GROWTH OF *FUSARIUM GRAMINEARUM* ON WHEAT BRAN/AGAR CULTURES IN RELATION TO FUSARIUM HEAD BLIGHT SUSCEPTIBILITY

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Meliza Stephnie Abeyratne

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RELATION TO FUSARIUM HEAD BLIGHT SUSCEPTIBILITY

By

MELIZA STEPHNIE ABEYRATNE

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

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

DR. ŞENAY ŞIMŞEK

Chair

DR. PAUL SCHWARZ

DR. DOUGLAS DOEHLERT

DR. JAE OHM

DR. SHAOBIN ZHONG

Approved:

06/18/2012	DR. DELAND MYERS
Date	Department Chair

ABSTRACT

Research investigates the chemical basis for Fusarium head blight (FHB) resistance and initiating development of a screening test for resistant wheat genotypes. The focus is on minimizing cost of screening and gaining chemical approach against FHB. Wheat bran/agar plates (8% bran, w/v) prepared from hard red spring wheat with different susceptibility to FHB were inoculated with *F. graminearum*. *Fusarium* plaque diameters and ergosterol levels after 4 days of growth were significantly lower ($p < 0.05$) on plates prepared from genotypes with low FHB susceptibility than from high FHB susceptible genotypes. *F. graminearum* growth was lower, when methanol-soluble compounds (MSC) extracted from a low FHB susceptibility genotype, Glenn, were added to high susceptibility genotype, Samson. Wheat bran/agar plates enriched with linoleic acid significantly ($p<0.05$) reduced the growth rate of *F. graminearum* in both Glenn and Samson genotypes. Oxygenated fatty acids, including monohydroxy- and dihydroxy- fatty acids were identified in the MSC.

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TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES viii

LIST OF FIGURES x

LIST OF ABBREVIATIONS xii

LIST OF APPENDIX TABLES xiii

INTRODUCTION 1

HYPOTHESES 3

RESEARCH OBJECTIVE 4

LITERATURE REVIEW 5

- Fusarium Head Blight 5
  - Introduction to Fusarium Head Blight 5
  - Economic Importance of Fusarium Head Blight in Wheat 6
  - Management Practices and Breeding Techniques Used to Control Fusarium Head Blight 7
  - Resistant Wheat Genotypes 8
- Lipids in Wheat 10
  - Structure of the Wheat Kernel 10
  - Lipid Content in Wheat Bran 11
  - Lipid Cellular Arrangement in Wheat Bran 13
  - Lipoxygenase in Wheat 15
  - Mechanism of Lipoxygenase Reaction 17
  - Role of Lipoxygenase in Plant Defense 19

Need Statement 19

MATERIALS AND METHODS 21

Wheat Grain 21

Fusarium graminearum Culture 21

Chemicals and Reagents 21

Preparation of Carnation Leaf Agar Media Plates 23

Carboxymethylcellulose Stock Spore Suspension Preparation 23

Calculation for Spore Count 24

Sterile Wheat Bran/Agar Petri Plate Preparation for 19 Wheat Genotypes 24

Fusarium graminearum Plaque Diameter Measurement 25

Ergosterol Analysis 25

Reconstitution Study 26

Lipid Extraction 27

Fatty Acid Methylation 29

Total Fatty Acids 29

Esterified Fatty Acids 30

Free Fatty Acids 30

Silylation 30

Gas Chromatography-Mass Spectrometry 31

Quantification of Oxidized Fatty Acids in Samson and Glenn Genotypes 31

Spiking Study 32

Media Preparation. 32

Wheat Bran Plate Preparation 32

Statistical Analysis 33

RESULTS AND DISCUSSIONS 35

Growth of *Fusarium graminearum* in Wheat Bran/Agar Plates 35

Reconstitution Studies between Low and High Susceptibility Genotypes 43

Lipid Analysis 50

Identification of Oxidized Fatty Acids by Diazomethane Methylation and Gas Chromatography-Mass Spectrometry Analysis 50

Effect of Total FFA Percentage in Different Wheat Bran with *Fusarium graminearum* Plaque Diameter 55

Quantification of Oxidized Fatty Acids in Glenn and Samson Incubated and Non incubated Treatments 56

Spiking Experiments 57

Identification of Oxidized Fatty Acids from Diazomethane Methylation in the Spiking Study 60

Quantification of Oxidized Fatty Acids in Spiking Study 61

CONCLUSION 63

Future Work 65

REFERENCES 66

APPENDIX 75

LIST OF TABLES

Table Page

1. Distribution of crude fat in the wheat kernel 12

2. Fatty acid composition in whole wheat grain, endosperm, bran, and germ........................... 14

3. List of genotypes (categorized according to their susceptibility levels) used in this study.... 22

4. List of genotypes (categorized according to their susceptibility levels) used in this study.... 37

5. ANOVA table for plaque diameter readings using 19 wheat genotypes with various Fusarium head blight susceptibility (natural log transformation).. 38

6. Bartlett's Test for homogeneity of diameter error variance among day 2, 3, and 4................ 38

7. Analysis of ergosterol in wheat bran/agar plates using 19 hard red spring wheat genotypes inoculated with *Fusarium graminearum* after day 4 42

8. ANOVA table for ergosterol analysis in wheat bran/agar plates using 19 hard red spring wheat genotypes inoculated with *Fusarium graminearum* after day 4.................................. 43

9. ANOVA table for ergosterol analysis in wheat bran/agar plates using 19 hard red spring wheat genotypes inoculated with *Fusarium graminearum* after day 4.................................. 46

10. ANOVA table for plaque diameters of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study. 47

11. ANOVA table for ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study... 48

12. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study on the third day... 49

13. Total oxygenated free fatty acid percentage for Glenn and Samson incubated and non incubated bran. 56

14. Plaque diameters of *Fusarium graminearum* in wheat bran/agar plates in spiked study using low (Glenn) and high (Samson) susceptibility wheat genotypes. 59

15. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates in spiked study using low (Glenn) and high (Samson) susceptibility hard red spring wheat genotypes. 60

16. Total oxygenated free fatty acid percentage in Glenn and Samson bran in the spiking study. 61

LIST OF FIGURES

Figure Page

1. Representation of different organization of tissues in bran, endosperm, and germ in a wheat kernel 10
2. Representative mass spectrum of 9-hydroxy-10-oxo, cis-15-octadecadienoic acid after methylation and silylation. 17
3. Cascade of reactions in the formation of hydroperoxides from linolenic acid oxidation by Lipoxygenase. 18
4. Activation cycle of lipoxygenase enzyme in cereals. 19
5. Schematic representation of methanol extraction from wheat bran. 28
6. Representation of different treatment combinations used in reconstitution study. 29
7. Representation of the diazomethane apparatus used in free fatty acid analysis. 30
8. Experimental design of different treatments used in the spiking study. 33
9. *Fusarium graminearum* growth in wheat bran/agar using 19 hard red spring wheat genotypes after four days of inoculation. 39
10. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates using 19 hard red spring wheat genotypes after the day 4. 40
11. Plaque diameters of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study. 44
12. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study. 48
13. Standard gas chromatogram which represents 1-palmitic acid, 2-Internal Standard, 3-stearic acid, 4-oleic acid, 5-linoleic acid, 6-linolenic acid fatty acids. 51
14. Mass spectra of 12,13-dihydroxy-9-octadecanoic acid identified in Glenn incubated bran... 51
15. Fragmentation patterns of 12,13-dihydroxy-9-octadecanoic acid identified in Glenn incubated bran. 52

16. Mass spectra of 9-hydroxy-10,12-octadecadienoic acid in incubated Glenn bran. 53

17. Fragmentation consistent with 9-hydroxy-10,12-octadecadienoic acid in incubated Glenn bran.. 53

18. Correlation between total free fatty acid area percent (diazomethane methylation) and plaque diameter readings among all 19 wheat genotypes.. 55

19. Plaque diameters of *Fusarium graminearum* in wheat bran/agar plates spiked study using low (Glenn) and high (Samson) susceptibility hard red spring wheat genotypes......... 58

20. A model which represent overall goal of this project including severity of FHB in wheat and compounds that can be identified as a result of FHB in the wheat bran. 64

LIST OF ABBREVIATIONS

w/w, db- weight/weight, dry basis

wt- weight

µm- micrometer

Fe^{3+} - ferric

Fe^{2+} - ferrous

H_2O_2- hydrogen peroxide

r.p.m- revolutions per minute

mL- milliliters

g- grams

mg- milligrams

µL- microliters

v/v- volume/volume

M- molar

mm- milimeter

cm- centimeter

MSC- methanol soluble compound

LIST OF APPENDIX TABLES

Table Page

17. ANOVA table for *Fusarium graminearum* plaque diameter readings in wheat bran/agar plates on Day 2 for reconstitution study. 75

18. ANOVA table for *Fusarium graminearum* plaque diameter readings in wheat bran/agar plates on Day 3 for reconstitution study. 75

19. ANOVA table for *Fusarium graminearum* plaque diameter readings in wheat bran/agar plates on Day 2 for spiking study. 76

20. ANOVA table for *Fusarium graminearum* for plaque diameter readings in wheat bran/agar plates on Day 3 for spiking study. 77

21. ANOVA table for ergosterol values in the spiking study (analyzed after day 3). 78

INTRODUCTION

Fusarium head blight (FHB) is an important fungal disease that affects several crop species including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), sorghum (*Sorghum vulgare* L.), and other grains (Bechtel et al., 1985). This disease is associated with several *Fusarium* species, but the main causal agent in wheat is *F. graminearum* and most frequently found in temperate and warmer regions of the U.S., China, and the southern hemisphere (Osborne and Stein, 2007). FHB results in economic losses including, reduced grain yield, quality, and contamination from mycotoxins, leading to rejection or lowering the price of grain (Prandini et al., 2009). This disease is favored by warm and humid conditions in the field, and is believed to infect the wheat spikelets during flowering stage of grain development (Stack, 2003). Infected wheat grains often appear to be small, wrinkled, light (or degraded), and chalky white (Prandini et al., 2009). Since the mid 1990's, FHB of wheat and barley have caused extreme economic losses in North America (Windels, 2000).

Wheat, a major cereal crop, has a long history for being a staple food for human consumption and animal feed. Due to FHB, there have been tremendous capital losses as well as yield losses (McMullen et al., 2008). Impact of FHB upon wheat and processed foods from wheat can be disastrous and that is why resistant (low susceptibility) wheat genotypes are necessary to overcome FHB (McMullen et al., 1997). Excellent quality of wheat is required to produce food products with high end quality. New wheat genotypes should be highly resistant against diseases, such as FHB, with excellent agronomic and quality traits (Parry et al., 1995). However, it is difficult to achieve all these requirements. FHB issue is a high priority for breeders, and millions of dollars have been invested to develop new genotypes with high FHB resistance and high yield

potential (Pumphrey et al., 2007). Information gained from breeding programs can be used to categorize wheat into different susceptibility levels and allows selection for more highly resistant genotypes. In general, wheat genotypes can be separated into high, medium, and low levels of FHB susceptibility (Ransom, 2010; WestBred, 2012; Wiersma, 2006; and Seednet, 2005). A large amount of capital is also invested in screening breeding lines for resistance against FHB (Wilde et al., 2007). Conducting screening in the field is never a simple procedure. Screening for susceptibility and development of higher FHB resistance are high priorities for wheat breeding efforts.

Lipids are considered to be a minor constituent of wheat; however, they play a major role in wheat production, storage, processing, and defense against plant pathogens (Konopka et al., 2006). Bran, one of the major anatomical structures, contains 5.1 to 5.8 % lipids, by weight (Table 1). Overall, hard red spring wheat (HRSW) bran lipids contain more linoleic acid (Table 2) than any other fatty acid (FA) (Pomeranz et al., 1966). Enzymes involved in lipid degradation include lipase and lipoxygenase (LOX). Lipoxygenase catalyzes lipid peroxidation in wheat bran to form hydroperoxides (Graveland, 1973). These hydroperoxides are metabolized further to form hydroxyl fatty acids (Kim and Min, 2008). Doehlert et al (2011) suggested monohydroxy and dihydroxy FA may provide resistance to FHB in oats. However, the occurrences of such compounds have not been identified or studied in wheat in relation to their role in defense against FHB. This study was conducted to develop a preliminary screening technique that would be feasible to conduct under *in vitro* conditions with minimal use of labor and cost to characterize susceptibility of different wheat genotypes and to identify hydroxyl fatty acids formed in the wheat bran/agar suspension.

HYPOTHESES

There are three main hypotheses that were tested during this study;

I. *F. graminearum* growth rate on wheat bran/agar plate is inversely correlated with FHB susceptibility of the genotypes used.

II. Exchange of methanol soluble compounds between high and low susceptibility genotypes will affect the *F. graminearum* growth in wheat bran/agar plates.

III. Spiking wheat bran/agar plates with linoleic acid will result in less growth of *F. graminearum* in two genotypes (high susceptibility and low susceptibility).

RESEARCH OBJECTIVE

Specific objectives of this research were as follows;

I. To determine differences in the inhibition of *F. graminearum* growth in wheat bran/agar plates using several wheat genotypes with various levels of FHB susceptibility.

II. To determine the effect of methanol soluble compounds from wheat bran on *F. graminearum* growth in wheat bran/agar plates using two genotypes (high susceptibility and low susceptibility).

III. To confirm the role of linoleic acid oxidation products on *F. graminearum* growth in wheat bran/agar plates.

LITERATURE REVIEW

Among many other cereal crops, wheat is one of the most important staple grains, which has a history of 9,000 years (Osborne and Stein, 2007). Wheat is an essential crop to the economy of United States that is the third largest wheat producer in the world (McMullen et al., 1997). The FHB impact on wheat is devastating, as such, the primary focus on this review is to identify some of the current key features of FHB, economic importance of FHB, breeding techniques including current laboratory methods used in screening for FHB, resistant wheat genotypes, structure of wheat, wheat lipids, LOX, LOX activity against disease resistance, and hydroxyl fatty acids formed in wheat due to LOX activity.

Fusarium Head Blight

Introduction to Fusarium Head Blight

Fusarium Head Blight (FHB), also called Scab, is a major fungal diseases affecting several small grain crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), and sorghum (*Sorghum vulgare* L.) (Stack, 2003; Bechtel et al., 1985). Infected kernels are often referred to as scabbed, blighted, or tombstones kernels. In the 19th century, there were several *Fusarium* species identified, that included *F. graminearum*, *F. poae*, *F. avenaceu*, *F. triticum,* and *Microdochium nivale*, while *F. graminearum* was the most prevalent species that was associated with FHB (Bushnell et al., 2003). *F. graminearum* has the capability of producing number of mycotoxins which includes, deoxynivalenol (3, 7, 15-trihydroxy, 12, 13-epoxytrichothec-9-en-8-one) (DON) and nivalenol (NIV), and zearalenone (ZEA) all of which have a range of toxicity to humans as well as animals (Osborne and Stein, 2007).

Past literature reviews have explained that the Northern Great Plains often have ideal environmental conditions for disease residues. In no-till cropping systems large quantities of inoculum may be blown from colonies near the soil (on residue), or from leaves of maize or other cereal plants (Bushnell, et al., 2003). Conidial and ascospore inoculum can accumulate on uppermost and lowermost leaves of canopy (Jones and Mirocha, 1999), which suggests that inoculum deposition occurs from above and below. Rain-splash is another important dispersal mechanism of FHB. However, rain-splash is not common in the Northern Great Plains (Stack, 2003). There is a high probability that rain-splash can enable conidial movement from leaf-to-leaf and ultimately to susceptible spikes. In the FHB disease system, each individual fungus has its own requirement for environmental and biological needs. For example, *F. graminearum* grows well at 30° C and is associated with warmer regions (Champeil et al., 2004).

Economic Importance of Fusarium Head Blight in Wheat

During the years 1991, 1993, 1995, and 1996, FHB epidemic resulted in enormous losses in wheat yield in Illinois, Indiana, Kentucky, Michigan, Missouri, and Ohio (Windels, 2000). Among wheat types, HRSW is one of the major wheat types used in the food industry, which were heavily infected from 1993 through 1997 in Minnesota, North Dakota, and South Dakota (Bushnell et al., 2003; Windels, 2000). Severe FHB epidemics have been reported throughout United States, Canada, South America, Europe and Asia during the twentieth century (McMullen et al., 1997). Being a weather dependant disease, the occurrence of FHB outbreaks varies considerably among years and regions (Jones and Mirocha, 1999). Recent updates in 2011 from the University of Kentucky have reviewed on the economical loss due to FHB in wheat, suggesting excess loss of $3 billion in the US wheat industry and have also led to food safety concerns (USWBSI, 2012). Food safety is a major issue concerning DON levels in wheat, which

is a current topic of discussion in US wheat and barley initiative organization. The focus of this organization is to reduce the FHB disease rate and eliminate mycotoxins that can contaminate food processing facilities (Maiorano et al., 2008). However, our efforts in this research are to understand the physiological basis for FHB resistance in wheat genotypes and to develop preliminary data for a simplified screening technique.

Management Practices and Breeding Techniques Used to Control Fusarium Head Blight

There have been several management practices introduced to reduce or to control FHB in wheat and other cereal crops. Most common is the use of fungicides. Currently, the most productive fungicides used are Prosaro and Caramba (Carver, 2009). Apart from the use of fungicides, infection levels are also controlled by different post-harvest techniques including controlling air flow in combines (removing shriveled seed by increasing combine airflow), cleaning seed (remove as many small seed as possible by post harvest screening and cleaning), and other seed treatments (Carver, 2009). Other techniques including crop rotations among sesame, soybean, canola, and sunflower, and tillage are commonly used to get rid of corn or wheat residue.

The development of resistant wheat genotypes is considered to be the best strategy to control the disease. In the past, variety selection procedure for FHB resistance has been based on phenotypic evaluation of disease occurrence and severity in the field (Bai and Shaner 1994). However, phenotypic assessment of FHB consumes time, high cost, and frequently imprecise. Phenotypic expression of FHB resistance is also greatly exaggerated by the environmental conditions (Bai and Shaner, 2004). Among breeding techniques, molecular markers are used to introduce new FHB resistance into HRSW (Bitzer and Herbek, 1997). Alien resistant genes

(from high FHB resistant *Agropyron spp*.) are transferred into low resistance (high susceptibility) wheat genotypes (Wilde, 2007). These wide-cross breeding techniques have also resulted in highly resistant genotypes and good grain quality.

