying degrees of severity. As already discussed above, *F. avenaceum* was found to cause most severe rotting followed by *F. culmorum* and *F. graminearum*, and *F. sporotrichioides* (Table 1.2) as compared *F. solani* f. sp. *pisi*. These pathogens are frequently associated with the FHB complex on cereals. The other species isolated from field pea roots, *F. acuminatum, F. oxysporum, F. redolens*, and *F. equiseti*, were found to be weakly pathogenic (less severe rot). A similar trend in symptom severity caused by *F. culmorum, F. redolens* was observed by Persson et al., (23). The symptoms (lesion

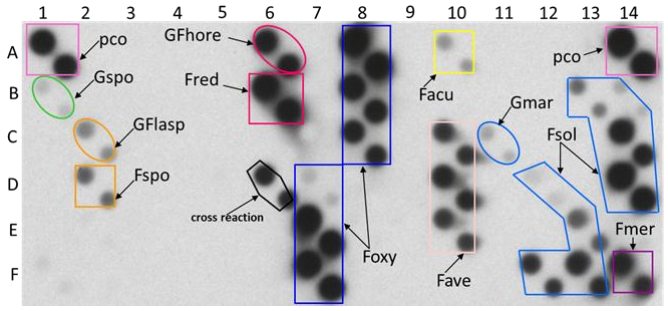
or rotting) caused by these different species were similar, and could not be differentiated. Our findings, along with other studies mentioned earlier, emphasize a possible change in *Fusarium* spp. associated with root rot in field peas with *F. solani* f. sp. *pisi*, the traditional root rotting *Fusarium* sp. reducing in prevalence. This change in species is likely to pose a challenge to the currently available management strategies recommended for reduction in root rot.

Results from our surveys, and those from different parts of the world that indicate the prevalence of *Fusarium* species like *F. avenaceum* highlight the need for identifying sources of resistance to this species and possibly re-evaluating the existing sources identified previously based on *F. solani* f. sp. *pisi* for resistance before incorporating them into breeding programs aimed at developing varieties for this region. Our findings from this study about the prevalence of F. avenaceum on dry peas, and its ability to cause severe root rot, and from previous reports discussed here emphasizes the possibility of this pathogen to emerge as a potential risk under the current cropping practices for pulse crops.

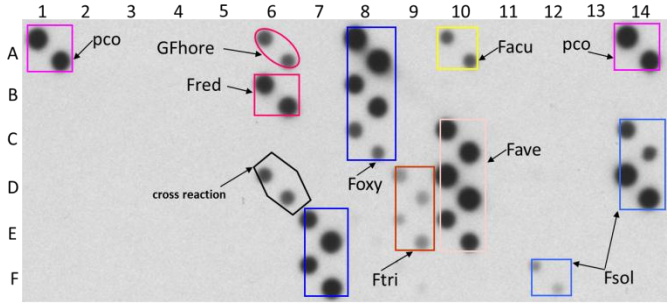
Acknowledgements

The *F. solani* f. sp. *solani* isolates used as positive control in these studies were kindly provided by Dr. Lyndon Porter, USDA-ARS, Prosser, WA.

a.



b.



c.

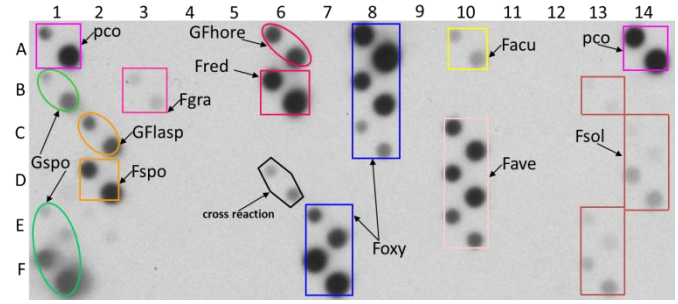


Figure 1.1. DNA array hybridization of pea root samples from 3 different fields located in northwestern ND severely affected by root rot. a. Sample 1615 (1), b. Sample 1550 ss, c. Sample 1616 (2). Pco=positive control; Gspo= group oligo section Sporotrichiella; Fgra=oligo for *F. graminearum*; GFlasp=group oligo for *F. langsthae* & *F. sporotrichioides*; Fspo= oligo for *F. sporotrichioides*; GFhore= group oligo for *F. hostae* & *F. redolens*; Fred=oligo for *F. redolens*; Foxy= oligo for *F. oxysporum* complex; Facu= oligo for *F. acuminatum*; Fave=oligo for *F. avenaceum* complex; Fsol = oligo for *F. solani* complex Gmar= oligo for section Martiella; Fmer= oligo for *F. merismoides* like.

Table 1.1. Number of fields, mean disease incidence, and mean disease severity observed during the surveys conducted in 2004, 2005, 2008, and 2009.

County	2004			2005			2008			2009		
	# of fields	Incidence[a]	Severity[b]	# of fields	Incidence	Severity	# of fields	Incidence	Severity	# of fields	Incidence	Severity
Benson	*	-	-	1	10	0.67	*	-	-	*	-	-
Bottineau	4	28.75	2.94	4	10	1.3	7	38.57	10.91	1	60.00	25.22
Burke	5	18	2.72	3	6.67	0.52	*	-	-	*	-	-
Cass	*	-	-	*	-	-	3	6.67	0	*	-	-
Divide	12	12.5	0.8	10	11.5	0.84	*	-	-	3	66.67	22.96
Foster	*	-	-	*	-	-	1	40	4	1	50	16.64
Hettinger	3	26.67	2.48	3	13.33	1.12	2	55	16.5	*	-	-
McKenzie	1	50	5.44	5	7	0.38	*	-	-	*	-	-
McLean	6	22.5	2.07	*	-	-	15	7.33	4.73	11	61.82	29.83
Mountrail	4	23.75	2.11	*	-	-	2	40	7.67	4	42.5	13.27
Ramsey	*	-	-	1	25	3.41	*	-	-	*	-	-
Renville	3	13.33	0.67	4	2.5	0.1	7	17.14	6.92	*	-	-
Sheridan	*	-	-	*	-	-	2	15	2.5	*	-	-
Ward	3	26.67	0.82	4	6.25	0.59	25	26	9.71	10	52	18.76
Williams	6	12.5	1.19	6	14.17	1.36	13	29.23	9.06	8	57.5	20.57

[a] and [b] : mean disease incidence, and mean disease severity expressed as %.

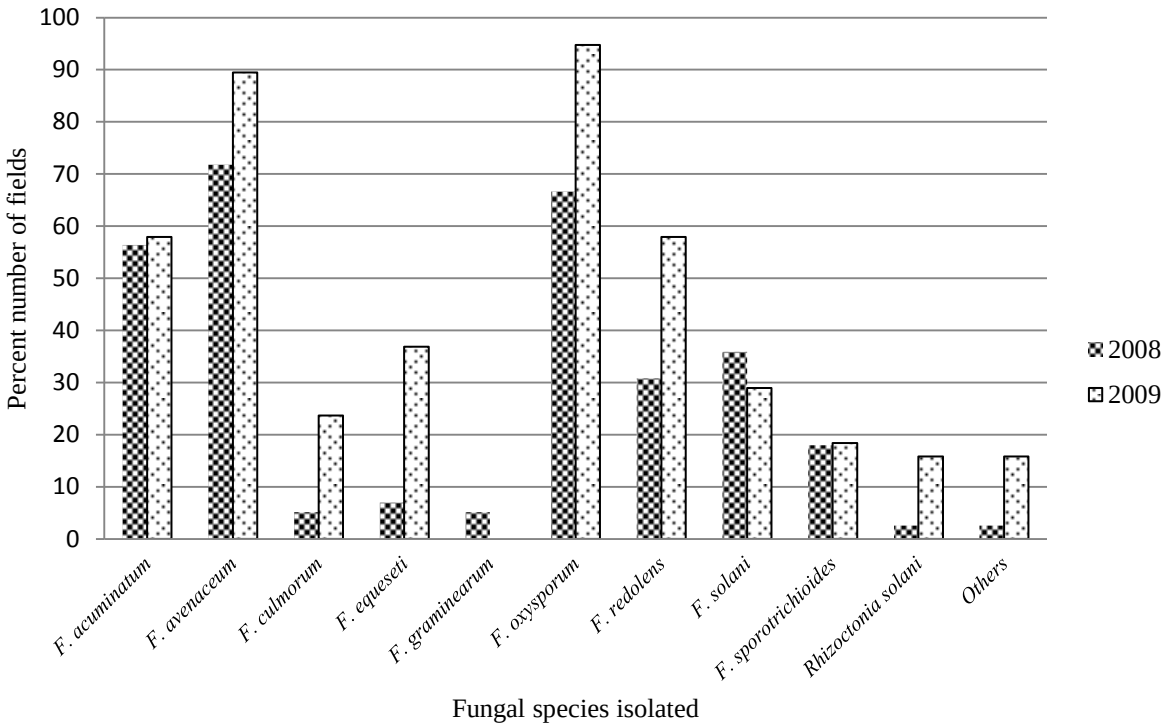


Figure 1.2. Fungal species isolated from symptomatic pea roots.

Table 1.2. Mean disease severity caused by different *Fusarium* species on cv. DS Admiral measured 10 days after inoculation.

***Fusarium* species**	**Mean Disease Severity (%)***
F.acuminatum	14.835 [ef]
F.avenaceum	60.347[a]
F.culmorum	45.723[b]
F.equiseti	6.521 [f]
F.aminearum	43.437 [b]
F.oxysporum	12.949 [ef]
F.redolens	6.841 [f]
F.solani	21.284 [de]
F.sporotrichioides	32.894 [c]
Control (*F. solani* f.sp. *pisi*)	25.226 [cd]

* numbers with same letters are not statistically different.

Table 1.3. Variation in aggressiveness among isolates of *F. avenaceum* obtained from field pea roots.

***F. avenaceum* Isolate**	**Mean Disease Severity (%)***
CTC 6c	46.71 [d, e, f]
CTC 6b	40.89 [f]
FPS M 60	86.65 [a]
PLE 1b SI	53.72 [c, d]
Pea 41	87.91 [a]
CTC 8G	49.07 [c, d, e, f]
Pea 47	6.47 [h, i]
Trt 6 NB (11)	26.66 [g]
Pea 47 (e)	42.50 [e,f]
Pea 47 (a)	6.25 [h, i]
Pea 9 (b)	29.85 [g]
FA 0601	88.67 [a]
FA 0602	46.06 [d, e, f]
FA 0604	9.97 [h]
FA 0606	24.39 [g]
Ave 1614	64.12 [b]
Ave 1550	51.14 [c, d, e]
F.s.pisi	56.64 [b, c]
Healthy control	0.00 [i]

* Numbers with same letters are not statistically different

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CHAPTER TWO. IDENTIFICATION OF SOURCES OF RESISTANCE TO *FUSARIUM AVENACEUM* IN COMMERCIAL FIELD PEA VARIETIES

Abstract

This study was conducted to identify sources of resistance to *F. avenaceum* within commonly grown varieties. 21 field pea varieties were screened for resistance against *F. avenaceum* and *F. solani* f. sp. *pisi* in growth chamber experiments and field trials. Growth chamber experiments were conducted using the sand cornmeal layer method. For *F. avenaceum,* cv. Majoret showed the lowest disease severity followed by 'Windham'. High disease severity was found on 'Toledo' and 'Stirling'. Similar to the response of varieties to *F. avenaceum,* cv. Windham had the lowest root rot severity with *F. solani* f. sp. *pisi* inoculation followed by 'SW Marquee' and 'Specter'. Significant variation in levels of resistance to root rot was observed in our field trials. Low levels of resistance were detected in a few cultivars, but no variety was found to be completely resistant to any of the pathogens tested. Winter pea variety 'Granger', with a mottled seed coat may have partial resistance to both pathogens *F. avenaceum* and *F.solani* f. sp. *pisi* as demonstrated under both inoculated and non-inoculated conditions.

Introduction

Dry pea or field pea (*Pisum sativum* L.) is an important cool season legume crop grown in the United States. The area under this crop has rapidly increased from 337,500 acres in 2003 to 756,000 acres in 2010. North Dakota, Montana and Washington are the leading dry edible pea producing states. North Dakota ranks first with an area of 430,000 acres planted to pea in 2010 and contributes to more than 55 per cent of value of production (NASS, 2010). Dry pea is susceptible to many root-rot pathogens including *Aphanomyces, Fusarium, Pythium,* and *Thielaviopsis.* Among these, Fusarium root rot is a serious disease present in all pea producing

areas in the United States (Kraft and Pfleger 2001) and traditionally, *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyder & Hans was considered to be the primary causal agent of pea root rot. There is a dearth of satisfactory methods to control this root rot disease.

Integration of different disease management strategies, including the use of resistant cultivars is often recommended for controlling soil borne pathogens. Use of resistant cultivars is a safe, economical, and effective method for crop disease management (Infantino et al., 2006). In the case of field peas, all Fusarium root rot resistance evaluations reported previously have been conducted using *F. solani* f. sp *pisi*. Currently there are no cultivars available with complete resistance to root rot, but sources of resistance (partial) to *F. solani* have been identified (Kraft and Pfleger, 2001). The genetics of resistance to Fusarium root rot in pea is quantitatively inherited (Infantino et al., 2006). In a recent study a microsatellite marker linked to a QTL which controls resistance to *F. solani* f.sp. *pisi* was identified (Feng et al., 2011). However, results from our surveys, and from different parts of the world that indicate the prevalence of *F. avenaceum* as the primary causal agent of this disease highlight the need for identifying sources of resistance to this species and possibly re-evaluating the existing sources identified previously based on *F. solani* f. sp. *pisi* for cross resistance before incorporating them into breeding programs aimed at developing varieties for this region.

Materials and methods

Growth chamber trials

Twenty-one pea varieties selected from commercially cultivated varieties were screened in the growth chamber using the sand cornmeal inoculum layer method (Bilgi et al., 2008). Three aggressive isolates of *F. avenaceum* (Pea 41, FA0601 and FPS M60), isolated from root rot affected field pea roots were used to screen the cultivars for disease reaction. The inoculum was

prepared by placing eight 5 mm plugs of *F. avenaceum* culture grown on half strength potato dextrose agar for 10 days under alternating dark and light cycles of 12 h each at 23ºC into 125 mL conical flasks containing a sterilized (at 15 lbs for 45 min) sand cornmeal mixture (45g of play sand, 5g of cornmeal and 10ml of distilled water). These flasks were incubated at room temperature for 7 to 10 days and shaken daily by hand to ensure uniform growth of the fungus. In 266 mL plastic drinking cups with small holes at the base to facilitate water drainage, 15g of sterilized (at 121ºC, 15 lbs for 45 min) premium grade coarse vermiculite was poured and compressed gently. This was followed by a 15g layer of inoculum and then covered by a layer of 8g of vermiculite. Three pre-germinated seeds of each variety were placed on this layer and were covered by another layer of 8g of vermiculite. Eighty mL of distilled water were added to each cup. Four cups of each variety were placed in trays and kept in growth chambers with a cycle of 14h light and 10h darkness with day and night temperatures of 21º and 18º C, respectively. The plants were watered daily and root rot severity was assessed 10 days after planting. Root rot severity was assessed as the length of the lesions compared to total root length, expressed as percentage. The experimental design was a completely randomized deisgn (CRD) with non-inoculated and positive controls (three aggressive *F. solani* f. sp. *pisi* isolates Fs 01.B1, F 54 and F215). The experiment was repeated two times.

Inoculated field trials

Disease nursery or sick plots were established by incorporating inoculum into soil. Inoculum was prepared by growing three aggressive isolates of the two pathogens (*F. avenaceum* and *F. solani* f. sp. *pisi*) on wheat for 10 days. After the pathogens completely colonized wheat grains they were dried in a greenhouse and the inoculum was incorporated into soil @ 1.5 g/ foot at the time of planting. Each plot contained three 7.5’ rows and 35 seeds were planted per row. The experimental design was a randomized complete block with four replications. This

experiment was repeated over two years.

Field trials under natural disease pressure

This experiment was conducted in Newburg, ND in a field with known history of root rot. The experiment was laid out as a randomized complete block design with four replications and 21 treatments, and conducted over a period of two years.

Sampling and disease rating

Sampling for root rot severity was conducted at the pre-flowering stage. Ten plants were randomly collected from each plot, placed in Ziploc bags and stored in coolers until analyzed. Roots were washed under running tap water and assessed for severity of root rot. Root rot severity was measured as the percentage of root length covered by lesions (length of lesions/total root length X 100).

Statistical analysis

Statistical analysis for the above studies was performed using SAS version 9.1 (SAS Institute Inc. 2002-2003). Homogeneity of variance between experiments was tested using Levene's test. Similar experiments were combined and analyzed using all replications using PROC GLM. Mean comparisons were performed using Fisher's protected least significant difference (LSD) test at $p = 0.05$.

Results

Growth chamber trials

Levene's test of homogeneity of variance was not significant for both *F. avenaceum* ($p= 0.065$) and *F. solani* f.sp. *pisi* ($p= 0.225$) trials, so the two experiments were combined for analysis. Significant differences in disease severity caused by *F. avenaceum* ($p = 0.04$, LSD=14.5) and *F. solani* f. sp. *pisi* ($p= 0.03$, LSD=21.9) were observed (Fig 2.1). For *F.*

avenaceum, cv. Windham showed the lowest disease severity followed by Nitouche, Franklin, K2, DS Admiral and Melrose. Highest disease severity was observed in Toledo, Stirling followed by Matrix, Granger, Lifter and Aaragron. Cv. Windham had lowest root rot severity with *F. solani* f. sp. *pisi* inoculation followed by SW Marquee, Specter, K2, Stirling, DS Admiral, Franklin, and Majoret. Highest disease severity was observed on SW Midas, Universal, Aragron, Nitouche, Majoret and Melrose. For both the pathogens, cvs. Windham, Franklin, K2 and DS Admiral consistently showed moderate levels of resistance.

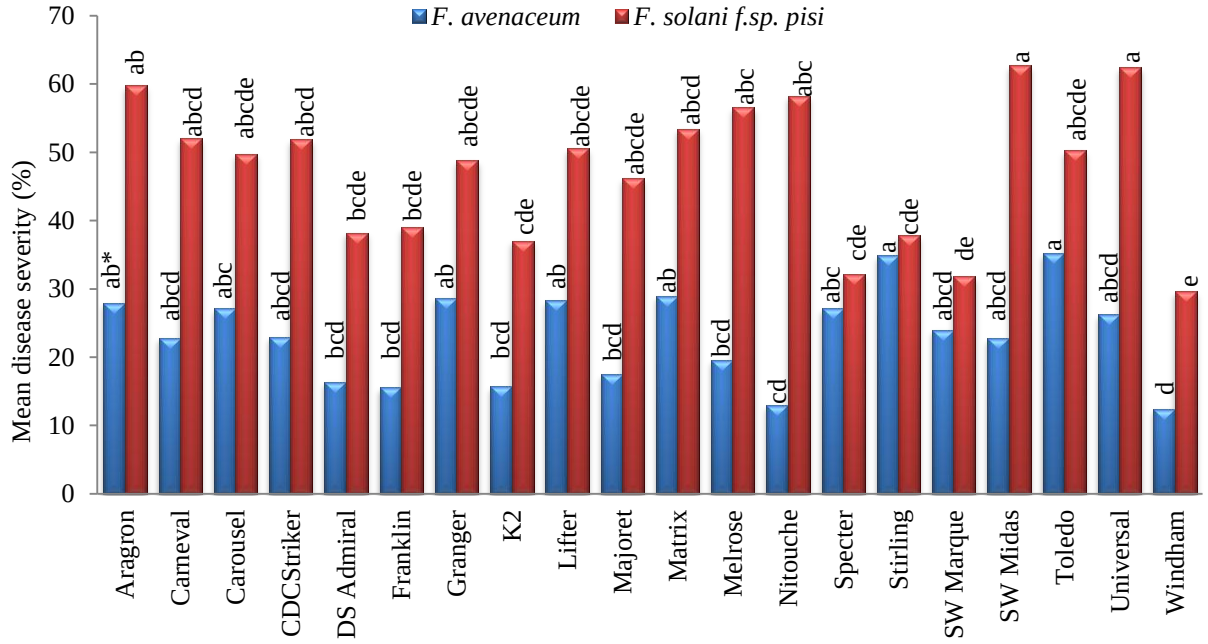

Figure 2.1. Response of field pea cultivars to root rot pathogens *F. avenaceum* and *F. solani* f. sp. *pisi.* * Bars with the same letter are statistically not different.

