WHEAT-HESSIAN FLY INTERACTIONS: FITNESS COSTS AND PHENOTYPIC CHARACTERIZATIONS

by

Verónica Campos Medina

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Entomology

West Lafayette, Indiana

December 2017

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Jeffrey Stuart, Chair
Department of Entomology
Dr. John Couture
Department of Entomology
Dr. Ian Kaplan
Department of Entomology
Dr. Steven Scofield
Department of Agronomy

Approved by:
Dr. Stephen Cameron
Head of the Graduate Program

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ABSTRACT

Author: Campos Medina, Verónica, A. PhD.
Institution: Purdue University
Degree Received: PhD
Title: Wheat-Hessian Fly Interactions: Fitness Costs and Phenotypic Characterizations.
Major Professor: Dr. Jeffrey Stuart

The Hessian fly-wheat relationship and its genetic tractability offer us a good system to address different questions that can help to improve not only the strategies used to control this pest, but also our understanding of the insect-plant relationship. Thus, taking advantage of these features in this study I examined the fitness cost of the loss of function of a virulence gene and I made a phenotypic characterization of this insect-host relationship.

First, this thesis has an introductory part, chapter one, presenting background information about the Hessian fly-wheat interaction. Chapter two explores an allele competition approach to detect fitness costs associated with loss of function of the *vH13* gene in virulent populations I observed that changes in allelic frequency of *vH13* over eight generations suggest no evidence of fitness cost. Plant damage caused by virulent Hessian fly populations has been associated with the fly's ability as a gall-maker to manipulate its host inducing morphological and physiological changes to wheat plants. In chapter three, the use of hyperspectral technology to phenotypically distinguish ungalled and galled plants is discussed. I was able to differentiate these two types of plants with high success. Additionally, I distinguished among galled plants induced by different Hessian fly populations. The Hessian fly's ability to induce galls depends not only on its own genome, but also on the plant genotype. Therefore, the same plant genotype may be susceptible or resistant depending on the insect genotype. Thus, in chapter four I discuss the characterization of the effects of compatible and incompatible interactions on the plant tissue above the soil using plants carrying the *H13* resistance gene. Nitrogen concentration, C:N ratio, LMA, and phenolics were predicted based on the near infrared reflectance of the leaves. My results suggest that under both interactions the complete seedling is experiencing changes which can be detected early in the infestation process. Additionally, I provide evidence that under a compatible interaction the insect manipulates its host in order to obtain the needed nutrition for its development.

1. INTRODUCTION

The extended phenotype hypothesis proposes that genes of one organism can have a phenotypic effect on a different organism. Insect-induced plant galls are a good example that support this hypothesis (Dawkins, 1999). It has been observed that while galls are composed only of plant tissue, their phenotype is shaped by the gall-maker genotype (da Silva Carneiro et al., 2015; Weis & Abrahamson, 1986). Although many studies assume that the gall is the result of the interaction between plant and gall maker genomes, investigating these two variables has been difficult because of the lack of genetic tractability for those relationships. In this context, the Hessian fly-wheat interaction is an excellent model to study this hypothesis because of the knowledge that we have about both the Hessian fly and wheat genomes and genotypes and how their interactions can be experimentally manipulated.

i. The Hessian Fly And Its Effect On Wheat (Host Manipulation)

The Hessian fly (*Mayetiola destructor*) is one of the most destructive pests of wheat in the United States, western Asia, and northern Europe (Harris et al., 2003). In the United States, since it was first reported in the 1770s in New York (Pauly, 2002), this pest has spread to all wheat producing areas of the country (Cambron et al., 2010). This fly, like almost all other members of the subfamily Cecidomyiinae, causes the formation of galls on plant tissue (Harris, 1966; Stuart et al., 2012). Thus, the damage caused by the Hessian fly is associated with its ability as a gall-maker to manipulate its host to obtain the needed nutrition for its development.

The Hessian fly attacks mainly wheat, although it is able to complete its life cycle on other grasses, most of which are closely related to wheat (Chen et al., 2009; Harris et al., 2003). Host selection occurs during adulthood, and even though the females do not feed on the plants, they are responsible for finding and selecting the plant to lay their eggs (Kanno & Harris, 2000). About one hour after mating, adult females fly from plant to plant carrying approximately 250 eggs. They typically oviposit two to five eggs on the adaxial surface of a young grass leaf (Harris & Rose, 1990). After hatching, first instar larvae select a feeding site, near the meristematic region, where they feed for approximately 6 days before molting. The second instar larvae feed for approximately another 4 days. After this period, the larval integument darkens and at the 10th or 11th day it is

possible to see the third instar developing inside the puparium formed by the second larval cuticle (Gagne & Hatchett, 1989). Although there are three larval instars, only the first two feed on the plant (Gagne & Hatchett, 1989; Hatchett et al., 1990; Stuart & Hatchett, 1987). Thus, the second instars have greater size than the third, reaching a length of 1.7-4.0 mm (Gagné & Hatchett, 1989).

The first two feeding larval instars have different feeding behaviors, which is reflected in their head morphology. The head capsule is directed anteriorly in the first instar while it is directed ventrally beneath the first thoracic in the second instar. In addition, the first instar head capsule appears less sclerotized than that of the second instar (Gagné & Hatchett, 1989). Most notably, the mouth parts of the first and second instars, especially the mandibles, suggest they have different feeding behaviors. The mandibles terminate in a prominent tooth in the first instar and a foreshortened tooth in the second instar (Hatchett et al., 1990), suggesting that the sessile second instar only drinks plant fluids whereas the first instar uses its mandible to penetrate cell walls and inject salivary fluids into wheat cells (Hatchett et al., 1990).

After examining salivary gland morphological changes through larval development, Stuart and Hatchett (1987) proposed that the anterior basal region of the gland is responsible for the secretion of substances injected in the plant. They came to this conclusion because of the change in the relative size of the basal and filament regions of the salivary gland in feeding (first and second instar) and non-feeding (third instar) larvae. During the early feeding period, the basal region constitutes the largest part of the gland. As the larvae develop, the basal region diminishes in size and volume until the filament region has greatly expanded in the non-feeding larvae.

Searching for the substances that the salivary gland secretes, Chen et al. (2004) found putative proteins with secreted signal peptides encoded by genes expressed only in first instar larvae. Finding expression of these genes in this larval stage is important because it is a critical stage that determines if the interaction between a specific Hessian fly biotype and specific wheat plant is compatible (Stuart et al., 2012). The hypothesis that salivary substances play a critical role in successful host colonization is supported by examples of plant pathogenic organisms that suppress plant defenses and alter the structure and function of the host cells. These substances are called effector molecules (Bent & Mackey, 2007; Jones & Dangl, 2006). Zhao et al. (2015) examined the Hessian fly genome for genes encoding effector proteins, finding that at least 7% of the genes encode putative effector proteins with N-terminal signal peptides that display evidence of rapid evolution.

Effector proteins secreted by the salivary gland are likely responsible for host-plant manipulation and the plant-gall development (Harris et al., 2003; Stuart et al., 2012). Thus, after only a few days Hessian fly attack is initiated, it is possible to detect changes in the plant. For example, larvae feeding for just two or three days induce plant nutritive tissue that supports larval development (Harris et al., 2006) and it is possible to observe morphological changes in seedling wheat plants: in comparison to uninfested plants, the second leaf is shorter, it shows a spiral curling, and it has a darker green color. Moreover, sheath elongation and the third leaf length are severely affected (Cartwright & et al., 1959). The infestation also causes physiological modifications manifested as differences in plant protein content (Shukle et al., 1992), and wheat leaf permeability (Kosma et al., 2010). These morphological and physiological changes are permanent and irreversible after the fourth day of feeding (Byers & Gallun, 1972). These changes are consistent with observed differences in gene expression profiles. Compared to uninfested plants, genes involved in the transport of nutrients infested are up-regulated and genes involved in cell wall metabolism and synthesis of defense molecules are down-regulated in susceptible infested plants (Liu et al., 2007). These changes are probably essential to normal larval development and the completion of the Hessian fly life cycle (Mittapalli et al., 2006).

ii. Control Of Hessian Fly Using Resistant Varieties

Plants are able to defend against the attack of pathogens. Based on the four-phased plant inmune system model, plants have two levels of immunity: basal and resistance-gene-mediated immunity. In the basal immunity, transmembrane receptors recognize conserved pathogen associated molecular patterns (PAMPs) and induce PAMP-triggered immunity (PTI). In resistance-gene-mediated immunity, most of the resistance (*R*) genes code for NB-LRR proteins that recognize an effector and induce effector triggered immunity (ETI) (Jones & Dangl, 2006). From the standpoint of the plant parasite, this kind of effector has an undesirable "avirulence" function, as pathogens producing the effector are avirulent on a host that carries the corresponding *R* gene (Ellis et al., 2009). Thus, genes that encode effectors that have avirulence functions are called *avr* genes.

To date four *avr* effector-encoding genes, *vH13*, *vH9*, *vH6*, and *vH24* have been identified in the Hessian fly (Aggarwal et al., 2014; Zhao et al., 2015; Zhao et al., 2016). Flies that express

these genes cannot survive on plants carrying the corresponding resistance gene; for example, flies that express the vH13 protein are avirulent on plants that carry the *H13 R* gene (Stuart et al., 2012).

Thus, the Hessian fly is not able to successfully manipulate some wheat genotypes, making resistant varieties of wheat the most reliable and cost effective method of managing this pest (Harris et al., 2003; Ratcliffe et al., 2000; Stuart et al., 2012). Resistance varieties are a useful control alternative in non intensive production, such as wheat, where although insecticides have been introduced in the United States (Jarman & Ballschmiter, 2012), their use is marginal (Berzonsky et al., 2003).

In wheat, there are at least 35 different Hessian fly *R* genes (H1-H34, and *Hdic*) (Hao et al., 2013; Li et al., 2013). Most of these are dominant or semi-dominant genes (Stuart et al., 2012). While some resistance genes have been identified in common wheat (H1, H2, H3, *h4*, H5, H7, and H8), others have been transferred from other grass species, for example *Triticum turgidum* var *durum* (H6, H9, H10, and H11), *Triticum tauschii* (H13), and *Secale cereale* (H25 and H26) (Friebe et al., 1996; Gill et al., 1987).

In general, there are 3 classes of host plant resistance mechanisms: antixenosis, tolerance and antibiosis. The last one is used in Hessian fly control, which includes any mechanism that reduces the growth or survival of the feeding stages of the insect (Berzonsky et al., 2003). Hessian fly females are not able to discriminate between resistant and susceptible wheat plants when they lay their eggs on resistant wheat (Harris et al., 2003). However, on resistant plants larvae neither grow nor develop and the majority die by the fourth day after egg hatch. By the seventh day there are no living larvae (Chen et al., 2009).

Although resistant plants do not prevent infestation or the initial larval attack, they do prevent the negative effects that larval feeding has on plant growth and yield by stopping gall induction (Anderson & Harris, 2006). On resistant wheat, an increase in the expression of the lectin-encoding *Hfr-1* gene occurs (Williams et al., 2002). Lectins have been proposed to have insecticidal or anti-nutritional effects in insects. This corresponds with the observation that three hours after larval feeding on resistant wheat begins, the larvae have inflamed microvilli with bulbous terminals. In some cases, microvilli were completely lacking. Six hours after larval feeding begins on resistant plants, the larvae lack microvilli and lumen content. The lectin proteins HFR1 and HFR2 are thought to be mainly responsible for this damage to the larval gut (Shukle et al., 2010).

iii. Virulent Population Evolution And Its Effect On Wheat Production

As with any pest population, Hessian flies have the ability to adapt to the anthropogenic selection pressures and develop resistance to the management strategies used to control them. This is most evident in the virulent Hessian fly genotypes that have evolved as a result of the deployment of resistant wheat varieties (Harris et al., 2003). Typically, Hessian fly populations overcome plant resistance in only six to eight years (Chen et al., 2009). Such as in other phytopathogen-plant relationships, where the capability to grow on resistant plants is due to the modification or loss of *avr* genes (Martin et al., 2003; Bent & Mackey 2007), the resistant biotypes of the Hessian fly carry recessive mutation in the genes, which allow the flies to avoid the ETI elicited by wheat resistance (R) proteins (Stuart et al., 2012). For example, congruent with the gene-for-gene interaction model between the Hessian fly and wheat (Lobo et al., 2006), recessive loss-of-function mutations in the *avr* gene *vH13* allow the larvae to survive on wheat plants carrying the *R* gene *H13* (Lobo et al., 2006; Harris et al., 2012). In fact, although there are several genes conferring resistance to Hessian fly, only a few of them are still effective in North America (Cainong et al., 2010). Moreover, Cambron et al. (2010) found that in southeastern United States only five genes (*H12*, *H18*, *H24*, *H*25, and *H26*) of 21 evaluated provide effective resistance against the Hessian fly. For this reason, is important to examine Hessian fly populations periodically to provide wheat breeders and growers information on the effectiveness of wheat resistance genes and biotype compositions in regional Hessian fly populations (Cambron et al., 2010; Chen et al., 2009).

vi. Outstanding Questions

The studies cited above make it clear that during the past years several important advancements have been made relative to our understanding of Hessian fly biology, genome content and its compatible and incompatible interactions with wheat. However, before this knowledge can be more fully applied to the practical management of this insect pest, additional questions must be addressed. First, are there fitness costs associated with identified Hessian fly virulence alleles that can be used in combination with molecular diagnostics to maintain avirulence

in Hessian fly populations? Second, outside of the *avr* genes themselves, does genetic variability influence the galling response and the extended phenotypes observed in the wheat-Hessian fly interaction? Do these genes influence Hessian fly fitness? And third, is it possible to observe these interactions earlier and with greater precision using modern plant phenotyping and imaging technologies? If so, can these observations elucidate the plant physiological changes that occur during wheat-Hessian fly interactions? The investigations described in this thesis were specifically performed to address these questions.

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2. EVALUATION OF FITNESS COSTS ASSOCIATED WITH THE ALLELE THAT PERMITS HESSIAN FLIES TO SURVIVE ON PLANTS CARRYING THE *H13* RESISTANCE GENE

2.1 Introduction

The first effector of the Hessian fly and of any insect was discovered as the product of an avirulence (*avr*) gene, *vH13*, which has been genetically and physically positioned in the Hessian fly genome. The *vH13* effector gene is located on the short arm of Hessian fly X2 chromosome. The expression of this gene, which occurs just in the salivary glands of the first larval instar, produces a unique protein, vH13 effector, which does not share sequence similarity with any other protein (Aggarwal et al., 2014).

It has been proposed that effector trigger immunity (ETI) is the mechanism behind wheat resistance to the Hessian fly (Zhao et al., 2015). Thus, plants that carry the *H13* resistance (R) gene, a dominant *R* gene located on the 6DS chromosome, can recognize directly or indirectly the vH13 effector protein and elicit ETI. Plants carrying the *H13* gene show a high level of antibiosis against susceptible Hessian fly populations (Liu et al., 2005). However, this *R* gene is not effective against some Hessian fly populations (Ratcliffe et al., 2000), called ¨virulent¨ populations or biotypes.

Virulent individuals in these populations have loss-of-function insertions in the *vH13* gene, which allow the fly to survive and manipulate the development of wheat plants that carry the *H13* *R* gene. Two insertions have been identified in exon 1 (256 bp and 5Kb) and another in exon 2 (461bp). These mutations prevent the proper transcription of the *vH13* genes in virulent larvae (Aggarwal et al., 2014).

Loss of function to a gene normally expressed in the Hessian fly suggests that a fitness cost may be associated with the loss-of-function alleles. From a practical standpoint, this is an important possibility because such a fitness cost might serve to drive the functional avirulent allele into the population in the absence of resistant plants. Thus, like insecticide resistance alleles in insecticide-free environments (Hardstone et al., 2009; Rinkevich et al., 2013), it may be possible that virulent Hessian fly alleles will have fitness costs in the absence of resistance plants. If this

were the case, the pest population might be managed by removing resistant plants before the loss-of-functional alleles become fixed in the population.