Screening techniques include spraying plants with *Fusarium* inoculum consisting of *F. graminearum* spores that are cultured in the laboratory. These techniques are more prevalent among greenhouse studies and in field screening nurseries (Evans and Dill-Macky, 2004). Data from these screenings are also used in genetic studies that allow breeding programs to identify resistance genes and conduct molecular mapping studies (Kolb et al., 2001).

Approximately 2,100 pots of wheat may be screened with the above mentioned technique every season in any program (Evans and Dill-Macky, 2004). It becomes difficult when there are too many pots in different locations to screen. Screening takes long periods of time and is labor intensive and is therefore expensive. This expense increases the amount of capital invested on these greenhouse studies (Evans and Dill-Macky, 2004). A simple *in vitro* laboratory scale technique to screen different wheat genotypes for susceptibility against FHB could save significant resources and accelerate the development of FHB resistant genotypes.

Resistant Wheat Genotypes

Defense related genes can be activated in wheat, barley, and in other cereal grains in response to attack by fungi and in wheat these genes can be activated by inoculating *F. graminearum* into wheat heads (Bacic, 1988). As the ovary develops into the caryopsis, it becomes less susceptible to fungal invasions (Walter et al., 2009). After the fungus begins to colonize floret tissue, a biotrophic interaction with the host is established, in which the fungus grows intercellularly for a day or two without producing symptoms. Then the growth becomes

necrotrophic, in which the fungus grows abundantly within cells, including chlorosis, browning, and in some cases, cell death (Kolb et al., 2001).

Resistance in wheat to FHB is classified as either type I or type II resistance (Osborne and Stein, 2007). Type I resistance is defined when there is resistance to a initial infection from the pathogen and type II resistance is when there is a resistance to spread of the pathogen in the spike after initial infection. These types of resistance are measured differently. Type I is measured by spray inoculating spikes with a suspension of macroconida and assessing the infected spikelet (Osborne and Stein, 2007). Type II is measured by inoculating a single floret within the center of a spike and then assessing the spread of infection.

Resistance to FHB is known to be complex and is significantly affected by many factors (Jones and Mirocha, 1999). Important genetic sources of resistance to FHB have been identified in wheat genotypes from Asia, Brazil and Europe, as well as the USA (McCartney et al., 2004). The most widely used source of resistance is the Chinese cultivar Sumai-3, derived from a cross between Funo and Taiwan Xiaomai (Rudd et al., 2001; McCartney et al., 2004). Massive outbreaks of FHB in wheat have motivated breeders to improve resistance in wheat genotypes. The resistant (low susceptibility) genotypes have the capacity of controlling the FHB disease and have high quality traits for the wheat industry (Windels, 2000). The National FHB Forum meetings held at USWBSI in 2011 confirms that there is much need for increasing FHB resistance in HRSW genotypes in particular regions. Our studies investigate how chemical components in the wheat grain may provide resistance and how simple tests may indicate their presence. Therefore it is important to identify some of the characteristic compounds and structures of the wheat kernel as this disease is more prevalent among wheat genotypes.

Lipids in Wheat

Structure of the Wheat Kernel

The wheat grain is more or less oval in shape with a crease on the ventral side of the kernel (Bechtel et al., 2009). Mill fractions of wheat are the bran, endosperm, and the germ. The embryo (or germ) of the wheat kernel is located on the dorsal side of the grain (Hemery et al., 2011). It contains B vitamins, vitamin E, antioxidants, phytonutrients, and unsaturated fats (Verma et al., 2009). The endosperm contains aluerone layer and the starchy endosperm. Together the endosperm comprises of more than 80% of the grain mass.

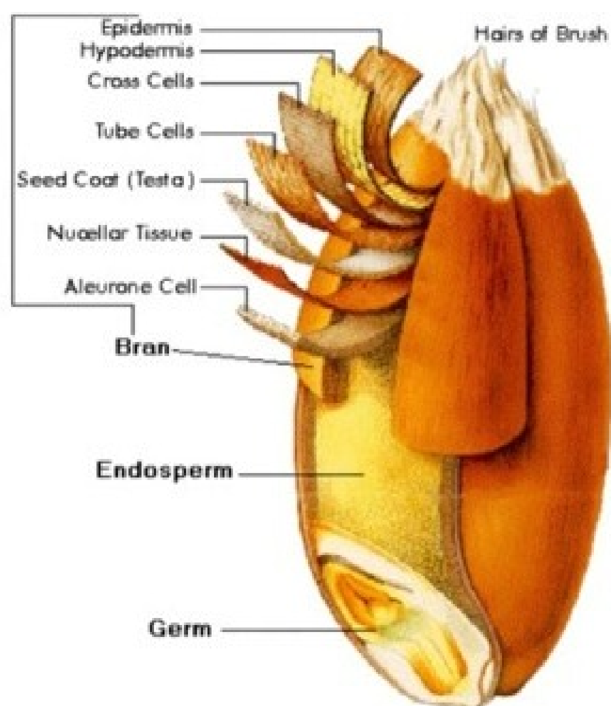


Figure 1. Representation of different organization of tissues in bran, endosperm, and germ in a wheat kernel.
(http://www.classofoods.com/page1_1.html)

Bran, which represents about 10-11.5 % of the wheat grain weight, is a composite multi-layer material made up of several adhesive tissues: outer pericarp, inner pericarp, testa, nucellar epidermis, and aleurone layer (Figure 1), with attached starchy endosperm residues (Verma et

al., 2009). An increasing number of studies have lately underlined the high nutritional potential of wheat bran which is currently an undervalued by-product of wheat milling (Hemery et al., 2011).

Wheat bran is highly enriched with cell wall materials and can be heterogeneous with complex polysaccharides, proteins, lipids, and lignin (Lagaerta et al., 2009). Main components of primary polysaccharides are cellulose, hemicellulose and several other pectic polysaccharides. In addition, primary cell walls often contain structural proteins such as the hydroxyproline-rich glycoprotein extensions (Bacic et al., 1988). Cellulose is a linear polymer of (1–4)-linked β-D-glucose, and in the cell walls several cellulose chains are packed into tightly ordered crystalline aggregates called as microfibrils. Microfibrils are deposited around each cell in a random framework and interlocked by hemicelluloses, leading to a strong network (Walter et al., 2009).

Lipid Content in Wheat Bran

Wheat lipids have been studied intensely over many years. Lipids in wheat or wheat flour are considered a minor compositional constituent, and yet they play a major role in wheat production. Whole wheat grain contains about 2.1-3.8% (w/w, db) crude fat. Lipids are most highly concentrated in the germ and bran (Table 1) and the flour contains only 0.75-2.2% (w/w, db) of the crude fat (Strokes et al., 1986). Overall, lipids can be distributed in biomembranes and organelles including spherosomes (membrane bound oil droplets) in oil rich tissues (aleurone and embryo) (Quartacci et al., 1995). Past studies have classified wheat bran lipid compounds into free, i.e. compounds that can be easily extracted by less-polar solvents, (e.g. petroleum ether and diethyl ether) and bound, that is readily extracted by more polar solvents including alcohol mixtures, water-saturated butanol, and by hydrolysis methods (Stokes,1985).

Table 1. Distribution of crude fat in the wheat kernel.

Tissue	Proportion of whole kernel (%)	Crude fat (%)
Whole grain	100	2.1-3.8
Bran	10-11.5	5.1-5.8
Endosperm	74.9-86.5	0.75-2.2
Germ	1.1-2.0	12.6-32.1

Adopted from Morrison, 1978.

Polar lipids (PoL) contain phospholipids (PL) and glycolipids (GL). The PL include phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, and phosphotidylinositol (Carver, 2009). Glycolipids are lipid compounds containing one or more monosaccharide residues linked by a glycosyl linkage to a lipid and are found in the whole kernel and endosperm. Digalactosyldiglyceride (DGDG) and monogalactosyldiglyceride (MGDG) are two common glycolipids (Carver, 2009). The NL and PoL are separated according to their structure and polarity.

Nonpolar lipids (NL) or neutral lipids include triacylglycerides (TAG), monoacylglycerides (MAG), and diacylglycerides (DAG), hydrocarbons, sterol esters (tocopherols and carotenoids), and free fatty acids (FFA). Fatty acids are the main concern in this research. MAG, DAG, and TAG fall into the major category of acylglycerols (Baysal and Demirdoven, 2007). All three carbons in the glycerol structure of the TAG are esterified with FA. The FA's are simple form of lipids that have a carboxylic acids along with hydrocarbon chains and can be in the form of unsaturated (presence of one of more double bonds) or saturated

(with all bonding positions among carbons occupied by hydrogen). Predominant fatty acids in wheat are myristic, stearic, palmitic, palmitoleic, oleic, linoleic, and linolenic acids (Baysal and Demirdoven, 2007).

The degree of unsaturation in lipids and the availability of these fatty acids in mature grains are significantly affected by the environmental conditions during growth, total lipid content in grain, and the activity of lipid-associated endogenous enzymes found in grain (Levand et al., 2009). The most common fatty acid in wheat bran is linoleic acid (Morrison, 1988) and other fatty acids found in wheat bran are listed in the Table 2.

Lipid Cellular Arrangement in Wheat Bran

The epidermis of wheat bran is a very specialized tissue, which plays a critical role in the development and survival of the whole organism (Vick, 1993; Stintzi et al., 2001). Through development, wheat has a compositional and structural variation of the epidermal cell wall, which is known as the cuticle (Quartacci et al., 1995).

Among other functions, the cuticle enables prevention of water loss and provides an effective barrier, which resists attacks from wheat pathogens (Vorwerk et al., 2004). Cuticle differs between species, organs, and developmental stages of wheat, but it is composed of components such as cutin and wax (Quartacci et al., 1995).

Single polyester chains are building blocks of cutin, and are made of hydroxy and epoxy C_{16} and C_{18} fatty acids and few glycerol structures (Dembitsky and Maoka, 2007). However, recent classification of the cuticle in *Arabidopsis thaliana* has discovered that cutin may also contain dicarboxylic acids, which are considered to be components of suberin, the other major polyester in plants (Konopka et al., 2006).

Table 2. Fatty acid composition in whole wheat grain, endosperm, bran, and germ.

Fatty Acid	Whole Grain (%)	Endosperm (%)	Bran (%)	Germ (%)
Myristic 14:0	0.1	Trace	Trace	Trace
Palmitic 16:0	24.5	18.0	18.3	18.5
Palmitoleic 16:1	0.8	1.0	0.9	0.7
Stearic 18:0	1.0	1.2	1.1	0.4
Oleic 18:1	11.5	19.4	20.9	17.3
Linoleic 18:2	56.3	56.2	57.7	57.0
Linolenic 18.3	3.7	3.1	1.3	5.2
Arachidic 20:0	0.8	Trace	Trace	Trace
Other	1.1	1.1	Trace	0.8

Adopted from Morrison, 1988.

Waxes are entrenched in the cuticular matrix or deposited on the surface and consist of a various mixture of very long-chain fatty acids (VLCFAs) and their monomeric derivatives consist of carbon chain lengths ranging from C_{20} to C_{40} (Stokes et al., 1986).

In plants, there are several enzymes that reacting with lipids to form products that are important in providing function to the plant. Among these, LOX is an important enzyme that breaks down fatty acids in wheat bran. As this research is focused on wheat lipids and their role in disease defense, it is important to understand the LOX reactions and the products (hydroxyl fatty acids) formed from the wheat bran fatty acids.

Lipoxygenase in Wheat

Lipoxygenase is found in nearly all plants and animals, including legumes, such as soybeans, mungbeans, navy beans, green beans, peas, and peanuts, and in cereals, such as rye, wheat, oats, barley, and corn (Simone et al., 2010). Past studies have shown that LOX has been shown to hydrolyze a number of polyunsaturated fatty acids (Barnes and Galliard, 1991). According to Auerman et al (1971), there are 17 fold and 4 fold more LOX activity in wheat germ and bran, respectively, than LOX activity in the endosperm. The enzyme LOX hydrolyzes specifically cis, cis-1, 4-pentadiene system, such as linoleic, linolenic, and arachidonic acid, to produce hydroperoxides with a cis, trans conjugated diene structure (Berry and Larreta-Garde, 1997). Linoleic acid makes up about half the total fatty acids in wheat grains (Graveland, 1973). Unlike lipase, LOX, like many other enzymes, is accelerated by adding water to cereal products (Barnes and Galliard, 1991). Wheat LOX has an optimum acidic pH of 5-7 (Baysal and Demirdoven, 2007).

Wheat LOX oxidizes linoleic acid to form the hydroperoxide primarily at carbon-9 (85%) or secondarily at carbon-13 (15%). Metabolism of hydroperoxide generates a variety of

compounds. For example in wheat, a mixture of 9, 10, 13-trihydroxy-trans-11 and 9, 10, 13-trans-10-octadecenoic acid (Tri-OH) has been reported (Berry and Larreta-Garde, 1997). Further, Levandi et al (2009) has identified cis, cis-9, 12-octadecadienoic acid, 9, 10-dihydroxy-12-octadecenoic acid, and 12, 13-dihydroxy-9-octadecenoic acid using LC-MS in different wheat genotypes. Figure 2, is an example of a mass spectra for 9-hydroxy-10-oxo, cis-15 octadecadienoic acid a product of the LOX pathway in wheat after methylation and silylation.

Apart from wheat, Graveland (1973) had identified 2-trans nonenal and 2-methyl furfural in a suspension of barley and further identified as α-hydroxy-keto acids, two isomeric γ-hydroxy-keto acids, and two isomeric dihydroxy acids. Studies have reported that in beans 13-hydroperoxide is capable of forming cis-3-hexanal and 12-oxo-cis-9-dodecenoic acid (Konopka et al., 2006). Oxidation of 9-hydroperoxides forms trans-2, cis-6-nonadienal compounds in beans, which are important plant defense mechanisms against disease (Berry and Larreta-Garde, 1997).

Lipoxygenase from different sources are able to catalyze oxygenation at specific points along the carbon chain, and are referred to as “positional” or “regio” points. Such specifications have significant input for the metabolism of the resultant hydroperoxides into a number of important secondary metabolites, including aldehydes and oxygenated fatty acids (Pomeranz et al., 1966). These end products are often harmful to wheat quality, and additionally, may play a role in the functioning of the wheat (Walter et al., 2009). As LOX is an important part of this discussion it is important to understand the basic principles of LOX activity and how compounds are formed within wheat bran.

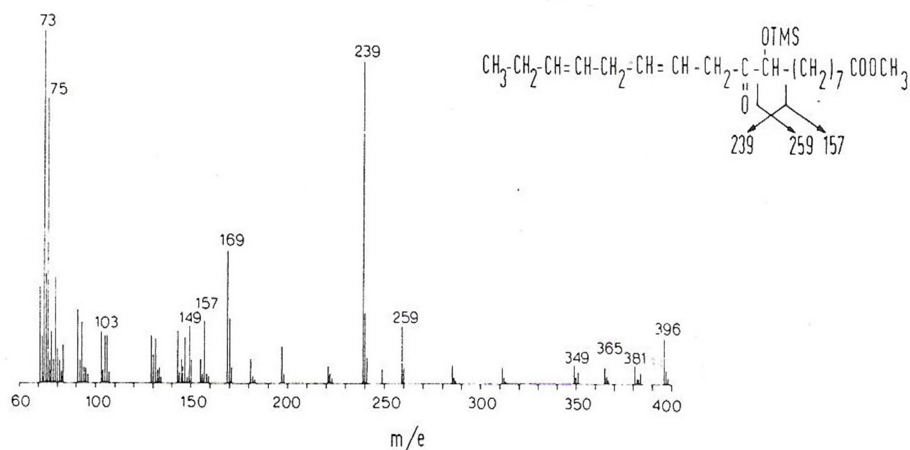
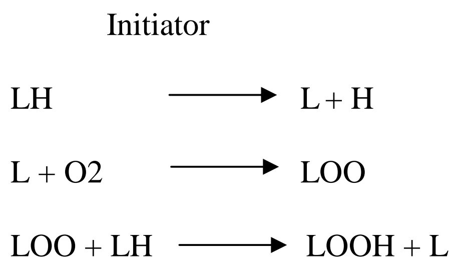


Figure 2. Representative mass spectrum of 9-hydroxy-10-oxo, cis-15-octadecadienoic acid after methylation and silylation.
(Graveland, 1973).

Mechanism of Lipoxygenase Reaction

There are three main reactions that take place in the presence of LOX within the lipid system, i.e. free radical initiation, propagation, and termination (Kim and Min, 2008). Initiation reaction proceed by losing a hydrogen free radical (H•). The resulting lipid free radicals (L•) react with oxygen to form peroxy radicals (LOO•). In this propagation process, LOO• react with more LH to form lipid hydroperoxides (LOOH), the fundamental primary products of autoxidation (Konopka et al., 2006) as depicted in the following scheme**:**



The decomposition proceeds by homolytic cleavage of LO-OH to form alkoxy radicals LO•. These radicals undergo C-C cleavage to form products such as aldehyde, ketones, alcohols,

and hydrocarbons (Figure 3) (Dembitsky and Maoka, 2007). LOX catalyzes the addition of oxygen to the chain reaction to form hydroperoxides. The complete lipid oxidation is composed of four parts (Konopka et al., 2006), (a) the activation of enzyme, (b) the aerobic pathway, (c) the anaerobic pathway, and (d) the non enzymatic pathway. The reactions are similar to those happening during autoxidation, but LOX can act faster than autoxidation and is more precise in terms of end products.

Lipoxygenase oxidizes polyunsaturated fatty acids and is specific for the kind of substrate it oxidizes and how the substrate is oxidized (Dembitsky and Maoka, 2007). LOX should be in the oxidized (Fe^{3+}) form for the oxidation reaction to continue as shown in Figure 4.