Inoculated field trials

Levene's test for homogeneity of variance for severity data was significant (p = 0.93), therefore the experiments conducted over two years were analyzed separately. There were significant differences between inoculum and varieties as well as the interaction between inoculum and varieties in both years. In 2010, *F. solani* f. sp. *pisi* (31.9%) had significantly

higher root rot severity compared to the *F. avenaceum* (11.8%) and non-inoculated control plots (13.0%) (Table 2.1).

Table 2.1. Severity of root rot caused by *F. avenaceum, F. solani* f. sp. *pisi,* and non-inoculated control.

	2010		2011
Pathogen	**Severity**	**Stand Count**	**Severity**
F. avenaceum	11.8 [b]	50.4[b]	15.5[b]
F. solani f. sp. *pisi*	31.9[a]	58.9[a]	25.2[a]
Control	13.0 [b]	62.9[a]	3.9[c]
LSD	2.3	8.1	3.2

* numbers followed by the same letter are not statistically different

The differences in severity caused by *F. avenaceum* and control plots were not significant. In the case of *F. avenaceum,* no significant differences in disease severity between the varieties were observed (Table 2.2). However, significant differences were found between varieties in response to *F. solani* f. sp. *pisi.* Among the 21 commercial varieties, Stirling, SW Marquee, Windham and Granger had the lowest disease severity followed by Lifter and Universal. Cultivars Midas and Carneval had greatest root rot severity followed by Aragron, Majoret, and CDC Striker. In the non-inoculated control similar trend was observed. Lowest disease severity was found on Universal followed by Toledo and Granger, SW Marquee and Midas. Nitouche had highest amount of disease followed by Matrix, Franklin and Admiral. In 2011 also *F. solani* f. sp. *pisi* resulted in higher disease severity (25.2%) followed by *F. avenaceum* (15.5%). Disease severity was lowest in the non-inoculated control plot (3.9%) as expected. In the *F. avenaceum* inoculated plot highest disease severity was recorded on Midas, Toledo, Matrix, Lifter, Aragron, Franklin, and Universal. Disease severity was lowest on Granger and Specter followed by K-2, Melrose, Admiral, and Stirling (Table 2.2). In the *F. solani* f. sp. *pisi* inoculated plot, higher disease severity was observed on CDC Striker, Majoret,

Windham, Aragron, Matrix, Midas and Specter. Cultivars Franklin, Lifter, Granger, Melrose, Universal, DS Admiral, Nitouche, Cranveval, Stirling, K-2 and Carousel had lower disease severity. In the control plot, the lowest amount of disease was found on Melrose, Granger, and Specter. Root rot severity was highest on Matrix followed by Toledo, DS Admiral, Majoret, K-2, carousel, Stirling, Prodigy, and Nitouche.

Table 2.2. Response of commercial pea varieties to root rot caused by *F. avenaceum, F. solani* f. sp. *pisi.*

	2010			2011		
Variety	***F.avenaceum***	***F.solani* f.sp. *pisi***	**Control**	***F.avenaceum***	***F.solani* f.sp. *pisi***	**Control**
Aragon	12.8	38.5^{ab}	13.2^{bcdef}	20.2^{bac}	30.3^{abcd}	3.1^{bcdef}
Carneval	13.1	42.8^{a}	13.7^{bcde}	12.8^{bcdef}	23.2^{cdefgh}	2.8^{cdef}
Carousal	11.1	31.1^{bcde}	13.3^{bcdef}	15.7^{cdef}	$25.6^{bcdefgh}$	4.5^{bcd}
CDCStriker	12.0	36.2^{abcd}	13.7^{bcde}	13.4^{cdef}	37.6^{a}	3.4^{bcdef}
DSAdmiral	14.9	30.3^{bcde}	13.9^{bcd}	11.9^{def}	19.9^{defghi}	5.9^{ab}
Franklin	14.0	33.1^{bcde}	16.4^{bc}	18.4^{abcd}	12.2^{i}	3.2^{bcdef}
Granger	8.4	26.9^{e}	$8.9d^{ef}$	8.2^{f}	17.5^{ghi}	1.3^{ef}
K-2	10.6	30.8^{bcde}	11.8^{cdef}	10.7^{def}	$25.0^{bcdefgh}$	4.9^{bcd}
Lifter	14.6	27.4^{de}	11.3^{cdef}	20.2^{abc}	15.4^{hi}	4.3^{bcde}
Majoret	9.4	37.6^{abc}	12.1^{bcdef}	15.6^{bcdef}	34.1^{ab}	5.0^{bc}
Marquee	10.7	26.4^{e}	11.0^{def}	14.2^{cdef}	26.1^{bcdefg}	3.5^{bcdef}
Matrix	11.7	32.8^{bcde}	17.0^{b}	$20.8ab^{c}$	29.8^{abcde}	8.4^{a}
Melrose	7.6	29.8^{bcde}	12.4^{bcdef}	11.9^{def}	18.7^{fghi}	1.1^{f}
Midas	13.8	42.9^{a}	10.4^{def}	24.4^{a}	29.7^{abcde}	3.5^{bcdef}
Nitouche	13.4	29.4^{cde}	26.2^{a}	13.4^{cdef}	$21.7^{cdefghi}$	4.1^{bcdef}
Prodigy	14.6	29.5^{cde}	13.3^{bcdef}	18.2^{abcd}	27.2^{bcdefg}	4.3^{bcde}
Specter	11.5	32.7^{bcde}	13.73^{bcde}	9.9^{ef}	27.9^{bcdefg}	1.9^{def}
Stirling	12.3	26.1^{e}	12.4^{bcdef}	12.8^{cdef}	23.5^{cdefgh}	4.4^{bcde}
Toledo	9.2	31.3^{bcde}	8.6^{ef}	22.4^{ab}	26.9^{bcdefg}	5.9^{ab}
Universal	11.1	27.4^{de}	8.1^{f}	17.6^{abcde}	19.6^{efghi}	3.9^{bcdef}
Windham	10.3	26.7^{e}	11.9^{bcdef}	16.2^{abcdef}	31.1^{abc}	3.4^{bcdef}
LSD	NS	8.88	5.3	8.1	10.5	2.0

* numbers followed by the same letter are not statistically different

Field trials under natural disease pressure

In 2010, no significant difference in disease severity was observed between the 21 varieties tested (Table 2.3). However, significant differences in root rot severity between varieties were detected in 2011. Disease severity was lowest on Granger, and Windahm followed by Carousel, K-2, Specter, Universal and Toledo. Varieties Aragron recorded the highest root rot severity followed by Matrix, DS Admiral, CDC Striker, Lifter, and Franklin.

Table 2.3. Response of field pea varieties to root rot under natural disease pressure.

Cultivar	**Mean Disease Severity**	
	2010	**2011***
Aragorn	31.30	40.67[a]
CDCStriker	21.31	32.12 [abc]
Carneval	45.37	23.39 [bcdefg]
Carousel	27.76	16.45 [fg]
DSAdmiral	26.17	32.96 [ab]
Franklin	27.16	29.77 [abcde]
Granger	21.19	12.17 [g]
K2	23.45	17.26 [fg]
Lifter	27.62	30.58 [abcd]
Majoret	30.07	21.94 [bcdefg]
SW Marquee	22.72	28.18[bcdef]
Matrix	33.08	33.42 [ab]
Melrose	37.70	22.67 [bcdefg]
SW Midas	36.71	30.36 [abcd]
Nitouche	47.17	#
Prodigy	28.17	20.21[cdefg]
Specter	39.97	17.38[efg]
Stirling	26.97	27.63 [bcdef]
Toledo	23.21	19.15[defg]
Universal	22.91	18.17[defg]
Windham	26.53	14.98 [g]
LSD	NS	12.48

* numbers followed by the same letter are not statistically different
# not included due to lack of seed

Discussion

Use of resistant cultivars is a cost effective method for managing plant disease (Infantino et al., 2006). For development of resistant varieties, identification of sources of resistance is very important. Traditionally, *F. solani* f. sp. *pisi* was considered as the primary causal agent of the disease in many parts of the world. Commercial varieties were commonly evaluated for resistance to this pathogen. *F. avenaceum* was reported as the most predominant and aggressive *Fusarium* spp involved with root rot from our surveys and from different parts of the world. In this study, we attempted to screen selected commercial field pea varieties for resistance to root rot disease caused by *Fusarium avenaceum.* We could not find higher levels of disease resistance in the growth chamber trials. From the growth chamber experiments, the winter pea cultivar Windham showed relatively lower disease severity compared to the other varieties used in this study followed by Franklin, a green pea variety to both *F. avenaceum* and *F. solani* f. sp. *pisi*. Franklin is known to have higher levels of resistance to Fusarium root rot caused by *F. solani* f. sp. *pisi* and wilt caused by *F. oxysporum* (McPhee and Muehlbauer, 2002a). However, this finding did not appear to hold true under field conditions. Windham is resistant to wilt caused by race 1 of *F. oxysporum* (McPhee et al., 2007). Significant variation in levels of resistance to root has been recorded in our field trials. None of the varieties tested were found to be completely resistant to root rot. Similar to our results, Hwang et al., (1995) observed that all the twenty cultivars tested in a study under field conditions in Alberta, Canada were susceptible to moderately susceptible to *Fusarium* root rot. In a study conducted on the *Pisum* core collection 44 plant introduction lines with a disease severity rating of 2.5 or less among 387 accessions screened were identified as being partially resistant to root rot (Grünwald et al., 2003). Ondrej et al., (2008) identified a higher level of resistance by the accessions LPKE 36, Herold, Kamelot

and Gotik from the 19 selected pea accessions. However, we have observed partial resistance in the Austrian winter pea variety, Granger. The response of this variety was consistent over the locations, years and pathogens tested. Granger is also known to have resistance to *F. oxysporum* f. sp. *pisi* race 1 causing Fusarium wilt (Muehlbaur et al., 1998). Similarly, another winter pea variety used in this study, Windham also showed partial resistance to root rot. The level of resistance among yellow and green peas from susceptible to moderately susceptible. The varieties Aragron, Matrix, DS Admiral, SW Midas, CDC Striker and Franklin showed consistently higher disease severity. From the inoculated field experiments, we also observed a significant variety x pathogen interaction, signifying the variable response of cultivars to the two different pathogens used in this study. Considering the prevalence of *F. avenaceum* in this region, efforts to develop root rot resistant varieties should include this pathogen in resistance evaluations. In this study, we observed that the severity of disease observed in *F. avenaceum* inoculated plots was less compared to the *F. solani* f. sp. *pisi* inoculated plots. However, the stand emergence data from 2010 (Table 2.1) indicated that *F. avenaceum* resulted in significantly lower seedling emergence which might be due to pre-emergence seed rot. Results from this suggest that winter pea variety Granger, with a mottled seed coat, may have partial resistance to both *F. avenaceum* and *F. solani* f. sp. *pisi* the pathogens as demonstrated under both inoculated and non-inoculated conditions. Among the two pathogens, *F. avenaceum* appears to have a greater impact on plant stand than *F. solani* and can cause higher disease severity as demonstrated in seedling evaluations conducted under green-house conditions.

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CHAPTER THREE. DETERMINING EFFICACY OF PRECIPITATED CALCIUM CARBONATE IN MANAGING *FUSARIUM* ROOT ROT OF FIELD PEA

Abstract

The efficiency of PCC in controlling *Fusarium* species most commonly associated with root rots was evaluated under *in vitro* and field conditions. No significant reduction in the radial growth and growth rate of the *Fusarium* species evaluated were recorded in the PCC amended plates except for *F. acuminatum* and *F. oxysporum.* However, significant differences in sporulation were detected in all species where sporulation was observed. Spore germination was also affected by the presence of PCC in the growth media. In studies conducted using liquid growth media, the dry mycelial weight of all the *Fusarium* species was found to be significantly lower in the presence of PCC compared to the control. In greenhouse experiments, PCC efficacy was tested by applying varying rates of lime (equivalent to 0, 1, 2.5, 5, and 10 t/ac) to pots containing soil inoculated with *F. avenaceum* and *F. solani*, the most common pathogens associated with root rot. Significant reduction in root rot disease severity was observed in both *F. avenaceum*, and *F. solani* inoculated pots at all the rates of PCC application compared to control. Reduction in root rot severity associated with application of PCC was also observed in field trials conducted in two locations and two years.

Introduction

Dry pea or field pea (*Pisum sativum* L.) is an important cool season legume crop grown in the United States. North Dakota, Montana and Washington are the leading dry edible pea producing states. The area under this crop has rapidly increased from 337,500 acres in 2003 to 756,000 acres planted to pea in 2007 nationwide and, from 166,000 acres to 430,000 acres during the above period in North Dakota (NAAS, 2010). Dry pea is susceptible to many root-rot

pathogens including *Aphanomyces*, *Fusarium*, *Pythium* and *Thielaviopsis*. Among these, Fusarium root rot is a serious disease present in all pea producing areas in the United States (Kraft and Pfleger 2001), and has become a major constraint in dry pea production in the North Central region over the past years. Traditionally, *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyder & Hans was considered to be the primary causal agent of this disease, but recent surveys conducted in North Dakota identified *Fusarium avenaceum* as being the most prevalent pathogen associated with pea root rot in this state (Mathew et al., 2008). There is a dearth of satisfactory methods to control root rot and no cultivars with complete resistance to Fusarium root rot are currently available (Kraft and Pfleger 2001), seed treatments used are also limited in their efficacy (Samuel Markell, personal communication). The importance of this crop to North Dakota and prevalence of the disease necessitates development of an integrated disease management program to reduce losses associated with Fusarium root rot in dry peas. Precipitated Calcium Carbonate (PCC) also known as spent lime, a byproduct of sugar industry is used as soil pH amendment in Europe. It is reported to be effective in reducing the severity of root rot in sugar beet and spinach caused by *Aphanomyces* (Windels et al., 2004, and Ingemarsson 2004). The assumption is that PCC could be effective for managing pea root rot disease too, because of the soil borne nature of the root rot pathogen and similar pH requirements. Further, spent lime also improves soil structure and tilth. Besides neutralizing pH, spent lime contains nitrogen (0.6%), phosphorous (0.7%), potassium (0.05%), and magnesium (1.1%) and enriches the soil (Ingemarsson, 2004). It has been successfully used in soils with a pH up to 8 (Windels et al., 2006) and since the pH of most pea growing soils in North Dakota ranges from 5 to 8 (Mathew, 2006) it was believed that the use of PCC could be effective in reducing losses in this crop as well. A large quantity of spent lime is produced by sugar

industries and is available free of cost to growers, e.g. 500,000 tons of PCC is produced annually by seven factories in the Red River Valley and Southern Minnesota. The only cost to be incurred by growers is that of transportation. The advantages of spreading PCC in root rot management would be two fold, providing a cheaper method for controlling the disease, and as amendment that improve the soil chemical and physical conditions.

Previous reports suggest that spent lime has been effective in reducing root rot and/or increases yield in sugar beet grown in North Dakota and Minnesota (Windels et al., 2004). PCC when applied at 3t/ac or 10 t/ac reduced Aphanomyces root rot of sugar beet from 93-100% to 62% compared to non-limed plots (Windels et al., 2004) and a reduction in *Aphanomyces* soil index values was recorded in sugar beet as a result of this application (Windels, 2004). In greenhouse studies in Sweden application of lime has resulted in reduced root rot severity of sugar beet and caused increase in plant fresh weight (Ingemarsson, 2004). Apart from these, lime has also been used to control club root of cabbage in US (Campbell and Greathead, 1989). Efficacy of lime in reducing root diseases has been documented in peas, tomato, potato, red clover (Allmaras et al., 1987, Sonoda, 1978, and Smith et al., 1976, Steiner and Alderman, 2003). The objective of this study was to determine the efficacy of PCC as an alternative strategy for managing root rot disease in field peas.

Materials and methods

Laboratory experiments

Effect of PCC on mycelial growth, spore production, spore germination and dry mycelial weight

Laboratory experiments were conducted to study the effect of PCC on mycelial growth, sporulation, spore germination, and reduction in dry mycelial weight of different *Fusarium*

species that are commonly associated with field pea root rot in the region *viz. F. avenaceum, F. acuminatum, F. solani, F. graminearum, F. redolens,* and *F. oxysporum* isolated from pea roots. Radial growth, sporulation, and spore germination were studied using Potato Dextrose Agar (PDA) media amended with PCC at concentrations equal to 1, 2, 5, and 10 t/ac. The amount of spent lime to be added to PDA plates that corresponded to field application rates was determined considering that 1 acre of furrow slice (afs) would weigh approximately 906,500 kg (1 ha furrow slice = 2.24×10^6 kg, and 1 ha = 2.471 acres).

Amount of PCC to be added per liter of media

$$= \frac{\text{Field application rate of PCC in } mg}{906{,}500}$$

For example, 1t/ac will be, $\frac{1000{,}000{,}000}{906{,}500} = \sim 1100$ mg /Kg of soil.

Using the above mentioned afs weight, 1, 2.5, 5, and 10 t/ac corresponded to approximately 1100, 2750, 5500, and 11,000 ppm respectively. Calculated amounts of PCC were added to potato dextrose agar (PDA) to give concentration equivalent to field application rates of PCC per petri plate. PDA plates without any PCC added served as control.

For studying the effect of spent lime on mycelial growth, 5mm plugs of actively growing cultures (5-7 days old) were excised and transferred on to petri plates (100 x 15 mm) containing PCC amended PDA and incubated in the growth chamber with a cycle of 14h light and 10h darkness with day and night temperatures of 21º and 18º C respectively. Observations on radial growth were measured from 2 days after inoculation (DAI) to 6 DAI. Two perpendicular readings of colony diameters were made for each petri plate. Radial growth at 6 DAI was expressed as maximum radial growth, and growth rate was estimated from the observations from

2^{nd} to 6^{th} DAI using PROC REG procedure of SAS version 9.1. Spore production or number of conidia produced at 6 DAI was counted using a haemocytometer. Five 5mm diameter plugs from the plates were cut from each plate and transferred to test tubes with 5ml sterile distilled water, and were shaken thoroughly. Aliquots from the tubes were transferred onto haemocytometer and number of conidia per ml was determined. From each plate, spores were counted two times. Experiment was laid out as completely randomized design, with three replications for each concentration, and each experiment was repeated three times.

Ability of PCC to inhibit spore germination was assayed using petri plates (65 x 15 mm) containing PDA amended with spent lime. The media and concentrations were prepared as mentioned above. Spores were obtained from each isolate and the spore concentration was adjusted to 1000 spores/ml using a haemocytometer, 200 µl of the spore suspension was uniformly spread on Petri dishes (65 × 15 mm) and incubated at room temperature for 24 h. A total of 100 spores per isolate were counted and the number of spores germinated at each concentration was expressed as the percentage of germinating spores. The experiment was laid out in a completely randomized design with three replications per concentration and was repeated two times.

Effect of spent lime on fungal biomass reduction was evaluated using liquid culture. Conical flasks containing 50ml of potato dextrose broth were prepared and calculated amounts of PCC was added to each flask to give a concentration of PCC per flask equivalent to 0, 1, 2.5, 5, and 10 t/ ac. These flasks were inoculated with 5mm discs of actively growing culture and incubated at room temperature on a rotary shaker at 120 rpm for 6 days. After 6 days the mycelium was harvested, dried in a hot air oven at 70ºC for 24h and the dry mycelial weight was

recorded. The experiment was laid out in a completely randomized design, each treatment was replicated three times and the experiment was repeated three times.

Greenhouse experiments

Efficacy of PCC in reducing root rot severity caused by the two pathogens most commonly associated with root rot of field peas in ND, *F. avenaceum,* and *F. solani* f.sp. *pisi* was assessed in the greenhouse studies. Three aggressive isolates of each pathogen species (Pea 41, FPS M60 and FA0601 of *F. avenaceum* and Fs 01.B1, F215, F54 of *F.solani* (Grünwald et al., 2003)) were used for this study. The inoculum was prepared by placing eight 5 mm plugs of actively growing *F. avenaceum* and *F. solani* f.sp. *pisi* cultures grown on half strength potato dextrose agar for 10 days under alternate dark and light cycles of 12 h each at 23ºC into a 125 ml conical flasks containing a sterilized (at 15 lbs for 45 min) sand cornmeal mixture (45g of play sand, 5g of cornmeal and 10ml of distilled water). These flasks were incubated at room temperature for 7 to 10 days and shaken daily by hand to ensure uniform growth of the fungus (Bilgi et al., 2008). All the three isolates of each species were mixed together before inoculating the soil. The inoculum was mixed with field soil sterilized at 121^0 C, 20 lbs for 2 hours on two consecutive days in the ratio of 1 incoulum : 20 soil w/w and filled into rectangular pots. The pots were watered thoroughly and covered with a plastic wrap for 48 hours to stabilize the inoculum (Sagar and Sugha, 2004). The amount of PCC to be added in this study was calculated based on the amount available to each plant under field conditions based on the surface area. Different rates of PCC equivalent to 0, 1, 2.5, 5, and 10t/ac were applied to the pots containing soil inoculum mixture, mixed thoroughly to ensure uniformity, and the pots were left for 24 hours before planting. Treatments with pots containing inoculum but no PCC and only autoclaved soil were used as controls. Each pot was planted with 15 seeds, and thinned to 10

seedlings after germination. DS Admiral, a known susceptible field pea cultivar to root rot was used for this study. After 14 days after planting, the plants were carefully removed from the pots, and roots were washed with tap water to remove soil adhering to the surface. Washed roots were rated for root rot severity.