With this in mind, Zhang et al. (2011) evaluated the fitness costs associated with Hessian fly virulence to *R* genes *H9* and *H13*. They found a decrease in adult size when virulent flies were reared on plants lacking resistance genes. He also found that virulent flies had a shorter wing length, which correlated with lower fecundity (Bergh et al., 1990; Harris et al., 2001). Unfortunately, because this study used distinct populations instead of isogenic Hessian fly strains, it was impossible to determine whether the *avr* genes in question were responsible alone or if other genes in the genomes of the insects examined were responsible for the fitness costs observed.

Here, we used allele competition experiments as an alternative approach to test for fitness costs associated with *H13*-virulence. This approach has been successfully used to measure the fitness costs associated with insecticide resistance alleles (Hardstone et al., 2009; Rinkevich et al., 2013). These experiments determined fitness costs as a function of allele loss from a population in the absence of selection pressure, e.g. insecticide or plant resistance. If alleles have associated benefits or neutrality, their frequencies increase or remain constant through generations. However, if alleles have detrimental effects, their frequencies decrease over generations (Hardstone et al., 2009). This approach is only possible because a molecular diagnostic for the alleles in question is available. Because *vH13* has been cloned (Aggarwal et al., 2014), molecular diagnostics for both virulent and avirulent *vH13* alleles exist, thereby making the allele competition experiment for *H13*-virulence and avirulence possible. Using this approach we put virulent and avirulent alleles in competition in a population exposed to susceptible plants. We hypothesized that *vH13*-loss-of-function would have fitness costs and the frequency of *H13*-virulent alleles would decrease over generations. We additionally contrasted the allele competition result with some insect performance traits evaluated previously to estimate virulence cost to *R* genes in Hessian fly populations.

2.2 Material and Methods

Plants

Newton plants were used. Newton is a commercial hard red winter cultivar, which is susceptible to all the Hessian fly genotypes that have been tested on it (Patterson et al., 1994).

Insects

Two strains of Hessian fly were used, GP and vH13. GP is a susceptible strain, unable to survive on wheat plants with any resistance genes. vH13 is a homozygous virulent strain, able to survive on wheat plants that carry the *H13 R* gene.

Allele Competition Experiment

To evaluate changes in the *vH13* virulence allele frequency in the absence of resistance plants, 200 females of GP and 200 vH13 were crossed with 200 GP and 200 vH13 males. The F_1 population was split into 5 different replicates. Each replicate was allowed to freely interbreed for 7 generations on susceptible wheat plants under controlled conditions (20ºC and 14:10 L:D). Thirty adult females and 30 adult males were collected each generation to be genotyped for *vH13*. In order to genotype the insects the genomic DNA was isolated, as previously described by (Morton et al., 2011), and used to amplify *vH13* using PCR with oligonucleotides containing the forward primer (5'-TCGAACAGGATGCCGAAACG-3') and reverse primer (5'-TGCAAAACAATGAATGTCAC-3') sequences. That combination of primers allows all the avirulent alleles and 2 virulent alleles be amplified. In order to amplify the allele associated with the 5Kb insertion, another reverse primer, located within the insertion, was included: 5'-TTGAATGTGCCGCGAGAGC-3'. Additionally, we used a putatively neutral marker located on the X1 chromosome to genotype the same individuals using the forward primer and reverse primer sequences, 5'-CGTGTGCTGCTACTATTTTG-3' and 5'- GCCCATTATTCGTTTGTTAG-3', respectively. To determine linkage disequilibrium between the *vH13* and the neutral loci, male genotypes were evaluated using the normalized disequilibrium parameter D', which is defined as D'=Dij/Dijmax.

Fitness Components

To estimate female fecundity and male fertility, females from the F_{13} population described above were allowed to lay their eggs on Newton plants. Three days after oviposition, neonate first instar larvae were collected in an aqueous solution of 0.02% NP-40. These larvae were transferred to Newton plants at the two-leaf developmental stage in the same solution. Each plant was infested with 1 larva. Plants were maintained at 20±2ºC with 80% humidity and L14:D10 photoperiod. The adult that emerged from each plant was collected and kept at -80ºC.

The right wing of each dead adult was removed from the thorax and mounted in Canada balsam on a microscopic slide. The samples were examined under a microscope (Fig. 2.1) and the wing lengths were measured in micrometers (μm) as the distance from the proximal end of the brown axillary sclerite to the point of radial sector vein at the margin of the wing apex (Bergh et al., 1990). The rest of the body was used to extract DNA in order to genotype each individual for *vH13* alleles.

Additionally, to evaluate the possibility of mating advantage, 50 adult females, 25 virulent and 25 avirulent, were released individually into a caged pot of wheat seedlings containing 2 males, one virulent (vH13) and one avirulent (GP). Because Hessian fly females mate only once during their lives, examining the genotype of female's offspring determined whether the female mated with the vH13 male or the GP male.

Data Analysis

To determinate the changes in virulence allele frequency, the 5 replicates of each generation were combined, and statistical differences between means were determined using one-way ANOVA and Tukey's *post hoc* test ($p<0.05$). To compare the wing lengths among genotypes, a one-way ANOVA was performed followed by Tukey's test ($p<0.05$). To compare the insemination success between virulent and avirulent males, a chi-squared analysis was performed ($p<0.05$).

2.3 Results

In the absence of resistant plants, the frequency of virulent alleles did not show a clear trend over the generations (d.f.=5; F=12; p-value<0.001; Fig. 2.2; Fig. 2.3). Changes in frequency of the major allele of the neutral marker and the virulent alleles for *vH13* showed a similar pattern. Both alleles showed a decrease in frequency from generation 2 to generation 3 (Fig. 2.4). This observation is consistent with the decrease in linkage disequilibrium observed from the same two generations. This suggested that from the original strains used to create the populations examined, the virulent alleles of *vH13* were in linkage disequilibrium with the major allele of the neutral marker and some recombination events in the females were necessary to break the linkage. If the decrease observed from generation 2 to generation 3 was due to selection against *vH13* we should expect to increase the linkage disequilibrium between these two markers because the virulent alleles and the major allele had been unpaired from the populations increasing the abundance of the other haplotypes. Additionally, from generation 3 both markers segregated independently and they behaved similarly. Therefore, we did not observe any evidence of a change in the allele frequency of *vH13* over time.

Examining the length of the wings, as a morphological index of adult performance we found no differences between genotypes in both males (d.f.=1; F=0.06; p-value=0.6992; Fig. 2.5) and females (d.f.=2; F=0.09; p-value=0.9137; Fig. 2.6).

Additionally no difference in insemination success was observed between avirulent or virulent males (χ^2=0.1379; p-value=0.7).

2.4 Discussion

The durability of resistance is associated with the fitness cost of virulence, and the durability and efficacy of *R* genes is central for control management. Thus, knowledge regarding how virulent alleles decrease in different Hessian fly populations once selection pressure is relaxed is important. Fitness cost of the *vH13* virulence alleles are expected to occur when individuals carrying loss-of-function *vH13* alleles have lower fitness on plants lacking the *H13 R* gene compared with insects carry functional *vH13* alleles on the same type of plant.

In this study we evaluated the fitness cost associated with the loss of function of *vH13* gene. Unlike other investigations that attempted to address this question, we used an allele competition approach to overcome the limitation of developing Hessian fly isogenic lines. After eight generations, no evidence of fitness cost was observed when the population was not exposed to selection pressure under greenhouse conditions. Under the same conditions the mating advantage and the flies' size in female and males, which is an estimate of female fecundity and male fertility, respectively, were consistent with our result of the allele competition experiment.

At least two possible explanations for the lack of an observable fitness cost exist. These include a functional redundancy of effectors in the Hessian fly genome and a fitness cost that is not observed under the environmental conditions tested. In examining these possibilities, we pay particular attention to the fact that virulence to *H13 R* gene for the Hessian fly is due to a loss of function of the *vH13* gene. In other words, with respect to larval fitness and gall formation, the *vH13* gene appears to have no functionality.

Stergiopoulos and de Wit (2009) proposed that lack of functionality of effector genes may be associated with an indirect recognition of the effector protein by the corresponding R protein or that the pathogenic function of these effectors may be redundant or compensated for by other effectors. Functional redundancy can allow pathogens to escape plant detection without compromising their fitness if other effectors provide the same pathogenic function. Thus, the loss of function of some effectors will have no fitness costs for the pathogen (Birch et al., 2008; Win et al., 2012). Functional redundancy of effector proteins has been observed in the interaction between *Pseudomonas syringae* and *Arabidopsis.* This bacterium has two type III effectors, AvrRpm1 and AvrB, which have the same target in the host the RIN4 protein (Grant & Lamb, 2006). For gall makers like the Hessian fly, a certain amount of redundancy might be expected due to the complexity of the galling process, as host-cell reprograming probably requires many effector proteins (Oates et al., 2016). Consistent with this idea, it has been estimated that at least 7% of Hessian fly genes encode effector proteins (Zhao et al., 2015). Thus, it seems reasonable that some functional redundancy might exist for some Hessian fly effector proteins.

Previous studies have also evaluated the *vH13* gene for pathogenic functionality without success. Harris et al. (2012) studied the interaction between *vH13* and *H13* in both compatible and incompatible interactions. They found that the loss-of-function of *vH13* effector gene does not compromise the insect's ability to manipulate the plant or the larva's capacity to grow on plants

that lack the *H13 R* gene. Based on their experiments, they suggested that *vH13* lacks a colonization function and does not affect basal resistance.

The results of the present investigation contrast with Zhang et al. (2011), who after evaluating wing length as a function of body size, concluded that adults lacking functional *vH13* alleles are smaller than adults carrying functional *vH13* alleles. Body size can affect three different fitness components, male mating advantage, female fecundity and male fertility, in animal species (Andersson, 1994). In addition, Bergh et al. (1990) found a strong correlation between Hessian fly body size and both maximum fecundity potential in females, and the number of females inseminated by males. Thus, Zhang et al. (2011) concluded that there is a reproductive fitness cost associated with *H13*-virulence. In light of the results of the present investigation, because the genetic differences between the *H13*-virulent and –avirulent in the flies that they examined extended well beyond just the *vH13* locus itself, we suggest that a combination of many genes in the individuals they examined were probably responsible for the differences in body size they observed.

The possibility that the environmental conditions examined were not conducive to the observation of a fitness cost is another possible reason that no fitness cost was observed. However, in this context it is important to point out that loss-of-function of *vH13* alleles are commonly observed in low frequency in field populations that have not been exposed to the *H13 R* gene. Using the diagnostic *vH13* markers and Hessian fly pheromone traps in combination, a number of southeastern U.S. populations previously unexposed to *H13* were examined for the presence of virulence to *H13*. Each of these populations contained the *vH13*-avirulence alleles described by Aggarwal et al. (2014) at a 1 to 10% frequency. A similar situation was observed in Australian populations of *Tribolium castaneum,* where populations had alleles that confer resistance to Malathion insecticide before this pesticide was released to the market (Hartley et al., 2006). This observation raises the important possibility that preexisting mutations may result from unrelated selection pressure on the population (Ffrench-Constant, 2013). In the case of *Tribolium*, the selection pressure may derive from chemicals produced by fungi or other organisms living in stored grain. In the case of the Hessian fly, there may be selection pressure from genes carried by alternative plant hosts.

Thus, the present study impacts two important assumptions underlying resistance management. First, we normally assume that virulence to an *R* gene develops in a population as a

consequence of the selection pressure that *R* gene exerts on the pest population. Second, we assume that in the absence of this selection pressure, the frequency of virulent individuals will decrease. If no fitness costs are associated with loss-of-function *vH13* alleles, we should expect that the frequency of functional *vH13* alleles will rapidly decrease in the population when *H13* resistant plants are deployed. Additionally, we would expect that rotation with plants lacking the *H13* gene would have little impact on the frequency of the loss-of-function *vH13* allele frequency in the population.

One more complexity in the wheat-Hessian fly interaction must be considered: systemic wheat susceptibility and its corresponding impact on the survival of avirulent genotypes on resistant plants. Systemic susceptibility is induced by a single virulent larva and this allows the survival of what would otherwise be avirulent larvae on the same resistant plant (Baluch et al., 2012; Grover et al., 1989). Baluch et al. (2012) proposed that this susceptibility or "resistance obviation" acts as a refuge for avirulent genotypes and thereby delays the evolution of virulence in the population. Although we still lack knowledge regarding the number of virulent and avirulent larvae a plant can support, and whether systemic susceptibility is equally beneficial for avirulent homozygotes and heterozygotes, the potential impact of systemic susceptibility remains an important question. It may be possible that systemic susceptibility masks the fitness costs associated with virulence alleles at some level if avirulent larval interactions benefit virulent larvae feeding on the same plant.

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Fig. 2.1. Wing of Hessian fly examined under microscope.

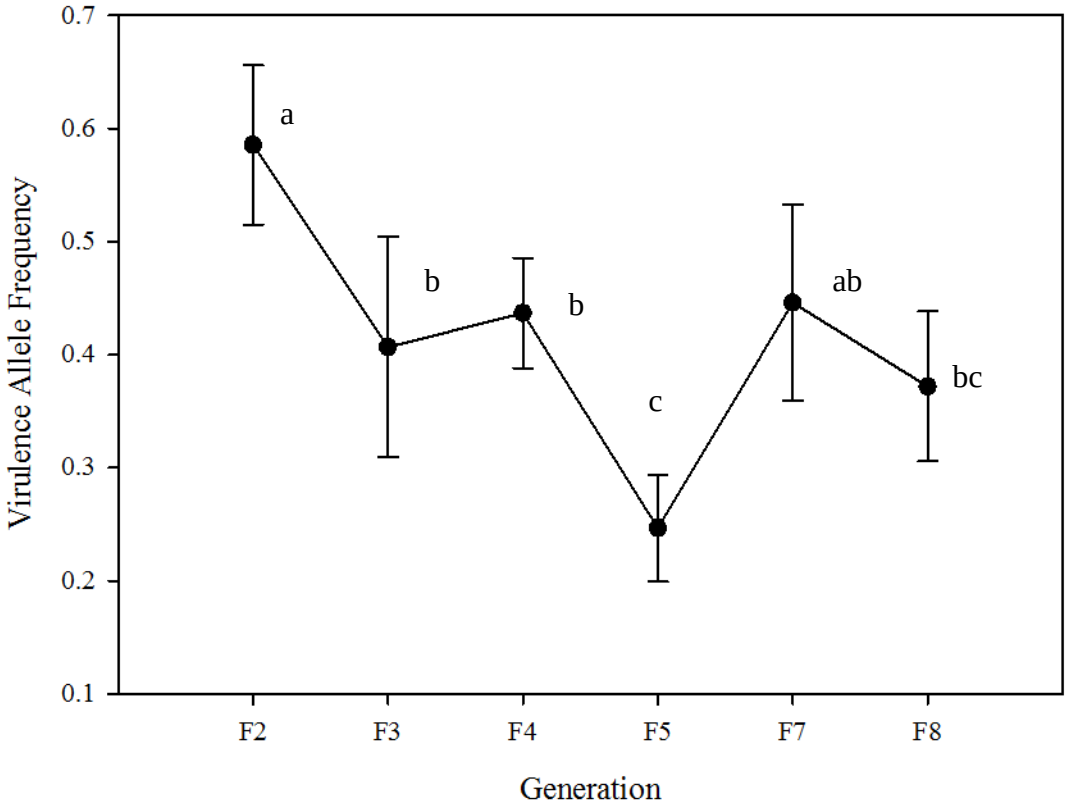

Fig. 2.2. Changes in virulence allele frequency over 8 generations. Result are averages of the five replicates, bars are the SD. Different letters indicate statistical differences ($p<0.05$) between means.