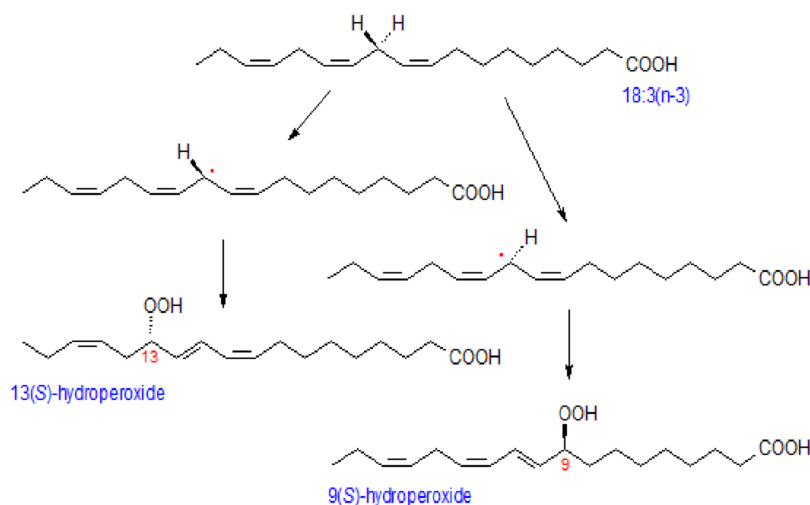


Figure 3. Cascade of reactions in the formation of hydroperoxides from linolenic acid oxidation by Lipoxygenase.
(http://lipidlibrary.aocs.org/lipids/eicplant/index.htm).

The oxidized form (Fe^{3+}) of LOX can catalyze the elimination of hydrogen from the C-11 methylene group of linoleic acid (C18:2) or linolenic acid (C18:3) (Berry and Larreta-Garde, 1997). LOX is reduced to the Fe^{2+} form (Figure 4) and reused in the cycle again (Dembitsky and Maoka, 2007).

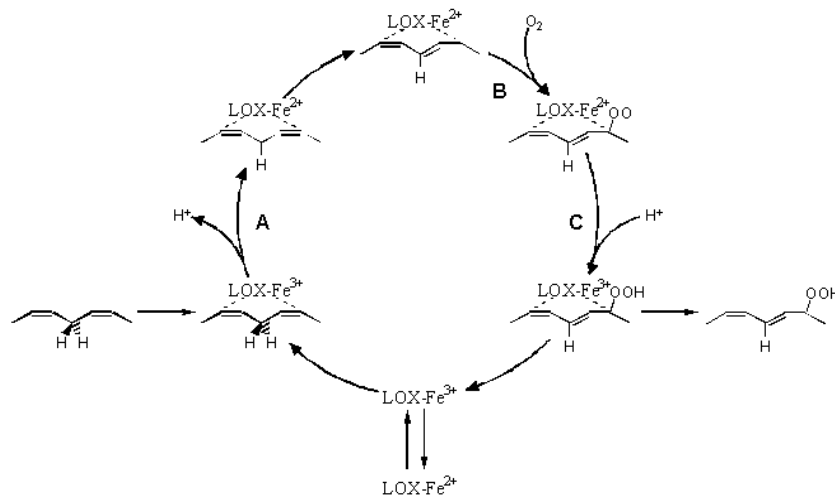


Figure 4. Activation cycle of lipoxygenase enzyme in cereals.
(Peng-Fei et al., 2011).

Role of Lipoxygenase in Plant Defense

There is abundant evidence that LOX is a critical element of the plant defense mechanism (Esaka et al., 1986). Many plants react to insect injure or wounding by the production of jasmonate and the activation of proteinase inhibitor genes in both wounded and non-wounded leaves (Esaka et al., 1986). Removal of wound-induced LOX activity from potato leaves reduces the production of jasmonate in response to wounding, which leads to high susceptibility to insect or fungal attack (Punja, 2004). Although recently jasmonic acid (JA) has been found to be a key regulator in the physiology, reproduction, and resistance in plants (Stintzi et al., 2001). Lipoxygenase in vegetative tissues provides hydroperoxide substrates that can be metabolized to compounds that play important roles in plant defense (Baysal and Demirdoven, 2007).

Need Statement

During the last three decades, wheat breeding has focused more on characterization and identification of genetic resources for reducing susceptibility to FHB (Stack, 2003). However,

only a limited number of genotypes with low susceptibility to FHB have been identified; and the molecular mechanisms of their low susceptibility are still unknown.

The notion that the LOX pathway controls certain physiological events in plants is receiving an increased attention in the academia and research. Primarily, the work is focused on plant senescence, control of enzymatic activity, and wound response (Prost et al., 2005). Products of the LOX pathway may also be produced as defense substances against diseases. For example, oxygenated fatty acids were found to be produced by the rice plant in defense against rice blast disease (Kato et al., 1983). Compounds consisting of monohydroxy, dihydroxy, and epoxy fatty acids were identified in oat/water slurries and speculated to act against FHB (Doehlert et al., 2011).

Therefore, more research needs to be conducted in order to understand the biochemical basis for FHB susceptibility and to identify chemical compounds, such as oxygenated fatty acids, and their possible relationship to FHB susceptibility in wheat. As far as breeding techniques are concerned, screening for resistant wheat genotypes against FHB in the field is relatively difficult and time consuming. Currently, only genetic marker based laboratory tests are available for FHB resistance. Genetic based selections are useful as long as markers are identified for a specific form of resistance. This research investigates the chemical basis for FHB resistance and represents the initiation of the development for a laboratory test for wheat *F. graminearum* resistance based on wheat grain chemistry.

MATERIALS AND METHODS

Wheat Grain

Wheat (HRSW) grains were obtained from the Langdon Research Extension Center in 2010. The genotypes were Samson (Helm et al., 1986), Reeder, Trooper, Dapps (Mergoum et al., 2005), Briggs (Devkota et al., 2007), Steele-ND (Mergoum et al., 2005), Howard (Mergoum et al., 2006), Granger, Granite, Ada (Anderson et al., 2007), Alsen (Frohberg et al., 2006), Barlow, Faller (Mergoum et al., 2008), Glenn (Mergoum et al., 2006), Traverse, Sabin, Jenna, Brennan, and Brick (Glover et al., 2010). Wheat grains were milled by using a Brabender Quadramat Jr. laboratory mill in the NDSU HRSW laboratory. Further, the wheat bran particle size was reduced (screen size 35) using a UDY cyclone mill and stored frozen at -20 to -21°C.

Fusarium graminearum Culture

Fusarium graminearum 08/RG/BF/51 strain was obtained from Dr. Rubella Goswami in the Plant Pathology Department, North Dakota State University, Fargo. This isolate was obtained from North Dakota and was capable of infecting wheat in green-house trials and produced about 255 ppm of DON on wheat.

Chemicals and Reagents

Agar (microbiological agar), carnation leaf and agar, carboxymethylcellulose (CMC) media- ammonium nitrite (NH_4NO_3), potassium phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4.7H_2O$), yeast, distilled water, High-pressure Liquid chromatography (HPLC) grade methanol, potassium hydroxide, diethyl ether, hexane, chloroform, methyl heptadecanoate, acetic acid, sodium sulfate, toluene, sulfuric acid (H_2SO_4), sodium methoxide, pyridine,

Table 3. List of genotypes (categorized according to their susceptibility levels) used in this study.

High Susceptibility [a]	Medium Susceptibility [b]	Low Susceptibility [c]
Samson	Steele-ND	Alsen
Reeder	Howard	Barlow
Trooper	Granger	Faller
Dapps	Granite	Glenn
Briggs	Ada	Traverse
	Sabin	Brick
	Brennan	
	Jenna	

[a] High Susceptibility genotypes including,

Samson (WestBred Wheat Variety Selection Guide, 2012), Trooper, Briggs, Reeder, Dapps (Ransom et al., 2010).

[b] Medium Susceptibility genotypes including,

Howard, Steele ND (Ransom et al., 2011), Granger, Granite, Ada, (Wiersma, 2006), Sabin (Wiersma, 2010), Brennan (Seednet Wheat Variety Selection Guide, 2005), Jenna (Minnesota Wheat Variety Selection Guide, 2011).

[c] Low Susceptibility genotypes including,

Glenn, Barlow, Faller, Alsen, Traverse, and Brick (Ransom et al., 2011).

hexamethyldisilazane, trimethylchlorosilane, and linoleic acid were from Sigma Aldrich (St. Louis, MO).

Preparation of Carnation Leaf Agar Media Plates

F. graminearum cultures were grown on a carnation leaf agar media for good growth, sporulation, and maintenance of the original culture. The carnation leaf agar was prepared with 20 g of agar per liter of distilled water. Agar was autoclaved and slants were made. Forceps were soaked in 100 % ethanol for about a minute and then flamed using a Bunsen burner. The forceps were cooled for about 20 seconds and then at least two of irradiated carnation leaves were carefully placed into each slant tubes. Sterile techniques were used for all operations. The cultures were left in direct light for at least 7 days with loose caps until there is visible growth (Leslie and Summerell, 2006).

Carboxymethylcellulose Stock Spore Suspension Preparation

Stock suspension was prepared by dissolving 0.25 g of NH_4NO_3, 0.25 g of KH_2PO_4, 0.125 g $MgSO_4.7H_2O$, 0.25 g of yeast, and 3.75 g of CMC in 250 mL of distilled water using a 500 mL volumetric flask. Media was boiled on a hot plate by mixing constantly using a stir bar. The flask media was allowed to cool for 5-8 minutes. Media was then autoclaved at 121° C for 15 minutes. After autoclaving, media was allowed to cool for 20 minutes. A carnation leaf was then transferred aseptically into the CMC media. The CMC media with a carnation leaf was then incubated in a shaker (250 r.p.m.) at room temperature and in sun light for 7-14 days until there was sufficient *F. graminearum* spore production. Media was filtered using sterile cheesecloth, glass funnels, and sterile media bottles. Once filtered, 100 mL was transferred into sterile 250 mL centrifuge bottles. These bottles were centrifuged at 13000 r.p.m. for 45 minutes. The

supernatant was decanted and discarded. Pellets of spores were placed in 50 mL sterilized 15% glycerol solution. Bottles were vortexed to re-suspend the spore pellets and frozen for future use (Myrvik et al., 1976).

Calculation for Spore Count

Spore count was determined using a simple heamocytometer, by adding 40 μL of the spore suspension to each side of the measuring plates. Number of macroconidia was observed under 4 x magnification power through the microscope. Due to high concentration of spores the count was taken only in 5 corner squares for the calculations. Each count of five squares was then added and multiplied by five to the dilution factor 10^4 (Myrvik et al., 1976).

Calculation for spore concentration = 137 in five squares

$$= 137 \times 10^4 \text{ cells/ mL}$$

$$= 1.37 \times 10^6 \text{ cells/1000μL}$$

$$= 1/1.37 \times 1000 = 730 \text{ μL}$$

$$=730 \text{ μL (spore volume)} +270 \text{ μL (water)} = 1000 \text{ μL}$$

This calculation was used to determine the spore concentration from the original spore concentration. Wheat bran plates were incubated with 30 μL of the diluted spore suspension.

Sterile Wheat Bran/Agar Petri Plate Preparation for 19 Wheat Genotypes

Wheat bran (8 g) was weighed into a 250 mL glass bottle for each of the 19 wheat genotypes. Agar powder (2 g) was added to each bottle along with the bran samples, and 100 mL of distilled water was added before autoclaving. Autoclaving (121° C) was done in a wet cycle for one hour. Once autoclaving was complete, the media bottles were allowed to cool slightly for

pouring. The media was then poured into sterile plates with an approximate volume of 25 mL in each plate. Sterile plates with the media were covered were left for cooling in the laminar air flow hood and stored bottom side up in a -20 °C cold room to avoid water condensation.

***Fusarium graminearum* Plaque Diameter Measurement**

A day or two prior to inoculating, wheat bran media plates were prepared. From the diluted suspension, 30 µL of the *Fusarium* conidia were inoculated to the center of the media plate. After inoculation, the plates were kept still for 5-10 minutes so that the conidial drop would not spread. The inoculated plates were incubated at 21-23°C until readings were completed. All the plates were prepared in four replicates and data was analyzed for three consecutive days measured in centimeters.

Ergosterol Analysis

Ergosterol was extracted and analyzed as described by Schwarz et al (1995) with modifications. The entire contents of the fungal plates were emptied into 500 mL glass jars and homogenized for 1 minute in 100 mL of HPLC grade methanol using a Polytron homogenizer. Homogenates were centrifuged for 10 minutes at 1000 x g. Twenty five milliliters of methanol supernatant was removed and 3 g of solid KOH was added to the above supernatant. Ten milliliters of hexane was added to the supernatant and incubated at 70°C for 30 minute, then cooled. After cooling, 5 mL of distilled water was added and mixed well. The top hexane layer was removed and the methanol was extracted twice more with 5 mL of hexane. Hexane fractions were pooled into one tube and evaporated using a nitrogen gas. The residual was dissolved in 2 mL of HPLC grade methanol and analyzed by HPLC. The extracts (1 mL) were loaded on a reverse-phase column (3 µm particle size) and the mobile phase was methanol-water (96:4 v/v)

at a flow rate of 1.2 mL/min. The column temperature was maintained at 50°C and ergosterol was detected at an absorbance of 282 nm.

Reconstitution Study

A reconstitution study was preformed according to Doehlert et al (2011) with modifications. Wheat bran (8g) was weighed into a glass bottle with 100 mL of distilled water and autoclaved immediately at 121°C for 15 minutes and another glass bottle with same content of wheat bran, agar, and distilled water was used for the incubated treatment. The mixture of bran and water was incubated for 4 hours at room temperature. Then the mixture was divided into tubes for freeze drying. The freeze dried samples were used for oil extraction. For 8 g of bran, 35 mL of methanol was added and kept in a shaker for 20 min (Figure 5). After the mixing process the tubes were centrifuged at 4,000 x g for 10 min. The supernatants were decanted into 25 mL volumetric flasks. The methanol extraction was repeated and the supernatants were pooled in one tube. Extracts were evaporated to dryness in a Rotovap evaporator, under a vacuum created by an aspirator in a water bath at 60°C. Samples were re-suspended in 10 mL of diethyl ether and added to dry defatted wheat bran. The ether was allowed to evaporate overnight before water was added to flour for incubation and media process. Methanol was used to extract lipids from wheat bran and because other compounds could possibly be found in this extract, it will be referred to as “methanol soluble compound” or “MSC” through the rest of the study. The MSC were reconstituted with the bran between the two wheat genotypes as shown in Figure 6. The plates were inoculated with fungal spores as previously described in wheat bran/agar plates with and without incubation in water for 4 hours. Plates were inoculated and plaque diameter readings were taken for 3 consecutive days and ergosterol was analyzed as mentioned previously on the third day the plaque diameters readings were taken.

The experimental design for the reconstitution study is shown in Figure 6. Treatments were applied to Samson (high susceptibility) and Glenn (low susceptibility) wheat bran. The control and non-incubated treatments were non-defatted wheat bran and all the other treatments were defatted. Glenn MSC is where Glenn MSC were added into the Glenn or Samson defatted wheat bran. Samson MSC is where Samson MSC were added into Glenn or Samson defatted wheat bran. Removed MSC is where MSC were removed from Glenn or Samson wheat bran. The methanol added treatment was the addition of methanol to Glenn or Samson wheat bran allowing for its complete evaporation.

Lipid Extraction

Lipids were extracted according to Bligh and Dyer (1959) with few modifications. One mg methyl heptadecanoate (internal standard) was added to each sample before extraction. Wheat bran (0.25 g) was homogenized in a mixture of 1.5 mL of water and 6 mL of chloroform-methanol (1:2 v/v) using a Polytron homogenizer (speed 5) for 2 minutes. After homogenization, 2 mL of water and 2 mL of chloroform were added; the mixture was shaken vigorously and then centrifuged for 10 minutes at 1200 x g. The bottom organic layer was transferred into a new tube and 6 mL of chloroform and 0.2 mL of acetic acid were added to the aqueous phase. After vigorous mixing, the tubes were again centrifuged and the bottom layers combined with the first layers. Extracts were then dried using sodium sulfate and filtered into 15 mL test tubes. Samples were finally flushed with nitrogen and stored at -18°C.