The experiment was set up as randomized complete block design with four replications per treatment and the experiment was repeated two times. Root rot severity was measured as the length of lesions on tap root compared to whole root length, expressed as percentage.

$$\text{Root rot severity} = \frac{\text{lesion length on tap root}}{\text{Total tap root length}} \times 100$$

Field experiments

Field studies were conducted in two grower's fields at Hickson, ND and Moorhead, MN with known history of root rot incidence. PCC was applied at the rates of 0 (untreated control), 5, 10, and 15t/ac of wet weight to the experimental plots. This was done by spreading and incorporating PCC shipped in from nearby sugar mills into the soil with a chisel plough in the fall of 2009. DS Admiral, a known susceptible field pea cultivar to root rot was used. Each treatment was replicated four times. Stand counts were recorded three weeks after planting on the four inner rows. Root rot severity was assessed at the pre-flowering stage. Ten plants were collected from each plot from inner 20ft in the center of the 60 ft long inner four rows by destructive sampling. Roots were washed under running tap water and assessed for incidence and severity of root rot. Root rot severity was measured as the percentage of root length covered by lesions (length of lesions/total root length X 100). Yield data was recorded at the end of crop season. The plots were 44' wide x 60' long, observations on stand counts, disease severity, and yield were recorded from the inner 20' of the 60' long rows. The experiment was laid out as

randomized complete block design, conducted at two different locations *viz.* Hickson, ND, and Morehead, MN and repeated over two years, in summer of 2010 and 2011.

Statistical analysis

Statistical analyses for the above studies was performed using SAS version 9.1 (SAS Institute Inc. 2002-2003). Homogeneity of variance between experiments was tested using LEVENE's test. Similar experiments were combined and analyzed using all replications using PROC ANOVA procedure. Mean separation was performed using Fisher's protected least significant difference (LSD) test.

Results

Laboratory experiments

Effect of PCC on mycelial growth, growth rate, spore production, spore germination, and fungal biomass

Test for homogeneity of variance between experiments showed no significant differences between experiments, so all the three experiments were combined and analyzed. Except for *F. acuminatum,* and *F. oxysporum,* no significant differences between lime rates were observed for maximum mycelial growth for all the *Fusarium* spp. *viz* . *F. avenaceum, F. solani, F. graminearum,* and *F. redolens,* tested in this study. The results are shown in table 3. 1. Mycelial diameter of *F. acuminatum* and *F.oxysporum* was highest on plates with PCC amended PDA compared to non-lime amended control plates. However, though differences in mycelial growth were not statistically significant, a similar trend was observed for the other *Fusaria* in this study.

Analysis of growth rate showed a trend similar to maximum growth. Except for *F. acuminatum,* and *F. oxysporum,* no significant reduction in the growth rate of the fungi was recorded in the lime amended plates (Table 3.2). Growth rate was higher in lime treated plates compared to non-lime amended control.

Table 3.1. Effect of PCC on maximum mycelium radial growth (cm).

Fusarium spp	PCC application rate (t/ac)*					
	0	1	2	5	10	LSD
F. acuminatum	2.16[b]	2.67[a]	2.78[a]	2.75[a]	2.74[a]	0.21
F. avenaceum	3.58	3.64	3.43	3.5	3.69	NS
F. graminearum	4.25[b]	7.2[a]	7.18[a]	7.16[a]	7.29[a]	0.96
F. redolense	6.56	6.71	6.77	6.56	6.53	NS
F. solani	5.18	5.59	5.66	5.68	5.64	NS
F. oxysporum	5.22[b]	6.44[a]	6.61[a]	6.71[a]	6.87[a]	0.44

* numbers followed by the same letter are statistically not different

Table 3.2. Effect of PCC on growth rate.

Fusarium spp	PCC application rate (t/ac)*					
	0	1	2.5	5	10	LSD
F. acuminatum	2.67[b]	3.79[a]	4.08[a]	4.03[a]	3.64[a]	0.07
F. avenaceum	5.67	5.73	5.32	5.45	5.85	NS
F. graminearum	15.94[b]	27.44[a]	27.41[a]	27.00[a]	28.39[a]	2.53
F. redolens	10.2	10.61	11.13	10.89	10.95	NS
F. solani	8.28	10	10.21	10.57	10.37	NS
F. oxysporum	8.25[b]	10.82[a]	11.33[a]	11.51[a]	12.07[a]	1.39

* numbers followed by the same letter are statistically not different

Significant differences in spore production were detected in all species where sporulation was observed, except *F. redolens,* (Table 3.3).Number of spores reduced with increasing rate of PCC. Spore production was significantly reduced at all concentrations of lime tested i.e., 1, 2.5, 5, and 10 t/ac in *F. acuminatum*, *F. avenaceum,* and *F. oxysporum.* In case of *F. solani*, reduction in spore production compared to control was significant only at higher concentrations of 2.5, 5,

and 10t/ac. No sporulation was observed for *F. graminearum* in any of the treatments including the control. Incubation of the plates for seven more days longer also did not result in any spore production.

Table 3.3. Effect of PCC on spore production.

Fusarium spp	Spore production (x 10^6 /ml)*					
	0	1	2.5	5	10	LSD
F. acuminatum	522.4[a]	5.9[b]	2.3[b]	4.2[b]	1.5[b]	268.65
F. avenaceum	40.67[a]	1.5[b]	1.33[b]	1.17[b]	0.83[b]	28.13
F. graminearum	0	0	0	0	0	NS
F. redolens	119.08	104.83	62.83	48.83	37.33	NS
F. solani	207.42[a]	136.25[ab]	116.75[b]	74.08[b]	58.17[b]	90.22
F. oxysporum	491.1[a]	211.7[b]	186.1[b]	179.9[b]	147.9[b]	236.11

* numbers followed by the same letter are statistically not different

Spore germination was also affected by the presence of lime in the growth media. As the *F. graminearum* isolate used in this study failed to produce spores on PDA, spores for this isolate were obtained by growing the culture on Mung Bean Agar (MBA). The germination percentage of spores of *F.graminearum,* and *F.oxysporum* was reduced only at higher concentrations of 5, and 10 t/ac, but in the other species studied, spore germination was significantly reduced at all concentrations i.e., 1, 2.5, 5, and 10 t/ac (Table 3.4) compared to the control plates.

Table 3.4. Effect of PCC on spore germination.

Fusarium spp	Spore germination (%)*					
	0	1	2.5	5	10	LSD
F. acuminatum	98.11[a]	92.33[b]	88.44[c]	87.44[c]	87.00[c]	2.69
F. avenaceum	98.44[a]	94.78[b]	93.11[bc]	93.78[bc]	92.44[c]	2.14
F. graminearum	99.11[a]	97.89[a]	96.89[a]	90.89[b]	89.00[b]	4.10
F. redolens	98.67[a]	96.56[b]	95.33[b]	93.22[c]	91.67[c]	2.08
F. solani	68.78[a]	44.33[bc]	47.33[b]	40.78[c]	41.44[c]	4.07
F. oxysporum	98.56[a]	98.11[ab]	98.11[ab]	96.89[b]	96.67[b]	1.60

* numbers followed by the same letter are statistically not different

Effect of PCC on fungal biomass production was studied using liquid media. Fungal biomass production was measured in terms of dry mycelial weight. The dry mycelial weight of all the *Fusarium* species studied in this experiment was found to be significantly lower in the presence of lime as compared to the control (Table 3.5). In general, dry mycelial weight decreased as the lime application rate increased.

Table 3.5. Effect of PCC on fungal biomass production.

Fusarium spp	Dry mycelial weight (mg)*					
	0	1	2.5	5	10	LSD
F. acuminatum	55.5^{a}	35.7^{b}	27.89^{bc}	19.41^{c}	26.76^{bc}	10.13
F. avenaceum	67.42 ^{a}	31.01^{c}	43.83^{bc}	56.88^{ab}	39.55^{c}	14.63
F. graminearum	88.46^{a}	54.19^{c}	67.88^{b}	58.07^{bc}	34.65^{d}	11.89
F. redolens	34.08^{a}	23.18^{b}	15.82^{b}	16.78^{b}	17.97^{b}	7.89
F. solani	48.38^{a}	29.8^{c}	16.76^{c}	16.48^{c}	5.79^{d}	10.49
F. oxysporum	33.31^{a}	13.43^{bc}	20.49^{b}	7.22^{c}	6.75^{c}	8.27

* numbers followed by the same letter are statistically not different

Greenhouse experiments

Greenhouse experiments were conducted to ascertain the efficacy of PCC in reducing root rot severity on field peas. Levene's test for homogeneity of variance showed that there were no significant differences between the two experiments. Therefore, data from the two experiments were combined to determine the effect of lime in reducing root rot severity. The experiments showed significant reduction in root rot disease severity, in both *F. avenaceum*, and *F. solani* treated pots at all the rates of lime application i.e., 1, 2.5, 5, and 10 t/ac compared to control (Table 3.6). Inoculated pots with no lime applied, always had recorded highest root rot severity (17.66% and 16.47% for *F. avenaceum* and *F. solani* respectively). For both pathogens, disease severity decreased in response to lime application.

Table 3.6. Effect of PCC on root rot disease severity caused by *F. avenaceum* and *F. solani* f. sp *pisi* in greenhouse trials.

	Mean Disease severity (%)*	
PCC rate (t/ac)	*F. avenaceum*	*F. solani* f.sp. *pisi*
0	17.66 [a]	16.47[a]
1	11.37 [b]	11.92[b]
2.5	8.32 [bc]	10.82[b]
5	6.09 [cd]	7.72[bc]
10	3.91 [d]	5.37[c]
Control	0.04 [e]	0.04[d]
LSD	3.29	4.33

* numbers followed by the same letter are not statistically different

Field experiments

Significant differences in root rot severity were recorded between the treatments in 3 of the 4 field trials conducted over two years (Table 3.7). Lime applied at higher rates always had less root rot severity as compared to the control. In 2010, at the Hickson site, lime application at all the rates i.e., 5, 10, and 15t/ac showed significantly lower amounts of disease compared to no lime application. However, at the Moorhead site, lime application only at the rates of 10, and 15t/ac showed significant reduction in root rot severity compared to the control. In 2010, the trials were affected by high weed pressure at both locations and no significant differences in stand counts (Table 3.8) or yield (Table 3.9) were observed with the application lime at either of the two sites. In 2011, statistically significant differences in disease severity were observed only at Hickson field trial. Disease severity in the plot with no PCC application had significantly high compared to the lime applied plots. In Moorhead, the differences in disease severity were not significant. The trials at both locations were affected by heavy rain and water logging in 2011.

No significant differences between treatments were observed for stand counts (Table 3.8) and yield (Table 3.9).

Table 3.7. Table showing the effect of PCC on root rot severity on field pea in field trials conducted over two years.

PCC rate (t/ac)	Mean disease severity (%)			
	Hickson		Moorhead	
	2010	2011	2010	2011
0	15.55[a]	17.61[a]	12.22[a]	10.04
5	10.41[b]	11.69[b]	9.01[ab]	7.61
10	6.99[c]	7.15[c]	6.17[bc]	6.56
15	4.51[d]	5.76[c]	3.71[c]	4.88
LSD	1.79	3.17	3.5	NS

* numbers followed by the same letter are not statistically different

Table 3.8. Table showing the effect of PCC on stand counts in field trials conducted over two years.

PCC rate (t/ac)	Stand count (plants/20ft)			
	Hickson		Moorhead	
	2010	2011	2010	2011
0	39.75	26.37	39.50	45.31
5	39.50	29.62	39.25	44.56
10	37.62	25.44	40.37	48.50
15	43.37	25.75	43.13	49.13
LSD	NS	NS	NS	NS

* numbers followed by the same letter are not statistically different

Table 3.9. Table showing the effect of PCC on yield of field pea in field trials conducted over two years.

PCC rate (t/ac)	Yield (g/20ft)			
	Hickson		Moorhead	
	2010	2011	2010	2011
0	3475.7	595.7	-	1120.5
5	2651.4	484.0	-	1194.8
10	2681.4	846.7	-	1108.5
15	2670.8	880.3	-	1409.8
LSD	NS	NS	-	NS

* numbers followed by the same letter are not statistically different

Discussion

The area under field pea production in North Dakota has increased rapidly in recent years. Field surveys conducted in the state indicated that root rot is a growing concern for field pea production in the state. From the field surveys, it has been identified that *Fusarium* spp are the major pathogens involved in causing root rot (Mathew et al., 2008). Currently there are no completely resistant cultivars available against this disease (Kraft and Pfelger 2001). Fludioxonil, Pyraclostrobin, and Trifloxystrobin are labeled for seed treatment for controlling seed borne and soil borne fungal (*Fusarium* and *Rhizoctonia*) diseases in ND (McMullen and Markell, 2010). However, seed treatments often fail to provide satisfactory control of the disease (Samuel Markell, personal communication). In this study we evaluated the effectiveness of PCC or spent lime, a byproduct from sugar beet industry as an alternative management strategy for controlling Fusarium root rot of field peas. Effectiveness of spent lime in controlling *Aphanomyces* root rot in sugar beet and spinach has been demonstrated under field and greenhouse conditions (Windels et al., 2010; Ingemarsson, 2004). However, in the available literature, no information on the influence of PCC on plant pathogenic *Fusarium* spp under *in vitro* conditions is available. Therefore, we initiated a study to evaluate the effect of PCC on mycelial growth, spore production, spore germination, and fungal biomass production. From our laboratory experiments, except for *F. acuminatum,* and *F. oxysporum,* we have observed no significant differences in either radial growth of growth rate of *F. avenaceum, F. solani, F. graminearum,* and *F. redolens*. However, visible differences in the mycelial density were observed. The mycelium in the lime amended plates was always sparse, i.e. less dense compared to the control plates, similar to kind of growth that can be observed when the cultures were grown on water agar, and a tendency towards aerial growth appeared to be favored. These visible

differences in mycelial density were further quantified in terms of dry mycelial weight or fungal biomass using potato dextrose broth amended with PCC. Application of lime at all the rates resulted in significant reduction in fungal biomass compared to PCC non-amended flasks. More than 50% reduction in dry mycelial weight was observed for *F. acuminatum, F. graminearum, F. solani* and *F. oxysporum*.

Addition of lime to the media resulted in increase in pH (data not presented). In this study we also observed significant reduction in spore production and spore germination in presence of spent lime in the media. Allmaras et al., (1987), in a field study reported a 37% reduction in propogule density of *F. solani* f.sp. *solani* over three years with single application of lime. In a laboratory study, Ulfig (2006) observed that application of lime to increase the pH to 12 resulted in elimination of keratinophilic *F. solani* from the sludge. This study was focused on keratinolytic and keratinophilic fungi, which are of less significance to agriculture, and reported that addition of lime to sludge resulted in either complete elimination or significant reduction in keratinolytic and keratinophilic fungi. In the greenhouse and field trials, application of PCC resulted in significant reduction in root rot severity compared to no lime applied plots. *F. avenaceum* and *F. solani* f.sp. *pisi* are considered as major root rot pathogens in the region (Mathew et al., 2008, Kraft and Pfelger, 2001), and therefore they were used in greenhouse trials for testing the efficacy of PCC in controlling the disease. Addition of PCC to pots resulted in increase of pH (6.86, 7.42, 7.48, 7.57, and 7.61 for 0, 1, 2.5, 5, and 10 t/ac respectively). Our data from greenhouse trials showed that root rot severity was significantly less in lime treated pots at all the rates of application compared to the control pots for both the pathogens. Disease severity decreased with increasing rates of lime application. Disease severity was lowest at 10 t/ac for *F. avenaceum* inoculated pots (3.91%, table 3.6), however, there were no significant

differences between 5 and 10t/ac treatments for *F. solani.* Decrease in *Aphanomyces* disease severity index values with application of slaked lime and factory lime was reported by Ingemarsson (2004). Efficacy of spent lime in controlling *Aphanomyces* root rot has been well studied (Windels et al., 2004-2010). Efficacy of liming has been successfully demonstrated in either reducing propogule density or control soil borne diseases in many crops including field pas, tomato, potato, red clover, and canola (Allmaras et al., 1987, Sonoda, 1978., and Smith et al., 1976; Woltz, et al., 1992; Steiner, and Alderman. 2003; Arshad, 1997; Hibbel et al., 2001). However, there are contrary reports where liming has resulted in increase of disease severity in field pea and red clover (Lyndon Porter, unpublished data; Steiner, 2003). PCC application, apart from resulting in suppression of the disease, has also resulted in enhancing the yield and quality of sugar beet (Windels et al., 2009). We have observed similar reduction in disease severity in our field experiments. However, we believe that our results were insufficient for establishing the effect on stand count and yield (Table 3.8). Stand counts were affected by high weed pressure in 2010 and water logging in 2011 (Table 3.9). In 2010 yield data could not be collected due to heavy rains and water logged conditions. In 2011, though the yield differences were not significant, higher yields were recorded with 15 t/ac application compared to the control plots.

PCC adsorbs various micro nutrients during the clarification process, and is considered enriched with nutrients compared to the commercial lime (Ingemarsson, 2004; Sims, 2010). It is known to supply Phosphorus to sugar beet, and response of sugar beet to PCC application was similar to phosphorus fertilizer application. (Sims, 2007, Salisbury and Hills 1987). In addition to supplying phosphorus, PCC also provides Ca, Mg, Na, and K (Sims, 2010, Ingemarsson, 2004). Based on our results, and other published information about PCC efficacy and contrasting reports, we hypothesize that effect of PCC in reducing root rot severity involve more than just

alteration (increase) of soil pH. Several effects of pH in suppression of disease have been reported including direct affect on pathogen growth, alteration of the soil conditions to make them favorable for the plant, and antagonistic microbial growth, or change in the nutrient availability (Crowley and Alvey, 2002). Suppression of Fusarium wilt is thought to be the result of increased competition for carbon (C) and Iron (Fe) in the soil by non pathogenic *Fusarium* and other microbial antagonists (Alabouvette, 1999). Allmaras et al., (1987) proposed that the reduction in propogule density of *F. solani* in lime applied field pea plots may be due to increased exchangeable Ca, which might have improved resistance to pathogen attack, and impaired saprophytic survival ability of the pathogen or by favoring the growth of microbial antagonists. The pH of the experimental sites at both locations was slightly alkaline (7.5 at Hickson and 7.8 at Moorhead), and we did not see any significant increase in soil pH with application of lime. Therefore, we believe that the reduction in root rot disease severity under field conditions may have involved multiple factors such as improved root growth and thus resistance to pathogen attack by supplying nutrients, and by favoring the growth of antagonistic microbes in rhizosphere. In North Dakota, zero tillage is a common practice in field pea. Addition of lime to soil reduces soil bulk density, i.e. soil become less compact, improves soil aeration, and prevents soil crusting (Webster, and Nyborg. 1986). Fusarium root rot in field pea is favored by soil compaction and agronomic practices like zero tillage (Tu, 1994; Fernandez et al., 2008). Thus we believe that PCC in addition to bringing favorable changes in soil chemical and biological properties, also alters physical properties that are favorable plant growth and unfavorable for pathogen growth. In this study we evaluated the efficacy of PCC as an alternative control strategy for managing Fusarium root rot of field pea. Our results indicate that PCC can be a potential alternative strategy for this disease control.