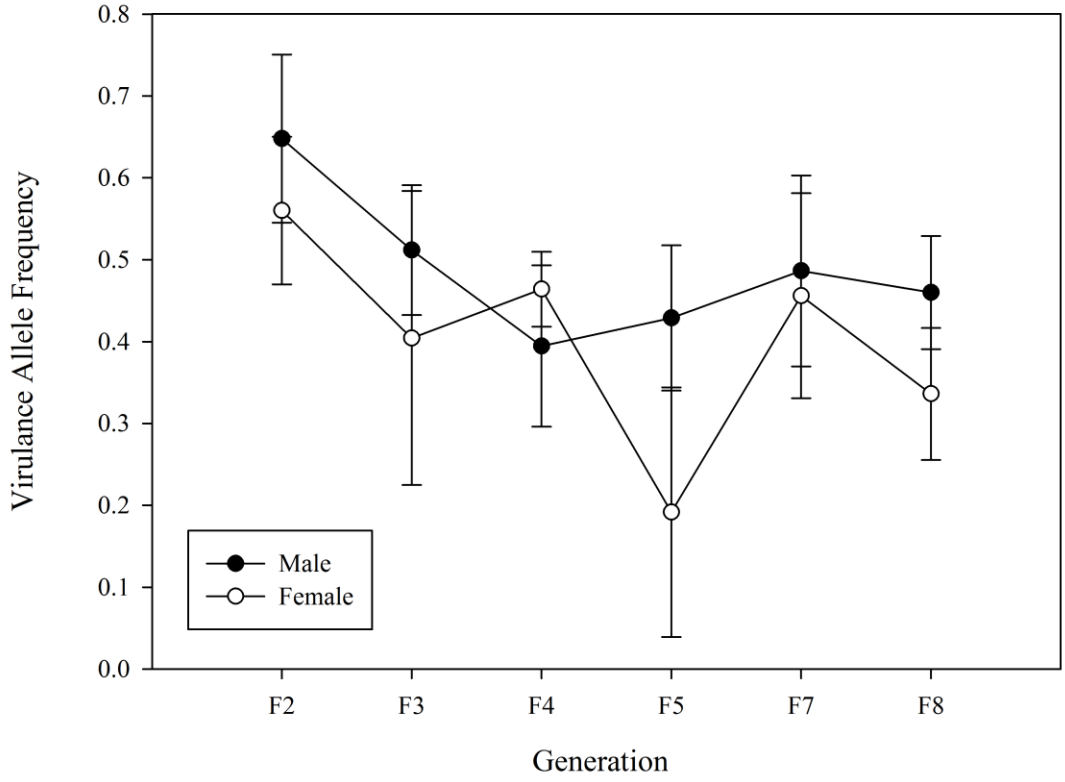

Fig. 2.3. Changes in virulence allele frequency for males and females over 8 generations. Result are averages of the five replicates, bars are the SD.

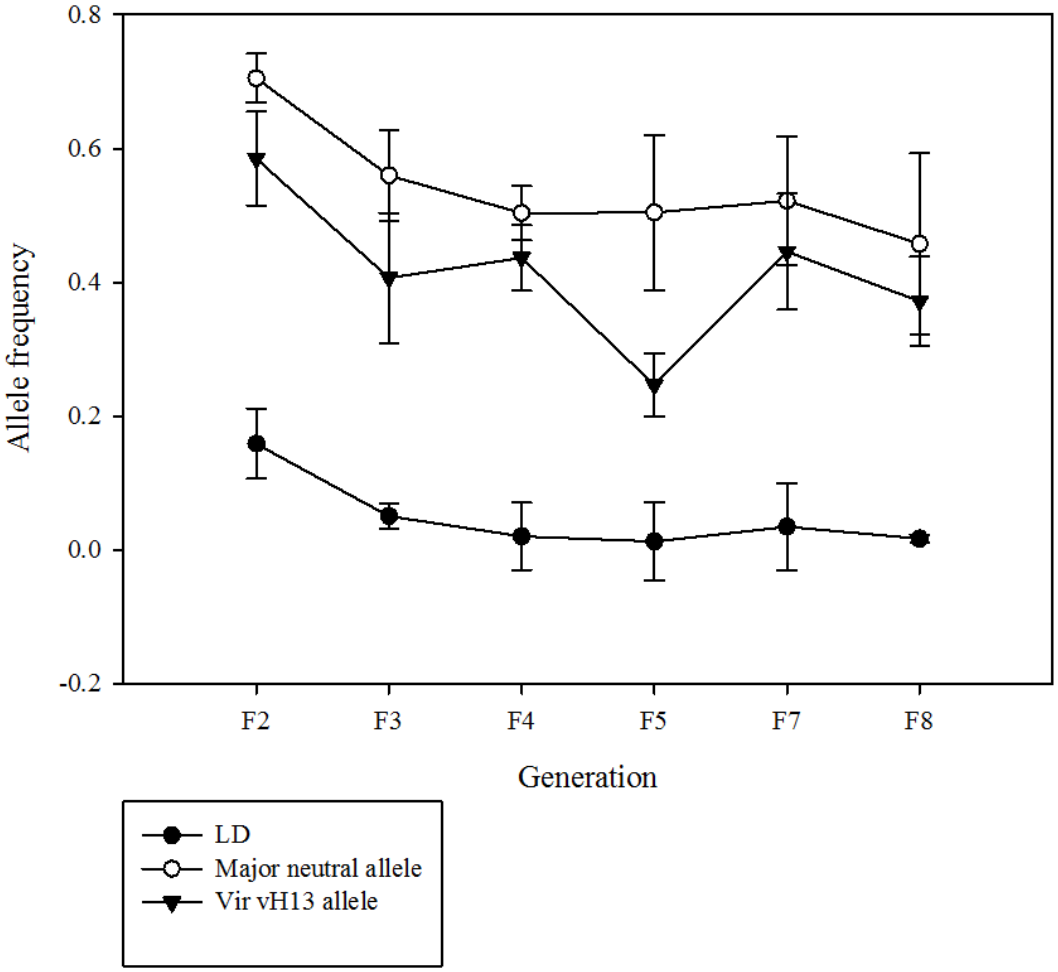

Fig. 2.4. Changes in allele frequency over 8 generations of the *vH13* virulent alleles and the major allele of the neutral marker. The linkage disequilibrium (LD) between the vH13 loci and the neutral loci are showed. Result are averages of the five replicates, bars are the SD.

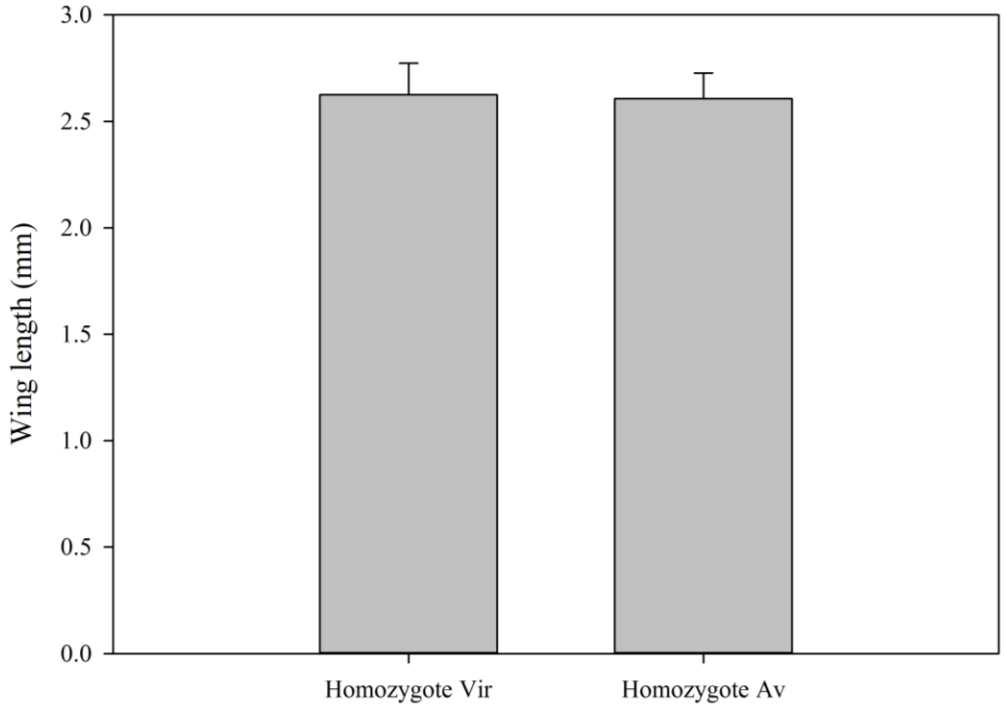

Fig. 2.5. Mean (± SD) wing length of the two male genotypes.

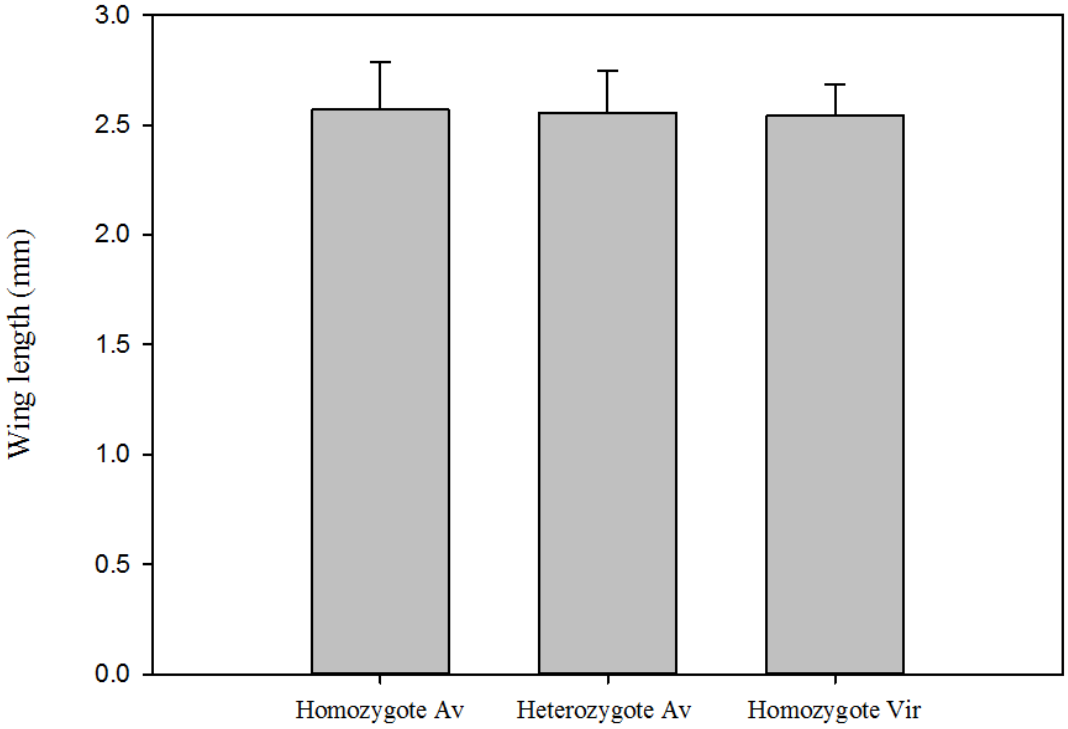

Fig. 2.6. Mean (± SD) wing length of the three female genotypes.

3. SPECTRAL PHENOTYPING OF HESSIAN FLY-WHEAT INTERACTIONS

3.1 Introduction

Herbivores influence the rate and form of plant growth, either directly through damage and consumption or indirectly through changes in resource allocation (Tscharntke, 1989). Gall-maker species are examples of organisms that indirectly alter the morphology and physiology of the host (Harris et al., 2003).

The ability to induce plant galls has evolved independently in seven different arthropod orders: Thysanoptera, Hemiptera, Homoptera, Lepidoptera, Coleoptera, Hymenoptera, and Diptera (Dreger-Jauffret & Shorthouse, 1992; Shorthouse et al., 2005). Diptera is the order with greatest number of gall making species. These belong to two suborders: Cyclorrhapha and Nematocera. The gall midges (Cecidomyiidae) belong to Nematocera and compose the most successful group of gall-maker insects in terms of both species number (>6000) and host range (Gagne & Hatchett, 1989).

The Hessian fly (*Mayetiola destructor*) is the most studied and one of the most economically important gall midges (Harris, 1966; Stuart et al., 2012). It is an important pest in common wheat (*Triticum aestivum*) and durum wheat (*T. turgidum*), and found almost everywhere in the world where wheat is grown (Berzonsky et al., 2003). The damage to wheat is caused by larvae, which feed near meristematic tissue for approximately 10 days (Chen et al., 2009). Although there are three larval instars, only the first two feed on the plant (Gagne & Hatchett, 1989; Hatchett et al., 1990; Stuart & Hatchett, 1987). First instar larvae feed for approximately 6 days and second instar larvae feed for approximately another 4 days (Gagne & Hatchett, 1989). During this short period, the insect is able to produce major physiological and morphological changes in its host (Kosma et al., 2010).

It has been hypothesized that Hessian fly first-instar larvae secrete effector proteins that elicit morphological changes in the plant (Harris et al., 2003; Stuart et al., 2012; Zhao et al., 2015). Genomic analysis indicated that at least 7% of all genes encode effectors (Zhao et al., 2015) and that the larval salivary gland probably produces those effectors which are injected into plant tissues via the larval mouthparts (Stuart et al., 2012; Zhao et al., 2015). Gene expression studies also

indicate that the genes encoding putative effector proteins are primarily expressed in first instars (Aggarwal et al., 2014; Chen et al., 2004). This pattern of expression corresponds with the stage in which either a compatible or incompatible wheat-Hessian fly interaction is established (Stuart et al., 2012).

Morphological and physiological changes in the plants induced by herbivores may affect their reflected spectra (Lin, 2015). Particularly, it has been observed that reflectance in the visible and near infrared light can be useful to determine the health of plants and permit the detection of biotic and abiotic stressors (Mirik et al., 2007; Peñuelas et al., 1994). Thus, spectral analysis offers a new method to examine the Hessian fly-wheat interaction. Additionally, the effect of different Hessian fly genotypes on the wheat phenotype has not been examined, although there is evidence that suggests that the gall-maker genotype can influence the changes observed in the host (Weis & Abrahamson, 1986).

Here we examine the spectral profile of ungalled plants (uninfested) and galled plants (infested) induced by two different Hessian fly populations to determine whether i) the reflected spectrum is able to detect differences in the galled and ungalled plant phenotypes and ii) whether different virulent Hessian fly populations induce different reflected spectra. To address these questions, we used wheat plants that carry the *H13* resistant gene and two different *H13*-virulent Hessian fly populations, one from the United States and another from Israel, that have been separated for at least 200 years. In order to account for the phenotypic variation associated with the gall induction process in wheat, a time series approach was used.

3.2 Materials and Methods

Plants

Patterson et al. (1994) developed near isogenic lines resistant to Hessian fly transferring resistance genes to a susceptible winter cultivar called Newton. The isogenic line that carries the *H13* resistant gene, Molly, hereafter referred as H13-present, was used in this experiment. Individual seedlings of this variety were germinated in plastic cones with a 14-cm diameter and a 14-cm depth in autoclaved soil under greenhouse conditions.

Insects

Two populations of Hessian fly were used, vH13-Israel and vH13-US. Both populations are able to induce gall formation on H13-present plants.

Insect-plant Infestation

Gravid females from both populations were allowed to lay their eggs on H13-present seedlings. Three days after oviposition, neonate first instar larvae were collected in an aqueous solution of 0.02% NP-40. These larvae were transferred to H13-present plants at the two-leaf developmental stage in the same solution. Each plant was infested with 2 larvae. Sixty plants were infested with each insect population. Additionally, 40 uninfested plants (ungalled plants) were used as a control. Plants were maintained at 20±2ºC with 80% humidity and L14:D10 photoperiod.

Hyperspectral Imaging System And Image Acquisition

A VNIR hyperspectral camera (Middleton Spectral Vision, Middleton, WI) covering the spectral range of 400-1000 nm with spectral resolution of 0.6 nm was used for taking plant images. The hyperspectral camera was set on a linear gantry system on top of an imaging box (28”L x 17”W x 48”H). To image the plants, they were placed flat at the bottom of the imaging box and the camera moved along the linear gantry scanning the plants.

Hyperspectral images were collected from the same galled and ungalled plants at eight different times during the gall formation period: 1, 2, 3, 4, 5, 8, 10 and 12 days post infestation (DPI).

Image Segmentation

Raw hyperspectral images were preprocessed in order to separate plant pixels from the background pixels according to their spectra. Green vegetation has a distinguished red-edge slope near 680-780 nm, which can be used to segment the plant pixels from the background (Filella & Penuelas, 1994). Image convolution filtering with a ramp vector was used at the wavelength range from 680 nm to 710 nm to enhance pixel regions with red-edge slope. The image convolution step provided a heat map of pixel regions with higher values at the pixels regions with red-edge slope

and lower values elsewhere. Thus, by choosing a suitable threshold value, only plant pixels were examined in subsequent analyses.