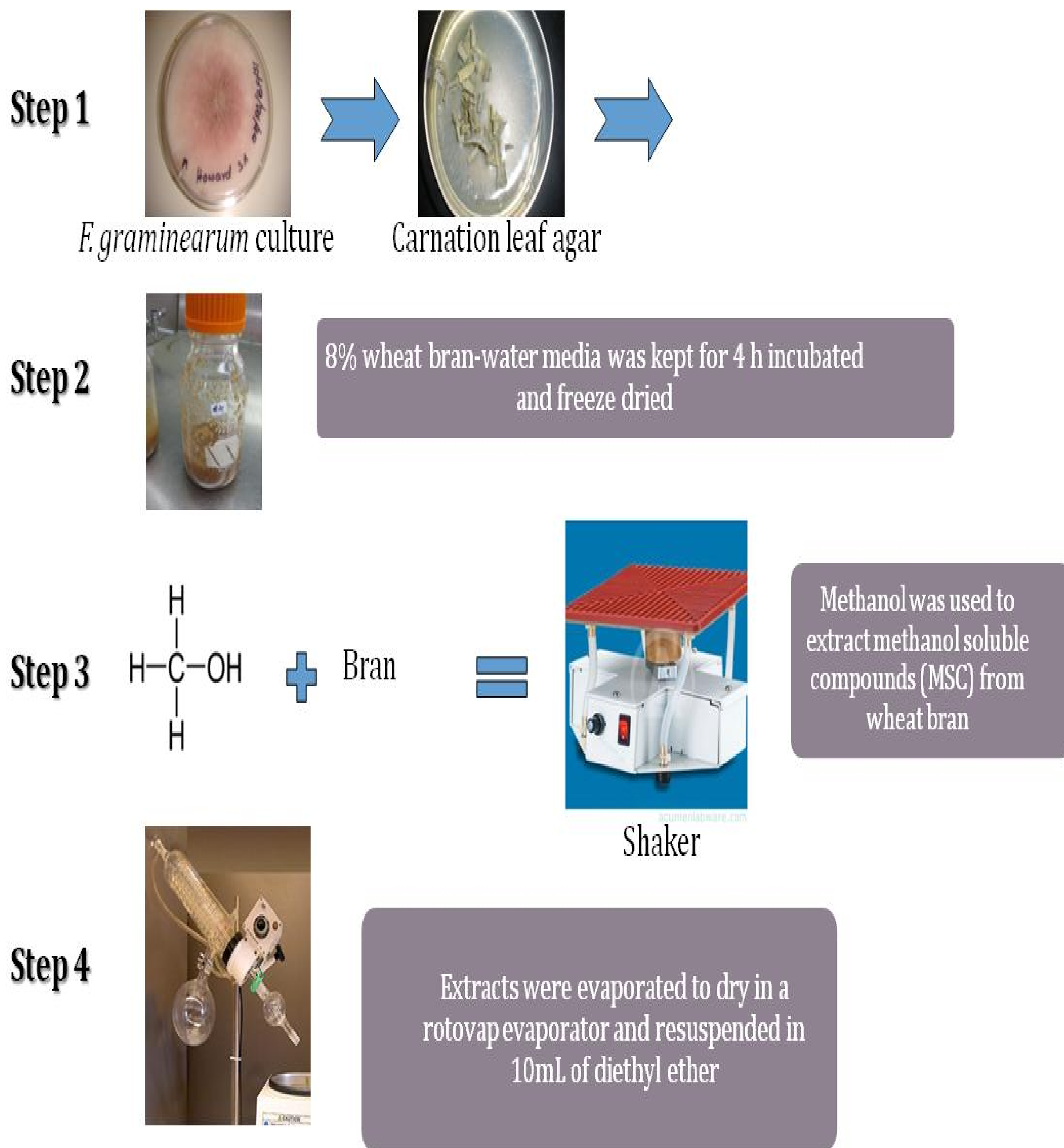


Figure 5. Schematic representation of methanol extraction from wheat bran. CMC-carboxymethylcellulose and MSC-methanol soluble compounds.

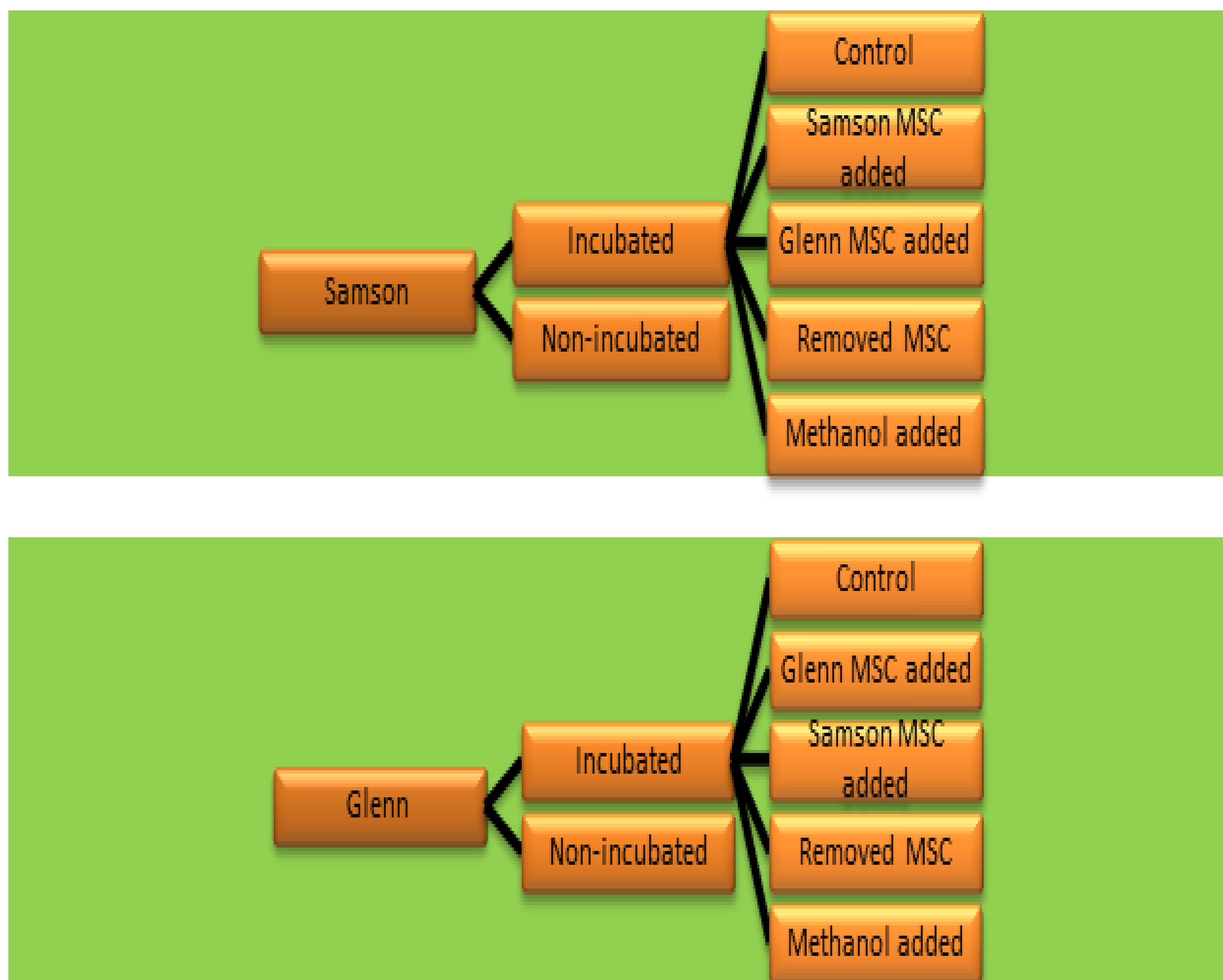


Figure 6. Representation of different treatment combinations used in reconstitution study. MSC-Methanol soluble compounds.

Fatty Acid Methylation

Total Fatty Acids (TFA)

TFA were methylated with acidic methanol. A lipid sample was dissolved in 1 mL of toluene, and 2 mL of 2% sulfuric acid in methanol was added. The mixture was left for 1 hour in a stoppered tube at 100°C and the esters were extracted in hexane as described by Doehlert et al (2010) with few modifications.

Esterified Fatty Acids (EFA)

Esterified fatty acids in the lipid samples were methylated by base-catalyzed transesterification (Doehlert et al., 2010). The lipid samples were dissolved in 1 mL of toluene. A mixture of 0.5 M sodium methoxide in anhydrous methanol (2 mL) was added and the solution maintained at 50°C for 10 min. As a result the nonesterifed free fatty acids were removed into high pH water phase during extraction of methyl esters with hexane.

Free Fatty Acids (FFA)

Free fatty acids were methylated with diazomethane as described by Christie (1989). Unmethylated fractions were not separated before injection.

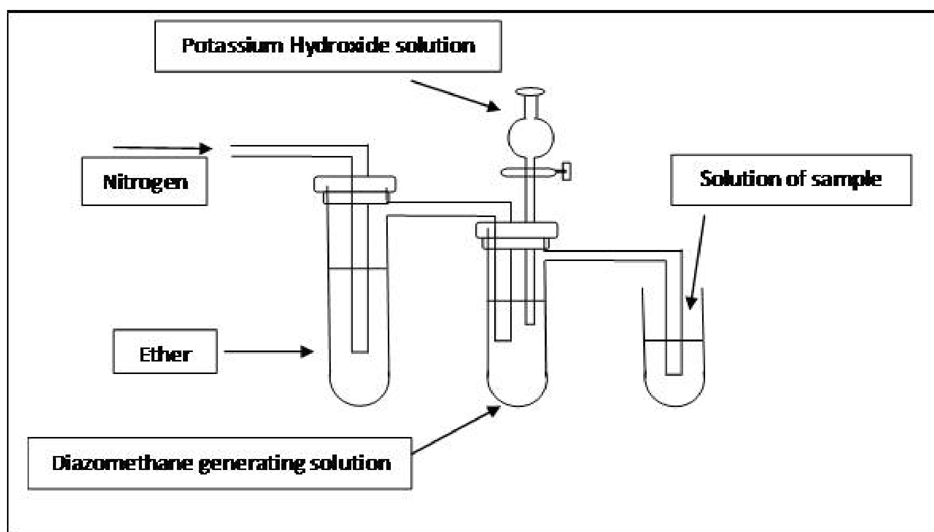


Figure 7. Representation of the diazomethane apparatus used in free fatty acid analysis.

Silylation

To determine the oxidized fatty acids by gas chromatography/mass spectroscopy (GC/MS), it was necessary to silylate all free hydroxyl groups. The methylated dried lipid

samples were dissolved in 1mL pyridine, and 0.3 mL hexamethyldisilazane and 0.1 mL trimethylchlorosilane were added. The mixture was allowed to stand for 15 minutes and taken to dryness by flushing with nitrogen. It was then dissolved in 2 mL of hexane, transferred into gas chromatography 2 mL vials, sealed, and stored at -18°C until analysis (Doehlert et al., 2010).

Gas Chromatography-Mass Spectrometry

Hewlett-Packard (Chemical Analysis Group, San Fernando, CA) 5890 series II gas chromatograph with a 7673 autosampler and a Hewlett-Packard 5971 mass selective detector used to quantify the methylated and silylated fatty acids. A Supelco (Supelco Park, Bellefonte, PA) capillary column (SP-2560; 100 m x 0.25 mm i.d) was used, with He as carrier gas. Injector and detector temperature were 260°C. Oven temperature gradient was 140-240°C at 4°/minutes. The results are expressed as % of the total ion signal. Methylated fatty acids were identified by comparing the retention times of the peaks in the samples with those of pure fatty acids. Oxidized fatty acids were identified from mass spectra.

Quantification of Oxidized Fatty Acids in Samson and Glenn Genotypes

Oxidized fatty acid were quantified using the following equation (Doehlert et al., 2010),

$OFA_{FFA} = (OFAS_{FFA}/TFAS_{FFA}) \times (FFA/TFA)$

$OFA_{EFA} = (OFAS_{EFA}/EFAS_{EFA}) \times (EFA/TFA)$

$OFA_{TFA} = OFA_{FFA} + OFA_{EFA}$

OFA_{FFA} = Percentage of oxygenated fatty acid from free fatty acids

OFA_{EFA} = Percentage of oxygenated fatty acid from esterified fatty acids

OFA_{TFA} =Percentage of oxygenated fatty acids from Total fatty acids

$TFAS_{FFA}$ = Free fatty acids percentage in total fatty acids

$EFAS_{EFA}$= Free fatty acids percentage in esterified fatty acids

Above calculations were based on the total fatty acid content in the incubated Glenn and Samson bran treatments. Values were analyzed using their main fatty acid (linoleic) peak areas in both genotypes. Oxygenated fatty acids from free fatty acids and oxygenated fatty acids from esterified fatty acids were calculated individually and added together to find the percentage of total oxygenated fatty acids.

Spiking Study

Media Preparation

Carboxymethylcellulose was prepared as previously explained and fresh F. graminearum spore stock solution was made for spiking method.

Wheat Bran Plate Preparation

Plates were prepared with same concentration as mentioned in previous methods; however, there was 320 mg/g of linoleic acid added into each plate. Similar to reconstitution method, spiked samples had incubated and non-incubated both treatments (Figure 8). Plates were prepared as described earlier with same conditions using autoclaving, pouring of plates, and storage until use. Plates were inoculated using the same fungus with 30 µL in the center and plaque diameter readings were taken for three consecutive days with four replicates and ergosterol was analyzed for each sample with the use of HPLC on the third day of plaque diameter readings were taken. Incubated and non-incubated samples were analyzed through GC-

MS. Wheat bran/ agar media with water were left for 4 hours at R.T. and this is referred to as incubated and mixture with wheat bran/ agar media with water when not left for 4 hours at R.T. is referred to as non-incubated.

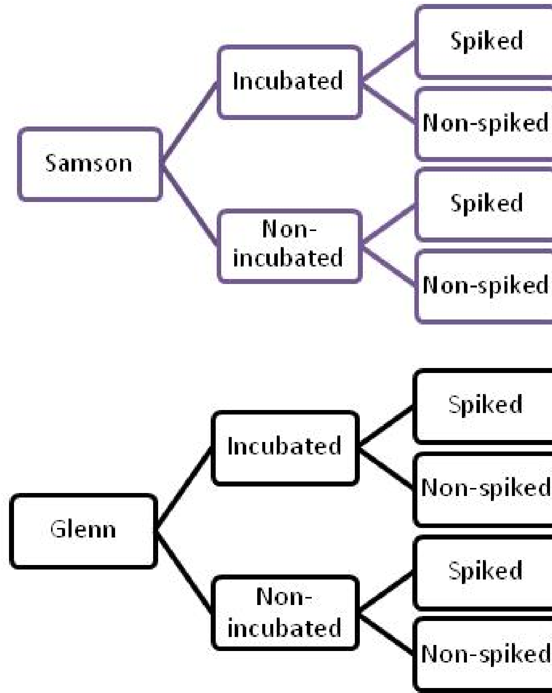


Figure 8. Experimental design of different treatments used in the spiking study.

Statistical Analysis

Study one with 19 HRSW genotypes with various FHB susceptibilities was analyzed statistically using randomized complete block design (RCBD) with four replicates. Bartlett's test was conducted to determine the error variance homogeneity. Since the error of homogeneity was not significant, data was transformed using natural log transferring system. Transformed data was used for ANOVA. Study two was again performed with RCBD split-split-plot design, genotype being the main plot, treatment being the sub-plot, and day being sub-sub-plot. Study three was the spiking experiment and the design used was a RCBD with split-split-plot design,

genotype being the main plot, incubation time being the sub plot, and treatment being the sub-sub-plot. For comparison of means, least significant (LSD) was applied. Correlation between total FFA percentage and the growth of *F. graminearum* in wheat/bran plates were examined. The Statistical software (SAS) for windows (V.9.2) (SAS institute, Cary, NC) used with 'MIXED' procedure with 'satterth' option. In this model cultivar and other treatments are assumed as fixed effects and replication is considered as a random effect.

RESULTS AND DISCUSSIONS

There are three main sections in this research, which are as follows;

1. *F. graminearum* growth in wheat bran/ agar plates was measured using nineteen wheat genotypes with various FHB susceptibilities: Plaque diameters were determined, ergosterol analysis was performed and fatty acid profiles in bran samples were analyzed with GC-MS.
2. A reconstitution study was performed using one low and one high susceptibility wheat genotypes where lipids were exchanged from high susceptibility genotypes with low susceptibility genotypes: Plaque diameters were determined and ergosterol analysis was performed.
3. A spiking study was performed using one low and one high susceptibility wheat genotypes where bran slurries were enriched with linoleic acid: Plaque diameters were determined, ergosterol analysis was performed and fatty acid profiles in bran samples were analyzed with GC-MS.

Growth of *Fusarium graminearum* in Wheat Bran/Agar Plates

Nineteen genotypes (Table 3) were used to prepare wheat bran/agar plates which were inoculated with *F. graminearum* spore suspension. Plaque diameter readings were taken for 4 consecutive days. In all of these microbiological experiments the nutrition source is the wheat bran which supplies the survival of the fungus whereas, agar is used as a source of solid and uniform media for the fungal growth. Based on the plaque diameters (Table 4), *F. graminearum* had the most growth on day 4 compared to days 1, 2 and 3 on wheat bran/agar plates. *F. graminearum* growth had a positive correlation in relationship to disease susceptibility in the

three categories as shown in figure 9, which represents the pooled distribution of the three susceptibility categories. The slopes were mainly linear and each day was analyzed separately as shown in the ANOVA tables (Table 5). Since the Bartlett's test (Table 6) was not homogenous, the data was transformed using the log natural transformation for statistical analysis using SAS. Results will be discussed following the order of high, medium and low susceptibility genotypes.

The high susceptibility category consisted of Samson, Reeder, Trooper, Dapps, and Briggs. Overall, all the genotypes in this category had a significantly higher growth rate of *F. graminearum* in wheat bran /agar plates than that of medium and low susceptibility categories. The highest plaque diameter on day 4 was recorded from Samson genotype at 7.80 cm (Table 4). No growth could be observed at day 1. *F. graminearum* plaque diameter readings were observed on day 2; however the diameter readings were slow in growth. This could be due to the time taken for the *F. graminearum* to grow on the wheat bran/agar culture plates. As seen in figure 9, day 3 and day 4 had significantly different ($p<0.05$) results with increased level of fungal plaque diameter. It is likely that in the field when exposed to this fungus, high susceptibility genotypes will have a higher growth rate.

For the medium susceptibility category, the growth rate of *F. graminearum* was significantly different after four consecutive days ($p<0.05$). This category includes Steele-ND, Howard, Granger, Granite, Ada, Sabin, Brennan, and Jenna. Day 2 had the same effect of increasing growth rate of fungus, but the plaque diameter growth rate of *F. graminearum* in the medium susceptibility genotypes was lower than the high susceptibility category.

Table 4. List of genotypes (categorized according to their susceptibility levels) used in this study.