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CHAPTER FOUR. ASSESSMENT OF FUNGAL GENE EXPRESSION PATTERN ASSOCIATED WITH ROOT INFECTION OF FIELD PEA BY *F. GRAMINEARUM*

Abstract

Field pea (*Pisum sativum*), an important rotational crop with cereals is greatly affected by root rots. *Fusarium graminearum*, commonly known as a cereal pathogen, has recently been associated with this disease along with a few other toxigenic *Fusarium* spp. To better understand the interaction between field pea and this pathogen, a study was conducted to elucidate mechanisms associated with *F. graminearum* infection on this crop. Fungal gene expression in artificially infected field pea roots and *F. graminearum* grown in culture was assessed using the Illumina mRNA-Seq technology. Three biological replications of *in vitro* and *in planta* libraries were sequenced, generating ~ 50 million single reads. A total of 613 *F. graminearum* genes were found to be differentially expressed *in planta* on pea. Among these, 237 genes were up-regulated and 376 were down-regulated. Functional classes of differentially expressed genes were determined using MIPS FunCatDB. Functions of 40% and 38% of the up-regulated and down-regulated genes were unknown. Within the up-regulated genes, functional classes associated with amino acid metabolism, nitrogen metabolism, extracellular polysaccharide degradation, detoxification by degradation and defense related proteins were significantly enriched. Homology search for the up-regulated genes *in planta* with characterized pathogenicity, virulence or effector genes in the PHI-base led to the identification of 53 genes with similarity to the genes in the PHI-base. Four of these were identified as having effector roles in other host-pathogen interactions. RT-PCR of selected up-regulated genes was conducted to validate mRNA-Seq analysis results. Overall, this study led to the identification of several genes involved in important molecular processes during *F. graminearum* – dry pea interaction.

Introduction

Dry Pea or field pea (*Pisum sativum* L.) is an important cool season legume crop grown in the United States. North Dakota ranks first with an area of 430,000 acres planted to pea in 2010 and contributes to more than 55 per cent of value of production (NASS, 2010). Root rots are one of the major diseases affecting this crop. A significant rise in the root rot incidence was observed in the state in recent years and *Fusarium* species were identified as the most common pathogens associated with the disease according to a state-wide survey conducted over the past three years (Mathew et al., 2008). *F. avenaceum* was found to be the most prevalent among the *Fusarium* species. Among the several other *Fusarium* spp, *F. graminearum*, the pathogen associated with Fusarium head blight of cereals (FHB) was also found to be involved in causing root rot of field pea. *F. graminearum* is one of the most extensively studied *Fusarium* speciesand the whole genome sequence of this pathogen is available along with a repertoire of information regarding gene expression on cereals (Cuomo et al., 2007 and Ma et al., 2010). Based on these findings, a study was initiated to evaluate gene expression patterns associated with interaction between field peas and *F. graminearum* with an aim to identify potential pathogenicity genes specifically linked to this host-pathogen system.

Next generation sequencing (NGS) has brought revolutionary changes in the status of biological research. The key features that made the extensive use of NGS popular are lower sequencing cost per base pair and the large amount of biological data generated per run. NGS has found successful applications in various fields of biological research like whole genome re-sequencing, *de novo* genome sequencing, transcriptome characterization, gene expression profiling, novel gene discovery, SNP detection, metagenomic, and epigentic analysis. It is believed that NGS will have huge implications in the field of plant pathology in the coming

yearsTranscriptome profiling and gene expression analysis that can help to advance our understanding of plant- pathogen interactions during disease development and enable identification of novel targets for disease management have been identified as a key area where NGS would be highly applicable. Therefore, the objective of this study was to identify fungal genes associated with the development of root rot in field peas using NGS technology. It was believed that findings from this study could lead to the identification of novel pathogenicity genes, provide a better understanding of the genes involved in disease development on leguminous hosts and also provide useful information regarding potential targets for designing disease management strategies.

Materials and methods

Inoculum preparation

The *F. graminearum* isolate PH-1 (NRRL 31084) used for whole genome sequencing was used in this study. Inoculum was prepared by following the method described by Bilgi et al., (2008). Briefly, 25 g of wheat seed was soaked in 50 ml sterilized water for 24 h, after which the excess water was decanted and autoclaved (121 ^{o}C for 20 min at 15 lbs). The flasks were allowed to cool down and five 5mm plugs of actively growing cultures were transferred to each flask. The flasks were incubated for 7 days at room temperature with periodic shaking to allow uniform growth of the inoculum.

cDNA library preparation and sequencing

In vitro cDNA libraries for the pathogen *F. graminearum* were prepared from axenic cultures grown on complete media (Leslie et al., 2006) (CM) as described in Güldener et al., (2006). Two 250 ml tissue culture flasks (BD Falcon) containing 100 ml CM were inoculated

with one ml suspensions of 10^6 macroconidia of *F. graminearum* and grown at 25 °C for 24 h with shaking at 150 rpm. Mycelia of each flask were harvested and washed with sterile distilled water. The harvested mycelia were re-inoculated into 100 ml CM and grown for another 12 h at 25 °C with shaking at 150 rpm, mycelia was harvested and used for RNA extraction. For *in planta* cDNA library preparation, root inoculations were performed using modified paper towel method (Bilgi et al., 2008). Ten days old seedlings of root rot susceptible field pea cv. DS Admiral, which were grown in 256 ml plastic cups containing sterilized vermiculite, were used for root inoculations. Three roots placed on a layer of four sterile paper towels were inoculated with 5g of inoculum. The roots were sampled at 24, 48, 72, 96, and 120 hours after inoculation and were stored at -80^{o}C until RNA extraction. Each sample for RNA extraction constituted a pool of six roots. Total RNA was extracted using the RNeasy mini kit (Qiagen Inc., Valencia, CA) and mRNA was isolated using the Oligotex mRNA mini kit (Qiagen Inc., Valencia, CA) following manufacturer's instructions. cDNA libraries for sequencing on Illumina platform were prepared using NEBNext mRNA Sample Prep Kit (New England Biolabs, Ipswich, MA) and Illumina multiplex adapters (Illumina Inc, San Diego, CA). Three biological replications for each condition i.e., *in planta* and *in vitro* libraries were sequenced on Illumina HiSeq 2000 platform.

mRNA-Seq data analysis

Quality checks for the RNA-Seq libraries were performed using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Estimates of over-represented sequences and quality score distribution were obtained from FastQC. FastX_clipper, and Fastx_quality_trimmer programs of FastX tool kit were was used to remove adaptor sequences and retain reads that had and minimum length of 36bp with a minimum quality score of 20. mRNA-Seq read mapping,

transcript abundance estimations, and differential gene expression analysis was performed according to Trapnell et al., (2012). The reference genome of *F. graminearum* PH-1 (fusarium_graminearum_ph-1_3_supercontigs.fasta) and the corresponding annotations (fusarium_graminearum_ph-1_3_transcripts.gtf) were downloaded from the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiDownloads.html). Reads were mapped to the reference genome using Bowtie v 0.12.7 (Langmead et al., 2009) and then TopHat v 1.3.2 (Trapnell et al., 2009), which performs spliced alignments of Bowtie unmapped reads to the reference genome. Transcript abundances were estimated using Cufflinks v 2.0.2 (Trapnell et al., 2009). The gene expression level data was normalized and expressed as fragments per kilobase per million mapped reads (FPKM). Differential gene expression analysis was conducted using CuffDiff program of Cufflinks (Trapnell et al., 2010) at a false discovery rate (FDR) of 0.05 after Benjamini-Hochberg correction for multiple testing. R package cummeRbund v 2.0.0 (Goff et al., 2012) was used for visualization of differential expression analysis results generated by CuffDiff.

Functional classification of differentially expressed genes

Functional categories of differentially expressed genes were determined according to the Functional Catalog annotation scheme 2.1 of gene products from *F. graminearum* genome using FunCatDB (Ruepp et al., 2004). A functional category was considered statistically enriched only when the *P* value for that category was < 0.05.

Comparative analysis of the up-regulated genes with characterized pathogenicity, virulence, effector genes

A homology search of up-regulated genes *in planta* was conducted with experimentally verified pathogenicity, virulence, and effector genes from fungal, oomycete, and bacterial pathogens in the Pathogen Host Interaction database (PHI-base) (Winnenburg et al., 2006). Protein sequences of the genes contained in the database were downloaded from the PHI-base website (http://www.phi-base.org/download.php), and a stand-alone BLAST search of up-regulated genes was performed against PHI-base protein sequences using the BLASTX function implemented in NCBI BLAST-2.2.26+ at an e-value threshold of 10E-4.

Validation of mRNA-Seq analysis by RT-PCR

Sequence analysis results were validated by performing RT-PCR of selected up-regulated genes and comparing to expression bar plots from differential expression analysis. The list of selected genes and the primers used are listed in Table 4.1. β-tubulin (FGSG_09530) was used as control. The reverse transcription reaction was performed on 1 µl total RNA from the *in vitro* and *in planta* libraries using QuantiTect® Reverse Transcription Kit (#205311, Qiagen, Chatsworth, CA) following manufacturer's instructions and 1 µl cDNA was used as template for RT-PCR. Each reaction mixture contained 2 µl of 10x Top Taq buffer, 1 µl dNTP (10mM each), 1.2 µl $MgCl_2$ (25mM), 2 µl of each of forward and reverse primers (2.5µM), 0.5 µl Top Taq DNA polymerase, and 10.3 µl of nuclease free water. The PCR cycling conditions were: one cycle of initial denaturation 94°C for 5 min followed by 30 cycles of 94°C for 30 sec (denaturation), 58 or 61°C for 30 sec (annealing), 72°C for 1 min (extension) and one cycle of final extension at 72°C for 10 min. For FGSG_02117 and FGSG_13459 annealing temperature of 58 °C was used. For FGSG_01767 and FGSG_04580, annealing temperature was 61 °C. 5 µl of each PCR reaction was loaded onto a 1% agarose (#v3125, Promega Corporation, Madison,WI.) gel and visualized with AlphaDigiDoc® Pro gel doc system (Alpha Innotech Inc).

Table 4.1. List of genes used for validation of sequence analysis using RT-PCR, and their function.

Gene	Function	Primers (5' →3')
FGSG_01767	Related to pisatin demethylase	F- CAGACTCTGATCCATACGGCTTC
		R- TAGCGTGCTCGGACGTTGTC
FGSG_02117	Related to cytochrome P450	F- TGCCGTAGACTCTTTCTCGAAG
	monooxygenase (lovA)	R- CCTTTATCGATTTCACGAAATCC
FGSG_04580	Probable ABC1 transport	F- GTATTCTGTTCAATGTCACGCTG
	protein	R- GCATGTAATCCACTGGAACGAC
FGSG_09530	Beta tubulin	F- GACAGCAATGGTGTTTACAACG
		R- GATTGACCGAAAACGAAGTTG
FGSG_13459	Related to pisatin demethylase	F- CAGCGTCGGCTTTTCTACAG
	cytochrome P450 CYP57	R- GCCGTATGATGAGATTGACCC

Results

Sequence data analysis

A total of 50,339,882 single read (SR) reads were generated from three biological replicates of infected and culture libraries. This constituted of 7,340,557 75bp reads and 16,825,868 76bp reads, and 26,173,457 50bp reads. After the strict quality control step 33,287,338 were retained that had a minimum quality score of 20 for each base and at least 36 bp long (Table 4.2). Reads were mapped to *F. graminearum* genome using TopHat v.1.3.2. Of the total quality filtered reads 28.3 to 91.4% of the reads were mapped to the genome. Percentage of mapped reads for culture libraries was higher compared to the infected libraries. The average mapped read percentage for culture libraries was 75.32 per cent (57.6 to 91.4%) as compared to the relatively lower per cent mapped reads for infected libraries (28.3 to 47.8%) with an average of 39.06% (Fig 4.1). Experimental variation resulting from biological variation was estimated using gene expression pattern of genes in biological replicates. High levels of correlation for

biological replicates (Infected 0.91 to 0.96, and culture 0.7 to 0.79) was observed indicating the robustness of sampling, assay and analysis methods (Fig 4.2).

Table 4.2. Read length, reads (total number, and QC filter passed reads) in each library.

Library	Replication	Read length (bp)	Total number of reads	QC passed reads
In planta (Infected_0)	1	75	7,340,557	5,883,892
In planta (Infected_1)	2	76	7,270,097	4,747,334
In planta (Infected_2)	3	50	15,302,469	10,638,794
In vitro (Culture_0)	1	76	9,555,771	5,544,552
In vitro (Culture_1)	2	50	5,907,254	3,545,292
In vitro (Culture_2)	3	50	4,963,734	2,927,474

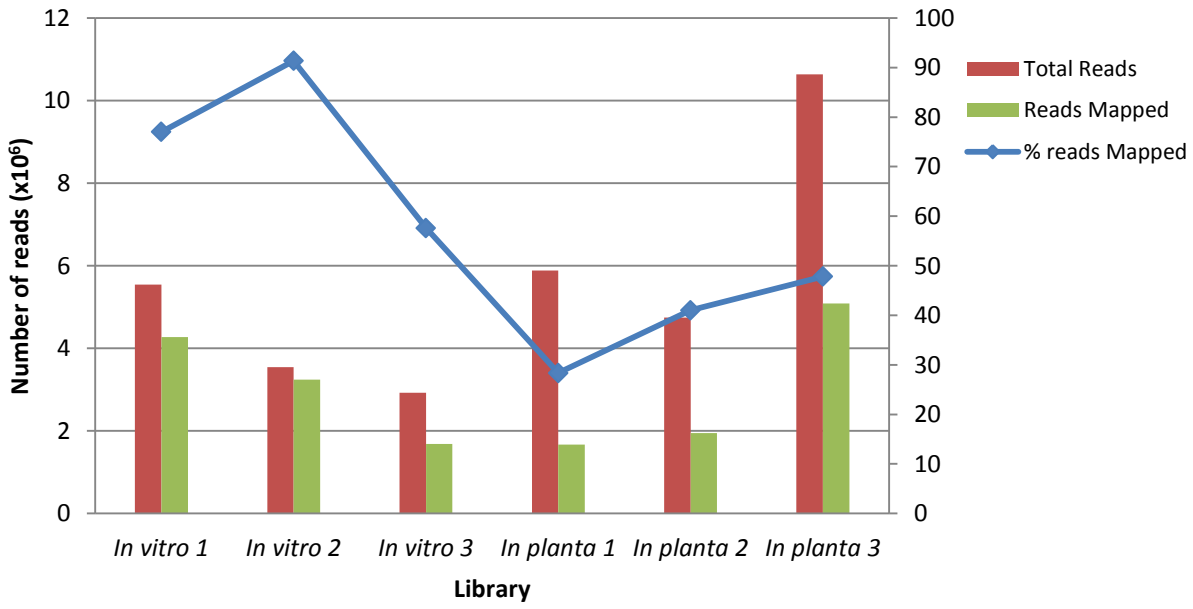

Figure 4.1. Number of quality filtered mRNA-Seq reads, and number of reads mapped to reference genome. Quality filtered reads were obtained after passing the total mRNA-Seq reads through pipeline of fastx_clipper and fastq_quality_trimmer of FastX tool kit. Reads were mapped to *F. graminearum* genome using Bowtie v 0.12.7 and Tophat v 1.3.2.

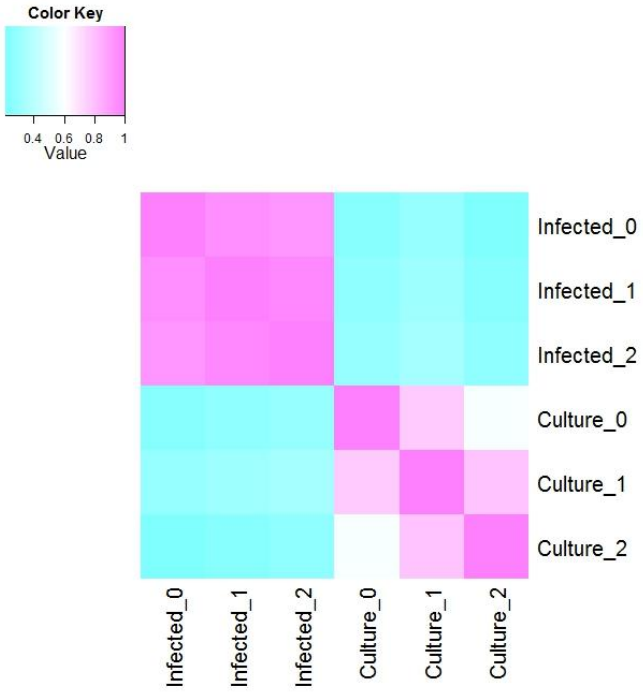

Figure 4.2. Heat map depicting Pearson's correlation coefficient values of normalized transcript abundances between replications of infected and culture libraries. Transcript abundances were obtained as normalized counts using Cufflinks v 2.0.2. Pearson correlation coefficients of normalized counts were calculated for all pairwise comparisons using R.

Differential expression analysis

Differential gene expression pattern between *in vitro* and *in planta* conditions was analyzed to identify specific genes involved in the infection process. This analysis led to the identification of a set of 613 *F. graminearum* genes that were differentially expressed *in planta* during the infection process compared to *in vitro* conditions (Fig 4.3). Among these, 237 genes were up-regulated and 376 genes were down-regulated *in planta*. During infection process 69 genes were found to be exclusively expressed *in planta*. The gene IDs and their functions are presented in Appendix Table A1.

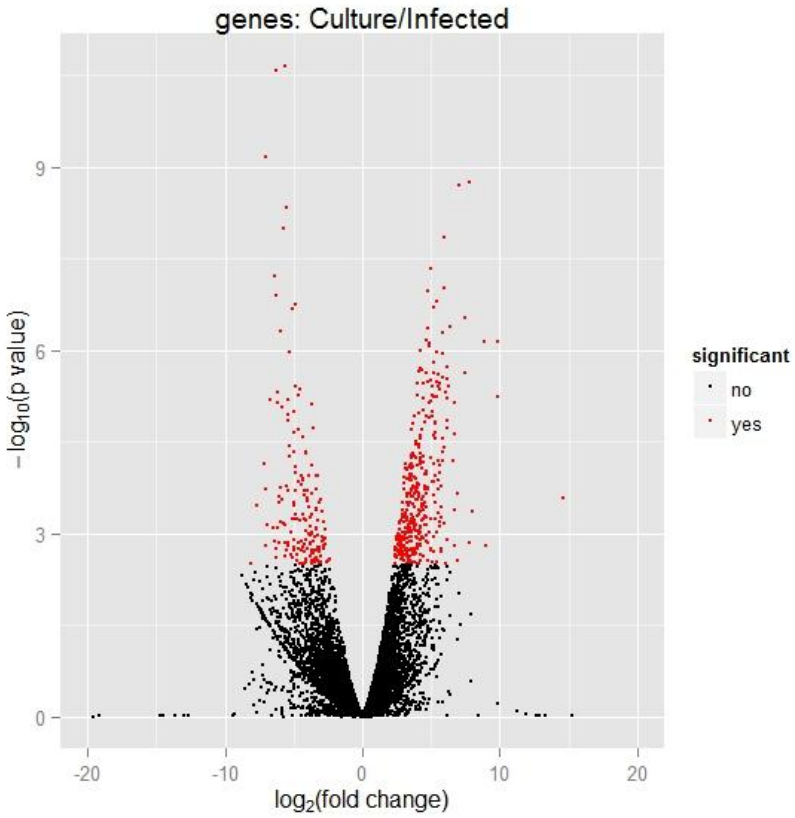

Figure 4.3. Volcano plot for differentially expressed *F. graminearum* genes. Log2 fold change of FPKM values between *in planta* and *in vitro* conditions is plotted on x-axis against log10 (*P* value) on y-axis. Red dots represent significantly differentially expressed genes at p=0.05.

Functional class distribution of differentially expressed genes

Differentially expressed genes were grouped into functional classes according to Munich Information Center for Protein Sequences (MIPS) Functional Catalog Database (FunCatDB). Functional category distribution using FunCatDB of the differentially expressed genes *in planta* compared to *in vitro* is presented in Fig 4.4. A large portion of both the up-regulated and down-regulated genes *in planta*, (40%, P = 0.98, and 38%, P = 0.99 respectively) were found to encode unclassified proteins with unknown functions. Significantly enriched functional categories in both up-regulated and down-regulated genes *in planta* are presented in Appendix Tables B1 and B2. There were similarities and differences in the major functional classes and

sub-classes between up-regulated and down-regulated genes. In both the up-regulated and down-regulated gene sets, the functional class associated with metabolism was significantly enriched. However, within this class , majority of the down-regulated genes *in planta* were involved in C-compound and carbohydrate metabolism (P = 0.0001), whereas in the up-regulated genes, in addition to C-compound and carbohydrate metabolism, genes involved in amino acid metabolism (P = 0.006), nitrogen, sulfur and selenium metabolism (P = 0.002), and amine metabolism (P = 0.047) were significantly enriched indicating nutrient starvation in general and nitrogen starvation in particular during infection process (Stephens et al., 2008). Gene FGSG_11164 was categorized into the functional class involved in protease mediated signal transduction (P = 0.016) which may play an important role in disease development. Another important functional sub-class consisting of genes responsible for extra cellular polysaccharide degradation (P = 4.09E-05) was exclusively enriched in up-regulated genes within the functional category metabolism. Genes belonging to the cell rescue, defense and virulence category were also significantly enriched in both the up-regulated (P = 0.029) and down-regulated (P = 6.21E-05) gene sets. In contrast to the down-regulated gene set, where genes involved in detoxification by export were enriched (P = 0.034), the up-regulated set was enriched with genes involved in detoxification by degradation (P = 0.041) and those coding for defense related proteins (P = 1.9E-04).