Data Analysis

To account for the effect of treatments and date in the spectrum profile, a two-way PERMANOVA was performed using 1000 permutations based on Euclidean distances with $\alpha = 0.05$. When differences among treatments were detected, a pairwise PERMANOVA was completed (Anderson, 2001). Additionally, a principal coordinate analysis (PCoA), also known as metric multidimensional scaling, was used to visualize the difference between treatments (Anderson & Willis, 2003). PCoA was performed against the spectral profile for all dates together. From this analysis a two-dimensional plots was produced using the first two PCoA scores.

To evaluate the ability of the spectral profile to distinguish among different treatments, a classification model using the partial least square-discriminant analysis (PLS-DA) was built. This analysis was first performed using all the data collected, and then subsequently performed for each time point that images were collected. Each time the analysis was performed, 85% of the image data was randomly assigned to a training data set and 15% of the image data was assigned to a testing data set. The training set was used to build the classification model. The testing set was used to validate that model. Cross validation was performed using 5 groups. In each run, one group, the cross validation group, was removed from the training set. The model was calibrated on the remaining training set and then the predictions were performed on the cross validation group. This process was repeated 100 times. Thus the number of latent variables was optimized to minimize error. The model was then applied to the testing set, which lacked treatment labels, in order to evaluate the model's performance. This information was summarized in a confusion matrix, which shows the number of correct and incorrect predictions (Ballabio & Consonni, 2013). The pls-da was performed using the *mixOmics* package in R (www.r-project.org).

Additionally, the ability of ten different reflectance indices to distinguish among treatments was evaluated. The indices were estimated using the approach Devadas et al. (2009):

1.Normalized difference vegetation index (NDVI)

$$NDVI = \frac{(NIR - R)}{(NIR + R)}$$

2.Narrow-band normalized difference vegetation index (NBNDVI)

$$NBNDVI = \frac{(R850 - R680)}{(R850 + R680)}$$

3.Transformed chlorophyll absorption in reflectance index (TCARI)

$$TCARI = 3[(R700 - R600) - 0.2(R700 - R550)\frac{R700}{(R850 + R670)}]$$

4.Structural independent pigment index (SIPI),

$$SIPI = \frac{(R800 - R445)}{(R800 + R680)}$$

5.Nitrogen reflectance index (NRI),

$$NRI = \frac{(R570 - R670)}{(R570 + R670)}$$

6. Anthocyanin Reflectance Index (ARI)

$$ARI = R550^{-1} - R700^{-1}$$

7. Plant senescence reflectance index (PSRI)

$$PSRI = \frac{(R678 - R500)}{(R750)}$$

8. Photochemical Reflectance Index (PRI)

$$NBNDVI = \frac{(R570 - R531)}{(R570 + R531)}$$

9.Physiological reflectance index mean with standard error (PhRI)

$$PhRI = \frac{(R550 - R531)}{(R550 + R531)}$$

10.Normalized Pigment Chlorophyll Ratio Index (NPCI)

$$NPCI = \frac{(R680 - R430)}{(R680 + R430)}$$

To account for the effect of treatments and date in the partial spectrum (10 indices) a two-way PERMANOVA was performed using 1000 permutations based on Euclidean distances with $\alpha = 0.05$. Thus I identified the index with the biggest contribution to distinguish among treatments. Then a Kruskal-Wallis test was performed to check the difference in this index among treatments, followed by Tukey's test ($p<0.05$).

3.3 Results

Insect-plant Infestation

At least one vH13-Israel larva survived to gall 52 (87%) H13-present seedlings, and at least one vH13-US larva survived to gall 43 (72%) H13-present seedlings.

Preprocessing

The segmentation process separated plant pixels from the background (Fig 3.1). Segmented images (Fig. 3.1b) were used as a mask to extract the reflectance values of all plant pixels from each band of the hyperspectral image.

The head and the end of the spectra were excluded from the analysis because of the noise, thus the full spectrum data collected included the spectra between 461.0979 and 984.8065 nm, hereafter called full spectral profile.

Full spectral profile

Once visual symptoms appeared, galled and ungalled plant phenotypes were easily distinguished (Fig. 3.2). Thus, at the beginning, ungalled plants look like the plants where the galling process were induced by the Hessian fly (Fig. 3.2 A, B and C); however, 12 DPI galled and ungalled plants have different phenotypes (Fig. 3.2 D, E and F).

From the spectral profile collected from the first 12 days that larvae are feeding on wheat it was possible to observed that the spectral profile in some regions was different between galled and ungalled plants. Interestingly, it was also possible to distinguish between seedlings galled by vH13-Israel and vH13-US larvae (Fig. 3.3). Examining the same data set, we found that the spectral profile was affected by treatment, date and treatment x date interaction (Table 3.1). The first two spectral axes explained 96.4% of the variability observed. Using these axes, it was possible to separate the spectral profiles of each treatment into a distinctive group. The first spectral axis shows separation between galled and ungalled plants, and the second axis makes a clear separation between the vH13-Israel and vH13-US galled plants (Fig. 3.4). The ability of the spectral profile to classify between galled and ungalled plants was evaluated using a discrimination model (Table 3.2). This model was able to differentiate these two groups of plants with an error rate of 6.66%. Another model was performed to evaluate the ability of the spectral profile to classify between ungalled and galled induced by different Hessian fly populations (Table 3.3). Although the error rate of the model increased when 3 groups of plants were included our result suggest that the spectral profile of H13-present plants changes as a result of the galling process, but this changes were not independent of who is inducing this process. Additionally, both models were performed for each post infestation day in order to describe the effect of date in the prediction. It is interesting to see that the prediction was more accurate when vH13-Israel flies were inducing the galling process compared with vH13-US induction (Table S3.1-S3.12).

Spectral Indices

A partial spectrum was used to compute the 10 indices (Fig 3.5). This partial spectrum was affected by treatments, date and their interaction (Table 3.4). ARI was the index with the biggest contribution to overall dissimilarity between treatments. Additionally, this index allows us to discriminate between treatments (df=2, K-W chi square=58.1, p-value<0.001; Fig. 3.6).

3.4 Discussion

The morphologies of many cecidomyiid-induced galls on many plant species have been described both macroscopically and microscopically (Harris et al., 2006; Stone & Schönrogge,

2003; Weis et al., 1988). However, this is the first time hyperspectral data have been used to describe the gall-induction process. This investigation demonstrates that this technology permits the differentiation of galled and ungalled plants earlier than a visual examination, and distinguishes between the galled plant phenotype induced by different Hessian fly populations.

Full Spectral Profile

Although similar morphological changes associated with the infestation in wheat have been described for Asian and American Hessian fly populations (El Bouhssini et al., 2009), the spectral profile between vH13-US and vH13-Israel showed differences (Fig. 3.3, 3.4, and 3.6). There is evidence that suggests that the gall-maker genotype can influence the changes observed in the host. For example, Weis and Abrahamson (1986) evaluated the interaction between the gall forming insect *Eurosta solidaginis* (Dipera:Tephritidae) and its host *Solidago altissima* (Asteraceae). They found that gall size is a heritable trait to which both insect and plant genomes contribute. Therefore, while the gall is composed only of plant tissue, its phenotype is influenced by the gall-maker genome.

It has been proposed that effector proteins would be responsible for the gall-maker manipulation. Due to the complexity of the process that gallers induce in their host to reprograming their cells, it is probably that gall-makers have many effector proteins to employ to induce the gall developing process (Oates et al., 2016).

In the Hessian fly genome at least 7% of all genes appear to encode effector proteins. Some of these proteins are very similar to wheat proteins involved in ubiquitination. These proteins apparently help the insect promote the formation of nutritive tissue and the immune suppression of wheat(Zhao et al., 2015). The nutritive tissue has been observed in wheat plants three days after infestation (Harris et al., 2010). After the first three days it is possible to detect changes in carbon and nitrogen: a reduction in the C-compounds and an increase in N-compounds (Zhu et al., 2008). Thus, using sensitive tools, such as hyperspectral phenotyping, we expected to see differences between ungalled and galled plants even during the earliest states of infestation (Tables S 3.1-S 3.8).

Johnson et al. (2012) examined vH13-US and vH13-Israel and found that mutations in the *vH13* gene were responsible for *H13*-virulence in both populations. Although both populations

have lost the functionality of the vH13 effector protein, we expect that due to these populations having evolved under different conditions that they may not share all effector proteins and that they may not share the same alleles for the effector encoding genes involved in gall induction. Thus, these two *H13*-virulent populations are expected to have sufficient differences in their genome to induce different infested plant phenotypes on the same plant genotype. This explains the different spectrum profiles observed between populations (Fig. 3.3 and 3.4;Table S 3.9-3.16). Hence, the Hessian fly-wheat interaction is another example of the extended phenotype hypothesis, in which genes of one organism have a phenotypic effect on a different organism (Dawkins, 1999). Thus, this hypothesis is now validated at the spectral profile level.

The ability of spectral data to distinguish between galled and ungalled plants and between galled plants induced by different Hessian fly populations opens the possibility of using this approach in the context of pest control. The Hessian fly has been controlled using mainly preventive strategies (Berzonsky et al., 2003), host plant resistance being the most reliable and cost effective means (Buntin, 1999). Unfortunately, one result of the use of resistant plants has been the development of Hessian fly biotypes able to survive on resistant wheat varieties. Typically, Hessian fly populations overcome plant resistance in six to eight years (Chen et al., 2009). Thus, Hessian fly populations should be periodically analyzed to provide wheat breeders and growers information on the effectiveness of wheat resistance genes and on biotype composition in regional populations (Cambron et al., 2010; Chen et al., 2009). However, standard biotype analysis is time consuming and labor-intensive, and it is impractical to check every field population (Chen et al., 2009). Therefore, it would be interesting to explore the potential of using phenotyping technology to detect infestation early in the season. Thus, farmers and plant breeders would have a new tool to make decisions with respect to the durability of the resistance before the symptoms are visible or the adverse effects become established.

This is a need not just for the Hessian fly, but also for other insect species. For example the soybean aphid (*Aphid glycines*), originally reported in United States in 2000, has become an important pest in soybean production due to yield losses reported, which can reach as high as 50%. Resistant plants have been used to control this aphid, but to date, four different biotypes have been described (Alt & Ryan-Mahmutagic, 2013). Thus, with respect to this insect, it would also be important to detect changes in biotype composition in the field that threaten resistance.

Spectral Indices

Several spectral indices have been used to interpret spectral data in relation to plant health. In wheat plants, these indices have been used to explore insect infestation, such as pathogen infection (Devadas et al., 2009; Mirik et al., 2006).

Because the Hessian fly causes little mechanical damage to the plant at the feeding site, the Hessian fly-wheat relationship has been compared with pathogen-plant interactions (Stuart et al., 2012). Larvae make just small perforations in the epidermal cells to inject saliva that carries effector proteins. Nevertheless, when we compared the plant physiological effects caused by the Hessian fly with those induced by wheat rust (*Puccinia spp.*), opposite spectral profiles are observed in the indices (Fig. 3.5) (Devadas et al., 2009).

Part of the spectral profile, the one used to calculate the indices, was able to differentiate between galled and ungalled plants. Anthocyanin reflectance index (ARI) was the index that made the greatest contribution to the difference between treatments. Devadas et al. (2009), evaluating wheat leaves with rust, also found that ARI was the most reliable index for discriminating between infected and healthy leaves. Additionally this index was able to distinguish among galled plants induced by different Hessian fly populations (Fig. 3.6). The ARI index was developed to estimate the amount of anthocyanin pigment in plant tissue (Gitelson et al., 2001). Thus, based on our results, uninfested plants have a greater concentration of anthocyanin compared with infested plants. Among the morphological changes associated to the Hessian fly infestation, the change in leaf color is one that has been widely described. Infested plants have greener leaves compared to uninfested plants. Robinson et al. (1960) found that leaves of infested plants have more chloroplasts than uninfested plants, and proposed that this could be the reason why greener leaf color is observed in infested plants. Because of this difference, we hypothesize that ARI best distinguishes between galled and ungalled plants because there is less anthocyanin relative to chlorophyll in galled plants. Thus, it is possible that because of the way that this index is computed, using wavelengths from 550 to 700 nm, greener plants have lower ARI values.

Our results suggest that using the full spectral profile and a subset of this profile the hyperspectral technology is able to detect phenotypic changes in wheat seedlings, under controlled conditions, as a result of the galling process. We learned that this technology discriminated galled and ungalled plants, and between galled plants induced by vH13-US and vH13-Israel. This approach looks like a promising tool to examine the Hessian fly-wheat interaction and should be

explored in field conditions in future investigations. Thus, this study stands as the first step in reaching the long-term goal of including phenotyping technology to detect Hessian fly-induced galls, as well as galled plant tissue induced by other plant parasites, as a diagnostic in the field. We anticipate therefore, that this technology will improve the durability of resistant plants and other pest management strategies.

3.5 References

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Table 3.1

Result of two-way PERMANOVA using the full spectrum collected from the first 12 DPI

	F	p-value
Treatment	87.9	<0.001
Date	108.5	<0.001
Treatment*Date	7.8	<0.001

Table 3.2

Confusion matrix of classification model between galled and ungalled plants

	Actual class	
	Galled	Ungalled
Predicted as Galled	120	9
Predicted as Ungalled	4	59
Error rate (%)	6.66	

Table 3.3

Confusion matrix of classification model between ungalled and galled induced by different Hessian fly populations.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	41	13	10
Predicted as vH13-Israel	3	60	2
Predicted as Control	7	0	56
Error rate	18.22		

Table 3.4

Result of two-way PERMANOVA from the spectral indices.

	F	p-value
Treatment	41.3	<0.001
Date	60.4	<0.001
Treatment*Date	4.7	<0.001

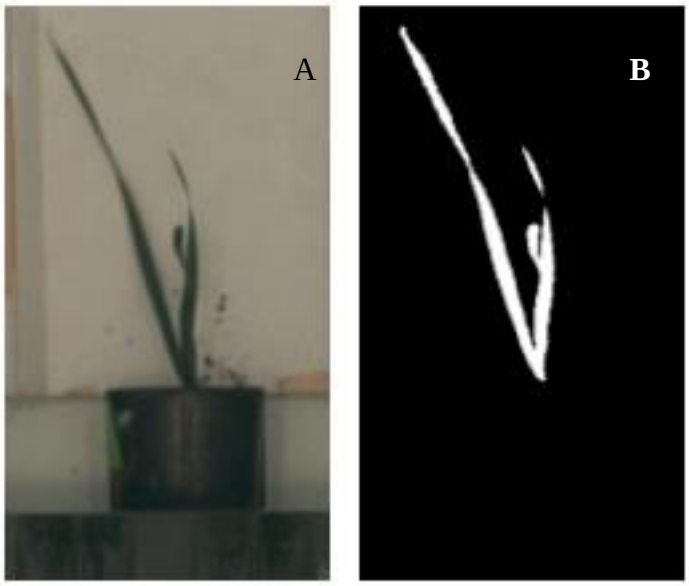

Fig. 3.1. A) Raw RGB image showing the wheat plant and the background, B) segmented image showing pixels from the plant.