Genotype	Day 2 (cm)	ln	Day 3 (cm)	ln	Day 4 (cm)	ln
Samson	3.70	1.30	5.80	1.76	7.80	2.05
Reeder	3.10	1.14	5.50	1.70	7.40	2.00
Trooper	2.80	1.02	5.40	1.68	7.30	1.99
Dapps	2.70	0.99	5.30	1.67	7.10	1.96
Briggs	2.70	0.99	5.20	1.65	6.70	1.91
Steele	2.50	0.92	5.10	1.63	6.60	1.89
Howard	2.40	0.88	5.10	1.62	6.50	1.87
Granger	2.40	0.89	4.90	1.60	6.20	1.83
Granite	2.40	0.88	4.80	1.56	6.20	1.82
Ada	2.40	0.86	4.70	1.54	6.20	1.82
Sabin	2.40	0.85	4.60	1.52	6.20	1.82
Brennan	2.30	0.82	4.50	1.50	6.00	1.79
Jenna	2.30	0.81	4.40	1.48	6.00	1.79
Alsen	2.10	0.75	4.30	1.47	5.80	1.76
Barlow	2.10	0.75	4.30	1.45	5.70	1.74
Faller	2.10	0.72	4.20	1.44	5.60	1.72
Glenn	2.00	0.71	4.10	1.42	5.50	1.70
Traverse	2.00	0.70	4.00	1.40	5.40	1.69
Brick	2.00	0.67	3.90	1.36	5.30	1.67
LSD (5 %)	-	0.03	-	0.01	-	0.01

Table 5. ANOVA table for plaque diameter readings using 19 wheat genotypes with various Fusarium head blight susceptibility (natural log transformation).*

Source	Deg of Freedom	Sum of Square	Mean Square	F Value	Pr > F
Day 2					
Gtype	18	1.8375	0.1021	220.70	<0.0001
Rep	3	0.0014	0.0005	1.00	0.4045
Error	54	0.0250	0.0005		
Day 3					
Gtype	18	0.9389	0.0522	521.53	<0.0001
Rep	3	0.0003	0.0001	1.04	0.3825
Error	54	0.0054	0.0001		
Day 4					
Gtype	18	0.8906	0.0495	679.60	<0.0001
Rep	3	0.0006	0.0002	2.96	0.0403
Error	54	0.0039	0.0001		

*Day 1 had zero growth, Gtype-Genotype, Rep-Replication.

Table 6. Bartlett's Test for homogeneity of diameter error variance among day 2, 3, and 4.*

Source	DF	Chi-Square	Pr > ChiSq
Day 2	18	10.0475	0.9303
Day 3	18	17.6986	0.4757
Day4	18	18.0161	0.3878

*Day 1 had zero growth, DF-degree of freedom.

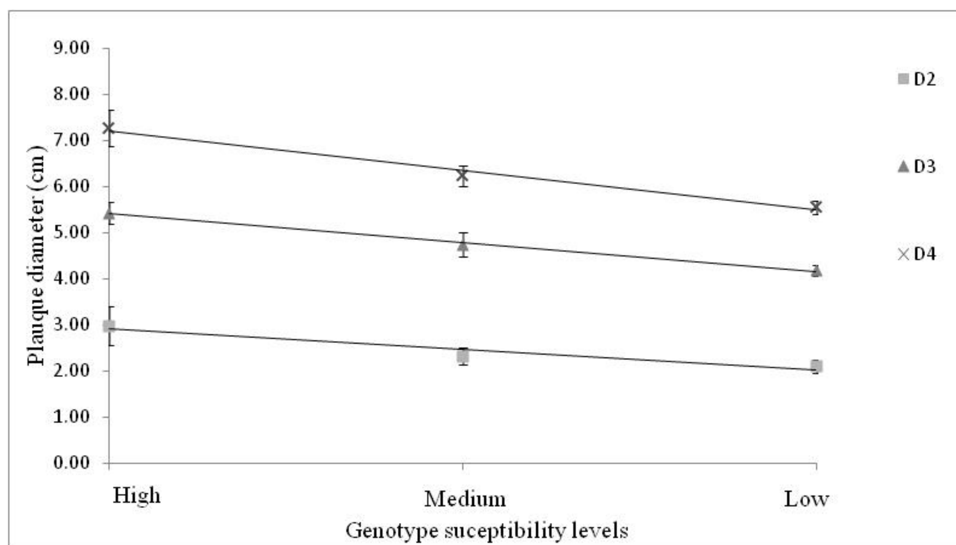


Figure 9. *Fusarium graminearum* growth in wheat bran/agar using 19 hard red spring wheat genotypes after four days of inoculation.
Each day was individually analysed by SAS and significanly different at $p<0.05$. D1 had zero growth.

Low susceptibility wheat genotypes had the least *F. graminearum* growth on the wheat bran/agar plates in this experiment. These wheat genotypes consisted of Alsen, Barlow, Faller, Glenn, Traverse, and Brick. Day 2, 3, and 4 had a relatively lower amount of the fungus growth. Regression analysis of plaque diameter and genotypic FHB susceptibility yield values of 0.9204 at day 2, 0.964 at day 3, and 0.9857 at day 4 (Figure 9). All these results were confirmed at a 95% significance confidence level.

Biomass of *F. graminearum* in each wheat bran/agar plate was confirmed by analysis of ergosterol. Ergosterol is a sterol (component of fungal cell membrane) that is found only in fungi and it does not occur in plant or animal cells (Pitt and Hocking, 2009). Generally, ergosterol is a highly unstable structure to quantify, and it is light sensitive and proper storage conditions must be maintained once it has been extracted. Ergosterol analysis was performed after taking the diameter readings on day 4. Results from wheat bran/agar plate ergosterol analysis (Figure 10)

showed that there is a significant difference ($p<0.05$) between genotypes of varying susceptibility. The data from diameter readings and ergosterol analysis showed similar trends for the growth of *F. graminearum* (Figures 9 and 10).

Samson, high susceptibility wheat genotype had wheat bran/agar plates with 0.62 mg/ per culture of ergosterol. The medium susceptibility genotypes had ergosterol levels of 0.42 mg/ per culture to 0.52 mg/ per culture (Table 7) ranging from lowest value to highest. Lowest susceptibility genotypes had lower values recorded from the ergosterol analysis ranging from 0.18 mg/ per culture to 0.39 mg/ per culture.

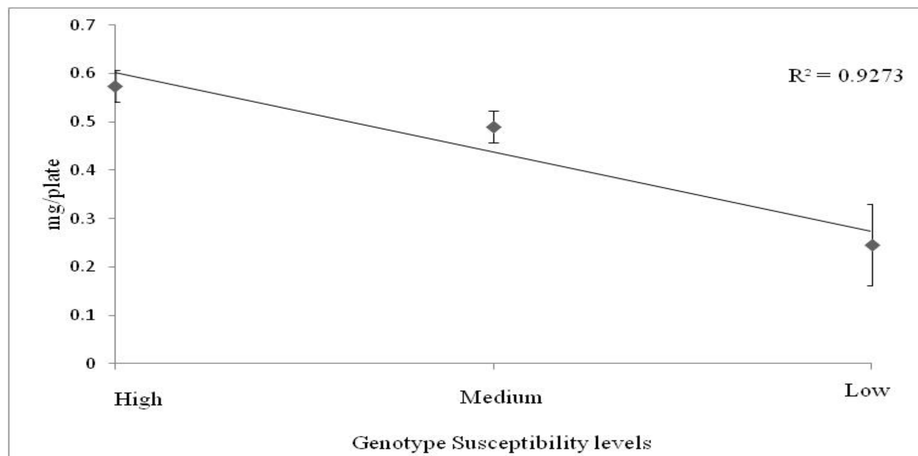


Figure 10. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates using 19 hard red spring wheat genotypes after the day 4.
Regression was significantly different at $p<0.05$.

Statistically, wheat genotypes within each category did not show a large variation. All of these results were confirmed at a 95% significance confidence level (Table 8). Thus, ergosterol analysis has confirmed that there is lower growth of *F. graminearum* on cultures made from low susceptibility genotypes than that of cultures made from high and medium susceptibility

genotypes. Also, the medium susceptibility genotypes had moderate growth of *F. graminearum* as compared to the high susceptibility genotypes.

Overall, the *F. graminearum* plaque diameter growth on wheat bran/agar plates was positively correlated with susceptibility levels of the genotypes. High plaque diameter growth and low plaque diameter growth was observed in high susceptibility genotypes and low susceptibility genotypes, respectively. A similar study was reported by Doehlert et al (2011), in which they tested *Fusarium* growth on flour cultures of oat, wheat, and barley. Their results reported higher growth of *F. graminearum* in wheat and barley cultures than those of oats. Oats are known to be less susceptible to FHB than wheat and barley. Ergosterol levels in oat cultures were also lower than those in wheat and barley (Doehlert et al., 2011).

The correlation between *Fusarium* growth on bran/agar plate cultures and FHB susceptibility as determined in the field suggests that some chemical component in the wheat bran was responsible for the variation is susceptibility; we speculate that products of lipid oxidation by LOX may contribute to the inhibition of *Fusarium* growth.

Doehlert et al (2011) incubated the cultures by exposing oat flour with water. Some scientists claim that incubating plant tissue in water provide time and proper environment for enzymes to function (Barnes and Galliard, 1991). For example, LOX in cereals can be activated by addition of water.

Since there are studies that show adding water and incubating for a number of hours could possibly increase the activity of the enzymes. The effect of incubating wheat bran/ agar media with water for 4 hours was tested in the next section of the thesis.

Table 7. Analysis of ergosterol in wheat bran/agar plates using 19 hard red spring wheat genotypes inoculated with *Fusarium graminearum* after day 4.

Genotype	Ergosterol (mg/Plate)
Samson	0.62
Reeder	0.60
Trooper	0.56
Dapps	0.56
Briggs	0.54
Steele	0.52
Howard	0.51
Granger	0.53
Granite	0.50
Ada	0.48
Sabin	0.48
Brennan	0.48
Jenna	0.42
Alsen	0.39
Barlow	0.30
Faller	0.21
Glenn	0.20
Traverse	0.19
Brick	0.18
LSD (5 %)	0.02

Table 8. ANOVA table for ergosterol analysis in wheat bran/agar plates using 19 hard red spring wheat genotypes inoculated with *Fusarium graminearum* after day 4.

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Replication	3	0.000	0.000	0.7	0.5357
Genotype	18	1.532	0.085	423.9	<.0001
Error	54	0.011	0.003		

Reconstitution Studies between Low and High Susceptibility Genotypes

Wheat bran/agar plates were prepared as described in the previous study, however the mixture of wheat bran, agar, and water was kept for 4 hours at room temperature. The 4 hour time period will be later referred to as incubation. After the incubation period, the plates were autoclaved and stored in a cold room (-20°C) and brought into room temperature before inoculation. The plates were inoculated with the *F. graminearum* spore suspension and diameter readings were taken for 3 consecutive days. A reconstitution study was conducted with the selected wheat genotypes Glenn and Samson. These genotypes were selected according to the FHB susceptibility. Samson was used as a high susceptibility genotype and Glenn was used as a low susceptibility genotype.

This study would help detect the presence of chemical components of the bran from low susceptibility genotypes which may be involved in FHB susceptibility. Methanol was chosen as the solvent to extract more of the highly polar oxygenated fatty acids. The fungal plaque diameter growth was significantly decreased (Figure 11 and Table 9) when MSC from Glenn were added to the defatted Samson bran. When MSC from Samson were added to defatted Glenn

bran, *F. graminearum* growth was significantly increased. Pure methanol was added and evaporated from the wheat bran to prevent speculation that the methanol could be involved with inhibition of *F. graminearum* growth. Addition of pure methanol to the bran had no effect, after evaporation, ($p <0.05$) on *F. graminearum* growth in wheat bran/agar plates.

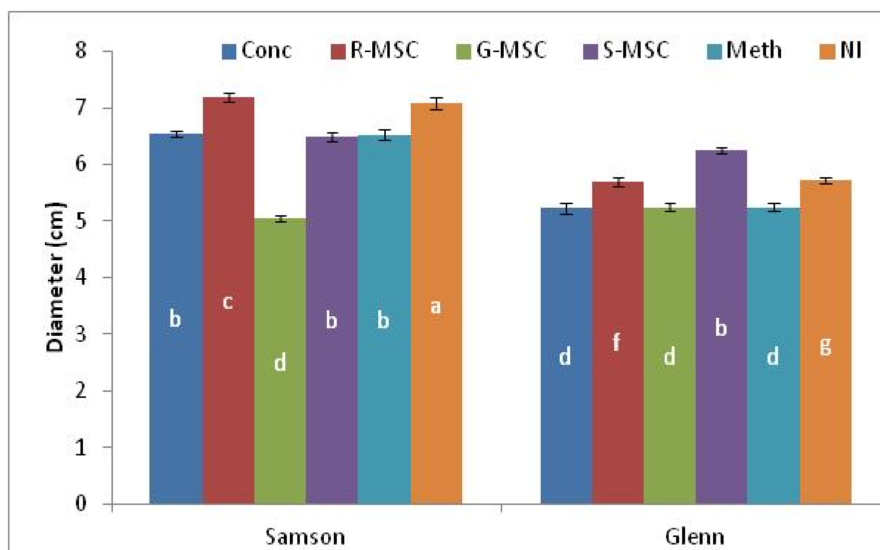


Figure 11. Plaque diameters of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study.
Diameters with same letters are not significantly different at $p<0.05$. Conc= Control (incubated), R-MSC=Removed MSC, S-MSC=Samson MSC, G-MSC=Glenn MSC, Meth= Methanol added, NI= non-incubated.

As expected, the growth of fungus on wheat bran/agar plates treated with pure methanol had similar diameters as the plates without any methanol treatment (measured as control). Fungal diameters were significantly higher when MSC were removed from both Glenn and Samson genotypes than the control samples, pure methanol treatment or samples with the MSC added back to the same genotypes. The non-incubated wheat bran treatment had higher fungal growth than incubated control treatment and these results were significantly different at 95% confidence level (Table 10). As expected the plaque diameter values and ergosterol content of the wheat

bran/agar plates followed the same trends. Treatments with MSC added to the same wheat bran was recorded as 1.06 mg/ per culture for Glenn and 3.81 mg/ per culture for Samson and which were not significantly different from the control and methanol added treatments (Figure 12 and Table 11). Non-incubated wheat bran plates had significantly higher *Fusarium* growth compared to all the other treatments ($p<0.05$) (table 12).

Detection of significant difference in terms of *F. graminearum* growth between the incubated and non-incubated wheat bran/agar media indicated that there may be formation of compounds during the incubation that inhibit the fungus growth (Figure 11).

In this study, we speculate that the products of LOX reactions could be partially responsible for this inhibition. In summary, according to statistical analysis there was significant difference in *Fusarium* growth ($p<0.05$) in cultures made from high and low susceptibility genotype bran. From what was observed in the reconstitution study, there appear to be compounds found in these genotypes which contribute to fungal inhibition.

We can suspect that some of these compounds could be found in the outer layers of wheat bran since the bran was used as the primary source of nutrient in the culture media. The study conducted by Doehlert et al (2011) had identified a few compounds such monohydroxy, dihydroxy, and epoxy fatty acids from oats that would possibly prevent the FHB disease at a certain level. In their study hexane-soluble compounds were extracted from oats and added into wheat. This reconstitution of wheat and oat hexane extracts had resulted in inhibition of *Fusarium* growth on wheat cultures. However, there are also similar studies showing that addition of salicylic acid to an agar culture media can reduce the production of DON, which is a mycotoxin produced by *F. graminearum* (Peng-Fei et al., 2011).

Table 9. ANOVA table for ergosterol analysis in wheat bran/agar plates using 19 hard red spring wheat genotypes inoculated with *Fusarium graminearum* after day 4.*

Genotype	Day	Treatment	Diameter (cm)
Glenn	3	Con	5.23
	3	G-MSC	5.25
	3	Meth	5.25
	3	NI	5.73
	3	R-MSC	5.70
	3	S-MSC	6.25
Samson	3	Con	6.55
	3	G-MSC	5.05
	3	Meth	6.53
	3	NI	7.08
	3	R-MSC	7.20
	3	S-MSC	6.50
		LSD (5 %)	0.107

*Day 1 had zero growth. Con = Control (incubated), G-MSC= Glenn methanol soluble compounds, Meth= Methanol added, NI= Non incubated, R-MSC= Removed methanol soluble compounds, S-MSC= Samson methanol soluble compounds. LSD 0.107 is used to compare between the two genotype treatments.

Table 10. ANOVA table for plaque diameters of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study.*

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Rep	3	0.06	0.02	6.30	0.0832
Gtype	1	12.26	12.26	3565.10	<0.0001
Error (a)	3	0.01	0.00		
Trt	5	11.29	2.26	389.30	<0.0001
Gtype *Trt	5	5.76	1.15	198.80	<0.0001
Error (b)	30	0.17	0.01		
Day	1	133.25	133.25	25413.80	<0.0001
Gtype *Day	1	0.98	0.98	186.90	<0.0001
Trt*Day	5	2.30	0.46	87.90	<0.0001
Gtype *Trt*Day	5	0.89	0.18	33.80	<0.0001
Error (c)	36	0.19	0.01		

*Gtype- genotype, Trt- Treatment, Rep- Replication, DF-degree of freedom.

Some of the inhibition observed in this research needed to be further clarified and identified, using an analytical technique such as GC-MS in incubated and non-incubated wheat bran, which is discussed in the next section of this thesis.

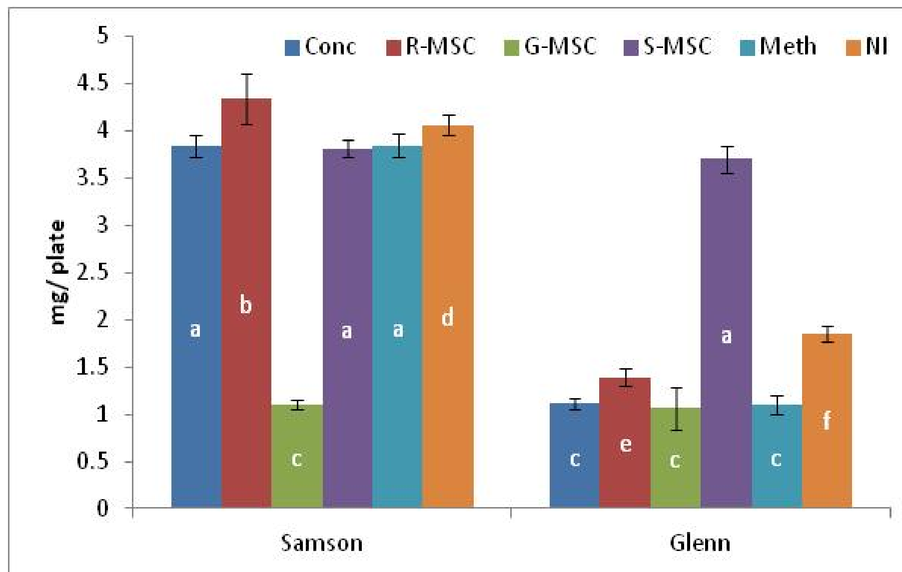


Figure 12. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study.
Ergosterol with same letters are not significantly different at $p<0.05$. Conc = Control (incubated), R-MSC=Removed MSC, S-MSC=Samson MSC, G-MSC=Glenn MSC, Meth= Methanol added, NI= non-incubated.