Comparative analysis of the up-regulated genes with characterized pathogenicity, virulence and effector genes

To gain further understanding of possible roles of differentially expressed genes in pathogenicity, a homology search of the up-regulated genes with the characterized pathogenicity, virulence, or effector genes was performed using stand-alone BLAST search against PHI-base.

Among the 237 up-regulated genes, 63 genes had homologs in the PHI-base. Ten of the 63 genes were characterized to have no effect on pathogenicity (Appendix Table C1). Fifty three genes had homologs to known pathogenicity, virulence or effector genes of either *Fusarium spp.* (Table 4.4) or other plant pathogenic fungi (Appendix Table C2). Four *F. graminearum* genes viz., FGSG_02251, FGSG_10990, FGSG_11164 and FGSG_13878 had homologs in other fungi with known effector functions. Two of these genes, FGSG_02251, FGSG_10990 and FGSG_13878 had similarity to *ACE1* gene (Böhnert et al., 2004) of the rice blast pathogen *Magnaporthe grisea.* FGSG_11164, a trypsin precursor has similarity to *GIP1* of oomycete pathogen *Phytophthora sojae* (Rose et al., 2002). As a result of the comparative analysis of differentially expressed genes against PHI-base, *F. graminearum* genes that are similar to genes conferring resistance to chemicals in other plant pathogenic fungi were also identified. These genes could potentially have a role in detoxification of host defenses.

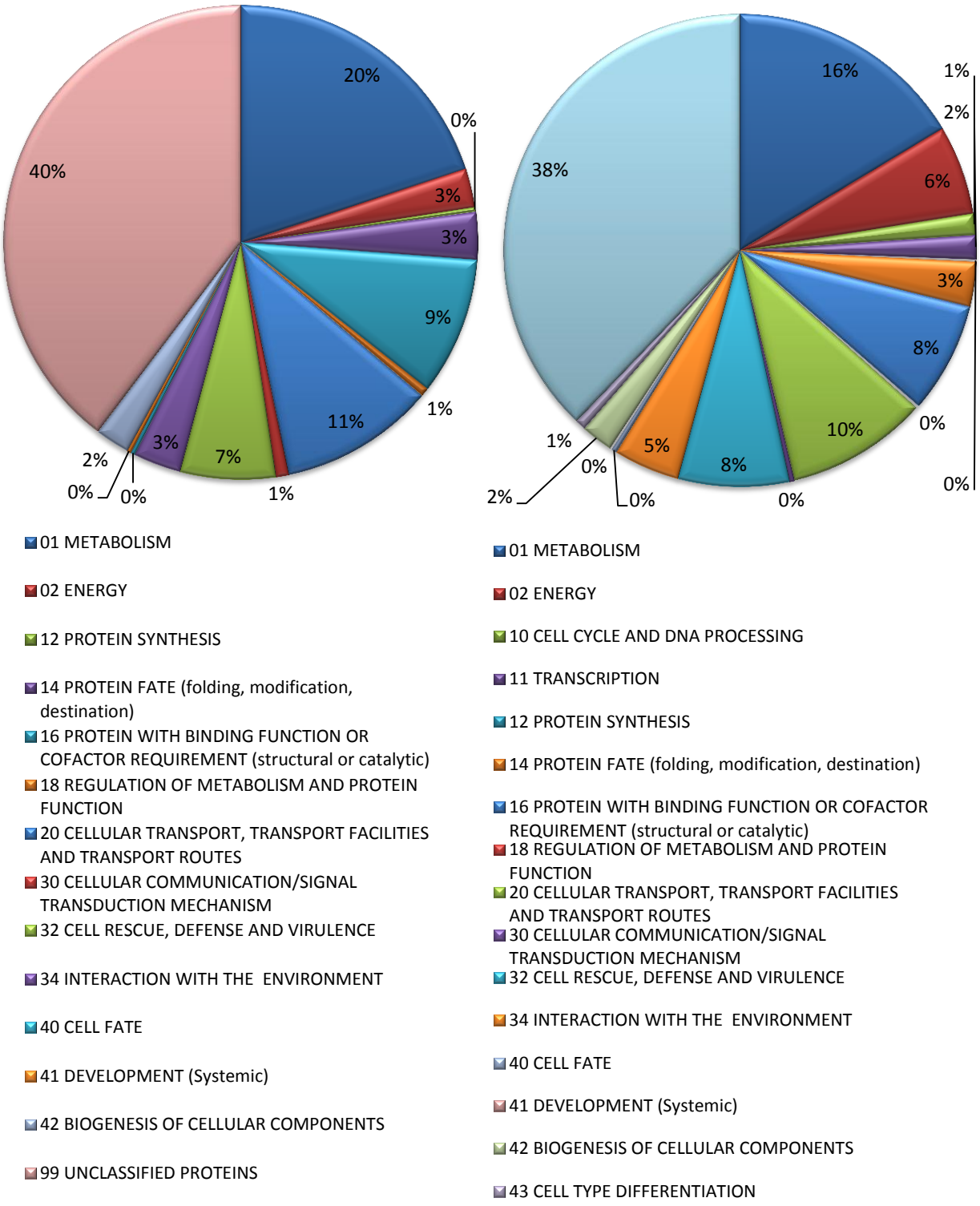

Figure 4.4. Functional category distribution of differentially expressed genes *in planta* compared with *in vitro* according to MIPS functional classification system FunCatDB. **A**. Significantly up-regulated *in planta*, and **B**. significantly down-regulated *in planta*.

Table 4.3. Upregulated genes with similarity to known pathogenicity and virulence genes of *Fusarium graminearum* and other plant pathogenic *Fusarium* species**.**

FGSG_ID	PHI-base Hits description
FGSG_01249	FGB1: *Fusarium oxysporum*, Reduced virulence; CGB1: *Cochliobolus heterostrophus*, Loss of pathogenicity; CPGB-1: *Cryphonectria parasitica*, Reduced virulence; MGB1: *Magnaporthe oryzae*, Loss of pathogenicity.
FGSG_04580	GPABC1 : *Gibberella pulicaris*, Reduced virulence; ABC1 : *Magnaporthe grisea*, Reduced virulence; BcatrB : *Botrytis cinerea*, Reduced virulence; MgAtr4 : *Mycosphaerella graminicola*, Reduced virulence
FGSG_04581	FOW2: *Fusarium oxysporum*, Loss of pathogenicity
FGSG_05906	FGL1: *Fusarium graminearum*, Reduced virulence
FGSG_06550	Umchs6: *Ustilago maydis*, Reduced virulence; CgCHSV: *Colletotrichum graminicola*, Reduced virulence; CHSV: *Fusarium oxysporum*, Increased virulence (Hypervirulence); umCHS5: *Ustilago maydis*, Reduced virulence
FGSG_07067	MGG_09263: *Magnaporthe oryzae*, Reduced virulence; FOW2: *Fusarium oxysporum*, Loss of pathogenicity; CLTA1:*Colletotrichum lindemuthianum*, Loss of pathogenicity
FGSG_08312	GPABC1 : *Gibberella pulicaris*, Reduced virulence; ABC3 : *Magnaporthe grisea*, Loss of pathogenicity; ABC1 : *Magnaporthe grisea*, Reduced virulence; BcatrB : *Botrytis cinerea*, Reduced virulence; MgAtr4 : *Mycosphaerella graminicola*, Reduced virulence
FGSG_08830	GPABC1 : *Gibberella pulicaris*, Reduced virulence; ABC1 : *Magnaporthe grisea*, Reduced virulence; BcatrB : *Botrytis cinerea*, Reduced virulence; MgAtr4 : *Mycosphaerella graminicola*, Reduced virulence
FGSG_13878	GzCPS1: *Fusarium graminearum*, Reduced virulence; HTS1: *Cochliobolus carbonum*, Loss of pathogenicity; AMT: *Alternaria alternata*, Loss of pathogenicity; NPS6: *Cochliobolus heterostrophus*, Reduced virulence; NPS6: *Cochliobolus miyabeanus*, Reduced virulence; NPS6: *Alternaria brassicicola*, Reduced virulence;

Validation of mRNA-Seq analysis by RT-PCR

For validation of RNA-Seq data analysis results, 5 genes including β-tubulin (FGSG_09530) as a control were selected. The selected genes for RT-PCR confirmation constituted of 4 genes that had hits in PHI-base, and were shown to have effect on virulence or pathogenicity in other *Fusarium* spp or plant pathogenic fungi. Intensity of amplification signal from the RT-PCR assay of selected upregulated genes was in concordance with log2fpkm values (Fig 4.5). As expected, primers designed to amplify β-tubulin gene produced bands in both *in vitro* and *in planta* libraries.

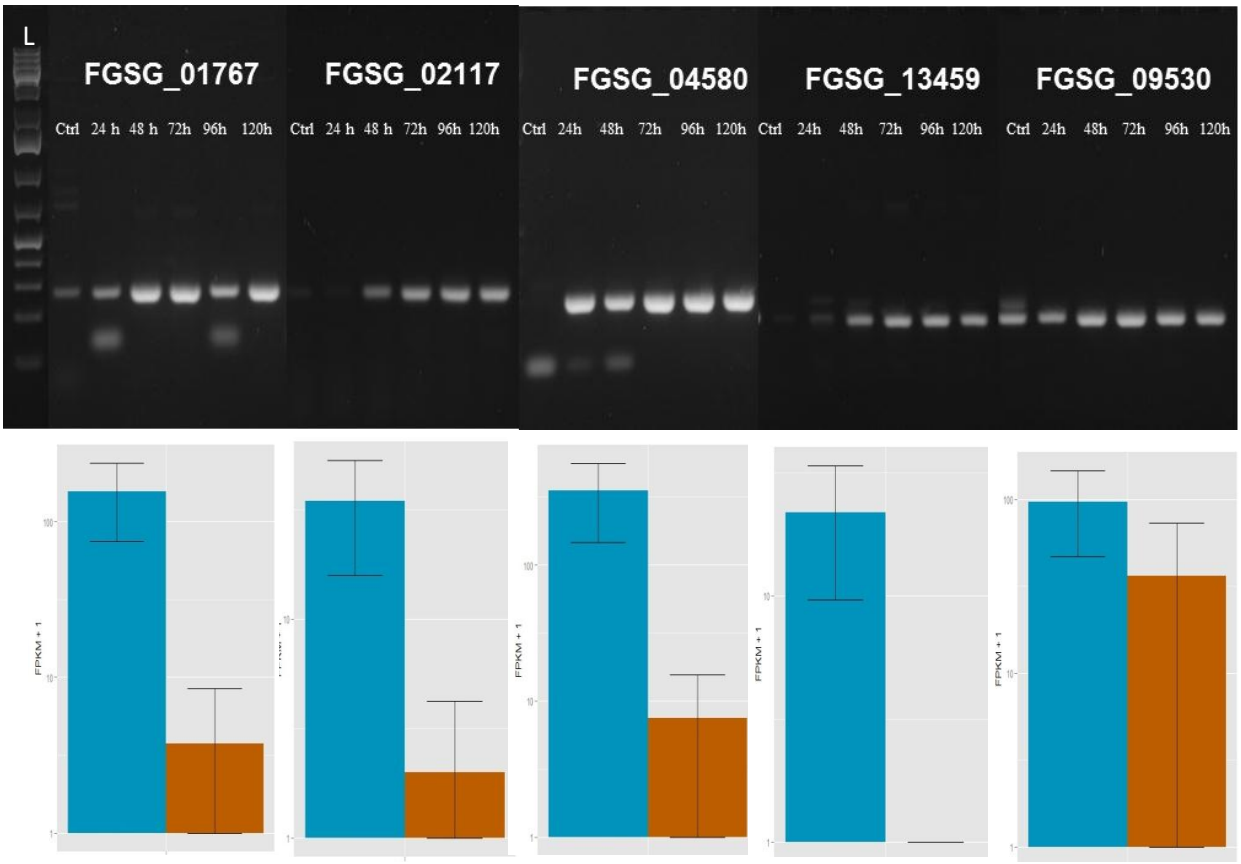

Figure 4.5. RT-PCR validation for expression analysis of selected up-regulated genes. A. Gel electrophoresis images of the selected genes. Lane L is ladder, for each gene ctrl represents *in vitro*, and 24, 48 72, 96 and 120h represents 24, 48 72, 96 and 120 hpi *in planta.* B. Expression bar plots for corresponding genes, log10fpkm values are (y-axis) are plotted against the libraries. Bars in cyan color represent expression *in planta* and golden bars represent expression *in vitro*.

Discussion

Next generation sequencing technologies have become very popular due to the low sequencing cost per base pair, massive amount of data generated per sequencing run and the resulting sequence depth. Illumina's mRNA-Seq is a powerful technology for full transcriptome sequencing and gene expression analysis. The goal of this study was to exploit the available genomic resources for *F.graminearum,* recently associated with root rots of field pea, and use the power of next generation sequencing to obtain a better understanding of genes involved in the interaction of this important group of pathogens with legumes that leads to disease development. This research was initiated to analyze the differential gene expression pattern of *F. graminearum* during infection of field pea roots using mRNA-Seq technology. Overall, three biological replications of *in planta* and *in vitro* cDNA libraries were sequenced generating 50,339,882 single reads. After applying quality checks, clipping adaptor sequences and trimming sequences with quality scores less than 20, 33,287,338 sequences that were at least 36 bp in length reads were obtained.

Approximately 57% of the quality filtered reads were mapped to the reference *F. graminearum* genome. While 75.32% of the *in vitro* library reads were mapped to the reference, only 39.06% of the *in planta* librarie reads mapped to it. This was expected because two of the *in planta* libraries were prepared from plant tissue infected with the pathogen. We found good correlation between biological replications of both *in vitro* and *in planta* libraries for the normalized mapped read counts (Fig 4.2). Data analysis led to the identification of613 *F. graminearum* genes differentially expressed during infection that were significant at 5% false discovery rate (FDR) following Benjamini-Hochberg procedure implemented in CuffDiff (Trapnell et al., 2010). Among these, 237 genes were found to be up-regulated during the

infection process compared to auxenic culture conditions and 376 genes were down regulated. FunCat is a tool for functional classification scheme of proteins, and this is an organism independent classification system (Ruepp et al., 2004). Functional distribution of the differentially expressed gene sets was determined using the FunCatDB scheme FG3 for *F. graminearum* where 40% and 38% of the genes from up regulated and down regulated gene sets respectively, coded for proteins that belonged to unclassified protein category. In previous gene expression studies, Stephens et al., (2008) and Lysøe et al., (2011), had also reported a high proportion of 59% and 72% of genes categorized as unclassified proteins from their studies on *F. graminearum* wheat interaction. This suggests that our understanding of the cellular processes occurring during infection process is fairly limited to date. Significantly enriched functional classes of the differentially expressed genes *in planta* give an indication of the important molecular processes active during pathogenesis. In the up-regulated gene set, within the major functional class metabolism (01, P = 1.07E-06), genes involved in amino acid metabolism (01.01, P = 0.006), nitrogen, sulfur and selenium metabolism (01.02, P = 0.002), secondary metabolism (01.20, P =0.001) were significantly enriched, suggesting active involvement of these processes during the general nutrient limited conditions that exist during infection process (Stephens et al., 2008). Enrichment of genes associated with extracellular polysaccharide degradation (01.25.01, P = 4.09E-05) exclusively *in planta* indicates the active involvement of cell wall degrading enzymes during the infection process. Twenty two (7%) of the up regulated genes were placed in cell rescue, defense and virulence category (32, P = 0.029). Within this category, proteins with detoxification function were enriched (32.07, P = 0.0008), followed by defense related proteins (32.05.03, P = 1.9E-04). It has been shown earlier that detoxification of host phytoalexins is necessary for successful infection (Fleissner et al., 2002 and Coleman et al.,

2011). These findings are also in agreement with the two previously reported studies mentioned above.

We performed a homology search using BLASTX with the PHI database which includes majority of characterized genes involved in host pathogen interaction, using the set of 236 up-regulated genes *in planta*. Sixty three of the genes had homologs among the genes in PHI-base and 10 of these had sequence homology with entries in PHI-base that were previously characterized as not affecting pathogenicity or virulence in other pathogens. The remaining 53 genes had similarity to known pathogenicity and virulence genes of either *Fusarium* spp. or other plant pathogens. It is interesting that 26 of the genes that had homology with PHI-base genes were categorized into unclassified proteins in the FunCat classification. From the comparative analysis to PHI-base, we identified 4 genes that have homologs in other plant pathogenic fungi with effector roles. Three of these genes, FGSG_02251, FGSG_10990 and FGSG_13878 had similarity to *ACE1* gene of the rice blast pathogen *M. grisea*. *ACE1* is a polyketide synthase (PKS) fused to nonribosomal peptide synthetase (NRPS). FGSG_02251 is a putative NRPS with an AMP binding site and thioester reductase domain. FGSG_10990 is related to AM toxin synthetase (AMT) with an amino acid adenylation (AA) domain. FGSG_13878 is 33 kb long with condensation domain, AMP-binding site, phosphopantethiene attachment site, NRPS domain, AA domain, and thioester reductase domain. FGSG_11164, a trypsin precursor has similarity to *GIP1* of oomycete pathogen *Phytophthora sojae*. *GIP1* is homologous to the trypsin class of serine proteases and has been shown to interact with soybean endoglucanases during pathogenesis in soybean roots while causing root rot. According to FunCatDB, this gene has been assigned to the functional class with protease mediated signaling. From the PHI-base homology search we have identified several genes with homology to genes that confer resistance

to chemicals in other pathogen-host systems. These are the genes that are probably involved with detoxification of host phytoalexins. The gene FGSG_01767 is annotated as related to pisatin demethylase. In studies conducted on other *Fusarium* spp infecting pea, pisatin demethylase (PDA) of *Nectria haematococca* (teleomorph of *F. solani*), was shown to be a host-specific virulence factor on pea (Wasmann and VanEtten 1996). This gene has sequence homology to the BcBOT1 (related: CND5) gene of *Botrytis cinerea,* deletion of which is shown to result in reduced virulence on bean and tomato. This gene appears to be expressed at very low levels in the control with expression level going up with progress of time after inoculation (Siewers et al., 2005). FGSG_02117 is related to cytochrome P450 monooxygenase (lovA), it had sequence homology to BcBOT1 (related: CND5) of *Botrytis cinerea,* and rg11/cyp51 of *Mycosphaerella graminicola* which give resistance to chemical (sterol 14α-demethylation inhibitors) (Leroux et al., 2007). This gene was not found to be expressed in the control and its expression also appeared to increase with time after infection. FGSG_04580 is a probable ABC1 transporter with very high sequence similarity with *GpABC1* of *Gibberella pulicaris,* and *NhABC1* of *Nectria haematococca.* Both *GpABC1* (Fleissner et al., 2002) and *NhABC1* (Coleman et al., 2011) have been shown to be necessary for phytoalexin tolerance and virulence on their respective hosts potato and field pea while causing tuber rot and root rot respectively. Coleman et al., (2011) reported that the ABC transporter (*NhABC1*) and a cytochrome P450 (pisatin demethylase) gene are virulence factors. Cytochrome P450 acts in detoxification of the phytoalexin 'pisatin' produced by pea, and the ABC transporter *NhABC1* confers tolerance to pisatin. The expression of this gene was not observed in the control but appeared to be strong at all the time points after infection.FGSG_04919 is a probable Na+ transporting ATPase ENA-1, which has match in PHIbase blast search with CLAP1 of *Colletotrichum lindemuthianum,* a gene encoding a putative

copper transporting ATPase, insertional mutants of which resulted in loss of pathogenicity that would be associated with melanin production (Parisot et al., 2001). This gene appeared to be turned on during initial infection with expression levels increasing with time. FGSG_08066 is related to ADH3- alcohol dehydrogenase III. According to BLAST, this gene matched the MGG_04556 gene from the rice blast fungus *Magnaporthe grisea,* disruption of which resulted in reduced virulence on its host rice (Jeon et al., 2007). We have validated our mRNA-Seq differential analysis results by performing RT-PCR on selected up-regulated genes. We observed a strong concordance between the amplification signal from RT-PCR and expression bar plots of FPKM values from Cuffdiff analysis for all the 4 up-regulated genes and the control β-tubulin gene.