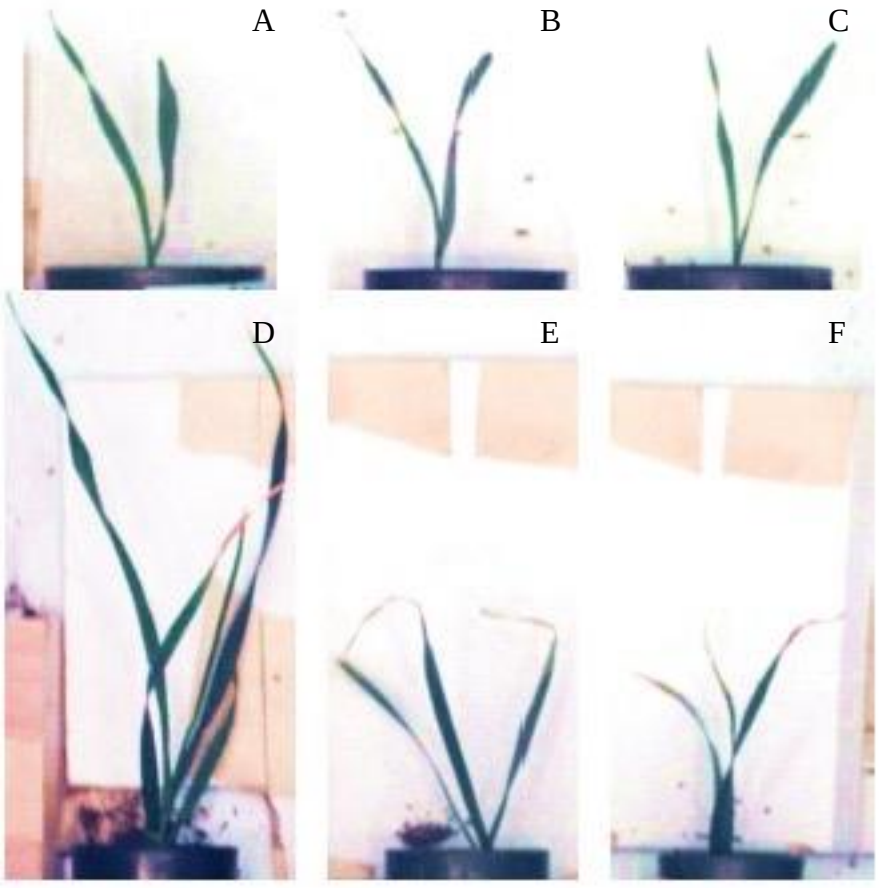

Fig. 3.2. RGB images illustrating the changes in plant phenotypes from 1DPI and 12 DPI between ungalled and galled plants. A and D Control sample 4 1DPI and 12 DPI, respectively; D and E vH13-US sample 2 1DPI and 12DPI, respectively; C and F vH13-Israel sample 18 1DPI and 12DPI, respectively.

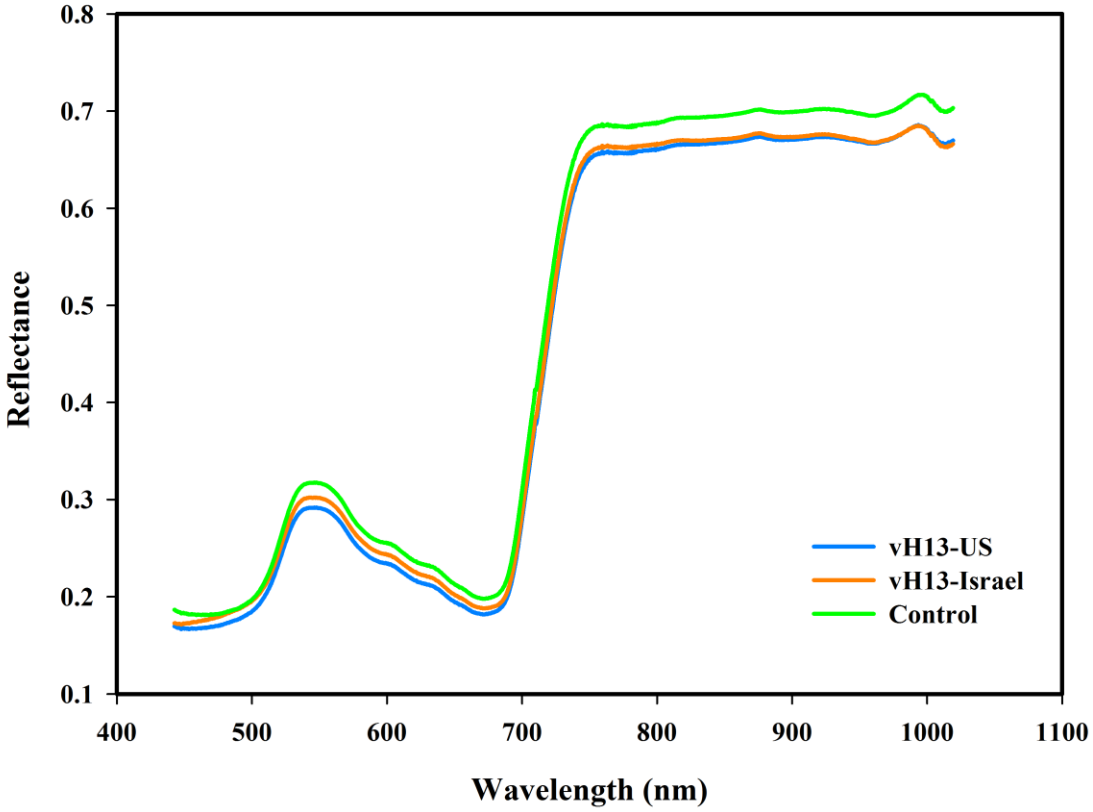

Fig. 3.3. Reflectance mean of H13-present wheat under different treatments.

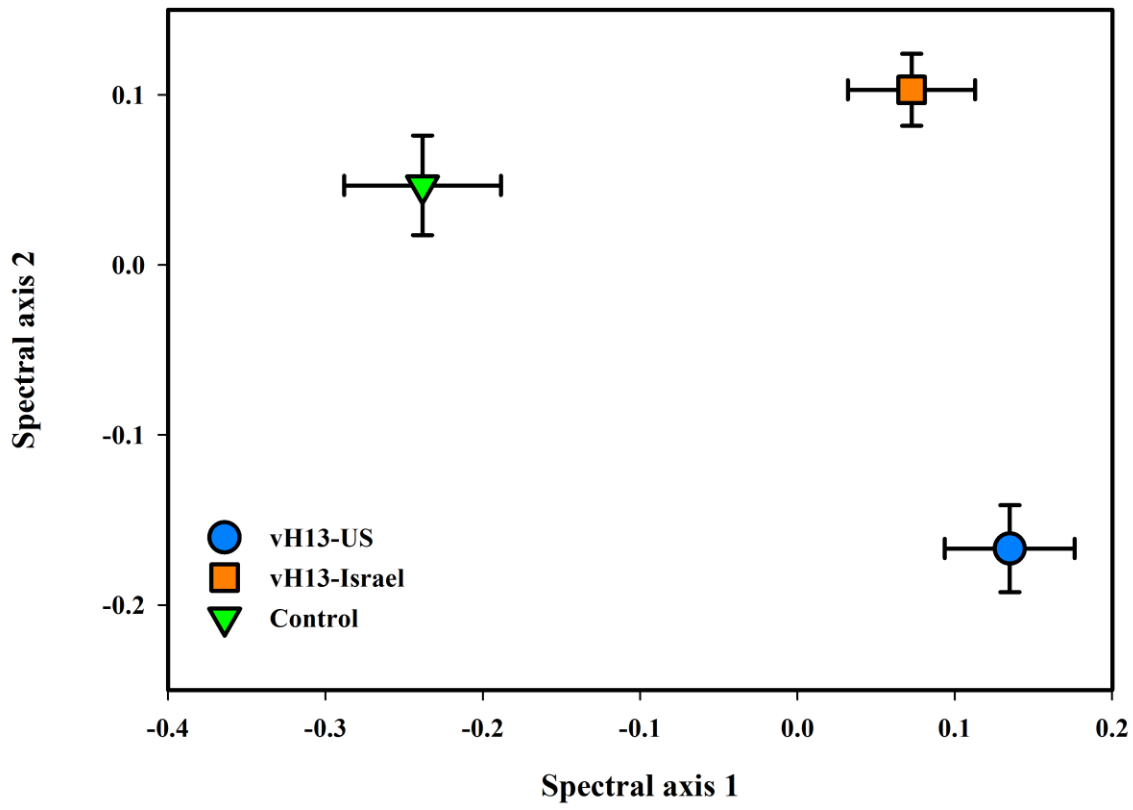

Fig. 3.4. Treatment means ± SE from principal coordinates analysis (PCoA) using the full spectrum for vH13-US, vH13-Israel and Control. Spectral axes 1 and spectral axes 2 explain 71.5%, and 25.1% of the variability observed among treatments, respectively.

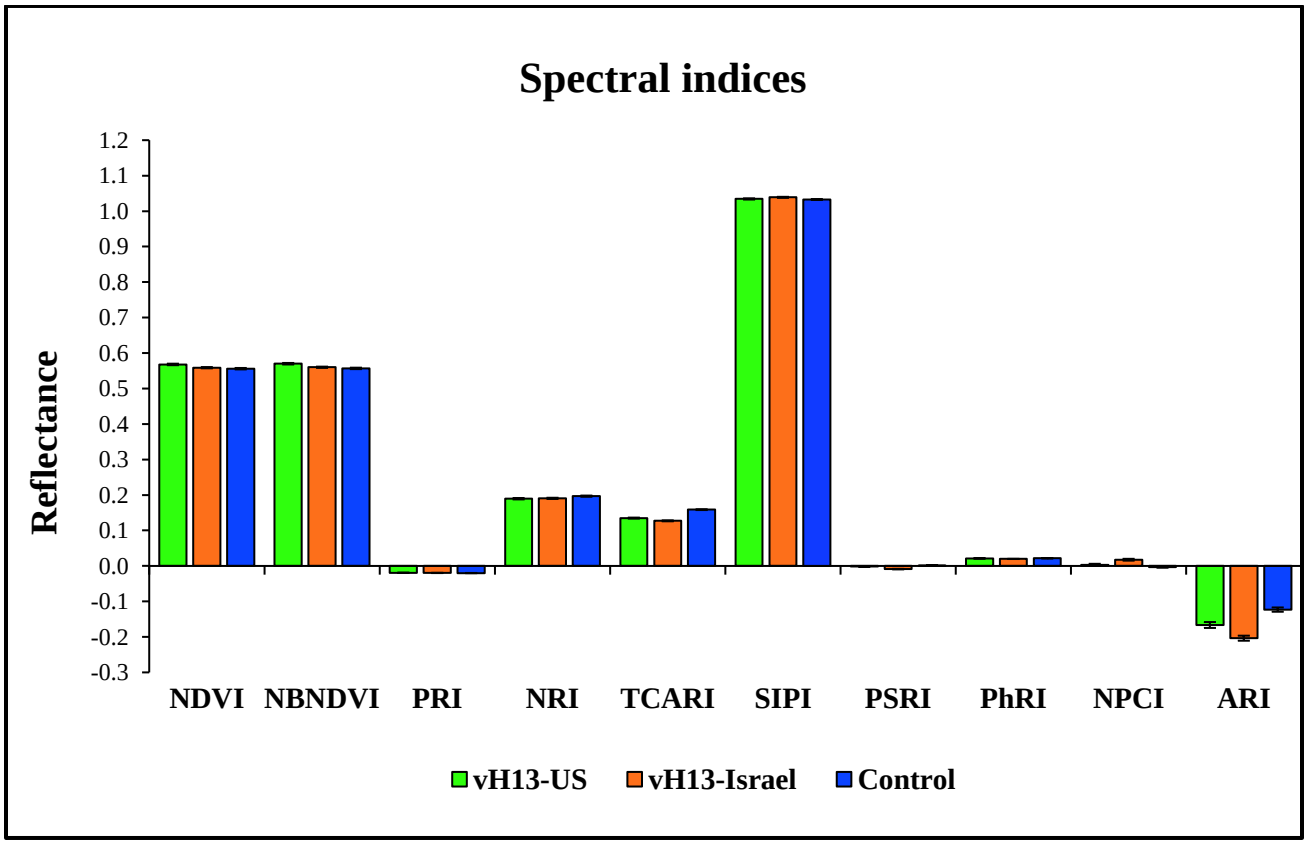

Fig. 3.5. Mean values ± SE of spectral indices for vH13-US, vH13-Israel and control.

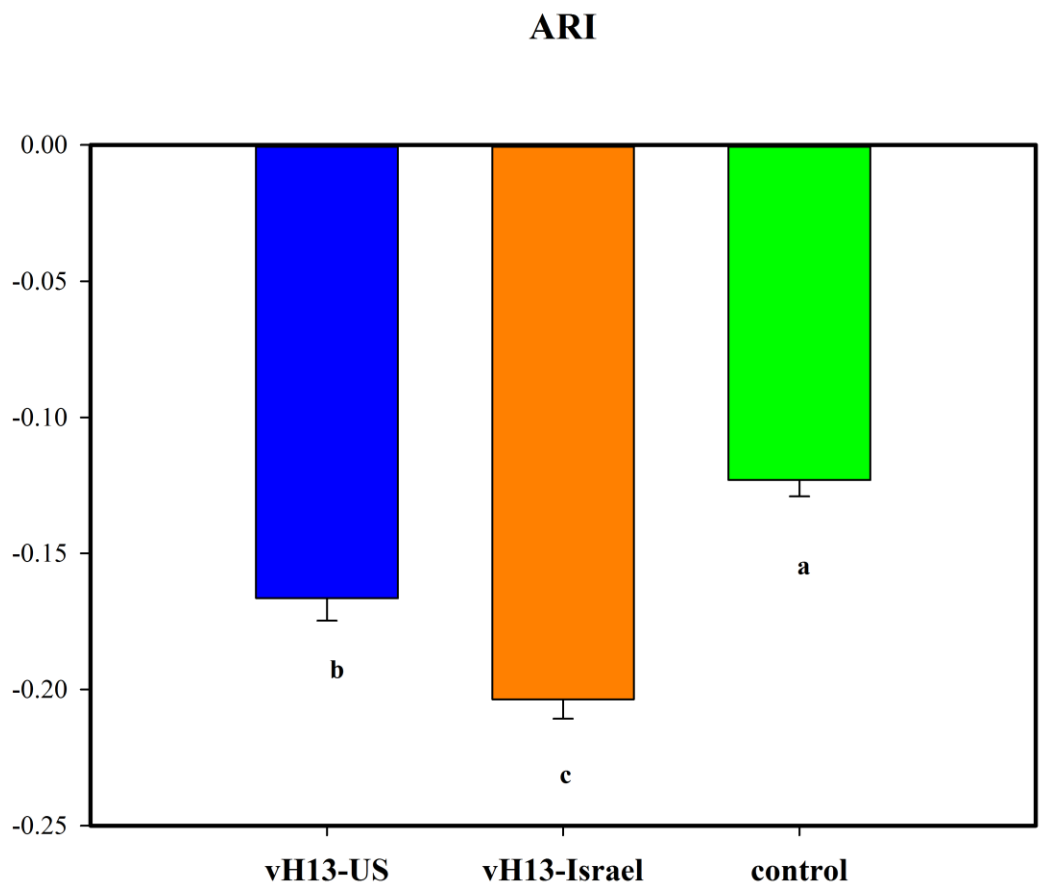

Fig. 3.6. Comparison of the anthocyanin reflectance index (ARI) among treatments. Mean values ± SE. Mean with different letters were significantly different (Tukey test $p<0.05$).

3.6 Appendix: Supplementary data for Chapter 3

Table S 3.1

Confusion matrix of classification model predicting galled and ungalled plants using 1DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	17	0
Predicted as Ungalled	0	10
Error rate (%)	0	

Table S 3.2

Confusion matrix of classification model predicting galled and ungalled plants using 2DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	15	2
Predicted as Ungalled	2	8
Error rate (%)	15.88	

Table S 3.3

Confusion matrix of classification model predicting galled and ungalled plants using 3DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	17	0
Predicted as Ungalled	2	8
Error rate (%)	10	

Table S 3.4

Confusion matrix of classification model predicting galled and ungalled plants using 4DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	17	0
Predicted as Ungalled	1	9
Error rate (%)	5	

Table S 3.5

Confusion matrix of classification model predicting galled and ungalled plants using 5DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	17	0
Predicted as Ungalled	0	10
Error rate (%)	0	

Table S 3.6

Confusion matrix of classification model predicting galled and ungalled plants using 8DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	16	1
Predicted as Ungalled	1	9
Error rate (%)	7.9	

Table S 3.7

Confusion matrix of classification model predicting galled and ungalled plants using 10DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	16	1
Predicted as Ungalled	0	10
Error rate (%)	2.9	

Table S 3.8

Confusion matrix of classification model predicting galled and ungalled plants using 12DPI data.