Table 11. ANOVA table for ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study.

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Rep	3	0.08	0.03	5.05	0.11
Gtype	1	38.78	38.78	7227.82	<.0001
Error (a)	3	0.02	0.01		
Trt	5	30.95	6.19	320.76	<.0001
Gtype *Trt	5	18.47	3.69	191.49	<.0001
Error (b)	30	0.58	0.02		

Table 12. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates prepared using Glenn (low susceptibility) and Samson (high susceptibility) genotypes in the reconstitution study on the third day.*

Genotype	Treatment	Ergosterol (mg/plate)
Glenn	Con-I	1.11
	G-MSC	1.06
	Meth	1.10
	NI	1.85
	R-MSC	1.39
	S-MSC	3.70
Samson	Con-I	3.84
	G-MSC	1.10
	Meth	3.84
	NI	4.07
	R-MSC	4.34
	S-MSC	3.80
	LSD (5 %)	0.20

*Con-I= Control-incubated, G-MSC= Glenn methanol soluble compounds, Meth= Methanol added, NI= Non incubated, R-MSC= Removed methanol soluble compounds, S-MSC= Samson methanol soluble compounds. LSD 0.2 is used to compare data between the two genotype treatments.

Lipid Analysis

Identification of Oxidized Fatty Acids by Diazomethane Methylation and Gas Chromatography-Mass Spectrometry Analysis

Three fatty acid methylation methods were used for derivitization, 1) methylation by acid catalysis (for determination of total fatty acids), 2) methylation by base-catalyzed transesterification (for determination of esterified fatty acid), and 3) methylation by diazomethane (for determination of free fatty acids). Gas chromatography-mass spectrometry (GC-MS) of methylated wheat bran samples indicated the presence of the following fatty acids in decreasing abundance: linolenic acid, linoleic acid, oleic acid, stearic acid, and palmitic acid (Figure 13).

Methylation by diazomethane analysis indicated the presence of oxygenated fatty acids from linolenic (18:3) and linoleic (18:2) acid. For structural identification, these fatty acids were methylated and silylated. Silylation converts methyl esters with hydroxyl groups into their trimethylsilyl (TMS) derivatives, which are more volatile. The hydrogen of the hydroxyl group is substituted with a TMS group [$-Si(CH_3)_3$] (Christie, 1989). In general, the mass spectra of all silylated hydroxyl methyl esters exhibit a major peak in their mass spectra at m/z 73 [$(CH_3)_3Si^+$] (Kleiman and spencer, 1973).

After silylation, there were additional peaks detected by the GC-MS analysis, which is an indication of presence of oxidized fatty acids or fragments. GC-MS results will be discussed for the compounds that elute at specific retention times in chromatograms. The selected elution times for each mass spectrum are 34.4 minutes and 35.3 minutes.

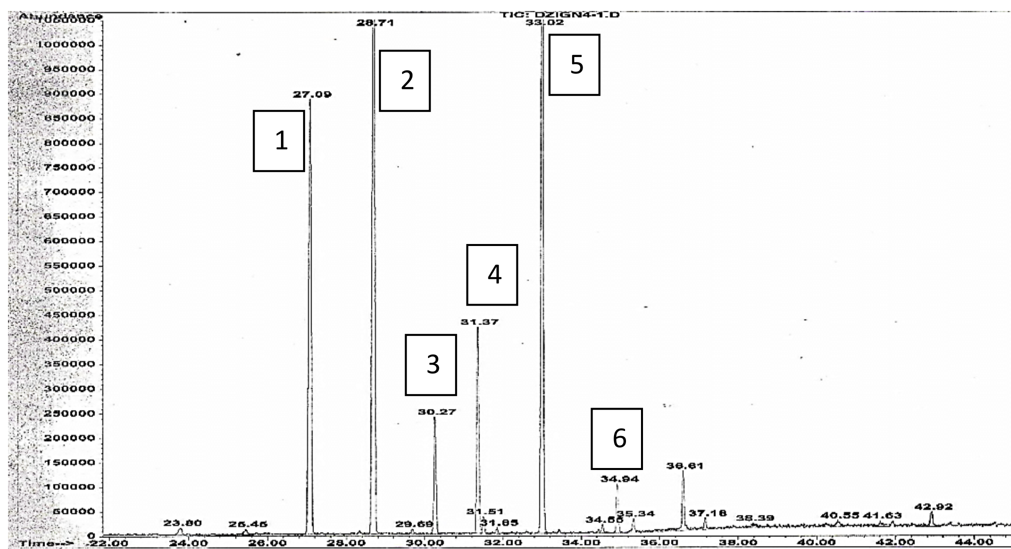


Figure 13. Standard gas chromatogram which represents 1-palmitic acid, 2-Internal Standard, 3-stearic acid, 4-oleic acid, 5-linoleic acid, 6-linolenic acid fatty acids.

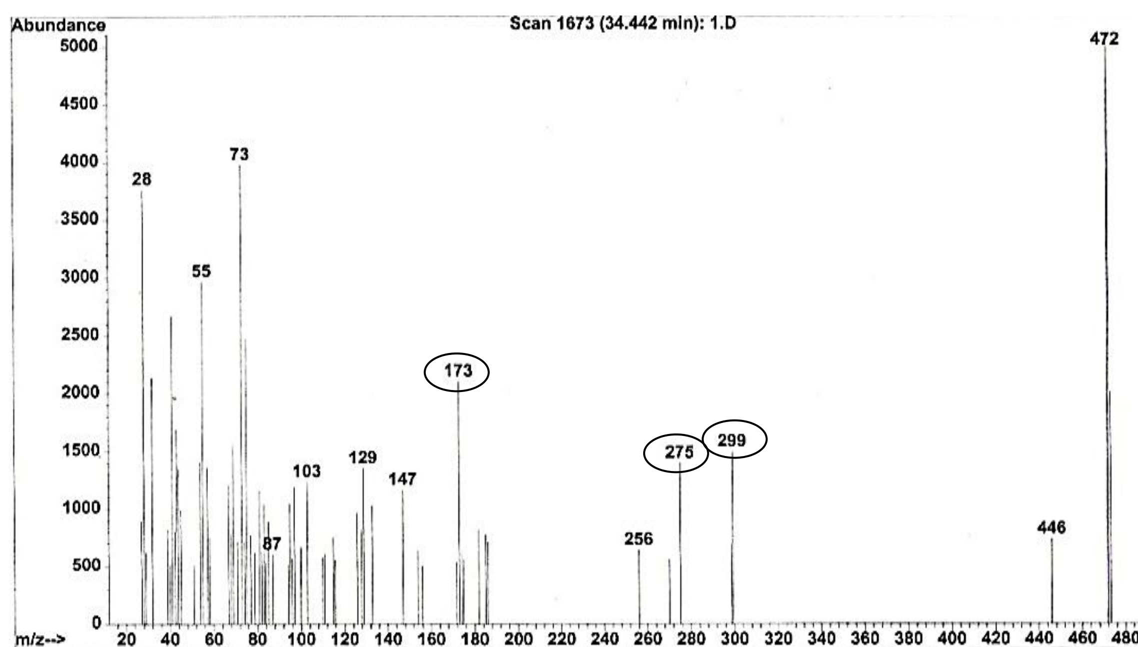


Figure 14. Mass spectra of 12,13-dihydroxy-9-octadecanoic acid identified in Glenn incubated bran.

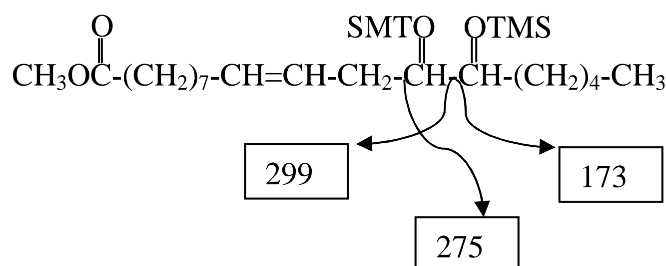


Figure 15. Fragmentation patterns of 12,13-dihydroxy-9-octadecanoic acid identified in Glenn incubated bran.

The mass spectrum of the compound (elutes at 34.4 min, Figure 14) 12, 13-dihydroxy-9-octadecanoic acid, consist of characteristic fragments observed at m/z 173 [TMSiO$^+$-CH-$(CH_2)_4$-CH_3], m/z 299 [$COOCH_3$-$(CH_2)_7$-CH=CH-CH_2-CH-OTMSi], m/z 275 [CH_3O-CH-CH (OTMSi)-CH-OTMSi] (Figure15). These fragments, m/z 299 and 173 are formed by the cleavage between C_{12}-C_{13} bond in and each fragment has a TMS group attached.

GC-MS results indicated the presence of above fragments in Glenn incubated wheat bran. This compound was originally identified as 12,13-dihydroxy-9-octadecanoic acid (Figure 14) by Kleiman and Spencer (1973). The mass spectrum of 12,13-dihydroxy-9-octadecanoic acid was previously provided by Doehlert et al (2011) in oat slurries, as well. Therefore, presence of 12,13-dihydroxy-9-octadecanoic acid is confirmed in incubated Glenn wheat bran. The same fatty acid was identified in other low susceptibility cultivars including Alsen, Traverse, and Faller incubated bran (data not shown).

The presence of 9-hydroxy-10,12-octadecadienoic acid (Figure 16, eluted at 35.3 min) was determined from two major fragments, m/z 225 [M-$(CH_2)_7COOCH_3$], m/z 311 [M-C_5H_{11}] (Figure 17).

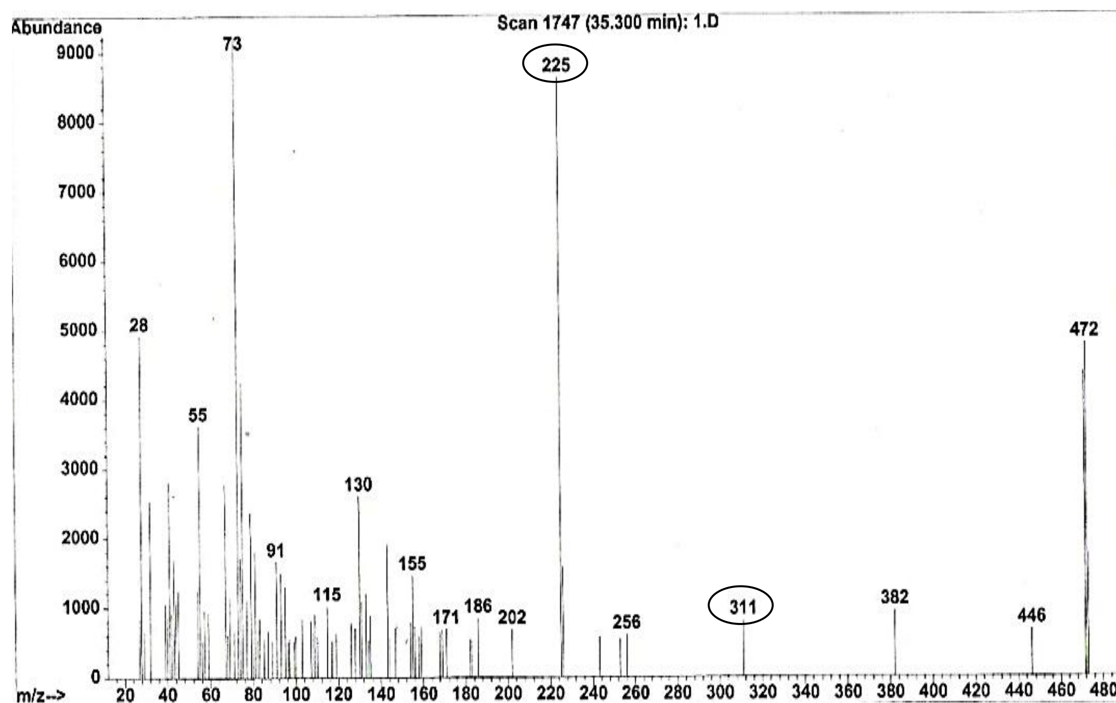


Figure 16. Mass spectra of 9-hydroxy-10,12-octadecadienoic acid in incubated Glenn bran.

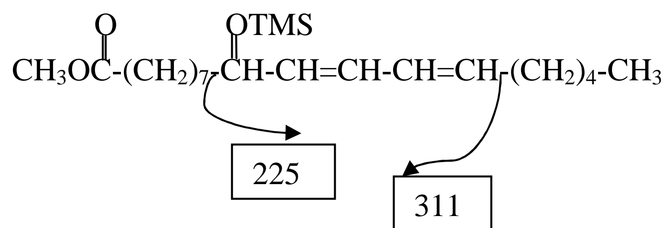


Figure 17. Fragmentation consistent with 9-hydroxy-10,12-octadecadienoic acid in incubated Glenn bran.

Fragment m/z 225 is formed from cleavage of the C_8-C_9 bond and m/z 311 is formed by cleavage of the C_{13}-C_{14} bond, which has the TMS group attached. The m/z 382 is the molecular ion and was identified in Figure 16. The characteristic peak m/z 73 is also present.

GC-MS results indicated the presence of above fragments in Glenn incubated wheat bran (Figure 17). The mass spectrum of 9-hydroxy-10,12-octadecadienoic acid was previously provided by Kleiman and Spencer (1973). Therefore, presence of 9-hydroxy-10,12-octadecadienoic acid is confirmed in incubated Glenn wheat bran.

The same fatty acid was identified in medium susceptibility category including Grainger and Ada incubated bran (data not shown). Some of the compounds eluted at 36.61, 38.15, 41.92, and 42. 91 minutes appeared with the chromatogram of fatty acids included the peak 73 suggested silylation, but precise structures could not be identified from the mass spectra.

GC-MS of methylated and sylated oxygenated fatty acids resulted in the identification of monohydroxy (9-hydroxy-10,12-octadecadienoic acid**)** and dihydroxy (12,13-dihydroxy-9-octadecenoic acid) fatty acids.

These have been identified in previous studies (Doehlert et al., 2011; Kleiman and Spencer, 1973) we hypothesis that oxygenated fatty acids identified in this study could be partially responsible for the growth inhibition of *F. graminearum* in wheat bran/agar plates. However, production and presence of oxygenated fatty acids do not form the complete basis for FHB inhibition.

When the methanol-soluble compounds (MSC) from the bran, which contained fatty acids and other compounds, were exchanged between low and high susceptibility wheat, there was less growth of *F. graminearum* in Samson wheat bran/agar plates containing the MSC from Glenn.

Effect of Total FFA Percentage in Different Wheat Bran with *Fusarium graminearum* Plaque Diameter

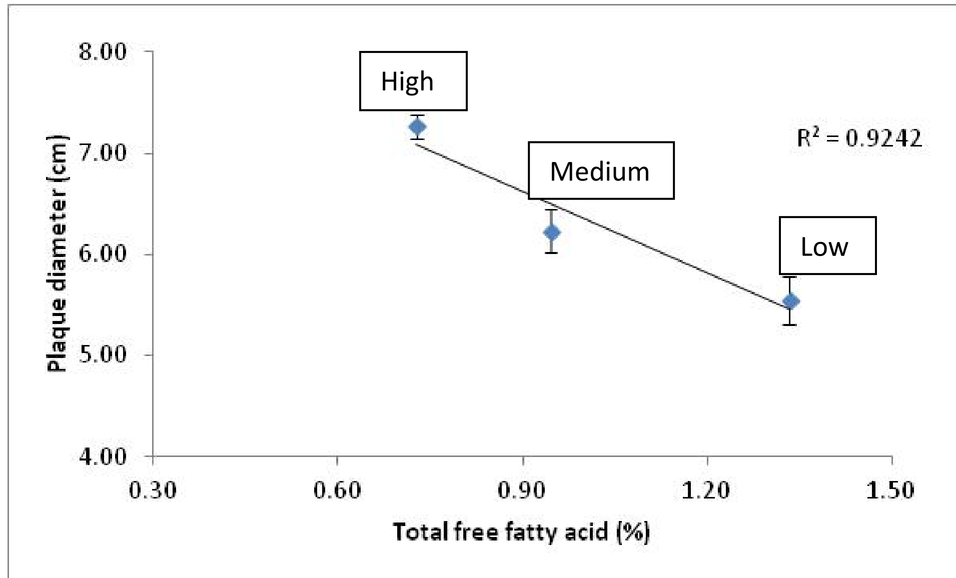


Figure 18. Correlation between total free fatty acid area percent (diazomethane methylation) and plaque diameter readings among all 19 wheat genotypes.

A negative correlation between total FFA % (incubated wheat bran) and plaque diameter readings of 19 wheat genotypes (pooled together within each susceptibility category) is represented from Figure 18. As the total FFA % increases, *F. graminearum* plaque diameter decreases. There were significant differences among wheat genotypes used in this study in terms of fatty composition and plaque diameter, and the correlation and regression analysis indicated high significance (R^2= 0.92, $p<0.05$). This correlation has a similar trend to the correlation between the degree of susceptibility of these wheat genotypes and the plaque diameter. As the level of susceptibility increased, the plaque diameter increased and the amount of total FFA (%) decreased, whereas, when the susceptibility decreased the plaque diameter decreased and the amounts of total FFA (%) increased. Therefore, genotypes such as Samson had high *F.*

graminearum growth and Glenn had lower fungal growth, which correlates with the amount of total FFA % in each of those genotypes. Similar correlations were previously determined by Doehlert et al (2011). Results from that study reinforced that lipid compounds were responsible, at least in part, for the inhibition of *F. graminearum* growth in oats. To determine the effect of free fatty acids in FHB inhibition oxidized fatty acids in Glenn and Samson genotypes were quantified using a proportionate equation.