The work presented in this chapter provides a preliminary analysis of gene expression patterns associated with infection of a leguminous crop such as field pea by *F. graminearum.* We have identified genes associated with protease mediated signaling, effector functions, extracellular polysaccharide degradation and detoxification to be up-regulated during pathogenesis. Knock out mutants of several selected genes are being created to characterize their role in pathogenicity and further elucidate the pathways associated with disease development in this pathosystem

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APPENDIX A. *F. GRAMINEARUM* GENES EXCLUSIVELY EXPRESSED *IN PLANTA*

Table A1. List of *F. graminearum* genes exclusively expressed *in planta* and their putative functions.

Gene ID	Putative Function
FGSG_00105	bnr repeat-containing glycosyl hydrolase
FGSG_01549	hypothetical protein
FGSG_01593	glucose galactose transporter
FGSG_01631	integral membrane protein
FGSG_01763	hypothetical protein
FGSG_01861	g-protein coupled receptor
FGSG_01994	hypothetical protein
FGSG_02110	proteinase inhibitor kazal
FGSG_02131	geranylgeranyl pyrophosphate synthetase
FGSG_02185	rhomboid family protein
FGSG_02216	lipoxygenase 1
FGSG_02251	nrps-like enzyme
FGSG_02387	flavoprotein involved in k+ transport
FGSG_02819	lea domain protein
FGSG_02875	tpa: beta-lactamase family protein (afu_orthologue afua_5g07500)
FGSG_02944	acetylcholinesterase precursor
FGSG_03052	hypothetical protein
FGSG_03099	hypothetical protein
FGSG_03128	hypothetical protein
FGSG_03365	tpa: endonuclease exonuclease phosphatase family protein (afu_orthologue afua_1g01540)
FGSG_03486	lipase 1 precursor
FGSG_03598	glycoside hydrolase family 93
FGSG_03614	gpi anchored protein
FGSG_03655	nad-binding rossmann fold oxidoreductase family protein
FGSG_03865	nhl repeat-containing protein
FGSG_03956	mfs transporter
FGSG_04302	fad binding domain-containing protein
FGSG_04518	af149296_1hard-surface inducible protein
FGSG_04581	fungal specific transcription factor domain-containing protein
FGSG_04590	benzoate 4-monooxygenase cytochrome p450
FGSG_04615	hypothetical protein
FGSG_04662	tat pathway signal sequence
FGSG_04695	hypothetical protein
FGSG_04871	hypothetical protein
FGSG_05868	hypothetical protein

Table A1. List of *F. graminearum* genes exclusively expressed *in planta* and their putative functions. (Continued)

Gene ID	Putative Function
FGSG_06468	major facilitator superfamily transporter
FGSG_06888	endo-1,3(4) -beta-glucanase
FGSG_07668	carboxypeptidase 2
FGSG_07735	nacht domain protein
FGSG_07794	pectate lyase
FGSG_07861	cap22 protein
FGSG_07997	hypothetical protein
FGSG_08101	hypothetical protein
FGSG_08147	kinesin light chain
FGSG_08178	acetoacetate decarboxylase
FGSG_09054	hypothetical protein
FGSG_09056	hypothetical protein
FGSG_09088	short chain dehydrogenase reductase family
FGSG_10183	cellobiose dehydrogenase
FGSG_10465	duf218 domain protein
FGSG_10595	alkaline protease
FGSG_10999	endo-1,4-beta-xylanase
FGSG_11270	flavin-binding monooxygenase
FGSG_11306	aggrecan core protein isoform 1 precursor
FGSG_11366	glycosyl hydrolase family 43 protein
FGSG_11377	hypothetical protein
FGSG_11383	transcription factor cys6
FGSG_11396	asparagine synthase
FGSG_11554	phenylacetone monooxygenase
FGSG_11981	major facilitator superfamily transporter
FGSG_12256	glucooligosaccharide oxidase
FGSG_12342	cytochrome p450 3a5
FGSG_12402	nadph-dependent fmn reductase
FGSG_12830	hypothetical protein
FGSG_13212	penicillin-binding protein
FGSG_13441	hypothetical protein
FGSG_13459	cytochrome p450
FGSG_13788	g-protein beta wd-40 repeats containing
FGSG_13939	hypothetical protein
FGSG_14001	endonuclease exonuclease phosphatase family protein

APPENDIX B. FUNCTIONAL CLASSES OF SIGNIFICANTLY ENRICHED *F. GRAMINEARUM* GENES

Table B1. Functional classes of significantly enriched *F. graminearum* up-regulated genes *in planta.*

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
01 METABOLISM	FGSG_05882 FGSG_02117 FGSG_04590 FGSG_01283 FGSG_04953 FGSG_00490 FGSG_09940 FGSG_12669 FGSG_05140 FGSG_16338 FGSG_17117 FGSG_02216 FGSG_04214 FGSG_04302 FGSG_08830 FGSG_10960 FGSG_11032 FGSG_05906 FGSG_01675 FGSG_04580 FGSG_11228 FGSG_11249 FGSG_04610 FGSG_10993 FGSG_01767 FGSG_07794 FGSG_02202 FGSG_16843 FGSG_02880 FGSG_13169 FGSG_10995 FGSG_01285 FGSG_00571 FGSG_01765 FGSG_06397 FGSG_02504 FGSG_02668 FGSG_05554 FGSG_03307 FGSG_06068 FGSG_05663 FGSG_09684 FGSG_09121 FGSG_16432 FGSG_07765 FGSG_16111 FGSG_12519 FGSG_09364 FGSG_10999 FGSG_00979 FGSG_08312 FGSG_02944 FGSG_11396 FGSG_03212 FGSG_12402 FGSG_13630 FGSG_05374 FGSG_04848 FGSG_17337 FGSG_01826 FGSG_02160 FGSG_05847 FGSG_04155 FGSG_09483 FGSG_10677 FGSG_11270 FGSG_11147 FGSG_09088	1.07E-06
01.01 amino acid metabolism	FGSG_04214 FGSG_00490 FGSG_12519 FGSG_01767 FGSG_07765 FGSG_05554 FGSG_11228 FGSG_16338 FGSG_01826 FGSG_09483 FGSG_11396 FGSG_02160 FGSG_00979 FGSG_17117 FGSG_01285 FGSG_05374	0.006094
01.01.03.03 metabolism of proline	FGSG_02160 FGSG_00979 FGSG_16338	0.010017
01.01.03.03.01 biosynthesis of proline	FGSG_02160 FGSG_00979	0.039435
01.01.09 metabolism of the cysteine - aromatic group	FGSG_00979 FGSG_01285 FGSG_17117 FGSG_02160 FGSG_11228 FGSG_05374 FGSG_07765 FGSG_09483 FGSG_01767	0.008079
01.01.09.04 metabolism of phenylalanine	FGSG_01285 FGSG_07765 FGSG_02160 FGSG_00979 FGSG_17117 FGSG_01767	0.0028
01.01.09.04.01 biosynthesis of phenylalanine	FGSG_00979 FGSG_17117 FGSG_07765 FGSG_01767 FGSG_02160	0.002998
01.01.09.04.02 degradation of phenylalanine	FGSG_07765 FGSG_01767 FGSG_17117	0.043593
01.01.11 metabolism of the pyruvate family (alanine, isoleucine, leucine, valine) and D-alanine	FGSG_00490 FGSG_02160 FGSG_01826 FGSG_00979	0.035225
01.02 nitrogen, sulfur and selenium metabolism	FGSG_02880 FGSG_02160 FGSG_09684 FGSG_13630 FGSG_10993 FGSG_00979 FGSG_05554 FGSG_07765 FGSG_06068 FGSG_17117 FGSG_10677	0.002155

Table B1. Functional classes of significantly enriched *F. graminearum* up-regulated genes *in planta.* (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
01.05 C-compound and carbohydrate metabolism	FGSG_16843 FGSG_04610 FGSG_11032 FGSG_09483 FGSG_05374 FGSG_04214 FGSG_10677 FGSG_08312 FGSG_09364 FGSG_11147 FGSG_00490 FGSG_09088 FGSG_10999 FGSG_01283 FGSG_17117 FGSG_04848 FGSG_04953 FGSG_04580 FGSG_02504 FGSG_03212 FGSG_05847 FGSG_07794 FGSG_04302 FGSG_08830 FGSG_01765 FGSG_00979 FGSG_03307 FGSG_02202 FGSG_11228 FGSG_05882 FGSG_00571 FGSG_05554 FGSG_01826 FGSG_09940 FGSG_01767 FGSG_05663 FGSG_02160 FGSG_13169 FGSG_12669 FGSG_06397	1.46E-06
01.05.03 polysaccharide metabolism	FGSG_13169 FGSG_05663 FGSG_04953 FGSG_01283 FGSG_07794 FGSG_02202 FGSG_00571 FGSG_05847 FGSG_03212 FGSG_06397	0.002128
01.06 lipid, fatty acid and isoprenoid metabolism	FGSG_17337 FGSG_01675 FGSG_05906 FGSG_01283 FGSG_02216 FGSG_09121 FGSG_02504 FGSG_16432 FGSG_02668 FGSG_02944 FGSG_02160 FGSG_09364 FGSG_07765 FGSG_00979 FGSG_11270 FGSG_10960 FGSG_02117 FGSG_01826	0.010212
01.20 secondary metabolism	FGSG_02117 FGSG_07765 FGSG_11228 FGSG_02160 FGSG_04302 FGSG_04590 FGSG_00979 FGSG_09364 FGSG_16111 FGSG_01285 FGSG_17117 FGSG_06068 FGSG_11270 FGSG_05554 FGSG_05374 FGSG_09121 FGSG_09940	0.001203
01.20.17 metabolism of secondary products derived from primary amino acids	FGSG_05374 FGSG_05554 FGSG_00979 FGSG_02160 FGSG_11228 FGSG_01285 FGSG_16111	0.000542
01.20.17.03 metabolism of amines	FGSG_11228 FGSG_05374	0.047391
01.20.29 metabolism of secondary products derived from L-glutamic acid, L-proline and L-ornithine	FGSG_02160 FGSG_00979	0.043344
01.25 extracellular metabolism	FGSG_04848 FGSG_05847 FGSG_11249 FGSG_02202 FGSG_06397 FGSG_05663	0.000243
01.25.01 extracellular polysaccharide degradation	FGSG_04848 FGSG_05663 FGSG_06397 FGSG_05847 FGSG_02202	4.09E-05
02.16.01 alcohol fermentation	FGSG_04214 FGSG_00979 FGSG_09364 FGSG_02160	0.015027
14.13.04 lysosomal and vacuolar protein degradation	FGSG_10595 FGSG_00806 FGSG_03315	0.020336
16.05 polysaccharide binding	FGSG_05663 FGSG_05847 FGSG_11164	0.003121
16.17.05 sodium binding	FGSG_11164	0.049294
16.21 complex cofactor/cosubstrate/vitamine binding	FGSG_11228 FGSG_02160 FGSG_09940 FGSG_00979 FGSG_02117 FGSG_00490 FGSG_01767 FGSG_05554 FGSG_02504 FGSG_05374 FGSG_16432 FGSG_13630 FGSG_09483 FGSG_10993 FGSG_05140 FGSG_12402 FGSG_01826	0.000118
16.21.05 FAD/FMN binding	FGSG_13630 FGSG_11228 FGSG_05374 FGSG_05140 FGSG_16432	0.014533

Table B1. Functional classes of significantly enriched *F. graminearum* up-regulated genes *in planta.* (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
16.21.07 NAD/NADP binding	FGSG_02504 FGSG_01826 FGSG_00490 FGSG_09483 FGSG_05374 FGSG_02160 FGSG_00979 FGSG_11228 FGSG_12402 FGSG_09940	0.001704
18.02.10 regulation of channel activity	FGSG_11164	0.049294
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	FGSG_04431 FGSG_16338 FGSG_15865 FGSG_11228 FGSG_03956 FGSG_08312 FGSG_06468 FGSG_08830 FGSG_04610 FGSG_16432 FGSG_07765 FGSG_00490 FGSG_05882 FGSG_04637 FGSG_01675 FGSG_01593 FGSG_02820 FGSG_07631 FGSG_01767 FGSG_09684 FGSG_04580 FGSG_01826 FGSG_02504 FGSG_02160 FGSG_00136 FGSG_02839 FGSG_03172 FGSG_06068 FGSG_05374 FGSG_11147 FGSG_00979 FGSG_10995 FGSG_11164 FGSG_05140 FGSG_17117 FGSG_17177	0.005181
20.01 transported compounds (substrates)	FGSG_02504 FGSG_07765 FGSG_04431 FGSG_03172 FGSG_17177 FGSG_08312 FGSG_00490 FGSG_05882 FGSG_11164 FGSG_05140 FGSG_04637 FGSG_16338 FGSG_06468 FGSG_02839 FGSG_10995 FGSG_00979 FGSG_02160 FGSG_01826 FGSG_17117 FGSG_16432 FGSG_03956 FGSG_01767 FGSG_15865 FGSG_05374 FGSG_04580 FGSG_09684 FGSG_04610 FGSG_00136 FGSG_11147 FGSG_11228 FGSG_01675 FGSG_02820 FGSG_01593 FGSG_07631 FGSG_08830 FGSG_06068	4.80E-05
20.01.01 ion transport	FGSG_11164 FGSG_01675 FGSG_04580 FGSG_08830 FGSG_03172 FGSG_08312 FGSG_04637 FGSG_02820	0.038715
20.01.01.01 cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.)	FGSG_08830 FGSG_04637 FGSG_01675 FGSG_11164 FGSG_08312 FGSG_02820 FGSG_04580	0.032461
20.01.03 C-compound and carbohydrate transport	FGSG_05882 FGSG_17177 FGSG_08312 FGSG_08830 FGSG_04431 FGSG_07631 FGSG_10995 FGSG_01593 FGSG_06468 FGSG_11147 FGSG_15865 FGSG_04637 FGSG_04610 FGSG_04580 FGSG_01675	8.71E-05
20.01.15 electron transport	FGSG_00490 FGSG_17117 FGSG_07765 FGSG_05374 FGSG_11164 FGSG_16432 FGSG_16338 FGSG_01826 FGSG_00979 FGSG_02504 FGSG_05140 FGSG_02160 FGSG_09684 FGSG_06068 FGSG_11228 FGSG_01767	2.99E-05
20.01.23 allantoin and allantoate transport	FGSG_03956 FGSG_15865 FGSG_06468 FGSG_04431	0.028205
20.01.25 vitamine/cofactor transport	FGSG_04431 FGSG_10995 FGSG_15865 FGSG_01675 FGSG_06468	0.008339
20.03 transport facilities	FGSG_07631 FGSG_03172 FGSG_04637 FGSG_02820 FGSG_06468 FGSG_16432 FGSG_15865 FGSG_01675 FGSG_05882 FGSG_08830 FGSG_11147 FGSG_04610 FGSG_04580 FGSG_02839 FGSG_08312 FGSG_04431 FGSG_10995	0.008511
20.03.02.02 symporter	FGSG_03172 FGSG_04610 FGSG_11147	0.045945
20.03.25 ABC transporters	FGSG_08830 FGSG_10995 FGSG_08312 FGSG_04580	0.04487

Table B1. Functional classes of significantly enriched *F. graminearum* up-regulated genes *in planta.* (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
20.09.16.01 Type I protein secretion system (ABC-type transport systems)	FGSG_08830 FGSG_04580 FGSG_08312	0.021917
20.09.18.07 non-vesicular cellular import	FGSG_06468 FGSG_15865 FGSG_04431 FGSG_04637 FGSG_01675 FGSG_17177 FGSG_04610 FGSG_03956 FGSG_11147 FGSG_02839	0.006581
30.01.05.03 protease mediated signal transduction	FGSG_11164	0.016708
32 CELL RESCUE, DEFENSE AND VIRULENCE	FGSG_04848 FGSG_01675 FGSG_17117 FGSG_09054 FGSG_02117 FGSG_11270 FGSG_10999 FGSG_06014 FGSG_02160 FGSG_09121 FGSG_11554 FGSG_07765 FGSG_09364 FGSG_01767 FGSG_08312 FGSG_08830 FGSG_04580 FGSG_00979 FGSG_10995 FGSG_03212 FGSG_02672 FGSG_01765	0.029323
32.01.01 oxidative stress response	FGSG_04580 FGSG_00979 FGSG_08312 FGSG_02160 FGSG_08830	0.025122
32.05 disease, virulence and defense	FGSG_00979 FGSG_04848 FGSG_08312 FGSG_09054 FGSG_01767 FGSG_02117 FGSG_17117 FGSG_04580 FGSG_02672 FGSG_02160 FGSG_08830 FGSG_03212	0.005847
32.05.01 resistance proteins	FGSG_00979 FGSG_08830 FGSG_08312 FGSG_09054 FGSG_04580 FGSG_02160	0.030126
32.05.01.03 chemical agent resistance	FGSG_08312 FGSG_04580 FGSG_08830	0.034812
32.05.03 defense related proteins	FGSG_02160 FGSG_17117 FGSG_02117 FGSG_00979 FGSG_02672 FGSG_03212 FGSG_01767	0.000194
32.07 detoxification	FGSG_00979 FGSG_17117 FGSG_11270 FGSG_10995 FGSG_09364 FGSG_01767 FGSG_08830 FGSG_01765 FGSG_09121 FGSG_08312 FGSG_11554 FGSG_02160 FGSG_07765 FGSG_04580 FGSG_09054	0.000862
32.07.09 detoxification by degradation	FGSG_01765 FGSG_02160 FGSG_00979	0.041303
34.01.01.01 homeostasis of metal ions (Na, K, Ca etc.)	FGSG_08312 FGSG_01283 FGSG_08830 FGSG_04637 FGSG_01675 FGSG_04580 FGSG_02820	0.03003
41.01.03 tissue pattern formation	FGSG_07067	0.049294
41.01.03.01 fruit body development (sexually or asexually derived spores)	FGSG_07067	0.016708

Table B2. Functional classes of significantly enriched *F. graminearum* down-regulated genes *in planta*.