	Actual class	
	Galled	Ungalled
Predicted as Galled	16	1
Predicted as Ungalled	1	9
Error rate (%)	7.9	

Table S 3.9

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 1DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	4	1	0
Predicted as vH13-Israel	1	11	0
Predicted as Control	0	0	10
Error rate (%)	9.44		

Table S 3.10

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 2DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	4	0	1
Predicted as vH13-Israel	3	9	0
Predicted as Control	1	0	9
Error rate (%)	18.33		

Table S 3.11

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 3DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	5	0	0
Predicted as vH13-Israel	0	12	0
Predicted as Control	1	0	9
Error rate	3.33		

Table S 3.12

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 4DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	3	1	1
Predicted as vH13-Israel	4	8	0
Predicted as Control	2	0	8
Error rate	31.11		

Table S 3.13

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 5DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	5	0	0
Predicted as vH13-Israel	2	10	0
Predicted as Control	1	0	9
Error rate	8.88		

Table S 3.14

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 8DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	4	0	1
Predicted as vH13-Israel	0	12	0
Predicted as Control	1	0	9
Error rate	10.00		

Table S 3.15

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 10DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	4	0	1
Predicted as vH13-Israel	0	12	0
Predicted as Control	0	0	10
Error rate	6.66		

Table S 3.16

Confusion matrix of classification model predicting ungalled and galled by two different Hessian fly populations using 12DPI data.

	Actual class		
	vH13-US	vH13-Israel	Control
Predicted as vH13-US	5	0	1
Predicted as vH13-Israel	0	10	1
Predicted as Control	0	0	10
Error rate	8.59		

4. CHARACTERIZATION OF HESSIAN FLY INFESTATION ON WHEAT FUNCTIONAL TRAIT RESPONSES

4.1 Introduction

Parasites must manipulate theirs host`s physiology and immunity in order to survive. From a morphological standpoint this is perhaps most evident in the manifestation of insect-induced plant galls (Tooker et al., 2008). However, the precise mechanisms used and their timing are poorly understood (Giron et al., 2016; Oates et al., 2016). To improve our understanding, model systems can be combined with more sensitive methodologies. Here we examine the galling process in the model system wheat-Hessian fly using reflectance spectroscopy.

The model organism, the Hessian fly (*Mayetiola destructor*), is a destructive insect pest of bread wheat (*Triticum aestivum*) and durum wheat (*T. turgidum*; Berzonsky et al., 2003). In the United States, since its first report in the 1770s in New York (Pauly, 2002), this pest has spread to all of the wheat producing areas of the country (Cambron et al., 2010). The damage to wheat caused by the Hessian fly is associated with the insect´s ability as a gall-maker to manipulate its host´s development.

The Hessian fly and wheat have a gene-for-gene interaction. Thus, for each resistance (*R*) gene in the plant there is an avirulence *(avr)* gene in the insect. To date one of these *avr* genes, *vH13*, has been genetically and physically positioned in the Hessian fly genome (Aggarwal et al., 2014). *H13*-avirulent flies carry the dominant "H13-avirulence" allele ($vH13^{v}$) at the *vH13 avr* locus. These alleles produce the vH13 effector protein, which is recognized by the H13 R protein in wheat. This recognition triggers a plant defense response that kills Hessian fly larvae (Stuart et al., 2012). However, flies that do not express the vH13 effector protein escape H13 R protein detection, and the larvae survive and gall *H13* plants. Thus, the Hessian fly's ability to induce a gall depends not only on the insect genotype, but also on the plant genotype. When a gall is induced the interaction between the insect and the plant is called "compatible", whereas if the gall cannot be induced and the larva dies then the interaction is called "incompatible" (Mittapalli et al., 2006).

Under compatible interactions, Hessian fly larvae induce changes that affect the whole plant seedling. Galled plants exhibit stunted growth, dark green leaves, and eventually die. A reason that could explain these changes could be that the quality of the growth of nutritive tissue

induced by the larvae is changed at the beginning of the infestation. This galled tissue acts as a sink for nutrients that are mobilized through the vascular system, supplying high quality resources to the larvae with a detrimental effect on plant development (Bronner, 1992; Harris et al., 2006). On the other hand, under incompatible interactions the *R* gene prevents the induction of nutritive tissue, thus the plants do not experience the changes that occur in a compatible interaction (Anderson & Harris, 2006).

Reflectance spectroscopy has been used as an effective method to estimate the concentrations of various plant compounds, including nutrients and secondary metabolites (Couture et al., 2016; Peñuelas & Filella, 1998). This methodology is a less expensive, less time consuming and a more reproducible alternative to standard chemical analyses of plant tissue. The ability of reflectance spectroscopy to identify and quantify plant compounds lies in the differential absorption of light depending on chemical structures and functional groups. The light reflected by the plant tissue is affected by the concentration of biochemical compounds because the bonds between different atoms, as a consequence of the radiation, produce differential vibrational waves. Thus, the light that matches the frequency of these vibrational waves is absorbed while the unmatched light is reflected or transmitted (Peñuelas & Filella, 1998). Organic components can be identified and quantified using near-infrared radiation (750-2500 nm) because this is absorbed by C-H, N-H, and O-H bonds, which are the main bonds associated with organic compounds. Thus, this technology is emerging as a valuable alternative to other methods that describe changes in plant chemical composition in plant-insect interactions.

4.2 Material and Methods

Plant

Patterson et al. (1994) developed near isogenic lines resistant to Hessian fly transferring resistance genes to a susceptible winter cultivar called Newton. The variety of wheat that carries the *H13* resistant gene, Molly, was used in this experiment. Individual seedlings of Molly were germinated in plastics cones with a 4 cm diameter and 14 cm depth in autoclaved soil under greenhouse conditions.

Insects

Two virulent strains of Hessian fly to Molly were used. One strain comes from US population, hereafter referred as vH13-US, and the other one from Israel populations, hereafter referred as vH13-Israel. Additionally, one avirulent strain, GP, was used. GP is a susceptible strain, unable to survive on wheat plants with any resistance genes.

Infestation Protocol

Gravid females from the 3 strains were allowed to lay their eggs on Molly seedlings. Three days after oviposition, neonate first instar larvae were collected in an aqueous solution of 0.02% NP-40. These larvae were transferred to Molly plants at the two-leaf developmental stage in the same solution. Each plant was infested with 2 larvae. Sixty plants were infested with vH13-Israel and 60 vH13-US flies (compatible interaction). Sixty plants were infested with GP flies (incompatible interaction). Additionally, forty uninfested plants served as a control. Plants were maintained at 20±2ºC with 80% humidity and L14:D10 photoperiod.

Collection of Spectral Measurements

We collected leaf reflectance using a high resolution full-range (350-2500 nm) spectroradiometer (1024i; Spectral Vista Corporation, Poughkeepsie, NY USA) using a reflectance probe with a leaf clip attachment. Because of the width of the young wheat leaves, it was necessary to construct leaf mats (Fig. 4.1) to fill the field of view. Reflectance measurements were collected on the foliar adaxial surface and two replicate measurements were collected for each mat. We collected leaf reflectance on plants prior to infestation and also incompatible interactions (i.e., avirulent flies), compatible interactions (i.e., virulent flies), and control treatments at two different time periods (4 and 12 days post infestation).

After the spectral measurements, foliar samples were flash-frozen with liquid nitrogen and stored at -20 °C until the leaves were lyophilized and then ground to a homogenous particle size via ball milling.

Chemical Analyses

Standard analytical determination of carbon and nitrogen was performed using a Thermo Finnigan Flash 1112 elemental analyzer. Total phenolic contents were quantified colorimetrically

according to Ainsworth and Gillespie (2007) with minor modifications. Twenty mg of dried leaf samples were extracted with 1.9 ml of 95% (vol/vol) methanol at room temperature for 48 h in the dark. Extracts were centrifuged for 5 min at 13,000*g* and room temperature. Then, 100 µl of each sample supernatant were mixed with 200 µl of 10% (vol/vol) Folin-Ciocalteu reagent and 800 µl of 700 mM Na_2CO_3. After a 2-h incubation at room temperature, 200 µl of each sample were transferred in a clear 96-well microplate and absorbance of each sample was recorded at 765 nm using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale CA). The blank-corrected absorbances were quantified using gallic acid a standard curve (0-0.7 mg/ml) and are reported as gallic acid equivalents.

Chemometric Modeling Approach

We generated models to predict nitrogen, sugar, starch, and phenolic concentrations (% dry mass) from fresh leaf spectral data using partial least squares regression (PLSR) (Wold et al., 1984; Wold et al., 2001). In cases where predictor variables are highly correlated, such as with spectral data, traditional regression techniques produce unreliable coefficients as predictor variables lead to bias in coefficients and error estimates (Grossman et al., 1996). PLSR, in contrast with standard regression techniques, reduces a large number of collinear predictor variables into a relatively few, uncorrelated number of latent variables, and has become the preferred method for chemometric analyses (Asner & Martin, 2008, 2011; Atzberger et al., 2010; Bolster et al., 1996; Serbin et al., 2014) We based the number of latent variables on reduction of the predicted residual sum of squares (PRESS) statistic (Chen et al., 2004) using leave-one-out cross validation. We combined the final set of extracted factors into a linear model predicting chemical concentrations. Examination of prediction residuals was used to identify outliers following Couture et al. (2016) which were subsequently removed from further analyses (Nitrogen= 6% of dataset; Carbon =5, 6% of dataset; leaf mass area (LMA) 33, 8% of dataset; Phenolics = 4, 5% of dataset).

We used wavelengths between 1500 and 2400 to build models for nitrogen and carbon, 400-2400 for LMA, and 1100-2400 for phenolics. We focused on the SWIR region for nitrogen, carbon, and phenolics because 1) our previous research has shown strong relationships in this wavelength range with our target constituents and 2) it avoids leveraging correlations between our target constituents and pigments in the visible wavelengths, instead focusing on the physical

relationship of absorbance of organic compounds and the functional groups associated with these compounds (Couture et al., 2016).

We assessed model performance by conducting 500 randomized permutations of the dataset using 70% of the data for internal calibration and withholding the remaining 30% for external validation. For each model permutation, we tracked the model fit (R^2), root mean square error (RMSE), and bias to assess model performance as applied to the held-out dataset. Here, we report the RMSE as both the determined value and as a percentage of the range of data (%RMSE) for each dependent variable. The latter metric is useful to assess the predictability error of the model within the data range and is also comparable across different models. An additional benefit %RMSE is that while it is functionally similar to the classical chemometric statistic residual prediction deviation (RPD), which assesses model error within the context of the reference data, %RMSE avoids the distribution assumptions and subjective model classifications associated with RPD (Couture et al., 2016). In addition, we determined the strength of the contribution of PLSR coefficients by individual wavelengths using the variable important to the projection (VIP) statistic (Wold et al., 1984; Wold et al., 2001). The VIP indicates the importance of an individual wavelength in explaining the variation in the response and predictor and response variables, such that larger weightings confer greater influence of individual wavelengths to the predictive model (Chong & Jun, 2005; Wold et al., 2001). Model performance statistics can be found in Supporting Information Figures 4.1-4.4. The modeling approach and model performance analyses were performed using the *pls* package (Mevik & Wehrens, 2007) in R (www.r-project.org).

Statistical Analyses

We analyzed leaf nitrogen concentration, C:N ratios, LMA, and phenolic content by repeated measures analysis of variance (RM-ANOVA) following the model $y_{ij} = T_i + D_j + TD_{ij}$. In this model, T represents treatment i, D represents day post infection j, and TD represents the interaction between treatment i and day post infection j. Treatments for this analysis included an uninfested control, incompatible (GP flies), vH13-US compatible and vH13-Israel compatible. To examine the changes in the above mentioned constituents, relative to the average pre-infestation value, we used a similar RM-ANOVA approach as above with the exception that plant responses to individual fly populations were averaged and used as a single treatment. Treatments for this analysis included a, uninfested control, incompatible, and compatible (vH13-US and vH13-Israel)

combined. We additionally compared the plant responses to the different fly populations using a separate analysis but a similar RM-ANOVA as above with the exception that the treatment contrasted only relative changes between the two fly populations compared to the average pre-infestation value. Treatments for this analysis included individuals from the vH13-US flies, and vH13-Israel flies.

4.3 Results

Prediction models characterized nitrogen, carbon, LMA, and phenolics on leaves of wheat under different infestation status (uninfested, compatible, and incompatible) very well (Fig.S4.1-S4.4). Mean validation values for nitrogen are as follows: R^2, 0.85; RMSE, 0.425; bias, 0.009; %RMSE, 10%. Mean validation values for carbon are as follows: R^2, 0.84; RMSE, 1.13; bias, -0.084; %RMSE, 22%. Mean validation values for LMA are as follows: R^2, 0.94; RMSE, 0.40; bias, -0.005; %RMSE, 6%. Mean validation values for phenolics are as follows: R^2, 0.69; RMSE, 0.83; bias, -0.01; %RMSE, 20%.

Infestation status and day post infection independently and interactively affected the four plant traits in response we measured (Fig. 4.2, Table 4.1). Relative changes showed a similar trend among all treatments for nitrogen, C:N ratio, and LMA over time, but the magnitude of the change was greater in the incompatible interactions than wither the control or compatible interactions, which were similar. However, for phenolics the relative change over time was similar between the control and compatible interaction, but increased under the incompatible interaction. All plant traits exhibited a similar pattern at the onset of infestation, where four days post infestation responses for the compatible interaction behave more similar to the control compared with the incompatible interaction, which responded differently (Fig. 4.3, Table 4.2). This pattern, however, was not observed consistently at twelve days post infestation (Fig. 4.3, Table 4.2).

Plant traits responded differently between fly populations and days post infestation except for LMA (Fig. 4.4, Table 4.3). Four days post infection, plant nitrogen content and C:N levels were similar between fly populations, but at twelve days the magnitude of change was greater for vH13-US than vH13-Israel populations (Fig. 4.4). Changes in LMA were similar between both fly populations (Table 4.3). The relative change in phenolics between fly populations was different than changes for plant nitrogen and C:N such that at four days post infestation plant phenolics

were approximately 3 times lower in vH13-Israel population than the vH13-US populations, but this response disappeared twelve days post infestation (Fig. 4.4).

4.4 Discussion

Near infrared reflectance spectroscopy has been successfully used to predict the composition of various compounds in plant tissues (Foley et al., 1998). Here, we used this approach to examine the changes in wheat plants responding to both compatible (plant galling) and incompatible (plant resistance) wheat-Hessian fly interactions. Previous investigations have characterized the microscopic and macroscopic changes in wheat morphology, specifically near the larval feeding site, and the gene expression profiles associated with these interactions. In combination with these studies, the ability of near infrared reflectance spectroscopy to accurately predict the relative abundance of plant compounds associated with the wheat response to the infestation from plant tissue above the soil provides new insight into the physiological processes underlying both interactions.

Incompatible Interaction

Plants have three general mechanisms they can use to resist insect attack: 1) they can act to limit access to the resource, 2) they can reduce the nutritive value of the available resource, and 3) they can produce toxic or anti-nutritive compounds in the resource (Chen, 2008). This investigation discovered evidence of all three mechanisms acting in wheat-Hessian fly incompatible interactions.

Among the mechanisms that limit the resource supply are the hypersensitive response (HR) and cell wall fortification. HR causes localized cell death that isolates the parasite from nutritive tissue. It has been widely observed as an induced defense response against gall-maker insects (Fernandes, 1990; Hoglund, 2014). Although dead tissue at the feeding sites of incompatible wheat-Hessian fly interactions is difficult to detect, larval mortality and an oxygen burst on resistant plants suggests that HR may be associated with the wheat-Hessian fly interactions involving at least some R genes (Grover, 1995; Shukle et al., 1992).

Our investigation does not provide additional clarity to the question of an HR reaction in the case of the *H13* R gene. However, we note that Santos et al. (2017) studying changes in leaf nutrients of *Bauhinia brevipes* as a result of the stress induced by gall-maker *Schizomyia macrocapillata* in resistant plants found that HR was associated with low levels of nitrogen. They suggested that nitrogen could be involved in the pathways related to HR. Our prediction for the changes in nitrogen concentration is consistent with this observation. Four days post infestation under incompatible interactions the relative reduction in nitrogen concentration was greater than both compatible interactions or uninfested treatments.