Quantification of Oxidized Fatty Acids in Glenn and Samson Incubated and Non incubated Treatments

Table 13. Total oxygenated free fatty acid percentage for Glenn and Samson incubated and non incubated bran.*

Genotype	Treatment	OFA_{FFA}	OFA_{EFA}	OFA_{TFA}
Samson	Non-incubated	1.19b	0.79ab	1.82d
Glenn	Non-incubated	1.29b	0.89a	2.25c
Samson	Incubated	2.38a	0.52b	2.57b
Glenn	Incubated	1.75b	0.73ab	2.89a

*Values in the same column with the same letter are not significantly different ($p<0.05$). OFA_{FFA} = Oxygenated fatty acid from free fatty acids, OFA_{EFA} = Oxygenated fatty acid from esterified fatty acids, OFA_{TFA} = Oxygenated fatty acids from total fatty acids.

Oxygenated total FFA % was quantified using the above proportionate equation to determine their levels in low and high susceptibility wheat genotypes. Significant differences ($p<0.05$) were observed in the data analyzed using FFA % from Glenn and Samson genotypes. The total FFA % of Glenn incubated wheat bran was significantly higher than Glenn non-incubated wheat bran (Table 13). Glenn incubated had considerably higher oxygenated FFA% than all the other treatments. Oxidized free fatty acid (OFA) from esterified fatty acids (EFA) was comparatively lower than oxidized fatty acids (OFA) from free fatty acid content (FFA).

Similar results were reported previously by Doehlert et al (2010). They found epoxy fatty acids were significantly higher in EFA than FFA in groats. However in wheat there was no epoxy fatty acids identified in wheat bran. Results in this study explain that Glenn has more FFA than Samson which could contribute to the low FHB susceptibility. The presence of high FFA in these low susceptibility genotypes suggests that they may be related to the inhibition of fungal diseases. Since we know that linoleic acid is an abundant fatty acid in wheat bran the next step was to use linoleic acid to spike these wheat bran/agar plates and inoculate them with *F. graminearum* to observe the fungal growth and to identify any similar oxidized compounds identified in earlier GC-MS analysis.

Spiking Experiments

Several oxygenated fatty acids were identified by GC-MS in oats in a study conducted by Doehlert et al (2011) and they speculated that these compounds could be inhibiting fungal growth. Some of the compounds identified were derived from oxidation of linoleic acid, which is the most predominant fatty acid found in wheat bran. Therefore, further research was expanded to perform a spiking study to confirm whether there are similar compounds identified when wheat bran slurries were enriched with linoleic acid. Glenn and Samson were used in this study to determine whether the oxidation products of linoleic acid could inhibit fungal growth.

Data obtained from the spiking experiment is shown in Figure 19 and Table 14. Treatments in this experiment were further separated into incubated (I) and non-incubated (NI) with spiked (S) and non-spiked (NS). NS treatment did not have linoleic acid added to the wheat bran/agar media, but the S treatment was spiked with linoleic acid. Linoleic acid was used in the spiking study since it is the primary fatty acid present in wheat and in bran. In study 1 and 2, we

hypothesized that some compounds, such as, oxygenated fatty acids produced from oxidation of linoleic acid could be inhibiting fungi. These compounds were identified as products formed from linoleic acid. Therefore, wheat bran was enriched with linoleic acid and is hypothesized to enhance the inhibition of *F. graminearum* from the products of linoleic acid oxidation.

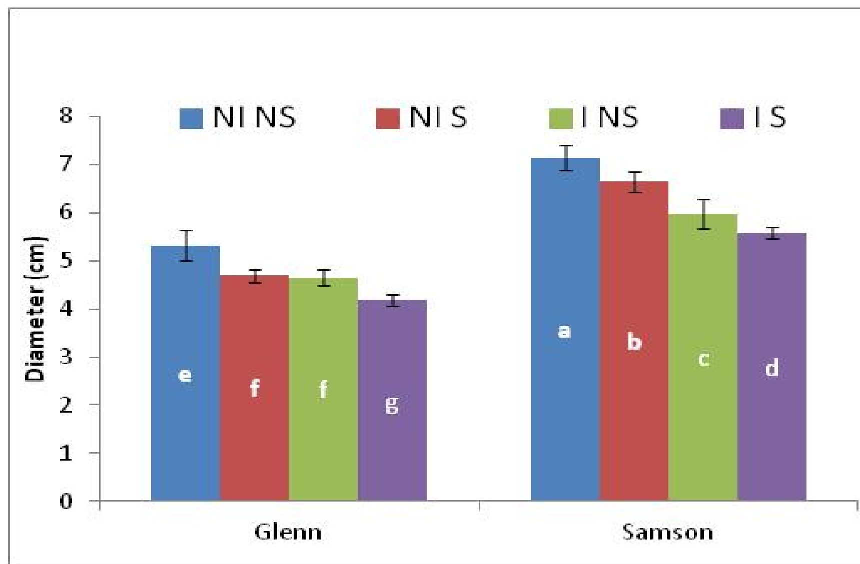


Figure 19. Plaque diameters of *Fusarium graminearum* in wheat bran/agar plates spiked study using low (Glenn) and high (Samson) susceptibility hard red spring wheat genotypes. Diameters with same letters are not significantly different at $p<0.05$. NI-NS= non-incubated non-spiked, NI-S=non-incubated spiked, I-NS=incubated non-spiked, I-S=incubated spiked.

At day 4, all Samson culture plates had significantly larger plaque diameter than Glenn plates. Among genotypes with the spiking treatment, all incubated plates had lower plaque diameters than any non-incubated treatment. Finally, within every genotype and incubation treatment spiked plates had lower plaque diameter than non-spiked plates. Thus, both spiking and incubation resulted in inhibition of *Fusarium* growth in bran/agar plates.

These results are consistent with the hypothesis that products of linoleic acid oxidation inhibit *Fusarium* growth. Ergosterol analyses indicated all Glenn wheat bran plates had lower

ergosterol than Samson. With every genotype and incubation treatment, all spiked wheat bran plates had less ergosterol and with every genotype and spiking treatment, non-incubated wheat bran plates had higher ergosterol ($p < 0.05$) (Table 15).

Table 14. Plaque diameters of *Fusarium graminearum* in wheat bran/agar plates in spiked study using low (Glenn) and high (Samson) susceptibility wheat genotypes.*

Genotype	I/NI	Trt	Diameter day 2 (cm)	Diameter day 3 (cm)
Glenn	I	NS	3.65e	4.65f
Glenn	I	S	3.00h	4.18 g
Glenn	NI	NS	4.59b	5.32e
Glenn	NI	S	3.79f	4.69f
Samson	I	NS	4.38c	5.97c
Samson	I	S	3.45g	5.5 7d
Samson	NI	NS	5.04a	7.13a
Samson	NI	S	4.01d	6.64b

*Day 1 had zero growth. Values in the same column with the same letter are not significantly different ($p<0.05$). I-incubated, NI-non incubated, NS-non spiked, S-spiked, and Trt-treatment.

We speculate that incubated samples had longer time for LOX activity during incubation time that can enhance their metabolic functions. The treatments with linoleic acid and incubation with water lead to important investigation of identifying compounds in wheat bran that may be involved with inhibition of fungal growth. Further research was needed to identify some of these compounds found in the spiked bran. Therefore, GC-MS was used to identify some of these compounds.

Table 15. Ergosterol analysis of *Fusarium graminearum* in wheat bran/agar plates in spiked study using low (Glenn) and high (Samson) susceptibility hard red spring wheat genotypes.*

Genotype	I/NI	Treatment	Ergosterol (mg/plate)
Glenn	I	NS	0.16f
Glenn	I	S	0.10g
Glenn	NI	NS	0.22d
Glenn	NI	S	0.18e
Samson	I	NS	0.25c
Samson	I	S	0.21d
Samson	NI	NS	0.34a
Samson	NI	S	0.28b

*Values in the same column with the same letter are not significantly different ($p<0.05$). I-incubated, NI- non incubated, S-spiked, and NS-non spiked.

Identification of Oxidized Fatty Acids from Diazomethane Methylation in the Spiking Study

Based on the data from spiking study, we can conclude that linoleic acid and its oxidized products have tendency to inhibit the fungal growth in wheat bran/agar plates. We attempted to identify fatty acid structures by GC-MS to detect abundance of oxygenated fatty acid. Therefore, the spiked and non-spiked samples were analyzed by GC-MS. Fatty acids including 9-hydroxy-10,12-octadecadienoic acid, and 12,13-dihydroxy-9-octadecenoic acid were identified by GC-MS in the samples. We suggest oxidized products from linoleic acid inhibit *F. graminearum* growth in Glenn and Samson bran/agar plates.

Quantification of Oxidized Fatty Acids in Spiking Study

Oxidized fatty acids in the spiking samples were quantified using the same proportionate equation as earlier which quantified oxidized fatty acids in incubated and in non-incubated bran.

Table 16. Total oxygenated free fatty acid percentage in Glenn and Samson bran in the spiking study.*

Genotype	Treatment	OFA_{FFA}	OFA_{EFA}	OFA_{TFA}
Glenn	NI-NS	1.37c	1.02ac	2.18c
Samson	NI-NS	0.77a	0.95ab	1.72d
Glenn	NI-S	1.45f	1.08ac	2.53e
Samson	NI-S	0.98ab	0.86a	1.84d
Glenn	I-NS	1.82d	0.80a	2.62e
Samson	I-NS	0.88b	0.90ab	1.78d
Glenn	I-S	2.34e	1.02ac	3.36a
Samson	I-S	2.11d	0.55d	2.66e

Values in the same column with the same letter are not significantly different ($p<0.05$*). NI-NS-non incubated non spiked, NI-S- non incubated spiked, I-NS- incubated non spiked, I-S-incubated spiked, OFA_{FFA} = oxygenated fatty acid from free fatty acids, OFA_{EFA} = oxygenated fatty acid from esterified fatty acids, and OFA_{TFA} = oxygenated fatty acids from total fatty acids.

The above calculation was performed using area percentage of linolenic (peak areas from 34.9 and 35.1 min) and linoleic acid (peak area 34.1 min) in each different Glenn and Samson treatments. Treatments with NI-NS (Glenn and Samson) were significantly different (*$p<0.05$*) from IS (Glenn and Samson) in total FFA content (Table 16). Samson IS was significantly higher than Samson I-NS, NI-S, NI-NS, and Glenn NI-S, NI-NS. Glenn IS had considerably higher oxygenated FFA% than all the other treatments. Patterns of OFA concentrations were inversely

related to plaque diameter and ergosterol concentrations. These results support the hypothesis that the oxidation of linoleic acid contributes to the inhibition of *Fusarium* in culture plates and may contribute to the resistance of Glenn wheat to FHB in the field.

CONCLUSION

This *in vitro* microbiological study provided strong preliminary evidence that HRSW genotypes can be screened for FHB susceptibility by measuring *F. graminearum* growth on agar plates which incorporate bran from the corresponding genotypes. *F. graminearum* grew better in wheat bran/agar plates prepared from bran of high susceptibility genotypes including Samson, Reeder, Trooper, Dapps, and Briggs. The fungus had moderate growth in medium susceptibility genotypes including Steele-ND, Howard, Granger, Granite, Ada, Sabin, Brennan, and Jenna. The third category, which is the low susceptibility genotypes, includes Alsen, Faller, Glenn, Traverse, and Brick had the lowest growth of *F. graminearum*. This *in vitro* microbiological study led us to investigate the biochemical composition in wheat bran that may be partially responsibility for the level of FHB susceptibility in wheat.

Methanol soluble compounds (MSC) present in wheat bran inhibited growth of *F. graminearum* to a certain extent in wheat bran/agar plates. Monohydroxy and dihydroxy fatty acids were identified from GC-MS analysis in incubated wheat brans. These fatty acids were also present in non-incubated wheat bran, but in lower levels. The OFA may be a result of LOX enzyme acting on 18:2 (linoleic acid) and 18:3 (linolenic acid) fatty acids. Most of these hydroperoxides were formed in the presence of linoleic acid. The oxidized fatty acids detected in this study were consistent with the structures identified by Doehlert et al. (2010 and 2011), and Kleiman and Spencer (1973). Some of the compounds in incubated bran identified by GC-MS were the same as those indentified from the spiking study. Fragments from 9-hydroxy-10,12-octadecadienoic acid and fatty acid 12,13-dihydroxy-9-octadecenoic acid were identified and confirmed by GC-MS.

Some studies indicated that hydroxyl and epoxy fatty acids could be associated with inhibition of the fungi of rice blast disease (Kato et al., 1984). Doehlert et al. (2011), also suggested that compounds including monohydroxy, dihydroxy, and epoxy fatty acids could be inhibiting the growth *F. graminearum* in oats. Therefore, this is the first study to associate involvement of these compounds in wheat against FHB.

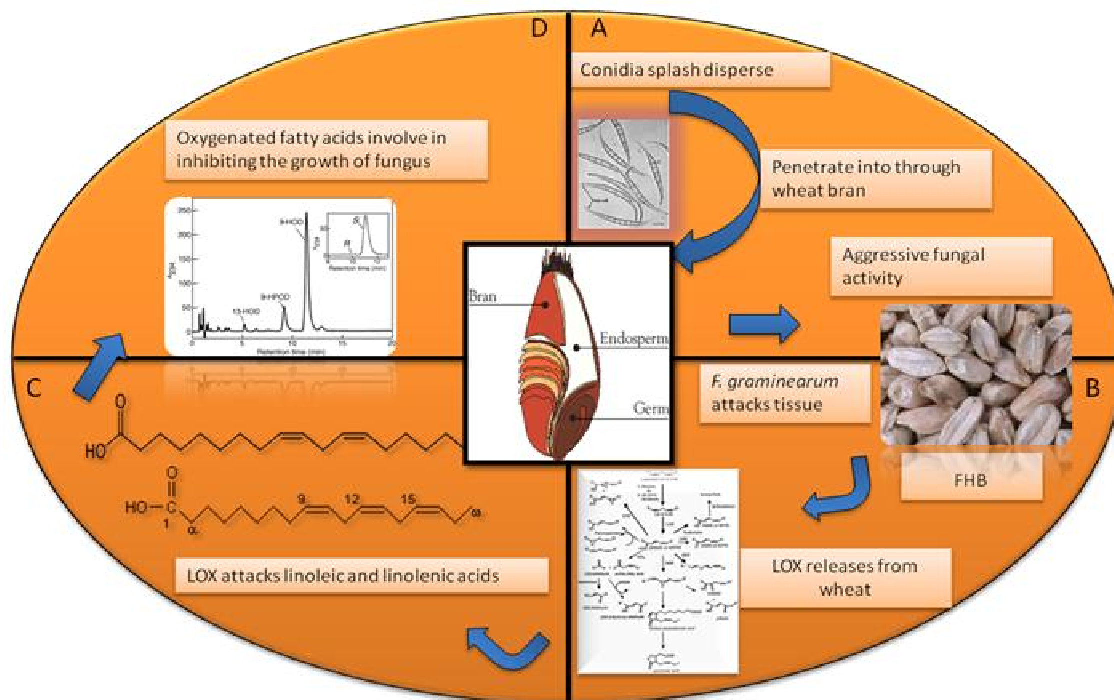


Figure 20. A model which represent overall goal of this project including severity of FHB in wheat and compounds that can be identified as a result of FHB in the wheat bran.

Figure 20 is a proposed model of FHB resistance in wheat representing the summary of findings presented in this research. This summary provides possible mechanisms that could be initiated within in the wheat bran during fungal diseases (FHB). These mechanisms are associated with activation of certain lipid-related enzymes like LOX that can lead to breakdown components in the wheat bran. These components break down into formation of secondary

products which have the potential to increase inhibition activity against *F. graminearum* in wheat. During this research some of these secondary products were identified in various susceptibility wheat genotypes. Therefore this research provides strong preliminary data for a laboratory scale screening test for FHB susceptibility.

Future Work

The following are the future work that can be drawn from this study, in order to strengthen the applicability of the microbiological *in vitro* study for a quick screening method for FHB susceptibility, the following areas should be extensively investigated.

1. Optimization of the incubation time in wheat bran/agar media (incubation time 4 hour).
2. Utilization of advanced analytical tools, such as, LC-QTOF would allow for identification of other unknown compounds and save time by using less labor intensive sample preparation.
3. Extraction of individual fractions from wheat bran and incorporate into wheat bran/ agar culture plates (Rescue studies).

REFERENCES

1. Anderson, J.A., R. Dill-Macky, J.V. Wiersma, G.A. Hareland, J.J. Wiersma, G.L. Linkert, D.V. McVey, R.H. Busch, Y. Jin, and J.A. Kolmer. 2007. Registration of 'Ada' Wheat . Crop Science. 47:434-435.
2. Auerman, L., A. Samsonov, M.M., Dubtsov, and M.P. Popov. 1971. Lipoxygenase activity of wheat grain and wheat flour. Applied biochemistry and microbiology 6: 573-576.
3. Bacic, A.P., J. Harris, and B.A. Stone. 1988. Structure and function of plant cell walls. The biochemistry of plants. APS Press, New York. p. 297–371.
4. Bai, G., and G. Shaner. 2004. Management and Resistance in Wheat and Barley to Fusarium Head Blight. Annu. Rev. Phytopathol. 42:135-161.
5. Barnes, P., and T. Galliard, 1991. Rancidity in cereal products. Lipid Technology. 3:23-28
6. Baysal, T., and A.h. Demirdoven. 2007. Lipoxygenase in fruits and vegetables: A review. Enzyme & Microbial Technology. 40:491-496.
7. Bechtel, D.B., J. Abecassis, P.R. Shewry, and A.D. Evers. 2009. Development, Structure, and Mechanical Properties of the Wheat Grain. p. 51-95. Wheat Chemistry and Technology, Fourth Edition. Grain Science References. AACC International, Inc.
8. Bechtel, D.B., L.M. Seitz, R.L. Gaines, and L.A. Kaleikau. 1985. The effects of *Fusarium graminearum* infection on wheat kernels. Cereal Chemistry. 62:191-197.
9. Berry, H., H. Debat, and V. LarretaGarde. 1997. Excess substrate inhibition of soybean lipoxygenase-1 is mainly oxygen-dependent. FEBS Letters. 408:324-326.