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
01 METABOLISM	FGSG_01973 FGSG_03513 FGSG_16069 FGSG_06370 FGSG_01349 FGSG_06051 FGSG_03120 FGSG_16657 FGSG_06528 FGSG_11386 FGSG_00578 FGSG_02034 FGSG_09677 FGSG_05292 FGSG_07539 FGSG_12704 FGSG_17214 FGSG_03061 FGSG_16595 FGSG_01445 FGSG_09456 FGSG_04458 FGSG_01015 FGSG_02658 FGSG_05921 FGSG_03941 FGSG_01157 FGSG_05683 FGSG_15955 FGSG_09805 FGSG_12331 FGSG_02432 FGSG_08774 FGSG_03127 FGSG_01621 FGSG_04223 FGSG_15910 FGSG_05330 FGSG_08980 FGSG_09101 FGSG_10433 FGSG_16527 FGSG_08979 FGSG_10739 FGSG_12745 FGSG_01069 FGSG_03882 FGSG_03066 FGSG_16400 FGSG_09155 FGSG_16340 FGSG_09639 FGSG_02291 FGSG_02273 FGSG_13755 FGSG_09402 FGSG_10444 FGSG_03984 FGSG_00150 FGSG_07678 FGSG_06081 FGSG_09374 FGSG_06553 FGSG_03775 FGSG_05467 FGSG_00323 FGSG_03014 FGSG_02019 FGSG_09279 FGSG_00278 FGSG_11081 FGSG_01686 FGSG_02145 FGSG_04270 FGSG_13120 FGSG_03498 FGSG_05597 FGSG_09947 FGSG_05514 FGSG_10005 FGSG_03186 FGSG_07908 FGSG_01743 FGSG_08402 FGSG_06612 FGSG_08377 FGSG_06598 FGSG_03710 FGSG_03779 FGSG_02978 FGSG_07783	2.84E-05
01.03.16.03 DNA degradation	FGSG_09374 FGSG_06081	0.017243
01.04 phosphate metabolism	FGSG_09805 FGSG_01157 FGSG_12704 FGSG_09155 FGSG_07783 FGSG_01445 FGSG_03779 FGSG_01015 FGSG_12745 FGSG_01069 FGSG_03127 FGSG_08774 FGSG_04270 FGSG_09456 FGSG_03882 FGSG_09402 FGSG_03014	0.013086
01.05 C-compound and carbohydrate metabolism	FGSG_06553 FGSG_05330 FGSG_13755 FGSG_09456 FGSG_02432 FGSG_16595 FGSG_09947 FGSG_02291 FGSG_03127 FGSG_16657 FGSG_06598 FGSG_08980 FGSG_04223 FGSG_06081 FGSG_07908 FGSG_05467 FGSG_06051 FGSG_02978 FGSG_03014 FGSG_16527 FGSG_06612 FGSG_10433 FGSG_02034 FGSG_08774 FGSG_03775 FGSG_02273 FGSG_12331 FGSG_01349 FGSG_07783 FGSG_01686 FGSG_01445 FGSG_03513 FGSG_03941 FGSG_03984 FGSG_02658 FGSG_03061 FGSG_00150 FGSG_11081 FGSG_03779 FGSG_10444 FGSG_16400 FGSG_03882 FGSG_00578 FGSG_09101 FGSG_01743 FGSG_05292 FGSG_01621 FGSG_00323 FGSG_09374	0.00011
01.05.02 sugar, glucoside, polyol and carboxylate metabolism	FGSG_06612 FGSG_04223 FGSG_03061 FGSG_03014 FGSG_07908 FGSG_10444 FGSG_05467 FGSG_02291 FGSG_00578 FGSG_01445 FGSG_06051 FGSG_03127 FGSG_09456 FGSG_05330	0.004888
01.05.02.04 sugar, glucoside, polyol and carboxylate anabolism	FGSG_03127 FGSG_02291 FGSG_03061 FGSG_01445 FGSG_03014 FGSG_05330 FGSG_09456 FGSG_06051	0.005291

Table B2. Functional classes of significantly enriched *F. graminearum* down-regulated genes *in planta*. (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
01.05.02.07 sugar, glucoside, polyol and carboxylate catabolism	FGSG_09456 FGSG_05330 FGSG_00578 FGSG_03061 FGSG_03014 FGSG_06051 FGSG_01445 FGSG_02291 FGSG_04223 FGSG_06612 FGSG_07908 FGSG_03127 FGSG_10444	0.002701
01.05.06 C-2 compound and organic acid metabolism	FGSG_02034 FGSG_01743 FGSG_02291 FGSG_03984 FGSG_02273 FGSG_06612	0.004706
01.05.06.07 C-2 compound and organic acid catabolism	FGSG_02034 FGSG_02273 FGSG_02291 FGSG_01743 FGSG_06612	0.010485
01.05.25 regulation of C-compound and carbohydrate metabolism	FGSG_03014 FGSG_02978 FGSG_01445 FGSG_06081 FGSG_06051 FGSG_08774 FGSG_09456 FGSG_09374	0.016259
01.06 lipid, fatty acid and isoprenoid metabolism	FGSG_16340 FGSG_02273 FGSG_03120 FGSG_05921 FGSG_09402 FGSG_03066 FGSG_10005 FGSG_00578 FGSG_01349 FGSG_05597 FGSG_07539 FGSG_07908 FGSG_02019 FGSG_16069 FGSG_03498 FGSG_09639 FGSG_03984 FGSG_03513 FGSG_01973 FGSG_16400 FGSG_00150 FGSG_15955 FGSG_05514 FGSG_04223 FGSG_08377 FGSG_01743 FGSG_02034 FGSG_02291 FGSG_06370	0.000861
01.06.06.13 tetraterpenes (carotinoids) metabolism	FGSG_03066 FGSG_16340	0.002015
01.06.10 regulation of lipid, fatty acid and isoprenoid metabolism	FGSG_03120 FGSG_02019 FGSG_07539	0.032715
01.07 metabolism of vitamins, cofactors, and prosthetic groups	FGSG_10739 FGSG_16340 FGSG_16069 FGSG_00578 FGSG_17214 FGSG_04223 FGSG_07783 FGSG_07678 FGSG_01743 FGSG_02273 FGSG_00278 FGSG_03710 FGSG_07908 FGSG_09639	0.01919
01.20 secondary metabolism	FGSG_08980 FGSG_04223 FGSG_00323 FGSG_13755 FGSG_02273 FGSG_16400 FGSG_02291 FGSG_09279 FGSG_00578 FGSG_07908 FGSG_11386 FGSG_10739 FGSG_08979 FGSG_03779 FGSG_17214 FGSG_12331 FGSG_03775 FGSG_01686	0.042268
01.20.17.09 metabolism of alkaloids	FGSG_07908 FGSG_13755 FGSG_04223 FGSG_16400	0.003243
01.20.19.05 metabolism of cobalamins	FGSG_17214	0.026183
02 ENERGY	FGSG_00556 FGSG_04458 FGSG_07908 FGSG_01342 FGSG_07539 FGSG_10444 FGSG_03779 FGSG_02273 FGSG_06598 FGSG_08343 FGSG_01445 FGSG_06528 FGSG_08980 FGSG_01743 FGSG_04223 FGSG_08774 FGSG_03355 FGSG_06051 FGSG_03127 FGSG_02291 FGSG_03061 FGSG_06553 FGSG_03984 FGSG_02477 FGSG_06612 FGSG_03014 FGSG_16527 FGSG_01686 FGSG_00578 FGSG_16595 FGSG_12331 FGSG_02034 FGSG_02432 FGSG_09456	4.07E-07

Table B2. Functional classes of significantly enriched *F. graminearum* down-regulated genes *in planta*. (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
02.01 glycolysis and gluconeogenesis	FGSG_09456 FGSG_01445 FGSG_01743 FGSG_02291 FGSG_10444 FGSG_16595 FGSG_08980 FGSG_04223 FGSG_03014 FGSG_01686 FGSG_02034 FGSG_03127 FGSG_08774	7.56E-07
02.01.03 regulation of glycolysis and gluconeogenesis	FGSG_08774 FGSG_01445 FGSG_09456 FGSG_03127 FGSG_03014	1.97E-05
02.16 fermentation	FGSG_02291 FGSG_03061 FGSG_02034 FGSG_04223 FGSG_01743 FGSG_06598 FGSG_02432 FGSG_07908 FGSG_02273 FGSG_08980 FGSG_10444 FGSG_01686 FGSG_06553	1.06E-05
02.16.01 alcohol fermentation	FGSG_02432 FGSG_06598 FGSG_02034 FGSG_08980 FGSG_01686 FGSG_02291 FGSG_02273	0.000682
02.16.03 lactate fermentation	FGSG_06553 FGSG_10444	0.032163
16.17 metal binding	FGSG_03061 FGSG_08980 FGSG_02034 FGSG_02749 FGSG_01686 FGSG_09456 FGSG_08402 FGSG_00150 FGSG_09515 FGSG_02432 FGSG_16595 FGSG_12331 FGSG_02291 FGSG_08466 FGSG_10739 FGSG_16526 FGSG_09805	0.01064
16.17.09 heavy metal binding (Cu, Fe, Zn)	FGSG_08980 FGSG_03061 FGSG_08402 FGSG_01686 FGSG_02291 FGSG_10739 FGSG_02034 FGSG_02749 FGSG_00150 FGSG_12331 FGSG_02432 FGSG_08466 FGSG_16526	0.009728
16.21.07 NAD/NADP binding	FGSG_05467 FGSG_07908 FGSG_16526 FGSG_03061 FGSG_00578 FGSG_04223 FGSG_10444 FGSG_00150 FGSG_16400 FGSG_02273 FGSG_12331	0.014273
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	FGSG_00578 FGSG_09402 FGSG_06508 FGSG_02139 FGSG_02322 FGSG_02281 FGSG_06528 FGSG_12331 FGSG_10375 FGSG_03347 FGSG_12745 FGSG_00748 FGSG_16526 FGSG_06081 FGSG_09155 FGSG_02675 FGSG_12704 FGSG_03984 FGSG_16362 FGSG_03882 FGSG_09515 FGSG_07587 FGSG_03744 FGSG_06108 FGSG_01349 FGSG_08466 FGSG_03014 FGSG_12179 FGSG_02273 FGSG_01015 FGSG_04943 FGSG_00152 FGSG_15955 FGSG_02978 FGSG_02749 FGSG_08055 FGSG_08774 FGSG_04370 FGSG_04458 FGSG_08377 FGSG_02145 FGSG_03162 FGSG_07539 FGSG_03125 FGSG_03355 FGSG_00556 FGSG_10809 FGSG_09374 FGSG_08343 FGSG_13641 FGSG_16803 FGSG_17257 FGSG_07564 FGSG_16069	0.001997
20.01 transported compounds (substrates)	FGSG_02273 FGSG_03744 FGSG_04370 FGSG_00578 FGSG_03882 FGSG_16803 FGSG_02145 FGSG_12331 FGSG_09374 FGSG_17257 FGSG_08055 FGSG_03014 FGSG_04458 FGSG_06108 FGSG_12745 FGSG_06081 FGSG_00556 FGSG_06528 FGSG_09515 FGSG_10809 FGSG_02978 FGSG_07564 FGSG_16526 FGSG_03347 FGSG_00152 FGSG_03162 FGSG_07539 FGSG_02139 FGSG_02281 FGSG_12179	1.22E-05

Table B2. Functional classes of significantly enriched *F. graminearum* down-regulated genes *in planta*. (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
	FGSG_13641 FGSG_08343 FGSG_08774 FGSG_12704 FGSG_08377 FGSG_09155 FGSG_07587 FGSG_02322 FGSG_02749 FGSG_16362 FGSG_01349 FGSG_10375	
	FGSG_16069 FGSG_02675 FGSG_03355 FGSG_03125 FGSG_04943 FGSG_15955 FGSG_06508 FGSG_03984 FGSG_01015 FGSG_08466	
20.01.01 ion transport	FGSG_12704 FGSG_02749 FGSG_16803 FGSG_00556 FGSG_09374 FGSG_09155 FGSG_09515 FGSG_03162 FGSG_06081 FGSG_08343 FGSG_04943 FGSG_06508 FGSG_03744 FGSG_13641 FGSG_03882 FGSG_08466 FGSG_15955	0.000113
20.01.01.01 cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.)	FGSG_12704 FGSG_04943 FGSG_15955 FGSG_03744 FGSG_08466 FGSG_02749 FGSG_06081 FGSG_00556 FGSG_09515 FGSG_13641 FGSG_09374 FGSG_03882 FGSG_08343 FGSG_06508 FGSG_16803	8.38E-05
20.01.01.01.01 heavy metal ion transport (Cu+, Fe3+, etc.)	FGSG_02749 FGSG_08466 FGSG_15955 FGSG_04943 FGSG_06508 FGSG_03744 FGSG_00556	0.004273
20.01.03 C-compound and carbohydrate transport	FGSG_06108 FGSG_12704 FGSG_03014 FGSG_03162 FGSG_08774 FGSG_09374 FGSG_06081 FGSG_07587 FGSG_02281 FGSG_10809 FGSG_02978 FGSG_03882 FGSG_04943 FGSG_06508	0.017694
20.01.07 amino acid/amino acid derivatives transport	FGSG_04943 FGSG_02145 FGSG_06508 FGSG_10375 FGSG_15955 FGSG_08055	0.025202
20.01.11 amine / polyamine transport	FGSG_02145 FGSG_17257 FGSG_04370 FGSG_16362	0.011533
20.01.15 electron transport	FGSG_03125 FGSG_02273 FGSG_16526 FGSG_03355 FGSG_03984 FGSG_04458 FGSG_08377 FGSG_00578 FGSG_16069 FGSG_12179 FGSG_01349 FGSG_08343 FGSG_12331 FGSG_06528	0.02078
20.01.27 drug/toxin transport	FGSG_02675 FGSG_17257 FGSG_02139 FGSG_03882 FGSG_04370 FGSG_16362 FGSG_12704 FGSG_02322 FGSG_07564 FGSG_00152	0.001208
20.03 transport facilities	FGSG_02978 FGSG_00152 FGSG_03882 FGSG_17257 FGSG_09515 FGSG_02322 FGSG_09155 FGSG_09374 FGSG_02749 FGSG_06081 FGSG_13641 FGSG_10809 FGSG_16362 FGSG_03162 FGSG_12704 FGSG_02139 FGSG_06508 FGSG_06108 FGSG_02675 FGSG_08466 FGSG_04370 FGSG_07587 FGSG_03347 FGSG_08343 FGSG_04943	0.003766
20.03.02 carrier (electrochemical potential-driven transport)	FGSG_16362 FGSG_13641 FGSG_09155 FGSG_17257 FGSG_00152 FGSG_06081 FGSG_09374	0.008231
20.03.02.03 antiporter	FGSG_13641 FGSG_16362 FGSG_17257 FGSG_06081 FGSG_00152 FGSG_09374	0.00064
20.03.02.03.01 proton driven antiporter	FGSG_06081 FGSG_09374 FGSG_17257 FGSG_16362 FGSG_13641	0.000994

Table B2. Functional classes of significantly enriched *F. graminearum* down-regulated genes *in planta*. (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
20.09.16 cellular export and secretion	FGSG_07564 FGSG_00152 FGSG_17257 FGSG_06508 FGSG_02322 FGSG_02978 FGSG_04370 FGSG_03882 FGSG_12704 FGSG_09374 FGSG_16362 FGSG_04943 FGSG_15955 FGSG_08466	0.000566
32 CELL RESCUE, DEFENSE AND VIRULENCE	FGSG_01621 FGSG_00150 FGSG_03385 FGSG_07908 FGSG_00578 FGSG_06554 FGSG_02139 FGSG_03125 FGSG_02034 FGSG_07564 FGSG_12179 FGSG_04023 FGSG_15955 FGSG_06612 FGSG_01015 FGSG_04370 FGSG_08377 FGSG_17257 FGSG_06051 FGSG_12745 FGSG_01157 FGSG_11080 FGSG_02675 FGSG_16362 FGSG_04458 FGSG_01440 FGSG_12704 FGSG_02291 FGSG_08448 FGSG_03064 FGSG_03498 FGSG_02749 FGSG_11386 FGSG_02322 FGSG_05514 FGSG_08979 FGSG_02273 FGSG_04223 FGSG_16526 FGSG_03882 FGSG_09279 FGSG_01158	6.21E-05
32.01 stress response	FGSG_12745 FGSG_16526 FGSG_02273 FGSG_03064 FGSG_15955 FGSG_08979 FGSG_03385 FGSG_01157 FGSG_03125 FGSG_04223 FGSG_01440 FGSG_04458 FGSG_06051 FGSG_02139 FGSG_06554 FGSG_03882 FGSG_07908 FGSG_01015 FGSG_01158 FGSG_08448	0.015422
32.01.01 oxidative stress response	FGSG_16526 FGSG_03882 FGSG_06554 FGSG_02273 FGSG_03125 FGSG_01158	0.045512
32.01.05 heat shock response	FGSG_01015 FGSG_01158 FGSG_03385 FGSG_04223 FGSG_12745 FGSG_07908	0.001063
32.05 disease, virulence and defense	FGSG_04370 FGSG_02322 FGSG_11386 FGSG_12704 FGSG_02749 FGSG_11080 FGSG_08377 FGSG_02273 FGSG_06612 FGSG_05514 FGSG_04458 FGSG_08448 FGSG_03882 FGSG_02139 FGSG_00578 FGSG_16362 FGSG_04023	0.003264
32.05.01 resistance proteins	FGSG_02322 FGSG_02749 FGSG_04370 FGSG_12704 FGSG_03882 FGSG_08448 FGSG_16362 FGSG_02273 FGSG_02139 FGSG_04458	0.003776
32.05.01.03 chemical agent resistance	FGSG_04458 FGSG_08448 FGSG_02749 FGSG_03882	0.025567
32.07 detoxification	FGSG_03125 FGSG_09279 FGSG_12704 FGSG_02749 FGSG_17257 FGSG_02273 FGSG_02322 FGSG_03498 FGSG_16362 FGSG_02291 FGSG_02675 FGSG_02034 FGSG_00150 FGSG_06554 FGSG_08448 FGSG_02139 FGSG_12179 FGSG_16526 FGSG_07564 FGSG_03882 FGSG_04370	0.000399
32.07.05 detoxification by export	FGSG_02749 FGSG_16362 FGSG_04370 FGSG_07564 FGSG_12704 FGSG_17257 FGSG_03882 FGSG_02322	0.003432
32.07.07 oxygen and radical detoxification	FGSG_03125 FGSG_06554 FGSG_09279 FGSG_16526	0.034089
32.07.07.01 catalase reaction	FGSG_06554 FGSG_16526	0.013159

Table B2. Functional classes of significantly enriched *F. graminearum* down-regulated genes *in planta*. (Continued)

FUNCTIONAL.CATEGORY	genes.SET	P.VALUE
34 INTERACTION WITH THE ENVIRONMENT	FGSG_02978 FGSG_10375 FGSG_07908 FGSG_08466 FGSG_08343 FGSG_01349 FGSG_04943 FGSG_15955 FGSG_01157 FGSG_00556 FGSG_02749 FGSG_12704 FGSG_03882 FGSG_16362 FGSG_08055 FGSG_03744 FGSG_09155 FGSG_03064 FGSG_13641 FGSG_08456 FGSG_16803 FGSG_06508 FGSG_04223 FGSG_09515 FGSG_07832	0.016784
34.01 homeostasis	FGSG_08466 FGSG_13641 FGSG_06508 FGSG_02749 FGSG_03882 FGSG_09155 FGSG_09515 FGSG_01349 FGSG_03064 FGSG_00556 FGSG_07832 FGSG_16803 FGSG_04943 FGSG_03744 FGSG_15955 FGSG_08343	0.003384
34.01.01 homeostasis of cations	FGSG_08466 FGSG_07832 FGSG_04943 FGSG_00556 FGSG_15955 FGSG_09515 FGSG_06508 FGSG_08343 FGSG_03882 FGSG_16803 FGSG_03064 FGSG_03744 FGSG_02749 FGSG_13641	0.007641
34.01.01.01 homeostasis of metal ions (Na, K, Ca etc.)	FGSG_09515 FGSG_08466 FGSG_07832 FGSG_15955 FGSG_03882 FGSG_16803 FGSG_02749 FGSG_06508 FGSG_13641 FGSG_00556 FGSG_03744 FGSG_04943	0.002554

APPENDIX C. COMPARATIVE ANALYSIS OF THE UP-REGULATED GENES WITH PHI-BASE

Table C1. List of up-regulated genes with PHI-base homologs that have been characterized as having no effect on pathogenicity or virulence in other host pathogen interactions.

Gene_ID	PHI-base Hit Description	e-value
FGSG_02839	PHI:1085|Ptr2|AAO31597|TX:13684|Stagonospora nodorum|Unaffected pathogenicity	9.3E-111
FGSG_02944	PHI:541|LIP1|AAU87359|TX:332648|Botrytis cinerea|Unaffected pathogenicity	1.98E-25
FGSG_04610	PHI:538|FRT1|AAU87358|TX:40559|Botrytis cinerea|Unaffected pathogenicity	3.83E-13
FGSG_11147	PHI:538|FRT1|AAU87358|TX:40559|Botrytis cinerea|Unaffected pathogenicity	1.9E-18
FGSG_00571	PHI:569|XYL3|AAC06239|TX:5507|Fusarium oxysporum|Unaffected pathogenicity	1.22E-08
FGSG_08003	PHI:569|XYL3|AAC06239|TX:5507|Fusarium oxysporum|Unaffected pathogenicity	1.25E-07
FGSG_10999	PHI:570|XYL4|AAK27975|TX:5507|Fusarium oxysporum|Unaffected pathogenicity	5E-119
FGSG_03307	PHI:716|ZEB1|ABB90284|TX:5518|Fusarium graminearum|Unaffected pathogenicity	2.76E-80
FGSG_09088	PHI:714|PKS4 (related: ZEA1)|ABB90283|TX:5518|Fusarium graminearum|Unaffected pathogenicity	0.002825
FGSG_12256	PHI:716|ZEB1|ABB90284|TX:5518|Fusarium graminearum|Unaffected pathogenicity	1.13E-06

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions.