A more compelling case can be made for limiting the insect from its food source via cell wall thickening and cell fortification. Harris et al. (2010) observed that the cell walls surrounding the larval feeding site showed thicker cell walls in incompatible interactions associated with three different R genes (*H6, H9* and *H13*). Our finding of increased LMA in plants under incompatible interactions is consistent with this result (Fig. 4.3), but also suggests that cell fortification is occurring in cells beyond the feeding site.

It also appears that additional defense against Hessian fly infestation involves decreasing the quality of the food available to the insect. Zhu et al. (2008) evaluated changes in C:N ratio on Molly plants under compatible and incompatible interactions. In this study they found that three days post infestation the plant tissue near the larval feeding place had a C:N ratio of 0.75 for uninfested plants, 0.46 for incompatible interactions, and 0.33 for compatible interactions. These relative ratios among treatments are similar to our prediction at twelve days post infestation. However, at four days post infestation we found that relative changes in C:N ratio between control and compatible interactions were smaller and more similar to the incompatible interactions. Considering that nitrogen is a limiting nutrient for herbivorous insect growth and development, because of the mismatch between insect physiological demands and plant content (Fagan et al., 2002; Mattson, 1980), an increase in the C:N ratio makes it more difficult for the insect to obtain the necessary quantity of the nitrogen necessary for insect growth.

Finally, there is also evidence that the plant produces toxins that adversely affect the insect´s ability to survive on resistant plants. We found an increase in the level of phenolic compounds at four days post infestation. The negative effects of phenolics in general are seen in the midgut of insects (Simmonds, 2003), which is consistent with the observation of midgut disruption early in the Hessian fly infestation process (Shukle et al., 2010).

The midgut seems an important target for plant resistance products, because the Hessian fly lacks a peritrophic membrane, and other protective structures such as the perimicrovillar membrane (Shukle et al., 2010). Therefore, the microvilli is exposed to food and toxic compounds such as the phenolics (Nation, 2008).

Thus our results suggest that, from a nutritional standpoint, under incompatible interactions, wheat limits the acquisition and quality of food. This in turn, potentially has negative effects on larval performance. Depending on the *R* gene, more of the 80% of larvae die within four days of infestation and no larvae survive after 7 days (Chen et al., 2009). Therefore, an early response to the infestation would be critical in order to minimize the negative effects on plant fitness, especially considering that has been described that changes in the plant are irreversible after four days of infestation (Byers & Gallun, 1972).

Although, larvae die under incompatible interactions, the resistance genes do not prevent wheat plants from early attack on the epidermal cells. Actually, it has been observed that the larvae try several times but are unable to establish a proper feeding site (Grover, 1995). Anderson and Harris (2006) proposed that this early attack could explain the growth deficit observed on resistance plants at the beginning of the interaction with avirulent larvae. Therefore, the complete seedling experiences changes as result of the resistance, which exceeds the feeding region and can be detected in the plant tissue above the soil, especially in the early infestation stages.

Compatible Interaction

Plants under stress negatively affect the survival and colonization of gall-maker species (Koricheva et al., 1998). Under compatible interactions, our results suggest that the Hessian fly larvae interfere with the capacity of wheat to detect and respond to larval infestation.

Although the Hessian fly larvae have been feeding for several days, and the manipulation of wheat is visible through the development of nutritive tissue (Harris et al., 2006), we found that in galled plants nitrogen concentration, C:N ratio, LMA and phenolic concentration are more like uninfested plants than ungalled plants, especially at the beginning of gall development. Thus, it appears that gall makers modify the physiological responses of the plant so that they exploit the resources they need for successful development while avoiding exposure to wheat defense responses. This is consistent with the nutrition hypothesis (Price et al., 1987), which suggests that the adaptive significance of galls is related to the ability of the gall maker to increase nutrient

quality and availability and decrease exposure to plant defense mechanisms (Gange & Nice, 1997; Hartley & Lawton, 1992).

From the previous chapter we concluded that the Hessian fly wheat interaction would be another example of the extended phenotype hypothesis. Thus, the phenotypic changes in Molly plants due to the galling process depends in part on the Hessian fly genotype. Trying to elucidate the reason behind these differences we used the same Hessian fly populations in this study (Fig. 4.4). At four days post infestation plants from different populations of compatible interactions differed only in the phenolic content, with a lower phenolic content in plants infested with vH13-Israel than vH13-US. Lower phenolic concentrations can be associated with higher infestation success observed in chapter 3. In addition, at twelve days post infestation, the magnitude of decrease in nitrogen concentrations and increase in C:N ratios, compared to control plants, were greater in vH13-US than vH13-Israel (Table 4.3).

Our results suggest that near infrared reflectance spectroscopy is able to detect changes in plant physiology on the wheat tissue above the soil to distinguish resistant and susceptible plants. Additionally, we learned that the four traits evaluated in this study play an important role in the wheat's capacity to resist Hessian fly infestation. Therefore, physiological changes early in the infestation process may be promising areas to explore subsequent questions remaining about the mechanisms associated with the Hessian fly-wheat interaction. While approximately 7% of the Hessian fly genome encodes effector proteins and several candidate effector genes have been identified (Aggarwal et al., 2014; Zhao et al., 2015; Zhao et al., 2016) the function of effector proteins is still unknown. To date, bio-assays to express these proteins inside the plant have been designed (Navarro, 2016); however, accurate tools to detect their effect were previously unavailable. Thus, reflectance spectroscopy could be a powerful tool to better understand the manipulation process induced by this fly in its host and the evolution of the Hessian fly-wheat relationship.

4.5 References

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Table 4.1

Two-way ANOVA examining the effect of infestation status (uninfested, incompatible, and compatible interactions), and days post infestation (DPI), and their interaction on plant traits.

Effects	**df**	**Nitrogen**		**C:N**		**LMA**		**Phenols**	
		F	**p-value**	**F**	**p-value**	**F**	**p-value**	**F**	**p-value**
Infestation status	3	7.8	<0.001	4.5	0.004	469.0	<0.001	279.8	<0.001
DPI	1	529.3	<0.001	180.4	<0.001	885.5	<0.001	0.9	0.344
Infestation x DPI	3	16.0	<0.001	5.0	0.002	36.4	<0.001	12.1	<0.001

Table 4.2

Two-way ANOVA examining the effect of infestation status (uninfested, incompatible, and compatible), days post infestation (DPI), and their interaction on the relative changes in plant traits

Effects	**df**	**Nitrogen**		**C:N**		**LMA**		**Phenols**	
		F	**p-value**	**F**	**p-value**	**F**	**p-value**	**F**	**p-value**
Infestation status	2	9.4	<0.001	5.4	0.005	705.1	<0.001	278.2	<0.001
DPI	1	448.3	<0.001	167.1	<0.001	625.5	<0.001	0.002	0.901
Infestation x DPI	2	20.8	<0.001	6.1	0.002	54.0	<0.001	7.1	<0.001

Table 4.3

Two way ANOVA examining the effect of insect populations (vH13-US or vH13-Israel), days post infestation (DPI), and their interaction on relative changes in plant traits.

Effects	**df**	**Nitrogen**		**C:N**		**LMA**		**Phenols**	
		F	**p-value**	**F**	**p-value**	**F**	**p-value**	**F**	**p-value**
Insect Population	1	4.4	0.036	3.3	0.068	0.006	0.935	19.9	<0.001
DPI	1	343.8	<0.001	111.5	<0.001	939.0	<0.001	7.9	0.006
Infestation x DPI	1	6.3	0.013	3.9	0.049	1.6	0.202	22.5	<0.001

Fig. 4.1. Example of a leaf mat. The leaves are arranged to fill the field of view of the plant probe used to measure the reflectance of each plant.

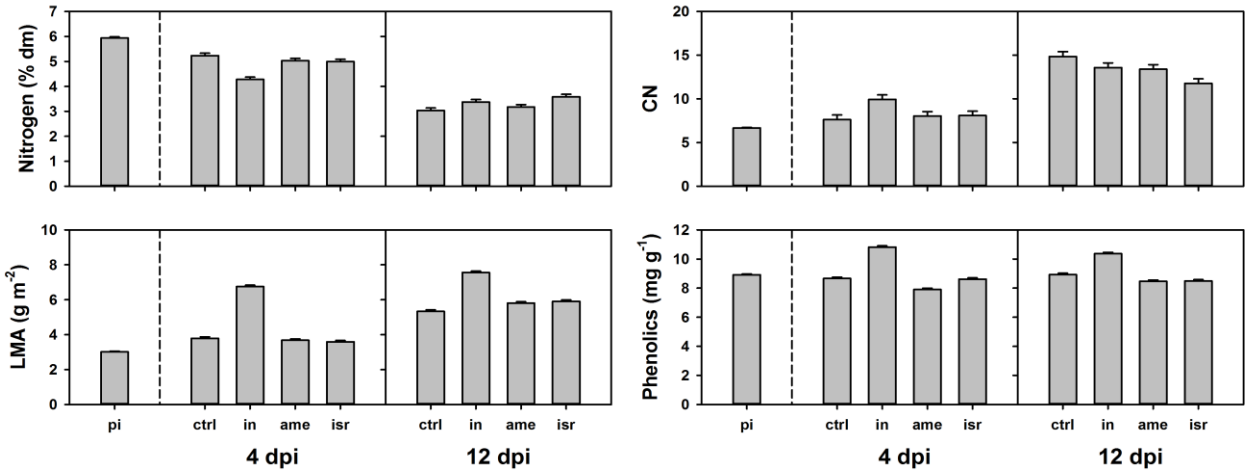

Fig. 4.2. Mean ± SD of predicted plant traits pre infestation (pi) and at four and twelve days post infestation (dpi) for the non-infested control (ctrl), incompatible interactions (in), vH13-US (ame), and vH13-Israel (isr). Statistical analysis includes only ctrl, in, ame, and isr.

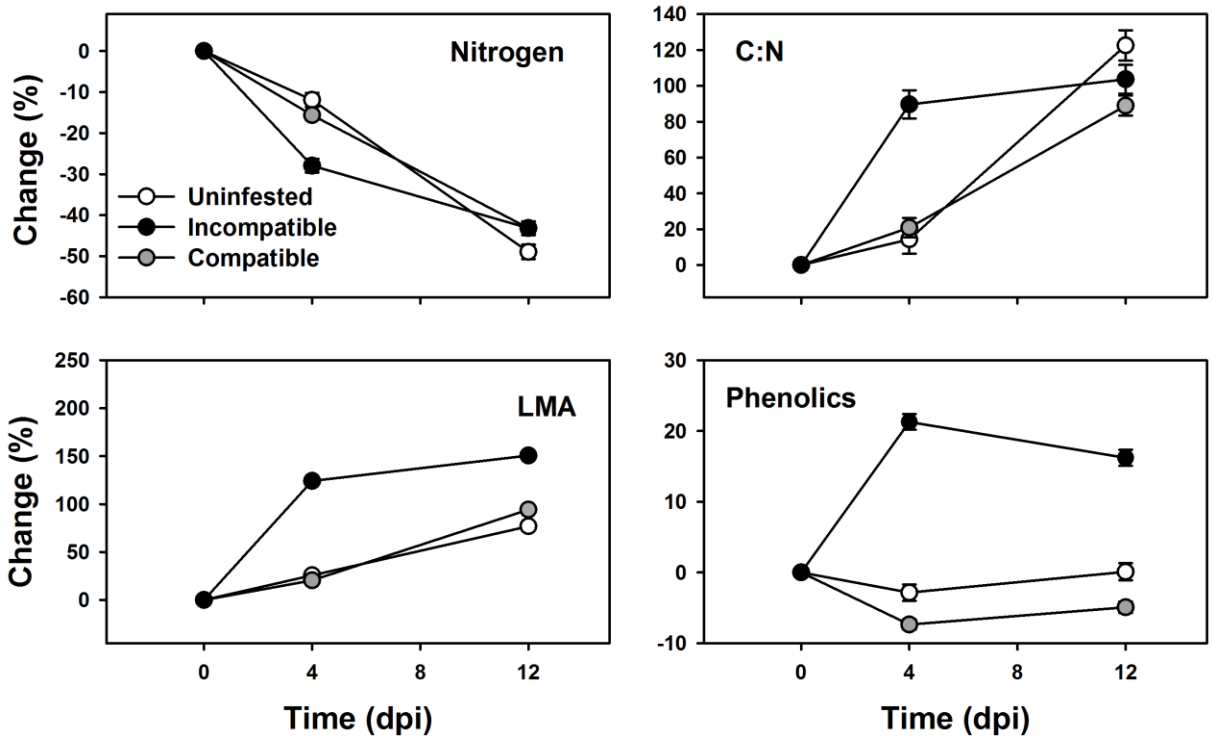

Fig. 4.3. Mean ± SD of the relative changes of nitrogen concentration, C:N ratio, LMA and phenolic concentration for uninfested plants, compatible interactions and incompatible interactions at four- and twelve-days post infestation (dpi).

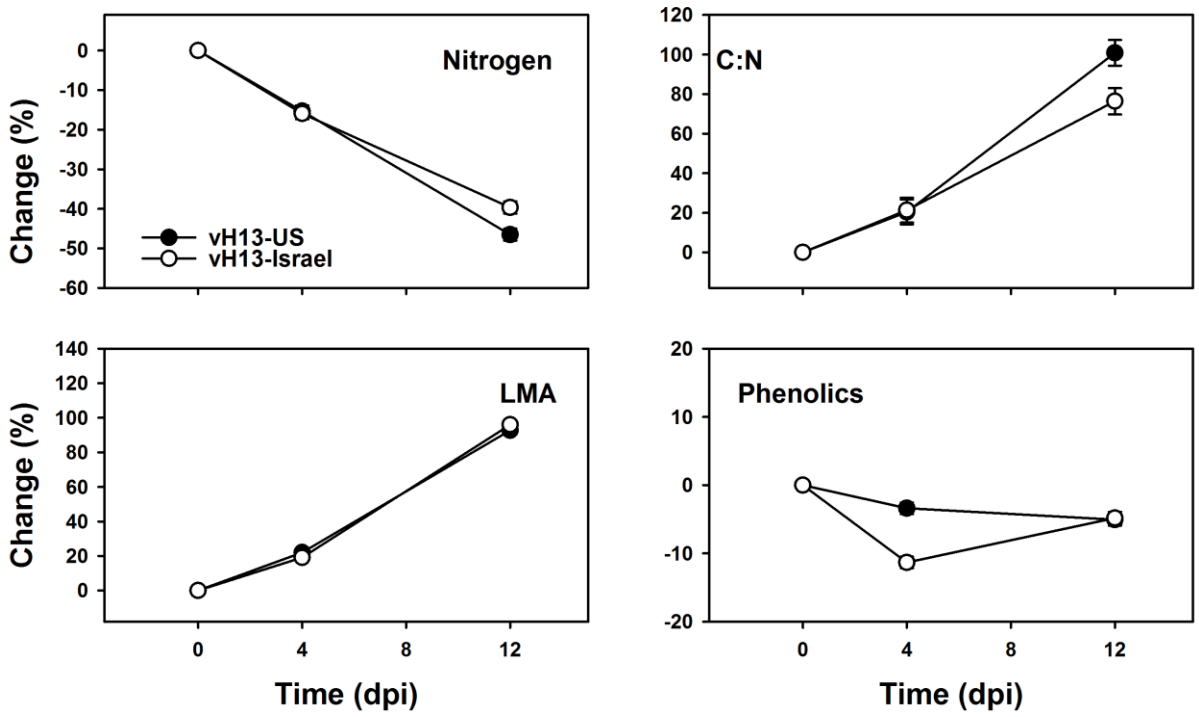

Fig. 4.4. Mean ± SD of the relative changes of nitrogen concentration, C:N ratio, LMA and phenolic concentration for uninfested plants, for vH13-US and vH13-Isreal at four- and twelve-days post infestation (dpi).