10. Bitzer, M.J., and J. Herbek. 1997. A comprehensive guide to wheat management. Cereal Chemistry. 2:120-125.

11. Bligh, E.G. and J.W. Dyer. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37:911-917.

12. Bread and the technology of bread production. Assessed on 14th January, http://www.classofoods.com/page1_1.html

13. Brennan. 2005. Seednet. Grain Research and Development Corporation. Assessed on the 20th February, http://www.seednet.com.au/NR/rdonlyres/88B74969-843E-4C28-A612-A761FA6BF58B/0/Brennan_5.pdf

14. Bushnell, W.R., and K.J. Leonard. 2003. Fusarium head blight of wheat and barley / edited by Kurt J. Leonard and William R. Bushnell. APS Press, St. Paul, Minn. p. 14:45-48.

15. Carver, B.F. 2009. Wheat science and trade. Blackwell pub. Iowa. p. 21:24-26.

16. Christie,W.W. 1989. The preparation of methyl and other esters of fatty acids. In Gas Chromatography and Lipids. A Practical Guide. The Oily Press. p. 2:66-80.

17. Dembitsky, V.M., and K. Maoka. 2007. Allenic and cumulenic lipid. Progress in Lipid Research. 46:328–375

18. De Simone, V., V. Menzo, A.M.De Leonardis, D.B. Maria-Ficco, D. Trono, L. Cattivelli, and P.De Vita. 2010. Different mechanisms control lipoxygenase activity in durum wheat kernels. Journal of Cereal Science. 52:121-128.

19. Desjardins, A.E., T.M. Hohn, and S.P. Mccormick. 1993. Trichothecene Biosynthesis in *Fusarium* Species - Chemistry, Genetics, and Significance. Microbiological Reviews. 57:595-604.

20. Devkota, R.N., J.C. Rudd, Y. Jin, K.D. Glover, R.G. Hall, and G.A. Hareland. 2007. Registration of 'Briggs' wheat. Crop Science. 47:432-434.

21. Doehlert, D.C., S. Angelikousis, and B. Vick. 2010. Accumulation of Oxygenated Fatty Acids in Oat Lipids During Storage. Cereal Chemistry. 87:532-537.

22. Doehlert, D.C., P. Rayas-Duarte, and M.S. McMullen. 2011. Inhibition of *Fusarium graminearum* Growth in Flour Gel Cultures by Hexane-Soluble Compounds from Oat (Avena sativa L.) Flour. Journal of Food Protection. 74:2188-2191.

23. Esaka, M., K. Suzuki, and K. Kubota. 1986. Inactivation of Lipoxygenase and Trypsin-Inhibitor in Soybeans on Microwave Irradiation. Agricultural and Biological Chemistry. 50:2395-2396.

24. Evans, C. K., and R. Dill-Macky. 2004. Screening for FHB Resistance. University of Minnesota small grains pathologists, prairie Grains Magazine. p. 14-20.

25. Frohberg, R.C., R.W. Stack, T. Olson, J.D. Miller, and M. Mergoum. 2006. Registration of 'Alsen' wheat. Crop Science. 46:2311-2312.

26. Glover, K.D., J.C. Rudd, R.N. Devkota, R.G. Hall, Y. Jin, L.E. Osborne, J.A. Ingernansen, J.R. Rickertsen, D.D. Baltensperger, and G.A. Hareland. 2010. Registration of 'Brick' Wheat. Journal of Plant Registrations. 4:22-27.

27. Gordon, M.H. 1984. Rancidity in foods: Edited by J.C. Allen and R.J. Hamilton. Applied Science Publishers, London. p. Food Chemistry. 14:233-234.

28. Graveland, A. 1973. Analysis of Lipoxygenase Nonvolatile Reaction-Products of Linoleic Acid in Aqueous Cereal Suspensions by Urea Extraction and Gas-Chromatography. Lipids. 8:599-605.
29. Graveland, A. 1973. Enzymatic Oxidation of Linolenic Acid in Aqueous Wheat-Flour Suspensions. Lipids. 8:606-611.
30. Helm, J.H., D.H. Dyson, and W.M. Stewart. 1986. Registration of Samson Barley. Crop Science. 26:384.
31. Hemery, Y., M. Chaurand, U. Holopainen, A.M. Lampi, P. Lehtinen, V. Piironen, A. Sadoudi, and X. Rouau. 2011. Potential of dry fractionation of wheat bran for the development of food ingredients, part I: Influence of ultra-fine grinding. Journal of Cereal Science. 53:1-8.
32. Horberg, H.M. 2002. Patterns of splash dispersed conidia of *Fusarium* poae and *Fusarium* culmorum. European Journal of Plant Pathology. 108:73-80.
33. Jenkinson, P., and D.W. Parry. 1994. Splash Dispersal of Conidia of *Fusarium*-Culmorum and *Fusarium*-Avenaceum. Mycological Research. 98:506-510.
34. Johnson, D.D. 1998. Economic impacts of fusarium head blight in wheat. Journel of Cereal Science. 103: 24-30.
35. Jones, R.K., and C.J. Mirocha. 1999. Quality parameters in small grains from Minnesota affected by Fusarium head blight. Plant Dis. 83:505-511.
36. Kato, T., T. Yokoyama, T. Namai, Y. Yamaguchi, and T. Uyehara. 1983. Defense mechanism of the rice plant against rice blast disease. Naturwissenschaften. 70:200-201.

37. Kim, H.J., and D.B. Min. 2008. Chemistry of Lipid Oxidation. Food lipids chemistry, nutrition, and biotechnology. CRC press. APS Press. New York. 299-320.

38. Kleiman, R. and Spencer, G.F. 1973. Gas chromatography-mass spectrometry of methyl esters of unsaturated oxygenated fatty acids. J Amer Oil Chem Soc. 2:31-38.

39. Kobrehel, K., and Y. Sauvaire. 1990. Particular lipid composition in isolated proteins of durum wheat. Journal of agricultural and food chemistry. 38:1164-1171.

40. Konopka, I., S. Czaplicki, and D. Rotkiewicz. 2006. Differences in content and composition of free lipids and carotenoids in flour of spring and winter wheat cultivated in Poland. Food Chemistry. 95:290-300.

41. Kolb, F.L., G.H. Bai, G.J Muhelbauer, J.A. Anderson, K.P. Smith, and G. Fedak. 2001. Host plant resistance genes for Fusarium head blight: mapping and manipulation with molecular markers. Crop Sci. 41:611-619

42. Lagaert, S., T. Belien, and G. Volckaert. 2009. Plant cell walls: Protecting the barrier from degradation by microbial enzymes. Seminars in Cell & Developmental Biology. 20:1064-1073.

43. Leslie, J.F., and B.A. Summerell. 2006. The *fusarium* laboratory manual. Blackwell Pub. Iowa. p. 45-56.

44. Maiorano, A., M. Blandino, A. Reyneri and F. Vanara. 2008. Effect of maize residues on the *Fusarium spp*. infection and deoxynivalenol (DON) contamination of wheat grain.Crop Protection 27:182-188.

45. McCartney C.A., D.J. Somers, G. Fedak, and W. Cao. 2004. Haplotype diversity at *Fusarium* head blight resistance QTLs in wheat. Theor. Appl. Genet. 109:261-271

46. McMullen, M., R. Jones, and D. Gallenberg. 1997. Scab of wheat and Barley: A Reemerging Disease of Devastating Impact. Plant Dis. 81:1340-1348.

47. McMullen, M., S.Halley, B.Schatz, S.Meyer, J.Jordahl, and J. Ransom. 2008. Integrated strategies for *Fusarium* head blight management in the United States. 3rd Int. FHB Symposium. Szeged, Hungary. p. 342-356.

48. Mergoum, M., T.L. Friesen, J.B. Rasmussen, T. Olson, R.C. Frohberg, and R.W. Stack. 2006. Registration of 'Glenn' wheat. Crop Science. 46:473-474.

49. Mergoum, M., T. Olson, J.B. Rasmussen, R.C. Frohberg, and J.D. Miller. 2005. Registration of 'Dapps' wheat. Crop Science. 45:420-421.

50. Mergoum, M., J.B. Rasmussen, T.L. Friesen, R.C. Frohberg, and R.W. Stack. 2006. Registration of 'Howard' Wheat. Crop Science. 46:2702-2703.

51. Mergoum, M., R.W. Stack, J.D. Miller, and R.C. Frohberg. 2005. Registration of 'Steele-ND' wheat. Crop Science. 45:1163-1164.

52. Mergoum, M., J.W. Rasmussen, T.L. Friesen, R.C. Frohberg, and R.W. Stack. 2008. Registration of 'Faller' Spring Wheat. Journal of Plant Registrations. 2:224-229.

53. Morrison, W.R. 1988. Lipids in cereal starches: A review. Journal of Cereal Science. 8:1-15.

54. Minnesota Wheat Verity Slection Guide. 2011. Fact sheets. Assessed on 24th January, http://www.smallgrains.org/2011WheatSurvey.pdf

55. Myrvik, A., J. Whitaker, and R. Cannon. 1976. The use of cellulose products to reduce agar concentration in microbiological media. Canadian Journal of Microbiology. 22:1002-1006.

56. Parry, D. W., P. Jenkinson and L. McLeod. 1995. *Fusarium* ear blight (scab) in small

grain cereals-a review. Plant Path. 44:207-23

57. Peng-Fei, Q., A. Johnston, M. Balcerzak, H. Rocheleau, J. Harris, L. Longx, W.Y. Zheng, Y. Ouellet. 2011. Effect of salicylic acid on *Fusarium graminearum*, the major causal agent of Fusarium head blight in wheat. Fungal biology. 30:1-14.

58. Pitt, J., and A. Hocking. 2009. Fungi and food spoilage. Methods of isolation, enumeration, and identification. Canadian Journal of Microbiology. 2:36-38.

59. Pomeranz, Y. 1971. Wheat Chemistry and Technology, American Association of Cereal Chemists, St. Paul, MN. 56-78.

60. Prandini, A., S. Sigolo, L. Filippi, P. Battilani, and G. Piva. 2009. Review of predictive models for Fusarium head blight and related mycotoxin contamination in wheat. Food & Chemical Toxicology. 47:927-931.

61. Prost, I., S. Dhondt, G. RotheVicente, J. Rodriguez, M.J. Kift, N. Carbonne, F. Griffiths, G. Rosahl, S. Castresana, C. Hamberg, and J. Fournier. 2005. Evaluation of the Antimicrobial Activities of Plant Oxylipins Supports Their Involvement in Defense against Pathogens. Plant Physiology, 1902–1913.

62. Pumphrey, M.O., R. Bernardo, and J.A. Anderson. 2007. Validationg the Fhb1 QTL for *Fusarium* head blight Resistance in near-isogenic wheat lines developed from breeding populations. Crop Sci. 47:200-206.

63. Punja, K.Z. 2004. Fungal disease resistance in plants. The hypersensitive responses and its role in disease resistance. Food product press. New York. p. 57-84.

64. Quartacci, M. F., C. Pinho, C.L.M. Sgherri, and F. Navari-lzzo. 1995. Lipid Composition and Two Wheat Cultivars Protein Dynamics in Thylakoids of Differently Sensitive to Drought. Plant Physiology. 108:191-197.

65. Ransom, J.K., B. Sorenson, and M. Mergoum. 2010. Trooper, Briggs, Reeder, Dapps, Howard, Steele ND, Glenn, Barlow, Faller, Alsen, Traverse, and Brick (Assessed on 14 the December,, http://www.maes.umn.edu/vartrials/swht.pdf

66. Rudd, J.C., R.D. Horsley, A.L. McKendry, and E.M. Elias. 2001. Host plant resistance genes for *Fusarium* Head Blight: Sources, Mechanisms and Utility in Conventional Breeding. Crop Sci. 41:620-627.

67. Seednet Wheat Variety Selection Guide. 2005. Grain Research and Development Corporation,assessed on 13th march, http://www.seednet.com.au/NR/rdonlyres/88B74969-843E-4C28-A612-A761FA6BF58B/0/Brennan_5.pdf

68. Schwarz, P.B., S. Beattie, and H.H. Casper. 1995. Fate and development of naturally occurring Fusarium mycotoxins during malting and brewing. American Society of Brewing Chemists. Journal of the American Society of Brewing Chemists. 53:121-127.

69. Stack, W. R. 2003. Fusarium Head Bligh of Wheat and Barley. History of Fusarium Head Blight with emphasis on North America. APS. St. Paul, Minnesota. 5: 456-459.

70. Stintzi, A., H. Weber, P. Reymond, J. Browse, and E.E. Farmer. 2001. Plant defense in the absence of jasmonic acid: The role of cyclopentenones. Proceedings of the National Academy of Sciences of the United States of America. 98:12837-12842.

71. Stokes, D.N., T. Galliard, and J.L. Harwood. 1986. Changes in the lipid composition of developing wheat seeds. Phytochemistry. 25:811-815.

72. US Wheat and Barley Scab Initiative. 2012. Reaction of Kansas, Nebraska, and South

Dakota winter wheat accessions to Fusarium head blight. Assessed on 10th March. http://scabusa.org/pdfs_dbupload/pdmr_nursery-report_11.pdf

73. Verma, B., P. Hucl, and R.N. Chibbar. 2009. Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. Food Chemistry. 116:947-954.

74. Vick, B.A. 1993. Lipid Metabolism in Plants, ed. Moore, T. S. CRC Press, Boca Raton, FL, 167–191.

75. Vorwerk, S., S. Somerville, and C. Somerville. 2004. The role of plant cell wall polysaccharide composition in disease resistance. Trends in Plant Science. 9:203.

76. Walter, S., P. Nicholson, and F.M. Doohan. 2010. Action and reaction of host and pathogen during Fusarium head blight disease. New Phytologist. 185:54-66.

77. Wiersma, J. 2010. Granger, Granite, Ada, and Sabin wheat varieties. Minnesota Agriculture Experiment Station. Assessed on 24th march. http://www.maes.umn.edu/releases/Ada.pdf

78. WestBred. 2012. Samson. Assessed on 23rd February. https://www.westbred.com/Products/North/ProductDocuments/SAMSON.pdf

79. Wilde, F., V. Korzum, E. Ebmeyer, H.H. Geiger, and T. Miedaner. 2007. Comparison of phenotypic and marker-based selection for Fusarium head blight resistance and DON content in wheat. Mol. Breed. 19:357-370.

APPENDIX

Table 17. ANOVA table for *Fusarium graminearum* plaque diameter readings in wheat bran/agar plates on Day 2 for reconstitution study.*

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Replication	3	0.06	0.02	3.60	0.1603
Gtype	1	3.15	3.15	546.83	0.0002
Error (a)	3	0.02	0.01		
Treatment	5	3.61	0.72	137.23	<.0001
Gtype *Trt	5	1.62	0.32	61.62	<.0001
Error (b)	30	0.16	0.01		

*Gtype-genotype, Trt-treatment, and DF-degree of freedom

Table 18. ANOVA table for *Fusarium graminearum* plaque diameter readings in wheat bran/agar plates on Day 3 for reconstitution study.*

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Replication	3	0.028	0.009	17.000	0.0219
Gtype	1	10.083	10.083	18150.000	<0.0001
Error (a)	3	0.002	0.001		
Trt	5	9.978	1.996	352.150	<0.0001
Gtype *Trt	5	5.029	1.006	177.500	<0.0001
Error (b)	30	0.170	0.006		

*Gtype-genotype, Trt-treatment, and DF-degree of freedom

Table 19. ANOVA table for *Fusarium graminearum* plaque diameter readings in wheat bran/agar plates on Day 2 for spiking study.*

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Rep	3	0.03	0.01	0.45	0.7383
Gtype	1	5.13	5.13	213.66	0.0007
Error (a)	3	0.07	0.02		
IC	1	13.05	13.05	556.14	<.0001
Gtype *IC	1	0.40	0.40	17.06	0.0061
Error (b)	6	0.14	0.02		
Trt	1	17.51	17.51	1049.24	<.0001
Gtype *Trt	1	0.40	0.40	23.99	<.0001
IC*Trt	1	0.09	0.09	5.62	0.0203
Gtype *IC*Trt	1	0.00	0.00	0.22	0.6368
Error (c)	76	1.27	0.02		

* IC- Incubated, Trt-treatment, Gtype-genotype, and rep-replication

Table 20. ANOVA table for *Fusarium graminearum* for plaque diameter readings in wheat bran/agar plates on Day 3 for spiking study.*

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Replication	3	0.18	0.06	0.7	0.626
Genotype	1	62.57	62.57	707.5	0.000
Error (a)	3	0.27	0.09		
IC	1	17.43	17.43	266.5	<.0001
Genotype*IC	1	1.68	1.68	25.7	0.002
Error (b)	6	0.39	0.07		
Treatment	1	5.85	5.85	133.0	<.0001
Genotype*Trt	1	0.07	0.07	1.5	0.228
IC*Trt	1	0.09	0.09	2.0	0.162
Genotype*IC*Trt	1	0.01	0.01	0.2	0.663
Error (c)	76	3.34	0.04		

*IC- Incubated, Trt-treatment, and DF-degree of freedom.

Table 21. ANOVA table for ergosterol values in the spiking study (analyzed after day 3).*

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Replication	3	0.21	0.07	1.1	0.4699
Genotype	1	28.11	28.11	447.1	0.0002
Error (a)	3	0.19	0.06		
IC	1	12.83	12.83	465.54	<.0001
Genotype*IC	1	0.12	0.12	4.28	0.0839
Error (b)	6	0.17	0.03		
Treatment	1	6.25	6.25	140.06	<.0001
Genotype*Trt	1	0.03	0.03	0.63	0.4281
IC*Trt	1	0.00	0.00	0.06	0.8097
Genotype*IC*Trt	1	0.12	0.12	2.79	0.0991
Error (c)	76	3.39	0.04		

*IC- Incubated, Trt-treatment, and DF-degree of freedom