Gene_ID	PHI-base Hit Description	e-value
FGSG_00523	PHI:350|EMP1|AAR06609|TX:318829|Magnaporthe oryzae|Reduced virulence	4.30184E-13
FGSG_01249	PHI:211|CaTUP1|AAB63195|TX:5476|Candida albicans|Reduced virulence	2.52822E-07
	PHI:300|FGB1|AAO91808|TX:5507|Fusarium oxysporum|Reduced virulence	0.003288
	PHI:311|MGB1|BAC01165|TX:318829|Magnaporthe oryzae|Loss of pathogenicity	0.005727
	PHI:334|CGB1|AAO25585|TX:5016|Cochliobolus heterostrophus|Loss of pathogenicity	0.00106845
	PHI:65|CPGB-1|AAC49838|TX:5116|Cryphonectria parasitica|Reduced virulence	0.003288
FGSG_01379	PHI:885|MGG_02240|EDJ98842|TX:318829|Magnaporthe oryzae|Reduced virulence	0.00169363
FGSG_01549	PHI:891|MGG_01748|EDK04531|TX:318829|Magnaporthe oryzae|Reduced virulence	0.0032716
FGSG_01767	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	6.971E-07
FGSG_02110	PHI:272|SOWgp|AAL09436|TX:5501|Coccidioides immitis|Reduced virulence	0.00781878
FGSG_02117	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	2.9305E-06
	PHI:838|erg11/cyp51|ABO93363|TX:54734|Mycosphaerella graminicola|Sensitive to chemical	2.43133E-07
	PHI:839|erg11/cyp51|ABO93364|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.22648E-07
	PHI:840|erg11/cyp51|ABO93365|TX:54734|Mycosphaerella graminicola|Resistant to chemical	7.37534E-07
	PHI:841|erg11/cyp51|ABO93366|TX:54734|Mycosphaerella graminicola|Resistant to chemical	2.43133E-07
	PHI:842|erg11/cyp51|ABO93367|TX:54734|Mycosphaerella graminicola|Resistant to chemical	1.05722E-07
	PHI:843|erg11/cyp51|ABO93368|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.16928E-07
	PHI:844|erg11/cyp51|ABO93369|TX:54734|Mycosphaerella graminicola|Resistant to chemical	1.05836E-07
	PHI:845|erg11/cyp51|ABO93370|TX:54734|Mycosphaerella graminicola|Resistant to chemical	2.43133E-07
	PHI:846|erg11/cyp51|ABO93371|TX:54734|Mycosphaerella graminicola|Resistant to chemical	7.99112E-08
FGSG_02251	PHI:325|ACE1|CAG28797|TX:318829|Magnaporthe oryzae|Effector (plant avirulence determinant)	7.00421E-05
FGSG_02267	PHI:59|THR1|BAA18962|TX:5462|Colletotrichum lagenarium|Reduced virulence	8.63202E-07
	PHI:784|MGG_00056|EDK03390|TX:318829|Magnaporthe oryzae|Reduced virulence	4.2704E-08
FGSG_02668	PHI:494|PPOA|EAL89712|TX:5085|Aspergillus fumigatus|Increased virulence (Hypervirulence)	3.0732E-178
	PHI:496|PPOC|EAL92371|TX:5085|Aspergillus fumigatus|Increased virulence (Hypervirulence)	1.2583E-177
FGSG_03212	PHI:144|CHT42|AAC05829|TX:29875|Trichoderma virens|Reduced virulence	2.06531E-12

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions. (Continued)

Gene_ID	PHI-base Hit Description	e-value
	PHI:409|BbCHIT1|AAN41259|TX:176275|Beauveria bassiana|Increased virulence (Hypervirulence)	0.0011338
FGSG_03518	PHI:175|HWP1|AAC96368|TX:5476|Candida albicans|Reduced virulence	3.96845E-07
	PHI:183|RBT1|AAG09787|TX:5476|Candida albicans|Reduced virulence	6.20425E-08
	PHI:517|CaEAP1|EAK95520|TX:5476|Candida albicans|Reduced virulence	1.86788E-05
	PHI:527|ALS3|AAO72959|TX:5476|Candida albicans|Reduced virulence	0.00165687
	PHI:805|MGG_00124|EDK03309|TX:318829|Magnaporthe oryzae|Reduced virulence	0.00629264
	PHI:816|MGG_04582|EDJ95999|TX:318829|Magnaporthe oryzae|Reduced virulence	4.88766E-10
FGSG_03969	PHI:135|BAD1 (related: WI-1)|AAA91036|TX:5039|Blastomyces dermatitidis|Loss of pathogenicity	0.00136271
	PHI:272|SOWgp|AAL09436|TX:5501|Coccidioides immitis|Reduced virulence	3.62039E-08
FGSG_04214	PHI:587|PSPTO0834|AAO54374|TX:223283|Pseudomonas syringae|Unaffected pathogenicity	1.25236E-05
	PHI:668|GNO1|AAP41027|TX:178876|Cryptococcus neoformans|Reduced virulence	1.26508E-08
FGSG_04302	PHI:1048|CTB7|ABK64184|TX:29003|Cercospora nicotianae|Reduced virulence	0.00386014
FGSG_04452	PHI:481|KLAP1|AAX14039|TX:27357|Colletotrichum acutatum|Loss of pathogenicity	2.152E-102
FGSG_04518	PHI:166|CHIP2|AAD53262|TX:5457|Colletotrichum gloeosporioides|Unaffected pathogenicity	1.31589E-35
	PHI:359|KER1|AAK14386|TX:5476|Candida albicans|Reduced virulence	1.59908E-05
	PHI:464|KIN1|AAB63336|TX:5270|Ustilago maydis|Unaffected pathogenicity	0.00025151
	PHI:465|KIN2|AAB63337|TX:5270|Ustilago maydis|Reduced virulence	1.11151E-07
FGSG_04580	PHI:1018|ABC3|AAZ81480|TX:318829|Magnaporthe oryzae|Loss of pathogenicity	1.6204E-08
	PHI:1030|bcatrA|CAA93142|TX:40559|Botrytis cinerea|Unaffected pathogenicity	4.7333E-122
	PHI:132|ABC1|AAB86640|TX:318829|Magnaporthe oryzae|Reduced virulence	0
	PHI:202|BcatrB|CAB52402|TX:40559|Botrytis cinerea|Reduced virulence	0
	PHI:258|GPABC1|CAC40023|TX:5128|Gibberella pulicaris|Reduced virulence	0
	PHI:310|MgAtr4|AAK15314|TX:54734|Mycosphaerella graminicola|Reduced virulence	0
	PHI:391|ABC2|BAC67162|TX:318829|Magnaporthe oryzae|Unaffected pathogenicity	0
	PHI:543|BCATRD|CAC41639|TX:40559|Botrytis cinerea|Unaffected pathogenicity	0
	PHI:867|MgAtr7|ABN41482|TX:54734|Mycosphaerella graminicola|Unaffected pathogenicity	0
FGSG_04581	PHI:734|FOW2|BAE98264|TX:5507|Fusarium oxysporum|Loss of pathogenicity	0.00431247

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions. (Continued)

Gene_ID	PHI-base Hit Description	e-value
FGSG_04590	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	4.7598E-17
	PHI:838|erg11/cyp51|ABO93363|TX:54734|Mycosphaerella graminicola|Sensitive to chemical	0.000910968
	PHI:839|erg11/cyp51|ABO93364|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000524329
	PHI:840|erg11/cyp51|ABO93365|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000910968
	PHI:841|erg11/cyp51|ABO93366|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000910968
	PHI:842|erg11/cyp51|ABO93367|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000910968
	PHI:843|erg11/cyp51|ABO93368|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000519521
	PHI:844|erg11/cyp51|ABO93369|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000692067
	PHI:845|erg11/cyp51|ABO93370|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00119987
	PHI:846|erg11/cyp51|ABO93371|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.000909766
FGSG_04852	PHI:500|YHB1|EAK91807|TX:5476|Candida albicans|Reduced virulence	3.94911E-07
FGSG_04953	PHI:191|TOM1|AAB08446|TX:39703|Septoria lycopersici|Unaffected pathogenicity	0
	PHI:24|Avenacinase gene|AAB09777|TX:29850|Gaeumannomyces graminis|Loss of pathogenicity	0
	PHI:748|um00446|Not available|TX:5270|Ustilago maydis|Unaffected pathogenicity	5.0285E-168
FGSG_05374	PHI:199|AOX1|AAF82788|TX:5499|Cladosporium fulvum|Reduced virulence	2.99514E-24
	PHI:922|um03615||TX:5270|Ustilago maydis|Unaffected pathogenicity	1.94418E-25
FGSG_05906	PHI:175|HWP1|AAC96368|TX:5476|Candida albicans|Reduced virulence	0.00129022
	PHI:432|FGL1|AAQ23181|TX:5518|Fusarium graminearum|Reduced virulence	7.6353E-179
FGSG_06068	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	1.05751E-19
	PHI:838|erg11/cyp51|ABO93363|TX:54734|Mycosphaerella graminicola|Sensitive to chemical	0.00165527
	PHI:839|erg11/cyp51|ABO93364|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00125533
	PHI:840|erg11/cyp51|ABO93365|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00217972
	PHI:841|erg11/cyp51|ABO93366|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00165527
	PHI:842|erg11/cyp51|ABO93367|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00165527
	PHI:843|erg11/cyp51|ABO93368|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00284315
	PHI:844|erg11/cyp51|ABO93369|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00125777
	PHI:845|erg11/cyp51|ABO93370|TX:54734|Mycosphaerella graminicola|Resistant to chemical	0.00165527

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions. (Continued)

Gene_ID	PHI-base Hit Description	e-value
FGSG_06486	PHI:1071|Gas1|CAF05793|TX:5270|Ustilago maydis|Loss of pathogenicity	2.36732E-22
	PHI:526|GAS1|CAF05793|TX:5270|Ustilago maydis|Reduced virulence	2.36732E-22
FGSG_06550	PHI:1056|CgCHSV|AAL23719|TX:31870|Colletotrichum graminicola|Reduced virulence	2.33068E-25
	PHI:285|CHSV|AAO49384|TX:5507|Fusarium oxysporum|Increased virulence (Hypervirulence)	3.67599E-24
	PHI:31|CHS3|BAA02707|TX:5476|Candida albicans|Reduced virulence	1.48529E-22
	PHI:389|Umchs6|AAB84285|TX:5270|Ustilago maydis|Reduced virulence	3.11294E-28
	PHI:390|WdCHS5|AAL79830|TX:5970|Wangiella (Exophiala) dermatitidis|Reduced virulence	3.68843E-24
	PHI:98|umCHS5|AAB84284|TX:5270|Ustilago maydis|Reduced virulence	4.43353E-19
FGSG_06676	PHI:527|ALS3|AAO72959|TX:5476|Candida albicans|Reduced virulence	0.00611003
FGSG_07067	PHI:169|CLTA1|AAG25917|TX:290576|Colletotrichum lindemuthianum|Loss of pathogenicity	0.00788531
	PHI:734|FOW2|BAE98264|TX:5507|Fusarium oxysporum|Loss of pathogenicity	0.00195975
	PHI:889|MGG_09263|EDJ97928|TX:318829|Magnaporthe oryzae|Reduced virulence	0.000962179
FGSG_07530	PHI:199|AOX1|AAF82788|TX:5499|Cladosporium fulvum|Reduced virulence	0
	PHI:922|um03615||TX:5270|Ustilago maydis|Unaffected pathogenicity	5.87736E-14
FGSG_07631	PHI:538|FRT1|AAU87358|TX:40559|Botrytis cinerea|Unaffected pathogenicity	0.000640337
FGSG_07735	PHI:481|KLAP1|AAX14039|TX:27357|Colletotrichum acutatum|Loss of pathogenicity	1.14273E-06
FGSG_07765	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	1.5583E-11
FGSG_07794	PHI:222|PELB|AAD09857|TX:5457|Colletotrichum gloeosporioides|Reduced virulence	7.7574E-31
FGSG_08178	PHI:250|DEC1|AAM88291|TX:5016|Cochliobolus heterostrophus|Reduced virulence	3.44687E-37
FGSG_08245	PHI:175|HWP1|AAC96368|TX:5476|Candida albicans|Reduced virulence	4.1627E-06
	PHI:517|CaEAP1|EAK95520|TX:5476|Candida albicans|Reduced virulence	2.77443E-13
	PHI:816|MGG_04582|EDJ95999|TX:318829|Magnaporthe oryzae|Reduced virulence	4.7182E-08
FGSG_08312	PHI:1018|ABC3|AAZ81480|TX:318829|Magnaporthe oryzae|Loss of pathogenicity	3.90487E-10
	PHI:1030|bcatrA|CAA93142|TX:40559|Botrytis cinerea|Unaffected pathogenicity	0
	PHI:132|ABC1|AAB86640|TX:318829|Magnaporthe oryzae|Reduced virulence	0
	PHI:202|BcatrB|CAB52402|TX:40559|Botrytis cinerea|Reduced virulence	0
	PHI:258|GPABC1|CAC40023|TX:5128|Gibberella pulicaris|Reduced virulence	0

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions. (Continued)

Gene_ID	PHI-base Hit Description	e-value
	PHI:310|MgAtr4|AAK15314|TX:54734|Mycosphaerella graminicola|Reduced virulence	0
	PHI:391|ABC2|BAC67162|TX:318829|Magnaporthe oryzae|Unaffected pathogenicity	0
	PHI:543|BCATRD|CAC41639|TX:40559|Botrytis cinerea|Unaffected pathogenicity	0
	PHI:867|MgAtr7|ABN41482|TX:54734|Mycosphaerella graminicola|Unaffected pathogenicity	0
FGSG_08519	PHI:784|MGG_00056|EDK03390|TX:318829|Magnaporthe oryzae|Reduced virulence	7.19203E-10
FGSG_08830	PHI:1018|ABC3|AAZ81480|TX:318829|Magnaporthe oryzae|Loss of pathogenicity	0.000978636
	PHI:1030|bcatrA|CAA93142|TX:40559|Botrytis cinerea|Unaffected pathogenicity	0
	PHI:132|ABC1|AAB86640|TX:318829|Magnaporthe oryzae|Reduced virulence	0
	PHI:202|BcatrB|CAB52402|TX:40559|Botrytis cinerea|Reduced virulence	0
	PHI:258|GPABC1|CAC40023|TX:5128|Gibberella pulicaris|Reduced virulence	0
	PHI:310|MgAtr4|AAK15314|TX:54734|Mycosphaerella graminicola|Reduced virulence	0
	PHI:391|ABC2|BAC67162|TX:318829|Magnaporthe oryzae|Unaffected pathogenicity	0
	PHI:543|BCATRD|CAC41639|TX:40559|Botrytis cinerea|Unaffected pathogenicity	0
	PHI:867|MgAtr7|ABN41482|TX:54734|Mycosphaerella graminicola|Unaffected pathogenicity	0
FGSG_09045	PHI:226|PEX6|AAK16738|TX:5462|Colletotrichum lagenarium|Loss of pathogenicity	0.00521086
FGSG_09364	PHI:587|PSPTO0834|AAO54374|TX:223283|Pseudomonas syringae|Unaffected pathogenicity	2.43844E-06
	PHI:668|GNO1|AAP41027|TX:178876|Cryptococcus neoformans|Reduced virulence	2.38228E-13
FGSG_09364	PHI:881|MGG_04556|EDJ96020|TX:318829|Magnaporthe oryzae|Reduced virulence	1.7149E-116
FGSG_10960	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	0.00572224
	PHI:494|PPOA|EAL89712|TX:5085|Aspergillus fumigatus|Increased virulence (Hypervirulence)	0
	PHI:496|PPOC|EAL92371|TX:5085|Aspergillus fumigatus|Increased virulence (Hypervirulence)	0
	PHI:59|THR1|BAA18962|TX:5462|Colletotrichum lagenarium|Reduced virulence	1.31271E-06
	PHI:1008|NPS6|ABI51982|TX:101162|Cochliobolus miyabeanus|Reduced virulence	1.9247E-101
	PHI:1009|NPS6|ABI51983|TX:29001|Alternaria brassicicola|Reduced virulence	2.50187E-95
	PHI:12|HTS1|AAA33023|TX:5017|Cochliobolus carbonum|Loss of pathogenicity	8.8875E-100
	PHI:133|AKT1|BAA36588|TX:5599|Alternaria alternata|Loss of pathogenicity	0.00687927
	PHI:160|AMT|AAF01762|TX:5599|Alternaria alternata|Loss of pathogenicity	1.21272E-94

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions. (Continued)

Gene_ID	PHI-base Hit Description	e-value
	PHI:325|ACE1|CAG28797|TX:318829|Magnaporthe oryzae|Effector (plant avirulence determinant)	5.06772E-33
	PHI:416|NPS6|AAX09988|TX:5016|Cochliobolus heterostrophus|Reduced virulence	1.9247E-101
FGSG_10991	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	1.23066E-19
FGSG_10995	PHI:1018|ABC3|AAZ81480|TX:318829|Magnaporthe oryzae|Loss of pathogenicity	5.12116E-22
	PHI:267|MLT1|AAD51594|TX:5476|Candida albicans|Reduced virulence	2.86133E-41
FGSG_11032	PHI:352|GLO1|CAD79488|TX:5270|Ustilago maydis|Loss of pathogenicity	2.38805E-14
FGSG_11164	PHI:652|GIP1|AAL11720|TX:67593|Phytophthora sojae|Effector (plant avirulence determinant)	3.36333E-12
	PHI:653|GIP2|AAL11721|TX:67593|Phytophthora sojae|Effector (plant avirulence determinant)	9.02929E-11
FGSG_11228	PHI:199|AOX1|AAF82788|TX:5499|Cladosporium fulvum|Reduced virulence	1.35795E-20
	PHI:517|CaEAP1|EAK95520|TX:5476|Candida albicans|Reduced virulence	0.00732198
	PHI:922|um03615||TX:5270|Ustilago maydis|Unaffected pathogenicity	1.52596E-56
FGSG_11398	PHI:59|THR1|BAA18962|TX:5462|Colletotrichum lagenarium|Reduced virulence	0.00152477
FGSG_12342	PHI:838|erg11/cyp51|ABO93363|TX:54734|Mycosphaerella graminicola|Sensitive to chemical	8.59079E-09
	PHI:839|erg11/cyp51|ABO93364|TX:54734|Mycosphaerella graminicola|Resistant to chemical	6.49488E-09
	PHI:840|erg11/cyp51|ABO93365|TX:54734|Mycosphaerella graminicola|Resistant to chemical	1.49497E-08
	PHI:841|erg11/cyp51|ABO93366|TX:54734|Mycosphaerella graminicola|Resistant to chemical	8.59079E-09
	PHI:842|erg11/cyp51|ABO93367|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.93574E-09
	PHI:843|erg11/cyp51|ABO93368|TX:54734|Mycosphaerella graminicola|Resistant to chemical	3.36762E-08
	PHI:844|erg11/cyp51|ABO93369|TX:54734|Mycosphaerella graminicola|Resistant to chemical	6.52025E-09
	PHI:845|erg11/cyp51|ABO93370|TX:54734|Mycosphaerella graminicola|Resistant to chemical	1.1333E-08
	PHI:846|erg11/cyp51|ABO93371|TX:54734|Mycosphaerella graminicola|Resistant to chemical	3.73093E-09
FGSG_13459	PHI:438|BcBOT1 (related: CND5)|AAQ16576|TX:40559|Botrytis cinerea|Reduced virulence	1.25242E-27
	PHI:838|erg11/cyp51|ABO93363|TX:54734|Mycosphaerella graminicola|Sensitive to chemical	4.13857E-06
	PHI:839|erg11/cyp51|ABO93364|TX:54734|Mycosphaerella graminicola|Resistant to chemical	2.37922E-06
	PHI:840|erg11/cyp51|ABO93365|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.13857E-06
	PHI:841|erg11/cyp51|ABO93366|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.13857E-06
	PHI:842|erg11/cyp51|ABO93367|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.13857E-06

Table C2. List of up-regulated genes with PHI-base homologs that have been characterized to have effect on pathogenicity or virulence in other host-pathogen interactions. (Continued)

Gene_ID	PHI-base Hit Description	e-value
	PHI:843|erg11/cyp51|ABO93368|TX:54734|Mycosphaerella graminicola|Resistant to chemical	3.09524E-06
	PHI:844|erg11/cyp51|ABO93369|TX:54734|Mycosphaerella graminicola|Resistant to chemical	5.45773E-06
	PHI:845|erg11/cyp51|ABO93370|TX:54734|Mycosphaerella graminicola|Resistant to chemical	5.45276E-06
	PHI:846|erg11/cyp51|ABO93371|TX:54734|Mycosphaerella graminicola|Resistant to chemical	4.13086E-06
FGSG_13878	PHI:1008|NPS6|ABI51982|TX:101162|Cochliobolus miyabeanus|Reduced virulence	1.45568E-96
	PHI:1009|NPS6|ABI51983|TX:29001|Alternaria brassicicola|Reduced virulence	1.13324E-89
	PHI:12|HTS1|AAA33023|TX:5017|Cochliobolus carbonum|Loss of pathogenicity	0
	PHI:133|AKT1|BAA36588|TX:5599|Alternaria alternata|Loss of pathogenicity	2.32459E-12
	PHI:160|AMT|AAF01762|TX:5599|Alternaria alternata|Loss of pathogenicity	6.7726E-120
	PHI:293|CPS1|AAG53991|TX:5016|Cochliobolus heterostrophus|Reduced virulence	0.00640174
	PHI:304|GzCPS1|AAP12366|TX:5518|Fusarium graminearum|Reduced virulence	0.00218177
	PHI:325|ACE1|CAG28797|TX:318829|Magnaporthe oryzae|Effector (plant avirulence determinant)	4.67555E-39
	PHI:416|NPS6|AAX09988|TX:5016|Cochliobolus heterostrophus|Reduced virulence	2.7498E-97
	PHI:508|AFT1|BAB69076|TX:5599|Alternaria alternata|Loss of pathogenicity	1.84989E-10