4.7 Appendix: Supplementary Data For Chapter 4

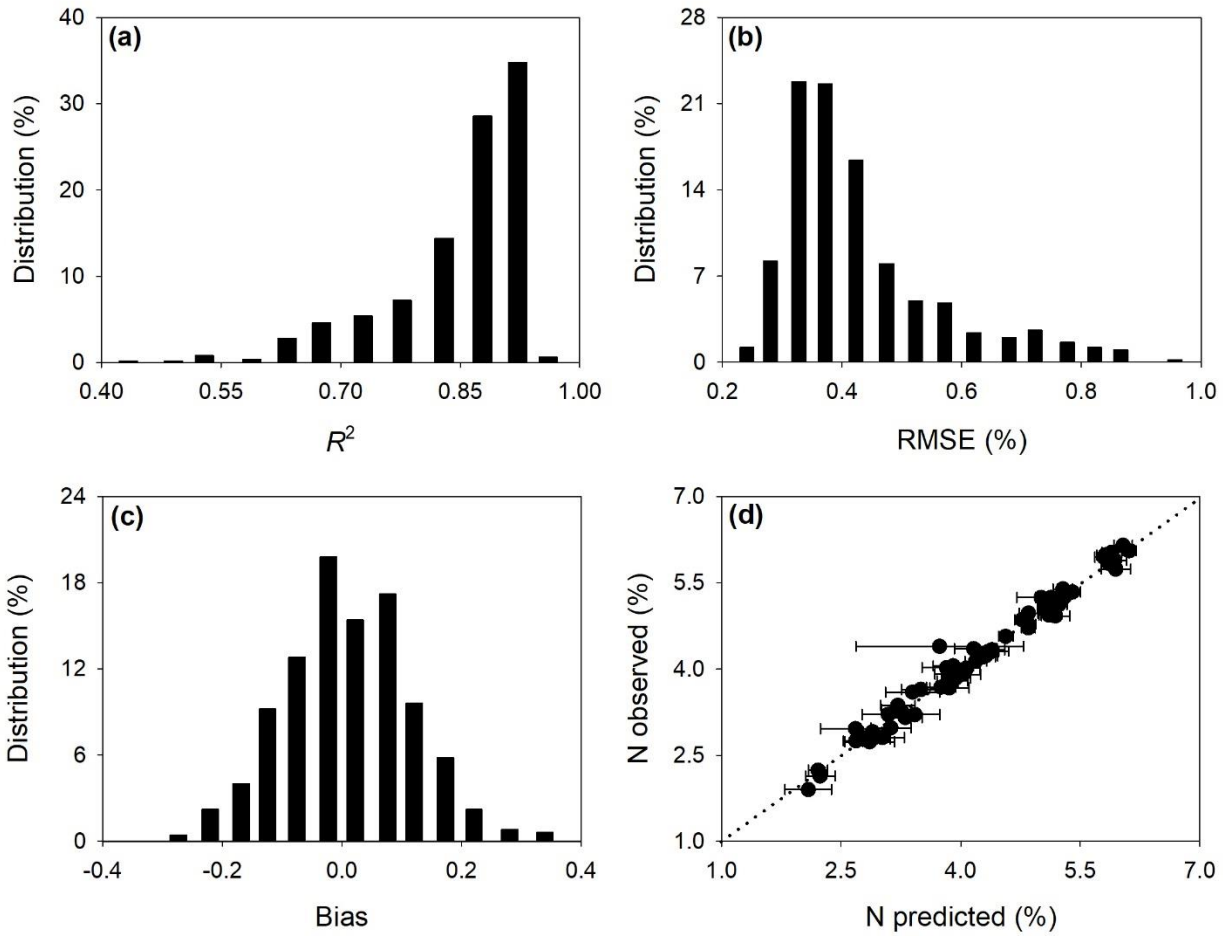

Fig. S4.1. Model diagnostic statistics generated through the evaluation of 500 random subsets, split 80:20 for calibration and validation, respectively, of nitrogen concentration predictions. Wavelength range used was 1500-2400, and was built using partial least squares regression. Individual boxes depict performance metrics for (a) R^2, (b) root mean square error (RMSE), (c) bias, and (d) observed vs. predicted (± SD) measurements from the validation data generated from the 500 iterations of the data set. Dashed black line is 1:1 line.

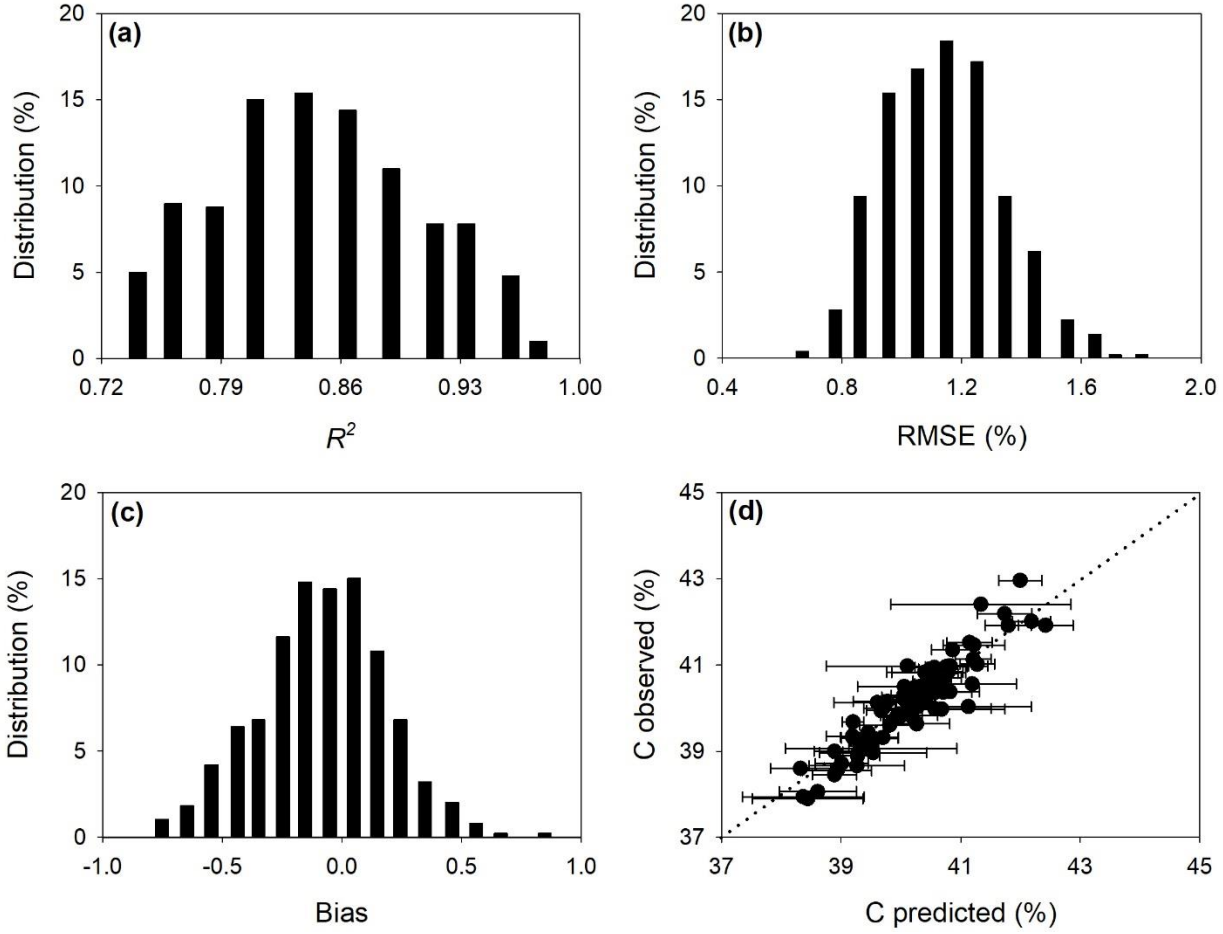

Fig. S4.2. Model diagnostic statistics generated through the evaluation of 500 random subsets, split 80:20 for calibration and validation, respectively, of carbon concentration predictions. Wavelength range used was 1500-2400, and was built using partial least squares regression. Individual boxes depict performance metrics for (a) R^2, (b) root mean square error (RMSE), (c) bias, and (d) observed vs. predicted (± SD) measurements from the validation data generated from the 500 iterations of the data set. Dashed black line is 1:1 line.

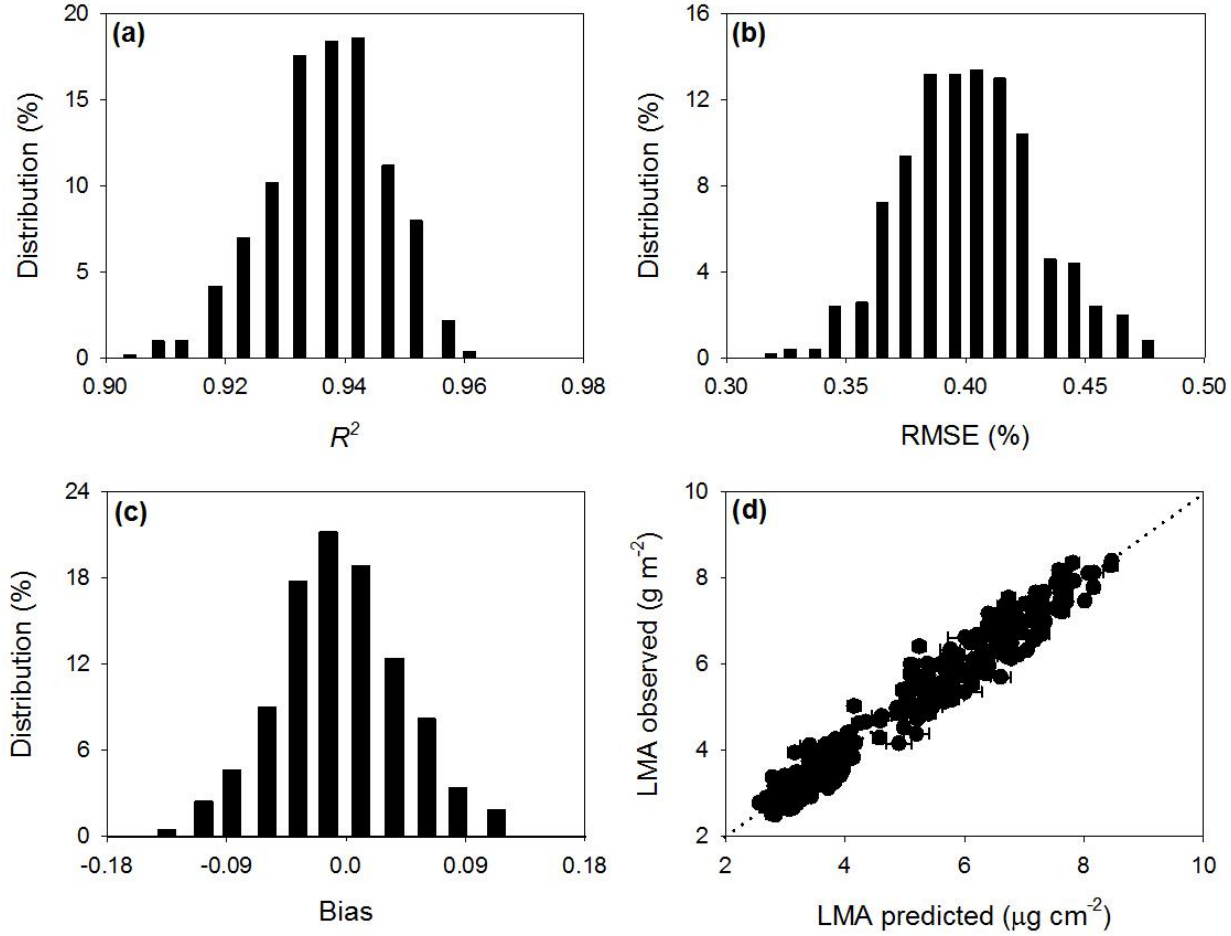

Fig. S4.3. Model diagnostic statistics generated through the evaluation of 500 random subsets, split 80:20 for calibration and validation, respectively, of leaf mass per area (LMA) predictions. Wavelength range used was 1500-2400, and was built using partial least squares regression. Individual boxes depict performance metrics for (a) R^2, (b) root mean square error (RMSE), (c) bias, and (d) observed vs. predicted (± SD) measurements from the validation data generated from the 500 iterations of the data set. Dashed black line is 1:1 line.

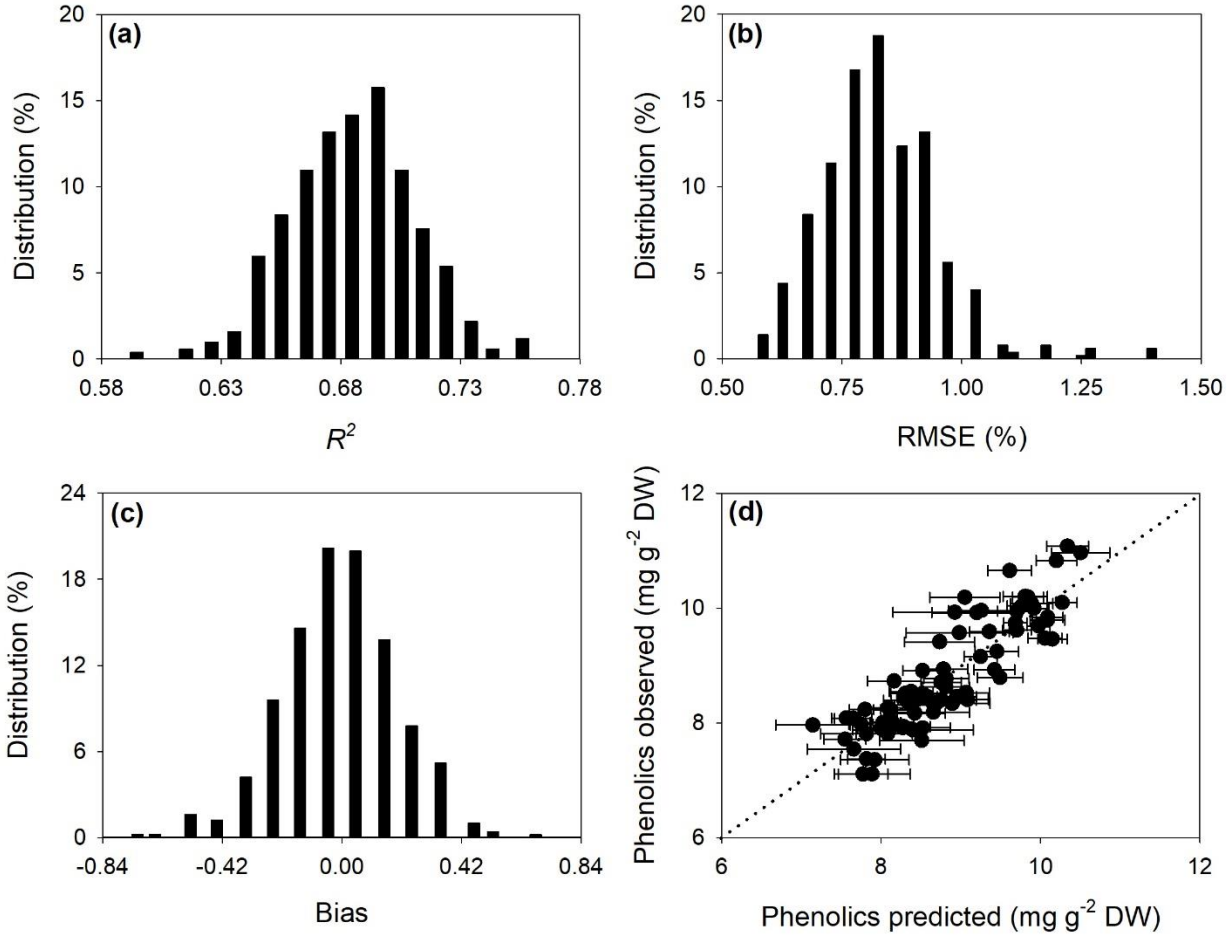

Fig. S4.4. Model diagnostic statistics generated through the evaluation of 500 random subsets, split 80:20 for calibration and validation, respectively, of phenolic concentration predictions. Wavelength range used was 1500-2400, and was built using partial least squares regression. Individual boxes depict performance metrics for (a) R^2, (b) root mean square error (RMSE), (c) bias, and (d) observed vs. predicted (± SD) measurements from the validation data generated from the 500 iterations of the data set. Dashed black line is 1:1 line.